UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

School of Medicine

Development of DNA vaccines for patients following stem cell transplantation

By

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ABSTRACT

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It is clear from murine models that DNA vaccines can induce specific immune responses against antigens from tumours or infectious organisms. We aim to develop DNA vaccine strategies to benefit patients who suffer with haematological malignancies. For B-cell tumours, the idiotype (Id), derived from the immunoglobulin (Ig) variable (V) genes of the malignant clone, is a tumour specific antigen. Our DNA vaccines, in which the relevant V gene sequences are fused to that of the Fragment C (FrC) portion of tetanus toxin, protect against follicular lymphoma and multiple myeloma (MM), and are in current clinical trials.

One goal of this project was to optimize the setting for DNA vaccination of patients with MM. Vaccination is most likely to be effective when the tumour burden is low. In MM, this state is commonly achieved after high dose chemotherapy and autologous stem cell transplantation (ASCT). Clearly, sufficient time must be allowed to elapse after ASCT for immune reconstitution. I have used the ability to respond to vaccination with tetanus toxoid as an indicator of immune capacity. Investigation of FrC specific $CD4^+$ T-cell responses also provides information relevant to our DNA fusion vaccine. By measuring antibody and cellular responses to tetanus toxoid vaccination I assessed recovery of myeloma patients (n=25) post-ASCT (range 6-81 months). Responses of MM patients compared favourably with those of patients with monoclonal gammopathy of undetermined significance (MGUS) (n=11) and healthy volunteers (n=12). This information will help to guide the timing of therapeutic anti-idiotype DNA vaccination in our clinical trial.

For intracellular tumour or infectious disease antigens, induction of a cytotoxic lymphocyte (CTL) response is desirable. To optimise induction of CTL, a modified DNA fusion vaccine has been developed: the C-terminal domain of FrC was removed to delete competitive epitopes. Epitope presentation was then enhanced by re-positioning the peptide sequence of interest to the C-terminus of the remaining FrC domain. We have previously shown in murine models that this vaccine (pDOM.peptide) successfully induced CTLs specific for a chosen tumour peptide. In order to test the operation of this design in the clinic, an epitope from human cytomegalovirus, NLVPMVATV was chosen. We have previously shown in mice that the DNA vaccine p.DOM-NLVPMVATV can induce CTL specific for the naturally processed peptide. As part of a phase I clinical trial we have now safely vaccinated 3 stem cell transplant donors. Cellular and humoral immune responses were monitored following vaccination and the results are presented.

Finally, to gain more insight into the ability of the new vaccine design to induce significant levels of specific CTL, I have developed the DNA vaccine pDOM-GILGFVFTL. This vaccine induced CTLs specific for the epitope which is immunodominant in the response of HLA-A0201 individuals to Influenza A virus. These CTLs could lyse cells infected with influenza virus and showed some efficacy in protecting mice from challenge with a lethal dose of influenza A virus.

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F.P.McNicholl, H. McCarthy, C.H. Ottensmeier, A.S. Duncombe, K.H.Orchard, A.G. Smith, F.K. Stevenson.
Poster 0509, 8th annual congress of the European Haematology Association, Lyon, France, June 2003.

Immune responses to tetanus vaccination in myeloma patients post autologous transplantation provide information on timing of vaccine-based immunotherapy.
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Tetanus toxoid vaccination of myeloma patients following autologous stem cell transplantation: lessons for vaccine based immunotherapy.
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APC	Antigen presenting cell
ATP	Adenosine tri-phosphate
BCR	B-cell receptor
BSA	Bovine serum albumin
CALLA	Common acute lymphobalstic leukaemia antigen
CD	Cluster of differentiation
CLIP	Class-II-associated invariant chain peptide
CDR	Complementarity determining region
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
D	Diversity
DC	Dendritic cell
DC-SIGN	Dendritic cell specific intracellular adhesion molecule-grabbing nonintegrin
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAP	Endoplamic reticulum aminopeptidase
FACS	Fluorescence-activated cell sorter
FrC	Fragment C
GI	Gastro-intestinal
GM-CSF	Granulocye macrophage colony stimulating factor
gp	glycoprotein
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
HA	Haemagglutinin
HCMV	Human cytomegalovirus
HEVM	Herpesvirus entry mediator
HHD	Human β2m-HLA-A2.1 (α1α2)- H-2Db (α3 transmembrane cytoplasmic)

HIV	Human immunodeficiency virus
HLA	Human lymphocyte antigen
HPV	Human papilloma virus
hsp	Heat shock protein
ICOS	Inducible T-cell costimulator
Id	Idiotype
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISS	Immuno-stimulatory sequence
IVTT	In-vitro transcription and translation
J	Junctional
LB	Luria broth
LCMV	Lymphocytic choriomenigitis virus
LICOS	Ligand of inducible T-cell costimulator
LPS	Lipo-polysaccharide
MCA	Medicines Control Agency
MCM	Monocyte conditioning medium
MCMV	Murine cytomegalovirus
MGUS	Monoclonal gammopathy of undertermined significance
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
N nucleotide	Non-template encoded nucleotide
NA	Neuraminidase
NK	Natural killer
P nucleotide	Palindromic nucleotide
PAMPs	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBSCT	Peripheral blood stem cell transplant
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PRR	Pattern-recognition recptor

PV	Pichinde virus
RAG	Recombinase activating gene
RNA	Ribonucleic acid
RSS	Recombination signal sequences
scFv	Single chain variable fragment
SCT	Stem cell transplant
SI	Stimulation index
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TD	T-cell dependent
TdT	Terminal deoxy-nucleotidyl-transferase
TI	T-cell independent
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TREC	T-cell rearrangement excision circlet
TT	Tetanus toxoid
V	Variable
WHO	World Health Organisation

1 Vaccination of myeloma patients following autologous stem cell transplantation

1.1 Introduction

The modern discipline of immunology grew from the discovery by Edward Jenner that "vaccination" with harmless cowpox infection prevented subsequent deadly smallpox infection. Vaccination had induced immunological memory which is the central characteristic of the adaptive immune system. By using attenuated or killed organisms, vaccines have been developed which protect against many infectious agents. The development of protective vaccines is one of the greatest successes of modern medicine, as illustrated by the global eradication of smallpox.

The successful manipulation of the immune system by vaccination against infections raised hopes that a similar approach could be used to develop treatments against cancer. The experiments of Klein over 40 years ago indicated that the immune system could recognise and reject tumours [1]. Since then much effort has been invested in the development of treatments which harness the immune system to combat cancer.

The adaptive immune system can respond to antigens, derived from either pathogens or tumours, in several different ways. In principle, the type of response which is likely to be effective against a given antigen depends on the location and nature of the antigen rather than whether it is derived from a pathogen or a tumour. Our research group has been involved in the development of DNA vaccines which are designed to induce immune responses against malignant cells [2]. The initial focus was on haematological malignancies, such as myeloma. Patients with this condition are often immunosuppressed, either as a result of the disease itself or the therapy administered to treat the disease. High dose chemotherapy followed by autologous stem cell transplantation is one approach currently used in the routine treatment of patients with this condition [3]. A successful anti-cancer vaccine strategy will require the immune system of the patient to be capable of mounting an immune response to the vaccine.

This thesis is divided into three main parts. In the first part I have investigated the immune capacity of patients with myeloma after autologous transplantation to determine if vaccination against tumour may be effective in this setting. In my introduction I will discuss the manner by which the key cells of the adaptive immune response, lymphocytes, generate specificity for the vast array of antigens which the adaptive immune system can recognise and respond to. I will then discuss the immunological principles which underlie

vaccination. This is followed by a discussion on the development of DNA vaccines. The final part of this introduction involves a review of the literature regarding immune reconstitution after autologous transplantation.

The second part of this thesis is concerned with the study of responses to a DNA vaccine designed to induce cytotxic lymphocytes (CTL) against human cytomegalovirus (HCMV) in allogenic stem cell transplant donors. In the final part of this thesis I will discuss the production development of a DNA vaccine designed to induce CTL against Influenza A virus, the use of which may facilitate the application of DNA vaccination in the clinic.

1.1.1 The immune system

The human immune system is comprised of the innate immune system and the adaptive immune system. The innate immune system is crucial in limiting the early replication and spread of infectious agents. It is manifested in some form by most organisms on the planet. The human innate immune system is complex. Neutrophils, eosinophils, basophils, mast cells, macrophages, dendritic cells and natural killer cells are highly specialised cells which are part of the innate immune system. In addition, soluble factors such as cytokines, chemokines, interferons and complement contribute to its effectiveness. The adaptive immune system has evolved on top of the innate system and responds to pathogens in a highly specific manner. As discussed later, the effective functioning of the adaptive immune system is however dependent upon and facilitated by innate cells and their products.

An adaptive immune system is found in all jawed fish and higher vertebrates. The term arises from the observation that this kind of response occurs during the lifetime of an individual as an *adaptation* to a previous infection with a pathogen. Following an initial infection, the adaptive immune system allows an individual to develop immunological memory to that pathogen, conferring the ability to respond more rapidly and effectively to a subsequent challenge by the same pathogen. The cells which are responsible for this remarkable faculty are called lymphocytes. Lymphocytes detect the presence of antigens in their environment by means of antigen receptors on their surface. The receptors produced by each lymphocyte exhibit a unique specificity for a particular antigen. As each person possesses billions of lymphocytes, a staggering number of antigens can therefore be detected and, under the appropriate circumstances, responded to. Lymphocytes are unique in their ability to generate such highly variable receptors. This is achieved during the development of each lymphocyte by a process in which the genes which encode the

variable region of the antigen receptor are rearranged. This process is known as somatic recombination and the mechanisms by which this is accomplished by lymphocytes are discussed below.

There are two major types of lymphocytes: T-cells and B-cells. T-cells can be further subdivided into cytotoxic T-cells, which primarily kill malignant cells or cells infected with viruses, and helper T cells whose main role is to activate other cells such as B-cells and macrophages. Following activation, B-cells can differentiate into plasma cells which secrete antibody. Antibody binds to extra-cellular pathogens or their toxic products. B-cells are the key cells of the humoral arm of the immune system and secretion of antibody is their main effector function.

1.1.1.1 T cells

T-cells possess an antigen specific, clonally restricted antigen receptor – the T cell receptor (TCR). Each T-cell bears approximately 30, 000 TCRs on its surface. The TCR allows T-cells to recognise pathogen derived peptides of the correct length when they are presented in association with the appropriate MHC molecule.

1.1.1.1.1 Generation of TCR diversity

The TCR complex consists of a membrane bound heterodimer composed of two polypeptide chains linked by a disulphide bond and associated with a non-polymorphic cytoplasmic membrane bound complex of proteins collectively known as CD3 [4]. The heterodimer is responsible for ligand recognition whilst CD3 induces the signalling events that result from receptor engagement [5]. Two distinct lineages of T cells can be distinguished by differences in the polypeptide chains which make up their TCR. Less than 10% of T-cells circulating in the blood express a TCR which is comprised of γ- and δ-chains whilst the vast majority of circulating T-cells, including all cytotoxic T-cells and helper T-cells, express a TCR composed of α- and β- chains. Nonetheless, each TCR chain consists of a variable amino-terminal region and a constant carboxy-terminal region.

The TCR genes are located on chromosomes 7 and 14 [6]. The β-chain complex is located at band q35 on the long arm of chromosome 7 and consists of two closely linked genes, each capable of encoding a β-chain constant region. The α-chain complex is located at band q11.2 on the long arm of chromosome 14 and is linked to the immunoglobulin heavy chain-complex. The gene complex encoding the δ genes is located entirely within

the α-chain complex whilst the γ-gene complex is located at band p15 on the short arm of chromosome 7.

The variable regions of each β- and δ- chain are encoded by a variable (V), a diversity (D) and a junctional (J) gene, whilst the variable regions of α- and γ- chains are only encoded by a V and a J gene. The number of gene segments available at each locus is shown below (Table 1) [6].

Table 1 The number of gene segments encoding the T-cell receptor chains.

TCR gene	V segments	D segments	J segments	C segments
α	70	0	61	1
β	67	2	13	2
γ	14	0	5	2
δ	8	3	4	1

1.1.1.1.2 Somatic (VDJ) recombination

TCR gene rearrangement is mediated by enzymes which are collectively called VDJ recombinase. Some of these enzymes are ubiquitous DNA repair enzymes whilst others are found only in developing lymphocytes. One member of the latter group is a complex of two proteins encoded by the recombination activating genes, RAG 1 and RAG 2 [7]. RAG genes are only expressed in immature lymphocytes and these genes are silenced in proliferating cells, limiting the risk of inappropriate DNA breaks during normal DNA replication. The rearrangement of TCRβ and TCRδ chains is a two step process (FIGURE 1). Firstly, a D gene recombines with a J chain and then a V chain recombines with the D-J block. Rearrangement of TCRα and TCRγ chains requires only a V-J recombination event.

Each TCR gene segment is flanked by sequences called recombination signal sequences (RSSs). These serve as recognition sites for VDJ recombinase and mark the points at which somatic recombination may occur. The RSS consists of two conserved sequences, a heptamer (CACA/TGTG) separated by either 12 or 23 random nucleotides from a consensus nonamer sequence (ACAAAAACC or GGTTTTTGT). Recombination can only take place between gene segments which are flanked by spacers of different nucleotide lengths. This accounts for the "12-23" rule [8].

VDJ recombinase introduces double-stranded breaks in the DNA between the RSS and the adjacent V, D or J gene segment. Temporary hairpin loop structures are formed which connect the parallel DNA strands on either side of the breaks. The ends of each excised stretch of DNA are joined to form extra-chromosomal DNA circles known as T-

cell rearrangement excision circlets (TRECs) whilst the two broken ends of the gene segments are joined.

TRECs do not replicate and are therefore diluted out by each T cell division[9]. The D to J gene rearrangement produces a TREC with a unique signal joint sequence. This initial recombination event is identical in approximately 70% of $\alpha\beta$ T cells and therefore the resultant TREC can be used as a molecular marker of thymic function. TRECs in circulating T lymphocytes mark these cells as recent thymic emigrants.

1.1.1.1.3 Junctional Diversity

The junctional region (V (D) J) encodes the hypervariable complementarity determining region 3 (CDR3) of each TCR polypeptide chain. Even greater diversity is introduced to this region during somatic recombination by several mechanisms. Gene encoded nucleotides may be removed by the exonucleolytic activity of as yet unidentified exonucleases. Nucleotides may also be added at junctions. The added nucleotides are known as P- and N- nucleotides [6]. P-nucleotides are so called because they make up palindromic sequences added to the ends of gene segments. Asymmetrical DNA cleavage by the RAG protein complex may result in two DNA strands of unequal length. The shorter strand must be extended with nucleotides complementary to the longer strand before the gene segments can be ligated. The inserted nucleotides are known as P nucleotides as their sequence is identical when read in either direction i.e. palindromic. N nucleotides are GC rich non-templated nucleotides added at random to the cut ends of the DNA strands by the enzyme terminal deoxynucleotidyl transferase (TdT). The addition of P- or N- nucleotides may sometimes introduce frameshifts or stop codons resulting in a non-functional gene.

The peptide binding groove of the TCR is formed by amino acids of the first, second and third complementarity-determining regions (CDR) of variable domain of each polypeptide chain. The centre of the antigen binding site of a TCR is formed by the CDR3 present in each polypeptide chain. The CDR3 is encoded by the junctional region which is initially formed by (V (D) J) recombination and then acted upon by the processes outlined directly above. The CDR1 and CDR2 regions are encoded within the germline V gene segment and form the periphery of the antigen binding site. Much less diversity is exhibited by the CDR1 and CDR2 regions.

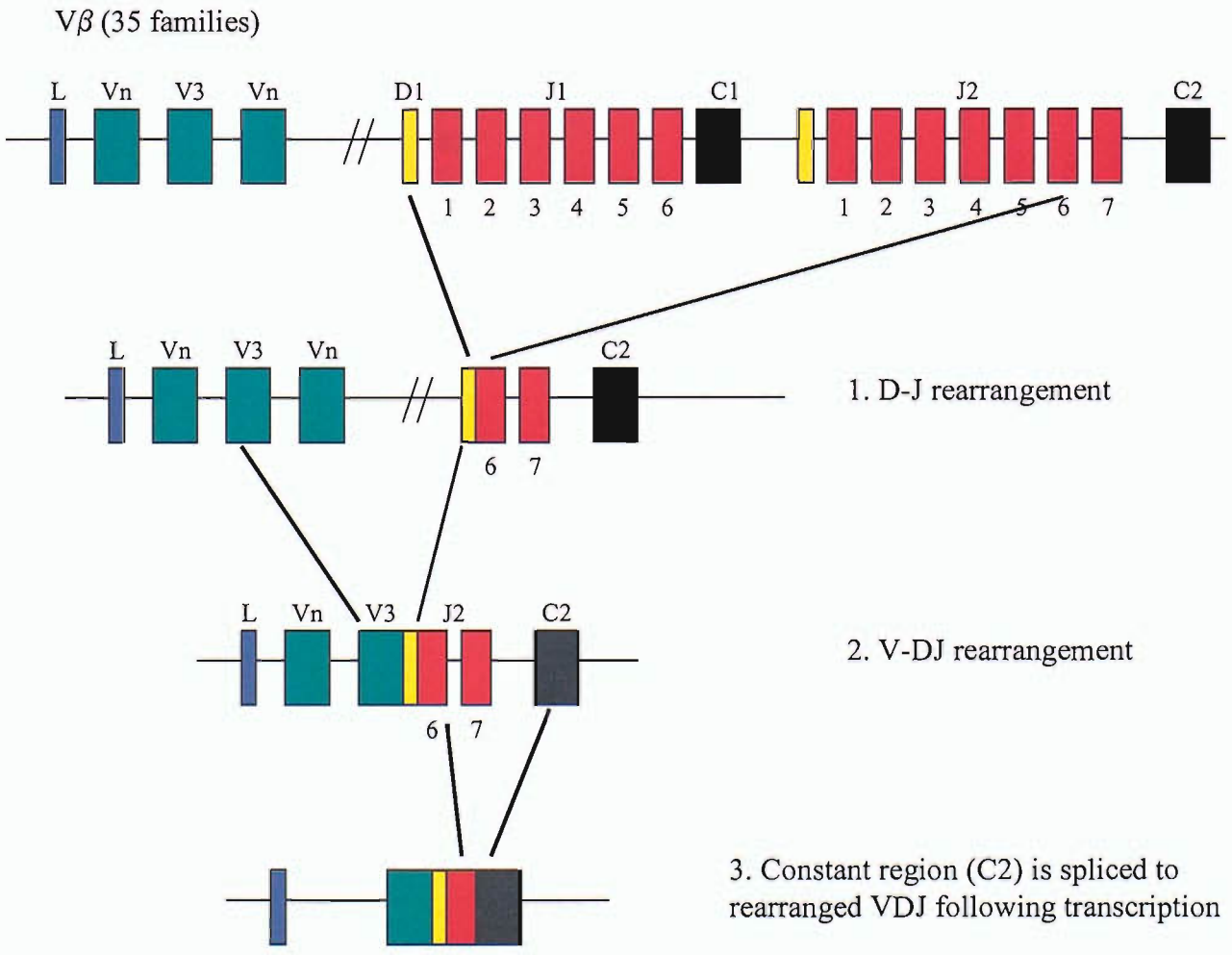


Figure 1. TCR β gene rearrangement

1.1.1.1.4 Normal T-cell development

T-cells develop from common lymphoid progenitors which reside in the bone marrow and are derived from pluripotent haematopoietic stem cells. Cells which are destined to become T-cells leave the marrow and migrate to the thymus. At this stage the cells lack the surface molecules which characterise T-cells and their T-cell receptor genes are in germline configuration. In the thymus these cells rearrange their TCR genes and then undergo a process involving differentiation, proliferation and selection which is dependent upon IL-7 production by a subset of thymic epithelial cells [10]. Cells which develop T-cell receptors incapable of recognising self MHC molecules fail a positive selection process and perish. Survivors mature and express high levels of T-cell receptor. The cells among this group which respond to self antigens are negatively selected and eliminated. The vast majority of T cells are therefore deleted in the thymus but the end result is the selection of viable T cells expressing T cell receptors capable of responding to foreign antigens when presented by self MHC molecules. Newly produced naïve T cells are exported from the thymus and travel to peripheral lymphoid tissues where they await an encounter with antigen whilst being sustained by repeated contact with MHC:self peptide complexes similar to those that originally positively selected them.

1.1.1.2 B cells

B-cells possess a unique antigen binding site on their cell surface – the B-cell receptor (BCR) [11]. The BCR consists of membrane bound immunoglobulin (sIg) complexed to CD79a (Igα) and CD79b (Igβ). Apart from a small portion of the C-terminus of the heavy-chain constant region, secreted immunoglobulin is identical to the membrane bound immunoglobulin portion of the BCR on the B-cell from which the plasma cell that secretes the immunoglobulin molecule was derived.

1.1.1.2.1 Immunoglobulin

Immunoglobulin molecules are Y-shaped glycoproteins composed of two identical heavy polypeptide chains and two identical light polypeptide chains. The antigen binding site is located at the apex of each arm of the Y. Each arm of the Y consists of a complete light chain linked by a disulphide bond to the amino-terminal part of a heavy chain. The stem of

the Y consists of the carboxy-terminal portions of the two heavy chains, again linked by disulphide bonds.

Light chains are smaller than heavy chains and consist of one variable (V_L) domain and one constant (C_L) domain. Two types of light chains are found in humans; kappa (κ) and lambda (λ). An immunoglobulin can have either two κ light chains or two λ light chains but never one of each. Heavy chains are composed of one V_H domain and three or four C_H domains.

Located in the N-terminal portion of the variable domains are three hypervariable loops of amino acids, termed complementarity determining regions (CDRs)[11]. These are separated by relatively conserved areas called framework regions. The three CDRs in each heavy chain and the three CDRs in the partner light chain form the antigen binding surface. Every Y-shaped Ig molecule therefore has two identical antigen-binding sites, one at the end of each arm.

Differences in the constant part of the heavy chain allow five different classes of immunoglobulin or isotypes to be identified; IgA, IgD, IgE, IgG and IgM. A lower case Greek letter is used to identify the corresponding heavy chain (α, δ, ϵ, γ, μ). In addition to the number of domains, other features which differ between isotypes include the number and location of disulphide bonds, the presence or absence of a hinge "region" and the distribution of N-linked carbohydrate groups. Great functional differences exist between isotypes.

IgD makes up less than 1% of serum immunoglobulin levels. It is found on the surface of naïve B-cells and a biological function has not been identified.

5-10% of immunoglobulins in the serum are of the IgM isotype. As outlined below, IgM is found on the surface of developing and antigen naïve B-cells. The antibody is secreted as a pentamer held together by disulphide bonds. IgM predominates in the early stages of the humoral response to an antigen and is very effective at activating the classical complement pathway.

IgG is the most abundant isotype in serum and may be further divided into 4 subclasses based on differences in their γ chain and numbered (IgG1-4) according to decreasing serum concentration. IgG1 and IgG3 bind to Fcγ receptors with high affinity whereas IgG3 binds with medium affinity and IgG2 with low affinity. IgG molecules which bind Fcγ receptors at high affinity are very effective at inducing opsonisation and antibody dependent cellular cytotoxicity.

IgA constitutes approximately 10% of serum immunoglobulin but is the predominant isotype found in secretions. It exists mainly as a monomer but may form dimers, trimers or tetramers.

IgE exists at only trace levels in the serum. It binds with high affinity to Fcϵ receptors on mast cells and basophils resulting in the release of histamine and other substances from these cells which are responsible for immediate hypersensitivity reactions.

1.1.1.2.2 Generation of Immunoglobulin/ BCR diversity

Like T-cells, B-cells also employ the processes of somatic recombination and junctional diversity to generate immunoglobulin diversity. They are able to generate further diversity by somatic hypermutation after an encounter with antigen.

1.1.1.2.3 Immunoglobulin genes

The immunoglobulin genes are located on chromosomes 14, 2 and 22. The heavy-chain gene complex is located at band q32 of the long arm of chromosome 14. This complex is composed of 51 potentially functional variable region genes, 27 diversity region genes, 6 joining region genes and the genes which encode the constant regions of the immunoglobulin heavy chain isotypes. The κ light-chain gene complex is contained within band p12 on the short arm of chromosome 2. This complex is contains 40 variable region genes, 5 joining region genes and the κ constant region. The λ light-chain gene complex is located at band q11.12 on the long arm of chromosome 22. It consists of 31 variable region genes, 4 joining region genes and 4 constant region genes (Table 2).

Table 2 The number of potentially functional gene segments for the V regions of human heavy and light chains.

Ig gene	V segments	D segments	J segments
κ	40	0	5
λ	31	0	4
H	51	27	6

The immunoglobulin genes rearrange during B-cell development to form complete V-domain exons. The mechanics of somatic recombination and junctional diversity in B-cells are similar to those outlined above for T-cells. Creation of an intact immunoglobulin heavy chain requires two rounds of gene recombination. The first recombination joins a D and J_H segment. The combined DJ_H segment then joins to a V_H segment to create a continuous heavy chain exon. Creation of a light chain variable gene requires only a V_L-J_L recombination event.

1.1.1.2.4 Development of mature B-cells

Human B-cells initially develop in the fetal liver and omentum but from the end of the second trimester, and throughout adult life, B-cells are exclusively and continuously produced in the bone marrow. Each stage of B-cell development is defined by changes in the expression of Immunoglobulin genes and cell-surface molecules (Figure 2).

The earliest lineage B-cells, which arise from pluripotent haematopoietic stem cells, are called progenitor (pro-) B cells. Pro-B-cells express CD19 and CD45R on their cell surface. These molecules are expressed throughout B cell development and are involved in B-cell receptor signalling. Pro-B cells also express CD10, CD34, CD38 and receptors for the growth factors Kit and IL-7. RAG proteins and TdT are first expressed at this stage. DJ_H recombination occurs in early pro-B-cells followed by V_H to DJ_H recombination in late pro-B-cells [11]. A successful gene rearrangement, termed a productive rearrangement, results in the production of a μ heavy chain and delineates the start of the pre-B cell stage. Unsuccessful rearrangements, termed non-productive rearrangements, do not result in the formation of protein. The first attempt at V_H to DJ_H rearrangement is non-productive in at least two out of three cases. Rearrangement then occurs on the other chromosome but if this is also unsuccessful then the lymphocyte dies.

The newly formed μ-chain then associates with a surrogate light chain, which is structurally homologous to a κ or λ light chain, to create a pre-B cell receptor (pre-BCR). Expression of the pre-BCR at low levels on the cell surface in association with Igα and Igβ produces a functional receptor complex. Expression of the pre-BCR is associated with cell enlargement followed by a burst of proliferation before pre-B-cells undergo the transition to small resting pre-B-cells. It is not clear what the pre-BCR receptor complex recognises, but it signals to the cell which then inhibits rearrangement of the heavy chain locus on the other

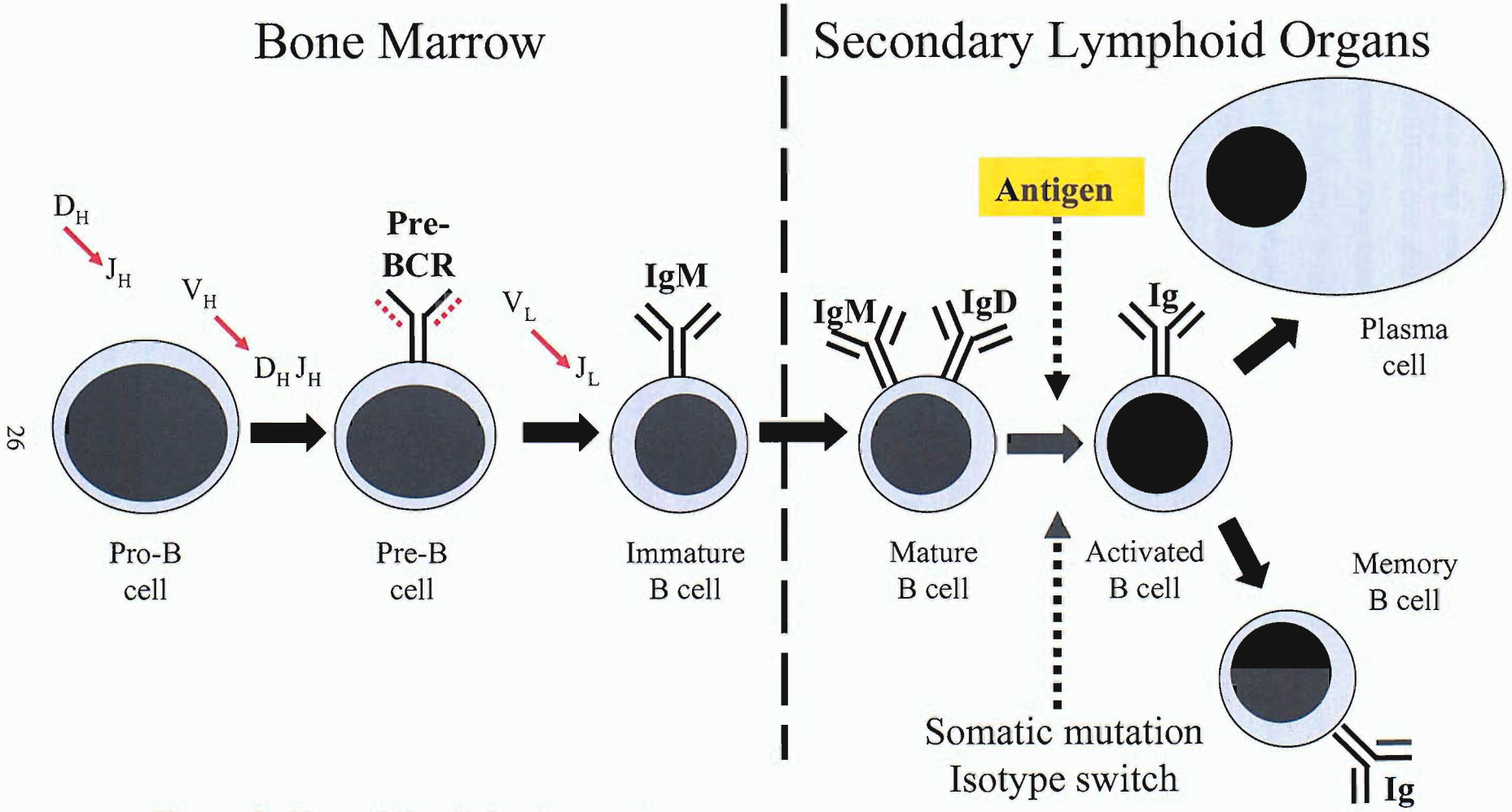


Figure 2 . Normal B-cell development

chromosome. This process is called allelic exclusion and ensures that each B-cell produces Ig of a single specificity.

Next, light chain gene rearrangement occurs. In humans, κ gene recombination usually occurs before that of the λ gene locus. A successful κ gene rearrangement inhibits λ gene rearrangement. If a κ light chain cannot be produced then the λ locus undergoes recombination. Individual B-cells can therefore only produce either a κ or λ light chain but not both. This is called isotypic exclusion. Approximately 2/3rds of mature human B-cells express κ light chains. Several successive attempts at a productive rearrangement can be made at each locus but if these fail then the cell dies. A successful V_L-J_L rearrangement results in the production of a light chain.

Each light chain molecule then combines with a heavy chain which has a μ or δ constant region to form IgM or IgD respectively. Expression of IgM on the cell surface signifies that the pre-B cell has become a mature B-cell. In addition to IgM, mature B-cells also express IgD of the same specificity on the cell surface. If the B-cell does not react with self antigen, IgM on the surface is not cross-linked and gene rearrangement ceases. These cells leave the marrow, enter the bloodstream and make their way to the peripheral lymphoid organs.

Meanwhile IgM undergoes strong cross-linking on the surface of bone marrow B-cells which react against self antigens. Some of these cells undergo apoptosis. This process is termed clonal deletion. However, as these self-reactive B-cells still express RAG genes, further light-chain gene rearrangement may occur. Another successful light chain rearrangement can allow the cell to replace the self-reactive receptor with an alternative receptor. If this subsequent receptor does not react strongly against a self-antigen then the cell does not undergo apoptosis. This process is termed receptor editing. Some B-cells encounter self-antigens which cause only weak cross-linking of their sIgM. These cells are inactivated and enter a permanent state of unresponsiveness termed anergy.

1.1.1.2.5 Post bone marrow B-cell development

Further B-cell development is dependent upon an encounter with antigen. An antigen can be classified as T cell dependent (TD) or T-cell independent (TI) [12]. By definition, the immune response to TI antigens does not require T-cell help. Memory cells are not induced in the immune response to TI antigens and IgM is the predominant antibody formed. Two types of TI antigen are recognised. TI-1 antigens are potent mitogens which cause non-specific polyclonal activation of most B-cells. Lipopolysaccharide (LPS) present in the cell membrane of gram negative bacteria is a TI-1 antigen. TI-2 antigens only activate mature

B-cells. They cross-link Ig on the B-cell surface. TI-2 antigens are typically repetitive carbohydrate or protein epitopes present at high density on the surface of microorganisms. Certain groups of individuals do not respond well to TI-2 antigens, such as children <2 years of age, adults >65 years of age, or asplenic or HIV seropositve patients [12].

Helper T-cells play an essential role in the generation of an immune response to a TD antigen. Memory B and T cells specific for the antigen are formed. In addition, high affinity antibodies of multiple isotypes are produced in response to a TD antigen by the processes of somatic hypermutation and class-switch recombination.

1.1.1.2.6 Somatic hypermutation and class-switch recombination

Following an encounter with antigen presented by helper T-cells in the in the geminal centres of lymph nodes, spleen or gut associated lymphoid tissue, B-cells can undergo the processes of somatic hypermutation and isotype switching [11]. Somatic hypermutation involves the introduction of point mutations into the V regions of the rearranged heavy and light chain genes at a very high rate. This process provides a means for generating further Ig diversity and it gives rise to mutant Ig molecules on the surface of B-cells, some of which bind to antigen with a higher affinity than the original receptors. The preferential selection of cells with a higher affinity for antigen to mature into antibody secreting cells is termed affinity maturation.

B-cells gain the ability to produce Ig of the isotypes IgG, IgA or IgE only after they have been stimulated with antigen in a germinal centre. This change is known as class switching and enables the production of Ig molecules with the same specificity but with different effector functions. The enzyme activation-induced cytidine deaminase (AID) plays an essential role in both class-switching and somatic hypermutation [13].

1.1.2 Immunotherapy of malignancy

Several immunotherapeutic treatments are currently used in the routine treatment of malignant disease. For example the survival of patients with large B-cell lymphoma has been prolonged by infusion of Rituximab [14]. This is a chimeric human/murine monoclonal antibody which binds to CD20, a molecule expressed on the surface of lymphoma cells.

Perhaps the most impressive example of passive immunotherapy in routine clinical use is allogeneic stem cell transplantation, which can cure patients with haematological malignancies. The use of this procedure in the treatment of cancer had originally been conceived simply as a method to overcome the acute and profound pancytopenia resulting from bone marrow toxicity which had, until then, limited the doses of chemotherapy and

radiotherapy which could be administered to patients with malignant diseases [15]. Curative therapy then became a realistic prospect for those patients with malignancies in which dose escalation resulted in significantly greater tumour-cell killing.

In addition to a beneficial effect on the underlying disease, the chemotherapy/radiotherapy was thought to have an important role in eradicating the recipient's immune system thereby preventing rejection of the transplant from a HLA matched sibling. The procedure was also associated with considerable treatment related mortality to which the immunologically mediated graft versus host disease was a major contributor. The requirements for the development of graft versus host disease were formulated by Billingham in 1966: the graft must contain immunologically competent cells, the recipient must express tissue antigens that are not present in the transplanted donor and the recipient must also be incapable of mounting an effective response to destroy the transplanted cells [16].

GvHD remains a significant barrier to allo-SCT today and efforts to control it continue. One approach which can decrease the incidence of GvHD involves depleting the stem cell graft of T-cells prior to infusion into the recipient. However, patients who receive T-cell depleted grafts have been noted to have a higher incidence of disease relapse than those who receive a non-T-cell depleted graft and who also experienced GvHD [17] This indicated that a clinically relevant anti-leukaemic effect (GvL) was associated with GvHD.

The importance of GvL had been recognised almost 50 years ago by Barnes and colleagues who had observed that that leukaemic mice treated with a subtherapeutic dose of radiation and a syngeneic graft transplant were more likely to relapse than mice given an allogeneic transplant [18,19]. It is now clear that it is the immune mediated graft-vs-malignancy effect that results from allo-SCT rather than the high dose therapy which is largely responsible for eradication of the malignancy [20]. Allogeneic transplantation is now available to older patients through the recent development of reduced intensity conditioning protocols which rely solely upon GvL for disease control. Despite this, allo-SCT is a realistic treatment option for a small proportion of patients with malignant disease, such as those with chronic and acute leukaemias. In addition only 20-30 % of patients would be expected to have a HLA-matched sibling to act as a stem cell donor but it provides impetus for other approaches which seek to attack malignancies by immunological mechanisms.

The goal of active vaccination strategies against cancer is to induce immune responses which can control malignant disease without life threatening side effects. Ideally, immunological memory against tumour antigens would be stimulated which could then prevent tumour re-emergence after initial control. In addition, it is desirable that

vaccination would be applicable to most patients with malignant disease rather than the small proportion for whom immunotherapy is currently a realistic option. Although immunotherapeutic vaccination strategies are not yet in routine clinical use, some tumour regressions have been seen in clinical trials which have built upon the promising results achieved in basic pre-clinical research.

The development of vaccination strategies against cancer is being helped by an improving understanding of how immunological memory is generated and maintained. All successful vaccines currently in clinical use against infection provide protection by inducing neutralising antibodies. There are many infections against which an effective vaccine does not yet exist, such as TB, leprosy, HIV, HPV and HCMV. A common feature of these infections is that control or eradication of disease cannot be achieved by neutralising antibodies alone but requires additional specific T-cell mediated effector mechanisms. This is analogous to the interaction between the immune system and malignancy where cellular immune responses and in particular $CD8^+$ cytotoxic T cell responses are of most importance. The immunological mechanisms which must be exploited in the development of vaccines which induce cellular responses against these viruses are therefore likely to be similar to those which will underlie successful vaccination against malignancy.

1.1.3 Principles of vaccination

There are several key steps which result in generation of a successful adaptive immune response to antigens delivered by vaccination. Firstly the antigen must be taken up by professional antigen presenting cells. This must occur in an immunological setting which facilitates APC activation. The APC must process and transport the antigen to organised lymphatic tissues and then interact with and activate antigen specific effector cells. On subsequent encounter with the relevant antigen, effector cells must then respond in a manner which successfully destroys the antigen and with it the organism or tumour with which it is associated. The immunological mechanisms which underlie each of these steps are outlined below.

1.1.3.1 Antigen uptake

Although dendritic cells, macrophages and B-lymphocytes are all classed as professional antigen presenting cells, mature dendritic cells are the most potent stimulators of naïve T cells. Dendritic cells are a heterogeneous family of leucocytes which are generated in the

bone marrow and then migrate to sentinel sites in the peripheral tissues, such as the skin and the gut mucosa, where infection is likely to occur. Here they exist in an immature state characterised functionally by a high capacity to acquire antigens but a low capacity to stimulate T cells.

Immature DCs can internalise exogenous antigen by endocytosis, pinocytosis, macropinocytosis and phagocytosis. The route employed to take up a particular antigen is determined by characteristics exhibited by the antigen such as its form, solubility or whether it is part of an immune complex. Endocytosis involves the formation of vesicles of approximately 150-200nm at the site of membrane invaginations called coated pits. Antigens induce coated pit formation by clustering specific cell surface receptors. Pinocytsis is the process by which soluble antigen within the extra-cellular fluid in the vicinity of a budding endosome can be taken up. Macropinocytosis involves the non-specific engulfing of large volumes of extracellular fluid by large vacuoles, macropinosomes, which form at the plasma membrane. Macropinosomes are 200-500nm in diameter and seem to form at the sites of membrane ruffling. Phagocytosis is a clathrin–independent process in which large extracellular particulate material is internalised into a phagosome.

Some of the receptors involved in phagocytosis and receptor mediated endocytosis are specific to dendritic cells, such as CD 205 (DEC 205) and DC- specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN), whilst others, of which the mannose receptor (CD 206) is an example, can be found on a variety of cells [21]. Immune complexes can be internalised via Fc receptors for IgE and IgG [21]. It appears that the α2-macroglobulin receptor, CD91, on the DC surface can act as a receptor for the heat shock proteins gp96, hsp90 and hsp70 [22,23] thereby mediating the internalisation of antigen located in hsp-peptide complexes released from damaged cells. The phagocytosis of apoptotic cells is aided by CD36 and $\alpha_v\beta_5$ integrin receptor on the DC surface[24].

1.1.3.2 Antigen processing and presentation to T cells

T cells can only recognise antigens when they are presented as peptides of the correct length in the groove of MHC molecules. $CD8^+$ T cell receptors recognise peptides which are usually 8-10 amino acids in length presented in the groove of MHC class I molecules. $CD4^+$ T cells receptors recognise peptides presented in the groove of MHC class II molecules. These peptides are usually 13-17 amino acids long but may be much longer. APCs must process antigen and deliver peptides in this format in order to stimulate a T cell

response. APCs possess two distinct pathways by which antigen is processed and presented. These are termed the endogenous pathway and the exogenous pathway. The route employed to process a particular peptide antigen is dictated by the intracellular compartment in which the peptide is located.

1.1.3.2.1 Endogenous pathway

Endogenously synthesised proteins located within the cytosol are degraded into short peptide fragments by cytoplasmic peptidases or by large cytosolic protease complexes termed 'proteosomes'. Through co-ordinated double cuts, the proteosome generates protein fragments of approximately 9-15 amino acids that contain the hydrophobic or positively charged COOH-terminal anchor residues required for MHC class I binding. Proteosomes can be transformed into immunoproteosomes by the presence of the immunomodulatory cytokine interferon-γ which is produced by activated T helper 1 lymphocytes, $CD8^+$ CTLs and natural killer cells. The peptide cleavage preferences of the immunoproteosome differs slightly from those of constitutive proteosome thereby facilitating the generation of some antigenic peptides whilst destroying others [25].

The pathway by which endogenously generated peptides are processed and subsequently presented on the cell surface in association with MHC class I molecules is represented schematically in Figure 3A. Peptides generated by this process then bind to the "transporter associated with antigen processing" (TAP), which transports the peptide across the endoplasmic reticulum membrane [26]. Further trimming in the lumen of the ER by the aminopeptidase ERAP1 generates peptides of 8-10 amino acids in length [27-29]. TAP and ERAP1 expression is also upregulated by Interferon-γ [30]. Peptides which then successfully bind MHC class I molecules are transported to the cell surface where they are available for interaction with $CD8^+$ T cells.

1.1.3.2.2 Exogenous Pathway

Extracellular antigens internalised into endocytic vesicles are degraded by acidic proteases known as cathepsins. This generates peptides which have the characteristics required to bind to MHC class II molecules. The pathway by which exogenous antigens are processed and subsequently presented on the cell surface in association with MHC class II molecules is represented schematically in Figure 3B.

MHC class II αβ dimers are synthesised in the ER of mature DCs and associate with a chaperone molecule called the invariant chain (Ii). The invariant chain has several functions. It prevents peptides present in the ER from binding to the newly formed αβ

dimer. It is also involved in transporting the $\alpha\beta$ dimer to late endocytic vesicles known as MIIC (MHC class II compartments) where peptide loading occurs [31]. The invariant chain is degraded by aspartic and cysteine proteases leaving the class-II-associated invariant chain peptide (CLIP) fragment in the peptide binding cleft of the MHC class II complex. CLIP is subsequently exchanged for an antigenic peptide in a reaction that is catalysed by HLA-DM [32 33]. DM also edits the peptide repertoire by exchanging peptides that bind poorly for peptides that fit more tightly in the peptide binding groove [32]. Expression of HLA-DM is also increased by interferon-γ.

Antigens derived from phagocytosed particles can be loaded directly onto MHC class II molecules in a similar manner in the phagosome [34] or released and transferred to the classical endocytic compartments for loading onto MHC class II molecules [25]. MHC class II-peptide complexes are transported to the cell surface where they are then available for interaction with $CD4^+$ T-cells.

1.1.3.3 Cross Presentation

Cross presentation was first described by Bevan and colleagues [35,36]. It is the term given to the phenomenon by which exogenous antigen is acquired by DCs and then presented in the groove of MHC class I to $CD8^+$ T cells. Cross presentation by professional APCs therefore breaks the rule which stated that only endogenously synthesised proteins could serve as the source of peptides presented in association with MHC-class I molecules on the surface of a cell. The mechanisms by which exogenous antigens in the phagosome crossed over into the MHC class I pathway were previously unclear. It had been observed that proteins could escape from the phagosomes and enter the cytosol [37]. Once in the cytosol, it was thought that phagosome derived proteins could then potentially join the pathway used to process endogenous antigens.

Recent observations have led to a revised model of cross-presentation. The pathway by which exogenous antigens are now thought to be processed and subsequently presented on the

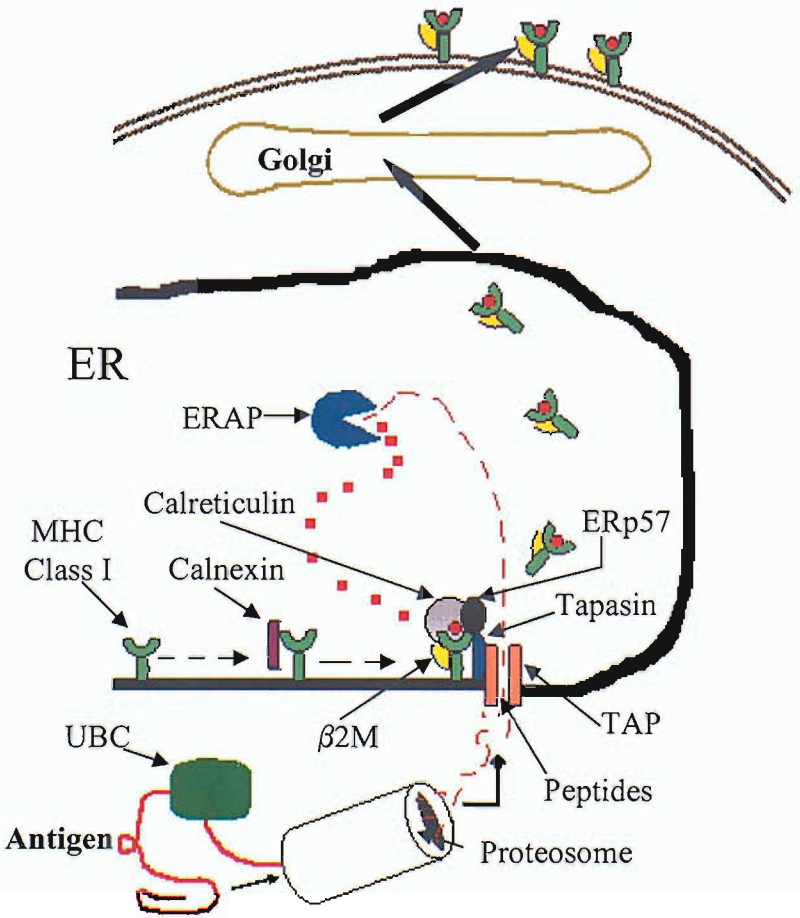


Figure 3A. Processing of endogenous antigen for MHC class I Presentation. Antigen is ubiquinated by the ubiquitinating enzyme complex (UBC) and degraded by the proteosome. Peptides are transported into the endoplasmic reticulum (ER) via transporter associated with antigen presentation (TAP). They associate with MHC class I molecules and are transported to the cell surface.

