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The role of the Lymph Node in the Establishment of an Adaptive Immune Response to Vaccination

Short Title: Lymph Nodes and Adaptive Immunity

by

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ABSTRACT

FACULTY OF MEDICINE

Immunology

Thesis for the Degree of Doctor of Philosophy

THE ROLE OF THE LYMPH NODE IN THE ESTABLISHMENT OF AN ADAPTIVE IMMUNE RESPONSE TO VACCINATION

Mr David Michael Layfield

Follicular T-helper (T_{FH}) cells are a subpopulation of CD4+ lymphocytes, which within germinal centres, determine differentiation of B-cells into memory cells and antibody-secreting plasmacytes. T_{FH} are therefore critical players in vaccine-induced immunity. Study of T_{FH} has been limited, as they are thought to be tissue resident cells, which do not normally re-circulate. While accessing blood is straightforward, access to lymph node tissue responding to vaccine is very limited. Therefore data on functions of tissue-resident human T_{FH} cells remains sparse.

This thesis details establishment of an ethically approved peri-surgical window of opportunity study and development of novel tissue processing techniques and laboratory assays designed to overcome this hurdle. I randomized 42 consenting breast cancer patients due to undergo sentinel lymph node biopsy to be vaccinated with combined tetanus/diphtheria/polio vaccine ipsilateraly, contralateraly or not at all prior to surgery. A vaccine draining, non-sentinel node was studied in the context of vaccine-specific antibody and circulating lymphocyte response over the seven weeks following vaccination.

Only lymph nodes draining the ipsilateral vaccine site were enriched for two CD4+derived populations; T_{FH} (CD45RO+CXCR5+ICOS+PD1+) and pre-T_{FH} (CD45RO+CXCR5+ICOS+PD1-). In blood, transient increases in absolute numbers of these same populations were observed one week following vaccination (mean-fold-increase: T_{FH} = 6.3; P = 0.002. Pre-T_{FH} = 4.0; P=0.002). In contrast a related population (CD45RO+CXCR5+ICOS-PD1+) showed no enrichment within vaccine-draining nodes or changes in circulating numbers post-vaccine.

Total IgG, IgM and IgG1-4 isotype immunoglobulin vaccine response was assessed. Response correlated with predominant cell-type increase in blood: CD45RO+CXCR5+ICOS+PD1- were prevalent in slow-responders, correlating with increases in immunoglobulin-switched plasmablasts (r = 0.90; 95%CI 0.74-0.97. P<0.0001), whereas CD45RO+CXCR5+ICOS+PD1+ were prominent in fast-responders, associated with increasing unswitched plasmablasts (r = 0.79; 95%CI 0.51-0.90. P<0.0001) and plasma cells (r = 0.57; 95%CI 0.17-0.81. P = 0.007).

Dichotomisation of response according T_{FH} sub-population tallies with measurable B-cell antibody and blast changes following vaccination. This possibly reflects memory state, suggesting different roles of T_{FH} and pre-T_{FH} in primary and secondary responses. Further study of the function of T_{FH} in lymphoid tissue-should focus on these two dynamic populations.

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DECLARATION OF AUTHORSHIP

I,……[please print name]

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research:

"The role of the Lymph Node in the Establishment of an Adaptive Immune Response to Vaccination"

I confirm that:

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Abbreviations

CRF	Case Report Form
CR UK	Cancer Research UK
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot assay
FBC	Full Blood Count
FFPE	Formalin fixed paraffin embedded
FrC	Fragment C (of Tetanus Toxin)
Her2	Human Epidermal Growth Factor Receptor 2
HTA	Human Tissue Authority
ICH-GCP	International Conference on Harmonisation Good Clinical Practice guidelines
INR	International Normalised Ratio
IRAS	Integrated Research Application System
IU	International Unit
MDT	Multi-Disciplinary Team
NHS	National Health Service
PBMC	Peripheral Blood Mononuclear Cells
PVDF	Polyvinyldene difluoride
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
R&D	Research and Development
REC	Research Ethics Committee
RGFHSC	Research Governance Framework for Health and Social Care
RNA	Ribonucleic Acid
RPMI/cRPMI	(complete)Roswell Park Memorial Institute cell culture media
SLN/SLNB	Sentinel Lymph Node (Biopsy)
SOP	Standard Operating Procedure
TAA	Tumour Associated Antigen
^{99m}Tc	Technetium-99
T_{CM}	Central memory T-lymphocyte
T_{EM}	Effector Memory T Lymphocyte
T_{EMRA}	Terminally differentiated CD45RA positive effector T Lymphocyte
T_{FH}	Follicular T Helper Cell
UHS	University Hospital Southampton
UoS	University of Southampton

1 Introduction

1.1 Antigen Presentation and the Adaptive Immune Response

The immediate response to an insult to the body is the generation of a co-ordinated response by a variety of chemical mediators, acute phase proteins and specialised cells of the innate immune system (1). The nature of an individual response is dependent on the degree of activation of individual components of each cascade and the sub-types of recruited immune cells, which is heavily influenced by the nature and site of insult itself. The innate response is generic and relatively untargeted, designed to deal with an immediate threat.

The adaptive immune response comprises of the generation of a reaction specific to individual antigens. It is distinguishable from the innate response in that it targets individual antigens and it requires time to respond to the presence of said antigen (1). The adaptive immune system also records the antigens it has previously been exposed to, allowing an accelerated response should the same antigen be encountered again; this is termed immune memory.

1.1.1 Lymphocytopoiesis

Lymphocytes are the active cellular components of the adaptive immune system which are produced within the bone marrow. They are divided into two broad classes according to their site of maturation; T-Lymphocytes mature within the thymus whereas B-Lymphocytes mature within bone marrow. The two sub-populations are defined by the specialist antigen receptors on their surface, which determine their function.

During haematopoiesis, progenitor B-lymphocytes and progenitor T-lymphocytes are produced within the bone marrow. Progenitor T-cells do not express T-cell receptors and leave the bone marrow to migrate to the thymus where they mature. The maturation process involves the generation of unique individual T-cell receptors (TCR) through genetic recombination within the variable regions of the DNA encoding the TCR. Once a maturing T-lymphocyte begins to express its unique T-cell receptor it is subjected to a stringent selection process coordinated by the thymic stromal cells.

Thymic stromal cells are specialised cells which express high levels of MHC Class I and MHC Class II. Any maturing lymphocyte which is unable to bind to the self-MHC molecules is identified and eliminated by induction of apoptosis (positive selection). Similarly, any maturing lymphocytes which demonstrate high affinity for self-peptide-MHC complexes presented by the stromal cells are also destroyed (negative selection). Up to 99% of maturing lymphocytes will be destroyed in this way. Those which remain are allowed to reach maturity. They possess T-cell receptors (TCR) capable of recognising self-MHC but are not active against self-peptides

presented upon these molecules, i.e. they carry a receptor which, potentially, recognises foreign antigen and is “self-tolerant”.

The TCR is complexed on the cell surface with 3 additional dimers collectively termed CD3 (the combination of the TCR and CD3 is termed the “TCR complex”). CD3 is a complex of polypeptide chains with long cytoplasmic tails which allow transmembrane signal transduction following interaction between TCR and its antigen; it comprises a heterodimer of gamma and epsilon chains (γε), a heterodimer of delta and epsilon chains (δε) and either a homodimer of zeta chains (ζζ) in 90% of complexes or a heterodimer of zeta and eta chains (ζη) in the remaining 10% of complexes. The CD3 complex is essential for TCR expression and signal transduction. The presence of CD3 can therefore be used to differentiate T-lymphocytes from other Lymphocyte sub-populations.

Unlike progenitor T-lymphocytes, progenitor B-lymphocytes remain within the bone marrow whilst reaching maturity. However, similarly to progenitor T-lymphocytes, immature B-lymphocytes undergo genetic re-arrangement to produce a unique membrane bound immunoglobulin which is complexed to a disulphide linked Ig-α/Ig-β transmembrane heterodimer constituting the B-cell receptor. Maturing B-cells with a high affinity for self-peptide are selected out following receptor-antigen interaction following self-peptide presentation by stromal cells within the bone marrow (1), leaving a population of mature, naïve B-lymphocytes possessing membrane bound immunoglobulin which has the potential to identify foreign antigen.

Once matured, the naïve lymphocyte will only recognise a specific antigen i.e. they are antigenetically committed. Unless this antigen is encountered the cell will remain in an unprimed state. The life-span of such unprimed cells is limited, so that if such a cell fails to encounter its respective antigen, it will apoptose. Naïve lymphocytes are released from the sites of their maturation into the circulation and typically congregate within the organs and vessels of the lymphatic system, as well as other sites throughout the body.

1.1.2 Antigen Presentation and Major Histocompatibility Complex (MHC) molecules

T-cell receptors and antibodies, either in soluble or membrane bound (B-cell receptor) form, only recognise discrete sites on individual antigens, rarely the whole molecule. Such sites are termed epitopes. B-cell receptors and antibodies are capable of identifying epitopes unaided, T-cell receptors require an epitope to be presented bound to Major Histocompatibility Complex (MHC) molecules.

MHC molecules are cell-membrane bound glycoproteins which externalise antigens to allow interrogation by T-cell receptors. There are two sub-types of MHC molecules; Class I MHC molecules are present on all nucleated cells and function as facilitators of surveillance by T-Lymphocytes. In broad terms, they present intracellular peptides formed within the endoplasmic reticulum to the cell surface so that any alteration in peptides produced by the cell, be it by genetic mutation or pathogen, can be recognised and eliminated. This mechanism of antigen presentation is termed the “cytosolic pathway”.

Class II MHC molecules are expressed predominantly by specialist antigen presenting cells. Such cells are able to internalise an exogenous antigen through phagocytosis or endocytosis, degrade it into peptide fragments within lysozymes, align it with MHC Class II molecules and present the resulting complex on its surface (the “endolytic pathway”), together with appropriate co-stimulatory signals. Once presented in this fashion, naïve T-lymphocytes specific to the presented antigen can recognise it, become activated, differentiate according to the co-stimulatory signals received and clonally expand to allow a specific, targeted response. Cells capable of processing and presenting antigen in this fashion include B-Lymphocytes, macrophages and dendritic cells, although dendritic cells are the only cells typically termed “professional” antigen presenting cells (2).

Mature activated dendritic cells constitutively express high levels of Class II MHC and co-stimulatory molecules. Doing so makes them effective and efficient antigen presenters capable of activating naïve T-lymphocytes. Macrophages do not constitutively express Class II MHC or co-stimulatory molecules until they become activated through phagocytosis and exposure to pro-inflammatory cytokines such as IFNγ (1). Their prominence within end-organs suggests a function as APCs within frontline tissues at the later, effector stage of the immune response (3). B-lymphocytes do constitutively express Class II MHC but do not express co-stimulatory molecules unless activated, making them less effective initiators of the primary immune response (2).

In addition, various other “non-professional” antigen presenting cells can be induced to express Class II MHC and co-stimulatory signals; however this ability is transient and only inducible by prolonged inflammatory stimulation. Such cells include fibroblasts, glial cells, vascular endothelial cells, pancreatic beta cells, thymic endothelial cells and thyroid epithelial cells.

T-Lymphocyte sub-types are restricted to which MHC class they are capable of recognising by the presence or absence of CD4 and CD8 co-receptors. CD4 is a membrane bound glycoprotein,

which acts as a co-receptor for Class II MHC; whereas CD8 is a dimeric membrane bound protein which is a co-receptor for Class I MHC. CD8+ve/CD4-ve lymphocytes can therefore only interact with cells expressing Class I MHC (termed “Class I restricted”) and CD4+ve/CD8-ve can only interact with cells expressing Class II MHC (“Class II restricted”). The expression of CD4 or CD8 therefore allows the identification of sub-populations of T-lymphocytes with distinct functional differentiation.

CD1 molecules are a third pathway through which antigens are presented. CD1 molecules have a structure similar to Class I MHC but are expressed by dendritic cells and B-lymphocytes only. They present exogenous non-peptide antigen processed through the endolytic pathway and allow recognition of lipid and glycolipid components of bacterial cell walls by the adaptive immune system. CD1d molecules in particular, are implicated in antigen presentation to Natural Killer T-cells (NKT-cells); a subgroup of lymphocytes’ which recognise non-peptide antigens presented on CD1d molecules, but also possess TCRs on their surface. The TCR profile of NKT cells is far more limited than the general T-cell population and is biased towards the recognition of non-peptide antigens from intracellular pathogens including mycobacteria.

1.1.3 Lymphocyte Maturation into Naïve Cells

The T-cell receptor is a heterodimer comprised of either an α and a β unit (αβ T-lymphocytes) or a γ and a δ unit (γδ T-lymphocytes). In adult humans, the vast majority of T-cells express αβ receptors, with less than 5% expressing γδ receptors. The generation of individual T-cell receptors is a multistep process which occurs within the thymus. Upon its arrival within the thymus, a progenitor T-cell has not undergone any genetic re-arrangement of its TCR genes which remain unexpressed. In addition, the progenitor T-cell does not express CD4 or CD8, i.e. it is a “double negative” cell.

These double negative cells undergo genetic re-arrangement within the variable regions of genes encoding the β-chain of the TCR. The β-chain is then expressed by the cell complexed with CD3 and a pre-α-chain to form a pre-T cell receptor (pre-TCR). If the rearranged β-chain is “productive” (i.e. potentially functional or valid) it is able to bind to ligands expressed by the thymic stromal cells. Such interaction induces signal transduction via CD3 which stops further re-arrangement of the β-chain genes, promotes clonal expansion of the cells with functional β-chains, and induces expression of CD4 and CD8. The dual expression of CD4 and CD8 means these cells are now termed “double positive” (CD4+8+).

Once the CD4+8+ cells stop proliferating, genetic re-arrangement of the variable regions of the genes encoding the α-chain can occur. This two stage process ensures maximal variability in the TCR by ensuring that every functional β-chain has the opportunity to be linked with all

variations of the α-chain. The α-chain rearrangements allow the expression of TCRs which then attempt to interact with MHC molecules expressed by thymic stromal cells – failure of the new αβ-heterodimer to effectively bind to MHC results in the genetic re-arrangement of the α-chain continuing; i.e. a single cell can "submit" several attempts at an effective αβ combination in an effort to avoid apoptosis. Failure to "find" an effective combination results in a failure of positive selection and apoptosis.

Once positive selection is "passed" the TCR is genetically fixed within each cell. The resulting cells are antigenetically committed. They undergo negative selection by dendritic cells and macrophages which are resident within the medulla of the thymus. CD4+8+ cells which express TCR which binds with high affinity to self-antigens expressed by the thymic cells are identified and selectively apoptosed. This process ensures that populations of T-cells active against the body's own tissues are not produced. T-Lymphocytes which survive both positive and negative selection go on to lose expression of either CD4 or CD8, to become single-positive naïve T-lymphocytes, prior to their release and circulation. The mechanism which dictates which co-receptor's expression is turned off is unclear.

B-lymphocytes mature within the bone marrow. Progenitor B-cells rely on the bone-marrow stromal cells to provide the supportive environment for their development. Progenitor B lymphocytes express a number of adhesion molecules which interact with ligands on the bone marrow stromal cells. Interaction between cell-adhesion molecules bring the cells into close proximity, allowing interaction between c-Kit on the progenitor B-lymphocyte and Stem Cell Factor (SCF) on the stromal cells. c-Kit – SCF interaction activates c-Kit which acts as a tyrosine kinase and promotes cell division and rearrangement of the genes encoding the heavy chain of the cell's immunoglobulin. A recombined heavy chain is expressed on the cell surface with a surrogate light chain in a fashion analogous to the progenitor-T lymphocytes expressing a β-chain with a pre-α-chain to form a pre-receptor. In this fashion, the pre-B receptor is expressed by the maturing progenitor B-cell to check the viability of the recombined heavy chain prior to further maturation. Only once a productive re-arrangement is achieved which allows expression of the recombined μ-heavy chains with the surrogate light chain (the pre-B-cell receptor) will a pro-B cell progress to become a Pre-B cell.

Pre-B lymphocytes express receptors for IL-7, secreted by the stromal cells, which drives further maturation and down-reregulation of adhesion molecules. The pre-B lymphocytes are now independent of cell-to-cell contact with the bone marrow stroma, but remain dependent on IL-7. In addition, the Pre-B lymphocyte begins to express CD25 which is the α-chain of the cell-surface receptor for IL-2. IL-2 and IL-7 drive the rearrangement of the light chain encoding

genes; successful recombination to produce productive light-chain results in the expression of the combined heavy and light chains on the surface of the cell as membrane bound IgM (mIgM) and the cessation of CD25 expression. The cells are now termed “immature B-lymphocytes”. Once immature B-cells express their mIgM, they undergo a process of negative selection; the cells are exposed to self-antigens on the surface of the bone marrow stromal cells. Recognition of the self-peptides by the immature cells results in rearrangement of genes encoding the variable component (both heavy and light, although light chain most notably) of the B-cell receptor utilising the same recombination mechanisms integral to primary gene arrangement in a process known as “receptor editing” (4). Failure to produce a self-tolerant receptor results in apoptosis. In this fashion, 90% of immature B-lymphocytes are destroyed.

Maturation completes once the mIgM is co-expressed with mIgD targeting the same antigen epitope; these cells are therefore mature, antigenetically committed, naïve B lymphocytes. They are released from the bone marrow and locate to the lymphatic tissues.

1.1.4 The Lymphatic System and Lymph Nodes as a Site of Antigen Presentation

The lymphatic system is a network of vessels which collect extravasated plasma (lymph) and return it to the circulation. Lymph is rich in soluble proteins, other antigens and lymphocytes. Lymph nodes take advantage of this by acting as nursery sites for naïve lymphocytes, which are therefore exposed to the antigens washed through the lymphatic system from the upstream tissues which they drain, maximising their chances of encountering the specific antigen against which they are active. In addition, B-lymphocytes and dendritic cells encountering antigen in the periphery can migrate to the lymph node in order to increase their chances of acquiring T-cell help. The primary draining node is therefore responsible for the majority of the co-ordination of response to an antigen (5)

As lymph flows through a lymph node, any particulate matter is captured by a network of resident macrophages and interdigitating dendritic cells. Such antigens are then phagocytosed, processed into peptides and presented by MHC Class II Molecules. Dendritic cells from the peripheral tissues (including interstitial and circulating dendritic cells, as well as Langerhans cells) (2) and macrophages(1) also migrate through the lymphatic system to present antigen encountered elsewhere within the lymph nodes.

A lymph node is made up of three distinct cellular regions; the medulla, the paracortex and the cortex. The medulla is closest to the efferent lymphatic vessels and contains few naïve or proliferating lymphocytes. It is populated predominantly by plasma cells. The paracortex contains interdigitating dendritic cells – antigen presenting cells – which process antigen and present it to naïve T-Lymphocytes. The paracortex is therefore a site within which naïve T-

lymphocytes congregate, interacting with the professional antigen presenting cells in a bid to find their antigen.

The cortex is the region of the node closest to the afferent lymphatic vessels; the cortex consists of macrophages and B-lymphocytes arranged within primary follicles. Follicular dendritic cells also reside within primary follicles, however in comparison to interdigitating dendritic cells follicular dendritic cells express smaller quantities of MHC molecules. They provide a reservoir of antigen and support naïve and proliferating B-lymphocytes. Lymph continually washes through the cortex, allowing the residing naïve B-Lymphocytes the opportunity to be exposed to their specific antigen.

1.1.5 Lymphocyte Activation and Clonal Proliferation

Each individual naive lymphocyte's receptor is specific to a single antigenic sequence. A naïve lymphocyte will only become active if its receptor encounters its specific antigen (although cross reactivity can occur – see section 1.5.2). Furthermore, upon activation a lymphocyte will up-regulate its expression of cytokine receptors and decrease its expression of BCL-2; doing so puts the lymphocyte in a vulnerable state. It requires continued exposure to appropriate cytokine activation signalling; failure of this leads to the decreased levels of BCL-2 causing apoptosis. This mechanism allows population control of clonally expanded activated lymphocytes; once the activation signals are turned off, the cellular response is reduced by controlled cell death.

1.1.5.1 CD4+ T-Lymphocyte Activation, Clonal Proliferation and Differentiation

Activation of naïve T-lymphocytes occurs within the paracortex of a lymph node. Naïve CD4+ve T-lymphocytes interact with processed antigenic peptides bound to Class II MHC molecules expressed on the surface of APCs. Subsequent gene activation, cell proliferation and differentiation are dependent on the co-stimulatory signals exchanged between the naïve T-lymphocyte and the APC.

Intracellular signalling is initiated following the cross-linking of the TCR α and β-chains by a MHC-peptide complex. This activates the G-protein Ras, which induces the MAP (Mitogen-activated Protein) kinase cascade that phosphorylates transcription factors which promotes cell proliferation and the production of Interleukin 2 (IL-2). Cross-linking of the TCR complex also results in translocation of the co-receptor CD4 to the vicinity of the TCR-CD3 complex. The intracellular component of CD4 bears a protein tyrosine kinase named Lck. Another protein tyrosine kinase, named Fyn, is also recruited. Both Lck and Fyn require activation by removal of a phosphate from its inhibitory site; this is performed by CD45, a transmembrane tyrosine

phosphatase which is constitutively expressed by leukocytes (otherwise termed the “leukocyte common antigen”).

Once the inhibitory phosphate has been removed, Lck and Fyn are able to associate with the cytoplasmic tails of CD3, phosphorylating the Immunoreceptor Tyrosine-based Activation Motif (ITAM) sequences borne there. Phosphorylation changes the conformation of the ITAMs, exposing docking sites for other signalling molecules such as ZAP-70 (zeta-associated protein which binds to ITAMs of the CD3 ζ chains). In turn, ZAP-70 activates phospholipase-C through phosphorylation, which hydrolyses the membrane phospholipid PIP_2 (phosphatidylinositol 4,5-biphosphate) into IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). IP_3 increases intracellular calcium, which activates calcineurin (a calmodulin-dependent phosphatase), which in turn activates NF-AT by de-phosphorylation. DAG activates protein kinase C (PK-C) which phosphorylates a number of intermediaries resulting in NF-κB activation. Both NF-AT and NF-κB enter the nucleus, activating various genes which, along with other products, produce IL-2.

In addition to TCR-cross-linking, activation of CD4+ cells is reliant on co-stimulation received following cell-to-cell interactions via cell-surface receptors and their membrane-bound ligands as well as various soluble signalling molecules. The interaction between stimulatory and inhibitory signals is multifaceted and further complicated by the various safety mechanisms used to prevent inappropriate or overzealous activation of immune cells.

B7 is a membrane bound signalling molecule which is constitutively expressed by dendritic cells and can be induced on activated B-cells and macrophages. It provides a co-stimulatory signal to lymphocytes undergoing activation. Naïve CD4+ T-lymphocytes constitutively express CD28, a receptor for B7. Therefore, co-stimulation of a naïve CD4+ cell through cross-linking of its TCR and through interaction between CD28 and B7 will result in activation. However, CD28-B7 interaction induces the CD4+ T-lymphocyte to express CTLA-4 which is also a receptor for B7. CTLA-4 has a higher affinity for B7 than CD28 and acts to antagonise the actions of CD28. The CD28 induced expression of CTLA-4 therefore acts as a brake, meaning that there is a mechanism through which activated cells can the switched off in the absence of continued stimulation.

The failure of an adequate or sustained co-stimulation of a naïve T-lymphocyte in the event of TCR-cross-linking by its target antigen induces a state of anergy within the cell. This prevents activation of T-lymphocytes in the absence of pro-inflammatory stimuli and avoids an inappropriate response to antigen.

Upon release from the thymus, naïve CD4+ lymphocytes are in a resting state and constitutively express CD45RA and CD28, which are available to allow TCR-mediated activation signalling and co-stimulation respectively, as well as the chemokine receptor CCR7 which mediates chemotaxis to the lymphatics. Upon exposure to its target antigen and appropriate co-stimulation through CD28-B7 interaction the cell produces IL-2 and expresses the IL-2 receptor. The effect of IL-2 on the cell is to prepare the cell for division and differentiation. After 48 hours of IL-2 stimulation the activated cell will enter the G_1 phase of its growth cycle. However this is dependent on the activation stimulus continuing, thereby preventing the CTLA-4-B7 interaction from arresting activation.

This clonal expansion of CD4+ cells specific to the instigating antigen gives rise to effector and memory sub-populations. Memory sub-populations are addressed in the section below. Effector cells carry out functions specific to their terminal differentiation, itself determined by the cocktail of co-stimulatory and co-inhibitory signals received during their genesis. The differentiation of a CD4+ cell presented with its antigen by a dendritic cell may therefore be determined by the activation state of the dendritic cell (6, 7). Several such effector sub-populations exist.

1.1.5.1.1 CD4+ Subset polarisation

CD4+ lymphocytes are Class II restricted; i.e. they only recognise antigen presented by cells expressing Class II MHC molecules. As such, they do not act as direct-action cells. Instead their role is in the co-ordination of the immune response, and the selective promotion of the most appropriate effector response, be that a particular immunoglobulin production, a cytotoxic T-lymphocyte response, recruitment of phagocytic cells or a suppression of response to an antigen.

Historic sub-division of CD4+ lymphocytes was binary, dividing CD4+ cells into either those promoting a cell-mediated response (T-Helper 1 lymphocytes (T_H1 lymphocytes)) or those promoting an antibody-mediated response (CD4+ T-Helper 2 lymphocytes (T_H2 lymphocytes)) (1, 8). These subsets were defined according to their cytokine profile; T_H1 cells secrete IL-2, IFN-γ and TNF-β which promotes cytotoxic T-lymphocyte activity against the target antigen. T_H2 cells were defined as those which secreted IL-4, IL-5, IL-6 and IL-10 and were associated with a B-cell response to antigen and the production of antigen specific antibodies (1, 8).

Contemporary understanding of the breadth of CD4+ T_H-cell subtypes has unveiled a more complicated picture, with at least six functionally distinct subsets termed T_H1, T_H2, T_H9 (pro-inflammatory cells associated with allergenic responses) (9), T_H17 (a subset of cells which facilitate immunity at the epithelial/mucosal barrier) (10), T_{REG} (regulatory T_H cells, a diverse

sub-group of cells of heterogeneous origins collectively thought to promote self-tolerance and quell over-reactivity to pathology) (11) and T_{FH} (follicular helper T-cells) (12-14). T_{FH} cells are the most recently described subset and their role is thought critical to the regulation of B-cell effector and memory responses.

Central to subset determination are distinct transcription factors which regulate differentiation; within the five established CD4+ subtypes these factors are T-bet (T_H1), GATA3 (T_H2), PU.1 (T_H9), $ROR_{\gamma T}$ (T_H17) and Foxp3 (iT_{REG}) (9, 13). Bcl-6 is widely accepted as the master-regulator transcription factor for T_{FH} cells (14-19) although other regulatory factors (IRF4, c-Maf, Batf, STAT1/3/5 and Ascl2) have also been proposed as important in this subset (18-21). Bcl-6 is a transcriptional repressor which has long been established as a critical regulator within germinal centre B-cells (22-24); it has a reciprocal and antagonistic relationship with Blimp-1 (15) and its constitutive expression effectively inhibits differentiation into alternative CD4+ subsets (16, 25). Conversely, Bcl-6 knockout has been associated with exaggerated response within T_H2 (22) and T_H17 (26) subsets, although conditional knockout of Bcl-6 from CD4+ lineage is not associated with non-T_{FH} subset hyper-immunity, suggestive of the importance of Bcl-6-mediated suppression of myeloid-derived pro-inflammatory factors in the normal immune response (27).

Fate determination is influenced by cytokines (28-32) and naïve T-cells are capable of differentiating into either subset at the point of activation (i.e. they are not fate determined prior to activation) (13, 33, 34). Furthermore plasticity exists between T_H1, T_H2, T_H17, iT_{REG} and T_{FH} cells, suggesting reversibility of subtype commitment and type-conversion of cells given appropriate stimuli (35-43), the propensity for which is thought to be mediated through epigenetic factors, with increased stability achievable following repeated and consistent polarising events *in vivo* (44-47). Such plasticity following exposure to polarising cytokines is also appreciable within the corresponding CD4+ memory cell subtypes (35, 48, 49) (expanded on in Section 1.2.2.1). However cytokine-mediated induction or repression of transcription factors may not be sufficient to induce subtype differentiation in certain instances (13, 14, 50); in particular initial interaction between dendritic cells and CD4+ cells followed by cell-to-cell mediated bi-directional feedback between antigen-specific B-cells and CD4+ cells appears critical for sustained T_{FH} derivation and normal germinal centre formation (14, 15, 51-53) – see Section 1.3.

Therefore localisation of immune cells during antigenic response influences fate determination. CXCR5 expression allows CD4+ cells to migrate in response to CXCL13 produced predominantly by follicular dendritic cells, but also by activated T_{FH} cells and myeloid and Plasmacytoid

dendritic cells resulting in accumulation around follicular boundaries (54-56), although the propensity to do so is also influenced by concomitant expression of other chemotactic receptors such as CCR7, PSGL1 and S1PR1 (57-61); BCL-6 is closely associated with CXCR5 expression, has also been demonstrated to have a direct influence on PSGL1 expression (60) and is positively correlated with that of CD69, an inhibitor of S1PR1-mediated egress (61). Achievement of a balance of these interacting factors which favours follicular localisation allows co-localisation of CD4+ cells with B-cells, and the necessary reciprocal stimulation through ICOS-ICOSL. Knockout or blockade of either ICOS or ICOSL results in deficient germinal centre formation and B-cell response in mice (62-65) and absence of germinal centres and T_{FH} cells is notable in patients with congenital ICOS deficiency (66, 67). B cell deficiency is associated with the absence of T_{FH} cells (15, 59, 68) underlining the importance of B-T interaction in T_{FH} generation, although initial early appearance of T_{FH}-phenotype cells is B-cell independent, being mediated through ICOS signalling during T-DC interaction (15, 53, 61), and need for B-cells for T_{FH} development can be abrogated by repeated antigen delivery (53), suggesting persistent exposure of the T cell to antigen is of primary importance, and that B-cells are in some fashion best suited to this purpose.

However at what point do naïve cells become fate determined? Downstream the BCL-6-BLIMP-1 axis may be important in skewing to either a non-T_{FH} or a T_{FH}-phenotype (and the latter's subsequent localisation/secondary interaction with B-cells) (14), however an initial switch must precede this. Indeed association has been made between pMHCII-TCR-binding affinity and CD4+ fate-determination at the point of T_{naive}-DC interaction (69-72) and the degree of antigen load (73, 74). Proposed mechanisms underlying such influence include differences in the degree of induced signalling through the Lck-MEK-ERK pathway, with stronger signals resulting in loss of TCR-driven GATA-3 expression and inhibition of STAT5 phosphorylation, reducing responsiveness to IL-2 and preventing the synergistic STAT5-GATA-3 mediated induction of IL-4 expression (50) leaving the cell open to influences from IFNγ/STAT1 and IL-12/STAT4, resulting in T-bet expression and T_H1 induction (50). Similar cytokine/STAT/transcription factor relationships have been mapped for the other CD4-effector subsets; IL-6/IL-23/STAT3/RORγt and TGF-β/SMADs/RORγt in T_H17 cells and IL-2/STAT5/Foxp3 and TGF-β/SMADs/Foxp3 in iT_{REG} (50). In the case of T_{FH} cells (IL6, IL21/STAT3/Bcl-6) (14, 19, 63), high-affinity TCRs have been shown to preferentially differentiate into T_{FH} phenotype and therefore TCR repertoires of T_{FH}-differentiated cells are more restricted compared with other subtypes (75). Factors which increase the drive towards T_{FH}-phenotype include antigen dose and therefore p:MHCII density on APC and TCR-pMHCII dwell-time (76). Said data supports the premise that innate properties of the individual TCR upon a naïve cell biases its fate-

determination (“induction phase”), however continued appropriate cytokine and cell-to-cell signalling (both autocrine and from partner cells), and implicit maintenance of appropriate balance of transcription factors throughout antigen response is required to stay the course (“polarisation phase”) (50).

1.1.5.2 CD8+ T-Lymphocyte Activation, Clonal Proliferation and Differentiation

Naïve CD8+ lymphocytes require several sequential stimuli prior to activation. Prior to activation, naïve CD8+ lymphocytes are incapable of cytotoxicity, proliferation or differentiation, and do not express IL-2 or its receptor. For activation to occur, naïve CD8+ cells must first become exposed to their target antigen presented by Class I MHC molecules on a cells surface. This interaction induces the expression of IL-2 and IL-2 receptors by the cell. For a naïve CD8+ cell to become activated, additional co-stimulation is required in the form co-receptor molecules (CD28/B7 interaction) on the cell surface, as well as additional IL-2 often supplied by activated (“effector”) CD4+ T_H lymphocytes. Optimal CD8+ effector response requires CD4+ T_H help (77, 78)

Appropriate antigen exposure in the presence of co-stimulatory signalling from the antigen presenting cell and in the presence of IL-2, induces the naïve CD8+ lymphocyte to become either a cytotoxic T-Lymphocyte (CTL) or a memory CD8+ lymphocyte. Activation of naïve CD8+ Lymphocytes can be supported by CD4+ T_H Lymphocyte activation of dendritic cells through CD40/CD40L interaction (77, 79). Once activated the effector cell up-regulates its expression of CD2, LFA-1 (CD18/CD11a heterodimer) and CD45RO.

CD2 and LFA-1 are adhesion molecules expressed on the CTL; upon antigen interaction with the TCR, these two molecules become activated, binding to intercellular cell adhesion molecules (ICAMs) on the target cell, forming a conjugate. The close approximation of the CTL and the target cell bearing its specific target antigen allows formation of the “Immune synapse”; a link between the two cells. Through this structure the CTL delivers perforins and granzyme proteases; the effector molecules which induce the target cell to apoptose. Another mechanism through which CTL can induce cell death is through Fas-FasL interaction; again this is reliant on close approximation between the CTL and its target cell through CD2/LFA-1 – ICAM interaction induced by TCR cross-linkage.

CD45 exists in several isoforms; CD45RA has a low affinity for the TCR complex, meaning it is slow to activate the cell, and is preferentially expressed on naïve T-cells. Once activated, the CD45 isotype is switched to CD45RO, which has a higher binding affinity; this lowers the activation threshold for “primed” (i.e. effector/memory) T-cell populations. For CD8+ memory

cells, this means that further interaction with its target antigen can induce a proliferation of cytotoxic effector cells without the need for CD4+ T_H lymphocyte IL-2.

1.1.5.3 B-Lymphocyte Activation, Clonal Proliferation and Differentiation

Two mechanisms exist for the activation of a B-cell response to an antigen; Thymus dependent and thymus independent. The majority of antigens are thymus-dependent; i.e. co-stimulation of the naïve B-cell by direct contact with a similarly specific CD4+ T_H Lymphocyte is required to generate an antibody response. This is dealt with in detail below. However some common antigens are thymus-independent; the antibody response to these does not require CD4+ co-stimulation. Thymus-independent (TI) antigens can induce production of antibodies either through direct stimulation of B-cells via Toll-Like Receptor (TLR) activation (TI-1 antigens) or through direct cross-binding of a number of B-cell receptors on the same cell due to an antigens large size and highly repetitive structure (TI-2 antigens) (80). Examples of such antigens include lipopolysaccharide cell wall components from gram-negative bacteria, which, in high doses, can instigate a broad polyclonal increase in serum antibody levels, not necessarily targeted against lipopolysaccharide (TI-1 antigen). These processes are counterintuitive given the classical concept of the adaptive immune response being targeted and antigen specific, however presumably confers a survival advantage in the event of a severe infection with bacteria bearing these antigens. Other thymus-independent antigens include highly repetitious molecules such as flagella or bacterial cell wall polysaccharides (TI-2 antigens). Exposure to these antigens can induce an antibody response from antigen-specific B-cells in the absence of direct contact from CD4+ T_H Lymphocytes, however cytokines derived from CD4+ T_H Lymphocytes are still required for efficient proliferation and class-switching and the process is generally weaker, with less class switching and fewer memory cells produced, when compared with an effective thymus-dependent antigen response. When IgG is produced by TI antigens, it is typically galactosylated and sialated, conferring an immunosuppressive function (80); the biological significance of these processes are currently unresolved.

Furthermore B-cells can acquire alternative help from cells other than T-cells; such responses are typically against TD-antigens but are augmented by the actions of highly activated macrophages and/or dendritic cells (81, 82). Recently B-cell help mediated by natural killer cell subtypes and neutrophils has also been described, however detailed review of said processes is outside of the scope of this thesis.

Thymus-dependent antigen responses require interaction of a B-cell with a T-cell of equivalent antigen specificity; as detailed above, activation of naïve T-lymphocytes occurs within the paracortex. During the generation of an antibody response to a thymus-dependent antigen,

naïve B-lymphocytes are dependent on exposure to their antigen in solution, typically within the cortex of the lymph node, followed by migration and interaction with their counterpart T-helper lymphocytes. This interaction occurs within the paracortex and results in small foci of proliferating T-helper and B-lymphocyte clones on the outermost aspect of the paracortex.

Exposure of a resting naïve B-Lymphocyte to its antigen causes cross-linking of its mIgM or mIgD and signal transduction via the associated Ig-α/Ig-β heterodimer, which carries various Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) analogous to those borne on the cytoplasmic tails of the CD3 complex. Tyrosine phosphorylation within the ITAM regions of the Ig-α/Ig-β heterodimer is, like in T-cell activation, dependent on de-phosphorylation of key tyrosine kinases by CD45. CD45 therefore removes the inhibitory phosphate from the protein tyrosine kinase Lyn, which phosphorylates the ITAMs on the Ig-α/Ig-β heterodimer. Phosphorylation exposes docking sites within the ITAMs which activates the Ras/MAP kinase cascade, as well as recruiting Syk, which induces nucleic factor production through activation of Phospholipase C (1).

Similarly to T-cell activation, effective activation of naïve B-Lymphocytes is assisted by co-stimulatory signals received through co-receptors. The co-receptor complex on B-lymphocytes consists of a complex of CD19, CD21 (CR2) and CD81 (TAPA-1), with or without CD225 (Leu 13). CD19 is a co-receptor which augments the ability of B-cell receptors to cluster on the cell surface, enhancing activation. Its cytoplasmic tail bears a number of tyrosine residues providing a substrate for the phosphorylation reactions on the Ig-α/Ig-β heterodimer ITAMs. CD21 is a receptor for a complement degradation product (C3d); complement opsonised antigen can therefore be bound in closer proximity to the B-cell receptor. CD81 is a transmembrane receptor which augments signal transduction. CD225 is a transmembrane protein, the expression of which is induced by the pro-inflammatory cytokine Interleukin-17 (IL-17). Counteracting the pro-activation receptors is the constitutional co-expression of CD22 which is present associated with the B-cell receptor in resting cells. Various ligands (Grb2, PTPN6, LYN, SHC1 and INPP5D) of CD22 provide inhibitory signals to the resting B-cells, promoting negative feedback and preventing inappropriate activation (83).

Once an antigen cross-links the B-cell receptor, the antigen-immunoglobulin complex is internalised (endocytosis) and the antigen processed and presented on the B-cells surface complexed with Class II MHC. In addition, B7 expression on the B-cell surface is induced. Coupling of a naïve B-cell with its antigen-specific T_H-lymphocyte counterpart forms a T-B conjugate. This induces conformational changes within both cells; the T_H-lymphocyte re-orientates its golgi apparatus and microtubule structure toward the junction with the naïve B-

lymphocyte. CD28-B7 interaction provides the necessary activation signal to the T_H cell; once activated, the T_H-lymphocyte up-regulates its expression of CD40-ligand (CD40L; CD154). CD40L is an essential co-stimulatory molecule which interacts with CD40 on the surface of the naïve B-lymphocytes to promote activation; both mIgM cross-linkage and CD40-CD40L interaction are required for a naïve B-lymphocyte to enter the cell cycle. The CD40-CD40L interaction provides the stimulatory signals to the naïve B-cell necessary to induce blast formation and proliferation. CD40-CD40L also induces expression of cytokine receptors on the B-cell membrane (1).

Although able to proliferate following CD40-CD40L interaction, the B-lymphocytes are unable to differentiate into antibody secreting plasma cells or memory cell progeny unless cytokine signalling is also present. Activated T_H-Lymphocytes provide the necessary pro-proliferation signals (1).

The initial proliferation of the B-cell progeny occurs within foci on the edge of the paracortex. Plasma cell differentiation occurs in these areas, with production of IgM antibody. Under the correct cytokine stimulation from the accompanying T_H Lymphocytes, the variable region of the heavy chain can be recombined with any constant region. This results in a change in the sub-type of antibody ("Isotype switching") which alters the function of said immunoglobulin. Isotype switching is thought to be mediated by class-specific recombinase enzymes, the expression of which is influenced by cytokines and other signalling molecules released by the co-stimulating T-helper lymphocytes. Therefore, the different isotypes of immunoglobulin present in the serum changes over time from the initial high prevalence of IgM, to a greater proportion of other isotypes, including IgG (1).

Small numbers of activated T-helper and B-lymphocytes will then migrate back to the primary follicle. Presence of these activated cells induces transformation of the primary follicle into a secondary follicle. The activated B-lymphocyte undergoes clonal expansion, generating plasma cells which secrete antibody targeting the antigen. A small number of B-lymphocytes will migrate to the centre of the secondary follicle; termed the germinal centre. The germinal centre contains activated helper T-lymphocytes (i.e. T_{FH}), follicular dendritic cells and both proliferating and non-proliferating B-lymphocytes. Those B-lymphocytes which are proliferating are undergoing somatic hypermutation within their variable genes. The resultant B-cell progeny therefore have slightly different antigen specificity from the original. A selection process then occurs within the germinal centre, which identifies those offspring which no longer bind the target antigen and causes them to apoptose ("affinity maturation"). Those that remain are specific to the stimulating antigen, but not necessarily identical to the original naïve

B-lymphocyte, thus widening the humoral response to a stimulus. This mechanism ensures a variety of antibodies are produced against a single antigen. The result is a heterogeneous, polyclonal serum antibody response. The B-lymphocytes which are produced by this process may then undergo isotype switching, however this process, and the B-cell progeny's survival, is dependent on interaction with the CD4+ T_H-lymphocytes within the germinal centre. The process of isotype switching and differentiation into plasmablasts and memory B-cells is not unique to the germinal centre (as mentioned above) however the effectiveness of the process is much higher in germinal centres compared with elsewhere – See section 1.3.3.

Isotype switching changes the functional Fc region of the antibody, without altering the antigen specificity. This process is dependent on CD40-CD40L interaction (i.e. direct cell-to-cell contact between activated T_H and B lymphocytes) and which sub-type of immunoglobulin is produced is dictated by the cytokines provided by the CD4+ T_H Lymphocytes. The role of CD4+ follicular helper T-cells is expanded upon within Section 1.3.3.2.

1.2 T-Lymphocyte Memory

Following successful activation of a naïve lymphocyte, the population of antigen specific lymphocytes undergoes vast expansion. This expansion phase lasts approximately 14 days in humans (84) during which time an activated lymphocyte will generate 15 generations of decedents (85). Once the initial stimulus wains, the fall in circulating cytokines and absence of antigens results in a contraction phase, where the majority of the expanded population will undergo apoptosis (1). However a proportion of the population will avoid apoptosis, differentiating into memory cells (1, 85).

It is unclear whether memory cell populations are derived from clonally expanded naïve lymphocytes or from effector cells which have encountered antigen (1) (see Sections 1.2.2.1.1 and 1.2.2.2.1). The generation of memory cells following initial ("primary") exposure to an antigen ensures a more rapid response on re-encountering the same antigen; a so called "secondary response"(1). Like naïve lymphocytes, memory cells persist in the G_0 stage of the cell cycle, however exposure to their specific antigen will induce colonial proliferation and production of effector cells without the barriers which are in place for naïve cells to do so (1). Various mechanisms exist which contribute to this lower threshold, however CD45 isoform switch is used in the experimental determination of memory sub-populations (84).

1.2.1 Conditions required for memory cell generation

Memory cell differentiation appears to be influenced by different factors depending on the type of lymphocyte. In CD8+ lymphocytes, generation of CTL and CD8+ memory cells seems independent of the length of time naïve cells are exposed to the antigen (85-88), i.e. memory

and effector cells are produced by both short and long exposure to antigen, however the magnitude of the effector/memory cell proliferation is dependent on duration of exposure (86, 87) and continued antigen presentation via dendritic cells and CD28 co-stimulation (87). Furthermore, repeat antigen exposure at a future point generates a secondary memory response, the nature of which is unaffected by whether the initial antigen exposure was short-lived or prolonged (86). However if antigen exposure is curtailed to less than 24-48 hours, differentiation of memory cells is impaired (89). The affinity of the TCR for a given antigen also influences the magnitude of cell proliferation following antigen-CD8+ cell interaction (90). Although the numbers of effector and memory cells induced by low-affinity antigen-TCR interactions are reduced, their function (the ability to generate memory cells, their properties, and the ability for memory cells to generate a secondary response) remains similar to cells produced from high-affinity interactions (90).

The response of CD4+ lymphocytes to antigen differs from that of CD8+ lymphocytes. The magnitude of the clonal proliferation and the differentiation of effector and memory sub-sets require persistent antigen exposure (91). Low dose antigen stimulus results in delayed and slower cell division, as well as a reduced recruitment of naïve cells (91). In addition, a maximal CD4+ expansion and effector response is dependent on continued antigen exposure (89, 91), and the number of CD4+ memory cells generated is reduced when exposure to antigen is transient (<48 hours) (89). Transient exposure to antigen also influences the CD4+ memory response, with reduced numbers of responsive lymphocytes following repeated antigen challenge (89). Such a difference is most marked within non-lymphoid organs, suggesting that although central memory cells are generated following transient antigen exposure, a full repertoire of memory cells with the ability to translocate to secondary sites fails to develop (89). The negative effect of transient antigen exposure on the generation of effector and memory cell populations can, however, be mitigated by a greater amplitude of antigen exposure (89), i.e. a greater amplitude of antigen stimulus which is transient (<24 hours) can generate effector and memory CD4+ cell populations equivalent to the same large stimulus experienced over 72 hours (89).

1.2.2 Memory cell sub-populations

A period of population contraction occurs following a primary antigen response (“Contraction Phase”), leaving a residual population of memory cells. These cells share common characteristics: they are predominantly quiescent, capable of intermittent self-renewal and survive in the absence of sustained exposure to their target antigen (92). However memory cells are not a homogenous population, consisting of sub-populations delineated by their cells of origin, but also by functional differentiation. Two broad classifications of memory cells exist

(93-95) which are characterised by their distinct tissue-homing tendencies as well as their response to repeat antigen challenge; Central Memory T-Cells (T_{CM}) express CCR7, mediating their preferential migration to lymphoid tissues (93). T_{CM} cells respond to antigen challenge by production of IL-2 and extensive proliferation; their progeny acquire effector function at a later time point, therefore T_{CM} cells reside in secondary lymphoid tissues and need to proliferate, differentiate and migrate prior to exacting an effector response (92). Although T_{CM} cells share their migratory sites with naïve T-cells, expression of additional receptors (e.g. CCR4, CCR6 and CXCR3) augment their response to cytokines and enhance their interaction with APCs (93). In contrast, Effector Memory T-cells (T_{EM}) do not express CCR7. Instead T_{EM} cells express a higher level of β-integrins as well as other tissue specific receptor molecules such as CCR5 which direct their migration to sites of inflammation (93, 96). The function of T_{EM} cells is distinct from T_{CM} cells as proliferation on exposure to antigen is less marked (97) and production of effector cytokines (IFNγ, IL-4, IL-5) and other effector molecules, such as perforins in cells of CD8+ linage, is more immediate (94). T_{EM} are therefore proposed to provide the immediate response to antigen at the site of injury/inflammation, whereas T_{CM} cells concern themselves with preparing the sustained response against insult (96). The origin of each sub-set is debated and differences exist between separate T-cell lineages.

1.2.2.1 CD4+ Memory T-Cells

The genesis of CD4+ memory T-cell sub-populations is a subject of debate with at least two theories proposed, but conflicting evidence supporting each. Unanswered questions include whether T_{EM} cells derive only from T_{CM} cells, whether effector cells give rise to T_{EM} and T_{CM} or whether T_{EM} and T_{CM} cells derive from different progeny determined at the time of antigen presentation, with or without direction from APCs. Such questions are further complicated by the different functional sub-populations of CD4+ cells, including T_H1, T_H2, T_H17, iT_{REG} and T_{FH} subtypes.

1.2.2.1.1 CD4+ Memory T-Cells: Generation

The fact that the homing strategies and receptor profile expressed by different CD4+ memory cell sub-populations reflects those displayed by the spectrum of effector CD4+ cells produced following antigenic stimulation bore the theory that memory sub-sets derived from the selective survival of effector cells arrested at various points throughout their differentiation (98-100) through a process of asymmetric division (101).

The division rate of sub-populations of human CD4+ cells was determined through in vivo labelling with deuterated glucose (102). The division rate of T_{EM} cells was measured to be 4.7% of the population each day, far exceeding that of T_{CM} (1.5%) and non-stimulated naïve CD4+

cells (0.2%) (102). The rate of proliferation of T_{EM} cells is suggestive of a means to replenish stocks of cells from a central reserve. The most popular theory is that differentiation from T_{CM} cells maintains the population of T_{EM} cells in the absence of on-going antigen stimulation. In this fashion T_{CM} cells act as memory-stem cells capable of differentiating into any "down-stream" effector memory cell (103). In-vitro stimulation of human CD4+ T_{CM} cells using IL-7, IL-15, TNFα, IL-6 and IL-10 resulted in a proportion of such cells down-regulating their CCR7 expression, and increasing their expression of CCR5, IFNγ and IL-4; i.e. these cells achieved a T_{EM} phenotype (104). This process was TCR-independent (104) but was attenuated in the absence of DC-derived cytokines; this hints at a role for T_{CM}-DC interaction in the maintenance of CD4+ T_{EM} populations.

The theory of CD4+ T_{EM} population replenishment by upstream CD4+ T_{CM} cells led to attempts to identify cytokine signals necessary to encourage differentiation into individual subtypes. Surprisingly, sub-divisions of CD4+ T_{CM} cells identified appeared to have pre-determined preference to differentiate into either T_H1- or T_H2-type progeny following exposure to homeostatic signals (IL-7 and IL-15) in the absence of additional polarising cytokines (48). Furthermore, which T_H subset a T_{CM} cell would differentiate into was predictable based on the expression of CXCR3 and CCR4, leading to these cells being termed pre-T_{H1CM} and pre-T_{H2CM} cells respectively (48). In addition, a third sub-set of cells with no pre-determined lineage preference was identifiable by the absence of both CXCR3 and CCR4 and the expression of CXCR5; CXCR3- CCR4- CXCR5+ CD4+ T_{CM} cells failed to differentiate under the influence of homeostatic cytokines in isolation (48). However, pre-T_{H1CM}, pre-T_{H2CM} and CXCR5+ T_{CM} cells are all capable of differentiation into either T_H1 or T_H2 cells following stimulation by polarising cytokines (49). These findings not only support the idea of T_{EM} cell replenishment from upstream T_{CM} cells acting as T_{EM} precursors, but also support memory cell generation as a product of cell-arrest throughout the differentiation of effector cells.

Further evidence for the latter can be derived by looking at the spectrum of memory sub-sets detectable following different antigen stimuli. Tetanus toxoid specific memory cells are detectable across the range of memory sub-populations (T_{CM}, T_{H1CM}, T_{H2CM} and T_{EM}) whereas CMV infection results in T_{CM}, T_{H1CM} and T_{EM} only (48). Similarly, influenza has been demonstrated to generate a memory cell population profile reflective of the effector response, with sub-types which do not contribute to the primary response remaining unrepresented by memory-cell equivalents (100). This would imply that memory cells are derived from effector populations which acquire memory characteristics.

An alternative view is forwarded by Professor Marc Jenkins, University of Minnesota. Tracking CD4+ cells using a tetramer-based method, this group found that the ratio of T_{H1EM} and T_{CM} cells specific to a single antigen was 50:50 throughout the contraction phase and then maintained at this ratio for up to a year following initial antigen challenge (105). This led to the conclusion that T_{EM} cells may not derive from T_{CM} cells, and that instead the two populations are metastable and non-convertible (92). In addition, the absence of B-cells has been demonstrated to limit the formation of CD4+ memory cells, the mechanism for which is demonstrably not a consequence of absence of secreted antibody (106). CD4+ T_{CM} are particularly affected by the absence of B-cells, with CD4+ T_{H1EM} cell development being maintained (92); again, if T_{EM} cell population was dependant on derivation from a T_{CM} pool, it would be expected that any factor which negatively effects T_{CM} differentiation would have a consequent effect on the T_{H1EM} cell population "downstream". A possible model for memory cell development suggests T_{CM} cell genesis is dependent of B-cell-$T_{CM\text{-}precursor}$ interaction, possibly mediated by ICOS (CD28 family member) (92), drawing interesting parallels with T_{FH} generation.

Such a theory is supported by observations of the dynamics of antigen presentation and the consequent memory cell progeny. Strong antigen stimulation preferentially induces IFNγ-producing CD4+ T_{EM} formation (107) whereas T_{CM} generation is more prominent in the later stages of response, when antigen clearance results in a weaker stimulation (108). Strong induction by activated APCs induces expression of tissue homing receptors and loss of CCR7 (89, 109). In the absence of such stimulation, CCR7 expression persists and cells remain within secondary lymphoid tissue, interact with B-lymphocytes and differentiate into T_{CM} cells. T_{FH} cells differentiate in such a fashion, leading to speculation that CXCR5+ T_{CM} cells may represent T_{FH} cells which have acquired a memory phenotype, the alternative theory being that in a sub-population of T_{CM} cells CXCR5 is induced, allowing a more rapid re-population of a germinal centre during the secondary B-cell response to antigen (92). This sub-set of T_{CM} cells expressing CXCR5 are implicated in facilitating secondary humoral response (110, 111) – see Section 1.3.4.

1.2.2.1.2 CD4+ Memory T-Cells: Selection and survival

What determines whether a cell becomes a memory cell? It is widely believed that selection of cells destined to become memory cells is dependent on induction of anti-apoptotic molecules (e.g. Bcl-2, Bcl-x_L) (112, 113) and augmentation of their response to homeostatic cytokines (IL-7 (114) and IL-15 (115-117)) (118). Cells which acquire these properties are selected out of the population following antigen clearance as they are the only cells with the capacity to survive (118). T-cell stimulation/activation is mediated by dendritic cells and other antigen presenting cells in vivo. Therefore the nature of the T-cell-DC interaction can be assumed to influence the

destiny of T-cells (95). Dendritic cells within a resting state continually process antigen yet fail to become activated (119). So called "immature" dendritic cells (iDC) have been demonstrated to be "tolerogenic"- i.e. they induce immune tolerance towards the antigen they present through deletion of antigen specific T-cells and induction of T_{REG} cells (119, 120). Dendritic cells become activated following exposure to lipopolysaccharides (121), inflammatory cytokines (e.g. TNFα, IL-3, IL-4, among others) (122-124) and/or CD40-CD40L interaction (125-127). The function of dendritic cells has been demonstrated to evolve over the 48 hours following their activation (128); a dendritic cell's immediate response to activation is polarising cytokine production and during this period they are effective inducers of an effector response (128). The capacity for cytokine production is diminished within 24-48 hours (128) leading to these cells being termed "exhausted DCs". These exhausted cells still maintained co-stimulatory receptor expression and were found to be more effective at inducing a T_{CM} phenotype (128), together with low antigen dose (108, 128) and transient DC-T-cell interaction (128, 129). Therefore iDC, mature DC and exhausted DC interactions with naive T-cells does influence their differentiation into memory subsets. Interaction with dendritic cells of differing activation states has been demonstrated to effect cytokine receptor and BCL expression in vitro (118) in a fashion consistent with such a hypothesis.

Human CD4+ antigen specific memory cell populations decline following the contraction phase in the absence of further exposure to their specific antigen, with a half-life of approximately 10 years (130, 131). This is in contrast with the population dynamics observed in human memory B-cells and CD8+ T cells, where the number of antigen-specific memory cells is maintained in the absence of further exposure (130, 132) – See section 1.2.2.2. Homeostatic cytokine signalling is important in the maintenance of CD4+ memory cell populations; blockade of IL-7 receptor has been demonstrated to result in population decline in in vivo murine models (133). Similarly, members of the TNF receptor family such as CD27 have been implicated in driving homeostatic proliferation: $CD27^{-ve}$ CD4+ cells demonstrate reduced survival and lower rates of proliferation (105) whereas OX40 signalling has been shown to induce expression of the anti-apoptotic molecules BCL-2 and BCL_{XL} through TRAF-dependant induction of NF-κB (134) with OX40 knock out cells demonstrating adequate primary response to antigen but failure of longevity (135, 136). Supportive OX40-OX40L signalling is thought to be provided by accessory cells with high constitutive expression of OX40 and CD30 within secondary lymphoid tissue (136, 137).

In addition to cytokine and receptor-ligand derived homeostatic signalling, CD4+ memory T-cell survival is also dependant on T-cell receptor signalling. TCR knock-out results in decline in

some sub-populations of CD4+ memory cells in mice, however this effect was not demonstrated population wide (138). Downstream signalling interruption (SLP-76 knockdown) mirrored these findings suggesting a role for TCR-mediated signalling in the maintenance of cell turn over in response to homeostatic cytokines (139). Interruption of TCR signalling results in a reduced rate of homeostatic turn over (139, 140), reduced responsiveness to IL-7 (139) and impaired memory cell persistence (139). Homeostatic signalling in CD4 cells is therefore considered TCR dependant and although populations of CD4 cells can be maintained in absence of TCR signalling (140, 141), the quality of response is considered to be reduced (141). Whether TCR signalling is antigen dependant is unclear; weak transient non-specific binding to MHC-antigen complexes may be sufficient to generate the homeostatic signals necessary for population maintenance in a fashion analogous to the non-antigen specific B-cell receptor interactions necessary for memory B-cell survival (142). Such an arrangement seems likely given the persistence of CD4+ memory T-cells specific to smallpox for up to 60 years following immunisation (130).

1.2.2.1.3 CD4+ Memory T-Cells: In the presence of persistent antigen

The generation of a sub-population of CD4+ cells with a memory phenotype is impaired when the antigen stimulus persists i.e. when antigen clearance fails. From a mechanistic view-point, it would be logical that the body would not wish to "remember" a response which failed to clear a stimulus. Furthermore, the observation that T_{CM} cells are preferentially generated once antigen stimulus is on the wane (see Section 1.2.2.1.1) and effector phenotype generation requires persistent antigen stimulus (91) (see section 1.2.1) hints at reasons for persistent antigen failing to induce memory cells. Chronic infection in humans is associated with predominance of CD4+ effector cells which fail to express markers associated with T_{CM} phenotype (143-145). Amyes *et al.* analysed antigen specificity of CD4+ subsets in patients with chronic EBV and CMV infection. They noted increased CD45RA expression in CD4+ cells specific to EBV epitopes which are persistently expressed compared with "latent" antigens (145). Harari *et al.* used a variety of infectious processes to model different modes of antigen presentation; they noted the cytokine profile of antigen specific CD4+ memory cell sub-populations was predominantly CCR7+ IL-2 producing (T_{CM} phenotype) following transient (cleared) antigen exposure, whereas strong persistent antigen exposure was associated with CCR7- IFNγ producing (T_{EM}) cells (144). Persistent low-level antigen exposure gave rise to CCR7-CD45RA+ cells in an advanced state of maturation as well as a paucity of T_{CM} cells (143); the inference is that persistence of antigen necessitates a greater commitment of cells into effector differentiation at the expense of the T_{CM} subset. The significance of CD4+ CCR7-

CD45RA+ cells is unclear and it is thought they represent terminally differentiated effector T-cells (94, 146).

1.2.2.2 CD8+ Memory T-Cells

1.2.2.2.1 CD8+ Memory T-Cells: Generation

The lineage of CD8+ T-lymphocytes is unclear; controversy exists regarding whether sub-populations of T_{CM} and T_{EM} derive from one another or arise independently. Furthermore whether differentiation between the two sub-sets occurs uni-directionally, bi-directionally or not at all has been debated.

Some clonal preservation between the two sub-sets was noted in humans, however such observations were limited by the limited sampling of blood possible in human volunteers and the inaccessible nature of lymphoid tissue compartments (147). Using murine models, the same group identified preservation of two-thirds of TCR clones within both T_{CM} and T_{EM} subsets confirming that both divisions must arise from common precursor cells (97). Furthermore, clones present in both sub-populations were more numerous (97), suggesting the ability to establish both memory sub-populations is linked to proliferative capacity, maybe as a consequence of affinity to antigen. In addition, those clones which were unique to the T_{EM} subset were less persistent over time, suggesting lasting memory is dependent on T_{CM} differentiation (97). Conversely, clones unique to T_{CM} sub-population were markedly less capable of generating effector cells following re-stimulation, the mechanism behind which remains unclear (97).

Preservation of TCR clones suggests common ancestry countering the idea that the destination of a naïve cell is determined at its point of activation. Mechanistically, clonotypic preservation suggests either unequal stimulation of sister cells (101), progressive differentiation (148) or reversion (149); CD8+ T_{CM} cells dividing in the absence of antigen and under the influence of IL-7 and IL-15 can spontaneously lose CCR7 expression and gain tissue homing receptors such as CCR4 (103, 148, 150), i.e. they acquire an T_{EM} phenotype. As in CD4+ equivalents, such differentiation was augmented by dendritic cells and dendritic cell-derived cytokines (103, 109, 148). Furthermore, a CCR4-positive sub-population of CD8+ T_{CM} cells are pre-programmed to preferentially differentiate into cytotoxic cells (identified by down-regulation of CCR7 and raised expression of perforin) under homeostatic stimulation with IL-7 and IL-15 (148). Therefore is seems that sub-populations of CD8+ T_{CM} with pre-selected effector function exist, in a fashion analogous to the presence of CD4+ T_{H1CM} and T_{H2CM} cells.

Whether reversion from an effector memory phenotype to a less differentiated T_{CM} form occurs is controversial. The demonstration in mice that the quality of recall, survival in the absence of antigen and ability to proliferate under homeostatic stimulation of CD8+ memory T-cells was impaired following failure of antigen clearance led to the hypothesis that memory T-cells derived from effector cells (151). However although reversion of T_{EM} cells to T_{CM}-like (CD62 expressing) cells has been demonstrated in other experimental systems, the resultant cells fail acquire the full proliferative capacity of normal T_{CM} cells (97). Further evidence for unidirectional differentiation is available, with T_{CM} cells readily differentiating to effector subsets without evidence of reciprocal differentiation of T_{EM} or effector T-cells under similar conditions (152). The demonstration that memory recall and survival fail to develop in the presence of antigen persistence does, however, support the notion that antigen clearance is critical in the development of a memory phenotype.

As in CD4+ T-cells, memory cell phenotype is characterised by responsiveness to homeostatic cytokines (153) and resistance to apoptosis (154), as well as the ability to mount a recall response. Significant heterogeneity exists between sub-populations of long-lived CD8+ T-cells; the proliferative capacity in response to antigen is reduced in T_{EM} and CCR7-CD45RA+ T_{EMRA} cells (a population of terminally differentiated effector memory cells) (93, 148). Proliferation in response to IL-7 is low in all subsets (148). Proliferation following IL-15 exposure is intermediate in T_{CM} cells but high in T_{EM} and T_{EMRA} cells, which correlates with each sub-populations expression of IL-15R (148). Conversely, expression of anti-apoptotic markers are reduced in the more differentiated cells, with T_{EM} and T_{EMRA} cells being more prone to apoptosis (148). Cytokine stimulation induced differentiation of T_{CM} cells into either T_{EM} cells or T_{EMRA} cells expressing high levels of perforin; T_{EM} cells under similar conditions failed to differentiate (148), suggesting unidirectional differentiation and reduced capacity for phenotypic change under cytokine stimulation in the differentiated cell subtypes.

Receptor expression can therefore be used to characterise these cells; IL-7Rα expression predicts cells destined to differentiate into memory cells (155-157). Survival traits are acquired during antigen clearance (158) by an IL-7 dependant mechanism – possibly mediated through Bcl-2 (114) – however what induces IL7R expression is unknown. Both IL-7 and T_H-cells are dispensable during the immediate response to immunogenic pathogens, however CD8+ response to protein/cell associated antigen is dependent on CD4+ T_H-induction of dendritic cell maturation (95).

The degree to which generation of CD8+ memory cells is influenced by the availability of CD4+ cell help is uncertain (95, 156, 159). One way of modelling CD8 response in the absence of

CD4+ help is through the use of MHC Class II knockout mice: these mice generate a normal primary response to infection however fail to generate a recall response to listeria infection (160); deleterious effect on secondary CD8+ cell response is not seen following depletion of CD4+ cell help at a time point delayed following primary exposure (160). The absence of CD40/CD40L mimics MHC Class II deletion, however whether CD40/CD40L CD4+ activation of DCs or direct CD40/CD40L interactions between CD4+/CD8+ cells occurs is uncertain (156). Absence of CD4+ T_H-mediated help does not impede the expression of IL-7R immediately post-infection, however the persistence of IL-7R is reduced (161). Transplantation of CD8+ T-cells activated within a MHC Class II deficient host into a MHC Class II replete recipient eight days following infection results in a normal recall response to secondary challenge (161); conversely, transfer from a MHC-class II replete primary host to a deficient recipient results in functional impairment on repeated antigen challenge (161). This implies critical interaction with CD4+ T_H-cells to generate CD8+ memory subsets occurs following the contraction phase, as opposed to the priming or expansion phase. However such results must be interpreted with caution, since the infection-model as well as the mouse model itself has been demonstrated to influence the pattern of both the primary and recall responses of CD8+ cells (162).

Illustrating this point, and in contrast to findings described above, other groups have demonstrated altered functional properties of CD8+ memory subsets generated in the absence of T_H-cells (163). Utilising mice depleted of CD4+ cells following intra-peritoneal instillation of an ablative antibody, the balance of apoptotic mediators within expanded CD8+ populations was markedly shifted towards pro-apoptosis in the absence of CD4+ mediated help at the point of antigen priming (163). In the absence of help, CD8+ memory cells were able to mount a recall cell-specific cytotoxic response, however the ability to clonally expand was reduced (163). The inability to clonally expand during recall response was attributed to a TNF-related apoptosis inducing ligand (TRAIL) dependent restriction on cell proliferation, the inference being that CD4+ T_H-cell help works to lift restrictions on cellular proliferation during the priming of progenitor CD8+ memory cells (163).

Consequently, in the presence of conflicting evidence, the exact role of CD4+ T_H-cells in the generation of CD8+ memory remains controversial. It is thought that the role of CD4+ cells varies dependent on the nature of the pathological process (162) with some conditions being CD4+ dependant (156, 159, 164-166), and others being CD4+ independent (162). What factors determine the need for CD4+ help, and the nature of the help required, is ill-defined.

1.2.2.2.2 CD8+ Memory T-Cells: Selection and survival

Resting memory CD8+ T-cells are dependent on IL-7 (114, 117, 167, 168) mediated survival signalling via the up-regulation of anti-apoptotic molecules such as Bcl-2 (114, 169), and IL-15 mediated proliferation (115, 117, 168). Because of the influence cytokine-mediated signalling has over CD8+ memory cells, these populations are able to survive and divide under cytokine stimulation in the absence of TCR- mediated signalling (115, 117, 138, 167, 168, 170-172). This contrasts with CD8+ naive cells, the maintenance of which depends on non-specific, short-lived self-MHC-TCR interaction (173, 174).

CD8+ memory cell populations demonstrate more stable dynamics than their CD4+ counterparts (130, 132). CD8+ cells would seem to have a lower threshold of stimulation in response to homeostatic cytokines; numbers of self-specific CD8+ cells which spontaneously develop memory cell phenotype during normal homeostasis exceeds incidence of similar CD4+ cells (175), consistent with the notion that CD4+ cells are the “gate-keepers” of adaptive immunity, with tighter regulation of their generation and survival than CD8+ or B-cells. The mechanism for greater homeostatic stability of memory CD8 cells is thought to be a consequence of greater efficiency of activation of STAT5, a signal transducer common to both IL-7R and IL-15R, secondary to higher expression of IL-15 receptor (176). Dependence on the STAT5 signalling pathway is not demonstrable for CD4+ memory cells (176), a finding consistent with the reduced importance of IL-15R mediated homeostasis in maintaining CD4+ memory cells (see Section 1.2.2.1.2).

The underlying mechanisms influencing survival and resistance to apoptosis within cells selected to acquire a memory phenotype are varied and thought to involve regulators of apoptosis, metabolism and transcription. As alluded to previously, regulation of the apoptotic balance is thought to be integral, with pro-homeostatic receptor expression being a hallmark of memory cells. Memory cells exhibit a higher level of expression of BCL-2 (114, 154), conferring resistance to down-stream mediators of apoptosis (154), resulting in selective survival during contraction phase (177). Induction of Bcl-2 and other anti-apoptotic molecules is attributed to the JAK3/STAT5 pathway through IL-7 and IL-15 signalling (176-178).

Metabolic switch from “catabolic” oxidative phosphorylation to “anabolic” glycolytic ATP production is a feature of effector cell clonal expansion (aka aerobic glycolysis) (179, 180) and this switch is mediated by cytokines such as IL-2 (181) and co-stimulation via CD28 (182). Pearce *et al.* demonstrated that TNF-associated receptor factor 6 (TRAF6) knockout in mice resulted in normal CD8+ effector response but failed to generate long-lived memory cell subpopulations (183). Deficiencies in the fatty-acid metabolism were implicated; TRAF6

deficient CD8+ cells were less able to switch back to oxidative metabolism following the withdrawal of IL2 support (183). AMP-activated kinase acts as a metabolic switch, inhibiting glycolysis in the presence of high quantities of AMP and encouraging a return to oxidative phosphorylation. TRAF6 deficiency resulted in reduced levels of AMP-activated kinase and, consequently, impaired ability to switch (183). Furthermore, activation of AMP-activated kinase using metformin or inhibition of mTOR, a downstream regulator of the same pathway, using rapamycin successfully augmented metabolic switch within expanded T-cells (183). In vivo administration of these drugs during the expansion phase restored the acquisition of memory cells in TRAF6 knockout mice and even increased the recall response in wild-type mice (183, 184). Rapamycin increases the proportion of antigen-specific CD8+ cells expressing memory phenotypic markers such as IL-7R, CD62 and Bcl2 (184). This held true irrespective of whether rapamycin was given only at the point of antigen exposure or withheld until the contraction phase, suggesting a dual role in the acquisition of a pre-memory cell phenotype and in effector-to-memory switch; these findings were not a result of increased cell proliferation, suggesting true augmentation of phenotype acquisition (184). Blockade of mTOR therefore improved the quality of the recall responses, implying that the ability to switch metabolic pathways is crucial for a cell to acquire memory cell survival traits. Factors influencing mTOR activity in vivo are incompletely characterised; IL-12 has been demonstrated to promote mTOR activity through phosphorylation, suggesting a possible mechanism through which IL-12 mediates effector differentiation in naive CD8+ cells (185). Persistent stimulation of CD8+ cells following failed antigen clearance would therefore enhance mTOR activation, impairing memory phenotype differentiation (see Section 1.2.2.2.3).

The wider influence of the mTOR pathway and its influence on sub-type switch of CD4+ cells, and B-cells, is the subject of on-going contemporaneous research (186). The inference being that the understanding of the metabolic processes implicit in the establishment of memory cell populations and the derivation of sub-population could be critically important in the design of immunotherapy. One downstream mediator of the mTOR pathway is the t-box transcription factor T-bet; T-bet has been demonstrated to promote effector differentiation at the expense of developing CD8+ T_{CM} cells (187) and is induced by pro-effector cytokines such as IL-12 (188). mTOR inhibition with rapamycin decreases expression of T-bet, increases eomesodermin and augments memory differentiation (185). T-bet has been shown to be increased in CD8+ cells activated in the absence of CD4+ help (187) which alludes to a possible mechanism underlying the reduced memory cell differentiation in the absence of T_H-cells (see Figure 1)

T-bet has been demonstrated to induce BLIMP-1 (B-lymphocyte-induced maturation protein 1) expression in mice (189); otherwise known as PR domain zinc finger protein 1 (PRDM1) in humans, BLIMP-1 represses β-interferon gene expression and is known to be critical in effector cell maturation in B lymphocytes (190, 191) and differentiation of effector CD4+ subsets (15). BLIMP-1 knockout results in a reduction in effector function (192) and augmentation of switch to memory phenotype in CD8+ cells (193). BLIMP-1 limits memory cell differentiation via repression of DNA-binding inhibitor Id3; Id proteins (a.k.a. “Inhibitor of DNA binding proteins”) are a family of proteins which heterodimerise with basic Helix-Loop-Helix (bHLH) transcription factors and inhibit their DNA binding (194). The universal importance of BLIMP-1 in determining lymphocyte differentiation into memory and effector subtypes is gradually coming to light (24, 195).

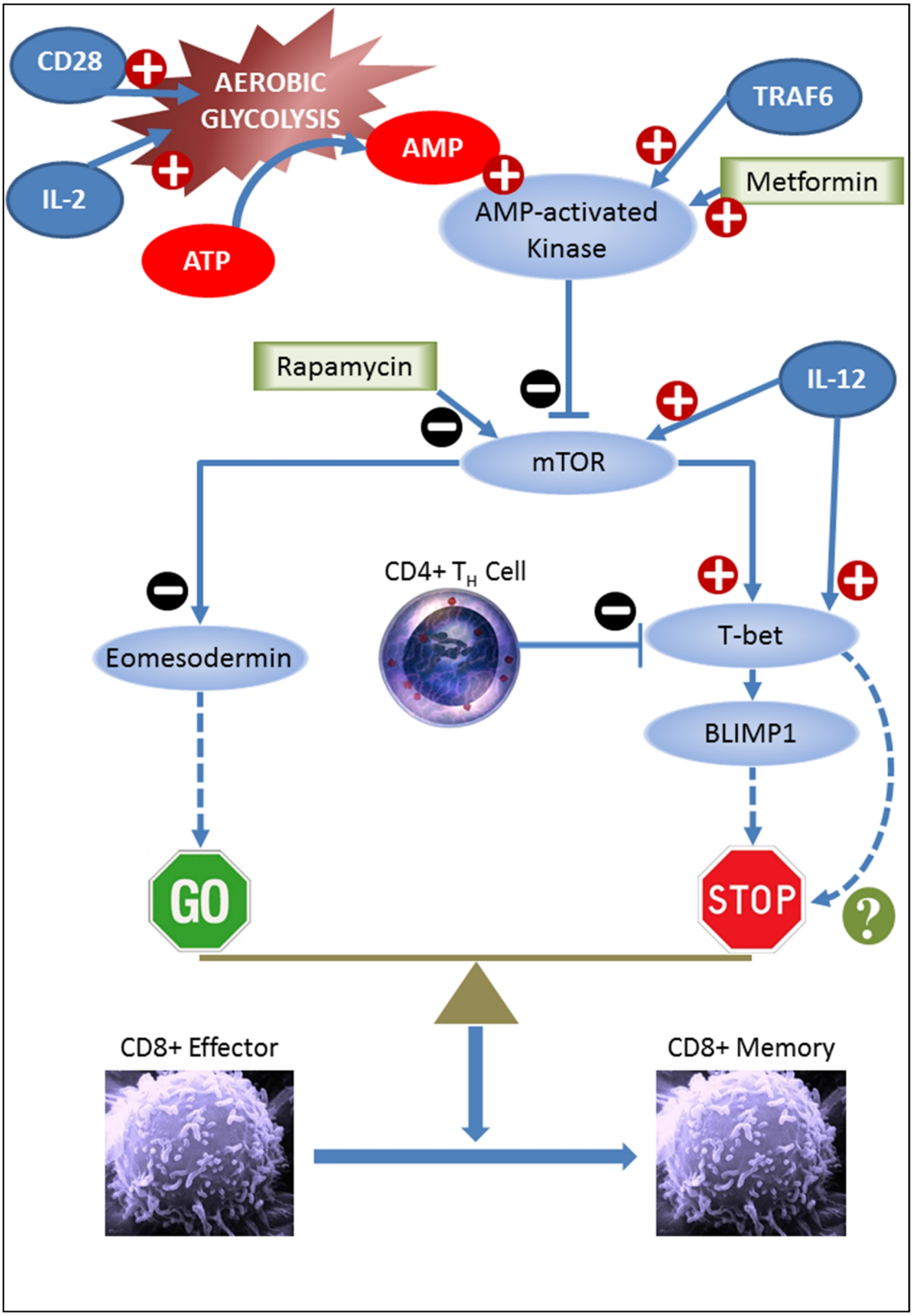


Figure 1: The effector/memory switch in CD8+ lymphocytes. Several pathways converge on BLIMP-1 to promote terminal differentiation of CD8+ T cells into effector cells at the expense of memory cell development. Metformin and rapamycin act at different points to counteract this effect. CD4+ T_H Cells promote memory phenotype switch via an incompletely understood mechanism which is reliant on the reduced presence of T-bet.

1.2.2.2.3 CD8+ Memory T-Cells: In the presence of persistent antigen

The persistence of antigen alters the resulting memory cell population; maturation of CD8+ memory cells in the presence of persistent antigen results in those cells failing to acquire the ability to persist in the absence of antigenic stimulation (151). The underlying reason for this appears to be that these cells are deficient in their response to homeostatic cytokines IL-7 and IL-15 as they fail to replicate once the chronic antigenic stimulus is removed (151). Chronic antigen stimulus results in the development of memory cells with low expression of IL-7R, IL-15R and their downstream signalling mediators STAT5 and Bcl2 (151). Antigen activation of the T-cell receptor results in IL-7R down regulation (196); during transient antigen exposure this is overcome by the subpopulation of cells destined to become memory cells which re-instate IL-7R expression, however in the presence of persistent antigen stimuli IL-7R down-regulation is exacerbated (196). In humans, chronicity of antigen exposure is associated with CD8+ terminal differentiation to a T_{EMRA} and short-lived effector phenotype and a paucity of long-lived T_{CM} cells (197).

In the presence of persisting antigen, CD8+ antigen specific T-cells eventually enter an altered state termed “exhaustion” (198). Most studies evaluating T cell exhaustion have used chronic viral infection as a model; exhaustion is characterised by gradual degrading of the functional potential of CD8+ T-cells (199), with loss of IL-2 production, proliferative capacity, homeostatic responsiveness and cytotoxicity occurring prior to the eventual inability to degranulate or produce TNFα, IFNγ and beta chemokines (198, 199). The eventual endpoint is an antigen specific cell which is functionally deplete, which may even undergo selective deletion (198). Development of exhausted phenotype is therefore associated with a resurgence of the antigen following loss of suppressive control (198).

Another feature of T-cell exhaustion is the induced expression of inhibitory molecules such as PD-1 (Programmed Death-1) (200) and LAG-3 (Lymphocyte Activation Gene-3) as well as other inhibitory pathways (201, 202). CTLA-4 has been demonstrated to be an important marker in CD4+ cells (203) however its relevance in CD8+ cells remains unproven (200, 203). Blockade of these pathways can rescue an exhausted cell’s function (200, 204-206) leading to the possibility of therapeutic application (206, 207). Intriguingly, chronicity of antigen is associated with BLIMP-1 overexpression, with a positive correlation between BLIMP-1 expression and the accrual of functional impairments typical of T-cell exhaustion (208). Deletion of BLIMP-1 restores the acquisition of memory cells and impairs expression of exhaustion markers (208) suggesting a role for activation of the BLIMP-1 pathway through chronic antigen stimulation in the induction of CD8+ T-cell exhaustion. However, since BLIMP-1 has a role in promoting

acquisition of an effector phenotype (192, 193), complete deletion had a negative impact on long-term viral control (208).

1.3 B-T cell interaction and Follicular T-Helper cells

1.3.1 Follicular T-Helper cell origins

It remains contentious whether T_{FH} cells are a separate subset or a phenotypic state of effector cells within the other subsets (13, 14). Cytokine profile of T_{FH} cells are often polarised in a fashion analogous to other subsets and this polarisation is determined by the nature of the instigating stimulus (64, 68, 209, 210). Similarly, iT_{REG} cells seem polarised in their specialisation in regulating either T_H1, T_H2 or T_H17 effector subsets (211-213) with the same transcription factors being important in both subset and corresponding iT_{REG} sub-phenotype (13). The parallels between effector subsets (T_H1, T_H2 and T_H17) and accompanying iT_{REG}/T_{FH} in terms of transcription factor and cytokine production suggest common upstream priming and signalling events.

As alluded to in section 1.1.5.1.1, T_{FH} cell differentiation under normal conditions is critically dependent on initial T-DC interaction followed by B-cell interaction; initial T-DC interaction requires strong TCR signalling resulting in inhibition of IL-2/STAT5 mediated induction of BLIMP-1 (214-217), thereby allowing Bcl-6 expression (15) in a fashion comparable to that delineated in T_H1/T_H2 fate determination (50). This Bcl-6 induction is ICOS dependent (61) and augmented by IL-6/STAT3 (paracrine) and IL-21/STAT3 (autocrine) signalling, which redundantly promote T_{FH} subtype differentiation (14, 60, 218).

Bcl-6 expression and subsequent CXCR5-mediated migration to the T-B boundary region allows B-T interaction; as previously mentioned, although the need for B-cell interaction can be dispensed with if antigen delivery can be sustained (53), under normal conditions the presence of B-cells is critical for T_{FH} development (59, 68). This interaction is dependent on ICOS-ICOSL signalling (62, 65, 67); initial localisation of pro-T_{FH} cells requires B-cell expression of ICOS-L at the T-B boarder (“capture”) which is not dependent of B-cell antigen specificity (219) whereas establishment of mature T_{FH} cells requires the assisting B-cell to share antigen specificity (15).

Bcl-6 is widely accepted as the master-regulator transcription factor for T_{FH} cells (14-19). Bcl-6 is a transcriptional repressor which has long been established as a critical regulator within germinal centre B-cells (22-24); it has a reciprocal and antagonistic relationship with Blimp-1 (15) and its constitutive expression has been demonstrated to inhibit differentiation into alternative CD4+ subsets (16, 25). Blimp-1 plays a critical role in promotion of effector phenotype in CD4, CD8 and B-cell lineages (see above) (15, 190, 191, 193), antagonising

memory cell phenotype acquisition in CD8 cells (193) and promoting B-cell terminal differentiation into plasma cells (220, 221). This retained function of Blimp-1 across all lymphocyte lineages belies its broad range of transcriptional influence, including inductive regulation of exocytosis and secretive capacity (221). Therefore although T_{FH} cells may occasionally and variably be demonstrated to share cytokine and transcriptional characteristics of other CD4 subtypes, Bcl-6-mediated Blimp-1 suppression maintains the non-effector phenotype common to all T_{FH} cells (14).

Achaete-scute homologue 2 (Ascl2) transcription factor has recently been identified as an early transcriptional regulator inducing CXCR5 expression (20) explicating the observable CXCR5 expression on activated cells in the absence of Bcl-6 expression (222). CXCR5+Bcl-6- cells can be demonstrated to express Ascl2 at levels exceeding CXCR5-Bcl-6- cells (20), and Ascl2 inhibits non-T_{FH} cell related gene expression (20). Ascl2 deletion results in impaired T_{FH} development (20) the implication being that Ascl2 induction is the first step in cells achieving T_{FH} phenotype, encouraging follicular migration through CXCR5 up-regulation and CCR7 suppression prior to Bcl-6 expression.

Expression of other transcription factors also shape the establishment of the T_{FH} phenotype; Maf co-expression augments Bcl-6-induced CXCR5, CXCR4, PD-1 and ICOS expression and enables IL-21 expression (18). Interferon regulatory factor (IRF)4 is a ubiquitous transcriptional-regulatory co-factor which is essential for normal development of the full range of CD4+ subtypes (19). In T_{FH} cells deficient IRF4 results in defective cellular function and subsequent impairment of B-cell immunity through diminished STAT3 binding (19, 21, 223). STAT5 functions as a negative regulator of T_{FH} development through IL-2 driven STAT5-mediated promotion of Blimp-1 expression (19, 214, 215, 217).

1.3.2 Human markers for T_{FH} cells

Two major subsets of human T_{FH} cells have been proposed, as well as several sub-phenotypes, according to their differential expression of surface markers, their cytokine expression profile and their anatomical localisation (224). In addition some evidence suggests said subsets differ in their functional capabilities (225, 226) in terms of provision of B-cell help.

Phenotypic strategies used to delineate T_{FH} subtypes have previously relied upon differential CXCR5 expression; Bentebibel *et al.* defined CD4+ cells isolated from human tonsil as $CXCR5^{Hi}$, which express PD-1, ICOS, CD40L and Bcl-6 to a greater degree than $CXCR5^{LO}$ cells (226) and are capable of supporting Ig-class switching and antibody secretion by naïve, memory and GC-B-cell populations, whereas $CXCR5^{LO}$ CD4+ cells are only able to support naïve and memory B-cells in vitro (226), promoting predominantly IgM secretion following exaggerated production

of IL-21 and IL-10. The authors interpretation was that CD4+CXCR5^{Lo}ICOS^{Lo} cells represented a distinct subset of T_{FH} cells specialised to provide help to B-cells outside of follicles; this was based on Bcl-6 expression on RT-PCR equivalent to that of CD4+CXCR5^{Hi}ICOS^{Hi} cells, however CD4+CXCR5^{Lo}ICOS^{Lo} had low expression of Bcl-6 protein. As such this population does not bear the hallmarks of T_{FH} cells as defined by other authors and, in light of the perfuse cytokine response to stimulation (226), which is uncharacteristic of T_{FH} cells (14), might be better considered to be pre-T_{FH} cells, capable of being induced to provide support for B-cells in vitro but of uncertain significance in vivo.

Kroenke *et al.* identified CD45RO+CXCR5$^{int.}$Bcl-6+ cells as in possession of the phenotypic profile of T_{FH} cells, but with a lower expression of key markers (ICOS, PD-1, SAP, CXCL13) compared with CD45RO+CXCR5^{Hi}Bcl-6+ cells; these were defined as T_{FH} and GC-T_{FH} cells respectively (18). Both were capable of inducing IgM expression by naïve B-cells following in vitro co-culture (18). However CXCR5+ has been previously demonstrated to be widely up-regulated following in vivo induction of memory response (227), therefore whether said cells represent an activated phenotype relocating to B-cell follicles is undetermined. Furthermore CXCR5^{Hi}ICOS^{Hi} CD4+ cells have been demonstrated to be unique in their ability to induce IgG expression following B-cell co-culture independent of additional stimulation (57). Therefore the CXCR5^{Hi}ICOS^{Hi} phenotype seems fully capable of undertaking the T_{FH} role whereas CXCR5$^{Lo/int}$ICOS$^{Lo/int}$ CD4+ cells may represent proto- or pre-T_{FH} cells. Supportive of this supposition is data demonstrating conversion of CXCR5$^{Lo/int}$ICOS$^{Lo/int}$ CD4+ cells to a CXCR5^{Hi}ICOS^{Hi} phenotype in vitro following B-cell co-culture (226) and following viral induction of Bcl-6 (18).

PD-1 is a critical marker of T_{FH} cells which is directly up-regulated by Bcl-6 expression (18). The proposed role for PD-1 overexpression is the suppression of proliferation in T_{FH} cells in the presence of continuous TCR-stimulation during the germinal centre reaction (18). Blockade of PD-1/PD-L1/2 signalling results in over-expansion of T_{FH} cells (228) and reduction in long-lived plasma cell generation following GC-reactions (229). Rasheed *et al.* demonstrated different proliferative capacity between CXCR5^{Lo}ICOS$^{Lo/int}$ cells and CXCR5^{Hi}ICOS^{Hi} cells, with the latter far less likely to undergo division following stimulation (57). Although the study did not measure PD-1 expression within the two cell groups, subsequent studies have shown a strong link between CXCR5^{Hi}ICOS^{Hi} phenotype and concomitant high PD-1 expression (14, 18, 224).

CD57 has also been proposed as a marker for GC-T_{FH} cells; CD57 (aka B3GAT1, HNK-1, Leu7) is a marker found on subpopulations of NK and CD8 cells (230) and its induction has been attributed to maturation into a more cytotoxic phenotype in the former, and prolonged

antigen exposure, telomere shortening, reduced replication potential and senescence in the latter. In follicular CD4+ cells, expression is closely correlated with phenotype defined according to CXCR5 and ICOS expression, with expression absent on CXCR5-ICOS- cells, and a significantly higher incidence of CD57 within $CXCR5^{Hi}ICOS^{Hi}$ population compared with $CXCR5^{Lo}ICOS^{Lo/int}$ cells (57, 231). CD57-expressing cells have previously been demonstrated to exist exclusively within the germinal centre (225), however CD57 expression has not been demonstrated to distinguish functional subsets (57), therefore its significance remains undefined.

The presence of CXCR5+ CD4 cells within the peripheral blood in humans continues to be of debated significance; low incidence of such cells are identifiable in individuals with congenital deficiencies in GC formation (67) suggestive of a dependency on germinal centre reactivity for their existence and leading to speculation that such cells may represent circulating T_{FH} lineage cells. However circulating CXCR5+ cells share few attributes of lymphoid-tissue resident T_{FH} cells, with low expression of ICOS and PD-1 and high expression of CCR7 (232), and the absence of Bcl-6 expression (14). Furthermore, as already mentioned, exposure of memory CD4 cells to their antigen can induce CXCR5 expression in a wide range of newly-generated effector and memory subsets (222, 227). Therefore the presence of CXCR5 on circulating CD4 cells cannot be taken to denote history of germinal centre residency. That said, in vitro co-culture with B-cells following CD4+ activation does demonstrate increased propensity of circulating CXCR5+ cells to induce Ig secretion, plasmablast differentiation and class switching compared to CXCR5- cells (232), although this could also be an activation state phenomena and is not achievable without CD4+ activation, in contrast to the capabilities of tissue-derived CXCR5+ICOS+ CD4+ cells (55, 57). More recently, circulating $CCR7^{Lo}PD\text{-}1^{Hi}$CXCR5+ cells have been demonstrated within both humans and mice transiently following vaccination (233); the presence of these cells correlated with active parallel T_{FH} differentiation within lymphoid tissues and predicted vaccine response and severity of antibody mediated autoimmune disease (233). They expressed intermediate levels of T_{FH} phenotypic and cytokine markers despite being deficient in the key marker Bcl-6. Furthermore the generation of $CCR7^{Lo}PD\text{-}1^{Hi}$CXCR5+ cells was independent of SAP, suggesting said cells are generated and released outside of the germinal centre (233) and, together with their intermediate expression of T_{FH}-defining markers , this was taken to indicate that true, GC-experienced T_{FH} cells were not detectable within peripheral blood (233). However $CCR7^{Lo}PD\text{-}1^{Hi}$CXCR5+ cells were able to accelerate the GC-reaction upon antigen re-encounter suggesting that these cells function to re-circulate from the primary site of antigen encounter and patrol the lymphatic follicles, accelerating the B-cell response to antigen should it be re-encountered at a different site (233).

In summary, $CXCR5^{Hi}ICOS^{Hi}$ cells bear all the hallmarks of functionally active T_{FH} cells, with concomitant high Bcl-6 and PD-1 expression (termed GC-T_{FH} cells within the literature). $CXCR5^{Lo/int}ICOS^{Lo/int}$ cells also express lower levels of T_{FH}-associated markers and can be induced to reflect the full $CXCR5^{Hi}ICOS^{Hi}$ phenotype, however they are functionally deplete in vitro. They have been variably classed as T_{FH} cells and are thought to congregate in the T-B boarder; whether they provide biologically relevant extra-follicular help to B-cells is controversial, however they are most likely to represent a pre-T_{FH} state, with the potential for incorporation within the germinal centre reaction, given the necessary signals. CD57 defines a sub-phenotype of both subsets with germinal centre residency, however it is not expressed by the majority of germinal centre CD4+ cells and its relevance is unknown.

1.3.3 Follicular Helper T cells and their function in B- cell interaction

T-B interactions commence within 2-3 days of antigen exposure following co-localisation of activated cells of both lineages to the T-B boarder (51, 234). B-cell maturation and antibody production can subsequently occur either through extrafollicular proliferation, resulting in rapid release of low-affinity antibody with minimal evident class-switching (235), or through re-entry into B-cell follicle with their T_{FH}-counterpart to proliferate within germinal centre reaction, with implicit somatic hypermutation and affinity maturation producing high-affinity, class-switched immunoglobulin (236-238) as well as long-lived memory B-cells and plasma cells (239). Both of these pathways are influenced through T_{FH}-mediated help (14). The signals which determine whether B-cells follow an extrafollicular or germinal centre pathway are poorly understood, however fate decision is thought to be influenced by the Bcl-6-Blimp-1 axis (235), with Bcl-6-mutations common within haematological malignancies of germinal centre origin and expression within B-cells essential for germinal centre B cell development (22, 240). Conversely, elevated Blimp-1 is associated with plasma-cell differentiation and extrafollicular proliferation (190, 241). Furthermore, high BCR-antigen affinity or high antigen frequency is associated with extrafollicular response whereas low-affinity/frequency favours germinal centre B-cell response (242). This draws an interesting parallel with T-cell fate determination, albeit with reversed orientation with respect to antigen affinity and its influence over the Bcl-6-Blimp-1 axis. Strong BCR-binding induces greater magnitude ERK1/2 signalling, resulting in inhibitory phosphorylation of PAX5 transcription repressor allowing increased expression of Blimp-1 (243) which, alongside increased IRF4 expression and subsequent augmentation of Blimp-1 signalling (244), encourages plasmablast differentiation. Similar pathways are thought to be implicated in the termination of the germinal centre response – see below.

Extrafollicular differentiation occurs within the junctional zone of the spleen or within the extramedullary cords of the lymph node (235) and can be critical in inferring early protection

prior to germinal centre maturation (245, 246) and precedes germinal centre formation (247). Early differentiation of activated, co-stimulated B-cells gives rise to short-lived, antibody secreting plasmablasts (235); whether biologically significant memory-B cells are generated from such reactions is controversial (248). Although IgM production is predominant within extrafollicular reactions isotype switch can occur (249) and the presence of Bcl-6-expressing CD4+ cells is necessary for optimal antibody production of all subtypes (241). The requirement of IL-21 remains controversial however, with conflicting reports both supporting (241, 250) and refuting (251, 252) the requirement of IL-21 for extrafollicular immunoglobulin response. The phenotype of CD4+CXCR5+Bcl-6+ cells implicated within extrafollicular differentiation is, as alluded to above, less defined than GC-T_{FH} cells, with low expression of Bcl-6, CXCR5, ICOS and PD-1 (241, 253). Since said cells demonstrably do not function within the germinal centre (although they may be inducible to a germinal centre compatible phenotype) they have variably been referred to as pre-GC or pre-T_{FH} cells.

The archetypal role of T_{FH} cells is, however, the establishment of a germinal centre, within which B-cells undergo somatic hypermutation, affinity maturation and class-switch recombination with resultant production of high-affinity, class switched, immunoglobulin-producing, long-lived plasma cells and memory B-cells (236, 237, 254, 255). T-B cell interaction is reliant on surface molecule and cytokine cross-talk, with common transcription-factor targets within the two cell lineages (14, 24).

Several surface markers are of primary importance in the establishment of the germinal centre. T_{FH} cell adoption of $CXCR5_{Hi}ICOS_{Hi}PD\text{-}1_{Hi}$ phenotype is reliant on ICOS-ICOSL signalling (62, 65, 67) from its antigen-specific B-cell partner (15). ICOS signalling, although critical for T_{FH} differentiation, is not thought to induce signalling within the B-cell (14); ICOS-ligand interaction is thought critical for co-localisation at the T-B boarder (219). Although its role within the germinal centre has not been fully established, recent study suggests ICOS-signalling induces IL-4 production by T_{FH} cells, which would ensure localised delivery confined to the T-B conjugate (256).

Once co-localised SAP (Signalling lymphocytic activation molecule (SLAM) Associated Protein) is absolutely critical in stable T-B conjugate formation (257); the defect associated with SAP-deficiently is a specific failure of protracted interaction between the two cell types which, individually, are functionally replete (51, 257, 258). The deficiency is critical only within the CD4+ population (259). Without stable T-B conjugation germinal centre formation is absent (257) and T_{FH} differentiation is incomplete. SAP signalling is induced by SLAM-family proteins (including CD84, SLAM, Ly108 and Ly9); deficiency of these proteins cause partial defects in

germinal centre and T_{FH} function suggesting redundancy despite use of a common signalling intermediary (SAP) (14). SLAM-family proteins each have different binding affinities and functions; CD84 and Ly108 are predominant in the mediation of T-B conjugation (258).

Once the germinal centre reaction is established the T-B cell conjugate migrates to the follicle to establish a germinal centre (260). Within the germinal centre the role of T_{FH} cells can be divided as follows:

1.3.3.1 Promotion of survival, proliferation and affinity selection

Bcl-6 and Blimp-1 are the regulators common to both cell types; within B-cells, the mechanisms and determinants underpinning levels of Bcl-6 expression are poorly understood (261) however its presence is critical for germinal centre B cell development (24, 248). Bcl-6 places plasmacyte and memory cell differentiation on hold (248, 262) whilst limiting the DNA-damage response during somatic hypermutation class-switch recombination and preventing global apoptosis of B-cells (24, 263). Blimp-1, however, drives plasma cell differentiation (190, 263) – see below. IL-21 has been shown to directly induce Bcl-6 expression within B-cells (251) and thereby plays a critical role in their regulation (264). In the absence of IL-21 germinal centres form normally, with normal formation of T_{FH} cells, however there is early termination of the germinal centre reaction, due to loss of IL-21 driven B-cell proliferation, with premature release of both plasma cells and memory B-cells of reduced antigen affinity (251, 252). IL-21 also induces CD86 expression on B-cells, which provides a reciprocal pro-activation countersignal to the T_{FH} counterpart, propagating CD4 signalling and proliferation (265).

The germinal centre is histologically divisible into dark and light zones; light zones contain a higher proportion of follicular dendritic cells (presenting retained antigen) and are sites of B-cell activation biased towards those cells best-capable of competing for antigen (237, 266, 267). Once activated, B-cells migrate to the dark zone and undergo rapid proliferation and hypermutation (237, 266). High affinity is optimally acquired following successive re-cycling between the two zones, with only the higher-affinity progeny successfully re-entering the dark zone following positive selection within the light zone (266). Germinal centre B-cells strongly express Fas and are reliant on pro-survival signals from T_{FH} cells; although the mechanism for positive selection of B-cell clones remains uncertain, indirect data based on mathematical modelling (268, 269) suggests the limiting factor to be T-cell help, with higher affinity B-cells best able to compete for antigen displayed by follicular dendritic cells, and therefore able to display a greater density of pMHC complexes and attract more T_{FH} help (237, 270). Experimentally, it is possible to circumvent the need for BCR-stimulation and cause an artificial over-expression of pMHC and ensure the positive selection of such clones, irrespective of

antigen affinity (266). Such T-cell derived survival signals are delivered by CD40L, PD-1 and BAFF (14), as well as various soluble mediators – see Table 1; B-cells are therefore in competition for T_{FH}-derived survival signals (271, 272) with failure to acquire said signals resulting in apoptosis (14, 237, 273). Another level of competition driving increased affinity is that posed by circulating antibody; the presence of antibody from existing or recently graduated plasma cells can sequester antigen, driving cells towards a higher affinity (274). Such a mechanism might also partially explain separate germinal centres simultaneously acquire similar levels of affinity (274), although alternative explanations would include the free access into germinal centres of all B-cells and their ability to enter into a germinal centre reaction in the event they can out-compete resident incumbents (237)

CD40L is expressed by activated CD4+ cells and its absence or inhibition is associated with failure to form germinal centres through B-cell intrinsic mechanisms (275, 276). CD40L provides pro-survival signals and can rescue B-cells from apoptosis (277, 278). IL-21 promotes rapid proliferation of CD40L-stimulated B-cells (279), however cytokine exposure (IL-21, IL-10) in the absence of CD40L results in rapid differentiation into plasma cells (280, 281), therefore CD40L provides a vital mechanism preventing terminal differentiation within the germinal centre (280). Furthermore exposure of B-cells to IL-21 in the absence of BCR engagement results in induction of a pre-apoptotic state regardless of CD40L interaction, suggestive of a mechanism by which cells can be selected according to affinity within the germinal centre in an IL-21 dependant manner (282)

Germinal centre B-cells express increased levels of PD-L2 when compared with naïve B-cells, with both PD-1 and PD-L1 expression equivalent between both cell types (229). Memory B-cells express all three markers to a greater degree than either naïve or germinal centre cells (229). Dynamic changes in PD-L2 expression hint at an important role in the selection and survival of germinal centre cells (229); absence of PD-1 signalling from CD4+ cells is associated with an isolated failure of long-lived plasma cell production during the late stage of the germinal centre reaction, with an increase in apoptosis of germinal centre B-cells (229) and a reduction in IgG production (283). It remains unclear whether this effect is due to intrinsic functional deficit within T_{FH} cells lacking PD-1 signals (which have been shown to under-express genes for IL-4 and IL-21 (229)) or due to B-cell intrinsic deficits incurred through absence of PD-L1 and PD-L2-mediated signalling.

1.3.3.2 Support of somatic hypermutation and class-switch recombination

The presence of T_{FH} cells and their cytokine derivatives are essential for the support of somatic hypermutation and class-switch recombination. The importance of IL-21 as the principle

cytokine mediating germinal centre reactions (264) is reflected by aberrance of germinal centre B-cells in the absence of T_{FH}-derived IL-21 (251, 252, 284). Without IL-21 there is a curtailment of affinity maturation due to early abortion of hypermutation (251, 252), and select B-cell intrinsic aberrations in immunoglobulin isotype generation (250, 284).

In addition, T_{FH} cells promote and direct class-switch recombination through various factors (12); study utilising gene-reporter mice allowing identification of cells both transcribing genes encoding cytokine and producing cytokine have shed light on the timeline of such processes. IL-4 transcription was noted within T-cells at the B-T boarder however active secretion was localised to CD4+ cells within the germinal centre (64, 210) and was restricted to cells with a T_{FH} phenotype (68)[1]; this is in line with the concept of priming a pre-GC T_{FH} population during initial B-T contact and later acquisition of the full GC-T_{FH} phenotype within the germinal centre. IL-4 production, although not transcription, was noted to be sensitive to ICOS blockade, suggesting a mechanism for localised cytokine release confined to stable conjugates (64, 256). In line with this isolated conjugates demonstrate an increased expression of AID and greater extent of hypermutation (64). Critically direction of class-switch recombination (i.e. the isotype induced within the B-cell) was predictable based on the cytokine production of its T_{FH} partner (64).

Cytokine influence of isotype-determination is complex and multifactorial, with a multitude of different cytokines shown to have an effect – see Table 1. In line with this, cytokine production by T_{FH} cells is variable and influenced by factors at the time of initial DC-T priming (see Section 1.3), drawing parallels with the polarisation of more established CD4+ subsets (T_H1, T_H2, T_H17, iT_{Reg}) in terms of their cytokine profiles and transcription factor expression (12, 68, 232). Subtype-polarised T_{FH} cells induced different profiles of immunoglobulin specialised according to subtype-function (232).

Direction of isotype switch is likely to be dictated by the balance of a multitude of cytokine influences. An example of the suspected complexity of such a system is isotype outcomes following in vitro exposure to IL-21 and IL-4 in various combinations; exposure to IL-4 in the absence of IL-21 generates predominantly IgE and IgG_4 isotypes and a paucity of IgG_1 (250, 284). Conversely, IL-21 in the absence of IL-4 induces IgA production (285) and both cytokines in combination synergistically promote IgG_1 switch (285). Given the differential effect of many different cytokines (Table 1), each with their own pathways for induction, the complexity of such a system is, at best, barely understood. Furthermore available in vivo data are on vaccine

[1] This functional restriction was not paralleled within the periphery, where $PD^{Lo}CXCR5^-$ T_H2 effector cells readily secreted IL-4 (Zaretsky 2009). Therefore site-restriction of function seems peculiar to T_{FH} cells

induced changes in expression within these follicular populations is exclusively murine, human data being restricted to in vitro studies.

As well as stimulation through soluble mediators, germinal centre B-cells also require other means of support to maintain the germinal centre reaction which are also thought to be derived from T_{FH} cells (14). Somatic Hypermutation and class-switch recombination within germinal centre B-cells are dependent on CD40–CD40L interactions (275, 286, 287); following inhibition ongoing reaction stops and germinal centres dissolve within 24 h (238, 275). Furthermore deficiency in ICOS or ICOS ligand also results in impaired germinal centre formation, isotype switching and impaired recall response, probably through a T-cell intrinsic deficit (288, 289).

T_{FH} induction of B-cell expression of Bcl-6 through an as yet incompletely understood mechanism thought to include IL-21 (251, 264) is required to quell the DNA-damage response and promotion of apoptosis during DNA-recombination (24, 263). In the absence of Bcl-6, and therefore in the absence of germinal centre reactions, class-switch can occur through extrafollicular reactions (248) however it is sub-optimal (241) and with minimal establishment of long-term antibody responses due to a failure of long-lived plasma cell generation (248). Additionally, both somatic hypermutation and class-switch recombination are absolutely dependent on B-cell expression of AID (Activation-induced cytidine Deaminase) (263, 290). Mechanisms underlying AID induction by T_{FH} cells also remain undetermined, although T_{FH} derived IL-4, IL-10, IL-13 and IL-21 have been implicated in its induction (14, 64, 264).