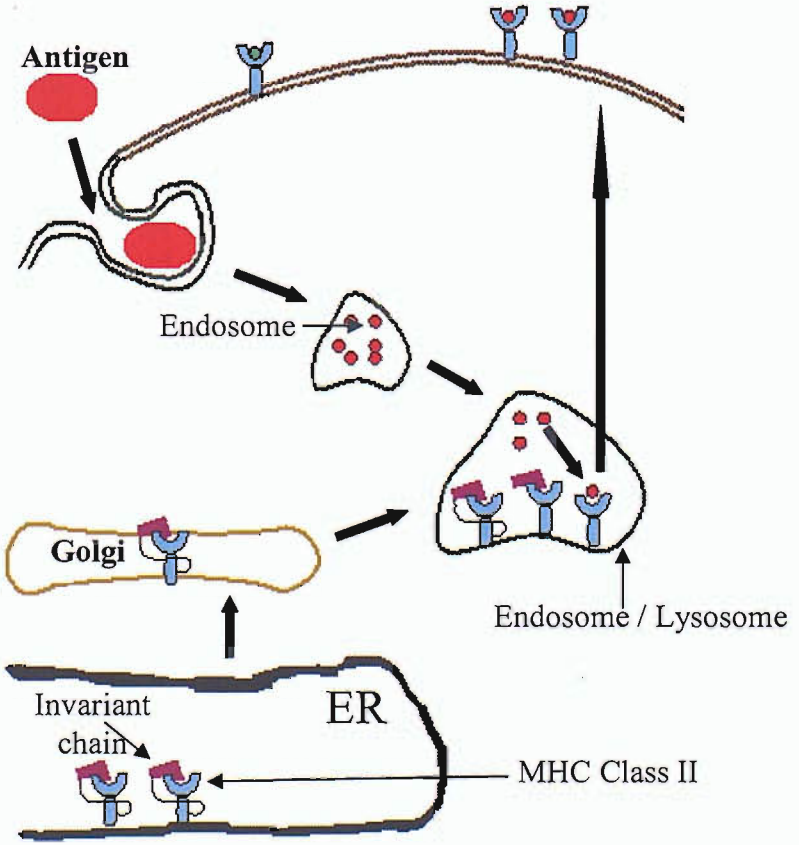


Figure 3B. Processing of exogenous antigen for MHC class II presentation. Exogenous protein is internalised into endosomal compartments and degraded by proteolytic enzymes and low pH. MHC class II synthesised in the ER associates with invariant chain and is directed through the Golgi to the endosomal/lysosomal compartments. Invariant chain is degraded and HLA-DM catalyses the exchange of CLIP for antigenic peptides which are then transported to the cell surface.

cell surface in association with MHC class I molecules is represented schematically in Figure 4. First, the presence of several ER-resident proteins were detected within the phagosome using mass–spectrometery and two-dimensional gel electrophoresis [38]. This was followed by experiments which demonstrated that phagosomes fuse with the ER during particle engulfment [39]. As a consequence of phagosome-ER membrane fusion, phagosomes are bound by membranes largely derived from the ER and contain the molecules involved in the MHC class I pathway and other elements involved in peptide processing and translocation [40,41]. One of these molecules is a protein-translocation channel, the Sec61 complex, which translocates proteins from the phagosome into the cytosol for degradation by the proteosome.

It has also been shown that proteosomes associate with the cytosolic side of the phagosome membrane [41]. Peptides produced by the proteosome are then imported back into the phagosome by TAP, which was acquired during the earlier fusion of the ER-phagosome membranes. Peptides are then loaded onto MHC class I molecules on the inside of the phagosome membrane and subsequently presented on the cell surface in the normal manner. These findings indicate that the phagosome can be regarded as an autonomous cross-presentation organelle. The ability to present exogenous antigens by both MHC class I and II increases the repertoire of peptides which can be presented to T-cells thus improving the efficiency of the adaptive immune response.

1.1.3.4 Activation of immature dendritic cells to mature dendritic cells

Activation of immature dendritic cells can occur by several mechanisms, most of which are associated with the presence of infection or other inflammatory stimuli. DCs possess pattern-recognition receptors (PRRs) which recognise pathogen associated molecular patterns (PAMPs) exhibited by viruses, bacteria, fungi and protozoa. The best studied PRRs are the Toll-like receptors (TLRs). Different DC subsets express unique patterns of TLRs indicating underlying functional distinctions. For example, human blood plasmacytoid DCs do not express TLR-4 but do express TLR-9, which allows them to respond to synthetic CpG- containing oligonucleotides. $CD11c^{+}$ DCs and monocyte-derived DCs do not express TLR-9 but can recognise and respond to lipopolysachharide via TLR-4. These distinctions in TLR repertoire expression among DC subtypes are not conserved across species boundaries as illustrated by the observation that murine non-plasmacytoid spleen DCs express TLR-9 [42] whilst murine plasmacytoid DCs have been found to express both TLR-4 and TLR-9 [43,44] [45].

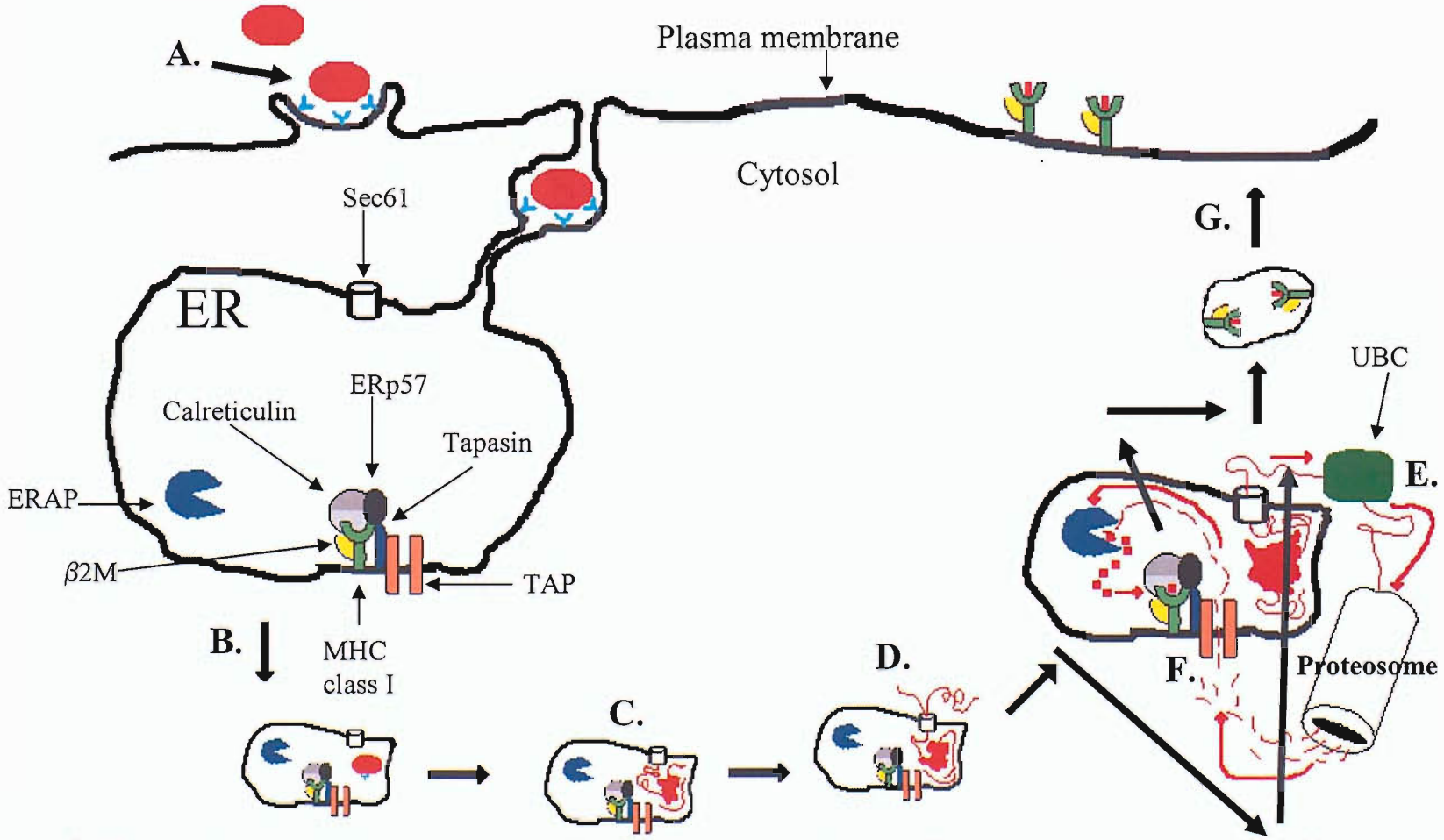


Figure 4. Processing of exogenous antigen for MHC class I cross presentation. **A)** Exogenous antigen is internalised by phagocytosis. **B)** ER membranes fuse with the plasma membrane to form a phagosome. **C)** Antigen is degraded by hydrolases and **D)** exported into the cytosol by Sec61. **E)** Cytosolic peptides are ubiquinated by ubiquinating enzyme complex (UBC) and then degraded by the proteosome. **F)** Processed peptides are re-imported via TAP and trimmed by ERAP. Peptides are loaded onto MHC class I molecules by the MHC class I loading complex and **G)** transported to the cell surface for cross presentation to $CD8^+$ T cells.

DCs also possess pattern recognition receptors other than TLRs. For example protein kinase R can recognise dsRNA, representing another pathway for DC activation by viruses [46]. C-type lectins may act as PRRs, as illustrated by DC-SIGN signalling induced by *Mycobacterium tuberculosis*. Inflammatory cytokines, such as IL-1β or TNF, produced by the innate immune system also trigger DC maturation. Matzinger has proposed that DC activation involves the recognition of danger signals released by damaged cells [47]. It is possible that some endogenous danger signals may act as ligands for PRRs whilst others such as bradykinins or ATP act via other specific receptors. Successful vaccines activate immature DCs using either some of the mechanisms listed above or by other as yet undiscovered methods.

The key result is that antigen uptake is followed by DC maturation. Maturation occurs during DC migration from peripheral tissues to draining lymph nodes [48]. The maturation process involves considerable changes during which the immature DC, adept at sampling antigens from the external environment, transforms into a mature DC which effectively presents antigens to T and B cells and is uniquely capable of activating naïve T-cells. Mature DCs extend long "dendritic" processes which increase their ability to interact with T cells. Efficient antigen presentation is facilitated by the expression of high levels of MHC class I and II on the mature DC cell surface. They also secrete high levels of IL-12 which stimulates T, B and NK cells [49]. In addition, mature DCs express accessory molecules such as ICAM-1/CD54, LFA-3/CD58 and B7.2/CD86 which interact with T cells to enhance adhesion and provide co-stimulation [50].

1.1.3.5 Co-stimulation of naïve T-cells

On its own, interaction of an appropriate MHC class II associated peptide on the DC surface with the T-cell receptor (signal 1) is unable to trigger the activation of a naïve T-cell. For this to occur, additional signals from co-stimulatory receptors are required [51].

T-cell co-stimulatory molecules are cell-surface molecules that cannot functionally activate T cells on their own, but amplify or counteract signals provided by the T-cell receptor [52]. In the absence of a positive co-stimulatory signal, T cells become anergic which can lead to the development of antigen tolerance. Signal 2 was previously thought to involve only one membrane bound receptor and to activate intra-cellular signal-transduction pathways that are distinct from those transduced through the TCR [53].

Many co-stimulatory receptors have since been identified and they can be divided into two main groups: the immunoglobulin superfamily and the tumour-necrosis factor

receptor (TNFR) superfamily. Included in the immunoglobulin superfamily are CD28 and inducible T-cell co-stimulator (ICOS). The TNFR superfamily includes the co-stimulatory receptors OX40 (CD134), 4-1BB (CD137), CD27, CD30 and herpesvirus entry mediator (HEVM). Although naïve T-cells constitutively express CD28, CD27 and HVEM, most T-cell co-stimulators are induced after antigen recognition by the TCR. For example expression of OX40, 4-1BB and CD30 is induced several hours after antigen recognition and peaks 2-4 days into the response.

Mature DCs express high levels of the molecules CD80 and CD86 which provide a co-stimulatory signal when they bind to CD28 on the T-cell surface. CD28 is somewhat unusual in having more than one ligand. Most co-stimulatory receptors have only one ligand. Mature DCs also express the ligands for the co-stimulatory receptors OX40 (OX40-L), 4-1BB (4-1BBL), CD27 (CD70) CD30 (CD30L) and ICOS (LICOS) enabling them to deliver signals which are important in the clonal expansion and survival of T-cells [51]. It has been suggested that the expression by immature DCs of LIGHT, the ligand for HVEM, indicates a possible role for this interaction in the early activation of T cells and APCs [51].

Interactions between DCs and T-cells are not unidirectional. CD40 is another member of the TNFR superfamily which is constitutively expressed by DCs. Expression of the ligand for CD40 (CD40L, also known as CD154) on the surface of a CD4 T-cell is strongly upregulated by CD28 and ICOS. CD40L is not itself a co-stimulatory molecule but by binding to CD40 it sends a potent signal to the DC to upregulate the expression of CD 80, CD86 and LICOS [52]. This "licenses" DCs to a higher state of activation and provides a positive feedback loop for CD28 and ICOS on the T cell [52 54-57]. DC licensing is also facilitated by the action of type I interferons (IFN-α/β) which may be produced by activated T-cells [58].

CD80 and CD86 on the DC can also engage with cytotoxic T lymphocyte antigen 4 (CTLA-4) receptors on T cells. CTLA-4 is only expressed at high levels after T-cell activation. It binds CD80 and CD86 with ~10 fold higher affinity than CD28 and exerts negative influences on T-cell activation.

1.1.3.6 Effector $CD8^+$ T cells

Following activation by DCs, naïve $CD8^+$ T cells proliferate and differentiate into effector CTLs which leave the lymphoid tissues and can destroy cells bearing its specific antigen without requiring further co-stimulation. Effector CTLs kill target cells either by the release of preformed granules which contain perforin and granzymes. Perforin creates holes in the lipid bilayer of the target cell allowing the granzymes to enter and activate

enzymes which induce programmed cell death. Recognition of antigen on the surface of a target cell also induces the expression of Fas-ligand (Fas-L) by the CTL. Apoptosis of the target cell is induced when Fas expressed on the cell surface binds to Fas-L expressed by the CTL. Production of the cytokines IFN-γ, TNF-α and TNF-β by activated CTLs also contributes to the host immune response.

1.1.3.7 Effector $CD4^+$ cells

Naïve $CD4^+$ T cells can differentiate into either T_H1 or T_H2 cells upon activation. A key factor which influences the differentiation path followed by the CD4 cell is the cytokine profile elicited by the infectious agent or tumour. Cells stimulated by IL-12 or IFN-γ tend to develop into T_H1 cells whilst those stimulated by IL-4 develop into T_H2 cells, especially if IL-6 is also present. These cells have important functional differences and the manner of the immune response generated against a pathogen or tumour is dictated by which T_H subtype gains ascendancy. Essentially, T_H1 cells drive a cell mediated immune response whereas T_H2 cells direct a humoral response. It must be stressed that although a T_H2 response can suppress a T_H1 response and vice-versa, the two are not mutually incompatible and the immune system may react to pathogens with a mixed T_H1/T_H2 response.

1.1.3.7.1 T_H1 cells and the cellular immune response

Upon activation, T_H1 cells stimulate macrophages and phagocytes by cellular interactions and by the production of interferon-γ and TNF-β. T_H1 cells also activate B cells to produce the opsonizing antibody subtypes IgG1 and IgG3. Fas ligand may be expressed on the surface of activated T_H1 cells. The interaction of Fas ligand with Fas on the surface of a target cell enables T_H1 cells to mediate direct cytotoxicity.

1.1.3.7.2 T_H2 cells and the humoral immune response

Activated T_H2 cells inhibit macrophages by secreting TGF-β, IL-4, IL-10 and IL-13. Antibody responses by B-cells to protein antigens require antigen-specific T cell help and this is the main role of activated T_H2 cells. Antigen is captured by B-cell receptor (BCR) located on the B-cell surface. BCR-Ag complexes are internalised into endosomes and then transported into class II compartments where antigens are loaded onto MHC class II. This process is aided by HLA-DO, an accessory molecule which inhibits the peptide exchange

activity of DM thereby promoting the presentation of antigens derived from BCR-internalised antigens [59].

Effector T_H2 cells which recognise the peptide:MHC class II complex then deliver activating signals to the B cell which stimulates proliferation and isotype switching. The interaction of CD40L on the T cell with CD40 on the surface of the B cell is an important signal in this process. IL-4 produced by the T_H2 cell acts synergistically with CD40L. This cytokine is produced in a polar fashion and thereby acts selectively on the antigen specific B cell. B cell activation generates two separate lineages of cells, plasma cells and memory B-cells. Plasma cells are terminally differentiated effector cells which produce antigen specific antibodies. The secondary humoral response to antigen is dependant upon reactive memory B cells which can rapidly differentiate into antibody producing plasma cells. During the secondary response greater amounts of higher affinity IgG antibodies are produced more rapidly than is seen in the primary response.

1.1.3.8 Characterisation of memory T lymphocytes

The goal of vaccination is to successfully induce immunological memory against a chosen antigen. The development of effective vaccines is therefore aided by an ever improving understanding of how memory responses are generated. The distinction between memory and effector B-cells, as outlined above, has been much easier to define than has been the case for T-cells. A recently proposed model suggests that designated cells with corresponding functions also exist in the T cell system [60]. Effector memory T-cells (T_{EM}) migrate to inflamed tissue and exhibit immediate effector function which provides 'protective' memory whilst central memory cells (T_{CM}) home to lymphoid organs, exhibit little effector function but proliferate and differentiate into effector cells in response to antigen thereby providing 'reactive' memory.

These cells can be distinguished by their surface expression of activation molecules, lymph node homing receptors and molecules associated with co-stimulation. This phenotypic characterisation of T_{EM} and T_{CM} applies only to resting cells as many of these markers are modulated upon cell activation. Central memory cells are $CD45RO^+$ and express the receptors CCR7 and CD62L. CD62L interacts with peripheral-node addressin (PNAd) on high endothelial venules, which mediates attachment and rolling [61,62]. CCR7 binds the chemokines CCL19 and CCL21 found on the luminal surface of endothelial cells in lymph nodes enabling firm arrest of the cell and the initiation of extravasation [63].

Effector memory T-cells have ceased to express CCR7 and are heterogeneous for CD62L expression [60]. The chemokine receptors and adhesion molecules expressed by T_{EM} cells facilitate homing to inflamed tissues. Functionally, T_{EM} are characterised by rapid

effector function and in keeping with this, CD8 T_{EM} contain large amounts of perforin and produce IFN-γ within hours of antigenic stimulation. A subgroup of CD8 T_{EM} which expresses CD45RA contains the largest amount of perforin. Similarly, CD4 T_{EM} cells rapidly produce either IFN-γ or IL-4 and IL-5 after exposure to antigen. The expression of chemokine receptors allows further subdivision of T_{EM} cells. The expression of CCR5 and CXCR6 distinguishes T_H1 and CTL cells within the T_{EM} pool from T_H2 cells which can be identified by the presence of CCR3 and CRTh2 [64-67].

Expression of co-stimulatory markers helps to identify distinct functional subsets of T_{EM} and T_{CM}. CD27 and CD28 are expressed by naïve T cells but are not present on CD8 memory cells characterised by high effector function and CD45RA expression. The fact that cells cannot be defined by one marker alone is highlighted by the observation that some CD45RA $CD8^+$ T-cells express CD27. The functional properties of these cells are intermediate between naïve and effector cells [68]. The phenotype of memory cells which control pathogens can differ between various infections. The majority of HIV specific $CD8^+$ memory cells found in the peripheral blood exhibit the phenotype associated with T_{EM} ($CD45RA^-$ $CCR7^-$) whereas those which control CMV are also $CCR7^-$ but express CD45RA [69].

1.1.3.9 Generation of memory T-cells

The mechanisms which underlie the generation of effector and central memory T-cells are being elucidated. After initial expansion in response to antigen, >90% of antigen specific cells die over the weeks which follow pathogen clearance. The number of antigen specific memory T cells formed is to be influenced by the "burst size" of the original response to the antigen [70]. The implication is that vaccination strategies should aim to induce as large an initial effector T-cell population as possible.

The strength of the initial signal delivered through the TCR has a major bearing on subsequent T cell differentiation and may play an important role in directing the cell to develop into a memory cell. An in-vitro system has been developed in which signal strength delivered to T cells can be modulated by altering the number and type of co-incubated DCs. These experiments showed that cells which received a strong signal in the presence of appropriate cytokines developed into effector cells. T-cells which received a weak signal proliferated but did not develop effector function. Upon in-vivo transfer these cells migrated to lymph nodes and rapidly proliferated and differentiated in response to antigen, characteristics associated with central memory cells. This was shown for both $CD8^+$ and $CD4^+$ T-cells.

Results from other experiments performed with $CD8^+$ cells are consistent with the view that memory cell generation occurs during the primary response. It was shown that $CD8^+$ cells which express the receptor for IL-7 during the effector phase of the primary response to LCMV go on to become memory cells [71]. These IL-7R cells were also found to express CD8$\alpha\alpha$. Signalling through CD8$\alpha\alpha$ leads to enhanced survival and differentiation into memory cells. It is also now clear that $CD4^+$ T-cell help is absolutely required during the initial antigen response if antigen specific memory $CD8^+$ T-cells are to be generated [72].

A better understanding of the mechanisms by which central memory cells are generated should enable the optimal conditions in which to prime and expand T_{CM} cells to be established. This will be of obvious benefit programmes such as ours that are involved in the development of vaccination strategies designed to induce cellular immune responses against tumour antigens.

1.1.3.10 Challenges specific to vaccination against malignancy

There are several important differences between current vaccines against infectious disease and vaccines which would be envisaged to provide successful control of malignancy. All vaccines against infectious disease which are currently in routine clinical use induce neutralising antibodies which subsequently protect hosts during a future encounter with that pathogen. These antibodies destroy the invading organism before it has caused disease [2]. However, to succeed against cancer or persistent viruses, a vaccine must be effective against a tumour or virus *in situ*. This is not an insurmountable hurdle as evidence that therapeutic vaccination against active disease is possible was provided many years ago by Louis Pasteur, who successfully vaccinated patients already infected with rabies by injecting spinal cord from a rabies infected rabbit [73].

In comparison to antigens from infectious disease, most potential tumour antigens are relatively weak immunogens [2]. Many are autoantigens which are over-expressed by tumour cells but may be present at low levels in normal cells. The ability of the immune system to respond to such antigens may be affected by pre-existing tolerance. The deletion of T-cells specific for "self" tumour antigens may have previously occurred in the thymus during the development of the adaptive immune system. This is known as central tolerance and whilst it is unlikely that such cells can be regenerated, the efficacy of deletion depends on the avidity of T-cells for the antigen and may not therefore be complete [74]. Peripheral tolerance may result from several processes, including regulation or anergy of tumour antigen specific T-cells [2]. Anergy occurs when antigens are presented to T-cells in the absence of appropriate co-stimulatory stimuli.

Even if immune responses are generated against tumour antigens, the tumour cells may evade attack by down regulation of MHC molecules, production of immunosuppressive cytokines, up-regulation of anti-apoptotic factors or down regulation of the chosen tumour antigen [74]. In addition, patients with malignant diseases may have a reduced immune capacity as a result of the disease process itself or treatments such as chemotherapy administered to decrease the tumour burden. Our group has explored the use of DNA vaccines to overcome these obstacles and induce an effective immunological attack on malignant diseases.

1.1.4 DNA vaccines

A new approach to vaccination was initiated by Wolff et al. They demonstrated that intramuscular injection of naked plasmid DNA encoding β-galactosidase resulted in transfection of muscle cells and production of the encoded protein [75]. It has since been shown that DNA vaccines can induce humoral and cellular immune responses against a chosen antigen. DNA vaccines have provided protection against a variety of infectious diseases in animals [76]. Considerable interest has been generated by the ability of DNA vaccines to induce immune responses against cancer in pre-clinical models [77]. The development of effective vaccines against cancer has been the primary goal of our research group. Although progress has been made towards this objective, an ever improving understanding of how the immune system responds to DNA vaccination should assist the future generation of effective therapeutic DNA vaccines.

1.1.4.1 DNA vaccine structure

A DNA vaccine consists of bacterial plasmid DNA into which a sequence encoding an antigen of interest has been inserted using the techniques of molecular biology [78]. The plasmid must contain an origin of replication sequence in order to allow plasmid growth in bacteria. The inclusion of a bacterial antibiotic resistance gene facilitates the selection of bacteria containing the desired plasmid during culture. Expression of the gene of interest is driven by a promoter. In practice, this promoter is usually derived from cytomegalovirus. The presence of polyadenylation sequences, such as simian virus 40, plays an important role in stabilising mRNA transcripts. The residual bacterial plasmid backbone is not simply a transport vehicle for the components listed above. It contains intrinsic adjuvant properties known as immunostimulatory sequences (ISS) which contribute greatly to the ability of the vaccine to induce an immune response.

1.1.4.2 DNA vaccine mechanism of action

DNA vaccines are most often administered by intramuscular injection, although intradermal injection, topical administration to mucous membranes or delivery directly into the skin using a biological ballistic device (the "gene gun") have all been employed. Regardless of the route of administration, the result is transfection of host cells by DNA from the vaccine. Intradermal delivery of a DNA vaccine directly transfects the skin-derived DCs (Langerhans cells) which are then thought to play a key role in antigen presentation to T cells [79-81].

Vaccine delivery by IM injection predominantly results in direct transfection of myocytes which then synthesize the encoded antigen [82,83]. However, muscle cells do not express MHC class II and co-stimulatory molecules that are required for effective T-cell priming. Evidence indicates that after IM delivery of a DNA vaccine, it is bone marrow derived antigen presenting cells, presumably dendritic cells, which activate T cells through "cross-priming" [84-87]. In this process, antigen is released by transfected myocytes, taken-up by bone marrow derived professional APCs, processed and then presented to T cells. Intramuscular injection may also result in the direct transfection of a small number of bone-marrow derived APCs which may then activate T cells without prior cross priming [88,89] (FIGURE 5).

As indicated above, immunostimulatory sequences in the bacterial plasmid DNA backbone play an important role in facilitating an immune response to the vaccine. The immunostimulatory sequences are composed of unmethylated CpG dinucleotides (CpG motifs) with specific flanking nucleotides [90]. CpG motifs are present in bacterial DNA at a 20-fold higher frequency than in vertebrate genomes. They are recognised by the mammalian immune system as a pathogen-associated molecular pattern (PAMP).

Toll-like receptor 9 (TLR9) has been identified as a possible pattern recognition receptor for bacterial DNA in both mice and humans [91,92]. It is expressed by B cells, dendritic cells and other cells of the innate immune system. Interaction between TLR-9 and DNA containing CpG motifs activates the Toll/IL-1 signalling pathway and triggers an inflammatory response characterised by the production of interleukin-6, IL-12, IL-18, TNF-α, IFN-γ, IFN-α and chemokines [93]. The observation that DNA vaccines can induce responses in TLR-$9^{-/-}$ mice indicates that additional receptors are involved [94 73] ISSs can also induce polyclonal B cell proliferation and stimulate DC maturation which is characterised by upregulated expression of MHC class II and co-stimulatory molecules. Dendritic cells stimulated by CpG motifs have been shown to produce factors including

IL-6 which may block the suppressive effect of $CD4^+$ $CD25^+$ regulatory T cells on T cell activation [95]. In summary, DNA vaccines exploit the ability of the immune systemto recognise the presence of bacterial DNA and activate a co-ordinated innate and adaptive immune response (FIGURE 5).

1.1.4.3 Development of a DNA vaccine against B cell malignancies

A vaccination strategy against any cancer first requires the identification of a clear tumour specific antigen against which an immune response can be directed. For this reason the development of DNA vaccines in our laboratory was initially focused on B-cell malignancies. Although these are a clinically heterogeneous group of malignancies, they are all thought to arise from B-lymphocytes at different stages of differentiation.

As previously discussed, Ig embedded in the surface of a normal B-lymphocyte is a unique receptor which enables the B-cell to recognise exogenous antigens. Under the correct circumstances, engagement of surface Ig by the antigen for which it is specific causes the B-cell to proliferate, generating a clone of effector cells (plasma cells), which produce the equivalent antibody, and some memory B-cells. Neoplastic transformation may occur at any stage of B-cell development. Some malignancies which are derived from B-cells, such as follicular lymphoma, express clonal surface Ig. Each Ig, whether derived form a normal or malignant B-cell, is itself inherently antigenic and antibodies can therefore be induced against it.

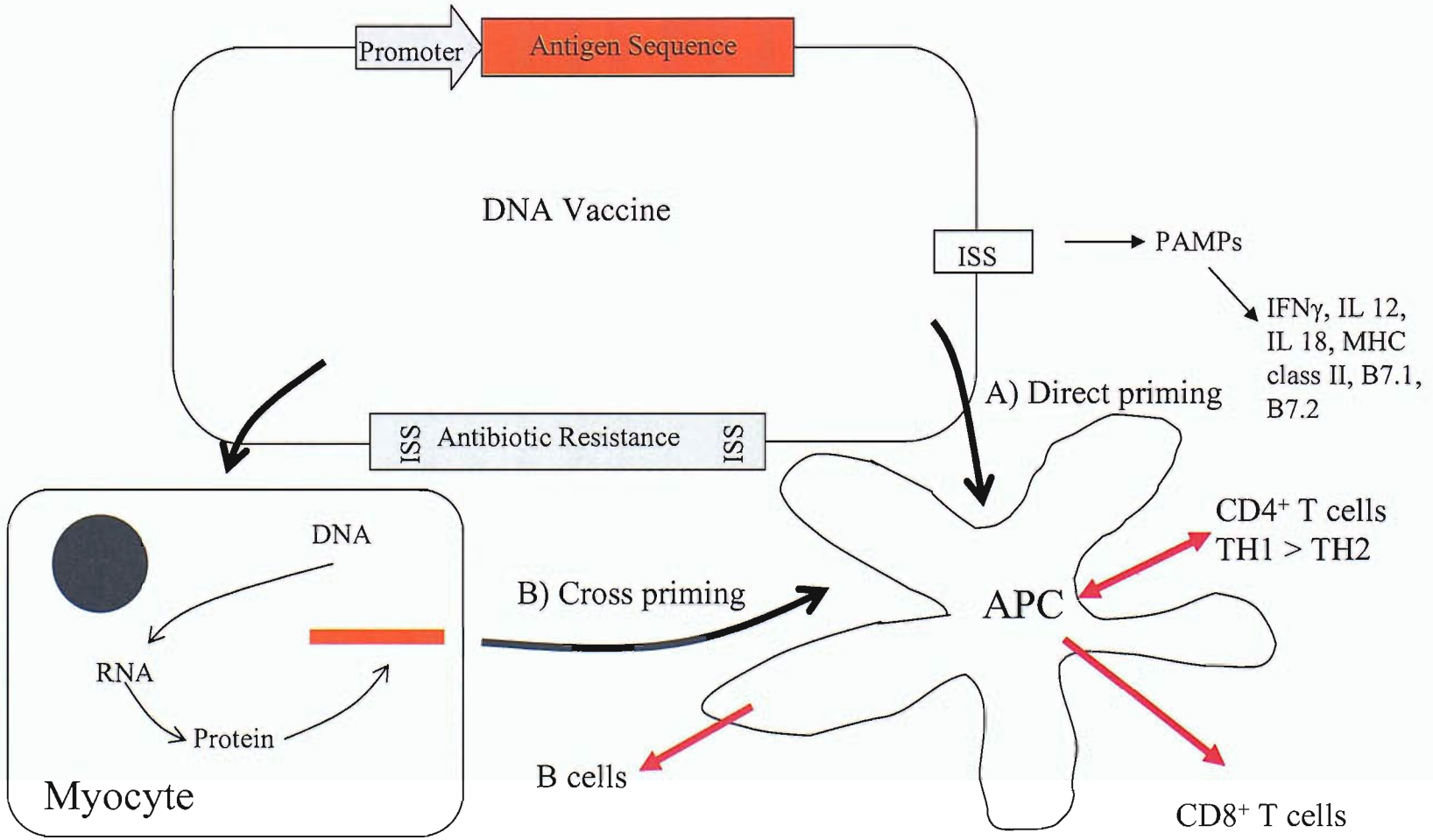


Figure 5. A).Injected naked DNA directly transfect myocytes or APCs. The promoter gene facilitates transcription of encoded antigen in transfected cells. Antigen produced within transfected APCs is then presented to specific T and B cells. B). Alternatively, antigen initially produced by transfected myocytes and subsequently acquired by APCs can be cross presented thereby stimulating a specific immune response. Immuno-stimulatory sequences within the plasmid backbone further activate APCs by signalling through pattern recognition receptors (PAMPs)

Each individual antigenic determinant of the variable region is referred to as an idiotope. The actual antigen binding site of Ig may be an idiotope, as can some other portions of the variable domains which lie outside of the antigen-binding site [96]. Antigenic determinants unique to a particular Ig are called private idiotopes whilst those antigenic determinants which are shared between different Igs are termed public idiotopes. Antibodies directed against public idiotopes can be responsible for serological cross-reactivity sometimes seen between antibodies. Each Ig contains multiple idiotopes, the sum of which is called the idiotype (Id).

The idiotype of Ig on the surface of malignant B-cell therefore represents a tumour specific antigen which is unique to the malignant clone. The ability of anti-Id antibodies to attack sIg-expressing tumours has clearly been shown in preclinical models [97]. A recent summary of clinical trials of Id Ig protein vaccines in follicular lymphoma shows that most patients generate an appropriate immune response [98]. As the production of individual patient-specific Id vaccines is technically challenging, our group sought to develop a DNA vaccine against idiotype.

As previously discussed, idiotypic determinants are encoded by the immunoglobulin variable heavy (V_H) and light (V_L) chain genes of the malignant clone. By using the techniques of molecular biology, the V_H and V_L genes can be recovered from the malignant cells found in the blood, bone marrow or lymph nodes of an individual with a B-cell tumour. These sequences can then be manipulated to create a single sequence which encodes the idiotype in the form of a single chain (scFv) [99]. By inserting a scFv sequence into a DNA plasmid a DNA vaccine designed to induce an immune response against the encoded idiotype was constructed. Although this vaccine could stimulate production of correctly folded scFv proteins when injected into mouse muscle, it only induced low levels of anti-Id antibody [100].

Efforts to improve the antibody inducing capacity of the vaccine took advantage of the immunological principle of "linked recognition". It was known that the covalent conjugation of a weak T-cell independent antigen to a carrier protein converted that antigen into a much more immunogenic T-cell dependent antigen. This approach had previously been employed in the development of protein vaccines effective against organisms such as *Haemophilius influenza* type B (Hib). Capsular polysaccharides from Hib did not stimulate the production of protective antibodies when administered as a vaccine to young children. Tetanus toxoid (TT) is a non-toxic but extremely immunogenic protein which has been successfully used as a carrier protein in conjugate vaccines. It is produced by the formaldehyde inactivation of the tetanus toxin, a potent polypeptide

neurotoxin produced by *Clostridium tetani* [2]. Conjugation of Hib antigens to TT amplifies production of anti Hib antibodies by B cells as a result of cognate T cell help received from helper T cells which recognise antigens presented on the B cell surface derived from TT element of the fused molecule [74].

Fusion of the DNA sequence of the non-toxic, highly immunogenic Fragment C (FrC) portion of tetanus toxin to scFv created a scFv-FrC fusion vaccine[101]. This vaccine was designed to induce FrC specific $CD4^+$ T-cells which would then provide cognate T-cell help for T and B cells specific for the linked antigen, scFv (FIGURE 6). As a result of this modification, the weak scFv vaccine was transformed into a powerful scFv-FrC fusion vaccine which could induce high levels of anti-idiotypic antibodies [101]. Mice injected with this vaccine were protected against subsequent tumour challenge in the A31 lymphoma model [77]. The fusion of scFv-FrC was demonstrated to be critical feature of the vaccine by the inability of scFv and FrC delivered on separate plasmids or even within the same plasmid to protect against tumour [77].

In parallel experiments, a scFv-FrC fusion vaccine was able to induce idiotype specific protective immunity in the 5T33 myeloma model [77]. This was somewhat surprising as myeloma cells do not express idiotype on the cell surface and are not susceptible to attack by anti-idiotype antibodies. Depletion experiments have established that $CD4^+$ T cells were essential for protection in this model *(unpublished)*. A mechanism which may explain the induction of this enhanced idiotype specific $CD4^+$ T cell response was proposed by Gerloni et al. They suggested that $CD4^+$ T cells reactive with a dominant determinant provide help to other $CD4^+$ T cells recognising weaker epitopes by up-regulating the co-stimulatory ability of APCs [102]. It is thought that malignant plasma cells are subsequently attacked directly by $CD4^+$ T cells via Fas/Fas ligand interaction, with a possible contribution from cytokines secreted by activated CD4 cells [103].

The results achieved in these pre-clinical models have paved the way for clinical trials in humans of DNA scFv-FrC vaccines against the B-cell malignancies follicular lymphoma and myeloma. It is expected that vaccination strategies against myeloma are most likely to succeed when the malignant clone has been reduced to a minimal residual disease state.

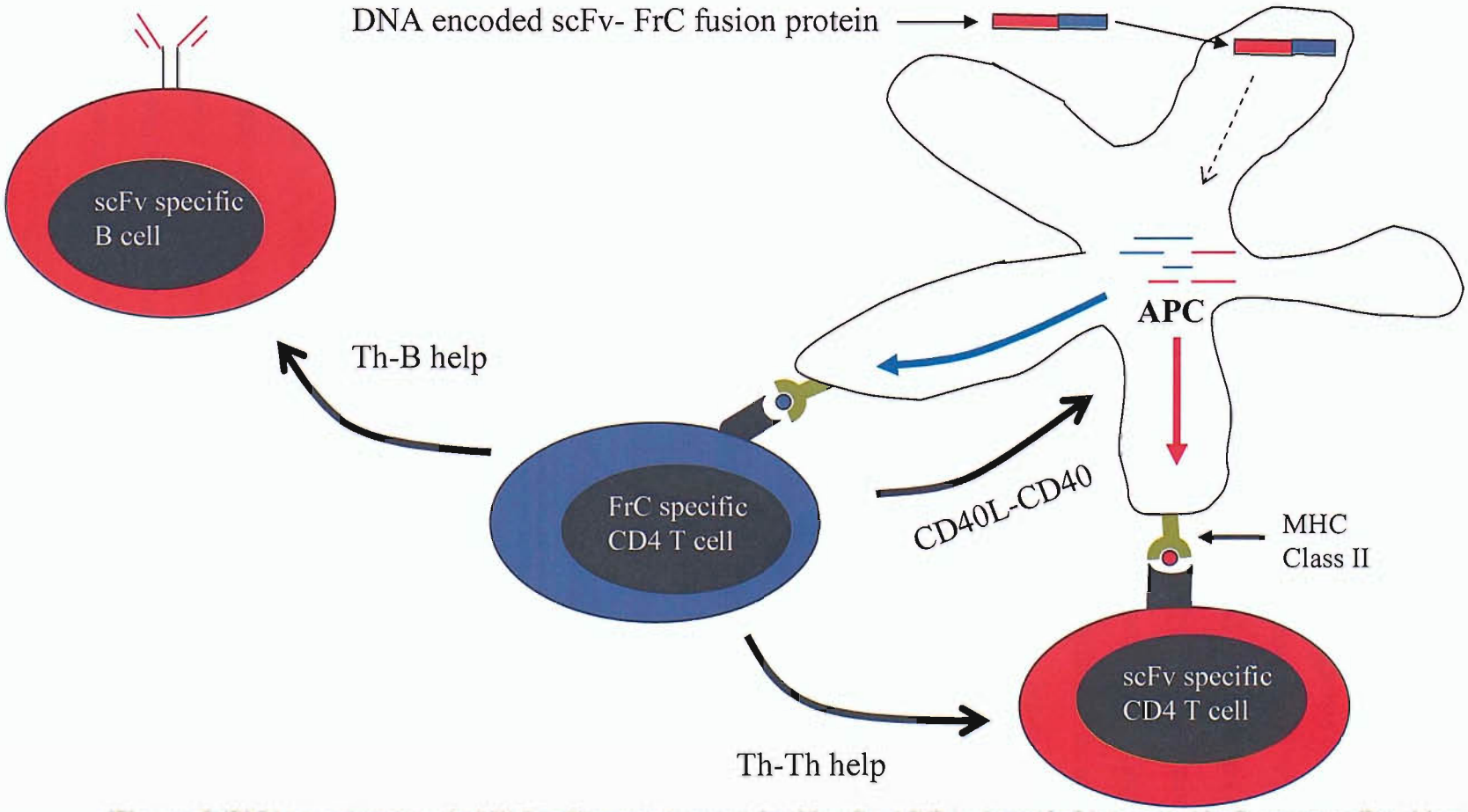


Figure 6. DNA vaccine encoded FrC-scFv protein accquired by the APC is degraded into peptide fragments. Peptides derived from FrC and scFv are presented on the APC surface in association with MHC class I and II molecules. The ability of the APC to activate scFv specific $CD8^+$ and $CD4^+$ T-cells is augmented via CD40L-CD40 signalling from $CD4^+$ T-cells specific for FrC, which also release cytokines that promote the activation of scFv specific B and T-cells.

1.1.5 Multiple myeloma

Multiple myeloma results from the malignant transformation of plasma cells or their precursors. It is a fatal plasma cell neoplasm associated with anaemia, osteolytic bone lesions, renal failure and immunodeficiency. Approximately 2500 new cases of myeloma are diagnosed each year in the UK [3]. The median age at diagnosis is 60-65 years [3]. Patients under the age of 65 are usually initially treated with 4-6 courses of "VAD" chemotherapy over a 3-4 month period. VAD comprises vincristine and doxorubicin (Adriamycin^{TM}) given by a continuous 4 day infusion together with high-dose dexamethosone [104]. This achieves a reduction Haematopoietic stem cells are subsequently collected. High dose melphalan chemotherapy is then routinely administered followed by an autologous stem cell transplantation procedure. This approach is not curative, but is associated with complete remission rates varying between 24% and 75% with a median survival of 4-5 years [3,105].

It has been demonstrated that improved complete response rates can be achieved by another bout of high dose therapy with autologous SCT [106], but this approach remains controversial as an overall survival benefit has not been consistently shown [107]. The only potentially curative therapy currently available for multiple myeloma is an allogeneic stem cell transplant but this is associated with a high risk of transplant-related death [108].

New, effective treatment strategies for myeloma are required. Thalidomide has recently been shown to produce responses in relapsed/refractory patients[109]. Responses in clinical trials have also been seen to the thalidomide derivatives such as Revimid^{TM}, but with less side effects[110]. Some patients have responded to the proteosome inhibitor Bortezomib [111]. Many other drugs are also under investigation and development [112].

We plan to determine if the impressive results achieved in the murine model with our scFv DNA vaccine can be translated into clinical practice to directly benefit patients with myeloma. It is anticipated that the reduction in disease burden achieved by high dose chemotherapy with autologous transplantation may provide a window of opportunity for anti-myeloma vaccine based immunotherapy before disease relapse occurs. This was therefore chosen as the setting for our clinical trial, entitled "Multiple Myeloma Idiotype FrC of Tetanus Toxoid" (MMIFTT).

Successful vaccination will require sufficient reconstitution of the patient's immune system to have taken place after transplantation to generate an effective immune response. The timing of scFv DNA vaccination after transplantation is therefore critical if the vaccine is to have the best chance of success. To guide our decision regarding when to

vaccinate, we examined the existing literature on immune reconstitution after autologous transplantation to ascertain when sufficient cellular and humoral immune reconstitution would be expected to have been achieved in the majority of patients. As the protection against myeloma induced by the scFv-FrC DNA fusion vaccine was dependent upon CD4 T-cells, we were particularly interested in the timing of reconstitution of these cells after transplantation. Unfortunately many of the studies which have examined immune reconstitution contained small numbers of patients, included both allogeneic and autologous transplant patients who had received various conditioning regimes in their analysis and did not assess cellular immune function. In addition, patients with a variety of haematological diseases were recruited into the studies, some of whom suffered from very aggressive conditions and relapsed soon after transplantation. Furthermore, most studies were concerned with the early post-transplant period when $CD4^+$ T-cell immune reconstitution is evolving. A summary of the useful literature is included below.

1.1.6 Haematological recovery after stem cell transplantation

Following stem cell transplantation, haematological recovery occurs rapidly, with the reappearance of neutrophils and platelets approximately 2-3 weeks after stem cell infusion. The patient is then no longer dependent on antibiotic and blood product support and is usually fit to be discharged home. However, lymphocyte reconstitution is only commencing at this stage and many months must pass before maximal reconstitution has taken place. Information gathered from immunophenotyping and, to a lesser extent, functional studies indicate that B-cells, CD4 and CD8 T-cells exhibit individual kinetics of reconstitution. Although the successful response of post-transplant patients to vaccination is likely to require the functional reconstitution of the immune system as a whole, the literature regarding lymphocytic reconstitution is best examined by considering B-cells and T-cells separately.