Function	Cytokine requirement	References
IgM Production	➢ IL-10 ➢ IL-15 ➢ IL-6 + IL-12 promotes IgM production (DC-derived)	(291, 292) (293, 294)
IgG_1 Production	➢ IL-4 ➢ IL-10 ➢ IL-15 ➢ IL-21 augmented by IL-4	(250, 285, 291, 292, 295)
IgG_2 Production	➢	
IgG_3 Production	➢ IL-10 ➢ **IL-21**	(295)
IgG_4 Production	➢ IL-4 + IL-13 synergistically promote IgG_4 and IgE production	(296-301)
IgA Production	➢ IL-10-mediated IgA switch augmented by TGFβ ➢ IL-15 ➢ IL-21 in the absence of IL-4	(291, 292, 302) (285)
IgE Production	➢ IL-4 + IL-13 synergistically promote IgG_4 and IgE production ➢ Ablation of both IL-4 and IL-13 results in loss of IgE ➢ IL-4 induction enhanced by IL-6, TNFα ➢ IL-4 induction inhibited by IL-8, IL-12, IFNα, IFNγ, TGFβ ➢ SUPPRESSED by IL-12, IFNα/γ, IL-21	(250-252, 294, 296-300, 303-305)
GC-B cell Proliferation and survival	➢ IL-2, IL-4, IL-13 and IL-15 promote proliferation of activated B-cells ➢ IL-6 provides survival signals ➢ IL-21 through STAT3 mediated signalling ➢ BAFF/APRIL ➢ INHIBITED by IFNα/γ	(292, 300, 306-311) (312-314) (273, 315)
PC differentiation	➢ IL-10 promotes GC B-cells and memory B-cells to differentiate into plasma cells through Blimp-1 induction ➢ IL-21 through STAT3-mediated up-regulation of Blimp-1 ➢ Encourages plasmablast differentiation	(310, 316) (314, 317, 318)

Table 1: Cytokine mediation of isotype switch in human B-cells. References largely derived from Moens and Tangye 2014 (264). Experiments performed in vitro using co-culture or activation via functional anti-CD40 antibody, soluble CD40L with or without functional anti-BCR antibody.

1.3.3.3 Termination of GC response

Germinal centre reactions spontaneously self-terminate. In addition a selection of individual germinal centre B-cells for terminal differentiation either into long-lived plasma cells or memory B cells occurs. The mechanisms underlying these processes are variably understood.

Bcl-6 down-regulation within B-cells is required prior to terminal differentiation into plasma cell lineage (317). Plasmacyte differentiation is entirely restricted to high-affinity clones, therefore a robust mechanism must exist through which only highly competitive cells achieve this status. Competition might be mediated directly through BCR-affinity or through competition for CD4 signalling. Direct BCR-signalling can induce inhibitory phosphorylation of PAX5 and in doing so alleviate repression of Blimp-1 (243). However evidence implicated CD40 mediated signalling, and therefore T_{FH} interactions, as key in plasma cell selection. CD40-mediated induction of IRF4 has been shown to down-regulate Bcl-6 expression through an NF-κB dependent pathway (237, 319-321) releasing Blimp-1 from Bcl-6 suppression; IRF4 is indispensable in plasma cell differentiation suggesting its regulation to be a major factor in plasma cell fate-determination (321). Furthermore, CD40 signalling augmented IL-21 induced STAT3 signalling within GC B-cells, resulting in competitive inhibition of Bcl-6 binding to an inhibitory loci within the Blimp-1 promoter region (322). This suggests a dual role for CD40 signalling, both maintaining germinal centre response whilst selecting cells for exit; the determining factor seems to be a threshold of signalling, within only high-affinity B-cells capable of attaining enough CD40-mediated stimulation to suppress Bcl-6 (321). Blimp-1 is the key transcriptional promoter of *Xbp1*, which induces conformational change and establishment of the secretory apparatus for large volume antibody production (220, 221, 323) and induces a non-proliferative state through suppression of pro-proliferative signals such as Myc and Bcl-6 (324). In addition, Blimp-1 down-regulates MHC and CIITA, resulting in loss of B-cell phenotype, has a reciprocal negative-feedback relationship with PAX5 (and therefore antagonises the propagation of the germinal centre response) and its functions are augmented by the co-expression of IRF4 (264, 325, 326). Deletion of Blimp-1 results in loss of short-lived plasma cells, enlarged germinal centres and loss of terminally differentiated long-lived plasma cells (190), suggesting a critical role in extrafollicular and GC-derived plasma cell generation as well as the appropriate termination of the germinal centre response (325).

Factors which determine B-memory (B_{MEM}) differentiation are poorly understood (254, 255). Few if any differences are appreciable in terms of the recombinant state and genetic profile of cells positively selected for recycling within the germinal centre and cells which become memory B-cells (237). This is in apposition with the observable differences apparent within

cells selected to become plasma cells, which universally possess a greater antigen affinity than either positively selected germinal centre B-cells or B_{MEM} cells (237). The selection criteria for plasma cell differentiation and B_{MEM} generation therefore is fundamentally different. The role of Bcl-6 within B_{MEM} cells is also unclear, with conflicting findings demonstrating enforced expression of Bcl-6 results in failure to generate B_{MEM}-phenotype cells in vivo (327) contrasting with findings that STAT5 mediates a pro-survival and self-renewal behaviour within B-cells dependent on induction of Bcl-6 (328). Possible mechanisms underlying B_{MEM} differentiation therefore include a default state in the absence of plasma cell differentiation and following the avoidance of apoptosis (237) or as a bimodal fate decision dependent on Bcl-6-Blimp-1 axis in a fashion as yet undefined (255). Greater understanding of germinal centre regulation at the micro-RNA level may unveil as yet unrecognised mechanisms of fate determination (329).

Termination of the germinal centre reaction has been postulated to be through various negative feedback mechanisms. Plasma cell-mediated suppression of T_{FH} phenotype through down-regulation of Bcl-6 and IL-21 expression within the CD4+ germinal centre cells provides one possible mechanism (330). An alternative of competition for antigen by circulating antibody would lead to spontaneous resolution of germinal centre response once quantity and affinity of immunoglobulin exceeds the capacity of incumbent germinal centre B-cells to compete (274). The predominant mechanism in vivo remains undetermined.

1.3.4 Memory Follicular Helper T cells

As discussed above, circulating CXCR5+ cells are detectable within the peripheral blood although their significance remains unclear. Initially such cells were suggested to represent memory counterparts of T_{FH} cells due to their expression of CD45RO (55, 92), a marker of antigen experience (224, 331). However the heterogeneity of CXCR5+ cells and the ready induction of CXCR5 following activation (222, 227) and through non-Bcl-6 mediated transcription (20) would imply such classification is too simplistic.

T_{FH} cells are enriched for high affinity T-cell receptors compared non-T_{FH} effector cells (57, 58); T_{FH} cells have also been demonstrated to up-regulate CD69 expression and down-regulate S1PR1 transcription (57, 58) aiding their persistence within the lymphoid follicle and allowing protracted interaction with B-cells. It is uncertain whether the retention of these antigen specific cells within the responding lymphoid tissue is due to retention of antigen depots within the lymph node (58); persistence of antigen-specific CD4+ T_{EM} cells has been demonstrated to be biased in favour of the site of original antigen exposure resulting in accelerated antigen response to re-exposure through the same route compared with re-exposure through a disparate route (58). In mice, regional draining lymph nodes maintained

the highest population of antigen-specific T_{FH} cells, with greater tetramer-binding affinity than those detectable in spleen. No antigen specific T_{FH} cells were detectable in non-draining lymph nodes or blood; this suggests high-affinity T_{FH} cells are retained more effectively within the sites of antigen encounter; lower-affinity cells escape and re-circulate, accumulating in the spleen (58). Similarly, antigen specific Memory B cells are more prevalent within draining lymph nodes than non-draining lymph nodes.

Data from Fazilleau et al. suggest two subpopulations of CXCR5+ve T_{EM} cells existent within lymph nodes responding to antigen; $ICOS^{Hi}$ $CXCR5^{+}$ T_{EM} express high levels of mRNA for OX40, IFNγ, IL-4 and IL-21 and seem to be $T_{FH\text{-}Effector}$ cells actively involved in the evolving B-cell response (58). $ICOS^{Lo}$ $CXCR5^{+}$ T_{EM} cells do not express high levels of mRNA for OX40, IFNγ, IL-4 and IL-21, however can be induced to express said marker mRNA following re-stimulation with antigen suggesting that these cells may be precursors to $T_{FH\text{-}Effector}$ cells (58). Termed $T_{FH\ Memory}$ - cells by Fazilleau et al. these cells are theorised to promote an accelerated memory B-cell response to antigen re-challenge, however they appear non-migratory; i.e. they remain within lymph nodes which originally drained initiating antigen. An alternative interpretation is that these cells represent cells recently stimulated by persistent antigen within the node, which may explicate why experimental models using antigen likely to be cleared in its entirety fail to demonstrate long-standing CXCR5+ T_{CM} populations (54) whereas those using models with viral or intracellular pathogens prone to chronicity or recurrent infection tend to demonstrate CXCR5 expression on T_{CM} cells (224). Indeed in the absence of persistent antigen, T_{FH} phenotype is rapidly lost, suggesting reliance on antigen rather than a true memory subtype (332).

Intrinsic difficulties remain regarding the evaluation of CD4+ memory subsets due, in part, to their preferential residence within bone marrow niches and subsequent inaccessibility (333). Over 80% of CD4+ memory cells reside within the bone marrow niche and these cells are capable of rapidly up-regulating necessary co-stimulatory molecules, cytokines, and effectively provide B-cell help (333). However whether a memory B-cell response is dependent on memory-CD4-help at all is debated (334, 335) and therefore uncertainty as to whether $T_{FH\ Memory}$ cells exist persists.

Within a murine model, there appears to be no difference in the prevalence of T_{FH} cells between mice in receipt of memory T-cells vs naïve mice following immunisation, suggesting there is no transfer of an accelerated T_{FH} (110). However T_{FH} cells and memory precursor CD8 T cells have been demonstrated to share a 140 common gene expression changes, and the Bcl-6-Blimp-1 axis is important in both cell lineages (336). Transferred T_{FH} cells showed a greater

propensity for persistence than transferred T_H1 cells (336), however this could be due to increased persistence of CXCR5+ T_{FH} cells within lymphatic tissue and retainment secondary to antigen persistence.

Within, again, murine models, CXCR5+ memory cells identified up to 100 days post-infection with similar gene expression profiles to effector-phase T_{FH} cells despite the loss of their surface phenotype (337). This is consistent with other groups who suggest CXCR5+ cells have a greater propensity to become T_{CM} cells and therefore do not persist as a separate memory sub-population; instead T_{FH} cells are proposed to constitute the T_{CM} pool and then reconstitute themselves from the same following secondary immune responses (338, 339). This would be consistent with the observation that CXCR5+PD-1+ effector cells transferred into naïve hosts can reconstitute the full range of T-cell lineages upon re-stimulation (339).

When compared to memory cells with a T_H1 lineage commitment, CXCR5+ cells demonstrated a greater capacity to re-generate a T_{FH}-phenotype population following antigen-re-challenge following adoptive transfer into a naïve host (337, 339), although this would be predicted based on reduced pluripotency of a more differentiated subset. However CXCR5+ memory cells have been demonstrated to possess repressive epigenetic changes which reduce their capacity to differentiate into T_H1 cells, suggesting these cells are pre-programmed to follow a T_{FH} pathway upon re-activation (337). Furthermore CXCR5 expression is maintained within the CD4+ population during the recall response following transfer of CXCR5+ memory cells into B-cell deplete mice; this would suggest these cells have lost their dependence on B-cell mediated antigen presentation to retain CXCR5 (337), however the ability to attain other features of T_{FH} phenotype has not been demonstrated. Finally, circulating $CCR7^{Lo}PD\text{-}1^{Hi}CXCR5+$ cells have been proposed as a contender for the identity of $T_{FH\ Memory}$ cells, however their presence within the blood is transient, persisting for less than 3 weeks, therefore their function is likely to be the systemic propagation of a response to antigen in the event of system-wide exposure, and the presence of a persistent, long-lived differentiated $T_{FH\ Memory}$ cell population remains unconfirmed.

1.3.5 Regulatory Follicular Helper T cells

Initial study of regulatory cells proposed to limit T_{FH} cell response focused on the presence of MHC Qa1-restricted CD8+ cells within germinal centre reactions (340, 341). MHC Qa1 is a class I MHC molecule expressed on activated B and T lymphocytes, presenting antigen peptides to CD8 cells and has been linked to immune-regulation (340). Knock out or disruption of MHC-Qa1-restricted CD8 cells, identifiable by their expression of CXCR5 and ICOS, and termed $CD8_{Reg}$ cells, resulted in expansion of T_{FH} cells and fatal autoimmune disease (341). Their effect

was through perforin-mediated cytotoxicity and they were functionally dependent on the presence of IL-15. Since B-cells express significant quantities of MHC Qa1 (340), and B-cell derived IL-15 has been demonstrated to promote CD8+ cell cytotoxicity (342) induction of CD8-mediated T_{FH}-suppression may be a mechanism through which B-cell numbers negatively-regulate the T_{FH} response. This would complement other mechanisms proposed through which B-cells mediate negative-feedback of T_{FH} cells, which include plasma-cell suppression of T_{FH} phenotype and differentiation (330).

CD4+ T_{Reg} cells have been demonstrated to selectively regulate the activity of specific T_H subsets according to their own transcription factor profile (reviewed within (343)) and T_{Reg} cells have been demonstrated to migrate to the germinal centre (344). Bcl-6 co-expressing CD4+CXCR5+Foxp3+ T_{Reg} cells are associated with suppression of the germinal centre response (345, 346); these cells demonstrate a comparable surface phenotype to T_{FH} effector cells, Bcl-6 dependence for generation and CXCR5 expression, as well as Bcl-6-mediated suppression of non-T_{FH}-related transcription factors and Blimp-1 (345, 346), however Blimp-1 expression is significantly higher in regulatory T_{FH} cells (T_{FR}) in line with its role in the constraining natural T_{Reg}-effector development (347). However despite these similarities they seemed to originate from induction of Bcl-6 within CXCR5-Foxp3+ natural T_{Reg} cells in the periphery rather than induction during differentiation of naïve T cells (i.e. they are not iT_{Reg} cells and therefore do not represent T_{FH} effector cells induced to express Foxp3) in mice (345, 346, 348). In addition to Bcl-6 induction, CD4+CXCR5+Foxp3+ T_{Reg} cells were dependent on the presence of SAP for differentiation, demonstrating an induction pathway which parallels that of T_{FH} effector cells (345). Said cells were capable of suppressing both affinity maturation and the generation of plasma cells and memory-B cell subsets (346) and limited T_{FH} populations and germinal centre responses (345, 348). Importantly, depletion of T_{FR} cells did not increase high-affinity antibody production and, in fact, had the opposing effect of increasing low-specificity antibody and the presence of extra-follicular origin plasma cells. This was despite a florid germinal centre response in these models, suggesting a critical role of T_{FR} cells in supressing proliferation of low-affinity T_{FH} and B-cell clones (345).

More recently, PD-1 has been demonstrated to be a key regulator of T_{FR} activity (349); although expressed by both T_{FH} and T_{FR} cells, PD-1 is typically associated with induction of iT_{Reg} cells from effector sub-populations (343). In T_{FH} cells PD-1 provides a key role in limiting TCR-mediated proliferative signals (18). Interestingly, deletion of PD-1 results in an increase in the number of T_{FR} cells possessive of enhanced suppressive abilities in terms of naïve T-cell

activation and antibody production (349). This would suggest a role for PD-1 in the suppression of T_{FR} cells, limiting their generation and function (349).

1.4 B-Cell Sub-populations and Memory B-Cells

1.4.1 Immunoglobulin Isotypes

Functionality of the humoral response is determined by the actions of antibody – the effector molecules of the B-cell response. Antibodies mediate their actions through their relative and isotype-intrinsic capabilities in terms of three main functions – see Table 2: complement activation, resulting either in membrane disruption and cell toxicity through the classic pathway, and immune-complex formation and opsonisation for macrophage-phagocytosis through C3b binding. Antibody-directed opsonisation for phagocytosis by neutrophils and macrophages following immunoglobulin-Fcγ receptor (FcγR) interaction and antibody dependent cellular toxicity directed by immunoglobulin Fc-binding to FcγR present on NK cells.

Observation that the degree of VDJ-mutation within genes encoding the different immunoglobulin subclasses correlates with position of the subclass-genes within the heavy-chain constant region locus and the time-point of emergence from the germinal centre has led to the conception of a temporal model dictating function (350). This is proposed to work in tandem with the directed subclass differentiation mediated by cytokines, with early antibody production favouring lower-affinity, shorter lived IgM, low-affinity IgE and IgG_3 subclasses, specialised in rapid pro-inflammatory response, later superseded by higher affinity, more cell directed IgG_1-dominent production. Progression to IgG_2 production, which is the second most prevalent isotype at rest, is proposed to serve to dampen the response, with persistence of antigen (i.e. failure to clear) associated with anti-inflammatory IgG4 production (350, 351).

However such a model is undoubtedly a simplification since isotype of immunoglobulin also follows the nature of the instigating antigen; for example IgG_2 predominates responses to non-peptide antigens (1). Furthermore the functional properties of immunoglobulin are also dictated by their sialylation state; immunoglobulins whose Fc portions are more sialylated are produced in the presence of tolerising conditions and are proposed to have regulatory properties (80). Finally immunoglobulins play a role in the regulation of primary (274) and secondary (352) germinal centre reactions via Fc-mediated binding of immune complexes to follicular stromal cells (353).

The variability of isotype response within a population to a vaccine has been commented on previously and attributed to differing memory state with resultant variation in Th1/Th2 cytokine profile (354). However its significance in terms of efficacy is unclear. There exists little information regarding immunoglobulin isotype variability following booster vaccination even to relatively commonly administered vaccines such as tetanus/diphtheria/polio. Original studies demonstrating a stepwise increase in anti-toxin titre did not take into account

immunoglobulin isotypes (355) whereas those that did distinguish immunoglobulin isotypes tended to be animal studies (356). Given the assumed functional significance of different isotypes further study into immunoglobulin induction following repetitive vaccination episodes and the factors determining variability in response in humans would seem prudent.

	IgM	IgE	IgG3	IgG1	IgG2	IgG4
Affinity	Low	Low	Low	Intermediate	High	Highest
Half-Life (days)	5	3	7	23	23	23
Time Point	Early	Low-affinity early High affinity Late	Early	Intermediate	Late. Predominant in TI, extra-follicular responses	Latest
Abundance at rest (Approx)	10%	Rare	5%	45%	20%	2.7%
Complement dependent cytotoxicity	++++		++++ -Highest affinity for C1q	+++ -High affinity for C1q	Poor	-
FcγR Interaction			Highest affinity for FcγRIIIA and FcγRIIIB High affinity for FcγRIIA	Highest affinity for FcγRIIA, FcγRI High affinity for FcγRIII	Weak	High affinity for FcγIIB (inhibitory)
Antibody Dependent cellular cytotoxicity			+++	+++	+/-	+/-
Opsonisation	+		++	+++	+	+

Table 2: Immunoglobulin subsets, features and function (1, 350, 357, 358)

1.4.2 Long term B-cell derived immunity

The thymus-dependant humoral response to antigenic challenge can be broadly divided into a rapid, short-lived, low-affinity, IgM-rich, non-persistent extrafollicular response and a later, higher-affinity, more targeted response generated through germinal centre reactions (235, 237). The long-lasting memory components of B-cell derived humoral memory can be divided into two broad forms: persistent serum immunoglobulin produced by long-lived plasma cells resident within survival niches located in various tissues, as well as bone marrow and spleen, provides preventative protection against re-exposure but has a limited capacity for adaption. Whereas memory B-cells rapidly differentiate into plasma cells following antigen re-challenge and are also thought to re-enter germinal centre reactions upon re-exposure generating high affinity antibody through hypermutation and affinity maturation. As alluded to within Section 1.3.3.3, selection of germinal centre cells for either plasma cell or memory cell differentiation are different, with the threshold for affinity set at a lower level for cells destined for memory cell differentiation (237). Reasons for this would include the need to maintain a broad affinity-repertoire within the B_{MEM} compartment to take account of antigenic drift within pathogens, which can subsequently be fine-tuned on germinal centre re-entry following secondary exposure (359). Conversely, immunoglobulin is an effector molecule with a range of immunological functions (Table 2), and the plasma cell-producers are terminally differentiated, therefore tighter control of affinity is required to prevent auto-reactivity (237). B_{MEM} cells and serological immunity therefore achieve different functions.

Consistent with the two branches of B-cell mediated memory being distinct is the observation that there is an absence of correlation between presence of antigen specific B_{MEM} cells with quantity of circulating antibody in a resting state (360-363). Furthermore, in memory responses against an antigen stimulus which induces a T-cell dominant response (e.g. tuberculosis; PPD) although antibody levels are unchanged between vaccinated and control patients, the numbers of B_{MEM} cells were notably different, suggesting an effector function of B_{MEM} cells which is not reliant on maintaining serum antibody levels at rest (363). Therefore fundamental questions remain regarding the role of B_{MEM} cells and their function within a recall response; each faction of the B-cell mediated memory response is poorly understood, due to intrinsic difficulties in terms of cell frequency, lineage traceability, phenotyping and access to cells resident within their central niche.

1.4.2.1 Antibody secreting cells

Differentiation between plasma cell progenitors and subtypes is challenging but best considered a continuum with 4 recognised levels of differentiation (325). An activated B-cell displays surface Ig and MHCII, is negative for CD138 and CXCR4 and has high expression of

PAX5. A pre-plasmablast cell reduces its MHCII and PAX5 expression whilst up-regulating IRF4. Plasmablast cells continue to express surface Ig and low quantities of MHCII, whilst also expressing CXCR4 and CD138. Within these cells PAX5 expression is negligible whilst IRF4 expression is markedly raised and low levels of Blimp-1 are also expressed. Short-lived plasma cells lose surface Ig whilst retaining low MHCII and CD138 expression and increasing CXCR4 and Blimp-1. Long-lived plasma cells have absent MHCII and surface Ig, minimal CD19, and have the highest expression of CD138, CXCR4 and Blimp-1. The higher levels of Blimp-1 correspond with the greatest repression of the B-cell phenotype; within humans fully differentiated, mature, bone marrow resident plasma cells express the least CD19, CXCR5, CD79, CD86, CD22, CD21, CD20, B220 and MHCII (325, 364, 365) and increased CD27, CD38 and CD138 (325, 366).

The dichotomy between short-lived plasma cells/plasmablasts and long-lived plasma cells can therefore be defined according to differential Blimp-1 expression; within mice, Blimp-1^{Hi} plasma cells (slowly replicating, stable population, antigen-quiescent) were confined exclusively to the spleen and bone marrow with only Blimp-1^{Int} plasma blast/cells (more rapid division, migratory, antigen-reactive) present within the circulation (364). However this compartmentalisation has been shown not to be absolute, with human studies demonstrating that cells of a mature plasma cell phenotype can be induced from the bone marrow following immune response to vaccination (367, 368); the release of these cells coincided with an outpouring of vaccine-specific plasmablasts from secondary lymphoid tissues, however the antigen specificity of circulating plasma cells was demonstrably not towards the vaccine and unaccompanied by an increase in non-specific serum immunoglobulin, leading to speculation that the plasma cell repertoire within long-term survival niches is plastic, subject to competition, and modulated according to ongoing antigen-experience (367, 369-371). The fate of these non-vaccine specific cells was undefined; the presumption is that they apoptose, although why such cells would recirculate rather than apoptose in situ is unexplained.

Through such competition total plasma cell numbers remain relatively static over an individual's lifetime despite repeated antigenic episodes (325). However the rules which define this competition remain poorly understood (369). In addition, no clear precursor-product relationship has been defined for bone-marrow resident plasma cells. Although long-lived bone marrow resident plasma cells are virtually exclusively composed of graduates from germinal centre reactions, whether they emerge from the germinal centre fully differentiated or as plasmablasts which then have to compete to acquire bone marrow residency is unclear. Following vaccination, both low and high affinity circulating antibody-secreting cells can be

identified however they are distinguishable by their relative propensity to undergo apoptosis (372), therefore despite lower affinity cells being released from the germinal centre they were inherently less capable of survival. Three possible explanations exist; these cells might have received different priming signals within the germinal centre making them less survival prone, alternatively, these cells may have been released earlier from the germinal centre and failed to acquire a survival niche, subsequently becoming more pro-apoptotic over time. A third possibility is that these cells represented different origins, either direct differentiation from pre-established B_{MEM} or newly affinity maturated through germinal centre reactions (either from naïve cells or following B_{MEM} re-entry), although this was discounted by the authors (372).

However, whether direct competition even occurs is uncertain, since inflammation itself has been shown to clear bone marrow niches through lymphocyte mobilisation (373, 374). The concept of population-replenishment from B_{MEM} cells was the accepted model; however this seems unlikely due to the different affinity profiles of the two cohorts. The alternative is that plasma cells either undergo homeostatic replication or are eternalised; evidence for either argument is inconclusive (375), although the prevailing view is that they are not generally capable of cell division (325, 376). Therefore a third model is that survival potential is imprinted at the time of induction, with some plasma cells intrinsically less survival prone than others (376, 377).

1.4.2.2 Memory B-lymphocytes

Memory B-lymphocytes support the secondary response to antigen through proliferation and rapid plasma cell differentiation and are found predominantly within the spleen in humans (378, 379). Their propensity to contribute to the secondary immune response is derived from a lack of negative-regulation in comparison with naïve cells, making them prone to rapid re-activation (380). Persistence of memory B-cells for up to the life-time of the host (130, 131) has generated speculation regarding mechanisms through which the population is preserved. Initial observations suggested persistence of memory B-cells requires the reservation of activating antigen within secondary lymphoid tissue (381-383). However in vivo study has demonstrated B_{MEM} cells can persist in the absence of their activating antigen in a quiescent state (384, 385). Cell-turnover of the resting B_{MEM} compartment has been estimated to equate to 2.66% per day compared with 0.46% per day of the naïve cell population (where the two cell populations were defined by CD19/CD27 expression on peripheral blood PBMCs) (386). However the difficulty in interpreting such data is that circulating B_{MEM} cells cannot assume to be representative of B_{MEM} cells within their niche.

Whether the B_{MEM} population in humans is fully quiescent or subject to homeostatic proliferation therefore remains unclear. Certainly resting B_{MEM} cells can be induced to proliferate and differentiate into antibody secreting cells following non-specific stimuli in the absence of their specified antigen in vivo (368). This has been suggested as a possible means through which the wider B_{MEM} population can be maintained as bystanders to ongoing immune responses, however in vivo differentiation to antibody-secreting cells and proliferation of B_{MEM} in the absence of specific antigen has been directly refuted (387, 388). Importantly, homeostatic proliferation has been demonstrated within the naïve-B cell population and is thought to contribute to their maintenance (389) however whether similar behaviour is demonstrable within the B_{MEM} population is unknown. B_{MEM} cells do have a higher constitutional level of activation-cascade related mediators compared with naïve counterparts (390). This is speculated to confer a lower threshold for activation, but might also confer longevity (390), allowing homeostatic proliferation following exposure to supportive cytokines.

1.4.2.2.1 Memory B-lymphocyte sub-populations and functions

The degree to which memory B-cells undergo somatic hypermutation, and at what point in their development this occurs, is also debated; original thoughts that somatic hypermutation was confined to the first two weeks of the immune response has given way to the consensus view that repeated rounds of mutation can occur throughout the duration of the germinal centre response (391) which can last for several months (237, 362). Hence memory B-cells generated in the early stages of the GC reaction demonstrate a lower frequency of mutations within their V-gene segments, with later populations emerging with more extensive changes as the response continues (392, 393).

Previous study in man has demonstrated differences in the memory recall of different IgG-isotypes (394). Primary response was associated with comparative levels of IgG1 and IgG2, with low levels of IgG3 and undetectable IgG4. Recall responses in the same individuals one year post primary exposure where predominantly IgG1, with a marked increase in IgG4 production relative to baseline (but still low comparatively). In contrast, the kinetics of IgG2 and IgG3 demonstrated a slow rate of increase (394), therefore the selection for B_{MEM} phenotype may preferentially be skewed towards isotypes at the extremes of functionality (IgG1 = cell-directed, high affinity. IgG4 = high affinity, neutralising, inhibitory). Consequently, and due to the high prevalence of serum IgG, research into long-term B-cell memory has generally focused on CD19+IgG+ cells which are detectable for several months following antigen exposure (391) and the cytoplasmic component of the IgG receptor which contributes to the survival signals required for long-term persistence of memory cells (395-397). However more recently other non-IgG/A/E subsets of long-lived GC-experienced B-cells have been

identified and are also considered to be memory B-cell subtypes (391). Therefore contemporary research has focused on functional differences between the various memory B-cell subtypes, which are characterised by their differing isotype, somatic hypermutation and surface marker expression.

Unfortunately there exist inherent difficulties in the study of human B-cells, and human B_{MEM} cells, since surface marker expression fails to consistently and invariably identify distinct subsets, although subsets demonstrably exist. These can be overcome in murine models using insertions of various reporter genes, however findings in mice do not necessarily translate to humans. The most commonly used marker to identify memory B cells in humans is surface CD27 expression (398, 399); although a reasonable robust marker of prior somatic mutation (i.e. activation) the water is muddied by the presence of both isotype-switched CD27- cells and non-switched CD27+ cells (400). Significant uncertainty persists regarding the function and relative importance of B_{MEM} cells of different CD27 expression and isotype, although CD27+ cells of both switched and un-switched isotypes respond more effectively to BCR-mediated stimulation than their CD27- counterparts, suggesting significant difference (390).

Analysis of replication history, somatic hypermutation and class-switch profiles demonstrate different origins of separate memory B-cell classes; CD27-IgA+ cells are thought to arise from germinal centre independent pathways within the gut. CD27+IgM+IgD+ “natural effector B-cells” are thought to arise from both brief GC responses and germinal centre independent pathways within the marginal zone of the spleen (401-403); the relative importance of each pathway is debated (402). It is uncertain whether this population could be further sub-divided according to CD43 expression into TI and TD-GC sub-populations(401, 404), however controversy remains whether CD43+ B-lymphocytes are a true B-cell sub-population (405). CD27-IgG+ and CD27+IgM+ cells are thought to require GC pathways (391, 402), but tend to have less somatic hypermutations than CD27+IgA/IgG+ cells; both CD27-IgG+ and CD27+IgM+ cells are thought to arise early from primary GC responses and be able to re-enter germinal centres to become CD27+IgA/IgG+ cells during secondary exposures (406). CD27+IgA/IgG+ cells demonstrate the highest level of proliferation capacity and greatest degree of B-cell receptor maturation (401) and are therefore thought to result solely from secondary GC responses.

The effector function of the subsets has been demonstrated to differ with respect to their response to re-challenge; IgG+ memory subsets differentiate directly into plasma cells without necessarily re-entering the GC (391) (this paper related to a murine model and therefore made no distinction between CD27 expression on IgG+ memory cells). This correlated with findings

from other groups demonstrating CD27+IgG B_{MEM} cells do not re-enter germinal centre reactions (387). Conversely, IgM+ memory subsets (defined, in this study, as cells exhibiting a knock-in transgenic marker for GC engagement) re-entered the GC, underwent proliferation and produced either IgM-producing cells, or isotype-switched to IgG+ memory subsets (391). Interestingly, within this murine model, IgG+ memory cells were noted to be less prevalent and persistent than their IgM+ counterparts; IgM+ cells were four times more prevalent up to 180 days post immunisation, with this predominance increasing thereafter and IgG+ cells becoming undetectable 6 months post vaccine (391). These findings were corroborated by other investigators who demonstrated affinity matured IgM+ B_{MEM} cells emerge from the germinal centre and are persistent. However alternate investigators refute the supposition that re-activation of germinal centre responses rarely involve switched B_{MEM} cells; using a murine adoptive transfer model switched B_{MEM} cells harvested 70 days post primary exposure were not only capable of germinal centre re-entry, but may also out-perform IgM+B_{MEM} cells in their capacity to do so (407). However such models differ from normal immune conditions, as memory cells are transferred to a host with no circulating immunoglobulin of equivalent specificity.

This is relevant as the actions of switched and non-switched B_{MEM} cells seem dependent on the quantity of circulating immunoglobulin, which draws interesting parallels with work demonstrating antibody feedback control of germinal centres (274); non-switched memory cells appear to be more active in secondary responses in the absence of circulating antibody at baseline whereas in the presence of pre-existent high titre of high affinity immunoglobulin the ability of these cells to generate a germinal centre response is diminished (408). Switched memory cells rapidly differentiated into plasmablasts; these cells produced large quantities of high affinity antibody which was correlated with a failure to induce germinal centres, presumably through immunoglobulin-mediated inhibition (408). Mechanisms of modulation of the recall germinal centre response include interaction between immune complexes bound to the FcγIIB receptors on the surface of follicular dendritic cells and B_{MEM} cells (352, 353); germline mutations of these FcγIIB inhibitory receptors is associated with a failure of self-tolerance in a variety of autoimmune diseases associated with abnormal and enhanced production of high-affinity antibody (409).

Additional challenges are presented in the identification and characterisation of transitional cells and germinal centre cells and distinguishing them from B_{MEM} cells. Transitional B-cells are naive B-cells which have recently left bone marrow and are yet to be exposed to antigens

within lymphoid tissue. Similar therefore to naïve B-cells, they express IgD, IgM and CD24, however they can be distinguished from mature naïve cells by their expression of CD38 (410).

CD38 has an additional association in humans (but not mice) with germinal centre cells. CD38 is an ectoenzyme which catalyses the synthesis and hydrolysis of cADP-ribose thereby influencing the regulation of intracellular Ca^{2+} and protection from apoptosis (411). It is more accurately thought of as an activation marker (412) and is up-regulated following stimulation by T-cell derived factors (413). It is also expressed by plasmablasts and plasma cells, as is CD27. Therefore phenotypically distinguishing these populations according to marker expression can be challenging (414). However CD38 is not expressed on resting human B_{MEM} cells, making CD38 a valuable negative discriminator (412, 413, 415). Additional sub-categorisation has been attempted within the germinal centre primarily due to the distinct dark and light zones present there and their proposed functional differences in terms of affinity selection and proliferation (See Section 1.3.3) CD77 has historically been touted as a distinguishing marker (centroblasts (CD77+) vs (Centrocytes) CD77-) (267) however lack of defining differences in terms of function or gene expression between CD77- and CD77+ germinal centre cells has cast doubt on validity as a marker (416-418). More recently, delineation according to CXCR4, CD83 and CD86 expression has been proposed (centroblast ($CXCR4^{Hi}CD83^{Lo}CD86^{Lo}$) vs centrocyte ($CXCR4^{Lo}CD83^{Hi}CD86^{Hi}$) (237, 266), which successfully delineates cells according to their propensity to follow the chemotactic gradient controlling intra-germinal centre migration (CXCL12 favouring dark zone, CXCL13, CCR6 and $S1P_3$ redundantly favouring light zone) (419, 420).

The persistent issue with understanding B-cell biology is the heterogeneity of different sub-sets according to a variety of markers. Eliciting the significance of these subsets and sub-subsets is also challenging considering their potential effector sites are in tissues which are unattainable in humans and unrepresentative in animal models given their limited immune-history and different phenotypic markers (414). Given these difficulties, the functional significance of various B-derived populations remains of interest to contemporary research (421), particularly given the speculated immune-modulatory role some of these tissue-resident cells are suspected to play; sub-populations can be identified according to Fc-receptor homologue expression profile and cytokine production (422) with distinct transcription factor expression and in vivo response to stimulation, although the function of such cells within an active immune response is unknown (422).

The non-immunoglobulin derived functions of B_{MEM} cells are thought to contribute to the pathogenesis of SLE (423), as well as influencing the differentiation of the T-cell response (424,

425). Despite the known relevance of B-cell derived cytokines to disease states, and the speculated sub-populations identifiable based on differential expression of cytokine, little research has been completed in the area (425). Growing interest in the subject has been stimulated by the observations of non-antibody mediated effects on T-cell populations following B-cell depletion therapy and the speculated importance of B_{REG} and anergic B-cells in cancer and other disease states.

1.5 Bystander Activation in T-Lymphocytes

Bystander activation of T-lymphocytes is characterised by the induction of phenotypic and functional changes within a lymphocyte in the absence of specific TCR stimulation (426). It is distinct from processes such as “cross-presentation” (MHC Class I presentation of exogenous antigen), “molecular mimicry” (cross reactivity between different antigen epitopes as a consequence of structural similarity) and “epitope spreading” (activation against epitope B due to its unveiling as a consequence to the primary immune response against epitope A) because these processes rely on antigen-TCR interaction (426). Therefore bystander responses are thought to be mediated by soluble signalling molecules and membrane bound receptor-ligand interactions distinct from those of MHC-antigen-TCR interaction. Bystander activation/proliferation is proposed to be a mechanism underlying population maintenance and control within the systemic memory lymphocyte population.

The concept of non-specific lymphocyte activation and proliferation initially arose from observations that only a small proportion of viral-induced expanded CD8 T-cell populations were antigen specific (427-430). These initial studies estimated that only 5-20% of the expanded lymphocyte population was specific for the target antigen. These estimates were changed following the development of more sensitive assays capable of defining epitope specificity more accurately. Use of ELISpot, intracellular cytokine staining and tetramer staining revised the estimate to up to 70% of expanded population comprising of antigen specific cells (431-435). Such work has called into question the degree of the bystander response in an immune-competent host and its biological significance in healthy individuals (436). Nonetheless an argument remains that bystander activation may be important in special circumstances such as HIV infection and autoimmune diseases (426) and may contribute to the pathogenesis of sepsis (437).

The mechanisms underlying bystander proliferation are unclear, as are the prerequisites for a cell population to be susceptible to bystander expansion. Whether naive cells are capable of undergoing bystander expansion is uncertain and is, at most, a rare event (431), however

memory CD8+ T cells have been shown to expand in vivo following exposure to IFNγ (430) and cytokines known to induce IFNγ such as IL-12 and IL-18 (438).

The activation status and functional capabilities of bystander expanded population is controversial (432); are such cells capable of cell-mediated effector functions or are they simply more numerous? Does the apparent increase within the circulating blood belie proliferation and population expansion or mobilisation of non-dividing cells from a tissue niche? In addition, is a similar response demonstrable within all lymphocyte sub-populations and what interactions play out between these cellular compartments? Are there phenotypic features which distinguish bystander cells from antigen-activated cells?

1.5.1 Proposed mechanisms of bystander response

1.5.1.1 Cell-to-Cell mediated signalling

Mature T-cells can be activated under experimental conditions through cross-linking of membrane bound CD2 (LFA-2; LFA-3 receptor) by CD58 (LFA-3) or by anti-CD2 antibodies (439). This appears dependent on association with CD3ζ chain of the TCR and shares down-stream components of TCR activation but doesn't require antigen. TCR-independent activation can also be induced through cross-linking of CD28 (440), with the additional crosslinking of CD27 augmenting this process (441). This occurs via an NFκB activation pathway independent from ZAP-70 and CD3ζ (downstream signalling molecules associated with TCR activation) (440).

CD40 agonists have been demonstrated to amplify response to weak TCR signalling (442). CD40-CD40L interaction has been demonstrated to be integral to CD4-helper function; CD40L expression by CD4 T_H cells allows interaction with both B-cells in the promotion of class switching, germinal centre formation and somatic hypermutation (287, 443-445) and DCs, the activation of which promotes a robust CD8+ response to antigen (446-448). CD40 agonists promote antigen independent proliferation of CD4+ and CD8+ cells with memory phenotype (induced in the absence of antigen), but not antigen-experienced memory T cells, via a mechanism dependent on APC CD40 expression (442).

Toll like receptor agonist induced bystander activation of CD4+ cells has been demonstrated to be reliant on cell-to-cell contact with LPS-activated dendritic cells (449); expression of activation markers and induction of IFNγ and TNFα production was achieved only when LPS-activated CD4-CD8-(double negative) splenocytes were in direct contact with purified CD4+ cells (449). This process was more efficient in memory CD4+ lymphocytes than naive populations (449). The efficacy of cell-to-cell mediated bystander activation was diminished but not ablated using combinations of blocking antibodies directed against B-7 family co-

stimulatory molecules (ICAM-1:LFA-1, CD80:CD86, ICOS:ICSOL, CD28:CTLA4) (449). Whether APC mediated activation of lymphocytes is dependent on cell-to-cell contact has been questioned by other work (see Section 1.5.1.3) which has successfully demonstrated cytokine (IFNαβ and IFNγ) mediated effects of LPS-activated DCs on lymphocyte activation markers, but did not assess cytokine production by the activated cells (450). The same work did allude to partial dependence on cell-to-cell contact in the induction of NK cell IFNγ production by LPS-stimulated DCs (450).

1.5.1.2 TCR Tickling

Although integral to the provision of survival signals to naive lymphocytes, TCR tickling as such is not thought to play a significant role in bystander activation or proliferation. However the theory of heterologous immunity (see Section 1.5.2) does suggest a role for TCR-cross reactivity in mediating survival following lymphocyte activation in the context of infection.

1.5.1.3 Cytokine Signalling

Initial work detailing bystander proliferation centred on response to viral antigens and therefore CD8+ T-lymphocytes. Type 1 interferons (IFNα and IFNβ; IFNαβ) produced by activated dendritic cells are known to drive the CD8 response to viral infection. Exposure to exogenous IFNαβ stimulates proliferation of CD8+ cells in the absence of TCR stimulation (430). The proliferative effect of IFNαβ is mediated through IL-15 activation of IL-2Rβ, the expression of which is up-regulated on memory CD8 cells (115). The source of IL-15 in vivo is thought to be macrophages stimulated by IFNαβ, bacterial lipopolysaccharide or viral infection (115), however IL-15 is also produced by blood-derived dendritic cells (451) and follicular dendritic cells (452). Antigen-independent alteration of lymphocyte tracking and activation state has been demonstrated in response to endotoxin in humans (437).

Toll-like receptor agonists mediate their induction of lymphocyte activation marker expression indirectly in a rapid yet transient manner, with activation peaking within 24 hours and returning to baseline after 7 days (450). DCs activated by toll-like receptor agonists induced antigen-independent activation of both naive and memory T-cells through an IFNαβ dependant mechanism which acted directly and indirectly through the induction of IFNγ production by NK cells and through autocrine stimulation of DC production of IL-12 and IL-18 (450).

The similarities between the proposed mechanisms of homeostatic proliferation and those of bystander proliferation resulted in theories that non-antigen specific population expansion following infection was a result of homeostatic proliferation following lymphocyte depletion. An alternative view was that expansion of lymphatic tissue during the course of infection

increased the space available in which homeostatic proliferation could occur. However other investigators maintain that homeostatic proliferation and bystander proliferation are distinct processes; Gilbertson *et al.* demonstrated induction of IFNγ production in CD8 cells of irrelevant specificity in response to *Mycobacteria avium* infection in mice (453). The model used attempted to distinguish between homeostatic and bystander proliferation by demonstrating the kinetics of cellular division was independent of lymphocytic depletion (453), however these experiments relied on transfer of transgenic cells between naive hosts, raising questions regarding the applicability to normal immune systems. IL-2Rγ ligation, IL-12 and IL-18 have been implicated in the induction of IFNγ production in the absence of antigen exposure (454-456); however the ability of lymphocytes to rapidly produce IFNγ in the absence of antigen seems to vary between individuals (456). In addition, the consequences of IFNγ production remains uncertain since non-antigen specific IFNγ production by lymphocytes has been implicated in hyper-immune pathology (455, 457) whilst also being integral during the depletion of cells during the contraction phase following antigen response (458). The long-term fate of cells induced to produce IFNγ in the absence of antigen therefore requires further evaluation.

1.5.2 Bystander response or Heterologous Immunity?

A body of evidence supports promiscuous binding of HLA-molecules to a number of peptides (459-461), suggesting a role for antigen cross-reactivity in the mediation of apparent bystander proliferation (462-464); mice with a prior exposure to LCMV demonstrated augmented response to vaccinia compared with those without, and this protection was transferrable only when both CD4+ and CD8+ cells were transplanted into a naive recipient (465). Cytokine mediated bystander effect was disparaged since protection was dependant of the sequence of viral infection, the pathogens involved and was not necessarily reciprocal (463, 465). Proposed explanation included non-antigen specific recruitment of IFNγ producing memory T-cells and IFNγ mediated augmentation of the primary response (465). However mortality following vaccinia exposure is reduced by pre-existing immunity to LCMV; such protection is dependent on the presence of memory cells specific to LCMV as demonstrated by adoptive transfer experiments (466). Proliferation of LCMV epitopes was not universal; i.e. memory T-cells specific to a certain LCMV epitopes proliferated more than other LCMV epitopes, hinting at selectivity of heterologous stimulation of memory cells (466). Furthermore, whilst certain LCMV epitopes were selectively expanded, others were depleted following vaccinia infection (466) which indicates TCR specificity influences response to heterogeneous antigen and implicates cross-reactivity as a mechanism. Cross-reactive epitopes are identifiable between the two viruses; the prominence of memory cells bearing TCRs specific to these epitopes

within immune mice responding to primary exposure to the second virus was increased relative to immune mice undergoing successive exposure to the same virus, with increased representation within the resultant memory pool thereafter (464). Cytokine simulation following administration of IFNαβ promoter Poly(I:C) failed to mimic heterologous immunity; IFNαβ exposure resulted in a lesser degree of cell division than that induced by cross-reactivity, failing to increase total cell numbers or influence the resultant memory population in terms of epitope hierarchy or quantity in numbers (463).

In the absence of cross-reactivity, established immunity to a primary antigen can be curtailed by subsequent exposure to a second antigen (467). CD8+ mediated cytotoxicity and CD4+ and CD8+ IFNγ production in response to *Listeria monocytogenes* (LM) was reduced in mice immune to LM following BCG exposure (467). This effect was mitigated, even reversed, when both pathogens were engineered to express common epitopes (467).

These results indicate response to antigen is influenced by the history of previous antigen exposure within a host and the pre-existent memory-T cell repertoire. In human subjects, BCG immunisation can affect both B-cell and T-cell responses to subsequent immunisations with unrelated antigens (468). Experimentally using mouse models, serial antigen exposure can be seen to influence the memory repertoire of previously encountered antigens (464, 466, 469), changing cellular distribution and sub-population composition, altering the immunodominance of epitopes (464) and even instigating the deletion of memory cells epitope subsets (464, 469). The mechanisms for such observations are unclear; heterologous TCR-cross reactivity may select promiscuous cells for survival whereas cytokine mediated mobilisation and division in the absence of TCR-stimulation could increase susceptibility to deletion. Whether such mechanisms are relevant in normal human immunity is unknown, however such observations draw interesting parallels with observations within the B-cell compartment, where plasma cells of disparate antigen specificity are mobilised and presumably replaced following vaccination/infection with another agent (see Section 1.4.2.1).

1.5.3 Bystander response within the CD8 T-Lymphocyte population

Observational data exists attesting to the potential for CD8+ cell activation in response to disparate antigen stimulation in humans; a patient with chronic hepatitis C apparently cleared hepatitis C viral antigens following superimposed infection with hepatitis B (470). IFNα treatment has become integral in the treatment of hepatitis C, the mechanism of which is proposed to be a non-specific augmentation of the immune response to viral antigen. A possible explanation for the clearance of hepatitis C in the presence of new exposure to hepatitis B is bystander activation of CD8+ response to hepatitis C antigen through IFNαβ with

or without other mediators. Patients with HIV infection demonstrate increased activation of CD8+ memory cells specific to EBV, CMV and influenza however activation did not translate into raised proliferation, perforin expression or improved survival (471). Non-antigen specific CD8+ cells migrate to response sites during infection but do not proliferate and are less persistent than antigen-specific counterparts (472).

Bystander CD8+ T-cell activation by IFNαβ (430) and IL-15 (115) results in proliferation of the memory sub-population, but can also result in controlled cell death (473, 474), which has been proposed as a possible mechanism of controlled attrition of memory cell populations (148, 469, 474-476). The stimulation of pre-activated CD8+ memory cells by inflammation in the absence of their specific antigen has been demonstrated to induce proliferation, however these proliferating cells do not appear to contribute to the memory populations (477). When compared with pre-activated CD8+ memory cells stimulated by inflammation in the presence of their antigen, the resultant memory cell population is markedly reduced (477). Resting memory cells exposed to the same conditions were not stimulated to proliferate (477). Therefore cells driven to continued proliferation in the absence of persistent antigen fail to generate memory populations.

CD8+ memory cells can be depleted by recurrent infection with an unrelated antigen (475). Khan *et al.* looked at the prevalence of antigen specific CD8+ T lymphocytes in CMV positive patients; they noted CMV positive patients' accumulated CD8+ lymphocytes specific to CMV-associated antigens with age, presumably as a result of repeated antigenic challenge and persistence of proliferation. Sub-population analysis supports such an assumption, demonstrating a high prevalence of differentiated T_{EM} and T_{EMRA} cells (475). The proportion of these cells which were capable of mounting a lytic or cytokine (IFNγ) response to antigen declined with age (475). In addition, patients with CMV positivity had reduced populations of CD8+ cells reactive towards unrelated EBV-associated antigens, the inference being that activation and proliferation of CMV-specific CD8+ cells diminished the other populations (475). Cells stimulated through antigen-independent means display higher sensitivity to Fas/Fas-L-induced apoptosis (474); infection with LCMV can induce apoptosis in CD8+ cells which are not specific for the virus in mice (473), a finding which can be recreated when exogenous IFNαβ is used to stimulate the same cells in vivo (473). However these findings persisted in mice which were deficient in FasL, NK cells or perforin (473). Selin *et al.* observed that populations of CD8+ memory cells declined following subsequent infection by a different agent, however they noted population stability for prolonged periods in the absence of subsequent heterogeneous antigen challenge (469, 476). They propose bystander mediated apoptosis as a mechanism to

clear space for memory cell "storage", as well as mediating the preferential survival of populations of T-cells bearing TCRs reactive towards more than one antigen (464, 469). In humans, acute infection has been demonstrated to induce up-regulation of PD1 expression on non-antigen specific CD8+ cells (478); PD1 expression is thought to be induced early following cytokine activation and is moderated following subsequent TCR-interactions (479). PD1 expression was associated with population decline in non-antigen specific CD8+ cells which could be mitigated by PD1 blockade (478).

Bystander activation induced apoptosis is not a universal observation. Chronic infection with *Leishmania donovani* in Listeria-immune mice resulted in an increase in listeria specific CD8+ memory cells with induction of T_{CM} bias within the population and enhancement of IFNγ production (480). Increased proliferation and numbers of CD4+ memory cells specific to listeria were also demonstrated (481). The authors proposed that splenomegaly induced by *L. donovani* increased the availability of protective lymphoid niche allowing survival of bystander-expanded population within this experimental model. Other possible explanations include the existence of unrecognised epitope cross-reactivity or the induction/augmentation of survival signalling by the persistence of infection; what would happen to the expanded population should the infection become cleared was not evaluated. Similarly, CD8+ response to primary EBV infection in humans was noted to induce activation of cells specific to unrelated CMV and influenza epitopes without subsequent depletion (482). Such results suggest the effect of bystander activation on the long term homeostasis of non-antigen specific populations may either be pathogen specific or effected by some as yet undetermined difference between the immune response to different pathogens.

Longitudinal studies demonstrating the dynamism of individual epitope-specific lymphocyte populations in humans (483) highlight a problem with applying findings in mouse studies to human biology; it is likely that the normal population dynamics of the immune system in the presence of day-to-day exposure to multiple epitopes is incompletely modelled by the simplistic controlled immune environment within laboratory mice.

Induction of antigen-independent proliferation of CD8+ memory cells is not unique to IFNαβ or IL-15; proliferation has been described in CD8+ cells in the presence of IFNγ and IL-12 production by activated NKT cells and antigen stimulated T-cells (484). Similarly, memory CD8+ cells can be stimulated to proliferate following intravenous infusion of IL-12 or IL-18 through an IFNγ dependant, IFNαβ independent mechanism; no such proliferation was demonstrable for CD4+ cells (438). These findings could not be reproduced following direct exposure of cells to IL-12 or IL-18 in vitro suggesting dependence on an unidentified in vivo intermediary; IFNγ,

IL-18 and IL-12 selectively stimulated IL-2Rβ positive cells suggesting IL-15 dependence (438) and IL-15 is known to be induced by IFNγ (115). However what happens to such cells following IFNγ induced proliferation is unclear and whether the memory population becomes depleted as a result of such stimulation remains unknown.

1.5.4 Bystander response within the CD4 T-Lymphocyte population

Bystander activation of CD4+ T-lymphocytes is less well characterised and less efficient than bystander proliferation in CD8+ cells. One theory behind this is that CD4+ memory cells express IL15R less frequently than their CD8+ counterparts (485). Nonetheless there is evidence supporting the existence of CD4+ bystander proliferation in several different models; immunopathology caused by non-antigen specific CD4+ lymphocyte recruitment and activation has been shown in mice (486-488). In addition viral infection results in non-specific recruitment of activated but not naive CD4+ cells to sites of inflammation, however such cells do not proliferate in the absence of their specific antigen (489).

In humans augmentation of IFNγ response to tetanus toxoid (TT) has been demonstrated following an unrelated viral illness (490). Furthermore healthy immune-competent adult human volunteers, in receipt of TT booster immunisation, demonstrate expansion of memory CD4+ cells specific to TT, as well as CD4+ memory cells specific to herpes simplex (491) purified protein derivative to tuberculin (PPD) (371, 492) and *Candida albicans* (371). In this model, there was no concomitant antibody response to any antigen save for TT, and the CD4+ bystander proliferation was confined to pre-existent memory cells (371).

CD4+ lymphocytes undergoing bystander proliferation are thought to be responding to stimuli other than the IL-15 dependent mechanisms proposed for CD8+ cells. Candidate pathways include IFN-γ derived from NK cells, NKT cells and other T-lymphocytes activated following specific activation or cytokine-mediated stimulation by IL-12 or IL-18 derived from activated APCs (484, 485).

An alternative, or co-existent, pathway implicates IL-2 and IL-7 produced by antigen-specific activated CD4+ memory lymphocytes responding to antigen presented by APCs inadvertently stimulating nearby CD4+ memory cells specific to alternative antigens via a “para-autocrine” mechanism (493). Bystander CD4+ proliferation of activated memory cells specific to an antigen unrelated to TT is observed in mice immune to TT who receive a booster immunisation of TT (493); no similar proliferation is seen when the same cells are observed following TT inoculation of TT-naive mice, nor following the inoculation of control peptide or saline (493). The magnitude of bystander proliferation of the unrelated antigen-specific CD4+ cells was proportional to that of the TT-specific memory response and the magnitude of IL-2 and IFNγ

production (493). Proliferation in vitro could be reproduced by IL-2 and IL-7 (493). However in this experimental system the cells observed to undergo bystander proliferation were pre-activated and therefore not fully representative of resting memory cells in vivo. In addition, the in vivo destiny of the bystander population was not evaluated and it is therefore unknown whether their non-antigen specific stimulation resulted in attenuated survival in a fashion analogous to the dynamics of CD8+ cells (477).

Although non-antigen specific CD4+ T-lymphocytes are activated in the presence of infection, their behaviour is not analogous to antigen-stimulated counterparts. Although vaccination against yellow fever induced an increase in prevalence and cytokine production by tetanus toxoid specific CD4+ cells, proliferation assays demonstrated these cells were not dividing at a rate comparable with the antigen-specific response (494). Survival of bystander-activated cells is significantly reduced relative to antigen-specific T-cells, leading to selective deletion of non-antigen specific cells and re-constitution of lymphoid compartments with antigen-specific cells (495). However this mouse study failed to distinguish between memory and naive cells, so whether such population clearance is seen in human patients with a diverse history of antigen exposure and a heterologous population of memory cells is unclear.

Therefore, as for CD8+ cells, evidence exists demonstrating CD4+ cells undergoing bystander activation become increasingly pro-apoptotic (495-497), with implications for some disease processes such as HIV (426). Cells undergoing antigen independent activation demonstrated increased susceptibility to apoptosis compared with resting cells, an effect dependent on lymphocyte IFNγ receptor expression (496). Bangs *et al.* developed a co-culture technique utilising transwell plates designed to share soluble mediators between two populations whilst preventing cell-to-cell interaction (497). They demonstrated up-regulation of activation markers (CD69) on CD4+ lymphocytes, which were demonstrably non-responders to Staphylococcal Enterotoxin B (SEB), when co-cultured in the presence of CD4+ lymphocytes responding to SEB antigen stimulation (497). This response was almost entirely confined to the memory-cell sub-population, with naive cells remaining uninvolved. The absence of cell-to-cell contact implicated soluble factors as the underlying mediators, however the response was not impaired by IL-15 and IL-23 neutralisation (497). Interestingly, gene analysis demonstrated differences in expression between cells activated through direct antigen interaction and those activated through bystander effects; genes expressed with greater frequency in directly activated cells tended to be associated with intrinsic cell death pathways (both proapoptotic (HtrA serine peptidase 2, caspase 9) and antiapoptotic (baculoviral inhibitor of apoptosis repeat-containing 5 (survivin), and myeloid cell leukemia sequence 1, Bcl-2) and the common

mediator caspase 3 (497). Genes up-regulated in the bystander-activated cells relative to those cells directly activated by antigen included components of the extrinsic cell-death pathway factor 1 and BH3-interacting domain death agonist (497). However interpretation of these results must be treated with caution; the bystander activated cells were denied cell-to-cell interaction, which might have influenced their expression of apoptotic regulators and were activated in the absence of APC or stromal support, which can be assumed to play a role in vivo which the experimental conditions cannot replicate. Such findings must therefore be ratified through in vivo study, preferably in an immune competent host.

Recent work within our own laboratory has looked at the proliferative and expression differences between bystander cells and antigen specific cells (498). Findings suggested bystander cells expressed surface CCR7 and IL-7R, as well as higher levels of Bcl-2, suggestive of central origin and pro-survival phenotype. Such expression was not paralleled within antigen-specific cells initially, however the IL-7R/Bcl-2 phenotype became increasingly prevalent within this population over time, suggesting an initial effector phenotype which gradually matured into a survival-prone long-lived set-up. In addition, antigen-specific cells were highly activated and proliferative whereas the bystander population was quiescent, suggesting the increase in bystander cells was not a proliferative phenomenon, rather a mobilisation from central niche. This draws obvious and interesting parallels with mobilisation phenomena within the B-cell compartment, however the apoptotic potential of the two cell populations differ, according to our study and that of previous authors (367). The potential function of this recirculation of established memory cells would be the surveillance of lymphoid tissue for relevant antigen or to provide space within central niche.

1.6 Studying vaccine response within human lymph nodes

Research detailing the response to vaccination within human lymph nodes is sparse. A group developing melanoma cancer vaccines lead by Dr Craig Slingluff (University of Vaginia, VA, USA) has used cell responses within lymph nodes to direct vaccine development. Cells isolated from vaccine draining lymph nodes from 5 patients with stage 4 melanoma were analysed for cytotoxic CD8+ T-cell response following vaccination against melanoma associated antigens (499); their strategy more readily identified cells responding to vaccine peptides within lymph node derived cells than matched peripheral blood. Their premise was that looking within vaccine-draining lymph nodes would give a more complete picture of the immunogenicity of a vaccine and therefore guide vaccine development.

Later publication by the same group details their experience of applying this technique to 113 trial patients undergoing vaccination therapy for metastatic melanoma within 4 separate

multicentre trials (500). The authors limited their investigation to CD8+ ELISpot assays using vaccine-derived peptides as stimulus with accompanying histopathology. All patients had advanced melanoma, variably resected, and were in receipt of variable adjuvants (500). Most recently, the same group published data relating to directed T_H1 response to peptide vaccination with CD4+ cells derived from both lymph node and blood (501).

The application of analysis of lymph node derived lymphocytes in the evaluation of vaccine response to anti-cancer immunotherapy has therefore directed development of these treatment strategies. The value of studying human patients rather than rodent or simian models is the more complete modelling implicit in patients with diverse antigen experience. In addition human study avoids the uncertainty of cross-species applicability. Advantages such as these mean that human-derived data complements animal study in developing our understanding of the immune system. Despite this, ex-vivo study of human lymph nodes is limited to the studies mentioned above, which deal exclusively with melanoma derived peptide response in patients treated for advanced melanoma.

Studying the wider response to vaccine within lymph nodes of immunologically normal human patients has therefore not previously been attempted. Breast cancer patients undergo sentinel node biopsy for early stage disease prior to tumour dissemination. Therefore, in the absence of lymphatic involvement, such patients are a good model for a normal immune system (502). Development of techniques to look at antigen specificity within the lymph node derived CD4 compartment, and sub-phenotypes thereof, will provide unique insight into the establishment of a normal humeral response to vaccination. Identifying what cellular changes within the node induce desirable outcomes in terms of isotype switch and memory generation may direct novel vaccine designs which are more efficient in stimulating an effective response (503). Such models can also be used to directly assess developmental vaccine efficacy, as demonstrated by Slingluff *et al.*

Coupled with the potential for translation of such techniques into vaccine design is the more fundamental, and contemporary, interest in Follicular Helper T cells as a distinct subset of CD4 cells. Murine studies as well as studies using human tonsil tissue and peripheral blood have provided interesting clues into their role, although no information is yet available on the response of these cells within human tissue following vaccination; investigation within human patients will provide valuable opportunity to further evaluate and validate the findings of previous study. The first step in doing so is the establishment of working investigational models which can be refined according to preliminary work.

1.7 Summary

Contemporary immunological research is focused on the interactions between T and B-cell compartments, with relevance to the response to immunotherapies for autoimmune diseases and cancer. Vaccine design, and the generation of an optimal desirable effect from vaccines, requires an intimate knowledge of the instigating processes within the responding tissues. B-lymphocytes are thought to have their fates determined before leaving the germinal centre, and T-lymphocytes shape and influence the cell- and humoral response to antigen. The priming events for both of these lineages occur within the lymphatic tissues draining antigen. Through acquisition of lymphatic tissue responding to vaccine, and comparison of the cell populations within said tissue with the response observable within the peripheral blood, I hope to gain valuable information regarding the normal response to vaccination, but also the factors which influence its outcome.

2 Aims and hypothesis

2.1 Aims:

- To phenotype the lymphocyte populations within the peripheral blood and lymph node following vaccination and profile the antigen-response of the CD4+ component and the antigen specificity of the B-cell component.
    - Detailing of the immune cell response through application of three 8-colour flow cytometry panels designed to numerate the memory T-cell populations, the B-cell populations and the T_{FH} populations. This will allow the description of the immune response within the study cohort and comparison cell changes demonstrable within lymph nodes where patients have received vaccination either ipsilateral or contralateral to the node. Although much work has previously focused on changes observable within human blood, this study will be unique in detailing the parallel response within nodal tissue.
- To characterise the antibody response to vaccination and correlate "outcomes" from vaccination (antibody response, isotype profile, memory B-cell response, T-cell response) with "predictors" (circulating T_{FH} cells, pre-existing immune cells/antibody isotype profile).
    - Previous authors have demonstrated correlation between subtypes of circulating CXCR5+ cells with antibody response to antigen. Correlations have also been drawn between the emergence of such cells and the pre-existing memory cell populations (both T_{MEM} but also B_{MEM}). This study will allow analysis along similar lines with the added, novel dimension of incorporation of cellular changes within the draining or non-draining secondary lymphoid tissue.
- To identify cell-populations of interest which can then be further analysed using techniques such as RNA expression profiling and ChIP sequencing.
    - Defining "cells of interest" will follow achievement of the primary two aims. Further analysis of these populations with the techniques mentioned will provide valuable information regarding these populations' transcription profile as well as the epigenetic changes important in fate determination.

2.2 Hypothesis:

The central hypothesis is that lymphocyte phenotypic profile and relative proportions of antigen specific B- and T-lymphocytes within the lymph node will reflect changes in cell populations and antibody response describable within the peripheral blood. Furthermore, population differences will be appreciable between vaccine-draining and non-vaccine-draining

lymph nodes. It is hypothesised that key lymph node resident cell types will be identifiable based on phenotypic profile which, when subjected to expression profiling, will demonstrate functions which reflect the antibody/cellular responses detailed within the peripheral blood.

3 Study Design, Development and Regulatory Approval

"Understanding immunity in lymph nodes: correlation between tissue and blood effects" (Short title: "Understanding immunity in lymph nodes") was set up as a randomised peri-surgical window study. The principle objective of this study was evaluation of the adaptive-immune sequel following vaccination through collection of blood and lymph node samples from women undergoing axillary sentinel lymph node biopsy during the treatment of breast cancer. The two principle foci of investigation centred upon the role of lymph node-resident and circulating follicular CD4+ helper-T cells (T_{FH} cells) in the developing humoral response to vaccination and bystander activation of cellular and humoral immune compartments within secondary lymphoid tissue. This section outlines the study's design and the rationale for decisions made with regards to protocol details. The second part of this section then details the development process and the necessary regulatory approvals which were obtained. Copies of submitted forms and finalised study-specific documents are included within Appendix A.**Study Protocol**

3.1.1 Study Overview

This study coordinated routine preventative vaccination using licenced vaccines containing tetanus toxoid with sentinel lymph node biopsy (SLNB) at a time-point when SLNB is clinically required for the staging of patients' breast cancer.

Patients diagnosed with breast cancer, and in whom a SLNB was planned as part of surgical treatment, were considered for study recruitment. Patients who had not received tetanus vaccination within the last 10 years were eligible for inclusion. Those patients who were willing to participate were randomised into one of three groups; test group, control group 1 or control group 2 (see "Study Population" below). Patients randomised to test group or control group 1 received a vaccine containing tetanus toxoid when they attended the pre-admission clinic visit approximately one week before surgery. All participants had a blood sample taken at the same pre-admission visit for study purposes.

At SLNB surgery, a second blood sample was obtained, and two further blood samples were collected post-surgery. The SLNB was performed as usual, but with a single additional lymph node taken for study purposes.

To reduce the risk of losing prognostic information from the study node in the event of a positive SLN biopsy, the sentinel nodes were processed on the same day as surgery using quantitative RT-PCR (qRT-PCR). In the event of a SLN biopsy positive for metastasis, the non-sentinel node taken for the purpose of the study was surrendered to the hospital pathologist for standard clinical processing (together with specimen(s) taken following completion axillary

clearance). If the sentinel node biopsy was negative for cancer the non-sentinel study node was submitted for research and processed according to protocols optimised using anonymously donated lymphatic tissue, stored within the Southampton Human Tissue Bank (HTA Licence Number 12009).

Once the tumour was excised, 2 core needle biopsy samples were taken from the tumour prior to it being submitted for histological assessment.

3.1.2 Study population:

Test group: Patients diagnosed with breast cancer in whom a SLNB was planned as part of surgical treatment received a vaccination against tetanus/diphtheria/polio administered subcutaneously into the arm on the same side as the surgery.

Control group 1: Patients diagnosed with breast cancer in whom a SLNB was planned as part of surgical treatment received a vaccination against tetanus/diphtheria/polio administered intramuscularly into the arm on the opposite side as the surgery.

Control group 2: Patients diagnosed with breast cancer in whom a SLNB was planned as part of surgical treatment received no vaccination prior to surgery.

3.1.3 Study plan: (See Appendix B)

Time point 1, Week 0: A blood sample was taken at the pre-admission clinic visit. Patients randomised to test group or control group 1 received a single, standard dose of a vaccine against tetanus/diphtheria/polio.

Time point 2, Week 1: A 2nd blood sample was taken at this time.

At surgery, SLNB was performed as usual, with one additional LN taken for study purposes. Prior to processing the additional non-sentinel node, the sentinel node(s) was analysed to ensure there was no evidence of metastatic disease. In the event of metastatic disease within the sentinel node(s), the node taken for study purposes was surrendered to the hospital pathologist for standard clinical procession rather than research. In addition, 2 core needle biopsy samples were taken from the tumour specimen for study purposes. One was snap frozen, whilst the other was lysed in RLT buffer (Qiagen, Düsseldorf, Germany) supplemented with 1% β-Mercaptoethanol.

In addition, homogenate generated for the purposes of the sentinel node biopsy, which remains surplus to clinical requirements, was retained for the purpose of the study

Time point 3, Week 3: Patients with positive sentinel nodes were informed that their node was not used for research and given the option to exit the study at this stage. In all remaining participants a 3rd blood sample was taken at the post-operative visit two weeks following surgery.

Time point 4, Week 6-7: A 4th and final blood sample was taken at 6-7 weeks post-vaccination. In addition, simple histopathological details were recorded.

3.1.4 Clinical Sampling

3.1.4.1 Patient identification and recruitment

Eligible patients were identified from the outpatient clinics at University Hospital Southampton or Princess Anne Hospital by members of the Breast Cancer Multidisciplinary Team. Patient information sheets were provided to eligible patients inviting them to consider participation. All patients were given a minimum of 24 hours to consider entry to the study. Interpreters were provided where possible for participants who were non-English speakers. Having received prior written and verbal explanation of the study, patients who wished to participate were required to provide written informed consent. Consent included permission for storage of samples for analyses relating to this study and for the use of FFPE histopathology material, where adequate surplus material existed following completion of full diagnostic work. Informed consent was obtained by members of the research team.

3.1.4.2 Inclusion criteria

- Patients due to undergo Sentinel Lymph Node Biopsy for breast cancer staging.
- Patients aged 18 years or older.
- Patients with the ability to understand the study requirements, provide written informed consent and comply with the study protocol procedures.

3.1.4.3 Exclusion criteria

- Presence of axillary node disease at presentation
- Presence of metastatic disease at presentation
- Treatment with primary hormone or chemotherapy prior to surgery
- Taking of immune-suppressing medications including oral, but not inhaled, steroids
- Taking or oral anticoagulants precluding intra-muscular injections
- History of hypersensitivity to tetanus containing vaccines
- History of previous malignancy (excluding previously treated skin basal cell carcinoma or squamous cell carcinoma)
- Vaccination with tetanus-containing vaccine within the preceding 10 years

3.1.4.4 End of Study Participation

Patients' active participation in the study ceased following the collection of the 4th blood sample at week 6-7 post-vaccination. Patients were asked to consent to the collection of clinical and histopathological data by research staff which continued following completion of patient's active participation.

3.1.5 Clinical and histopathological data collection

Where available, clinical and histopathological information was extracted and recorded onto study specific case report forms (CRF) by a member of the CRUK Clinical Research Unit. Data points recorded included age at diagnosis, tumour characteristics (such as grade, size, histological sub-type, hormone receptor status and Human epidermal growth factor receptor 2 (HER2) expression status).

Data were extracted from patients clinical records (generally from computer based records of histopathology reports) held by Southampton University Hospitals NHS Trust by clinically trained research personnel with authorisation to access such details and stored within a secure facility within the Southampton CRUK Clinical Centre. Data were anonymised at the point of entry onto the CRF to allow linkeage to the relevant sample, but with all identifying information removed. Patients were asked to consent to the research team contacting their general practitioner in order to establish their vaccination history.

3.1.6 Sample processing and storage

All samples were anonymised upon receipt in the laboratory and identified only by a unique patient identification number according to the standard operating procedure in place for this purpose (CSD/SOP/003). The sample number and date of receipt were also recorded. On conclusion of the study any remaining samples or tissue were donated to the University of Southampton Research Tissue Bank (Human Tissue Act License: 12009, Southampton and South West Hampshire Research Ethics Committee: 280/99).

3.1.6.1 Blood collection

Each blood collection consisted of 60mL anti-coagulated blood and 10mL clotted blood.

The total number of venesections were 4 over a period of 6-7 weeks amounting to 280mL of blood in total. Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated blood by centrifugation over Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and either used immediately or cryopreserved in 50% decomplemented human Ab serum, 40% complete RPMI 1640 medium and 10% DMSO. Cells were transferred to liquid nitrogen after 24 hours. Serum was isolated from clotted blood samples by centrifugation and stored at -80°C.

3.1.6.2 Processing Study Tissue:

A single, additional non-sentinel LN was taken during surgery following the SLNB by the responsible surgeon. The study node was kept in complete RPMI 1640 medium supplemented with antibiotics (penicillin and streptomycin) whilst the sentinel node was processed by the molecular pathology department of University Hospital Southampton according to clinically validated standardised protocol using quantitative RT-PCR to identify the presence or absence of metastasis.

In the event of the sentinel nodes proving negative for metastasis the study node was processed fresh on the day of surgery by dissociation into a single cell suspension using a validated technique optimised using donated tissue from the Southampton Human Tissue Bank (see section 5.1). Cells were cryopreserved as per PBMCs (see section 3.1.6.1).

Two core needle biopsies were taken from the tumour specimen following surgical excision and prior to preservation in formalin. No additional tissue was removed from the patient. One was snap frozen, whilst the other was lysed in RLT buffer (Qiagen, Düsseldorf, Germany) supplemented with 1% β-Mercaptoethanol. The tumour specimen itself was formalin fixed, paraffin embedded prior to histological examination. Tissue surplus to clinicopathological requirements was made available for the purposes of the study.

Following SLNB, the sentinel node was processed by the hospital pathology laboratory according to their validated clinical protocols. Briefly each node was sectioned at 2mm intervals, with alternate sections being homogenised into RLT buffer (Qiagen, Düsseldorf, Germany) supplemented with 1% β-Mercaptoethanol. The homogenate was then subjected to RNA extraction and analysed using qRT-PCR for markers indicative of metastatic involvement of the node. The homogenate surplus to requirements was frozen at minus 80°C. The sections which were not homogenated were formalin fixed, paraffin embedded and examined within the histopathology laboratory. Both the tissue slides and homogenate were available for use for the purpose of the study in the event of material being surplus to the clinical requirements of the patient.

Patient material collected during this study was stored within a secure facility in the Southampton CRUK Clinical Centre. Material was held as linked anonymised samples and labelled with a study specific number. The chief investigators and co-investigators had access to the samples for analyses relating to this study. Patients were asked to provide signed consent for the indefinite storage of samples for use by the investigators for analyses relating to the objectives of this study, or in the event of samples or tissue remaining on conclusion of

the study, for donation to the University of Southampton Tissue bank for use in future ethically approved research.

3.1.6.3 Use of Banked Human Tissue:

The human tissue bank held on site within the University of Southampton stores samples of lymphatic tissue including lymph nodes from non-breast cancer patients. Patients donating these specimens have consented to their use in relevant, ethically approved studies.

The techniques and assays intended for use during the main work of the study were first validated using this anonymous tissue which was surplus to clinical or diagnostic requirements.

3.1.6.4 Data Storage

All essential documents, including source documents, will be retained for a minimum period of 15 years following the end of the study. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. The Chief Investigators and Co-Investigators will have access to the data for analyses.

Patient confidentiality was maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples. No personally identifying information was released in any report or publication relating to this work.

Data was collected and retained in accordance with the Data Protection Act 1998.

3.1.6.5 Monitoring and audit

The study was monitored and audited in accordance with UHS procedures. All trial related documents will be made available on request for monitoring and audit by the relevant REC and other licensing bodies in the event that external audit be deemed necessary.

3.1.6.6 Ethics and R&D approval

The study has been performed following Research Ethics Committee (REC) approval, Site Specific Assessment and local Research and Development approval.

3.1.6.7 Research governance

The study was conducted in accordance with The Medicine for Human Use (Clinical Trial) Amendment Regulations 2006 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care.

3.1.6.8 Indemnity

This was an NHS-sponsored research study. For NHS sponsored research, HSG(96)48 reference no. 2 applies. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS Indemnity covers NHS staff, medical academic staff with honorary contracts, and those conducting the trial. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim.

3.2 Rationale of protocol design

3.2.1 Inclusion and exclusion criteria

During the design phase of the study, the inclusion and exclusion criteria were set to allow recruitment of the widest possible set of suitable patients. The target population were patients undergoing axillary sentinel node biopsy. However the study aimed to investigate the immunological sequeli of a recall (or memory) response to vaccination in the presence of cancer, therefore it was important to ensure patients were not immune compromised from other innate or external factors.

The inclusion criteria selected (see 3.1.4.2) define a broad potential cohort of the majority of patients who are to undergo SLNB and who are therefore suitable for recruitment. We used the term “Sentinel lymph node biopsy for cancer staging” as this can be considered inclusive of said procedure being undertaken in patients with both invasive disease and pre-invasive disease (such as ductal carcinoma in situ). We also avoided specifying the type of cancer; although our primary intention is to investigate immune response to vaccination in the presence of breast cancer, other cancers, such as melanoma, also use SLNB as a technique for staging. In the event of findings/results requiring further validation within another disease-setting, the broad inclusion criteria would allow the study to shift to recruitment of patients with non-breast cancer with minimal additional regulatory approval. This concept was also applied when writing the wider protocol, Research Ethics Committee (REC) application and Research and Development (R&D) documentation, and when using non-gender specific language throughout.

We wished recruitment to be unrestricted according to patient age, however to simplify Research Ethics Committee considerations recruitment was limited to patients over 18. In practical terms, it is unlikely that such restriction would limit recruitment considering the more typical age-demographics associated with cancers managed using SLNB. Similarly, recruitment was limited to those able to give full consent for inclusion within the study without background

of cognitive impediment or risk-factors for vulnerability, such as incompletely treated mental health diagnoses.

Given the wide scope of the inclusion criteria, restriction of patients unsuitable for recruitment was necessary through application of exclusion criteria (see 3.1.4.3). Sentinel node biopsy is typically reserved for patients who do not have any evidence of lymph node involvement following pre-operative axillary ultrasound assessment with or without cytological or histological analysis as appropriate (504). For clarity, we included an exclusion criteria stating explicitly that presence of nodal disease at presentation precluded the patient from being recruited into the study. In addition, patients with metastatic disease are often not considered appropriate for sentinel lymph node biopsy. Again, for clarity, such patients were specifically excluded from recruitment. Exclusion of such patients was considered extremely important, since use of an axillary node in these patients could conceivably hinder the histological staging of these patients following lymph node block dissection (i.e. axillary node clearance in the case of breast cancer patients).

Patients undergoing primary chemotherapy prior to surgical management were also excluded from recruitment. Use of SLNB in patients who have received primary chemotherapy is contentious (505-508) however it is increasingly being accepted in selected patients who presented with node-negative disease prior to commencement of primary chemotherapy. Given the deleterious effect chemotherapy has been demonstrated to have on immune response to vaccination (509), exclusion of such patients was deemed prudent.

Given the primary objectives of the study were to evaluate the normal immune response to vaccination steps were taken to exclude patients likely to mount an altered or atypical response to vaccination. Therefore those taking immune-suppressing or immune-modulating medications including oral steroids were excluded from participation. Patients taking inhaled steroids were eligible due to the low systemic availability of such preparations. Although cancer itself is considered an immune-modulating disease (510-513) through selection of patients who have not developed lymphatic spread (i.e. those thus far resistant to metastatic dissemination) it was hoped that the patient cohort recruited would have an immune system as close to “normal” as possible. Supporting the use of breast cancer patients as suitable models of a “normal” immune-response are two pilot studies demonstrating an immune-response to vaccination comparable to that of normal controls (502, 514); the limitation of extrapolating said previous work to our own is our intention to study the cellular-response to vaccine within draining lymph nodes, which could reasonably be considered to be more

directly influenced by tumour-derived factors. This influenced the design of our study in terms of our choice on control cohorts (see 3.2.3).

In an effort to normalise a baseline for all patients, those who have received a tetanus-containing vaccine within ten years were excluded. The rationale for this was to attempt to ensure that patients recently exposed to tetanus toxoid antigen, who would be expected to have a high baseline and more exaggerated response to antigen re-challenge, were excluded. Those patients remaining would, hopefully, give a more uniform "long-term recall" response to antigen re-challenge.

At the time of the studies conception, a key point of interest was the immune-response to Tumour Associated Antigens (TAAs) and how that might be influenced by concomitant vaccination to unrelated peptides. In an effort to simplify the model as much as possible, patients with previous history of malignancy were excluded from recruitment. This was to avoid previous anti-tumour antigen responses against their original cancer manifesting as spurious results. Prior history of squamous cell carcinoma and basal cell carcinoma of the skin was not thought to be relevant in this context and therefore was not considered to constitute a reason for exclusion.

Consideration was given to factors likely to influence the safety of patients participating within the study. Patients on anticoagulants were considered higher risk; patients on warfarin are typically only given intramuscular injections cautiously and when required. Some manufacturers recommend use of subcutaneous vaccination in preference to intramuscular inoculation to avoid deep muscular haematoma complicating intramuscular injection (515). In all cases, patients coagulation status (i.e. an up-to-date measurement of International Normalised Ratio (INR)) would be necessary to safely administer a vaccine to a warfarinised patient, something not practically possible given the necessity to vaccinate the patient at the same time as their pre-admission clinic appointment. Therefore the decision to exclude patients taking warfarin or equivalent oral anticoagulant was made. Furthermore, patients with a self-reported history of hypersensitivity to any vaccine containing tetanus toxoid were excluded.

3.2.2 Vaccine Choice

Our group is currently working on the development of an anti-tumour vaccine against Human epidermal growth factor receptor 2 (HER2/neu), a receptor overexpressed in 20-30% of breast cancers and the therapeutic target of trastuzumab (516). Previously our group used DNA-fusion genes utilising a sequence encoding a non-pathogenic protein component of tetanus toxin (Fragment C; FrC – see also Section 4.12.1) as an immune enhancer within a vaccine

targeting B-cell lymphoma (517). A similar strategy is now being applied to develop an anti-HER2/neu vaccine for use in breast cancer. Therefore studying the response to tetanus toxoid within the context of this project should yield useful information regarding the factors implicit in promotion of a desirable adaptive immune response to vaccination, which may facilitate future vaccine design.

Tetanus toxoid vaccination is undertaken as part of the UK childhood vaccination programme and following the risk of possible exposure (518). Therefore the majority of patients within the UK will have a history of exposure to tetanus toxoid, making it a suitable candidate antigen when studying recall response. Tetanus toxoid induces both a strong cellular and humoral immune response, meaning both T-cell and B-cell reactivity can be studied. This was particularly desirable considering the attainment of a lymph node responding to vaccination would allow evaluation of cells derived from the follicular regions of the lymph node; these regions are the site of B-cell maturation and isotype switching, influenced by the actions of specialist subsets of CD4+ helper T-cells known as follicular helper T-cells (T_{FH} cells). Therefore a vaccine which induces a strong humoral response was desirable. Furthermore our research group has previous experience of using tetanus toxoid vaccine to study the bystander response to recall vaccination observed in the peripheral blood compartment (371, 493); unanswered questions from previous work include whether the increased frequency of bystander-specific CD4+ T-cells within the peripheral blood is also detectable within the lymph nodes draining vaccine, or in non-vaccine draining lymph nodes. Tetanus toxoid therefore provided an opportunity to study both questions within a single project.

Because of our intent to study the bystander response to vaccination, a simple vaccine containing a single antigen was desirable. Previous work was performed using healthy volunteers vaccinated with single-agent tetanus toxoid vaccination (371). However in the intervening years the licence for single-agent vaccines against tetanus has been withdrawn. Following discussion with the Medicines and Healthcare products Regulatory Agency (MHRA) it transpired that import and administration of such a vaccine for the purposes of such a study would not be possible (see 3.3). Therefore choice of vaccines was limited to products licenced within the UK. REVAXiS (519) was selected as the most suitable vaccine as it contained the least number of additional antigens compared with other tetanus-containing vaccines (such as REPEVAX, which is combined with additional antigens isolated from pertussis) and an equivalent dose of tetanus toxoid to that used in our groups previous studies. Components included purified diphtheria toxoid (minimum 2 international units (IU)), purified tetanus toxoid (minimum 20 IU), inactivated polio virus type 1 (40D antigen units), inactivated polio

virus type 2 (8D antigen units), inactivated polio virus type 3 (32D antigen units), aluminium hydroxide (0.35mg), phenoxyethanol, formaldehyde and medium 199 (a complex of amino acids, mineral salts, vitamins, polysorbate 80 dissolved in water, as well as other substances (519)).

The choice of REVAXiS was also supported by the low population prevalence of all three constitutive pathogens within the UK. This minimised the possibility of unrecognised exposure within the 10 years preceding recruitment.

3.2.3 Control groups and vaccination sites

The primary aim of the study was to compare the adaptive immune response within a vaccine draining lymph node with that seen in the peripheral blood compartment. An important comparator would therefore be the cellular changes seen within the lymph node during systemic response to vaccination i.e. the changes within a lymph node following vaccination at a site distant from its lymphatic drainage. In addition since the patients would be undergoing surgery (an external, pro-inflammatory stimulus) recruitment of patients undergoing surgery, but who do not receive vaccination, would be required. Therefore three groups were planned:

Test group: 30 patients diagnosed with cancer in whom a SLNB is planned as part of surgical treatment. These patients receive a vaccination against tetanus administered into the arm on the same side as the surgery.

Control group 1: 15 patients diagnosed with cancer in whom a SLNB is planned as part of surgical treatment. These patients receive a vaccination against tetanus administered into the arm on the opposite side as the surgery.

Control group 2: 15 patients diagnosed with cancer in whom a SLNB is planned as part of surgical treatment. These patients receive no vaccination prior to surgery vaccination.

Vaccine administration is typically through deep intramuscular injection, most commonly within the deltoid or superolateral aspect of the gluteal muscle. Lymphatic drainage of the deltoid is variable; although some lymphatic drainage through the lateral group of axillary lymph nodes is typical, primary drainage is through the deltoideopectorial glands (sited alongside the cephalic vein, between Pectoralis major and Deltoid, immediately deep to the clavicle) (520). From the deltoideopectorial glands, drainage is to the subclavicular lymph node group, deep to the base of the axilla (520).

An alternative administration strategy is via deep subcutaneous injection; most vaccines recommend intramuscular injection due to the reduced incidence of injection-site reactions.

However the majority of vaccines are suitable for subcutaneous administration since this route is utilised in patients at high risk of bleeding complications. Available data suggests no difference in the systemic effect of vaccination between the two routes following vaccination (521). The lymphatic drainage of the skin of the medial aspect of the upper arm drains more reliably to the lateral group of axillary lymph nodes (520); this is desirable considering the aim of the study is to analyse the cellular response within vaccine-draining lymph nodes. The position of lymph nodes draining the vaccine following subcutaneous injection within the medial aspect of the upper arm (i.e. lateral axilla) is also more safely accessible during surgery than nodes within the subclavicular group.

For these reasons it was decided that patients receiving the ipsilateral vaccination ("Ipsilateral Cohort" – IC) would receive that vaccination at an "atypical" injection site, i.e. subcutaneous, at the medial aspect of the upper arm. The added discomfort incurred by such a strategy to participants was considered justifiable considering the reasons mentioned. The additional incidence of injection-site reactions was highlighted within the REC submission prior to approval, and sufficient mention of said reactions was made within the information leaflets provided to potential participants (see appendix A.8.).

Although theoretically vaccination subcutaneously compared to intramuscularly might influence the mode of antigenic presentation to the cells within the lymph nodes, this was considered an insufficient reason to subject the contralateral-immunisation-control-cohort ("Contralateral Cohort" – CC) to the added incidence of injection site reactions. Therefore patients within CC were to receive vaccination via intramuscular injection. Including the CC cohort allows evaluation of the "systemic" lymphatic response to vaccination whilst controlling for changes induced by the vaccine which are not dependent on antigen drainage. The non-vaccinated cohort controls for both groups by providing "normal resting" lymph node cells for comparison and detail of post-surgical change within the peripheral blood in the absence of vaccination.

3.2.4 Recruitment and Randomisation

Consideration was given to two recruitment strategies (see Figure 2); initially, the possibility of recruiting all patients suitable to the study regardless of vaccination history, then allocation according to the strategy outlined within Figure 2a. was considered. Although advantageous in terms of total potential numbers of patients available for recruitment, this strategy was considered flawed in terms of the likely temporal bias toward the non-vaccinated cohort (NC). More rapid recruitment into NC would have left the study vulnerable to confounding variables such as changes in prevalence of different community-acquired antigenic stimuli (such as the

seasonal flu virus, rotavirus, etc.) and operator-dependent factors (i.e. changes in anaesthetic/surgical approach etc.).

Although such factors were considered to have a marginal risk of influencing results, the alternate strategy outlined in Figure 2b. was deemed to be more scientifically sound. By recruiting only patients who had not received a tetanus-containing vaccination in the last 10 years to all three cohorts normalised the baseline in all groups insofar as possible. Furthermore it avoided the possibility of confounding bias as detailed above.

Randomisation was undertaken following consultation with a medical statistician. Blinding of the study co-ordinator was deemed impractical since assay design required knowledge of the vaccination state. However research nurses were blinded to which group a patient was to be assigned up to the point of recruitment to avoid unduly influencing patient choice as to whether to consent to the study. A randomised allocation list was generated by the medical statistician kept as a locked file by the study co-ordinator. Following patient recruitment the recruiting research nurse would contact the study co-ordinator and be informed of the patients' allocation.

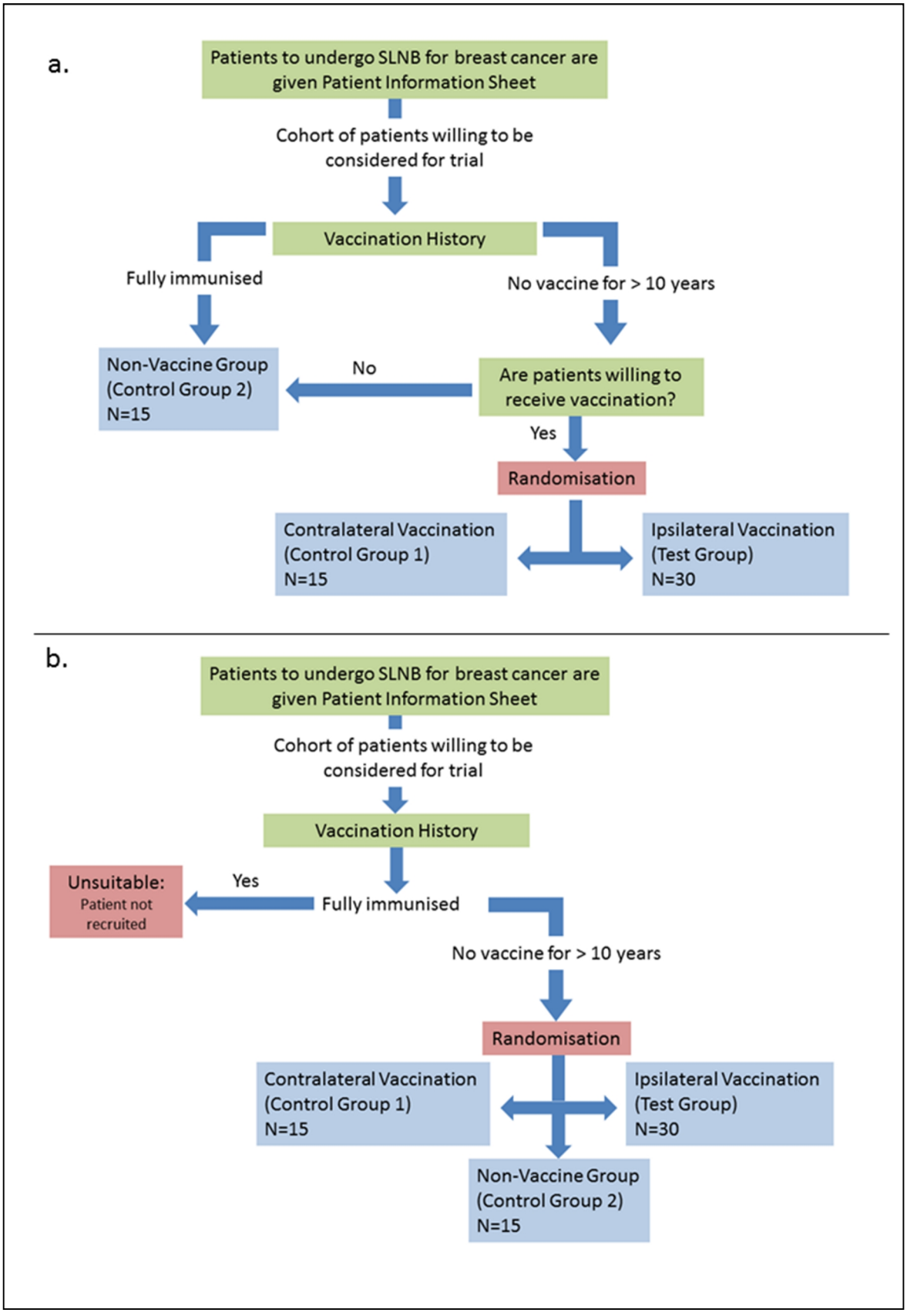


Figure 2: Recruitment and Randomisation strategies. a. Initial strategy was designed to recruit the widest scope of patients and reserved randomisation for those patients willing/suitable for vaccination. b. Final adopted strategy randomised all patients recruited.

3.2.5 Timing of recruitment, vaccination and subsequent blood samples

Due to patient considerations, blood and tissue collection time points were dictated by normal clinical care and taken opportunistically at the time of routine perioperative appointments whenever possible.

Due to the necessity to recruit and immunise a patient in the time between the decision to operate being taken and the operation taking place, the most pragmatic solution was to attempt to recruit patients at the time of their pre-admission clinic appointment. Due to centralised government targets set for maximum waiting times between diagnosis and first definitive treatment of any cancer (522), patients are typically operated on quickly leaving a potential pre-operative window of less than two weeks. Pre-admission clinics are planned to take place one week prior to surgery, however logistically there is a lot of variability with many patients undergoing pre-admission assessment less than one week prior to surgery. Altering patients planned dates of surgery in the case of inconvenient timings was deemed inappropriate. However for the purposes of the study, and to ensure that interpretable date could be derived from across the cohort, it was desirable restrict the timeframe between vaccination and surgery. Therefore for sake of feasibility, patients recruited into the study were required to undergo pre-admission assessment within 5-8 days of their surgery, with the optimum target of 7 days. Where possible, pre-assessment dates were changed to fit this ideal, with the strict provision that there was no possibility of negatively influencing the patients preparation for surgery, and that the patients operation would not be delayed. Therefore some patients were excluded from the study if their pre-admission assessment appointment was too close to their planned date of surgery and no alternative date for their pre-admission assessment was possible.

The third time point was planned to coincide with the patients' standard post-operative appointment (2 weeks following surgery). As well as being convenient for the patient, the three-week post-vaccination time point was considered to be optimal for assessment of the cell-mediated response to vaccination, taking into account previous findings (371). The final time point was planned for 6-7 weeks post vaccination; this time point was selected to avoid confounding influence from post-operative adjuvant chemotherapy/radiotherapy, which typically was not commenced until this point in time. Therefore the study aimed to take final blood samples prior to onset of adjuvant therapy. Additionally, this time point coincided with peak cell-mediated response seen within a subset of patients (termed "late responders") observed within our groups previous study (371).

3.2.6 Removal of an additional non-sentinel lymph node

A breast sentinel node biopsy generally involves the removal of between one and three sentinel nodes (mean two) to stage the axilla. However, prior to the introduction of SLNB, an axillary clearance was performed on all breast cancer patients, which involves the removal of all LNs within a defined anatomical boundary, typically between10 to 20. Modern surgical practice is to perform SLNB on all suitable patients in an attempt to avoid the more extensive clearance procedure and limit procedural-associated morbidity whilst still providing accurate staging of the axilla (504). The study requires the removal of a single additional lymph node at the time of surgery. Theoretically the negative consequences of removing an extra node might include a possible increase in risk of upper limb morbidity such as lymphoedema, shoulder pain/stiffness and intercostobrachial nerve injury.

Comparison of patient-reported quality-of-life outcomes between SLNB and axillary clearance does demonstrate differences, but these are shown to resolve with time (523). Similarly, the ALMANAC trial demonstrates differences in morbidity between the two surgical approaches, but the rate of arm lymphoedema, as measured by mean change in arm volume, was not significantly greater with axillary clearance compared to SLNB at 12 months (524). Therefore removal of a single, additional non-sentinel node at the time of SLNB is felt unlikely to cause significant additional morbidity.

With regards to the potential for under-staging, in sentinel node negative cases, no further nodes would normally be removed so removal of a single non-sentinel node will not detract from the axillary staging. In node positive cases patients would normally proceed to axillary clearance and the total number of involved nodes would be taken into account when staging the axilla. For the purposes of the study, the non-sentinel lymph node removed for analysis would not be processed until intra-operative analysis of the sentinel lymph nodes had taken place. Therefore in the event of a positive sentinel node identified intra-operatively, the study node would be returned for inclusion with the subsequent axillary clearance sample.

Intra-operative analysis of sentinel lymph nodes is associated with a false-negative rate, the precise value of which is technique dependent (525). Therefore in the event of a false-negative sentinel node result, the use of the non-sentinel node for the purposes of the study would result in the loss of staging information from that node during a subsequent completion axillary clearance. However data from a trial of axillary radiotherapy demonstrates that avoiding an axillary clearance does not alter decision making regarding adjuvant therapies (526). Therefore loss of data from a single node in a minority of patients within our study is

unlikely to negatively influence the provision of care to these patients. On this basis we successfully applied for and were granted Research Ethics Committee approval for the study.

3.2.7 Core-biopsy of tumour specimen

At the time of surgery two 14G core needle biopsy specimens are taken. Each core needle biopsy will remove only a small quantity of tissue which will not hinder histological staging of the tumour. Removal of tumour tissue in this fashion is standard practice in several large multi-centre peri-surgical studies, for example the POETIC breast cancer trial (527).

3.3 Study timeline, regulatory approvals and commencement of recruitment

The study was set up in line with local (528) and national guidelines (529) on the establishment of clinical study. Following initial conception in March 2011 recruitment opened in July 2013. This sub-section and accompanying appendices detail the steps taken to attain regulatory approval. Figure 3 details the major milestones leading to commencement of recruitment. The final protocol as detailed above in Section 3.1 evolved throughout the process. For clarity only the final versions of the Protocol, Patient Information Sheet, GP Information sheet, Consent Form and Case Report Form are included within the Appendices (See Appendix A.8.).

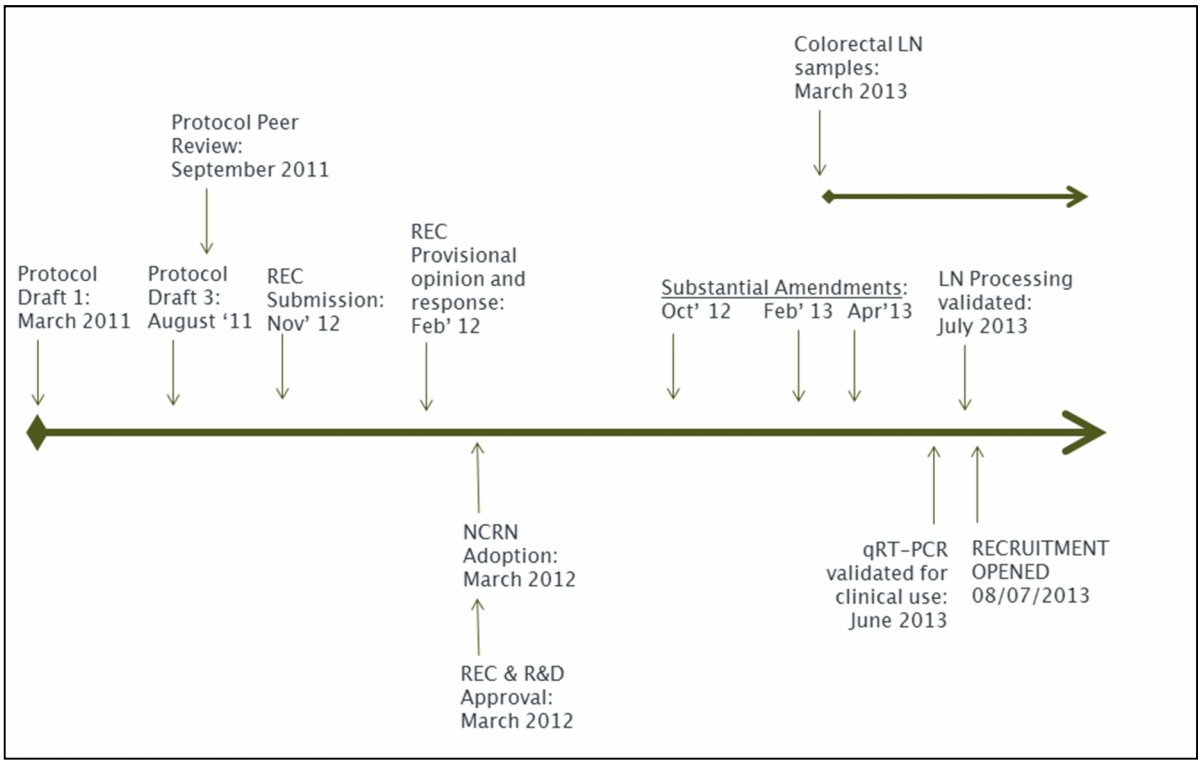


Figure 3: Timeline of study set-up detailing major milestones between the completion of the first draft protocol (March 2011) and the commencement of recruitment (July 2013).

3.3.1 Local protocol review, approval and sponsorship agreement

Prior to central approval through completion of the Integrated Research Application System (IRAS) it was necessary to secure a sponsorship agreement. A condition for all research conducted within the National Health Service (NHS) is the securement of a sponsor, as stipulated within the Research Governance Framework for Health and Social Care (RGFHSC), second edition (530). The role of a sponsor is to ensure that proper arrangements are in place to initiate, manage, monitor, finance, deliver and report proposed research. In addition, the sponsor takes on responsibility for reviewing and approving any modifications in the research plan prior to the attainment of any necessary approval from a regulatory authority (530). In the absence of an external third party sponsor, University Hospital Southampton NHS Foundation Trust will act as a research sponsor, subject to certain criteria (531). As a qualifying study, NHS sponsorship of the project was sought.

A key responsibility of a research sponsor is to undertake "an appropriate process of independent expert review [which] has demonstrated the research proposal to be worthwhile, of high scientific quality and good value for money." (quoted from RGFHSC (530)). Requirements for application for NHS sponsorship therefore included submission of the study protocol together with evidence of independent scientific peer review. The necessary application forms and subsequent approval documentation is included within Appendix A.1.

3.3.2 Radiation Protection concerns and Environment Agency approval

Following peer review of the protocol, concerns were raised regarding the handling of tissues recently removed from patients administered a radioisotope. Sentinel lymph node biopsy utilises albumin colloid radiolabeled with technetium-99 (^{99m}Tc) (^{99m}Tc Human Serum Albumin nanocolloid) administered via subcutaneous injection into the breast. Radioactivity is traceable intraoperatively using a gamma probe and used to identify specific lymph nodes with a high uptake of the radio-tracer. These node(s) are termed the "sentinel lymph node(s)" and analysed for the presence of tumour spread. Non-sentinel lymph nodes therefore, by definition, have a low uptake of radioisotope.

^{99m}Tc has a physical half-life of 6.0058 hours (532, 533) meaning 93.7% of ^{99m}Tc will decay to ^{99}Tc within 24 hours. There are no restrictions placed on patient activities following administration of radioisotope due to the low dose administered and the rapid rate of decay. However although the exposure to laboratory workers, operating theatre staff and patient is considered low (533) standard practice within our institution and nationally is to ensure all potentially contaminated material (including patient derived tissue) is kept for a minimum of 72 hours to allow radioisotope decay, prior to disposal.

To address the concerns raised following protocol-peer review we contacted the Environment Agency through the University of Southampton's Radiation Protection Advisor. Following the application for, and approval of, a variation in the University's Environmental Permit through the Environment Agency, we received approval to progress with the study. A copy of the Radiation Protection Advisors notice is included within Appendix A.2.

3.3.3 National Institute for Health Research Comprehensive Research Network portfolio adoption and Research Ethics Committee submission through the Integrated Research Application System

In order to access funding available to support research nurse and service support, it was necessary to apply for National Institute for Health Research (NIHR) Comprehensive Research Network (CRN) portfolio adoption status.

In addition, for the study to go ahead the study plan had to be reviewed by a NHS Research Ethics Committee (REC); under UK legislation any study conducted within the NHS (involving NHS patients or their carers) which involves the collection of patient information or the analysis of human tissue must be approved by a Research Ethics Committee (534). The purpose of REC review is to ensure the ethical conduct of research with specific focus on ensuring minimal risk and preservation of patient dignity, rights, safety and well-being. To this end all study-related documents, including patient literature, consent forms, protocol

documents and case-report forms are reviewed, along with documents demonstrating local peer-review, adequate available funding, sponsorship agreement in principle and established indemnity policy are included with a completed, structured application form.

Application for NIHR CRN adoption and REC approval were completed and submitted through the Integrated Research Application System (IRAS) run by the Health Research Authority (HRA). Original documentation submitted is included within Appendix A.3. Following initial application and meeting with the REC, queries were raised in the form of a Provisional Opinion from the REC – see Appendix A.3.III.

The majority of queries raised were minor, requiring simple alterations of the patient literature. Concern was raised regarding cohort allocation; following this query the decision was made to implement randomisation across the study population, as detailed in Section 3.2.4. The committee raised the question of histological analysis of the non-sentinel node (see the third paragraph on page 3 of the Provisional Opinion Document, Appendix A.3.III); the response to the query made during the meeting was misinterpreted by the committee. Clarification was made during subsequent correspondence that in the absence of a positive result from the sentinel node biopsy, no additional analysis (histological or cytological) would be performed on the non-sentinel node. The rationale for this was that doing so would constitute a deviation from normal staging practice and the clinical significance or otherwise of any result taken from the non-sentinel study node would therefore be unknown.