1.1.6.1 B-cell reconstitution after autologous HSCT

The observation that stem cell transplant recipients were similar to normal infants in their susceptibility to infections with encapsulated bacteria suggested that parallels existed in the B cell function of both groups. Further evidence gained from immunophenotyping and functional studies indicate that B-cell recovery after transplantation recapitulates normal B-cell ontogeny [113-116]

1.1.6.1.1 Immunophenotypic studies

The kinetics of B-cell reconstitution has been extensively charted using fluorescent monoclonal antibodies to characteristic surface markers. The numbers of $CD19^+$ and $CD20^+$ B cells are low for the first 2 months after transplantation although they usually reach normal levels by 3-6 months. [113,115,117-119]. B-cell numbers then rise to supranormal levels before progressively falling back into the normal range [113,120,121]

CD10 is expressed at the early stages of B cell development. As early as one month after autologous or allogeneic transplantation many patients have a marked increase in bone marrow $CD10^+$ B cell precursors. These cells may have the morphological characteristics of lymphoblasts and their immunophenotype (CD19+ CD10+ surface Ig – ve) is often indistinguishable from CALLA pre B cell acute leukaemia. The presence of such cells can cause concern in patients in whom recurrent ALL is a consideration, leading to further investigations such as immunoglobulin gene rearrangement studies, to clarify that the cells are indeed polyclonal. Increased numbers of CD10 cells persist in the marrow for at least one year after transplantation [114]. Expression of CD10 is not found on reconstituting B cells in the peripheral blood [113,122]. However it has been observed that the reconstituting peripheral blood B-cells are characterised by the expression of increased levels of the surface markers CD5, CD23 and CD38 during the first 12 months after transplantation [113-115]. Cells displaying this phenotype usually comprise a small proportion of B-cell numbers in adults and older children but are commonly found in healthy young children and in cord blood illustrating another similarity between the early humoral immune system and that which develops after transplantation [115].

Expression of IgD and absence of CD27 on the cell surface can be used to distinguish naïve B cells from memory B cells [123,124]. Both naïve and memory cells are almost undetectable at two months after autologous or allogenic BMT. Naïve cell (IgD^+) numbers are in the normal range at one year post allograft and have risen to supranormal levels 12 months after autologous BMT. Memory B cell reconstitution is much slower, with numbers returning to normal 5 years after allogeneic transplantation [123].

A correlation between the numbers of reconstituting B-cell and CD4 T-cell numbers has been observed following transplantation [113]. Interactions between these cells may facilitate their reconstitution. It is interesting that a similar correlation was not detected between CD8 cells and B cells [113].

Expression of IgM on the cell surface signifies that the pre-B cell has become a naïve B cell. Naive B cells have unmutated Vgenes and their phenotype is $CD19^+$, IgM^+ and IgD^+. Approximately 80% of the B cells of normal healthy adults exhibit this phenotype compared with >95% of B cells in marrow transplant recipients [113,125].

1.1.6.1.2 Immunoglobulin gene rearrangement studies

Immunoglobulin gene rearrangement studies also indicate that post-transplant B-cell development retraces B-cell ontogeny [126,127]. The V_H repertoire after BMT exhibits a relative increase in the V_H2, V_H4, V_H5 and V_H6 gene families. This pattern is also seen in normal B-cell ontogeny. Although V_H3 and V_H4 gene usage of patients 3 months after BMT is similar to that of normal adults, fewer somatic mutations are detected in the rearranged V_H genes of BMT recipients suggesting that their ability to generate high affinity antibodies may be impaired [128].

1.1.6.1.3 B cell function after transplantation

The measurement of immunoglobulin levels in patients after transplantation gives some indication of B-cell function. Some experiments which assess the function of B-cells from post-transplant patients by measuring responses to various in-vitro stimuli have also been performed.

Immunoglobulin levels

A limited degree of information regarding B-cell function can be acquired by measuring serum immunoglobulin levels after transplantation. It has been demonstrated that by one year after allogeneic transplantation IgM, IgG1 and IgG3 levels are often normal whilst IgG2 and IgA levels remain suppressed [113]. It is interesting to note that infants achieve normal adult serum immunoglobulin levels in a similar sequence (IgM $\rightarrow$ IgG_1 and IgG_3 $\rightarrow$ IgG_2, IgG_4 and IgA)[113]. Post-transplant antibody levels have been shown to correlate with pre-transplant levels [129], raising concern about using antibody levels as an indicator of B-cell reconstitution.

Reconstituting plasma cells which are derived from transplanted stem cells represent only one possible source of antibody after transplantation. Bone marrow plasma cells which have survived the conditioning regimen represent another source [129] whilst mature B cells present in the transplant may also subsequently differentiate into plasma cells and contribute to post transplant immunoglobulin levels. This is illustrated by the transfer of humoral immunity from donor to recipient that has been demonstrated after allogeneic transplantation.[130-133].

When these factors are taken into consideration it is perhaps not surprising that discrepant data exists in the literature regarding post-transplant immunoglobulin levels. For example, Alpek and colleagues investigated the immune recovery of patients with AL-amyloidosis after autologous PBSCT. All patients had normal serum immunoglobulin

levels prior to transplant and although lower at 12 months post transplant, they remained within the normal range [134]. Reimer, on the other hand, found that immunoglobulin levels remained unchanged in the first year after transplantation [119] whilst the IgA and IgM levels of autologous PBSCT patients investigated by Nauchbaur were suppressed and had not recovered by 6 months after transplantation [135].

In-vitro B cell functional studies

A better insight into deficiencies in B cell function after autologous or allogeneic transplantation has been provided by in-vitro studies. The proliferative response to the B cell mitogen SAC was found to be low for the first 3 months post-transplantation before returning to normal [115,136]. The production of IgM in response to a combination of PWM and SAC returned to normal by 4-6 months post-transplant whereas IgG production remained below the normal range until 19-24 months [115]. Again, these results parallel ontogeny as IgM secretion in response to polyclonal B-cell activators is present at birth with the ability to produce IgG is gradually attained by 2 years of age [137 138].

The function of B-cells one year after transplantation has been investigated using CD40L and IL-10 stimulation. This demonstrated an intrinsic B cell defect manifested by a lack of isotype switching and subsequent IgG production [139].

A decreased ability to acquire somatic mutations in rearranged V genes has also been found in B-cells from patients who were one year after allogeneic transplant [125]. These cells were co-cultured with either autologous T cells or those from a healthy volunteer. Patient T cells could support somatic hypermutation in volunteer B cells, indicating that the functional deficit was intrinsic to the post-transplant B cells. Patients later in the post-transplant period have not been examined in this manner nor have similar studies been performed on patients after autologous transplantation.

By stimulating B cells separately with IL-2 and IL-10 Korholz and colleagues also showed that post-transplant patients have defective B cell function [140]. They observed an isotype switching defect in 4/24 patients after allogeneic transplant whilst defective terminal differentiation was seen in six patients. Included in their study were seven patients who were 0.9 – 6.1 years post-autologous transplantation. Defective terminal differentiation of B cells was demonstrated in three of this group. These individuals were 0.9, 1 and 1.5 years post transplant respectively whilst the sub-group with normal B-cell function were 1.3, 2.1, 4.5 and 6.1 years post transplantation. The small number of post-autograft patients in these studies makes it difficult to draw definitive conclusions regarding the timing of immune reconstitution.

1.1.6.2 T-cell reconstitution after autologous HSCT

Studies have shown that T cells can be regenerated by a thymic dependent and a thymic independent pathway following myeloablative therapy and HSCT. Although there is evidence in humans that specialised T-cells can develop in the liver and gut by thymic independent mechanisms and that this process may also occur in the murine bone marrow, there is no evidence that a broad range of normal TCR$\alpha\beta$ T cells can develop in extra-thymic sites in humans.

Initially after stem cell transplantation, T-cell numbers increase by a thymic independent pathway. This process involves the expansion of pre-existing memory T-cells. It is known as peripheral expansion as it occurs in the peripheral tissues rather than in the thymus. Peripheral expansion of mature T cells within the host or the graft was demonstrated to be the main route of T-cell regeneration in athymic hosts after T-cell depletion [141]. It seems to occur in response to diminished numbers of thymic progeny suggesting co-regulation of the two pathways. The peripheral pathway is also increased in euthymic BMT recipients when they are treated with cyclosporine A which inhibits the generation of T cells via the thymic pathway [142]. Peripheral expansion is increased in older animals after BMT and it has been suggested that this may be a consequence of an age related decrease in thymic function [143]. Although this provides a mechanism for athymic mice to increase their T cell numbers after BMT, total T cell numbers do not reach normal levels. This cannot be overcome by simply increasing the size of the initial T-cell innoculum [144]. A T-cell repertoire resulting solely from the peripheral expansion of pre-existing lymphocytes is not ideal as it is obviously limited by the TCR diversity present in the initial innoculum [145].

The thymic dependent pathway is essentially a recapitulation of ontogeny. This pathway was elucidated by experiments which compared the T-cell regeneration of euthymic and athymic mice which underwent myeloablative irradiation followed by T-cell depleted bone marrow transplantation [142]. Euthymic recipients regained normal T cell number and function 12 weeks after BMT. They were able to subsequently respond to neoantigenic challenges unlike the athymic mice [146]. Anti-idiotype vaccination strategies are more likely to be successful following transplantation if the thymic dependent pathway regains function after transplantation. This would then allow the generation of T-cell clones against "neo-antigens" such as idiotype.

1.1.6.2.1 Immunophenotypic analysis of the T-cell content of stem cell transplants

The kinetics of T-cell reconstitution following peripheral blood stem cell transplantation has been extensively studied using FACS analysis of cell surface markers. Lymphocyte subset frequencies in the first few days post transplant reflect the composition of the apharesis product. However, considerable variability of the T-cell content of the transplant itself appears to exist [136]. Steingrimsdottir found that the CD4/CD8 ratio in the graft was similar to that of normal peripheral blood [117]. This was also reported by Tamladge et al [147], whilst in the experiments performed by Ashihara and colleagues, the numbers CD4+ T cells were much greater than CD8+ T cells resulting in an elevated CD4:CD8 ratio [118].

1.1.6.2.2 Immunophenotypic analysis of $CD8^+$ T-cell reconstitution

It is a consistent finding that CD3+ T cell numbers return to normal values in the peripheral blood of patients at 2- 3 months after transplantation. This is driven by the rapid peripheral expansion of CD8+ T-cells. $CD8^+$ cell numbers reach normal levels by 1-2 months and then rise to supra-normal levels before returning to baseline again by approximately one year post-transplantation [117,148].

There has been considerable interest in using surface phenotypic markers expressed on the cell surface to compare the immune reconstitution of naive T cells with those which are antigen experienced. Although a definitive marker which can distinguish between these cells does not exist, a frequently used approach involved analysis of the expression of different isoforms of the leucocyte common antigen, CD45. Differential splicing of the exons A, B and C, which encode part of the N-terminal extracellular domain, results in the production of isoforms of different molecular weight. The high molecular weight isoform, referred to as CD45 RA, includes either exons A, B and C or exons A and B. The low molecular weight isoform, CD45 RO, lacks exons A, B and C. The different isoforms of CD45 regulate the responsiveness of T cells to stimulation. Cells which express CD45RO mount greater proliferative responses to suboptimal anti-CD3 stimulation than CD45 RA cells.

At the time when many of the immunophenotyping studies investigating immune reconstitution were performed it was thought that expression of CD45 RA identified naïve $CD4^+$ cells, whilst antigen experienced cells expressed CD45 RO on the cell surface. This concept had grown from the observation that most neonatal CD4 T cells expressed CD45 RA [149] [122] whilst a high proportion of adult CD4 cells expressed CD45 RO [150]. These markers were thought to identify two distinct populations of $CD4^+$ cells [150]. Other studies

also indicated that $CD4^+$ T-cells recently exported from the thymus expressed CD45RA [151,152].

As previously discussed, it is now clear that a distinction between naïve and memory cells can not be made using these markers alone as $CD45RO^+$ cells can regain the expression of CD45RA. In addition, a significant number of human peripheral T-cells express both isoforms [153]. These studies still provide some insight into the timing of immune reconstitution as it remains likely that cells which are truly antigen naïve are contained within the CD45RA subset [153]. The reconstitution of both subsets of CD8 cells occurs quickly, reaching normal levels by 100 days after transplantation [122].

It has been consistently observed that the reconstituting CD8 cells have an activated phenotype, expressing high levels of HLA-DR [117,119,154]. This peaks at 6 months and then gradually falls, typically reaching reference values by one year post-transplantation. The significance of this finding is unclear but it suggests that the CD8+ cells are experiencing antigenic stimulation. T-cells reacting against antigen encountered in the early post-transplant period may therefore be selectively expanded representing a mechanism for the skewed spectratype histograms which have been observed after allogeneic transplantation.

1.1.6.2.3 Immunophenotypic analysis of $CD4^+$ T-cell reconstitution

CD4 T-cell reconstitution occurs much more slowly than that of CD8 T-cells. Most reports in the literature have been concerned with the early stages of immune reconstitution and have therefore only analysed the lymphocyte phenotypes up to 1 year after stem cell transplantation [117-119,122,134,136,147].

It is consistently found that CD4 numbers have not returned to normal by this time. CD4+ CD45RA+ cell numbers are noted to be particularly low. Based on these studies it has been suggested that it might take several years for CD4+ T cell counts to normalise [155]. There is little actual data available regarding the $CD4^+$ T-cell reconstitution of patients who are several years post-transplantation. When Nordoy et al examined patients 4-10 years after transplantation with autologous bone marrow they found that CD4+ T cell counts were still only approximately 2/3rds that of normal controls. The percentage of CD4+ T-cells that were also CD45RA+ was particularly depressed at only half of the value observed in the normal control group [156].

Considerable controversy has existed regarding the degree to which thymic or non-thymic pathways contribute to CD4 T-cell reconstitution in adults after stem cell transplantation. The replacement of thymic tissue with fat during puberty results in a substantial decrease in thymic size. Thymic involution has been interpreted as an

indication that the thymus fulfilled a minor role in the maintenance of adult T-cell populations. This view was supported by observation that immunodeficiency did not occur in older children or adults who had undergone thymectomy [157].

Evidence suggesting limited thymic function in adults after stem cell transplantation came from studies which differentiated naïve T-cells from memory or effector T-cells by their cell surface molecule phenotype using flow cytometry. Mackall found the rate of CD4 T cell recovery of children under 15 years of age who have received cytotoxic anti-neoplastic therapy was inversely related to age [158]. Children exhibited a particularly brisk recovery of the "naïve" CD45RA subset and this was temporally associated with transient thymic enlargement, termed "thymic rebound". Neither feature was seen in older children and adults. They typically showed persistently low numbers of CD4 cells, the vast majority of which displayed the "memory" CD45RO phenotype. Although thymic enlargement was not seen in the adults, their CD45RA cell numbers gradually rise, suggesting that the thymic pathway may be involved CD4 T cell reconstitution but at a relatively low level.

The ability of these studies to differentiate between naïve and memory T cells was based on the assumption that the thymus was the major source of CD45RA T cells and therefore detectable numbers of cells with this phenotype could be used as an indicator of thymic function. This is in doubt as it is now clear that CD4 CD45RO cells may reacquire CD45RA, blurring the distinction between "naïve" and "memory cells" as determined by this approach [153]. The accuracy of conclusions drawn from these data is further undermined by the suggestion that recent thymic emigrants may also rapidly undergo peripheral expansion in the post-transplant setting. This is accompanied by the conversion of CD45RA to CD45RO. Consequently, phenotypic analysis of CD45RA cell numbers may underestimate thymic activity [159].

1.1.6.2.4 TRECs measurement and spectratyping to assess T cell reconstitution

Evidence that the adult thymus may contribute substantially to immune reconstitution after SCT can be found in recent studies which, in addition to phenotypic analysis of T-cells, employ new techniques such as spectratyping and TRECs measurement.

In patients with myeloma, TREC levels fell to undetectable numbers in the first weeks following myeloabaltive chemotherapy and autologous transplantation [160]. However, by day 100 after transplantation, TREC levels were detectable again in most patients. Numbers of TREC then continued to increase until 1 year post-transplant, after which they either remained constant or decreased slightly.

In addition to TREC measurement, the T-cell diversity of these myeloma patients was also analysed using a direct methodology called "spectratyping". This technique is based on the observation that variable lengths of CDR3 segments are created in different T–cells during the recombination process and by the addition of N-nucleotides [161]. The TCR CDR3 regions are amplified by PCR using primers which anneal to the variable and constant regions. The result is a spectrum of PCR products of different lengths [162]. A spectratype is produced when the PCR product lengths are plotted against fluorescence intensity. Thus far, the technique has predominately been applied to analysis of the β chain as a result of its prevalence and because it contains a smaller number of variable region gene families than the TCRα locus. The spectratype of T-cells from normal cord blood shows a Gaussian distribution [163]. With age, antigenic stimulation causes changes in the heights of the spectratype peaks and a normal "polyclonal" profile can be identified. Although the CD4 TCRBV repertoires of the myeloma patients studied by Douek et al remained skewed at 1 and 2 years post transplant, patients who had increases in TREC number also had substantially higher numbers of TCRBV families detected in their repertoire [160].

1.1.6.2.5 T cell function after autologous transplantation

Studies employing techniques such as the immunophenotyping of lymphocytes in the peripheral blood of patients after stem cell transplantation have transformed our understanding of the kinetics of immune reconstitution. However, these data do not inform us about the functional capacity of the transplanted immune system. Studies which assess T-lymphocyte function following transplantation are very limited in number. Of those which do exist, few have been recently published.

Lymphocyte proliferation

The ability of lymphocytes to undergo extensive proliferation after an encounter with a specific antigen is an essential feature of the adaptive immune response. This enables low frequency lymphocytes to generate sufficient numbers of effector cells to combat a pathogen expressing a specific antigen. Proliferation in response to specific antigen can be assessed *in-vitro* by incubating lymphocytes with an antigen and subsequently measuring the incorporation of ^{3}H-Thymidine into DNA. Incubation with polyclonal mitogens induces mitosis in many lymphocytes of different specificities. An indication of lymphocyte function can be gained by measuring responses to polyclonal mitogens. The information gained from this approach can be focused by using a polyclonal mitogen to which only a certain subset of lymphocytes respond. For example Phytohaemagglutinin is

derived from the red kidney bean and induces responses in T lymphocytes. Pokeweed mitogen on the other hand induces both T and B lymphocytes to respond.

Using this method, T cell proliferation has been found to be impaired in the early post-transplant period. At 100 days after autologous PBSCT, the proliferative responses of peripheral blood leucocytes to PHA have been shown to be significantly lower than those of normal individuals but higher than those of patients at the same stage following an autologous BMT [136,147,164,165].

Cayeux et al also demonstrated that PHA and IL-2 induced T cell proliferative responses of 25 patients in the first 8 weeks after autologous BMT were much lower than normal [166]. Similar results were achieved when anti-CD3 and IL-2 were used to stimulate the cells instead of PHA and IL-2. The responses of each of these patients had increased when tested later in the post-transplant period but unfortunately the time points chosen to retest the patients ranged from 55 to 912 days after transplantation, preventing a meaningful interpretation of the kinetics of functional immune reconstitution.

Cytokine synthesis

Activated T cells produce interleukin-2. IL-2 causes T-cell proliferation and differentiation. The measurement of IL-2 production by T cells after incubation with PHA and PMA gave Cayeux and colleagues another indication of their ability to respond to non-specific stimuli [166]. They showed that five patients who were less than 6 weeks post autologous BMT were unable to produce IL-2. Cells from the same individuals regained this capacity when tested later in the post-transplant period, when their CD4:CD8 ratio had increased somewhat, but remained subnormal. The data from this study do not allow us to deduce the time at which normal IL-2 production is regained point at which these patients were assessed ranged from 62 to 802 days after transplantation. Production of other cytokines other than IL-2 has also been found to be abnormal after transplantation. For example, deficient production by lymphocytes of IFN-γ, GM-CSF, IL-3 and IL-4 was noted in the first 6 moths after autologous peripheral blood or marrow transplantation [167].

1.1.7 Conclusion

For therapeutic DNA vaccination to be successful, the patient's immune system must have recovered sufficiently from the stem cell transplant procedure to mount a functional response to vaccination. As previously discussed, the protection provided by the scFv-FrC

DNA vaccine developed in our laboratory against myeloma in the 5T33 model was dependent upon $CD4^+$ T cells. Information regarding the cellular immune function of patients after transplantation is limited, as indicated above. Many of the above studies which have examined immune reconstitution contained small numbers of patients, included both allogeneic and autologous transplant patients who had received various conditioning regimes in their analysis and did not assess cellular immune function. In addition, patients with a variety of haematological diseases were recruited into the studies, some of whom suffered from very aggressive conditions and relapsed soon after transplantation. As previously mentioned, most studies were also concerned with the early post-transplant period when $CD4^+$ T-cell immune reconstitution is evolving. A thorough examination of the literature does not allow us to accurately predict when the functional immune reconstitution of myeloma patients occurs following high dose chemotherapy and autologous peripheral blood stem cell transplantation. This knowledge is critical for the success of therapeutic vaccination strategies aimed at inducing an immune response against residual disease present after transplantation. We therefore chose to assess the functional immune capacity of these patients to respond to a T-cell dependent vaccine, tetanus toxoid.

1.2 Assesment of the immune capacity of myeloma patients following autologous stem cell transplantation

1.2.1 Study Aim

The aim of this study was to assess the capacity of myeloma patients, who are several months after high dose chemotherapy and stem cell transplantation, to mount an effective immune response to vaccination with tetanus toxoid. In this context tetanus toxoid is a protein recall antigen, as all patients had previously received standard primary and booster tetanus toxoid vaccinations. The degree to which patients at various time points after transplantation have functionally reconstituted their tetanus specific memory immune response can then be determined by measuring the responses to TT in humoral and cellular in-vitro assays after vaccination.

1.2.2 Materials and methods

1.2.2.1 Vaccination schedule

After obtaining informed consent, patients were vaccinated intramuscularly (Deltoid) with 0.5ml TT (Pasteur Merieux). Blood samples were collected from patients prior to vaccination and one month post-vaccination. Anti-TT antibody levels were measured by ELISA on serum samples. The cellular responses of patient PBMCs to TT and FrC were assessed by measuring lymphocyte ^{3}H-Thymidine incorporation in a lymphoproliferation assay and by the production of interferon-γ and interleukin-13 (IL-13) Elispot assays.

1.2.2.2 Anti-TT ELISA

Anti-TT IgG antibodies were measured using TT and WHO human antitoxin standards (NIBSC Herts, UK). Briefly, ELISA plates were coated with 0.5 LFU/ml TT in coating buffer, 200 μl per well and incubated at 4^{0}C overnight. They were washed with PBS/Tween and blocked with blocking buffer for 1 hour at 37°C. The TT anti-sera standard (IgG) at 10 iu/ml was diluted in PBS starting at 1/100 dilution. Patient samples

and an internal control serum were also diluted in PBS. Plates were washed with PBS/Tween and then doubling dilutions of standard and test samples added, 200 μl per well. Plates were then incubated for 1.5 hours at 37°C. They were then washed x4 in PBS/Tween before the addition of anti-Fc γ-HRP (Sigma) at a 1/2000 dilution and incubated for a further hour at 37°C. After 4 washes, 200 μl of fresh substrate buffer was added to each well. The colour was allowed to develop and the reaction was then stopped by the addition of 80μl per well of 2.5M H_2SO_4. The optical density was read on an automatic ELISA reader.

1.2.2.3 Preparation of peripheral blood mononuclear cells (PBMCs)

PBMC were isolated from heparinised blood by centrifugation over Lymphoprep. They were frozen at 0.5-1x10^7/ml in 50% decomplemented human AB serum (Sigma-Aldrich), 40% RPMI (Invitrogen) and 10% DMSO (Sigma-Aldrich), and stored in liquid-nitrogen. Prior to cellular assays, samples were thawed at 37°C and washed in RPMI medium (Invitrogen).

1.2.2.4 Lymphoproliferative assay

Using 96-well micro-titre plates, 2 x 10^5 viable lymphocytes in 200μl of RPMI-1640 + 10% AB-serum (C-RPMI) were cultured for 5 days in triplicate with either 10μg/ml TT (Not absorbed, code 02/232, NIBSC, UK), 20μg/ml FrC (in house) or medium alone (negative control).

On day 2, 5μg/ml Phytohaemagglutinin (PHA-P from *Phaseolus Vulgaris*, Sigma-Aldrich, UK) was added to positive control wells. On day 5, wells were pulsed with 37Kbq ^{3}H-Thymidine (Amersham Biosciences) and left overnight. On the following day, proliferation was measured by ^{3}H-Thymidine incorporation using a "Top Count" microplate scintillation counter (Packard Biosciences, Berkshire UK). The stimulation index (SI) was calculated as the mean counts per minute (cpm) of TT-stimulated cells minus that of unstimulated cells, divided by the mean cpm of untreated cells. An SI of 3 or higher was considered a positive result.

1.2.2.5 Interferon-γ Elispot assay

96-well ELISPOT plates were pre-coated overnight at 4°C with 100 μl per well of murine anti-human IFN-γ IgG1 antibody (mAb 1-DIK, Mabtech, Nacka, Sweden) at 10μg/ml. Platers were washed 3 times with sterile PBS and then 2 x 10^5 PBMC in 200μl of C-RPMI

/10% decomplemented pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either 10μg/ml TT (NIBSC, UK), medium alone (negative control) or 5μg/ml PHA (Sigma-Aldrich). After incubation for 48 hours at 37°C in 5% CO_2, the cells were discarded and the wells were washed 6 times with 150μl of filtered PBS/0.1% Tween-20 (Sigma-Aldrich). Subsequently, a biotinylated murine anti-human IFN-γ IgG1 antibody which recognises a different epitope of human IFN-γ than mAB 1-D1K (7-B6-1, Mabtech) at 1μg/ml in filtered PBS/1%BSA (Sigma-Aldrich) was added (100μl per well) and the plates were incubated for 90 minutes at 37°C in 5% CO_2. The wells were then washed another 6 times with filtered PBS/ Tween. Strepavidin conjugated alkaline phosphatase (Mabtech) diluted 1:1000 in filtered PBS/1%BSA was then added to each well (100μL) and the plates were again incubated for 1 hour at 37°C in 5% CO_2. After 6 further washes with filtered PBS/Tween, IFNγ spots were detected using an alkaline phosphatase-conjugate substrate kit (Zymed Laboratories Inc. San Francisco CA). Once spot development had finished the reaction was stopped by washing the plates with tap water. Once the plates had dried, a computer assisted video image analyser (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used to count the spots. Background spots were subtracted. Results are reported as the number of spot forming units / 1 x 10^6 lymphocytes. Thirty spots per 10^6 lymphocytes (5 spots per well) was regarded as a positive result.

1.2.2.6 IL-13 Elispot assay

96-well ELISPOT plates were pre-coated with overnight at 4°C with 100μl per well of murine anti-human IL-13 IgG1 antibody (h-IL-13 I, Mabtech, Nacka, Sweden) at 10 μg/ml. Plates were then washed 3 times with sterile PBS and then 2 x 10^5 PBMC in 200μl of C-RPMI /10% decomplemented pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either 10μg/ml TT (NIBSC, UK), medium alone (negative control) or 5μg/ml PHA (Sigma-Aldrich). After incubation for 48 hours at 37°C in 5% CO_2, the cells were discarded and the wells were washed 6 times with 150μl of filtered PBS/0.1% Tween-20 (Sigma-Aldrich). A biotinylated murine anti-human IL-13 IgG1 antibody which recognises a different epitope of human IL-13 than h-IL-13 I antibody (h-IL-13 II, Mabtech) at 1μg/ml (Mabtech) in filtered PBS/1%BSA (Sigma-Aldrich) was added (100μl per well) and the plates were incubated for 90 minutes at 37°C in 5% CO_2. The wells were then washed another 6 times with filtered PBS/ Tween. Strepavidin conjugated alkaline phosphatase (Mabtech) diluted 1:1000 in filtered PBS/1%BSA was then added to each well (100μL) and the plates were again incubated for 1 hour at 37°C in

5% CO_2. After 6 further washes with filtered PBS/Tween, IL-13 spots were detected using an alkaline phosphatase-conjugate substrate kit (Zymed Laboratories Inc. San Francisco CA). Once spot development had finished the reaction was stopped by washing the plates with tap water. After the plates had dried, a computer assisted video image analyser (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used to count the spots. Background spots were subtracted. Results are reported as the number of spot forming units / 1 x 10^6 lymphocytes. Thirty spots per 10^6 lymphocytes (5 spots per well) was regarded as a positive result.

1.2.2.7 Patient details

1.2.2.7.1 Myeloma patients

Myeloma patients (n=25) who had previously undergone an unmanipulated autologous peripheral blood stem cell transplantation procedure were studied. In all cases, the conditioning regimen had been Melphalan 200mg/m^2 and high dose methyl prednisolone. The transplants had contained at least 2x10^6/kg $CD34^+$ cells or 1x10^8/kg mononuclear cells. No patient had received any other anti-myeloma therapy following transplantation. The median age of the cohort was 60 years (range 41-69 years). Only one patient had received a tetanus vaccine since the transplant procedure. At least 7 years had passed since the other patients had been vaccinated against tetanus. Patient clinical features are presented in Table 3.

1.2.2.7.2 MGUS patients

Monoclonal gammopathy of undertermined significance (MGUS) is defined by the presence of serum monoclonal protein at a concentration of 30 g per litre or less; no monoclonal protein or only moderate amounts of monoclonal light chains in the urine; the absence of lytic bone lesions, anemia, hypercalcemia, and renal insufficiency related to the monoclonal protein. [168] Patients with MGUS do not exhibit the paresis of serum immunoglobulin levels which is often observed in multiple myeloma.

All of the MGUS patients (n=11) in this study fulfilled these diagnostic criteriae. The median age of the MGUS cohort was 69 years (range 57-77 years). MGUS patient clinical features are presented in Table 4.

Patient	Age (years)	Sex	Type	Best response to PBSCT	Last TT vaccine (years)	At time of Vaccination		
						Months post PBSCT	Lymphocyte ($x10^9$/l)	Paraprotein (g/dl)/ BJP (mg/24 hrs)*
1	65	F	IgGκ	CR	>10	6	2	0
2	60	M	IgGκ	CR	>10	7	2.2	0
3	59	M	IgGκ	CR	>10	7	1.6	0
4	64	M	IgGκ	PR	>10	8	1.6	4.1
5	54	F	IgGλ	PR	>10	10	3.6	3
6	54	F	κ BJP	CR	>10	10	1.5	0
7	63	M	IgGκ	CR	>10	11	1.2	0
8	65	M	IgGλ	CR	>10	14	1.2	0
9	41	M	IgAκ	CR	>10	15	2	0
10	54	F	IgAκ	PR	>10	17	2.9	2.1
11	56	F	NS	PR	>10	18	2.5	N/A
12	55	F	IgAλ	PR	>10	19	1.9	6.5
13	64	M	IgGκ	PR	>10	20	1.1	0
14	52	M	IgGλ	PR	1	20	4.3	5.5
15	55	M	IgGκ	PR	>10	23	1.8	1.3
16	63	M	IgGκ	PR	>10	27	0.9	25.3
17	66	F	λ BJP	PR	>7	31	2.2	354 BJP
18	67	M	IgGκ	CR	>7	31	1.6	0
19	69	M	IgGκ	CR	>10	35	2.2	0
20	63	F	IgGλ	PR	>10	35	1.8	0
21	61	F	λ BJP	PR	>10	56	1.4	0
22	63	M	IgGκ	PR	>10	57	1.5	0
23	57	F	IgGλ	PR	>10	64	3.6	24.9
24	58	F	IgGκ	NC	>10	64	1.9	N/A
25	53	M	IgGκ	PR	>10	81	3.2	0

Table 3. Clinical and laboratory characteristics of post-ASCT myeloma patients

Patient	Age	Sex	Subtype	Last Tetanus vaccination	At the time of vaccination	
					Lymphocytes ($x10^9/l$)	Paraprotein (g/l)
1	57	F	IgGκ	>10 years	1.7	5.3
2	57	F	IgAλ	>10 years	2.9	6.6
3	60	F	IgGκ	3 years	2.9	13.8
4	65	M	IgGκ	>10 years	3.1	3.1
5	66	M	IgGλ	>10 years	4.2	5.3
6	69	F	IgGκ	12 years	2.2	6.2
7	71	F	IgGλ	>10 years	3.1	7.4
8	72	F	IgGλ	>10 years	4.3	17.7
9	73	F	IgAκ	5 years	1.2	11.2
10	76	F	IgGλ	>10 years	1.7	3.4
11	77	M	IgGκ	>10 years	1.9	9.7

Table 4 Clinical and laboratory characteristics of MGUS patients

1.2.2.7.3 Healthy volunteers.

The control group of healthy volunteers (n=12) were either partners of myeloma patients (n=3) or colleagues from our laboratory (n=9). The median age of the cohort was 38 (range 26-61).

1.2.3 Results

1.2.3.1 Antibody responses

Protection against infection with *Closridium tetani* is provided by anti-tetanus antibodies. Prior to vaccination, 8/25 myeloma patients had TT-antibody levels below the WHO minimum protective value of 0.01 IU/ml (Figure 7) *(Galazka A. The immunological basis for immunisation. Module 3: Tetanus. In: Expanded Programme on Immunisation. World Health Organisation: Geneva,; 1993).* The pre-vaccination anti-TT levels of the myeloma cohort were significantly lower than those of the MGUS patients and normal volunteer group ($p=0.009$ and $p=0.016$ respectively) (Figure 8). As expected, a significant difference could not be detected between the anti-TT levels of the MGUS and normal volunteer groups either pre- or post-vaccination.

The anti-TT level of all three groups rose significantly after vaccination (Figure 9). Of the eight myeloma patients with baseline anti-TT levels below 0.01IU/ml, seven had antibody levels above the protective threshold by one month after TT vaccination (Figure 7). Although each myeloma patient raised their anti-TT level after vaccination, the post–vaccination antibody levels of the myeloma patients were significantly lower than those of the MGUS patients or normal volunteers ($p=0.0002$ and $p=0.003$ respectively) (Figure 8).

The median post-vaccination antibody level of the myeloma group was 26 times higher than the median pre-vaccination value. This fold increase was higher than the corresponding values observed for the MGUS and normal volunteer groups who exhibited a 15 and 10 fold rise in median antibody levels respectively.

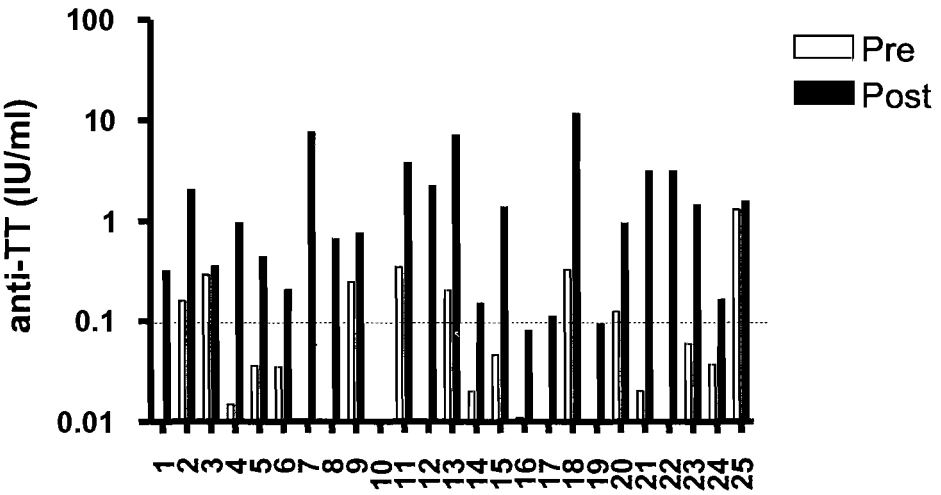


Figure 7. Anti-TT antibody levels of post-autograft myeloma patients pre (□) and post (■) and post tetanus toxoid vaccination. The World Health Organisation (WHO) minimum protective anti-TT level of 0.01IU/ml is shown by a broken line.

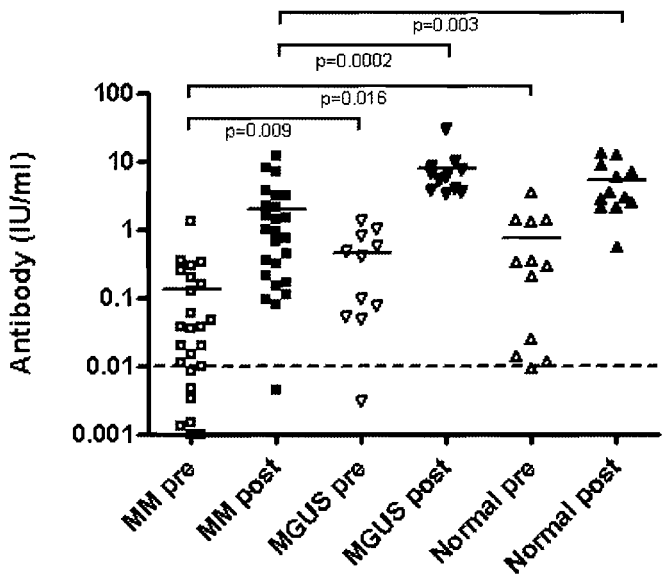


Figure 8. Anti-TT antibody levels pre (□) and post (■) TT vaccination of myeloma, MGUS and normal volunteers. The World Health Organisation (WHO) minimum protective anti-TT level of 0.01IU/ml is shown by a broken line

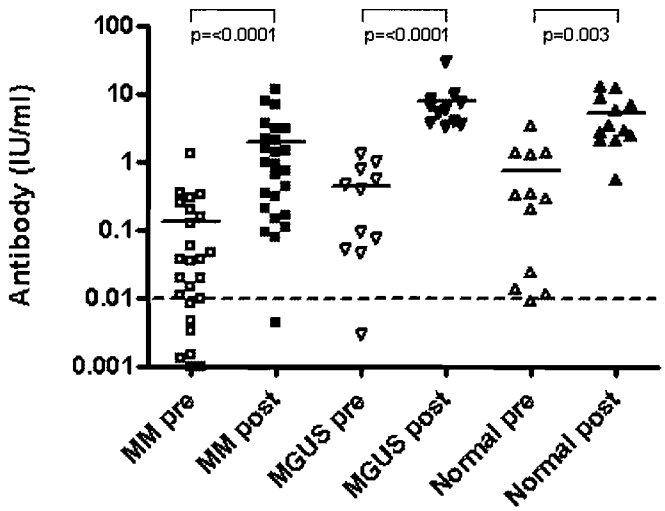


Figure 9. Anti-TT antibody levels pre (□) and post (■) TT vaccination of myeloma, MGUS and normal volunteers. The World Health Organisation minimum protective Anti-TT antibody level is indicated by a broken line.

1.2.3.2 Lymphoproliferation results

As tetanus toxoid is a recall antigen, it was anticipated that low level TT specific lymphoproliferation would be detected in samples taken from normal subjects prior to vaccination. Indeed, the TT specific memory response (stimulation index>3) was revealed in the pre-vaccination samples of all 11 MGUS patients and 8/12 normal volunteers (Figure 10). However, TT-specific lympho-proliferation was only detected in 8/25 myeloma patients before vaccination. The initial SI of the myeloma cohort was significantly lower than that of MGUS and normal groups ($p=0.002$ and $p=0.014$ respectively) (Figure 11). Following the high dose chemotherapy and ASCT procedure which the myeloma patients had undergone, it was expected that TT specific T-cells would be present at a lower frequency in the peripheral blood of these pateints than in that of the control subjects. Although the post-autograft myeloma patients exhibited a lower level of immunity to TT prior to vaccination it is clearly much more important for the potential success of our FrC-idiotype fusion vaccine strategy that these patients are able to mount an effective response to vaccination.

In that respect it was encouraging that the LPA was positive (SI>3) for 20 myeloma patients after vaccination (Figure 10A). All of the myeloma patients with a positive pre-vaccination lymphoproliferative assay (LPA) exhibited a higher SI when tested one month after vaccination (Figure 10A). In total, All MGUS patients and normal volunteers had a positive post-vaccination LPA (Figure 10B&10C). Despite the fact that the initial LPA of the myeloma group was significantly lower than that of the MGUS and normal volunteer cohorts, the post vaccination LPA results of the three groups were similar. This may indicate that a "ceiling" of maximal response exists in the lymphoproliferative assay and that the myeloma group reached this level after vaccination, as did the MGUS and healthy volunteer groups (Figure 11).

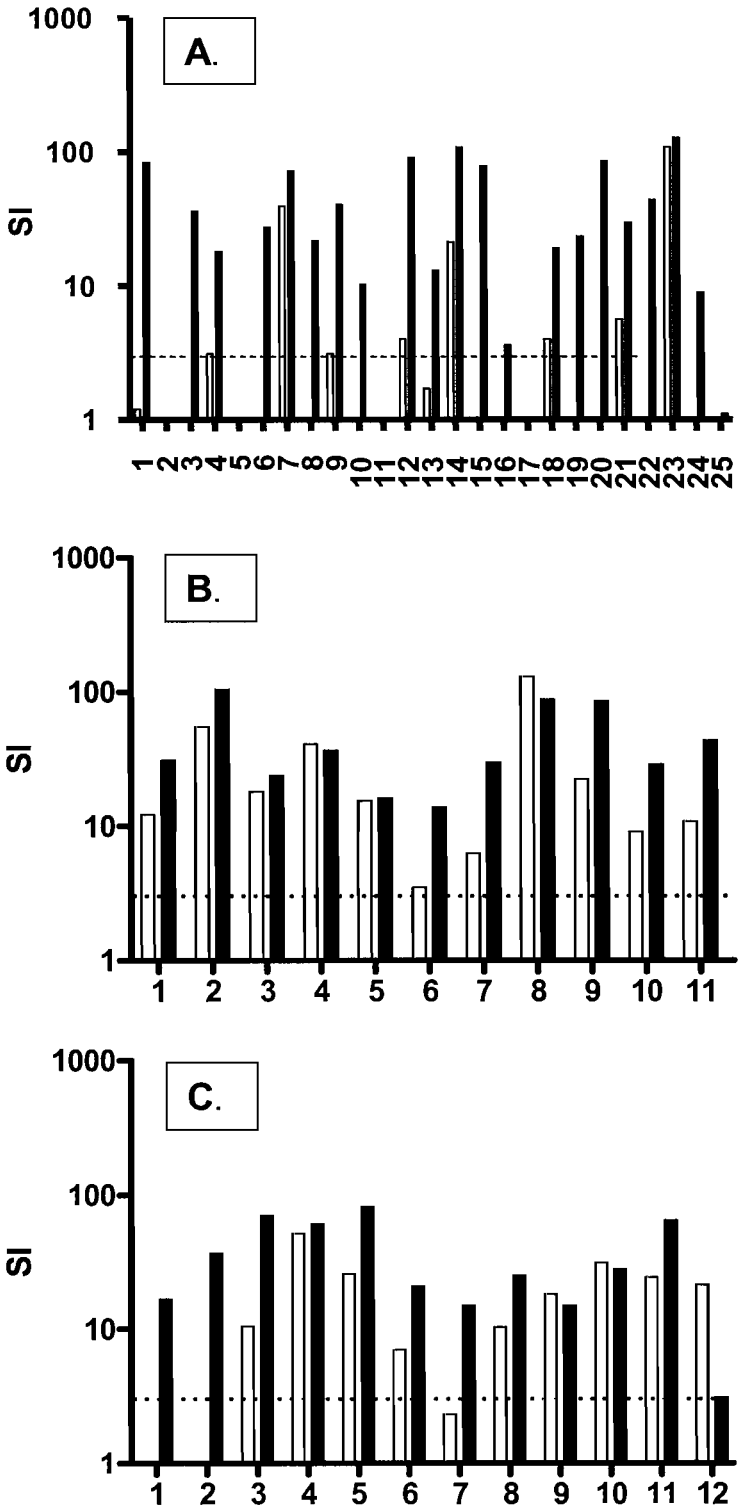


Figure 10 . Lymphoproliferative responses pre □ and post ■ TT vaccination. A) Myeloma patients, B) MGUS patients C) Normal volunteers. SI= stimulation index. SI results >3 are considered positive. This level is indicated by a broken line.

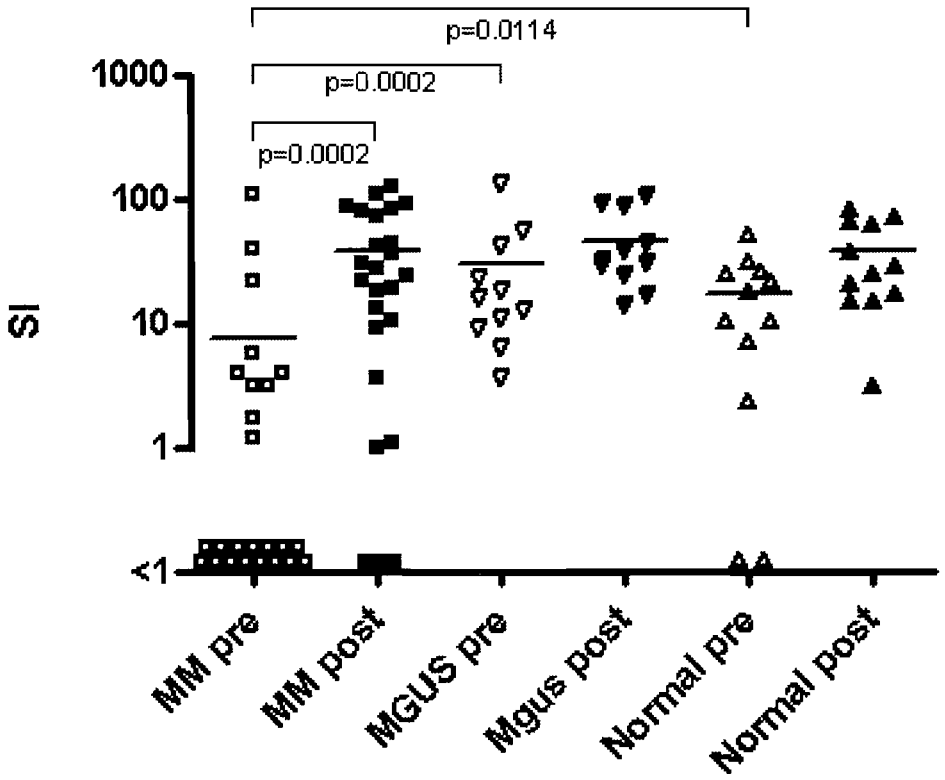


Figure 11. Lymphoproliferative responses of myeloma (MM), MGUS and normal volunteers, pre (□) and post (■) TT vaccination. SI= stimulation index.

1.2.3.3 Interferon-γ Elispot results

We were able to further dissect the cellular response to TT vaccination by using Elispot assays which detected Interferon γ, a Th-1 cytokine, or IL-13, characteristically produced by Th-2 lymphocytes. IFN-γ production in response to incubation with TT (>30 spots/10^6 cells) was elicited in 3/25 myeloma patients, 6/11 MGUS patients and 8/12 normal volunteers prior to vaccination (Figure 12). The IFN-γ elispot assay was significantly lower in pre-vaccination samples from the myeloma patients than in the MGUS patients and healthy volunteers (p=0.046 and p=0.02 respectively) (Figure 13A).