The issue remaining unaddressed was the REC's concern that the study would be better classified as a Clinical Trial of an Investigational Medicinal Product (CTIMP):

3.3.4 Correspondence with MHRA – confirmation study did not constitute a CTIMP

A CTIMP is defined as follows by the National Institute for Health Research (535):

> *"An investigation in human subjects, other than a non-interventional trial, intended:*
>
> *a) to discover or verify the clinical, pharmacological and/or other pharmacodynamic effects of one or more medicinal products,*
>
> *b) to identify any adverse reactions, or*
>
> *c) to study absorption, distribution, metabolism and excretion, with the object of ascertaining the safety and/or efficacy of those products."*

Classification of a study as a CTIMP infers additional regulation subject to the European Commission Directive 2001/20/EC ("The Clinical Trials Directive") implemented through The Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 1031) (536). The Medicines and

Healthcare Products Regulatory Agency (MHRA) provides a tool to help define whether a clinical study should be subject to clinical trials regulations (537).

As our intent was to use a tetanus containing vaccine to induce an immune response in order to study the nature of the response, we felt that classification as a CTIMP was inappropriate. Since the efficacy of the vaccine was not under question (the efficacy was considered proven as the products are all in common use) and we had no intention of comparing vaccine efficacy we felt we did not meet the criteria for classification as a CTIMP. Following correspondence with the Clinical Trail Helpline at the MHRA, we received confirmation of our position which was fed back to the REC (see Appendix A.3.IV.).

3.3.5 Receipt of favourable opinion, R&D approval and completion of set-up

Confirmation of a favourable REC opinion was received following the correspondence detailed above. The favourable opinion letter is included within Appendix A.3.V.

Following completion of REC approval, submission through IRAS for local Research and Development (R&D) was made, inclusive of Site-Specific Information (SSI) form, additional supportive documentation and NHS costings, as contained within Appendix A.4. R&D application ensures the legal obligations of the site of research are adhered to, as per the guidance issues within the Research Governance Framework (530). As such, the sponsorship agreement and REC approval are subject to satisfactory application for R&D approval.

Following successful completion of the R&D application process the study was approved for commencement of recruitment.

3.3.6 Delayed commencement of recruitment: Rationale

Recruitment was delayed following successful completion of NIHR CRN, REC and R&D approval process for 16 months (see Figure 3). Partly this was to allow time for the study co-ordinator to apply for admission as a post-graduate student at the University of Southampton (enrolment October 2012). However additional factors subsequently made delay in recruitment desirable:

3.3.6.1 Substantial amendments: Rationale and submission

Once a protocol has been granted REC and R&D approval, any substantial changes to the planned protocol or documentation relating to the study have to be ratified by the same regulatory authorities. Guidelines are available regarding what constitutes a substantial, and what constitutes a non-substantial amendment (538).

The process requires initial review of the changes by the study sponsor. Once local approval for the changes has been attained, application to the REC can be made using the standardised "Notice of Substantial Amendment Form". Following successful outcome, the REC will issue a

"Notice of Favourable Ethical Opinion" which can then be forwarded to the R&D department overseeing the study. Only following acceptance by the local R&D department can the changes be implemented.

Prior to commencement of recruitment, it became necessary to make three substantial amendments to the study. The details of each are outlined below:

3.3.6.1.1 Amendment 1: addition of core-biopsy to protocol

Following continuing work within the wider research group, interest grew regarding tumour-related factors associated with the tumour-specific immune response and the immune-modulatory effect of cancer. The original protocol allowed for the retrieval of histological specimens and other material surplus to clinical requirements from the pathology laboratories following analysis. This would allow further research orientated histological analysis of the tumour specimens. However gene-expression analysis is increasingly being used as a tool to evaluate the tumour micro-environment. These techniques are reliant on isolation of RNA from tissue, a process which requires either extraction from fresh or freshly snap-frozen tissue.

The Breast Surgical Department were in the process of making a transition in their provision of intra-operative analysis of sentinel lymph node biopsies at the time of study set up. Introduction of an intraoperative quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)-based "molecular analysis" of sentinel node biopsy specimens meant that part of the sentinel node specimens were homogenised for RNA extraction. Surplus homogenate would be available for study purposes and might provide an interesting comparison when compared with RNA extracted from the tumour specimens.

It was therefore deemed important to acquire fresh tumour specimens for study purposes to allow RNA extraction. The most pragmatic way to achieve this was to perform core-biopsy on the tumour once the surgical specimen had been removed from the patient. Precedent for this had been previously set by the POETIC trial (527). An application for a substantial amendment to the study protocol was submitted and approved on this basis; the submitted Notice of Substantial Amendment form, sponsor approval letter and subsequent Notice of Favourable Ethical Opinion relating to the amendment are included within Appendix A.5.

3.3.6.1.2 Amendment 2: correction of REC form and addition of "use of banked human tissue"

Upon review of the original REC form submitted, we noted that a checkbox had been erroneously completed. The section concerned the use of "surplus tissue"; throughout the original protocol and REC application form reference had been made on multiple occasions to the planned use of tissue surplus to clinical or diagnostic requirements. As a key planned part

of the wider study project, it was important to clarify this error prior to commencement of the study. Therefore request for correction was submitted as part of amendment 2.

Isolation of viable cells from the study lymph node was a key technique which required optimisation. Early observations of the results achievable using the cell-isolation protocol detailed within the standard operating procedure (SOP) used by the University of Southampton Human Tissue Bank demonstrated the viability and functionality of cells isolated through purely mechanical means to be impaired (see Section 5.1.1). It was therefore necessary to optimise a new isolation procedure.

It was deemed important to establish this optimised procedure prior to recruitment of study patients if possible. Patients undergoing surgery for inflammatory bowel disease (a benign condition caused by idiopathic inflammation of the bowel wall) have lymph nodes removed with the surgical specimen which, unlike in cases of bowel cancer, are of no histopathological interest. The University of Southampton Human Tissue Bank sited at University Hospital Southampton (Human Tissue Authority Licence Number 12009) has established ethical approval to collect tissue surplus to clinical or diagnostic requirements and store said tissue in anonymised form. This tissue is available for use by any appropriately approved study.

Our original application did not include permission to use banked human tissue samples. Substantial amendment 2 was therefore necessary to allow access to lymph nodes removed during surgery for benign gastrointestinal conditions. The submitted Notice of Substantial Amendment form, sponsor approval letter and subsequent Notice of Favourable Ethical Opinion relating to the amendment are included within Appendix A.6. Following successful completion of this process it was possible to optimise a new isolation procedure, completion of which preceded the study commencing recruitment (see Figure 3).

3.3.6.1.3 Amendment 3: alteration to allow use of multi-agent tetanus vaccine

The original intention was to utilise a single agent tetanus vaccine for the purposes of the study. The vaccine intended for use was a product produced by Sanofi Pasteur MSD, manufactured in Germany. A supplier was identified (IDIS Pharma Limited, UK) and the product was confirmed to be European Medicines Agency licenced.

However import of the product was not given MHRA approval; single-agent tetanus vaccines are no longer licenced for use in the UK. Single-agent vaccines can be imported following MHRA approval, however this requires justification on a case-by-case basis. Following discussion with the MHRA it was made clear that scientific validity was not sufficient justification and a clinical reason for administration of a single agent would need to be made

prior to approval. Alternatively, the study classification could be changed to a CTIMP, through which administration of unlicensed medicinal product could be authorised.

Decision was therefore taken to administer licenced vaccines which restricted choice to multi-agent products (see Section 3.2.2). Substantial amendment 3 was therefore necessary to alter the protocol and patient information literature to reflect the change in vaccination strategy. The submitted Notice of Substantial Amendment form, sponsor approval letter and subsequent Notice of Favourable Ethical Opinion relating to the amendment are included within Appendix A.7.

3.3.6.2 Pharmacy

Administration of a vaccine to study patients required an agreement with the pharmacy department at University Hospital Southampton regarding vaccine acquisition and dispensation. The studies status as a non-CTIMP meant Clinical Trails Pharmacy was initially reluctant to take on responsibility for vaccine supply. This resulted in various difficulties which stemmed from the fact that the normal hospital pharmacy, as separate entity, did not have any established protocols for dispensing medications for the purposes of research, nor the pre-existing financial arrangements to source remuneration from University-held grant funding.

Following an appeal to senior-management within pharmacy, an agreement was reached that the supply of vaccine, and its dispensation for administration to study participants, would be coordinated through Clinical Trails Pharmacy.

3.3.6.3 Delay to allow establishment of molecular analysis of sentinel lymph node biopsy

A key principle of the study is that only nodes taken from patients with a negative sentinel lymph node biopsy would be used for the purposes of research. The difficulty in ensuring this was that at the time of study conception the procedure in place for analysis of the sentinel node at University Hospital Southampton was histological analysis of formalin fixed, paraffin embedded (FFPE) lymph nodes. Histological analysis of FFPE specimens remains the histological gold standard for analysis of the sentinel nodes however this procedure takes a minimum of 48 hours. Therefore patients whose tissue is analysed in this fashion and who are found to have positive lymph nodes (i.e. histological evidence of tumour spread into the node) are required to undergo a second surgical procedure to completely remove remaining lymph nodes.

The initial solution to this problem was to introduce intraoperative analysis for use in patients undergoing surgery within the study. Intra-operative analysis of the sentinel node allows the diagnosis of metastasis to be made during the patient's primary surgery, and therefore

completion axillary clearance can be performed immediately, without need to undergo a second operation under general anaesthetic. Techniques available include histological, cytological and molecular-pathological options (525); the initial plan was to utilise imprint cytology since this disrupted less tissue than fresh-frozen sectioning and would therefore be less compromising to the FFPE results. However since intra-operative analysis was not standard practice within the unit, it was felt that the service would not be amenable to the routine audit and quality control procedures which would typically be implemented for standardised clinical diagnostic testing procedures and therefore the patients should not have management decisions made on the basis of such results. Furthermore, since the service was not being provided for the purpose of patient care, the prioritisation of tissue handling was low and we anticipated delay in gaining results from the cytological analysis, particularly in the event of tissue specimens being delivered to the laboratory late in the day or outside of normal working hours. This would result in the study node being kept on ice for a protracted length in time, since processing the node would be precluded until analysis of the sentinel node was available. The potential detrimental effect on cell acquisition was therefore a concern.

Molecular techniques utilising qRT-PCR to identify gene expression considered characteristic of metastatic involvement of lymphatic tissue by breast cancer are considered more sensitive and specific than fresh-frozen sectioning and imprint cytology (525). The Breast Surgical Unit at University Hospital Southampton decided to introduce this technology for routine use for all patients undergoing sentinel lymph node biopsy in 2012. This was advantageous to the study in that it guaranteed a result from the sentinel node biopsy at the time of patient surgery, thereby minimising the time the study node would be left prior to processing. In addition the added accuracy of the technique again reduced the risk of the study node detracting from final histological staging in the event of a false negative result. Thirdly, since qRT-PCR was being introduced as the new standard of care across all patients undergoing surgery within the unit, there was no longer the concern of performing non-standard procedures on diagnostic tissue from study patients.

Considering the advantages of waiting for the qRT-PCR technique to be implemented prior to recruitment commencing, the decision was made to delay recruitment to allow the setup of the new service. Unfortunately this delayed recruitment until July 2013.

3.3.7 Final Documentation and commencement of recruitment

Following completion of the above amendments and the establishment of an agreement with pharmacy and implementation of the qRT-PCR intraoperative analysis service, recruitment

opened 10/07/2013. The first patient was recruited into the study 28/07/2013. The final study-related documents are included within Appendix A.8.

4 Methods

4.1 Patient Identification, Recruitment and Data collection

Patients were identified as potentially suitable following multidisciplinary team (MDT) discussion regarding diagnostic imaging, pathological results and management options. Patients identified as appropriate for sentinel lymph node biopsy were flagged and their case notes reviewed by the study coordinator. In the absence of exclusion criteria the patient was identified as suitable for approach; this was noted on the patients MDT meeting outcome sheet and a Patient Information Sheet (PIS) placed within the patients notes by a member of the research team.

Patients were offered a Patient Information Sheet during their next clinic consultation; the details of patients who were willing to take a patient information sheet were passed on to the coordinating research nurse practitioner. Patients were subsequently contacted by a qualified research nurse practitioner by phone after 48-72 hours to gauge interest and given an opportunity to ask further questions at this stage. Patients willing to consider recruitment were met by qualified research nurse practitioner prior to their pre-admission clinic appointment. The nurse practitioner then conducted a screening interview to ensure the patient did not meet any exclusion criteria prior to consenting the patient for inclusion within the study. Data collected was recorded upon the Case-Report Form and linked-anonymised at the point of collection.

Following completion of the consent process, the patient was randomised into one of the three cohort groups. A request for vaccine was sent to clinical trials pharmacy whilst bloods were taken for study purposes. In the event of randomisation to receive a vaccine, administration was performed following venesection.

At each subsequent time point additional clinical data was collected relevant to pathological diagnosis and management. In addition any potential confounding (such as infectious episode, commencement of adjuvant treatment, etc.) or adverse events were recorded. All data was collected on linked-anonymised Case-Report forms. Said forms were held by study research nurse practitioners in a secure facility according to established University of Southampton Standard Operating Procedures.

4.2 Patient blood and tissue sampling

4.2.1 Venesection for study purposes

Procedure was performed as an aseptic procedure by individuals trained and practiced in the technique. Verbal consent was gained for performing the procedure at each stage. A

tourniquet was placed at the mid-point of the upper arm and tightened. Site for venesection was selected. A steret soaked in 70% isopropyl alcohol was used to clean site prior to venepuncture using an 18G vacutainer needle. Blood samples were taken as follows; 10ml clotted blood (Plain bottle, red top), 4ml EDTA anti-coagulated blood (purple top) and 60ml lithium heparin anti-coagulated blood (green top). Upon completion tourniquet was released, needle withdrawn and pressure applied to the site using sterile dressing.

A sample (EDTA anti-coagulated blood) and request were submitted for Full Blood Count (FBC) with differential white blood cell count at each time point; this was performed by the University Hospital Southampton Haematology Department.

4.2.2 Surgical acquisition of tissue samples

The surgical operation performed on study patients was essentially unchanged from that performed on non-study patients. Briefly, pre-operatively patients received a peri-areolar sub-dermal injection of radiolabeled human albumin to the affected breast. Lymphangioscintigraphy was performed as per departmental protocol and in accordance with British Nuclear Medicine Society Guidelines (532). In cases where patients were to undergo breast conserving surgery, the position of the tumour was marked by a radiologist using either ultrasound and skin marking or stereotactic wire localisation. Blood samples were acquired prior to general anaesthetic as per Section 4.2.1.

Patients were anaesthetised according to the favoured practice of the consultant anaesthetist responsible for each individual patient. The exact anaesthetic techniques, and pharmaceuticals used, were recorded within the case-report form.

For the majority of cases, a peri-areolar sub-dermal injection of 2ml of Patent Blue V dye was performed; in a minority of cases this was omitted according to the preferred practice of the operating consultant surgeon. The surgical site was prepared using antiseptic solution and draped to preserve a sterile operating field. Sentinel lymph node biopsy preceded surgery to the breast; an incision was made into the hair-bearing skin of the axilla along a convenient skin crease. Dissection was continued through the superficial fascia to gain access to the axillary fat. Gamma probe was used to ascertain the position of the sentinel node, indicated by the strongest recordable signal from the probe. Following localisation, the sentinel node was removed and the axilla re-examined for radio-activity using the gamma probe. In the event of above-background residual activity, further sentinel nodes were located and excised using the same localisation technique until radioactivity returned to background levels. In addition, any lymph nodes which were stained blue (in cases where Patent Blue V dye was used) were also considered to be sentinel nodes and were removed. After completion of the sentinel node

biopsy an additional non-sentinel node was located using digitation of the axilla and removing a prominent node. The non-sentinel "study" node was then placed in cold cRPMI until the result of the sentinel node biopsy was known. In the event of a negative result the non-sentinel study node was transferred to the University of Southampton laboratory facilities for processing (see Section 4.4.4).

The sentinel nodes were transferred to the molecular pathology laboratory where they were processed as per the standard operating procedure developed by the University Hospital Southampton Pathology Department utilising the Metasin qRT-PCR test (539). Briefly each node received was processed as a separate specimen. Nodes were striped of surrounding axillary fat and sectioned at 2mm intervals. Alternate sections were homogenised in RLT buffer (Qiagen, Düsseldorf, Germany) supplemented with 1% β-Mercaptoethanol using an Omni homogenizer system (Omni International, Kennesaw, GA, US). 200µl of homogenate, positive and negative controls were mixed with an equal quantity of 70% ETOH. RNA extraction was then performed using a QIAvac 24 Plus vacuum manifold (Qiagen, Düsseldorf, Germany) and RNA extraction column from RNeasy Miniprep kit (Qiagen. Cat:74106) according to manufacturer's instructions and without a DNA-removal step. Analysis using qRT-PCR was then performed through addition of 4µl of prepared RNA to a pre-prepared master mix containing 2.5pm/µl of each forward and reverse primer for internal control gene (PBGD), mammaglobin (MG) and Cytokeratin 19 (CK-19) and 1pm/µl of each fluorescently-labelled probe for PBGD, MG and CK-19, Light Cycler^{R} 480 RNA Master Hydrolysis probes and Activator (Roche Diagnostics Ltd.) loaded into Smartcycler PCR machine (Cepheid Inc. Sunnyvale, CA, US). A positive result was defined as detection of CK-19/MG RNA above normal threshold in the presence of positive housekeeper gene expression and positive and negative control validation.

Whilst the sentinel nodes were undergoing analysis, the breast tumour was excised, either by wide local excision or mastectomy. Following excision the breast specimen was examined and 2 core biopsies taken from the tumour using 14G, 9cm semi-automated Achieve Core Biopsy needle (UK Medical Limited. Cat: A1409). One core biopsy was placed directly into 1ml RLT buffer (Qiagen, Düsseldorf, Germany) supplemented with 1% β-Mercaptoethanol, whilst the other was placed into a cryovial containing cold RPMI culture medium (to prevent desiccation). Tumour biopsies were then transferred to the University of Southampton laboratory facilities – see Section 4.4.2.

The remaining tumour specimen, sections from lymph nodes not homogenised and all other tissue removed during the course of surgery were immediately fixed in formalin and processed

by the pathology laboratories at University Hospital Southampton according to established protocol.

4.2.3 Sample labelling and storage

Processing records for all samples received during the course of the study were kept logging lot numbers and dates of opening for all reagents used. All samples were labelled with date of receipt, patient identification number, sample type, vial number and, if relevant, cell concentration.

Storage location was logged on the sample processing sheet and on electronic files maintained on a secure university computer. All sample logs were maintained in an anonymous state, identifiable only by the patient's study-specific number.

4.3 Blood sample processing

(Performed in sterile conditions in a class II laminar flow safety cabinet)

4.3.1 Reagents

- Lymphoprep (**Axis-Shield. Cat: 1114547**)
- RPMI (25mM HEPES, without L-glutamine). (**PAA. Cat: E15-041**)
- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- Trypan Blue (**Sigma Cat: T8154**)
- Dimethyl Sulfoxide (DMSO) (**Sigma Cat:472301**)

4.3.2 Serum isolation from whole blood

Blood was collected as per Section 4.2.1 into 10ml Plain (Red top) vacutainer tube. Sample was allowed to clot at room temperature for minimum 1 hour (maximum 2 hours). After clot had formed sample was centrifuged at 485g at 4°C for 15 minutes. Serum was drawn off from above compacted clot-layer using a Pasteur pipette and divided into five 500µl aliquots. Aliquots were then frozen immediately and stored at -80°C.

4.3.3 Isolation of Peripheral Blood Mononuclear Cells from whole blood

Blood was collected as per Section 4.2.1 into 6x10ml Lithium Heparin (Green top) vacutainer tubes. 10ml of room temperature Lymphoprep was measured into 6 universal containers. 10ml of anti-coagulated blood was then gently layered on top of the Lymphoprep within each universal. Blood was then separated according to density across the Lymphoprep by

centrifugation at 950g for 23 minutes at room temperature. The centrifuge was set to minimum rate of deceleration to avoid vortexing.

Following layering across Lymphoprep the blood is separated into a plasma layer overlying the mononuclear cells which are held above the Lymphoprep. Erythrocytes pass through the Lymphoprep layer and form a pellet below. Plasma was then removed from above the mononuclear layer and processed as per Section 4.3.4.

10ml of RPMI pre-warmed to 37°C was measured into 3x 50ml Falcon tubes. Using a Pasteur pipette the mononuclear layer was removed from each universal and decanted into the RPMI-containing Falcon tubes. Each Falcon tube was then topped up to 50ml with pre-warmed RPMI. Samples were centrifuged at 700g for 8 minutes at room temperature using a moderate deceleration.

Supernatant from each tube was discarded leaving the cell pellet which was re-suspend by gentle agitation. 10ml of pre-warmed RPMI was added to each tube prior to combining into one single tube. The remaining two tubes were washed with additional pre-warmed RPMI and this added to the cell-containing-tube to make up a volume of 50ml. The sample was then centrifuged at 485g for 8 minutes at room temperature using a moderate deceleration.

Supernatant was discarded leaving the cell pellet. A cell count and viability assessment was performed as per Section 4.5.1 and cells then cryopreserved as per Section 0.

4.3.4 Isolation of Plasma from whole blood

Following the separation of anti-coagulated blood using centrifugation over Lymphoprep, the plasma layer was retrieved from above the mononuclear layer using a Pasteur pipette with care being taken to avoid incorporation of cells. Aliquots of 1ml of plasma were measured into 5 vials. These were frozen immediately and stored at -80°C.

4.4 Tissue sample processing

4.4.1 Reagents/Equipment

- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- Liberase Research Grade Enzyme Blend, Dispase Low (**Roche. Cat:05401160001**)
- Deoxyribonuclease 1 (**Sigma. Cat:D4527**)
- RLT Buffer (from RNeasy Mini kit, **Qiagen. Cat:74106**)
- β-Mercaptoethanol (**Sigma. Cat: M6250**)
- Sterilised fine stainless steel wire mesh

- 70µl Cell Strainer (**BD Falcon. Cat:352350**)

4.4.2 Handling of Core Biopsy Samples from Tumour

Two tumour samples from the operating theatre were received from each patient (as per Section 4.2.2). The cryovial containing the core biopsy in RPMI had the culture medium removed and the core biopsy was snap-frozen within the vial by emersion into liquid nitrogen. The tumour biopsy in RLT +βME was vortexed for 1 minute, then frozen at -80°C.

4.4.3 Creating a Single Cell Suspension from Human Lymph Node – Mechanical

(Performed in sterile conditions in a class II laminar flow safety cabinet)

The lymph node was received from theatre in cold cRPMI. The node was tipped onto a sterile wire mesh held above a petri dish containing 10ml of cRPMI. All fat and associated non-nodal tissue was excised. The bung from a 50ml syringe was removed from the syringe-body and used to desegregate the node through the wire mesh using gentle pressure. The mesh was then washed through with 15ml total of cRPMI (front and back).

The resulting suspension was collected using the re-assembled 50ml syringe and transferred into a 50ml Falcon tube through a 70µn cell strainer. The petri dish was washed twice with 10ml of cRPMI, which was also added to the cell suspension through the 70µn cell strainer.

The sample was centrifuged at 700g for 8 minutes at room temperature using a moderate deceleration. The supernatant was discarded leaving the cell pellet. A cell count and viability assessment was performed as per Section 4.5.1 and cells then cryopreserved as per Section 4.5.2.

4.4.4 Creating a Single Cell Suspension from Human Lymph Node – Enzymatic-digest

(Performed in sterile conditions in a class II laminar flow safety cabinet)

The lymph node was received from theatre in cold cRPMI. The node was tipped onto a petri dish. All fat and associated non-nodal tissue was excised.

The node was transferred to a 50ml Falcon tube containing 1ml of digest solution (1ml cRPMI pre-warmed to 37°C containing 0.15 Wünsch units (WU) of Liberase DL and 800 Kunitz units (KU) DNAse 1). A sharp scalpel was then used to macerate node within tube. The node was left within the digest solution for 1 hour at 37°C on an agitation plate set to 450 RPM.

The resultant suspension was decanted into a 50ml falcon through a 70µn cell strainer. 5ml of cRPMI was used to wash the digest tube and was then added to the sample through the cell strainer. The node remnant was tipped from the cell strainer onto a sterile wire mesh above a petri dish. The cell strainer was then washed with 10ml cRPMI onto mesh and returned to the

50ml Falcon tube. The bung from a 50ml syringe was removed from the syringe-body and used to desegregate the node through the wire mesh using gentle pressure. The mesh was then washed through with 15ml total of cRPMI (front and back).

The resulting suspension was collected using the re-assembled 50ml syringe and transferred into a 50ml Falcon tube through a 70µn cell strainer. The petri dish was washed twice with 10ml of cRPMI, which was also added to the cell suspension through the 70µn cell strainer.

The sample was centrifuged at 700g for 8 minutes at room temperature using a moderate deceleration. The supernatant was discarded leaving the cell pellet. A cell count and viability assessment was performed as per Section 4.5.1 and cells then cryopreserved as per Section 4.5.2.

4.5 Cell count, Viability assessment, Cryopreservation and Sample thawing

(Performed in sterile conditions in a class II laminar flow safety cabinet)

4.5.1 Cell count and assessment of viability through Trypan Blue Exclusion Assay

4.5.1.1 Principle

Certain compounds are prevented free translocation across cell membranes. Trypan blue is a diazo dye with a strong negative charge. As such this compound does not cross the cell membrane of the majority of viable cells (540). However non-viable cells lose the integrity of their cell walls, allowing the dye to traverse into the cytoplasm. Non-viable cells can subsequently be identified using light-microscopy as those with blue cytoplasmic staining. Numeration is through use of a haemocytometer.

4.5.1.2 Reagents/Equipment

- Culture medium
- Trypan Blue (**Sigma Cat: T8154**)
- Neubauer Haemocytometer

4.5.1.3 Protocol

The cell population was suspended in a known volume of culture medium (typically 1ml however more or less was used depending on predicted numbers of cells). 10µl of cell suspension was diluted with filtered trypan blue dye. Dilution factor used depended on projected numbers of cells; 1:2 dilution was used in instances where predicted cell concentration was $10x10^6$/ml or less. Numeration of higher concentrations of cells required 1:5 dilution or greater. When using a higher proportion of trypan blue dilution of trypan blue using culture medium was necessary to reduce the dark background of the dye (i.e. for a 1:5 dilution,

10µl of cell solution was added to 20µl of trypan blue and 20µl culture medium). Cells were carefully suspended within the dye solution to ensure homogeneity.

10µl of resultant cell/dye suspension was applied to a neubauer haemocytometer. The neubauer haemocytometer has a central grid of 25 large squares each containing 16 small squares. Total area under the central grid is $10^{-4}cm^3$. The number of cells within the central grid were counted and then applied to the following calculation:

Number of cells per ml ($x10^6$) = *cell count* x *dilution factor* x *conversion factor*

Where:

Cell count = average number of cells per large square

Dilution factor = dilution of cells in trypan blue (i.e. 1:2, 1:5)

Conversion factor = multiplication factor to convert volume assessed during cell count to 1ml. (typically $1x10^4$)

Percentage cell viability was calculated as equal to the number of viable cells/total cells x100.

4.5.2 Cryopreservation of PBMCs and Lymph node single-cell suspension

4.5.2.1 Reagents/equipment

- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- Dimethyl Sulfoxide (DMSO) (**Sigma Cat:472301**)
- Nalgene Cryocontainer (**Sigma C1562-1EA**) containing isopropyl alcohol (**Sigma W292907**)[2]
- Cryovials

4.5.2.2 Protocol

A cell solution of known cell concentration was attained as per sections 4.3.3, 4.4.3 or 4.4.4. The desired freezing concentration was determined with an optimal concentration considered to be between 5-$10x10^6$ cells/ml. Each vial requires 1ml of cell/freezing medium suspension, therefore the total final volume required in millilitres was equal to the concentration of cell stock divided by the desired cell concentration for freezing. The cell solution was made up to the desired final volume ensuring the final composition of the cell/freezing medium suspension was as follows:

[2] Isopropyl alcohol requires replacement following 5 freeze-thaw cycles

- 40% cRPMI
- 50% Decomplemented Human Ab Serum
- 10% DMSO

The DMSO was added last whilst gently vortexing the cell suspension (DMSO is detrimental to cell viability, therefore time between addition of DMSO and freezing was minimised). The cell suspension was divided into 1ml aliquots per cryovial. cryovials were placed into a cryocontainer insulated with isopropyl alcohol and then into a -80°C freezer. The cryocontainer functioned to ensure a steady cooling rate of 1°C/minute. After 24 hours vials were transferred into liquid nitrogen storage.

4.5.3 Thawing of cryopreserved cells

4.5.3.1 Reagents:

- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- Fetal Calf Serum (**Sigma. Cat: F2442**)

4.5.3.2 Protocol:

cRPMI was pre-warmed to 37°C and supplemented with either 5-10% decomplemented human antibody serum or 10% Fetal Calf Serum, depending on desired cell-use. 10ml of resulting culture medium was then dispensed into 15ml Falcon tubes, one for each cell sample being thawed.

Once receiving media had been prepared, sample vials were removed from liquid nitrogen and placed within insulated carriage-containers filled with dry ice for transport to the laboratory.

Samples were rapidly thawed by placement in a water bath set to 37°C. Care was taken to ensure that vials were not fully immersed due to potential for contamination. Once nearly fully thawed, vials were dried then wiped with tissue soaked in 70% ethanol. Vials were transferred immediately into a class II laminar flow safety cabinet. 1ml of culture medium from pre-prepared tubes was added to vial to complete thawing process, dilute vial contents and minimise osmotic shock, then entire vial contents was transferred to pre-prepared falcon tube. Cryovial was washed once with culture media and this was added to falcon tube.

Sample(s) were then centrifuged at 350g for 5 minutes. Supernatant was discarded from above cell pellet and cell count and viability assessment was performed as per Section 4.5.1.

4.6 RNA Extraction Protocol

4.6.1 Reagents:

- RNAse-Zap (**Ambion. Cat:AM9780**)
- RNAse-free DNAse set (**Qiagen. Cat:79254**)
- RNeasy Mini kit (**Qiagen. Cat:74106**)
- Ethanol **(Fisher Chemicals. Cat: E/0650DF/17)** (diluted to 70% using ddH_2O)

4.6.2 Protocol:

All areas of the bench top and equipment used throughout the process of isolating RNA were pre-treated using RNAse-Zap SDS solution.

Tumour biopsy samples or sentinel node homogenate were received frozen. Samples were defrosted and then vortexed thoroughly. Samples were centrifuged at 14,000RPM for 3 minutes at 4°C. Supernatant was transferred to an Eppendorf centrifuge vial and an equal volume of 70% ethanol added. 700µl of the resultant solution was added to an extraction column and centrifuged at 14,000RPM for 15 seconds. The filtrate was discarded and the process repeated until the original sample/ethanol solution was exhausted.

DNA was removed from the extraction column as follows: 350µl of RW1 buffer was added to the column which was then centrifuged at 14,000RPM for 15 seconds. Filtrate was then discarded. DNAse solution made up as per manufacturer's instructions (10µl of DNAse1 combined with 70µl of RDD buffer) and then 80µl was added to the column and left at room temperature for 15 minutes. After 15 minutes a further 350µl of RW1 buffer was added to the column which was then centrifuged at 14,000RPM for 15 seconds. The filtrate was then discarded. The column was washed twice with 500µl of RPE buffer; during each wash step the column was centrifuged at 14,000RPM for 15 seconds and the filtrate discarded. Following the final spin-step the collection tube was changed and the column re-centrifuged at 14,000RPM for 60 seconds. The collection tube was then discarded.

RNA was harvested from the column as follows: the column was fixed into an Eppendorf and then loaded with 35µl of RNAse-free water. This was left for 1 minute then centrifuged at 14,000RPM for 60 seconds. The filtrate was retrieved and re-loaded onto the column, which was then re- centrifuged at 14,000RPM for 60 seconds. The final filtrate was stored at -80°C until use.

Prior to storage a 5µl aliquot of filtrate was subjected to quantification analysis using Nanodrop microvolume UV-Vis spectrophotometer and RNA-integrity scoring.

4.7 Enzyme Linked Immunosorbent Assay (ELISA)

(Performed in sterile conditions in a class II laminar flow safety cabinet)

4.7.1 Principle:

An indirect ELISA technique was utilised to ascertain the presence or otherwise of serum immunoglobulin specific to a given antigen. The technique relies on non-specific binding of the antigen of interest to the surface of a polystyrene microtiter plate; following dissolution within a suitable buffer the antigen will bind to the plate through charge interactions. Unbound areas of the plate are "blocked" from binding subsequent proteins by use of an irrelevant protein, such as Bovine Serum Albumin (BSA). This prevents non-specific binding of the antibodies within the serum samples to be tested. Bare wells within the plate plan (wells with no antigen which are therefore coated with the blocking protein only) are used to exclude non-specific binding of antibody within the patients sera to the plate or the irrelevant protein.

Dilutions of serum are then added to the plate; all antibodies specific to the antigen bound to the plate will adhere. These can then be detected using a secondary antibody specific to human antibody. Detection of different antibody isotypes is achievable by using different secondary antibodies specific to the isotype of interest. Read-out is achievable since the secondary antibody is conjugated with horse-radish peroxidase (HRP); its reaction with hydrogen peroxide alters the colour of the substrate buffer, changing the solutions absorbance characteristics, which can be measured using a microplate reader.

Quantification of serum antibody concentration can be performed by comparison across a standard curve generated using a standard with a known concentration of antibody to the specific antigen of interest, which is typically expressed as an "International Standardised Unit" (IU). Semi-quantification can be achieved through comparison of test samples with a standard curve generated by a constant sample; as long as the same constant sample is used to compare all other samples, the quantity of antibody can be expressed relative to the constant as "Arbitrary Units" (AU).

4.7.2 Reagents:

- ELISA plates (96-well, MaxiSorp Immunoplates. (Thermo Scientific. **Nunc: Cat:442404**)
- Quality Control Sera:
  - Samples of previously defined antibody titre used to define equivalence between assays. A high, medium and low titre quality control is used.

- O-phenylenediamine tablets (OPD) (**Sigma Cat:P-4664**)
- Tween 20 (**Sigma Cat:P-1379**)
- PBS (10x concentration) (**BDH Cat:43711 7K**)
- BSA (**Sigma Cat:A-7906**)
- Coating Buffer (in 500ml ddH_2O, pH 9.5) (0.79g Sodium Carbonate + 1.46g sodium bicarbonate)
  - 14.9mM Na_2CO_3 (anhydrous) (**Sigma Cat:S-7795**)
  - 34.75mM $NaHCO_3$ (**Sigma Cat:S3817**)
- Blocking Buffer (1% BSA in 1x PBS)
- Substrate Buffer (in 500ml ddH_2O, pH 4.5) (2.34g citric acid 3.65g dibasic sodium phosphate)
  - 24.3mM Citric Acid (anhydrous) (**Sigma Cat:C-0759**)
  - 51.4mM $NaHPO_4$ (**Sigma Cat:S-0876**)
- Sulphuric Acid (H_2SO_4) 2.5Mol/L (**BDH Prolabo Cat:4.80364.1000**)
- H_2O_2 (**Sigma Cat:H-1009**)
- For a list of coating antigens, anti-sera and detection antibodies used, and their respective stock and working concentrations, see Table 3:

4.7.3 Protocol:

Day 1:

The coating antigen of interest is diluted in coating buffer to the pre-determined, optimised coating concentration. 200µl of antigen/coating buffer is then added to each well of a 96 well MaxiSorp Immunoplate with the exception of "bare" wells (see sample Plate Plan, Appendix C), into which coating buffer only was added. The antigen is allowed to adhere to the plate overnight, which is left overnight at 4°C (covered with Parafilm to prevent contamination).

Day 2:

Excess coating antigen was removed by washing wells four times with 200µl PBS-0.1% Tween (PBS-T). 200µl of blocking buffer was added to each well and plate incubated at 37°C for 1 hour.

Dilutions of reference standard, quality control serum and patient samples were prepared; in the case of antigens where reference standards were available, stock solution was prepared at 100x starting concentration. Where arbitrary serum standards were used, starting concentration was according to the specifics of each batch. For clarity the following method describes use of a reference standard at a stock concentration 100x use:

Reference standards were diluted by a factor of 50 to twice usable concentration. Quality control serum samples at high, intermediate and low activity were diluted as per their individual batch instructions. Patient serum samples were diluted by a factor of 25 and by a factor of 50. All dilutions were performed in PBS-T. Excess blocking buffer was then removed from the plate by washing wells twice with 200µl/well PBS-T.

For an assay performed in duplicate: 200µl of diluted reference standard was added to wells A_{1-2} of the plate. Similarly, 200µl of patient samples diluted by a factor of 25 were added to wells A_{5-6}, A_{7-8}, A_{9-10} and A_{11-12}, and 200µl of patient samples diluted by a factor of 50 were added to the bare wells, as per the plate plan (Appendix C). 200µl of diluted QC sera were added to columns 3-4 as per plate plan. All empty wells received 100µl of PBS-T. Serial double-dilutions of columns 1-2, 5-12 were then performed by taking 100µl from row A and mixing into Row B, then repeating down to row H. Following removal of 100µl from row G_{1-2} the samples were discarded, leaving row H_{1-2} with plain PBS-T (blank wells). All deficient wells were then topped up with a further 100µl of PBS-T, leaving a final top dilution within Row A of 1:100 for the standard and 1:50 for the serum samples.

The plate was incubated at 37°C for 1 hour to allow binding of immunoglobulin. Excess immunoglobulin was removed by washing wells four times with 200µl PBS-T. The desired secondary ("detection") antibody was diluted to its working concentration in PBS-T and 200µl added to each well. This was allowed to bind at 37°C for 1 hour. Excess detection antibody was removed by washing wells four times with 200µl PBS-T. One OPD tablet was dissolved in 25ml of substrate buffer to which 25µl of H_2O_2 was added. 200µl of this solution was added to each well and allowed to develop. The reaction was stopped by adding 80µl of 2.5M H_2SO_4 to each well. Absorbance was measured within 30 minutes using a Bio-rad iMark Microplate Absorbance Reader set to 490nm detection.

Table 3: Reagents for ELISA: a) coating antigen, b) standard sera[3] and c) detection secondary antibodies

a)

Antigen	Source	Stock	Dilution for Coating Concentration
1. Tetanus Toxoid	NIBSC (04/150)	1mg/ml	2.0ug/ml
2. Diphtheria Toxoid	NIBSC (02/176)	1mg/ml	2.0ug/ml
3. PPD	SSI C/O NIBSC (RT27 SSI (2391) (RT50)	666.6mg/ml 1mg/ml	10ug/ml 10ug/ml
4. FHA	ENZO (ALX-630-123-0100)	1mg/ml	2.5µg/ml
5. Fragment C	In House	1mg/ml	2µl/ml

b)

Standard	Catalogue Number	Stock concentration
Tetanus Anti-Toxin	NIBSC TE-3	1IU/ml
Diphtheria Anti-Toxin	NIBSC 10/262	1IU/ml
FHA Anti-Serum	NIBSC 06/140	100IU/ml

c)

Antibody	Catalogue Number	Working Dilution
Anti-Human IgG (Fc Specific) – HRP, Goat.	Sigma A0170-1ml	1:10,000
Anti-Human IgM (µ Specific) –HRP, Goat.	Sigma A0420-1ml	1:10,000
Anti-Human IgG1 (Fc specific) – HRP, Mouse.	Life Science Tech. MH1715	1:2000
Anti-Human IgG2 (Fd specific) – HRP, Mouse.	Life Science Tech. MH1722	1:2000
Anti-Human IgG3 (Hinge specific) – HRP, Mouse.	AbCam Ab99829	1:1000
Anti-Human IgG4 (Fc specific) – HRP, Mouse.	Life Science Tech. MH1742	1:2000
Anti-Human IgE (ε-chain) – HRP, Goat	Sigma A9667-2ml.	1:8000

[3] Serum standards to normalise to an "international Unit" of serum anti-toxin/anti-body are available for total IgG levels against tetanus toxoid, diphtheria toxoid and FHA. No similar standards are available for IgG-isotypes or IgM antibodies of these antigens, and no standards are available for PPD. Therefore for these assays it was necessary to normalise to a positive serum sample and express titres as "Arbitrary Units" (AU).

4.7.4 Analysis

Analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, US) and GraphPad Prism® v6.0 (GraphPad Software Inc., San Diego, Cl., US).

An average absorbance within the blank wells was subtracted from all remaining wells to account for background. Mean absorbance readings were calculated for each dilution of standard and these figures plotted against their known concentration (in IU or AU) to form a standard curve.

Validity of the assay was confirmed through quantification of the antibody content of the quality control sera by comparison across the standard curve (see below); assay was accepted as representative if values achieved for quality control sera fell within 25% of the known true value. In addition, the background absorbance from bare wells was checked to ensure non-specific binding to the plate or irrelevant blocking protein did not account for absorbance within the test wells.

For each sample concentration the measured absorbance was compared with that achieved within the straight portion of the standard curve; the conversion calculation was performed based on the highest concentration of test serum whose absorbance remained within the straight portion of the standard curve. Absorbance values from these wells were converted to AU or IU via interpolation using non-linear regression across a hyperbolic (concentration) curve. This gave a quantification of the antigen-specific immunoglobulin content within said wells, which was then scaled up according to the wells dilution factor to achieve a figure for the undiluted sample.

4.8 CD4-cell Enzyme-Linked Immunosorbent Spot (ELISpot) assay

(Performed in sterile conditions in a class II laminar flow safety cabinet)

4.8.1 Principle:

ELISpot assays utilise a sandwich enzyme-linked immune-assay platform to detect cellular production of a specific analyte of interest. Through stimulation of a cell population it is possible to induce release analytes such as cytokines. This can be done using non-specific stimulants such as Staphylococcal Enterotoxin B (SEB) or by using individual antigens which will only stimulate CD4+ cells specific to that given antigen. The assay is then used as a read out to numerate the number of cells specific to the stimulating antigen which produce the analyte of interest.

ELISpot plates consist of a 96-well format plate with Polyvinyldene difluoride (PVDF) membranes at the base of each well. The PVDF membranes bind amino acids non-specifically

and with a high binding capacity/affinity due to their porous structure. However due to the hydrophobic properties of PVDF, proteins within an aqueous solution will only gain maximal access to the binding surface of the membrane. Activation with ETOH changes the properties of the membrane making its surface hydrophilic and improving protein binding. Antibodies or antigens can then be coated to the plate surface.

The assays specificity to a given analyte is dependent on the specificity of the antibodies used to coat the plate. For example, coating the plate with an antibody specific to IFNγ would mean that any IFNγ produced by cells following stimulation would be captured onto the plate (hence alternative nomenclature for coating antibody is "capture" antibody in this system). Once the cells have been washed off, the analyte remains bound to the analyte-specific antibodies on the plate. The presence of the analyte can then be detected using a secondary antibody specific to the same analyte. The result is the analyte sandwiched between two antibodies; the capture and the detection antibody, hence the term "sandwich" immune-assay. Read out is possible due to the secondary antibody being conjugated to biotin (aka Vitamin B7); biotin readily forms strong association with streptavidin (bacterial protein derived from *Streptomyces avidinii)* (541) which is conjugated to the enzyme alkaline phosphatase for use in the assay. The plate is developed by addition of a substrate buffer containing 5-Bromo-4-chloro-3-indolylphosphate p-Toluidine Salt (BCIP) and Nitroblue Tetrazolium Chloride (NBT) (BCIP/NBT); alkaline phosphatase dephosphorylates BCIP resulting in a reduction reaction with NBT which forms an insoluble purple precipitate which stains the membrane of the well. This is seen as a localised spot where the analyte binds to the plate. Each spot represents the position of a single cell which produced the analyte of interest upon stimulation with the specific antigen. Read out is expressed as "number of [analyte]-producing, [antigen]-specific cells per well".

4.8.2 Reagents:

- ELISpot plates (96-well, 0.45um Hydrophobic High Protein Binding Plates. Supplier **Merck Millipore. Cat: MAIPS4510**)
- ddH_2O – certified endotoxin free
- Ethanol (**Fisher Chemicals. Cat: E/0650DF/17**) diluted to 35% using endotoxin-free ddH_2O
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- RPMI (25mM HEPES, without L-glutamine). (**PAA. Cat: E15-041**)

- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- BCIP/NBT Substrate Kit (**Invitrogen. Cat: 00-2209**)
- Bovine Serum Albumin (BSA) (**Sigma. Cat: A-7906**)
- Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- Tween 20 (**Sigma. Cat:P1379**)
- Streptavidin (**Mabtech. Cat: 3310—10**)
- For a list of coating antibodies, antigens for stimulation and biotinylated antibodies and their respective stock and working concentrations, see Table 4:

Table 4: Reagents for CD4 cell ELISpot: a) coating antibodies, b) antigens for stimulation and c) biotinylated secondary antibodies

a)

Antibody	Source	Stock	Dilution for Coating Concentration
1. Anti-human IFNγ mAb 1-D1k	Mabtech (3420-3-1000)	1mg/ml	5ug/ml
2. Anti-human IL-2 mAbs IL2-I/249	Mabtech (3440-2-1000)	500ug/ml	5ug/ml

b)

Antigen	Source	Stock	Working concentration
1. Tetanus Toxoid	NIBSC (04/150)	1mg/ml	20ug/ml
2. Diphtheria Toxoid	NIBSC (02/176)	1mg/ml	20ug/ml
3. PPD	SSI C/O NIBSC	666.7ug/ml	10ug/ml
4. SEB	Sigma (S4881)	1mg/ml	1ug/ml
5. Anti-CD3 and anti-CD28	BD Biosciences (555336 and 555725)	1mg/ml	2ug/ml each

c)

Antibody	Source	Stock	Dilution for Coating Concentration
1. Anti-human IFNγ mAb 7-B6-1 Biotinylated	Mabtech. (3420-6-1000)	1mg/ml	1ug/ml
2. Anti-human IL-2 mAb IL2-II, Biotinylated	Mabtech (3440-6-1000)	500ug/ml	1ug/ml

4.8.3 Protocol:

Day 1:

The plate plan was laid out on a 96 well plate; each stimulation required replication in triplicate and each cell sample subjected to a negative (unstimulated) control for each cell concentration and at least one positive control (either SEB or CD3/CD28). Coating antibody was diluted in 1xPBS (sterile) according to its optimised working concentration (see Table 4).

Plate membrane was activated by addition of 50µl of 35% ETOH to each well which was allowed to sit within the well for a maximum of 60 seconds. ETOH was removed from the plate membrane by washing five times using 150µl of endotoxin free ddH_2O. 100µl of Coating Antibody solution was then added to each well and allowed to bind to the plate overnight at 4°C.

Day 2:

Sample cells were defrosted into cRPMI + 5% Human Antibody Serum as per Section 4.5.3. Cells were subsequently diluted to 4x10^6 cells/ml in cRPMI + 5% Human Antibody Serum and rested for 4 hours at 37°C, 4% CO_2.

2 hours prior to use, the excess coating antibody was removed from the plate by washing five times using 150µl of 1xPBS. Unbound areas of the plate were then blocked with irrelevant protein to prevent non-specific plate binding through addition of 100µl of cRPMI + 10% Human Antibody Serum, which was allowed to bind for 2 hours at 37°C + 4% CO_2.

Following their 4 hour resting phase, cells were centrifuged at 485g for 5 minutes and counted as per Section 4.5.1. Cells were then diluted in cRPMI + 5% Human Antibody Serum to desired concentration according to the number of cells per well to be plated. For identification of antigen-specific PBMCs, cells were diluted to 4x10^6 cells/ml. For identification of antigen-specific cells isolated from a reactive lymph node, cells were diluted to 1-2x10^6 cells/ml.

Immediately prior to use solutions of antigens diluted to 2x working concentration (see Table 4) in cRPMI + 5% Human Antibody Serum were made. Excess blocking medium was then removed from the plate by washing five times using 150µl of 1xPBS. 100µl of the antigen solutions were added to each well according to the plate plan. Negative control wells received 100µl of cRPMI + 5% Human Antibody Serum. 100µl of the cell solutions were then added to their respective wells (therefore for cells diluted to a concentration of 4x10^6 cells/ml, each well received 4x10^5 cells) with the exception of the positive control wells which received 12.5-25µl/well (i.e. 5-10 x10^4 cell per well when cell stock was diluted to 4x10^6 cells/ml) to ensure these wells remained readable. The volume of the positive control wells was then made up to a total of 200µl using

additional cRPMI + 5% Human Antibody Serum. Cells were then cultured in their stimulating antigens at 37°C + 4% CO_2 for 48 hours.

Day 4:

Cells were removed from the plate by washing five times using 150µl of 1xPBS supplemented with 0.1% Tween (PBS-T). The secondary biotinylated antibody corresponding to the initial coating antibody was then diluted to its working concentration in PBS + 1% BSA and 100µl of the solution added to each well. The secondary antibody was allowed to bind for between 90 – 120 minutes at 37°C + 4% CO_2.

Excess secondary antibody was removed from the plate by washing five times using 150µl of 1xPBS-T. Streptavidin was diluted to 1:1000 concentration in PBS + 1% BSA and 100µl added per well. This was allowed to bind at 37°C + 4% CO_2 for 60 minutes.

Excess streptavidin was removed by washing five times using 150µl of 1xPBS-T. The under-tray of the plate was removed, blotted dry and replaced (to reduce background). BCIP/NBT substrate buffer was made up to manufacturer's instructions and 100µl added per well. Spots were allowed to develop and the reaction stopped through overriding dilution using tap water. Plate was dried and then read using ELISpot plate reader ELR04 (Autoimmun Diagnostika GmbH, Straßberg, Germany) running ELISpot Reader 7.0 software (Autoimmun Diagnostika GmbH, Straßberg, Germany).

4.8.4 Analysis:

Analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, US).

The number of spots within each well was normalised to number of spots per million (SPM) by multiplication of the number of spots by $1x10^6$ / number of cells plated per well. The SPM within the negative control wells were averaged and subtracted from all remaining wells to exclude background. SPM from each triplicate were averaged and compared across cell samples.

4.9 B-cell lineage Enzyme-Linked Immunosorbent Spot (ELISpot) assay

(Performed in sterile conditions in a class II laminar flow safety cabinet)

4.9.1 Principle:

The major methodological difference between B-cell and CD4 cell ELISpot is the use of antigen to coat the plates. Antibody produced by the cells which is specific to the antigen bound to the plate then adheres and can be detected using a secondary biotinylated antibody specific to the

isotype of antibody of interest. In this fashion the assay is analogous to the indirect principle employed in the ELISA method described in Section 4.7, except the read out is in a solid phase.

Using this plate technique circulating antibody secreting cells (ASCs) specific to a given antigen can be identified readily, with resultant spots representing either circulating plasma cells or plasmablasts. The number of spots can be expressed as a proportion of total cells plated. However through use of a "positive control well" coated with anti-human IgG or anti-human IgM, the total number of ASCs can be numerated; by using a coating anti-human immunoglobulin any target isotype produced by a cell will bind to the plate and be detected in a fashion analogous to the sandwich enzyme-linked immune-assay principle employed for CD4 ELISpot. Through this technique, the antigen-specific ASCs can be expressed as a percentage of total ASCs for a given isotype.

Memory B cells however do not produce antibody at rest. Therefore the assay uses stimulation through combination of R848 and recombinant human interleukin 2 ($_{rh}$IL2). This technique has been previously described in the literature as robust in selective induction of antibody production by memory B-cells (542, 543). Following simulation the memory b-cells are numerated by subtracting the number of ASC within the unstimulated population from the number within the stimulated population (see Section 4.9.5)

4.9.2 Reagents:

- ELISpot plates (96-well, 0.45um Hydrophobic High Protein Binding Plates. Supplier Merck Millipore. Cat: MAIPS4510)
- Ethanol (**Fisher Chemicals. Cat: E/0650DF/17**) diluted to 35% using endotoxin-free ddH_2O
- RPMI (25mM HEPES, without L-glutamine). (**PAA. Cat: E15-041**)
- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat: P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- Fetal Calf Serum (**Sigma. Cat: F2442**)
- BCIP/NBT Substrate Kit (**Invitrogen. Cat: 00-2209**)
- Bovine Serum Albumin (BSA) (**Sigma. Cat: A-7906**)
- Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- Tween 20 (**Sigma. Cat:P1379**)
- R848 (542) – resiquimod (TLR7 & 8 agonist) (**Mabtech. Contained within Cat:3850-2HW-Plus**)
- rhIL-2 (**Mabtech. Contained within Cat:3850-2HW-Plus**)
- Streptavidin (**Mabtech. Cat: 3310—10**)

- For a list of coating antibodies/antigens and biotinylated antibodies with their respective stock and working concentrations see Table 5:

Table 5: Reagents for B cell ELISpot: a) coating antigens / antibodies, b) biotinylated secondary antibodies

a)

Antibody	Source	Stock	Dilution for Coating Concentration
1. Anti-human IgG mAb MT91/145	Mabtech (3850-3-1000)	0.5ug/ml	10ug/ml
2. Anti-human IgM mAb	Mabtech (3840-2AW)	0.5ug/ml	10ug/ml
3. Tetanus Toxoid	NIBSC (04/150)	1mg/ml	3.5ug/ml
4. Diphtheria Toxoid	NIBSC (02/176)	1mg/ml	3.5ug/ml
5. PPD	SSI RT50	1mg/ml	10ug/ml
6. FHA	ENZO (ALX-630-123-0100)		5ug/ml
7. Fragment C	In House	1mg/ml	3.5 ug/ml

b)

Antibody	Source	Stock	Dilution for Coating Concentration
1. Anti-human IgG mAbs MT78/145; biotin	Mabtech (3850-6-1000)	0.5mg/ml	1ug/ml
2. Anti-human IgM mAb; Biotin	Mabtech (3840-2AW)	0.5mg/ml	1ug/ml

4.9.3 Protocol for numeration of antigen specific antibody secreting cells only:

Day 1:

The Plasma Cell plate plan was laid out on a 96 well plate; each antigen specificity required replication in triplicate and each cell sample subjected to a negative (blank, uncoated well) control. Positive control wells (coated with either anti-human IgG or anti-human IgM) were included to provide numeration of "Total IgG (or IgM) -producing cells" – an example plate plan is included in Appendix D. Coating antigens and antibody was diluted in 1xPBS (sterile) according to its optimised working concentration (see Table 5).

Plate membrane was activated by addition of 50µl of 35% ETOH to each well which was allowed to sit within the well for a maximum of 60 seconds. ETOH was removed from the plate membrane by washing five times using 150µl of endotoxin free ddH_2O. 100µl of Coating Antigen or antibody solution was then added to each well and allowed to bind to the plate overnight at 4°C.

Day 2:

2 hours prior to use, the excess coating antigen/antibody was removed from the plate by washing five times using 150µl of 1xPBS. Unbound areas of the plate were then blocked with irrelevant protein to prevent non-specific plate binding through addition of 100µl of cRPMI + 10% Fetal Calf Serum (FCS), which was allowed to bind for 2 hours at 37°C + 4% CO_2.

One hour prior to use sample cells were defrosted into cRPMI + 10% FCS as per Section 4.5.3 with the addition of a further wash step prior to cell counting (to remove residual human antibody serum). Cells were subsequently diluted to $1x10^6$ cells/ml in cRPMI + 10% FCS and rested for 1 hour at 37°C, 4% CO_2.

Immediately prior to use cells were washed in cRPMI + 10% FCS and re-counted as per Section 4.5.1 prior to re-dilution to a concentration of $1x10^6$/ml. Excess blocking media was removed from the plate by washing five times using 150µl of 1xPBS. 200µl of cell suspension was then added to each well ($2x10^5$ cells/well), with the exception of positive control wells which received 25µl of cell suspension and 175µl of cRPMI + 10% FCS. Cells were then cultured on the plate for 24 hours at 37°C, 4% CO_2.

Day 3:

Cells were removed from the plate by washing five times using 150µl of 1xPBS supplemented with 0.1% Tween (PBS-T). The secondary biotinylated antibody corresponding to the initial coating antibody used for the positive control was then diluted to its working concentration in

PBS + 1% BSA and 100µl of the solution added to each well. The secondary antibody was allowed to bind for between 90 – 120 minutes at 37°C + 4% CO_2.

Excess secondary antibody was removed from the plate by washing five times using 150µl of 1xPBS-T. Streptavidin was diluted to 1:1000 concentration in PBS + 1% BSA and 100µl added per well. This was allowed to bind at 37°C + 4% CO_2 for 60 minutes.

Excess streptavidin was removed by washing five times using 150µl of 1xPBS-T. The under-tray of the plate was removed, blotted dry and replaced (to reduce background). BCIP/NBT substrate buffer was made up to manufacturer's instructions and 100µl added per well. Spots were allowed to develop and the reaction stopped through overriding dilution using tap water. Plate was dried and then read using a (Autoimmun Diagnostika GmbH, Straßberg, Germany).

4.9.4 Protocol for numeration of antigen specific antibody secreting cells and memory B-cells:

Day 1:

Plasma cell plate was prepared as per the protocol for numeration of antibody secreting cells (see Section 4.9.3).

Day 2:

The plate was washed and blocked, and the cell-samples thawed, as per the protocol for numeration of antibody secreting cells.

Prior to the rest phase cells were divided for use within the plasma cell numeration plate and for use in the memory B-cell numeration plate; those cells apportioned for use within the plasma cell assay were diluted to $1x10^6$/ml and rested/plated as per the protocol for numeration of antibody secreting cells (see Section 4.9.3).

Those cells apportioned for use within the memory B-cell assay were diluted to $2x10^6$ cells/ml in cRPMI + 10% FCS. Each sample was divided into 2 wells of a 6-well plate, with each well receiving $5x10^6$ cells in a total of 2.5ml of culture medium. One well was then supplemented with 1µg/ml of R848 and 10ng/ml of $_{rh}$IL2 – designated "stimulated". The remaining well received no stimulation – designated "unstimulated". The cells were then cultured for 72 hours at 37°C, 4% CO_2.

Day 3:

Plasma cell plate was developed as per the protocol for numeration of antibody secreting cells (see Section 4.9.3).

Day 4:

The Memory B Cell plate plan was laid out on a 96 well plate; each antigen specificity, negative and positive control required replication in triplicate for both unstimulated and stimulated cells – an example plate plan is included in Appendix D. Coating antigens and antibody was diluted in 1xPBS (sterile) according to its optimised working concentration (see Table 5).

Plate membrane was activated by addition of 50µl of 35% ETOH to each well which was allowed to sit within the well for a maximum of 60 seconds. ETOH was removed from the plate membrane by washing five times using 150µl of endotoxin free ddH_2O. 100µl of Coating Antigen or antibody solution was then added to each well and allowed to bind to the plate overnight at 4°C.

Day 5:

2 hours prior to use, the excess coating antigen/antibody was removed from the plate by washing five times using 150µl of 1xPBS. Unbound areas of the plate were then blocked with irrelevant protein to prevent non-specific plate binding through addition of 100µl of cRPMI + 10% Fetal Calf Serum (FCS), which was allowed to bind for 2 hours at 37°C + 4% CO_2.

Immediately prior to use sample cells were harvested from the culture plate into cRPMI + 10% FCS and centrifuged 485g, 5 minutes prior to counting as per Section 4.5.1 and dilution to a concentration of $1x10^6$/ml. Excess blocking media was removed from the plate by washing five times using 150µl of 1xPBS. 200µl of cell suspension was then added to each well ($2x10^5$ cells/well), with the exception of positive control wells which received 25µl of cell suspension and 175µl of cRPMI + 10% FCS ($25x10^4$ cells/well). Cells were then cultured on the plate for 24 hours at 37°C, 4% CO_2.

Day 6:

Plate was developed as previously described (see Section 4.9.3).

4.9.5 Analysis:

Analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, US).

The number of spots within each well was normalised to number of spots per million (SPM) by multiplication of the number of spots by $1x10^6$ / number of cell plated per well.

For Plasma cell plates:

The SPM within the negative control wells were averaged and subtracted from all remaining wells to exclude background. SPM from each antigen-triplicate were averaged. The total number of antibody secreting cells within the sample was defined as equal to the average SPM count from the positive control wells. The antigen specific ASCs where then described as a percentage of the Total ASCs and as a proportion of cells plated.

For Memory B-cell Plates:

Antigen-specific plasmablasts were defined as the number of spots within antigen coated wells containing unstimulated cells, minus the number of spots within the blank wells containing unstimulated cells.

Antigen-specific memory B cells were defined as the number of spots within antigen-coated cells containing stimulated cells, minus the number of antigen-specific plasmablasts and minus the number of spots within the blank wells containing stimulated cells.

4.10 Flow Cytometry-based assays (Non-stimulated)

4.10.1 Reagents:

- FACS Buffer (900ml ddH2O, 100ml 10x Phosphate Buffered Saline, 5g Bovine Serum Albumin, 1g Sodium Azide)
  - Bovine Serum Albumin (BSA) (**Sigma Cat:A7906-100G**)
  - Sodium Azide (NaN_3) (**Sigma Cat:438456-25G**)
  - Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat:P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- 1% Paraformaldehyde (**Sigma. Cat:P6148**)
- BD CompBead Plus; Anti-mouse Ig, k (**BD Biosciences. Cat:560497**)
- BD Cytofix/Cytoperm kit with BD GolgiPlug (Brefeldin A) (**BD Biosciences. Cat:555028**)
  - Brefeldin A (BrefA) diluted to 1µl/ml in FACS buffer (BrefA Buffer)
  - Cytofix/cytoperm solution
  - Perm/wash buffer (10x) diluted in ddH_2O
- Biolegend Nuclear Factor Fixation and Permeabilisation Buffer Set. (**Biolegend Cat:422601**)
  - Nuclear Factor Fixation Buffer (4x) diluted with sterile-filtered PBS

- Nuclear Factor Permeabilisation buffer (10x) diluted with sterile-filtered PBS

- For a list of antibodies and florescent labels used see Table 6:

4.10.2 Protocol:

For all assays using fresh cells or when cells were cultured in medium devoid of Human Antibody Serum, cells were pre-conditioned in cRPMI with 10% Human Antibody serum to achieve Fc-receptor blockade. For assays using cryopreserved cells, cells were thawed into cRPMI containing 10% Human Ab Serum as per section 4.5.3.

All spin or wash steps were performed at 485g for 5 minutes at room temperature unless otherwise stated. Between steps and during incubation cells were maintained in the dark at 4°C unless otherwise stated. All flow Cytometry was performed on BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, US).

4.10.2.1 Establishment of new staining panel:

Flow cytometry staining panels were designed for use on BD FACS Canto II flow cytometer utilising 4-2-2 laser configuration. Candidate markers were identified and matched with available fluorochromes from suppliers. Compatibility was checked using on line software available from BD Biosciences (BD FACS Spectrum. Available at:

http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp). Candidate panels were compensated and assessed for suitability using the "fluorescence minus 1" technique; briefly, cells were stained with all antibodies within a panel save for one, resulting in up to eight tubes each lacking a different marker within the panel. Each fluorochrome was then plotted against the missing channel to ensure minimal "bleed-through", which might result in false positive characterisation. In the presence of "bleed-through" the panel was rejected and re-designed.

Set up for each panel was standardised over time through use of BD™ Cytometer Setup and Tracking beads.

Table 6: List of antibodies and florescent markers used

Target Antigen	Fluorochrome [4]	Isotype	Clone	Company	Catalogue Number	Quantity (for Staining)	Quantity (for Compensation using Beads)
Live/Dead Viability Markers							
Aqua Live/Dead Discrimination Dye	~Violet Green[5]			Life Technologies	L34957	1µl of reconstituted stock/1x10^6 cells	N/A
Annexin V (544)	FITC[6]			In House	N/A	1.25µl	N/A
PI	~Argon Yellow[7]			Life Technologies	P3566	0.45µg	
Antibodies							
CXCR5	AF 488	Rat IgG2,κ	RF8B2	BD Biosciences	558112	3µl	N/A
CD14	AF488	Mouse IgG1,κ	HCD14	Biolegend	325610	5µl	1.0µl
IgD	AF488	Mouse IgG2a, κ	IA6-2	Biolegend	348215	5µl	1.0µl
CD303	FITC	Mouse IgG2a, κ		Miltenylbiotech	130-090-510	6.6µl	0.5µl
CD154	PE	Mouse IgG1,κ	TRAP1	BD Biosciences	555700	5µl	1.0µl
CCR7	PE	Mouse IgG2a, κ	Go43H7	Biolegend	353203	5µl	0.25µl
CD1c	PE	Mouse IgG2a, κ	AD5-8E7	Miltenylbiotech	130-090-508	6.6µl	0.5µl
PD1	PerCP Cy5.5	Mouse IgG1,κ	EH12.2H7	Biolegend	329913	5µl	1.0µl
CD8	PerCP	Mouse IgG1,κ	RPA-T8	Biolegend	301029	5µl	1.0µl
CD4	PerCP	Mouse IgG2b, κ	OKT4	Biolegend	317431	5µl	1.0µl
CD14	PerCP	Mouse IgG2a, κ	RM052	Beckman Coulter	A07765	6.6µl	0.5µl
CD56	PerCP	Mouse IgG1,κ	HCD56	Biolegend	318341	5µl	1.0µl
CD19	PE-Cy7	Mouse IgG1,κ	HIB19	Biolegend	302216	4µl	0.5µl
ICOS	PE-Cy7	Mouse IgG1,κ	ISA-3	eBiosciences	25-9948-41	5µl	1.0µl

[4] The following abbreviations are used: AF488 – AlexaFluor 488, FITC – Fluroescein isothiocyanate, PE – phycoerythrin, PerCP – peridinin chlorophyll protein, APC – allophycocyanin, BV 421 – Brilliant Violet 421

[5] Aqua Live/Dead discrimination dye (Life Technologies) is an amine-reactive dye. Non-viable cells have permeable membranes allowing cytoplasmic staining and increased florescence-uptake in these cells. Multiple colours are available; "Aqua" is excited by UV laser and fluoresces green light.

[6] A protein which binds to phosphatidylserine and phosphatidlyethanolamine, two phospholipids which are present on the surface of dead or dying cells (Marino *et al.* 2013)

[7] Propidium iodine is an intercalating agent which binds to nucleic acids in non-viable cells. It is excitable by the 488 line of the argon laser and emits in the same channel as PE

CD137	APC	Mouse IgG1,κ	4b4-1	BD Biosciences	561702	5µl	1.0µl
Bcl-6	APC	Rat IgG2a, κ	7D1	Biolegend	358506	5µl	1.0µl
CD4	APC	Mouse IgG1,κ	SK3	Biolegend	344614	5µl	0.5µl
CD27	APC	Mouse IgG1,κ	O323	Biolegend	302810	5µl	0.5µl
IgM	APC-Cy7	Mouse IgG1,κ	MHM-88	Biolegend	314519	5µl	0.5µl
IgG	APC-Cy7	Mouse IgG2a, κ	HP6017	Biolegend	409314	6.6µl	1.0µl
CD3	APC-Cy7	Mouse IgG2a, κ	HIT3A	Biolegend	300318	4µl	1.0µl
CD45	APC-Cy7	Mouse IgG1,κ		BD Pharmagen	557833	1.6µl	0.8µl
CD45RA	BV 421	Mouse IgG2b, κ	HI100	Biolegend	304130	5µl	0.5µl
CD38	V450	Mouse IgG1,κ	HB7	BD Biosciences	646851	5µl	N/A
CD16	V500	Mouse IgG1,κ	3G8	BD Biosciences	561394	5µl	N/A
CD3	V500	Mouse IgG1,κ	UCHT1	BD Biosciences	561416	4µl	N/A
CD14	V500	Mouse IgG2b, κ	MφP9	BD Biosciences	562693	5µl	N/A
CD19	V500	Mouse IgG1,κ	HIB19	BD Biosciences	561121	5µl	

4.10.2.2 Protocol for Annexin/PI viability assessment:

$2x10^5$cells were washed in 2ml of Annexin V binding buffer (in house) (diluted in ddH_2O) and then re-suspended in 250µl of Annexin V Binding Buffer. Annexin V and Propidium iodide were added to the cells and flow Cytometry preformed following an incubation period of 10 minutes.

4.10.2.3 Protocol for phenotyping WITH Live/Dead discrimination staining:

Number of cells used was determined by intended use (see below).

Aqua Live/dead discrimination stain was diluted to working concentration by addition of 4µl of stock to 1ml 1xPBS. Cells were washed in 2ml 1xPBS and re-suspended in 250µl stain per $1x10^6$ cells. Stain was allowed to bind for 30 minutes at 4°C away from light. Cells were washed in 2ml FACS buffer and then underwent further staining as below:

4.10.2.4 Protocol for surface marker staining:

Number of cells used was dictated by the predicted number of events and whether intracellular staining was required; the additional wash-steps implicit in intracellular staining necessitate a larger starting population. Rare target populations would, again, require greater numbers of cells.

Cells were washed either following live/dead discrimination staining or immediately following Fc receptor blockade in 2ml FACS buffer. Antibodies were prepared in a total volume of FACS buffer equal to 100µl per tube. 100µl of the resultant antibody solution was added per tube and allowed to bind for 30 minutes at 4°C away from light. Unbound antibody was washed away using 2ml of FACS buffer and cells either re-suspended in 200µl of FACS buffer for immediate analysis or fixed as below for later analysis (Section 4.10.2.5) or processed for intracellular staining of additional markers (Sections 4.10.2.6 and 4.10.2.7).

4.10.2.5 Protocol for Fixation of Cells for Delayed analysis using Flow Cytometry:

Following the final wash step after surface staining cells were re-suspended in 100µl 1% paraformaldehyde which was allowed to work for 15 minutes at 4°C away from light. Paraformaldehyde was then removed by washing cells in 2ml FACS buffer. Cells were re-suspended in 200µl of FACS buffer and kept in the dark at 4°C until use (up to 1 week).

4.10.2.6 Protocol for Intracellular Staining for Cytokines and other extra-nuclear proteins (e.g. antibody)

A minimum of $2x10^6$ cells were used per tube. In the event of Live/Dead discrimination and/or surface staining being used, this was performed prior to intracellular staining as above EXCEPT instead of FACS Buffer BrefA Buffer was substituted for all washes and antibody staining steps during the surface staining (PBS was still used for Live/Dead discrimination staining, the protocol for which was unchanged).

Following surface staining cells were washed twice in 2ml BREFA Buffer. Cells were re-suspended in 100µl BD Cytofix/Cytoperm solution and incubated for 15 minutes. Cells were washed twice in 2ml BD Perm/Wash buffer and antibodies for use for intracellular stain prepared in a total volume of BD Perm/Wash buffer equal to 100µl per tube. 100µl of antibody solution was added to each tube and allowed to bind for 30 minutes at 4°C, away from light.

Excess antibody was removed by washing twice in 2ml BD Perm/Wash buffer. Cells were then re-suspended in 200µl of FACS buffer and analysed.

4.10.2.7 For nucleic factor staining

A minimum of $2x10^6$ cells were used per tube. In the event of Live/Dead discrimination and/or surface staining being used, this was performed prior to nucleic factor as above.

Following surface staining cells were re-suspended in 1ml Nuclear Factor Fixation Buffer (Biolegend) which was allowed to work for 20 minutes at room temperature and away from light. Cells were spun then washed using 1ml Nuclear Factor Permeabilisation buffer (Biolegend). Cells were re-suspended in 1ml Nuclear factor permeabilisation buffer which was allowed to work for 20 minutes at room temperature, away from light.

Antibodies for use for intra-nuclear staining were prepared in a total volume of Nuclear factor permeabilisation buffer equal to 100µl per tube. Cells were spun and re-suspend in 100µl of the antibody solution. Antibodies were allowed to bind at room temperature for 30 minutes away from light.

Excess antibody was removed by washing in 2ml FACS buffer. Cells were then re-suspended in 200µl of FACS buffer and analysed.

4.11 Ex-vivo re-stimulation assay (utilising Flow Cytometry)

4.11.1 Principle:

Antigen specificity of CD4 cells cannot be directly ascertained without use of Class II tetramer. However ex-vivo antigen stimulation and subsequent cell staining for markers of activation (i.e. CD154) or induced cytokine production has previously been utilised by our laboratory to determine cell specificity (371, 493, 498).

The ultimate aim of this study is to isolate live antigen-responding follicular helper T-lymphocytes for the purpose of RNA extraction. Intracellular staining for cytokine production requires permeabilisation of the cells, which is not therefore compatible with isolation for RNA-sequencing analysis. Cytokine-capture techniques are available, but rely on cells not only producing cytokines, but also secreting cytokine in significant quantities; such assays are also

selective for individual cytokines and may not be reflective of the broader population as a whole. Previous publications have detailed the use of both CD154 (CD40L) (545-547) and CD137 (4-1BB) (548) as markers for cell activation; both markers are reported to identify a broader population of antigen-responsive CD4+ cells which produce a variety of cytokines and in variable quantities, thereby partially avoiding the selection bias of cytokine-based techniques whilst identifying the majority of cells induced to produce cytokines.

CD137 is expressed on the cell surface of recently activated lymphocytes whereas CD154 is up-regulated on the cell surface, but rapidly cycled. Therefore whilst CD137 can be identified by surface staining, optimal CD154 staining requires either co-culture with the antibody in the presence of the protein transport inhibitor monensin (545, 546), or intracellular staining following co-culture with either monensin (545, 546) or an alternative protein transfer inhibitor brefeldin A (498). Again intracellular staining would not be compatible with live-cell sorting however co-culture of live cells with antibody would be suitable for isolation for RNA extraction.

Monensin is an inhibitor of exocytosis whereas Brefeldin A is an inhibitor of the golgi apparatus itself. Therefore Monensin is suitable for co-culture assay as it allows protein to the surface but not its release, whereas Brefeldin A causes confinement to the cytoplasm. However Monensin is associated with worse cell viability when compared with Brefeldin A and is less effective at retaining cytokine (549). The following assay was therefore devised to assess the utility of ex-vivo antigen re-stimulation as an tool for assessing antigen specificity of tissue derived follicular cells. Co-stimulation was provided using a combination of functional human anti-CD28 and anti-human CD49d antibodies. Both CD154 and CD137 were assessed for suitability as markers of cell activation.

4.11.2 Reagents:

- FACS Buffer (900ml ddH2O, 100ml 10x Phosphate Buffered Saline, 5g Bovine Serum Albumin, 1g Sodium Azide)
  - Bovine Serum Albumin (BSA) (**Sigma Cat:A7906-100G**)
  - Sodium Azide (NaN_3) (**Sigma Cat:438456-25G**)
  - Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat:P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))

- 1% Paraformaldehyde (**Sigma. Cat:P6148**)
- BD CompBead Plus; Anti-mouse Ig, k (**BD Biosciences. Cat:560497**)
- Functional mouse anti-human CD28 (clone CD28.2 (RUO), mouse IgG1, k) (**BD Biosciences. Cat: 555725**)
- Functional mouse anti-human CD49d (clone 9F10 (RUO), mouse IgG1, k) (**BD Biosciences. Cat: 555501**)
- Tetanus Toxoid (**NIBSC. Cat: 04/150**)
- Diphtheria Toxoid (**NIBSC. Cat: 02/176**)
- Purified Protein Derivative from Tuberculin (PPD) (**Statens Serum Institute. Cat: 2391, Batch RT50**)
- Staphylococcal Enterotoxin B (**Sigma. Cat: S4881**)
- Monensin ("Golgistop") (**BD Biosciences. Cat:554724**)
- Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- BD Cytofix/Cytoperm kit with BD GolgiPlug (Brefeldin A) (**BD Biosciences. Cat:555028**)
  - Brefeldin A (BrefA) diluted to 1µl/ml in FACS buffer (BrefA Buffer)
  - Cytofix/cytoperm solution
  - Perm/wash buffer (10x) diluted in ddH_2O

For a list of antibodies and florescent labels used see Table 6.

4.11.3 Protocol:

Cryopreserved cells were thawed into cRPMI containing 5% Human Ab Serum as per section 4.5.3 and made up to a concentration of $20x10^6$ cells/ml. 100µl of cell medium was added to each well of a 96-well culture plate. Double concentration stimulation medium was prepared consisting of cRPMI containing 5% Human Ab Serum supplemented with either 2µg/ml Staphylococcal Enterotoxin B (positive control), 40µg/ml Tetanus Toxoid, 40µg/ml Diphtheria Toxoid or 20µg/ml of PPD. 100µl of stimulation medium was added to each well, with control wells receiving un-supplemented cRPMI with 5% human Ab serum, leaving a total volume of 200µl containing $2x10^6$ cells in each well.1µg/ml of anti-CD28 and anti-CD49d were added to all stimulated wells with the exception of the Staphylococcal Enterotoxin B. Addition of anti-CD28 and anti-CD49d alone provided a negative co-stimulated control, whilst an un-supplemented well provided a negative control. Cells were cultured at 37°C, 4% CO_2 for either 6, 12, 24 or 48 hours according to the individual assay requirements. For each experiment an uncultured cell sample provided a "baseline" control.

When used, monensin was added at a final concentration of 2µM at the beginning of the cell culture. When used, Brefeldin A was added at a final dilution of 1:1000 (as per manufacturers guidance) 2 hours following antigen exposure. When either agent was used, unexposed cells receiving the same stimulation were used as controls to assess effect of exposure.

Following completion of culture, cells were harvested from wells by repeat washing with 200µl sterile PBS. Cells were then stained for analysis as per protocols detailed in Section 4.10.2.

4.11.4 Analysis

Activation marker up-regulation was calculated in terms of percentage positive events within the total CD4+ population, or within individual sub-populations, minus the percentage positive events within the same population within the co-stimulated control wells.

4.12 B-lymphocyte staining with fluorescently labelled antigen

STATEMENT: the principles behind this section, and the design of assays used in the development and validation of this assay, were contributed, in part, by a post-doctoral colleague (Dr Alexandra Allen). Laboratory work necessary to the production of fluorescently labelled antigen, and the optimisation of its conditions of use, was performed in part by a student (Miss Catherine Pointer) working under our joint supervision. Data relevant to this has previously been submitted to the University of Southampton as part of Miss Catherine Pointer's Integrated PhD, within which I was acknowledged as a co-supervisor.

4.12.1 Principle:

B-lymphocytes recognise and bind their target antigen directly via the B-cell receptor which is essentially a membrane bound antibody. Therefore B-cells specific to a certain protein can be identified by labelling that protein with a florescent marker, and allowing said labelled protein to bind the B-cell receptor. The dye in its pre-reactionary state possesses a succinimidyl ester moiety which binds primary amines of the target protein. Due to the formalin treatment of toxoids (used to render them non-toxic) whole toxoids cannot be labelled using this method. Therefore a non-toxic protein derivative of the toxin is required for labelling.

Fragment C (FrC) is the C-terminal binding portion of tetanus toxin, with a molecular weight of approximately 47,000 daltons. It is antigenic, non-toxic and capable of inducing neutralising antibodies against tetanus toxin in vivo (550). It has also been utilised as an adjunct in vaccine design (see Section 3.2.2). These properties make FrC protein suitable for use as a surrogate for tetanus toxoid specificity, as well as being of interest to the wider work undertaken within our unit. Mouse Serum Albumin (MSA) is a protein to which there should have been no prior antigenic exposure within the majority of human patients. Therefore B-cells specific to MSA should be rare, allowing it to be used as a negative control stain.

4.12.2 Reagents:

- Fragment C from Tetanus Toxoid (**In house reagent, curtesy of Dr Patrick Duriez**)
- Mouse Serum Albumin (MSA) (**Merck Millipore. Cat: 126674-25MG)**
- AlexaFluor 647 Protein Labelling kit (**Invitrogen. Cat: A20173**)

  Containing:
  - Alexa Fluor 647 Reactive dye provided within vial containing self-contained magnetic stir bar
  - Sodium bicarbonate
  - Purification resin
  - 10x Elution buffer
  - Purification columns + funnels
- Ultra-pure Nuclease-Free Water (**ThermoFisher Scientific. Cat: AM9932**)
- 10x Nuclease-free Phosphate Buffered Saline (**ThermoFisher Scientific. Cat: AM9625**)

4.12.3 Protocol:

1mg FrC protein or 1mg of Mouse serum albumin was dissolved in 500µl of ultra-pure, nuclease-free PBS. To this 50µl of 1M sodium bicarbonate was added and then the mix transferred to the vial of reactive dye. Using the integrated magnetic stir bar, the reaction mix was agitated continuously at room temperature for 1 hour.

Elution buffer was prepared by dilution with ultrapure nuclease-free H_2O. Purification column and funnel were assembled according to manufacturer's instructions and column loaded with purification resin. Protein reaction mix was then added to the column, elution buffer was then layered on top of the mix continuously for 30 minutes. Band separation of conjugated and unconjugated dye was observed on the column with good special separation; the first (faster moving) band represented the protein-bound dye which was collected for experimental use and stored at 4°C , protected from light. Using absorbencies measured via nanodrop, equivalent binding to dye per Mole of protein was confirmed between FrC and the control protein MSA.

These reagents can subsequently be used in a fashion similar to fluorescently labelled antibodies utilising the Alexa Fluor 647 channel.

4.13 Florescence-activated cell sorting

4.13.1 Principle

Flow cytometers with the ability to isolate individual cells according to their fluorescence characteristics can be used to separate individual populations with a high degree of purity.

These populations can subsequently be cultured or, in the case of this study, used to generate RNA or DNA libraries.

4.13.2 Reagents:

- 10x Nuclease-free Phosphate Buffered Saline (**ThermoFisher Scientific. Cat: AM9625**)
- Nuclease-Free Water (**ThermoFisher Scientific. Cat: AM9932**)
- MACS Buffer (900ml ddH2O, 100ml 10x Phosphate Buffered Saline, 5g Bovine Serum Albumin, 2mM EDTA)
    - Bovine Serum Albumin (BSA) (**Sigma Cat:A7906-100G**)
    - Ethylenediaminetetraacetic acid (EDTA) (**Invitrogen. Cat:15575-038**)
    - Phosphate Buffered Saline (PBS) (**BDH. Cat: 43711K**)
- Human AB Serum (**Sigma. Cat:4522, Lot: 051M0916**) – Batch Tested and decomplemented by heating to 56°C for 45 minutes prior to storage at -20°C in single-use aliquots.
- cRPMI (500ml RPMI + 5ml x100 Penicillin/Streptomycin (**Sigma. Cat: G7513** + 5ml x100 Glutamine (**Sigma. Cat:P4333**) + 5ml x100 Sodium Pyruvate (**Gibco. Cat:11360-039**))
- BD CompBead Plus; Anti-mouse Ig, k (**BD Biosciences. Cat:560497**)
- TRIzol LS (**ThermoFisher Scientific. Cat: 10296028**)
- Ultra-pure Nuclease-Free Water (**ThermoFisher Scientific. Cat: AM9932**)
- 10x Nuclease-free Phosphate Buffered Saline (**ThermoFisher Scientific. Cat: AM9625**)
- Nuclease free 1.25ml collection tubes (**ThermoFisher Scientific. Cat: AM12400**)
- Nuclease free 8x micro-tube collection strips (**ThermoFisher Scientific. Cat: AB-0778**)
- For a list of antibodies and florescent labels used see Table 6:

4.13.3 Protocol:

Staining of cells for florescence activated cell sorting follows the same protocols as per standard flow cytometry (see Section 4.10.2), however MACS buffer is used in place of FACS buffer in all instances. For the purposes of RNA isolation cell fixation, permeabilisation and/or intracellular staining were avoided.

All spin or wash steps were performed at 485g for 5 minutes at room temperature unless otherwise stated. Between steps and during incubation cells were maintained in the dark at 4°C unless otherwise stated. All flow Cytometry was performed on BD FACSAria I flow cytometer (BD Biosciences, Franklin Lakes, NJ, US). Machine set up, including fluidics optimisation was performed by an expert technician to ensure optimal conditions. Samples were sorted into a cooled, sterile medium to maintain RNA integrity.

4.13.3.1 Protocol for isolation of whole populations for RNA isolation

Technique was used to sort 4 populations for the purposes of RNA sequencing analysis. Populations were CD4+CD45RO+CXCR5+ cells expressing either a ICOS-PD1-, ICOS+PD1-, ICOS+PD1+ or PD1+ICOS- phenotype. A minimum of 5000 cells from each population were required for intended RNA sequencing analysis; the quantity of "whole sample" required to achieve this target was determined from original phenotypic analysis . To optimise yield and minimise run time two passages through the cytometer were performed for each sample. Initial passage was on a yield sort gated on all CD4+CD45RO+CXCR5+ cells sorted into MACS buffer at 4°C. These cells were then concentrated by centrifugation at 450g for 5 minutes and re-suspended in 1ml MACS buffer.

4-way cell sort on a high-purity setting was then used, gated on the 4 populations detailed above. Cells were sorted directly into 750µl TRIzol in nuclease-free collection tubes to a maximum of 50,000 events. Once sort was complete samples were vortexed and stored at -80°C.

4.13.3.2 Protocol for isolation of single cells

Technique was used to sort individual FrC-specific B-cells. Cells were surface stained with labelled antigen. Micro tube collection strips were prepared in 96-well configurations within a designated PCR hood; all necessary reagents and equipment was subjected to 30 minutes exposure to high intensity ultra violet light to avoid contamination. Each microtube was loaded with 5µl of 1x ultra-pure nuclease free PBS and then covered with an adhesive film.

Two passages through the cytometer were used to maximise yield and minimise run time. Initial passage was on a yield sort gated on all antigen positive CD19 cells. Collection, concentration and re-constitution of yield sorted cells was performed as per Section 4.13.3.1. Second passage was performed on the same gated population at the lowest possible flow rate using a single cell sort. Once 96 cells were captured the plate was re-covered and immediately placed on dry ice. Samples were then transferred to storage at -80°C.

4.14 Isolation and quantification of RNA from TRIzol LS-lysed samples

4.14.1 Principle

To ensure adequate RNA quantity and integrity was being achieved through the technique detailed in Section 4.13.3.1, trial samples were used to generate RNA which was subsequently subjected to quantification assay utilising primers for beta-2-microglobulin (ß2M). ß2M-gene encodes the ß2M protein which is universally expressed on the surface of nearly all eukaryote cells. It is therefore used as a positive control in PCR reactions. It can also be used as a surrogate marker for total RNA content of a sample.

4.14.2 Reagents:

- Direct-zol RNA MiniPrep Kit (**Zymo research. Cat: R2053**)

  Containing:

  - Direct-zol RNA PreWash
  - RNA Wash Buffer
  - DNase I
  - DNA Digestion Buffer
  - DNase/RNase free water
  - Zymo-Spin IIC Columns
  - RNase free collection tubes
- RNAse-Zap (**Ambion. Cat:AM9780**)
- Ethanol (**Fisher Chemicals. Cat: E/0650DF/17**)
- Ultra-pure Nuclease-Free Water (**ThermoFisher Scientific. Cat: AM9932**)
- Ultra-pure Nuclease-Free RT-PCR grade Water (**ThermoFisher Scientific. Cat: AM9935**)
- 10x Nuclease-free Phosphate Buffered Saline (**ThermoFisher Scientific. Cat: AM9625**)
- SuperScript III Reverse Transcriptase Kit (**Invitrogen. Cat: 18080-093**)

  Containing:

  - SuperScript III Reverse Transcriptase
  - 5x First Strand Buffer
  - 0.1M DTT
- RNAseOUT Recombinant Ribonuclease Inhibitor (**Invitrogen. Cat: 10777-019**)
- Oligo$(dT)_{20}$ Primer (**Invitrogen. Cat: 18418-020**)
- 10mM dNTP mix (**Invitrogen. Cat: 18427-013**)
- 25mM Magnesium chloride ($MgCl_2$) (**Thermo Scientific. Cat: R0971**)
- Random Hexamers (**Invitrogen. Cat: N8080127**)
- Forward and Reverse ß2M primers (**Sino Biological. Cat: HP100002**)
- GoTaq qPCR Master Mix (Promega. Cat: A6001)

  Containing:

  - GoTaq pPCR Master Mix
  - CXR Reference Dye
  - Nuclease-free water

4.14.3 Protocol:

RNA was purified from samples stored in TRIzol LS using the Direct-zol RNA MiniPrep kit according to the manufacturer's instructions. Briefly stored samples were defrosted and made up to 1ml using 1x ultrapure nuclease-free PBS. Samples were diluted with a further 1ml of

ethanol, transferred to a Zymo-Spin IIC column set within a collection tube and spun at 16,000g for 30 seconds (all subsequent spins were performed in this fashion unless otherwise stated). Flow through and collection tube were then discarded and the column reloaded into a new collection tube. 400µl of RNA wash buffer was added to the column and spun. 5µl of DNase I was added to 75µl DNA digestion buffer which was then loaded onto the column and incubated at room temperature for 15 minutes, then spun. 400µl of Direct-zol RNA pre-wash was added to the column and spun for 1 minute. This step was repeated and then 700µl of RNA wash buffer added to the column and spun for1 minute and flow through discarded. The empty column was then spun for a further 2 minutes. The column was then transferred to a RNase-free collection tube and 30µl of DNase/RNase free water added to the column and spun for 1 minute to elute RNA. Flow through was re-loaded onto column and re-spun for 1 minute to maximise yield.

Quantitative RT-PCR was performed on ice within a designated PCR laboratory, using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories Incorporated, Berkeley, California. USA), according to protocols previously optimised by Mr Oliver Wood and Dr Tilman Sanchez-Elsner, University of Southampton. Quantities given are per sample.

Briefly, primer mix was made using 0.25µl Oligo(dT), 0.25 µl random hexamer, 0.5 µl 10mM OligoNTP and 3 µl RT-PCR grade H_2O. 1 µl of eluted RNA was added to the primer mix and annealed at 65°C, 5 minutes then 4°C, 5 minutes. Superscript mix was made using 1 µl Reaction Buffer, 2 µl 25mM $MgCl_2$, 1 µl 0.1M DTT, 0.5 µl RNAseOUT and 0.5 µl SuperScript III Reverse Transcriptase and added to the annealed primer mix, then run at 25°C, 10 minutes, 50°C, 50 minutes, 85°C 5 minutes. This “Reaction Mix” was then cooled to 4°C until use.

Quantification of ß2M was performed in duplicate. Reaction mix was diluted ten times with qRT-PCR grade H_2O. PCR mix was made using 0.625 µl 5 µM Forward ß2M Primer, 0.625 µl 5 µM Reverse ß2M Primer, 5µl GoTaq Master Mix, 0.1µl CXR reference dye, 1.65µl RT-PCR grade H_2O and 2µl of the diluted reaction mix. PCR mix was then thermically cycled and fluorescence measured.

4.14.4 Analysis:

Florescence measured over time during the thermal cycling was compared to a standard curve to allow quantification of RNA within the original sample.

5 Methodological Optimisation

5.1 Technique Optimisation for generation of a single cell suspension from human lymph node

5.1.1 Established mechanical technique inadequate for purpose of study

Fresh human lymph nodes taken from colonic mesentery were subjected to mechanical dissociation as per Section 4.4.3 by the human tissue bank at University of Southampton. Tissue was processed immediately following excision. Isolated cells were assessed for viability before and after cryopreservation using trypan blue exclusion and through annexin V/Propidium Iodide (Ann/PI) viability assay analysed by flow Cytometry. Phenotypic analysis of lymphocyte sub-populations was performed through surface staining for CD3, CD4, CD8, CD14, CD19, CD45RA, CD56 and CCR7; Monocytes, B-lymphocytes and NK cells were identified through single marker staining for CD14, CD19 and CD56 respectively. Total CD4+ and CD8+ populations were numerated using duel staining for CD3/CD4 and CD3/CD8 respectively. CD45RA and CCR7 staining was used to identify memory phenotypes of both CD4 and CD8 populations; CCR7+CD45RA+ were designated naïve, CCR7+CD45RA- were designated T_{CM}, CCR7-CD45RA- were designated T_{EM} and CCR7-CD45RA+ were termed T_{EMRA}. Concurrently dendritic cell sub-populations were phenotyped through staining for CD1c, CD14, CD19, CD45 and CD303, where myeloid dendritic cells were defined as CD45+CD19-CD14-CD1c+ and Plasmacytoid dendritic cells as CD45+CD19-CD14-CD303+.

Initial cell retrieval was adequate with $10.2x10^7$ cells harvested from Node 1 (HLN1) and $8.6x10^7$ from Node 2 (HLN2). Viability assessment pre- and post- cryopreservation are presented in Table 7. Pre-cryopreservation, viability of freshly isolated cells was between 72-84%. Post-cryopreservation 67.1% of HLN1 and 37.8% of HLN2 cells frozen were retrieved in a viable state according to Ann/PI viability assessment.

Following phenotypic analysis, lymphocyte sub-populations pre-cryopreservation were compared with those present post-cryopreservation to ascertain whether preferential loss of particular sub-populations could account for loss of viability. Figure 4 illustrates the changes in lymphocyte sub-populations within HLN2 and can be considered representative of the data from both samples – no clear disproportionate loss was demonstrable however repetition of this comparison with further sample nodes was required.

ELISpot assay was performed to determine the functional state of isolated cells when compared to cryopreserved PBMCs (from an unrelated donor). Equal numbers of viable cells were plated for each sample. Marked differences were noted in the reactivity of lymph node (LN) cells when compared to PBMCs, with 5 to 50 fold difference in IFNγ production and 2.5 to

17 fold difference in IL-2 production seen following stimulation with SEB or functional anti-CD3/CD28 (see Figure 5). Flow Cytometry demonstrated proportional differences in CD4+ populations between the two samples; the lymph node isolates were B-cell and CD4+ cell rich compared with PBMCs which contained a greater number of monocytes and NK cells. However, a greater proportion of CD4+ cells within LN isolates (up to 50% of the total number of CD4+ cells) were naïve (CD45RA+CCR7+) whereas around 60% of circulating CD4+ cells were antigen-experienced (CD45RA-CCR7+/-).

Previous studies have demonstrated a high proportion of CD4+ cells isolated from lymph nodes do not produce cytokine following stimulation (146). In addition, the cytokine screen performed was limited to IFNγ and IL-2, therefore functional response through production of other cytokines could not be excluded. Furthermore, the differences in cytokine production could have been a consequence of different antigen-experience within LN and PBMCs. However the magnitude of the difference in addition to poor viability of cells isolated through mechanical means necessitated exploration of alternative approach.

Table 7: Cell viability and retrieval following mechanical dissociation of lymph node both before and after cryopreservation. Numbers of cells and their proportional viability given prior to and following cryopreservation. Far right column gives percentage of original cells retrieved in a viable state according to Annexin V/Propridium iodide staining following cryopreservation.

		Initial Viability (%)		Retrieval		Retrieval Viability (%)		% cells retrieved following freeze/thaw
	Number Frozen	T.Blue	Ann/PI	Number	%	T.Blue	Ann/PI	
HLN1	5.1×10^6	84	80	4.7×10^6	92	89.5	72.9	67.1
HLN2	5.06×10^6	72	72.9	3.7×10^6	72	79	52.5	37.8

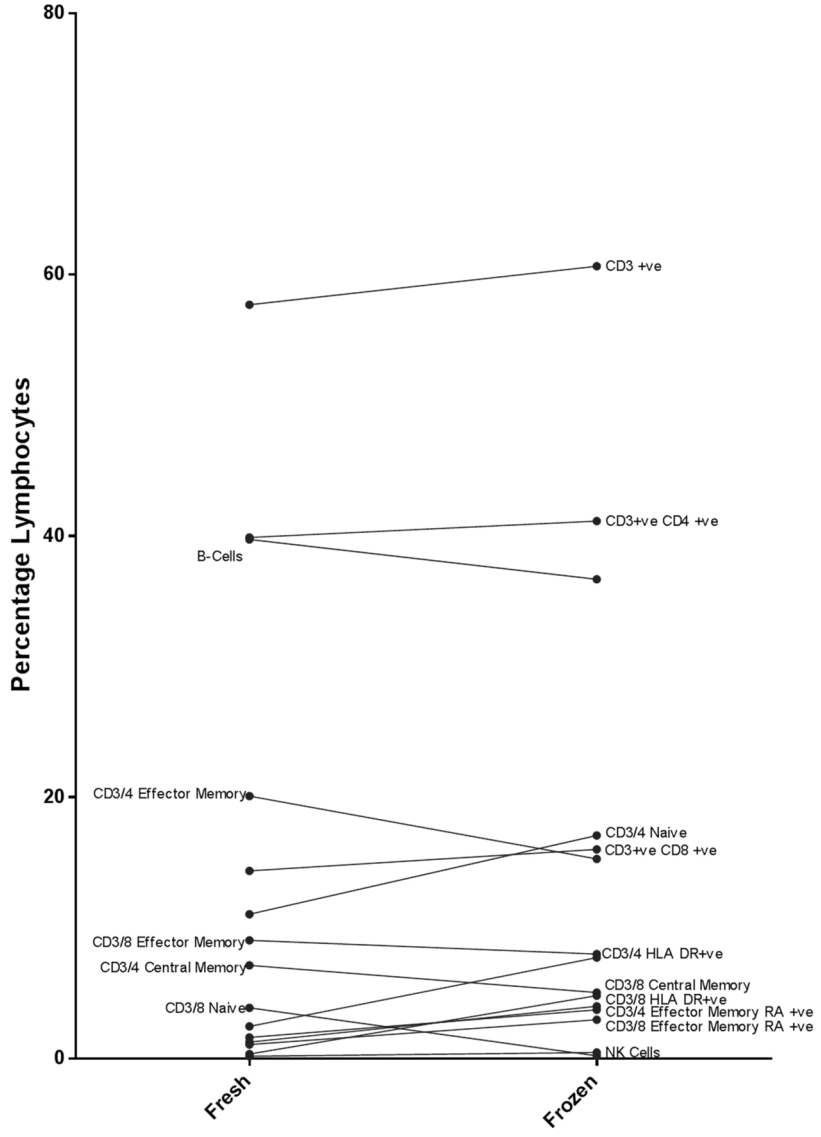


Figure 4: Change in lymphocyte sub-populations following cryopreservation within single cell suspension isolated from human lymph node (HLN2) using mechanical dissociation. Points plotted represent the percentage of the total lymphocyte population assigned to each phenotype. Cells increasing in their proportional representation within the total lymphocyte population are labelled on the right hand side, whereas those reducing are labelled on the left.

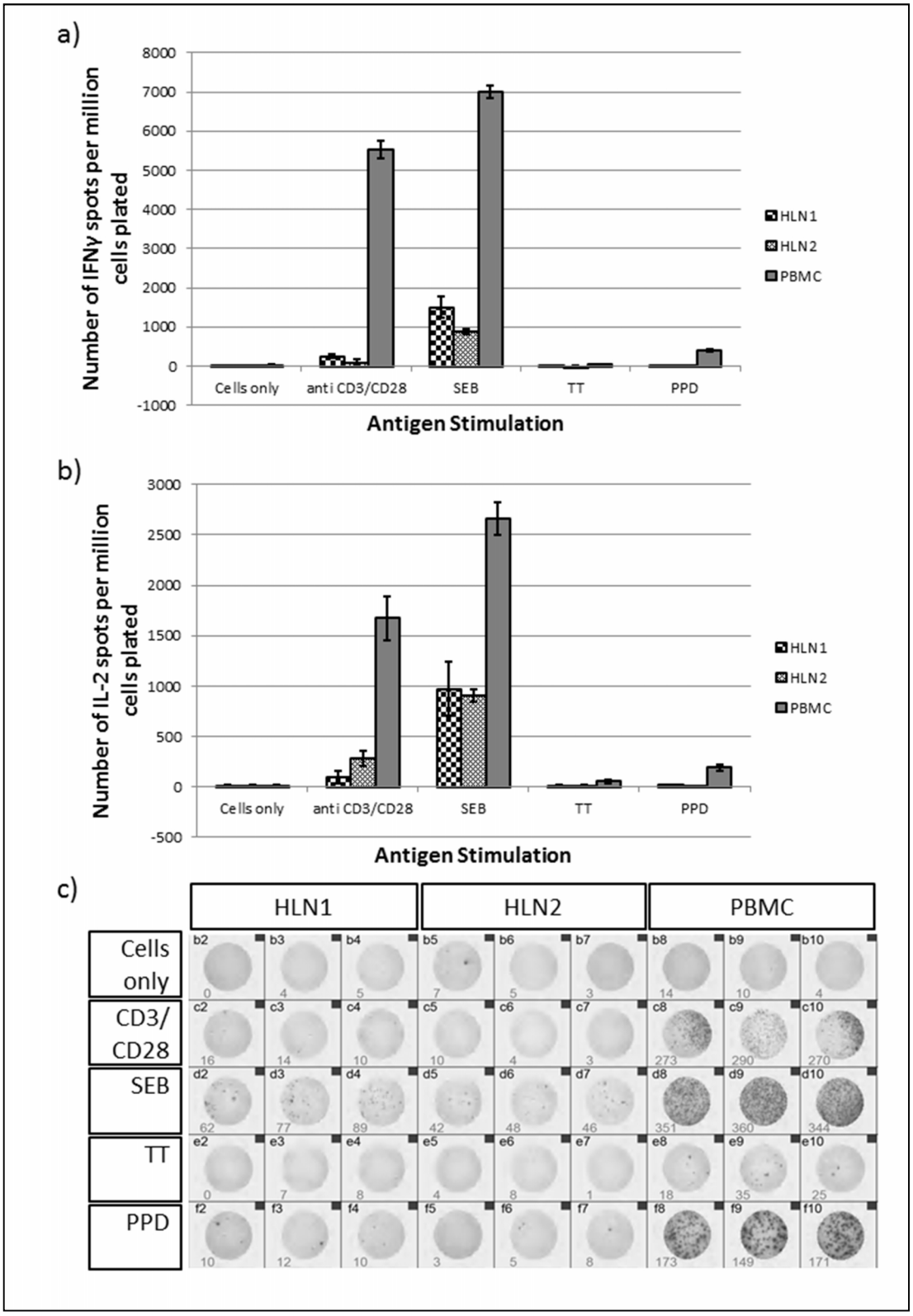


Figure 5: Comparison between PBMCs and cells derived from human lymph nodes (HLN1, HLN2) following mechanical disaggregation with regards to number of cytokine-producing cells per million plated following stimulation. Bar charts suggest a 5 to 50 fold difference in IFNγ production (a) and 2.5 to 17 fold difference in IL-2 production (b) following stimulation with positive controls. Data shown with error bars depicting 1x standard deviation about the mean. c) A representative ELISpot plate demonstrating IFNγ production following antigen stimulation (cell density $1x10^5$/well for CD3/CD28 and SEB, $4x10^5$/well for all other conditions).

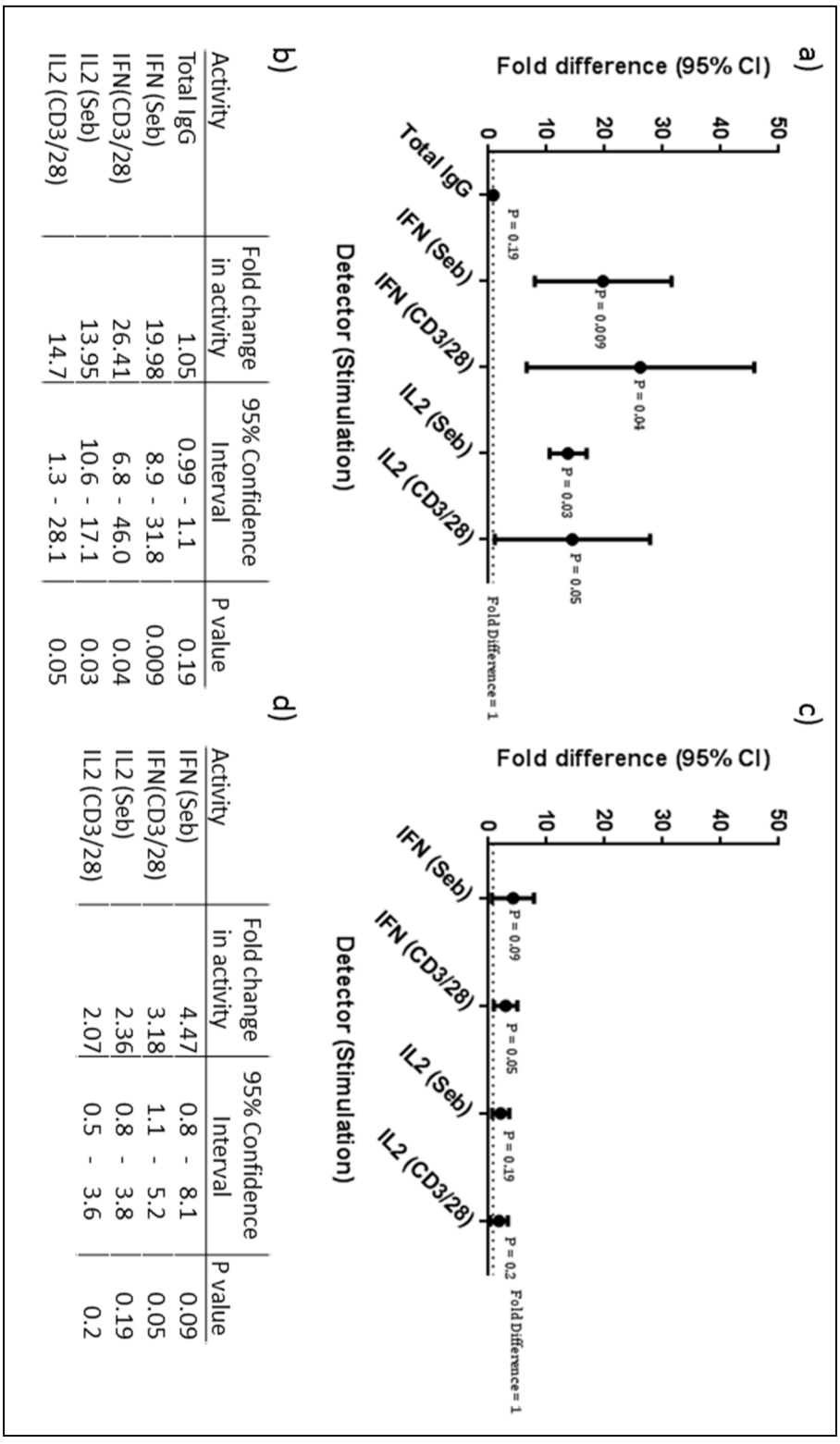
5.1.2 Use of enzymatic digestion step significantly improved IFNγ and IL2 production

To determine whether cell viability and functional state could be improved through alternative methods for creating a single cell suspension, the following strategy was followed: lymph nodes from bowel mesentery of patients undergoing gastrointestinal resection for active inflammatory bowel disease were bisected. One half was processed by mechanical means as per Section 4.4.3 whereas the other half was processed following enzymatic digest for 1 hour using 800KU/ml DNAse1 (Sigma. Cat: D4527) and 1mg/ml Collagenase IV (Sigma. Cat: C5138). Collagenase IV was selected as it was in use within protocols used for cell isolation from other tissues within our laboratory with good results. This enzyme is isolated from culture of *Clostridium histolytium* and contains at least 7 different contaminating protease enzymes undefined by the manufacturer (551). Technique used for tissue digest has been previously described within Section 4.4.4. with the notable difference concerning the enzyme preparations used.

Introduction of a Lymphoprep step was also attempted to reduce the quantity of debris and non-viable cells isolated. Single cell suspensions acquired through either mechanical or enzymatic means were halved and re-suspended in room temperature cRPMI; one half was layered over an equal quantity of Lymphoprep at room temperature centrifuged at 950g for 23 minutes. Cells were then retrieved from above the Lymphoprep layer. Processing in this fashion was performed on two lymph node specimens processed both mechanically and enzymatically, then each condition subjected to Lymphoprep step; in all instances substantial loss of cell numbers was incurred (mean number of cells (NoC) pre-Lymphoprep = $33.4x10^6$, NoC post-Lymphoprep = $2.9x10^6$; P = 0.004 (Students paired samples test)) with and average loss of 90.1% of the population (95% Confidence Interval = 82.2 – 98.0%). This was thought to be due to differences in cell density between lymph node-resident cells and PBMCs, and the process abandoned for future samples.

8 lymph node samples underwent mechanical and enzymatic treatment and were subjected to ELISpot before and after cryopreservation. Summary data are shown in Figure 6; following SEB stimulation, the number of IFNγ-producing cells detectable within cells isolated following enzyme pre-treatment was 20-fold greater than the number detectable following mechanical dissociation alone (95%CI: 8.9 – 31.8; P = 0.009) - Figure 6.a,b. Similarly, the number of detectable IL-2-producing cells was 14-fold greater (95%CI: 10.6 – 17.1; P = 0.03) - Figure 6.a,b. These findings were paralleled by stimulation with functional anti-CD3/CD28 [data not shown]. Conversely no change was seen in the total number of ASC present in mechanically vs enzymatically disaggregated cells - Figure 6.a,b.

Comparison of the detection of cytokine-producing cells within fresh compared with frozen cell suspensions demonstrated no significant difference (Figure 6.c), although there existed a general trend towards increased production of IFNγ per million cells plated within frozen cells - Figure 6.d. This was thought to likely be due to plating of cells of borderline viability within the fresh samples, which did not survive the cryopreservation process (i.e. cryopreservation posed a “survival challenge” and only viable cells went on to be counted prior to plating).



b)

Activity	Fold change in activity	95% Confidence Interval	P value
Total IgG	1.05	0.99 - 1.1	0.19
IFN (Seb)	19.98	8.9 - 31.8	0.009
IFN(CD3/28)	26.41	6.8 - 46.0	0.04
IL2 (Seb)	13.95	10.6 - 17.1	0.03
IL2 (CD3/28)	14.7	1.3 - 28.1	0.05

d)

Activity	Fold change in activity	95% Confidence Interval	P value
IFN (Seb)	4.47	0.8 - 8.1	0.09
IFN(CD3/28)	3.18	1.1 - 5.2	0.05
IL2 (Seb)	2.36	0.8 - 3.8	0.19
IL2 (CD3/28)	2.07	0.5 - 3.6	0.2

Figure 6: Fold-change in cytokine production by lymph node cells following non-specific stimulation with either SEB of functional anti-CD3/CD28; summary data from 8 nodal samples with mean fold change in activity and corresponding 95%CI. Assay read outs were either IFNγ or IL-2 production following stimulation, or total number of IgG producing cells following Plasma cell ELISpot. a) fold change in read out following enzymatic-digest compared to mechanical desegregation alone, where production by mechanically processed cells is taken as baseline. b) figures and P-values associated with data presented in a). c) fold change in read out following cryopreservation compared to fresh tissue, where production by fresh (i.e. cells which have not undergone cryopreservation) is taken as baseline. d) figures and P-values associated with data presented in c)

5.1.3 Enzymatic pre-treatment with non-purified Collagenase IV results in the loss of CXCR5 from the surface of cells, whilst improving retrieval of some memory-cell sub-populations

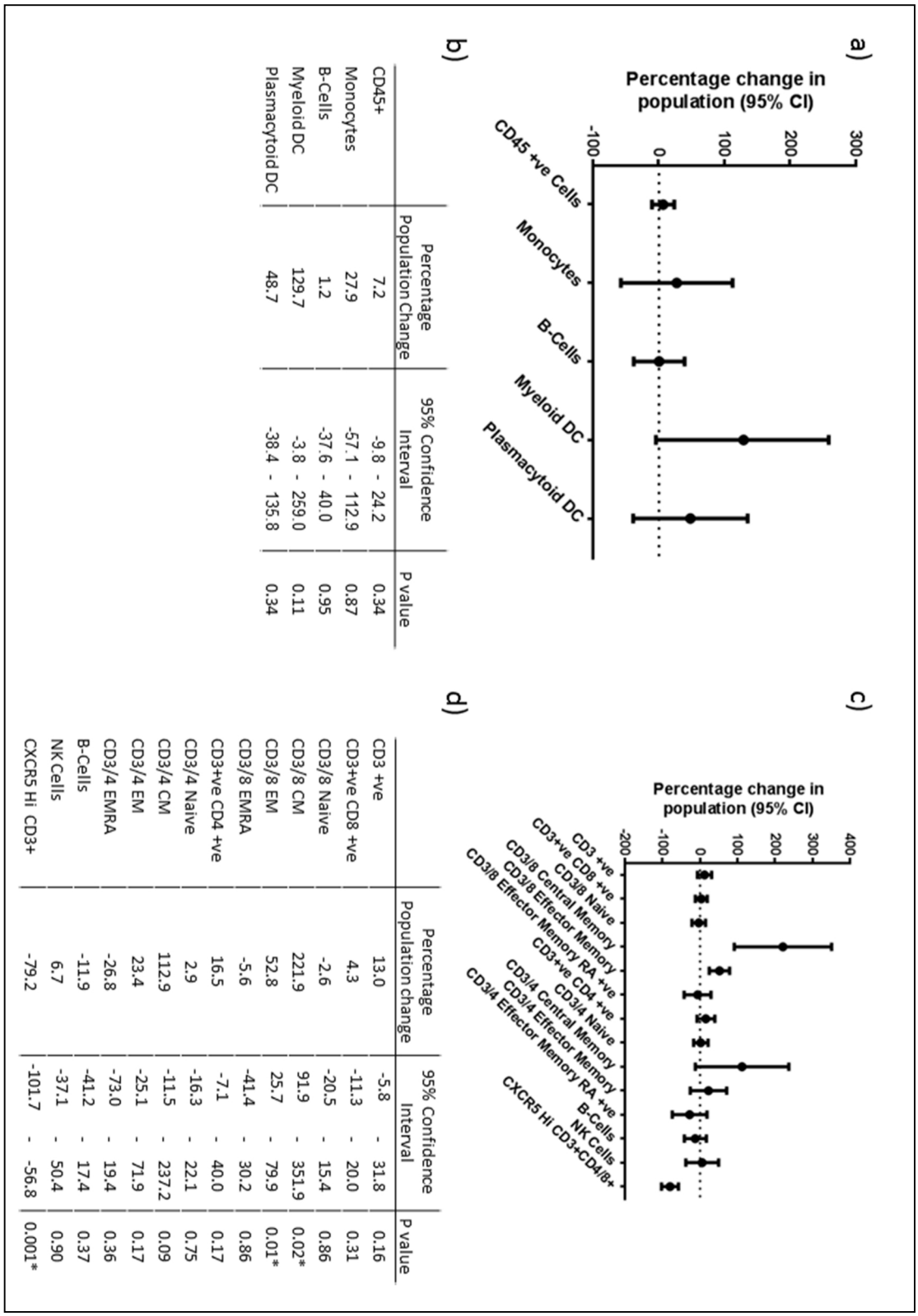
Cell isolates from lymph nodes processed by mechanical and enzymatic means were subjected to cell-surface staining and phenotyping by flow cytometry according to the two panels as previously described (see Section 5.1.1). CXCR5 was used as a marker for follicular cells and was included within the lymphocyte phenotypic panel.

The differences in cytokine production demonstrated through ELISpot could have been a consequence of improved retrieval of antigen-presenting cells (and consequent increase in efficiency of antigen presentation during antigen stimulation on the plate) or positive selection of CD4+ cells during enzyme digest. Analysis of 6 lymph node isolates demonstrated no difference in the proportional representation of non-T cell populations between mechanical and enzyme-treated cell isolates – see Figure 7.a,b. Furthermore no differences in the proportional representation of either total CD4+ T-lymphocytes or memory cell sub-sets thereof could be demonstrated (Figure 7.c,d).

Significant differences were demonstrable in the proportional representation of some cell sub-sets; the proportion of CD8-lineage memory cells was increased significantly in samples which had undergone enzymatic digest (Figure 7.c,d). Mean percentage change for CM and EM was 220% (95%CI = 92-352; P = 0.02) and 53% (95%CI = 26-80; P = 0.01) respectively. In addition, the proportion of cells expressing CXCR5 on their surface was significantly reduced by 79% (95%CI = 57-102; P = 0.001) – Figure 7.c.d.

To ascertain whether differences in proportional representation in these populations was due to differences in survival following enzyme isolation it was necessary to exclude direct action of the enzyme on the surface marker expression. An increase in memory-cell subsets could be due to enzymatic removal of CD45RA from naïve cells rendering the cell CD45RA-ve and resulting in miss-classification as an antigen-experienced memory lymphocyte. Cells from lymph nodes isolated through mechanical means were incubated for 30 minutes in the presence of 800KU/ml DNAse1 and 1mg/ml Collagenase IV. Flow cytometry was performed on the cell population before and after treatment. No difference in the number of cells gated as CD45RA- was discernable between cells before and after treatment (45.8% vs 47.8%).

Similarly, mechanically disaggregated cells were incubated for 30 minutes in the presence of either DNAse1 or Collagenase IV (See Figure 8.a.); no change in the proportion of lymphocytes expressing CXCR5 was discernable following DNAse 1 exposure, however collagenase IV caused a 96% loss of cells gated as CXCR5+.



b)

	Percentage Population Change	95% Confidence Interval	P value
CD45+	7.2	-9.8 - 24.2	0.34
Monocytes	27.9	-57.1 - 112.9	0.87
B-Cells	1.2	-37.6 - 40.0	0.95
Myeloid DC	129.7	-3.8 - 259.0	0.11
Plasmacytoid DC	48.7	-38.4 - 135.8	0.34

d)

	Percentage Population change	95% Confidence Interval	P value
CD3 +ve	13.0	-5.8 - 31.8	0.16
CD3+ve CD8 +ve	4.3	-11.3 - 20.0	0.31
CD3/8 Naive	-2.6	-20.5 - 15.4	0.86
CD3/8 CM	221.9	91.9 - 351.9	0.02*
CD3/8 EM	52.8	25.7 - 79.9	0.01*
CD3/8 EMRA	-5.6	-41.4 - 30.2	0.86
CD3+ve CD4 +ve	16.5	-7.1 - 40.0	0.17
CD3/4 Naive	2.9	-16.3 - 22.1	0.75
CD3/4 CM	112.9	-11.5 - 237.2	0.09
CD3/4 EM	23.4	-25.1 - 71.9	0.17
CD3/4 EMRA	-26.8	-73.0 - 19.4	0.36
B-Cells	-11.9	-41.2 - 17.4	0.37
NK Cells	6.7	-37.1 - 50.4	0.90
CXCR5 Hi CD3+	-79.2	-101.7 - -56.8	0.001*

Figure 7: Percentage differences in proportion representation of immune-cell sub-populations within lymph node single-cell suspensions generated through either mechanical or enzyme-digest disaggregation. Figures presented are percentage change using average proportional representation within mechanically disaggregated samples as a baseline. Therefore positive change represents an increase in proportional representation of a population following enzyme treatment. a) and b) detail changes within non-T lymphocyte populations in terms of total number of viable cells whereas c) and d) detail changes within lymphocyte sub-populations in terms of total number of viable lymphocytes. Mean values plotted with 95% confidence intervals represented by error bars.

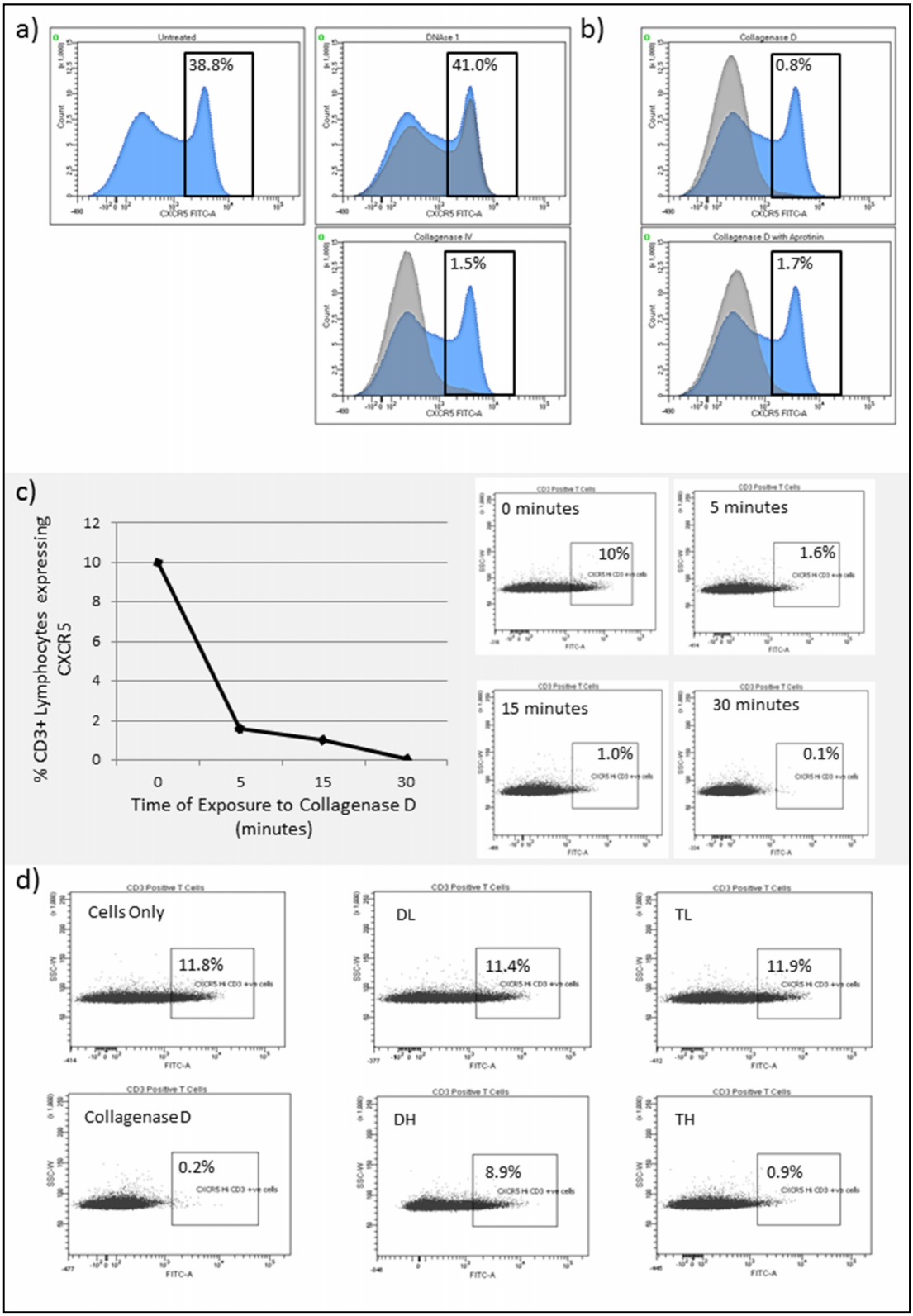


Figure 8: CXCR5 surface marker detection following cell-exposure to enzyme. a) Flow cytometry histogram plots demonstrating CXCR5 surface expression on untreated lymphocytes (top left) and following treatment with DNAse 1 (top right) and Collagenase IV (bottom right). Plots show untreated expression (blue) with treated cells overlaid in grey. Percentage treated population gated as CXCR5+ shown. b) Loss of marker following exposure to Collagenase D is not sufficiently attenuated through addition of protease inhibitor aprotinin. c) Line graph demonstrating percentage CD3+ lymphocytes expressing CXCR5 over time following collagenase D exposure d) Flow cytometry dot-plots showing CXCR5 expression on untreated cells, and cells exposed to different enzyme preparations (Liberase: DL = Dispase Low, DH = Dispase High, TL = Thermalysin Low, TH = Thermalysin High)

5.1.4 CXCR5 loss from cell surface is avoidable through use of protease-depleted enzyme preparations

Previously published work reporting flow-cytometry-based phenotypic characterisation of immune cells isolated from lymphatic tissue has described use of collagenase D combined with a protease inhibitor during cell isolation (146). Mechanically disaggregated cells were exposed to collagenase D (Roche Products Limited. Cat: 1088858001), with or without the protease inhibitor aprotinin (Roche. Cat: 10236624001), for 30 minutes (see Figure 8.b.); CXCR5 loss was comparable to that seen with Collagenase IV. Process was repeated using further addition of soya-bean trypsin inhibitor with similar results (data not shown).

To ascertain whether loss of CXCR5 could potentially be mitigated by altering duration of enzyme digest cells were exposed across a 30 minute time-course, illustrated within Figure 8.c. This demonstrated rapid loss of CXCR5 from the cell surface within 5 minutes of exposure.

Liberase (Roche Products Limited) enzyme blends are purified blends of collagenase I and collagenase II available with various levels of re-introduced protease (either dispase or thermalysin). Protease activity is graded as either low or high for dispase preparations (DL and DH respectively) and low, medium or high for thermalysin preparations (TL,TM, TH). Figure 8.d. depicts the effect of exposure of lymph node cells to DL, DH, TL and TH Liberase preparations compared to collagenase D. Although cell-surface loss was comparable to collagenase D in the TH treated cells, only partial loss was experienced with DH. No marker loss was discernable following exposure to DL and TL preparations.

5.1.5 Lymph node disaggregation using Liberase DL/DNAse1 enzyme digest preserves cytokine response to stimulation and antibody production without depletion of surface markers

Lymph node samples were bisected with half undergoing mechanical disaggregation and half processed according to methodology previously described within Section 4.4.4. CD4 ELISpot demonstrated the use of DL/DNAse enzyme pre-treatment resulted in preservation of cytokine production relative to mechanical disaggregation alone (Figure 9.a.) as previously demonstrated following Collagenase IV/DNAse digest, despite the low protease content of the preparation. In addition, surface marker CXCR5 was preserved as predicted (Figure 9.b.).

Six further lymph node samples from colorectal tissue were processed using the new enzyme blend and comparison made between mechanical and enzyme disaggregation with respect to B-cell markers and plasma cell function. No significant differences in B-cell sub-populations could be demonstrated (Figure 9.c.) and no difference in the number of IgG antibody secreting cells was apparent (Figure 9.d.).

To ascertain whether CXCR5 was unique in its susceptibility to enzyme activity, a panel of markers were exposed to collagenase D, collagenase IV and various Liberase preparations. CD21 and CD27 both demonstrated susceptibility to enzyme-mediated cell-surface loss comparable to that seen in CXCR5 following exposure to Liberase TH, but not Collagenase D or Collagenase IV. Therefore susceptibility of each individual marker to enzyme degradation is variable; prior to employing phenotypic markers in the evaluation of cell suspensions from tissue preparations, said markers should be screened for loss following exposure to enzymes employed in tissue desegregation process.

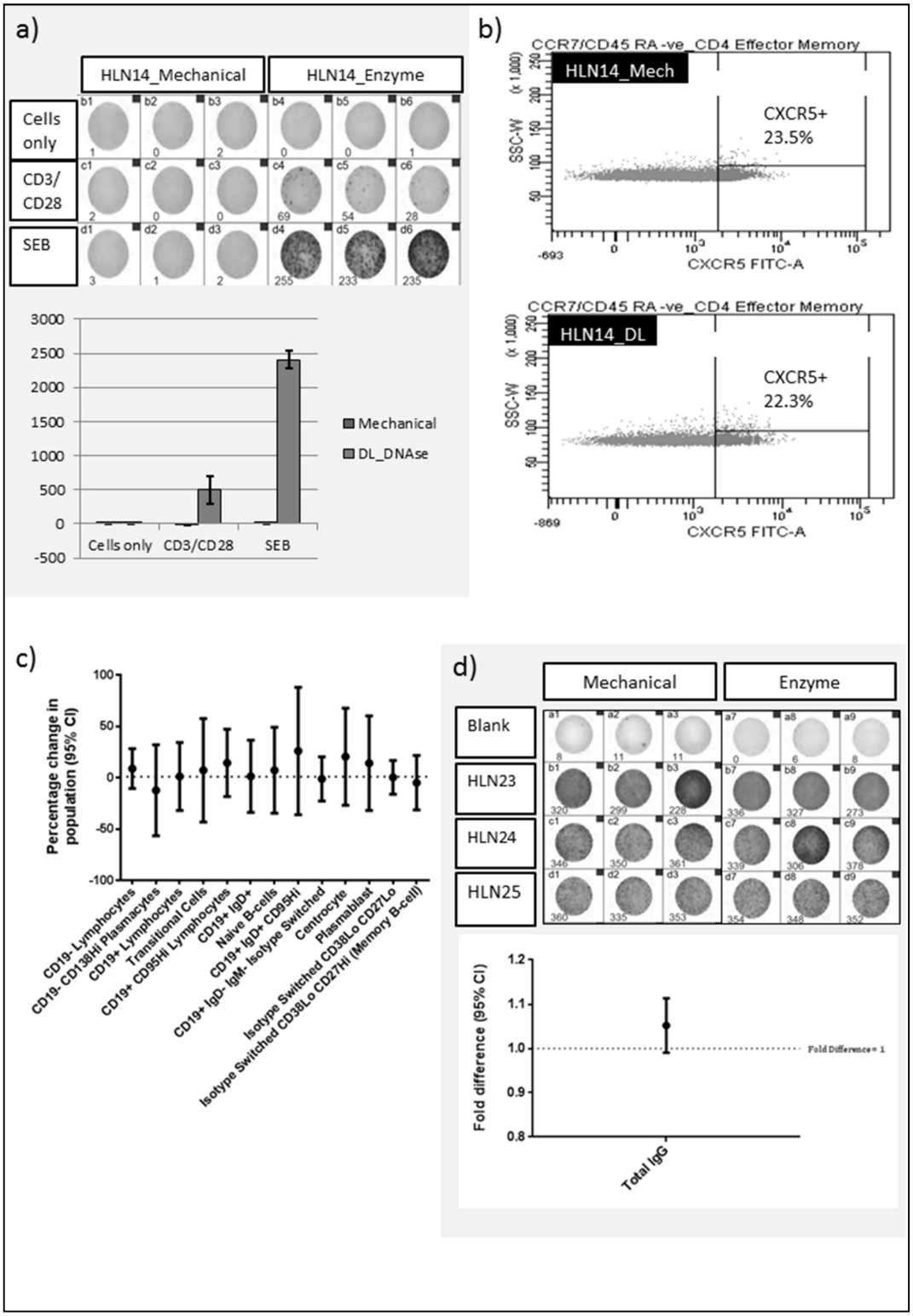
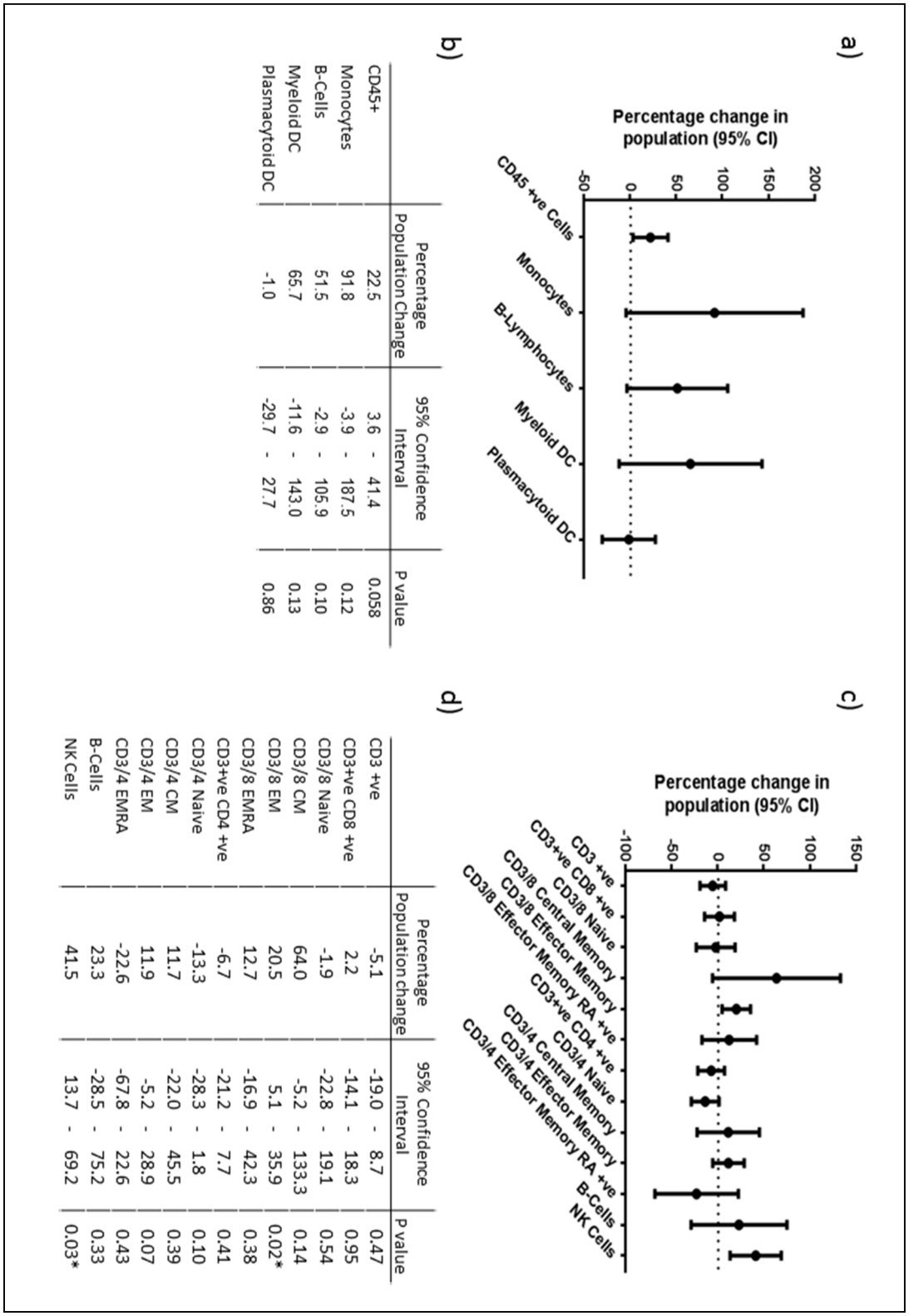


Figure 9: Phenotypic and functional properties of cells isolated from lymph nodes disaggregated mechanically compared to those isolated using new enzyme protocol. a) ELISpot plate and accompanying bar chart (mean + 95%CI) demonstrating number of IFNγ spots detectable within the same lymph node following either mechanical or enzymatic dissociation. b) CXCR5+ expression on lymph node cells following either mechanical dissociation (top) or dissociation following enzymatic digestion using Liberase DL (bottom). c) Percentage differences in proportion representation of B Lymphocyte sub-populations within samples. Figure presents mean percentage change (with error bars denoting 95% CI) using average proportional representation within mechanically disaggregated samples as a baseline. d) Total IgG antibody secreting cells within nodal samples desegregated either by mechanical or Liberase DL based enzyme digest as detected by B-cell ELISpot accompanied by summary graph demonstrating mean difference in numbers between samples in terms of fold change with mechanical technique serving as baseline.

5.1.6 Cryopreservation incurs change in proportional representation of immune-cell subpopulations

Cryopreservation was noted to trend towards significant alteration in CD4+ cytokine production (Section 5.1.2, Figure 6). Possible explanations for this would include positive or negative selection of key subpopulations following cryopreservation as a consequence of innate differences in survival potential. Constituent immune cell sub-populations within 8 lymph node specimens were compared pre-and post-cryopreservation (Figure 10); no differences could be demonstrated in the proportion of non-T-lymphocyte populations, suggesting availability of antigen presenting cells in fresh and frozen samples was comparable.

Significant increases in the proportional representation on CD8+ T_{EM} (mean percentage population increase = 20.5% (95%CI: 5.1 – 35.9; P = 0.02)) and NK-cells (41.5% (95%CI: 13.7 – 69.2; P = 0.03)) (Figure 10.d) following cryopreservation were observed. The significance of these changes remains unclear and may limit the interpretation of CD8+ and NK cell population data, however since these changes were outside of the CD4+ pool they should not influence CD4+ cell assays.



	Percentage Population Change	95% Confidence Interval	P value
CD45+	22.5	3.6 - 41.4	0.058
Monocytes	91.8	-3.9 - 187.5	0.12
B-Cells	51.5	-2.9 - 105.9	0.10
Myeloid DC	65.7	-11.6 - 143.0	0.13
Plasmacytoid DC	-1.0	-29.7 - 27.7	0.86

	Percentage Population change	95% Confidence Interval	P value
CD3 +ve	-5.1	-19.0 - 8.7	0.47
CD3+ve CD8 +ve	2.2	-14.1 - 18.3	0.95
CD3/8 Naive	-1.9	-22.8 - 19.1	0.54
CD3/8 CM	64.0	-5.2 - 133.3	0.14
CD3/8 EM	20.5	5.1 - 35.9	0.02*
CD3/8 EMRA	12.7	-16.9 - 42.3	0.38
CD3+ve CD4 +ve	-6.7	-21.2 - 7.7	0.41
CD3/4 Naive	-13.3	-28.3 - 1.8	0.10
CD3/4 CM	11.7	-22.0 - 45.5	0.39
CD3/4 EM	11.9	-5.2 - 28.9	0.07
CD3/4 EMRA	-22.6	-67.8 - 22.6	0.43
B-Cells	23.3	-28.5 - 75.2	0.33
NK Cells	41.5	13.7 - 69.2	0.03*

Figure 10: Percentage differences in proportion representation of immune-cell sub-populations within lymph node single-cell suspensions before and after cryopreservation. Figures presented are mean percentage change (with error bars denoting 95% CI) using average proportional representation within fresh, unfrozen samples as a baseline. Therefore positive change represents an increase in proportional representation of a population following cryopreservation. a) and b) detail changes within non-T lymphocyte populations in terms of total number of viable cells whereas c) and d) detail changes within lymphocyte sub-populations in terms of total number of viable lymphocytes.

5.2 Optimisation of ELISA protocol for TTd, DTd and PPD

5.2.1 New tetanus toxoid performs comparably to previously used antigen, although current serological anti-toxin standard and historic standard perform differently

Tetanus toxoid (TTd) ELISA has been used historically within our laboratory. TTd standard antigen (lot: 04/150) and anti-Tetanus serological standard (lot: TE-3) were sourced from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, UK) for the purposes of this current study and compared to reagents used previously (TTd: lot 02/232, Anti-Tetanus standard: lot 76/589, also from NIBSC).

Established and new TTd antigen use during ELISA was compared by coating plates with 2.5µg/ml concentration of antigen and use of established Tetanus-antitoxin serum control to generate a standard curve. Figure 11.a. demonstrates equivalent performance with generation of near-identical standard curves. Optimal coating concentration was ascertained through titration of antigen concentration at 0.5µg/ml intervals and use of a constant dilution of antitoxin serum control (Figure 11.b.); this demonstrated peak absorbance was achieved with a coating concentration between 1.5-2.0µg/ml.

Established and new tetanus-antitoxin serum controls were compared; both controls were diluted to stock concentration of 1IU/ml and diluted 1:100 for top concentration, then doubling dilutions thereafter as per Section 4.7. The standard curves generated (Figure 11.c.) were used to calculate the anti-TTd IgG content of two sets of quality control sera (QC1 and QC2 - Figure 11.d.); calculations from 76/589 estimated control sera to contain 49% less anti-TTd antibody than TE-3 (95%CI 47.2-51.2; P = 0.006). Despite the lack of consistency between the two standards, TE-3 was accepted since its use would be consistent across current study samples; comparison of current study samples with historic samples from previous study would require historic samples to be re-analysed using the new standard.

5.2.2 Diphtheria Toxoid and standard perform comparably to Tetanus Toxoid

Diphtheria Toxoid (DTd) ELISA has not previously been used within our laboratory. DTd standard antigen (lot: 02/176) and anti-diphtheria serological standard (lot: 10/262) were sourced from NIBSC. One half of an ELISA plate was coated with 2.5µg/ml DTd and the other half coated with 2.5ug/ml TTd; diphtheria serum antitoxin standard was diluted to 1 IU/ml and used at a top concentration of 1:100 with doubling dilutions thereafter to create a standard curve, which was run in parallel with optimised concentrations of tetanus-anti-toxin standard and developed for the same amount of time; resulting standard curves were comparable (Figure 12.a.). Optimal coating concentration was then determined for DTd in a fashion

analogous to that used for TTd (Figure 12.b.); peak absorbance was achieved with a coating concentration between 1.5-2.0µg/ml.

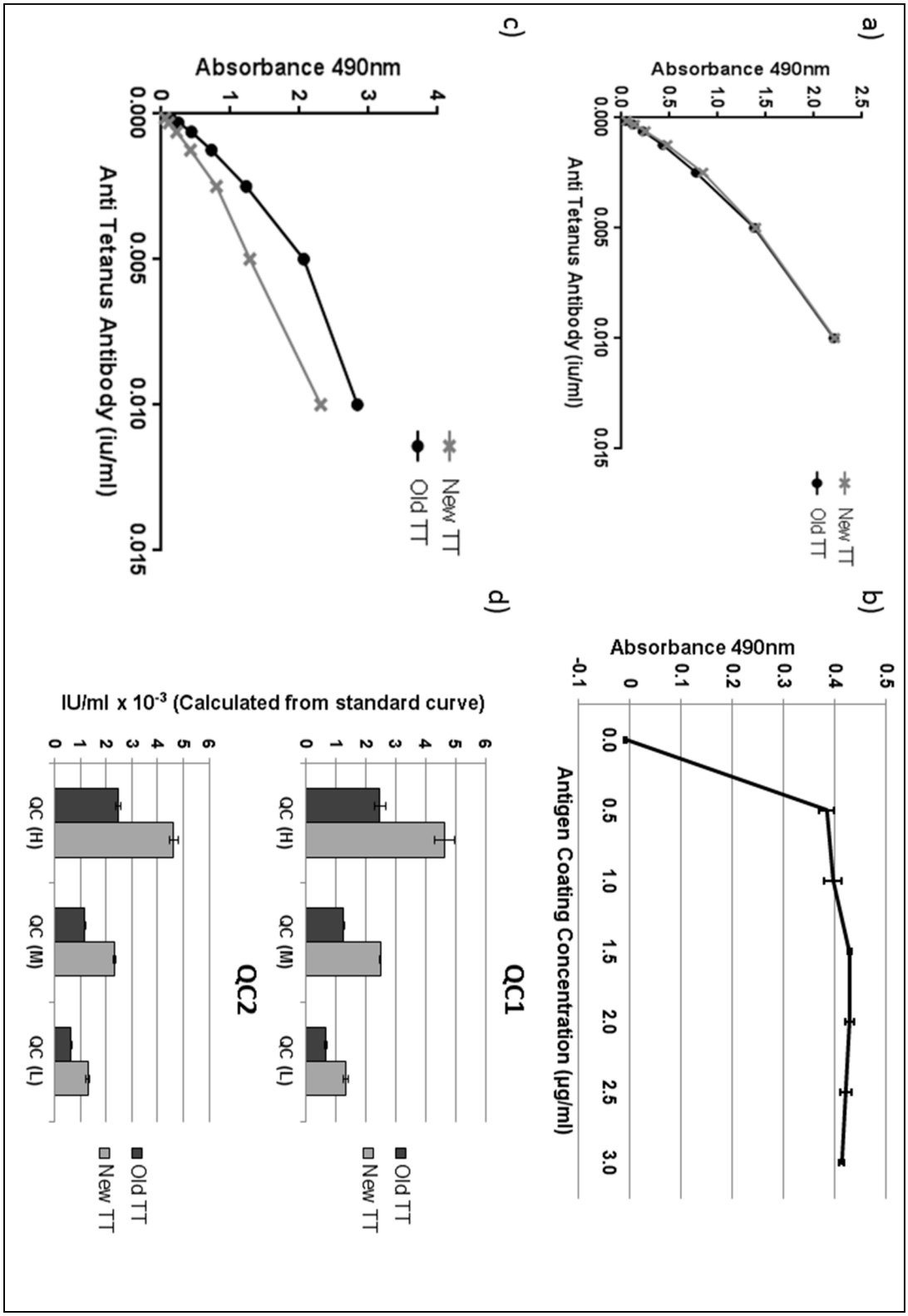


Figure 11: Optimisation of TTd ELISA. a) Equivalence of the standard curves achieved utilising historic anti-toxin standard on plates coated with both new (04/150) and old (02/232) TTd standards. b) Absorbance (mean +/- 95%CI) achieved following application of a 1:800 dilution of anti-toxin control (76/589) to wells coated with incremental concentrations of TTd antigen demonstrating peak absorbance following coating with 1.5-2.0µg/ml. c) Standard curves generated using established antitoxin standard ("old TT"; 76/589) and new standard ("new TT"; TE-3) and d) comparison of quantity values (mean +/- 95%CI) generated from curves for two sets of serum quality controls (QC1 and QC2) demonstrate non-equivalence.

5.2.3 PPD ELISA standardisation and establishment of IgG isotype and IgM standards

Purified Protein Derivative from tuberculin (PPD) ELISA has been used historically within our laboratory. PPD standard antigen was sourced from NIBSC (Lot: RT27) and from Statens Serum Institute (SSI) (Copenhagen, Denmark) (Lot: RT50) for the purposes of this current study. There are no serum standards available commercially for anti-PPD antibody; our laboratory has previously used serum from a healthy donor as a positive control and to allow semi-quantification using arbitrary units (AU). This historic in-house standard was used to compare the newly acquired PPD with originally used antigen (Figure 12.c.); serum standard was diluted to a top-use concentration of 1:4 as per previous use. Standard curves achieved using old and new PPD were comparable.

Attempt was then made to determine optimal coating concentration; both old and new PPD were coated to an ELISA plate at incremental concentrations between 0 – 15µg/ml (Figure 12.d.) and serum standard diluted to 1:8 used for detection. In the case of both antigens there was an absence of a dose-response curve with absorbance within blank wells (without antigen coating) being equivalent to wells coated with antigen.

A possible explanation for this finding was cross-reactivity between serum standard and blocking protein; standard blockade using 1% BSA was then compared with blockade using alternate irrelevant proteins (3% casein, 2% Normal Goat Serum (NGS)). In addition 2 further serum samples from healthy volunteers were screened (Serum 1, Serum 2, used at 1:8 dilution as per PPD standard) alongside the historic in-house serum standard for PPD and tetanus anti-toxin standard from NIBSC (at 1:800 dilution) (Figure 12.e.). With the exception of tetanus antitoxin standard, all serum samples at the concentrations used demonstrated binding within wells blocked with irrelevant protein. Furthermore, when comparing wells pre-coated with 10µg/ml PPD prior to 1%BSA blockade with wells simply blocked with 1%BSA there appeared to be little difference, suggesting that there was no detectable PPD-specific antibody binding.

Patient serum was therefore screened for IgG reactivity against PPD. In addition IgM, IgG1, IgG2, IgG3 and IgG4 reactivity against TTd, DTd and PPD was assessed in 12 study patients (numbers 001 – 012). Serum from time point 3 (3 weeks post immunisation) was used as it was anticipated to represent the peak of response. Serum was diluted to 1:40 and 1:400; each sample dilution was tested within antigen-coated wells and uncoated “bare” wells. Both antigen coated wells and bare wells were then blocked using 1% BSA. To ensure adequate compensation for non-specific binding, absorbance from uncoated “bare” wells was subtracted from absorbance within antigen-coated wells. Serum reactivity between patients was then compared in order to identify “high responders” suitable for use as standards - Figure

13. IgG2 and IgG3 responses were low throughout all 12 specimens. The highest responding patient/patients from all other isotype/antigen combinations were identified and standards formulated using either single patient's serum or a combination of individuals. Subsequent ELISA results are therefore expressed as proportion of standard response from these samples (termed "Arbitrary Units" (Au)).

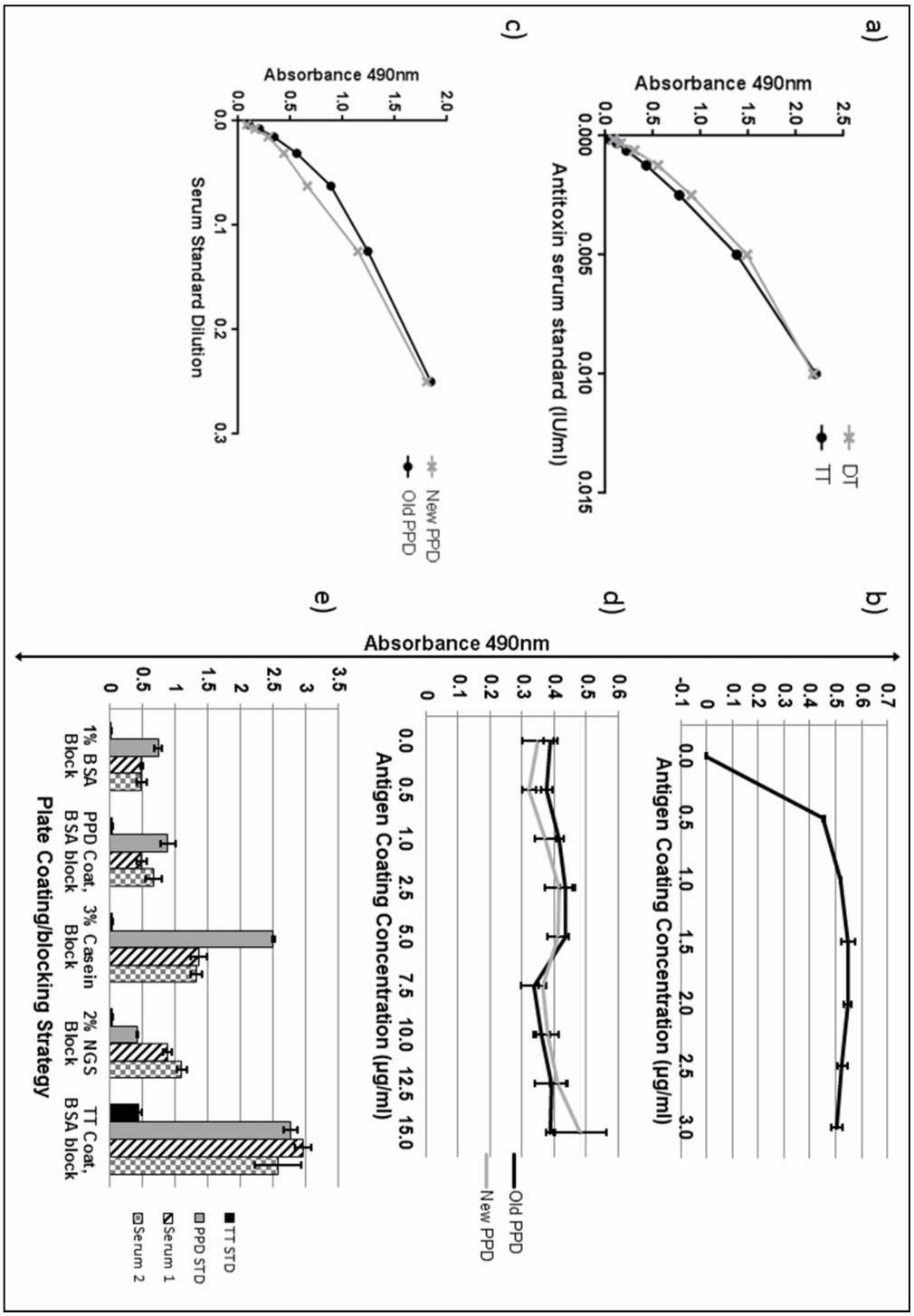


Figure 12: Optimisation of DTd and PPD ELISA. a) Comparison of TTd and DTd standard curves demonstrate equivalence. b) Absorbance (mean +/- 95%CI) achieved following application of a 1:800 dilution of anti-toxin control (10/262) to wells coated with incremental concentrations of DTd antigen demonstrating peak absorbance following coating with 1.5-2.0µg/ml., c) New PPD (RT27) compared to PPD previously used through generation of standard curve using historic serum standard; top concentration was a 1:4 dilution of serum, with doubling dilutions thereafter. d) Attempted optimisation of coating concentration using RT27 and original PPD using 1:8 dilution of historic serum standard and an incremental increase in PPD coating concentration demonstrating the absence of an increase in absorbance with increasing coating concentration, suggestive of non-specific binding. e) Comparison of different blockade strategies showing non-specific background with serum samples (historic PPD STD, Serum 1 &Serum 2) diluted to 1:8 vs TTd STD 1:800 demonstrating non-specific binding within all three serum samples when used at the concentrations previously employed during historic work.

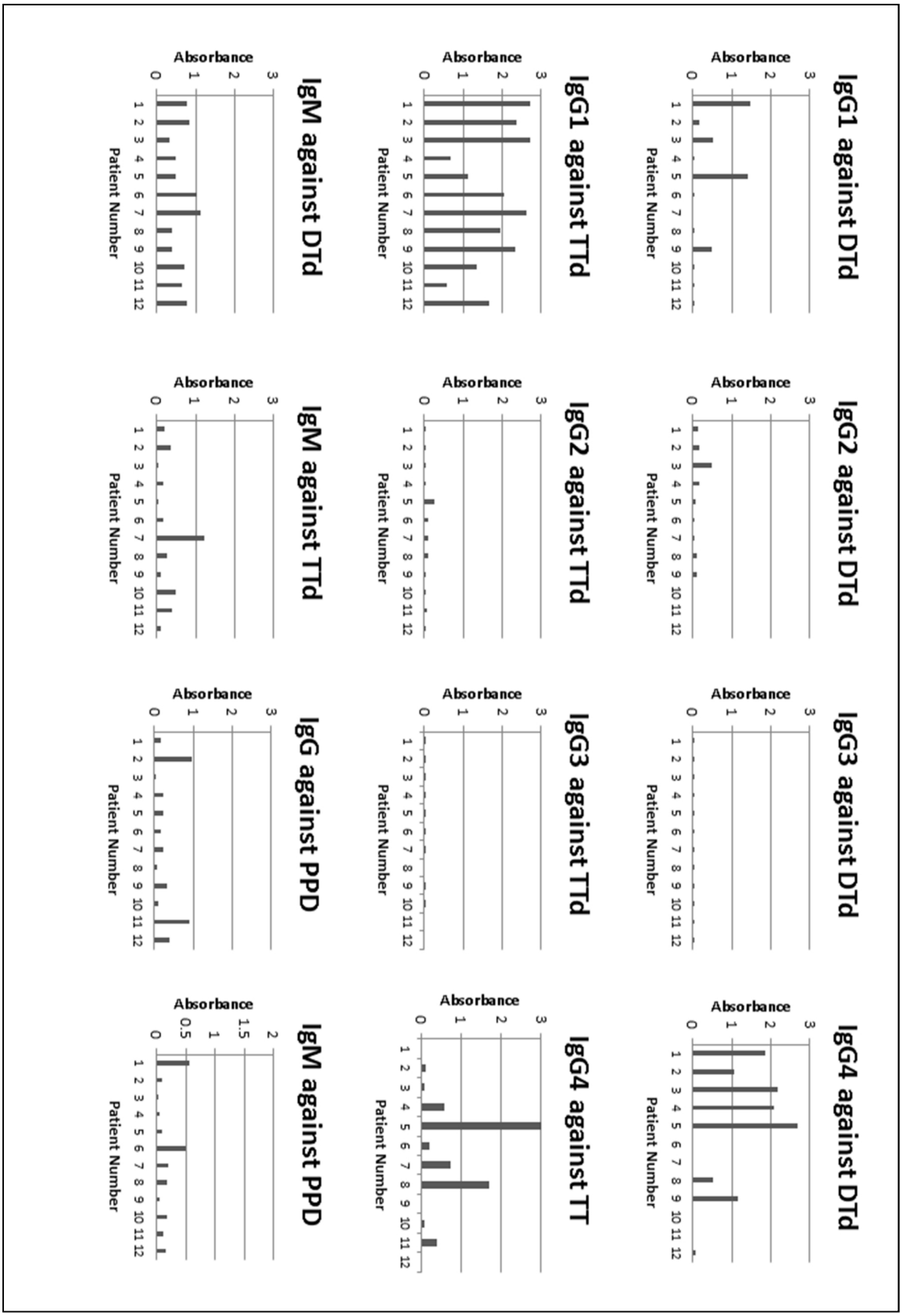


Figure 13: Screening ELISA looking at reactivity against either TTd, DTd or PPD according to isotype within the serum of patients 001 – 012isolated from blood taken at time point 3 (predicted peak response). Figures display absorbance at 490nm.

5.3 Optimisation of CD4 and B-cell ELISpot protocol

5.3.1 New tetanus toxoid and PPD perform acceptably within CD4 ELISpot compared to established antigens

TTd antigen (lot: 04/150) was compared to reagent used previously (TTd: lot 02/232) for the purposes of CD4 ELISpot. Similarly, PPD (SSI; Lot: RT50) was compared with stored PPD from previous study.

Comparison was made using IFNγ ELISpot according to protocol described within section 4.8. Cells from a stored leukapheresis sample were plated at $4x10^6$ cells per well. Original antigen was used to stimulate cells at previous coating concentration (20µg/ml for both PPD and TTd). New antigen was applied to cells at 10µg/ml, 20µg/ml and 40µg/ml and number of spots per million cells compared with old antigen - Figure 14.a.

Number of spots generated following stimulation using 20µg/ml of TTd (Lot: 04/150) was comparable to 20 µg/ml of TTd (Lot: 02/232). PPD (Lot: RT50) out-performed previously used antigen; 10µg/ml was sufficient to induce comparable IFNγ spots/million to 20µg/ml of the old antigen.

A similar dose titration was performed for stimulation with DTd (Lot: 02/176); no historic antigen was available for comparison. Stimulation using 20µg/ml of antigen out-performed 10µg/ml however use of 40µg/ml failed to significantly increase spot count further - Figure 14.b.***Fetal Calf Serum is required for optimal performance of B-cell ELISpot***

PBMCs from a healthy, immunised volunteer were used to evaluate kinetics of plasmablast response to vaccine. Blood samples were available prior to vaccination, 1 week post vaccination and 4 weeks post vaccination. Samples were assessed for total IgG producing cells, anti-TTd IgG producing cells and anti-DTd producing cells at the three time points as per protocol described within section 4.9.3. A notable increase in circulating Antibody Secreting Cells (ASCs) was appreciable at 1 week post vaccination with a return to baseline numbers noted by 4 weeks post vaccination (Figure 15.a).

PBMCs from 1 week post vaccination were subsequently used to identify optimum antigen coating concentration for both DTd and TTd; wells were coated with 2.5µg/ml, 3.5µg/ml, 5µg/ml, 7.5µg/ml, and 10µg/ml or left uncoated. Cells were plated in triplicate and number of ASCs evaluated. For both DTd and TTd, optimum number of spots was observed when antigen was coated at a concentration of 3.5µg/ml - Figure 15.b.

Optimised coating concentration was then applied during memory B-cell ELISpot performed using cells from a stored leukapheresis sample according to the protocol described with

section 4.9.4. Uncertainty existed regarding the optimal culture conditions during the stimulation phase. To compare cRPMI + FCS with a serum free alternative (AIM V serum free culture media, Life Technologies, California, US), cells were cultured in either media, and the number of ASCs generated compared - Figure 15.c. Incidence of plasmablasts producing TTd or DTd specific IgG was low in samples cultured in both media, as expected. Interestingly, survival of plasmablasts within samples cultured in AIM V was far inferior to samples cultured in FCS supplemented cRPMI. Similarly, number of induced ASCs (“memory B cells) specific to TTd and DTd was far higher in the cRPMI + FCS culture, as was the number of total IgG producing memory B-cells.

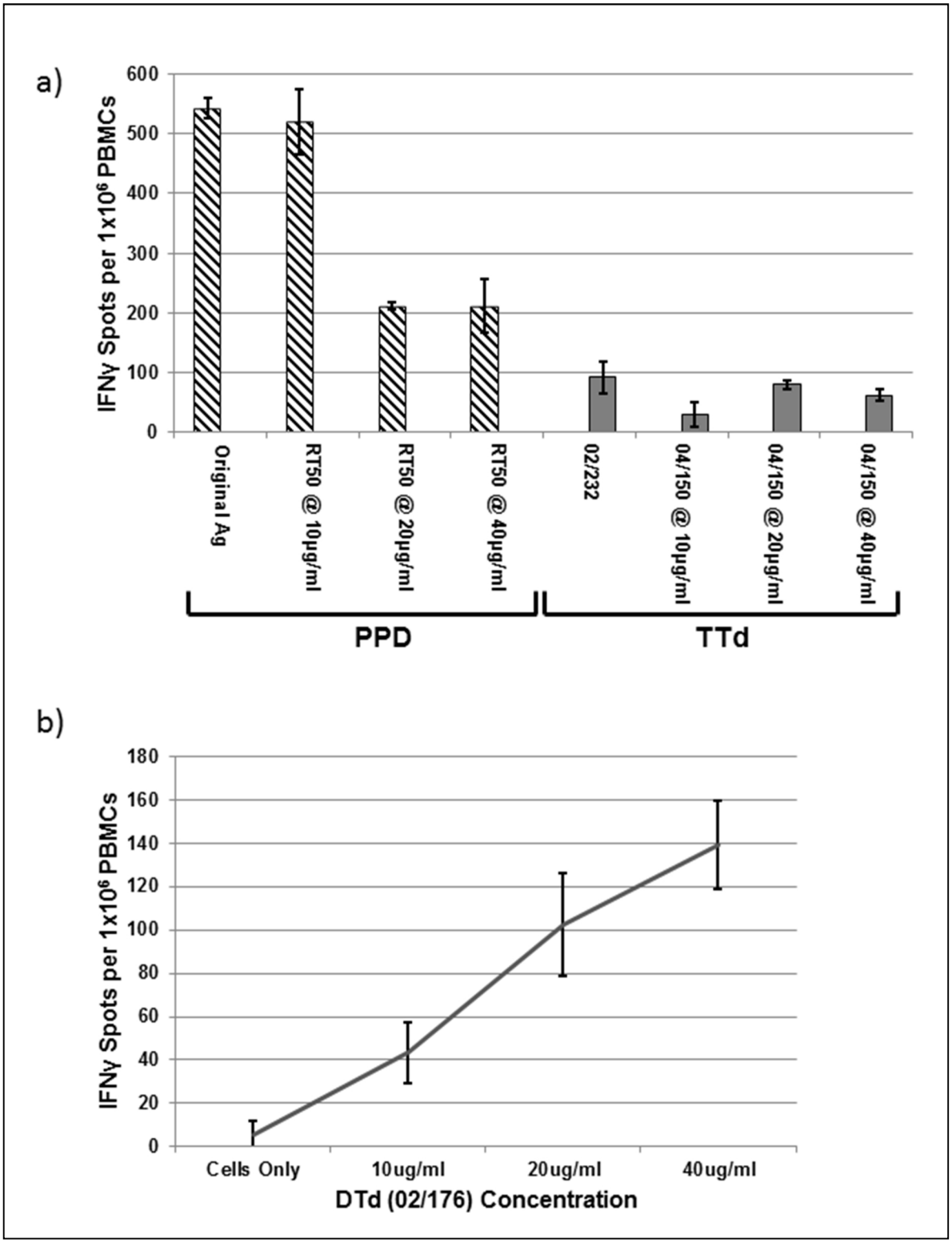


Figure 14: Optimisation of CD4 ELISpot antigen stimulation. a) Number of spots per million PBMCs plated (mean +/- 95%CI) achieved using newly acquired antigen compared to antigen previously used within our laboratory; new TTd (04/150) performed similarly to previously used antigen (02/232). New PPD (RT50) at 10ug/ml induced equivalent number of IFNγ spots/million cells plated when compared to previously used PPD at 20µg/ml. b)) Number of Spots achieved per million cells plated (mean +/- 95%CI) when stimulated with incremental concentrations of DTd antigen (02/176).

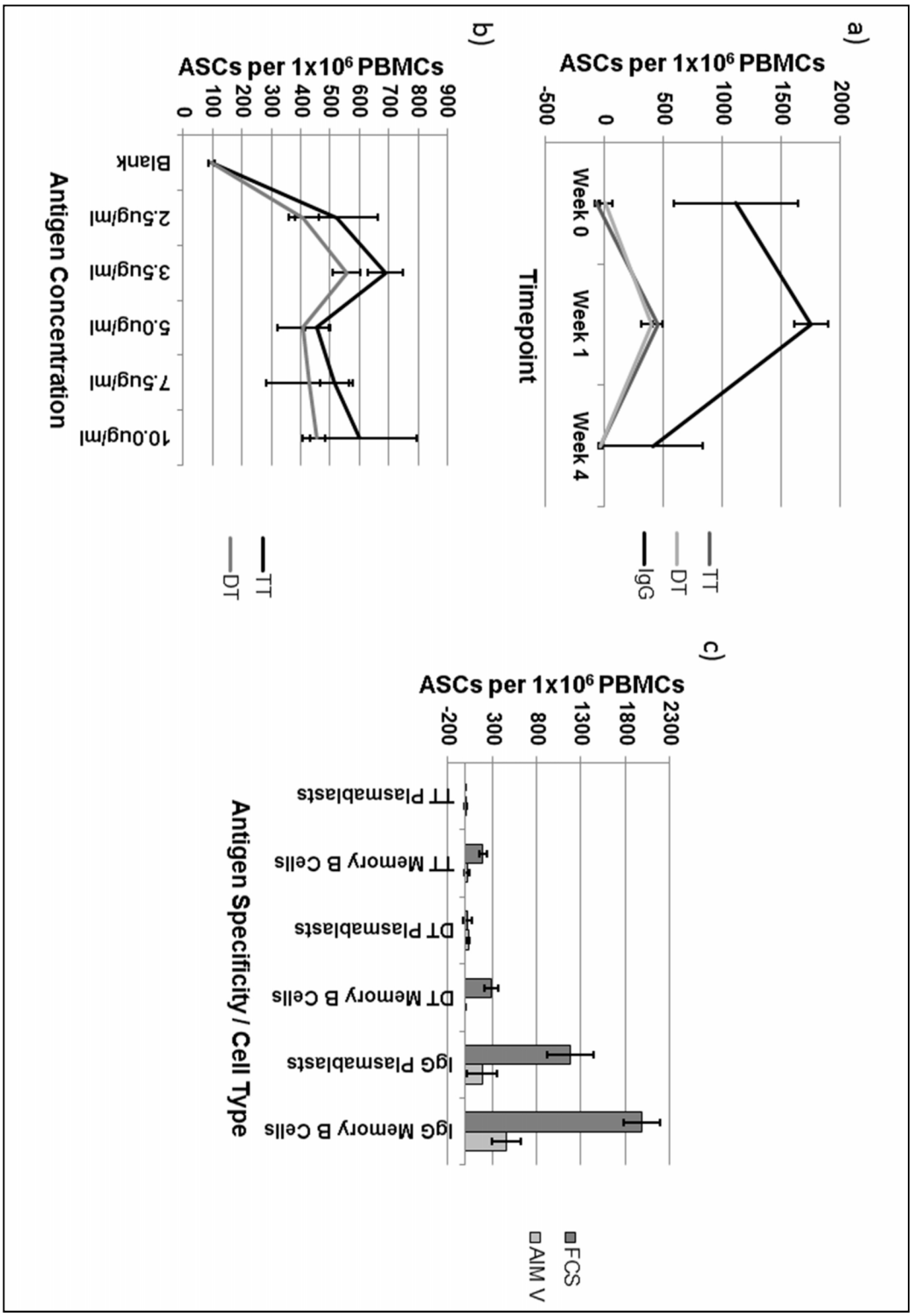


Figure 15: Optimisation of B-cell ELISpot. a) Following immunisation, maximum numbers of antibody secreting cells (ASCs) (mean +/- 95%CI) are observable at on week post immunisation with a return to baseline numbers within 4 weeks. b) Number of spots (mean +/- 95%CI) achieved following application PBMCs to wells coated with incremental concentrations of either TTd or DTd antigen, demonstrating peak number following coating with 3.5µg/ml. c) Comparison between number of ASCs per million PBMCs observed following parallel culture of PBMCs in either cRPMI +10% FCS or AIM V serum-free culture media; clear advantage demonstrable in terms of number of plasmablasts and number of memory B-cells following inclusion of FCS within culture.

5.4 Development of assay to determine antigen specificity of tissue-derived Follicular helper T-cells using ex-vivo antigen re-stimulation.

The issues implicit with development of this assay have previously been outlined in Section 4.11.1. The ideal assay would allow live cell harvest without unduly effecting cell viability or phenotype. For this cells would need to be co-cultured with monensin. To assess feasibility cell samples from two lymph nodes were cultured in the presence of monensin and their viability assessed at 6 and 24 hours using an amine reactive dye (Figure 16.b.) PBMCs were cultured alongside to provide a comparator (Figure 16.a.). PBMCs proved resistant to prolonged exposure to monensin, with number of non-viable cells not significantly increased over untreated controls. Lymph node derived cells, however, proved more sensitive; 20% of CD4+ lymphocytes were non-viable at baseline which increased to 45% at 6 hours and over 85% at 24 hours. The lethality of monensin treatment precluded anti-CD154 antibody co-culture assays as a practical means of identifying antigen specific cells.

Phenotypic changes in cultured cells over time were assessed by culturing lymph node derived cells in the presence or absence of SEB either 6 or 24 hours. Figure 16.c. illustrates the changes in composition of the CD4+ population in terms of surface expression of CXCR5, PD1 and ICOS. Although CXCR5+ICOS- populations remain unchanged, the number of cells expressing ICOS in the stimulated wells markedly changes at the 24 hour time point (i.e. both CXCR5+ICOS+PD1- and CXCR5+ICOS+PD1+ populations). This induction of phenotypic changes was not demonstrable in unstimulated control wells, nor was it apparent at the earlier 6 hour time point. Therefore 6 hour stimulation appeared to be optimal in terms of preservation of phenotype.

Figure 16.d. and Figure 16.e. illustrate the differing cell surface activation marker kinetics of PBMCs and lymph node derived cells respectively following exposure to SEB. CD154 was rapidly up-regulated on PBMCs following activation within 6 hours (peak expression: 9.1% of CD4+ cells) however fell relative to this by 24 hours. In contrast CD137 was on a continued upward trend at the later time point. Within lymph node derived cells the pattern was different; CD154 induction was poor compared to PBMCs and did not exceed a prevalence of 1.2% of CD4+ cells Lymph node derived cell's expression of CD137 exceeded this, however plateaued at an earlier point than in PBMC's.

In light of these findings simulation at 6 hours was chosen as the optimal time point to assess surface up-regulation of markers of activation on these cell types.

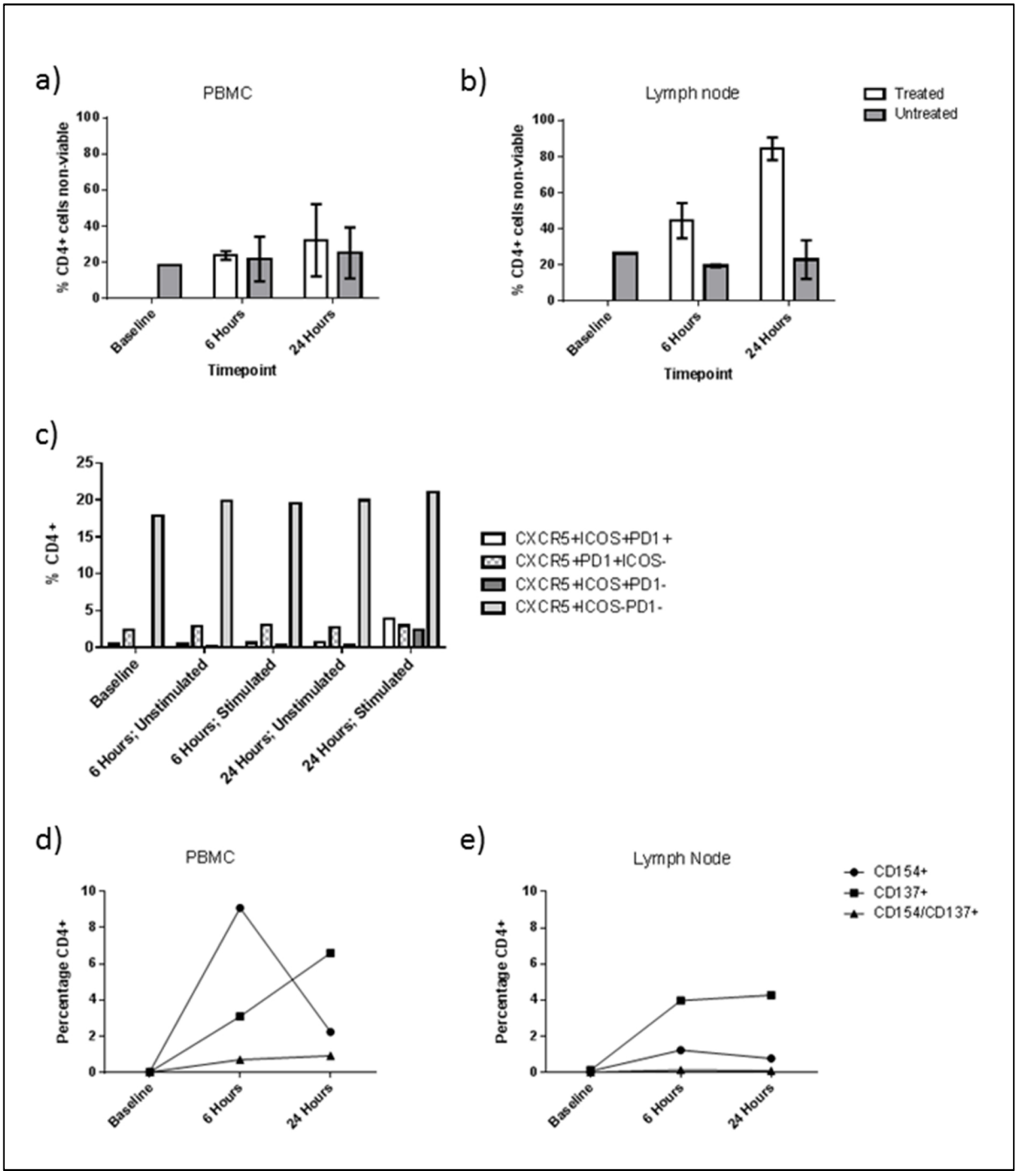


Figure 16: Comparison of conditions of stimulation in Lymph Node derived cells compared to PBMCs. a-b) effect of monensin exposure on cell viability over 6 and 24 hours as assessed using an amine exclusion assay on a) PBMCs and b) Lymph node derived cells. Bars plot mean values with error bars denoting 95% confidence interval. c) Changes in population composition of CD4+ cells following stimulation with Staphylococcal Enterotoxin B (SEB) for either 6 or 24 hours, compared with baseline population and changes noted in cells cultured without stimulation. d-e) changes in the surface expression of activation markers CD154 and CD137 following exposure to SEB for either 6 or 24 hours in d) PBMCs and e) Lymph node derived cells.

5.5 Optimisation of FrC labelling of B-cells

Conditions of use of the Alexafluor 647-congugated FrC reagent created in the processes outlined in Section 4.12 were determined by comparing assay function with that of B-cell ELISpot (see Section 4.9). Peripheral blood from a healthy volunteer taken one week following tetanus vaccination was used. Optimal coating concentration for FrC within the ELISpot was determined by titration (See Figure 17.a-b.). Number of FrC-specific plasma cells per $1x10^6$ PBMCs was calculated to be 127.

The same sample was then subjected to flow cytometry utilising the fluorescently labelled FrC and control antigen (labelled MSA). Titration of optimal concentration of use was performed (see Figure 17.d.); labelled antigen was used at two concentrations, 0.1µl per $2x10^6$ cells and 0.01µl per $2x10^6$ cells. At the higher concentration a high rate of staining was noted on CD19+$CD38^{Hi}$ lymphocytes for FrC, but not MSA. However FrC staining was noted on CD3+ lymphocytes. Since CD3+ lymphocytes should not stain with FrC, the positive signal was thought to be false positive. At 0.01µl per sample however FrC stained CD19+$CD38^{Hi}$ lymphocytes were noted in the absence of positively stained CD3+ cells. MSA staining remained negative.

Using 0.01µl per $2x10^6$ cells, the number of CD19+$CD38^{Hi}$ lymphocytes per 10^6 PBMCs was calculated for the test sample (see Figure 17.c.). The approximate number was $144/1x10^6$ PBMCs; this was considered to correspond closely with the number estimated by ELISpot.

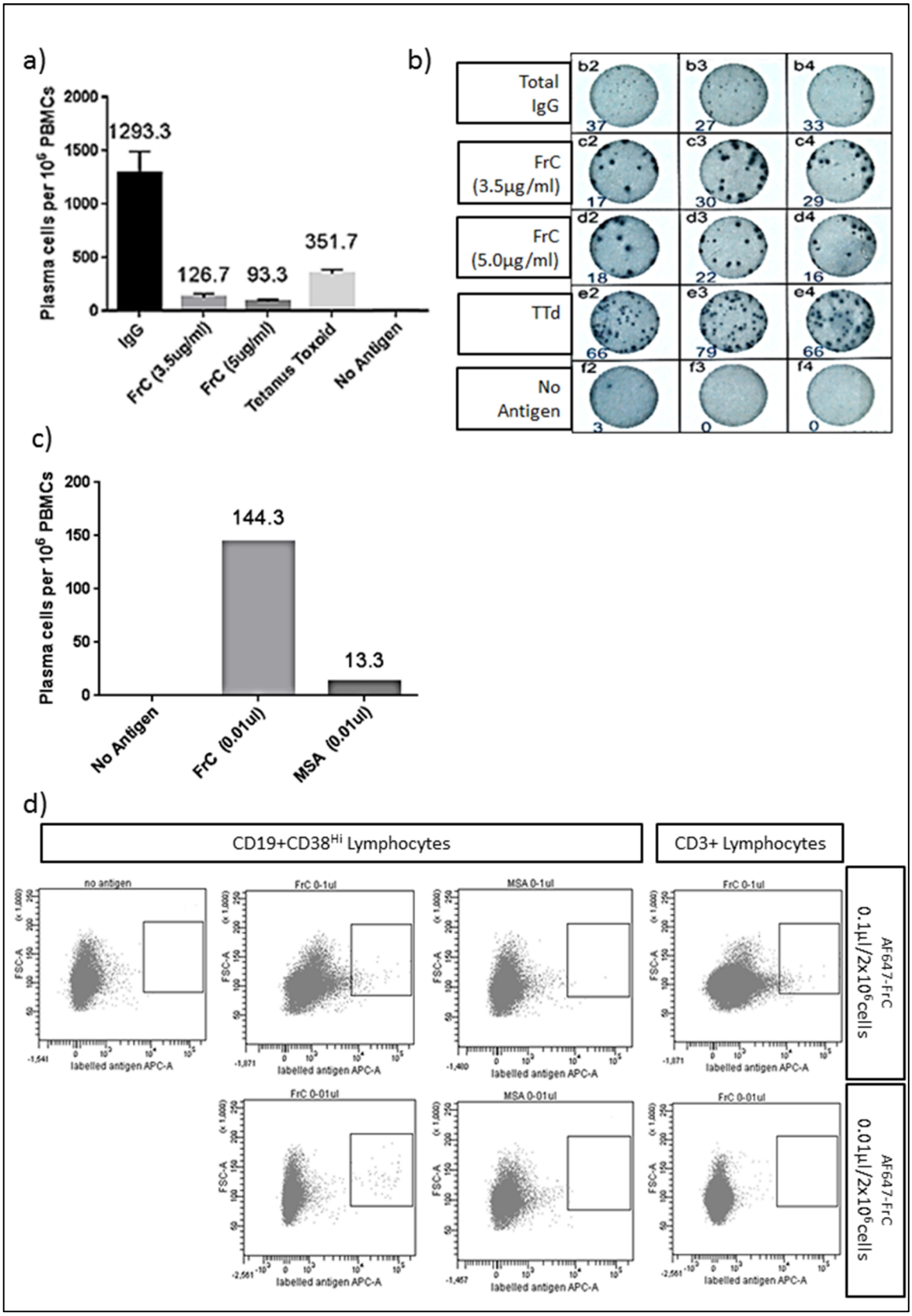


Figure 17: Optimisation of conditions of use of AlexaFluor 647-labelled Fragment C (AF647-FrC). a) Number of plasma cells per 1x10^6 PBMCs as calculated from B-cell ELISpot (b.); numbers calculated include Total IgG producing cells, total TTd specific cells and number of FrC-specific cells calculated from wells coated with either 3.5µg/ml of Frc, or 5µg/ml of FrC. c) Number of FrC-specific CD19+CD38^{Hi} lymphocytes calculated from flow cytometric analysis detailed in d). d) flow cytometry performed using AF647-FrC; top row performed using either no antigen (far left) or antigen at 0.1µl/2x10^6 cells. Bottom row stained using 0.01µl/2x10^6 cells. First three columns represent CD19+CD38^{Hi} lymphocytes whereas far right column represents CD3+ lymphocytes.

6 Recruitment and cohort comparability

6.1 Patient enrolment, randomisation and follow up

The study remained open for recruitment for 22 months. During that time 300 patients were assessed for eligibility (see Consort Diagram, Figure 18). Of those 258 were excluded; the most frequent reason was because patients did not receive the study information (n=69; 26.7%). 38 (55.1%) of these patients were not given information due to human error, 23 (33.3%) were deemed inappropriate for approach due to manifest psychological stress at the time of consultation and 8 (11.6%) were enrolled in an alternate study. 68/300 (22.7%) of patients screened declined involvement within the study; the majority of patients declining involvement who gave a reason cited their reluctance to undertake further commitments at a time of personal stress (n=18) whereas a smaller number declined because of the number of additional blood tests (n=11). 40 patients did not meet inclusion criteria following further screening whereas an additional 34 patients (11.3% of those screened) were excluded from the study because the dates of their pre-assessment and surgery were too close together to allow inclusion. 35 patients completed their treatment at alternative centres, 4 were to undergo mastectomy with immediate reconstruction by surgeons who felt the additional axillary dissection required by study participation increased risk of complications in such patients and 8 (2.7%) were excluded on grounds of co-morbidity (hidradenitis suppurativa, immunodeficiency disorder, haemochromatosis, COPD with restriction to wheel chair, HIV, neurodegenerative disorder, general frailty, axillary scaring following previous trauma).

42 patients were randomised for inclusion; 20 patients were randomised to receive ipsilateral subcutaneous vaccination and 10 patients were randomised to contralateral intramuscular vaccination. All patients randomised to receive a vaccine received one. 10 patients were randomised into the “no vaccine” control group. A further 2 patients received an alternative vaccine “Repevax™”; Repevax contains the same tetanus, diphtheria and polio antigens as REVAXiS, with the addition of pertussis antigens (pertussis toxoid (2.5mcg), filamentous Haemagglutinin (5mcg), pertactin (3mcg), fimbriae Types 2 and 3 (5mcg)). Repevax was used in these patients to provide positive control samples to optimise assays using reactivity against filamentous Haemagglutinin (FHA) as a read out. Use of said assays was abandoned when supply of FHA became non-viable due to cost restrictions.

The number of patients enrolled within the study from whom no lymph node was retrieved for study purposes following a positive qRT-PCR test equalled 13; this included 6/20 (30%) within the ipsilateral group, 3/10 (30%) within the contralateral group and 2/10 (20%) within the no vaccine group. Both patients who received Repevax were qRT-PCR positive. Additionally 2

patients within the no vaccine group were withdrawn early; one patient had poor venous access and proved impossible to venesect in sufficient volume to provide enough PBMCs for analysis whereas the second patient was withdrawn following time point 2 after she developed a surgical site infection within her axilla necessitating admission to hospital and intravenous antibiotics. This second patient was also one of the 2 patients who was qRT-PCR positive. The nodes taken from four patients were of insufficient size/cellularity for inclusion within the study (two within the ipsilateral cohort and one patient in both the contralateral and no vaccine groups). One patient randomised to the ipsilateral group had a previously unidentified microcytic anaemia – attempts to process blood from this patient proved unsuccessful due to altered cellular density impairing the Lymphoprep™ stage. Finally a single patient from the no vaccine group received a vaccine from their General Practitioner between TP3 and TP4; this patient therefore was excluded from the TP4 analysis, although their other samples were included within the study. Although nodal tissue was not retrieved, blood from all 4 time points was collected from 10 of the 11 patients who were randomised to one of the three study cohorts and had positive sentinel lymph node biopsies, as well as from all four patients from whom insufficient nodal tissue was retrieved and from the two patients who received Repevax.

Following omissions and exclusions as detailed above the numbers of patients within the three cohorts who were included in the full analysis (both tissue and PBMCs) were 11 within the ipsilateral cohort, 6 patients within the contralateral cohort and 6 within the no vaccine cohort.

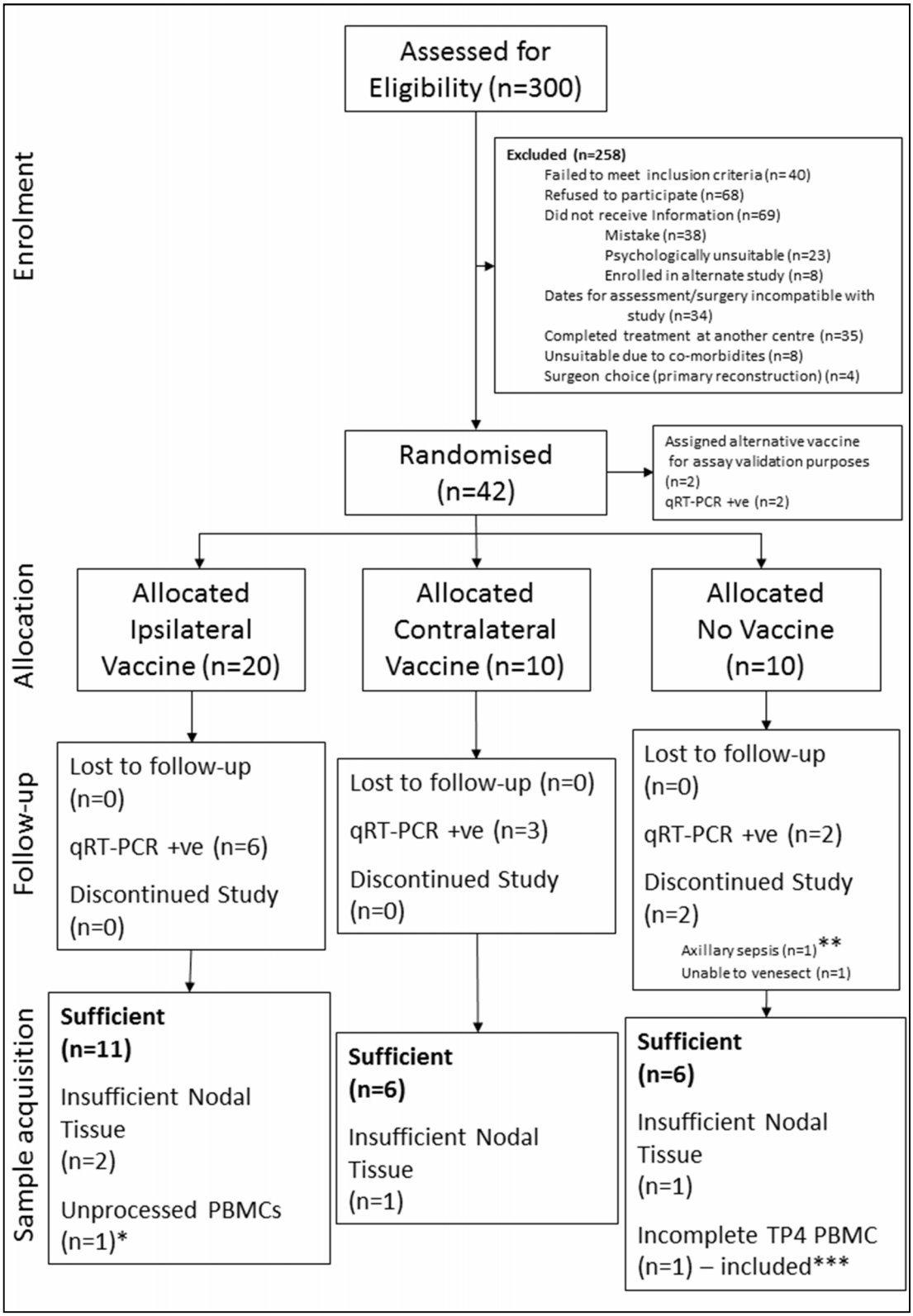


Figure 18: Consort diagram describing patient eligibility and recruitment into the study. Forty-two patients were randomised for study inclusion. Of those randomised, thirteen patients were excluded as they were found to have a positive sentinel node biopsy, five patients were withdrawn from the study due to inability to acquire sufficient samples (four – insufficient nodes, one – unable to acquire blood) with a further patient excluded following technical issues isolating PBMCs from blood, attributed to anaemia (*). One patient was withdrawn following a post-operative complication, however said patient also was found to have a positive sentinel lymph node (**). One patient was administered a vaccine by their General Practitioner during the study period (***); although their TP4 PBMCs could not be included within the analysis, all other samples were included within the study analysis.

6.2 Participant demographics, tumour characteristics and surgery within cohort

Mean age of patients within the study was 60 years (Range 35 – 79). Table 8 details and compares the characteristics of the patients randomised to each study group; details for the two patients assigned an alternative vaccine are not described. Distribution of each of the comparators across the three vaccine groups (Ipsilateral (I), Contralateral (C) and No Vaccine (N)) was equivalent.

Six patients underwent surgery for DCIS in the absence of invasion whilst 36 (85.7%) were being treated for invasive disease. Invasive ductal carcinoma was diagnosed in 24/42 (57.1%) of patients with Invasive Lobular Carcinoma accounting for 6/42 (14.3%) and mixed ductal and lobular carcinoma present in 3/42 (7.1%) of patients. 34/42 (81%) of patients had oestrogen receptor positive disease whereas Her2 overexpression was identified in 6/42 (14.3%) of patients.

Of the 42 patients described, 13 had positive sentinel lymph nodes (I=6, C=3, N=2 with both patients who received an alternative vaccine also having positive sentinel nodes). As expected, strong association was seen in those with evidence of lymphovascular invasion and nodal disease; 7/10 (70%) patients with lymphovascular invasion had positive nodes compared to 6/26 (23.1%) who did not have lymphovascular invasion identified within their tumour specimen (P=0.005).

Overall 14 (33.3%) patients underwent mastectomy of whom 3 underwent mastectomy with immediate reconstruction and 1 underwent bilateral mastectomy due to contralateral concomitant extensive DCIS. 28 (66.7%) of patients were treated with wide local excision.

6.3 Participant vaccination history

Patient vaccine history was gathered by direct patient interview and through corroboration with patient health records held by each individual's General Practitioner (GP). Unfortunately patient recall proved unreliable as a source of information and available GP held records were often incomplete. Consequently for 16.7% of participants the time since their last tetanus vaccination remains unknown, whilst time since last polio immunisation is unknown in 23.8% of the study participants and for tuberculosis the figure is 47.6% (see Table 9). Despite these limitations, no difference in the vaccine history between the three groups could be identified.

	Total Number (%)	Group			P-value
		Ipsilateral (%)	Contralateral (%)	No Vaccine (%)	
TOTAL	**42 (100)**	**20 (47.6)**	**10 (23.8)**	**10 (23.8)**	
Age					
Mean	57.9	57.8	61.4	55.4	0.59
Range	35 - 79	35 - 74	47 – 79	40 - 66	
Standard Deviation	10.1	10.0	12.4	8.6	
T stage of breast tumour					
Tis (Carcinoma in situ)	6 (14.3)	4 (20.0)	1 (10.0)	1 (10.0)	0.61
T1 (0.1-19.9mm)	15 (35.7)	8 (40.0)	4 (40.0)	3 (30.0)	
T2 (20.0-49.9mm)	17 (40.5)	7 (35.0)	5 (50.0)	5 (50.0)	
T3 (>50.0mm)	3 (7.1)	1 (5.0)	0 (0.0)	1 (10.0)	
Nodal Disease					
Node negative	29 (69.0)	14 (70.0)	7 (70.0)	8 (80.0)	0.21
1-3 Nodes involved	9 (21.4)	4 (20.0)	2 (20.0)	2 (20.0)	
>3	4 (9.5)	2 (10.0)	1 (10.0)	0 (0.0)	
Lymphovascular Invasion					
Present	10 (23.8)	4 (20.0)	3 (30.0)	1 (10.0)	0.09
Not seen	32 (76.2)	16 (80.0)	7 (70.0)	9 (90.0)	
Histological Type					
DCIS	6 (14.3)	4 (20.0)	1 (10.0)	1 (10.0)	0.57
IDC	24 (57.1)	11 (65.0)	4 (40.0)	7 (70.0)	
ILC	6 (14.3)	2 (10.0)	3 (30.0)	1 (10.0)	
IDC/ILC	3 (7.1)	3 (15.0)	0 (0.0)	0 (0.0)	
Other	3 (7.1)	0 (0.0)	2 (20.0)	1 (10.0)	
Grade					
DCIS in isolation	6 (14.3)	4 (20.0)	1 (10.0)	1 (10.0)	0.97
I	5 (11.9)	2 (10.0)	1 (10.0)	2 (20.0)	
II	21 (50.0)	10 (50.0)	5 (50.0)	4 (40.0)	
III	10 (23.8)	4 (20.0)	3 (30.0)	3 (30.0)	
Hormone Receptor Status					
ER+	34 (81.0)	16 (80.0)	8 (80.0)	8 (80.0)	0.38
Her2+	6 (14.3)	1 (5.0)	2 (20.0)	2 (20.0)	
Triple Negative	1 (2.4)	1 (5.0)	0 (0.0)	0 (0.0)	
Surgical Treatment					
Mastectomy	11 (26.2)	3 (15.0)	3 (30.0)	4 (40.0)	0.59
Mastectomy with reconstruction	3 (7.1)	2 (10.0)	1 (10.0)	0 (0.0)	
WLE	28 (66.7)	15 (75.0)	6 (60.0)	6 (60.0)	
cAXCL	13 (31.0)	6 (30.0)	3 (30.0)	2 (20.0)	

Table 8: Patient demographics, tumour characteristics and surgical treatment. Abbreviations: DCIS – ductal carcinoma in situ, IDC – Invasive ductal carcinoma, ILC – Invasive Lobular Carcinoma, IDC/ILC – mixed IDC and ILC, ER+ - Oestrogen Receptor positive, Her2+ - Human epidermal growth factor receptor 2 positive, "triple negative" – tumour negative for oestrogen receptor, Her2 receptor and progesterone receptor, WLE – Wide Local Excision, cAXCL – Completion axillary clearance. Continuous data analysed via one-way ANOVA. Nominal data analysed by either Chi squared or Fisher's exact test, as appropriate.

	Total Number (%)	Group			
		Ipsilateral (%)	Contralateral (%)	No Vaccine (%)	P-value
TOTAL	**42 (100)**	**20 (47.6)**	**10 (23.8)**	**10 (23.8)**	
Tetanus/Diphtheria					
Number previously vaccinated	35 (83.3)	15 (75.0)	9 (90.0)	9 (90.0)	0.75
Unknown	7 (16.7)	5 (25.0)	1 (10.0)	1 (10.0)	
Mean time to last known vaccine (Years): Range: SD:	19.7 11 – 33 5.6	20.4 12 – 33 6.7	19.3 11 – 26 5.8	19.0 13 – 25 4.6	
Polio					
Number previously vaccinated	32 (76.2)	13 (65.0)	8 (80.0)	9 (90.0)	0.50
Unknown	10 (23.8)	7 (35.0)	2 (20.0)	1 (10.0)	
Mean time to last known vaccine (Years): Range: SD	23.2 12 – 50 8.5	22.0 12 – 40 8.9	20.6 15 – 26 3.5	26.0 17 – 50 10.1	
Tuberculosis					
Number previously vaccinated	22 (52.4)	9 (45.0)	4 (40.0)	8 (80.0)	0.20
Unknown	20 (47.6)	11 (55.0)	6 (60.0)	2 (20.0)	
Mean time to last known vaccine (Years): Range: SD	39.5 21 - 53 7.7	36.3 21 – 45 8.2	45.0 43 – 47 1.8	40.0 26 – 53 8.8	
Other vaccinations					
Hepatitis B	7 (16.7)	3	2	2	N/A
Haemophilus influenzae	3 (7.1)	1	2	0	
Pneumococcus	12 (28.6)	4	5	2	
Meningitis C	0 (0.0)	0	0	0	
MMR	11 (26.2)	6	5	0	
Influenza	16 (38.1)	6	5	3	
Smallpox	4 (9.5)	1	2	1	

Table 9: Vaccine history of study participants. P-values relate to numbers vaccinated within each group, as calculated using Fisher's exact test. No differences in mean time since last know vaccine were identified (One-way Anova – data not shown).

6.4 Patient blood counts at baseline and throughout time course

Full blood counts were taken at each time point for all participants to allow comparison of absolute lymphocyte numbers. For the purpose of this analysis all available data from the 40 study participants randomised to either ipsilateral (I), contralateral (C) or no vaccine (N) cohorts were included; one patient did not have blood taken and a further patient was withdrawn following post-operative complications, therefore 38 patients were included (I = 20, C = 10, N = 8). Figure 19 provides comparison across a) haemoglobin, b) total white cell count, c) neutrophil count and d) lymphocyte count for each group at each time point. No differences between the gross counts were identifiable at any of the time points.

Dynamic change in cell counts was accessed using Friedman's paired test, relating change to baseline levels (TP1). A significant fall in haemoglobin was seen in all three groups at the post-operative time points (TP3 and TP4), with the most marked change occurring at TP3 (Figure 20). The magnitude of haemoglobin drop was equivalent across the three groups (I: mean drop = 5g/l (S.D. 9.8), C: 5.3g/l (4.7), N: 8.4g/l (6.6)). Time course changes in white cell count (Figure 20.b.) and neutrophil count (Figure 20.c.) were also observed across all three groups, however these failed to achieve statistical significance. A small significant increase in lymphocyte count was observed in vaccinated patients at TP2 but not in unvaccinated patients (Figure 20.d.) with an average increase of $0.16x10^9$cells/l (8.5%) in those vaccinated compared with -$0.12x10^9$cells/l (-2.4%) in those who did not receive a vaccination.

It was considered likely that more extensive surgery might have a more significant confounding effect on cell counts. Therefore changes in cell counts were compared between patients who had undergone mastectomy (n = 10) and those who had undergone wide local excision (WLE; n = 28). Absolute counts were equivalent for both groups across the time course (Figure 21.a-d.). When considering changes in counts across the time course, both patients undergoing mastectomy and those undergoing WLE dropped their haemoglobin significantly relative to baseline by TP3 (Figure 21.e.), however the magnitude of the drop was greater and more prolonged in the mastectomy cohort (Mastectomy: mean fall TP3 = 7.7%, SD 6.6. WLE: 2.1%, SD 6.4. P = 0.007). Furthermore patients undergoing mastectomy experienced a significant fall in their white cell count (Figure 21.f.) and neutrophil count (Figure 21.g.) by TP4, which was not reciprocated within patients undergoing WLE. When considering the changes in lymphocytes over the time course (Figure 21.h.), no significant difference in the gross cell dynamics could be identified between patients undergoing mastectomy, and those undergoing WLE.

It is probable that the fall in total white cell count and neutrophil count in patients undergoing mastectomy occurs as a consequence of the greater haemopoiesis required to reconstitute the red cell compartment following the greater losses sustained during surgery. Although one would anticipate a similar impact on lymphocyte numbers, no such findings were demonstrable within the study cohort and therefore sub-grouping of patients according to surgery undertaken was not considered necessary for study data analysis. The dynamic change in total lymphocyte counts observable in vaccinated patients at TP2 suggests an adequate gross peripheral blood response to vaccination within our study cohort at the point of tissue collection.

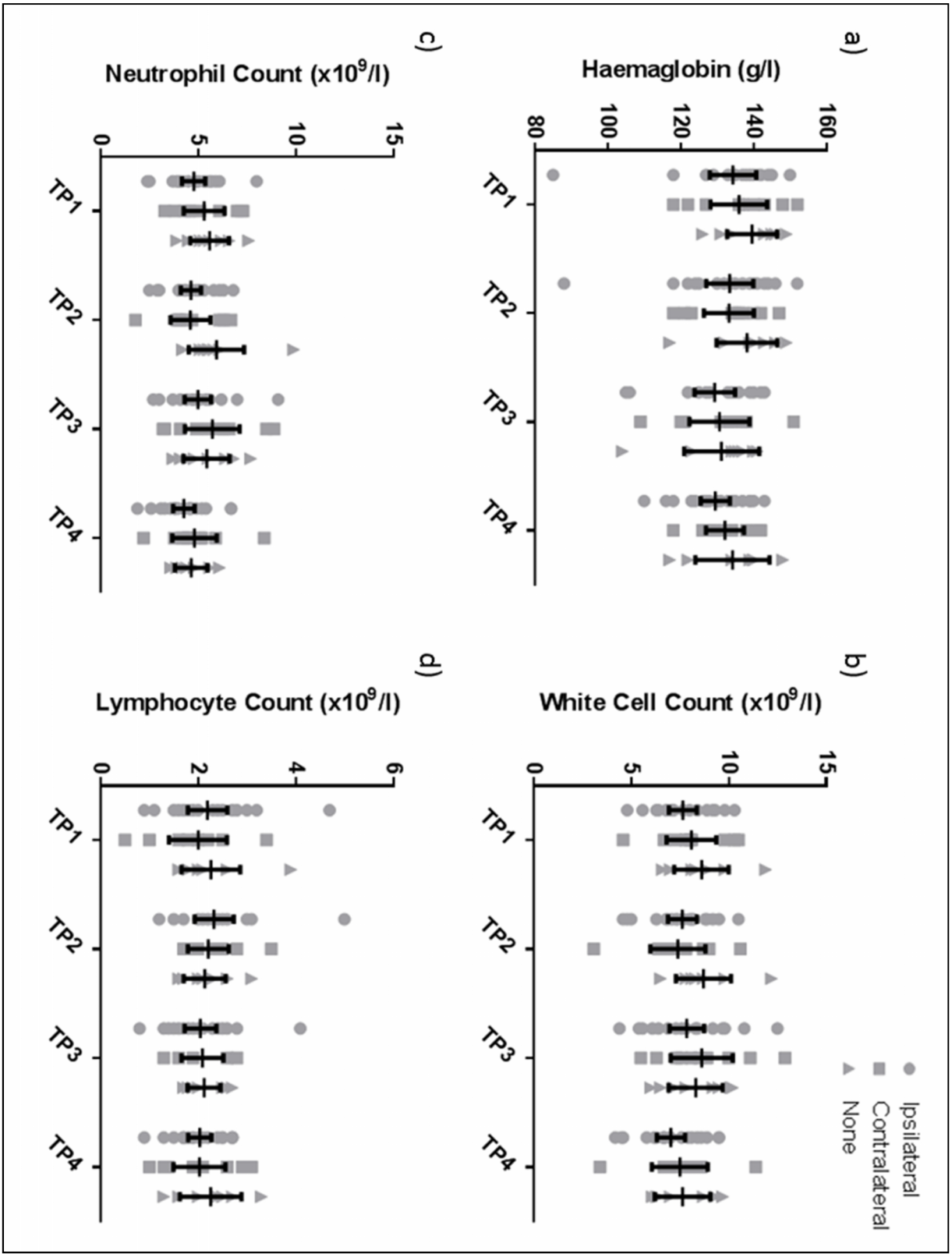


Figure 19: Blood counts across treatment groups (see legend) and time points (x-axis). Graphs illustrate mean with 95% Confidence intervals superimposed on the individual data points for each group. The blood counts were equivalent at all-time points across all three groups: a) illustrates haemoglobin, b) total white cell count, c) neutrophil count and d) lymphocyte count.

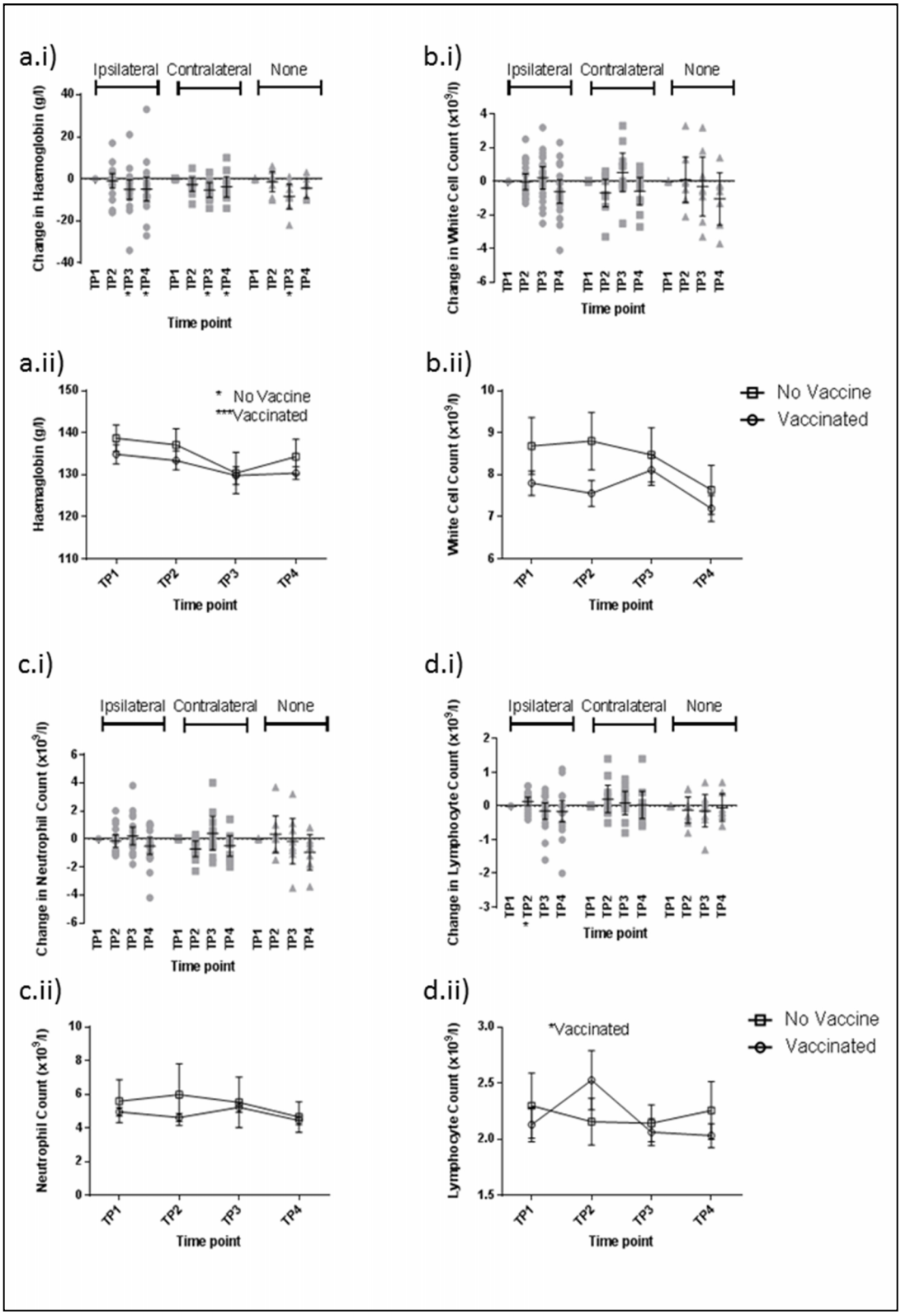


Figure 20: Changes in blood counts across time course within individual treatment groups (x.i) and within vaccinated versus unvaccinated patients (x.ii). a.i) and a.ii) detail haemoglobin levels across the time course, whereas b) details total white cell count, c) neutrophil count and d) lymphocyte count. In all cases mean values are plotted with error bars representing SEM. x.i) plots detail the change from baseline (TP1) in terms of absolute numbers whereas x.ii) graphics detail the absolute values. Significance of change over time course relative to baseline was assessed through non-parametric paired analysis utilising Friedman's test; for x.i) significant change is denoted by (*) under the x-axis label and for x.ii) degree of significance is highlighted within the plot above the relevant time point. Note the significant increase in lymphocyte count at time point 2 relative to baseline within patients receiving vaccination (d).

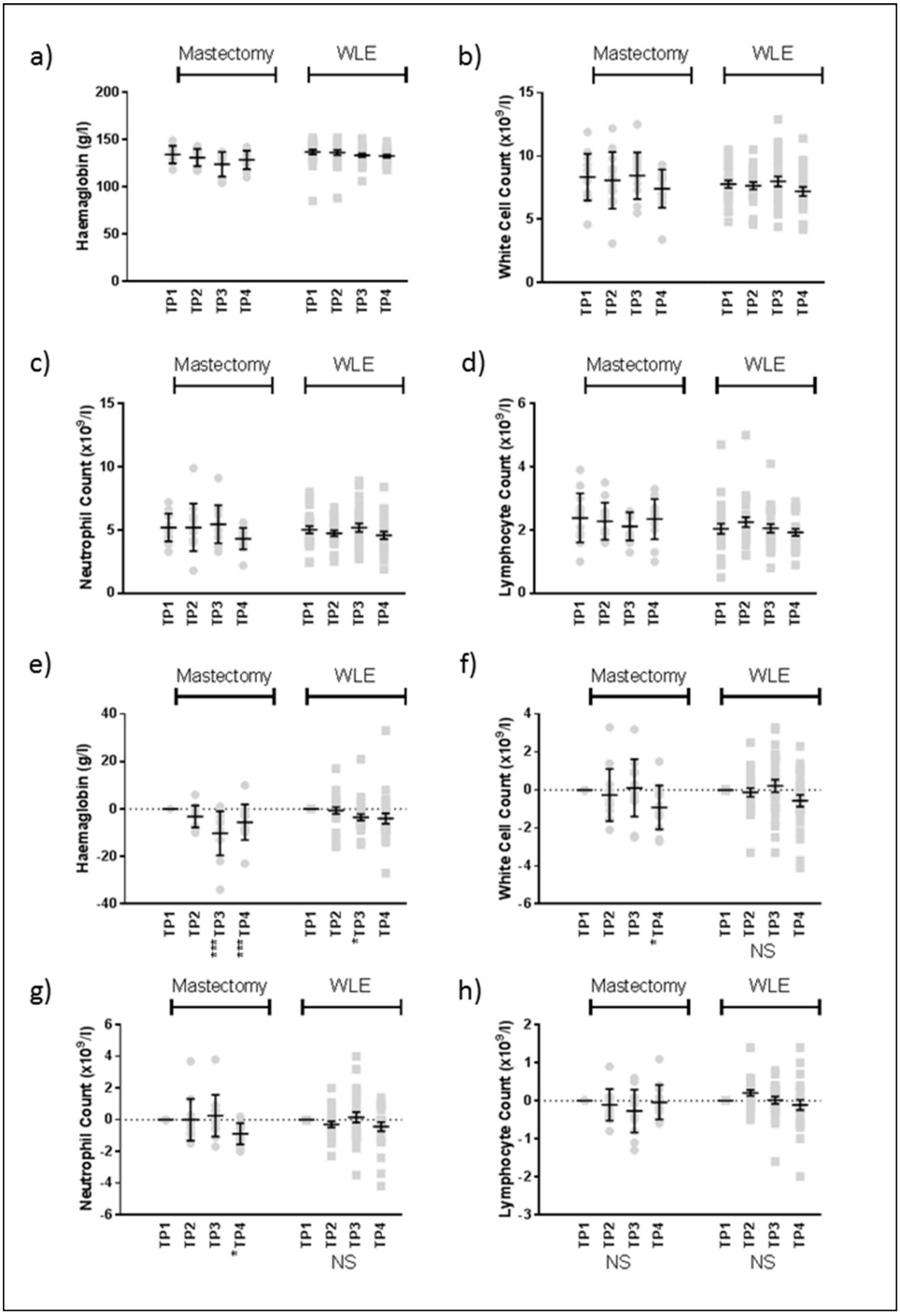


Figure 21: Comparison of blood trends in patients undergoing mastectomy versus those undergoing wide local excision (WLE). a) absolute values for haemoglobin b) white cell count c) neutrophil count and d) lymphocyte count within the two cohorts. e-h) values expressed as change in values relative to baseline (TP1); significance as assessed with Friedman's test utilising Dunn's multiple comparison test denoted by (*, * according to magnitude. NS = non-significant). All plots show mean values with error bars displaying SEM.**

7 The immunoglobulin response to vaccination

The serum from the twenty three patients within the final cohort (see Section 6.1) was analysed for the presence of antibody specific to TTd, DTd and PPD across all four time points (TP). For TTd and DTd, IgG isotypes (IgG_{1-4}) were also analysed. Serum from all 42 recruited patients was screened for presence of IgE specific to TTd and DTd; levels of vaccine-specific IgE were below detection threshold in all 42 patients.