Following vaccination, IFN-γ production by the myeloma patients was higher than the pre-vaccination level (p=0.002) (Figure 13A). In total, 15 myeloma patients had a positive IFN-γ Elispot assay after vaccination. The post-vaccination assay was positive for all MGUS patients and healthy volunteers (Figure 12B&12C). Although the post-vaccination IFN-γ production of the myeloma cohort remained below that of the normal volunteers (p=0.03) (Figure 13B), there was no significant difference between the MGUS and myeloma groups (Figure 13B).

1.2.3.4 IL-13 Elispot results

We were able to perform an IL-13 Elispot in all of the MGUS and normal volunteer groups and in 22/25 myeloma patients. The pre-vaccination IL-13 Elispot was positive (>30 spots/10^6 cells) for one MGUS patient and four normal volunteers (Figure 14B&14C). Whilst no myeloma patient had a positive IL-13 Elispot prior to vaccination, the assay was positive in 14 patients after vaccination (Figure 14A).

The increased IL-13 production by the myeloma cohort in response to vaccination was highly significant (p=0.0001) (Figure 15). The post-vaccination IL-13 results of the myeloma patients did not differ significantly from those of the MGUS or normal volunteer groups (Figure 15).

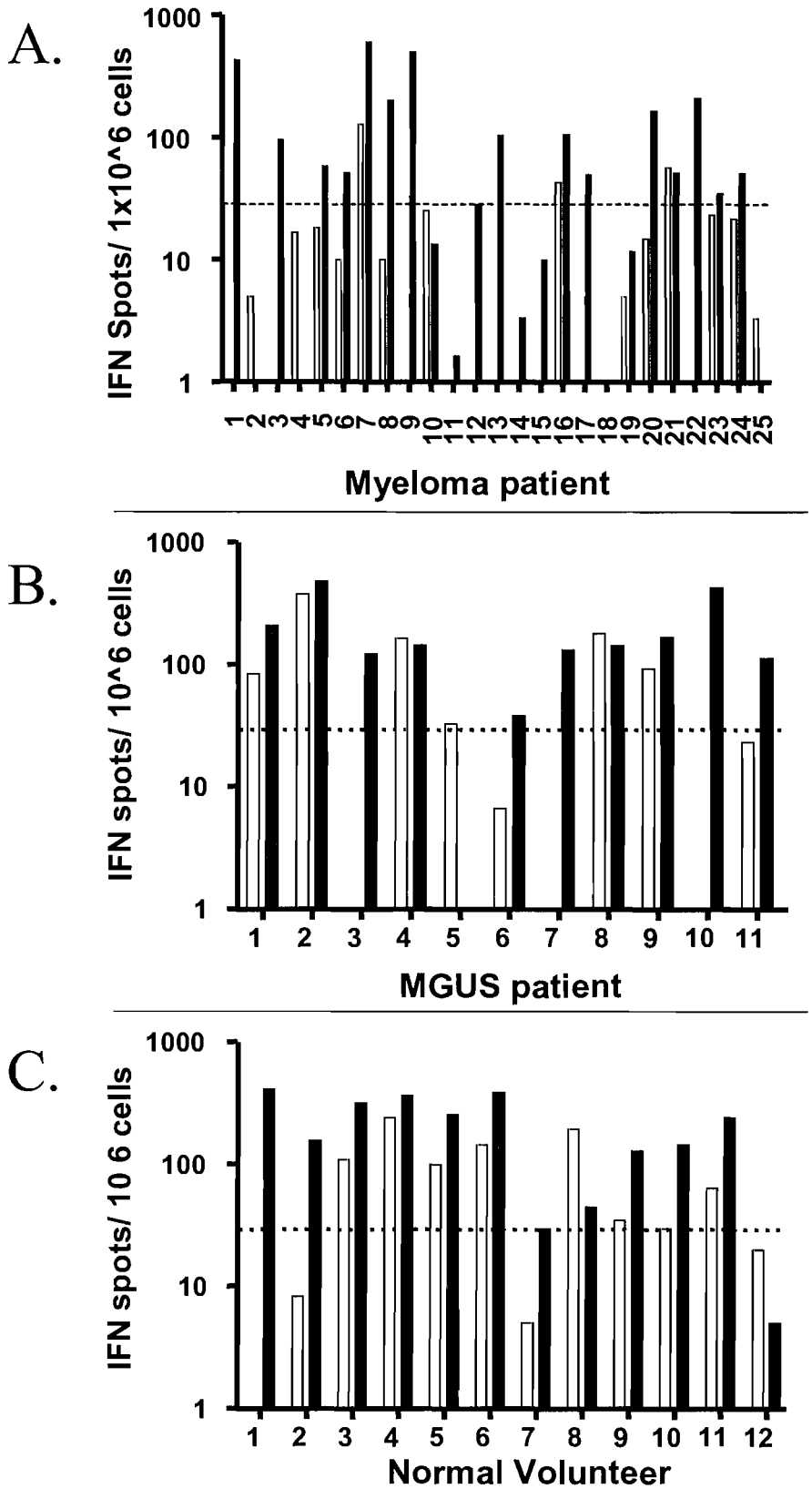
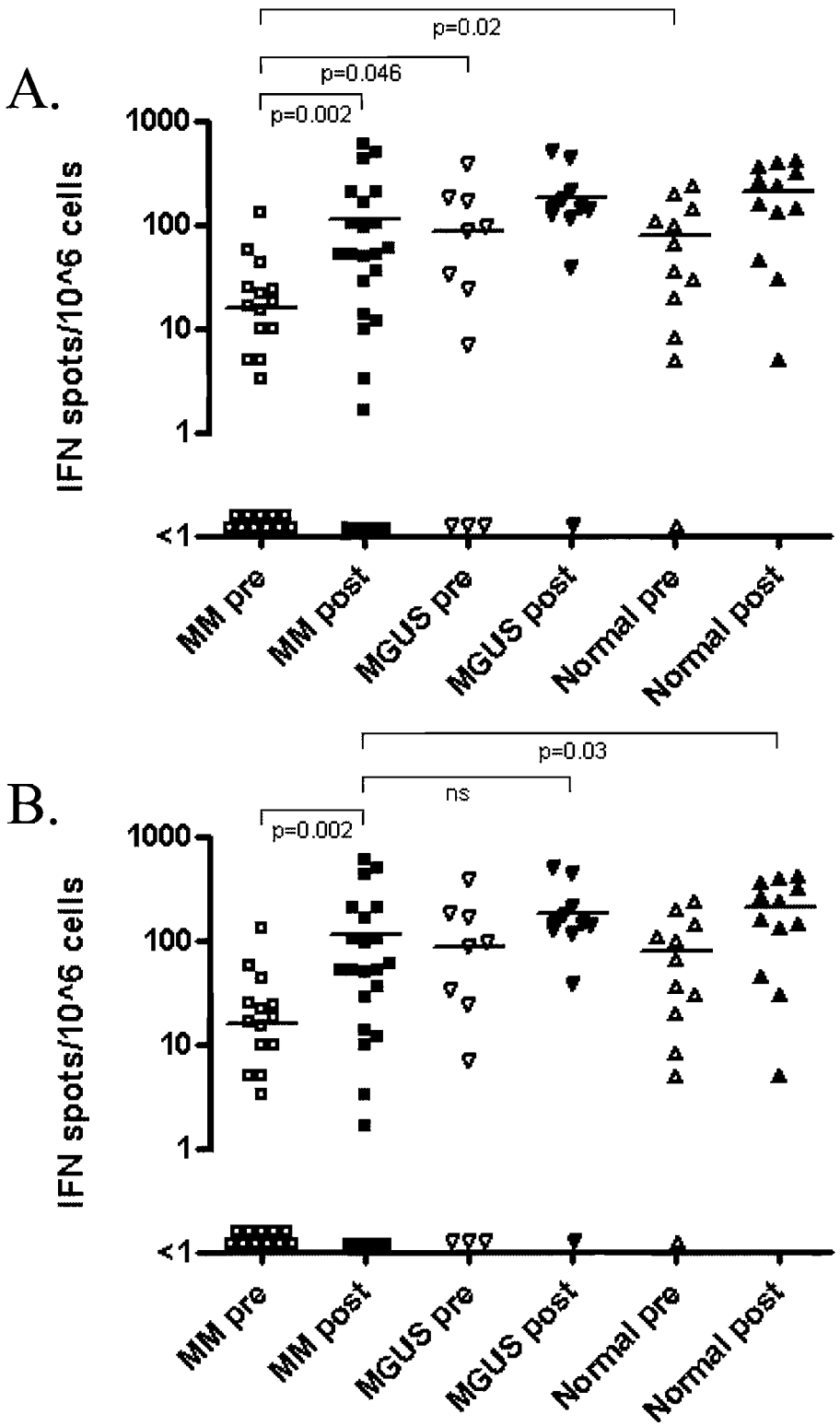


Figure 12. Interferon-γ Elispot responses pre (□) and post (■) TT vaccination. A) Myeloma patients. B) MGUS patients C) Normal volunteers.



Figures 13 A and B. Interferon-γ Elispot responses of myeloma (MM), MGUS patients and normal volunteers, pre (□) and post (■) TT vaccination. 13A and B differ only in the groups compared for statistical analyses.

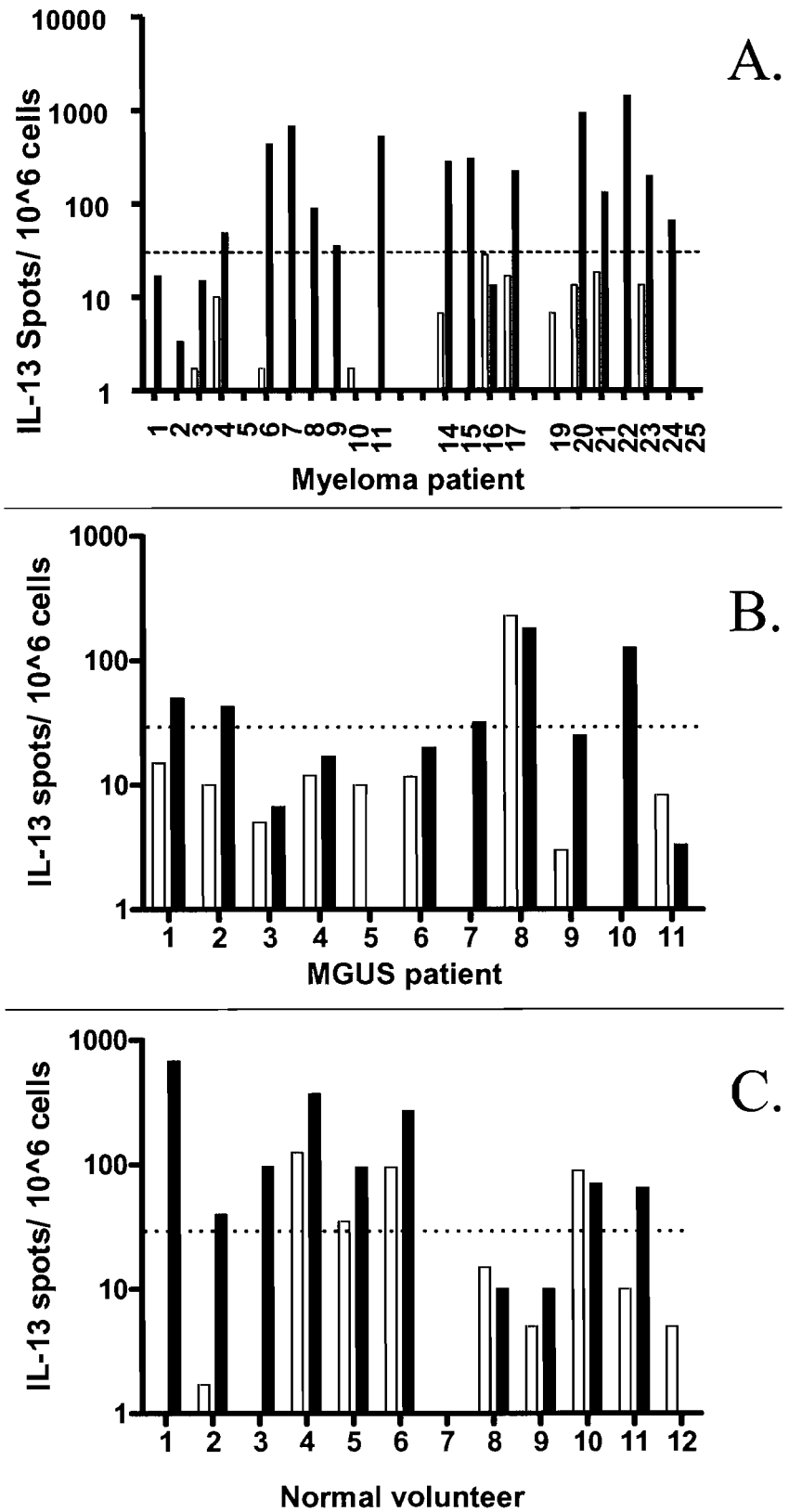


Figure 14. IL-13 Elispot responses pre (□) and post (■) TT vaccination. A) Myeloma patients, B) MGUS patients and C) Normal volunteers.

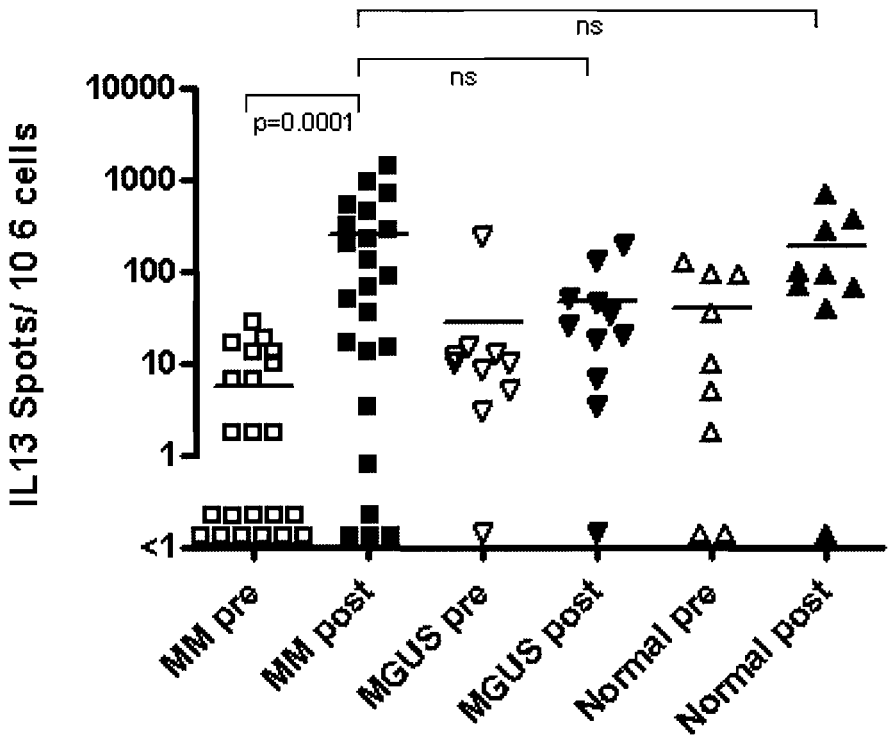


Figure 15. IL-13 Elispot responses of myeloma (MM), MGUS patients and normal volunteers, pre (□) and post (■) TT vaccination.

1.2.3.5 Myeloma patient subset analysis

All of the myeloma patients included in this study had previously been treated with high dose chemotherapy followed by an autologous stem cell transplant procedure. However, they varied greatly in the time which had elapsed since their transplant (range 6-81 months). The individual ELISA, LPA, IFN-γ Elispot and IL-13 Elispot results of each patient are depicted in Figure 19. As in previous figures, the myeloma patients are arranged chronologically; patient one was six months post-transplant whereas 81 months had passed since patient 25 had received an ASCT. As a result, we had the opportunity to compare the responses to vaccination of patients who were early in their post-transplant period (<20 months, n=13, range 6-19 months, median=10.5 months) with those for whom a longer time had passed (n=12, range 20-81 months, median=35 months).

Both groups significantly raised their anti-TT antibody levels after vaccination. Surprisingly, there was no significant difference between antibody levels of the two subgroups either before or after vaccination (Figure 16). An identical pattern was also seen when the cellular assays examined in this manner. The post-vaccination LPA, IFN-γ and IL-13 results within each group were significantly higher than the corresponding pre-vaccination values. Again, there was no significant difference between the two groups when corresponding results were compared (Figure 17).

Recent reports in the literature have suggested that myeloma patients who are less than 12 months post-autograft exhibit inferior immune function than patients who are greater than 12 months post-transplant. We had seven patients in our cohort who were less than 12 months post-transplant (myeloma patients 1-7). Again no significant differences were detected when the results of these patients were compared with the subset of patients for whom more than 12 months had elapsed since transplant (n=18) (Figure 18).

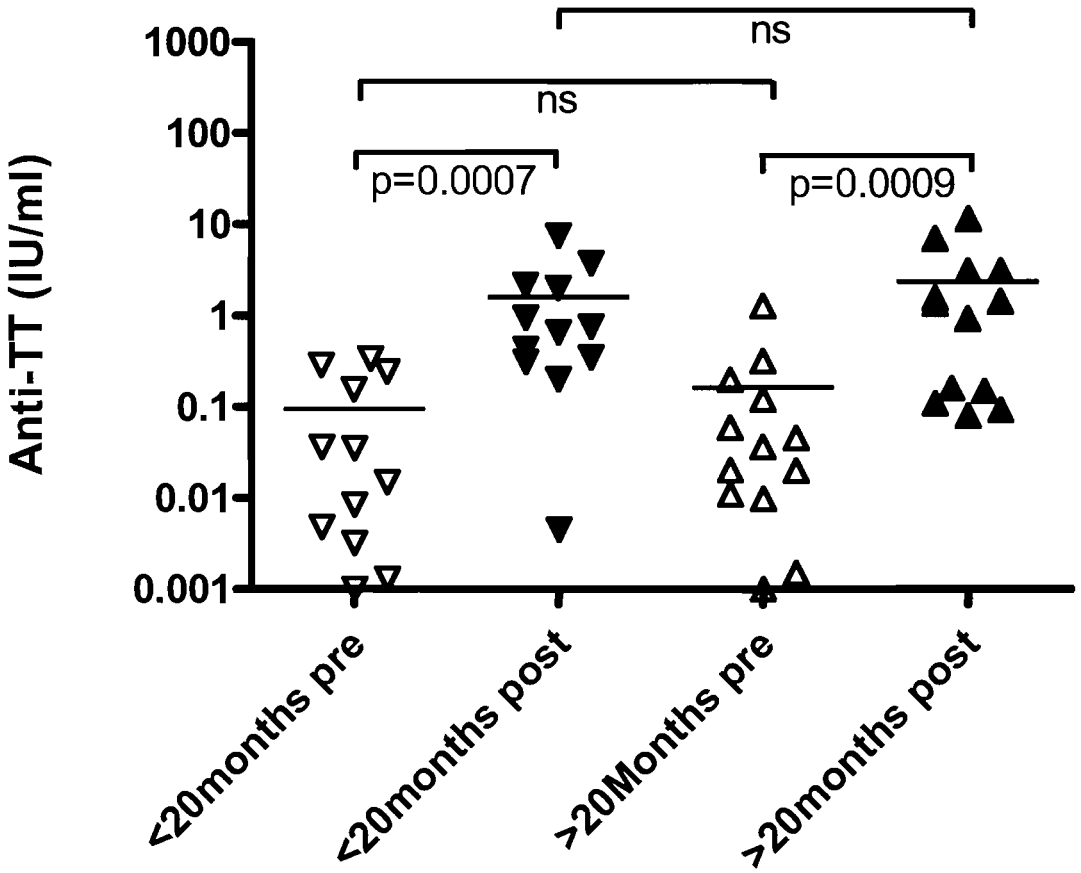


Figure 16. Anti-TT antibody results of myeloma patients pre (□) and post (■) TT vaccination. These patients were either <20 months or >20 months post-ASCT.

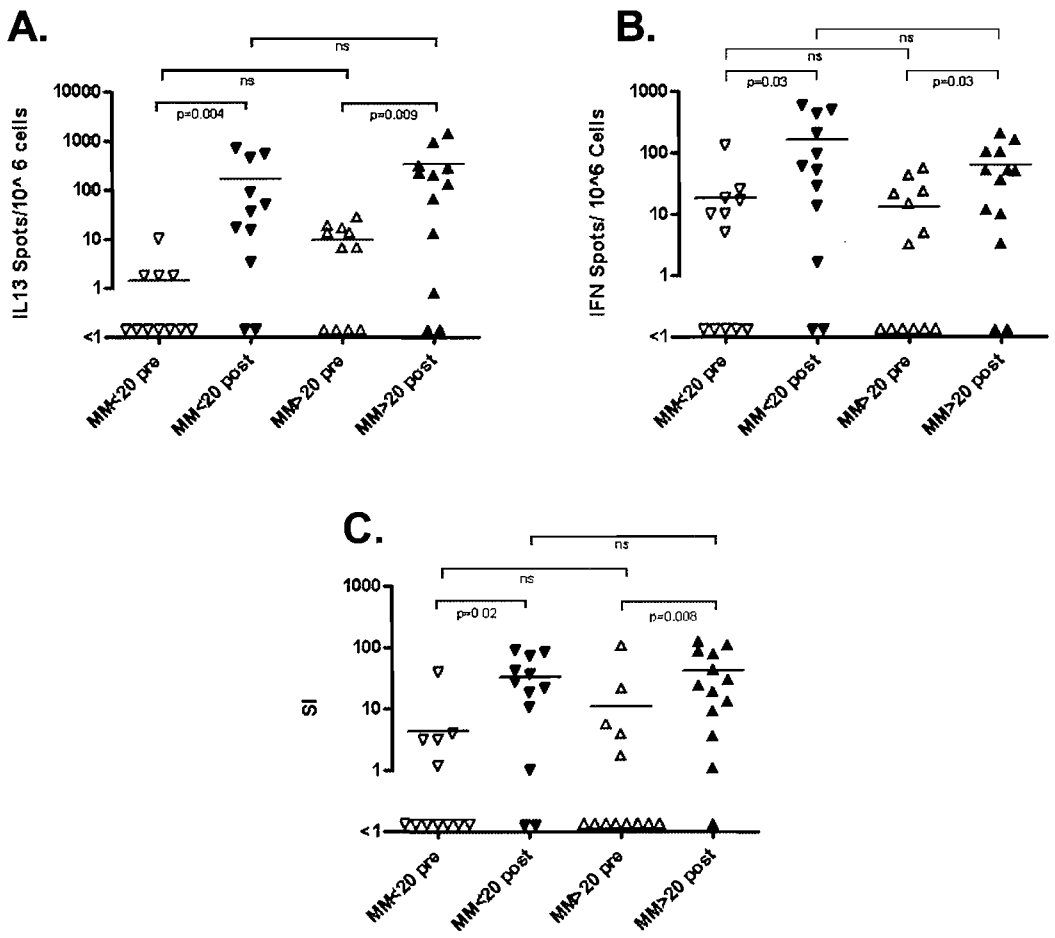


Figure 17. Cellular responses to vaccination of myeloma patients pre (□) and post (■) TT vaccination. A) IL-13 Elispot, B) IFN-γ Elispot and C) Lymphoproliferation assay. These patients were either <20 months (MM<20) or >20 months (MM>20) after ASCT.

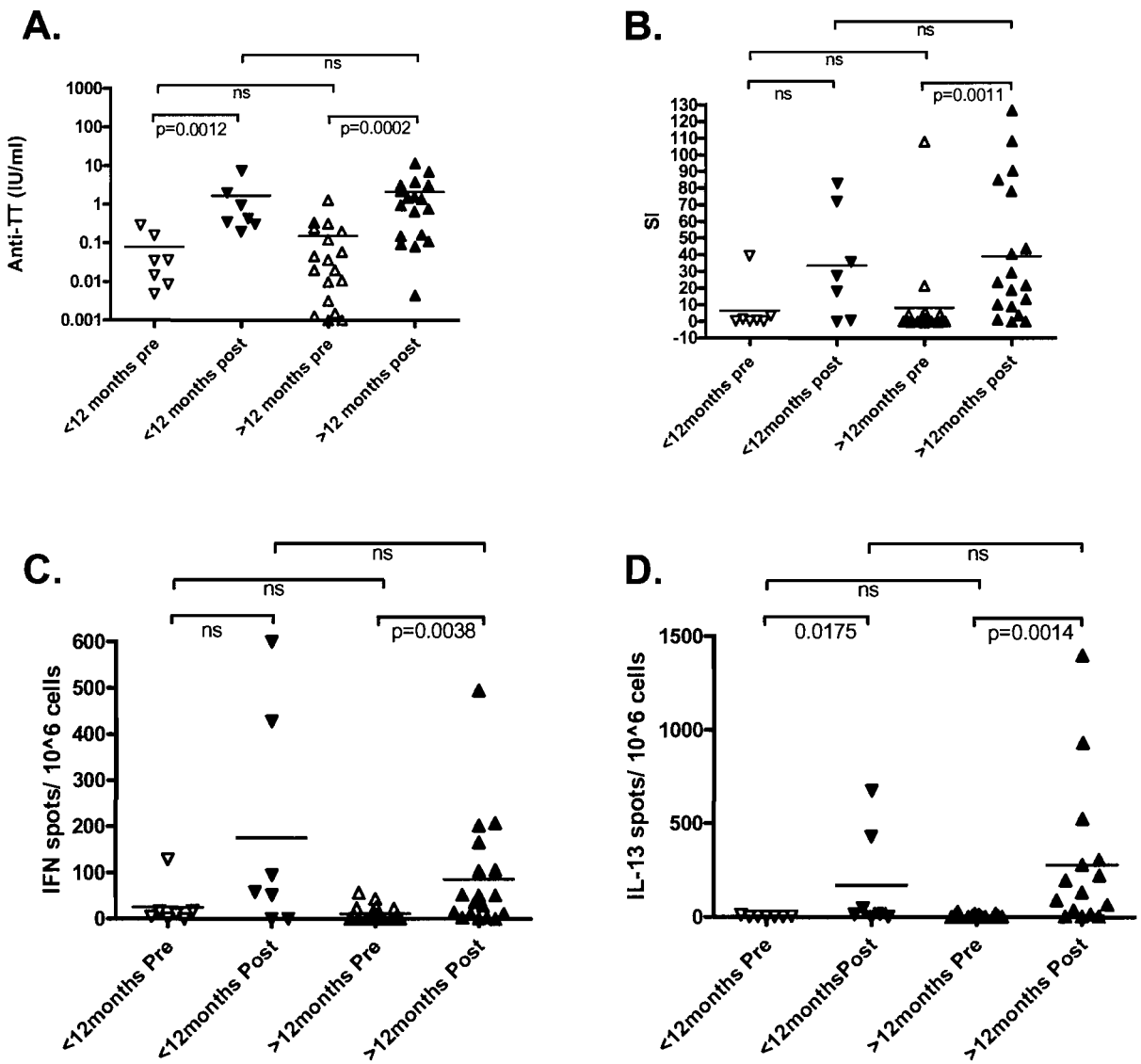


Figure 18. A) ELISA, B) Lymphoproliferation, C) IFNγ Elispot and D) IL-13 Elispot results of myeloma patients pre (□) and post (■) TT vaccination. These patients were either <12 months or > 12 months post-autograft.

1.2.4 Discussion

An allogeneic stem transplant is the only currently available therapy for multiple myeloma which has the potential to cure the disease. This procedure is associated with a high rate of transplant related mortality and a significant rate of myeloma relapse after transplantation. It is considered to be a realistic treatment option for the very small minority of myeloma patients who are less than 40-50 years of age and who also have a matched sibling donor. Myeloma therefore remains incurable for the vast majority of patients who suffer from the disease.

High dose chemotherapy followed by an autologous stem cell transplant is associated with a much lower transplant related mortality rate than an allogeneic SCT and may be considered for patients up to 70 years of age. The procedure can significantly reduce the volume of malignant plasma cells and has been shown to induce a complete remission in 24-75% of patients [3]. It has also been demonstrated to prolong progression-free and overall survival [169]. It is considered to be the treatment of choice for patients younger than 60 years of age [3]. Unfortunately, tumour cells contaminate the stem cell graft [170,171] and cause an inevitable relapse of myeloma.

In the interval between stem cell transplantation and eventual disease relapse there exists a "window of opportunity" during which a vaccination strategy against tumour associated antigens could be effective. The idiotype (Id) of the myeloma clone is an attractive tumour specific target for vaccine mediated immunotherapy.

There are many factors which must be considered in order to optimise the potential of an idiotype vaccination strategy in the post-transplant period. There are vaccine specific issues, such as vaccine design, construction and delivery, and factors which concern the ability of the immune system of the patient to mount a robust response to the vaccine. Following a stem cell transplant, adequate reconstitution of the patient's immune system must have occurred if an anti-idiotype vaccine is to stand any chance of inducing the desired immune response. The timing of vaccination after transplant is therefore of cardinal importance.

It is clearly desirable to vaccinate as early as possible in the post-transplant period. Disease burden is at its lowest at this point and the likelihood of myeloma relapse increases as each month passes. Once relapse has occurred, the disease often progresses at a considerable pace. This would present an even greater challenge to an anti-tumour vaccination strategy and would decrease the probability of a successful outcome. In addition, high levels of tumour Ig may be associated with a state in which immunological tolerance exits toward the tumour [172]. Another potential advantage of vaccination against a

tumour antigen early in the post transplant period is that the T-cell repertoire may be skewed towards antigens encountered during reconstitution [173].

Despite these concerns, it is clear that vaccination must be delayed until sufficient post-transplant immune reconstitution has occurred. With regard to our anti-myeloma vaccine strategy, we are particularly concerned that sufficient time has elapsed for $CD4^+$ T-cell reconstitution to take place. Although the clone of malignant cells in myeloma may contain circulating precursor B-cells, the most significant malignant cell in terms of number and clinical effect is the malignant plasma cell. Myeloma plasma cells may secrete Ig but do not express it on their cell surface and will not therefore be susceptible to attack by anti-idiotype antibodies. However, idiotype specific $CD4^+$ cells induced by our FrC-idiotype fusion DNA vaccine have been shown to suppress myeloma in a preclinical model. It is therefore essential for our strategy that myeloma patients are able to mount $CD4^+$ T-cell responses to vaccination in the post-transplant period. After injection of our DNA fusion vaccine, FrC specific $CD4^+$ T-cells are thought to provide linked T-cell help to anti-Idiotype T-cells. Consequently, it is crucial for our approach that post-autograft myeloma patients have recovered the ability to generate responses against FrC of Tetanus Toxoid at the time of anti-tumour vaccination. By vaccinating a cohort of myeloma patients with TT we were able to test the immune responsiveness of these patients to a relevant antigen at various time-points during their post-transplant recovery.

As TT is a T-cell dependent antigen, the antibody responses of our myeloma patients are a measure of functional T-cell reconstitution *in vivo*. The pre and post vaccination anti-TT levels of the myeloma group were lower than the corresponding figures of the MGUS and normal volunteer groups. This was unsurprising and indicates that a degree of immune dysfunction is still present in the post-transplant myeloma patients. For anti-tumour vaccine therapy to be effective in this clinical setting, it may not be necessary for vaccinated patients to mount the same degree of immune response which normal individuals may be capable of. In that respect the antibody responses of our myeloma patients are more encouraging. The post-vaccination anti-TT level of the myeloma group was much higher than the pre-vaccination figure ($p<0.0001$). All myeloma patients responded to a single TT vaccination by raising their anti-TT levels and only one patient had a post-vaccination anti-TT level below the antibody level defined by the WHO that guarantees protection against tetanus. Furthermore, the post TT antibody levels of the myeloma group were higher than the pre-vaccination levels of the MGUS and normal volunteer groups.

For reasons outlined above we were especially interested in the cellular responses to vaccination of the post-autograft patients. Pre-vaccination samples from the myeloma

patients yielded significantly lower levels of lymphoproliferation after incubation with TT than the equivalent samples from MGUS or normal volunteers. Specifically, a positive result was detected in only 8/25 myeloma patients prior to vaccination. We are more concerned with the ability of the patients to mount a robust cellular response to vaccination rather than pre-vaccination baseline levels per se. For that purpose the post-vaccination lymphoproliferation levels and, in particular, the increase in lymphoproliferation after vaccination relative to the pre-vaccination level is more meaningful. The degree of TT-induced lymphoproliferation exhibited by the myeloma patients was significantly higher after vaccination than prior to vaccination. In each of the myeloma patients with a positive pre-vaccination LPA we detected higher levels of TT-induced lymphoproliferation after vaccination. In total, the LPA was positive for 20 myeloma patients following vaccination. Most impressive was the observation that the post-vaccination LPA of the myeloma group was similar to that of the MGUS group and normal volunteers.

We were able to further investigate the vaccination induced cellular responses using Elispot assays which detected Interferon-γ or IL-13. Similar to the lymphoproliferation results, significantly lower levels of Interferon-γ were produced before vaccination by the myeloma patients compared to the MGUS patients or normal volunteers. Again, the myeloma patients responded to vaccination by producing higher levels of Interferon-γ. Although no difference was detected between the myeloma patients and the MGUS patients after vaccination, a degree of immune dysfunction is indicated by the observation that the normal volunteer group produced significantly more Interferon-γ after vaccination than the myeloma cohort.

Examination of the IL-13 results also showed that the post-autograft myeloma patients could respond to TT vaccination by increasing the number of TT-specific $CD4^+$ cells. As was the case with the lymphoproliferation assay, no difference could be detected between the myeloma, MGUS and normal groups after vaccination.

The antibody, lymphoproliferation, Interferon-γ and IL-13 level results indicated that our post-autograft myeloma patients could respond to TT vaccination by generating immune responses similar to those necessary if FrC-idiotype vaccination is to be successful. Some measurements unsurprisingly reveal that these patients continue to experience a degree of immune dysfunction when compared to MGUS patients or normal volunteers, but this was not the case for all of the assays employed.

Results from patient to patient were heterogeneous (Figure 19). Only myeloma patient 10 did not respond to vaccination by achieving a protective anti-TT antibody level. In addition, no cellular response was detected by LPA, IFN-γ or IL-13 elispot in this

individual's post-vaccination assays (Figure 19). This patient could be truly deemed to have mounted no response to vaccination.

All other patients exhibited a rise in anti-TT antibody level after vaccination. Although this usually occurred alongside a detectable rise in the cellular assays, it was not always the case. Some of the individual results were intriguing. In addition to myeloma patient 10, a response was not detected by any of our cellular assays in the post-vaccination samples from two other myeloma patients (patients 2 and 25) (Figure 19). One of these, myeloma patient 25, had the highest anti-TT level of the group prior to vaccination. Of all the post-autograft myeloma patients investigated, patient 25 also exhibited the smallest rise in antibody level (20%) after vaccination (Figure 19). It is perhaps unsurprising that a robust cellular response was not therefore detected. Pateint 2 however exhibited a >10 fold rise in his anti-TT level after vaccination (Figure 19). The reasons behind the negative cellular assays in the case of patient 2 are unclear. It is most likely that he did indeed generate a T-cell response which accompanied and facilitated the large rise in anti-TT antibodies. It is possible that vaccination induced an initial rise in the number of TT-specific cells in his peripheral blood but that the frequency of these cells had returned to baseline levels by the time of sampling 1 month after vaccination.

Almost every combination of result was observed in the cellular assays carried out on the myeloma pateints we studied. This can be clearly seen by looking at the IFN-γ and IL-13 elispot results of the 17 myeloma patients in whom the post vaccination LPA was positive. In nine of this group, the IFN-γ and IL-13 post-vaccination elispot assays were also positive. Another 4 pateints had a positive IL-13 Elispot and a negative IFN-γ elispot. Meanwhile, three of the patients had a negative IL-13 Elispot and a positive IFN-γ elispot. In one patient (patient 19) both the IL-13 and the IFN-γ elispots were negative whilst the LPA was positive (Figure 19). The explanation for these heterogenous individual results is unclear. It has been reported that TT vaccination can induce a range of TH-1 and TH-2 immune responses and it is therefore possible that some patient did experience a response biased the direction of one group of T-helper cells. It is also possible that different kinetics of response are exhibited by the cells detected in the LPA, IFN-γ and IL-13 Elispot assays.

The vast majority of our myeloma patients significantly raised their anti-TT antibody levels after vaccination and in most cases this was accompanied by a demonstable rise in the cellular assays. Our experiments indicate that myeloma patients can respond to TT vaccination from as early as 6 months after ASCT.

Lindemann and colleagues recently investigated the cellular *in-vitro* immune function of myeloma patients by measuring lymphoproliferative responses to mitogens and several recall antigens including TT[174]. After extensive statistical analysis they showed that

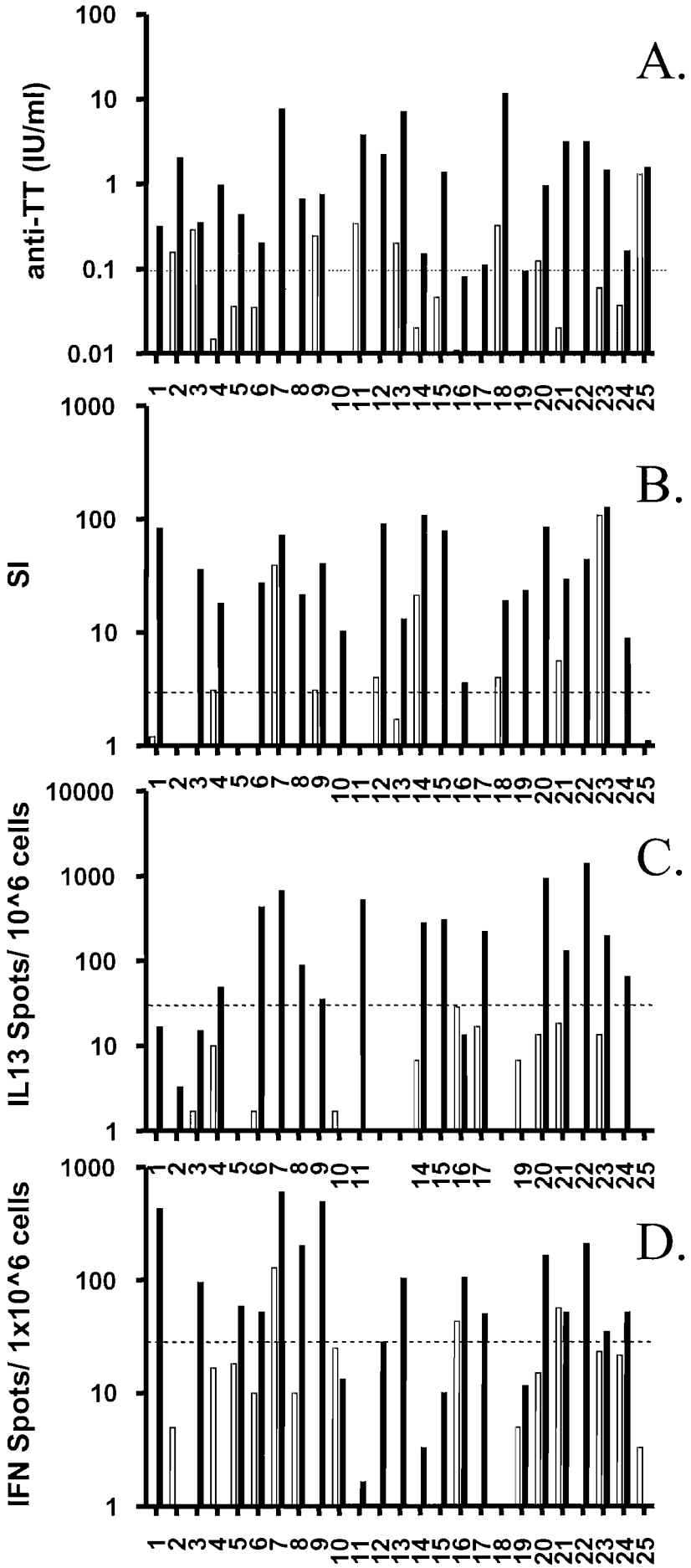


Figure 19. A) ELISA, B) LPA, C) IL-13 D) IFN-γ results of post autograft myeloma patients, pre (□) and post (■) TT vaccination.

myeloma patients who were less than 12 months post-transplant had impaired T-cell function compared with normal volunteers, myeloma patients receiving conventional chemotherapy or myeloma patients who were more than 12 months post-transplant. The authors noted that fewer anti-idiotypic responses were generated in a vaccination trial involving post-autograft myeloma patients than in another trial in which only two of a 12 patient cohort had received a transplant[174]. As the vaccinations in the post-autograft trial were commenced when the patients were only 4 months post-transplant, Lindemann speculated, perhaps correctly, that the inferior outcome in this trial was due to impaired lymphocyte function resulting from the autograft procedure. In addition to their own in-vitro findings, these observations prompted the authors to advise that a prolonged interval should be observed after ASCT to allow immune recovery before idiotypic vaccination is administered. Lindemann used lymphoproliferation assays to study the immune function of patients, giving us only a narrow view of immune competence of the patients[174]. Elispot assays would have yielded further interesting date. More importantly, the authors base their recommendations regarding the timing of idiotype vaccination after autologous transplantation on data which did not examine the ability of such patients to respond to vaccination[174].

Our results show that although baseline pre-vaccination lymphoproliferation levels of post-autograft myeloma patients are lower than those of MGUS patients or normal volunteers, the post-vaccination levels of all three groups are similar, indicating that post-autograft patients can mount a significant functional response to vaccination despite the observed pre-vaccination dysfunction.

The myeloma patients included in our study were between 6 and 81 months post-transplant. This allowed us to examine if differences in immune responsivness existed between those who were early and those who were later in their post-transplant recovery. We divided the cohort into two groups, those between 6 and 20 months post transplant and those for whom a longer interval had passed. We found no differences between the corresponding pre- or post-transplant antibody, LPA Interferon-γ or IL-13 levels of the two groups.

Lindemann and colleagues also reported that a significant difference in cellular in-vitro immune function existed between patients who were less than 12 months (median 3 months) and those greater than 12 months post-transplant (median 28 months)[174]. All of our myeloma patients were at least 6 months post transplant. Seven of this group were in their first year after transplantation at the time of vaccination. We compared the results of these patients with those of the myeloma patients who were greater than one year post-

transplant and again found no significant difference between the two groups. We note that the patients studied by Lindemann were only a median of 3 months post transplant whereas the patients in our <12 months group were a median of 8 months post transplant[174]. This may represent a considerable difference in terms of immune reconstitution after transplantation and could possibly account for the improved performance exhibited by our patients compared to what would have been predicted using Lindemann's data. It must also be noted that the number of patients in our study who were within one year of transplant was very small (n=7). However, some of the highest antibody, lymphoproliferative, Interferon-γ and IL-13 results which we observed after vaccination were from patients in this group.

Nine of our myeloma patients had disease detectable by serum or urine electrophoresis (albeit at low levels) at the time of vaccination. We found no differences in the vaccination responses of these patients compared with those without disease detectable by electrophoresis.

We used several different assays with which gave us a broad view of the various networks involved in the adaptive immune response to TT vaccination. It remains likely that some aspects of the immune response to TT were entirely undetected by our assays. For example, De Rosa et al have recently used 12 colour flow cytometery to show that a large number of $CD4^+$ T cells induced after TT vaccination generate IL-2 without co-existing Interferon-γ production[175].

In this study we have used TT vaccination to gauge the degree to which memory B and T-cell responses have reconstituted in the months following ASCT. It remains possible that some of the responses detected in our assays could be derived from TT specific naïve cells which have been newly produced during the reconstitution process. By vaccinating post-transplant patients with a strong neo-antigen, such as keyhole limpit haemocyanin (KLH), it would b possible to gain a clear impression of the degree to which the immune system has regained the ability to mount an immune response to antigens which it has not previously encountered.

In conclusion, we demonstrated that the post-autograft myeloma patients studied exhibited a significant ability to respond to TT vaccination. The ability of post-transplant myeloma patients to mount T-helper responses to the recall antigen FrC of tetanus toxoid is crucial to our idiotype vaccination strategy. The information gained in this study shall guide the design of future studies which will aim to assess the ability of FrC-idiotype fusion vaccines to induce immune responses in myeloma patients following autologous stem cell transplantation. Specifically, we plan to vaccinate myeloma patients from 6 months post-transplant.

2 DNA vaccination to induce a CTL response against human cytomegalovirus

2.1 Introduction

As a result of their intracellular location, many potential tumour antigens will only be expressed on the cell surface as peptides in the groove of the MHC class I molecules. Cell expressing such peptides/MHC class I molecules are therefore be susceptible to attack by $CD8^+$ cytotoxic lymphocytes (CTLs) specific for the expressed epitope. This also applies to persistent virus infections such as HIV or HCMV. Vaccine strategies which were successful against viral infections like varicella and polio worked primarily through the induction of antibodies. The realisation that CTLs induced by vaccination could potentially eradicate or control malignant disease or viral infections like HIV has led to intense interest in the rational development of such vaccines.

Although CTLs may not prevent the first wave of HIV infection or development of tumour, they may theoretically prevent resultant disease by eliminating infected or malignant cells. Another similarity exhibited by both HIV and malignant cells is that they can respond to selective pressure by undergoing frequent mutations. This provides another potential advantage for CTL vaccines as CTLs are often specific for epitopes generated from functionally constrained internal proteins rather than less essential surface proteins against which antibodies are directed. This may limit the escape of tumour/virus from immunological control.

The immunological principles which govern the development of a CTL response are the same regardless of whether the epitope against which the CTL reacts is derived from a virus or a tumour and therefore the knowledge gained in the development of CTL vaccines in one setting is also likely to be applicable to the other.

It has previously been demonstrated that DNA vaccines could induce efficient CTL responses [176]. Our group therefore endeavoured to design and produce DNA vaccines which could induce CTL responses against chosen tumour antigens. The scFv-FrC DNA vaccine had previously induced impressive antibody and $CD4^+$ T-cell responses against idiotype which were able to protect against lethal tumour challenge in the A31 and 5T33 models. The use of FrC as a promotional sequence for vaccines designed to induce CTL responses remained attractive as $CD4^+$ T cells are crucial in the establishment and

maintenance of CTL responses. Whilst developing FrC-tumour antigen fusion vaccines to induce CTL responses, our group not only found that several MHC class I-binding peptides were present in FrC, but also that the presence of such peptides could suppress CTL responses against weaker peptides from the fused tumour antigen [177].