The following subsections describe the serum immunoglobulin response to vaccination; eleven patients were immunised on the ipsilateral side to their cancer by subcutaneous injection, six received vaccination contralateral to their cancer via deep intramuscular injection and six received no vaccination.

Total IgG specific to DTd and TTd was quantified against an international standard and is therefore expressed in international units (IU). IgG specific to PPD, and all specificities of IgM, IgG_1, IgG_3 and IgG_4 are expressed as proportional to highest responders identified following serum screening (see Section 5.2.3) and are therefore expressed as Arbitrary Units (AU). None of the 23 patients had sufficient serum IgG_2 levels to serve as a standard, therefore IgG_2 is charted as absorbance at 490nm; because of this, no comparison can be made between patients regarding IgG_2 in terms of relative quantification, however changes in absorbance across the time course within the same patient can be commented upon.

7.1 Baseline levels of immunoglobulin were comparable between groups

Figure 22 summarises the baseline levels of vaccine specific immunoglobulin (total IgG and isotypes) specific to TTd, DTd and PPD present at TP1 (baseline; prior to vaccination) in all 23 patients. Of the 23 patients included within the final analysis three had unknown vaccination history (012, 022 and 027) and one patient denied previously receiving a vaccine (001). In all four cases no corroborating record was available from their general practitioner. Patients 012, 022 and 027 had serum anti-TTd and anti-DTd immunoglobulin levels comparable to those known to have previously received a vaccine. Patient 001 had the highest baseline levels of anti-TTd IgG of anyone from the cohort, and anti-DTd levels comparable to other subjects who had previously received a vaccine. Therefore despite these four patients not having a documented history of exposure to vaccine it was felt reasonable to assume prior exposure given serological evidence of antigen specificity. Given the lack of consistency between patient-reported vaccine history and the serological findings at baseline, and in light of the difficulty in accessing historic records of vaccination from our patients, no sub-analysis of the cohort according to vaccine history was attempted.

Comparison was made between baseline immunoglobulin levels in the three groups; Figure 23 summarises the mean levels of immunoglobulin isotypes present within patients recruited to the “ipsilateral”, “contralateral” and “no vaccine” groups. Due to the significant variability and limited group size, wide standard deviation was seen in all comparisons. Table 10 details outcome of two-sample student T-tests comparing levels across the three groups; a Holm-Šidák correction was used to adjust P-value to take account of multiple testing. No significant difference in the baseline levels of any isotype between of any of the groups was appreciable.

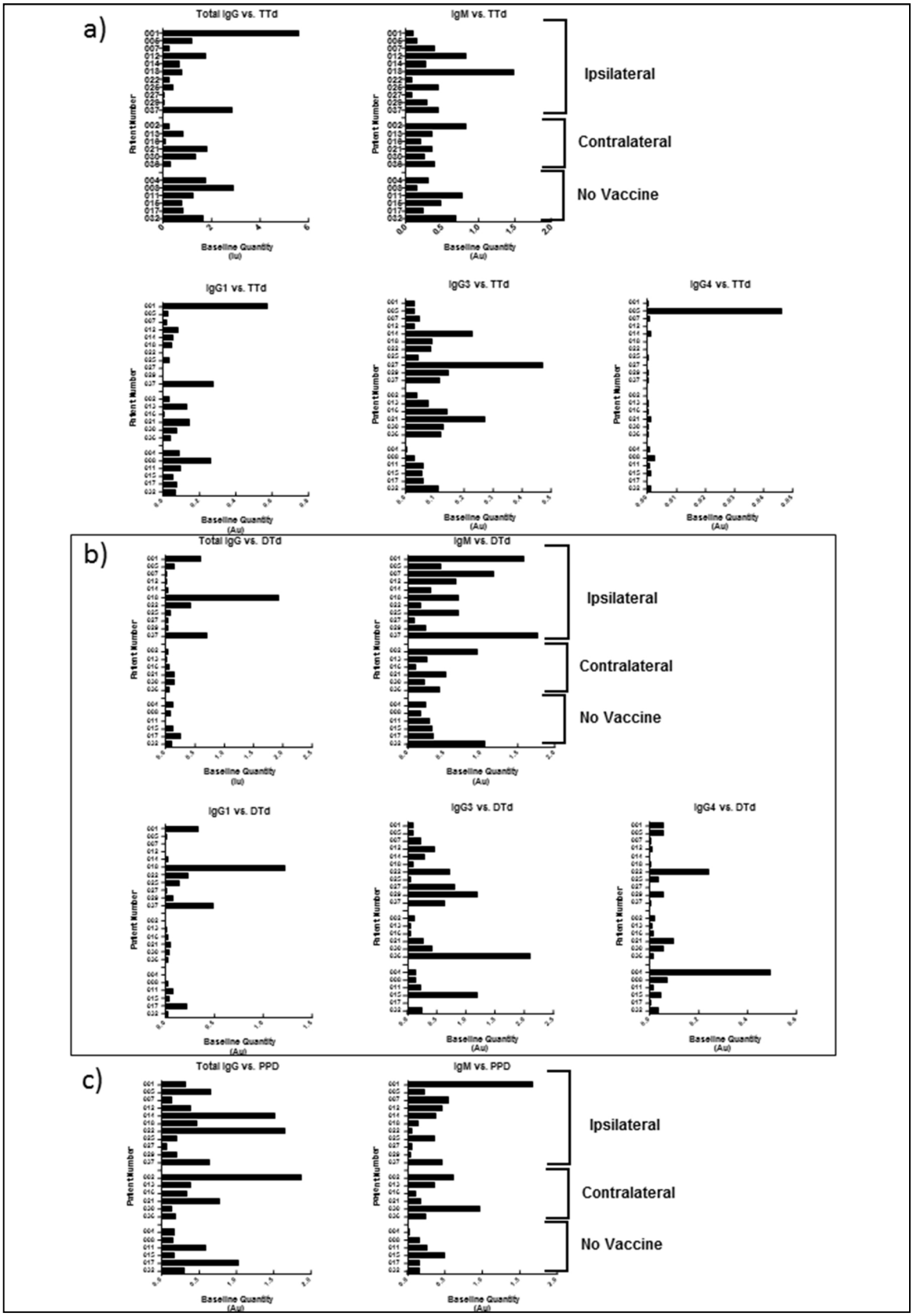
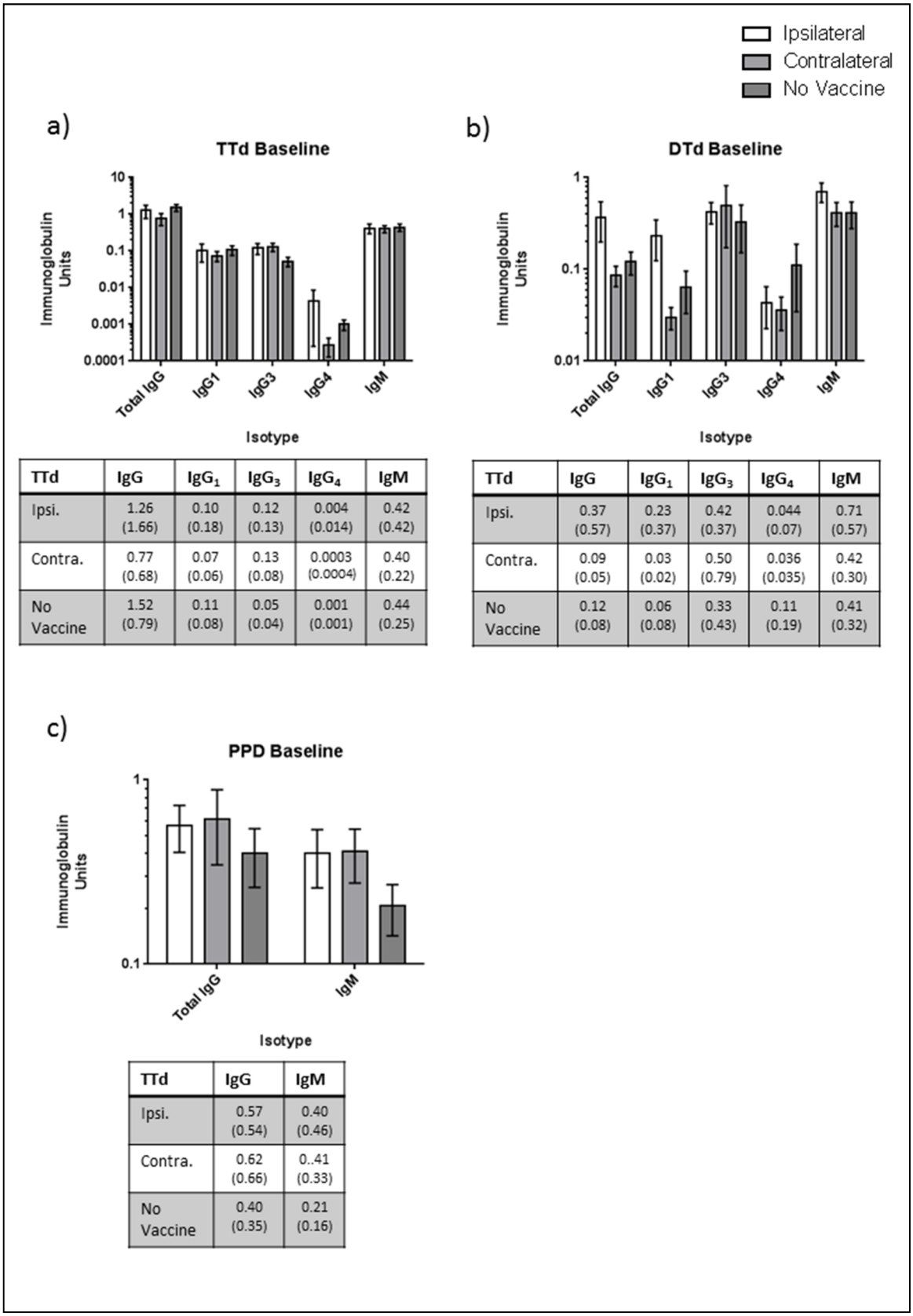


Figure 22: Quantity of immunoglobulin for each patient a) against TTd b) against DTd and c) against PPD at baseline. Patients received either ipsilateral (11 patients, 001 – 037; top of each chart) or contralateral vaccination (6 patients, 002 – 036; mid-section of each chart), or no vaccine ("No vaccine" - 6 patients, 004 – 032; bottom of each chart)). Units of quantification are either Iu (total IgG) or Au against an in house standard (IgM, IgG1, IgG3, IgG4). No in house standard was available for IgG2 for TTd or DTd due to universal low serum levels and therefore comparative data was not available.



TTd	IgG	IgG_1	IgG_3	IgG_4	IgM
Ipsi.	1.26 (1.66)	0.10 (0.18)	0.12 (0.13)	0.004 (0.014)	0.42 (0.42)
Contra.	0.77 (0.68)	0.07 (0.06)	0.13 (0.08)	0.0003 (0.0004)	0.40 (0.22)
No Vaccine	1.52 (0.79)	0.11 (0.08)	0.05 (0.04)	0.001 (0.001)	0.44 (0.25)

TTd	IgG	IgG_1	IgG_3	IgG_4	IgM
Ipsi.	0.37 (0.57)	0.23 (0.37)	0.42 (0.37)	0.044 (0.07)	0.71 (0.57)
Contra.	0.09 (0.05)	0.03 (0.02)	0.50 (0.79)	0.036 (0.035)	0.42 (0.30)
No Vaccine	0.12 (0.08)	0.06 (0.08)	0.33 (0.43)	0.11 (0.19)	0.41 (0.32)

TTd	IgG	IgM
Ipsi.	0.57 (0.54)	0.40 (0.46)
Contra.	0.62 (0.66)	0..41 (0.33)
No Vaccine	0.40 (0.35)	0.21 (0.16)

Figure 23: Comparison of baseline immunoglobulin levels between ipsilateral, contralateral and no vaccine groups. Comparison was made between average baseline quantities of a) TTd specific, b) DTd specific and c) PPD specific immunoglobulin isotypes. Graphs illustrate mean levels in each group on a logarithmic scale (Total IgG = Iu, all others = Au) with error bars describing Standard Error of Mean (SEM). Data tables display mean and (standard deviation) of the same data. No significant differences between groups were identified for any of the immunoglobulin isotypes (see Table 10).

Comparator	P-value	Mean 1	Mean 2	Difference	SE of Difference	T-ratio
TTd: Ipsilateral vs. Contralateral						
Total IgG	0.504	1.26	0.77	0.49	0.72	0.68
IgG1	0.708	0.10	0.07	0.03	0.07	0.38
IgG3	0.873	0.12	0.13	-0.01	0.06	0.16
IgG4	0.481	4.41E-03	2.76E-04	4.13E-03	0.01	0.72
IgM	0.926	0.42	0.40	0.02	0.18	0.09
TTd: Ipsilateral vs. No Vaccine						
Total IgG	0.729	1.26	1.52	-0.26	0.73	0.35
IgG1	0.955	0.10	0.11	0.00	0.08	0.06
IgG3	0.245	0.12	0.05	0.07	0.06	1.21
IgG4	0.560	4.41E-03	1.00E-03	3.40E-03	0.01	0.60
IgM	0.902	0.42	0.44	-0.02	0.19	0.12
TTd: Contralateral vs. No Vaccine						
Total IgG	0.110	0.77	1.52	-0.75	0.43	1.76
IgG1	0.420	0.07	0.11	-0.03	0.04	0.84
IgG3	0.058	0.13	0.05	0.08	0.04	2.14
IgG4	0.067	2.76E-04	1.00E-03	-7.27E-04	0.00	2.05
IgM	0.770	0.40	0.44	-0.04	0.14	0.30
DTd: Ipsilateral vs. Contralateral						
Total IgG	0.249	0.37	0.09	0.28	0.24	1.20
IgG1	0.199	0.23	0.03	0.20	0.15	1.34
IgG3	0.801	0.42	0.50	-0.07	0.28	0.26
IgG4	0.799	0.04	0.04	0.01	0.03	0.26
IgM	0.262	0.71	0.42	0.29	0.25	1.17
DTd: Ipsilateral vs. No Vaccine						
Total IgG	0.309	0.37	0.12	0.25	0.24	1.05
IgG1	0.285	0.23	0.06	0.17	0.15	1.11
IgG3	0.634	0.42	0.33	0.10	0.20	0.49
IgG4	0.294	0.04	0.11	-0.07	0.06	1.09
IgM	0.257	0.71	0.41	0.30	0.25	1.18
DTd: Contralateral vs. No Vaccine						
Total IgG	0.412	0.09	0.12	-0.03	0.04	0.86
IgG1	0.318	0.03	0.06	-0.03	0.03	1.05
IgG3	0.656	0.50	0.33	0.17	0.37	0.46
IgG4	0.355	0.04	0.11	-0.08	0.08	0.97
IgM	0.974	0.42	0.41	0.01	0.18	0.03
PPD: Ipsilateral vs. Contralateral						
Total IgG	0.872	0.57	0.61	-0.05	0.29	0.16
IgM	0.965	0.40	0.41	-0.01	0.21	0.04
PPD: Ipsilateral vs. No Vaccine						
Total IgG	0.513	0.57	0.40	0.16	0.24	0.67
IgM	0.344	0.40	0.21	0.19	0.20	0.98
PPD: Contralateral vs. No Vaccine						
Total IgG	0.500	0.61	0.40	0.21	0.30	0.70
IgM	0.201	0.41	0.21	0.20	0.15	1.37

Table 10: Multiple T-tests comparing mean immunoglobulin quantities within ipsilateral, contralateral and no vaccine groups at baseline. Values given to 2 decimal places except P-value (3 decimal places). P-values calculated using a two sample T test with a Holm-Šidák correction for multiple comparisons (alpha = 0.05).

7.2 Vaccine-specific Total IgG response in the absence of detectable change in IgM

Changes in quantity of circulating immunoglobulin specific to TTD, DTd and PPD within the serum of patients recruited to the ipsilateral, contralateral and no vaccine cohorts was assessed throughout the study time course. Paired analysis using Friedman's test was used to evaluate change in serum levels with a Dunn's post-test assessment of difference between individual time points.

Using this approach significant increase in serum IgG specific to TTd and DTd was appreciable within both the ipsilateral and contralateral cohorts at TP3 and TP4 relative to baseline. No such change in serum IgG specific to PPD was detectable. In addition no changes in immunoglobulin levels were detectable within those patients who were not vaccinated (see Table 11 and Figure 24.a.). Therefore patients recruited seemed to provide a reasonable model of immune response to vaccination, with the dynamics of their response to vaccine conforming to what one would expect.

Serum levels of vaccine specific IgM were not increased relative to baseline in the majority of patients for either TTd or DTd (Figure 24.b.). A minority of patients demonstrated a modest increase in IgM which peaked at TP2; an example of such a response is illustrated in Figure 24.c. The more common pattern of IgM response was a reduction in detectable levels at TP3 and TP4 relative to baseline as shown in Figure 24.d. Summary statistics of IgM levels therefore suggest a significant reduction of vaccine specific IgM within the serum of vaccinated patients at the later time points (Table 11, Figure 24). The most likely explanation for such an observation is that, during the ELISA assay, the low-affinity IgM is prevented from binding the plate-bound antigen by the more copious and higher-affinity IgG-isotypes. Such a theory is supported by the observation that apparent reduction in observable IgM levels occurs in parallel with multi-fold increases in IgG; a typical example of the parallel increase in IgG and decrease in IgM is shown in Figure 24.d.

7.3 Magnitude of response is similar between Ipsilateral and contralateral cohorts

To assess comparability between the ipsilateraly vaccinated and the contralateraly vaccinated cohorts the mean magnitude of peak immunoglobulin response was compared between the cohorts. Mean values for percentage increase over baseline at TP3 demonstrated no significant difference between the two cohorts (see Table 12). Analysis was repeated for TP2 and TP4 with the same result (data not shown). There is therefore no obvious difference between the serum immunoglobulin response seen in patients recruited to the ipsilateral and contralateral cohorts as expected.

Of note was the significant variability in magnitude of response within both cohorts and for both antigens, as evidenced by the wide standard deviation about the mean for all variables. See Section 7.5 for detailed description of variability of immunoglobulin response across cohort.

			TP1	TP2	TP3	TP4	Significance
Total IgG vs TTd	Ipsilateral	Mean	1.26	2.47	10.51	8.27	P<0.0001
		SD	1.66	3.07	15.70	11.93	Significant increase in IgG
		SEM	0.50	0.92	4.73	3.60	vs TTd at TP3 and TP4
	Contralateral	Mean	0.77	1.72	9.58	7.55	P=0.0015
		SD	0.68	1.73	10.85	5.53	Significant increase in IgG
		SEM	0.28	0.71	4.43	2.26	vs TTd at TP3 and TP4
	No Vaccine	Mean	1.52	1.24	1.23	1.21	NS
		SD	0.79	0.60	0.57	0.96	P=0.13
		SEM	0.32	0.25	0.23	0.39	
Total IgG vs DTd	Ipsilateral	Mean	0.37	0.64	1.40	1.34	P=<0.0001
		SD	0.57	0.88	1.24	1.26	Significant increase in IgG
		SEM	0.17	0.26	0.37	0.38	vs DTd at TP3 and TP4
	Contralateral	Mean	0.09	0.19	0.84	0.65	P=0.003
		SD	0.05	0.26	0.80	0.37	Significant increase in IgG
		SEM	0.02	0.11	0.33	0.15	vs DTd at TP3 and TP4
	No Vaccine	Mean	0.12	0.12	0.12	0.07	NS
		SD	0.08	0.09	0.09	0.06	P=0.77
		SEM	0.03	0.04	0.04	0.02	
Total IgG vs PPD	Ipsilateral	Mean	0.57	0.56	0.57	0.52	NS
		SD	0.54	0.51	0.54	0.49	P=0.25
		SEM	0.16	0.15	0.16	0.15	
	Contralateral	Mean	0.61	0.56	0.49	0.52	NS
		SD	0.66	0.54	0.45	0.63	P=0.35
		SEM	0.27	0.22	0.18	0.26	
	No Vaccine	Mean	0.40	0.41	0.42	0.22	NS
		SD	0.35	0.37	0.39	0.24	P=0.72
		SEM	0.14	0.15	0.16	0.10	
IgM vs TTd	Ipsilateral	Mean	0.42	0.37	0.34	0.38	P=0.01
		SD	0.42	0.36	0.45	0.53	Significant reduction in
		SEM	0.13	0.11	0.14	0.16	serum IgM vs TTd at TP3
	Contralateral	Mean	0.40	0.45	0.39	0.37	relative to baseline
		SD	0.22	0.17	0.19	0.16	
		SEM	0.09	0.07	0.08	0.07	
	No Vaccine	Mean	0.44	0.47	0.43	0.38	NS
		SD	0.25	0.25	0.25	0.29	P=0.13
		SEM	0.10	0.10	0.10	0.12	
IgM vs DTd	Ipsilateral	Mean	0.71	0.61	0.60	0.55	P=0.01
		SD	0.56	0.45	0.49	0.43	Significant reduction in
		SEM	0.17	0.14	0.15	0.13	serum IgM vs DTd at TP4
	Contralateral	Mean	0.42	0.40	0.40	0.32	relative to baseline
		SD	0.30	0.27	0.35	0.24	
		SEM	0.12	0.11	0.14	0.10	
	No Vaccine	Mean	0.41	0.43	0.44	0.38	NS
		SD	0.32	0.36	0.37	0.40	P=0.78
		SEM	0.13	0.15	0.15	0.16	
IgM vs PPD	Ipsilateral	Mean	0.40	0.39	0.41	0.42	NS
		SD	0.46	0.40	0.42	0.40	P=0.67
		SEM	0.14	0.12	0.13	0.12	
	Contralateral	Mean	0.41	0.42	0.39	0.42	
		SD	0.32	0.37	0.34	0.35	
		SEM	0.13	0.15	0.14	0.14	
	No Vaccine	Mean	0.21	0.22	0.19	0.16	NS
		SD	0.16	0.18	0.14	0.16	P=0.13
		SEM	0.06	0.07	0.06	0.06	

Table 11: Mean, standard deviation (SD) and Standard Error of Mean (SEM) of serum quantities of IgG and IgM immunoglobulins specific to TTd, DTd and PPD at each of the four time points within the ipsilateral, contralateral and no vaccine cohorts. P-values generated through Paired analysis using Friedman's test (non-parametric ANOVA analysis).

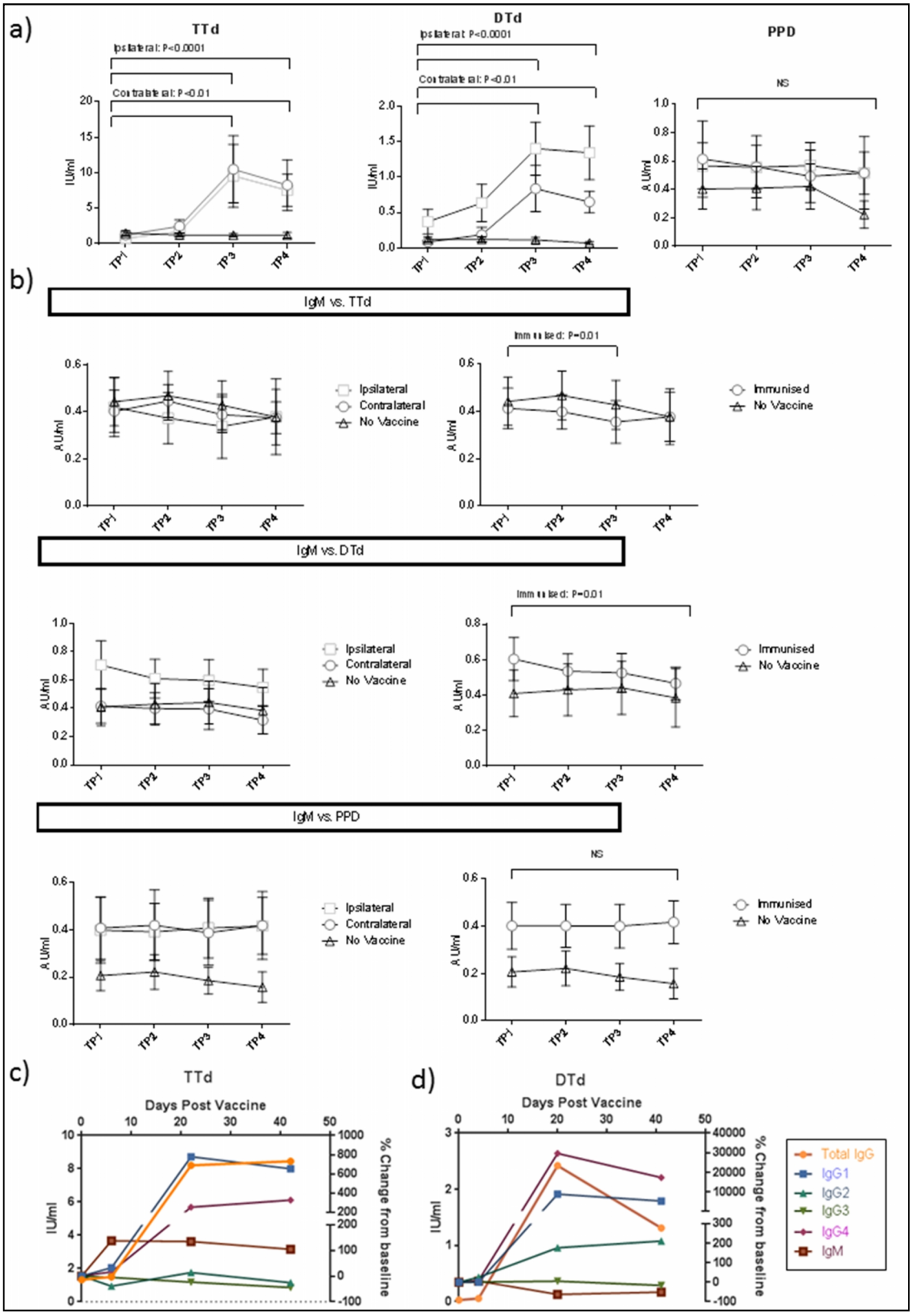


Figure 24: Serum immunoglobulin response to vaccination. a) Total IgG and b) IgM specific to TTd, DTd, and PPD at each time point. For b); left hand graph illustrates response within individual groups whereas right hand graph compares response within "immunised" (ipsilateral + contralateral) vs "no vaccine" groups. Graphs illustrate mean with error bars denoting SEM. P-values generated using paired analysis utilising Friedman's test with a Dunns multiple comparison correction. c-d) Dynamic change in serum immunoglobulin following vaccination in two example patients (c): Patient 030 response to TTd and d):Patient 013 response to DTd); Total IgG (yellow) expressed in IU (left "Y" axis); all other isotypes expressed as percentage change (right "Y" axis) according to the colour coded key (right hand side).

		Ipsilateral			Contralateral			SE of Difference	P-value
		Mean	SEM	SD	Mean	SEM	SD		
Total IgG	TTd	1136.9	435.6	1444.8	1679.9	569.6	1395.2	724.96	0.465
	DTd	828.1	361.4	1198.5	1824.0	1266.2	3101.6	1035.68	0.351
	PPD	-0.1	4.3	14.4	-12.1	5.4	13.1	7.09	0.111
IgG1	TTd	1547.1	540.3	1792.0	924.6	282.3	691.5	769.75	0.431
	DTd	578.0	246.3	816.8	2507.9	1416.1	3468.7	1071.27	0.092
IgG3	TTd	108.4	49.1	162.7	447.5	446.9	1094.8	327.80	0.317
	DTd	70.1	29.8	98.8	16.7	16.6	40.7	42.66	0.230
IgG4	TTd	2198.5	1608.7	5335.4	228.1	109.0	266.9	2212.33	0.387
	DTd	6280.7	2238.9	7425.6	5767.4	4793.0	11740.3	4615.48	0.913
IgM	TTd	-15.3	21.0	69.5	6.8	25.8	63.2	34.24	0.528
	DTd	-18.1	6.9	23.0	-9.6	11.1	27.2	12.41	0.506
	PPD	3.7	4.5	14.9	-5.5	11.8	28.8	10.44	0.392

Table 12: Comparison of peak increase in antigen-specific immunoglobulin levels between ipsilateral and contralateral cohorts. Valves given describe the percentage increase over baseline seen at TP3. No significant differences were identified between the peak increases seen in the two cohorts (two-sample T-test with a Holm-Šidák correction for multiple comparisons (alpha = 0.05).

7.4 IgG response to antigen across time course varies according to isotype and antigen

Given the comparability of the ipsilateral and contralateral cohorts in terms of their serological response to vaccination, it was deemed reasonable to group these cohorts into a "vaccinated" group to allow further comment on the dynamics of the vaccine response.

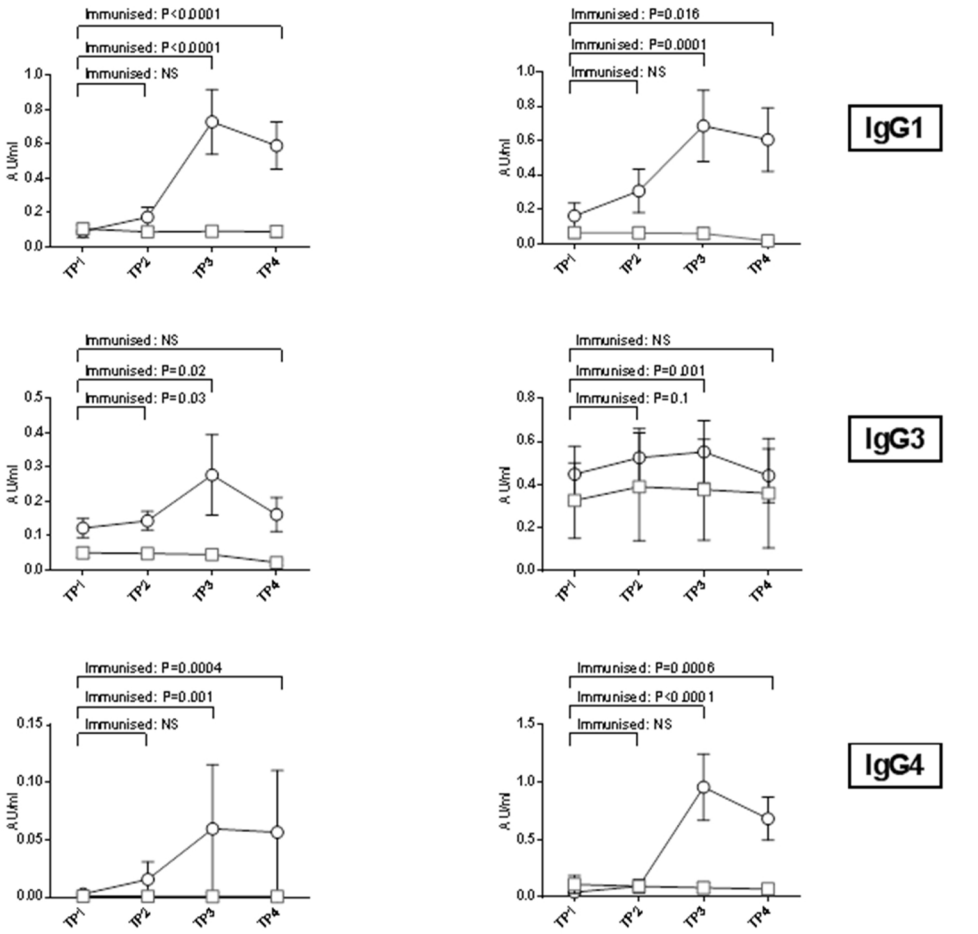
Figure 25 illustrates the change in quantity of vaccine specific IgG isotypes within vaccinated and unvaccinated patients. Change from baseline quantity was assessed using Friedman paired analysis incorporating a Dunn correction for multiple testing. Variation across the time course was seen for all three IgG isotypes within the vaccinated group; however no such variation was demonstrable within the unvaccinated patients (Figure 25.b.). At a population level, no significant increase in IgG_1 or IgG_4 specific to either TTd or DTd could be detected in the vaccinated patients prior to time point 3, however significant changes over baseline values were seen at time points 3 and 4 - Figure 25.a. A significant increase IgG_3 specific to TTd was noted at time point 2, with a corresponding borderline increase in anti-DTd IgG_3 at the same time point, although the response was of a low level and of similar kinetics to IgG_1. For both TTd and DTd the level of IgG_3 was significantly increased at time point 3 with respect to baseline, however by time point 4 levels had returned to approximately baseline in contrast with the observed IgG_1 and IgG_4 response.

The response to TTd and DTd was contrasted in the patients who received vaccine; due to the variability in the quantity of immunoglobulin present at baseline (see Figure 23) and the inability to relate standardised quantities directly due to differences in scaling etcetera, it was not possible to make direct comparison between absolute quantities (data not shown). Magnitude of response as described by percentage change relative to baseline (TP1) levels was therefore used to compare the response to individual antigens (See Figure 26). As expected, the increase in serum levels of IgG specific to TTd and DTd exceeded that of IgG specific against PPD control. This was evident at TP2 (mean increase IgG vs TTd = 150.7%; (Standard Error of Mean (SEM) = 71.0). PPD = 1.6% (3.7)), as well as TP3 (TTd = 1329.0% (341.7). PPD = -4.4% (3.6)) and TP4 (TTd = 1036.0% (248.4). PPD = -13.2% (4.8)). Conversely no significant difference could be discerned between changes in serum IgM specific to TTd/DTd compared to PPD (Figure 26). Despite this, individual patients did generate increased serum levels of IgM vs TTd, but not vs DTd.

The magnitude of total IgG, IgG_1 and IgG_3 response against TTd and DTd was of equivalent magnitude at all-time points (Figure 26). In contrast IgG4 response was of significantly greater magnitude against DTd compared to TTd at both TP3 (DTd: mean increase = 6100% (SEM =

2137). TTd: 1503.0 (1050.0); P = 0.026) and TP4 (DTd: 4037% (SEM = 1430). TTd: 1200.0 (692.1); P = 0.042), but not at the earlier TP2.

a)

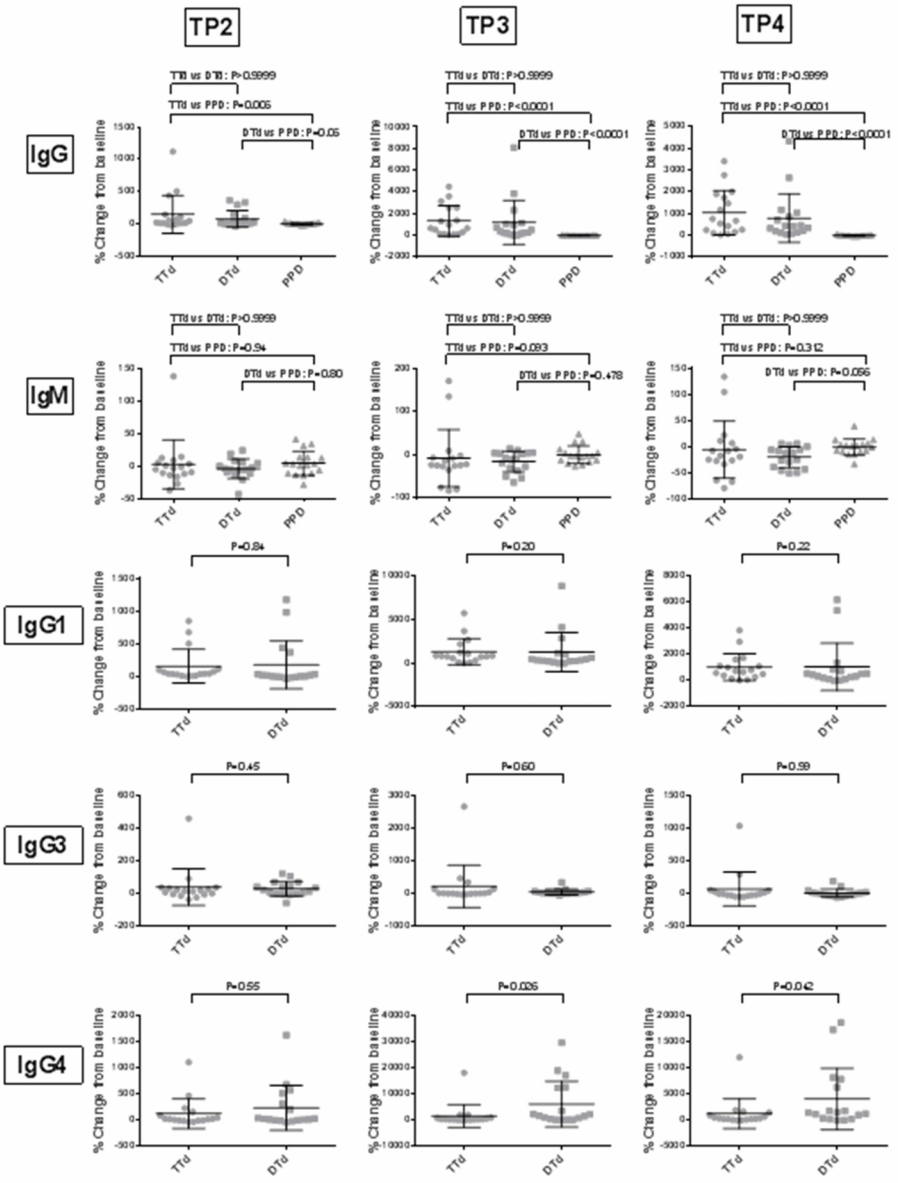


b)

		Immunised					No Vaccine				
		TP1	TP2	TP3	TP4	Friedman P-value	TP1	TP2	TP3	TP4	Friedman P-value
IgG1	TTd	0.09 (0.14)	0.17 (0.24)	0.73 (0.78)	0.59 (0.57)	<0.0001	0.11 (0.08)	0.09 (0.04)	0.09 (0.05)	0.09 (0.08)	0.31
	DTd	0.16 (0.31)	0.31 (0.52)	0.69 (0.85)	0.61 (0.76)	<0.0001	0.06 (0.08)	0.06 (0.07)	0.06 (0.08)	0.02 (0.02)	0.19
IgG3	TTd	0.12 (0.11)	0.14 (0.11)	0.28 (0.48)	0.16 (0.20)	0.0005	0.05 (0.04)	0.05 (0.03)	0.05 (0.03)	0.02 (0.02)	0.06
	DTd	0.45 (0.53)	0.53 (0.56)	0.55 (0.59)	0.44 (0.52)	0.0009	0.33 (0.43)	0.39 (0.62)	0.38 (0.57)	0.36 (0.62)	0.32
IgG4	TTd	2.95E-03 (0.01)	0.02 (0.06)	0.06 (0.23)	0.06 (0.22)	<0.0001	1.00E-03 (7.89E-04)	9.09E-04 (6.21E-04)	8.56E-04 (6.10E-04)	7.53E-04 (8.64E-04)	0.71
	DTd	0.04 (0.06)	0.09 (0.12)	0.96 (1.19)	0.68 (0.77)	<0.0001	0.11 (0.19)	0.09 (0.14)	0.08 (0.12)	0.07 (0.11)	0.54

Figure 25: IgG isotype response to vaccine components. Patients from ipsilateral and contralateral cohorts are grouped into "vaccinated" group; comparison is made to unvaccinated "no vaccine" patients. a) Change in quantity (AU) of antigen specific (Left: TTd, Right: DTd) IgG_1 (Top), IgG_3 (Middle) and IgG_4 (Bottom) over the vaccine time course. Mean antigen quantity plotted with error bars denoting SEM. Significance of change relative to baseline calculated through paired analysis utilising Friedman's test with a Dunns multiple comparison correction. No significant change relative to baseline was detectable in unvaccinated patients therefore values given are for the vaccinated cohort. b) Descriptive statistics for individual isotypes specific for TTd and DTd at each time point with mean AU and (standard deviation), as well as P-value calculated using Friedman's test stated.

a)



b)

		TT:d			DT:d			PPD		
		Mean % Increase	SD	SEM	Mean % Increase	SD	SEM	Mean % Increase	SD	SEM
IgG	TP2	150.7	292.8	71.0	77.8	129.2	31.3	1.6	15.4	3.7
	TP3	1329.0	1409.0	341.7	1180.0	2036.0	493.8	-4.4	14.8	3.6
	TP4	1036.0	1024.0	248.4	789.9	1112.0	269.6	-13.2	19.6	4.8
IgM	TP2	2.5	37.4	9.1	-4.3	15.1	3.7	4.1	18.7	4.5
	TP3	-7.5	66.2	16.1	-15.1	24.1	5.8	0.5	20.4	5.0
	TP4	-6.4	54.9	13.3	-20.5	20.0	4.9	-1.3	15.5	3.8
IgG1	TP2	151.8	258.8	62.8	173.9	367.3	89.1			
	TP3	1327.0	1500.0	363.9	1259.0	2254.0	546.7			
	TP4	1033.0	1035.0	251.1	1051.0	1822.0	441.9			
IgG3	TP2	41.1	112.5	27.3	30.5	44.6	10.8			
	TP3	228.1	647.3	157.0	51.3	85.5	20.8			
	TP4	73.0	262.4	63.7	10.1	61.7	15.0			
IgG4	TP2	121.1	283.7	68.8	231.2	429.6	104.2			
	TP3	1503.0	4331.0	1050.0	6100.0	8809.0	2137.0			
	TP4	1200.0	2854.0	692.1	4037.0	5898.0	1430.0			

Figure 26: Magnitude of response against different antigens. a) Comparison of percentage increase in serum concentration of immunoglobulin isotypes across time course in relation to baseline (TP1) levels. Non-parametric test used to compare magnitude of response between antigens; for IgG and IgM, Kruskal-Wallis test with Dunns correction for multiple comparisons used to compare TTd vs DTd, TTd vs PPD and DTd vs PPD. For IgG isotypes, Mann-Whitney U-test used to compare TTd vs DTd. b) Descriptive statistics for % increase over baseline (TP1) levels with mean, SD and SEM stated.

7.5 Comment on the variability of serological response across the cohort

As commented on earlier (see Section 7.3), and evidenced by summary statistics demonstrating wide standard deviations about mean values for serum quantities (Figures Figure 24,Figure 25 and Figure 26; Tables Table 10 and Table 11) and percentage change in immunoglobulin (Figure 26; Table 12), there exists vast heterogeneity in antigen-specific antibody levels prior to (Figure 22) and following vaccination. Therefore although summary statistics can be used to identify broad trends within the patient cohort (see above sections), it remains useful and necessary to visualise and comment upon serological response on an individual patient basis.

To enable this, a novel approach to the graphical representation of serological data was devised based on the concept of a heat map (see Figure 27). Termed an "up-regulation matrix", this method allows the visualisation of ELISA data from all 23 patients, at each time point, for TTd, DTd and PPD and for IgM, IgG and IgG_{1-4} in a single graphic. The data visually represented is increase of immunoglobulin of a particular isotype and specificity over baseline level (TP1). Although this methodology has its advantages as described, there are some drawbacks, namely the loss of information comparing absolute quantity of immunoglobulin between patients. This results in some aberrations in interpreting the data; Patient 005, for example, can be seen to have a moderate (5-20 fold) increase in serum IgG_4 specific to TTd following vaccination. However when taking into account baseline levels of antibody (see Figure 22) one notes that the same patient had an extremely high baseline. Therefore following vaccination patient 005 had a peak anti-TTd IgG_4 level 22-fold higher than the next nearest patient and 250-fold higher than the average peak value. Taking into consideration baseline values is therefore imperative.

Interpretation of such data is therefore complex. However visualisation in this fashion does allow commentary on several interesting observations within the cohort. Firstly a minority of patients can be seen to respond to vaccination earlier than the broader cohort (Figure 27); although summary statistics failed to show a significant increase in serum immunoglobulin at TP2 at a population level (Figure 24, Table 11), from Figure 27 it is possible to identify individuals who generated an early response to either TTd, DTd or both. Patients 001, 005, 021 and 025 had a robust (1-5-fold increase) response to both vaccine components at TP2 (example profile - Figure 27.c.) whereas patients 002 and 014 had a robust response to only TTd at TP2. The remaining patients failed to generate a robust increase in serum immunoglobulin until TP3. There was also significant heterogeneity in the predominant isotypes generated during vaccine response; Figure 27.b. illustrates a response with IgG2 and IgG4 predominance (Patient 029 responding to DTd) whereas Figure 27.c. illustrates a

response with IgG1 and IgG3 predominance (Patient 013 responding to TTd). Furthermore heterogeneity of response can be identified within a single individual (Figure 27. e-f.).

Graphical representation of the data in this fashion also allows easy identification of patients who have a less typical response to vaccination; Patient 007 and 029 have a robust IgG and IgG_4 response to DTd however no corresponding IgG_1 response. Patient 007 generates no response to TTd despite a robust DTd response. 6/17 (35%) and 5/17 (29%) of patients generate IgG_3 against TTd and DTd respectively with similar numbers producing low levels of IgG_2; a minority of patients generated both IgG_2 and IgG_3 against a single antigen. Two patients (007, 030) generated a detectable increase in anti-TTd IgM; both of these patients had low-magnitude increases in IgG_1 and/or IgG_4 when compared with patients where there was a marked reduction in detectable serum IgM; this would seem to corroborate the theory outlined in Section 7.2 that apparent reduction in IgM is as a result of competition on the plate for available antigen. However other patients with similarly moderate high-affinity responses to vaccination failed to generate vaccine-specific IgM. It is therefore undetermined to what degree IgM up-regulation contributes to recall vaccine response within the cohort.

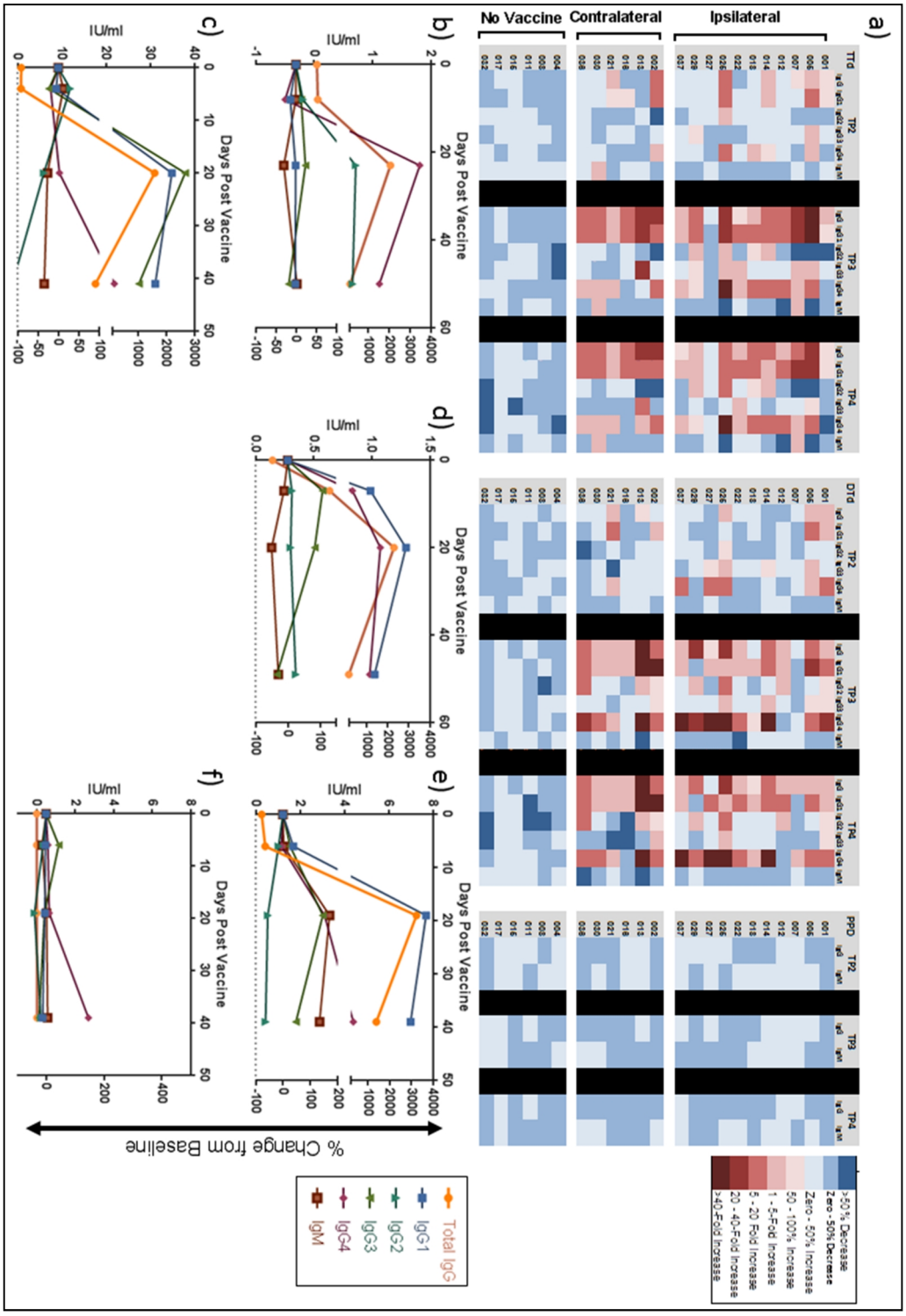


Figure 27: Immunoglobulin up-regulation across the cohort. a) Up-regulation matrix detailing response to TTd (left-hand block), DTd (middle block) and PPD (right-hand block) following vaccination. Each block is subdivided into response at Time Point 2 (TP2), TP3 and TP4, and within each sub-block each isotype of antibody (IgG (Total IgG), IgG1-4, IgM) is detailed by a single column. Up-regulation is represented relative to baseline levels according to the colour-gradient key (top right). Each row represents response within an individual patient, with "ipsilateral", "contralateral" and "no vaccine" cohorts having been grouped together. b-f) Examples of graphic representation of data presented in a) i.e. dynamic change in serum immunoglobulin following vaccination. Total IgG (yellow) expressed in IU (left "Y" axis); all other isotypes expressed as percentage change (right "Y" axis) according to the colour coded key (right hand side). b-c) Examples of patients responding to vaccination in a primary fashion (b – Patient 029 responding to DTd with IgG2 and IgG4 predominance, c – Patient 013 responding to TTd with IgG1 and IgG3 predominance). d) Example of a patient responding to TTd in a secondary fashion. e-f) Example of disparity within a single patient; Patient 007 responding against e) TTd and f) DTd, with a notable difference in the magnitude of the response against the two components of the vaccine tested.)

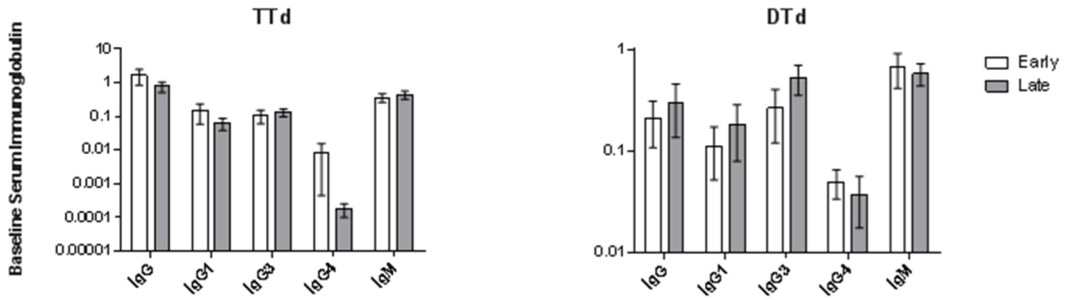
7.6 Baseline serum immunoglobulin levels fail to predict vaccine response

Given the unexpected variability in the response to vaccination within study patients, the factors which determined type of response became of interest. In a broad sense, it is possible to divide the patient cohort into “early” responders and “late” responders for analytical purposes. It would seem reasonable to assume that the underlying reason for the difference in response within these two groups is a difference in the memory state. With that in mind baseline immunoglobulin levels in “early” and “late” responders were compared to determine whether baseline serum abundance predicts vaccine response.

Figure 28.a. compares the baseline quantity of immunoglobulin specific against TTd and DTd in patients who generated an “early” response to vaccination, and those who generated a “late” response. Mann-Whitney U test was used to identify differences between the two groups; there was no identifiable significant difference in the serum levels of total IgG, IgM or individual isotypes between the two groups for either of the antigens. Therefore baseline serum antigen specific antibody was unable to predict the nature of the response to vaccination.

To establish whether baseline immunoglobulin levels at the time of vaccination could predict the magnitude of serological response, correlation analysis was performed. Spearman’s non-parametric test demonstrated no correlation between baseline levels of Total IgG, IgM or individual IgG isotypes with the peak levels of IgG, IgG_1 or IgG_4 (Figure 28.b.). Similar analysis of baseline levels versus magnitude of response one week following vaccination (TP2) revealed no relationship between baseline IgG_4 and early IgG_1 response for both TTd and DTd. Graphical representation (Figure 28.c.) demonstrates the wide spread of data points around the best-fit line.

a)



b)

Baseline			Early response			Peak Response		
			TP2 Total IgG	TP2 IgG1	TP2 IgG4	TP3 Total IgG	TP3 IgG1	TP3 IgG4
IgG	TTd	Spearman r	0.17	-0.01	0.05	-0.12	-0.19	0.08
		95% CI	-0.35 – 0.61	-0.50 – 0.48	-0.45 – 0.53	-0.58 – 0.40	-0.62 – 0.34	-0.43 – 0.55
		P-value	0.51	0.96	0.85	0.66	0.47	0.75
	DTd	Spearman r	0.34	0.38	0.33	-0.30	-0.23	-0.08
		95% CI	-0.18 – 0.71	-0.14 – 0.73	-0.20 – 0.71	-0.69 – 0.23	-0.65 – 0.30	-0.55 – 0.43
		P-value	0.18	0.14	0.20	0.24	0.38	0.75
IgG1	TTd	Spearman r	0.12	-0.12	-0.11	-0.13	-0.39	-0.06
		95% CI	-0.40 – 0.58	-0.58 – 0.40	-0.57 – 0.41	-0.59 – 0.39	-0.74 – 0.13	-0.53 – 0.45
		P-value	0.65	0.66	0.68	0.61	0.12	0.83
	DTd	Spearman r	0.23	0.04	0.13	-0.18	-0.45	0.12
		95% CI	-0.29 – 0.65	-0.46 – 0.52	-0.39 – 0.58	-0.62 – 0.34	-0.77 – 0.06	-0.40 – 0.58
		P-value	0.37	0.88	0.63	0.49	0.07	0.65
IgG4	TTd	Spearman r	0.36	**0.50**	-0.04	0.34	0.11	0.05
		95% CI	-0.17 – 0.72	**0.02 – 0.80**	-0.52 – 0.46	-0.18 – 0.71	-0.40 – 0.57	-0.45 – 0.53
		P-value	0.16	**0.04**	0.88	0.18	0.67	0.85
	DTd	Spearman r	**0.56**	**0.47**	-0.03	0.31	0.08	-0.24
		95% CI	**0.09 – 0.82**	**-0.03 - 0.78**	-0.51 – 0.47	-0.21 – 0.70	-0.43 – 0.55	-0.66 – 0.29
		P-value	**0.02**	**0.058**	0.92	0.22	0.77	0.35
IgM	TTd	Spearman r	0.10	-0.07	0.45	0.28	0.13	0.46
		95% CI	-0.41 – 0.56	-0.54 – 0.44	-0.05 – 0.78	-0.24 – 0.68	-0.39 – 0.58	-0.04 – 0.78
		P-value	0.71	0.79	0.07	0.27	0.63	0.06
	DTd	Spearman r	0.48	**0.63**	0.18	-0.04	0.01	-0.18
		95% CI	-0.02 – 0.79	**0.20 – 0.86**	-0.34 – 0.62	-0.52 – 0.46	-0.48 – 0.50	-0.62 – 0.34
		P-value	0.053	**0.008**	0.49	0.88	0.97	0.49

c)

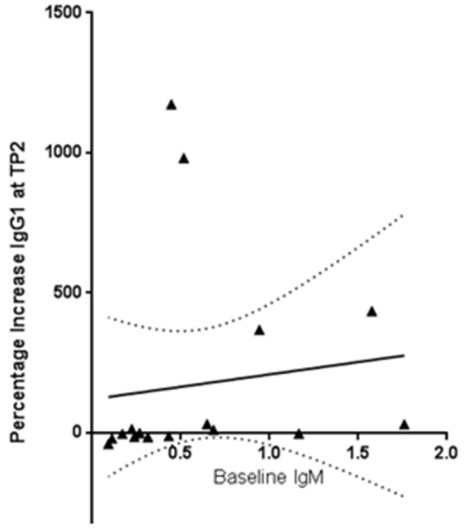


Figure 28: Predicting the serum response to vaccination. a) Comparison of baseline serum immunoglobulin levels specific to TTd (left) and DTd (right) in patients who responded early versus those who responded late. Graphs chart mean quantities in IU (total IgG) and AU (IgG_{1-4} and IgM) with error bars denoting SEM. No significant differences were identified. b) correlation analysis utilising Spearman's test comparing baseline immunoglobulin levels for Total IgG, IgG_1, IgG_4 and IgM with the percentage increase over baseline seen at TP2 ("early") and TP3 ("peak"); r-value, 95% CI and P-value are given for each comparison. c) Graphic representation of correlation between baseline IgM and early increase in serum IgG1 specific to DTd; graph illustrates line of best fit with 95% Confidence interval. .

8 Detailed description of peripheral blood responses to vaccination

Vaccination induces a complex immunoglobulin response as already demonstrated in section 7. Synchronously there is an induced change in the circulating lymphocyte populations. The novel focus of this thesis is the characterisation of lymphocyte populations within vaccine-draining nodal tissue, however to interpret findings in tissue it is necessary to detail response in blood, as this gives insight into the nature and scale of vaccine response. Gross changes in total lymphocyte numbers were appreciable by TP2 in the peripheral blood of patients who had undergone vaccination, however similar changes were not seen in the blood of non-vaccinated patients (see 6.4, Figure 20). In order to detail the dynamic cell populations within the peripheral blood three 8-colour flow cytometry panels were designed. Compatibility of the panel with cells isolated from lymph node tissue was ensured throughout to allow comparison between blood and node – see Section 9.

Figure 29 illustrates the characterisation strategy used; cryopreserved cells were prepared according to Section 4.10.2. Briefly, cells were thawed in CRPMI supplemented with 10% human antibody serum and divided unevenly so that Panels 2 and 3 received approximately twice the number of cells as Panel 1 (see Figure 29). For Panel 1, a minimum of $1x10^6$ cells were stained, with a minimum of $2x10^6$ cells stained for Panels 2 and 3. For peripheral blood Panel 1 was used to numerate the absolute numbers of major cell populations; calculation was based on percentage of the total lymphocyte population multiplied by total number of lymphocytes identified by synchronous full blood count, undertaken by the haematological laboratory of University Hospital Southampton NHS Foundation Trust. By taking the absolute number for the major population (e.g. CD3+CD4+) from Panel 1, it was possible to numerate subpopulations of said cells identified following application of Panels 2 and 3 (See Figure 29).

Absolute numeration of lymphocytes from lymphatic tissue was not possible as it would have been influenced by total node size, which would be subject to patient-to-patient variability, itself influenced by known and unknown variables. Therefore quantification of nodal populations is only expressible in terms of either percentage total lymphocytes or percentage of subpopulation.

Panel design was undertaken according to the principles outlined in Section 4.10.2.1. All panels utilised a “dump” strategy whereby a channel was used to positively exclude dead cells, as well as unwanted sub-populations. All three panels (see Figure 29) positively excluded monocytes and macrophages (using CD14) and NK cells (CD16) to minimise false-positive staining due to Fc-receptor binding of florescent antibodies. Panel 3 (CD4+) included a positive exclusion of CD19+ cells, as these also carry Fc-receptors. Panel 2 (B-cell characterisation) proved more

difficult as positive exclusion of CD3+ cells and selection of CD19 positive cells proved unreliable due to small populations of $CD38^{Hi}CD27^{Hi}CD19^{Lo/Intermediate}$ cells; it was unclear whether these represented highly activated B-cells or highly activated T-cells, since both populations would up-regulate CD38 and CD27 and down-regulate CD19 and CD3 respectively. Therefore for the B-cell panel it was necessary to positively exclude T-lymphocytes based on both CD3 and CD4/8 – see Figure 29.

In addition to numeration of the major lymphocyte subsets (B-cell, CD4+, CD8+) panel 1 also allowed numeration of memory cell sub-populations of T-lymphocytes defined according to their surface expression of CD45RA and CCR7 (see Section 1.1.5.2 and Section 1.2.2). The expression of CXCR5, a receptor which mediates cellular migration to follicular boundaries (See Section 1.1.5.1.1 and Section 1.3.2) was also incorporated into the panel to provide information on relative expression of said marker across the broader lymphocyte populations.

Panel 2 provided information about the sub-populations of B-lymphocytes, with particular focus on distinguishing activated cells undergoing blastic differentiation/proliferation (based on surface expression of CD27, CD38 and CD138 (325, 366) – See Section 1.4.2.1) and resting memory subsets expressing CD27 (390) in the absence of CD38 (414) – see Section 1.4.2.2.1. Through staining for surface IgM and IgD it was possible to identify "Switched" B-cells (i.e. double-negative) and unswitched B-cells (not double negative); positive staining for human IgG was attempted, but proved unreliable due to Fc-receptor binding of human IgG from storage media. The following cell populations were therefore identifiable: switched memory (CD38-CD27+), unswitched memory ($IgM+IgD^{Lo}CD27+$), naïve (unswitched, CD27-CD38-), activated naïve (unswitched, $CD27\text{-}CD38^{Int}$), IgM Blasts (unswitched, $IgD\text{-}CD38^{Hi}$), centrocytes/blasts (switched, $CD38^{int}$), plasmablasts (switched, $CD38^{Hi}$) and plasma cells (switched, $CD38^{Hi}$, CD138+).

Panel 3 further evaluated the follicular makers expressed within the CD4+ population (see Section 1.3.2); follicular helper T-cells are by definition CD45RO+ as they are activated, antigen experienced, differentiated cells. This property was therefore used in the panel to delineate the threshold for ICOS and PD1 expression on CXCR5+ cells (see Figure 29). T_{FH} cells were defined as CD4+CD45RO+CXCR5+ICOS+PD1+; associated cell populations lacking either ICOS or PD1 expression were also quantified, as they have variably be described in the literature as "$T_{FH\text{-}Memory}$ cells" and "pre-T_{FH} cells" respectively (see Section 1.3). For the duration of this thesis, these latter two terms were avoided as they remain highly contentious and said populations were described by their surface phenotype. Within the panel CCR7 was also included; the role of CCR7 expression on "follicular-type" CXCR5+ CD4 cells is unclear,

therefore clarity was sought through comparison of expression on tissue-derived cells and their circulating counterparts.

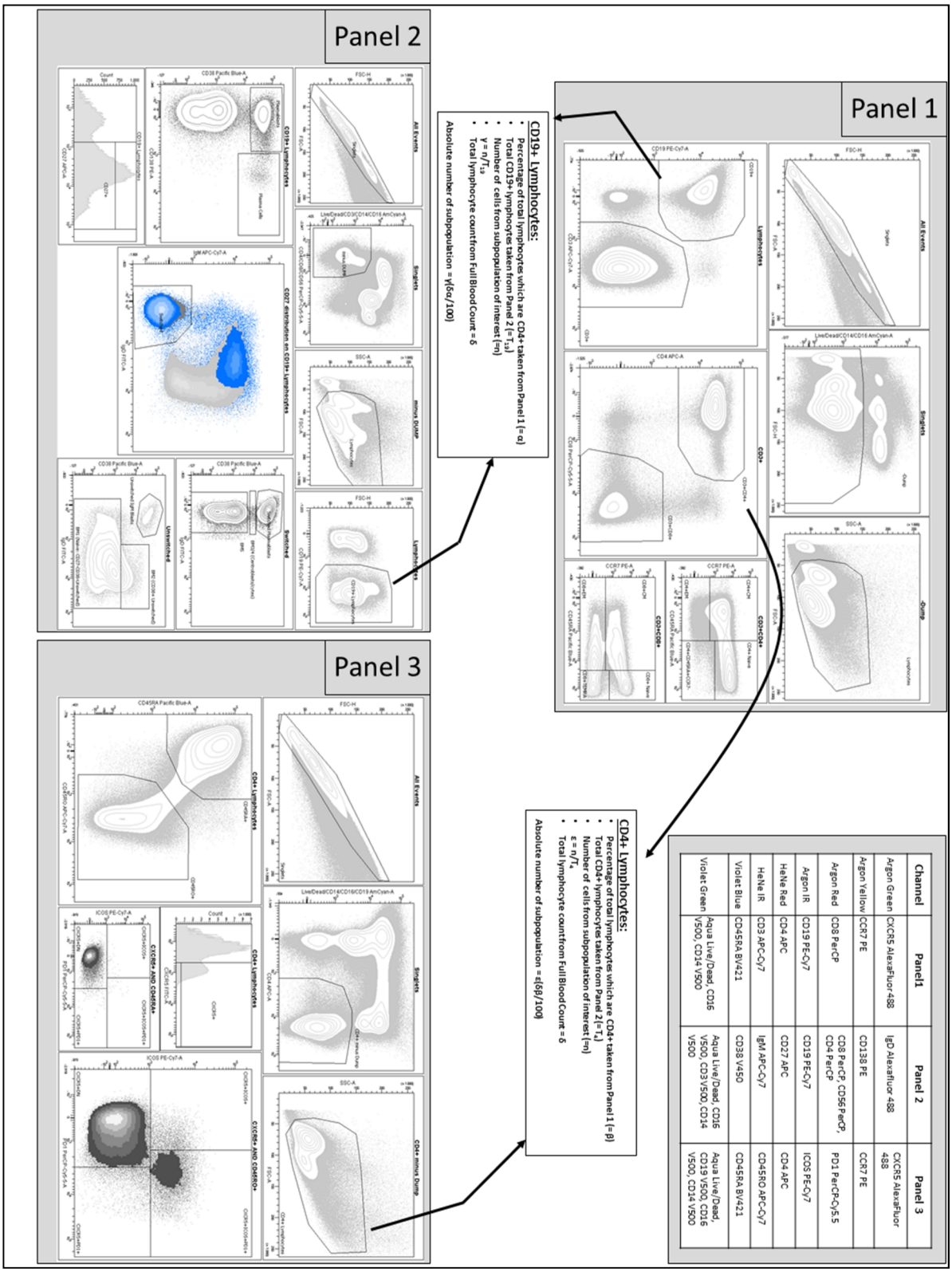


Figure 29: Phenotyping lymphocytes isolated from blood and lymphatic tissue. Three 8-colour flow cytometry panels were used to characterise lymphocytes from each compartment. Panel 1 numerated the major sub-populations of CD19, CD4 and CD8 lymphocytes, and also characterised the T-cell memory sub-populations. Panel 2 characterised the B-lymphocyte sub-populations according to their surface expression of immunoglobulin, CD27, CD38 and CD138. Panel 3 further characterised the CD4+ compartment, identifying sub-populations expressing chemokine receptor CXCR5 with or without the co-expression of T_{FH} surface markers (ICOS, PD1). In the case of peripheral blood, calculation of absolute numbers was achieved through application of calculation detailed within the two text boxes.

8.1 Baseline lymphocyte populations are comparable between the study cohorts

Baseline cell numbers were compared across the three patient cohorts. Kruskal-Wallis test was used to test for significance across the three groups, with additional multiple comparisons between pairs of groups performed utilising Dunn's test. Table 13 details the mean cell number per litre of blood, standard deviation and 95% confidence intervals for some of the major populations analysed for. Figure 30 and Figure 31 graphically illustrate the same data across the wider range of populations analysed. No significant difference between the groups was identifiable for any of the sub-populations analysed. Therefore the three groups were considered to have a similar baseline for subsequent analysis and were accepted to be comparable.

Across all patients, at baseline CD4+ T-cells made up 62% (SD 8.7) of circulating lymphocytes, with CD8+ T-cells accounting for 19% (7.24) and CD19+ B-lymphocytes 13% (6.4). The majority (61%, SD 12.3) of circulating CD4+ cells were naïve, whereas for CD19+ B-lymphocytes and CD8+ T-cells 48% (SD 23.3) and 39% (SD 16.5) of circulating cells were naïve respectively.

Within the study cohort, on average 16% (10.5) of circulating CD8+ T-cells were CCR7+CD45RA- (Central Memory, CM), 24% (11.7) were CCR7-CD45RA- (Effector Memory, EM) and 22% (14.0) were CCR7-CD45RA+ (terminally differentiated effector cells, TEMRA). Conversely, within the CD4+ T-cell compartment, 29% (10.0) were CM and 10% (4.6) were EM. 9.4% (3.9) of all circulating CD4+ lymphocytes were CXCR5+; T_{FH} (CD4+CD45RO+CXCR5+PD1+ICOS+) cells comprised only 0.023% (0.018) of circulating CD4+ cells. CD4+CD45RO+CXCR5+ICOS+PD1- cells were found with a similar frequency to T_{FH} cells (0.020, SD 0.016) however CD4+CD45RO+CXCR5+PD1+ICOS- cells were on average 20-times more numerous, accounting for 0.41% (0.22) of circulating CD4+ cells.

24% (12.1) of circulating CD19+ lymphocytes were switched memory cells. 12% (7.3) were IgM+CD27+ (unswitched memory) cells. Plasma cells (switched CD138+CD38^{Hi}) comprised just 0.02% (0.01) of circulating CD19+ cells whilst switched plasmablasts and IgM+ plasmablasts comprised 1.05% (0.7) and 0.16% (0.14) of the CD19+ population respectively.

Cell Population		Ipsilateral	Contralateral	No Vaccine
CD19+	Mean	333.5	271.0	275.4
	SD	221.9	90.5	216.1
	95%CI	184.4 - 482.5	176.0 - 366.0	48.7 - 502.2
CD4+	Mean	1447.0	1279.0	1406.0
	SD	680.7	583.9	443.8
	95%CI	990.0 - 1910.0	667.0 - 1890.0	940.6 - 1870.0
CD4+ Effector Memory	Mean	159.3	167.2	124.7
	SD	67.9	117.9	57.6
	95%CI	113.7 - 204.9	43.4 - 290.9	64.3 - 185.1
CD4+ Central Memory	Mean	442.9	454.4	399.1
	SD	264.0	209.4	276.5
	95%CI	265.9 - 620.0	234.6 - 674.1	108.9 - 689.3
CD4+CD45RA-CXCR5+	Mean	113.2	107.1	84.9
	SD	77.2	65.3	43.4
	95%CI	61.4 - 165.1	38.6 - 175.6	39.3 - 130.4
CD8+	Mean	470.6	454.3	480.2
	SD	213.6	248.0	228.8
	95%CI	327.0 - 614.0	194.0 - 715.0	240.0 - 720.0
CD8+ Effector Memory	Mean	112.6	130.2	84.4
	SD	81.2	97.7	33.7
	95%CI	58.1 - 167.2	27.7 - 232.7	49.0 - 119.7
CD8+ Central Memory	Mean	74.1	75.2	76.2
	SD	57.5	57.5	88.9
	95%CI	35.5 - 112.7	14.8 - 135.5	0.0 - 169.5
CD8+CD45RA-CXCR5+	Mean	5.5	6.5	3.2
	SD	4.2	5.8	1.6
	95%CI	2.6 - 8.3	0.4 - 12.7	1.6 - 4.9
CD19+ Switched Memory	Mean	94.8	72.4	69.1
	SD	130.9	46.4	68.3
	95%CI	6.9 - 182.8	23.7 - 121.0	0.0 - 140.7
CD19+IgM+CD27+	Mean	34.4	28.7	43.6
	SD	39.1	18.0	34.4
	95%CI	8.2 - 60.7	9.8 - 47.6	7.6 - 79.7
Plasmablasts	Mean	2.9	3.6	1.9
	SD	2.1	3.0	1.1
	95%CI	1.6 - 4.3	0.4 - 6.7	0.8 - 3.0
T_{FH} (CD4+ CD45RO+CXCR5+ PD1+ICOS+)	Mean	0.269	0.348	0.454
	SD	0.209	0.229	0.246
	95%CI	0.128 - 0.409	0.108 - 0.589	0.196 - 0.712
CD4+CD45RO+ CXCR5+ICOS+ PD1-	Mean	0.200	0.313	0.442
	SD	0.134	0.224	0.271
	95%CI	0.110 - 0.290	0.079 - 0.548	0.157 - 0.727
CD4+CD45RO+ CXCR5+PD1+ ICOS-	Mean	5.2	7.6	6.1
	SD	1.9	6.3	1.8
	95%CI	3.9 - 6.5	1.0 - 14.3	4.2 - 8.0

Table 13: Circulating populations at baseline within the three study groups. Figures are given as cell number/Litre blood x10^6. Difference between groups tested for using Kruskal-Wallis test with additional individual comparisons between each pair of groups performed utilising Dunn's correction for multiple comparisons. No significant difference was identified.

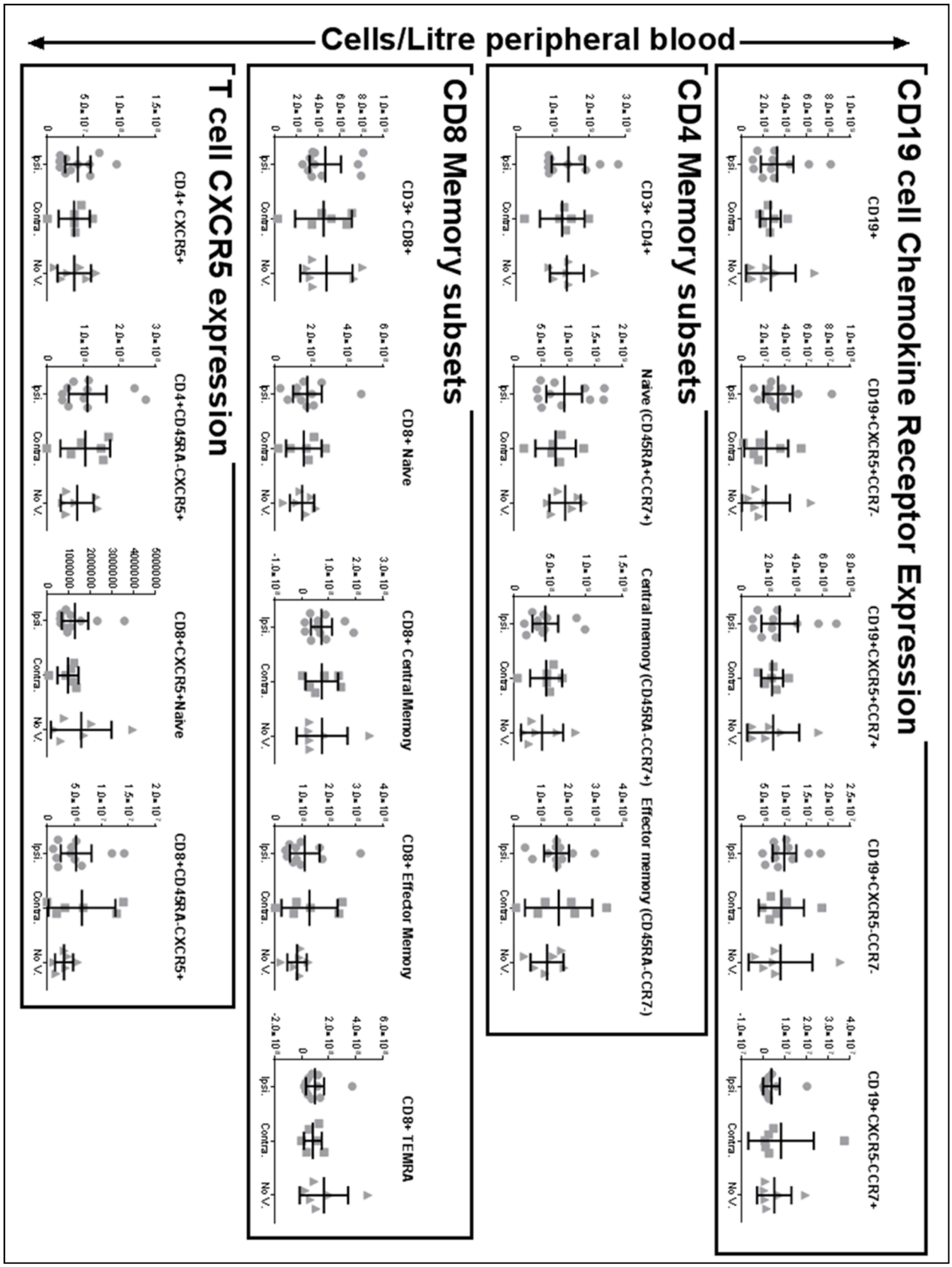


Figure 30: Circulating lymphocyte populations within the three patient cohorts at baseline. Mean cell numbers per litre blood plotted with 95% Confidence intervals. CCR7 and CXCR5 expression on circulating CD19+ cells represented in the top box, with memory cell subsets of CD4+ and CD8+ graphed in middle two boxes. CXCR5+ CD4 populations defined according to co-expression of CD45RA and CCR7 presented within the bottom box. No significant differences between the three groups ("Ipsi" = Ipsilateral, "Contra." = Contralateral, "No V." = No Vaccine) could be identified.

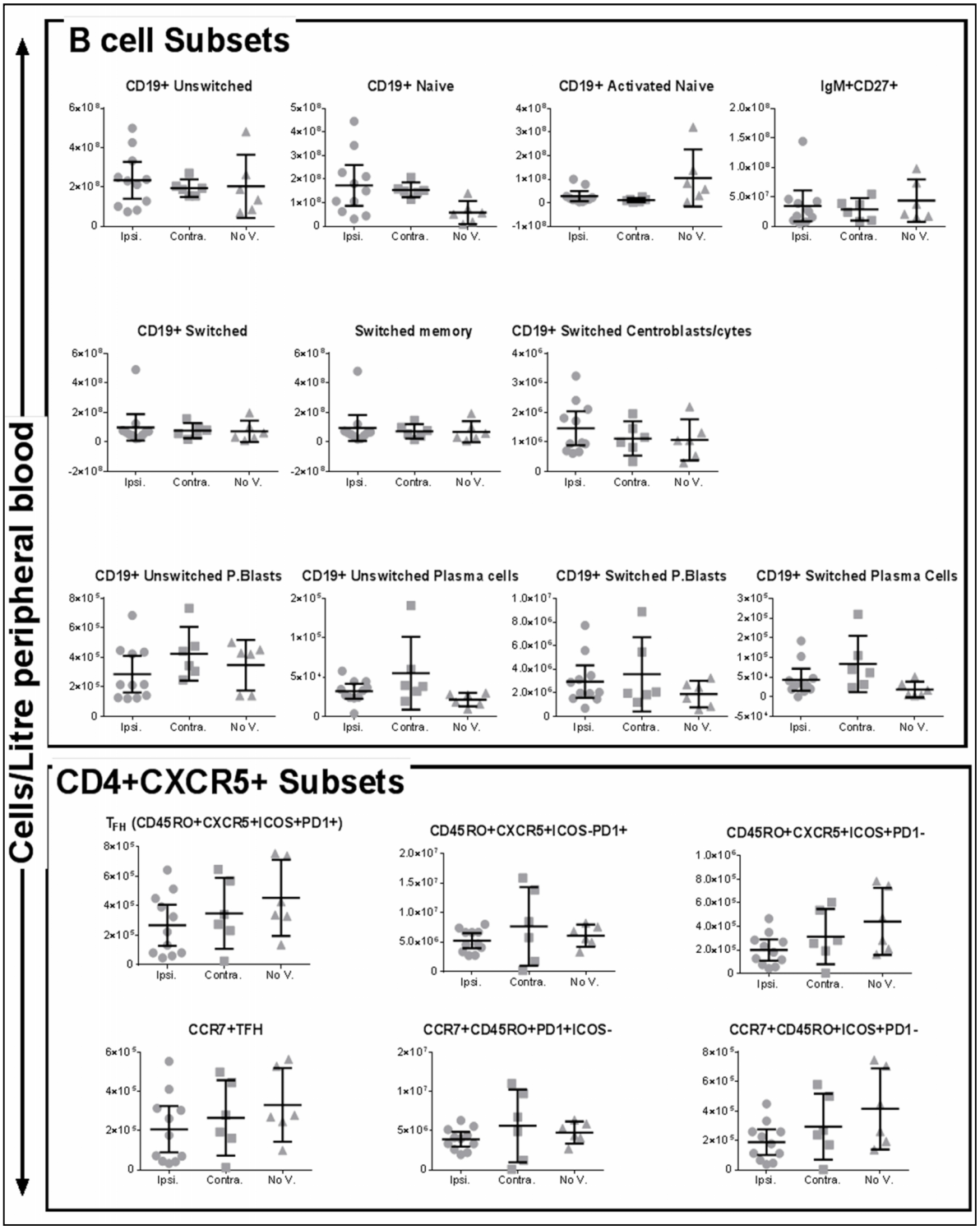


Figure 31: Circulating lymphocyte sub-populations within the three patient cohorts at baseline. Mean cell numbers per litre blood plotted with 95% Confidence intervals. No significant difference between the three groups could be identified with respect to number of circulating B-cell (top box) or CD4+ T-cell (bottom box) subpopulations.

8.2 Dynamics of circulating lymphocyte sub-populations following vaccination

To ascertain the relative dynamics of lymphocyte subpopulations within the peripheral blood following vaccination, phenotypic analysis was performed on PBMCs isolated from blood taken at all four time points.

Figure 32 illustrates the dynamic shift in the gross CD4+, CD8+ and CD19+ populations, and subpopulations thereof which express the chemokine receptors CXCR5 and CCR7, and CD45RA. For both CD4+ and CD8+ populations, the gross naïve and effector memory components remained static following vaccination. However in both CD4+ and CD8+ populations there was a slight fall in absolute numbers of central memory cells at TP4 (CD4+: mean fall = 16.5%; 95% CI 5.9 - 27.1%, P = 0.05. CD8+: 16.5; 95% CI 3.4 – 29.5%, P = 0.05) (Friedman paired analysis incorporating a Dunn correction for multiple testing). Similarly, in both the CD4+ and CD8+ compartments, total numbers of CD45RA-CXCR5+ cells fell significantly at TP3 relative to baseline (CD4+: 20.7%; 95% CI 0.6 – 40.7%, P = 0.035. CD8+: 24.5; 95% CI 3.9 – 45.2%, P = 0.03).There was no corresponding change in the same populations in the non-vaccinated group at these same time points. The total CD19+ population also remained static across the time course. There was no significant increase in the gross population counts at TP2 within the CD4+ or CD8+ compartments.

Despite the absence of gross increases in the total CD4+ population, notable changes in CD4+ sub-populations were recognisable following vaccination - Figure 33, Table 14. Analysis looked at sub-populations of CD4+ cells expressing either ICOS, PD1, or both markers (Figure 33); no significant increase in CD45RA+ cells expressing these markers was seen following vaccination. At TP2 a significant increase in CD4+CD45RO+ICOS+PD1- (mean increase = 132%; 95% CI 67 – 197%, P = <0.0001) and CD4+CD45RO+ICOS+PD1+ (246%; 95% CI 135 – 359%, P = 0.0001) was seen, with no detectable change in CD4+CD45RO+PD1+ICOS- cells (49%; 95% CI -14 – 111%, P = 0.56) and no similar increases in the non-vaccinated control patients. When considering only CD45RO+CXCR5+ CD4+ cells a similar pattern was seen however the amplitude of the response at TP2 was much greater, with a 654% increase in said cells with a ICOS+PD1- phenotype (95% CI 156 – 1153%; P = 0.0004) and an 848% (95% CI 350 – 1347%; P = 0.0043) increase in cells expressing a full T_{FH} phenotype (i.e. CD4+CD45RO+CXCR5+ICOS+PD1+). No concomitant significant increase in CD45RO+CXCR5+PD1+ICOS- was apparent (181%; 95% CI -67 – 428%, P = 0.14) and no change in unvaccinated controls was seen. Furthermore the responses in patients within both vaccinated arms were almost identical (Figure 33), demonstrating consistency across the treatment groups.

At TP2 there was also a notable increase in CD19+CXCR5- cells (mean increase = 138%; 95% CI 65 – 211%, P = <0.001) in the absence of any similar change in unvaccinated controls - Figure 32. This reflected the underlying increases in plasmablast and plasma cell numbers occurring at TP2 in the absence of any detectable changes in the number of circulating naïve or memory B-cell populations - Figure 34, Table 14. Switched plasmablast numbers increased by a mean of 546% (95% CI 113 – 980%, P = <0.0001), switched plasma cells increased by 871% (95% CI 103 – 1640%, P = 0.007) and unswitched plasmablasts increased by 317% (95% CI 53 – 582%, P = 0.007).

Figure 35.a. is an overlay plot of the B-cell populations within representative patient before (blue) and one week following (grey) vaccination. The $CD38^{Hi}$ plasmablast and plasma cell populations are clearly present at the later time point however they are poorly represented in the circulation of the individual at baseline. Although more subtle, the same principle can be used to illustrate the changing CD4+ populations (Figure 35.b.); here numbers of CD4+CD45RO+CXCR5+ cells expressing ICOS and/or PD1 can be seen to alter following vaccination. For clarity the same data has been presented without an overlay; Figure 35.c. shows the CD4+CD45RO+CXCR5+ population prior to vaccination and Figure 35.d. shows the same population at TP2. The increased representation of circulating T_{FH} phenotype cells following vaccination is clearly appreciable.

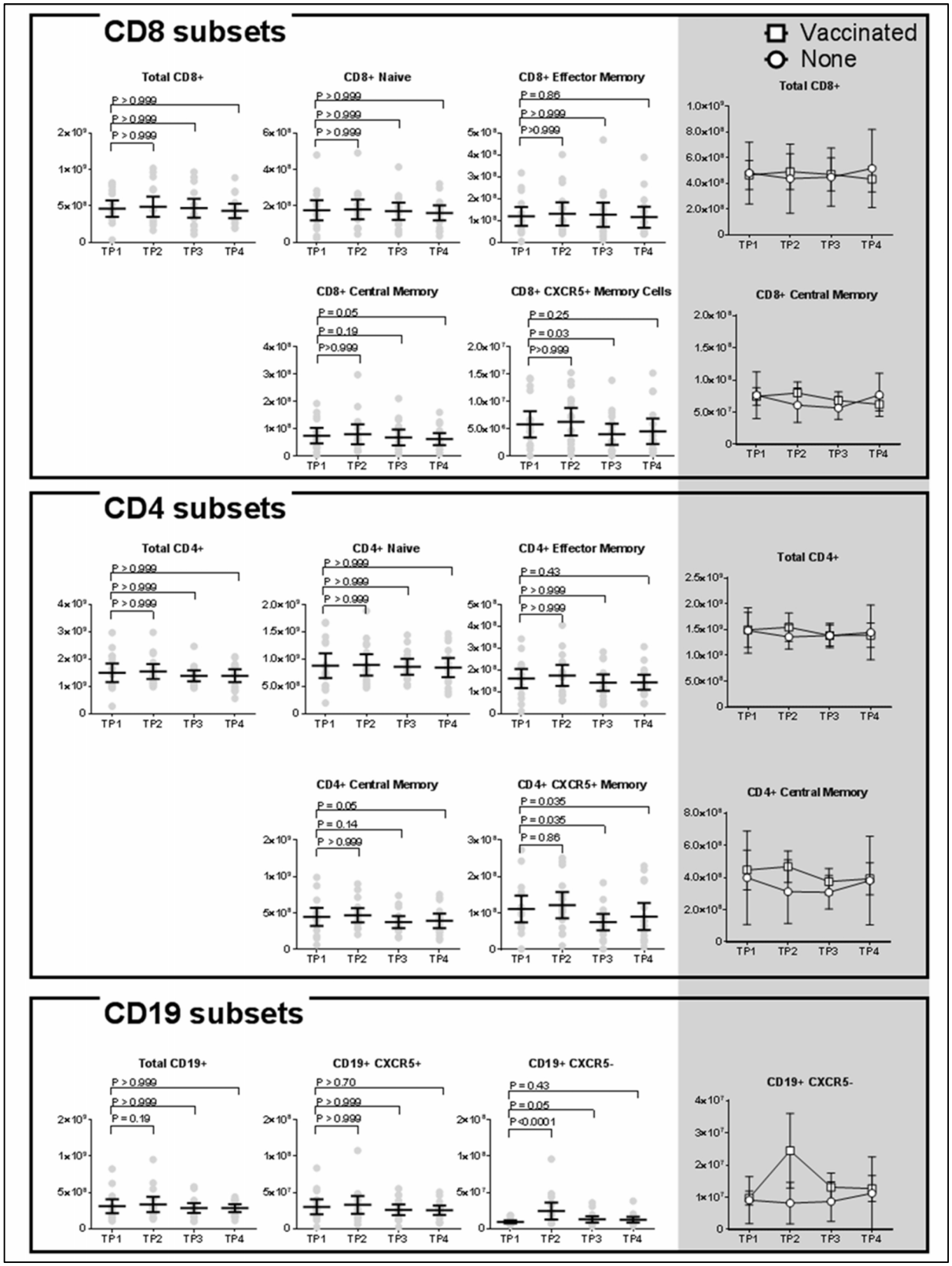


Figure 32**: Change in absolute numbers of circulating lymphocytes over the vaccine time course. Y-axis scale expressed in number of cells per litre of blood. Significance of change relative to baseline calculated through paired analysis utilising Friedman's test with a Dunns multiple comparison correction. White area: graphs detailing data from vaccinated patients with mean number plotted with error bars denoting 95% Confidence Intervals. Grey area: comparison between dynamics seen in key populations within vaccinated patients plotted alongside unvaccinated controls.**

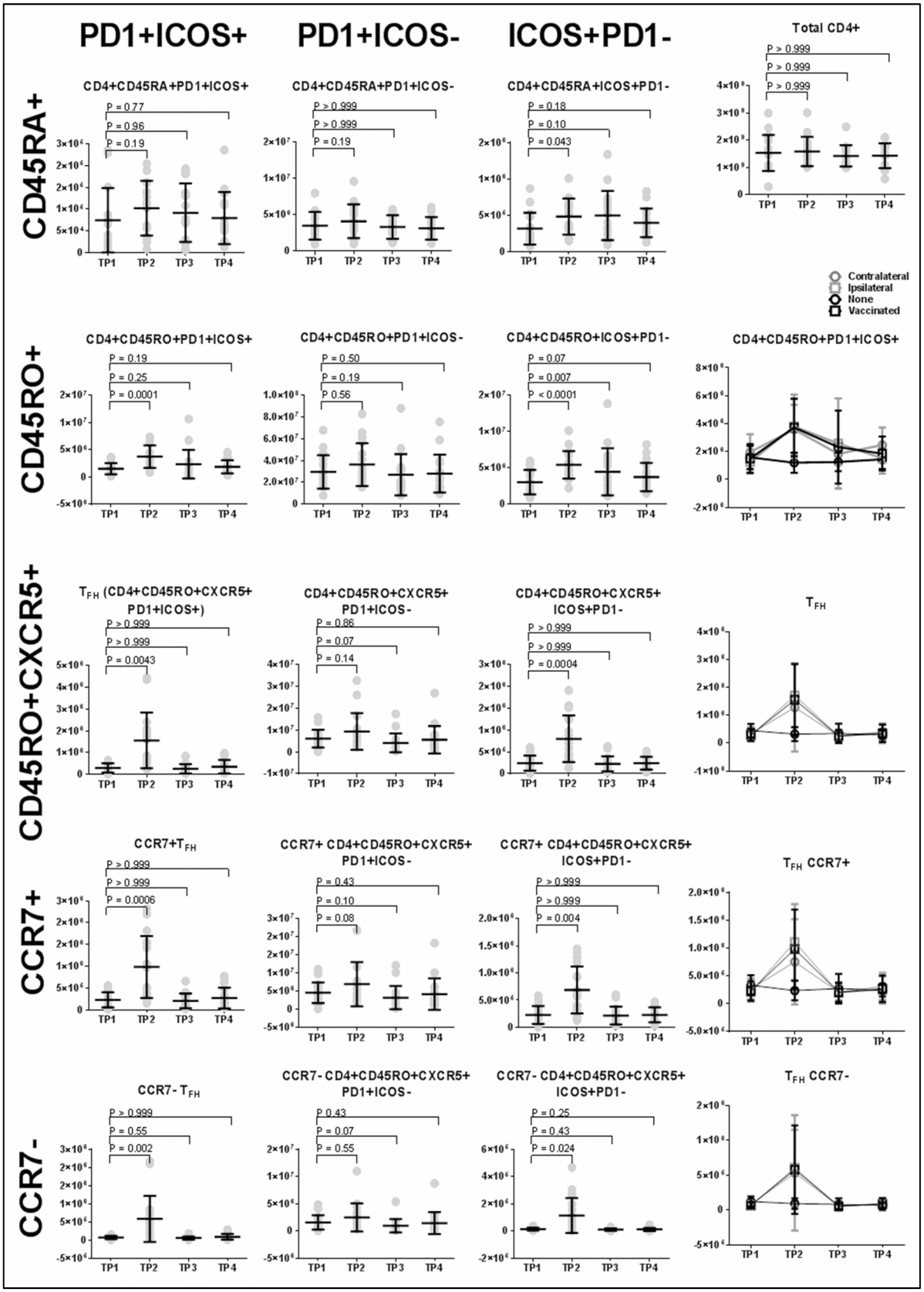


Figure 33**: Change in absolute numbers of sub-populations of circulating CD4+ lymphocytes (expressed as cells/litre of blood – Y-axis) over the vaccine time course. Significance of change relative to baseline calculated through paired analysis utilising Friedman's test with a Dunns multiple comparison correction. Graphs detail data from vaccinated patients with mean number plotted with error bars denoting 95% Confidence Intervals. Top far right-column demonstrates no change in total CD4+ cells. Remaining plots within far right column detail comparison between vaccinated patients from each group (ipsilateral, contralateral) plotted alongside pooled data as well as unvaccinated controls.**

Cell Population		Vaccinated				No Vaccine			
		TP1	TP2	TP3	TP4	TP1	TP2	TP3	TP4
CD19+	Mean	311.3	336.0	285.8	285.5	311.9	277.2	278.9	317.1
	SD	185.1	206.0	132.0	106.0	220.3	152.9	153.9	197.5
	95% CI	216.1 - 406.5	230.1 - 441.9	217.9 - 353.6	231.0 - 340.0	38.3 - 585.4	87.4 - 467.0	87.8 - 470.1	71.9 - 562.3
	% Increase		13.6	-1.9	0.5		0.7	2.1	4.3
	95% CI of % increase		-3.8 - 31.1	-12.9 - 9.1	-10.0 - 11.0		-19.6 - 21.1	-16.2 - 20.3	-11.7 - 20.4
Unswitched Naïve	Mean	166.8	169.8	154.9	165.2	68.2	60.8	70.1	74.7
	SD	103.8	98.1	87.3	86.6	44.9	31.8	53.9	63.2
	95% CI	113.4 - 220.1	119.4 - 220.3	110.0 - 199.8	120.7 - 209.7	12.5 - 123.9	21.3 - 100.3	3.2 - 137.1	-3.8 - 153.2
	% Increase		9.4	-2.6	3.7		-0.5	7.4	5.8
	95% CI of % increase		-7.6 - 26.4	-14.8 - 9.5	-8.6 - 16.1		-19.8 - 18.8	-12.4 - 27.2	-15.1 - 26.6
Unswitched Memory	Mean	32.4	35.4	29.9	26.8	48.2	47.3	42.3	49.5
	SD	32.6	36.1	23.4	18.6	36.3	33.9	26.2	36.3
	95% CI	15.6 - 49.2	16.9 - 54.0	17.9 - 42.0	17.3 - 36.4	3.2 - 93.3	5.2 - 89.3	9.8 - 74.8	4.4 - 94.6
	% Increase		8.1	-0.4	-4.7		7.3	4.4	3.3
	95% CI of % increase		-9.9 - 26.1	-14.0 - 13.1	-19.1 - 9.7		-14.8 - 29.5	-18.2 - 26.9	-6.5 - 13.1
Activated Naïve	Mean	22.4	22.7	18.0	18.2	114.9	99.0	96.3	118.2
	SD	26.4	23.0	14.9	14.4	126.2	91.5	95.8	113.3
	95% CI	8.8 - 36.0	10.9 - 34.5	10.3 - 25.7	10.8 - 25.6	-41.8 - 271.6	-14.7 - 212.6	-22.6 - 215.2	-22.6 - 258.9
	% Increase		20.3	-2.4	1.8		-1.5	-6.1	6.9
	95% CI of % increase		-3.2 - 43.8	-19.1 - 14.4	-19.2 - 22.8		-18.9 - 15.9	-20.7 - 8.5	-26.5 - 40.4
Unswitched Blasts	Mean	0.336	1.024	0.360	0.468	0.331	0.299	0.325	0.491
	SD	0.188	1.040	0.236	0.364	0.177	0.228	0.291	0.307
	95% CI	0.239 - 0.432	0.489 - 1.559	0.239 - 0.482	0.281 - 0.656	0.111 - 0.551	0.016 - 0.581	-0.036 - 0.686	0.110 - 0.872
	% Increase		**317.0**	21.7	64.3		-13.5	-12.0	39.2
	95% CI of % increase		**52.5 - 581.6**	-14.5 - 57.9	-13.3 - 141.8		-44.2 - 17.2	-53.0 - 29.1	0.0 - 78.4
Switched Memory	Mean	70.8	78.4	63.3	56.5	69.8	61.0	60.3	64.6
	SD	96.8	114.1	71.3	51.7	59.8	41.7	45.4	51.5
	95% CI	21.0 - 120.6	19.8 - 137.1	26.7 - 99.9	29.9 - 83.1	-4.5 - 144.0	9.2 - 112.7	4.0 - 116.7	0.7 - 128.5
	% Increase		17.1	-1.7	-0.9		4.1	2.7	-3.7
	95% CI of % increase		-15.1 - 49.4	-14.4 - 11.1	-20.6 - 18.9		-21.1 - 29.3	-25.5 - 30.9	-11.4 - 4.1
Switched Plasmablasts	Mean	3.167	12.990	4.406	4.492	2.153	1.635	1.667	2.201
	SD	2.355	12.910	4.027	4.058	0.961	0.634	0.460	1.274
	95% CI	1.956 - 4.378	6.353 - 19.620	2.335 - 6.476	2.406 - 6.579	0.960 - 3.347	0.847 - 2.423	1.097 - 2.238	0.619 - 3.783
	% Increase		**546.3**	74.5	100.3		-18.1	33.1	-4.4
	95% CI of % increase		**112.5 - 980.1**	-38.5 - 187.4	-37.0 - 237.6		-39.1 - 2.8	-76.6 - 142.9	-28.5 - 19.8
Switched Plasmacells	Mean	0.058	0.458	0.065	0.061	0.021	0.036	0.011	0.015
	SD	0.054	0.637	0.069	0.086	0.020	0.048	0.008	0.021
	95% CI	0.030 - 0.086	0.130 - 0.785	0.030 - 0.101	0.017 - 0.106	-0.003 - 0.046	-0.024 - 0.096	0.001 - 0.021	-0.011 - 0.042
	% Increase		**871.3**	8.3	-12.7		43.1	60.1	20.8
	95% CI of % increase		**102.8 - 1639.9**	-28.5 - 45.0	-38.1 - 12.7		-28.6 - 114.7	-112.8 - 233.1	-124.3 - 166.0
Total CD8+	Mean	464.8	490.7	469.1	432.1	506.1	470.3	479.1	515.2
	SD	218.6	269.5	250.5	192.2	246.0	271.0	224.4	244.0
	95% CI	352.4 - 577.2	352.2 - 629.3	340.3 - 597.9	333.4 - 530.9	200.7 - 811.6	133.8 - 133.8	200.5 - 757.8	212.2 - 818.2
	% Increase		23.9	12.5	22.7		-11.4	-5.0	2.8
	95% CI of % increase		-24.9 - 72.7	-18.3 - 43.4	-42.1 - 87.5		-36.4 - 13.5	-29.6 - 19.5	-14.4 - 19.9
CD8+ Naive	Mean	176.6	180.8	171.1	161.6	144.3	144.9	143.5	141.4
	SD	107.3	104.8	92.1	79.9	65.8	70.3	71.8	51.7
	95% CI	121.5 - 231.8	127.0 - 234.7	123.7 - 218.4	120.6 - 202.7	62.6 - 226.1	57.7 - 232.2	54.4 - 232.7	77.1 - 205.6
	% Increase		16.9	9.1	9.2		-1.8	-2.3	5.1
	95% CI of % increase		-14.8 - 48.7	-12.1 - 30.4	-23.8 - 42.2		-23.5 - 20.0	-34.4 - 29.8	-19.8 - 29.9
CD8+ Central Memory	Mean	74.5	79.8	68.1	62.1	85.5	68.8	63.3	76.9
	SD	55.7	71.2	56.1	42.8	96.1	70.0	45.0	75.1
	95% CI	45.9 - 103.1	43.2 - 116.3	39.3 - 96.9	40.1 - 84.1	-33.8 - 204.8	-18.1 - 155.8	7.5 - 119.1	-16.4 - 170.1
	% Increase		5.5	-14.6	**-16.5**		-13.8	-1.1	-3.1
	95% CI of % increase		-11.2 - 22.2	-28.3 - -0.9	**-29.5 - -3.4**		-36.4 - 8.8	-40.2 - 37.9	-37.1 - 31.0
CD8+ Effector Memory	Mean	118.8	130.3	126.5	115.0	84.4	66.9	72.9	85.2
	SD	84.7	103.8	108.5	94.5	33.7	47.2	36.4	13.9
	95% CI	75.3 - 162.4	77.0 - 183.6	70.7 - 182.3	66.4 - 163.6	49.0 - 119.7	17.4 - 116.5	34.8 - 111.1	68.0 - 102.4
	% Increase		51.4	31.2	74.8		-27.5	-14.5	-10.0
	95% CI of % increase		-44.3 - 147.0	-27.8 - 90.2	-99.0 - 248.7		-59.9 - 5.0	-43.1 - 14.0	-25.4 - 5.3
CD8+ CXCR5+ Memory	Mean	5.832	6.311	4.020	4.554	2.748	2.154	2.083	2.257
	SD	4.693	4.895	3.738	4.509	1.129	1.774	1.566	1.780
	95% CI	3.419 - 8.244	3.794 - 8.828	2.098 - 5.941	2.235 - 6.872	1.345 - 4.150	-0.049 - 4.357	0.138 - 4.027	0.047 - 4.468
	% Increase		27.9	**-24.5**	-2.9		-33.4	-31.5	-17.1
	95% CI of % increase		-12.6 - 68.3	**-45.2 - -3.9**	-50.9 - 45.0		-67.8 - 1.0	-61.2 - -1.7	-62.6 - 28.3
Total CD4+	Mean	1496.0	1547.0	1385.0	1391.0	1482.0	1323.0	1361.0	1445.0
	SD	660.6	534.4	394.6	459.5	469.9	235.1	244.5	427.6
	95% CI	1156.0 - 1836.0	1272.0 - 1821.0	1182.0 - 1588.0	1154.0 - 1627.0	898.2 - 2065.0	1031.0 - 1615.0	1057.0 - 1665.0	914.4 - 1976.0
	% Increase		24.5	9.7	15.2		-4.8	-1.0	0.0
	95% CI of % increase		-23.6 - 72.5	-22.1 - 41.5	-37.1 - 67.5		-24.2 - 14.6	-29.2 - 27.1	-26.9 - 26.9
CD4+ Naïve	Mean	882.1	897.8	862.1	849.0	886.0	854.2	906.1	930.5
	SD	441.1	380.9	278.3	338.6	242.7	207.5	245.2	240.9
	95% CI	655.3 - 1109.0	702.0 - 1094.0	719.0 - 1005.0	674.9 - 1023.0	584.6 - 1187.0	596.6 - 1112.0	601.7 - 1211.0	631.3 - 1230.0
	% Increase		19.2	14.1	15.9		0.7	5.3	8.1
	95% CI of % increase		-22.7 - 61.2	-13.9 - 42.1	-26.9 - 58.7		-19.0 - 20.5	-20.3 - 30.8	-21.4 - 37.5
CD4+ Central Memory	Mean	447.0	467.7	375.3	392.9	450.5	354.3	345.7	381.2
	SD	239.1	189.8	158.0	193.3	275.3	175.5	47.0	222.2
	95% CI	324.1 - 569.9	370.2 - 565.3	294.0 - 456.6	293.5 - 492.3	108.7 - 792.3	136.4 - 572.2	287.3 - 404.1	105.4 - 657.0
	% Increase		10.0	-12.2	**-16.5**		-18.1	-5.6	-11.9
	95% CI of % increase		-13.6 - 33.7	-26.5 - 2.1	**-27.1 - -5.9**		-39.8 - 3.5	-42.4 - 31.2	-40.0 - 16.2
CD4+ Effector Memory	Mean	162.1	176.2	143.2	144.5	141.4	110.1	106.1	129.3
	SD	85.1	93.7	72.8	67.7	45.0	36.4	34.3	57.5
	95% CI	118.3 - 205.8	128.0 - 224.4	105.8 - 180.7	109.7 - 179.3	85.5 - 197.4	65.0 - 155.3	63.5 - 148.7	58.0 - 200.7
	% Increase		37.5	12.3	31.9		-23.5	-19.8	-9.4
	95% CI of % increase		-20.1 - 95.1	-29.7 - 54.3	-56.0 - 119.9		-43.1 - -4.0	-42.3 - 2.7	-34.4 - 15.6
CD4+ CXCR5+ Memory	Mean	111.1	121.6	75.0	90.5	90.8	77.1	66.2	75.0
	SD	71.2	70.5	44.1	72.4	45.7	33.4	26.9	39.5
	95% CI	74.5 - 147.6	85.3 - 157.8	52.3 - 97.7	53.2 - 127.7	34.1 - 147.5	35.6 - 118.6	32.9 - 99.6	25.9 - 124.1
	% Increase		60.5	**-20.7**	6.6		-16.5	-19.2	-8.0
	95% CI of % increase		-28.3 - 149.3	**-40.7 - -0.6**	-57.0 - 70.1		-43.0 - 10.0	-56.2 - 17.8	-51.6 - 35.5
TFH	Mean	0.297	1.562	0.259	0.356	0.459	0.358	0.382	0.308
	SD	0.212	1.281	0.210	0.312	0.275	0.265	0.382	0.148
	95% CI	0.187 - 0.406	0.904 - 2.221	0.151 - 0.367	0.195 - 0.516	0.118 - 0.800	0.028 - 0.687	-0.092 - 0.856	0.124 - 0.491
	% Increase		**848.3**	12.4	49.7		-24.8	-25.0	-20.0
	95% CI of % increase		**349.7 - 1346.9**	-33.4 - 58.3	-15.6 - 114.9		-52.3 - 2.6	-66.8 - 16.7	-60.8 - 20.8
CD4+ CD45RO+CXCR5+PD1+ICOS-	Mean	6.071	9.396	4.151	5.604	5.771	4.401	3.817	4.130
	SD	4.033	8.433	4.309	6.271	1.851	1.945	1.441	1.331
	95% CI	3.998 - 8.145	5.060 - 13.730	1.936 - 6.367	2.379 - 8.828	3.472 - 8.069	1.986 - 6.816	2.027 - 5.606	2.477 - 5.783
	% Increase		180.9	-18.6	70.0		-29.0	-28.0	-15.8
	95% CI of % increase		-66.6 - 428.4	-54.0 - 16.7	-108.4 - 248.3		-61.6 - 3.6	-67.7 - 11.8	-73.5 - 41.9
CD4+ CD45RO+CXCR5+ICOS+PD1-	Mean	0.240	0.801	0.226	0.240	0.489	0.420	0.365	0.364
	SD	0.173	0.534	0.171	0.146	0.275	0.314	0.293	0.058
	95% CI	0.151 - 0.329	0.526 - 1.076	0.139 - 0.314	0.166 - 0.315	0.147 - 0.830	0.030 - 0.811	0.002 - 0.729	0.291 - 0.436
	% Increase		**654.3**	58.0	75.0		-26.4	-13.9	-3.4
	95% CI of % increase		**155.9 - 1152.7**	-46.4 - 162.4	-51.7 - 201.8		-49.8 - -3.1	-59.7 - 31.9	-51.0 - 44.1

Table 14: Change in lymphocyte populations during time course. Mean absolute numbers (with Standard deviation and 95% Confidence Intervals) given for each population at each time point, with percentage increase and 95% CI for increase. Significant change as identified through paired analysis utilising Friedman's test with a Dunns multiple comparison correction highlighted in blue.

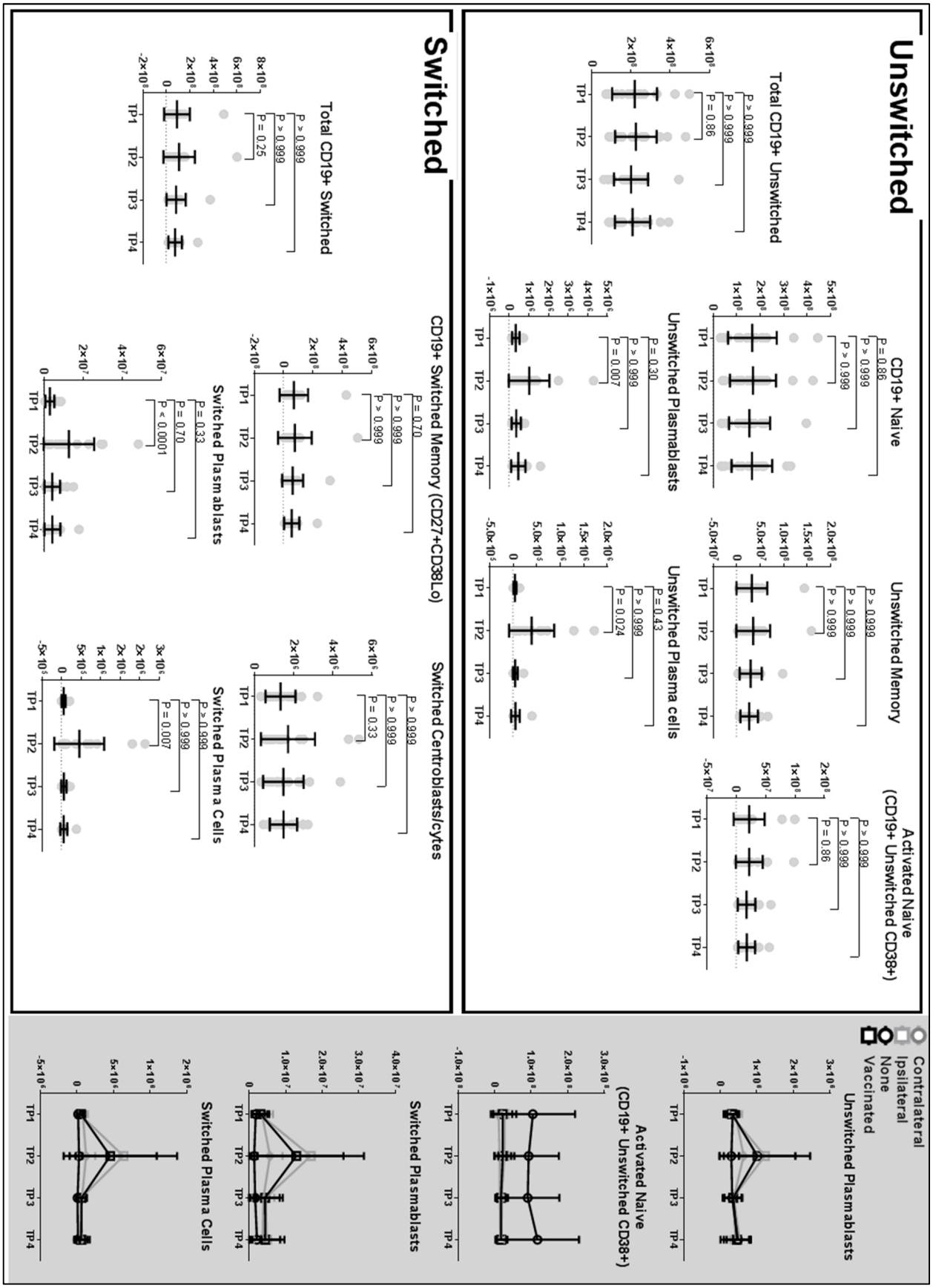


Figure 34**: Change in absolute numbers of sub-populations of circulating CD19+ lymphocytes over the vaccine time course. Significance of change relative to baseline calculated through paired analysis utilising Friedman's test with a Dunns multiple comparison correction. Graphs detail data from vaccinated patients with mean number plotted with error bars denoting 95% Confidence Intervals. Grey area: comparison between dynamics seen in key populations within the individual groups who received a vaccine plotted alongside unvaccinated controls.**

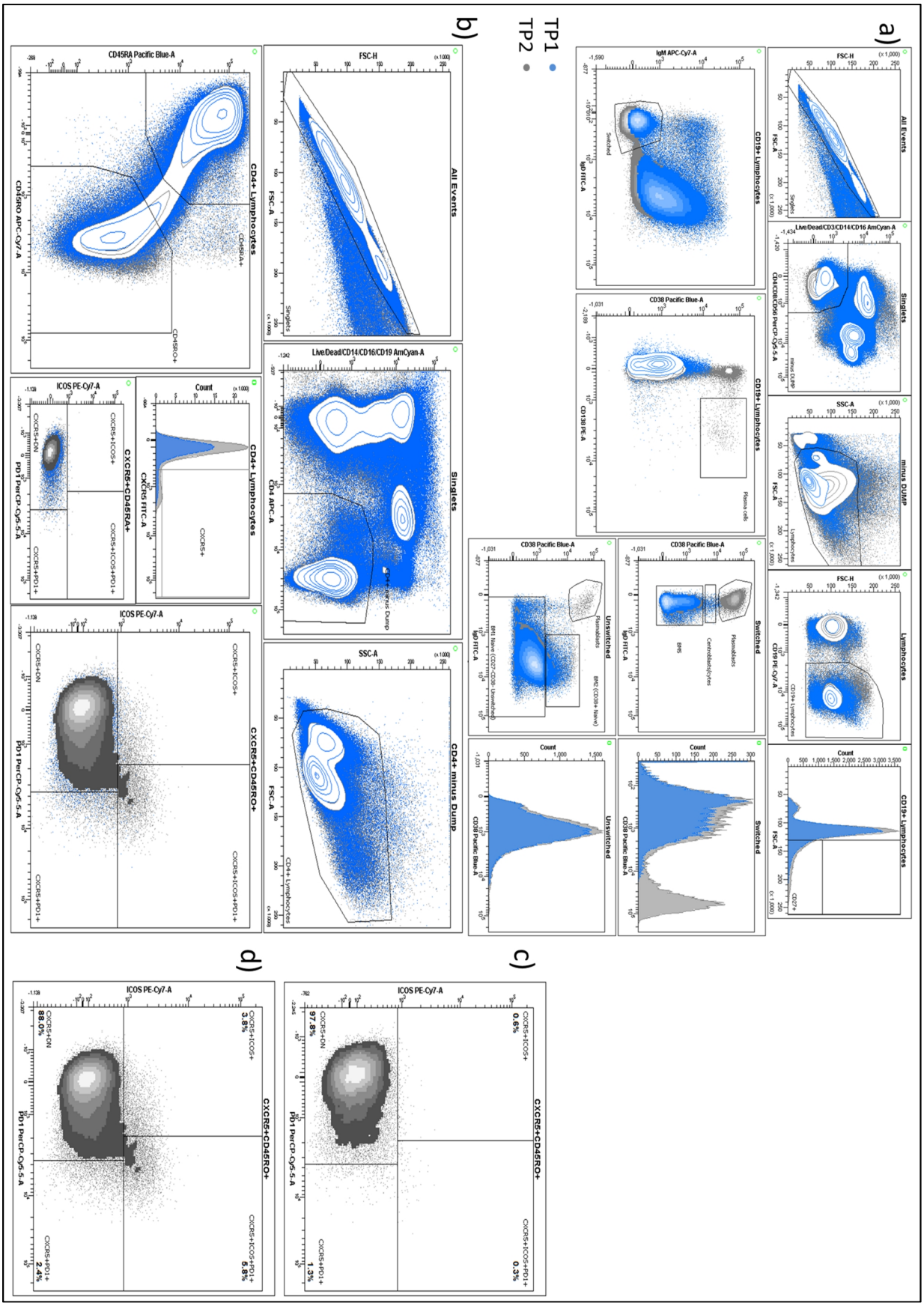


Figure 35**: example flow cytometry plot taken from a vaccinated patient. a-b) overlay plots for a) CD19+ and b) CD4+ lymphocytes at time point 1 (blue dots) and time point 2 (grey dots) demonstrating the change in profiles between the two time points. c-d) contour plot detailing ICOS and PD1 expression on CD4+CD45RO+CXCR5+ cells at c) TP1 and d) TP2 following vaccination. Note the increased prominence of the T_{FH} (ICOS+PD1+) cells at TP2.**

8.3 CCR7 expression on circulating CD4+CD45RO+CXCR5+ cells following vaccination

CCR7 is a chemokine receptor mediating chemotaxis to T-cell zones within lymphatic tissue. As such its expression of the surface of CD4+ cells is used to identify subtypes of CD4+ cells which migrate through lymph nodes, i.e. naïve and central memory cells. T_{FH} cells express CXCR5 and down-regulate CCR7 which results in their movement, in lymph nodes, from the paracortex to cortex, to allow interaction with B-cell counterparts during the response to vaccination. Therefore lack of CCR7 expression has been held as a hallmark of T_{FH} phenotype.

The role of CCR7 expression on the surface of the circulating counterparts of follicular-derived cells is unclear – see Section 1.3.2 and Section 1.3.4. Therefore dynamic change in CCR7 expression within the CD4+CD45RO+CXCR5+ compartment following vaccination was considered in more depth. Figure 36.a. details histogram plots of CCR7 expression on Total CD4+ cells, as well as CCR7 expression on T_{FH} (CD4+CD45RO+CXCR5+ICOS+PD1+), CD4+CD45RO+CXCR5+ICOS+PD1- and CD4+CD45RO+CXCR5+PD1+ICOS- cells, at baseline (blue) and one week following vaccination (grey). As per findings detailed in Section 8.2, the number of T_{FH} and ICOS+PD1- cells increase relative to baseline following vaccination whereas the PD1+ICOS- cell numbers remain static. Within the two dynamic populations both CCR7+ and CCR7- are seen to increase significantly (see Figure 33) (T_{FH}: CCR7+ mean increase: = 772%; 95% CI 294 – 1250%, P = 0.0006. CCR7- : 1587%; 95% CI 378 – 2795%, P = 0.002. ICOS+PD1-: CCR7+: = 605%; 95% CI 134 – 1077%, P = 0.004. CCR7- : 1085%; 95% CI 407 – 1763%, P = 0.024) whereas both CCR7+ and CCR7- PD1+ICOS- cell populations remained unaltered by vaccination. .

Although both CCR7+ and CCR7- cells within both populations increase, the increase in CCR7- cells was more marked with a resulting significant change in the composition of both populations - Figure 36.b. At baseline 73% (SD 12.5%; 95% CI 66.7 – 79.6%) of circulating "T_{FH}" cells were CCR7+ which decreased to 66% (SD 11.7%; 95% CI 60.4 – 72.4%) one week following vaccination (P = 0.01). 94% (SD 4.2%; 95% CI 91.6 – 95.8%) of ICOS+PD1- cells were CCR7+ prior to vaccination, which fell to 88% (SD 8.0%; 95% CI 84.1 – 92.3%) by TP2 (P = 0.007). There was no significant change in the PD1+ICOS- population composition, and no change in unvaccinated controls - Figure 36.b.

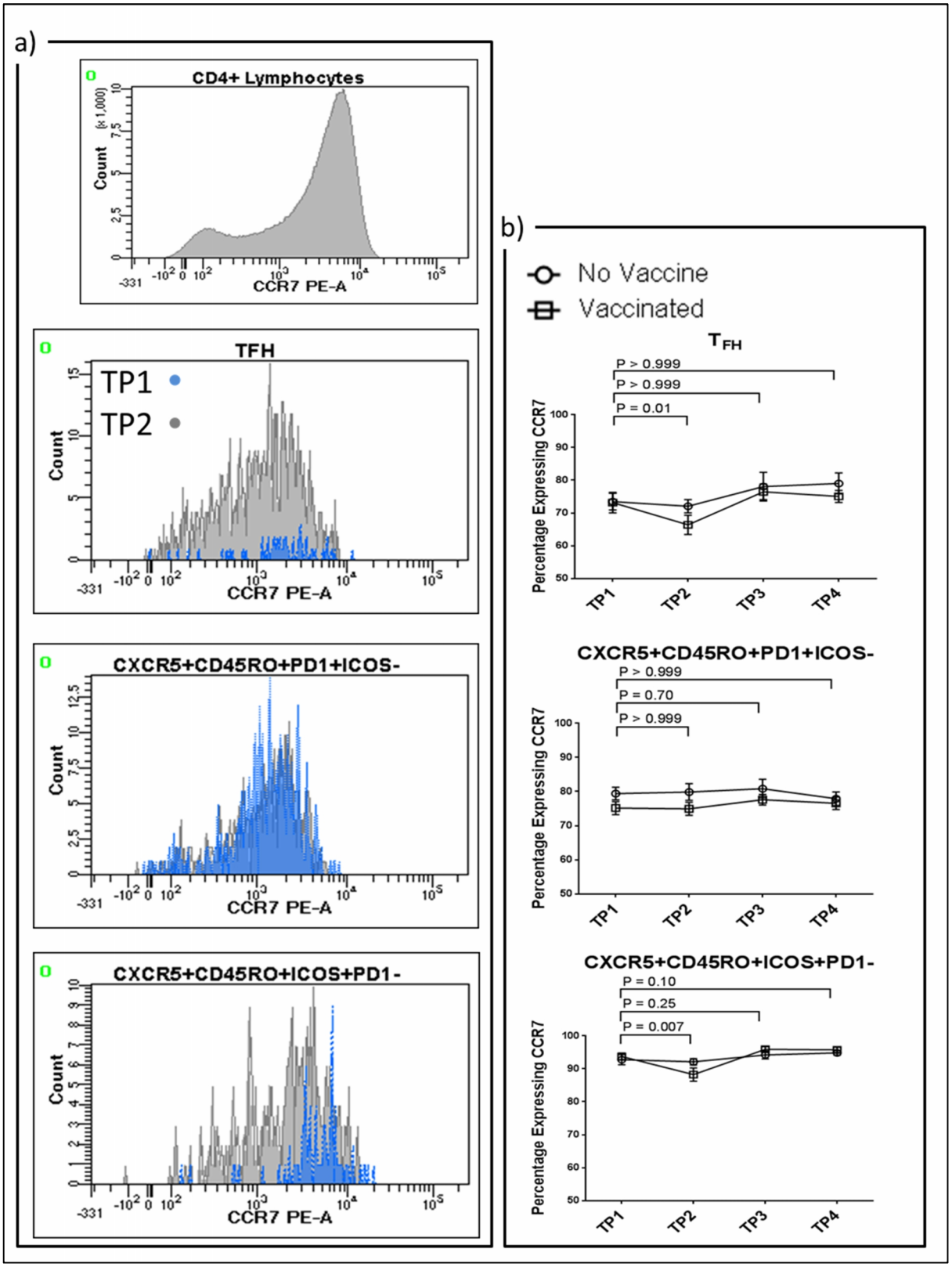


Figure 36: CCR7 expression on three sub-populations of CD4+CD45RO+CXCR5+ cells defined according to their expression of ICOS and PD1. a) histogram overlay plots from each population demonstrating change in population frequency and CCR7 expression at TP1 (blue) and TP2 (grey). b) Significance of change of the percentage of each subpopulation expressing CCR7 throughout the time course. Significance of change relative to baseline calculated through paired analysis utilising Friedman's test with a Dunns multiple comparison correction.

8.4 Correlation between B- and T-cell components of the vaccine response suggests differences in the type or the stage of cellular response to vaccination between patients

To determine whether the responses of the CD4+ and CD19+ cellular compartments were independent or interlinked correlation analysis was performed - Figure 37. Expectation was that there would be a tight correlation between dynamic components within a compartment however cross-compartment correlation would be poor. However contrary to this supposition no correlation was seen between the percentage increases in T_{FH} (CD4+CD45RO+CXCR5+ICOS+PD1+) and their CD45RO+CXCR5+ICOS+PD1- ("ICOS+PD1-") counterparts (Spearman r = 0.37; 95% CI -0.15 – 0.73. P = 0.14) - Figure 37.a. Furthermore no correlation was seen between increases in plasma cells and switched plasmablasts, nor between switched plasmablasts and unswitched plasmablasts. There was, however, a correlation between increase in plasma cells and unswitched plasmablasts (r = 0.64; 95% CI 0.12 – 0.87. P = 0.009).

There existed a strong correlation between separate components of the CD4+ compartment and the CD19+ compartment. Increase in T_{FH} cells was linked with increases in both unswitched plasmablasts (r = 0.79; 95% CI 0.51 – 0.90. P < 0.0001) and plasma cells (r = 0.57; 95% CI 0.17 – 0.81. P = 0.007), but not switched plasmablasts (r = 0.21; 95% CI -0.31 – 0.64. P = 0.42) - Figure 37.b. Conversely increase in ICOS+PD1- correlated strongly with switched plasmablasts (r = 0.90; 95% CI 0.74 – 0.97. P < 0.0001) but not with increases in unswitched plasmablasts or plasma cells - Figure 37.c.

It remained unclear whether these two different cellular responses (ICOS+PD1-/Switched plasmablasts ("type 1") and T_{FH}/Unswitched plasmablasts ("type 2")) represented different types of responses or whether they reflected a different time point in similar responses. Therefore the serum antibody responses of patients responding in the different ways were compared. The two responses occurred on a spectrum; to maximise the possibility of demonstrating a difference, patients with the most extreme responses were compared. Two patients had a very "type 1" response (patients 005 and 007), two patients had a marked "type 2" response (patients 012 and 022) and two patients had a mixed response (patients 014 and 037). The time between vaccination and TP2 in the 6 patients was between 6-7 days in all cases, without any preponderance to an earlier timescale in any one of the three groups.

The serum immunoglobulin responses to vaccination of these 6 patients is illustrated in Figure 37.d.; the two patients undergoing a "type 1" response (i.e. concomitant increase in circulating CD4+CD45RO+CXCR5+ICOS+PD1- and Switched plasmablasts) appear to have a lower amplitude response to vaccination at TP2, TP3 and TP4 compared to the other patients. Those

patients undergoing a “type 2” response (i.e. concomitant increase in T_{FH} and unswitched plasmablasts and plasma cells) appear to have an earlier response to vaccination, and to respond with greater magnitude.

To assess this observation further correlation analysis was performed looking at possible associations between magnitude of increase of individual cell types at TP2 with either peak serum vaccine-specific IgG increase (Figure 38.a.) or serum IgG increase at TP2 (Figure 38.b.); greater increases in ICOS+PD1- or switched plasmablasts (i.e. a “type 1” response) were negatively correlated with peak serum IgG increase. This implies that the significance of the “type 1” observation is not confined to the single time point at which the observation is made, and does effectively predict the ongoing nature of the vaccine response. This observation would suggest that those patients with greater increases in CD4+CD45RO+CXCR5+ICOS+PD1- and Switched plasmablasts at TP2 were likely to have a lower amplitude peak response to vaccination. However the converse cannot be held for patients with a more pronounced increase in T_{FH} and/or unswitched plasmablasts (“type 2”), as said increases poorly correlated with measured peak immunoglobulin response.

Increase in circulating T_{FH} cells at TP2 did, however, weakly correlate with increase in serum vaccine-specific IgG at the same time point ($r = 0.52$; 95% CI 0.03 – 0.80. $P = 0.03$) - Figure 38.b. However the same could not be demonstrated for unswitched plasmablasts. Therefore patients who demonstrate a marked increase in circulating T_{FH} cells at TP2 tend to also generate an earlier serum immunoglobulin response following vaccination.

These observations suggest that circulating CD4+CD45RO+CXCR5+ICOS+PD1- and T_{FH} (CD4+CD45RO+CXCR5+ICOS+PD1+) cells emerge at different times during the response, and may carry out different functions according to the type of response induced by vaccination. This demonstrates that despite the heterogeneity of individual serum responses to vaccination, broad trends can still be identified.

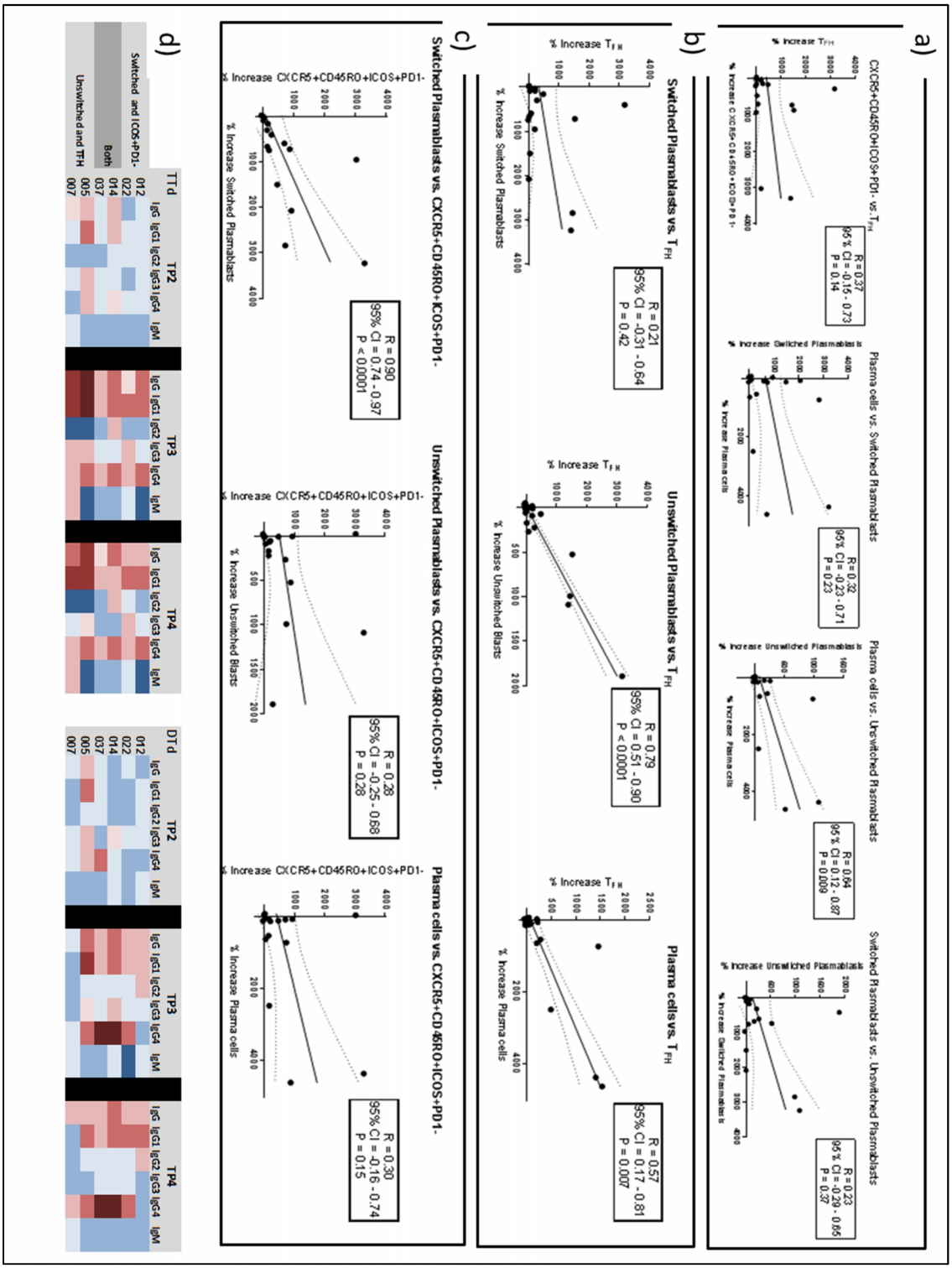


Figure 37: Correlation between change in circulating cell populations at Time Point 2. For a-c) Spearman correlation performed with plots showing line of best fit with 95% Confidence intervals. a) plots comparing cells from within same compartments (far left: T_{FH} vs CD45RO+CXCR5+ICOS+PD1-, left: plasma cells vs switched plasmablasts, right: plasma cells vs unswitched blasts, far right: switched plasmablasts vs unswitched plasmablasts. b) Correlation between CD19+ subsets and T_{FH} cell increase. c) Correlation between CD19+ subsets and CD45RO+CXCR5+ICOS+PD1- cell increase. d) Up-regulation matrix detailing response to TTd (left-hand block), DTd (right-hand block) following vaccination. Each block is subdivided into response at Time Point 2 (TP2), TP3 and TP4, and within each sub-block each isotype of antibody (IgG (Total IgG), IgG1-4, IgM) is detailed by a single column. Up-regulation is represented relative to baseline levels according to the colour-gradient key (top right). Each row represents response within an individual patient. 6 patients have been detailed according to the type of cellular response to vaccine (CD45RO+CXCR5+ICOS+PD1- and switched plasmablast upregulation (patients 012 and 022), T_{FH} and unswitched plasmablast upregulation (patients 005 and 007) or a combined response (patients 014 and 037).

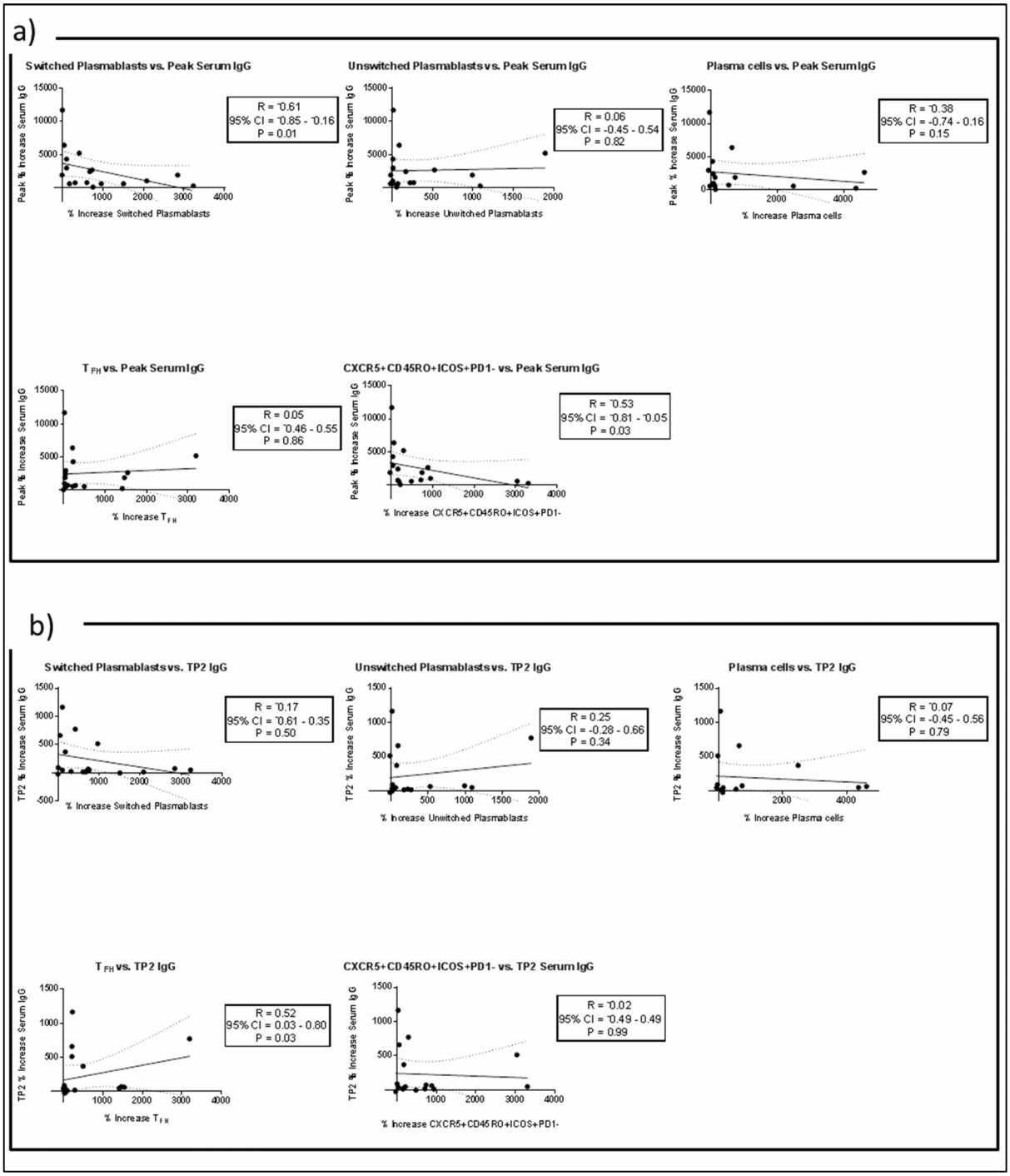


Figure 38: Correlation between change in circulating cell populations at Time Point 2 and immunoglobulin response. Spearman correlation performed with plots showing line of best fit with 95% Confidence intervals. a) Correlation between cell changes and peak IgG response. b) Correlation between cell changes and IgG response at time point 2.

9 Detailing lymphocyte populations isolated from nodal tissue

9.1 Differences in lymphocyte populations between peripheral blood and lymph node

Comparison was made between lymphocytes derived from the peripheral blood and those from lymph nodes following generation of a single cell suspension (see Section 5.1). The same three phenotyping panels were applied to both sets of samples (see Figure 29) however, as alluded to previously, absolute numbers within the blood and tissue could not be compared, therefore percentage composition of lymphocyte populations within each compartment was compared instead. In an attempt to ensure comparability, values for blood were taken at time point 1 (i.e. at rest) and values from node populations were derived from the contralateral and no vaccine control patients, to avoid comparing reactive tissue with "resting" blood.

As expected, differences were demonstrable in the populations existent within the circulation, and within the tissue, with some notable exceptions - Figure 39, Table 15. (all statistics quoted derived using Student T-test with Holm-Šidák correction for multiple comparisons (α = 5.0%), without equivalent standard deviation being assumed)

CD19+ cells comprised a greater proportion of lymphocytes within the lymph node (LN) than in the peripheral blood (PB) (mean percentage: LN = 23.4%; 95% CI 19.0 – 27.9, PB = 12.6%; 95% CI 9.9 – 15.4, P < 0.001). Conversely CD8+ T-cells made a smaller contribution to the total lymph node lymphocyte population (LN = 8.0%; 95% CI 5.9 – 10.0, PB = 19.4%; 95% CI 16.3 – 22.5, P < 0.001). The percentage of total lymphocytes which were CD4+ was equivalent between the two compartments (LN = 66.3%; 95% CI 61.6 – 70.9, PB = 61.7%; 95% CI 58.0 – 65.4, P 0.78).

Differences in terms of the constitutional make-up of the CD19+ and CD4+ populations were also apparent. For CD19+ lymphocytes naïve and IgM memory cells made similar contributions to total CD19+ numbers in the blood and in the tissue. Activated naïve B-cells (BM2) and both switched and unswitched plasmablasts were all more prominent within the blood (see Table 15). Plasma cells were rare in both blood and node. Switched memory B-cells (LN = 36.4%; 95% CI 31.0 – 41.7, PB = 20.0%; 95% CI 15.2 – 24.8, P < 0.001) and Centroblasts/cytes (LN = 0.86%; 95% CI 0.55 – 1.18, PB = 0.46%; 95% CI 0.38 – 0.54, P 0.02) were more frequent within the CD19+ populations derived from nodal tissue.

No differences could be demonstrated between the proportional make-up of the CD4+ populations within the peripheral blood and lymph node tissue in terms of the relative contribution of naïve, central memory and effector memory subsets to total CD4+ lymphocytes. However there was a significantly greater proportion of CD4+ cells expressing CXCR5 within

the lymph nodes (LN = 19.4%; 95% CI 16.8 – 21.9, PB = 9.5%; 95% CI 7.8 – 11.2, P < 0.001). This difference was not confined to any one subset; 7.2% of naïve CD4+ cells expressed CXCR5 within nodal tissue compared with 4.8% within the peripheral blood (P = 0.02). Similarly a significantly greater proportion of central memory CD4+ cells (LN = 38.3%, PB = 23.2%; P < 0.001) and, most markedly, effector memory cells (LN = 25.1, PB = 1.6%; P < 0.001) were CXCR5+ in the nodes than in the blood.

As detailed in Section 8, three follicular-associated CD4+CD45RO+CXCR5+ populations are of contemporary interest (ICOS+PD1+ (T_{FH}), ICOS+PD1-, PD1+ICOS-), two of which were seen to increase in number within the peripheral blood following vaccination. All three of these populations, as expected, comprised a greater proportion of the CD4+ population within lymphatic tissue than in the peripheral blood, however they remained rare in both compartments. T_{FH} cells comprised 0.56% of the CD4+ population within the lymph node (95% CI 0.40 – 0.72) compared with 0.02% (95% CI 0.02-0.03%) within the blood (P < 0.0001). CD4+CD45RO+CXCR5+ICOS+PD1- cells comprised 0.19% (95% CI 0.12 – 0.25) of lymph node CD4+ cells compared with 0.02% (95% CI 0.01 – 0.03) of circulating CD4+ cells (P < 0.0001), and CD4+CD45RO+CXCR5+ PD1+ ICOS- cells comprised 1.69% (95% CI 1.31 – 2.07) of lymph node CD4+ cells compared with 0.41% (95% CI 0.31 – 0.50) of circulating CD4+ cells (P < 0.0001).

As well as the numerical differences in these populations, the surface expression of CCR7 was also markedly different between tissue resident cells and their circulating counterparts - Figure 39.f., Table 16. 73.3% (95% CI 68.4 – 78.1%) of circulating T_{FH} cells, 76.3% (95% CI 73.2 – 79.3%) of CD4+CD45RO+CXCR5+ PD1+ ICOS- cells and 93.5% (95% CI 91.7 – 95.2%) of CD4+CD45RO+CXCR5+ICOS+PD1- cells express CCR7. Within the lymph node 14.5 % (95% CI 10.3 – 18.8%) of T_{FH} cells (P < 0.0001), 33.8% (95% CI 28.8 – 38.7%) of CD4+CD45RO+CXCR5+ PD1+ ICOS- cells (P < 0.0001) and 59.7% (95% CI 53.9 – 65.4%) of CD4+CD45RO+CXCR5+ICOS+PD1- cells (P< 0.0001) express CCR7.

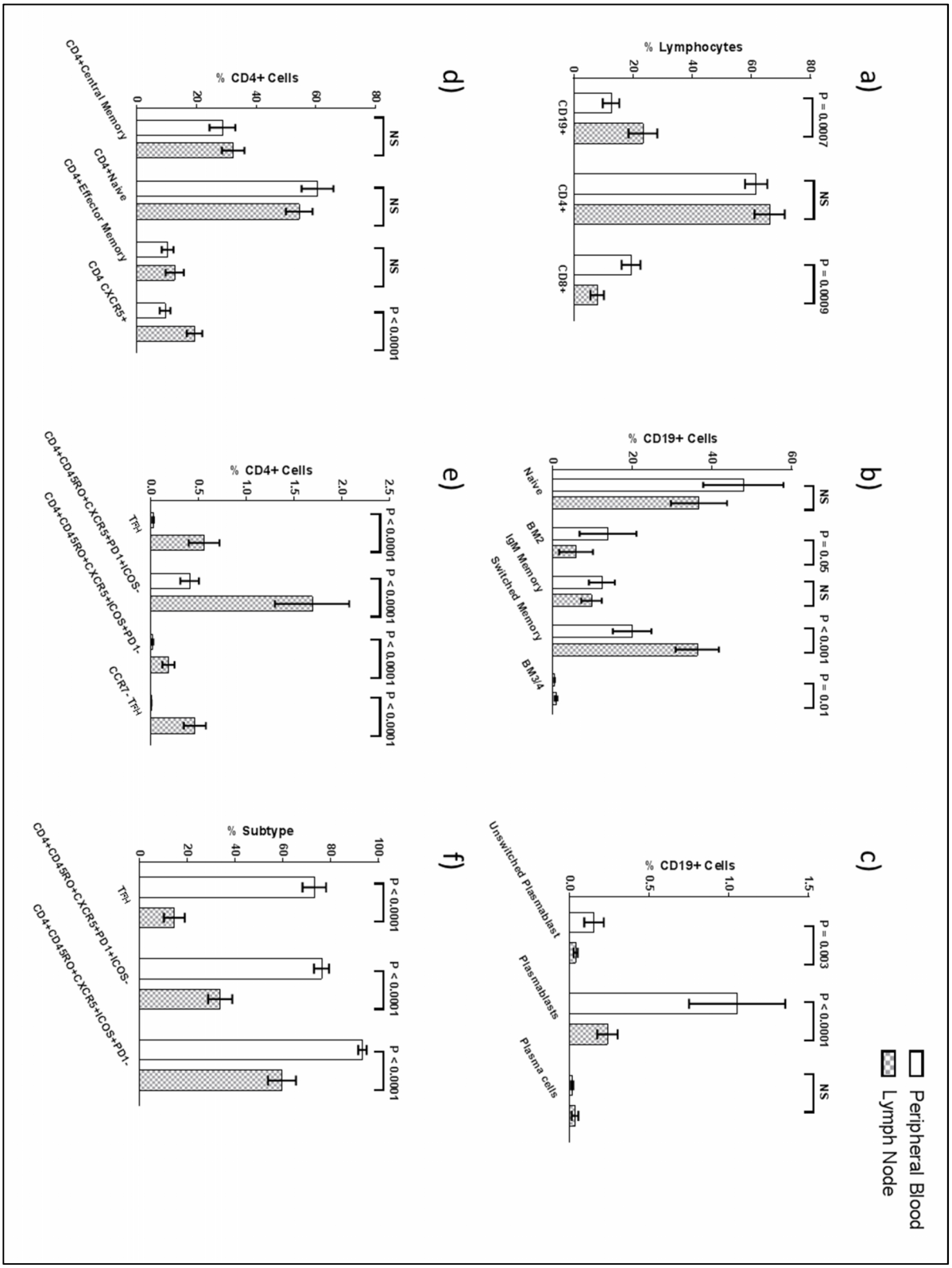


Figure 39: Comparing the proportional representation of individual lymphocyte sub-populations within peripheral blood and lymph node. Bar charts plotting mean values with error bars representing 95% confidence interval. a) gross CD19+, CD4+ and CD8+ subsets; plot describes the percentage of total lymphocytes made up by each phenotype. b-c) CD19+ subsets described as % of total CD19+ lymphocytes. d-e) CD4+ subsets described as % of total CD4+ lymphocytes. f) CCR7 expression on three CD4+CD45RO+CXCR5+ subsets demonstrating a greater level of expression of circulating cells compared with their tissue-derived counterparts. Data generated from blood at time point 1, and from lymph nodes taken from the contralateral and unvaccinated control patients. P-values generated by Student t-test with Holm-Šidák correction for multiple comparisons (alpha set to 5.0%) without assuming equivalent standard deviation between the two compartments.

		Blood	Node	Difference	SE of Difference	P-Value
CD19+	Mean	12.6	23.4			
	SD	6.3	4.2	-14.95	2.00	<0.001
	95% CI	9.9 - 15.4	19.0 - 27.9			
CD4+	Mean	61.7	66.3			
	SD	8.5	4.4	0.72	2.56	0.78
	95% CI	58.0 - 65.4	61.6 - 70.9			
CD8+	Mean	19.4	8.0			
	SD	7.2	2.0	10.47	1.67	<0.001
	95% CI	16.3 - 22.5	5.9 - 10.0			
B-lymphocyte sub-types as % Total 19+ cells						
Naive	Mean	47.9	36.8			
	SD	22.8	16.0	11.16	5.94	0.07
	95% CI	38.1 - 57.8	29.9 - 43.7			
BM2	Mean	13.9	5.9			
	SD	16.2	9.6	8.03	4.01	0.05
	95% CI	6.9 - 20.9	1.7 - 10.0			
IgM Memory	Mean	12.4	9.8			
	SD	7.3	5.8	2.59	2.00	0.20
	95% CI	9.2 - 15.6	7.3 - 12.3			
Unswitched Plasmablast	Mean	0.16	0.04			
	SD	0.14	0.03	0.12	0.03	0.0004
	95% CI	0.10 - 0.22	0.03 - 0.05			
Switched Memory	Mean	20.0	36.4			
	SD	11.0	12.3	-16.37	3.53	<0.001
	95% CI	15.2 - 24.8	31.0 - 41.7			
BM3/4	Mean	0.46	0.86			
	SD	0.19	0.74	-0.40	0.16	0.02
	95% CI	0.38 - 0.54	0.55 - 1.18			
Switched Plasmablasts	Mean	1.05	0.24			
	SD	0.68	0.14	0.81	0.15	<0.001
	95% CI	0.76 - 1.35	0.18 - 0.30			
Plasma cells	Mean	0.02	0.04			
	SD	0.02	0.05	-0.02	0.01	0.10
	95% CI	0.01 - 0.02	0.01 - 0.06			
CD4+ lymphocyte sub-types as % Total CD4+ cells						
CD4+ Central Memory	Mean	28.7	32.4			
	SD	9.8	8.6	-3.62	2.77	0.20
	95% CI	24.5 - 33.0	28.6 - 36.1			
CD4+ Naïve	Mean	60.6	54.5			
	SD	12.1	10.1	6.12	3.36	0.08
	95% CI	55.4 - 58.9	50.1 - 58.9			
CD4+ Effector Memory	Mean	10.3	12.7			
	SD	4.5	3.0	-2.39	1.75	0.18
	95% CI	8.4 - 12.3	9.7 - 15.7			
CD4+ CXCR5+	Mean	9.5	19.4			
	SD	4.0	5.9	-9.89	1.51	<0.001
	95% CI	7.8 - 11.2	16.8 - 21.9			
TFH	Mean	0.02	0.56			
	SD	0.02	0.36	-0.54	0.08	<0.001
	95% CI	0.02 - 0.03	0.40 - 0.72			
CD4+CD45RO+CXCR5+PD1+ICOS-	Mean	0.41	1.69			
	SD	0.22	0.88	-1.28	0.19	<0.001
	95% CI	0.31 - 0.50	1.31 - 2.07			
CD4+CD45RO+CXCR5+ICOS+PD1-	Mean	0.02	0.19			
	SD	0.02	0.14	-0.17	0.03	<0.001
	95% CI	0.01 - 0.03	0.12 - 0.25			

Table 15: Comparing the proportional representation of individual lymphocyte sub-populations within peripheral blood and lymph node. For gross CD19+, CD4+ and CD8+ subsets, figures given relate to the percentage total number of lymphocytes. CD19+ and CD4+ subsets are described according to the % total of each subset. Data generated from blood at time point 1, and from lymph nodes taken from the contralateral and unvaccinated control patients. P-values generated by Student t-test with Holm-Šidák correction for multiple comparisons (alpha set to 5.0%) without assuming equivalent standard deviation between the two compartments.

		Blood	Node	Difference	SE of Difference	P-Value
%CD4+ cells = CCR7- TFH	Mean	0.01	0.46			
	SD	0.00	0.26	-0.46	0.06	<0.001
	95% CI	0.00 - 0.01	0.35 - 0.58			
%TFH = CCR7+	Mean	73.3	14.5			
	SD	11.2	9.8	58.74	3.17	<0.001
	95% CI	68.4 - 78.1	10.3 - 18.8			
% CD4+CD45RO+CXCR5+PD1+ICOS- = CCR7+	Mean	76.3	33.8			
	SD	7.1	11.5	42.50	2.89	<0.001
	95% CI	73.2 - 79.3	28.8 - 38.7			
% CD4+CD45RO+CXCR5+ICOS+PD1- = CCR7+	Mean	93.5	59.7			
	SD	4.0	13.3	33.77	2.96	<0.001
	95% CI	91.7 - 95.2	53.9 - 65.4			

Table 16: Comparing CCR7 expression on three CD4+CD45RO+CXCR5+ subsets within peripheral blood and lymph node. Data generated from blood at time point 1, and from lymph nodes taken from the contralateral and unvaccinated control patients. P-values generated by Student t-test with Holm-Šidák correction for multiple comparisons (alpha set to 5.0%) without assuming equivalent standard deviation between the two compartments.

9.2 Comparison of lymphocyte sub-populations within lymph nodes taken from the ipsilateral, contralateral and unvaccinated patients

Lymphocyte population composition within the lymph nodes from each of the study cohorts were compared to identify any gross differences between populations within nodes taken from the vaccinated side, compared with those from nodes which are not thought to have been directly exposed to the vaccine (i.e. from the two control arms). Statistical analysis was performed utilising non-parametric Kruskal-Wallis test with a Dunn's correction for multiple comparisons.

Figure 40 compares the population constitution between the three cohorts. There was no statistical difference between any of the groups for any of the populations analysed with the exception of CD4+ lymphocyte size. When gated as either "small" or "large", a greater proportion of small lymphocytes were seen in the Contralateral (59.3%; 95% CI 50.1 – 68.5%) and No vaccine groups (45.7%; 95% CI 24.2 – 67.2) compared with the ipsilateral group (27.5; 95% CI 22.1 – 32.9) (P = 0.001).

Possible differences between the ipsilateral cohort and the two control groups were also observed in the percentage of CD4+ cells which were T_{FH} phenotype (Ipsilateral: 0.74; 95% CI 0.43 – 1.05. Contralateral: 0.37; 95% CI 0.23 0.51. No vaccine: 0.42; 95% 0.26 – 0.58) and CD4+CD45RO+CXCR5+ICOS+PD1- (Ipsilateral: 0.24; 95% CI 0.11 – 0.37. Contralateral: 0.10; 95% CI 0.05 0.16. No vaccine: 0.17; 95% 0.10 – 0.25) however these were not statistically significant. Therefore an alternative strategy was required to look for population differences between the cohorts.

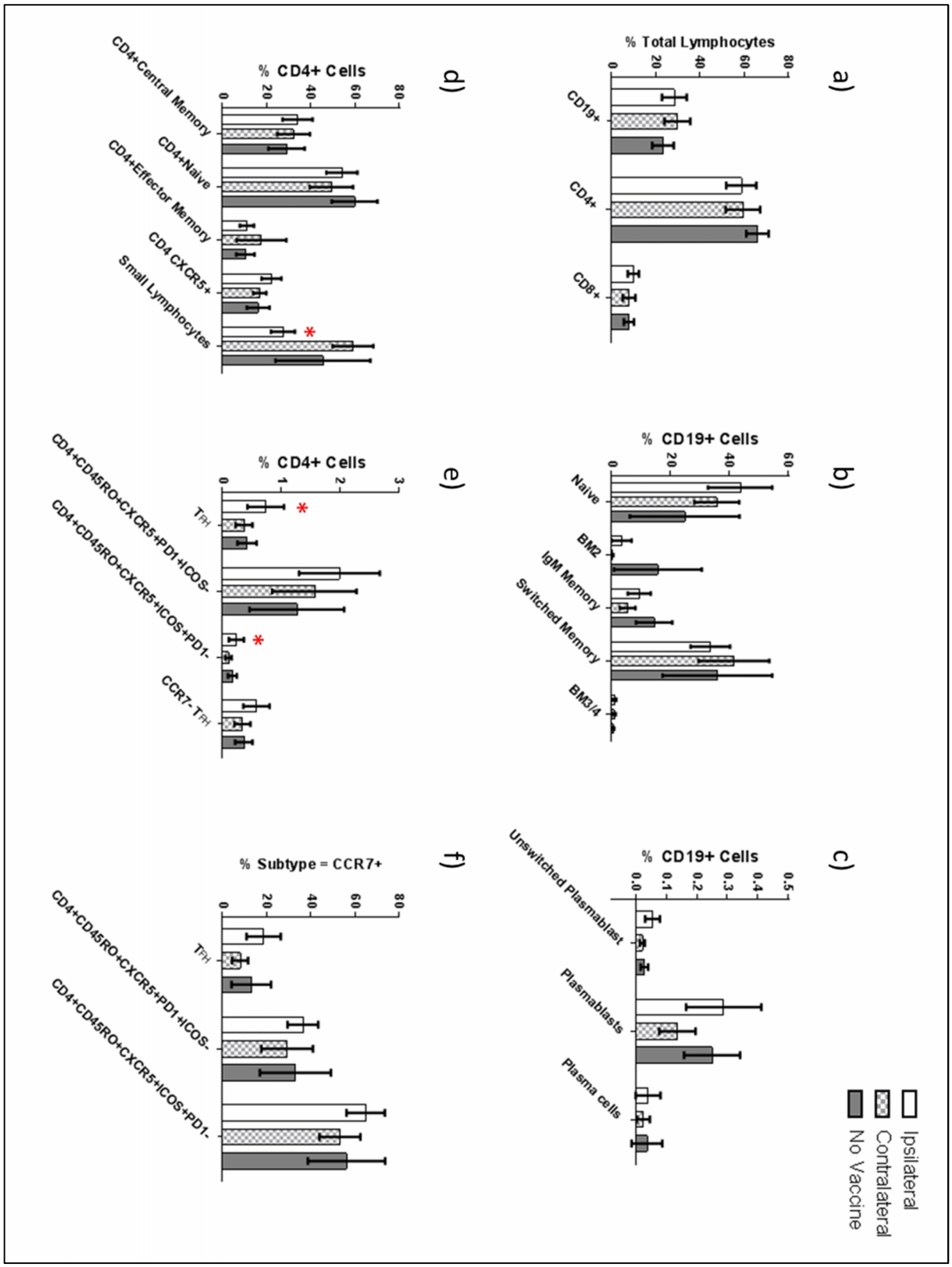


Figure 40: Population differences within the lymph nodes between study cohorts. Bar charts plotting mean values with error bars representing 95% confidence interval. a) gross CD19+, CD4+ and CD8+ subsets; plot describes the percentage of total lymphocytes made up by each phenotype. b-c) CD19+ subsets described as % of total CD19+ lymphocytes. d-e) CD4+ subsets described as % of total CD4+ lymphocytes. f) CCR7 expression on three CD4+CD45RO+CXCR5+ subsets. * denotes possible differences between the cohorts.

9.3 Surface activation marker expression does not allow delineation between ipsilateral and control nodes

CD154 and CD137 are markers of recent activation of CD4+ cells – see Section 5.4. Levels of expression on $T_{FH+,}$ CD4+CD45RO+CXCR5+ICOS+PD1- and CD4+CD45RO+CXCR5+PD1+ICOS- cells, as well as total CD4+ cells, was assessed at baseline - Figure 41.a. Surface expression of CD154 was low in all four cell types, although expression was highest in T_{FH} cells, being present on the surface of 0.60% of said cells (95% CI 0.42 – 0.77). Although more frequently expressed than CD154, CD137 expression was still infrequent, being present on 1.58 % (95% CI 0.84 – 2.32) of T_{FH} cells. Equivalent expression was seen on CD4+CD45RO+CXCR5+ICOS+PD1- cells (2.9%; 95% CI 1.7 – 4.2%), and both subsets had significantly greater expression of surface CD137 than CD4+CD45RO+CXCR5+PD1+ICOS- cells (0.35%; 95% CI 0.21 – 0.50%. $P < 0.01$) and total CD4+ cells (0.27%; 0.15 – 0.38%. $P < 0.001$).

Intracellular CD154 expression was, by far, the most abundant marker at baseline. 39.8% (95% CI 31.8 – 47.9%) of T_{FH} cells expressed intracellular CD154. T_{FH} expression of intracellular CD154 was significantly greater than levels seen in CD4+CD45RO+CXCR5+ICOS+PD1- cells (15.0%; 95% CI 9.0 – 21.0%. $P = 0.02$), CD4+CD45RO+CXCR5+PD1+ICOS- cells (15.4%; 95% CI 8.4 – 22.3%. $P = 0.02$) and CD4+ cells (3.6%; 95% CI 2.4 – 4.9%. $P < 0.001$).

Comparison of the expression of these markers was therefore performed to assess whether any differences between the study cohorts existed (Figure 41.b. and Figure 42.a-b). No significant differences between any of the cohorts for any of the markers on any of the cell subtypes could be identified.

Antigen stimulation during a 6 hour culture was attempted to delineate the different cohorts. Such techniques have been previously used within our laboratory to quantify numbers of antigen-specific CD4+ cells within peripheral blood samples. Figure 41.c-e. and Figure 42.c-d detail the results for intracellular CD154 expression, and surface expression of CD154 and CD137 respectively. In summary, co-culture with vaccine-derived antigen did not induce expression over and above baseline values for intracellular or surface CD154, and induced changes were no different from those demonstrable using a control antigen (PPD) in all cases. Furthermore, no combination of antigen, or activation marker, could delineate between patient cohorts. This strategy was therefore abandoned.

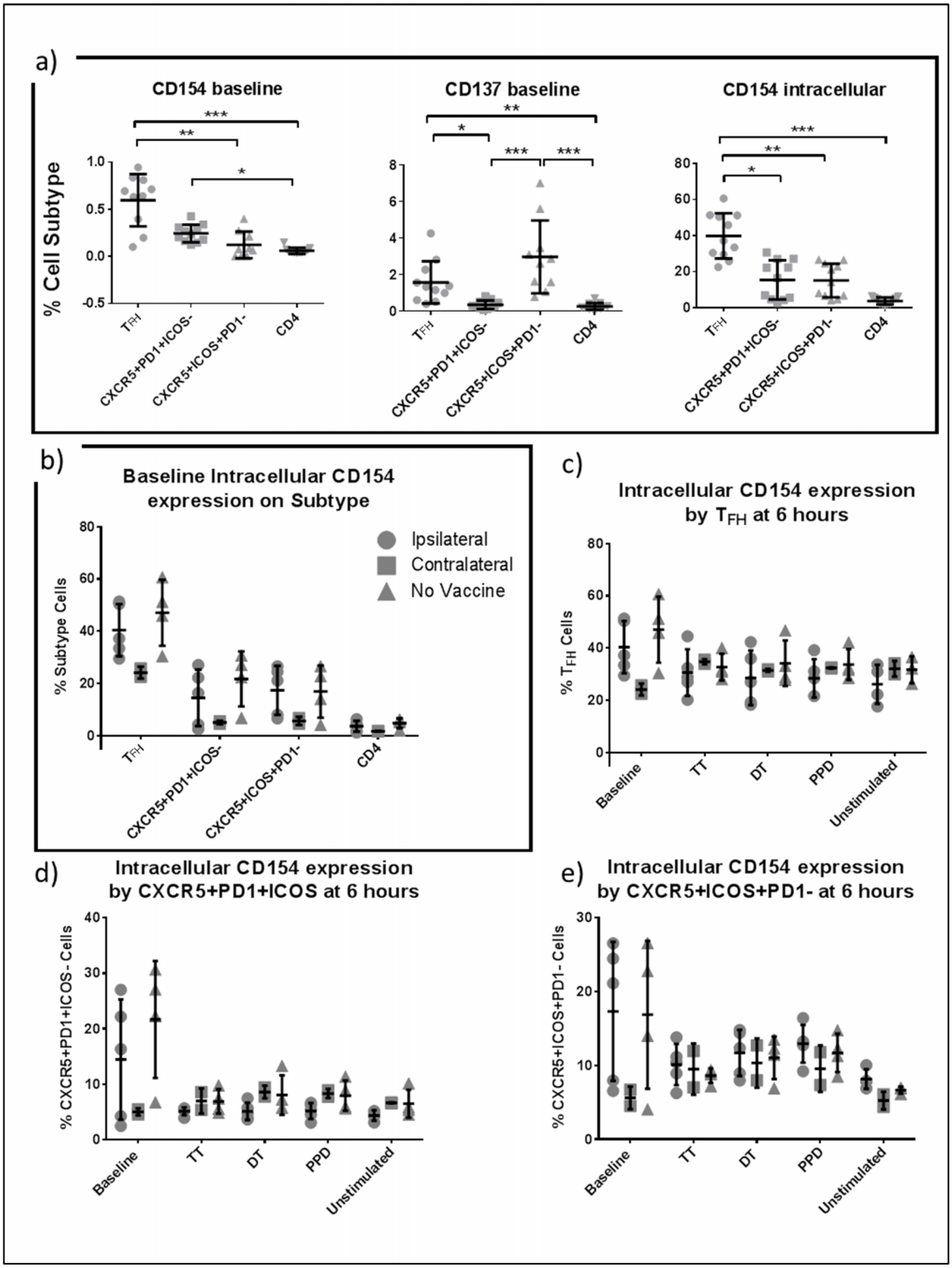


Figure 41: Expression of markers of activation within nodal-derived CD4+CD45RO+CXCR5+ lymphocytes. a) surface expression of CD154 and CD137, and intracellular expression of CD154 at baseline within each cell subtype. Significance calculated using Kruskal-Wallis test with Dunn's correction for multiple comparisons . b) Intracellular expression of CD154 according to study cohort at baseline. c-e) Effect of co-culture with antigen on the intracellular expression of CD154 within each cell subtype. Plots detail individual values with mean and 95% confidence intervals represented by overlaid lines.

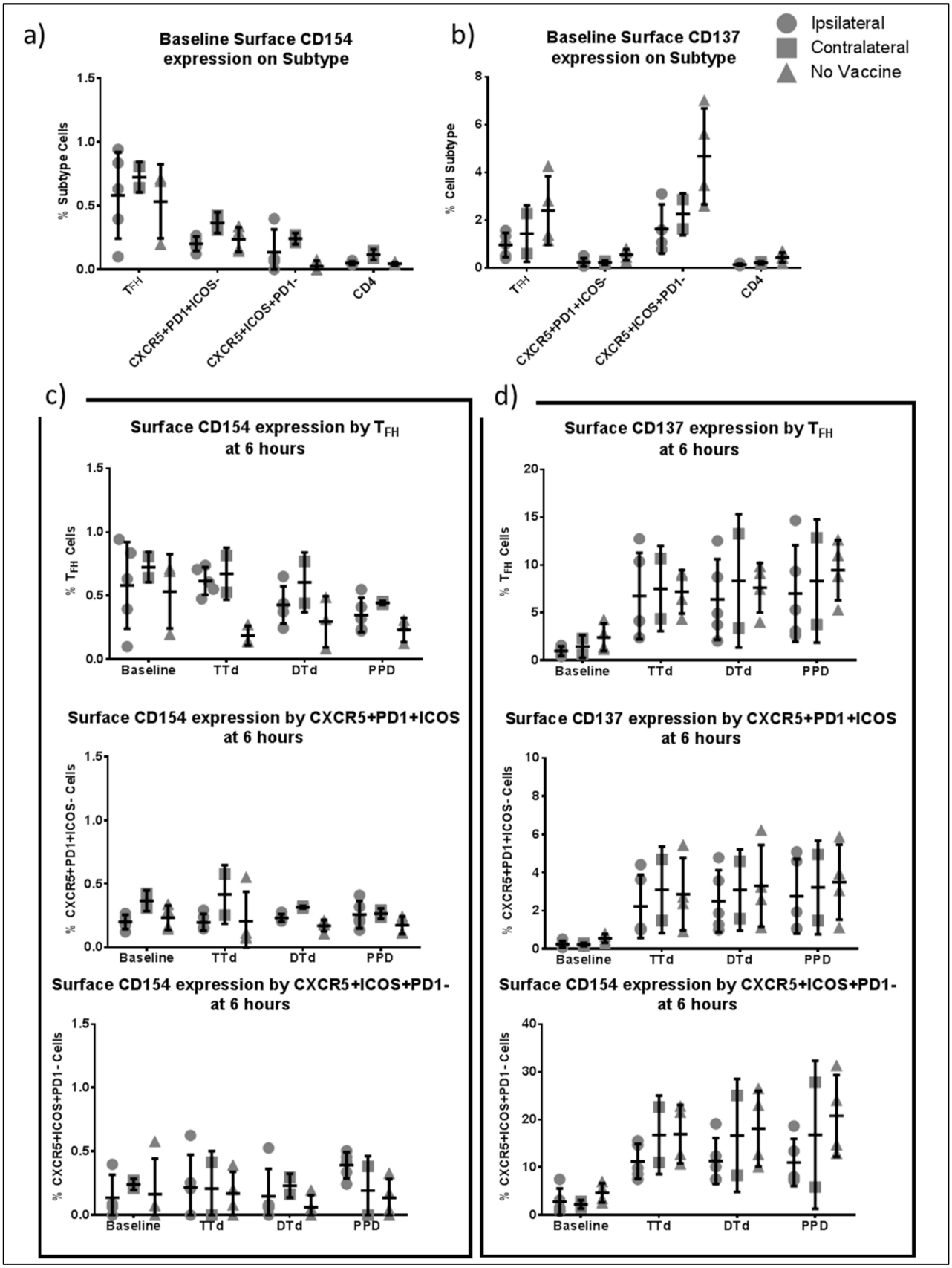


Figure 42: Expression of surface markers of activation within nodal-derived CD4+CD45RO+CXCR5+ lymphocytes according to study cohort. a) surface expression of CD154 at baseline. b) surface expression of CD137 at baseline. c-d) Effect of co-culture with antigen on the surface expression upon each cell subtype of CD154 and CD137 respectively. Plots detail individual values with mean and 95% confidence intervals represented by overlaid lines

9.4 Vaccine-specific B-lymphocytes delineate vaccine-draining and non-vaccine draining nodes

CD4+ cells cannot be easily stained according to the antigen specificity of their receptor – doing so requires Class II tetramer staining, with patient-to-patient variability in staining implicit according to MHC phenotype. B-cells, however, can be readily stained according to antigen specificity using fluorescently labelled antigen.

AlexaFluor 647 labelled Fragment C (FrC) (see Section 4.11) was therefore used to identify B-lymphocytes specific to this component of tetanus toxoid by surface staining. The phenotypic panel used is detailed in Figure 43.a.

A clear difference was seen in the number of FrC-specific B-lymphocytes present between nodes taken from the Ipsilateral cohort compared with the Contralateral and No vaccine groups, as well as irrelevant antigen (Mouse Serum Albumin, MSA) and unstained control samples - Figure 43.b. There appeared to be a separation of at least 4 patients within the Ipsilateral cohort whose nodes possessed considerably higher numbers of FrC-specific B-lymphocytes than other patients within the same cohort, and compared with the 4 control groups. Correlation between percentage of CD19+ cells specific to FrC and percentage of CD4+ cells which were T_{FH} cells (CD45RO+CXCR5+PD1+ICOS+) demonstrated a strong association between the presence of both cell subsets (Pearson r = 0.85; 95% CI 0.68 – 0.94, P < 0.0001). The same four FrC-specific B cell high nodes also contained the highest numbers of T_{FH} cells. There was a single outlier with raised T_{FH} cell numbers in the absence of high Total FrC-specific B-lymphocytes (marked with an asterisk (*) within Figure 43 and within Figure 44); this patient underwent surgery at day 5 post vaccination, at least 24 hours earlier than the remaining cohort. Although total FrC-specific B-cells was not greatly raised, when considering FrC-specific Unswitched cells numbers these were comparable with the other four “raised FrC” patients, and indeed the number of FrC-specific BM2 cells within this same patient was the highest of any of the 23 patients within the study.

These five patients from the Ipsilateral cohort with high representation of both T_{FH} cells and FrC-specific CD19+ cells were therefore considered to be different; the node-acquisition at surgery is untargeted – i.e. the “vaccine-draining” node is not selectively removed. Therefore there is no guarantee that a node taken from the ipsilateral side is draining, and therefore responding to, the vaccine. These five samples were therefore considered to be “vaccine-draining” nodes, as they were enriched for antigen-specific B-cells and had a greater density of T_{FH} cells within them, the nodes from the remaining 6 patients within the Ipsilateral cohort were therefore considered to be “non-vaccine draining”.

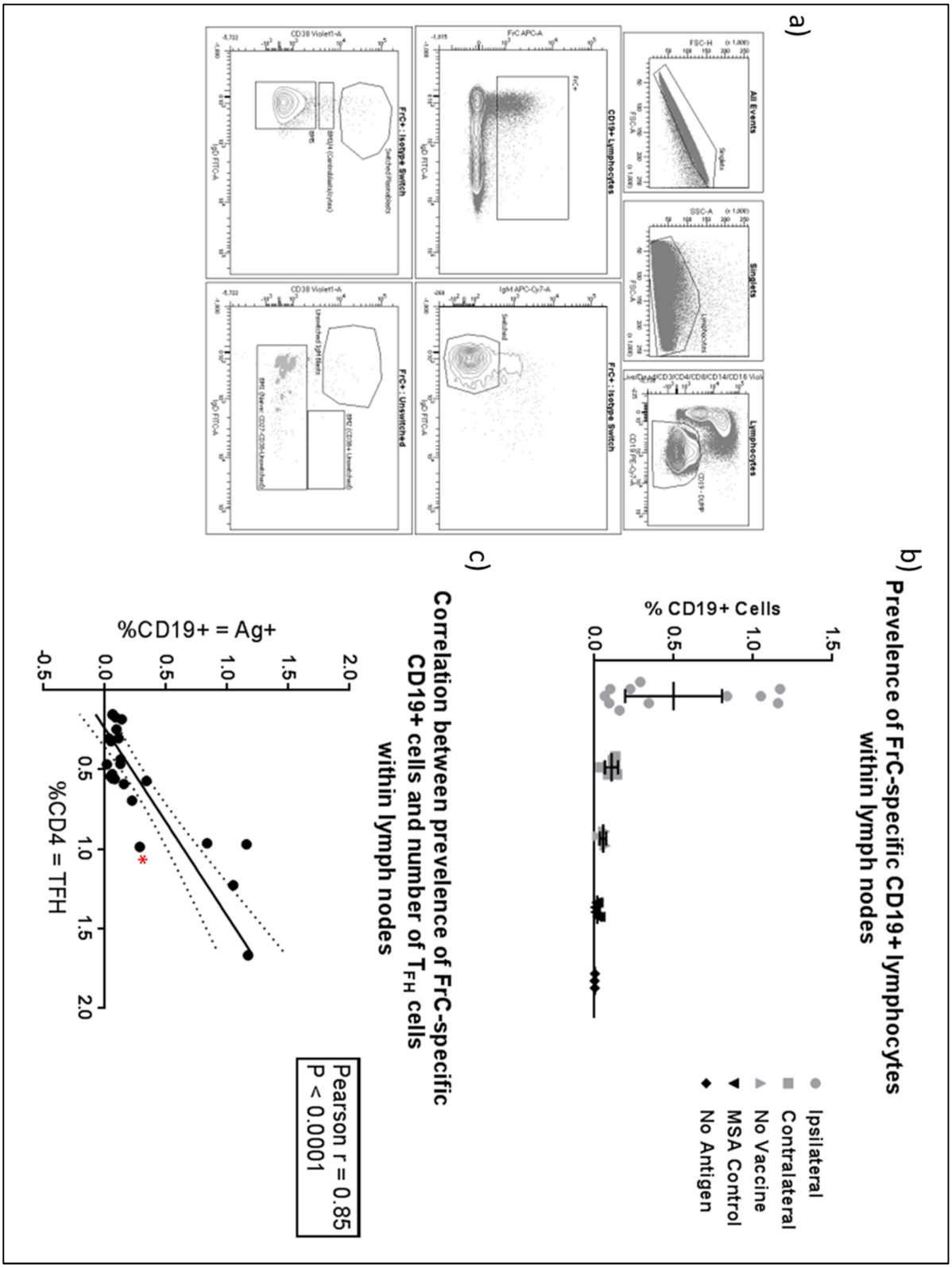


Figure 43: Identifying FrC-specific B-lymphocytes and the relationship of their prevelance to the proportional representation of T_{FH} cells within the CD4+ compartment within lymph nodes. a) phenotyping strategy demonstrating FrC staining and characterisation of antigen-specific B-cell subsets. b) prevelence of FrC-specifc B-cells within lymph nodes takenfrom patients delineated according to study cohort; plot details individual values with mean and 95% confidence intervals represented by overlaid lines. c) Pearson correlation between prevelance of FrC-specific B-cells and T_{FH} cells within lymph nodes. Plot is line of best fit with 95% confidence interval.

9.5 Population differences between "Vaccine draining" and "Non-vaccine draining" nodes

Comparison was made between the B-cell (Figure 44) and CD4+ lymphocyte (Figure 45 and Figure 46) populations within the vaccine draining, non-vaccine draining, contralateral and no vaccine groups. Sub-categorising the Ipsilateral cohort in this fashion unveiled delineation in the proportional representation of T_{FH} cells within the CD4+ compartment according to vaccine-draining state (Figure 44). 1.17% (95% CI 0.79 – 1.54) of Total CD4+ cells within draining nodes were T_{FH}, compared with 0.41 (95% CI 0.16 – 0.67) within the non-draining, 0.37% (95% CI 0.23 - 0.51) within the contralateral and 0.42% (95% 0.26 – 0.58) within the no vaccine groups (P = 0.001).

The antigen-specific B-subtypes present with greatest prevalence within the draining nodes were switched memory (mean percentage CD19+ = 0.48%; 95% CI 0.07 – 0.90) and naïve cells (0.19%; 95% CI 0.09 – 0.30) whilst other subsets were less prevalent (IgM Memory and IgM plasmablasts). By comparing non-draining nodes taken from the vaccinated side with the other two control groups, it was also possible to identify some differences in the populations of B-lymphocytes within lymph nodes from the responding lymphatic drainage field, which were not directly involved in the on-going vaccine response – these nodes were enriched for FrC-specific activated naïve (BM2) and Centroblasts/cytes (BM3/4) relative to the Contralateral and No Vaccine control arms, although the frequency of these cells still remained low (Figure 44).

As alluded to in Section 9.4, on an individual basis some differences could be speculated on in terms of the relative preponderance of different FrC-specific B-cell subsets. A single patient (marked *) was noted to have a paucity of switched FrC-specific cells but an abundance of FrC-unswitched sub-types, particularly BM2, and T_{FH} cells. Possible reasons for this might include the different time frame this node was harvested at, which may have preceded the time at which isotype switch occurred.

Despite delineating the Ipsilateral cohort according to vaccine-draining status the overall compositional make-up of the CD4+ and CD19+ populations remained comparable across the four groups in the majority of instances - Figure 45 and Figure 46. The notable exception to this being CD4+CD45RO+CXCR5+ICOS+PD1- cells which, in a fashion comparable to T_{FH} cells, were significantly more prevalent in vaccine draining nodes (mean percentage CD4+ = 0.38%; 95% CI 0.14 – 0.62%) compared with non-vaccine draining (0.12%; 95% CI 0.06 – 0.19), contralateral (0.10%; 0.05 – 0.16) and no vaccine groups (0.17%; 95% CI 0.07 – 0.02). Delineation according to size failed to demonstrate any difference according to vaccine-draining status, with both ipsilateral-draining and ipsilateral-non-draining having similar size profile, although both

ipsilateral groups were significantly different to contralateral and no vaccine groups – see Figure 46.

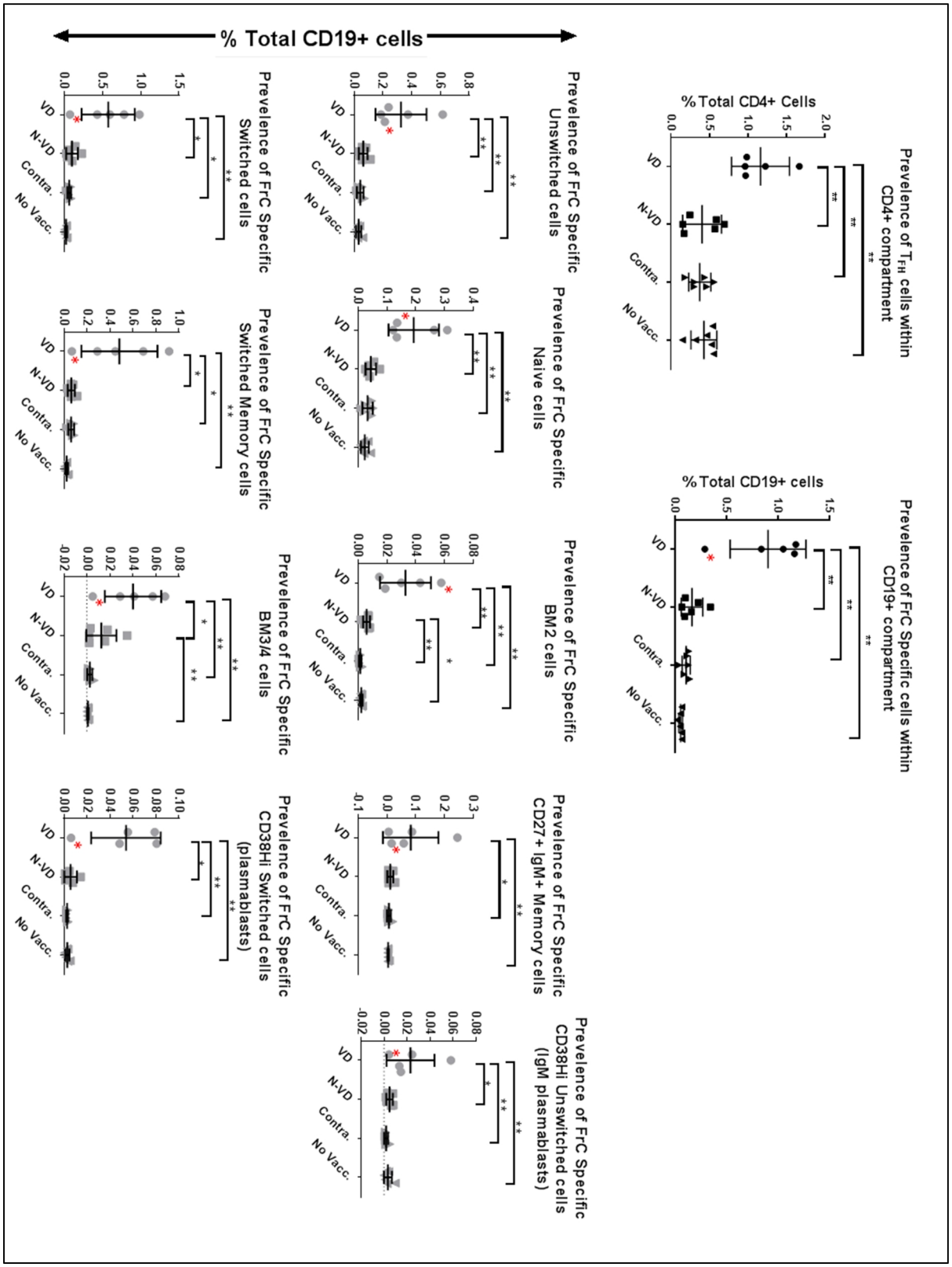


Figure 44: Comparison between lymphocyte populations within lymph nodes from vaccine draining (VD), non-vaccine draining (N-VD), contralateral and no vaccine groups. Plots detail individual values with mean and 95% confidence intervals represented by overlaid lines. Top-left: Number of T_{FH} cells within the CD4+ population. Top-right: Total number of FrC-specific cells within the CD19+ population. All other plots relate to number of FrC-specific cells within individual CD19+ subtypes. Significance calculated one-way ANOVA when Brown-Forsythe test demonstrated equivalence of standard deviations between groups. Otherwise Mann-Whitney test performed. * denotes the single outlier of interest's position within each plot.

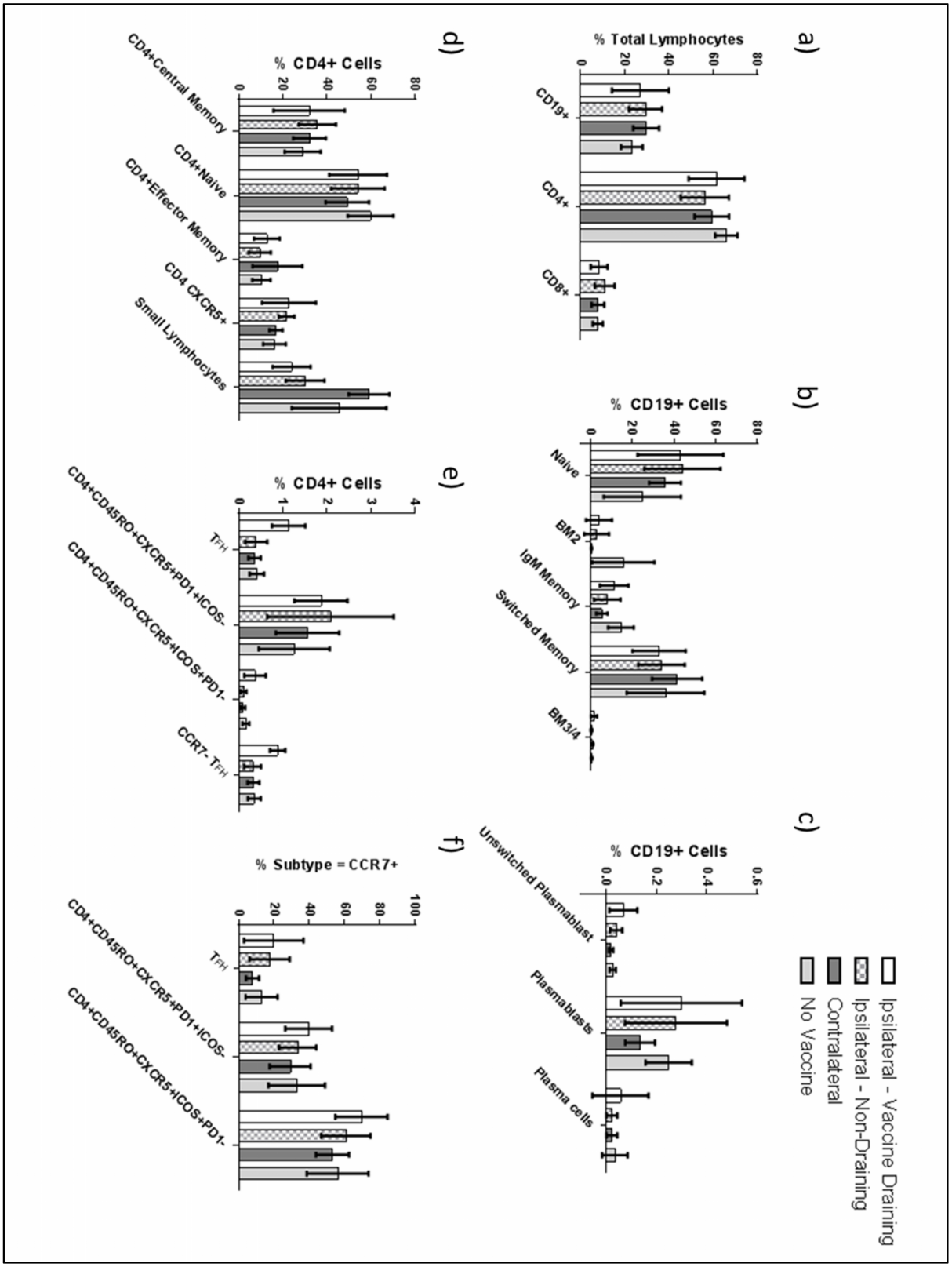


Figure 45: Comparison between lymphocyte populations within lymph nodes from vaccine draining, non-vaccine draining, contralateral and no vaccine groups. Bars indicate mean values with error bars denoting 95% confidence intervals

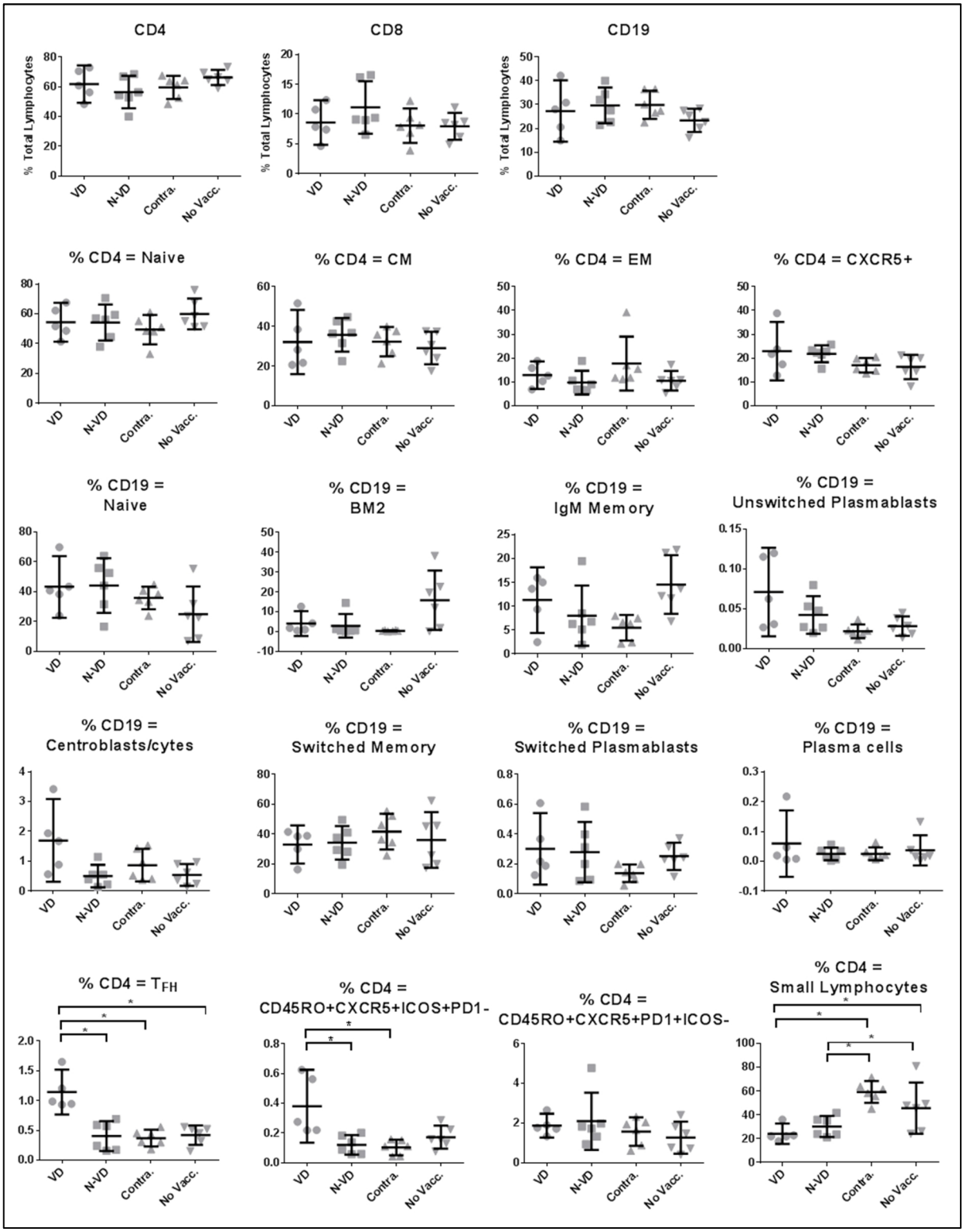


Figure 46**: Comparison between lymphocyte populations within lymph nodes from vaccine draining, non-vaccine draining, contralateral and no vaccine groups. Plots detail individual values with mean and 95% confidence intervals represented by overlaid lines. Significance (*) calculated using Kruskal-Wallis test with a Dunn's correction for multiple comparisons.**

10 Discussion

Lymphatic tissue is the staging point of the immune systems response to antigenic challenge. Specialist CD4+ follicular helper T-cells within lymphatic tissue are known to exert great influence over the ensuing humoral response, however the study of these cells in humans has been limited. Both ex-vivo co-culture and gene expression analysis has been used to define the functional capabilities of CD4+CXCR5+ populations derived from peripheral blood (226) or node (18, 57, 552). However it is difficult to extrapolate data derived from ex-vivo stimulation to normal in-vivo, tissue bound behaviour. Furthermore studies of gene expression within tissue-derived human T_{FH} cells have exclusively used cells derived from human tonsil (57, 553, 554) which has its own problems: tissue from tonsils has been subject to an unknown pathological infective/inflammatory process and therefore may not represent normal immune response . Furthermore the antigen exposure is unknown and uncontrolled, and therefore cannot be correlated to associated peripheral blood end points.

Through design and set up of a novel in vivo human study I have created an opportunity to examine previously inaccessible lymph node-based immune processes which underpin fundamental aspects of human immunity. Using my techniques it is now possible to look at the cell populations within vaccine draining lymph nodes, and compare these to the evolving immune response occurring within the peripheral blood. Using said techniques it will now, for the first time, be possible to corroborate data from animal studies of these same processes, leading to validation of some key assumptions regarding human adaptive immunity, as well as pursuing original discoveries.

Establishment of this study has required the optimisation of a series of novel assays with development of robust and repeatable processing techniques being the initial priority. Isolation of cells from lymph nodes without unduly effecting their phenotype and function proved problematic. The question of function was considered important since planned assays to determine antigen specificity relied on cytokine production. Although ex-vivo antigen re-stimulation of lymph node-derived cells ultimately failed to provide information regarding bystander activation of tissue-derived cell subsets (See Section 9.3), the successful preservation of function following enzymatic desegregation, as demonstrable by ELISpot assay (Figure 6), suggests cells isolated with said technique are less degraded by the process than those isolated through mechanical means. If we accept function as a surrogate marker of cell health then the use of enzymes in the isolation process remains of importance when considering gene expression of key populations derived from tissue. The relevance of this to planned and ongoing work will be evident below.

The disadvantage of enzyme desegregation is the deleterious effect on expression of some surface markers. Through screening of enzyme preparations it was possible to identify a suitable preparation without protease contamination, which did not cause surface loss. Subsequent comparison confirmed preservation of function was not dependent on the protease content of the enzyme used (Figure 9) and therefore it was possible to preserve both phenotype and function through careful selection of enzyme. All target markers selected for use in subsequent flow cytometry panels were therefore screened for susceptibility to enzymatic degradation using my selected enzyme cocktail (0.15 WU Liberase DL plus 800 KU DNAse 1); thus far no marker has shown measurable degradation to this combination, and therefore my observations regarding phenotype can be considered robust.

The key aim of the study was to attempt to compare changes within lymph nodes to peripheral blood "outcomes"; i.e. antibody and cellular responses to vaccination. In order to achieve this it was necessary to describe the changes occurring within the blood following vaccination in detail. Whole blood studies across the study time course confirmed a transient increase in total lymphocyte count in patients receiving a vaccination. No post-operative lymphopenia was demonstrable in mastectomised patients despite significant falls in post-operative haemoglobin (in line with previously published observations(555)) and neutrophil counts, suggesting perioperative blood loss failed to adversely affect response (see Figure 20 and Figure 21). Sub-group stratification according to surgical approach was therefore deemed unnecessary, which meant cohort size was sufficient for meaningful analysis.

Cohort variability in terms of baseline serum levels of vaccine-specific immunoglobulin was marked although no differences between study groups could be identified (Figure 22, Figure 23 and Table 10). Baseline levels also failed to predict the nature of the subsequent early or peak immunoglobulin response to vaccination (Figure 28) – see below. The response to vaccination varied according to isotype: IgM response proved difficult to detect; as discussed in Section 7.2 in the majority of patients IgM remained unchanged or even fell relative to baseline. Failure to detect an IgM response could indicate that the chosen time points missed the IgM increase in these patients since IgM is known to be an early, transient immunoglobulin (1).However the presence of IgM was seen to increase in a minority of patients as early as time point 2 therefore in at least some patients the chosen time points did intersect the IgM response. However this was only true for TTd since no IgM responders to DTd were identified (Figure 26 and Figure 27); why this should be the case is unclear since both antigens are similar in their nature. That said no direct comparison in terms of quantity of antigen-specific immunoglobulin can be inferred between the two antigens due to the restrictions of the

assays used, although one can comment that the profiles of isotype prevalence at baseline were not markedly different (Figure 22). Observation of a fall in levels relative to baseline is more indicative of a technical failing of the assay. As IgM levels were repeatable within individuals, showed variability across the cohort and demonstrated reactionary dynamics following vaccination in some individuals, we can infer that this failure was not absolute. The likelihood is that levels described at baseline are true reflections of vaccine-specific IgM serum levels. Reduced IgM detection at the later time points in the presence of high serum-levels of vaccine-specific IgG isotypes is, presumably, due to competition on the plate for antigen. The observation that there exists an increase in circulating IgM+ plasmablasts following vaccination would suggest that a detectable change in serum IgM should be present.

On a population level, IgG_1 and IgG_4 increased significantly at the later time points whilst IgG_3 response was demonstrable at weeks one and three post vaccination, and was more transient in nature (Figure 25). This correlates well with what is already known regarding vaccine response (Section 1.4.1) however individual response to vaccine is far less predictable; variability in response was one of the single most striking findings within the patient cohort. Despite patients being recruited from a reasonably homogenous target population (women with breast cancer without lymphatic involvement who have not been exposed to vaccine components for a minimum of 10 years) patients responded in markedly different ways to the same vaccine – Section 7.5. As alluded to earlier, analysis of individual responses demonstrates that variability between individuals, and within individuals to different vaccine components, exists. By making broad generalisations it is possible to categorise patients into, for example, “early” and “late” responders however, as previously mentioned, baseline levels were unable to predict response. The serological antibody levels at baseline can be considered a fair representation of the long-lived antibody-producing plasma cell population present within centralised niches, having previously been generated in response to historic exposure to the target antigen. In the absence of any strong serum-antibody predictors of response to vaccination, one must conclude that either circulating B_{MEM}-cells are not representative of the corresponding plasma cell population (360-363), or their response is tailored/modified during the germinal centre responses which follow re-exposure (387, 391, 407). We know from prior study that both of these suppositions are supportable on the basis of existing evidence; application of the techniques developed during the course of this project could allow insight into these processes. Through isolation of antigen specific peripheral blood and germinal centre B-cells it will be possible to compare V gene mutational state of these populations. This will allow comparison of the mutation state between these populations; by varying the time

point of lymph node acquisition it will be possible to map these processes during the recall response over time, given sufficient patient numbers.

However the focus of this present study was to evaluate the role of follicular CD4+ populations in the recall response to vaccine. Interestingly the presence of follicle-associated populations within the blood one week following vaccination appears to correspond to the immunoglobulin response. There exists an apparent dichotomy of the response to vaccination according to increase in either CD4+CD45RO+CXCR5+ICOS+PD1-/Switched plasmablasts or CD4+CD45RO+CXCR5+ICOS+PD1+/ Unswitched plasmablasts, the former cellular response being associated with a slower, lower amplitude immunoglobulin response whilst the latter is associated with a faster response, possibly of greater amplitude (Section 8.4,Figure 37, Figure 38). The fact that the polarisation of responses into “early” and “late” according to serum immunoglobulin levels also appears to crudely predict gross changes in total populations of follicular-associated CD4+ and CD19+ lymphocytes strongly supports fundamental differences in the immunological response between individuals. What determines the nature of the response however remains unclear; both responses were seen in patients at a similar time following vaccination, therefore sampling error, although possible, seems unlikely. Impressively, the type of cellular response also appears to correlate with immunoglobulin levels at the later time points, again suggesting that the observation reflects more than different “snap-shots” of the same reaction, since differences are maintained through to the later endpoints. True differences in the follicular responses to vaccination in these groups therefore seems likely, and may well derive from differences in memory state/recall.

However the immunological relevance of the observed changes remains unclear. The association between slow immunoglobulin responses and increased numbers of circulating CD4+CD45RO+CXCR5+ICOS+PD1- cells, and rapid responses with increased T_{FH} cells would appear to make sense; CD4+CD45RO+CXCR5+ICOS+PD1- are considered a more immature cell phenotype in the context of follicular response to vaccination (Section 1.3.2) (18, 57, 226). Therefore the abundance of these cells in the circulation of patients making a slower immunoglobulin response could reflect a more delayed evolution of the CD4+ follicular cell response, due to differences in memory recall (i.e. akin to a “primary-like” response). Conversely individuals making a more rapid immunoglobulin response also have a more predominant increase in circulating cells expressing a surface phenotype typical of mature germinal centre T_{FH} cells (CD45RO+CXCR5+ICOS+PD1+), indicating a more rapid establishment of follicular response (i.e. a probable “memory” response). Why such differences in response exist remains unclear.

Furthermore explanation of the association of a rapid immunoglobulin and T_{FH} response with the emergence of a greater number of unswitched plasmablasts into the circulation is less clear-cut. During the evolving germinal centre reaction cell populations remain in a continuous state of flux; cell apoptosis and egress from the nodal reaction is dependent on cells' ability to compete for antigen. Extrafollicular proliferation has been proposed as an important initial step in early vaccine response (235); said process typically involves low infinity, minimally class-switched B-cells. Extrafollicular proliferation self-terminates on establishment of germinal centre reactions; therefore the circulating unswitched plasmablasts seen may represent progeny released from these extrafollicular foci in patients whose germinal centres have rapidly established. However the association between emergence of switched plasmablasts and slower immunoglobulin/follicular CD4+ responses does not fit with the same theory. Previous authors have described vaccine-induced mobilisation of non-vaccine specific B-cells of a mature phenotype (Switched, CD38+) from long term survival niches (Section 1.4.2.1) (367, 368); these cells were termed plasma cells by the respective authors, however surface CD138 expression was not detailed. The phenotype of these cells was CD19+CD27+CD38+IgM/D -ve (i.e. the same as the population I have termed "plasmablasts"), with additional markers such as HLA-DR, CD95 and CD20 used to distinguish from blast cells. Due to the differences in the comparative phenotypic strategy used in my study it is not possible to draw direct comparisons between these previous findings and my own, however it is possible that the increased numbers of circulating switched "plasmablasts" seen within this study are comparable to those seen by previous authors, and there represent a mobilisation phenomenon rather than a vaccine specific response.

One major shortcoming of the data discussed above is the unknown antigen specificity within the circulating cell populations. Although dynamic changes in distinct sub-populations can clearly be demonstrated, whether or not the cells entering the circulation are vaccine-specific remains unproven. Work within our own laboratory, as well as that of others, has previously focused on non-vaccine-specific induction of circulating CD4+ populations; a so-called "bystander" response (Section 1.5.4). Therefore there remains a real, theoretical possibility that the induced changes seen in circulating CD4+ populations, as well as their B-cell counterparts, represent non-vaccine specific processes. During this study attempts were made to develop assays to assess antigen specificity of the populations which had proven to be dynamic following vaccination (Section 9.3). Previous work within our laboratory has utilised cytokine production and/or CD154 up regulation following ex vivo antigen stimulation as a method for identifying antigen specificity (498). However use of CD154, and an alternative surface activation marker CD137, to determine antigen specificity proved impossible within

the follicular-cell populations of primary interest to this current study. This was due to antigen-induced phenotypic changes on CD4+ cells including induction of CXCR5 and, notably, ICOS (see Figure 16.c.) which undermined the validity of resulting data regarding the antigen specificity of individual CD4+ subtypes. Characterising the antigen specificity of the B-cell populations was more straightforward (Section 4.11 and Section 9.4), however due to time and financial constraints was restricted to nodal tissue for the purpose of this study.

Therefore currently the antigen specificity of the vaccine-induced increases in CD4+CD45RO+CXCR5+ and B-cell populations remain unknown. Nonetheless other authors have previously reported similar findings to our study. He *et al.* reported a transient increase in the number circulating CD4+CXCR5+PD1+ $CCR7^{Lo}$ cells one week following vaccination (233) and suggested these circulating cells are not of germinal centre origin, but do function to survey and propagate the response to antigen should it be encountered at other sites. Bentebibel *et al.* (556) meanwhile noted correlation between numbers of circulating CD4+CXCR5+ICOS+ cells and antibody titre in autoimmune disease and following vaccination, with the same group recently publishing data supporting correlation between circulating T_{FH} populations at one week post vaccination, with anti-influenzae antibody titre at 28 days (557). Within the current study cohort CD4+CD45RO+CXCR5+ICOS+PD1- cells were found with a similar frequency to T_{FH} cells (0.020% of circulating CD4+ cells) whilst CD4+CD45RO+CXCR5+PD1+ICOS- cells account for 0.41% of circulating CD4+ cells. My work may well therefore represent a refinement on the understanding of vaccine-induced changes in gross CD4+ subsets within the circulation. Whereas He *et al.* considered the CD4+CXCR5+PD1+ population as a whole, this current work allowed sub-division of this population according to ICOS and CD45RO expression (Section 8.2, Figure 33). Data demonstrates that dynamism is confined to CD45RO+CD45RA- cells expressing either ICOS or ICOS and PD1. Cells expressing PD1 alone were not increased in the circulation by vaccination. Therefore the subsequent analysis performed by He *et al.* would have included both dynamic and non-dynamic populations, as their gating strategy did not differentiate between PD1+ICOS+ and PD1+ICOS- cells. This which might explain why He *et al.* found indeterminate expression of T_{FH}-associated genes in their target population.

Furthermore CCR7 expression on these same dynamic populations changed significantly one week following vaccination (Figure 36); for both dynamic CD4+CD45RO+CXCR5+ populations (i.e. ICOS+PD1+ and ICOS+PD1-) both CCR7+ and CCR7- subgroups increased in number, with CCR7- cells increasing by the greatest extent. Both CCR7- (233) and CCR7+ (232) cells have been shown to correlate with vaccine response and follicular cell proliferation within lymphatic

tissue, however the relative importance of these circulating cells remains unclear and a subject of continued debate. Analysis of gene expression within tonsillar-derived germinal centre T_{FH} cells demonstrated Bcl-6-mediated repression of the *CCR7* gene (554); since Bcl-6 is considered the key "phenotype defining" transcription factor in T_{FH} cells, the presence of CCR7 surface expression has been considered an exclusion marker for follicular T cells. Increased numbers of both CCR7+ and CCR7- follicular-type CD4+ cells suggests probable variance in Bcl-6 expression within the circulating cell population, presumably as a consequence of variation in TCR binding affinity. However actions of BCL6 have previously been detailed within T_{FH} cells (Section 1.3.1) and B-cells (22-24) and are known to be heavily cell context dependent, moderated through interactions with different binding motifs such as AP1 (554), which explains how the same factor can exert such disparate effects on different cell populations. Therefore the increase in CCR7+ and CCR7- cells following vaccination could also be due to variance in the upstream or downstream regulatory factors associated with Bcl-6 signalling.

Previous studies have looked at phenotypic differences between cells to determine the hallmarks of "T_{FH}" cells and "pre-T_{FH}" cells, as well as regulator and memory counterparts (section 1.3.2) – this study suggests that a number of phenotypes are present, and vary in terms of their relative numbers, which corresponds to differences in response. A more complete understanding of the significance, origin and functional properties for these circulating populations is required. Their roles are likely to be different and considering one the subsidiary of another is likely to be simplistic – analysis of gene expression and regulatory state may identify key similarities and differences between different circulating populations. Similar gene expression/regulation across the populations would imply that function is determined through surface phenotype as a consequence of cell location and proliferative capability. More likely is that the differences observed in the separate circulating populations in terms of phenotype and vaccine response reflect an underlying difference in the gene expression state.

Another unanswered question in immune biology is how circulating follicular-associated CD4+ cells compare to their lymph node-derived counterparts. Significant debate exists regarding the relevance of the circulatory changes and how they map onto their tissue-bound counterparts – my work suggests circulating populations change following vaccination, but are cells found in the blood fundamentally different to nodal cells? Circulating cells express CCR7 to gain access to lymphatic tissue via paracortex then rapidly transit to B-cell areas. Therefore are CCR7+ circulating follicular cells those which never made it to follicular areas due to reduced affinity (and therefore failed to up-regulate Bcl-6 sufficiently to lose CCR7 surface

expression)? Does their presence in the blood represent a recirculation event, intended to allow the surveyance of regional and systemic nodes for same/similar antigen?

In gross population terms differences in the total numbers of B cell subsets between the node and blood largely conformed to expectations; plasmablasts were more prominent in the blood whereas switched memory and BM3/4 (Centroblasts/centrocytes) made up a greater proportion of the CD19+ population within the node. Surprisingly no difference in the prevalence of IgM memory cells was demonstrable, but the trend was for increased predominance in the blood. Similarly, activated naïve B cells (BM2) made up a greater proportion of the CD19+ cohort in the blood than in the node; however interpretation of said information is difficult as comparison of absolute numbers is impossible. Therefore the observed increased predominance of these populations may simply reflect the overriding number of switched memory cells within the node, which constitute 36.4% of CD19+ cells in the node, but only 20.0% in the blood.

Within the CD4+ compartment (Section 9.1, Figure 39, Table 15) proportion of CXCR5+ CD4+ lymphocytes was higher in lymph nodes than peripheral blood. This observation held true for CD45RA+CCR7+, CD45RA-CCR7+ and CD45RA-CCR7- cells expressing CXCR5. CXCR5 expression can be induced following activation (227); since the lymph node acts as the site of antigen presentation and T-cell (and B-cell) activation it is possible that the differences in overall CXCR5+ expression reflect the activation states of the two compartments (despite efforts to avoid comparing activated nodal tissue (i.e. vaccine draining node) to resting state blood by only including data from unvaccinated and contralateral control nodes). However as expected populations expressing follicular markers (i.e. ICOS and/or PD1) made up a greater proportion of the total CD4+ population in the node than in the blood. CD4+CD45+CXCR5+PD1+ICOS- cells were the most common cells expressing either ICOS and/or PD1 in both the blood and the node whilst both ICOS+PD1- and ICOS+PD1+ cells were rare in the circulation but represented to a greater degree in the node. CCR7 expression varied between nodal populations, with 60% of ICOS+PD1- CXCR5+ cells expressing CCR7, compared with 34% of PD1+ICOS- and 15% of ICOS+PD1+ cells. These same populations in the blood also varied in their CCR7 expression, however circulating counterparts all had a higher CCR7 expression than node-derived cells. Different CCR7 expression is likely to reflect different functional state and location within the node however it is interesting to note that the most prevalent population in both blood and node is the population which has no dynamic response to vaccination.