It had been known for some time that the CTL response to a viral infection was narrowly focused upon only a few of the numerous potential epitopes present within viral proteins. This phenomenon is termed 'immunodominance' and occurs not only following microbial infection, but also after vaccination. Poor CTL responses against the linked tumour antigen encoded in our FrC fusion vaccine could therefore be explained by competition from immunodominant MHC class I epitopes within the FrC sequence.

2.2 Mechanisms of immunodominance

Many factors are likely to contribute to the immunodominance of a particular epitope. Although these include properties intrinsic to cytotoxic lymphocytes, the mechanisms which are involved in processing and presentation of antigen by APCs will be considered first. Competition between peptides can exist at every step in the processing pathway. Foreign antigen undergoes proteolysis within the proteosomes or endosomes of an APC resulting in the production of a variety of short peptides. Preferences for particular cleavage sites will influence the peptides generated by this process [178-180]. Peptides are then transported to the endoplasmic reticulum by Transporter associated with Antigen Processing (TAP). Peptides which are 8-16 amino acids long and which have either a hydrophobic or a positively charged carboxyl terminal residue will be most efficiently transported by TAP. On arrival in the ER peptides may be further altered by amino-peptidase N-terminal trimming [178,181].

Competition for binding of MHC class I molecules in the ER also influences which epitopes become immunodominant. Most peptides will not bind MHC class I molecules and are rapidly cleared out of the ER whilst those peptides with high affinity for MHC class I will bind thereby forming stable complexes which are successfully transported to the cell surface for presentation to CTLs.

Other factors must be involved in immunodominance, as relatively few peptides which are processed and presented stimulate strong CTL responses [182]. The available T-cell repertoire is important as an immune response to the peptide requires a CTL with a TCR recognising the MHC/ peptide complex. Competition then exists between CTLs recognising different epitopes. A hierarchy of dominance can be distinguished with peptides that induce strong CTL responses said to be immunodominant over subdominant peptides which induce weaker responses. The affinity with which the TCR binds its

MHC/peptide complex may play a role with high affinity CTLs having an advantage against low affinity CTLs [183]. Development of a dominant CTL clone is thought to require both the immunodominant and subdominant epitopes to be presented on the same APC [184]. A potential mechanism for this effect was suggested by studies which showed that high affinity CTLs may lyse the presenting APC during the secondary response to antigen, thus preventing activation of lower affinity CTLs [185,186]. This may be of particular importance if APCs presenting an antigen are limited in number. Although this mechanism is not thought to be of importance during the initial priming of CTL responses, it has a significant effect when a primed CTL encounters an APC expressing its specific epitope.

Another mechanism by which immunodominance may occur is suggested by the observation that T cells may remove MHC and other proteins from the surface of APCs, thereby limiting the opportunity for heterogeneous CTLs to be activated. Other T cell factors may contribute to immunodominance such as the frequency of CTL precursors specific for a particular epitope [183]. It is to be expected that the effect of this will be greatest in secondary responses [187] [188] but it has been demonstrated that cross reactivity between epitopes found in hetrologous viruses can have a key influence in shaping the hierarchy of CTL responses [182]. Many different mechanisms are involved in the complex process by which an epitope achieves immunodominance. It is clear that design of an epitope based vaccine must take immunodominance and its mechanisms into account.

2.3 Development of a DNA fusion vaccine designed to induce a CTL response

Modification of our vaccine design was required in order to improve its capacity to induce CTLs against a tumour antigen. The two MHC class I binding epitopes which had been identified were located in the second domain of FrC. These competing immunodominant epitopes were removed whilst the "promiscuous" helper epitope (p30) located in the first domain was retained (Figure 20). This was desirable as $CD4^+$ T cells are pivotal in the establishment and maintenance of CTL responses. The bcl-1 derived leader sequence present

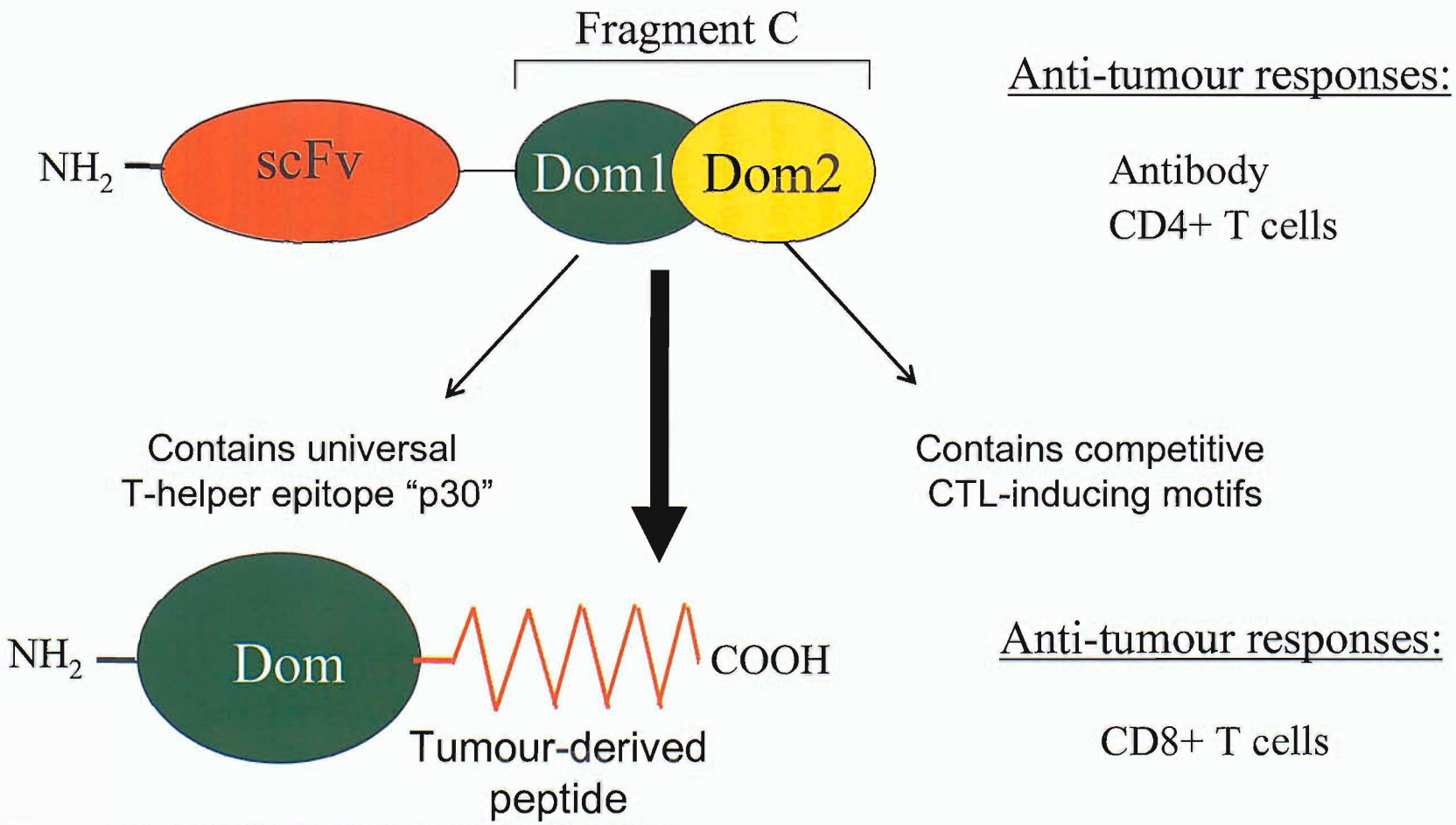


Figure 20. The scFv-FrC DNA vaccine design was modified by removing the second domain of FrC (DOM 2) and placing a CTL epitope at the C-terminus. This produced a DNA vaccine which could induce strong $CD8^+$ T-cell responses against the encoded CTL epitope.

in the scFv vaccine design was also retained as this would target the candidate peptide to the E.R. [177]. Repositioning of the peptide sequence to the 3' end of the first domain of FrC took advantage of the observation that proteolyic enzymes in the ER liberated peptides from the C-termini of delivered proteins (Figure 21) [189] . The mechanism of action of this new vaccine is again dependent upon cognate T cell help, this time provided by p.DOM specific $CD4^+$ cells, which facilitate an immune response against the fused CTL epitope (Figure 22). The p.DOM design was initially tested in the murine CT26 colorectal tumour model which expresses an endogenous retroviral glycoprotein (gp70). Within gp70 is a CTL epitope designated AH1. The p.DOM-AH1 vaccine induced AH1 specific CTLs which were detectable ex-vivo and able to lyse tumour cells in-vitro [190].

2.4 Translation of DNA vaccination from the laboratory to the clinic

As illustrated above, murine models have facilitated the rationale development of DNA vaccines. The aim of our programme is to translate the promising immune responses against target antigens demonstrated in experimental animal systems to clinical practice. This requires the evaluation of vaccine performance and safety in pilot clinical trials. Assessment of the capacity of the pDOM DNA vaccine design to induce a CTL response in humans first demanded the selection of a suitable vaccination target. Human Cytomegalovirus (HCMV) was chosen for several reasons. Disease resulting from this infection causes considerable morbidity and mortality among immunocompromised patients, such as those who have undergone allogeneic haematopoietic stem cell transplantation. CTLs are recognised to be crucial in the control of CMV in immunocompetent subjects. A vaccine which induces CMV specific CTLs may therefore be effective in preventing disease in the immunocompromised. Importantly, the epitope which is immunodominant in the CTL response of HLA-A*0201 individuals to CMV has been identified, providing an ideal target antigen against which the capacity of the vaccine to induce CTLs could be tested.

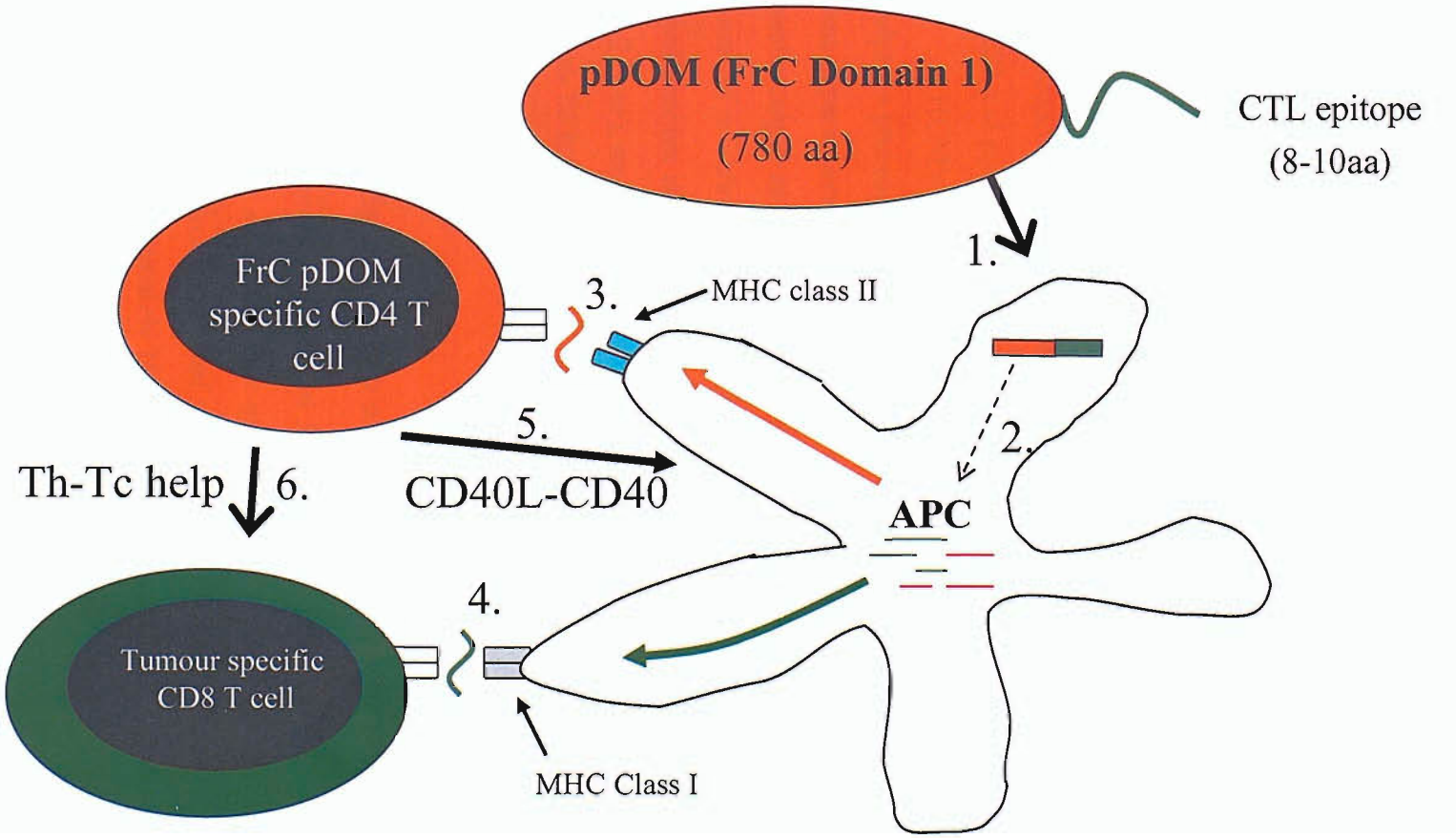


Figure 21. Mechanism of action of pDOM-tumour epitope DNA vaccine. DNA vaccine encoded pDOM-CTL epitope protein is 1) acquired by the APC and 2) degraded into peptide fragments. 3) Peptides derived from pDOM are presented on the APC surface in association with MHC class II whilst 4) the CTL epitope is presented in association with MHC class I molecules. 5) Interaction between CD40 ligand on the pDOM specific $CD4^+$ T cell with CD40 on the surface of the APC augments the ability of the APC to activate $CD8^+$ T cells specific for the tumour epitope. 6) T-helper- cytotoxic T cell help (Th-Tc) is also provided by cytokines released from the pDOM specific $CD4^+$ T-cell.

2.4.1 Human Cytomegalovirus

2.4.1.1 Virus structure and function

The HCMV virion is an icosahedral nucleocaspid, 100-nm in diameter, containing a 230-kbp double stranded linear DNA genome. The HCMV genome is the largest of all herpesviruses. It is surrounded by a proteinaceous "matrix" layer, which, in turn, is enclosed within a lipid bilayer. More than 30 proteins are found in the complete infectious virion. Four proteins contribute to the structure of the nucleocaspid whilst the phospholipids envelope contains six virus encoded glycoproteins. The major envelope glycoprotein is termed gB. The remaining 20-25 structural proteins, the majority of which are phosphorylated, are located in the matrix layer. The most abundant matrix proteins are pp150 and pp65.

HCMV initially attaches to the cell surface by low-affinity binding of gB to heparin sulphate proteoglycans [191]. Stable binding is then achieved by the interaction of gB with a non-heparin receptor. Fusion of the virus and cell membranes is followed by entry of nucleocaspid and matrix proteins into the cytoplasm. Mature pp65 is transferred at the onset of infection and can be can be detected in the nucleus within one hour [192,193]. The viral genome is then expressed sequentially, giving rise to production of immediate early (0-2 hour), early (<24 hour) and late (>24 hour) viral proteins [194]. Viral DNA synthesis occurs 16 hours after infection and the release of progeny virus commences 72 hours post infection.

2.4.1.2 Epidemiology and clinical features of HCMV infection

HCMV has successfully infected 50-90% of the adults worldwide. Infection rates are highest in lower socioeconomic groups. In industrialised countries, approximately 40% individuals are already infected with CMV by the time they reach adolescence. This figure increases by approximately 1% per year of life, resulting in a higher prevalence among the older population. HCMV can be acquired through contact with saliva, urine, semen, milk or cervical secretions. Virus usually enters through the epithelium of the upper GI, respiratory or genitourinary tracts. Less frequently, CMV infection may be acquired by blood transfusion, stem cell or organ transplantation.

Primary infection of immunocompetent hosts is usually asymptomatic although it may result in a mononucleosis syndrome. Complications including pneumonia, myocarditis, hepatitis, GI ulceration and retinitis can occur but are rare. Following

resolution of primary infection by the host immune response, the virus establishes lifelong latency in myeloid progenitor cells located in the bone marrow. Although latency is maintained when these cells develop into monocytes, CMV can reactivate after further differentiation to macrophages [195]. Following reactivation, infectious virions are shed from mucosal surfaces and can transmit infection to new hosts.

2.4.1.3 The immune response to HCMV

The humoral immune system pays a minor role in the control of HCMV infection. Virus neutralising antibodies are produced against envelope glycoproteins, with gB being the main target *(Pass RF Fields Virology p2675-2705, Philadelphia: Lippincott, Williams and Wilkins)*. Antibodies are also produced against the matrix proteins but unable to interact with the surface of virions and are of limited importance [193].

In contrast, the cellular immune response is crucial in the control of HCMV. Studies in the murine cytomegalovirus model showed that ablation of all T-cells resulted in MCMV reactivation and high levels of productive infection. This did not occur when only $CD4^+$ or $CD8^+$ cells were depleted, indicating some functional redundancy of both T-cell subsets. The importance of $CD4^+$ cells in the control of HCMV infection is suggested by the observation that up to 3% of $CD4^+$ cells in the blood of normal immunocompetent individuals are specific for HCMV [196]. Although help provided by $CD4^+$ cells is required for an optimal $CD8^+$ immune response to CMV [197] it is interesting to note murine experiments which demonstrated that transfer of $CD8^+$ MCMV-specific CTLs alone were sufficient to protect mice from fatal CMV infection [198].

The detection of a direct correlation between recovery of HCMV-specific CTL responses in patients following allogeneic stem cell transplantation and their protection from CMV disease also indicated that the CTL response to HCMV was of clinical importance [199]. Subsequently, the use of bulk cultures with subsequent cloning identified that the main target for HCMV-specific CTLs was the lower matrix protein, pp65 [200]. This was confirmed by a limiting-dilution analysis in which the frequency of CTL precursors specific for HCMV was determined [201].

Further investigation identified the epitope CMV $pp65_{495\text{-}503}$ (NLVPMVATV) as immunodominant in the context of HLA-A*0201 [202]. Using MHC tetramers, it has been demonstrated that several percent of $CD8^+$ cells in the peripheral blood of HLA-A2*0201 individuals may be specific for this epitope [203-205]. This population is maintained at a stable level for years and often contains only a few individual clones that have undergone massive expansion in vivo [206]. The high frequency of pp65 specific CTLS in healthy CMV

carriers suggests that CMV reactivation often occurs but remains subclinical due to rapid effective control by CTLs [207].

Although it is accepted that NLVPMVATV is usually immunodominant, high levels of CTLs to this epitope are not always detected in CMV seropositive HLA-A*0201 subjects. The immunodominance of HLA-B*07 restricted CTL responses to HCMV over HLA-A*02 results in a lower frequency of NLVPMVATV specific $CD8^+$ cells in subjects who co-express HLA-A*02 and HLA-B*07 [208]. In addition, the recent finding that as many as one third of HLA-A*0201 individuals have a higher frequency of CTLs recognising peptides within the IE-1 protein than those reacting against NLVPMVATV indicates that our understanding of the cellular immune response to CMV continues to evolve [205].

2.4.1.4 HCMV infection in the immunocompromised

Cytomegalovirus infection often causes severe disease in immunologically immature or immunocompromised individuals. Intra-uterine infection of the foetus can occur following primary or reactivation of latent maternal infection. 10 to 15% of congenitally infected foetuses suffer long term CNS damage, making HCMV the leading infectious cause of birth defects.

Studies in the 1980s identified cytomegalovirus as a major cause of morbidity and mortality in patients following allogeneic stem cell transplantation. Disease usually developed during the early post –transplantation period (<100 days) and was heralded by fever, cough and the development of a pneumonia which was often fatal. Retinitis and GI ulceration occurred less frequently. The incidence of early CMV disease in transplant recipients has been reduced from 20-30% to 5% by the use of anti-viral pharmaceutical agents.

A pre-emptive treatment strategy is currently most frequently employed in transplant centres to combat CMV. The pp65 antigen can be detected in a patient's blood prior to the onset of CMV disease using either an antigen immunofluorescence assay or a quantitative PCR assay. Therapy with Ganciclovir is then administered intravenously twice daily for 7 days and then once daily, five times per week, for an additional 2-5 weeks [209]. Ganciclovir is a nucleoside analogue and acts as a competitive inhibitor of viral DNA polymerase. Unfortunately, for several reasons, current treatment of HCMV is sometimes unsuccessful. Disease may occur in a substantial number of patients whose pp65 antigen or DNA assays are negative [210] or HCMV may develop ganiciclovir resistance [211]. Another problem is that prolonged ganciclovir use is toxic to the bone marrow and the resulting neutropenia is associated with a higher risk of invasive bacterial and fungal infections

[212,213]. In addition, the risk of late CMV disease (>100 days) has increased and remains a significant problem [214].

2.4.1.5 Risk factors for HCMV disease after allogeneic transplantation

Factors known to influence the risk of post-transplant CMV disease include the CMV serostatus of donor and recipient, the source and manipulation of stem cells, the conditioning regimen, post-transplantation immunosuppressive medication and the use of prophylactic or pre-emptive anti-CMV pharmaceutical agents. By examining these criteria, certain subgroups of patients can be identified as being at higher risk of developing CMV disease.

Most CMV infections after SCT result from reactivation of latent virus from the recipient. CMV seropositive stem cell recipients are at high risk regardless of the donors' serological status [215] whereas CMV seronegative recipients of stem cells from CMV seropositive donors are at lower risk [216]. Patients who receive a stem cell graft from an unrelated donor have an increased risk of CMV disease [217]. The immunosuppressive regimens that allow the recipient to retain the graft and avoid GvHD have a major impact on CMV disease incidence. $CD34^+$ selection or T-cell depletion of the graft significantly increases the risk of CMV disease [218 219]. A high incidence of CMV infection has been observed when high doses of alemtuzumab-1H (immunoglobulin G1 humanised monoclonal antiCD52) are employed in the conditioning regimen [220]. Recipients who are treated with high-dose corticosteroids are also considered to be at high risk [221].

2.4.1.6 HCMV specific T cell reconstitution after allogeneic SCT

Many of the factors listed above cause increased susceptibility to CMV disease by delaying or reducing CMV specific T-cell immune reconstitution. An association between CMV disease and poor T-cell proliferative responses to HCMV has been demonstrated following allo-SCT [222 223]. The return of functional T cell responses can usually be detected between 40-90 days after SCT although this is delayed in patients who receive ganciclovir [224]. Ganciclovir causes decreased viral replication thereby limiting the antigen available to drive the expansion of CMV specific T cells.

Reconstitution of CMV specific $CD8^+$ T-cells is rapid in recipients of a HLA-matched sibling transplant in which both the donor and recipient are CMV seropositive. Using MHC class I tetramers, $CD8^+$ cells specific for NLVPMVATV can be detected as

early as 21 days after transplantation in this setting [203]. Serial measurements show that these cells can subsequently increase rapidly in number, often comprising greater than 20% of the recipient's total $CD8^+$ cell population. Tetramer analysis does not identify whether the reconstituting $CD8^+$ T cells originate from the donor or the recipient. However, clonotypic probing has demonstrated that these CMV specific CTLs are derived from the donor [225]. This was found to be the case in all patients examined, regardless of the CMV serostatus of donor and recipient. Studies have suggested that recovery of CMV specific $CD8^+$ cells to levels above 10-20 $x10^6$/L is associated with protection from CMV disease. This suggests that interventions which rapidly increase the number of CMV specific CTLs to above this level may be effective in preventing or treating CMV disease.

2.4.1.7 The use of adoptive cellular therapy to treat HCMV disease after HSCT

Successful adoptive therapy of CMV has been reported by Riddell and co-workers. They administered ex-vivo generated, donor derived, CMV specific $CD8^+$ T cells to stem cell recipients [226,227]. This protocol is unsuitable for routine clinical use as CMV infected fibroblasts were used to stimulate in-vitro T-cell activation and expansion, exposing the patients to a potential biohazard. In addition, transfer of very large numbers of T-cells (over 10^9 per week) were required for therapeutic effect and CMV specific CTL responses were poorly maintained in the absence of CMV-specific T-helper responses. By using donor-monocyte-derived dendritic cells pulsed with viral lysate, another group controlled CMV infection with much lower numbers of CMV specific $CD8^+$ T cells (0.2-1.0 x 10^5/ m^2) and without the risk of viral infection [228 229]. Several patients experienced a further CMV reactivation, perhaps because the cells generated by in-vitro expansion were predominantly peripheral effector cells and therefore relatively few central memory T cells were transferred. These studies clearly illustrate the clinical potential of immunotherapeutic strategies which increase the numbers of CMV specific CTLs in allograft recipients.

Adoptive immunotherapy must resolve several problems before it will be suitable for widespread clinical use. These include technical and time dependent challenges, the frequent lack of appropriate donor material and the expense of current protocols. Progress continues to be made in this field. An interesting approach recently published involved the selection of CMV specific $CD8^+$ T-cells from a stem cell donor using HLA-peptide tetramers[230]. These cells were then purified by selection with magnetic beads and the CMV specific portion was then re-infused into the patient. All patients experienced a reduction in

the level of CMV viremia. This approach does not require expensive in-vitro culture and again highlights how effective CTLs can be in controlling HCMV infections.

2.4.1.8 Vaccination against HCMV

An alternative immunotherapeutic approach involves vaccination of stem donors (+/- stem cell recipient vaccination) to increase CMV specific CTL numbers. Although various approaches have been employed in the past, such as attenuated live vaccines, recombinant live vaccines, whole protein vaccines, peptide vaccines and DNA vaccines, an effective vaccine which induces either an antibody or cellular response against cytomegalovirus in humans is not currently available for clinical use.

Of the various strategies listed above, some have aimed to induce antibody responses against HCMV whilst others attempted to induce CTL responses. In some cases the vaccine was designed to activate both arms of the immune system. The attenuated Towne strain has been tested in healthy volunteers and in renal transplant recipients [231]. It induced neutralising antibodies, a CTL response and reduced HCMV disease in seronegative renal transplant patients but did not prevent CMV infection [232,233]. It was further examined in seronegative mothers of HCMV infected children and was found to be ineffective [234]*(erratum in J Infect Dis 1995 171(4):1080)* [235]. As it is a live vaccine, the use of the Towne strain in transplant patients presents an infectious risk to immunocompromised patients, in addition to the fears of possible oncogenicity which surround the use of all live attenuated herpesviruses.

Other groups employed a vaccination strategy which used a canarypox vector as a vehicle for the expression of recombinant gB and pp65 proteins [236 237]. Avian pox viruses have a limited potential for replication in humans and do not therefore carry the same infective risks to immunocompromised patients as live attenuated HCMV stains.

Non-viral approaches which pose no infective threat are ideally preferred for transplant patients. Vaccines composed of recombinant gB protein combined with either alum or MF59 were evaluated in 46 seronegative adults [238]. Although the MF59 formulation was found to be superior, inducing neutralising antibodies which were still present 12 months after vaccination, it has not been shown that antibodies induced by this vaccine are sufficient to prevent infection.

As outlined previously, anti-HCMV CTL responses are pivotal in the ability of the normal immune system to control HCMV. Much work has therefore focused upon the development of a vaccine capable of inducing effective CTLs against CMV. An approach developed by Diamond and colleagues which has shown some promise in preclinical models involves the use of a lipidated CTL epitope [204]. When tested in mice, a lipopeptide

vaccine incorporating the epitope NLVPMVATV induced CTLs which were cytotoxic for CMV infected fibroblasts. A key component of this lipopeptide vaccine design is the inclusion of a T_H epitope, PADRE, which was derived by synthetic alteration of optimal peptide binding motifs for human HLA class II DRb1 and b2 alleles. This augmented the CTL response to NLVPMVATV.

The ability of DNA vaccines to induce humoral or cellular responses against antigens derived from HCMV has been explored in mice by several investigators. Few studies have been published in which a DNA vaccine has been used to induce CTL responses. In one such study, in which a plasmid vector encoding pp65 from the Towne strain was used, Enderez et al induced CTLs in 6/10 mice which, after a 5 day in-vitro stimulation, could lyse target cells infected with a vaccinia virus expressing HCMV-pp65 [239].

2.4.1.8.1 Development of the DNA vaccine pDOM.NLVPMVATV

Our group had already demonstrated that the pDOM vaccine design could induce CTLs against an encoded tumour epitope. As outlined above, an effective vaccine which is capable of inducing CTLs against CMV could be of great clinical value. A vaccine with the potential to induce a CTL response against an epitope of CMV was produced in the laboratory of Prof. F.Stevenson by Mr. Andrew King. This vaccine was constructed by inserting the epitope which is immunodominant in the response of HLA-A*0201 individuals to HCMV infection (NLVPMVATV) into the pDOM DNA vaccine thereby creating p.DOM.NLVPMVATV.

Mr King subsequently tested the ability of p.DOM.NLVPMVATV to induce specific CTL responses in mice. Groups of HLA-A2 transgenic mice were injected with either p.DOM.NLVPMVATV or a control vaccine, pDOM. After 14 days the mice were sacrificed and splenocytes were cultured overnight with NLVPMVATV peptide in an elispot assay. CTLs specific for NLVPMVATV had clearly been induced by p.DOM.NLVPMVATV vaccination. No cells specific for NLVPMVATV were induced by vaccination with the control vaccine p.DOM (Figure 22). Further analysis with a PE-labelled tetramer specific for NLVPMVATV showed that 1-5% of splenic $CD8^+$ T-cells of mice which had been vaccinated

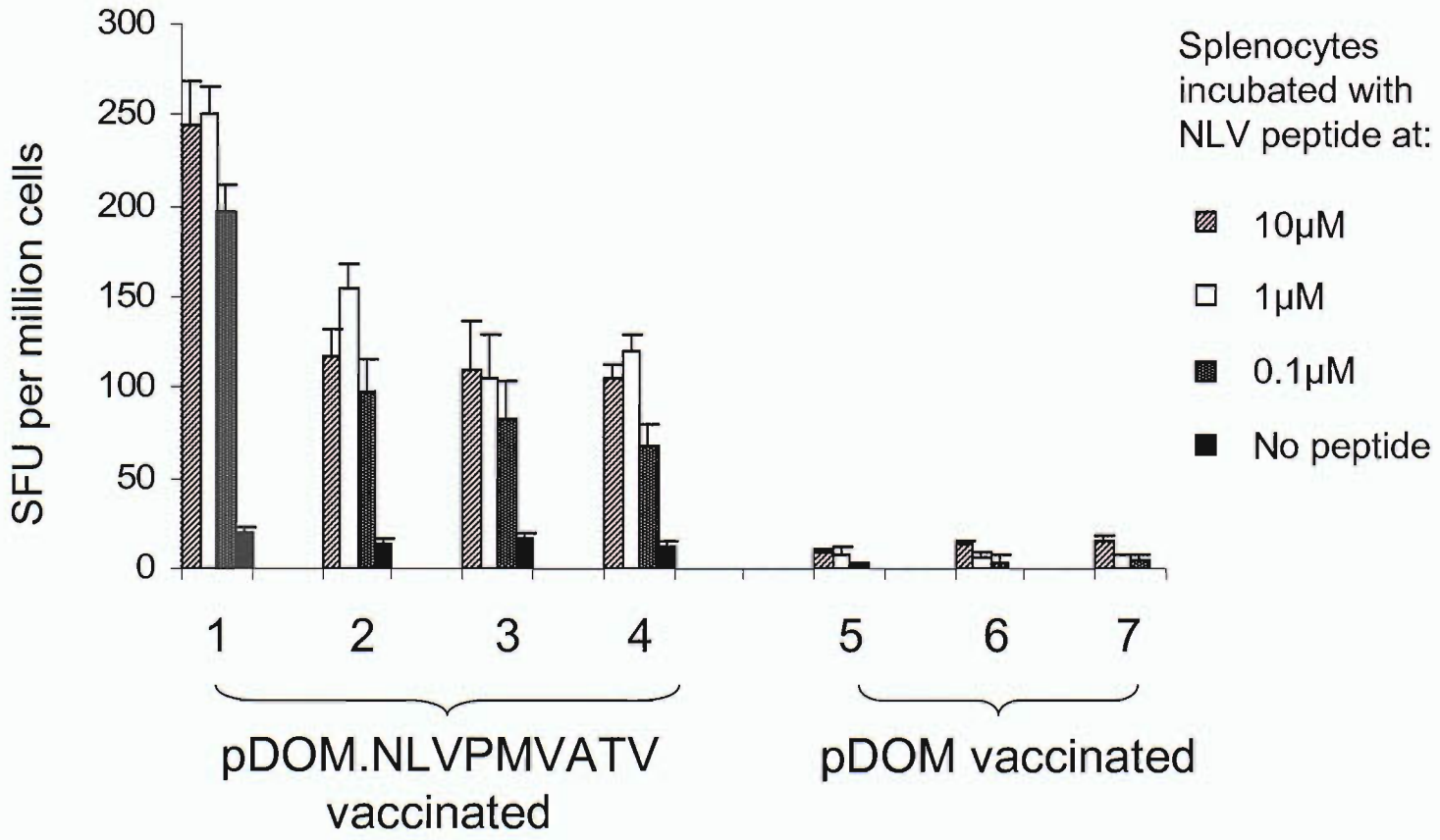


Figure 22. Ex vivo Interferon-γ elispot analysis of splenocytes of mice vaccinated 14 days previously with pDOM.NLVPMVATV or pDOM. After overnight incubation with NLVPMVATV peptide splenocytes from pDOM.CMV-NLV vaccinated mice produce Interferon-γ whilst cells from pDOM vaccinated mice do not. SFU= Spot forming units.

with p.DOM.NLVPMVATV were specific for NLVPMVATV. $CD8^+$ T-cells specific for NLVPMVATV were not detected among splenocytes from p.DOM vaccinated mice (Figure 23).

In addition, splenocytes from mice vaccinated with p.DOM.NLVPMVATV were able to specifically lyse Jurkat cells (A2/Kb) which had been infected with MVA expressing recombinant CMV pp65. Mice vaccinated with pDOM were unable to lyse these cells (Figure 24).

Having clearly demonstrated in a murine model that p.DOM.NLVPMVATV vaccination could induce an impressive CTL response against NLVPMVATV we then assessed the ability of this vaccine to induce or augment a CTL response to NLVPMVATV in humans.

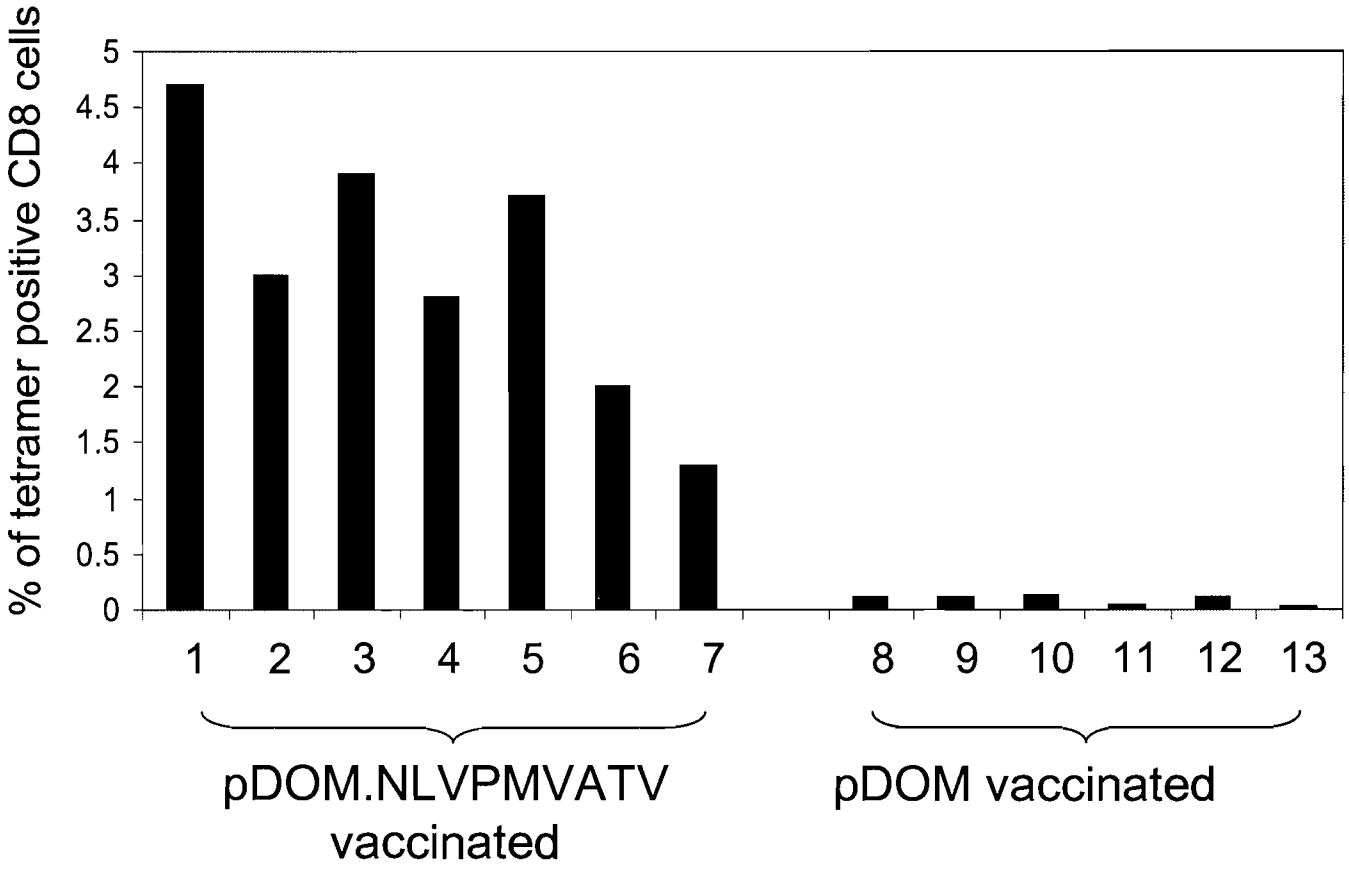


Figure 23. NLVPMVATV specific tetramer analysis of splenocytes from mice vaccinated 14 days previously with pDOM.NLVPMVATV or pDOM. Results are presented as the percentage of CD8+ splenocytes that were positive for the tetramer.

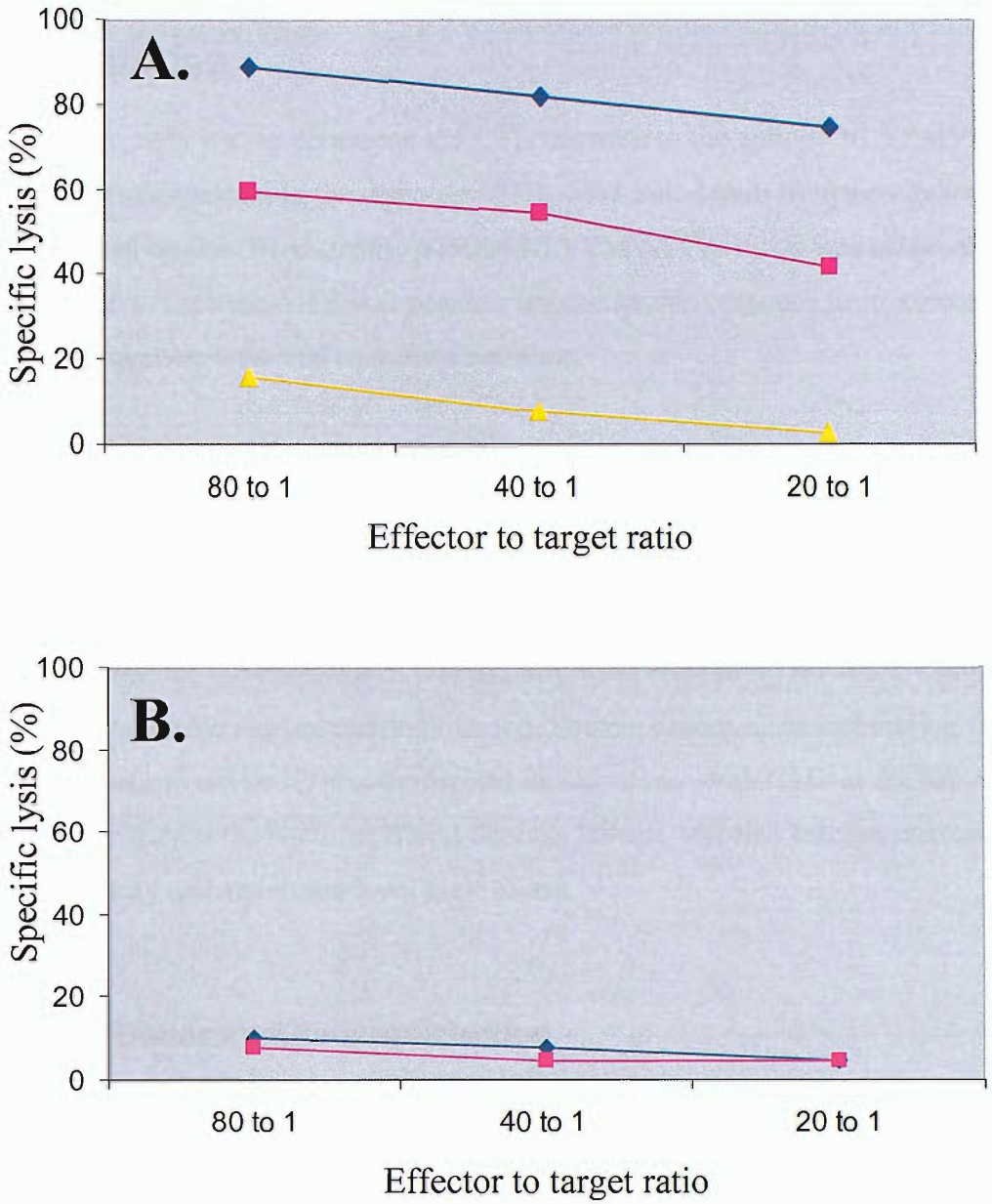


Figure 24. (A) Splenocytes from mice vaccinated with p.DOM.NLVPMVATV cause specific lysis of MVA pp65 infected target cells (◆) or NLVPMVATV pulsed target cells (■) but do not lyse MVA infected target cells (▲) in a ^{51}Cr cytotoxicity assay. **(B)** Splenocytes from pDOM vaccinated mice are unable to either any of these target cells.

2.5 Human immune responses to pDOM.NLVPMVATV vaccination

The aim of this study was to determine if a CTL response to the epitope NLVPMVATV (which is immunodominant in the response of HLA-A2 individuals to cytomegalovirus) could be induced by the DNA vaccine p.DOM.NLVPMVATV. If this was achieved, we then also aimed to determine if it was possible to transfer this response from a stem cell donor to an allogeneic stem cell transplant recipient.

2.5.1 Methods and materials

2.5.1.1 pDOM.NLVPMVATV vaccine preparation

The DNA vaccine for the clinical trial was initially was prepared by Mr Andy King in the laboratory of the Molecular Immunology Group, Somers cancer sciences building. The bulk preparation and sterile fill was performed in accordance with GMP at the MCA approved laboratory at the National Blood Service, Bristol. Vaccine batches were assessed for sterility, purity and endotoxin level prior to use.

2.5.1.2 Donor and Patient Selection

Eligibility of Donors

1. Donor for allogeneic transplantation of an HLA identical sibling.
2. Likely time to transplant $\geq$8 weeks
3. Donor is HLA A*0201 positive.
4. WHO performance score 0
5. Donor must be greater than 18 years of age.
6. No medical condition, requiring immunosuppressive/steroid treatment in the 2 months prior to vaccination. Inhaled steroids will be allowed.
7. Known donor CMV status; CMV sero-positive and CMV sero-negative donors will be allowed
8. Donor must be able and willing to travel to the centre for vaccination, blood tests and follow up.
9. The donor and patient must give informed consent to vaccination and GTAC monitoring of their health records.

10. If of child bearing age the donor must be willing and able to take contraceptive measures for a period of 6 months.

Eligibility of patients

1. HLA A*0201 positive recipient of allograft (stem cells or bone marrow).
2. Known CMV status; CMV sero-positive and CMV sero-negative – recipients will be allowed
3. Patient must be greater than 18 years of age.
4. Informed consent to study and GTAC monitoring of health records.
5. No uncontrolled sepsis or clotting disorder
6. Platelet count $>50x10^9/l$.

Exclusion criteria for donors and patients

1. Presence of clinically significant levels of anti-DNA antibodies, anti-muscle antibodies or rheumatoid factors or who have active autoimmune disease.
2. Presence of antibodies to HIV.
3. Presence of severe medical condition other than the cancer requiring transplant and transplant related problems in the patient.
4. Pregnancy, lactation or not using contraceptive measures.

2.5.1.3 Vaccination schedule

The DNA vaccine p.DOM.NLVPMVATV was investigated in the phase I/II study "DNA vaccination against a CMV/FrC of tetanus toxin fusion gene in allograft donors and recipients". The study was approved by the local ethics committee, the Gene Therapy Advisory Committee (GTAC) and the Medicines Control Agency (MCA, now known as the Medicines and Healthcare products Regulatory Agency, MHRA). In each case, informed consent was obtained from both the stem donor and recipient. HLA-A*0201 stem cell transplant donors were vaccinated intramuscularly with the DNA vaccine p.DOM.NLVPMVATV on weeks 0, 1, 2, 4, 8 and 12.

Each vaccine contained 1mg of DNA. We decided to use a dose of 1mg of DNA based on our experience from previous vaccine trials in lymphoma in which we found responses to FrC could be detected after vaccination with doses of DNA of 500μg and above. As FrC was a previously encountered antigen, DNA vaccination in the lymphoma trials needed only to stimulate the pre-existing memory response to this antigen. We felt that higher levels of antigen expression would be most likely required to prime an immune

response against the previously unencountered antigen NLVPMVATV and therefore chose a higher dose of 1mg for this trial.

The vaccine was supplied in standard PBS and the concentration of DNA was therefore 1mg/ml. Each vaccination involved a 1ml injection into either the right-sided or left-sided deltoid muscle. The side chosen for vaccination was alternated.

2.5.1.4 Preparation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from heparinised blood by centrifugation over Lymphoprep. Cells were washed twice with RPMI (Invitrogen) and counted. Following a further wash, a portion of fresh cells were analysed in an Interferon-γ Elispot assay or labelled with MHC class I tetramer complexes and analysed by flow cytometry. Remaining PBMCs were frozen at 0.5-1x10^7/ml in 50% decomplemented human AB serum (Sigma-Aldrich), 40% RPMI (Invitrogen) and 10% DMSO (Sigma-Aldrich), and stored in liquid-nitrogen for future use.

2.5.1.5 Interferon-γ ELISPOT to detect antigen specific CD8+ T-lymphocytes

96-well ELISPOT plates (Millipore, Bedford, USA) were precoated overnight at 4°C with 10 μg/ml anti-IFNγ antibody (mAb 1-DIK, Mabtech, Nacka, Sweden) in sterile filtered PBS. 1 x 10^5 and/ or 4x10^5 PBMC in 200μl of C-RPMI/10% decomplemented pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either NLVPMVATV or GILGFVFTL peptide (Peptide Protein Research Ltd. Eastleigh UK) at 1ug/ml or PHA (Sigma-Aldrich) 5μg/ml or medium alone. After incubation for 16 hours at 37°C in 5% CO_2, the cells were discarded. Wells were washed 6 times with PBS/0.1% Tween-20 (Sigma-Aldrich). Biotinylated anti-IFNγ antibody (7-B6-1, Mabtech) at 1μg/ml was added, and plates were then incubated at 37°C in 5% CO_2 for 90 minutes. Wells were again washed 6 times with PBS/0.1% Tween-20 and then strepavidin conjugated alkaline phosphatase (Mabtech) was diluted 1:1000 in PBS/ 1% BSA and added at 100μl per well. The plates were incubated 37°C in 5% CO_2 for 60 minutes. An alkaline phosphatase-conjugate substrate kit detected IFNγ-producing cells (Zymed Laboratories Inc. San Francisco CA). After spot development was complete, the reaction was stopped with tap water. The spots were counted by a computer assisted video image analyser (Autoimmun Diagnostika GmbH, Strassberg, Germany) using ELISPOT reader software (AID, version

3.1). Background spots were subtracted. Results are reported as the number of spot forming units / 1 x 10^6 PBMC.

2.5.1.6 Anti-TT and anti-FrC ELISAs

Anti-TT and anti-FrC IgG antibodies were measured using WHO human antitoxin standards (NIBSC Herts, UK). Briefly, ELISA plates were coated with 0.5 LFU/ml TT in coating buffer, or E coli expressed FrC (0.5ug/ml diluted 1/2000 in coating buffer) 200 µl per well and incubated at 4^0C overnight. They were washed with PBS/Tween and blocked with blocking buffer for 1 hour at 37°C. The TT anti-sera standard (IgG) at 10iu/ml was diluted in PBS starting at 1/100 dilution. Patient samples and an internal control serum were also diluted in PBS. Plates were washed with PBS/Tween and then doubling dilutions of standard and test samples added, 200 µl per well. Plates were then incubated for 1.5 hours at 37°C. They were then washed x4 in PBS/Tween before the addition of anti-Fc γ-HRP (Sigma) at a 1/2000 dilution and incubated for a further hour at 37°C. After 4 washes, 200 µl of fresh substrate buffer was added to each well. The colour was allowed to develop and the reaction was then stopped by the addition of 80µl per well of 2.5M H_2SO_4. The optical density was read on an automatic ELISA reader.

2.5.1.7 Interferon-γ ELISPOT assay for $CD4^+$ T-cell responses to FrC

96-well ELISPOT plates were pre-coated overnight at 4°C with 10 µg/ml anti-IFNγ antibody (mAb 1-DIK, Mabtech, Nacka, Sweden). 2 x 10^5 PBMC in 200µl of C-RPMI /10% decomplemented pooled human AB serum (Sigma-Aldrich) were incubated in triplicate with either FrC (at 20µg/ml, 2µg/ml or 0.2µg/ml), P30 (1µg/ml or 0.1µg/ml), medium alone (negative control) or 5µg/ml PHA (Sigma-Aldrich). After incubation for 48 hours at 37°C in 5% CO_2, the cells were discarded and the wells were washed 6 times with 150µl of filtered PBS/0.1% Tween-20 (Sigma-Aldrich). Biotinylated anti-IFNγ antibody at 1µg/ml (7-B6-1, Mabtech) in filtered PBS/1%BSA (Sigma-Aldrich) was added (100µl per well) and the plates were incubated for 90 minutes at 37°C in 5% CO_2. The wells were then washed another 6 times with filtered PBS/ Tween. Strepavidin conjugated alkaline phosphatase (Mabtech) diluted 1:1000 in filtered PBS/1%BSA was then added to each well (100µL) and the plates were again incubated for 1 hour at 37°C in 5% CO_2. After 6 further washes with filtered PBS/Tween, IFNγ spots were detected using an alkaline phosphatase-conjugate substrate kit (Zymed Laboratories Inc. San Francisco CA). Once spot

development had finished the reaction was stopped by washing the plates with tap water. Once the plates had dried, a computer assisted video image analyser (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used to count the spots. Background spots were subtracted.

2.5.1.8 Enumeration of NLVPMVATV-specific $CD8^+$ T lymphocytes with fluorescent MHC class I tetramer complexes.

PBMCs were re-suspended in FACS buffer (PBS with 1% BSA and 0.1% sodium azide) at $1x10^6$ cells per 100μl. PE-labelled HLA-A*0201/NLVPMVATV tetramer complexes (Proimmune, Oxford, UK) were diluted 1:10 in FACS buffer. PBMCs were then stained with the optimal quantity of tetramer (2.5μl per $1x10^6$ cells). One μl of anti-CD8 mAb conjugated with FITC (BD Pharmingen) and 2.5μl of anti-CD3 conjugated with PerCP (BD Pharmingen) were added to the cells. They were then incubated at room temperature for 30 minutes and washed in FACS buffer before analysis on FACScailibur using CELLQUEST software (Becton Dickson). Cells were gated on a live lymphocyte gate and then a CD3+ gate. The proportion of CD8+ cells that labelled with the tetramer was then calculated.

2.5.1.9 Enumeration of GILGFVFTL-specific $CD8^+$ T lymphocytes with fluorescent MHC class I tetramer complexes.

PBMCs were re-suspended in FACS buffer (PBS with 1% BSA and 0.1% sodium azide) at $1x10^6$ cells per 100μl. PE-labelled HLA-A*0201/GILGFVFTL tetramer complexes (Proimmune, Oxford, UK) were diluted 1:10 in FACS buffer. PBMCs were then stained with the optimal quantity of tetramer (5μl per $1x10^6$ cells). One μl of anti-CD8 mAb conjugated with FITC (BD Pharmingen) and 2.5μl of anti-CD3 conjugated with PerCP (BD Pharmingen) were added to $1x10^6$ cells. They were then incubated at 37°C for 30 minutes and washed in FACS buffer before analysis on FACScailibur using CELLQUEST software (Becton Dickson) as outlined above.

2.5.1.10 Selection of NLVPMVATV tetramer +ve $CD8^+$ T cells

PBMCs were re-suspended in FACS buffer at $1x10^7$ cells/ml and stained with PE-labelled HLA-A*0201/NLVPMVATV labelled tetramer complexes (5µl per $1x10^7$ cells). Cells were also stained with anti-CD8 mAb conjugated with FITC (BD Pharmingen) (10µl per 10^7 cells). They were incubated in the dark at 20°C for 30 minutes and washed in FACS buffer. Cells were sorted on a FACSvantage by gating on a live lymphocyte gate and then gating on $CD8^+$/tetramer positive or $CD8^+$/ tetramer negative cells. These samples were sent to the Wessex regional genetics laboratory where chimerism studies were performed to determine if the cells were of donor or recipient origin.

2.5.1.11 Generation of autologous dendritic cells

Dendritic cells (DCs) were generated by plating $1x10^7$ PBMCs (3mls at $3.3x10^6$/ml) in CRPMI with 5%AB serum (Sigma) in a 6-well tissue culture plate (Nunc, UK). The cells were allowed to adhere for 2 hours at 37°C. After this time the non-adherent fraction was gently washed off with warm RPMI. The adherent cells were cultured in CRPMI with 5%FCS (3mls per well) in the presence of 1000U/ml GM-CSF (Schering-Plough) and 500U/ml IL-4 (R&D). Cytokines were added to the cultures on days 0, 2, 4 and 6. On day 5 DCs were matured by one of two methods. In method 1, LPS (20ng/ml) and Interferon-γ (1000u/ml) were added. For method 2, monocyte conditioning medium mimic (MCM-mimic) was added. MCM mimic is a cocktail of the following cytokines: TNF-α 5ng/ml, IL-1β (5ng/ml), IL-6 (150ng/ml) and PGE_2 (1ug/ml). Mature DCs were collected on day 7.

2.5.1.12 Immunophenotyping of DCs

DCs were analysed on a FACScaliber both prior to and following maturation to ascertain their immunophenotype. Gating was performed on cells exhibiting a large forward scatter and side scatter profile so as to exclude contaminating lymphocytes. The following antibodies were used: anti-CD1a-FITC, anti-CD14-PE anti-HLA-DR-PE, anti-CD83-FITC, anti-CD80-FITC and anti-CD86-FITC. Non-reactive isotype-matched antibodies were used as controls (all antibodies were from BD Pharmingen). Cells were analysed on a FACScalibur.

2.5.1.13 DC culture with CD8 cells.

CD8 cells were isolated using a CD8 isolation kit (Miltenyi Biotec, UK) using the manufacturers instructions. Briefly the following hapten-conjugated antibodies were added to PBMCs: CD14, CD4, CD19 and CD56. Cells are then washed and anti-hapten beads are incubated with the cells. The cells are then run through a magnetic column and cells the CD14-,CD4-, CD19-, CD56- population that does not bind to the column is collected. These CD8 cells were then placed in a 96-well culture plate at $1x10^5$ cells per well with $1x10^4$ DCs and peptide (either NLVPMVATV or GILGFVFTL) at 10μM. Cells were co-incubated in the presence of interleukin-7 at a concentration of 5ng/ml. After 3 days IL-2 was added at 20 IU/ml. Cells were taken on day 7 for tetramer analysis and a ^{51}Cr release cytotoxicity assay. In a leter comparison, $CD8^+$ at $1x10^5$ per well were incubated with fresh PBMCs at $2x10^5$ per well in the presence peptide (as above), IL-12 (2ng/well), IL-7 (1ng/well), anti-CD28 (0.2ng/well) and CD49d (0.2ng/well)

2.6 Results

Three stem cell donor/ recipient pairs have been recruited to the study thus far. The CMV serostatus and HLA typing results for each individual are shown in tables 5 and 6. The ideal donor/recipient pairing in which to test the vaccine would involve a HCMV seronegative donor to a HCMV seronegative recipient. None of the pairings recruited thus far exhibit this combination.

2.6.1 Toxicity monitoring

Following vaccination, donors were monitored closely for at least one hour. Pulse, blood pressure and temperature were monitored at 15 minute intervals. No donor experienced an acute adverse reaction during these periods of observation. A physical examination of each donor was carried out at each clinic visit. Particular attention was paid to DNA vaccination sites. No donor exhibited any evidence of muscle damage at a DNA vaccination site. With the exception of an occasional ache for a few days at the vaccination site, no adverse effects were reported by any volunteer.

Donors also were monitoed with regular haematology, biochemistry and immunological tests. Full blood count, erythrocyte sedimentation rate, coagulation screen, renal function, liver function, muscle enzymes and auto-immune profile remained normal throughout follow-up. Stem-cell transplant recipients were closely monitored as is usual in the post-transplant period.

2.6.2 Tetramer and Elispot results

2.6.2.1 Donor 1

Donor 1 was CMV seronegative. Prior to vaccination , an NLVPMVATV (NLV) specific $CD8^+$ T cell population was not detected by either FACS analysis using PE-labelled HLA-A*0201/NLVPMVATV tetramer complexes (Figure 25) or in an Elispot assay for Interferon-γ production in response to overnight PBMC incubation with NLVPMVATV peptide (Figure 26). The donor was monitored until week 46 after vaccination. At no point was a response to NLV detected by either tetramer or Elispot analysis (Figures 25 and 26).

Table 5 Allogenic SCT recipient clinical characteristics

Recipient	Age	Sex	Diagnosis	Conditioning intensity	Stem cell source	Stem cell dose
1	23	F	ALL	Standard	BM	7.71 $x10^6$/kg
2	57	M	CLL	Reduced	PBSCs	5.38 $x10^6$ / kg
3	20	F	AA	Standard	BM	7.44$x10^6$/kg

Table 6 Donor/ Recipient HLA class I and class II type and CMV serostatus

Donor/ Recipient 1	
HLA-A	0201
HLA-B	44, 57
HLA-C	04, 06
HLA-DR	DRB1*07
HLA-DQ	DQB1*02, 0303
HLA-DP	DPA1*0103/05, DPB1*0401, 1301

	CMV Serostatus
Donor 1	Seronegative
Recipient 1	Seronegative

Donor/ Recipient 2	
HLA-A	0201
HLA-B	27, 35
HLA-C	01, 04
HLA-DR	DRB1*04, 11
HLA-DQ	DQB1*0301/04, 0302/07

	CMV Serostatus
Donor 2	Seropositive
Recipient 2	Seropositive

Donor/ Recipient 3	
HLA-A	01, 0201
HLA-B	08, 51
HLA-C	0701, 01
HLA-DR	DRB1*03011/0306, 04
HLA-DQ	DQB1*02,0301/0304

	CMV Serostatus
Donor 3	Seropositive
Recipient 3	Seropositive

Using similar techniques, a CD8+ T-cell population specific for a control epitope, GILGFVFTL (GIL), was detected prior to vaccination and showed only minor fluctuations throughout the observation period (Figures 25 and 26).

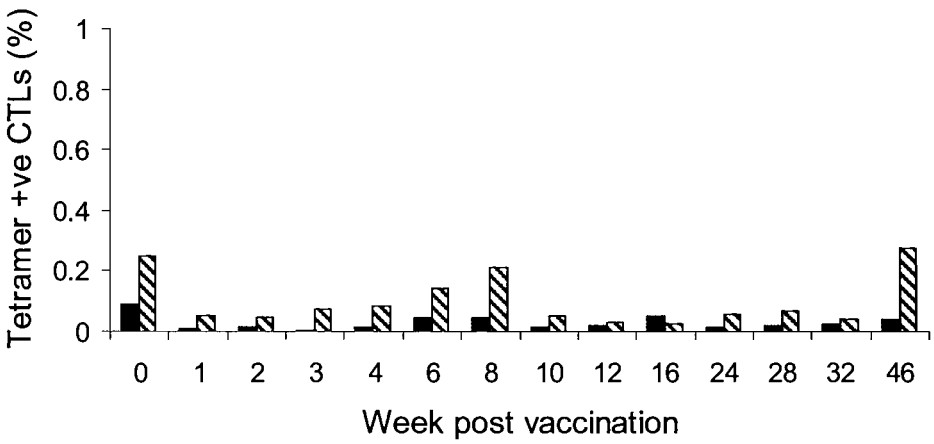


Figure 25. The proportion of donor 1's peripheral blood $CD8^+$ T cell population which was specific for NLVPMVATV (■) or GILGFVFTL (▨)

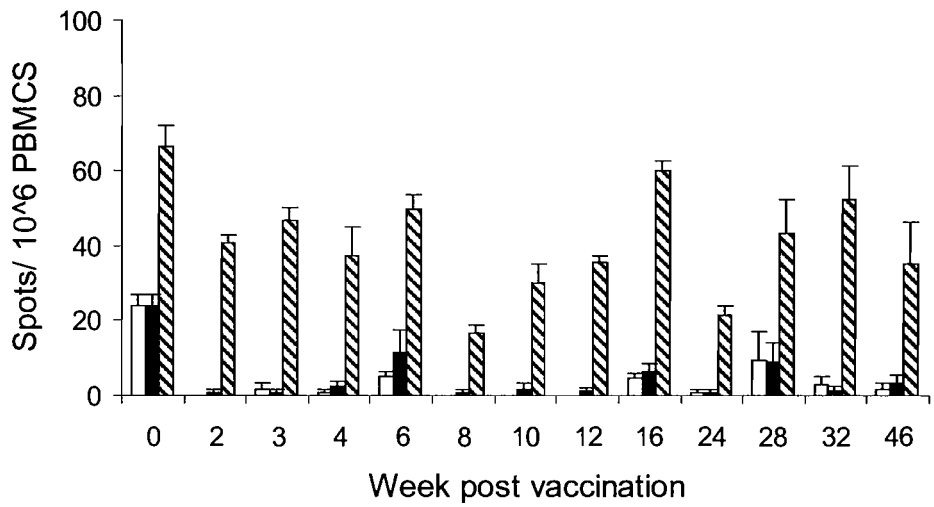


Figure 26. The number of Interferon-γ spots produced when PBMCs from donor 1 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▨).

2.6.2.2 Recipient 1

Recipient 1 was also seronegative for CMV. This patient was neutropenic and lymphopenic prior to transplantation. Tetramer analysis was performed on a very small number of cells pre-transplant. Again, an NLVPMVATV specific population was not detected (Figure 27 and 28). The response of this patient to NLV and GIL were measured by tetramer and Elispot until week 33 post-transplantation. Convincing populations of $CD8^+$ T-cells specific for NLVPMVATV were not detected at any point.

2.6.2.3 Donor 2

Donor 2 was CMV seropositive and was also homozygous for HLA-A*0201. CTLs specific for NLVPMVATV are often present at a high frequencey in such individuals. Prior to vaccination, 3.45% of $CD8^+$ cells were found to be specific for NLV by tetramer analysis (Figure 29). In contrast, only 0.1% of $CD8^+$ T-cells were specific for the control epitope GIL. After vaccination, NLV specific $CD8^+$ cells fell to 2% by weeks 2 and 3 before rising to 3.3% by week 8. This was followed by another fall at week 12 and 16 before a further rise at week 24 to their highest level, 3.9%. It is likely that the variation exhibited by donor 2 simply represents natural fluctuation in the level of NLV specific CTLs. It remains possible that pDOM.CMV-NLV vaccination could have contributed to these flucutations.

Fluctuation in the level of NLV specific CTLs was also noted in the elispot assay (Figure 30). The lowest number of NLV specific CTLs, 478 per 10^6 PBMCs, was detected at week 2 (Figure 30). The highest level, 1547 per 10^6 PBMCs, was observed at week 32. This latter result suggests that the week 32 tetramer result was erroneously low. All of these assays were performed on fresh cells. This approach has several advantages. Most notably, this makes more cells available for analysis as cells are inevitably lost in the freezing-thawing process. Performing the assays on fresh cells conserves this valuable resource and may be crucial when the cells of interest are present at a low frequency. The disadvantage of this approach is that an abberant result, such as the NLV tetramer result of Donor 2 at week 32, cannot simply be repeated and then substituted. CTLs specific for the control epitope, GILGFVFTL, were not detected in Donor 2 by elispot throughout follow-up. We experienced higher levels of background staining with the GIL tetramer than with the NLV tetramer. This accounts for the low level of GIL positive $CD8^+$ cells detected by tetramer analysis.

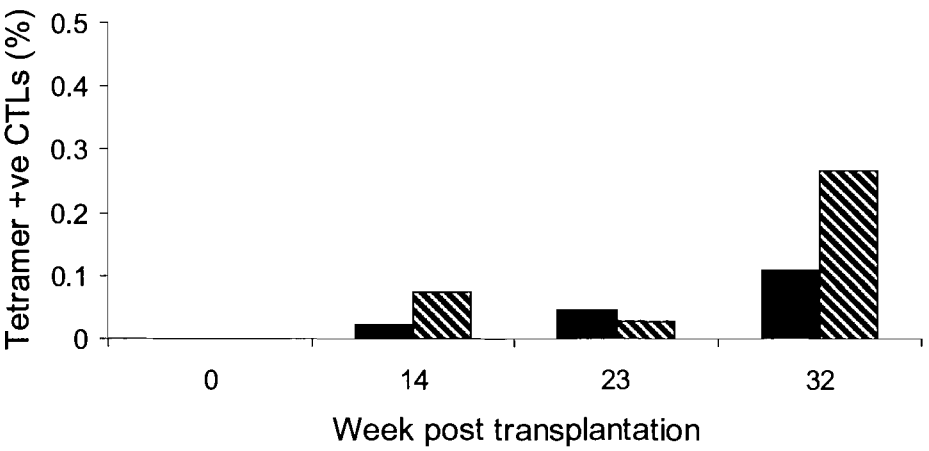


Figure 27. The proportion of peripheral blood CD8+ T cell population of recipient 1 which were specific for NLVPMVATV (■) or GILGFVFTL (▨).

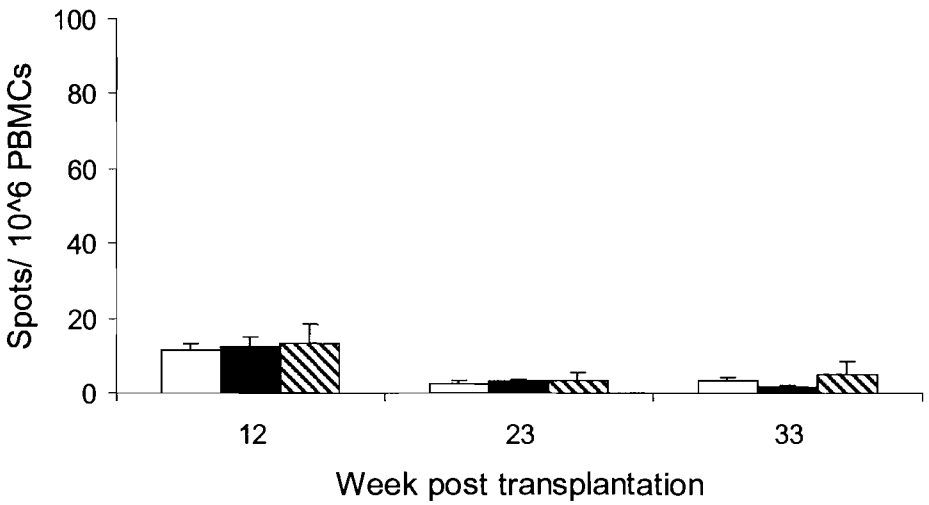


Figure 28. The number of Interferon-γ spots produced when PBMCs from Recipient 1 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▨).

2.6.2.4 Recipient 2

Recipient 2 was also CMV seropositive. The first NLV tetramer analysis was performed on his PBMCs at week 4 post-transplantation and we found that 11.6% of his $CD8^+$ T-cells were specific for NLV (Figure 31). By week 6, NLV specific cells comprised more than 19% of the total $CD8^+$ T-cells population. Immune reconstitution was at an early stage and the total number of $CD8^+$ T-cells was still at relatively low level. After transplantation, patients at risk for and subsequent CMV disease are monitored twice weekly for CMV reactivation using a PCR assay specific for CMV pp65. CMV reactivation was first detected by this technique at week 5 ½ post stem cell transplantation but returned to undetectable levels without requiring therapy. Reactivation was again detected at week 7. The patient commenced ganciclovir therapy at week 8 post transplant. CMV became undetectable again by PCR at week 9. Ganciclovir therapy was discontinued at week 10 after 2 negative PCR results had been received. The frequency of CTLs specific for NLV seemed to rise after each CMV reactivation before falling again to approximately 12%.

The Elispot assay confirmed that CTLs specific for NLV were present at a very high frequency in Recipient 2 (Figure 32). Again, there was some discordance between the elispot and the tetramer assays for individual weeks. As was found with donor 2, no CTLs specifc for GIL were detected by Elispot in Recipient 2 throughout follow-up (Figure 31 and 32).

2.6.2.5 Donor 3

Donor 3 was CMV seropositive. She was heterozygous for HLA-A*0201, unlike the first 2 donors who were both homozygous for HLA-A*0201. Tetramer analysis revealed that NLV specific $CD8^+$ cells comprised 0.17% of the total $CD8^+$ T-cell population prior to vaccination (Figure 33). This rose slightly to 0.31% at week 3. The level then fluctuated within the range 0.06-0.3% until week 44 when 0.7% of CD8 cells were found to be specific for NLV.

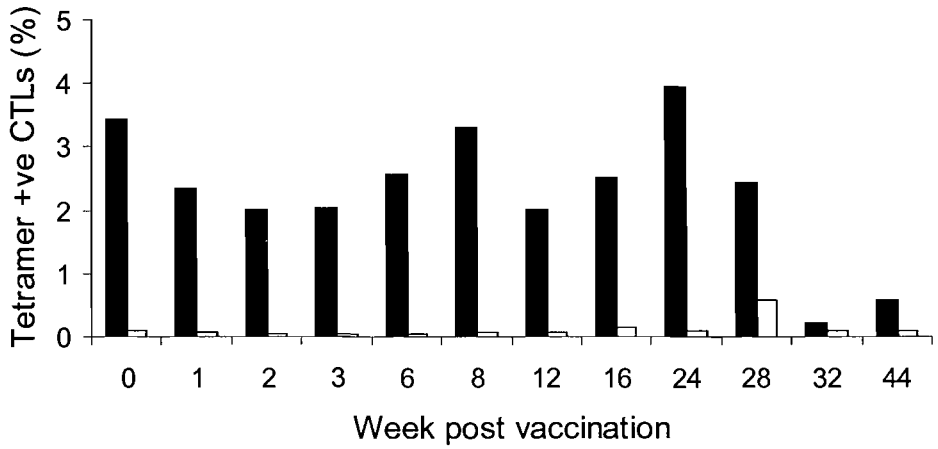


Figure 29. The proportion of donor 2 peripheral blood $CD8^+$ T cell population which was specific for NLVPMVATV (■) or GILGFVFTL (□)

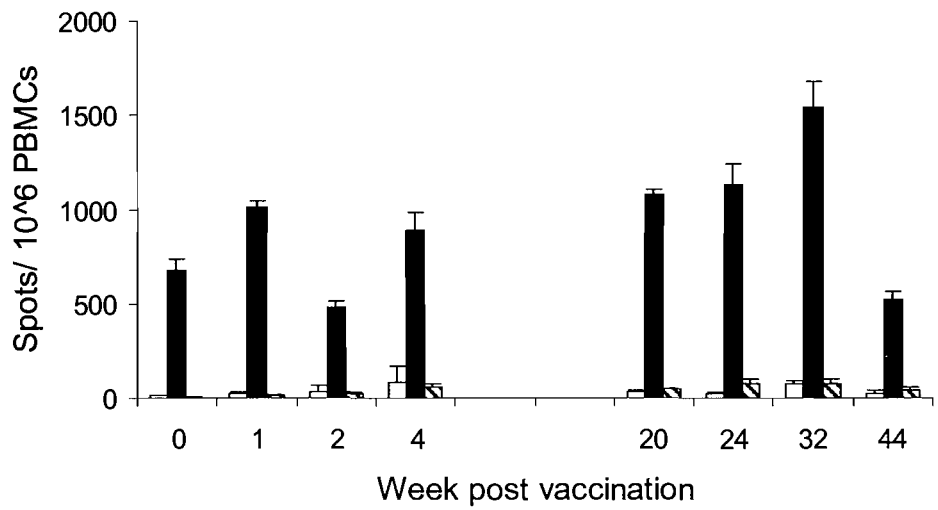


Figure 30. The number of Interferon-γ spots produced when PBMCs from donor 2 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▨).

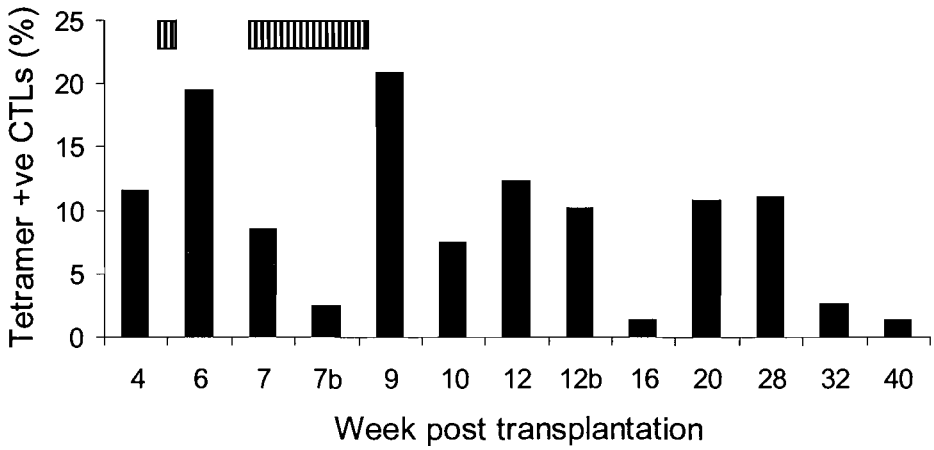


Figure 31. The proportion of peripheral blood CD8+ T cell population of recipient 2 which were specific for NLVPMVATV (■). HCMV reactivation is indicated above (▥).

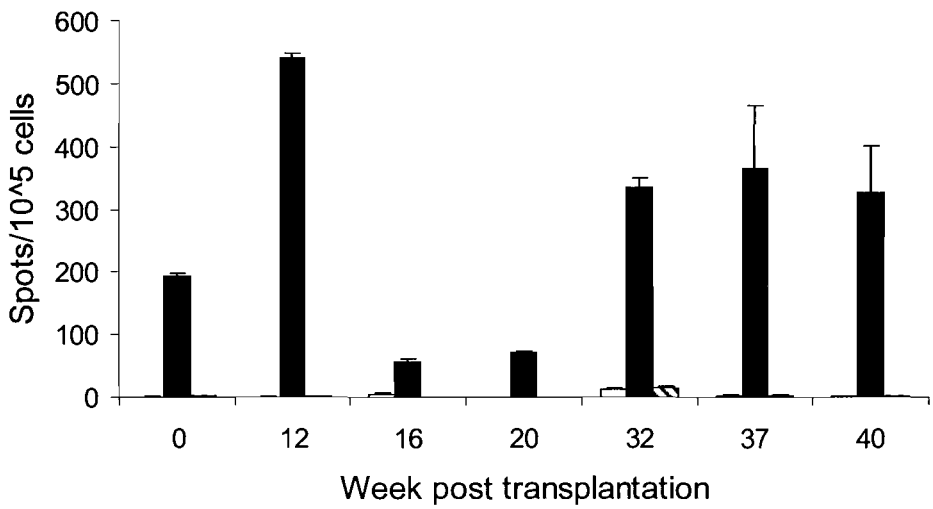


Figure 32. The number of Interferon-γ spots produced when PBMCs from recipient 2 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▨).

In concordance with the tetramer results of Donor 3, Interferon- γ producing $CD8^+$ cells specific for NLV were alsoconsistently detected in the Elispot assay (Figure 34). Pre-vaccination, 270 of 10^6 PBMCs were found to be NLV specific CTLs. The number of interferon-γ spots detected then fluctuated during follow-up from 100-572 per 10^6 PBMCs. NLV specific cells were detected in the Elispot assay of Donor 3 at a much higher frequency than those specific for GIL. This was not seen clearly in the tetramer assay. Again this was because the GIL tetramer assay suffered from higher levels of background staining. The Elispot assay was both more specific and more sensitive.

2.6.2.6 Recipient 3

Recipient 3 was found to be CMV seronegative when tested several months prior to transplantation. However, a baseline tetramer analysis performed 3 weeks prior to transplantation revealed that 2.5% of her $CD8^+$ T-cells were specific for NLV, a very high level to be found in a HLA-A*0201 heterozygote (Figure 35). Repeat serology was positive for CMV IgG and weakly positive for CMV IgM indicating a recent primary CMV infection. This recipient had a needle phobia and unfortunately developed a tunnel line associated thrombosis early in her post-transplant recovery. This made it very difficult to monitor her CTL responses to NLV and highlights another of the many difficulties encountered in performing clinical trials.

The level of NLV specific $CD8^+$ T-cells gradually fell and was 1.27% one week pre-transplant. Reactivation of CMV occurred very early in the post-transplant period. It was detected by pcr on day 14 and ganciclovir therapy was commenced on day 25. Tetramer analysis was unsuccessful during this period due to severely haemolysed blood samples and very low peripheral lymphocyte counts. Ten weeks after transplantation, 1.36% of her $CD8^+$ T cells were shown to be specific for NLV.Prior to transplant, NLV specific $CD8^+$ cells were also shown to be present at a high frqeuncy by the Elispot assay (Figure 36). At week -2, we observed that 897 of 10^6 PBMCs were NLV specific CTLs. This had fallen to $120/10^6$ PBMCs when the assay was repeated at 10 weeks post transplantation. CTLs specific for GIL were undetectable at both time points.

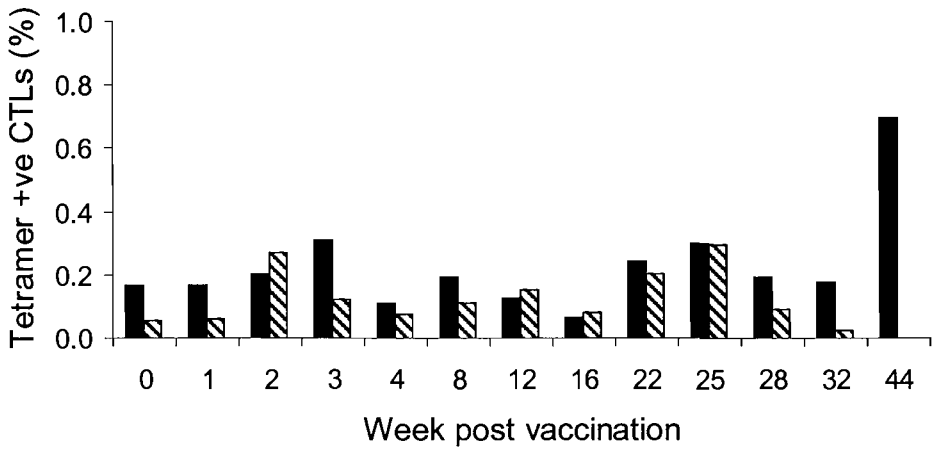


Figure 33. The proportion of donor 3 peripheral blood $CD8^+$ T cell population which was specific for NLVPMVATV (■) or GILGFVFTL (▧)

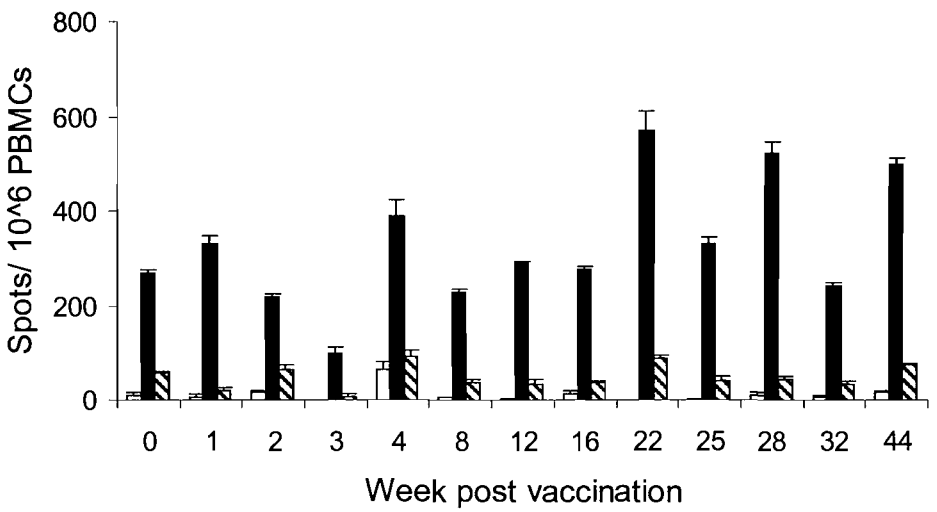


Figure 34. The number of Interferon-γ spots produced when PBMCs from donor 3 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▧).

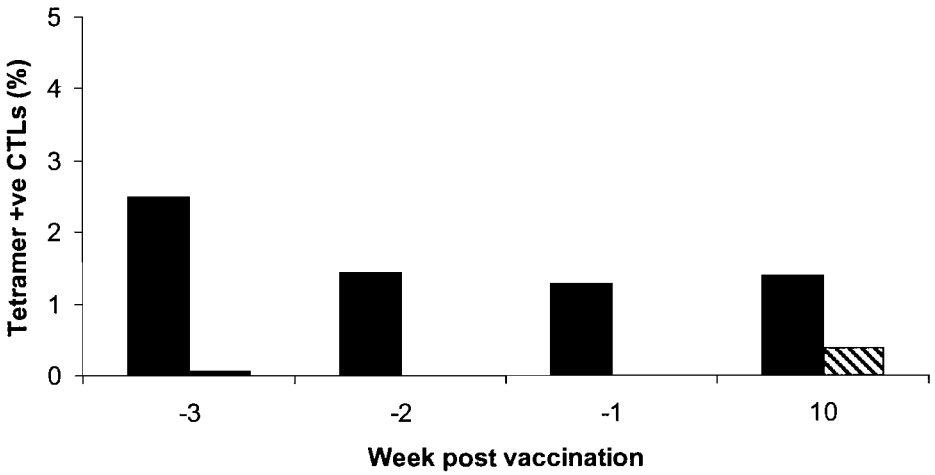


Figure 35. The proportion of peripheral blood CD8+ T cell population of recipient 3 which were specific for NLVPMVATV (■) or GILGFVFTL (▨).

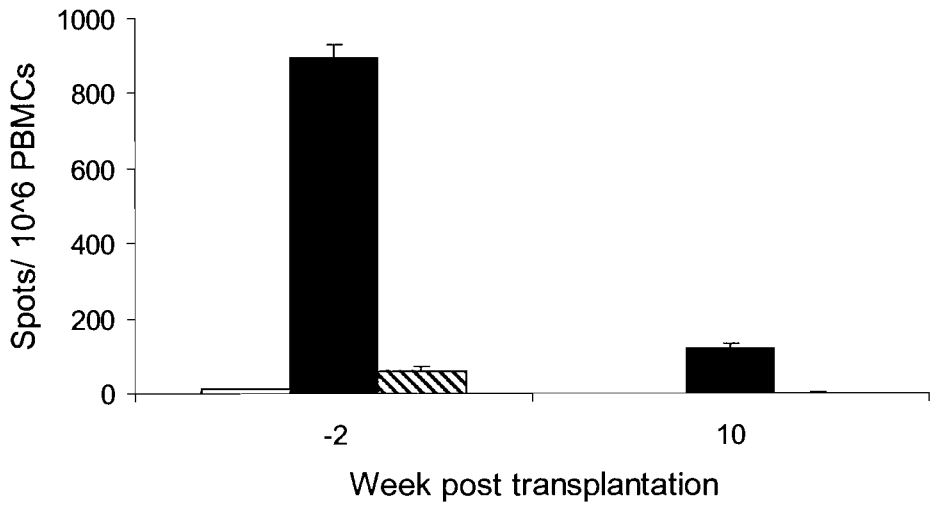


Figure 36. The number of Interferon-γ spots produced when PBMCs from recipient 3 were incubated over night with no peptide (□), NLVPMVATV (■) or GILGFVFTL (▨).

2.6.3 FrC Elispot

The pDOM vaccine is designed to induce CTLs against a defined epitope. However, as pDOM is derived from Fragment C of tetanus toxin, successful vaccination with pDOM may have influenced the numbers of circulating FrC specific $CD4^+$ T-cells. We therefore performed Interferon-γ Elispot assays to monitor these cells before and following p.DOM.NLVPMVATV vaccination.

2.6.3.1 Donor 1

$CD4^+$ T cells from donor 1 did not produce Interferon-γ in response to incubation with FrC or P30 either pre- or post-vaccination

The frequency of donor 1 $CD4^+$ T-cells specific for FrC was assessed before and after vaccination in an interferon-γ elispot assay. A response to incubation with FrC was not detected prior to vaccination or to week 8. A small response to FrC was detected at week 16 but this was not apparent at week 20 (Figure 37).

2.6.3.2 Donor 2

Prior to vaccination, a clearly defined population of FrC specific CD4+ T-cells was detected by interferon-γ Elispot at a frequency of 33 cells per 10^6 PBMCs (Figure 38). By week 4 post vaccination this had risen to 78 cells per 10^6 PBMCs. The frequency of these cells remained elevated when Donor 2 was reassessed at week 8 ($74/10^6$ PBMCs). The level had fallen by week 12 to $43/10^6$ PBMCs and then fluctuated from $34\text{-}59/10^6$ PBMCs throughout follow-up. The increased levels detected at weeks 4 and 8 be indicative of a small response to vaccination or could represent a natural fluctuation in the frequency of these cells in the peripheral blood of Donor 2

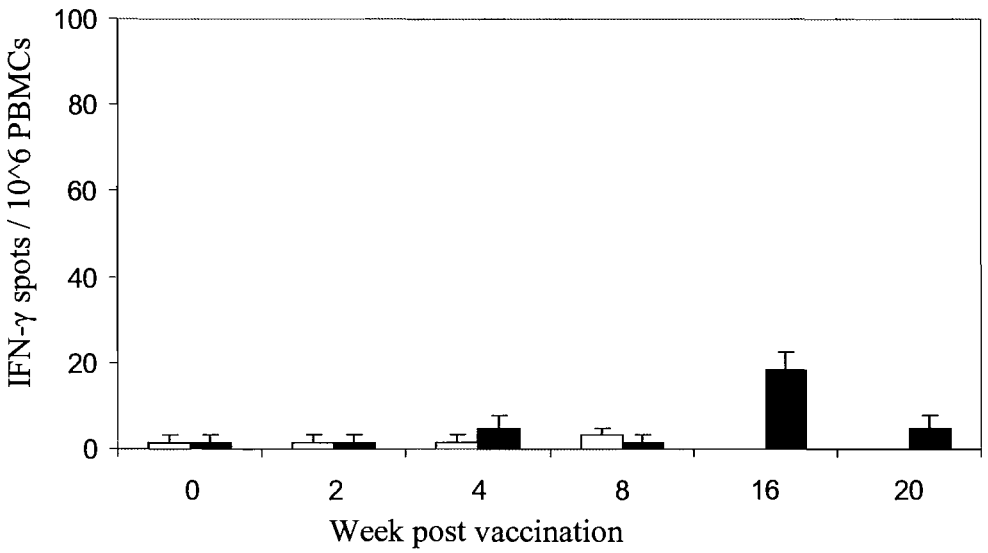


Figure 37. The number of Interferon-γ spots produced when PBMCs from donor 1 were incubated with no peptide (□) or FrC (■).

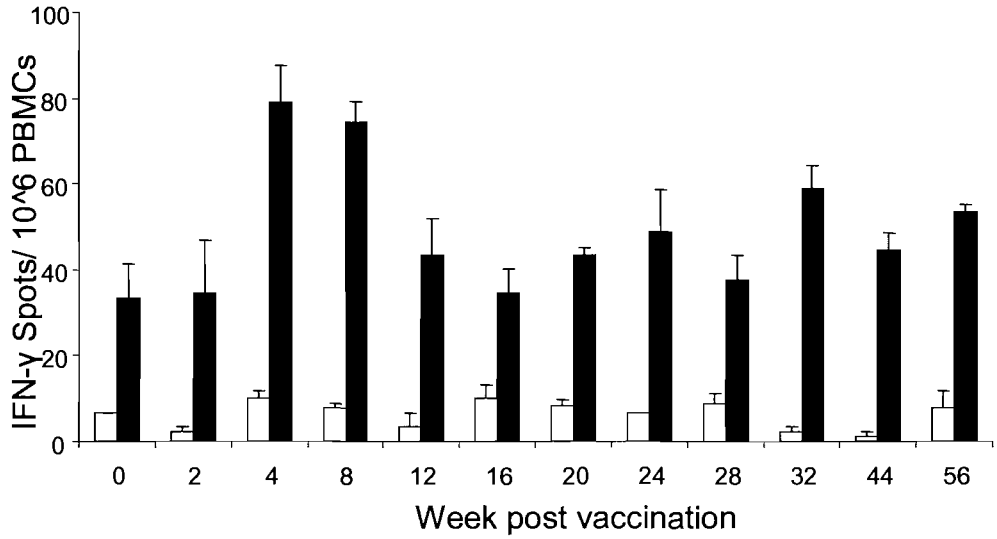


Figure 38. The number of Interferon-γ spots produced when PBMCs from donor 2 were incubated with no peptide (□) or FrC (■).

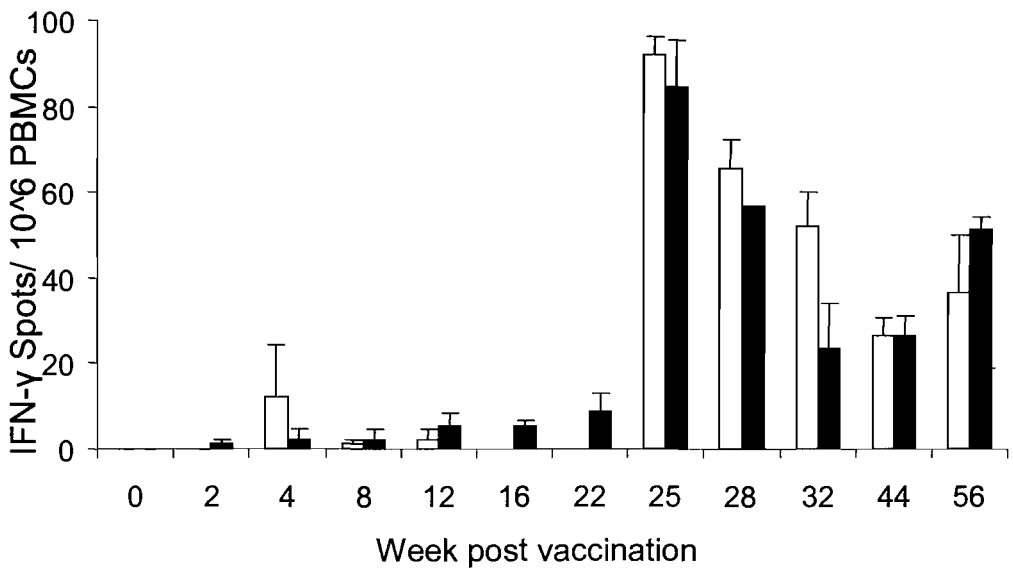


Figure 39. The number of Interferon-γ spots produced when PBMCs from donor 3 were incubated with no peptide (□) or FrC (■).

2.6.3.3 Donor 3

Donor 3 was similar to donor 1 in that a population of FrC specific CD4 T-cells could not be detected either prior to vaccination or during follow-up (Figure 39).

2.6.4 Antibody responses

Although the DNA vaccine p.DOM.NLVPMVATV was not designed to induce an antibody response, it does contain a large portion of FrC. We therefore monitored anti-FrC antibody levels prior to and following vaccination.

2.6.4.1 Donor 1

Vaccination with pDOM.CMV did not cause an increase in the levels of anti-TT or anti-FrC antibodies.