Furthermore both dynamic populations are more abundant in vaccine draining lymph nodes than non-vaccine draining nodes and control nodes (Figure 46) when draining status is

determined by number of antigen-specific B-lymphocytes. Induction of ICOS expression precedes that of PD1 and is more transient (Section 1.3.1); previous authors have variably labelled CD4+CXCR5+CD45RO+ subtypes as pre-GC, GC and $T_{FH\text{-}Memory}$, cells according to their differential expression of CXCR5 and other markers, as well as according to their ICOS+PD1-, ICOS+PD1+ and PD1+ICOS- status respectively. The observations above would seem to mirror this. ICOS+PD1- cells are less terminally differentiated than their counterparts, as evidenced by their differing functional capabilities and proliferative potential ex vivo (14, 18, 58, 224, 226). Here we find these cells are dynamic following vaccination, more prevalent in vaccine draining nodes and express CCR7 to a greater degree than PD1+ counterparts, suggesting although these cells are reactionary to vaccination, the actions of Bcl-6 within them are less complete. ICOS+PD1+ cells are also vaccine-responsive, however CCR7 expression is markedly less than that of ICOS+PD1- cells; Bcl-6 influence within these cells is therefore likely to be more complete, as it is known that Bcl-6 is a key negative regulator of CCR7 expression (554). Conversely PD1+ICOS- cells do not react to vaccine; their expression of CCR7 is equivalent to ICOS+PD1+ cells in the circulation, but is more prevalent in nodes. These cells are the most numerous subtype in both the circulation and the node. Their functional role, however, remains unclear. Similar cells have previously been labelled as “memory” cells (552); the observation that there is a lack of population response to vaccine in either the blood or the node would circumstantially support this possibility.

Ultimately full details of differential gene expression, and gene regulatory state, between these cell types would allow insight into the functional similarities and differences between not only the three different populations, but between the peripheral blood and node bound counterparts expressing the same surface phenotype (552). Comparison of activation state, TCR-affinity, apoptotic potential, memory-associated gene expression and regulatory gene expression would allow accurate speculation regarding relative importance and function within an evolving immune response. To this end florescence-activated cell sorting was utilised to isolate individual cell populations of interest (see Section 4.13).
CD4+CD45RO+CXCR5+ICOS+PD1-, ICOS+PD1+ and PD1+ICOS- cells were isolated from the nodes of all 23 study patients, as well as CD4+CD45RO+CXCR5+ICOS-PD1- control samples. In addition the same populations have been isolated from the peripheral blood of six patients from the ipsilateral group. Test samples were used to harvest RNA according to the protocols outlined in Section 4.14; from 5,000 cells the estimated yield of RNA was approximately 30ng, sufficient for RNA sequencing analysis. Cell samples have therefore been prepared for RNA isolation and transferred to a collaborating institution for RNA-sequencing analysis. The results of this are expected in the near future.

This ongoing work will aim to answer broad questions regarding the origin and function of the cells of interest identified within this current study. However future work will aim to uncover the determinants of the differing immune responses between individuals which has been uncovered and detailed by this study (Section 7). Understanding of these processes may lead to the development of novel strategies to manipulate and control the response generated by a vaccine in terms of the cellular response and/or the antibody isotype produced. Previously published findings have demonstrated the influence of antibody isotype on disease outcome in humans (558) and response to anti-tumour immunotherapy in mice (559). Detailing the processes which occur during T-B interaction, and specifically the role of T_{FH} cells, may allow new insight into how isotype switch is determined. This may have implications not only for anti-cancer vaccine therapy, but also vaccine design and strategy as a whole. For example, repeat vaccination and the induction of an IgG_4 response may detract from existing immunity whereas directed isotype switch to IgG_1 may induce a preferable response to vaccination. Conversely, the efficacy of a vaccine strategy against a certain cell target may be best served by a high affinity, functional blockade rather than a cytotoxic effect. We know there exist a variety of signals involved in directing humoral response to vaccine and that T_{FH} cytokine production can predict isotype response (64). However analysis of gene regulation and expression will provide insight into the up-stream processes and allow greater understanding of fate determination during a response. The significance of T_H1, T_H2 or T_H17 effector subset transcription factors in T_{FH} sub phenotypes (211-213) may well prove key: parallels between effector subsets (T_H1, T_H2 and T_H17) and accompanying iT_{REG}/T_{FH} in terms of transcription factor and cytokine production suggest common upstream priming and signalling events. How this maps onto humoral response is currently unclear however analysis of gene regulation/expression within vaccine-responding lymph node derived T_{FH} cells within the context of a measured humoral response is likely to provide vital insight.

Achieving this aim will require some modifications of the current study format; number of vaccine-draining nodes successfully isolated following recruitment of 20 patients into the ipsilateral cohort was five. Although sufficient to attempt to address some of the broader questions detailed above, these numbers are insufficient to compare even the differing processes in early versus late responders, let alone the intricacies of different isotype responses. Potential strategies to improve efficiency of sample collection would be to target the vaccine draining node specifically; any technique used would need to avoid interfering with the standard techniques used to isolate sentinel nodes during surgery (see Section 4.2.2). Recently a new technique for nodal mapping has become available which utilises fluorescence to guide surgical biopsy (560, 561). Incorporation of this technique to identify the vaccine

draining node would allow the reliable identification of nodal tissue relevant to addressing these objectives.

One of the key limitations of this work has been the technical inability to identify antigen-specificity within the CD4+ populations. Vaccine draining status was inferred by enrichment of FrC-specific B-cells within nodal tissue (Section 9.4). Through isolation of whole populations of T_{FH} and related cells from these nodes I hope to draw broad conclusions regarding their gene usage within the context of a vaccine response. However confirmation of vaccine-specificity within these nodes would lend further weight to conclusions drawn from this work, as would confirmation of the specificity of their circulating counterparts. Furthermore with the advent of single-cell sequencing based technologies (562, 563), the potential scope for analysis of these same antigen-specific populations at a much greater resolution is an exciting prospect. Such techniques would require use of Class II Tetramers specific to vaccine components; these tools exist and are commercially available (552), and would be suitable for use in a single-cell sorting strategy (although Class-II tetramer use is restricted to participants with compatible HLA-phenotypes). I have already employed a similar strategy in sorting individual antigen-specific B-lymphocytes from nodal tissue; analysis of the V-gene mutation state of these cells from vaccine-draining nodes is planned to gain information regarding maturity of the response within individual cells and how this maps onto their class-switch status, surface phenotype and the peripheral immunoglobulin response. Figure 44 demonstrates the variable nature of the populations within draining lymph nodes; the significance of these variations is unknown, and as alluded to earlier (see Section 9.4), may reflect different stages of an evolving response. Single-cell analysis with either V-gene analysis, or next-generation sequencing-based "-omic" technologies, will be perfectly suited to shed light onto these questions. Ultimately, given sufficient resources, genomic (i.e. T-/B-cell receptor recombination), transcriptomic and histomic analysis of T- and B-cell counterparts from vaccine draining nodes will allow the dissection and study of the interactions between these cells during a response to vaccine to a level of detail not previously thought possible. This study details the development of a unique strategy to study said processes within primary human lymph nodes.

There exist, however, some limitations to the work carried out; the inability to identify CD4+ antigen specificity has been discussed, as has the difficulty in selectively removing the vaccine-draining node during surgery. These limitations do not invalidate the work undertaken when looking at whole populations, and indeed it is anticipated that the comparison of vaccine draining versus non-vaccine draining nodes will provide an interesting control arm in the planned RNA-transcriptome analysis. However refinement of the study techniques, using the

strategies already mentioned, will be required for the anticipated future work on a single-cell level. Considering the vulnerability of "-omic" technology to degradation, particularly when applied to the small cell numbers envisaged, processing techniques may have to be adapted, as it is likely that cryopreservation will not be suitable. Other authors have previously used sorted cryopreserved cells during transcription analysis, however typically with greater numbers of cells than I have been able to generate (i.e. around 5,000 cells per population per sample). Whether meaningful data can be extracted from these samples is therefore uncertain, although I remain hopeful. Avoidance of the cryopreservation step would minimise cell stress, reduce time between tissue desegregation and population isolation and avoid exposure to foreign serum. The disadvantage to such a strategy is that each sample then becomes "single use" and requirements on laboratory staff increase markedly.

Another factor which makes this research strategy challenging is the difficulty implicit in recruitment. The recruitment of 42 patients required the screening of 300 patients over 22 months. Any work requiring the recruitment of human subjects is inherently more difficult; recruitment of patients at a time of personal stress is even more challenging. Surprisingly however only 23/300 patients were not approached due to concerns regarding their emotional state at the time of recruitment and only 18 patients stated stress as the reason for them declining enrolment. An almost equal number of patients were not recruited due to the planned dates of their surgery not conforming to the needs of the study (34/300, 11.3%); the importance of ensuring a set time frame between surgery and vaccination centres around conformity in terms of measured immunological end points, as well as the known time scales of T-B interactions within draining lymphatics (234, 257). Increasing the scale of investigation may well, therefore, necessitate restructuring of service provision within the scope of an academic clinical research unit, to increase recruitment without undue delay in surgery. Such strategies may include greater integration of the pre-assessment service and academic support staff, ring-fencing of certain theatre slots for the purpose of study patients and selective assignment of patients meeting study criteria to the clinics of academic staff.

Recruitment was further hindered by the inability to acquire complete sample sets from 19 of the 42 patients recruited (Section 6.1). The most frequent reason for incomplete sampling was the presence of axillary metastasis; 31.0% (13/42) incidence of positive sentinel node biopsy is slightly higher than expected, but within acceptable limits. This was compounded by withdrawal of 6 other patients from whom tissue or blood sampling proved insufficient. The decision not to include patients with positive sentinel nodes was made at the time of study set up in 2011. Since then new guidance from professional bodies such as the American Society of

Clinical Oncology has been published regarding the management of the axilla in patients with certain favourable prognostic features (564). Therefore it is no longer considered universally essential to clear the axilla in the presence of metastasis, which could, theoretically, mitigate the number of patients recruited to the study who do not end up providing nodal samples. This would aid resource planning and recruitment. Whether such a strategy is desirable, however, must be questioned, since the immune conditions within an involved axilla are less likely to be considered “normal” and may compromise the data gathered. To address this question, work is envisaged to assess the serum response to vaccination within the remaining blood samples unprocessed for the purposes of this thesis. As alluded to in Section 6.1, 14 patients had full sets of bloods taken across the four time points, but were not included in the current study either because their sentinel node biopsy was positive (n=10) or because insufficient nodal tissue was acquired for the study (n=4). Analysis of the vaccine response within these samples would clarify whether lymph node involvement has an undue effect on immunoglobulin or cellular response, and whether widening inclusion criteria to include said patients is desirable.

Data from human lymphatic tissue responding to vaccination is sparse. The problems implicit with such study are the confounding variables including the need for surgery during the vaccine course and the heterogeneity of the study population in terms of medical co-morbidities, medications, tumour stage, past vaccination history (as well as wider history of antigen exposure), HLA status and other factors such as post-operative complications and adjuvant treatment. By careful data collection and recruitment of significant numbers of patients I have attempted to account for these variables during my data analysis. This study has therefore provided, and will continue to provide, unique and novel information regarding the response to vaccination within human lymph nodes and increased our understanding of the role of human lymph nodes in the development of the adaptive immune response. It is anticipated that such study will compliment, validate and build upon observations made in murine studies to date, and in doing so provide a unique viewpoint into human immunobiology.

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UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Department of Cancer Sciences

Volume 2 of 2

Appendices

The role of the Lymph Node in the Establishment of an Adaptive Immune Response to Vaccination

Short Title: Lymph Nodes and Adaptive Immunity

by

Mr David Michael Layfield MRCS. AICSM. MBBS. BSc. (Hons.)

Thesis for the Degree of Doctor of Philosophy

June 2016

Table of Contents

Appendix A: Documentation relevant to study set-up and regulatory approval

1). Sponsorship application and approval

I. Trial Assessment form

CANCER RESEARCH UK | Southampton NHS University Hospitals NHS Trust | UNIVERSITY OF Southampton School of Medicine

CANCER CARE DIRECTORATE PROTOCOL REVIEW COMMITTEE

Trial Assessment Form

BACKGROUND					
Trial Name	UNDERSTANDING IMMUNITY IN LYMPH NODES.				
Principle Investigator	PROF C. OTTENSMEIER | MR R.I. CHRISTIE				
Study Phase	Phase I ☑	Phase II ☐	Phase III ☐	Other (comment)	
Design	LABORATORY ANALYSIS OF LYMPH NODE TISSUE SAMPLES FROM PATIENTS WHO HAVE RECEIVED TETANUS VACCINATION				
Primary Endpoint	- ASSESSMENT OF BLOOD AND LYMPH NODE FOR VACCINE SPECIFIC HUMORAL AND CELLULAR RESPONSE				
Secondary Endpoints	- IMMUNE RESPONSE CORRELATED TO TUMOUR STAGE / NODAL DISEASE - IMMUNE RESPONSE TO TUMOR-ASSOCIATED ANTIGENS SUCH AS CEA, HER2/NEU ETC. AS A BYSTANDER RESPONSE TO VACCINE ANTIGEN.				
Intervention/Arms	TWO STUDY GROUPS: 1) RECEIVE VACCINATION 2) NO VACCINATION RECEIVED. - COMPARISON OF HUMORAL/CELLULAR IMMUNE ACTIVITY BETWEEN GROUPS				
Sponsor	SUHT RESEARCH AND DEVELOPMENT				
Type	NIHR Portfolio ☐	NIHR commercial ☐	Academic ☑	Commercial ☐	
Funder	CANCER RESEARCH UK				
Previous External review	Yes ☐	No ☑	Reviewer:		
SUPPORT REQUIRED					
Staff (use WTE*)					
NHS Consultant Time	10 MINUTES PER CASE (0.0001 WTE)		Junior Doctor Time	ZERO	
Research Nurse Time	60 MINUTES PER CASE (0.3 WTE)		Additional Chemotherapy Nursing Time	ZERO.	
Facilities					
Where will research be conducted?	CANCER SCIENCES				
WTCRF	Yes ☐	No ☑	Number of visits to WTCRF		
Additional WTCRF nursing time?	Yes ☐	No ☑	WTCRF nursing time (WTE)		

Protocol Review Form
* WTE; whole time equivalent. This is required for SSI form. All trials must be conducted according to ICH GCP standards
Page 1 of 2

Support Departments				
Hospital Laboratories	Yes ☐	No ☑		
Radiology	Yes ☐	No ☑	Imaging modalities	
	Number of additional imaging procedures			
Pharmacy	Yes ☐	No ☑		
Pathology	Yes ☐	No ☑		
	Tumour block retrieval		Other	
Nuclear Medicine	Yes ☐	No ☑	Modality	
	Number of additional procedures			
	Who will hold ARSAC?			
Tissue Bank	Yes ☑	No ☐	- Apart study Complete	
Research lab	Yes ☑	No ☐		
EXCESS COSTS				
Drug costs	Yes ☑	No ☐	Cost of Vaccine	
Equipment costs	Yes ☐	No ☑		
Additional inpatient stay	Yes ☐	No ☑	Number of nights anticipated	
Additional outpatient visits	Yes ☑	No ☐	Number	1
Are there potential cost savings to the Care Group ?	No			
Are there resource implications for other Departments?	No			
TIMELINES				
Expected start date	January 2012		Expected date of last recruitment	January 2014
Expected Study Completion	January 2014			
ACCRUAL				
Annual recruitment	23		Total anticipated recruitment	45
Justify estimate	150 sentinel node Biopsies Performed Each Year			
Are there any studies open or in set-up that may compete for recruitment? Which studies will be prioritized in the research portfolio	No			

Protocol Review Form
* WTE; whole time equivalent. This is required for SSI form. All trials must be conducted according to ICH GCP standards
Page 2 of 2

II. Protocol Review Outcome Report

CANCER RESEARCH UK

UNIVERSITY OF Southampton
School of Medicine

Dr Ramsey Cutress
Mailpoint 824
Cancer Sciences
Somers Building
SGH
Tremona Road
Southampton

26th September 2011

Dear Ramsey

Re: Understanding Immunity in Lymph Nodes

The Protocol for this study was presented at the Cancer Sciences Division Protocol Review Meeting on 23rd August 2011.

There were no issues raised and you can now proceed for approval to undertake this study in Southampton.

Yours sincerely

Dr Andrew Davies
Honorary Consultant Medical Oncology
CRUK Senior Lecturer in Medical Oncology

Cancer Sciences Division
University of Southampton
Southampton General Hospital
Tel: 02380794546
Email: A.Davies@soton.ac.uk

Please reply to:
CRUK Research Centre, Cancer Sciences Division
School of Medicine, Southampton General Hospital, Mailpoint 824, Somers Cancer Research Building, Tremona Road, Southampton SO16 6YD United Kingdom

University of Southampton, Highfield Campus, Southampton SO17 1BJ United Kingdom
Tel: +44 (0)23 8059 5000 Fax: +44 (0)23 8059 3131 www.southampton.ac.uk

III. Sponsorship Request Form

UNIVERSITY OF Southampton | Southampton Clinical Research Institute (SCRI) | Southampton NHS University Hospitals NHS Trust

SUHT/UoS Sponsor Request Form

Version 1.4 05-09-08

NB Not for use for IVth year or Intercalated.

Section 1 – To be completed for all projects requesting SUHT to act as Sponsor[1]

1. Study title Is the project educational? Doctorate ☒ Masters ☐ n.a. ☐

Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

2. Proposed dates Start Date: 01/11/2011 End date: 01/11/2014

3. Chief Investigator contact details (for educational projects insert lead academic supervisor)

Title/Name	Prof. Christian Ottensmeier/Mr Ramsey Cutress	Principal employer: University of Southampton	
Work Address:	Cancer Sciences Division		
Department	Somers Cancer Research Building Southampton General Hospital Tremona Road, Southampton SO16 6YD	Telephone	02380796184
Email	r.i.cutress@soton.ac.uk	**CI holds SUHT Honorary Contract:** Yes ☒ Applying ☐ No ☐	

4. Principal Investigator for SUHT site contact details (for educational insert student investigator)

Title Name	Mr Ramsey Cutress	Principal employer: UoS /SUHT	
Work Address:	Cancer Sciences Division		
Department	Somers Cancer Research Building Southampton General Hospital	Telephone	02380796184
Email	r.i.cutress@soton.ac.uk	**PI holds SUHT Honorary Contract:** Yes ☒ Applying ☐ No ☐	

5. What type of Scientific Peer review has been performed?

Reviewed by:
☒ Non-commercial funder ☐ Commercial funder
☒ SUHT Care Group/Division ☐ Multi-centre research group
☐ Other, please state

6. Will the study involve SUHT i.e. staff, students, resources, premises?

Yes ☒ No ☐

[1] Not to be used for IVth year Medical Student Projects or Intercalated students. For these projects go to the IVth year Medical Students page.

Layfield - Sponsorrequestform 21082011.doc Page 1 of 2

UNIVERSITY OF Southampton | Southampton Clinical Research Institute (SCRI) | Southampton NHS University Hospitals NHS Trust

7. Will the study be managed by the Southampton Clinical Trials Unit?

Yes ☐ No ☒

8. Is this a study of a medical device(s), investigational medicinal product (IMP) or an invasive technique? *Please tick all that apply.*

Medical device ☐ IMP[2] ☐ Invasive technique ☐ None ☒

9. Is this study:

Single-centre?	Yes ☒	No ☐	
Multi-centre[3]?	Yes ☐	No ☒	If yes complete section 2
Pilot?	Yes ☐	No ☒	

Section 2 – To be completed for multi-centre studies

10. Will SUHT/UoS be the lead centre?

Yes ☐ No ☐

If No, which NHS Trust/University will be the lead centre?

11. Approximately how many centres do you anticipate becoming involved in the study? 1

Where will the sites be located: *Please tick all that apply*

Within the UK ☒ Outside UK, within EU[4] ☐ Other International ☐

Please identify non UK countries that will be participating:

Section 3 – Funding details, to be completed for all projects

12. Is the research or will the research be in receipt of any external funding?

Yes ☒ No ☐

13. If yes, which type(s) of funding organisation(s) is or will be funding the study?

National Institute of Health Research (NIHR)	☐	If NIHR which funding stream? e.g. Research for Patient Benefit	
Medical Research Council	☐	Department of Health	☐
Other Research Council	☐	Other Govt Dept/EU	☐
HEFCE	☐	Commercial Company	☐
Research Charities	☒	Other, please state:	

[2] Trials involving IMPs must obtain Clinical Trial Authorisation (CTA) from the MHRA prior to commencing. All costs associated with the CTA must be included as part of the funding application. For more information see the MHRA website.
[3] Multi-Centre Study: A study conducted according to a single protocol but carried out at more than one site and by more than one investigator.
[4] Full list of EU countries go to the UKTradeInfo site

IV. Sponsorship Approval Letter

Southampton University Hospitals NHS Trust NHS

Please reply to: Research and Development
SGH - Level E, Laboratory & Pathology Block, SCBR - MP 138
Southampton University Hospitals NHS

Telephone: 023 8079 5078
Fax: 023 8079 8678
E-mail: sharon.atwill@suht.swest.nhs.uk

Professor Christian Ottensmeier
Somers Cancer Research Building, MP 824
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

28 September 2011

Dear Professor Ottensmeier

ID: RHM CAN0817 Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

Re: NHS Research Governance and Identification of Nominated Research Sponsor

I am writing to confirm that Southampton University Hospitals NHS Trust is prepared to act, in principle, as Sponsor for this study under the terms of the Department of Health Research Governance Framework for Health and Social Care.
SUHT's final acceptance of sponsorship responsibilities is dependent on full R&D approval. We advise to seek early R&D guidance on adequate financial resources, as well as early submission of relevant NHS access permissions (Research Passport Form/Letter of Access/Honorary Contracts).

SUHT fulfills the role of research sponsor in ensuring management, monitoring and reporting arrangements for research. I understand that you will be acting as the principal investigator responsible for the daily management for this study, and that you will be providing regular reports on the progress of the study to the Trust on this basis.

I would like to take this opportunity to remind you of your responsibilities under the terms of the Research Governance Framework, that it is a requirement of the terms and conditions of approval that you become fully conversant with the Research Governance Framework on Health and Social Care document which is available from :
http://www.dh.gov.uk/en/Policyandguidance/Researchanddevelopment/index.htm

Please do not hesitate to contact R&D should you require any additional information or support.
May I also take this opportunity to wish you every success with your research.

Yours sincerely

Sharon Atwill
Research Governance Officer

Copy to: Mr Ramsey Cutress,
Mr David Layfield,

2). Radiation Protection Advice Notice following Environment Agency Approval

UNIVERSITY OF Southampton

RPA Advice Note

To: David Layfield **From:** Craig Morrissey, Radiation Protection Adviser

Date: 24 October 2011

Re: Non-sentinel lymph node acquisition

Ref:

This is to advise you that the University of Southampton is prepared to support the proposed non-sentinel lymph node trials in relation to the potential radioactive aspect of the study.

Due to the radionuclide being used in the study it is required to submit a variation in the University Environmental Permit for the use and disposal of radioactive material. Once the variation is approved by the Environment Agency the Cancer Sciences Division will be able to accept the non-sentinel lymph nodes. Subsequent use, storage and disposal will be managed by the Faculty of Medicine in accordance with University radiation management procedures.

As the potential level of radioactivity that will be transferred to the Faculty of Medicine will be low it is assessed that the standard control measures in place within the designated radiation laboratory in the cancer Sciences Division shall be suitable and sufficient. However, individuals shall be issued with personal dosimetry to monitor any potential exposure to ionising radiations and work activities shall be reviewed following the first trail.

Yours Sincerely

Craig Morrissey
Radiation Protection Adviser
Head of Radiation Protection
University of Southampton

3). NIHR CRN and REC application documentation and subsequent correspondence

I. NIHR CRN Portfolio adoption form

NIHR CRN Portfolio Application Form IRAS Version 3.2

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Understanding immunity in lymph nodes. V 1

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(●) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located?*(Tick all that apply)*

[✓] England
[ ] Scotland

1 72796/258584/20/939

NIHR CRN Portfolio Application Form IRAS Version 3.2

☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ Ministry of Justice (MoJ)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Do you want your NHS R&D application(s) to be processed through the NIHR Coordinated System for gaining NHS Permission?

◉ Yes ○ No

If yes, you must complete and submit the NIHR CSP Application Form immediately after completing this project filter, before proceeding with completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

(•) Yes ( ) No

Please describe briefly the involvement of the student(s):
Full involvement with research planning, experiment design and data analysis under close supervision

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

(•) Yes ( ) No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

( ) Yes (•) No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

( ) Yes (•) No

NIHR CRN Portfolio Application Form IRAS Version 3.2

Integrated Research Application System
Application Form for Research limited to working with human tissue samples and/or data

NIHR CSP APPLICATION FORM

Please complete some basic questions about your project to provide the information required by the Portfolio Adoption Team to assess whether your project is eligible for inclusion in the NIHR portfolio. The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Understanding immunity in lymph nodes. V 1

1. Full title of the research:

Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects

3. Please select the main subject area of research. Additional sub-topics may be selected, if required

- [ ] Age and Ageing
- [ ] Anaesthetics
- [x] Cancer (includes malignant haematology
- [ ] Cardiovascular
- [ ] Clinical
- [ ] Critical Care
- [ ] Dementias and Neurodegenerative Diseases
- [ ] Dermatology
- [ ] Diabetes
- [ ] Ear, Nose and Throat
- [ ] Gastrointestinal
- [ ] Genetics
- [ ] Health Services Research
- [ ] Hepatology
- [x] Immunology and Inflammation
- [ ] Infectious Disease and Microbiology
- [ ] Injuries and Accidents
- [ ] Medicines for Children (does not include Paediatrics)
- [ ] Mental Health
- [ ] Metabolic and Endocrine
- [ ] Musculoskeletal (Rheumatoid Arthritis is a separate category)
- [ ] Nervous System Disorders
- [ ] Non-malignant Haematology
- [ ] Ophthalmology
- [ ] Oral and Dental
- [ ] Paediatrics (does not include Medicines for Children)
- [ ] Primary Care

NIHR CRN Portfolio Application Form IRAS Version 3.2

- ☐ Public Health Research
- ☐ Renal
- ☐ Reproductive Health and Childbirth
- ☐ Respiratory
- ☐ Rheumatoid Arthritis
- ☐ Stroke
- ☑ Surgery
- ☐ Urogenital

4-1. Is this a single centre study?

◉ Yes ○ No

4-2. Where will the research take place? *(Tick as appropriate)*

- ☑ England
- ☐ Scotland
- ☐ Wales
- ☐ Northern Ireland
- ☐ Other states in European Union
- ☐ Other countries in European Economic Area
- ☐ USA
- ☐ Other international (please specify)

5-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		
Employer	University of Southampton		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Work E-mail	cho@soton.ac.uk		
* Personal E-mail	cho@soton.ac.uk		
Work Telephone	02380795161		
* Personal Telephone/Mobile			
Fax	02380795152		

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*

5-3. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

Title Forename/Initials Surname

NIHR CRN Portfolio Application Form IRAS Version 3.2

	Mrs Sharon Atwill
Address	Research Governance Officer-Division A, R&D Office
	E Level, Laboratory and Pathology Block
	SCBR - Mailpoint 138, Tremona Road, Southampton
Post Code	SO166YD
E-mail	sharon.atwill@suht.swest.nhs.uk
Telephone	02380795314
Fax	

6. Give details of the lead NHS R&D contact for this research:

Title	Forename/Initials	Surname
Mrs	Sharon	Atwill

Organisation	
Address	Research Governance Officer-Division A, R&D Office
	E Level, Laboratory and Pathology Block
	SCBR - Mailpoint 138, Tremona Road, Southampton
Post Code	SO166YD
Work Email	sharon.atwill@suht.swest.nhs.uk
Telephone	02380795314
Fax	
Mobile	

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

7. How long do you expect the study to last?

Planned start date: 01/04/2012
Planned end date: 01/04/2015

Duration:

Years:	3
Months:	0

9. Has external funding for the research been secured?

- [x] Funding secured from one or more funders
- [ ] External funding application to one or more funders in progress
- [ ] No application for external funding will be made

Please give details of funding applications.

Organisation	Cancer Research UK - Experimental Cancer Medicine Centre (ECMC) Network
Address	Angel Building
	407 St John Street
	London
Post Code	EC1V4AD
Telephone	02034695388
Fax	

Mobile
Email

Funding Application Status: (•) Secured ( ) In progress

Amount: £400,000 per annum

Duration
Years: 5
Months: 0

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?
Experimental Cancer Medicine Centre Grant (as from 01/04/2012)

What type of research project is this?

( ) Standalone project
(•) Project that is part of a programme grant
( ) Project that is part of a Centre grant
( ) Project that is part of a fellowship/ personal award/ research training award
( ) Other

Other – please state:

10. Please select the level of commercial participation in this project.

(•) None
( ) Industry funding, but not industry sponsored
( ) Industry funding and industry sponsored
( ) Industry sponsored, but not industry funded

II. NHS Research Ethics Committee Application form

NHS REC Form | Reference: 11/SC/0509 | IRAS Version 3.2

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Understanding immunity in lymph nodes. V 1

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(●) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located?*(Tick all that apply)*

[✓] England
[ ] Scotland

Date: 07/11/2011 | 1 | 72796/263866/1/607

☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ Ministry of Justice (MoJ)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Do you want your NHS R&D application(s) to be processed through the NIHR Coordinated System for gaining NHS Permission?

◉ Yes ○ No

If yes, you must complete and submit the NIHR CSP Application Form immediately after completing this project filter, before proceeding with completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

(•) Yes ( ) No

Please describe briefly the involvement of the student(s):
Full involvement with research planning, experiment design and data analysis under close supervision

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

(•) Yes ( ) No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

( ) Yes (•) No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

( ) Yes (•) No

NHS REC Form | Reference: 11/SC/0509 | IRAS Version 3.2

Integrated Research Application System
Application Form for Research limited to working with human tissue samples and/or data

National Patient Safety Agency
National Research Ethics Service

Application to NHS/HSC Research Ethics Committee

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Understanding immunity in lymph nodes. V 1

Please complete these details after you have booked the REC application for review.

REC Name:
Southampton A REC

REC Reference Number:
11/SC/0509

Submission date:
07/11/2011

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects

A2-1. Educational projects

Name and contact details of student(s):

Student 1

Title	Forename/Initials	Surname
Mr	David	Layfield

Address	Cancer Sciences Division Southampton University Hospital tremona Road, Southampton
Post Code	SO16 6YD
E-mail	david.layfield@doctors.org.uk
Telephone	07708533869
Fax	02380795152

Give details of the educational course or degree for which this research is being undertaken:
Name and level of course/ degree:
PhD

Name of educational establishment:
Southampton University

Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Mr	Ramsey	Cutress
Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
E-mail	r.i.cutress@soton.ac.uk		
Telephone	07979904339		
Fax	02380795152		

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mr David Layfield	☑ Mr Ramsey Cutress

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- ○ Student
- ○ Academic supervisor
- ◉ Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		
Employer	University of Southampton		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Work E-mail	cho@soton.ac.uk		

* Personal E-mail	cho@soton.ac.uk
Work Telephone	02380795161
* Personal Telephone/Mobile	
Fax	02380795152

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

Title	Forename/Initials	Surname
Mrs	Sharon	Atwill

Address	Research Governance Officer-Division A, R&D Office
	E Level, Laboratory and Pathology Block
	SCBR - Mailpoint 138, Tremona Road, Southampton
Post Code	SO166YD
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A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):	RMH CAN0817
Sponsor's/protocol number:	RMH CAN0817
Protocol Version:	Vs 1
Protocol Date:	15/01/2011
Funder's reference number:	C491/A11945

Project website: N/A

Additional reference number(s):

Ref.Number Description	Reference Number

Registration of research studies is encouraged wherever possible. You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you have registered your study please give details in the "Additional reference number(s)" section.

A5-2. Is this application linked to a previous study or another current application?

○ Yes ◉ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, this summary will be published on the website of the National Research Ethics Service following the ethical review.*

The body's response to "foreign" material (aka antigen; infection, vaccination or cancer) is co-ordinated by interactions between specialist cells within lymphatic tissues such as lymph nodes. Despite this our understanding of the immune system in humans is based almost entirely on studying blood samples, as lymphatic tissue is more difficult to access.

Patients undergoing treatment for breast cancer have a small number of lymph nodes (usually 2-3) removed from the armpit as a standard part of their operation; these are then studied to look for evidence of cancer spread.

Understanding how the lymph nodes contribute to and coordinate the immune response is of critical importance to the development of future vaccines including vaccines against cancer. During this study we wish to evaluate the response of lymph nodes to vaccination, and compare this with changes seen in peripheral blood samples.

To this end, we wish to recruit patients diagnosed with breast cancer in whom a limited removal of lymph nodes is planned as part of surgical treatment. We will ask for permission to record the anonymised clinical and pathology details for these patients for validation purposes. These patients will be divided into two groups according to their vaccination history; those who have not previously been immunised against a common antigen, such as tetanus or hepatitis B, will be offered vaccination a week prior to their surgery. Those who are already fully immunised will not receive additional vaccination, but instead will act as a comparison group. Blood tests will be taken before, during and after their surgery. In addition a single extra lymph node will be removed at the time of surgery for the purpose of the study.

This will allow us to compare the lymph node response to that seen in peripheral blood samples.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

The study raises the following issues:

1) Acquisition of human tissue

The study requires the removal of a lymph node from the patients' axilla (armpit) during their cancer operation. Current practice is to perform a sentinel lymph node (SLN) biopsy (SLNB) in patients with early breast cancer in whom there is no evidence of cancer spread. The SLN(s) is/are the first lymph node(s) which receive(s) drainage from the breast cancer. It is identified during the operation using a combination of radioisotope and blue dye injected around the tumour. Being the first node(s) to receive drainage from the cancer, it is accepted that the results from SLNB accurately predict the presence of tumour within the axilla.
SLNB is used because it avoids extensive removal of all lymph nodes (typically between 10 to 20 nodes) from the axilla, which is associated with arm swelling (Lymphoedema) and other complications. A breast sentinel node biopsy generally involves the removal of between two and three sentinel nodes to stage the axilla. Comparison of patient-reported quality-of-life outcomes between sentinel node biopsy and extensive removal of all lymph nodes (aka axillary clearance; AXCL) does demonstrate differences between sentinel node biopsy and AXCL but these resolve with time. Similarly the ALMANAC trial demonstrated differences in morbidity between the two surgical approaches but the rate of arm lymphoedema, as measured by mean change in arm volume, was not significantly greater with axillary clearance compared to sentinel node biopsy at 12 months. It is therefore felt that removal of a single additional node at the time of sentinel node biopsy is unlikely to cause significant additional morbidity.
The SLNB is used to stage the axilla. The removal of a single additional node which is not the sentinel node will therefore not devalue the prognostic information gathered from the SLNB. To further mitigate the risk of loosing prognostic information through the use of a non-sentinel node within our study, the study node will not be processed for the study until an analysis of the sentinel node(s) has been completed. In the event of the sentinel node biopsy proving positive for cancer spread, the study node will be surrendered back to the hospital pathologist and the patient will be removed from our study.

2) Acquisition of Blood Samples

The study requires acquisition of 4 blood samples;

The first sample will be acquired immediately prior to vaccination, during the patient's pre-operative assessment. These samples will be taken at the same time as routine pre-operative blood tests. Therefore extra samples will be taken however additional venesection will not be necessary, nor will an additional hospital/clinic visit.

The second sample will be taken at the time of surgery. This will be performed during routine venous access prior to anaesthetic. Therefore additional venesection will not be necessary, nor will an additional hospital/clinic visit.

The third sample will be taken 2 weeks following surgery. This will occur prior to the patients' routine follow up appointment. Therefore an additional hospital/clinic visit will not be necessary; however patient will undergo venesection which would not constitute a standard aspect of their treatment.

The fourth sample will be taken 5 weeks following surgery. This will require an additional clinic appointment and additional venesection which would not normally take place. However during this visit the patients will undergo an opportunistic wound review and be given an opportunity to ask questions of their clinicians/breast care specialist nurse.

3) Administration of vaccine and use of "atypical injection sites"

Patients who have an up-to-date vaccination history, and are therefore fully immunised, will not receive additional vaccination. They will still have an additional non-sentinel node removed and undergo blood sampling as detailed above.
Patients who are not immunised against a given antigen, such as tetanus toxoid, will receive immunisation during their pre-operative assessment; therefore an additional hospital/clinic visit will not be necessary. The administration of the vaccine will not directly benefit the patient in the absence of a subsequent exposure to the infective agent targeted by the vaccine. No adverse effect is predicted following vaccination with regards to their cancer treatment, which will follow a standard course.
The study protocol requires the administration of a single agent vaccine. The reasons for this are to avoid clouding of results with additional variables implicit in the use of multi-agent vaccines. The standard tetanus vaccine used within the NHS is the Diphtheria/Tetanus/Polio combined vaccine, therefore we intend to source a single agent vaccine from an approved German supplier (Impfstoff® tetanus absorbed 40iu).
The study protocol requires administration of vaccine in an area which will drain to the axilla. Standard administration of vaccine is to the deltoid (Shoulder) muscle using an intramuscular injection. This technique would not result in the vaccine draining to the lymph nodes within the axilla and therefore we would be unable to study the response of lymph nodes to vaccination by sampling the axillary node. Therefore it will be necessary to use atypical sites for inoculation. We intend to inoculate using a subcuticular injection either in the breast, inner aspect of the upper arm or forearm, where lymphatic drainage will carry the antigen to the axilla. Vaccines used will be suitable for subcuticular injection.

4) Handling of Radioisotope

Sentinel lymph node biopsy requires the administration of radioisotope. Tissue subsequently removed from the patient must be handled according to the Standard Operating Procedure (SOP) in place for radioactive tissue samples. Although the additional node removed for the study will be a non-sentinel node, and therefore, by definition, have very little radioisotope within it, the SOP will be adhered to by our study group. The hospitals Radiation Protection Officer (RPO) has been consulted during the study design and the appropriate liscences will be held by our group.

5) Anonymised clinical and pathology details

Patients included within the study will be required to give written informed consent to biopsy in theatre, storage of samples indefinitely for use in this project, recording of clinical and pathological details in a linked anonymised fashion and use of any surplus histopathological sample held by the hospital Histopathology Department following completion of normal diagnostic processing. Patients will have these issues discussed with them and will receive an information sheet. They will be given a minimum of 24 hours to consider entry. Written informed consent will then be taken prior to inclusion in this study.

A6-3. Proportionate review of REC application *The initial project filter has identified that your study may be suitable for proportionate review by a REC sub-committee. Please consult the current guidance notes from NRES and indicate whether you wish to apply through the proportionate review service or, taking into account your answer to A6-2, you consider there are ethical issues that require consideration at a full REC meeting.*

○ Yes - proportionate review ◉ No - review by full REC meeting

Further comments (optional):

Note: This question only applies to the REC application.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply:*

- [ ] Case series/ case note review
- [ ] Case control
- [ ] Cohort observation
- [x] Controlled trial without randomisation
- [ ] Cross-sectional study
- [ ] Database analysis
- [ ] Epidemiology
- [x] Feasibility/ pilot study
- [x] Laboratory study
- [ ] Metanalysis
- [x] Qualitative research
- [ ] Questionnaire, interview or observation study
- [ ] Randomised controlled trial
- [ ] Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

Response to antigen has previously been considered highly specific. Our study group has recently identified that this may not be the case; a "bystander" response, or activation of immune cells to other antigens, occurs following vaccination against a single agent.
Therefore, principle objective is to study the interaction between antigen presenting cells (APCs) and antigen specific CD4+ and CD8+ cell populations, looking particularly at how APCs promote a bystander response by memory cells specific to other antigens not present in the vaccination.

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

It is known that primary breast tumours and melanoma induce immune anergy to tumour antigens. This is thought to be through a complex mechanism which involves immune-suppressive cytokines (chemical cell signallers) and/or recruitment of immature dendritic cells (IDC) and induction of immune tolerance to the tumour via a regulatory T-cell dependant mechanism.
Therefore, secondary objective is to correlate the lymph node status of the patients with the reactivity to antigen, in order to evaluate whether immune response to vaccine is attenuated in the presence of tumour spread.

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

Our research group has shown that immunity in humans is much more dynamic than initially thought; T-cell responses against tetanus toxoid (TT) inoculation are accompanied by significant activation of T cells, mainly of a CD4+ phenotype, that were specific for other, unrelated Ags, such as proteins from Candida albicans (C. albicans) and tuberculosis.
In a follow-up study, a mouse model was used to confirm that transferred T cells, which had been pre-activated in vitro with a peptide from ovalbumin (pOVA), showed a bystander proliferative response during a parallel and unrelated memory immune response to TT. Bystander proliferation was dependent on boosting of the TT-specific memory response in the recipients, with no effect in naive mice. Bystander stimulation was also proportional to the strength of the TT-specific memory T-cell response. T cells activated in vitro displayed functional receptors for IL-2 and IL-7, suggesting these as potential mediators. These data confirm the crosstalk between a stimulated CD4+ memory T-cell response and CD4+ T cells activated by an unrelated Ag.

These observations shed light on a possible pathway by which our immune system is able to maintain immunological

T-cell memory over time as a bystander response to ongoing infections, even in the absence of exposure to the infectious agent against which protection is required.

We are currently examining whether the bystander effect observed after booster vaccination is also present during the immune response to a primary vaccine. Using healthy donors, we are evaluating bystander activation of memory CD4+ and CD8+ T cells in persons who are exposed to a hepatitis B vaccine for the first time (RHM CAN0489; REC 06/Q1702/155).

This current study will further our understanding of the mechanism of bystander maintenance of immune-memory by allowing direct evaluation of antigen-presentation within lymphatic tissue for the first time. Understanding these mechanisms might guide design of future vaccines which are better able to manipulate the immune system and promote more effective and incessant protection against infection.

In addition, knowledge of the unique immune environment of primary cancers draining lymphatics will guide the development of anti-cancer vaccines. Better understanding of the way in which cancer promotes immune anergy might shed light on therapeutic strategies to "de-cloak" tumour cells and activate immune cells to tumour-specific antigens, through novel vaccination techniques.

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

Patient Identification and Recruitment:

Eligible patients will be identified from the breast cancer SUHT outpatient clinics at Southampton General Hospital or Princess Anne Hospital by members of the Breast Cancer Multidisciplinary Team. Patient information sheets will be provided to eligible patients inviting them to consider participation. All patients will be given a minimum of 24 hours to consider entry to this study.

Inclusion Criteria:

1.Patients undergoing resection of invasive breast carcinoma (mastectomy or wide local excision) as confirmed by pre-operative core biopsy, who are to have a sentinel lymph node biopsy as part of their primary surgical procedure.
2.Aged 18 years or older
3.The ability to understand the study requirements, provide written informed consent and comply with the study

Exclusion Criteria:

1.Unwillingness or inability to consent to cancer resection or sentinel node biopsy
2.Unwillingness or inability to consent to inclusion within the study
3.Age under 18
4.Immune Modifying drugs (Including oral steroids)

Consent:

Having received prior written and verbal explanation of the study, patients will be required to provide written informed consent to participation and indefinite storage of biopsy samples for analyses relating to this study. Consent will include permission for the use of surplus histopathology samples, and the secure storage of anonymised case-linked clinical data, as detailed below. Informed consent will be obtained by the surgeon undertaking the patient's operation. Interpreters will be provided where possible for participants who are non-English speakers.

Study Numbers and Study Groups:

Participants will be divided into two groups according to their immunisation history. Upon agreeing to participate, patients will have their full immunisation history taken from them by the clinician responsible for their care. Those patients who have not previously been immunised against the study antigen (i.e. Tetanus Toxoid) will be offered an immunisation; those willing will be the immunised group (Group A). Participants who have previously received immunisation, or who are unwilling to receive immunisation, will be recruited to the control group (Group B).Group A will be further divided into patients receiving vaccination to the ipsilateral arm (i.e. the SAME side as the lymph node is removed from) (Group A1) and those receiving vaccination to the contralateral arm (Group A2). Group A2 will act as a positive control group, to ascertain whether any changes seen within the treatment group are the result of local or systemic immunological responses to vaccination.The study will require recruitiment of 30 responders into Group A1, 15 patients into Group A2 and 15 patients into Group B.

Study Protocol:

1) Patient Participation:

The study will require patients to attend a secondary care centre (SUHT outpatient clinics at Southampton General Hospital, the Royal South Hants Hospital or Princess Anne Hospital) on 4 separate occasions over a period of 6 weeks. Each blood collection will consist of 60mL anti-coagulated blood and 10mL clotted blood.

Week 0; Visit 1: Pre-assessment clinic; Consenting patients who have been recruited to either Group A or Group B attend clinic as part of their standard pre-assessment for surgery. All participants will have additional blood samples taken for study purposes at the time of their standard pre-operative screening blood tests. Additional venesection will not be necessary, nor will an additional hospital/clinic visit.
In addition, participants from Group A will receive sub-cutaneous vaccination. This will be administered at a site where the vaccine antigen will drain to the axillary lymph nodes.

Week 1; Visit 2: Day of operative procedure; during preparation for administration of an anaesthetic, patients have an intravenous cannula inserted. At this time, a second sample of blood will be taken for study purposes; therefore additional venesection will not be necessary.
During surgery, all participants will have a single additional non-sentinel lymph node removed from the axilla, following the completion of the sentinel lymph node biopsy.

Week 3; Visit 3: Post-operative follow-up clinic; at the time of routine follow up clinic appointment, a third sample of blood will be taken from study participants. An additional hospital/clinic visit will not be necessary; however patient will undergo venesection which would not constitute a standard aspect of their treatment.

Week 6; Visit 4: Additional clinic appointment; a fourth blood sample will be taken 5 weeks following surgery. This will require an additional clinic appointment and additional venesection which would not normally take place. However during this visit the patients will undergo an opportunistic wound review and be given an opportunity to ask questions of their clinicians/breast care specialist nurse.

2) Sample Handling and Analysis

Blood Samples:

Blood samples will be taken by clinic nurses, an anaesthetist or research nurses from the CRUK Clinical Research Unit and processed on the same day. Coordination between the research nurse and laboratory team regarding the timing of collections will occur prior to venesection. Peripheral blood mononuclear cells (PBMCs) will be isolated from anti-coagulated blood by centrifugation and transferred to liquid nitrogen for storage. Serum will be isolated from clotted blood samples by centrifugation and stored at -20∟C.

Sentinel Lymph Nodes:

These lymph nodes are removed for the purposes of diagnosis and staging of the axilla. They will undergo standard analysis as per standard operating procedure within the Department of Cellular Pathology, Southampton University Hospitals NHS Trust. Patients within the study will have their sentinel node samples processed on the same day of surgery.

Where possible, histopathological material (FFPE tissue blocks/slides) surplus to diagnostic requirements, that would otherwise be discarded, will be made available by the Department of Cellular Pathology, Southampton University Hospitals NHS Trust to the study. Tissue handling according to the Standard Operating Procedure for radioactive samples will be closely adhered to and monitored by the Radiation Protection Officer for Southampton University Hospitals NHS Trust.

Non-Sentinel Lymph Nodes:

Coordination between the surgical and laboratory teams regarding the timing of collections will occur prior to commencement of the surgical list. The lymph node (LN) removed for the specific purposes of the study will be stored on ice pending the results of the sentinel node biopsy. In the event of a positive sentinel node biopsy, the study node will be surrendered back to the hospital pathologist and processed as part of any subsequent completion axillary clearance.

In patients where the sentinel node biopsy proves negative, the study LN will be processed fresh on the day of surgery by mechanical dissociation into a single cell suspension and preserved in liquid nitrogen. Tissue handling according to the Standard Operating Procedure for radioactive samples will be closely adhered to and monitored by the Radiation Protection Officer for Southampton University Hospitals NHS Trust.

Storage:

Patient material collected during this study will be stored within a secure facility in the Southampton CRUK Clinical Centre. Material will be held as linked anonymised samples and labelled with a study specific number. The chief investigators and co-investigators will have access to the samples for analyses relating to this study. Patients will be asked to provide signed consent for the indefinite storage of samples for use by the investigators for analyses relating to the objectives of this study, or in the event of samples or tissue remaining on conclusion of the study, for donation to the University of Southampton Tissue bank for use in future ethically approved research.

Laboratory Analysis:

The primary tools for analysis are intended to be flow cytometry, proliferation and ELISPOT assays and ELISA, and will follow established SOPs in the Cancer Sciences Division. Assays will be performed by members of Professor Ottensmeier's group within the laboratories of the Cancer Sciences Division, University of Southampton School of Medicine. Initially, assays will focus on the following:

1. Identifying patient HLA A2 status
2. Assessment of blood and LN compartments for vaccine specific humoral and/or cellular immune responses
3. Assessment of blood and LN compartments for bystander humoral and/or cellular immune responses, for example, PPD, C.albicans, CMV, EBV, Influenza, etc.
4. Investigate immune responses to tumour-associated antigens such as CEA, HER2/neu, PASD1, WT1, MAGEA10, BAGE1, NY-ESO-1/CTAG1B, etc.
5. B-cell, NK, NKT and dendritic cell populations will be evaluated numerically, phenotypically and functionally where possible
6. FFPE tissue will be used for the analysis of proteins or genes relevant to the tumour/immune response by immunohistochemistry and gene expression, respectively; for example, the tumour markers HER2/neu, CEA, CA15.3, estrogen receptor, progesterone receptor, etc. Analysis of receptors for other key immunological molecules, such as cytokines (e.g. IFN-L, IL-2, IL-13, IL-5) or chemokines, will be undertaken where available material and existing technology allows.

Additional investigation will be directed by the results from initial studies.

3) Data Acquisition and Storage

Patient details relating to personal and histopathological characteristics will be recorded (taken from the final definitive histopathology report following surgical resection) on study specific case report forms (CRF), linked to anonymised samples by their study specific number. The CRFs will be completed by members of the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre. The Chief Investigator will retain overall responsibility for the recording and quality of the data.

Data points will include:
•Age
•Tumour grade
•Tumour size
•Presence of metastatic disease following sentinel node biopsy
•The extent of axillary nodal involvement in patients with positive sentinel node involvement who subsequently undergo completion axillary clearance as part of their management.
•Histological tumour type
•Oestrogen and progesterone receptor status
•HER2 over expression status

All essential documents including source documents will be retained for a minimum period of 15 years following the end of the study. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. The Chief Investigator and Co-Investigators
will have access to the data for analyses. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples. No personally identifying information will be released in any report or publication relating to this work.

4) End of Study Participation:
Following the 4th blood donation at week 6, patients' active participation will cease. No further collection of samples will occur beyond this point. Recording of age and histopathological details will occur once the final diagnostic histopathology report is available, typically 10-14 days following surgery.

5) Statistics and Sample Size:
Analysis endpoints will be exploratory in nature and thus formal statistical calculation of sample size has not been performed. Up to 60 patients will be recruited for the purposes of this study.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- ☐ Design of the research
- ☐ Management of the research
- ☐ Undertaking the research
- ☐ Analysis of results
- ☐ Dissemination of findings
- ☑ None of the above

Give details of involvement, or if none please justify the absence of involvement.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

1. Patients undergoing resection of invasive breast carcinoma (mastectomy or wide local excision) as confirmed by pre-operative core biopsy, who are to have a sentinel lymph node biopsy as part of their primary surgical procedure.
2. Aged 18 years or older
3. The ability to understand the study requirements, provide written informed consent and comply with the study

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

1.Unwillingness or inability to consent to cancer resection or sentinel node biopsy
2.Unwillingness or inability to consent to inclusion within the study
3.Age under 18
4.Currently taking immune modifying medication (Including oral steroids)

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Consent	1	0	10 minutes	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.
Clinic Appointments	3	2	10 minutes	Surgeon, research nurse and breast care specialist nurse

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Blood Tests	4	1	5 minutes	Surgeon or research nurse in outpatients prior to clinic appointment.
Immunisation	1	0	5	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.
Sentinel Lymph Node Biopsy	1	1	15 minutes	Surgeon in theatre
Excision of single additional non-sentinel axillary lymph node	1	0	2 minutes	Surgeon in theatre

A21. How long do you expect each participant to be in the study in total?

Each participant will be involved within the study for a total of 6 weeks from the point of recruitment (at the time of pre-assessment, initial blood sampling +/- immunisation) to the final point of contact (4th blood sample).

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Participants will be required to attend an additional outpatient clinic appointment. At this point in time they will undergo venesection. To compensate for the inconvenience of an extra clinic visit, patients will be offered an opportunistic additional wound review and be given the option of discussing any aspect of their care with either their surgeon or a Breast Care Nurse Specialist.
Participants in Group A will also receive an injection at the time of their pre-assessment clinic. Although there is no risk of harm from immunisation in terms of the patients' cancer diagnosis, a small number might experience short term discomfort or local site reactions, in line with the listed side-effects of each individual vaccine.

A24. What is the potential for benefit to research participants?

The administration of the vaccine will not directly benefit the patient in the absence of a subsequent exposure to the infective agent targeted by the vaccine.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used? *For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under*

arrangements with the responsible care organisation(s).

Patients with clinically node-negative breast cancer, who are due to undergo breast cancer resection (either mastectomy or wide local resection) and sentinel lymph node biopsy will be identified by a member of the Breast care MDT, typically the surgeon responsible for their care, during the patients visit to outpatient clinics at Southampton General Hospital or Princess Anne Hospital.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

○ Yes ◉ No

Please give details below:

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

○ Yes ◉ No

A29. How and by whom will potential participants first be approached?

The surgeon undertaking breast cancer resection (either mastectomy or wide local resection) and sentinel lymph node biopsy at the time he/she discusses the operation with the patient

A30-1. Will you obtain informed consent from or on behalf of research participants?

◉ Yes ○ No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Patient information sheets will be provided to eligible patients inviting them to consider participation. All patients will be given a minimum of 24 hours to consider entry to this study. Patients will be required to provide written informed consent to participation and indefinite storage of non-sentinel node biopsy samples and blood samples for analyses relating to this study. Consent will also be taken regarding the donation of sentinel node material surplus to diagnostic requirements for the purpose of this study. Consent will also be taken regarding the anonymised storage of case-specific data regarding patient details (e.g. age) and final histology (e.g. histological sub-type, grade, stage, hormone receptor status).

Informed consent for the surgical procedure will be obtained by the surgeon undertaking the patient's operation. Informed consent for inclusion within the study will be taken by a doctor participating within the research who is appropriately trained in consent for research purposes, or an appropriately trained and qualified research nurse.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

◉ Yes ○ No

A31. How long will you allow potential participants to decide whether or not to take part?

A minimum of 24 hours. Patients will be given information regarding the study at the time of discussion of their surgery. Consent for participation within the study will be taken at the time of their pre-assessment clinic appointment; typically patients will therefore have 1-2 weeks to consider involvement.

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters)*

Interpreters will be provided where possible for participants who are non-English speakers.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

- ○ The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.
- ◉ The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.
- ○ The participant would continue to be included in the study.
- ○ Not applicable – informed consent will not be sought from any participants in this research.
- ○ Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

If you plan to retain and make further use of identifiable data/tissue following loss of capacity, you should inform participants about this when seeking their consent initially.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)? *(Tick as appropriate)*

- ☑ Access to medical records by those outside the direct healthcare team
- ☐ Electronic transfer by magnetic or optical media, email or computer networks
- ☐ Sharing of personal data with other organisations
- ☐ Export of personal data outside the EEA
- ☐ Use of personal addresses, postcodes, faxes, emails or telephone numbers
- ☐ Publication of direct quotations from respondents
- ☐ Publication of data that might allow identification of individuals
- ☐ Use of audio/visual recording devices
- ☑ Storage of personal data on any of the following:
  - ☐ Manual files including X–rays
  - ☐ NHS computers
  - ☐ Home or other personal computers
  - ☑ University computers
  - ☐ Private company computers
  - ☐ Laptop computers

Further details:

Patient details relating to personal and histopathological characteristics will be recorded (taken from the final definitive histopathology report following surgical resection) on study specific case report forms (CRF), linked to anonymised samples by their study specific number. The CRFs will be completed by members of the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre.
Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples as per the standard operating procedure in place for this purpose (CSD/SOP/003).

A38. How will you ensure the confidentiality of personal data? *Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

Data relating to histopathological characteristics will be collected onto study specific CRFs by research staff from the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

The clinically trained staff involved in this study who are part of the patients direct healthcare team. This will include the surgeon, research nurses from the Cancer Research UK Clinical Centre and the Chief Investigator.

Storage and use of data after the end of the study

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ○ 6 – 12 months
- ○ 12 months – 3 years
- ◉ Over 3 years

If longer than 12 months, please justify:
Personal data will be accessed within a few weeks of the patient undergoing their surgical procedure. The data will be anonymised at this point. We will store and access this linked anonymised data indefinitely but will not go back to accessing identifiable data again.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

○ Yes ◉ No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

◉ Yes ○ No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

◉ Yes ○ No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50. Will the research be registered on a public database?

◉ Yes ○ No

Please give details, or justify if not registering the research.
research will be registered on the CLRN database

Registration of research studies is encouraged wherever possible.
You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you are aware of a suitable register or other method of publication, please give details. If not, you may indicate that no suitable register exists. Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- ☑ Peer reviewed scientific journals
- ☐ Internal report
- ☑ Conference presentation
- ☐ Publication on website
- ☐ Other publication
- ☐ Submission to regulatory authorities
- ☐ Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- ☐ No plans to report or disseminate the results
- ☐ Other (please specify)

A53. Will you inform participants of the results?

○ Yes ◉ No

Please give details of how you will inform participants or justify if not doing so.
Patients will be informed that the research will be published in the scientific literature. The information sheet provides contact numbers for the researchers involved if they wish to contact us about findings which we would of course be happy to provide. Beyond this we do not propose to actively contact them.

5. Scientific and Statistical Review

A54. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi–centre research group
- ☑ Review within the Chief Investigator's institution or host organisation
- ☑ Review within the research team
- ☐ Review by educational supervisor
- ☐ Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The project has been reviewed within the Cancer Sciences Division and additionally reviewed by the research team.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- ☐ Review by independent statistician commissioned by funder or sponsor
- ☐ Other review by independent statistician
- ☐ Review by company statistician
- ☐ Review by a statistician within the Chief Investigator's institution
- ☐ Review by a statistician within the research team or multi–centre group
- ☐ Review by educational supervisor
- ☐ Other review by individual with relevant statistical expertise
- ☑ No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Department	MD PhD FRCP		
Institution	University of Southampton		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Telephone	02380795161		
Fax	02380795152		
Mobile			
E-mail	cho@soton.ac.uk		

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Qualitative description of immunological events in the lymph node and blood to vaccination

A58. What are the secondary outcome measures? *(if any)*

The effect of stage/lymphatic spread of primary malignancy on the immunological response to vaccination witnessed within lymph nodes and peripheral blood.

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size:	60
Total international sample size (including UK):	60
Total in European Economic Area:	60

Further details:
Group A1 (N=30) Receiving vaccine in same arm as operation is to occur (Study Group)
Group A2 (N=15) Receiving vaccine into contralateral arm (Control group; to differentiate between systemic and local effects of vaccine)
Group B (N=15) Not receiving vaccine

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

Analysis endpoints will be exploratory in nature and thus formal statistical calculation of sample size has not been performed.

A61. Will participants be allocated to groups at random?

○ Yes ◉ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Simple descriptive statistics will be used to analyse and present the data from this study.

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co–applicants, protocol co–authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		
Employer	University of Southampton		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Telephone	02380795161		
Fax	02380795152		

Mobile	
Work Email	cho@soton.ac.uk

Title	Forename/Initials	Surname
Mr	Ramsey	Cutress

Post	Senior Lecturer and Consultant Surgeon
Qualifications	FRCS PhD MBBS
Employer	University of Southampton, Southampton University Hospital NHS Trust
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	07979904339
Fax	02380795152
Mobile	07979904339
Work Email	r.i.cutress@soton.ac.uk

Title	Forename/Initials	Surname
Mr	David	Layfield

Post	Research Fellow
Qualifications	MRCS AICSM MBBS BSc (Hons)
Employer	RHCH NHS Trust
Work Address	Cancer Sciences Division Southampton University Hospital tremona Road, Southampton
Post Code	SO16 6YD
Telephone	07708533869
Fax	02380795152
Mobile	07708533869
Work Email	david.layfield@doctors.org.uk

Title	Forename/Initials	Surname
Dr	Gianfranco	Di Genova

Post	Research Fellow
Qualifications	PhD
Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097
Work Email	g.di-genova@soton.ac.uk

Title	Forename/Initials	Surname
Dr	Kathy	McCann

Post	Research Fellow
Qualifications	PhD

Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097
Work Email	kjm8@soton.ac.uk

Title	Forename/Initials	Surname
Miss	Lindsey	Chudley

Post	Research Fellow
Qualifications	BSc
Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097
Work Email	l.chudley@soton.ac.uk

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status:
- (●) NHS or HSC care organisation
- ( ) Academic
- ( ) Pharmaceutical industry
- ( ) Medical device industry
- ( ) Local Authority
- ( ) Other social care provider (including voluntary sector or private organisation)
- ( ) Other

Commercial status: Non-Commercial

If Other, please specify:

Contact person

Name of organisation	Southampton University Hospitals NHS Trust
Given name	Sharon
Family name	Atwill
Address	Research Governance Officer-Division A, R&D Office
Town/city	E Level, Laboratory and Pathology Block
Post code	SO166YD
Country	UNITED KINGDOM

Telephone	02380795314
Fax	
E-mail	sharon.atwill@suht.swest.nhs.uk

Is the sponsor based outside the UK?
○ Yes ◉ No

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

A65. Has external funding for the research been secured?

☑ Funding secured from one or more funders
☐ External funding application to one or more funders in progress
☐ No application for external funding will be made

Please give details of funding applications.

Organisation	Cancer Research UK - Experimental Cancer Medicine Centre (ECMC) Network
Address	Angel Building 407 St John Street London
Post Code	EC1V4AD
Telephone	02034695388
Fax	
Mobile	
Email	

Funding Application Status: ◉ Secured ○ In progress

Amount: £400,000 per annum

Duration
Years: 5
Months: 0

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?
Experimental Cancer Medicine Centre Grant (as from 01/04/2012)
What type of research project is this?

○ Standalone project
◉ Project that is part of a programme grant
○ Project that is part of a Centre grant
○ Project that is part of a fellowship/ personal award/ research training award
○ Other

Other – please state:

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another

country?

◯ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
	Mrs	Sharon	Atwill
Organisation			
Address	Research Governance Officer-Division A, R&D Office E Level, Laboratory and Pathology Block SCBR - Mailpoint 138, Tremona Road, Southampton		
Post Code	SO166YD		
Work Email	sharon.atwill@suht.swest.nhs.uk		
Telephone	02380795314		
Fax			
Mobile			

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 01/04/2012
Planned end date: 01/04/2015
Total duration:
Years: 3 Months: 0 Days: 0

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)

The Last visit of the last patient undergoing the trial

A71-2. Where will the research take place? *(Tick as appropriate)*

- ☑ England
- ☐ Scotland
- ☐ Wales
- ☐ Northern Ireland
- ☐ Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
◯ Yes ◉ No

A72. What host organisations (NHS or other) in the UK will be responsible for the research sites? *Please indicate the type of organisation by ticking the box and give approximate numbers of planned research sites:*

☑ NHS organisations in England	1
☐ NHS organisations in Wales	
☐ NHS organisations in Scotland	
☐ HSC organisations in Northern Ireland	
☐ GP practices in England	
☐ GP practices in Wales	
☐ GP practices in Scotland	
☐ GP practices in Northern Ireland	
☐ Social care organisations	
☐ Phase 1 trial units	
☐ Prison establishments	
☐ Probation areas	
☐ Independent hospitals	
☐ Educational establishments	
☐ Independent research units	
☐ Other (give details)	
Total UK sites in study:	1

A76. Insurance/ indemnity to meet potential legal liabilities

***Note:** in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland*

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

☑ NHS indemnity scheme will apply (NHS sponsors only)
☐ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

☑ NHS indemnity scheme will apply (protocol authors with NHS contracts only)
☐ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

- [x] NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)
- [ ] Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood samples will be taken from participants, as well as lymph node samples as described in Part A.

2. Who will collect the samples?

Qualified practice/research nurse (First blood sample), Anaesthetist (Second blood sample), Research nurse (3rd and 4th blood samples) and Consultant Breast Surgeon (Lymph node samples).

3. Who will the samples be removed from?

- [x] Living donors
- [ ] The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?
(●) Yes ( ) No

In future research?
(●) Yes ( ) No ( ) Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

( ) Yes (●) No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*
( ) Yes (●) No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
(●) Yes ( ) No

If Yes, say who will have access to the code and personal information about the donor.

The chief investigator and a CRUK research nurse who will maintain the case report forms for patients within the study.

In a form in which the donor could be identifiable to researchers?
○ Yes ◉ No

9. What types of test or analysis will be carried out on the samples?

The primary tools for analysis are intended to be flow cytometry, proliferation and ELISPOT assays and ELISA, and will follow established SOPs in the Cancer Sciences Division. Assays will be performed by members of Professor Ottensmeier's group within the laboratories of the Cancer Sciences Division, University of Southampton School of Medicine.

Primary laboratory analyses:
1. Identify patient HLA A2 status
2. Assessment of blood and LN compartments for vaccine specific humoral and/or cellular immune responses
3. Assessment of blood and LN compartments for bystander humoral and/or cellular immune responses, for example, PPD, C.albicans, CMV, EBV, Influenza, etc.
4. Investigate immune responses to tumour-associated Ags, such as CEA, HER2/neu, PASD1, WT1, MAGEA10, BAGE1, NY-ESO-1/CTAG1B, etc.
5. B-cell, NK, NKT and dendritic cell populations will be evaluated numerically, phenotypically and functionally where possible
6. FFPE tissue will be used for the analysis of proteins or genes relevant to the tumour/immune response by immunohistochemistry and gene expression, respectively; for example, the tumour markers HER2/neu, CEA, CA15.3, estrogen receptor, progesterone receptor, etc. Analysis of receptors for other key immunological molecules, such as cytokines (e.g. IFN-ι, IL-2, IL-13, IL-5) or chemokines, will be undertaken where available material and existing technology allows.

10. Will the research involve the analysis or use of human DNA in the samples?

○ Yes ◉ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Tissue samples obtained within this study will be stored within a secure facility within the Southampton Cancer Research UK Centre. Samples will be held as linked anonymised samples and labelled by a study specific number. The Chief Investigator and Co-Investigators will have access to the samples for analyses relating to this study. Patients will be asked to provide signed consent for the indefinite storage of samples for use by the Investigators for analyses relating to the objectives of this study.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☑ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

☑ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☐ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

Details of intended tissue bank:

The University of Southampton Research Tissue Bank (Human Tissue Act License: 12009, Southampton and South West Hampshire Research Ethics Committee: 280/99)

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.*

Research site		Investigator/ Collaborator/ Contact	
Institution name	Southampton University Hospitals NHS Trust	Title	Professor
Department name	Cancer Sciences Center	First name/ Initials	Christian
Street address	Tremona Road	Surname	Ottensmeier
Town/city	Southampton		
Post Code	SO166YD		

PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.

4. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendment.

5. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.

6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2006.

7. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.

8. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 1998.

9. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:

    - Will be held by the main REC or the GTAC (as applicable) until at least 3 years after the end of the study; and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
    - May be disclosed to the operational managers of review bodies, or the appointing authority for the main REC, in order to check that the application has been processed correctly or to investigate any complaint.
    - May be seen by auditors appointed to undertake accreditation of RECs.
    - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

10. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

11. I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

Contact point for publication *(Not applicable for R&D Forms)*

NRES would like to include a contact point with the published summary of the study for those wishing to seek further information. We would be grateful if you would indicate one of the contact points below.

- [x] Chief Investigator
- [ ] Sponsor
- [ ] Study co-ordinator

☐ Student
☐ Other – please give details
☐ None

Access to application for training purposes *(Not applicable for R&D Forms)*
Optional – please tick as appropriate:

☐ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

This section was signed electronically by Professor Christian Ottensmeier on 29/10/2011 13:13.

Job Title/Post:	Professor
Organisation:	University of Southampton
Email:	cho@soton.ac.uk
Signature:	.....
Print Name:	
Date:	*(dd/mm/yyyy)*

D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co−sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.
3. Any necessary indemnity or insurance arrangements, as described in question A76, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.
4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
6. The duties of sponsors set out in the Research Governance Framework for Health and Social Care will be undertaken in relation to this research.
7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

This section was signed electronically by Mrs Hope Howard on 31/10/2011 14:02.

Job Title/Post:	Research Governance Officer
Organisation:	University Hospital Southampton NHS Foundation Trust
Email:	hope.howard@suht.swest.nhs.uk

NHS REC Form | Reference: 11/SC/0509 | IRAS Version 3.2

D3. Declaration for student projects by academic supervisor(s)

1. I have read and approved both the research proposal and this application. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level.

2. I undertake to fulfil the responsibilities of the supervisor for this study as set out in the Research Governance Framework for Health and Social Care.

3. I take responsibility for ensuring that this study is conducted in accordance with the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research, in conjunction with clinical supervisors as appropriate.

4. I take responsibility for ensuring that the applicant is up to date and complies with the requirements of the law and relevant guidelines relating to security and confidentiality of patient and other personal data, in conjunction with clinical supervisors as appropriate.

Academic supervisor 1

This section was signed electronically by Mr Ramsey Cutress on 25/10/2011 17:32.

Job Title/Post:	Senior Lecturer and Consultant Surgeon
Organisation:	University of Southampton and Southampton University Hospitals Trust
Email:	r.i.cutress@soton.ac.uk

III. REC Provisional Opinion Document

NHS
National Research Ethics Service

NRES Committee South Central - Southampton A
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1381
Facsimile: 0117 342 0445

28 December 2011

Christian Ottensmeier

MP 824, Somers Cancer Research Building, Southampton General Hospital
Southampton
SO16 6YD

Dear Ottensmeier

Study Title:	**Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects**
REC reference number:	**11/SC/0509**
Protocol number:	**RMH CAN0817**

The Research Ethics Committee reviewed the above application at the meeting held on 13 December 2011. Thank you for attending to discuss the study.

Documents reviewed

The documents reviewed at the meeting were:

Document	*Version*	*Date*
Covering Letter		31 October 2011
Evidence of insurance or indemnity	Sponsor Insurance	01 August 2011
GP/Consultant Information Sheets	5	31 October 2011
Investigator CV		29 March 2010
Letter from Sponsor		28 September 2011
Other: CV- Mr Ramsey Cutress		07 November 2011
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Participant Consent Form	5	31 October 2011
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Protocol	5	31 October 2011
REC application	1	07 November 2011
Referees or other scientific critique report		26 September 2011
Summary/Synopsis	5	31 October 2011

Ethical issues raised, resolved or noted in preliminary discussion

1. The Committee thought the researchers should exclude potential participants that had hypersensitivity to tetanus toxoid.

2. The Committee felt that it hadn't been explained clearly enough as to why it was necessary to conduct two extra blood tests.

3. The patient information sheet was clear, however, it was a little vague in explaining how participants would be assigned to each arm.

4. It was agreed that the risks to participants appeared minimal.

5. The Committee queried whether a sensitivity test to the toxoid was required, but concluded that this would ruin the results.

6. The Committee queried whether this study would be better classified as a CTIMP.

Ethical issues raised by the Committee in private discussion, together responses given by the researcher

1. The Committee asked the CI whether a hepatitis injection would also be given to participants.

   The CI confirmed that it would only be a tetanus injection.

2. As participants would be asked to attend clinic outside their routine visits, clarity was needed as to whether participants would be offered travel expenses.

   The CI confirmed that they would.

3. Clarification was needed in regards to the recruitment process.

   The CI explained that this study would be done in collaboration with breast surgeons. Participants would already have been diagnosed at the point when they would be introduced to the research. Participants would then be consented when they came in for reassessment. The researcher stated that there would be a common path of entry for participants. However, the feasibility had not been tested yet.

4. The Committee needed clarification as to whether researchers would inform participants' GPs as there was no option for this in the consent form.

   The CI confirmed that GPs would be contacted.

5. Committee queried the research as to the duration between participants' diagnoses and consent.

The CI confirmed that these are patients who would already know their diagnosis.

6. The CI was asked whether it was necessary for the research team to send participant nodes back to the pathology department and how this would be communicated to the participants.

   The CI stated that during surgery, an extra lymph node would be taken, although this would not be the sentinel node, i.e. the node that showed radioactive isotope first there is a remote possibility of it being positive for tumor cells even when the sentinel node was negative. For this reason, they plan to take a section and look for tumor cells. If it was positive they would return the node to pathology to help stage the tumor.

7. The CI was asked whether information should be included in the PIS to inform the participant that researchers may not use their nodes.

   The CI outlined that the participants would be informed when they returned for their results.

8. The CI was asked to explain how participants would be categorised into each arm.

   The CI stated that they had toyed with the idea of randomising participants, but thought this to be pragmatic. The team had settled on asking which group the participant wanted to be in. The Committee did not think that the method to allocate participants was defined enough.

9. The Committee queried what the extra blood test would be used for.

   The CI clarified that extra blood would be taken to study the immunes response.
10. The Committee thought that the level of discomfort to the participants had been underestimated by the researcher and this could be outlined a little clearer in the PIS.

Provisional opinion

The Committee is unable to give an ethical opinion on the basis of the information and documentation received so far. Before confirming its opinion, the Committee requests that you provide the further information set out below.

The Committee delegated authority to confirm its final opinion on the application to the Chair.

Further information or clarification required

Requirements:

1. In reference to the participant information sheet:

   a) Please include details about samples being stored in a tissue bank.
   b) If hepatitis is not being administered, please remove this.
   c) Include travel expense details.

d) Make participants aware that their lymph nodes may not be used for the study.

2. In reference to the Consent form:

   a) Make it an optional point for participants' samples to be transferred to a tissue bank for future studies.
   b) Create a point that allows participants to consent to their GPs being informed.

3. Clarity is needed about the method used to allocate participants to a particular arm. The Committee did not see this as an ethical issue, but something is needed in the PIS to reflect that participants have an option.

When submitting your response to the Committee, please send revised documentation where appropriate underlining or otherwise highlighting the changes you have made and giving revised version numbers and dates.

If the committee has asked for clarification or changes to any answers given in the application form, please do not submit a revised copy of the application form; these can be addressed in a covering letter to the REC.

The Committee will confirm the final ethical opinion within a maximum of 60 days from the date of initial receipt of the application, excluding the time taken by you to respond fully to the above points. A response should be submitted by no later than 26 April 2012.

The Committee nominated the Coordinator, Mrs Maxine Knight to be the point of contact should you require further clarification upon receipt of this letter.

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/SC/0509	**Please quote this number on all correspondence**

Yours sincerely

pp

Dr Iain MacIntosh
Chair

Email: scsha.SWHRECA@nhs.net

IV. MHRA – Confirmation that the study does not constitute a CTIMP

From: "Clinical Trial Helpline" <ctdhelpline@mhra.gsi.gov.uk>
Date: 15 December 2011 14:24:18 GMT
To: "David Layfield" <david.layfield@[redacted]>
Cc: <scsha.SWHRECA@nhs.net>, <r.l.cutress@soton.ac.uk>, <cho@soton.ac.uk>
Subject: RE: SCOPE 11/SC/0509 - Understanding immunity in lymph nodes. V 1

Notification that a Clinical Trial Authorisation (CTA) is not required

Dear Dr Layfield

Thank you for your email dated 08 December 2011.

I can confirm that your proposal is not a Clinical Trial of an Investigational Medicinal Product (IMP) as defined by the EU Directive 2001/20/EC and no submission to the Clinical Trials Unit at the MHRA is required.

Kind regards

Clinical Trial Helpline
MHRA

-----Original Message-----
From: David Layfield [mailto:david.layfield@[redacted]]
Sent: 08 December 2011 16:29
To: Clinical Trial Helpline
Cc: scsha.SWHRECA@nhs.net; r.l.cutress@soton.ac.uk; cho@soton.ac.uk
Subject: SCOPE 11/SC/0509 - Understanding immunity in lymph nodes. V 1
Importance: High

Dear Sir/Madam,

Please could you confirm the attached protocol does not constitute a clinical trial. The application for the study is due for ethics board review on the 13th December (Southampton A REC), so if possible, a response before this time would be greatly appreciated.

I have included correspondance I have received from the NRES Committee South Central below. The protocol has been flagged for possibly being applicable to Section D1 of the CTIMP algorithum.

Briefly, we intend to use a vaccine (tetanus or hepatitis) to induce an immune response. It is the immune response that we are studying. The efficacy of the vaccine is not under question (the efficacy is considered proven as the products are all in common use). We have no intention of trialling the vaccinations and comparison of efficacy of medicines is not our intention.

I am more than happy to answer/clarify any points raised and can be contacted through this email address or via my mobile - 0[redacted].

Once again, thank you for your time.

Yours Faithfully,

Dr David M Layfield

On Thu, 8 Dec 2011 15:29:20 +0000
"SWHRECA (SOUTH CENTRAL STRATEGIC HEALTH AUTHORITY)"
<scsha.SWHRECA@nhs.net> wrote:
>Dear David Layfield.
>
>Thank you for your research application. However, it has been picked up that the study possibly qualifies as a CTIMP. Please can you notify me whether this has been passed by the MHRA. If not, please can you get some form of written confirmation from the MHRA whether this is indeed a CTIMP or not.

>
>I have spoken with Miss Lindsey Chudley who is going to look into the matter.
>
>Thank you kindly
>
>
>
>Maxine Knight | Research Coordinator | NRES Committee South Central -
>Southampton A National Research Ethics Service (NRES)
>

V. REC Favourable Opinion Document

NHS
National Research Ethics Service

NRES Committee South Central - Southampton A
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1381
Facsimile: 0117 342 0445

12 March 2012

Professor Christian Ottensmeier
University of Southampton
MP 824,
Somers Cancer Research Building,
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Study title:	**Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects**
REC reference:	**11/SC/0509**
Protocol number:	**RMH CAN0817**

Thank you for your email dated 09 February 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of

the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		31 October 2011
Evidence of insurance or indemnity	Sponsor Insurance	01 August 2011
GP/Consultant Information Sheets	5.1	05 February 2012
Investigator CV		29 March 2010
Letter from Sponsor		28 September 2011
Other: CV- Mr Ramsey Cutress		07 November 2011
Other: CV- Mr David Layfield		07 November 2011
Other: R&D Project peer to peer report		23 August 2011
Participant Consent Form	5.1	05 February 2012
Participant Information Sheet	5.1	08 January 2012
Protocol	5	31 October 2011
REC application	1	07 November 2011
REC application		
Referees or other scientific critique report		26 September 2011
Response to Request for Further Information		
Summary/Synopsis	5	31 October 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/SC/0509	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp

Dr Iain MacIntosh
Chair

Email: scsha.SWHRECA@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mrs Sharon Atwill, University Hospital Southampton Foundation Trust*

4). Research and Development application documentation

I. R&D application form

NHS R&D Form IRAS Version 3.2

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Understanding immunity in lymph nodes. V 1

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(●) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located?*(Tick all that apply)*

[✓] England
[ ] Scotland

1 72796/264051/14/364

☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ Ministry of Justice (MoJ)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Do you want your NHS R&D application(s) to be processed through the NIHR Coordinated System for gaining NHS Permission?

◉ Yes ○ No

If yes, you must complete and submit the NIHR CSP Application Form immediately after completing this project filter, before proceeding with completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

(•) Yes ( ) No

Please describe briefly the involvement of the student(s):
Full involvement with research planning, experiment design and data analysis under close supervision

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

(•) Yes ( ) No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

( ) Yes (•) No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

( ) Yes (•) No

Integrated Research Application System
Application Form for Research limited to working with human tissue samples and/or data

NHS/HSC R&D Form (project information)

Please refer to the Submission and Checklist tabs for instructions on submitting R&D applications.

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Understanding immunity in lymph nodes. V 1

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects

A2-1. Educational projects

Name and contact details of student(s):

Student 1

	Title	Forename/Initials	Surname
	Mr	David	Layfield
Address	Cancer Sciences Division		
	Southampton University Hospital		
	tremona Road, Southampton		
Post Code	SO16 6YD		
E-mail	david.layfield@doctors.org.uk		
Telephone	07708533869		
Fax	02380795152		

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD

Name of educational establishment:
Southampton University

Name and contact details of academic supervisor(s):

Academic supervisor 1

NHS R&D Form IRAS Version 3.2

	Title	Forename/Initials	Surname
	Mr	Ramsey	Cutress
Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
E-mail	r.i.cutress@soton.ac.uk		
Telephone	07979904339		
Fax	02380795152		

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Mr David Layfield	☑ Mr Ramsey Cutress

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- ○ Student
- ○ Academic supervisor
- ◉ Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		
Employer	University of Southampton		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Work E-mail	cho@soton.ac.uk		
* Personal E-mail	cho@soton.ac.uk		
Work Telephone	02380795161		
* Personal Telephone/Mobile			
Fax	02380795152		

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

	Title	Forename/Initials	Surname
	Mrs	Sharon	Atwill
Address	Research Governance Officer-Division A, R&D Office		
	E Level, Laboratory and Pathology Block		
	SCBR - Mailpoint 138, Tremona Road, Southampton		
Post Code	SO166YD		
E-mail	sharon.atwill@suht.swest.nhs.uk		
Telephone	02380795314		
Fax			

A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):	RMH CAN0817
Sponsor's/protocol number:	RMH CAN0817
Protocol Version:	Vs 1
Protocol Date:	15/01/2011
Funder's reference number:	C491/A11945

Project website: N/A

Additional reference number(s):

Ref.Number Description	Reference Number

Registration of research studies is encouraged wherever possible. You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you have registered your study please give details in the "Additional reference number(s)" section.

A5-2. Is this application linked to a previous study or another current application?

○ Yes ◉ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, this summary will be published on the website of the National Research Ethics Service following the ethical review.*

The body's response to "foreign" material (aka antigen; infection, vaccination or cancer) is co-ordinated by interactions between specialist cells within lymphatic tissues such as lymph nodes. Despite this our understanding of the immune system in humans is based almost entirely on studying blood samples, as lymphatic tissue is more difficult to access.

Patients undergoing treatment for breast cancer have a small number of lymph nodes (usually 2-3) removed from the armpit as a standard part of their operation; these are then studied to look for evidence of cancer spread.

Understanding how the lymph nodes contribute to and coordinate the immune response is of critical importance to the development of future vaccines including vaccines against cancer. During this study we wish to evaluate the response

of lymph nodes to vaccination, and compare this with changes seen in peripheral blood samples.

To this end, we wish to recruit patients diagnosed with breast cancer in whom a limited removal of lymph nodes is planned as part of surgical treatment. We will ask for permission to record the anonymised clinical and pathology details for these patients for validation purposes. These patients will be divided into two groups according to their vaccination history; those who have not previously been immunised against a common antigen, such as tetanus or hepatitis B, will be offered vaccination a week prior to their surgery. Those who are already fully immunised will not receive additional vaccination, but instead will act as a comparison group. Blood tests will be taken before, during and after their surgery. In addition a single extra lymph node will be removed at the time of surgery for the purpose of the study.

This will allow us to compare the lymph node response to that seen in peripheral blood samples.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

The study raises the following issues:

1) Acquisition of human tissue

The study requires the removal of a lymph node from the patients' axilla (armpit) during their cancer operation. Current practice is to perform a sentinel lymph node (SLN) biopsy (SLNB) in patients with early breast cancer in whom there is no evidence of cancer spread. The SLN(s) is/are the first lymph node(s) which receive(s) drainage from the breast cancer. It is identified during the operation using a combination of radioisotope and blue dye injected around the tumour. Being the first node(s) to receive drainage from the cancer, it is accepted that the results from SLNB accurately predict the presence of tumour within the axilla.
SLNB is used because it avoids extensive removal of all lymph nodes (typically between 10 to 20 nodes) from the axilla, which is associated with arm swelling (Lymphoedema) and other complications. A breast sentinel node biopsy generally involves the removal of between two and three sentinel nodes to stage the axilla. Comparison of patient-reported quality-of-life outcomes between sentinel node biopsy and extensive removal of all lymph nodes (aka axillary clearance; AXCL) does demonstrate differences between sentinel node biopsy and AXCL but these resolve with time. Similarly the ALMANAC trial demonstrated differences in morbidity between the two surgical approaches but the rate of arm lymphoedema, as measured by mean change in arm volume, was not significantly greater with axillary clearance compared to sentinel node biopsy at 12 months. It is therefore felt that removal of a single additional node at the time of sentinel node biopsy is unlikely to cause significant additional morbidity.
The SLNB is used to stage the axilla. The removal of a single additional node which is not the sentinel node will therefore not devalue the prognostic information gathered from the SLNB. To further mitigate the risk of loosing prognostic information through the use of a non-sentinel node within our study, the study node will not be processed for the study until an analysis of the sentinel node(s) has been completed. In the event of the sentinel node biopsy proving positive for cancer spread, the study node will be surrendered back to the hospital pathologist and the patient will be removed from our study.

2) Acquisition of Blood Samples

The study requires acquisition of 4 blood samples;

The first sample will be acquired immediately prior to vaccination, during the patient's pre-operative assessment. These samples will be taken at the same time as routine pre-operative blood tests. Therefore extra samples will be taken however additional venesection will not be necessary, nor will an additional hospital/clinic visit.

The second sample will be taken at the time of surgery. This will be performed during routine venous access prior to anaesthetic. Therefore additional venesection will not be necessary, nor will an additional hospital/clinic visit.

The third sample will be taken 2 weeks following surgery. This will occur prior to the patients' routine follow up appointment. Therefore an additional hospital/clinic visit will not be necessary; however patient will undergo venesection which would not constitute a standard aspect of their treatment.

The fourth sample will be taken 5 weeks following surgery. This will require an additional clinic appointment and

additional venesection which would not normally take place. However during this visit the patients will undergo an opportunistic wound review and be given an opportunity to ask questions of their clinicians/breast care specialist nurse.

3) Administration of vaccine and use of "atypical injection sites"

Patients who have an up-to-date vaccination history, and are therefore fully immunised, will not receive additional vaccination. They will still have an additional non-sentinel node removed and undergo blood sampling as detailed above.
Patients who are not immunised against a given antigen, such as tetanus toxoid, will receive immunisation during their pre-operative assessment; therefore an additional hospital/clinic visit will not be necessary. The administration of the vaccine will not directly benefit the patient in the absence of a subsequent exposure to the infective agent targeted by the vaccine. No adverse effect is predicted following vaccination with regards to their cancer treatment, which will follow a standard course.
The study protocol requires the administration of a single agent vaccine. The reasons for this are to avoid clouding of results with additional variables implicit in the use of multi-agent vaccines. The standard tetanus vaccine used within the NHS is the Diphtheria/Tetanus/Polio combined vaccine, therefore we intend to source a single agent vaccine from an approved German supplier (Impfstoff® tetanus absorbed 40iu).
The study protocol requires administration of vaccine in an area which will drain to the axilla. Standard administration of vaccine is to the deltoid (Shoulder) muscle using an intramuscular injection. This technique would not result in the vaccine draining to the lymph nodes within the axilla and therefore we would be unable to study the response of lymph nodes to vaccination by sampling the axillary node. Therefore it will be necessary to use atypical sites for inoculation. We intend to inoculate using a subcuticular injection either in the breast, inner aspect of the upper arm or forearm, where lymphatic drainage will carry the antigen to the axilla. Vaccines used will be suitable for subcuticular injection.

4) Handling of Radioisotope

Sentinel lymph node biopsy requires the administration of radioisotope. Tissue subsequently removed from the patient must be handled according to the Standard Operating Procedure (SOP) in place for radioactive tissue samples. Although the additional node removed for the study will be a non-sentinel node, and therefore, by definition, have very little radioisotope within it, the SOP will be adhered to by our study group. The hospitals Radiation Protection Officer (RPO) has been consulted during the study design and the appropriate liscences will be held by our group.

5) Anonymised clinical and pathology details

Patients included within the study will be required to give written informed consent to biopsy in theatre, storage of samples indefinitely for use in this project, recording of clinical and pathological details in a linked anonymised fashion and use of any surplus histopathological sample held by the hospital Histopathology Department following completion of normal diagnostic processing. Patients will have these issues discussed with them and will receive an information sheet. They will be given a minimum of 24 hours to consider entry. Written informed consent will then be taken prior to inclusion in this study.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply:*

- [ ] Case series/ case note review
- [ ] Case control
- [ ] Cohort observation
- [x] Controlled trial without randomisation
- [ ] Cross-sectional study
- [ ] Database analysis
- [ ] Epidemiology
- [x] Feasibility/ pilot study
- [x] Laboratory study
- [ ] Metanalysis
- [x] Qualitative research
- [ ] Questionnaire, interview or observation study

☐ Randomised controlled trial
☐ Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

Response to antigen has previously been considered highly specific. Our study group has recently identified that this may not be the case; a "bystander" response, or activation of immune cells to other antigens, occurs following vaccination against a single agent.
Therefore, principle objective is to study the interaction between antigen presenting cells (APCs) and antigen specific CD4+ and CD8+ cell populations, looking particularly at how APCs promote a bystander response by memory cells specific to other antigens not present in the vaccination.

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

It is known that primary breast tumours and melanoma induce immune anergy to tumour antigens. This is thought to be through a complex mechanism which involves immune-suppressive cytokines (chemical cell signallers) and/or recruitment of immature dendritic cells (IDC) and induction of immune tolerance to the tumour via a regulatory T-cell dependant mechanism.
Therefore, secondary objective is to correlate the lymph node status of the patients with the reactivity to antigen, in order to evaluate whether immune response to vaccine is attenuated in the presence of tumour spread.

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

Our research group has shown that immunity in humans is much more dynamic than initially thought; T-cell responses against tetanus toxoid (TT) inoculation are accompanied by significant activation of T cells, mainly of a CD4+ phenotype, that were specific for other, unrelated Ags, such as proteins from Candida albicans (C. albicans) and tuberculosis.
In a follow-up study, a mouse model was used to confirm that transferred T cells, which had been pre-activated in vitro with a peptide from ovalbumin (pOVA), showed a bystander proliferative response during a parallel and unrelated memory immune response to TT. Bystander proliferation was dependent on boosting of the TT-specific memory response in the recipients, with no effect in naive mice. Bystander stimulation was also proportional to the strength of the TT-specific memory T-cell response. T cells activated in vitro displayed functional receptors for IL-2 and IL-7, suggesting these as potential mediators. These data confirm the crosstalk between a stimulated CD4+ memory T-cell response and CD4+ T cells activated by an unrelated Ag.

These observations shed light on a possible pathway by which our immune system is able to maintain immunological T-cell memory over time as a bystander response to ongoing infections, even in the absence of exposure to the infectious agent against which protection is required.

We are currently examining whether the bystander effect observed after booster vaccination is also present during the immune response to a primary vaccine. Using healthy donors, we are evaluating bystander activation of memory CD4+ and CD8+ T cells in persons who are exposed to a hepatitis B vaccine for the first time (RHM CAN0489; REC 06/Q1702/155).

This current study will further our understanding of the mechanism of bystander maintenance of immune-memory by allowing direct evaluation of antigen-presentation within lymphatic tissue for the first time. Understanding these mechanisms might guide design of future vaccines which are better able to manipulate the immune system and promote more effective and incessant protection against infection.

In addition, knowledge of the unique immune environment of primary cancers draining lymphatics will guide the development of anti-cancer vaccines. Better understanding of the way in which cancer promotes immune anergy might shed light on therapeutic strategies to "de-cloak" tumour cells and activate immune cells to tumour-specific antigens, through novel vaccination techniques.

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

Patient Identification and Recruitment:

Eligible patients will be identified from the breast cancer SUHT outpatient clinics at Southampton General Hospital or Princess Anne Hospital by members of the Breast Cancer Multidisciplinary Team. Patient information sheets will be provided to eligible patients inviting them to consider participation. All patients will be given a minimum of 24 hours to consider entry to this study.

Inclusion Criteria:

1.Patients undergoing resection of invasive breast carcinoma (mastectomy or wide local excision) as confirmed by pre-operative core biopsy, who are to have a sentinel lymph node biopsy as part of their primary surgical procedure.
2.Aged 18 years or older
3.The ability to understand the study requirements, provide written informed consent and comply with the study

Exclusion Criteria:

1.Unwillingness or inability to consent to cancer resection or sentinel node biopsy
2.Unwillingness or inability to consent to inclusion within the study
3.Age under 18
4.Immune Modifying drugs (Including oral steroids)

Consent:

Having received prior written and verbal explanation of the study, patients will be required to provide written informed consent to participation and indefinite storage of biopsy samples for analyses relating to this study. Consent will include permission for the use of surplus histopathology samples, and the secure storage of anonymised case-linked clinical data, as detailed below. Informed consent will be obtained by the surgeon undertaking the patient's operation. Interpreters will be provided where possible for participants who are non-English speakers.

Study Numbers and Study Groups:

Participants will be divided into two groups according to their immunisation history. Upon agreeing to participate, patients will have their full immunisation history taken from them by the clinician responsible for their care. Those patients who have not previously been immunised against the study antigen (i.e. Tetanus Toxoid) will be offered an immunisation; those willing will be the immunised group (Group A). Participants who have previously received immunisation, or who are unwilling to receive immunisation, will be recruited to the control group (Group B).Group A will be further divided into patients receiving vaccination to the ipsilateral arm (i.e. the SAME side as the lymph node is removed from) (Group A1) and those receiving vaccination to the contralateral arm (Group A2). Group A2 will act as a positive control group, to ascertain whether any changes seen within the treatment group are the result of local or systemic immunological responses to vaccination.The study will require recruitiment of 30 responders into Group A1, 15 patients into Group A2 and 15 patients into Group B.

Study Protocol:

1) Patient Participation:

The study will require patients to attend a secondary care centre (SUHT outpatient clinics at Southampton General Hospital, the Royal South Hants Hospital or Princess Anne Hospital) on 4 separate occasions over a period of 6 weeks. Each blood collection will consist of 60mL anti-coagulated blood and 10mL clotted blood.

Week 0; Visit 1: Pre-assessment clinic; Consenting patients who have been recruited to either Group A or Group B attend clinic as part of their standard pre-assessment for surgery. All participants will have additional blood samples taken for study purposes at the time of their standard pre-operative screening blood tests. Additional venesection will not be necessary, nor will an additional hospital/clinic visit.
In addition, participants from Group A will receive sub-cutaneous vaccination. This will be administered at a site where the vaccine antigen will drain to the axillary lymph nodes.

Week 1; Visit 2: Day of operative procedure; during preparation for administration of an anaesthetic, patients have an intravenous cannula inserted. At this time, a second sample of blood will be taken for study purposes; therefore additional venesection will not be necessary.
During surgery, all participants will have a single additional non-sentinel lymph node removed from the axilla, following the completion of the sentinel lymph node biopsy.

Week 3; Visit 3: Post-operative follow-up clinic; at the time of routine follow up clinic appointment, a third sample of blood will be taken from study participants. An additional hospital/clinic visit will not be necessary; however patient will undergo venesection which would not constitute a standard aspect of their treatment.

Week 6; Visit 4: Additional clinic appointment; a fourth blood sample will be taken 5 weeks following surgery. This will require an additional clinic appointment and additional venesection which would not normally take place. However during this visit the patients will undergo an opportunistic wound review and be given an opportunity to ask questions of their clinicians/breast care specialist nurse.

2) Sample Handling and Analysis

Blood Samples:

Blood samples will be taken by clinic nurses, an anaesthetist or research nurses from the CRUK Clinical Research Unit and processed on the same day. Coordination between the research nurse and laboratory team regarding the timing of collections will occur prior to venesection. Peripheral blood mononuclear cells (PBMCs) will be isolated from anti-coagulated blood by centrifugation and transferred to liquid nitrogen for storage. Serum will be isolated from clotted blood samples by centrifugation and stored at -20∟C.

Sentinel Lymph Nodes:

These lymph nodes are removed for the purposes of diagnosis and staging of the axilla. They will undergo standard analysis as per standard operating procedure within the Department of Cellular Pathology, Southampton University Hospitals NHS Trust. Patients within the study will have their sentinel node samples processed on the same day of surgery.

Where possible, histopathological material (FFPE tissue blocks/slides) surplus to diagnostic requirements, that would otherwise be discarded, will be made available by the Department of Cellular Pathology, Southampton University Hospitals NHS Trust to the study. Tissue handling according to the Standard Operating Procedure for radioactive samples will be closely adhered to and monitored by the Radiation Protection Officer for Southampton University Hospitals NHS Trust.

Non-Sentinel Lymph Nodes:

Coordination between the surgical and laboratory teams regarding the timing of collections will occur prior to commencement of the surgical list. The lymph node (LN) removed for the specific purposes of the study will be stored on ice pending the results of the sentinel node biopsy. In the event of a positive sentinel node biopsy, the study node will be surrendered back to the hospital pathologist and processed as part of any subsequent completion axillary clearance.

In patients where the sentinel node biopsy proves negative, the study LN will be processed fresh on the day of surgery by mechanical dissociation into a single cell suspension and preserved in liquid nitrogen. Tissue handling according to the Standard Operating Procedure for radioactive samples will be closely adhered to and monitored by the Radiation Protection Officer for Southampton University Hospitals NHS Trust.

Storage:

Patient material collected during this study will be stored within a secure facility in the Southampton CRUK Clinical Centre. Material will be held as linked anonymised samples and labelled with a study specific number. The chief investigators and co-investigators will have access to the samples for analyses relating to this study. Patients will be asked to provide signed consent for the indefinite storage of samples for use by the investigators for analyses relating to the objectives of this study, or in the event of samples or tissue remaining on conclusion of the study, for donation to the University of Southampton Tissue bank for use in future ethically approved research.

Laboratory Analysis:

The primary tools for analysis are intended to be flow cytometry, proliferation and ELISPOT assays and ELISA, and will follow established SOPs in the Cancer Sciences Division. Assays will be performed by members of Professor Ottensmeier's group within the laboratories of the Cancer Sciences Division, University of Southampton School of Medicine. Initially, assays will focus on the following:

1. Identifying patient HLA A2 status
2. Assessment of blood and LN compartments for vaccine specific humoral and/or cellular immune responses
3. Assessment of blood and LN compartments for bystander humoral and/or cellular immune responses, for example, PPD, C.albicans, CMV, EBV, Influenza, etc.
4. Investigate immune responses to tumour-associated antigens such as CEA, HER2/neu, PASD1, WT1, MAGEA10, BAGE1, NY-ESO-1/CTAG1B, etc.
5. B-cell, NK, NKT and dendritic cell populations will be evaluated numerically, phenotypically and functionally where

possible
6. FFPE tissue will be used for the analysis of proteins or genes relevant to the tumour/immune response by immunohistochemistry and gene expression, respectively; for example, the tumour markers HER2/neu, CEA, CA15.3, estrogen receptor, progesterone receptor, etc. Analysis of receptors for other key immunological molecules, such as cytokines (e.g. IFN-L, IL-2, IL-13, IL-5) or chemokines, will be undertaken where available material and existing technology allows.

Additional investigation will be directed by the results from initial studies.

3) Data Acquisition and Storage

Patient details relating to personal and histopathological characteristics will be recorded (taken from the final definitive histopathology report following surgical resection) on study specific case report forms (CRF), linked to anonymised samples by their study specific number. The CRFs will be completed by members of the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre. The Chief Investigator will retain overall responsibility for the recording and quality of the data.

Data points will include:
•Age
•Tumour grade
•Tumour size
•Presence of metastatic disease following sentinel node biopsy
•The extent of axillary nodal involvement in patients with positive sentinel node involvement who subsequently undergo completion axillary clearance as part of their management.
•Histological tumour type
•Oestrogen and progesterone receptor status
•HER2 over expression status

All essential documents including source documents will be retained for a minimum period of 15 years following the end of the study. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. The Chief Investigator and Co-Investigators
will have access to the data for analyses. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples. No personally identifying information will be released in any report or publication relating to this work.

4) End of Study Participation:
Following the 4th blood donation at week 6, patients' active participation will cease. No further collection of samples will occur beyond this point. Recording of age and histopathological details will occur once the final diagnostic histopathology report is available, typically 10-14 days following surgery.

5) Statistics and Sample Size:
Analysis endpoints will be exploratory in nature and thus formal statistical calculation of sample size has not been performed. Up to 60 patients will be recruited for the purposes of this study.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- ☐ Design of the research
- ☐ Management of the research
- ☐ Undertaking the research
- ☐ Analysis of results
- ☐ Dissemination of findings
- ☑ None of the above

Give details of involvement, or if none please justify the absence of involvement.

4. RISKS AND ETHICAL ISSUES

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RESEARCH PARTICIPANTS

A15. What is the sample group or cohort to be studied in this research?

Select all that apply:

- [ ] Blood
- [x] Cancer
- [ ] Cardiovascular
- [ ] Congenital Disorders
- [ ] Dementias and Neurodegenerative Diseases
- [ ] Diabetes
- [ ] Ear
- [ ] Eye
- [ ] Generic Health Relevance
- [x] Infection
- [x] Inflammatory and Immune System
- [ ] Injuries and Accidents
- [ ] Mental Health
- [ ] Metabolic and Endocrine
- [ ] Musculoskeletal
- [ ] Neurological
- [ ] Oral and Gastrointestinal
- [ ] Paediatrics
- [ ] Renal and Urogenital
- [ ] Reproductive Health and Childbirth
- [ ] Respiratory
- [ ] Skin
- [ ] Stroke

Gender:	Male and female participants
Lower age limit: 18	Years
Upper age limit:	Years

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

1. Patients undergoing resection of invasive breast carcinoma (mastectomy or wide local excision) as confirmed by pre-operative core biopsy, who are to have a sentinel lymph node biopsy as part of their primary surgical procedure.
2. Aged 18 years or older
3. The ability to understand the study requirements, provide written informed consent and comply with the study

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

1.Unwillingness or inability to consent to cancer resection or sentinel node biopsy
2.Unwillingness or inability to consent to inclusion within the study
3.Age under 18
4.Currently taking immune modifying medication (Including oral steroids)

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Consent	1	0	10 minutes	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.
Clinic Appointments	3	2	10 minutes	Surgeon, research nurse and breast care specialist nurse

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Blood Tests	4	1	5 minutes	Surgeon or research nurse in outpatients prior to clinic appointment.
Immunisation	1	0	5	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.
Sentinel Lymph Node Biopsy	1	1	15 minutes	Surgeon in theatre
Excision of single additional non-sentinel axillary lymph node	1	0	2 minutes	Surgeon in theatre

A21. How long do you expect each participant to be in the study in total?

Each participant will be involved within the study for a total of 6 weeks from the point of recruitment (at the time of pre-assessment, initial blood sampling +/- immunisation) to the final point of contact (4th blood sample).

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Participants will be required to attend an additional outpatient clinic appointment. At this point in time they will undergo venesection. To compensate for the inconvenience of an extra clinic visit, patients will be offered an opportunistic additional wound review and be given the option of discussing any aspect of their care with either their surgeon or a Breast Care Nurse Specialist.

Participants in Group A will also receive an injection at the time of their pre-assessment clinic. Although there is no risk of harm from immunisation in terms of the patients' cancer diagnosis, a small number might experience short term discomfort or local site reactions, in line with the listed side-effects of each individual vaccine.

A24. What is the potential for benefit to research participants?

The administration of the vaccine will not directly benefit the patient in the absence of a subsequent exposure to the infective agent targeted by the vaccine.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used? *For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under arrangements with the responsible care organisation(s).*

Patients with clinically node-negative breast cancer, who are due to undergo breast cancer resection (either mastectomy or wide local resection) and sentinel lymph node biopsy will be identified by a member of the Breast care MDT, typically the surgeon responsible for their care, during the patients visit to outpatient clinics at Southampton General Hospital or Princess Anne Hospital.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

○ Yes ◉ No

Please give details below:

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

○ Yes ◉ No

A29. How and by whom will potential participants first be approached?

The surgeon undertaking breast cancer resection (either mastectomy or wide local resection) and sentinel lymph node biopsy at the time he/she discusses the operation with the patient

A30-1. Will you obtain informed consent from or on behalf of research participants?

◉ Yes ○ No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Patient information sheets will be provided to eligible patients inviting them to consider participation. All patients will be given a minimum of 24 hours to consider entry to this study. Patients will be required to provide written informed consent to participation and indefinite storage of non-sentinel node biopsy samples and blood samples for analyses relating to this study. Consent will also be taken regarding the donation of sentinel node material surplus to diagnostic requirements for the purpose of this study. Consent will also be taken regarding the anonymised storage of case-specific data regarding patient details (e.g. age) and final histology (e.g. histological sub-type, grade, stage, hormone receptor status).

Informed consent for the surgical procedure will be obtained by the surgeon undertaking the patient's operation. Informed consent for inclusion within the study will be taken by a doctor participating within the research who is appropriately trained in consent for research purposes, or an appropriately trained and qualified research nurse.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

(●) Yes ( ) No

A31. How long will you allow potential participants to decide whether or not to take part?

A minimum of 24 hours. Patients will be given information regarding the study at the time of discussion of their surgery. Consent for participation within the study will be taken at the time of their pre-assessment clinic appointment; typically patients will therefore have 1-2 weeks to consider involvement.

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

Interpreters will be provided where possible for participants who are non-English speakers.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

( ) The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.
(●) The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.
( ) The participant would continue to be included in the study.
( ) Not applicable – informed consent will not be sought from any participants in this research.
( ) Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

If you plan to retain and make further use of identifiable data/tissue following loss of capacity, you should inform participants about this when seeking their consent initially.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

[x] Access to medical records by those outside the direct healthcare team
[ ] Electronic transfer by magnetic or optical media, email or computer networks
[ ] Sharing of personal data with other organisations

- [ ] Export of personal data outside the EEA
- [ ] Use of personal addresses, postcodes, faxes, emails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [ ] Manual files including X-rays
  - [ ] NHS computers
  - [ ] Home or other personal computers
  - [x] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details:
Patient details relating to personal and histopathological characteristics will be recorded (taken from the final definitive histopathology report following surgical resection) on study specific case report forms (CRF), linked to anonymised samples by their study specific number. The CRFs will be completed by members of the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre.
Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples as per the standard operating procedure in place for this purpose (CSD/SOP/003).