Antibodies against FrC were detected in Donor 1 at a level of 73IU/ml pre-vaccination. This level was found to fluctuate within the range 61-82IU/ml during follow-up. A significant increase in anti-FrC antibody levels was not induced by vaccination with p.DOM.NLVPMVATV (Figure 40).

2.6.4.2 Donor 2

Anti-FrC antibodies were present in Donor 2 at much lower levels than in Donor 1 prior to vaccination. Again, vaccination with p.DOM.NLVPMVATV did not induce a substantial rise in anti-FrC levels (Figure 41).

2.6.4.3 Donor 3

Before vaccination with p.DOM.NLVPMVATV Donor 3 also exhibited much lower anti-FrC antibody levels than Donor 1. At week 0, anti-FrC antibodies were present at 6.75 IU/ml. Anti-FrC levels fluctuated slightly during follow-up but no definitive rise in FrC antibodies was detected during the observation period (Figure 42).

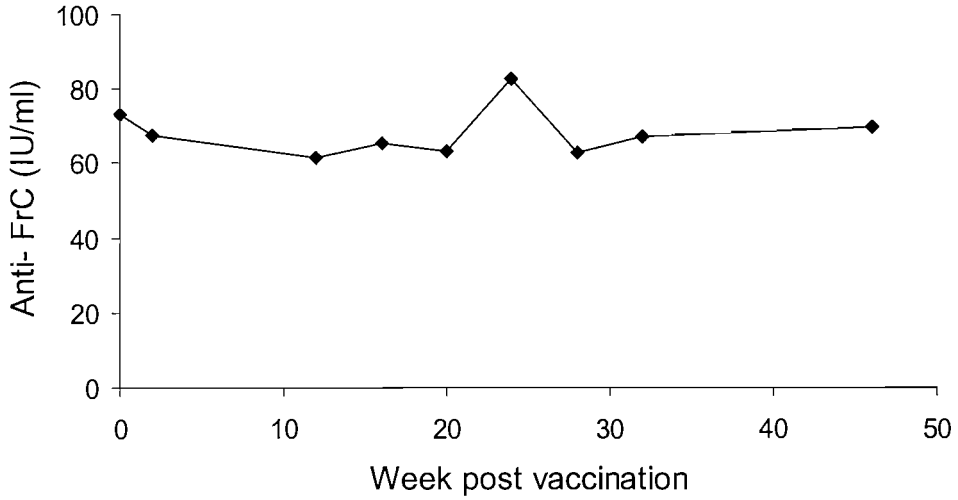


Figure 40. Donor 1 anti-FrC antibody levels

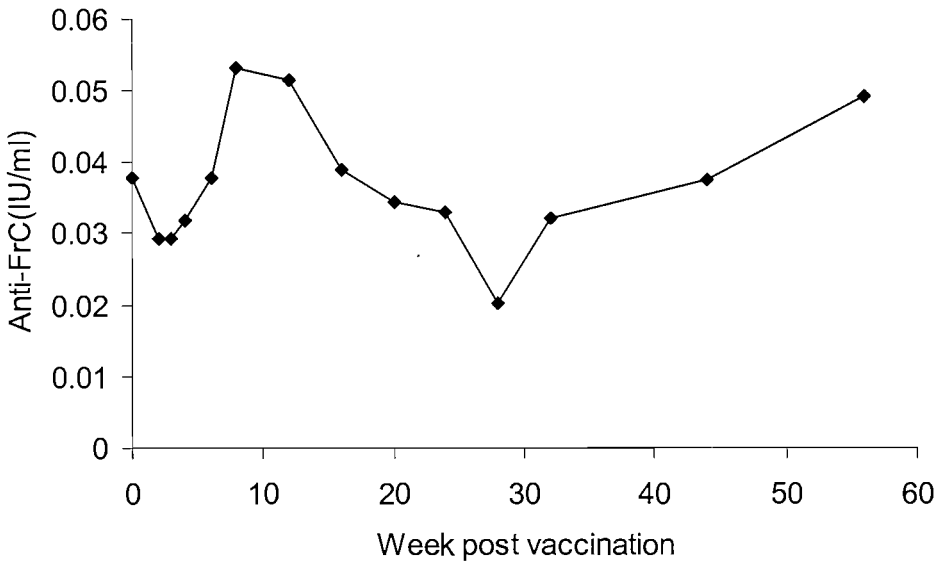


Figure 41. Donor 2 anti-FrC antibody levels

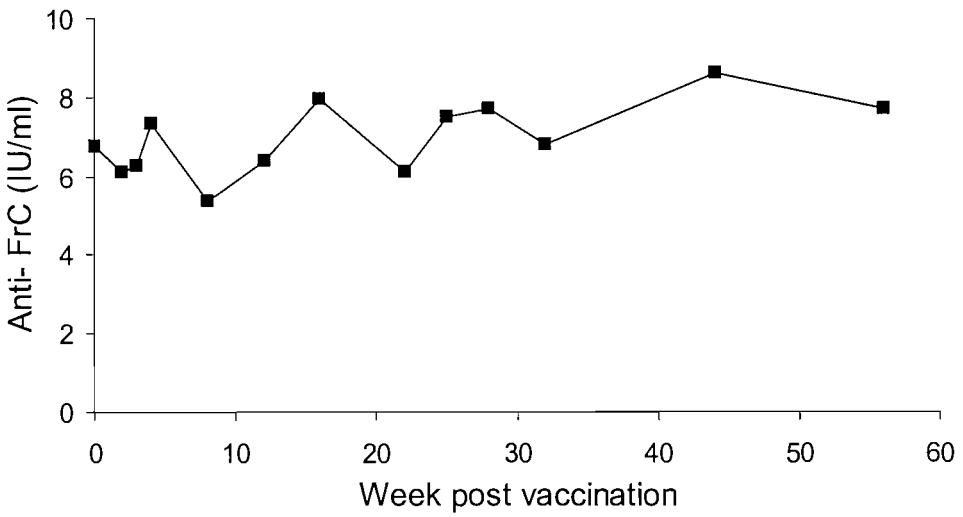


Figure 42. Donor 3 anti-FrC antibody levels

2.6.4.4 Recipient Anti-FrC levels

Anti-FrC antibody levels showed similar kinetics in Recipients 1 and 2 (Figures 43 and 44). Both recipients had anti-FrC levels of approximately 15IU/ml when first tested 6-12 weeks after transplantation. The level gradually tailed off during follow-up. The final sample taken from Recipient 2 at week 51 showed a slight increase in the level of anti-FrC antibodies.

Recipient 3 also showed a fall in antibody levels from week 10 to week 22 (Figure 45). We were able to measure anti-FrC levels in this individual at week 0. Recipient 3 experienced a significant rise in anti-FrC level from week 0 to week 4 and 10. The significance of this is unclear.

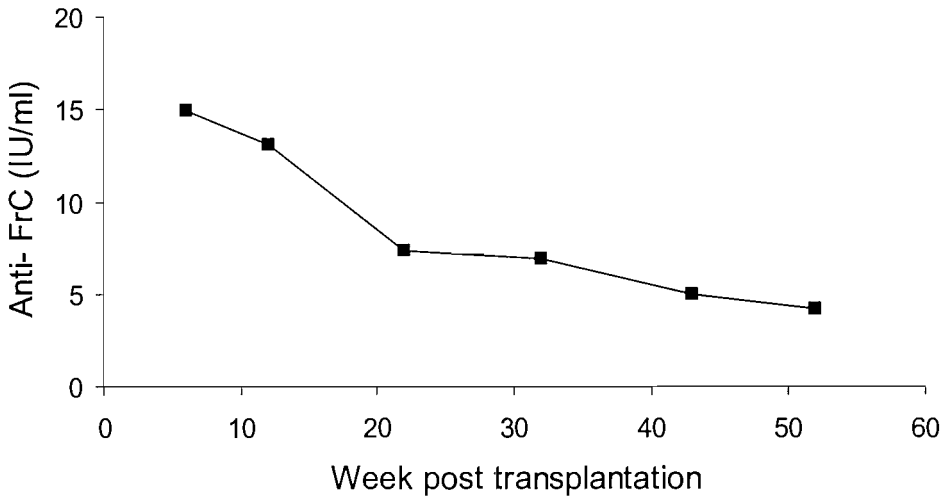


Figure 43. Recipient 1 anti-FrC antibody level

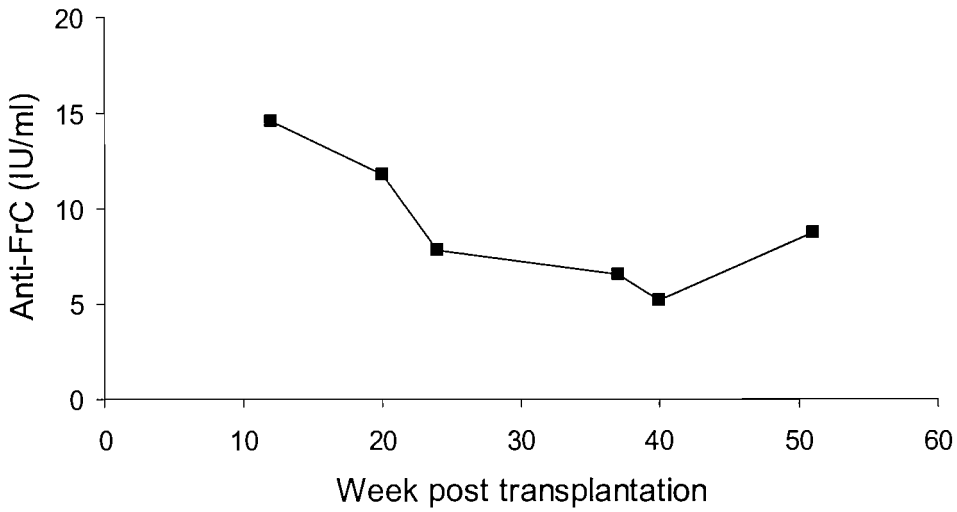


Figure 44. Recipient 2 anti FrC antibody level

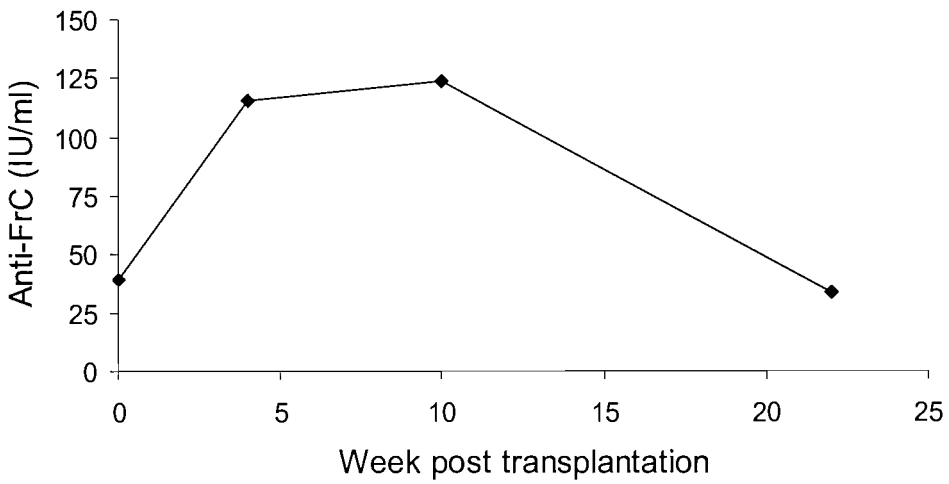


Figure 45. Recipient 3 Anti-FrC antibody level

2.6.5 Chimerism studies

2.6.5.1 Recipient 2

The $CD8^+$ T-cells specific for NLVPMVATV detected at high frequency in recipient 2 were of donor origin.

It was important to determine if the NLV specific T-cells detected at such high frequencies in recipient 2 were of donor origin. It is routine practice in transplant centres to identify polymorphic differences between the stem cell donor and recipient prior to transplantation. These differences can then be used in chimerism studies after transplantation to establish if full engraftment has occurred. Donor lymphocyte infusions can then be administered if cells of recipient origin are found to be increasing in number. Markers which distinguished between donor 2 and recipient 2 had therefore already been identified. Using a FACSvantage, NLVPMVATV specific $CD8^+$ T–cells from recipient 2 were separated from the total $CD8^+$ T-cell population. After selection, over 90% of the $CD8^+$ T-cells were specific for NLV (Figure 46). Both the NLV specific $CD8^+$ cells and the remaining $CD8^+$ cells were sent to the Wessex regional genetics laboratory for chimerism studies. This identified that 100% of the cells in both groups were of donor origin.

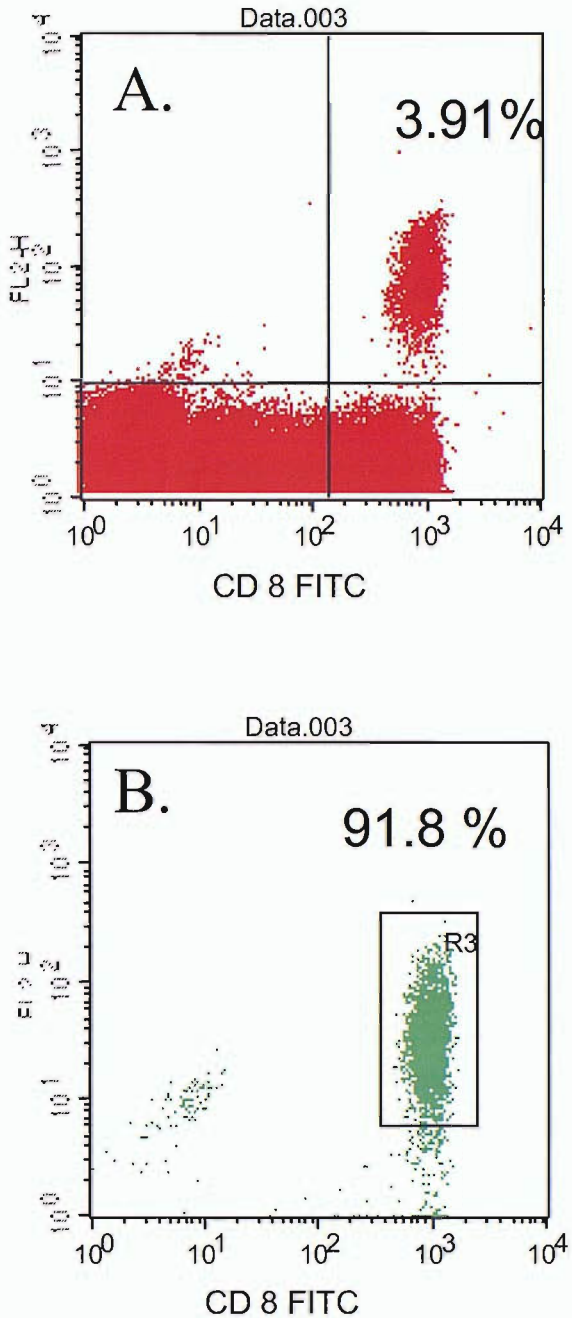


Figure 46. Analysis of peripheral blood of recipient 2 before and after PE-labelled NLVPMVATV tetramer FACS selection. A). Prior to selection 3.91% of the $CD8^+$ T-cell population of recipient 2 were specific for NLVPMVATV. B). Post selection over 90% of the $CD8^+$ T-cell population were NLVPMVATV specific. FL2-H = PE labelled NLVPMVATV tetramer.

2.6.6 Development of a method to expand peptide specific $CD8^+$ T-cells

The NLV tetramer assay and the interferon-γ Elispot assay are both designed to detect NLVPMVATV specific $CD8^+$ cells in the peripheral blood. The tetramer assay can detect cells at a frequency of 0.1% of the total $CD8^+$ population whereas the elispot is more sensitive and can detect specific a population which makes up 0.0025-0.01% of the total PBMC population. It remains possible that the pDOM.CMV vaccine had induced cells in donor 1 at a frequency that was below the sensitivity of our assays. We therefore sought to develop an assay which would improve our ability to detect cells present at frequency lower than that which can be confidently detected by the methods used above. We required a technique which could expand low numbers of specific cells to a level where they could be detected by tetramer or elispot. Mature dendritic cells are the most effective antigen presenting cells. Our aim was to develop a technique using mature dendritic cells to expand a low frequency population of specific $CD8^+$ cells to a much higher frequency.

2.6.6.1 Immunophenotype of dendritic cells matured with MCM-mimic or LPS/IFN-γ

Dendritic cells were generated by exposing adherent PBMCs from a healthy volunteer to GM-CSF and IL-4. The cells were immunophenotyped and found to express low levels of CD86, HLA-DR and CD83 in addition to high levels of CD1A, consistent with immature dendritic cells. Dendritic cells were matured with either Lipopolysaccharide and Interferon-γ or monocyte conditioning medium mimic (MCM-mimic). MCM-mimic is a defined cocktail of IL-1β, TNF-α, IL-6 and prostaglandin E_2. The immunophenotype of dendritic cells matured by each method was compared. Both groups of dendritic cells were found to express a mature phenotype. The cells which had been matured with MCM-mimic expressed higher levels of HLA-DR, CD86 and CD83 with lower levels of CD1A than those matured with lipopolysaccharide/ Interfeon γ (Figure 47). This result suggested that dendritic cells matured by MCM-mimic were more mature than those developed by lipopolysaccharide/ Interferon γ and therefore should also be more proficient at presenting antigens to T cells.

2.6.6.2 Ability of dendritic cells matured by MCM-mimic to expand specific CTLs compared with that of dendritic cells matured by LPS/IFN-γ.

CD8 cells from a HLA-A0201 individual (who was known to have a detectable peripheral blood CD8 population specific for GILGFVFTL) were incubated in a 96well U bottomed plate with GILGFVFTL loaded autologous dendritic cells in the presence of IL-2 and IL-7. The ratio was ten CD8 cells to one dendritic cell. Initially, GILGFVFTL specific CTLs comprised 0.1% of the CD8 population (Figure 48A). After 7 days, the dendritic cells matured by lipopolysaccharide and interferon-γ had expanded the GILGFVFTL specific CTLs to **7%** of the total CD8 positive cells (Figure 48C). Dendritic cells matured by MCM-mimic had only expanded GILGFVFTL specific CTLs to **1%** of the total CD8 positive cells (FIGURE 48B).

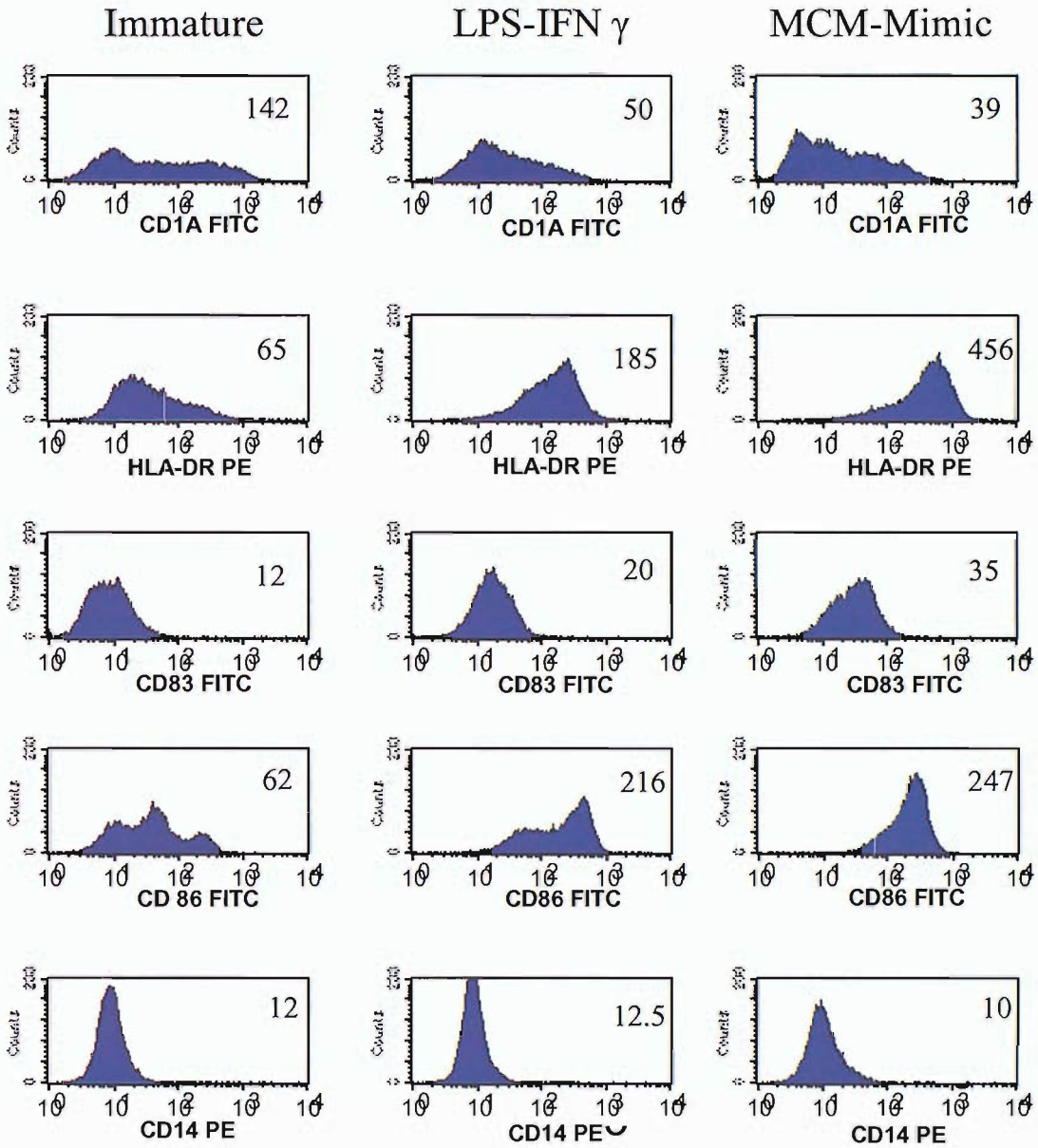


Figure 47. FACS analysis of immature, Lipopolysacharide/Interferon-γ matured and monocyte conditioning medium –mimic (MCM-mimic) matured dendritic cells.

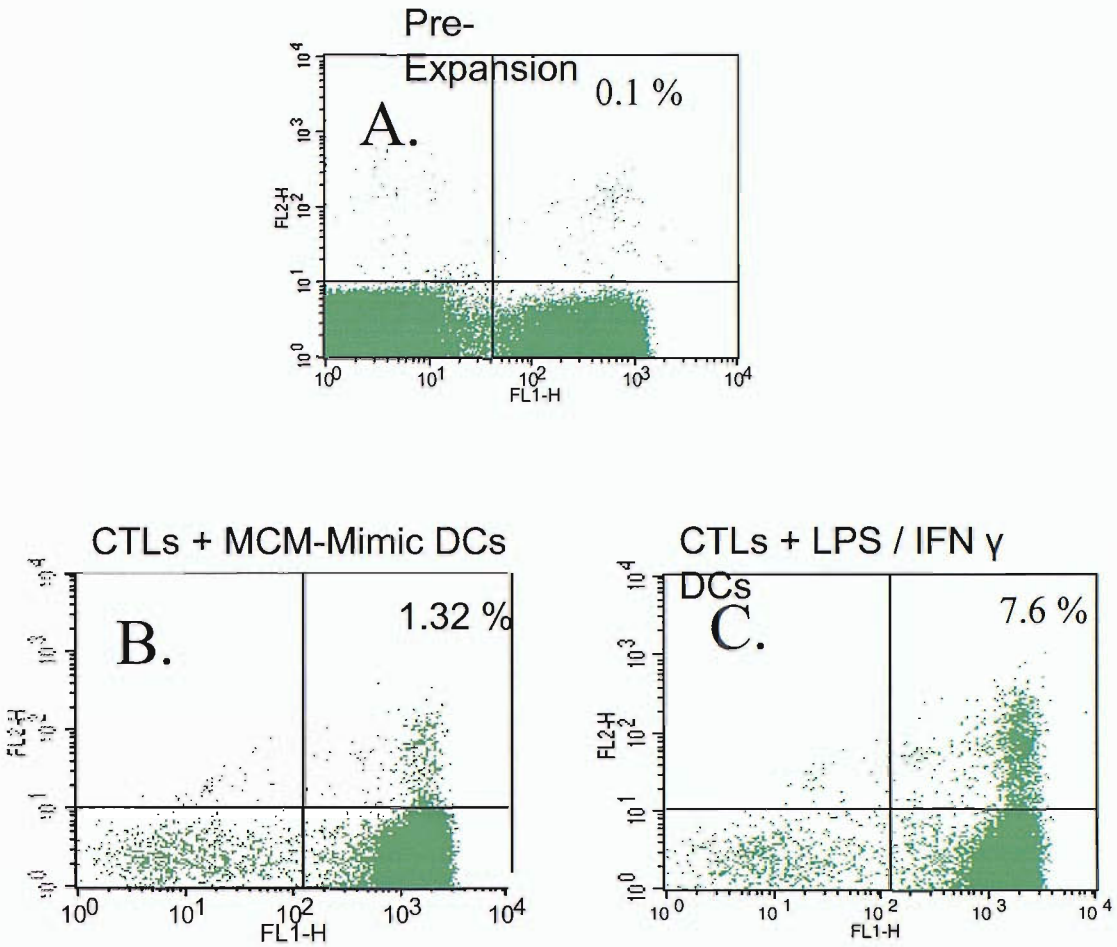


Figure 48. FACS analysis of CTLs pre and post expansion. FL1-H = CD8 FITC. FL2-H = GILGFVFTL PE labelled tetramer. **(A.)** Prior to expansion, GILGFVFTL specific CTLs comprise 0.1% of the $CD8^+$ population.
(B.) After a 7 day co-incubation with GILGFVFTL loaded DCs matured by MCM-Mimic, GILGFVFTL specific CTLs comprise 1.32 % of the $CD8^+$ population,
(C.) After co-incubation with GILGFVFTL loaded DCs matured by LPS / IFN γ, GILGFVFTL specific CTLs make up 7.6 % of the total $CD8^+$ population.

2.6.6.3 Comparison of the specific cytotoxicity of specific CTLs expanded by either MCM-mimic matured or LPS/IFN-γ matured dendritic cells.

The ability of the cytotoxic T cells which had been expanded by either the MCM-mimic or LPS/IFN-γ dendritic cells to lyse GILGFVFTL loaded T2 cells was assessed in a ^{51}Cr release cytotoxicity assay. The T2 cells had been loaded with GILGFVFTL at the following concentrations: A: 0.001uM, B: 0.1μM, C: 10μM or no peptide. The CTLs expanded by LPS/IFN-γ matured dendritic cells were superior to those expanded by MCM-mimic matured dendritic cells at causing cytolysis of T2 cells exposed to peptide at all three concentrations (FIGURE 49).

2.6.6.4 Comparison of the capacity of peptide pulsed dendritic cells to expand peptide specific CTLs compared with that of peptide pulsed PBMCs

The ability of peptide loaded dendritic cells, which had been matured with LPS/ IFNγ, to expand GILGFVFTL specific CTLs were compared with that of peptide loaded PBMCs. Previous assays had shown that GILGFVFTL specific CTLs comprised 0.1% of the volunteer's $CD8^+$ T cell population. Autologous selected CD8 cells at $1x10^5$ cells per well were incubated with $1x10^4$ dendritic cells or $2x10^5$ PBMCs. The capacity of PBMCs to expand specific CTLs was examined under four different experimental conditions. The first group of PBMCs were co-incubated with 10μM GILGFVFTL peptide only. The second group were incubated with peptide and autologous selected $CD8^+$ T cells at $1x10^5$ cells per well. The third group were incubated with peptide, $CD8^+$ cells, IL-12 and IL-7. Finally, the fourth group were incubated with peptide, $CD8^+$ cells, IL-12, IL-7, CD28 and CD49d.

CTLs specific for GILGFVFTL comprised 11.6% of the total CD8 population after expansion with peptide loaded dendritic cells. PBMCs in the presence of IL-12, IL-7, CD28 and CD49d also expanded CD8 cells specific for GILGFVFTL, but only to 2.09% of the total CD8 population. PBMCs with IL-12 and IL-7 expanded specific CD8 cells to 1.89% in the presence of IL-12 and IL-7 and to 1.86% without IL-12 or IL-7. PBMCs with GILGFVFTL peptide alone only expanded specific CD8 cells to 0.39% (repeated, 0.47%)

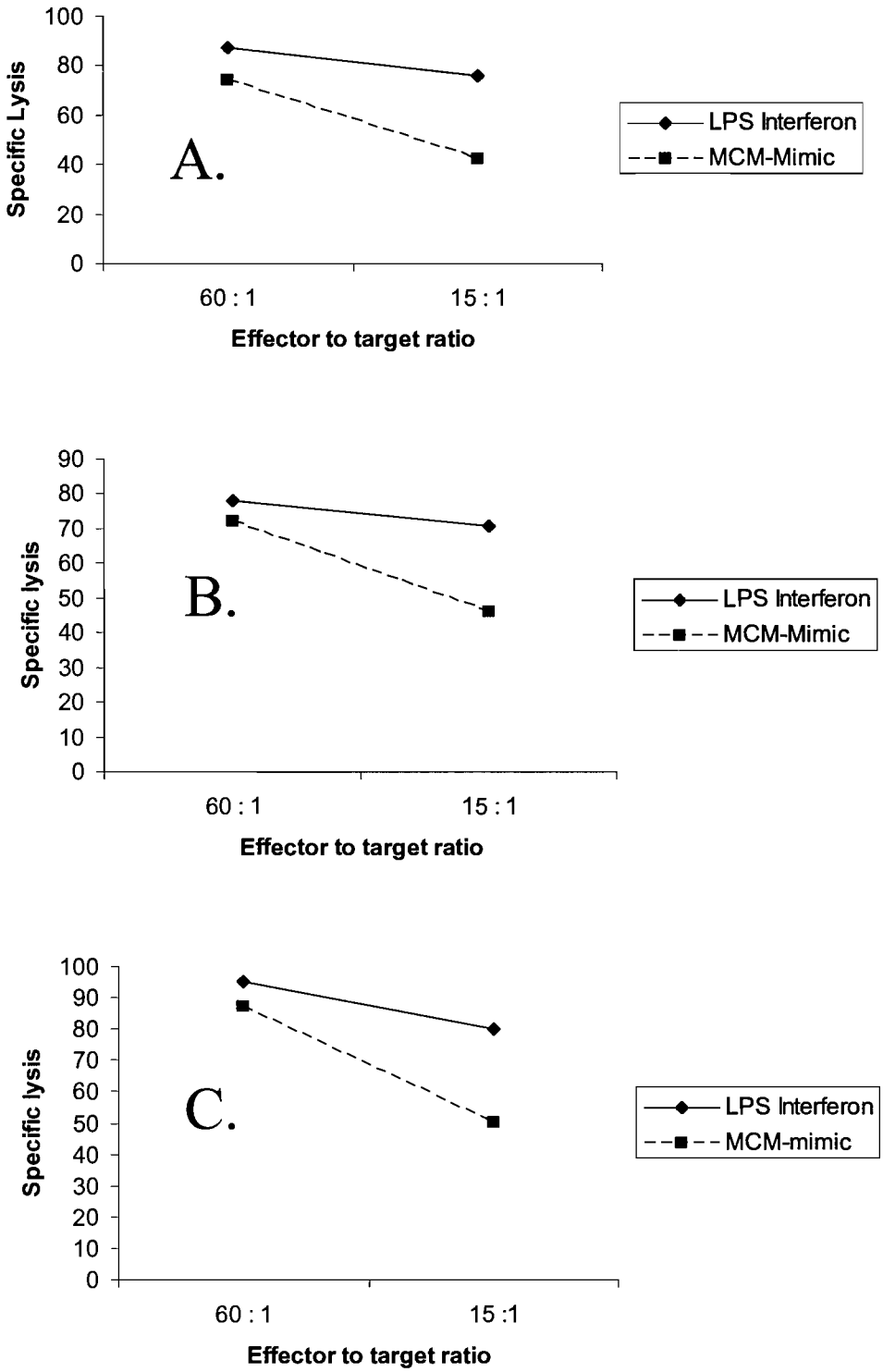


Figure 49. Specific lysis of GILGFVFTL loaded target cells by CTLs expanded by DCs which had themselves been loaded with GILGFVFTL at **A)** 0.001μM, **B)** 0.1 μM and **C)** 10μM. DCs matured by LPS / IFNγ shown by an unbroken line. DCs matured by MCM-Mimic shown by a broken line.

It was concluded that peptide pulsed dendritic cells which had been matured by LPS/ IFN γ were most effective at expanding a specific CTL population (Figure 50).

2.6.6.5 Incubation of CTLs from Donor 1 with NLVPMVATV pulsed autologous dendritic cells.

Dendritic cells were developed from fresh PBMCs of Donor 1. They were matured by exposure to Lipopolysaccharide and Interferon γ and then pulsed with NLVPMVATV peptide. The dendritic cells were then incubated with autologous $CD8^+$ T-cells which had been selected from PBMC samples collected and frozen at weeks 0, 4, 8 and 16 post-vaccination.

After 7 days incubation, FACS analysis was carried out to determine the number of NLVPMVATV specific CD8 cells present. The results were very similar for each time point. Analysis of the results indicated that approximately 0.05% of $CD8^+$ cells were specific for NLVPMVATV (Figure 51). This result seems to indicate that vaccination had not increased the number of NLVPMVATV specific CD8 detectable by week 16 in the peripheral blood of Donor 1. It remains possible that the true NLVPMVATV specific CTL population is masked by artefactual non-specific staining of cells which may prevented the detection of a small difference in the frequency of NLVPMVATV specific CTLs induced by vaccination.

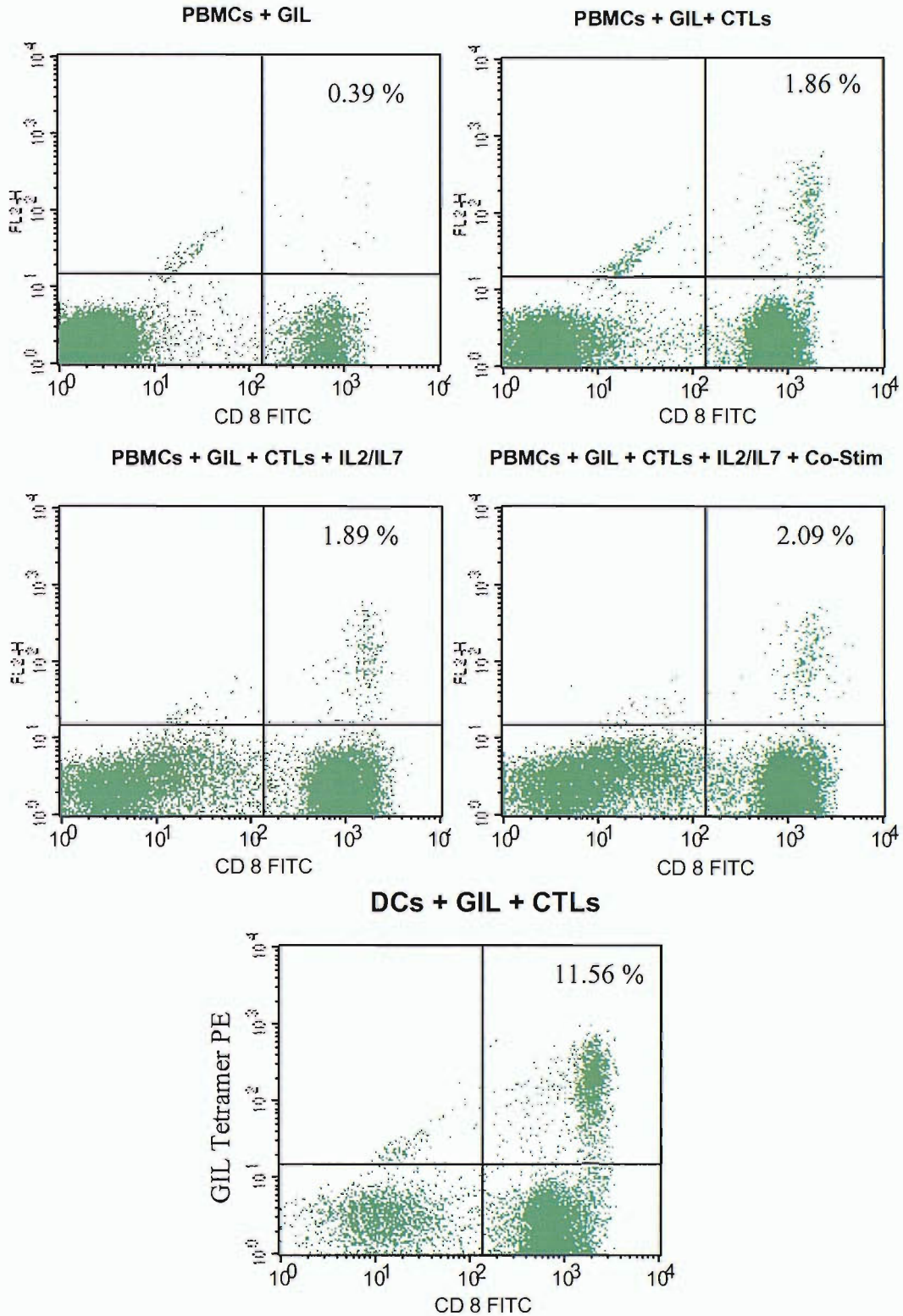


Figure 50. Following a one week co-incubation, GILGFVFTL loaded dendritic cells expanded greater numbers of GILGFVFTL specific CTLs than peptide loaded PBMCs in the presence or absence of cytokines and co-stimulatory molecules. FL2-H = PE labelled GILGFVFTL tetramer.

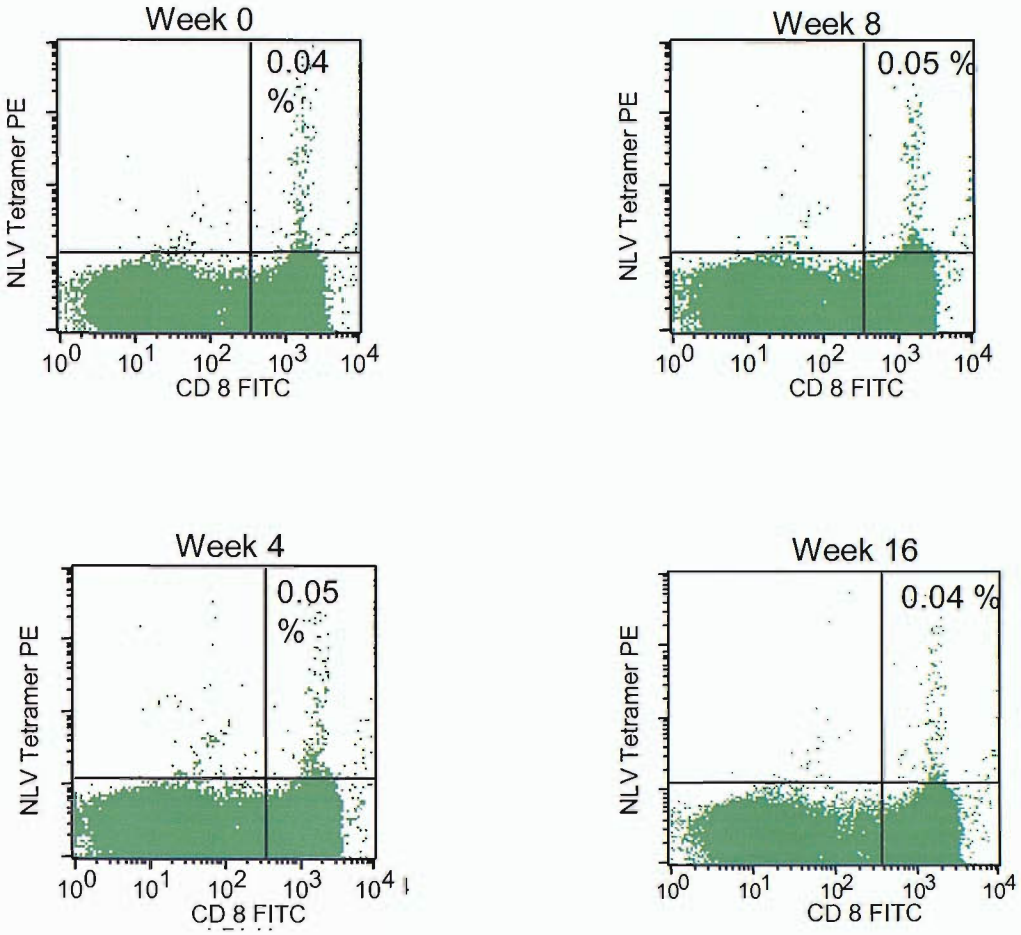


Figure 51. CTLs isolated from Donor 1 at weeks 0,4,8 and 16 were incubated with NLVPMVATV pulsed autologous dendritic cells. After 7 days the CTLs were analysed by FACS to asses the number of NLVPMVATV specific CTLs. Vaccination with pDOM.NLVPMVATV had not induced a specific CTL population which could be expanded in vitro.

2.7 Discussion

We aimed to use the DNA vaccine p.DOM.NLVPMVATV to induce or augment a CTL response against the epitope NLVPMVATV derived from the pp65 protein of CMV. The ability of the vaccine to induce immune responses was then evaluated in the laboratory by immunological assays. In humans we are limited to searching in samples of peripheral blood for responses to p.DOM.NLVPMVATV vaccination. This makes cellular immune responses, especially those of low magnitude, much more difficult to monitor in humans than in mice where responses in splenic or other lymphoid tissue can be assessed in a relatively easily.

Despite these limitations, the assays employed in this study allowed us to measure several components of the immune system which may have been affected by p.DOM.NLVPMVATV vaccination. The ex-vivo NLV tetramer and Elispot assays quantify the number of NLV specific effector CTL present in the peripheral blood. An attempt to detect the presence of central memory cells in the peripheral blood of donor 1 after vaccination was made by incubating CD8 T-cells with autologous NLVPMVATV pulsed mature dendritic cells. The FrC elispot assesses the frequency of T_H1 CD4 T-cells present in the peripheral blood that are specific for FrC. The p.DOM.NLVPMVATV vaccine contains the first domain of FrC against which a CD4 response may be stimulated by vaccination. Although the aim of the vaccine is to stimulate an effective CTL response, the presence of a portion of FrC could cause an increase in anti-FrC antibody titres. By extrapolating from experience in murine models, we would not expect succesful vaccination with p.DOM.NLVPMVATV to have significantly increased the level of FrC antibodies present in our vaccine recpients. Nonetheless, measurement of anti-FrC antibodies was clearly desirable for a complete evaluation of the immune response to p.DOM.NLVPMVATV vaccination.

In this study we vaccinated volunteers who were either CMV seropositive or seronegative. These are two very different settings in which to test our vaccine. The successful vaccination of a seronegative individual requires the vaccine to induce a response against an antigen which the immune system has not previously encountered. This is a considerable challenge. In comparison to standard protein vaccines, DNA vaccines, as currently administered, have proved in humans to be relatively weak. It was anticipated that an immune response induced by p.DOM.NLVPMVATV could therefore be quite small in magnitude and assays designed to detect such a response would have to be very sensitive.

The vaccination of CMV seropositive individuals presents an entirely different challenge both for the vaccine and for our ability to monitor the immune response to the vaccine. In this setting, memory and effector $CD8^+$ T-cells specific for NLVPMVATV are already present, often at very high levels. The immune system of such a volunteer would be expected to be much more responsive to a vaccine which delivered the NLVPMVATV epitope than that of an individual who had not previously encountered this antigen. However, the monitoring of a specific CTL response induced by p.DOM.NLVPMVATV is made very difficult by high numbers of specific CTLs already present before vaccination. In addition, the effect that successful vaccination might have on the peripheral blood frequency of NLVPMVATV specific CTLs is unclear. It would seem likely that the frequency these cells in the blood should rise if vaccination was succesful. However, it is also possible that successful vaccination would initially cause the levels of these cells in the blood to fall as they were recruited to the vaccination site or the lymphoid system. Perhaps the immune system could respond to succesful vaccination by increasing numbers of specific effector CTLs at the vaccine site only or by increasing the numbers of memory CTLs present in the lymphoid system. Both of these outcomes would represent "successful" vaccination but would be accompanied by minimal changes in specific CTL numbers detectable in the blood.

Of even greater concern is recent data from murine experiments which indicate that although DNA can be very effective in priming an immune response, it is ineffective when used as a boosting vaccination in heterologous prime-boost strategies[240-242]. This is thought to result from the ability of pre-existing CTL to kill APCs loaded with low amounts of antigen generated by DNA vaccination[242]. As donors 2 and 3 were CMV seropositive, the administration of the DNA vaccine pDOM.NLVPMVATV could be regarded as a DNA boost following a previous "virus prime" and may not now represent a strategy which is likely to be effective.

The development of a vaccine which could induce a specific CTL reponse against NLVPMVATV in mice was a significant achievement. To translate and reproduce this effect in humans presents considerable new challenges to our vaccine and to our ability to measure a successful response. The pilot clinical trial within which the results above were obtained represents our first attempts to wrestle with these challenges and the results must be interpreted in light of such considerations.

Donor 1 was CMV seronegative. As expected, CTLs specific for NLVPMVATV were not detected by tetramer or elispot prior to vaccination. The induction of a CTL response to this epitope was not detected by these assays at any point after vaccination. This can be interpreted in several ways. It seems likely that an immune response was not

induced in this individual by p.DOM.NLVPMVATV. It is also possible that an immune response did occur, but at a level below the sensitivity of our assays.

To increase our ability to detect a small response we sought to develop an assay in which specific CTLs could be expanded from low levels of central memory cells, which may have been induced by vaccination, to much higher levels. We chose to use autologous mature dendritic cells pulsed with specific peptide, as they are known to be the most effective antigen presenting cells. We compared two different methods of maturing dendritic cells. We found that DCs matured by the cytokine cocktail MCM-mimic (MCM-mimic DCs) had an immunophenotype which indicated that they were more mature than DCs matured by lipopolysaccharide and Interferon-γ (LPS-IFN-γ DCs). Consequently, we expected the MCM-mimic DCs to be more effective at expanding specific CTLs than DCs matured by LPS-IFN-γ. We were surprised to find that this was not the case. Greater numbers of specific CTLs were expanded by peptide pulsed, LPS-IFN-γ DCs than by MCM-mimic DCs. In addition, CTLs expanded by LPS-IFN-γ DCs were functionally superior, exhibiting increased killing of peptide loaded target cells than those CTLs expanded by MCM-mimic. We also showed that the ability of LPS-IFN-γ DCs to expand specific CTLs was much superior to that of PBMCs either with or without additional co-stimulatory cytokines. Although these experiments were performed using the epitope GILGFVFTL as a surrogate for NLVPMVATV, the immunological principles which underlie the expansion of specific CTLs would not be expected differ between these two epitopes.