A37. Please describe the physical security arrangements for storage of personal data during the study?

Locked in a swipe card protected facility (Cancer Sciences Building, Southampton General Hospital). Electronic data is on password protected computers.

A38. How will you ensure the confidentiality of personal data?*Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

Data relating to histopathological characteristics will be collected onto study specific CRFs by research staff from the CRUK Clinical Research Unit and stored within a secure facility within the Southampton CRUK Clinical Centre. Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

The clinically trained staff involved in this study who are part of the patients direct healthcare team. This will include the surgeon, research nurses from the Cancer Research UK Clinical Centre and the Chief Investigator.

Storage and use of data after the end of the study

A41. Where will the data generated by the study be analysed and by whom?

Within the Cancer Sciences Building at Southampton General Hospital. Data will be analysed by the Chief and Co-Investigators. It will be anonymised at this point.

A42. Who will have control of and act as the custodian for the data generated by the study?

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		
Work Address	Cancer Sciences Division		
	Southampton University Hospitals		
	Tremona Road, Southampton		
Post Code	SO16 6YD		
Work Email	cho@soton.ac.uk		
Work Telephone	02380795161		
Fax	02380795152		

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ○ 6 – 12 months
- ○ 12 months – 3 years
- ◉ Over 3 years

If longer than 12 months, please justify:
Personal data will be accessed within a few weeks of the patient undergoing their surgical procedure. The data will be anonymised at this point. We will store and access this linked anonymised data indefinitely but will not go back to accessing identifiable data again.

A44. For how long will you store research data generated by the study?

Years: 15
Months:

A45. Please give details of the long term arrangements for storage of research data after the study has ended. *Say where data will be stored, who will have access and the arrangements to ensure security.*

All essential documents including source documents will be retained for a minimum period of 15 years following the end of the study. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. It will be labelled only with a study number.

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

○ Yes ◉ No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g.

financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

◉ Yes ○ No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

◉ Yes ○ No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50. Will the research be registered on a public database?

◉ Yes ○ No

Please give details, or justify if not registering the research.
research will be registered on the CLRN database

Registration of research studies is encouraged wherever possible.
You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you are aware of a suitable register or other method of publication, please give details. If not, you may indicate that no suitable register exists. Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- ☑ Peer reviewed scientific journals
- ☐ Internal report
- ☑ Conference presentation
- ☐ Publication on website
- ☐ Other publication
- ☐ Submission to regulatory authorities
- ☐ Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- ☐ No plans to report or disseminate the results
- ☐ Other (please specify)

A52. If you will be using identifiable personal data, how will you ensure that anonymity will be maintained when publishing the results?

N/A

A53. Will you inform participants of the results?

○ Yes ◉ No

Please give details of how you will inform participants or justify if not doing so.
Patients will be informed that the research will be published in the scientific literature. The information sheet provides contact numbers for the researchers involved if they wish to contact us about findings which we would of course be happy to provide. Beyond this we do not propose to actively contact them.

5. Scientific and Statistical Review

A54. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi–centre research group
- ☑ Review within the Chief Investigator's institution or host organisation
- ☑ Review within the research team
- ☐ Review by educational supervisor
- ☐ Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The project has been reviewed within the Cancer Sciences Division and additionally reviewed by the research team.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- ☐ Review by independent statistician commissioned by funder or sponsor
- ☐ Other review by independent statistician
- ☐ Review by company statistician
- ☐ Review by a statistician within the Chief Investigator's institution
- ☐ Review by a statistician within the research team or multi–centre group
- ☐ Review by educational supervisor
- ☐ Other review by individual with relevant statistical expertise
- ☑ No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Department	MD PhD FRCP		
Institution	University of Southampton		
Work Address	Cancer Sciences Division		
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Fax	02380795152
Mobile	
E-mail	cho@soton.ac.uk

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Qualitative description of immunological events in the lymph node and blood to vaccination

A58. What are the secondary outcome measures? *(if any)*

The effect of stage/lymphatic spread of primary malignancy on the immunological response to vaccination witnessed within lymph nodes and peripheral blood.

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size:	60
Total international sample size (including UK):	60
Total in European Economic Area:	60

Further details:
Group A1 (N=30) Receiving vaccine in same arm as operation is to occur (Study Group)
Group A2 (N=15) Receiving vaccine into contralateral arm (Control group; to differentiate between systemic and local effects of vaccine)
Group B (N=15) Not receiving vaccine

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

Analysis endpoints will be exploratory in nature and thus formal statistical calculation of sample size has not been performed.

A61. Will participants be allocated to groups at random?

○ Yes ◉ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Simple descriptive statistics will be used to analyse and present the data from this study.

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co–applicants, protocol co–authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Post	Professor of Immunology and Oncology		
Qualifications	MD PhD FRCP		

Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795161
Fax	02380795152
Mobile	
Work Email	cho@soton.ac.uk

Title	Forename/Initials	Surname
Mr	Ramsey	Cutress

Post	Senior Lecturer and Consultant Surgeon
Qualifications	FRCS PhD MBBS
Employer	University of Southampton, Southampton University Hospital NHS Trust
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	07979904339
Fax	02380795152
Mobile	07979904339
Work Email	r.i.cutress@soton.ac.uk

Title	Forename/Initials	Surname
Mr	David	Layfield

Post	Research Fellow
Qualifications	MRCS AICSM MBBS BSc (Hons)
Employer	RHCH NHS Trust
Work Address	Cancer Sciences Division Southampton University Hospital tremona Road, Southampton
Post Code	SO16 6YD
Telephone	07708533869
Fax	02380795152
Mobile	07708533869
Work Email	david.layfield@doctors.org.uk

Title	Forename/Initials	Surname
Dr	Gianfranco	Di Genova

Post	Research Fellow
Qualifications	PhD
Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097

Work Email	g.di-genova@soton.ac.uk

Title	Forename/Initials	Surname
Dr	Kathy	McCann

Post	Research Fellow
Qualifications	PhD
Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097
Work Email	kjm8@soton.ac.uk

Title	Forename/Initials	Surname
Miss	Lindsey	Chudley

Post	Research Fellow
Qualifications	BSc
Employer	University of Southampton
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton
Post Code	SO16 6YD
Telephone	02380795097
Fax	02380795152
Mobile	02380795097
Work Email	l.chudley@soton.ac.uk

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status:
- (●) NHS or HSC care organisation
- ( ) Academic
- ( ) Pharmaceutical industry
- ( ) Medical device industry
- ( ) Local Authority
- ( ) Other social care provider (including voluntary sector or private organisation)
- ( ) Other

Commercial status: Non-Commercial

If Other, please specify:

Contact person

Name of organisation Southampton University Hospitals NHS Trust

Given name	Sharon
Family name	Atwill
Address	Research Governance Officer-Division A, R&D Office
Town/city	E Level, Laboratory and Pathology Block
Post code	SO166YD
Country	UNITED KINGDOM
Telephone	02380795314
Fax	
E-mail	sharon.atwill@suht.swest.nhs.uk

Is the sponsor based outside the UK?
◯ Yes ◉ No

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

A65. Has external funding for the research been secured?

☑ Funding secured from one or more funders
☐ External funding application to one or more funders in progress
☐ No application for external funding will be made

Please give details of funding applications.

Organisation	Cancer Research UK - Experimental Cancer Medicine Centre (ECMC) Network
Address	Angel Building 407 St John Street London
Post Code	EC1V4AD
Telephone	02034695388
Fax	
Mobile	
Email	

Funding Application Status: ◉ Secured ◯ In progress

Amount: £400,000 per annum

Duration
Years: 5
Months: 0

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?
Experimental Cancer Medicine Centre Grant (as from 01/04/2012)
What type of research project is this?

◯ Standalone project
◉ Project that is part of a programme grant
◯ Project that is part of a Centre grant

○ Project that is part of a fellowship/ personal award/ research training award
○ Other

Other – please state:

A66. Has responsibility for any specific research activities or procedures been delegated to a subcontractor (other than a co-sponsor listed in A64-1) ? *Please give details of subcontractors if applicable.*

○ Yes ◉ No

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

○ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
	Mrs	Sharon	Atwill
Organisation			
Address	Research Governance Officer-Division A, R&D Office		
	E Level, Laboratory and Pathology Block		
	SCBR - Mailpoint 138, Tremona Road, Southampton		
Post Code	SO166YD		
Work Email	sharon.atwill@suht.swest.nhs.uk		
Telephone	02380795314		
Fax			
Mobile			

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 01/04/2012
Planned end date: 01/04/2015
Total duration:
Years: 3 Months: 0 Days: 0

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)

The Last visit of the last patient undergoing the trial

A71-1. Is this study?

◉ Single centre
○ Multicentre

NHS R&D Form IRAS Version 3.2

A71-2. Where will the research take place? *(Tick as appropriate)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland
- [ ] Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
○ Yes ◉ No

A72. What host organisations (NHS or other) in the UK will be responsible for the research sites? *Please indicate the type of organisation by ticking the box and give approximate numbers of planned research sites:*

[x] NHS organisations in England	1
[ ] NHS organisations in Wales	
[ ] NHS organisations in Scotland	
[ ] HSC organisations in Northern Ireland	
[ ] GP practices in England	
[ ] GP practices in Wales	
[ ] GP practices in Scotland	
[ ] GP practices in Northern Ireland	
[ ] Social care organisations	
[ ] Phase 1 trial units	
[ ] Prison establishments	
[ ] Probation areas	
[ ] Independent hospitals	
[ ] Educational establishments	
[ ] Independent research units	
[ ] Other (give details)	
Total UK sites in study:	1

A73-1. Will potential participants be identified through any organisations other than the research sites listed above?

○ Yes ◉ No

A74. What arrangements are in place for monitoring and auditing the conduct of the research?

The study will be monitored and audited in accordance with SUHT procedures. All trial related documents will be made available on request for monitoring and audit by SUHT, the relevant REC and other licensing bodies.

A76. Insurance/ indemnity to meet potential legal liabilities

***Note:** in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland*

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

- [x] NHS indemnity scheme will apply (NHS sponsors only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

- [x] NHS indemnity scheme will apply (protocol authors with NHS contracts only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

- [x] NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)
- [ ] Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

A78. Could the research lead to the development of a new product/process or the generation of intellectual property?

○ Yes ◉ No ○ Not sure

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood samples will be taken from participants, as well as lymph node samples as described in Part A.

2. Who will collect the samples?

Qualified practice/research nurse (First blood sample), Anaesthetist (Second blood sample), Research nurse (3rd and 4th blood samples) and Consultant Breast Surgeon (Lymph node samples).

3. Who will the samples be removed from?

☑ Living donors
☐ The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?
◉ Yes ○ No

In future research?
◉ Yes ○ No ○ Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

○ Yes ◉ No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*
○ Yes ◉ No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
◉ Yes ○ No

If Yes, say who will have access to the code and personal information about the donor.
The chief investigator and a CRUK research nurse who will maintain the case report forms for patients within the study.

In a form in which the donor could be identifiable to researchers?
○ Yes ◉ No

9. What types of test or analysis will be carried out on the samples?

The primary tools for analysis are intended to be flow cytometry, proliferation and ELISPOT assays and ELISA, and will follow established SOPs in the Cancer Sciences Division. Assays will be performed by members of Professor Ottensmeier's group within the laboratories of the Cancer Sciences Division, University of Southampton School of Medicine.

Primary laboratory analyses:
1. Identify patient HLA A2 status
2. Assessment of blood and LN compartments for vaccine specific humoral and/or cellular immune responses
3. Assessment of blood and LN compartments for bystander humoral and/or cellular immune responses, for example, PPD, C.albicans, CMV, EBV, Influenza, etc.
4. Investigate immune responses to tumour-associated Ags, such as CEA, HER2/neu, PASD1, WT1, MAGEA10, BAGE1, NY-ESO-1/CTAG1B, etc.
5. B-cell, NK, NKT and dendritic cell populations will be evaluated numerically, phenotypically and functionally where possible
6. FFPE tissue will be used for the analysis of proteins or genes relevant to the tumour/immune response by immunohistochemistry and gene expression, respectively; for example, the tumour markers HER2/neu, CEA, CA15.3, estrogen receptor, progesterone receptor, etc. Analysis of receptors for other key immunological molecules, such as cytokines (e.g. IFN-L, IL-2, IL-13, IL-5) or chemokines, will be undertaken where available material and existing

technology allows.

10. Will the research involve the analysis or use of human DNA in the samples?

○ Yes ◉ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Tissue samples obtained within this study will be stored within a secure facility within the Southampton Cancer Research UK Centre. Samples will be held as linked anonymised samples and labelled by a study specific number. The Chief Investigator and Co-Investigators will have access to the samples for analyses relating to this study. Patients will be asked to provide signed consent for the indefinite storage of samples for use by the Investigators for analyses relating to the objectives of this study.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☑ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

☑ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☐ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

Details of intended tissue bank:

The University of Southampton Research Tissue Bank (Human Tissue Act License: 12009, Southampton and South West Hampshire Research Ethics Committee: 280/99)

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.*

Research site		Investigator/ Collaborator/ Contact	
Institution name	Southampton University Hospitals NHS Trust	Title	Professor
Department name	Cancer Sciences Center	First name/ Initials	Christian
Street address	Tremona Road	Surname	Ottensmeier
Town/city	Southampton		
Post Code	SO166YD		

PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.

4. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendment.

5. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.

6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2006.

7. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.

8. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 1998.

9. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:

    - Will be held by the main REC or the GTAC (as applicable) until at least 3 years after the end of the study; and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
    - May be disclosed to the operational managers of review bodies, or the appointing authority for the main REC, in order to check that the application has been processed correctly or to investigate any complaint.
    - May be seen by auditors appointed to undertake accreditation of RECs.
    - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

10. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

11. I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

Contact point for publication*(Not applicable for R&D Forms)*

NRES would like to include a contact point with the published summary of the study for those wishing to seek further information. We would be grateful if you would indicate one of the contact points below.

- [x] Chief Investigator
- [ ] Sponsor
- [ ] Study co-ordinator

☐ Student
☐ Other – please give details
☐ None

Access to application for training purposes *(Not applicable for R&D Forms)*
Optional – please tick as appropriate:

☐ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

This section was signed electronically by Professor Christian Ottensmeier on 29/10/2011 13:14.

Job Title/Post:	Professor
Organisation:	University of Southampton
Email:	cho@soton.ac.uk
Signature:	.....
Print Name:	
Date:	*(dd/mm/yyyy)*

D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co−sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.
3. Any necessary indemnity or insurance arrangements, as described in question A76, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.
4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
6. The duties of sponsors set out in the Research Governance Framework for Health and Social Care will be undertaken in relation to this research.
7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

This section was signed electronically by Mrs Hope Howard on 31/10/2011 14:00.

Job Title/Post:	Research Governance Officer
Organisation:	University Hospital Southampton NHS Foundation Trust
Email:	hope.howard@suht.swest.nhs.uk

D3. Declaration for student projects by academic supervisor(s)

1. I have read and approved both the research proposal and this application. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level.

2. I undertake to fulfil the responsibilities of the supervisor for this study as set out in the Research Governance Framework for Health and Social Care.

3. I take responsibility for ensuring that this study is conducted in accordance with the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research, in conjunction with clinical supervisors as appropriate.

4. I take responsibility for ensuring that the applicant is up to date and complies with the requirements of the law and relevant guidelines relating to security and confidentiality of patient and other personal data, in conjunction with clinical supervisors as appropriate.

Academic supervisor 1

This section was signed electronically by Mr Ramsey Cutress on 25/10/2011 17:34.

Job Title/Post:	Senior Lecturer and Consultant Surgeon
Organisation:	University of Southampton and Southampton University Hospitals Trust
Email:	r.i.cutress@soton.ac.uk

II. SSI application form

NHS SSI IRAS Version 3.2

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Understanding immunity in lymph nodes. V 1

1. Is your project research?

(•) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(•) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(•) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	( ) Yes	(•) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(•) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(•) Yes	( ) No

3. In which countries of the UK will the research sites be located?*(Tick all that apply)*

[x] England
[ ] Scotland

1 72796/270280/6/120/92652/229492

☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ Ministry of Justice (MoJ)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Do you want your NHS R&D application(s) to be processed through the NIHR Coordinated System for gaining NHS Permission?

◉ Yes ○ No

If yes, you must complete and submit the NIHR CSP Application Form immediately after completing this project filter, before proceeding with completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

(•) Yes ( ) No

Please describe briefly the involvement of the student(s):
Full involvement with research planning, experiment design and data analysis under close supervision

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

(•) Yes ( ) No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

( ) Yes (•) No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

( ) Yes (•) No

Site-Specific Information Form (NHS sites)

Is the site hosting this research a NHS site or a non-NHS site? *NHS sites include Health and Social Care organisations in Northern Ireland. The sites hosting the research are the sites in which or through which research procedures are conducted. For NHS sites, this includes sites where NHS staff are participants.*

- (●) NHS site
- ( ) Non-NHS site

This question must be completed before proceeding. The filter will customise the form, disabling questions which are not relevant to this application.

One Site-Specific Information Form should be completed for each research site and submitted to the relevant R&D office with the documents in the checklist. See guidance notes.

The data in this box is populated from Part A:

Title of research:
Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects

Short title: Understanding immunity in lymph nodes. V 1

Chief Investigator:	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier

Name of NHS Research Ethics Committee to which application for ethical review is being made:
Southampton A REC

Project reference number from above REC: 11/SC/0509

1-1. Give the name of the NHS organisation responsible for this research site

Southampton University Hospitals NHS Trust

1-2. In which country is the research site located?

- (●) England
- ( ) Wales
- ( ) Scotland
- ( ) Northern Ireland

1-3. Is the research site a GP practice or other Primary Care Organisation?

( ) Yes (●) No

2. Who is the Principal Investigator or Local Collaborator for this research at this site?

Select the appropriate title: (•) Principal Investigator ( ) Local Collaborator

	Title	Forename/Initials	Surname
	Mr	Ramsey	Cutress
Post	Senior Lecturer and Consultant Surgeon		
Qualifications	FRCS PhD MBBS		
Organisation	University of Southampton, Southampton University Hospital NHS Trust		
Work Address	Cancer Sciences Division Southampton University Hospitals Tremona Road, Southampton		
PostCode	SO16 6YD		
Work E-mail	r.i.cutress@soton.ac.uk		
Work Telephone	07979904339		
Mobile	07979904339		
Fax	02380795152		

a) Approximately how much time will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.25

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (•) Yes ( ) No

A copy of a current CV for the Principal Investigator (maximum 2 pages of A4) must be submitted with this form.

3. Please give details of all locations, departments, groups or units at which or through which research procedures will be conducted at this site and describe the activity that will take place.

Please list all locations/departments etc where research procedures will be conducted within the NHS organisation, describing the involvement in a few words. Where access to specific facilities will be required these should also be listed for each location.

Name the main location/department first. Give details of any research procedures to be carried out off site, for example in participants' homes.

	Location	Activity/facilities
1	Department of Breast Surgery, Princess Anne Hospital, Southampton General Hospital NHS Trust	Site of outpatient clinics, recruitment and surgery
2	Cancer Sciences Unit, Cancer Sciences Center, Southampton General Hospital, University of Southampton	Processing of samples, analysis, data storage, tissue storage

5. Please give details of all other members of the research team at this site.

1

	Title	Forename/Initials	Surname
	Professor	Christian	Ottensmeier
Work E-mail	cho@soton.ac.uk		
Employing organisation	University of Southampton		

Post	Professor of Immunology and Oncology
Qualifications	MD PhD FRCP
Role in research team:	other (please specify) Chief Investigator

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.05

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (•) Yes ( ) No

2

	Title	Forename/Initials	Surname
	Mr	David	Layfield
Work E-mail	david.layfield@doctors.org.uk		
Employing organisation	RHCH NHS Trust		
Post	Research Fellow		
Qualifications	MRCS AICSM MBBS BSc (Hons)		
Role in research team:	researcher		

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
1

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (•) Yes ( ) No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

3

	Title	Forename/Initials	Surname
	Dr	Gianfranco	Di Genova
Work E-mail	g.di-genova@soton.ac.uk		
Employing organisation	University of Southampton		
Post	Research Fellow		
Qualifications	PhD		
Role in research team:	researcher		

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.25

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (•) Yes ( ) No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

4

	Title	Forename/Initials	Surname
	Dr	Kathy	McCann
Work E-mail	kjm8@soton.ac.uk		
Employing organisation	University of Southampton		
Post	Research Fellow		
Qualifications	PhD		
Role in research team:	researcher		

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.25

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (●) Yes ( ) No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

5

	Title	Forename/Initials	Surname
	Ms	Meg	Gale
Work E-mail	m.l.gale@soton.ac.uk		
Employing organisation	Cancer Research UK Clinical Center, University of Southampton		
Post	Research Sister		
Qualifications	Batchelor of Theology - Southampton University, 1984 General Nursing Level 1(RGN) - Southampton 1988 EBN 237, Oncology Nursing (RGN) - SOuthampton 1990		
Role in research team:	research nurse		

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.25

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? (●) Yes ( ) No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

6

	Title	Forename/Initials	Surname
	Ms	Joanna	Wood
Work E-mail	jwly09@soton.ac.uk		
Employing organisation	Cancer Research UK Clinical Center, University of Southampton		
Post	Research Nurse		
	BSc (Hons) - Podiatry - University of Southampton 2007		

Qualifications	MSc - Ecology - University of Bangor 1993 BSc (Hons) - Environmental Science - University of Greenwich 1992
Role in research team:	research nurse

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
0.25

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? ◉ Yes ○ No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

6. Does the Principal Investigator or any other member of the site research team have any direct personal involvement (e.g. financial, share-holding, personal relationship etc) in the organisation sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

7. What is the proposed local start and end date for the research at this site?

Start date:	01/04/2012
End date:	01/04/2015
Duration (Months):	36

8-1. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *(These include seeking consent, interviews, non-clinical observations and use of questionnaires.)*

Columns 1-4 have been completed with information from A18 as below:

1. *Total number of interventions/procedures to be received by each participant as part of the research protocol.*
2. *If this intervention would have been routinely given to participants as part of their care, how many of the total would have been routine?*
3. *Average time taken per intervention (minutes, hours or days)*
4. *Details of who will conduct the procedure, and where it will take place*

Please complete Column 5 with details of the names of individuals or names of staff groups who will conduct the procedure at this site.

Intervention or procedure	1	2	3	4	5
Consent	1	0	10 minutes	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.	
Clinic Appointments	3	2	10 minutes	Surgeon, research nurse and breast care specialist nurse	

8-2. Will any aspects of the research at this site be conducted in a different way to that described in Part A or the protocol?

○ Yes ◉ No

If Yes, please note any relevant changes to the information in the above table.

Are there any changes other than those noted in the table?

9-1. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *(These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.)*

Columns 1-4 have been completed with information from A19 as below:

1. *Total number of interventions to be received by each participant as part of the research protocol*
2. *If this intervention would have been routinely given to participants as part of their care, how many of the total would have been routine?*
3. *Average time taken per intervention (minutes, hours or days)*
4. *Details of who will conduct the procedure, and where it will take place*

Please complete Column 5 with details of the names of individuals or names of staff groups who will conduct the procedure at this site.

Intervention or procedure	1	2	3	4	5
Blood Tests	4	1	5 minutes	Surgeon or research nurse in outpatients prior to clinic appointment.	
Immunisation	1	0	5	Surgeon or research nurse in outpatients prior to pre-assessment clinic appointment.	
Sentinel Lymph Node Biopsy	1	1	15 minutes	Surgeon in theatre	
Excision of single additional non-sentinel axillary lymph node	1	0	2 minutes	Surgeon in theatre	

9-2. Will any aspects of the research at this site be conducted in a different way to that described in Part A or the protocol?

○ Yes ◉ No

If Yes, please note any relevant changes to the information in the above table.

Are there any changes other than those noted in the table?

10. How many research participants/samples is it expected will be recruited/obtained from this site?

60 participants; 1 lymph node and 4 blood samples from each

11. Give details of how potential participants will be identified locally and who will be making the first approach to them to take part in the study.

Participants will be identified as suitable by their consultant. The consultant will make the initial approach and explain the nature of the study and supply the patient with an information sheet. Verbal consent will be sought for a research nurse to contact the patient and consent will be taken at the time of the patients pre-assessment clinic appointment.

12. Who will be responsible for obtaining informed consent at this site? What expertise and training do these persons have in obtaining consent for research purposes?

Name	Expertise/training
Mr Ramsey Cutress	Consent for routine surgical procedures, GCP training and consent for clinical trials
Professor C Ottensmeier	GCP training and consent for clinical trials
Joanna Wood	GCP training and consent for clinical trials
Meg Gale	GCP training and consent for clinical trials
David Layfield	Consent for routine surgical procedures

15-1. Is there an independent contact point where potential participants can seek general advice about taking part in research?

Yes. An independent research nurse is named on the patient information sheet and is trained to provide advice to patients considering involvement in research

15-2. Is there a contact point where potential participants can seek further details about this specific research project?

Yes. There is a named doctor (who is involved in the research) on the patient information sheet who will be able to give specific details regarding the project.

16. Are there any changes that should be made to the generic content of the information sheet to reflect site-specific issues in the conduct of the study? *A substantial amendment may need to be discussed with the Chief Investigator and submitted to the main REC.*

No

Please provide a copy on headed paper of the participant information sheet and consent form that will be used locally. Unless indicated above, this must be the same generic version submitted to/approved by the main REC for the study while including relevant local information about the site, investigator and contact points for participants (see guidance notes).

17. What local arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

Translators will be provided if required. All patients consent will be considered on an individual basis. If a patient is unable to understand written of verbal communication despite a translator then it is unlikely they will be asked to participate.

18. What local arrangements will be made to inform the GP or other health care professionals responsible for the care of the participants?

The GP will receive a separate information letter informing them of their patients participation with detail of the processes their patients will be involved in.

19. What arrangements (e.g. facilities, staffing, psychosocial support, emergency procedures) will be in place at the site, where appropriate, to minimise the risks to participants and staff and deal with the consequences of any harm?

Harm from participation is extremely unlikely. Standard follow up procedures will be followed and additional outpatient appointments will also be arrange as per the protocol discribed. All clinical activity (Pre-assessment, blood tests, immunisation, surgery) will be taking place on a hospital site with all the facilities available.

The patient information sheet contains advice to participants who feel they have come to harm from the study and contact details are provided in the event of any concerns or questions.

20. What are the arrangements for the supervision of the conduct of the research at this site? *Please give the name and contact details of any supervisor not already listed in the application.*

Mr Ramsey Cutress will supervise both the research and clinical aspects of the project. Professor C Ottensmeier will provide additional academic supervision. The student will be working within a team of experienced post-doctorial researchers (G. Di Genova, K. McCann) and will have weekly formal laboratory meetings with principle investigator Mr Ramsey Cutress

21. What external funding will be provided for the research at this site?

- ○ Funded by commercial sponsor
- ◉ Other funding
- ○ No external funding

Please give details of the funding:
Cancer Research UK - Experimental Cancer Medicine Centre (ECME) Network annual grant

Type of funding	Details (including breakdown over years if appropriate)
(i) Block grant	£400,000 per annum for 5 years from 01/04/2012
(ii) Per participant	
(iii) Other (give details)	

Which organisation will receive and manage this funding?
The funding is provided by CRUK to Professor Ottensmeiers' research fund and is managed through the facilities of the Cancer Sciences Division of Southampton University

23. Authorisations required prior to R&D approval

This section deals with authorisations by managers within the NHS organisation. It should be signed in accordance with the guidance provided by the NHS organisation. This may include authorisation by clinical supervisors, line managers, service managers, support department managers, pharmacy, data protection officers or finance managers, depending on the nature of the research. Managers completing this section should confirm in the text what the authorisation means, in accordance with the guidance provided by the NHS organisation.

This section may also be used by university employers or research support staff to provide authorisation to NHS organisations, in accordance with guidance from the university.

1. Type of authorisation:
Research and Development Lead

	Title	Forename/Initials	Surname
	Professor	Mike	Grocott
Post	Research and Development Lead -Division A		
Qualifications			
Organisation	Southampton University Hospitals NHS Trust		
Work Address	Department of Anaesthetics		
	Level D, Centre Block		
	Southampton General Hospital, Tremona Road, Southampton		
PostCode	SO166YD		
Work E-mail	mike.grocott@uhs.nhs.uk		
Work Telephone	02380777222		
Mobile			
Fax			

Signature: .....

Date: .....

2. Type of authorisation:
Care Group Manager

	Title	Forename/Initials	Surname
	Dr	Chris	Baughan
Post	Care Group Manager		
Qualifications			
Organisation	Southampton University Hospitals NHS Trust		
Work Address	Level B Radiotherapy Southampton General Hospital Mailpoint 301, Tremona Road, Southampton		
PostCode	SO166YD		
Work E-mail	chris.baughan@uhs.nhs.uk		
Work Telephone	02380777222		
Mobile			
Fax	02380796682		

Signature: .....

Date: .....

3. Type of authorisation:
Pharmacy Manager

	Title	Forename/Initials	Surname
	Ms	Joanna	Cantel
Post	Pharmacy Manager		
Qualifications			
Organisation	Southampton University Hospitals NHS Trust		
Work Address	Pharmacy Department Mail Point 40 Southampton General Hospital, Tremona Road, Southampton		
PostCode	SO16 6YD		
Work E-mail	joanna.cantel@uhs.nhs.uk		
Work Telephone	02380794223		
Mobile			
Fax			

Signature: .....

Date: .....

4. Type of authorisation:
Radiation Protection Officer

	Title	Forename/Initials	Surname
	Mr	Craig	Morrissey
Post	Radiation Protection Officer		
Qualifications			
Organisation	University of Southampton		
Work Address	26 University Road		
	Highfield Campus		
	University of Southampton, Southampton, Hampshire		
PostCode	SO17 1BJ		
Work E-mail	c.morrissey@soton.ac.uk		
Work Telephone	02380594337		
Mobile			
Fax			

Signature: .....

Date: .....

Declaration by Principal Investigator or Local Collaborator

1. The information in this form is accurate to the best of my knowledge and I take full responsibility for it.
2. I undertake to abide by the ethical principles underpinning the World Medical Association's Declaration of Helsinki and relevant good practice guidelines in the conduct of research.
3. If the research is approved by the main REC and NHS organisation, I undertake to adhere to the study protocol, the terms of the application of which the main REC has given a favourable opinion and the conditions requested by the NHS organisation, and to inform the NHS organisation within local timelines of any subsequent amendments to the protocol.
4. If the research is approved, I undertake to abide by the principles of the Research Governance Framework for Health and Social Care.
5. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to the conduct of research.
6. I undertake to disclose any conflicts of interest that may arise during the course of this research, and take responsibility for ensuring that all staff involved in the research are aware of their responsibilities to disclose conflicts of interest.
7. I understand and agree that study files, documents, research records and data may be subject to inspection by the NHS organisation, the sponsor or an independent body for monitoring, audit and inspection purposes.
8. I take responsibility for ensuring that staff involved in the research at this site hold appropriate contracts for the duration of the research, are familiar with the Research Governance Framework, the NHS organisation's Data Protection Policy and all other relevant policies and guidelines, and are appropriately trained and experienced.
9. I undertake to complete any progress and/or final reports as requested by the NHS organisation and understand that continuation of permission to conduct research within the NHS organisation is dependent on satisfactory completion of such reports.

NHS SSI IRAS Version 3.2

10. I undertake to maintain a project file for this research in accordance with the NHS organisation's policy.

11. I take responsibility for ensuring that all serious adverse events are handled within the NHS organisation's policy for reporting and handling of adverse events.

12. I understand that information relating to this research, including the contact details on this application, will be held by the R&D office and may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

13. I understand that the information contained in this application, any supporting documentation and all correspondence with the R&D office and/or the REC system relating to the application will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

Signature of Principal Investigator or Local Collaborator: .....

Print Name: Mr Ramsey I Cutress

Date:

III. University of Southampton Indemnity form

Insurance and Research Governance Application for Projects Requiring Approval by Ethics Committee and Involving Research on Human Subjects, their tissues, organs or data, by Staff and/or Students of the University of Southampton

The project must not commence until insurance, ethics approval and sponsorship are obtained

PART A - PLEASE COMPLETE ALL QUESTIONS

Ethics Submission Number: Unknown

1. Title of Study: Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

Start date: 01/11/2011 End date: 01/11/2014

2. **Researcher's Details**

Title: Profesor	Name: Christian Ottensmeier / Mr Ramsey Cutress
University School:	Southampton University
University Department/Division:	Cancer Sciences Division
Address:	Somers Cancer Research Building
	Southampton General Hospital
	Tremona Road, Southampton SO16 6YD
Tel: 02380796184	Email r.i.cutress@soton.ac.uk

3. Are student researchers involved with this project? Yes

4. Is the study based solely on questionnaires, or other research **not** involving invasive techniques or medicinal products? No

Please estimate numbers of volunteers participating in the study:

	Adults	Minors *
Patients	60	0
Healthy human volunteers	0	0

* Minors under 18 years of age

5. Is this a Multi Centre Trial? No

If yes and the trial is sponsored by UoS or SUHT, or managed by UoS, please estimate numbers of volunteers participating in the study overall:

	Adults	Minors
Patients		
Healthy human volunteers		

6. Does the study involve invasive techniques? Yes

7. Does the study involve the use of a medicinal product or the testing of a medical device? Yes – established product, NOT investigative.

IF AN INVESTIGATIVE MEDICINAL PRODUCT IS INVOLVED
Please indicate which phase category the study falls into N/A

8. Who is the Research Sponsor? SUHT

9. Who is the Funder? Grant Funded: Prof Ottensmeier CRUK ECMC Grant

10. **For Commercial trials only**, is an ABPI Indemnity being given? N/A

IF YES: the ABPI Indemnity form, preferably in triplicate, should be forwarded with this form for signature by an Authorised Signatory on behalf of the University.

Will any part of this study take place outside the UK? No

Layfield - UoS indemnity form 21082011.doc v2 08/05/2009
Page 1 of 2

If Yes, in which country(ies)?	N/A

PART B - PLEASE COMPLETE QUESTIONS AS APPLICABLE

11. **For Student projects** Student status: PG

Supervisor's Details			
Title:	Mr	Name:	Ramsey I Cutress
University School			Southampton University
University Department or Division			Cancer Sciences Division
Address:			Somers Cancer Research Building
			Southampton General Hospital
			Tremona Road, Southampton SO16 6YD
Tel:	02380796184	Email	r.i.cutress@soton.ac.uk

12. **For multi site studies**

How many sites are involved?	1
Is Southampton the lead site?	Yes
Are any sites outside the UK?	0
Are contracts/site agreements in place?	N/A

13. **For studies involving the NHS Patients, staff or resources**

Is the study approved by the NHS Trust R+D office?	Pending
Is the study approved by NHS ethics committee?	Pending

14. **For Clinical Trials involving drugs, devices or clinical interventions** **Reference Number**

Is the study registered with the MHRA?	N/A
Is the study registered on the European Clinical Trials (EudraCT) database?	N/A
Is the study registered on the National Research Register (Clinical trials database)?	N/A

15. **For studies using tissue samples**

Are the tissue samples accessed via a licensed tissue bank?	No
Are you seeking ethical approval for your study?	Yes

16. **For all studies, will the Applicant be responsible for:**

Reporting amendments to the protocol	Yes
Reporting adverse events and significant developments	Yes
If No, who will be responsible?	N/A

For Research Governance information, please contact:
Research Governance Office, Email: rgoinfo@soton.ac.uk Tel: 02380 598849
Website: http://www.soton.ac.uk/corporateservices/rgo/index.html

For Insurance information, please contact:
Finance Department, Insurance Services, Email: insure@soton.ac.uk Tel: 02380 592417
Website http://www.soton.ac.uk/finance/insurance/index.html

Please send this form with all other supporting documents to:
Research Governance Office, University of Southampton, B37/4055, Highfield, Southampton SO17 1BJ
or email to rgoinfo@soton.ac.uk.

IV. R&D Data protection registration form

Section B:

Data Protection Questionnaire SUHT

Will your computerised or paper filing system hold patient/person or other identifiable data – if yes please answer all the following by ticking the Yes/No box as appropriate

		Yes	No
1.	If you are collecting data directly from the patient, will you obtain explicit consent from the patient for the information to be stored and processed?	x	
2.	If you are collecting data directly from the patient, will you fully outline what and how you will use the data and will this be explicit on the form given to the patient?	x	
3.	If you are collecting data directly from the patient, will you ensure the data is not used by you or anyone else for other purposes?	x	
4.	Are you clear how each data item will contribute to your research studies?	x	
5.	If you are collecting data directly from the patient, will you have a method of checking accuracy of data?	x	
6.	Have you arranged full security precautions for data to the level described in Section 4 of the attached guidance (if you are not sure seek professional advice from relevant IT Department)	x	
7.	Will the data be held / processed on a SUHT NHS computer	x	
8.	If you are not using a SUHT computer, please indicate which of the following you will be using: b) University computer (totally anonymised data) **NB - If c), d), or e) is to be used, you MUST seek advice from IM&T**		
9.	Can you confirm you will **NOT** pass patient identifiable information via e-mail or on the Internet?	x	
10.	Do you hold a NHS contract or SUHT honorary contract – (if using Southampton University Hospitals NHS Trust patient data you must be an employee or honorary employee of SUHT. If not seek advice from SUHT personnel) * see note	x	

Note: *If you hold an honorary contract, a copy will need to be included with your application for Data Protection approval.*

Southampton University Hospitals NHS Trust
Data Protection Office

Version: 08/08

Data Protection Advice SUHT – Please complete Section C and D or E

Section C

Registration of Research project

Completed by: **Mr Ramsey Cutress**

NAME OF ENQUIRER: **Mr Ramsey Cutress**

DEPT/MAILPOINT/ADDRESS: **Southampton University, Cancer Sciences Division, Somers Cancer Research Building, Southampton General Hospital, Tremona Road, Southampton. SO16 6YD**

TELEPHONE NUMBER: **02380796184** BLEEP: **N/A**

RESEARCH & DEVELOPMENT NUMBER: To be confirmed

STUDY TITLE: **Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects**

DATA PROTECTION REFERENCE NUMBER:.....(for office use only).....

Please print your information

Section D

I have enquired about Data Protection for audit/research purposes. Having read the guidance provided, I am satisfied that I will manage my data in accordance with the Data Protection principles. I have completed the questionnaire in Section B of this form and have been instructed to complete Section D.

SIGNED.....DATE.....

Following completion of the form continue with your submission for Ethics via Research & Development. Please ensure you receive a written compliance letter with a DP reference number from IM&T.

Section E

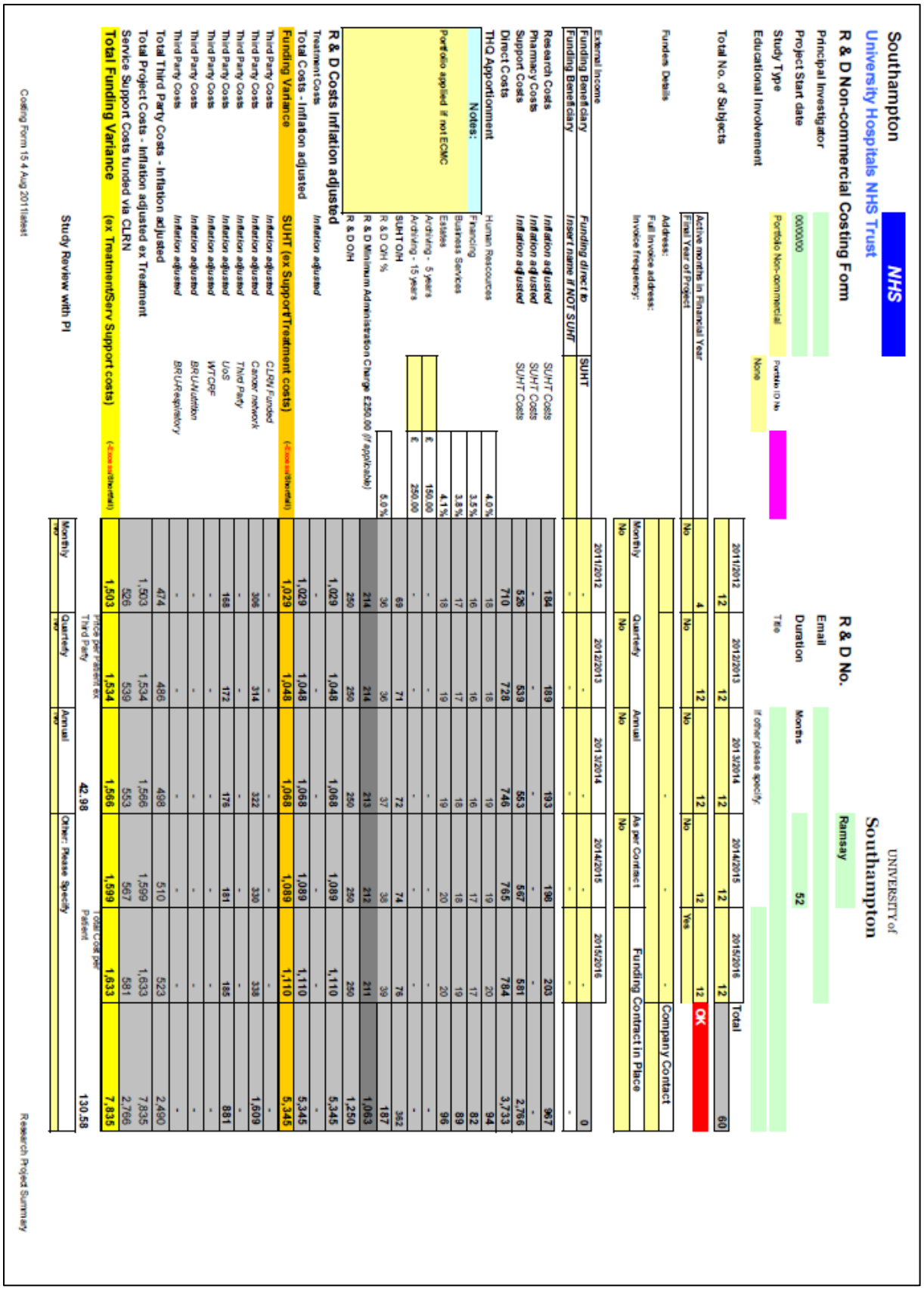
I am not sure whether I meet the criteria as outlined in the Trust Data Protection guidance or have been instructed to complete Section E by the enclosed questionnaire or flowchart. My reasons or issues are given below:

SIGNED.....DATE.....

Southampton University Hospitals NHS Trust
Data Protection Office

Version: 08/08

V. Costings


Southampton
NHS
University Hospitals NHS Trust
R & D Non-commercial Costing Form
UNIVERSITY of
Southampton
R & D No.
Ramsay
Principal Investigator
Email
Project Start date
00/00/00
Duration
Months
52
Study Type
Portfolio Non-commercial
Portfolio ID No
Title
Educational Involvement
None
If other please specify
Total No. of Subjects
2011/2012 12
2012/2013 12
2013/2014 12
2014/2015 12
2015/2016 12
Total 60
Active months in Financial Year
No 4
No 12
No 12
No 12
No 12
OK
Final Year of Project
Funders Details
Address:
Full Invoice address:
Invoice frequency:
Company Contact
Monthly
Quarterly
Annual
As per Contract
Funding Contract in Place
No
No
No
No
No
External Income
2011/2012
2012/2013
2013/2014
2014/2015
2015/2016
Funding Beneficiary
SUHT
Funding direct to
Funding Beneficiary
Insert name if NOT SUHT
Research Costs
Inflation adjusted
SUHT Costs
Pharmacy Costs
Inflation adjusted
SUHT Costs
Support Costs
Inflation adjusted
SUHT Costs
Direct Costs
THQ Apportionment
Human Resources
Financing
Business Services
Estates
Archiving - 5 years
Archiving - 15 years
SUHT O/H
R & D O/H %
R & D Minimum Administration Charge £250.00 (if applicable)
R & D O/H
Notes:
Portfolio applied if not ECMC
4.0%
3.5%
3.8%
4.1%
£ 150.00
£ 250.00
5.0%
R & D Costs Inflation adjusted
Inflation adjusted
Treatment Costs
Total Costs - Inflation adjusted
Funding Variance
SUHT (ex SupportTreatment costs)
(Excl VAT/Benefit)
Third Party Costs
Inflation adjusted
CLRN Funded
Third Party Costs
Inflation adjusted
Cancer network
Third Party Costs
Inflation adjusted
Third Party
Third Party Costs
Inflation adjusted
UoS
Third Party Costs
Inflation adjusted
WTCRF
Third Party Costs
Inflation adjusted
BRU/Institution
Third Party Costs
Inflation adjusted
BRU/Respiratory
Total Third Party Costs - Inflation adjusted
Total Project Costs - Inflation adjusted ex Treatment
Service Support Costs funded via CLRN
Total Funding Variance
(ex Treatment/Serv Support costs)
(Excl VAT/Benefit)
Price per Patient ex
Third Party
42.98
Total Cost per
Patient
130.58
Monthly
Quarterly
Annual
Other: Please Specify
Study Review with PI
Costing Form 15.4 Aug 2011latest
Research Project Summary

5). Documents relating to Substantial Amendment 1

I. Notice of substantial amendment letter

National Research Ethics Service

NHS Health Research Authority

NOTICE OF SUBSTANTIAL AMENDMENT (non-CTIMP)

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) available in the Integrated Research Application System (IRAS) at http://www.myresearchproject.org.uk or on the EudraCT website at https://eudract.ema.europa.eu/document.html.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.nres.nhs.uk/applications/after-ethical-review/notification-of-amendments/.

Details of Chief Investigator:	
Name:	*Professor Christian Ottensmeier*
Address:	Cancer Sciences Division University Hospital Southampton Tremona Road Southampton
Postcode:	SO16 6YD
Telephone:	02380 795161
Email:	cho@soton.ac.uk
Fax:	02380 795152

Full title of study:	Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects
Lead sponsor:	University Hospital Southampton NHS Foundation Trust
Name of REC:	South Central
REC reference number:	11 SC 0509
Name of lead R&D office:	University Hospital Southampton NHS Foundation Trust
Date study commenced:	Recruitment pending

Notice of substantial amendment (non-CTIMP), version 4.0 November 2011 1

Protocol reference *(if applicable)*, current version and date:	Understanding immunity in lymph nodes Protocol Version 5.2 31.10.2012
Amendment number and date:	Amendment 1 31.10.2012

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC Application Form

Yes ***No***

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes *No*

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes *No*

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes ***No***

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study.

If this is a modified amendment, please explain how the modifications address concerns raised previously by the ethics committee.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

We intend to take additional tissue samples (2x core needle biopsies) from some of the primary tumour specimens following their surgical excision and prior to their preservation in formalin. This will occur at the time of the patients' surgery. The core-needle biopsy specimens are small and will not impact on the histological assessment of the tumour. This method of gathering tumour tissue is standard protocol within several multi-centre peri-surgical trials, e.g. POETIC. The biopsies will be used to evaluate the populations of tumour infiltrating immune cells and their antigen specificity. This will guide evaluation of any bystander response to tumour antigens initiated by vaccination with an unrelated antigen, increasing the scientific validity of the study.

These changes are reflected in amendments to the documents listed below, which are highlighted in yellow for clarity.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

The amendment does not require any additional active participation by the patient.

The amendment requires additional tissue to be taken from the surgical specimen, but no additional tissue will be removed from the patient and doing so will not impact on the patients care.

List of enclosed documents

Document	*Version*	*Date*
31102012 SNB protocol	5.2	31.10.2012
31102012 patient information sheet – lymph node response to vaccine	5.2	31.10.2012
31102012 consent form – axillary node excision	5.2	31.10.2012
31102012 GP information sheet – lymph node response to vaccine	5.2	31.10.2012

Declaration by Chief Investigator

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: Professor Christian Ottensmeier

Date of submission: 31/10/2012

Declaration by the sponsor's representative

The sponsor of an approved study is responsible for all amendments made during its conduct.

The person authorising the declaration should be authorised to do so. There is no requirement for a particular level of seniority; the sponsor's rules about delegated authority should be adhered to.

- I confirm the sponsor's support for this substantial amendment.

Signature of sponsor's representative: .....

Print name: .....

Post: .....

Organisation: .....

Date: .....

II. Approval letter from study sponsor

University Hospital Southampton NHS
NHS Foundation Trust

Clinical Governance
R&D Department
University Hospital Southampton NHS Foundation Trust
Level E, Laboratory & Pathology Block
SCBR – Mailpoint 138
Tremona Road
Southampton SO16 6YD

sharon.atwill@uhs.nhs.uk
Tel: 023 8079 5078
Fax: 023 8079 8678

21 November 2012

Professor Christian Ottensmeier
Cancer Sciences Division
University Hospital Southampton
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Re: **11/SC/0509 – Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects (RHM CAN0817)**

Thank you for providing a copy of the documents for amendment number 1 dated 31 October 2012, these have been reviewed and as sponsor we are happy for these to be submitted to the Research Ethics Committee for review and approval.

Please ensure that a copy of the REC approval letter for the amendment is forwarded to myself to enable full R&D approval to be given for the amendment. Please note that the amendment should not be implemented until both REC and R&D approval are in place.

If I can be of any further assistance please do not hesitate to contact me.

Yours sincerely

Mrs Sharon Atwill
Research Governance Officer – Division A

www.uhs.nhs.uk

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III. Notice of Favourable Ethical Opinion

NHS
Health Research Authority

NRES Committee South Central - Southampton A
Bristol Research Ethics Committee Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1381
Fax: 0117 342 0445

17 December 2012

Professor Christian Ottensmeier
University of Southampton
MP 824,
Somers Cancer Research Building,
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Study title:	**Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects**
REC reference:	**11/SC/0509**
Protocol number:	**RMH CAN0817**
Amendment number:	**1**
Amendment date:	**31 October 2012**
IRAS project ID:	**72796**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
GP/Consultant Information Sheets	5.2	31 October 2012
Participant Consent Form	5.2	31 October 2012
Participant Information Sheet	5.2	31 October 2012

Protocol	5.2	31 October 2012
Notice of Substantial Amendment (non-CTIMPs)	1	31 October 2012
Covering Letter		21 November 2012

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

11/SC/0509:	**Please quote this number on all correspondence**

Yours sincerely

pp

Dr Simon Kolstoe
Vice Chair

E-mail: nrescommittee.southcentral-southamptona@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Sharon Atwill*

6). Documents relating to Substantial Amendment 2

I. Notice of substantial amendment letter

National Research Ethics Service

NHS Health Research Authority

NOTICE OF SUBSTANTIAL AMENDMENT (non-CTIMP)

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) available in the Integrated Research Application System (IRAS) at http://www.myresearchproject.org.uk or on the EudraCT website at https://eudract.ema.europa.eu/document.html.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.nres.nhs.uk/applications/after-ethical-review/notification-of-amendments/.

Details of Chief Investigator:

Name:	Professor Christian Ottensmeier
Address:	Cancer Sciences Division University Hospital Southampton Tremona Road Southampton
Postcode:	SO16 6YD
Telephone:	02380 795161
Email:	cho@soton.ac.uk
Fax:	02380 795152

Full title of study:	Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects
Lead sponsor:	University Hospital Southampton NHS Foundation Trust
Name of REC:	South Central
REC reference number:	11 SC 0509
Name of lead R&D office:	University Hospital Southampton NHS Foundation Trust
Date study commenced:	Recruitment pending

Notice of substantial amendment (non-CTIMP), version 4.0 November 2011 1

Protocol reference *(if applicable)*, current version and date:	Understanding immunity in lymph nodes Protocol Version 5.3 18.02.2013
Amendment number and date:	Amendment 2 18.02.2013

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC Application Form

Yes *No*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes *No*

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes ***No***

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes ***No***

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study.

If this is a modified amendment, please explain how the modifications address concerns raised previously by the ethics committee.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

The investigators would like to seek the following amendments to the agreed study plan:

1) The original REC form was completed with section 2a. Sub-section b. "Will you be using surplus tissue or existing stored samples identifiable to the researcher?" checked as "no". This was in error and the correct answer should have been "yes". At various points in the original REC application form (sections A6-2 and A30-1) and original protocol (Page 12, lines 19-21 and Page 13, lines 16-22) we make reference to the use of tissue surplus to diagnostic requirements being surrendered by the pathology department for use in the study. REC approval was originally granted based on these documents. We hope that this error on our part can be corrected, with the alteration of section 2a sub-section b to correctly reflect the contents of the original forms.

2) We intend to optimise the techniques that we are using to isolate cells from lymph nodes removed for the purposes of our study. It has come to our attention that the way in which we do this can influence the outcome of our experiments. It is therefore important to validate these techniques prior to recruitment into our study.

Patients undergoing surgery for benign colorectal disease (such as ulcerative colitis or Crohn's disease) have lymph nodes removed as part of their normal surgical treatment. These lymph nodes are surplus to diagnostic requirements and are often discarded. The tissue bank at Southampton General Hospital (HTA Licence 12009) has approval to collect lymphatic tissue for the purposes of ethically approved research. This material is banked with the patients consent in an anonymised form, unlinked to any clinical data. We would like permission to remove such tissue from the tissue bank for use in the validation of our techniques. This will allow our techniques to be optimised prior to receipt of the study tissue, thereby maximising the potential scientific gain of our research and increasing the scientific validity of the study, and eliminating any risk that study material may be lost through under-optimised processing. No data gained from this material will have any relevance to the patient's condition or prognosis. Techniques performed on such material will be limited to flow-cytometry to quantify cell sub-populations within lymph nodes and assays to evaluate the function of these cells during processing.

This change is reflected in amendments made to the protocol which are highlighted in yellow for clarity.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

The amendment does not require any additional active participation by patients.

The amendment requires the use of banked human tissue. This tissue has been stored within the Southampton Tissue Bank according to HTA guidelines. Patients from whom tissue has been taken have given their consent for any of their tissue taken which is surplus to their clinical or diagnostic requirements to be used in relevant ethically approved studies. All tissue is banked in a fully anonymised unlinked fashion. No new information relevant to the patients' health or condition will be attained through the intended work.

List of enclosed documents

Document	*Version*	*Date*
18022013 SNB protocol	5.3	18.02.2013

Declaration by Chief Investigator

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: Professor Christian Ottensmeier

Date of submission: 18/02/2013

Declaration by the sponsor's representative

The sponsor of an approved study is responsible for all amendments made during its conduct.

The person authorising the declaration should be authorised to do so. There is no requirement for a particular level of seniority; the sponsor's rules about delegated authority should be adhered to.

- I confirm the sponsor's support for this substantial amendment.

Signature of sponsor's representative: .....

Print name: .....

Post: .....

Organisation: .....

Date: .....

II. Approval letter from study sponsor

University Hospital Southampton NHS
NHS Foundation Trust

Clinical Governance
R&D Department
University Hospital Southampton NHS Foundation Trust
Level E, Laboratory & Pathology Block
SCBR – Mailpoint 138
Tremona Road
Southampton SO16 6YD

sharon.atwill@uhs.nhs.uk
Tel: 023 8079 5078
Fax: 023 8079 8678

27 February 2013

Professor Christian Ottensmeier
Cancer Sciences Division
University Hospital Southampton
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Re: **11/SC/0509 – Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects (RHM CAN0817)**

Thank you for providing a copy of the documents for amendment number 2 dated 18 February 2013, these have been reviewed and as sponsor we are happy for these to be submitted to the Research Ethics Committee for review and approval.

Please ensure that a copy of the REC approval letter for the amendment is forwarded to myself to enable full R&D approval to be given for the amendment. Please note that the amendment should not be implemented until both REC and R&D approval are in place.

If I can be of any further assistance please do not hesitate to contact me.

Yours sincerely

Mrs Sharon Atwill
Research Governance Officer – Division A

www.uhs.nhs.uk

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III. Notice of Favourable Ethical Opinion

NHS
Health Research Authority

NRES Committee South Central - Southampton A

Bristol Research Ethics Committee Centre

Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1381
Fax: 0117 342 0445

15 March 2013

Professor Christian Ottensmeier
University of Southampton
MP 824,
Somers Cancer Research Building,
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Study title:	**Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects**
REC reference:	**11/SC/0509**
Protocol number:	**RMH CAN0817**
Amendment number:	**2**
Amendment date:	**18 February 2013**
IRAS project ID:	**72796**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	5.3	18 February 2013
Notice of Substantial Amendment (non-CTIMPs)	2	18 February 2013
Covering Letter		27 February 2013

A Research Ethics Committee established by the Health Research Authority

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

11/SC/0509:	**Please quote this number on all correspondence**

Yours sincerely

Dr Simon Kolstoe
Vice-Chair

E-mail: nrescommittee.southcentral-southamptona@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Sharon Atwill, University Hospital Southamtpon Foundation Trust*

A Research Ethics Committee established by the Health Research Authority

7). Documents relating to Substantial Amendment 3

I. Notice of substantial amendment letter

National Research Ethics Service

NHS Health Research Authority

NOTICE OF SUBSTANTIAL AMENDMENT (non-CTIMP)

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) available in the Integrated Research Application System (IRAS) at http://www.myresearchproject.org.uk or on the EudraCT website at https://eudract.ema.europa.eu/document.html.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.nres.nhs.uk/applications/after-ethical-review/notification-of-amendments/.

Details of Chief Investigator:

Name:	Professor Christian Ottensmeier
Address:	Cancer Sciences Division University Hospital Southampton Tremona Road Southampton
Postcode:	SO16 6YD
Telephone:	02380 795161
Email:	cho@soton.ac.uk
Fax:	02380 795152

Full title of study:	Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects
Lead sponsor:	University Hospital Southampton NHS Foundation Trust
Name of REC:	South Central
REC reference number:	11 SC 0509
Name of lead R&D office:	University Hospital Southampton NHS Foundation Trust
Date study commenced:	Recruitment pending

Notice of substantial amendment (non-CTIMP), version 4.0 November 2011 1

Protocol reference *(if applicable)*, current version and date:	Understanding immunity in lymph nodes Protocol Version 5.3 18.02.2013
Amendment number and date:	Amendment 3 21.04.2013

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC Application Form

Yes *No*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes *No*

If yes, please submit <u>either</u> the revised protocol with a new version number and date, highlighting changes in bold, <u>or</u> a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes *No*

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes ***No***

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study.

If this is a modified amendment, please explain how the modifications address concerns raised previously by the ethics committee.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

The original study plan was to use a single agent tetanus vaccine to investigate the function of the immune system. Attempts to source this vaccine have been unsuccessful as the product does not have a licence for use in the United Kingdom. It has therefore become necessary for us to change our intended vaccine for use in the study from a single agent tetanus vaccine to another vaccine containing tetanus toxoid which is licenced for use within the United Kingdom.

Therefore the investigators would like to seek the following amendments to the agreed study plan:

Within section A6-2 of the original REC form, under the sub-section heading "3) administration of vaccine and use of atypical injection sites" we state our intent to source a single agent tetanus vaccine in preference to use of the Diphtheria/tetanus/polio vaccine available within the UK. We would appreciate it if this statement could be removed.

The following changes have therefore been made to the protocol, patient information sheet, G.P. information sheet and consent form as a consequence of the change in vaccine. These are highlighted in yellow for clarity:

- **Protocol:** where the protocol previously read "single agent tetanus vaccine", this has now been changed to "a vaccination against tetanus".
- **GP Information Sheet:** detail within the sheet has been changed to reflect the change of vaccine being used.
- **Patient Information Sheet:** No changes to the content were necessary. The version number and date have been changed to correspond to the date and version of the GP information sheet.
- **Consent form:** version number and date have been changed to correspond to the new version patient information sheet

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

The amendment does not require any additional active participation by patients.

This amendment changes the intended vaccine from a product unlicensed for use in the UK to one which is fully licenced for UK use and is regularly administered to NHS patients.

List of enclosed documents

Document	*Version*	*Date*
21042013 SNB protocol	5.4	21.04.2013
21042013 Consent form - axillary node excision	5.3	21.04.2013
21042013 GP information sheet – Lymph node response to vaccine	5.3	21.04.2013
21042013 Patient Information Sheet – Lymph node response to vaccine	5.3	21.04.2013

Declaration by Chief Investigator

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: Professor Christian Ottensmeier

Date of submission: 21/04/2013

Declaration by the sponsor's representative

The sponsor of an approved study is responsible for all amendments made during its conduct.

The person authorising the declaration should be authorised to do so. There is no requirement for a particular level of seniority; the sponsor's rules about delegated authority should be adhered to.

- I confirm the sponsor's support for this substantial amendment.

Signature of sponsor's representative: .....

Print name: .....

Post: .....

Organisation: .....

Date: .....

II. Approval letter from study sponsor

University Hospital Southampton NHS
NHS Foundation Trust

Clinical Governance
R&D Department
University Hospital Southampton NHS Foundation Trust
Level E, Laboratory & Pathology Block
SCBR – Mailpoint 138
Tremona Road
Southampton SO16 6YD

sharon.atwill@uhs.nhs.uk
Tel: 023 8079 5078
Fax: 023 8079 8678

24 April 2013

Professor Christian Ottensmeier
Cancer Sciences Division
University Hospital Southampton
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Re: **11/SC/0509 – Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects (RHM CAN0817)**

Thank you for providing a copy of the documents for amendment number 3 dated 21 April 2013, these have been reviewed and as sponsor we are happy for these to be submitted to the Research Ethics Committee for review and approval.

Please ensure that a copy of the REC approval letter for the amendment is forwarded to myself to enable full R&D approval to be given for the amendment. Please note that the amendment should not be implemented until both REC and R&D approval are in place.

If I can be of any further assistance please do not hesitate to contact me.

Yours sincerely

Mrs Sharon Atwill
Research Governance Officer – Divisions A&C

www.uhs.nhs.uk

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III. Notice of Favourable Ethical Opinion

NHS
Health Research Authority

NRES Committee South Central - Southampton A
Bristol Research Ethics Committee Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Tel: 0117 342 1381
Fax: 0117 342 0445

20 May 2013

Professor Christian Ottensmeier
University of Southampton
MP 824,
Somers Cancer Research Building,
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear Professor Ottensmeier

Study title:	**Understanding immune responses to prophylactic vaccination in lymph nodes: correlation between tissue and blood effects**
REC reference:	**11/SC/0509**
Protocol number:	**RMH CAN0817**
Amendment number:	**3**
Amendment date:	**21 April 2013**
IRAS project ID:	**72796**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

No ethical issues.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
GP/Consultant Information Sheets	5.3	21 April 2013
Participant Consent Form: Axillary node excision	5.3	21 April 2013

Participant Information Sheet: Lymph node response to vaccine	5.3	21 April 2013
Protocol	5.4	21 April 2013
Notice of Substantial Amendment (non-CTIMPs)	3	21 April 2013
Covering Letter		24 April 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

11/SC/0509:	**Please quote this number on all correspondence**

Yours sincerely

pp
Dr Simon Kolstoe
Vice Chair

E-mail: nrescommittee.southcentral-southamptona@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Sharon Atwill*
Sharon Atwill, University Hospital Southamtpon Foundation Trust

8). Final study-specific documentation

I. Study Protocol

PROTOCOL

Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

Short Title: Understanding immunity in lymph nodes

REC Reference Number: 11/SC/0509

Chief investigators:

Mr Ramsey Cutress
Cancer Research UK Clinical Centre
Somers Cancer Research Building, MP824
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD
Study related medical decisions should be addressed to Mr Cutress in the first instance.

Professor Christian Ottensmeier
Cancer Research UK Clinical Centre
Somers Cancer Research Building, MP824
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Co-investigators:

Mr David Layfield
Dr Katy McCann
Mrs Lindsey Chudley
Dr Gianfranco Di Genova

Study Sponsor:

R&D Office
E Level, Southampton centre for biomedical research
Centre Block
Southampton General Hospital
Tremona Road
Southampton SO16 6YD
Telephone (023) 8079 5314
Fax: 023 8079 8678
Email: R&Doffice@suht.swest.nhs.uk.

Study Sites:

Southampton General Hospital, Tremona Road, Southampton, SO16 6YD
Tel: 023 8077 7222

Princess Anne Hospital, Coxford Road, Southampton, SO16 5YA
Tel: 023 8077 7222

Departments:

Cancer Sciences Division, University of Southampton School of Medicine, Somers Cancer Research Building, MP824, Southampton General Hospital, Tremona Road, Southampton SO16 6YD

Southampton Breast Unit, C Level, MP132, Princess Anne Hospital, Coxford Road, Southampton, SO16 5YA

Abbreviations

Ag	Antigen
C. albicans	*Candida albicans*
CEA	Carcinoembryonic antigen
CMV	Cytomegalovirus
CRF	Case report form
DMSO	Dimethylsulphoxide
EBV	Epstein-Barr virus
FFPE	Formalin-fixed paraffin-embedded
Flu	Influenza
HER2/neu	Human epidermal growth factor receptor 2
LN	Lymph node
PASD1	PAS domain containing 1
PBMC	Peripheral blood mononuclear cell
PPD	Protein derivative of tuberculin
REC	Research ethics committee
SNB	Sentinel node biopsy
TT	Tetanus toxoid
WT1	Wilms' tumour 1

Contents

Background

The immunity raised by vaccination to infectious agents is well documented, but despite this, the literature describing the kinetics of humoral and cellular immune responses in humans is surprisingly sparse. A recent study undertaken in Southampton has shown that immunity in humans is much more dynamic than initially thought. The study revealed that T-cell responses against an antigen (Ag) included in a vaccine, in this case tetanus toxoid (TT), followed a predictable time-course that could be readily monitored in the blood. However, surprisingly, the study also revealed significant activation of T cells, mainly of a CD4+ phenotype, that were specific for other, unrelated Ags, such as proteins from *Candida albicans* (*C. albicans*) and tuberculosis.[1] This observation shed light on a possible pathway by which our immune system is able to maintain immunological T-cell memory over time as a bystander response to ongoing infections, even in the absence of exposure to the infectious agent against which protection is required.

In a follow-up study, a mouse model was used to confirm that transferred T cells, which had been pre-activated *in vitro* with a peptide from ovalbumin, showed a bystander proliferative response during a parallel and unrelated memory immune response to TT.[2] Bystander proliferation was dependent on boosting of the TT-specific memory response in the recipients, with no effect in naive mice. Bystander stimulation was also proportional to the strength of the TT-specific memory T-cell response. T cells activated *in vitro* displayed functional receptors for IL-2 and IL-7, suggesting these as potential mediators. These data confirm the crosstalk between a stimulated CD4+ memory T-cell response and CD4+ T cells activated by an unrelated Ag.

We are currently examining whether the bystander effect observed after booster vaccination is also present during a primary immune response to a previously unseen Ag. Using healthy donors, we are evaluating bystander activation of memory CD4+ and CD8+ T cells in persons who are exposed to a hepatitis B vaccine for the first time (RHM CAN0489; REC 06/Q1702/155).

All of our studies in humans to date have looked in the blood, as this is easily accessible. However, immune response do not develop in the blood but in the lymph nodes (LNs) draining the site of vaccination. Human data on immune responses to vaccination in the draining LNs are absent from the literature. In the proposed study, we wish to understand the immunological events in the LN compartment following prophylactic vaccination and to

compare the observations in the LN with those in the blood. Understanding these biological effects is a critical prerequisite for the understanding of immune responses induced by vaccination for the treatment of cancer and to better understand how we might successfully develop cancer vaccines.

Cancer is known to modulate the immune reactivity of its draining lymphatics by release of immune-suppressive cytokines and via its influence over antigen presentation by dendritic cells[3]. Tumour infiltrating lymphocytes are also thought to be important in anti-tumour response[4], as demonstrated by their apparent influence on disease outcome[5-7]. Understanding the mechanisms by which the bystander response re-arms the immune system against unrelated antigens may suggest mechanisms by which anti-tumour vaccination and other immunotherapies targeting cancer might reactivate anti-tumour immunity. We therefore also intend to analyse the antigen specificity of tumour infiltrating immune cells and correlate that with the bystander proliferation witnessed in our patients following vaccination.

Objectives

To characterise the response to vaccination, including that with tetanus, in the blood and draining LNs in patients undergoing sentinel node biopsy for cancer.

Study design

This study will coordinate routine preventative vaccination against infectious diseases such as tetanus, influenza (Flu) or hepatitis with sentinel node biopsy (SNB) at a time-point when SNB is clinically required for the staging of patients' cancer. The initial focus will be in patients with breast cancer. If successful, we intend to extend the study to patients having a SNB for other cancers, for example, melanoma.

Patients diagnosed with breast cancer, and in whom a SNB is planned as part of surgical treatment, will be offered vaccination in the ipsilateral arm to the breast cancer prior to surgery. The consultant surgeon or clinician responsible for the care of the patient will obtain a vaccination and medical history to determine if routine vaccination is appropriate. Patients who have not received tetanus vaccination within the last 10 years will be eligible for inclusion. Those patients who are willing to participate will be randomised into one of

three groups; test group, control group 1 or control group 2 (see "Study Population" below). Patients randomised to test group or control group 1 will be immunised when they attend the pre-admission clinic visit one week before surgery. All participants will have a blood sample will be taken at the pre-admission visit for analysis.

At SNB surgery, a second blood sample will be obtained, and two further blood samples will be collected post-surgery. The SNB will be performed as usual, but with a single additional LN taken for study purposes. A breast sentinel node biopsy generally involves the removal of between two and three sentinel nodes to stage the axilla. However, prior to the introduction of SNB, an axillary clearance was performed on all breast cancer patients, which involves the removal of all LNs within a defined anatomical boundary, typically between 10 to 20. Comparison of patient-reported quality-of-life outcomes between SNB and axillary clearance does demonstrate differences, but these are shown to resolve with time[8]. Similarly, the ALMANAC trial demonstrates differences in morbidity between the two surgical approaches, but the rate of arm lymphoedema, as measured by mean change in arm volume, was not significantly greater with axillary clearance compared to SNB at 12 months[9]. It is therefore felt that removal of a single, additional non-sentinel node at the time of SNB is unlikely to cause significant additional morbidity. In sentinel node negative cases, no further nodes would normally be removed so removal of a single non-sentinel node will not affect axillary staging. In node positive cases, data from a trial of axillary radiotherapy demonstrates that avoiding an axillary clearance does not alter decision making regarding adjuvant therapies[10]. We therefore feel that the removal of one additional non-sentinel node will not result in the loss of important prognostic information.

To reduce the risk of loosing prognostic information from the study node in the event of a positive SLN biopsy, the sentinel nodes will be processed on the same day as surgery. If the SLN biopsy is positive for metastasis, the non-sentinel node taken for the purpose of the study will be surrendered to the hospital pathologist for standard clinical processing (as per a completion axillary clearance). If the sentinel node biopsy is negative for cancer the non-sentinel study node will be submitted for research and processed according to protocols optimised using anonymously donated lymphatic tissue, stored within the Southampton Human Tissue Bank (HTA Licence Number 12009).

Once the tumour has been removed, 2 core needle biopsy samples will be taken from the tumour prior to it being submitted for histological assessment. Each core needle biopsy will remove only a small quantity of tissue which will not hinder histological staging of the tumour. Removal of tumour tissue in this fashion is standard practice in several large multi-

centre peri-surgical studies, for example the POETIC breast cancer trial[11]. These biopsies will allow analysis of the antigen specificity of the tumour infiltrating immune cells; one biopsy will be processed in "RNA later", the other will be processed for cell retrieval.

In this study, we wish to investigate humoral (memory B cell/plasmablasts and/or antibody) and cellular immune responses to vaccination in both the peripheral blood and LN compartments following vaccination. Furthermore, we intend to assess bystander immune responses to other antigens unrelated to vaccination, such as *Candia albicans* (*C. albicans*) and protein derivative of tuberculin (PPD) of *Mycobacterium tuberculosis*. Studies of bystander response to anti-tumour antigens will also be performed, guided by findings from biopsies taken from the primary site.

Study population:

Test group: 30 patients diagnosed with cancer in whom a SNB is planned as part of surgical treatment will receive a vaccination against tetanus administered subcutaneously into the arm on the same side as the surgery.
Control group 1: 15 patients diagnosed with cancer in whom a SNB is planned as part of surgical treatment will receive a vaccination against tetanus administered intramuscularly into the arm on the opposite side as the surgery.
Control group 2: 15 patients diagnosed with cancer in whom a SNB is planned as part of surgical treatment will receive no vaccination prior to surgery vaccination.

Study plan: (See Appendix A)

Week 0: A blood sample will be taken at the pre-admission clinic visit. Patients randomised to test group or control group 1 will receive a single, standard dose of a vaccine against tetanus.

Week 1: A 2nd blood sample will be taken at this time.
At surgery, SNB will be performed as usual, with one additional LN taken for study purposes. Prior to processing the additional non-sentinel node, the sentinel node(s) will be analysed to ensure there is no evidence of metastatic disease. In the event of metastatic disease within the sentinel node(s), the node taken for study purposes will be surrendered to the hospital pathologist for standard clinical procession rather than research. In addition, 2 core needle biopsy samples will be taken from the tumour specimen for study purposes.

Week 3: Patients with positive sentinel nodes will be informed that their node has not been used for research and given the option to exit the study at this stage. In all remaining participants a 3rd blood sample will be taken at the post-operative visit two weeks following surgery.

Week 6: A 4th and final blood sample will be taken at 6 weeks post-vaccination. In addition, simple histopathological details will be recorded and access to formalin-fixed paraffin-embedded (FFPE) tissue surplus to diagnostic requirements will be requested for immunohistochemistry and other studies.

Each blood collection will consist of 60mL anti-coagulated blood and 10mL clotted blood. Total venesections: 4 over 6 weeks; 280mL (a significantly smaller volume than that of a single blood donation). These blood samples are necessary to identify the antibodies produced during the response to vaccination, their titres and sub-types, in order to correlate these with the changes seen within the study node.

All samples will be anonymised upon receipt in the laboratory and identified only by a unique patient identification number according to the standard operating procedure in place for this purpose (CSD/SOP/003). The sample number and date of receipt will also be recorded. On conclusion of the study any remaining samples or tissue will be donated to the University of Southampton Research Tissue Bank (Human Tissue Act License: 12009, Southampton and South West Hampshire Research Ethics Committee: 280/99).

Clinical Sampling

Patient identification and recruitment

Eligible patients will be identified from the outpatient clinics at University Hospital Southampton or Princess Anne Hospital by members of the Cancer Multidisciplinary Team. Patient information sheets will be provided to eligible patients inviting them to consider participation. All patients will be given a minimum of 24 hours to consider entry to the study. Interpreters will be provided where possible for participants who are non-English speakers. Having received prior written and verbal explanation of the study patients who wish to participate will be required to provide written informed consent. Consent will include permission for storage of samples for analyses relating to this study and for the use of FFPE histopathology material, where adequate surplus material exists following completion of a full

diagnostic work. Informed consent will be obtained by members of the clinic team or the surgeon undertaking the patient's operation.

Inclusion criteria

1. Patients due to undergo SNB for cancer staging.
2. Patients aged 18 years or older.
3. Patients who have not received tetanus vaccination within the last 10 years
4. Patients with the ability to understand the study requirements provide written informed consent and comply with the study protocol procedures.

Exclusion criteria

1. Presence of axillary node disease at presentation
2. Presence of metastatic disease at presentation
3. Taking of immune-suppressing medications including oral, but not inhaled, steroids
4. Hypersensitivity to tetanus containing vaccines

End of Study Participation

Patients' active participation in the study will cease following the collection of the 4th blood sample at week 6 post-vaccination. No further collection of samples will occur beyond this point. Recording of age and histopathological details will occur once the final diagnostic histopathology report is available.
Patients found to have positive sentinel nodes will have the node they donated for study purposes returned to the hospital pathology department for standard processing and their nodal tissue will not be used for study purposes. These patients will be given the option to leave the study at the time of their 2 week post operative visit, which is when they will be informed of their pathology results (including that of their primary specimen and SLNB)

Clinical and histopathological data collection

Where available, clinical and histopathological information will be extracted and recorded onto study specific case report forms (CRF) by a member of the CRUK Clinical Research Unit. Data points recorded will include:

- Age at diagnosis
- Tumour grade
- Tumour size
- Histological tumour type
- Oestrogen and progesterone receptor status
- HER2 over-expression status

Data will be extracted from patients clinical records (generally from computer based records of histopathology reports) held by Southampton University Hospitals NHS Trust by clinically trained research personnel with authorisation to access such details and stored within a secure facility within the Southampton CRUK Clinical Centre. Data will be anonymised at the point of entry onto the CRF to allow it to be linked to the relevant sample, but with all identifying information removed. The Chief Investigators will retain overall responsibility for the recording and quality of the data.

Sample processing and storage

Use of Banked Human Tissue:

The human tissue bank held on site within the University of Southampton stores samples of lymphatic tissue including lymph nodes from non-breast cancer patients. Patients donating these specimens have consented to their use in relevant, ethically approved studies.

The techniques and assays intended for use during the main work of the study will first be validated using this anonymous tissue which is surplus to clinical or diagnostic requirements. Validation will ensure that the processes used within the study do not, in themselves, effect the results from the material retrieved from study participants. By validating the procedures prior to recruitment we hope to maximise the scientific validity of this work and minimise any possible loss of material from study participants which could occur if sub-optimal techniques were used.

Processing Study Tissue:

A single, additional non-sentinel LN will be taken during surgery following the SNB by the responsible surgeon. The node will then be placed in a sterile container and placed immediately on ice for collection from theatre reception by a member of the CRUK Clinical Research Unit. Coordination between the surgical and laboratory teams regarding the timing of collections will occur prior to commencement of the surgical list.

The sentinel nodes will be processed on the same day of surgery to look for metastasis. In the event of the sentinel nodes proving negative for metastasis the study node will be processed fresh on the day of surgery by mechanical dissociation into a single cell suspension, followed by cryopreservation in 50% decomplemented human Ab serum, 40% complete RPMI 1640 medium and 10% dimethylsulphoxide (DMSO), using a validated technique optimised using donated tissue from the Southampton Human Tissue Bank. Cells will be transferred to liquid nitrogen after 24 hours.

Two core needle biopsies will be taken from the tumour specimen following surgical excision and prior to preservation in formalin. No additional tissue will be removed from the patient. One biopsy specimen will be placed in "RNA later" and the other transferred on ice to the CRUK Clinical Research Unit where it will be processed in a fashion similar to the study lymph node, as described above.

Blood samples will be taken by research nurses from the CRUK Clinical Research Unit and processed on the same day. Coordination between the research nurse and laboratory team regarding the timing of collections will occur prior to venesection. Peripheral blood mononuclear cells (PBMCs) will be isolated from anti-coagulated blood by centrifugation over Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and either used immediately or cryopreserved in 50% decomplemented human Ab serum, 40% complete RPMI 1640 medium and 10% DMSO. Cells will be transferred to liquid nitrogen after 24 hours. Serum will be isolated from clotted blood samples by centrifugation and stored at -20°C.

Patient material collected during this study will be stored within a secure facility in the Southampton CRUK Clinical Centre. Material will be held as linked anonymised samples and labelled with a study specific number. The chief investigators and co-investigators will have access to the samples for analyses relating to this study. Patients will be asked to provide signed consent for the indefinite storage of samples for use by the investigators for analyses relating to the objectives of this study, or in the event of samples or tissue remaining on conclusion of the study, for donation to the University of Southampton Tissue bank for use in future ethically approved research.

Where possible, surplus diagnostic histopathological material (RNA lysate, FFPE tissue blocks/slides) will be made available by the Department of Cellular Pathology, Southampton University Hospitals NHS Trust.

Laboratory analysis

1. Identify patient HLA status.
2. Assessment of blood and LN compartments for vaccine specific humoral and/or cellular immune responses.
3. Assessment of blood and LN compartments for bystander humoral and/or cellular immune responses, for example, against PPD, *C.albicans*, CMV, EBV, Flu, etc.
4. Investigate immune responses to tumour-associated Ags, such as CEA, HER2/neu, PASD1, WT1, MAGEA10, BAGE1, NY-ESO-1/CTAG1B, etc.

We aim to assess the peripheral blood and LN for evidence of vaccine and cancer specific CD4+ and CD8+ T cells, their phenotype and function. Evidence for bystander activation of T cells with other specificities will be sought, including PPD and *C. albicans,* as well as other viral Ags. Furthermore, we aim to investigate evidence for tumour-specific immune responses. B cell, NK, NKT and dendritic cell populations will be evaluated numerically, phenotypically and functionally where possible.

Of interest will be to examine whether non-sentinel LNs are affected by the tumour in terms of their ability to respond to booster vaccination and whether there is evidence of immunosuppression exerted by the tumour, for example, by assessing the number of regulatory T cells or myeloid derived suppressor cells. Explorative correlations of the level and type of vaccine-induced immune response with tumour grade and stage will be undertaken.

The primary tools for analysis are intended to be flow cytometry, proliferation and ELISPOT assays and ELISA, and will follow established SOPs in the Cancer Sciences Division. Assays will be performed by members of Professor Ottensmeier's group within the laboratories of the Cancer Sciences Division, University of Southampton School of Medicine.

Where adequate surplus histopathology material exists, FFPE tissue will be used for the analysis of proteins or genes relevant to the tumour/immune response by immunohistochemistry and gene expression, respectively; for example, the tumour markers, CEA, CA15.3, etc. Analysis of receptors for other key immunological molecules, such as cytokines (e.g. IFN-γ, IL-2, IL-13, IL-5) or chemokines, will be undertaken where available material and existing technology allows. Additional techniques will be used as new technology becomes available.

Data Storage

All essential documents, including source documents, will be retained for a minimum period of 15 years following the end of the study. Analytical data from this study will be stored electronically on password protected data files on workstations within the Southampton CRUK Clinical Centre by the investigators. The Chief Investigators and Co-Investigators will have access to the data for analyses.

Patient confidentiality will be maintained by removing patient identifiable labels other than an assigned study specific number to create linked anonymised samples. No personally identifying information will be released in any report or publication relating to this work.

Data will be collected and retained in accordance with the Data Protection Act 1998.

Monitoring and audit

The study will be monitored and audited in accordance with SUHT procedures. All trial related documents will be made available on request for monitoring and audit by SUHT, the relevant REC and other licensing bodies.

Ethics and R&D approval

The study will be performed subject to Research Ethics Committee (REC) approval, including any provisions of Site Specific Assessment and local Research and Development approval.

Research governance

The study will be conducted in accordance with The Medicine for Human Use (Clinical Trial) Amendment Regulations 2006 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines; and the Research Governance Framework for Health and Social Care.

Indemnity

This is an NHS-sponsored research study. For NHS sponsored research, HSG(96)48 reference no. 2 applies. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS Indemnity covers NHS staff, medical academic staff with honorary contracts, and those conducting the trial. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim.

Funding

This study is funded through research grants held by Mr Cutress and Professor Ottensmeier.

II. Patient information sheet

CANCER RESEARCH UK

University Hospital Southampton NHS
NHS Foundation Trust

UNIVERSITY OF Southampton

Medicine

Cancer Research UK Centre
Somers Cancer Research Building
Mail Point 824
Southampton General Hospital
Tremona Road
Southampton SO16 6YD, UK

T 023 8079 6184
F 023 8079 5152

Director Professor Peter Johnson

PATIENT INFORMATION SHEET

Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

REC Reference Number: 11/SC/0509

You are being asked to consider taking part in a research study. Before you decide whether or not to take part, it is important for you to understand why it is being done and what it will involve. Please take time to read the following information, and discuss it with others if you wish. Ask us if anything is not clear.

1. What is the purpose of the study?

We are investigating how the body responds to vaccination. Vaccines work by exposing the immune system to foreign material (or "antigens") which the body then targets for removal. Although scientists have previously studied the white blood cells produced in response to vaccines by taking blood samples, no one has previously studied the role of lymph nodes following vaccination. Lymph nodes are known to be the processing centres for antigens and understanding how the cells within lymph nodes co-ordinate the immune system may be important in the future development of vaccines to cancer and other diseases. Additionally, cancer is known to interact with the immune system to allow its spread to the lymph nodes. Comparing tissue from tumours and correlating these findings with the changes seen within the lymph nodes may shed light onto this process.

This study will allow us to collect a single extra lymph node from patients during surgery and to take small samples of tumour tissue after the tumour has been surgically removed. We will then examine these samples in our laboratory to understand the body's response to vaccines and cancer.

2. Why have I been chosen?

You have been diagnosed with breast cancer and are about to undergo a sentinel node biopsy to remove 1-4 lymph nodes from your armpit to check for tumour spread. You have 15 – 20 lymph nodes in your armpit and we would like to take a single extra lymph node during the operation to use as part of our study.

3. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form to confirm that you understand what is involved when taking part in this study. If you decide to take part you are free to change your mind at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of care you receive.

4. What will happen to me if I take part?

If you would like to be considered to take part in this study, or would like to discuss it further, first you can contact the people listed at the end of this information sheet. If you agree to enter the study we will ask you or your General Practitioner (G.P.) (with your permission) about your vaccination history. This is because it will help us to understand the results if we know about your vaccination history.

Patients who agree to enter the study who have not received tetanus vaccination within the last 10 years will be assigned to one of three groups at random; the reason for needing three groups is that it is equally important to study what happens if the vaccination is given in a different place, or not at all, so that we can compare the changes seen in these patients to those witnessed in the "test group". The reason it is not possible to choose which group you would wish to be in is because this may introduce bias, or "prejudice", which would make the groups unequal in terms of the characteristics of the patients in each group. Randomly assigning patients to each of the three groups is seen by scientists as the "fairest" way to allocate patients to groups to avoid inadvertently affecting the results of the experiment.

You will therefore be randomly chosen to be in one of the following three groups:

Group 1: "Test Group" – Will receive a tetanus vaccination into the same arm as the surgery will be performed. This will be administered under the skin of the arm.
Group 2: "Control Group 1" – Will receive a tetanus vaccination into the opposite arm to which the surgery will be performed. This will be administered into the muscle of the arm.
Group 3: "Control Group 2" – Will receive no vaccination.

Before entering the study you will first be asked to sign a consent form. During your pre-assessment appointment one week prior to your surgery you may or may not receive a vaccination according to which group you will have been assigned and at the same time blood samples will be taken during your scheduled pre-operative screening blood test. Although extra blood will be taken, you will not need a separate blood test.

At the time of your operation, a second blood sample will be taken during your anaesthetic; again although a small amount of extra blood will be taken, you should not need to have an extra blood test. During your operation, immediately after the sentinel node biopsy has been performed, a single extra lymph node will be removed from the armpit. Once the tumour has been removed, two small needle biopsies of tissue will be taken from it. These tissue samples will give us information about the immune cells within the tumour, which will allow us to understand the effect the tumour has on the immune system, but they will not influence the treatment you receive and no additional tissue will need to be removed from you. The rest of your operation will be exactly as normal.

Two weeks following your operation you will be seen in the outpatients' clinic as part of your standard post-operative care. At this point in time we will take a third sample of blood for the study; this is a blood test you would not normally undergo if you were not part of the study. Finally, five weeks following your operation you will be

seen in outpatients and the last blood sample will be taken; this is a clinic appointment and blood test that you would not normally undergo if you were not part of the study.

All four blood samples, the needle biopsies from your tumour and the extra lymph node will be sent to our laboratory for use in our research. All other blood tests and tissues samples taken during your treatment will sent to the hospital pathologist as normal. We will keep a record of what the hospital pathologist finds when examining these samples, however our work will not influence the normal tests performed on your blood tests, sentinel node biopsy or the tumour specimen.

5. What will happen to my blood and the lymph node once it is removed?

A standard sentinel lymph node biopsy will be performed by your surgeon. The purpose of performing the sentinel lymph node biopsy is to see whether your cancer has spread to the armpit, as this will affect the choice of additional treatment your surgeon will recommend to you. At the same time a single extra node will be removed for the study.

On the day of your surgery the sentinel lymph node(s) will be tested for cancer in the standard way. If the biopsy result shows that cancer is present in the sentinel node then we will not use the node that you have donated for the study. This is because in the event of the sentinel node being positive, you will be advised that all the rest of the lymph nodes under the arm will need removal as well to establish the total number of involved nodes. It would therefore be necessary to return the study node to allow it to be included with this analysis rather than use it for our study. You will be given to option to leave the study at this point if your sentinel lymph node is positive, thereby avoiding the final two blood tests, however if you wished to remain within the study we can still include the blood samples you donate in our work.

However, we expect that 90% of patients entered into this study will have negative sentinel node biopsies. Following a negative sentinel node biopsy the donated study node will be analysed within our laboratory and compared with the blood samples you donate and the samples of tumour. The tissue and blood samples will be stored within the tissue bank at Southampton Cancer Sciences Centre. We will ask for your consent to store these tissues for the purpose of this study. In addition we will ask for your consent to use these tissues in future studies which may be related to, but not directly part of, this study. Your consent to this is entirely optional and will not affect your participation within this study.

6. What are the possible disadvantages and risks of taking part?

You will be asked to go through two blood tests that you would not normally have to have. There is no specific risk to you from having the blood taken, however there will be the normal discomfort associated with any blood test.

The removal of a single extra lymph node from the armpit should not increase your risk of complications from surgery.

For patients randomized to "Control Group 1", the vaccine you receive will be administered into the shoulder muscle of the arm on the opposite side to your surgery. This will be similar to previous vaccinations you will have received and possible consequences of having the injection include arm ache and soreness.

For patients randomized to the "Test Group", the vaccine you receive will be administered into the skin of the arm on the side of your surgery; the reason for injecting it under the skin is because this means it is likely that the vaccine will drain to the lymph nodes under the arm, one of which we will then remove at the time of your surgery. Normally, other vaccinations you will have received will have been given within the shoulder muscle. Because you will have your vaccination in the skin of the arm you may experience more redness and tenderness at the site of injection than if we had given you the vaccine in the shoulder.

7. What are the possible benefits of taking part?

You will be asked to attend an extra clinic appointment five weeks after surgery; this would give you an extra chance to ask questions of your surgical team and any concerns you might have can be addressed.
Those patients receiving a vaccination should gain immunity in the event of subsequent exposure.
More generally, we hope this study will allow us to better understand how the immune system works and how we can use it to fight infectious disease and cancer. However our results are unlikely to be of direct benefit to you.

8. What happens after I take part in the study?

All of your medical care will continue as normal.

9. What if something goes wrong?

It is very unlikely that you will be harmed by taking part in this research project, but we are advised to inform you that there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you have concerns about any aspect of the way you have been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD.

10. Will my taking part in this study be kept confidential?

Yes. Records for this study and your health records will remain strictly confidential at all times. However these will need to be made available to people performing the study. They may also be looked at by people from the regulatory authority. The purpose of this is to check that the study is being carried out correctly. Any information about you which leaves the hospital will have personal information removed so that your identity is protected. You will only be identifiable by select members of the clinical research team through an individual study number.

We intend to inform your General Practitioner (G.P.) of your participation within this study. If, for any reason, you do not wish your G.P. to be informed please let one of our research team know.

11. What will happen to the results of this clinical study?

The results of the study will be published in a scientific journal. You will not be identified in any report or publication.

12. Who is organising and funding this clinical trial?

This study is being organised by doctors and scientists from the Southampton Cancer Research UK Clinical Centre. Funding is provided by a research grant held by one of the scientists here, Professor Christian Ottensmeier. The doctors conducting the study are not being paid to do so.

13. Will there be any payment for taking part?

No payments will be made to you for taking part in this study. However we realise that you will incur additional travel expense from attending an additional outpatient appointment 5 weeks following your surgery. Compensation will be available to you and details on how to claim for travel expenses can be sought from the research nurses whose details can be found at the end of this document.

14. Who has reviewed the study?

This study was reviewed and approved by a protocol review committee of the Southampton Cancer Research UK Clinical Centre and by the Southampton & South West Hampshire Research Ethics Committee.

15. Contact for further information

If you require any further information or have any concerns while taking part in the study please contact one of the following people:-

Breast Research Nurses	Telephone: 02380 794618
Consultant: Mr Ramsey Cutress	Telephone: 02380 796184

If you take part in this study you will be given a copy of this information sheet and a copy of the signed consent form to keep.

Thank you for taking time to read this information sheet

III. GP information sheet

Cancer Research UK Centre
Somers Cancer Research Building
Mail Point 824
Southampton General Hospital
Tremona Road
Southampton SO16 6YD, UK

T 023 8079 6184
F 023 8079 5152

Director Professor Peter Johnson

Dear Doctor.....

RE: <<<Patients Details>>>

I am writing to inform you that the above named patient of your practice has kindly volunteered to participate in a research study being performed within University Hospitals Southampton NHS Foundation Trust. The study is entitled "Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects" (REC Reference Number: 11/SC/0509).

Your patient has been asked to participate as she is to undergo a sentinel lymph node biopsy as part of her surgical treatment for breast cancer. The aim of the study is to uncover the role that lymph nodes play in co-ordinating the immune response to antigen with the ultimate goal of future development of effective cancer vaccines. Prior to your patients' inclusion we clarified their vaccination history to ascertain whether they are suitable; only patients who have not received tetanus vaccination within the last 10 years can participate. It may have been necessary for our department to have contacted your practice, with your patients' prior consent, to establish their immunisation history.

During the course of this study 75% of patients will receive a vaccination against tetanus. This will be administered one week prior to sentinel node biopsy. During your patients subsequent surgery we will remove a single additional node for the purpose of the study and may take core needle biopsies of tumour tissue from the surgical specimen once resected. Participants who do receive vaccination have an additional node removed and tumour biopsies taken without preceding vaccination to provide a negative control group.

Your patient will receive a vaccination against Tetanus / no vaccination. (Deleted as appropriate)

As well as the removal of an extra lymph node and tumour tissue, 4 samples of blood will be taken from each patient. This will be done during clinic appointments at either University Hospital Southampton or Princess Anne Hospital.

The care of your patient will not be affected by their participation within the study and their surgery will be unchanged save for the removal of a single additional lymph node. Your patient has been provided with a Patient Information Sheet, a copy of which has been included with this letter.

If you have any questions regarding your patients participation or require any further information please contact feel free to contact me.

Yours Sincerely,

Mr Ramsey Cutress

IV. Consent form

CANCER RESEARCH UK

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CONSENT FORM

Title of Project: Understanding immune responses to prophylactic vaccination in the lymph node: correlation between tissue and blood effects

REC Reference Number: 11/SC/0509

Name of Researcher: **Mr R.I. Cutress**

Name of Patient: _____

Please initial box

1. I confirm that I have read and understand the information sheet (Version 5.3 dated 21st April 2013) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I agree to my General Practitioner (G.P.) being contacted to ascertain my full vaccination history. ☐
4. I agree to my General Practitioner (G.P.) being informed regarding my participation in this study and I agree to my G.P. being informed regarding any vaccination I may receive during this study, so that this information can be included in my vaccination record. ☐
5. I agree to the storage of my tissue and blood samples indefinitely by the researchers conducting this study for later use in this study and research relating to the aims of this study. ☐
6. I agree to the storage of my tissue and blood samples indefinitely by the researchers conducting this study for use in ethically approved research separate from this study. ☐
7. I understand that sections of any of my medical notes may be looked at by the researchers involved in this study. I give permission for these individuals to have access to my records. I understand that any data from my notes will only be stored by the researchers in an anonymous form. ☐
8. I have informed the researchers of my participation in any other research study ☐
9. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher/co-investigator	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes

V. Case report form

Initials:				D.O.B:	DD	MM	YYYY	Patient Trial Number:	

(Patient Trial Number will be issued on cohort assignment)

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PRIOR TO COHORT ASSIGNMENT: Day of Diagnosis

Date of Diagnosis (1st clinic appointment following MDT decision): DD MM YYYY

Inclusion / exclusion criteria:

Criteria:	Y	N
INCLUSION:		
Patients due to undergo SNB for cancer staging		
Patients aged 18 years or older		
Patients with the ability to understand the study requirements, provide written informed consent and comply with the study protocol procedures		
EXCLUSION:		
Current immune modulating drugs (Including oral (not inhaled) steroids)		
Tetanus vaccination within the last 10 years		
Presence of axillary nodal/metastatic disease		
Hypersensitivity to tetanus vaccine		

Patient offered a patient information sheet:	Y	N
Patient accepted a patient information sheet:	Y	N

Date of Completion:	DD/MM/YYYY	Completed By:(Print)		Signed:	

Understanding the body's immune response to vaccine
Case Report Form Version 5.0
10.01.2013

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Initials:				D.O.B:	DD	MM	YYYY	Patient Trial Number:	

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PRE-OPERATIVE ASSESSMENT: Date: DD MM YYYY

Past History of Malignancy										
Diagnosis	Date of Diagnosis	Surgery Performed		Chemotherapy Received		Radiotherapy Received			Hormone therapy	
		Date	Operation	Date*	Agent(s)	Date*	Site(s)	Dose/ Fraction	Date*	Agent(s)

* Date of Completion. If on-going state "OG"

Vaccination History		
Vaccine	**Completed childhood vaccination**	**Date of most recent vaccination**
Diphtheria/tetanus/pertussis (DTaP)	Y/N	DD/MM/YYYY
Polio (IPV)	Y/N	DD/MM/YYYY
Hepatitis B	Y/N	DD/MM/YYYY
Haemophilus influenzae type B (Hib)	Y/N	DD/MM/YYYY
Pneumococcal conjugate vaccine (PCV)	Y/N	DD/MM/YYYY
Meningitis C (MenC)	Y/N	DD/MM/YYYY
Measles, Mumps, Rubella (MMR)	Y/N	DD/MM/YYYY
Influenza	Y/N	DD/MM/YYYY
Tuberculosis (BCG)	Y/N	DD/MM/YYYY

Medication History		
Drug Allergies:		
Medication:	**Dose:**	**Regimen:**

Is the patient receiving hormone therapy (Tamoxifen/Aromatase Inhibitor) pre-operatively?	Yes	No
If yes, which agent?		

Vaccine Given:	Tetanus		None
Site of Vaccination:	Ipsilateral	Contralateral	
Blood Test Taken:	Yes	No	

Date of Completion:	DD/MM/YYYY	Completed By:(Print)		Signed:	

Initials:				D.O.B:	DD	MM	YYYY	Patient Trial Number:	

(Patient Trial Number will be issued on cohort assignment)

DAY OF SURGERY: Date: DD MM YYYY

Blood Sample Taken:	Yes	No				
Breast procedure performed	Wide Local Excision	Mastectomy				
Sentinel Node Biopsy:	Node Number:	Pink/Blue:	Count:	qRT-PCR:	Final Histology	
	1	Pink/Blue:		Positive/Negative	Positive/Negative	
	2	Pink/Blue:		Positive/Negative	Positive/Negative	
	3	Pink/Blue:		Positive/Negative	Positive/Negative	
	4	Pink/Blue:		Positive/Negative	Positive/Negative	
Non-Sentinel Node:	5	Pink				

Anaesthetic:

Type of anaesthetic: (Please circle all relevant)

GA Sedation LMA ETT

TIVA N2O Sevo Iso Des

Paravertebral Block PECS block

Paralysis: yes no

Local Anaesthetic wound infiltration: Dose: _____ml_____%Bupivicaine

Drug Administration:

	Pre-med:	Peri-op:	Total in Recovery:
Paracetamol (g)			
Diclofenac (mg)			
Ibuprofen (mg)			
Gabapentin (mg)			
Ketorelac (mg)			
Tramadol (mg)			
Fentanyl (mcg)			
Morphine (mg)			
Ondansetron (mg)			
Cyclizine (mg)			
Dexamethasone (mg)			
Other:			

Date of Completion:	DD/MM/YYYY	Completed By:(Print)		Signed:	

Initials:				D.O.B:	DD	MM	YYYY	Patient Trial Number:	

(Patient Trial Number will be issued on cohort assignment)

FOLLOW-UP APPOINTMENT 1:

Date: DD MM YYYY

Blood Sample Taken:	Yes	No	Patient Refused
Any Adverse Events?			

Histology of Primary Specimen					
Sub-type:	Invasive Ductal	Invasive Lobular	Isolated high Grade DCIS/LCIS	Other (please specify):	
Size of invasive component (mm):					
Grade of Invasive component:		I	II	III	
Hormone Receptor Status:		ER+ve	ER-ve	Quikscore:	
		PR+ve	PR-ve	HER2 +ve	HER2 -ve

Associated non-invasive component:	DCIS	LCIS	**Size (mm):**	
	Grade:	Low	Intermediate	High

Nottingham Prognostic Index:	

FOLLOW-UP APPOINTMENT 2:

Date: DD MM YYYY

Blood Sample Taken:	Yes	No	Patient Refused
Any Adverse Events?			
Patient Treatment to date:	Chemotherapy	Radiotherapy	Hormone Therapy
Date Started:			
Agents regimen and dosage used:			

Date of Completion:	DD/MM/YYYY	Completed By:(Print)		Signed:	

Initials:				D.O.B:	DD	MM	YYYY	Patient Trial Number:	

(Patient Trial Number will be issued on cohort assignment)

Differential Full Blood Count:

		Appointment 1: Pre-assessment	Appointment 2: Day of Surgery	Appointment 3: 2/52 post-surgery	Appointment 4: 6/52 post-surgery
Hb	g/l				
WBC	$*10^{9}$				
Neutrophils	$*10^{9}$				
Lymphocytes	$*10^{9}$				
Monocytes	$*10^{9}$				
Eosinophils	$*10^{9}$				
Basophils	$*10^{9}$				

Date of Completion:	DD/MM/YYYY	Completed By:(Print)		Signed:	

Appendix B: Study Map

Patient Recruitment:

Patients meeting the inclusion criteria are approached offered a patient information sheet by their consultant or breast care nurse during their consultation

Patients receive a follow up phone call by a trained Research Nurse Practitioner to assess interest and allow patient to ask questions/queries

Time point 1: Week 0 (Patient's pre-assessment appointment)

- Patient is met by research nurse and consented into study
- Patient is randomised into one of three cohorts
- First blood sample is taken at the same time as venesection for routine pre-operative bloods
- Vaccination administered (if appropriate)

Time point 2: Week 1 (Patient's day of surgery)

- Second blood sample is taken prior to anaesthetic
- During procedure, additional axillary lymph node is removed following completion of SLNB. Following a negative SLNB result the node is processed for study purposes. In the event of a positive SLNB the study node is surrendered for histopathological processing along with the completion axillary clearance specimen.
- Patients with a positive SLNB will be given the opportunity to discontinue with the study following this time point. However in the event the patient is willing to continue, time-points 3 and 4 will be collected.
- Two core biopsies are removed from the tumour specimen prior to formalin fixation.
- Homogenate generated for the purposes of the sentinel node biopsy, which is surplus to clinical requirements, will be retained for the purpose of the study

Time point 3: Week 3 (Patient's standard post-surgical follow up appointment)

- Patient attends standard follow up clinic appointment
- Met by research nurses and third blood sample is taken

Time point 4: Week 6-7

- Patient asked to attend additional hospital appointment
- Final blood sample is taken

Appendix C: Sample Plate plan for ELISA

Plate plan below is set for serial double-dilutions of up to 4 patient serum samples and includes bare wells for each sample to exclude non-specific binding.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1:100	STD 1:100	QC1	QC1	Pt1 1:50		Pt2 1:50		Pt3 1:50		Pt4 1:50	
B	STD 1:200	STD 1:200	QC2	QC2	Pt1 1:100		Pt2 1:100		Pt3 1:100		Pt4 1:100	
C	STD 1:400	STD 1:400	QC3	QC3	Pt1 1:200		Pt2 1:200		Pt3 1:200		Pt4 1:200	
D	STD 1:800	STD 1:800	Bare STD 1:100	Bare STD 1:100	Pt1 1:400		Pt2 1:400		Pt3 1:400		Pt4 1:400	
E	STD 1:1600	STD 1:1600	Bare Pt1 1:50	Bare Pt1 1:50	Pt1 1:800		Pt2 1:800		Pt3 1:800		Pt4 1:800	
F	STD 1:3200	STD 1:3200	Bare Pt2 1:50	Bare Pt2 1:50	Pt1 1:1600		Pt2 1:1600		Pt3 1:1600		Pt4 1:1600	
G	STD 1:6400	STD 1:6400	Bare Pt3 1:50	Bare Pt3 1:50	Pt1 1:3200		Pt2 1:3200		Pt3 1:3200		Pt4 1:3200	
H	Blank	Blank	Bare Pt4 1:50	Bare Pt4 1:50	Pt1 1:6400		Pt2 1:6400		Pt3 1:6400		Pt4 1:6400	

Appendix D: Sample Plate Plan for B-cell ELISpot

Plasma Cell Plate: For numeration of antibody producing cells specific for up to 6 different antigens, within up to 4 samples/time points. Blank wells included to allow normalisation of background and "positive control wells", coated with anti-human antibody, to allow numeration of total number of antibody producing cells.

		Time Point 1			Time Point 2			Time Point 3			Time Point 4		
		1	2	3	4	5	6	7	8	9	10	11	12
+ve Control	A												
TT	B												
DT	C												
PPD	D												
FHA	E												
Blank	F												
	G												
	H												

Memory – B Cell Plate: For numeration of the number of cells stimulated to produce antibody specific for up to two different antigens following stimulation within up to 4 samples/time points.

			Time Point 1			Time Point 2			Time Point 3			Time Point 4		
			1	2	3	4	5	6	7	8	9	10	11	12
+ve Control	**Unstimulated**	A												
	Stimulated	B												
TT	**Unstimulated**	C												
	Stimulated	D												
DT	**Unstimulated**	E												
	Stimulated	F												
Blank (no antigen)	**Unstimulated**	G												
	Stimulated	H												