We applied the technique developed above to determine if CTL specific for NLVPMVATV could be expanded from samples taken from donor 1 during follow-up. Autologous DCs were grown from from the peripheral blood of donor 1. These DCs were then matured with LPS-IFN-γ and pulsed with NLVPMVATV peptide. The DCs were subsequently co-incubated for 1 week with MACS selected $CD8^+$ T-cells derived from donor 1 at weeks 0, 4, 8, and 16 after vaccination. No difference was detected in the numbers of NLVPMVATV specific CTLs expanded from each time point, although a low level of background staining may have masked a small response.

A response to FrC of tetanus toxoid by $CD4^+$ T-cells from donor 1 was not detected either prior to or in the weeks following vaccination. A very small response to FrC was detected at week 16 but this was not seen at week 20. The significance of this result is unclear. Similar assays performed in samples from Donor 2 revealed an increase in the frequency of FrC specific $CD4^+$ T-cells at weeks 4 and 8 post vaccination. This could represent a response to the vaccine but could also simply reflect natural variation in the frequency of FrC specific cells in the blood. It could also indicate variability inherent

within the assay. Measurement of the numbers of FrC specific T-cells present at serial intervals in the blood of normal volunteers who have not received the p.DOM.NLVPMVATV vaccine are required to further investigate this. In addition, the vaccination of futher volunteers with pDOM-CMV-NLV would also shed light on this issue.

The ability of the pDOM vaccine to induce an immune response against the linked CTL epitope is crucially dependent upon the linked T-cell help provided by $CD4^+$ T-helper cells specific for epitopes derived from the first domain of FrC. In mice, pDOM is sufficiently immunogenic to induce an immune response even if these animals have not previously encountered FrC. We did not detect the presence of FrC specific CD4 T-cells in the peripheral blood of Donor 1 prior to or following vaccination. This may have impaired her ability to respond to the vaccine.

Although the vaccine p.DOM.NLVPMVATV is designed to induce CTL responses, it contains part of FrC of tetanus toxoid against which antibodies may be induced if the vaccine is successfully delivered. We found no change in the anti-FrC antibody levels of any of our donors after vaccination.

In conclusion, we have no evidence from any of the tests performed so far that an immune response was induced by the vaccination of donor 1 with p.DOM.NLVPMVATV.

Donor 2 was CMV seropositive and was also homozygous for HLA-A*0201. It was not a surprise that CTLs specific for NLVPMVATV were present in his peripheral blood at a very high frequency prior to vaccination. For the first 3 weeks after vaccination the frequency of these cells, as measured by the tetramer assay, fell lightly before rising again at week 6. They continued to fluctuate throughout follow up. This may simply reflect normal variation in the number of NLV specific cells present in the peripheral blood or it may reflect the exit of specific CTLs from the blood to the tissues in the first weeks after vaccination. Similar results were observed in the NLVPMVATV Elispot assay.

A reponse by Donor 2 to vaccination may have been detected in the FrC Elispot. At weeks 4 and 8 the frequency of FrC specific T-cells in the blood rose substantially before falling to baseline levels again by week 12. This could again represent merely natural fluctuation or assay variability. Investigation of unvaccinated normal volunteers will help to clarify this issue. No change in the level of anti-FrC specific antibodies was observed in Donor 2 following p.DOM.NLVPMVATV vaccination.

Donor 3 was similar to donor 2 in that she had previously been infected with CMV. However she was heterozygous for HLA-A2*0201 and therefore had a much lower level of NLVPMVATV specific CTLs in her peripheral blood prior to vaccination. The frequency of these cells, as measured by the tetramer assay, slowly rose in the first 3 weeks

after vaccination before falling at week 4 only to oscillate up and down after that. A definite pattern is difficult to discern. A particularly high frequency of NLV specific $CD8^+$ T-cells was observed at week 44. The significance of this is unclear. The NLVPMVATV Elispot did not confirm the presence of such a high frequency of specific T-cells at week 44. Overall, the NLVPMVATV elispot assay results are similar to those seen in the tetramer analysis in that the level of specific cells detected seems to oscillate up and down within a certain range. Again this may reflect either actual biological variation or assay variation.

As was the case with donor 2, FrC specific T-cells were not detected by Elispot prior to or following vaccination. No change in FrC antibody levels were identified either.

As a response to vaccination has not been demonstrated in donor 1 it is unsurprising that $CD8^+$ T-cells specific for NLVPMVATV have not been detected by tetramer or elispot in recipient 1, who was also CMV seronegative. Recipients 2 and 3 were both CMV seropositive. It is known that the proportion of $CD8^+$ T-cells which are specific for NLVPMVATV can reach very high levels soon after transplant in a CMV seropositive HLA-A*0201 homozygote who receives a transplant from a matched donor who is also CMV seropositive. This was the situation with Recipient 2. By day 20 after transplant, NLV specific cells comprised 11.6% of the total $CD8^+$ population. This rose to 19% by week 6. Despite this, CMV reactivation was first identified at week 5 post transplant. Further reactivation occurred at week 7 which required ganciclovir therapy by week 8. It was interesting to note the rise in NLVPMVATV specific Cd8 cells in the blood of Recipient 2 which followed each CMV reactivation. Recipient 3 also experienced CMV reactivation and required therapy with either ganciclovir or valganciclovir for 6 weeks. Neither donor 2 nor donor 3 developed CMV disease. We cannot discount the possibility that vaccination of the stem cell donors with p.DOM.NLVPMVATV aided the reconstitution of NLVPMVATV specific cells in these recipients but equally we cannot prove that such a link exists.

The ideal setting in which to test pDOM.NLVPMVATV would involve vaccination of a HCMV seronegative individual who would subsequently act as a stem cell donor for a HCMV seropositive recipient. This situation is known to be associated with a high risk of HCMV reactivation and disease in the recipient. In addition, NLV specific $CD8^+$ cells are normally detected in this setting much later in the post-transplant period than is seen in a HCMV +/+ transplant. The detection of NLV specific CTLs at a time point earlier than that anticipated could provide evidence that the donor had been successfully primed by p.DOM.NLVPMVATV.

In conclusion, this study must be regarded as a non-conclusive pilot assessment of the ability of pDOM vaccine design to induce CTL responses in humans. The clinical setting in which vaccination was performed differs greatly between donors who have previously experienced infection with HCMV and those who have not. Thus far, only one seronegative donor has been recruited. Although no response to vaccination was detected in this individual, a thorough evaluation of the capacity of p.DOM.NLVPMVATV to induce a specific CTL response will require the recruitment of further seronegative donors to the trial. If the lack of response exhibited by donor 1 is confirmed in subsequent seronegative donors, then additional procedures with the potential to improve the response to vaccination must be evaluated.

Electroporation of the vaccinated muscle is a technique which is showing promise as a method to boost immune responses to DNA vaccination [82,243,244]. Our group has found that mice which receive an initial DNA vaccination followed several weeks later by a combination of repeat DNA vaccination and muscle electroporation respond by generating much greater CTL and antibody responses than mice who receive repeat vaccination without electroporation [240]. Electroporation may therefore provide a method to improve responses to DNA vaccination in the clinic.

2.7.1 Future work

We plan to recruit further donor/recipient pairs into this trial. Electroporation will be incorporated into this study if other trials show that it significantly improves the ability of DNA vaccines to generate immune responses in humans.

3 A DNA vaccine to induce CTLs against Influenza A virus

Objective

The aim of this study was to develop a DNA vaccine designed to induce a CTL response against the epitope GILGFVFTL which is immunodominant in the context of the HLA-A*0201 response to influenza A viral infection.

3.1 Introduction

3.1.1 Influenza

Influenza is a highly infectious acute viral infection of the respiratory tract which is characterised by the abrupt onset of fever, chills, headache, myalgia and a dry cough. It is usually a self-limiting disease, with recovery within two to seven days, but may result in serious illness such as primary pneumonitis and secondary bacterial pneumonia. Encephalitis, Guillain-Barre syndrome, cardiac, renal and other complications occur less frequently. It is estimated that each year approximately 20% of children and 5% of adults develop symptomatic influenza. In England and Wales, 5870 excess deaths were attributed to influenza during the 2001/02 winter. Influenza incidence was not considered to be particularly high that year. Mortality is greatest among elderly individuals with underlying chronic respiratory and cardiac disease.

3.1.2 Influenza Virus

Human influenza viruses are classified as members of the Orthomyxoviridae family. These single stranded, negative sense RNA viruses have a segmented genome and are characterised by possession of a nucleocaspid with helical symmetry and an envelope with glycoprotein spikes. The family is divided into four genera which correspond to Influenza types A, B and C and Thogoto-like viruses.

Type A is the most clinically important of the influenza viruses, causing annual winter outbreaks of influenza. Influenza A viruses are divided into subtypes based on antigenic differences between their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Fifteen HA subtypes (H1-15) and nine NA subtypes (N1-9) have been identified.

Although influenza viruses incorporating all HA and NA subtypes have been recovered from aquatic birds, only three HAs (H1, H2 and H3) and two NAs (N1 and N2) have been detected in the viruses causing human pandemic and epidemic influenza since 1918.

3.1.2.1 Virus structure and function

Influenza virus particles are highly pleomorphic, spherical/ovoid in shape with a diameter of 80-120nm (Figure 52). Long filamentous particles may also occur. The outer surface of the particle consists of a lipid bilayer, from which glycoprotein spikes of haemagglutinin (HA) and neuraminidase (NA) project. Haemagglutinin binds to sialic-acid receptors on the host cell, facilitating entry of the virus. Neuraminidase prevents viral aggregation and assists the release of progeny virions from infected cells by cleaving glycosidic links to sialic acid. The M2 protein forms an ion channel between the virus and the external environment. It provides a low pH which is crucial for HA synthesis and virion uncoating during early virus replication. The influenza virus has two layers of protein shells which assemble around and protect the genomic RNAs. These are an outer matrix protein (M1) and nucleoprotein (NP) which coats the genomic RNAs to form RNA nucleoproteins. A polymerase complex for transcription is formed by the PB2-, PB1- and PA-encoded proteins which associate at one end of each gene segment. Finally, gene segment eight encodes non-structural export protein (NEP) and non-structural protein 1 (NS1) which are synthesised in an infected cell but are not incorporated into the virus structure.

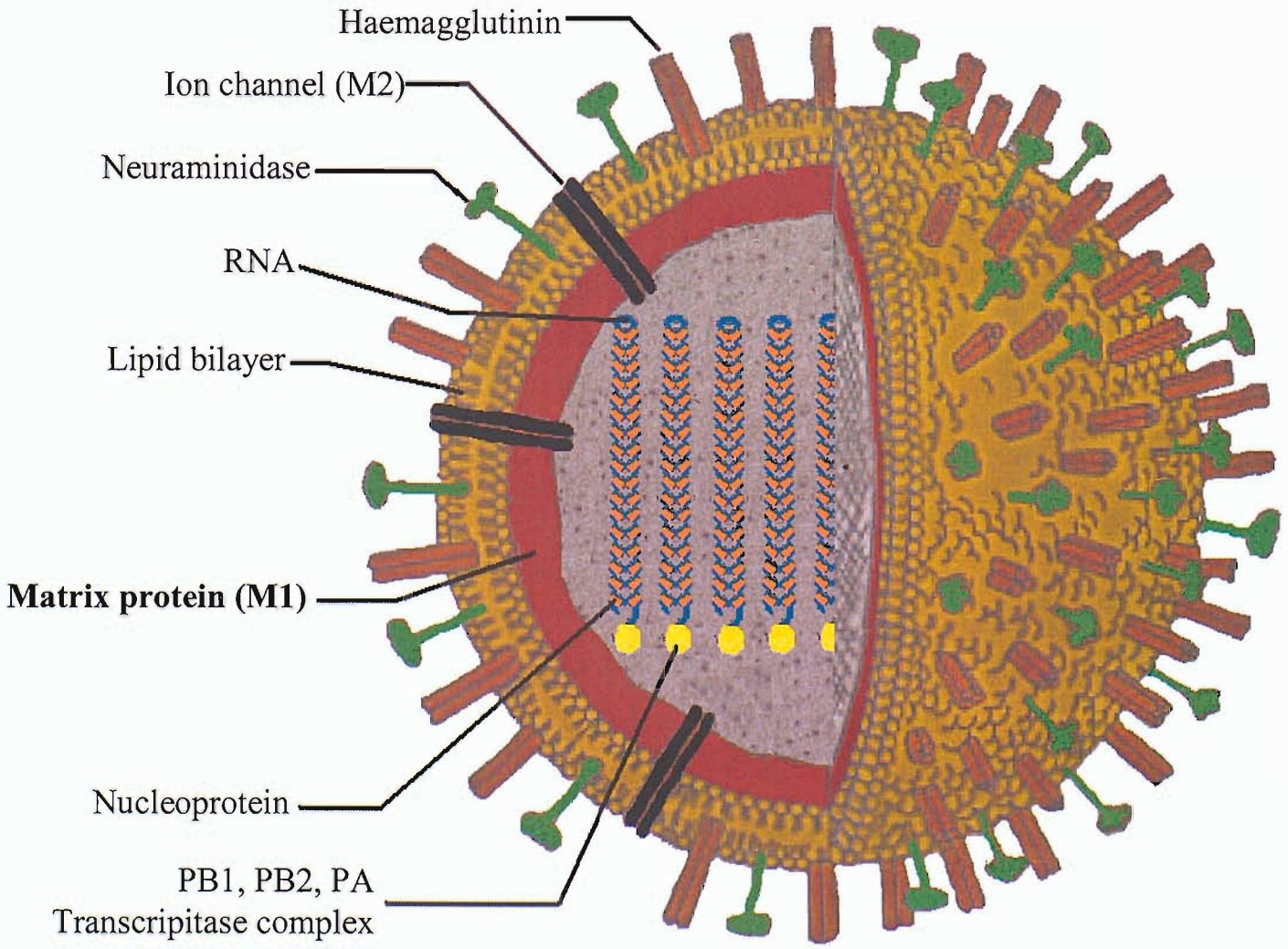


Figure 52. Schematic representation of Influenza A virus

3.1.2.2 Matrix protein M1

The M1 protein is encoded by RNA segment 7. In addition to protecting the virus RNA, M1 has the ability to bind to cell membranes and to RNA nucleoproteins. This function underlies the important role of M1 as a nuclear export factor and in viral assembly. In the nucleus of the host cell, M1 molecules bind to newly formed viral RNA nucleoproteins (RNPs) and assist their transport into the cytoplasm of the host cell. Upon reaching the cytoplasm, additional M1 molecules bind the RNPs and transport them to the region of cell membrane where membrane bound M1 is located. The new viral particle is then pinched off from the cell surface [245].

3.1.3 Influenza infection and the response of the immune system

Human influenza virus differs from animal strains in that infection is restricted to respiratory epithelial cells. In humans, the specific trypsin-like protease which cleaves viral haemagglutinin into two functional subunits is found only in respiratory epithelial cells and their secretions. This limits sites of influenza antigen presentation and processing to the respiratory mucosa and draining lymph nodes. Virus can be detected in secretions 24 hours before onset of illness. The viral load rises to $10^3 - 10^7$ $TCID_{50}$/ml of nasopharyngeal wash and remains high for 1-3 days before falling to a low level by the fifth day [246].
Influenza A is a virus of diverse genetic and antigenic composition which presents the human immune system with a "moving target". The outcome of infection is variable and depends on interactions between the virus and immune system within a restricted time frame. Although the degree of complementarity between the surface glycoprotein antigens and the antibodies against them which are current in the population determines whether an influenza outbreak occurs, cellular mediated responses are also important against influenza. Influenza infected cells are cleared by CTLs whilst antibody neutralises free virus leading to disease resistance.

3.1.3.1 Humoral immune response to influenza

Haemagglutinin and neuraminidase (NA) located on the virus surface are the main targets of the humoral response to influenza infection. Antibodies specific for NA reduce the spread of virus but do not prevent disease. IgA and IgG antibodies against haemagglutinin neutralise viral infectivity, providing complete protection during a future encounter with homologous virus.

Influenza viruses undergo frequent genetic variation. An accumulation of point mutations in genes coding for surface glycoproteins enables the virus to evade destruction by antibodies induced by previous infection. This process is known as antigenic drift and is responsible for epidemics of influenza.

Antigenic shift is heralded by the abrupt appearance of an influenza A virus with a new haemagglutinin. An antigenically shifted virus can occur during dual infection of a cell by a virulent wild human virus and a zoonotic influenza virus. This allows genetic reassortment between the viruses to take place resulting in a viable virus with new HA or HA and NA surface glycoproteins. An antigenically shifted virus can spread rapidly from person to person and has the potential to cause an influenza pandemic as there are no pre-existing antibodies specific for this virus in the human population.

Four pandemics occurred in the 20th century. The most devastating was the H1N1 Spanish influenza pandemic of 1918-19 which caused an estimated 20–40 million deaths. Asian 'flu (H2N2) which appeared in 1957 and Hong Kong 'flu (H3N2) of 1968 remain in circulation and are thought to have originated in birds. H1N1 influenza re-emerged in 1977 following escape from a laboratory. This caused a pseudo-pandemic and remains in circulation today.

3.1.3.2 Cellular immune response to influenza

Effector CTLs have been shown to have an important role in clearing influenza virus infections. The cellular response is directed against internal proteins, such as matrix protein, polymerases and nucleoprotein [247,248]. Gene mutations do not affect these antigens as frequently as the surface glycoproteins [249]. They are therefore more likely to be conserved between influenza subtypes. CTLs specific for cross-reactive epitopes can provide heterosubtypic immunity against serologically distinct viruses [250,251].

The role of CTLs in controlling primary influenza infection has been extensively studied in murine models. Effector CTLs are initially detected in the lungs 7 days after infection. The response is transient with a sharp rise and fall. The peak occurs by day 9 or 10 with viral clearance on day 8 or 9 [252].

In an attempt to delineate the role of CTLs in clearing influenza virus, normal mice were compared to mice with $CD8^+$ cell depletion or β_2-microglobulin gene disruption. Following viral infection, a potent CTL response was detected in the lungs of normal mice. As expected, there was minimal response in the $CD8^+$ T cell depleted mice. The $CD8^+$ deficient mice were still able to clear the virus, illustrating some plasticity and redundancy of the immune response [253].

Adoptive transfer experiments have also helped to establish the importance of CTLs against influenza. Transfer of a CTL clone specific for nucleoprotein into mice infected with influenza virus was shown to significantly reduce lung virus tires 6 days after viral infection [254]. When the lungs of these mice were examined histologically, they had decreased lung epithelial damage compared to controls. Infusion of clones with greater *in-vitro* cytotoxic activity was associated with more rapid viral clearance and epithelial recovery [254].

More recent analysis has shown that high levels of specific CTLs, previously expanded by infection with 2 different subtypes of influenza, provide substantial protection against respiratory challenge with a third influenza subtype [255]. 25 % of splenic $CD8^+$cells were specific for influenza nucleoprotein 2 weeks after secondary infection. Lung viral titres in double primed mice and naïve mice were similar 24 hours after infection illustrating that massive numbers of influenza specific CTLs are unable to prevent infection. However, virus was rapidly controlled and the double primed mice remained clinically normal. $CD8^+$ depletion experiments demonstrated that the protection was provided by CTLs.

Despite the presence of CTLs against epitopes conserved between serologically distinct virus strains, humans seem to remain susceptible to infection by viruses expressing these epitopes. The severity of influenza pandemics could, at first glance, be taken to suggest that memory CTLs provide minimal protection against subsequent influenza infection. This analysis does not take into account the suggestion that the morbidity caused by pandemics might have been much greater in the absence of CTLs providing some degree of heterosubtypic protection.

Measurable CTL memory induced by influenza infection in humans appears to be relatively short lived, with a half-life of 3-5 years [256]. Morbidity and mortality related to influenza is greatest among the elderly population. Older people are more likely to suffer from co-existing illnesses which weaken their capacity to overcome influenza infection. Immunosenescence is also likely to play a role [257]. Memory $CD8^+$ cells with hallmarks of replicative senescence have been identified in older individuals *in-vivo* [258]. Sub-optimal CTLs responses may be involved in an individual's increased susceptibility to severe influenza disease.

If the effect of an accelerated CTL response to human influenza infection mirrored that seen in mice [255], then a vaccine inducing such a response may be clinically useful. It is envisaged that such a vaccine would complement rather than replace strategies designed to induce protective antibodies against influenza.

3.1.3.2.1 Immunodominance of GILGFVFTL

T cell epitopes that bind to class I MHC molecules are usually peptide nonamers. For HLA-A2, the hydrophobic amino acids Leu, Met, and to a lesser extent Ile predominate at the second position with Val or Leu usually found at the anchor position (P9) [259]. As previously discussed, cytotoxic T cell responses to complex antigenic proteins are directed against a very small proportion of potential epitopes. This characteristic is termed immunodominance. Mice infected with influenza develop a CTL response against a single immunodominant epitope derived from the nucleoprotein (NP.366) [260].

The human CTL response to influenza infection follows the same principle, but is not as narrowly focused as in the mouse [248]. Investigation of 6 donors revealed that CTLs were directed primarily against matrix 1 protein, although responses against nucleoprotein and PB2 were also detected [261]. Further analysis revealed that target cells sensitized by a nonameric peptide corresponding to amino acids 58-66 of matrix 1 protein (GILGFVFTL) were most effectively lysed by influenza specific CTLs from HLA-A2 donors [262].

The immunodominance of this GILGFVFTL in the context of the human HLA-A2 response to influenza has been confirmed by Gianfrani and colleagues [248]. PBMCs from 8 HLA-A2 individuals were incubated for 15 days with GILGFVFTL peptide and then assayed for CTL activity. Cells from all eight individuals were found to demonstrate vigorous activity against influenza (PR8) infected target cells. Other influenza derived epitopes were recognised by some of the group but were subdominant to GILGFVFTL.

Immunodominance hierarchies may be altered by subsequent heterologous viral infection [182]. It is known that heterologous viral infection can negatively impact on the frequency of pre-existing memory cells, as shown by experiments in which mice sequentially infected with LCMV, Pichinde virus (PV), VV, MCMV and VSV experienced a reduction in memory T-cells specific for the previously encountered viruses [263,264]. Although infection of LCMV-immune mice with PV was found to lead to a decrease in CD8 T cells to most LCMV-encoded epitopes, cells specific for subdominant LCMV epitope, NP205, increased in frequency [182]. This epitope shares 6 of 8 amino acids with a corresponding epitope from PV (PV-NP205). Cross reactivity against epitopes from unrelated viruses therefore provides a mechanism for the maintenance of some memory T-cell populations whilst others are lost. It may also play a role in maintaining the immunodominant GILGFVFTL CD8 memory cell population at high levels in the absence of recurrent influenza infection. T-cells specific for GILGFVFTL have exhibited cross reactivity against an epitope of Epstein-Barr virus, EBV BMLF1-280 [265].

3.1.3.2.2 The epitope GILGFVFTL is conserved among influenza A viruses

The epitope GILGFVFTL seems to be conserved by influenza A viruses to a remarkable degree, making it an attractive candidate epitope for vaccination strategies. Analysis of the GenBank database verified that the GILGFVFTL epitope has been conserved among all strains of influenza virus which afflicted humans from 1970-2000 [248]. Subsequently the genome of the Influenza virus responsible for the 1918 pandemic has been analysed and also shown encode GILGFVFTL [266].

3.1.4 Vaccination against influenza

3.1.4.1 Current influenza vaccine

The trivalent influenza vaccine in current use is designed to induce humoral immunity against a H1N1, H3N2 and an influenza B virus. The WHO predicts which H1N1 and H3N2 virus will be in circulation using the worldwide influenza surveillance programme. Selection of viruses for manufacturing is made each spring, allowing only a few months to produce enough vaccine for the following autumn. Vaccines are then manufactured from the selected viruses grown in fertile hens' eggs and inactivated with formaldehyde. 234 million of the world's population of 6 billion were vaccinated in 2000 [246].

Unfortunately, protection induced by the vaccine is at best suboptimal, reaching 70-80% against well matched circulating strains in young adults. Protection in the elderly varies from 30-70% [246]. An additional weakness of current inactivated vaccines is that they do not induce potent cytotoxic responses [267].

3.1.4.2 DNA vaccination against influenza

Influenza provided the setting for some of the earliest work which documented the remarkable ability of DNA vaccination to mediate protective immunity in mice and chicken against an encoded antigen [268,269]. Since then, the capacity of DNA vaccines to induce humoral and cellular immune responses against influenza and other infectious diseases has been documented in various animal models [78 76].

3.1.4.2.1 DNA vaccination to induce antibodies against influenza

DNA vaccines have been produced which stimulate the production of haemagglutination inhibiting antibodies in mice [270-272] and primates [273]. Another group injected pigs with a DNA vaccine which induced antibodies against the extracellular domain of the M2 protein

(M2e) [274]. This protein is not accessible to antibody and therefore antibodies against it are not virus neutralising. However they can bind to M2e on infected host cells and reduce virus replication by interfering with virus budding. Vaccinated pigs were not protected against subsequent influenza infection. In fact they showed more severe clinical signs than control pigs. Only pigs in which an anti-M2e antibody response had been generated experienced a worse outcome and the authors felt that T helper cells may have played a role in the exacerbation.

3.1.4.2.2 DNA vaccination to induce CTL responses against influenza

The majority of studies in mice which show that DNA vaccination can induce influenza specific CTLs have focused on nucleoprotein, as this contains the immunodominant epitope of the murine CTL response to influenza infection. It was demonstrated that intramuscular injection of plasmid DNA encoding influenza nucleoprotein protected BALB/c mice against a subsequent influenza virus challenge [268]. The transfer of serum from vaccinated mice did not protect non-vaccinated mice, indicating that protection was not antibody mediated. Vaccination had induced a vigorous CTL response specific for a H-2 K^d restricted nucleoprotein epitope. By performing adoptive transfer of T cell subsets, the group later showed that $CD4^+$ and $CD8^+$ cells could confer protection after vaccination. In-vivo T cell subset depletion studies suggested that the critical cells were $CD8^+$ [275].

It was interesting to note that an almost identical DNA vaccine encoding nucleoprotein, without the immunodominant epitope, could still provide protection against lethal influenza challenge [276]. The protection was provided by CTLs, but on this occasion they were reactive against another epitope. The removal of the immunodominant epitope had allowed a CTL response to be induced against a subdominant epitope. This experiment added to mounting evidence that the implications of immunodominance must be considered in vaccination strategies designed to induce CTL responses.

Others continued to seek to stimulate CTL responses against a broad range of influenza epitopes by vaccinating mice with a mixture of DNA plasmids encoding full length matrix protein or nucleoprotein [277]. CTLs which lysed virus infected targets and afforded some protection against influenza challenge were induced. By using an interferon-γ ELISPOT assay, CTLs specific for the immunodominant nucleoprotein epitope were detected but the authors did not show evidence that CTLs specific for other nucleoprotein epitopes or epitopes derived from the matrix protein were stimulated by vaccination.

Intramuscular injection with a plasmid containing the influenza M gene induced antibodies and CTLs against matrix protein [278]. After vaccination with this plasmid, 70%

of mice survived a lethal challenge with influenza virus. Only 9% of vaccinated mice that received anti-CD8 mAb prior to viral challenge survived, indicating that it was CTLs rather than antibodies induced by vaccination which were critical in providing protection from influenza.

3.1.5 Objective of study

Taking account of the importance of immunodominance in CTL responses, we aimed to develop a DNA vaccine which would stimulate CTLs against influenza matrix 1 protein. Our vaccine was designed to induce CTLs specific for GILGFVFTL, which is the immunodominant epitope for CTL responses against influenza in HLA-A2 individuals. Potential vaccines designed for human use must first be tested for efficacy in murine models. The HLA-A2/Kb transgenic mouse has proven to be a very useful system in which to model HLA-A2 immune responses. The cellular immune response to influenza demonstrated by this model seems to closely mirror that of human HLA-A2 individuals as shown by experiments in which target cells pulsed with GILGFVFTL induced significant recall activity in CTLs from HLA-A2/Kb transgenic mice previously infected with influenza [248].

3.2 Materials and methods

The expression vector pcDNA3 (Invitrogen, Paisley, UK) was used for the construction of the DNA vaccines used in these studies. This vector consists of a multiple cloning site that is under the transcriptional control of the promoter from the immediate early gene of human cytomegalovirus (CMV). It also contains transcription termination and a polyadenylation sequence from the bovine growth hormone gene to enhance RNA stability. In addition there is a neomycin resistance gene in a separate expression cassette that is under the control of the SV40 promoter. This promoter is immediately preceded by the SV40 origin of replication. An ampicillin resistance gene enables selection of the plasmid in bacterial cultures. All vaccines in these studies were placed in the multiple cloning site with a Kozac sequence (GCC GCC ACC) placed at the beginning of the encoded sequence.

The vaccine pDOM was kindly provided by Dr J. Rice. This encodes a Kozac sequence upstream of the leader sequence. The leader sequence is derived from the IgM VH of the BCL1 tumour. This is fused to the beginning of the sequence of the first domain of Fragment C (FrC) of tetanus toxin (the amino terminal sequence, known as pDOM).

3.2.1 Plasmid DNA assembly

pDOM.FLU was constructed using pDOM as a template. A PCR SOEing procedure using the primers listed below was employed. The forward 5' primer was complementary to a portion of the T7 sequence. The reverse 3'primer encoded 18 overlapping nucleotides from the 3' terminus of pDOM, GILGFVFTL, a stop codon and the Not I restriction site.

Forward primer (5'-3') :
TAATACGACTCACTATAGGG

Reverse primer (5'-3') :
ATATGCGGCCGCTTAGAGCGTGAACACAAATCCTAAAATCCCGTTACCCCAGA
AGTCACG

A PCR reaction was carried out in a volume of 50μl with 2μl of pDOM plasmid (50μg/ml) as template. 1μl of each primer (10pmol) were used with 0.5ul DNTPs (25 mM) and 0.6μl

of Expand High Fidelity Taq polymerase (Roche Biochemicals UK) The 50μl reaction volume was completed by 5μl of the supplied reaction buffer and 39.9ul of sterile H2O. After an initial denaturing step of 94°C for 5 minutes, 5 cycles were performed with a denaturing step of 94°C for 30 seconds, an annealing step of 55°C and an extension step of 72 minutes for 1 minute. This was then followed by 25 cycles which differed only in that the annealing step was 60°C. A final extension step for 5 minutes at 72°C was performed.

The products of the PCR reaction were run on a 1% agarose gel and a band of approximately 800Kb was excised and purified using QIAquick Gel Extraction kit (Qiagen, UK).

The DNA was then digested with restriction enzymes NOT I and Hind III (New England Biolabs, UK). The reaction volume was 25μl which comprised 21.25μl of purified PCR product, 0.5μl of each restriction enzyme, 0.25μl bovine serum albumin and 2.5μl of reaction buffer. The digestion took place at room temperature over a 2 hour period. The product was again cleaned with a Quiagen kit.

An empty pcDNA3 vector was ligated to the PCR product in a final volume of 12μl containing 2μl pcDNA3 vector, 1μl T4 DNA ligase, 6μl of 2x Ligation Buffer (Promega, T4 DNA ligation kit, Southampton UK) and 3μl PCR product. The ligation was performed at 4°C for 16 hours.

3.2.2 Transformation of plasmid DNA into competent bacteria

The vaccine was transformed into XL 1 blue competent bacteria (Promega, Southampton, UK). 5μl of the above ligation product was incubated with 60μl of competent E.coli. This was left on ice for 20 minutes. Bacteria were subsequently heat shocked at 42°C for 45 seconds and then returned to ice for 2 minutes. 350μl of Luria Broth (LB) was added to the bacteria. This was followed by one hour incubation on a shaking platform at 37°C. The bacteria were then plated out onto LB agar. This was incubated at 37°C for 16 hours to allow selection of transformed bacteria.

Colonies that had grown over this time period were selected and added to 2mls LB medium containing 100μg/ml ampicillin. This was then cultured overnight at 37°C on a shaking platform. The cells were then lysed and the DNA purified using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was eluted into 100ul of Elution Buffer (EB).

DNA constructs were subjected to restriction digestion to confirm the presence of a cloned insert of the correct size and to asses the quality of the DNA. 6μl of the DNA was added to 0.2μl of both Hind III and Not I (New England Biolabs, UK), 1μl of the recommended reaction buffer, 0.1μl BSA and 2.5μl sterile H_2O. This was incubated at

37°C for 1 hour. Any clone that was found to the insert of the correct size was stored at -20°C.

3.2.3 Amplification of plasmid DNA

A clone with the correct insert was selected and 1ul was transformed into 50ul of XL-1 blue competent bacteria as above. The bacteria were again plated out overnight on LB agar containing 100ug/ml of ampicillin. A colony was selected and grown overnight at 37°C in 10mls LB medium containing ampicillin as above. Three 800ml flasks of LB/Ampicillin were then inoculated with this culture. The flasks were incubated for 18 hours at 37°C on a rotating platform. The DNA was extracted from these cultures using an ion-exchange resin (QIAfilter Giga Kit, Qiagen , UK). A 1 in 100 dilution of the final DNA in EB was quantitated using a biophotometer (Eppendorf AG, Germany). The amount of DNA produced was 11.5mg.

3.2.4 In vitro transcription /translation reaction

The TNT T7 Coupled Reticulocyte Lysate System (Promega) was used to determine if the vaccine could lead to the production of a protein product of the correct size. In this assay, radioactive methionine is incorporated into the final protein product which can then be detected on radiographic film. Plasmid DNA (500ng) was incubated with rabbit reticulocyte lysate (20ul of TNT T7 mastermix) and 100 uCi of ^{35}S-labelled methionine. The reaction was incubated at 37°C for 90 minutes. The product was denatured at 95°C for 5 minutes in the presence of 2-mercaptoethanol and NuPage loading buffer (Invitrogen). This was subjected to electrophoresis on a pre-cast Nupage 4-12% polyacrylamide gel (Invitrogen) for 50 minutes at 200V. The gel was subsequently placed on filter paper and vacumn dried for 1 hour. Rainbow markers (Amersham) were run alongside the samples to confirm the presence of a protein band which was of the expected size. Once dried, the gel was exposed to radiographic film overnight. The film was then developed to produce a record of the result.

3.2.5 Sequence analysis of DNA plasmid

The sequence of the cloned insert of DNA plasmid was determined using the Big Dye reaction kit (Applied Biosystems, UK). In this reaction 4ul (100μg/ml) of plasmid DNA was added to 1μl of primer (1.6pmol/μl), 2 μl of sequencing buffer and 2 μl Big Dye

reaction mixture. This mixture contains DNA polymerase and ddNTPs that are labelled with different fluorochromes. The primers listed below enabled the insert to be sequenced in both directions.

T7: 5' - TAATACGACTCACTATAGGG

pDOM.forward: 5' - TAACGAGTACTCCATCA

pDOM.reverse: 5' – AAACAGACCAACCAGAGC

PJR5p1: 5' - GGCACAGTCGAGGCTGATCA

The sequencing reactions were performed for 30 cycles on a GeneAmp PCR System 9700 (Applied Biosystems). Each cycle involved denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and finally extension at 60°C for 3 minutes. Samples were precipitated with 2ul of 3M sodium acetate and 25ul of ethanol on ice for 10 minutes. The precipitate was then centrifuged at 16000rcf for 30 minutes and washed once in 160ul of 75% ethanol. DNA was resuspended in 1.2ul of loading buffer and formamide and sequenced on an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequences were analysed using MacVector 4.5.3 (Oxford Molecular, UK) and Editview 1.0 software (Applied Biosystems).

3.2.6 Mice

The HLA-A2.K^b transgenic mouse model was initially used to test the ability of the vaccine to induce cytotoxic T cells which were specific for GILGFVFTL. These mice express a chimeric HLA class I molecule A2.1. K^b which is composed of the $\alpha 1$ and $\alpha 2$ domains of HLA-A2 and the $\alpha 3$ transmembrane and cytoplasmic domains of H-2 K^b with endogenous murine β_2 microglobulin on a predominantly C57BL/6 background [279]. K^b mice can mount HLA-A2 and H-2b restricted CTL responses. These mice were provided by Dr V. Cerundolo (Institute of Molecular Medicine, Oxford, UK). Although they provide a model in which to study HLA-A2.1 CTL responses, their use is limited by preferential usage by mouse CTLs of H-2 class I molecules for antigen recognition [280].

HHD transgenic mice became available during these experiments. These HLA-A2 transgenic $H2K^{b-/-}$ $\beta 2m^{-/-}$ double knockout mice express a chimeric MHC class I molecule in which the $\alpha 1 \alpha 2$ domains are derived from HLA-A2.1 whilst the $\alpha 3$ transmembrane

domain is derived from murine H-2D^b. They were created by transfecting H-2D^b and mouse β2 microglobulin double knockouts with a HHD (**H**uman β2m-**H**LA-A2.1 (α1α2)-H-2**D**^{b} (α3 transmembrane cytoplasmic)) monochain construct [281]. This forces the mouse CD8$^+$ repertoire to make use of the transgenic HLA class I molecules and restores a sizeable peripheral CD8$^+$ T-cell repertoire educated on the transgenic human molecule [281].These mice were obtained from Prof V Cerundolo, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford.

3.2.7 Vaccination

Mice were vaccinated with 50ul of plasmid DNA in saline at a concentration of 0.5mg/ml into both rear quadriceps muscles. Control mice were injected with a plasmid encoding p.DOM without the GILGFVFTL encoding sequence. Mice were subsequently sacrificed at day 17-18 and their spleens were harvested. Single cells suspensions were prepared by padding splenocytes through a 70um nylon mesh. Mononuclear cells were then isolated over a lymphoprep density gradient (AS Pharma, UK) at 800rcf for 20 minutes. The cells from each group were pooled for further experimentation.

3.2.8 Generation of cytotoxic lymphocyte cell lines

Splenocytes were cultured in RPMI 1640/10% FCS at a concentration of 5x10^6 in the presence of 20U/ml of IL-2 and GILGFVFTL peptide at 1uM/0.1μM /0.01μM for 7 days at 37°C with 5% CO_2.

3.2.9 Cytotoxicity Assays

Following 6 days of *in-vitro* stimulation as outlined above, cells were again isolated by density gradient centrifugation.

T2-A2 cells, kindly provided by Prof. T. Elliot, were used as target cells. The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and 25mM HEPES buffer (Invitrogen) (R-10). They were washed and resuspended in 300ul of R-10 which contained 200uCi of chromium-51. They were then incubated at 37°C with 5%CO_2

for 1 hour either in the presence, or absence, of GILGFVFTL peptide which was at a concentration of 10μM.

The targets were washed and then $5x10^3$ cells per well were placed in a 96 well U-bottomed plate. Effector cells from the CTL lines were added at a ratio of either 50:1 or 100:1.The effector and target cells were incubated for 5 hours at 37°C with 5% CO_2. Maximum lysis was determined by adding 2% Nonidet P40 to a portion of the wells which contained target cells only. The remaining "target cell only" wells were then used to measure spontaneous target cell lysis. The plates underwent centrifugation at 400rcf for 5 minutes. 100ul of the supernatant which resulted from this process was then transferred from each well to LMP3 tubes. The gamma activity produced by each tube over a 5 minute period was measured in a Wallac 1282 compugamma counter. Specific lysis was calculated by the standard formula below:

$$\frac{\text{Release by CTL} - \text{Release by targets alone}}{\text{Release by 2\% Nonidet P-40} - \text{Release by targets alone}}$$

3.2.10 Infection of Jurkat and L-929 cell lines with influenza virus

Mice which were transgenic for the human HLA-A2 molecule were vaccinated with either the pDOM.GILGFVFTL or pDOM DNA vaccine as previously described. The mice were sacrificed 16 days later. Splenocytes from pooled animals were stimulated for 6 days with 20u/ml IL-2 and the peptide GILGFVFTL at 1μM concentration.

Influenza infected and GILGFVFTL peptide loaded Jurkat cells and L929 cells served as target cells in this killing assay. Jurkat cells were originally derived from a human T-lymphoblast clone. The Jurkat cells used in these experiments had been stably transfected with a plasmid containing the chimeric class I molecule HLA-A2/Kb (α1 α2 domains from HLA-A2, α3 domain from murine Kb). They were a kind gift from Prof. L. Sherman, Scripps Institute, California.

L929 cells were originally derived from a murine connective tissue tumour. The L-929 cells used in these experiments had been transfected retrovirally with HLA-A2. They were a kind gift of Dr. Philip Stevenson, University of Cambridge. L929 cells were grown in Minimum essential medium (MEM) (Invitrogen) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate (Invitrogen), 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, supplemented with 10% heat-inactivated FCS. They were incubated at 37°C/ 5%CO_2. Prior to use, cells were rinsed with MEM medium

and incubated with trypsin/EDTA (1X, Invitrogen) at 37°C until the cells detached. To prepare the cells for experimental use they were washed three further times with MEM and resuspended in CRPMI at $1x10^7$/ml.

The Jurkat cells had been incubated in CRPMI/10%FCS (Invitrogen) with 0.5mg/ml geneticin (Invitrogen). They were washed three times with CRPMI and resuspended in CRPMI at $1x10^7$/ml prior to infection with influenza.

Jurkat and L929 cells were infected with 100ul X31 influenza A virus (H3N2) per 10^6 cells in serum-free medium for 2 hours. The virus was a kind gift from Dr. Philip Stevenson, University of Cambridge. After the first hour, chromium-51 (Amersham, UK) was added to the cell suspension (200uCi of chromium-51 per 300μl cell suspension ie 1x 10^6 cells). "Peptide loaded" Jurkat and L929 cells were each incubated with 10μM GILGFVFTL peptide at 37°C with 5%CO_2 for 1 hour. The targets were washed and then $5x10^3$ cells per well were placed in a 96 well U-bottomed plate. Effector cells from the CTL lines were added at ratios of 150:1, 100:1, 75:1, 50:1 and 25:1. The effector and target cells were incubated for 6 hours at 37°C with 5% CO_2. The killing assay protocol was then followed as above.

3.2.11 Dendritic cell generation

Dendritic cells were generated by plating PBMCs from a HLA A2 volunteer in a 6-well tissue culture plate (Nunc, UK). The cells were allowed to adhere for 2 hours at 37°C, 5% CO_2. The non-adherent fraction was then removed and the adherent cells cultured in 5%FCS with 1000U/ml GM-CSF (Schering-Plough) and 500U/ml IL-4(R&D). These Cytokines were added on days 0, 2, 4 and 6.

3.2.12 Production of In-Vitro transcribed mRNA

The insert from the plasmid pDOM.GILGFVFTL was amplified by PCR using the primers below:

GILPOLYA

Forward Primer (5'-3'): (T7-FrC)
TAA TAC GAC TCA CTA TAG GGA GAG CCA CCA TGG GTT GGA GCT GTA TCA

Reverse Primer (5'-3'):
TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TAG AGC GTG AAC ACA AAT CC

The Poly A tail incorporated into the PCR product leads to greater stability of the RNA produced later in the process. The PCR products were then run on a 1% agarose gel and purified with a QIAquick gel purification kit. The purified DNA was then subjected to phenol/chloroform precipitation to remove any protein contamination. This was achieved by mixing the DNA with an equal volume of phenol: chloroform: isoamyl alcohol solution (25:24:1, Sigma, UK). This was then centrifuged and the aqueous layer removed. Chloroform (99% solution, Sigma) was added and the mixture again centrifuged. The aqueous layer (45ul) was removed once more and the DNA precipitated with Ammonium acetate 5M solution (Sigma) and 100ul ethanol. The supernatant was removed and the pellet was washed again with 75% ethanol solution. The supernatant was removed after another centrifugation, and the pellet dried and re-suspended in RNA-ase free water (Ambion). DNA concentration was assayed by spectrophotmetrical analysis at Optical density (OD) 260.

Transcription of the purified DNA was carried out in a 40ul reaction for 2 hours at 37°C using a T7 MessageMachine kit (Ambion) to produce 5'm^7 GpppG-capped in-vitro transcribed mRNA. The resultant IVT mRNA sample was purified by DNAase I digestion followed by LiCl precipitation and 70% ethanol wash according to the manufacturer's instruction. RNA concentration was assayed by spectrophotometrical analysis at OD 260.

3.2.13 DC transfection

Immature DCs from a HLA-A2 donor were washed twice in serum free medium (EquiBio, Kent, UK) and resuspended at $2x10^6$ /ml in electroporation medium (EquiBio, Kent, UK) Subsequently 200ul of the cell suspension was mixed with 1.35μg of IVT mRNA in 100μl RNAase free water and electroporated using a Gene Pulse II (BIO-RAD, Herts, U.K.) in a 0.4-cm cuvette using an Easyject Plus device (EquiBio, Kent, U.K.).

A voltage of 300V was applied, combined with a capacitance of 150μF, in a total cuvette volume of 200μl.

After electroporation, the DCs were immediately placed in 3mls of pre-warmed CRPMI/5%FCS on a low adherence plate. They were incubated for 2 hours at 37°C/5%

CO_2. Lipopolysaccharide and IFN gamma were then added as maturation stimuli. 24 hours later the DCs were cultured with autologous CD8+ cells.

3.2.14 CD8+ cell culture with DCs

See method in CMV chapter.

3.2.15 Antigen priming

Mice were anesthetised with 150ul of 10mg/ml ketamine/ xylazine. Ketamine (2-[2-Chlorophenyl]-2-[methylamino]-cyclohexanone) Hydrochloride, (Sigma). Xylazine (2-[2,6-Dimethylphenylamino]-4H-5,6-dihydrothiazine), Hydrochloride (Sigma). Mice were then inoculated intranasally with 30µl of PBS containing a 1/5 dilution of X31 (H3N2) influenza A virus. The titre of the virus administered to the mice was 600 haemagglutinating units. The virus preparation was shown to be free of bacterial contaminants and endotoxin by the Limulus amebocyte lysate assay (Associates of Cape Cod Inc., Woods Hole, MA.

3.2.16 Tissue sampling

Mice were anesthetised with 150ul of 10mg/ml ketamine/xylazine. The mice were then bled out from the axilla into vacutainers containing lithium heparin (Becton, Dickinson. UK). The samples were then placed on ice for transport to Tenovus.

3.2.17 Bronchoalveolar lavage

The trachea was then exposed and a disposable plastic cannula with a 1.0-ml syringe attached was inserted through an incision immediately posterior to the larynx. The respiratory tract was then washed out in a standard reproducible manner with HBSS + 1% BSA, using three, separate 1.0ml aliquots, each of which was infused and withdrawn five times. Samples were transported on ice to Southampton. The BAL fluid was then spun at 1500g for 10mins. Each sample was resuspended in 5mls RPMI/10 % FCS. Adherent cells were removed by adsorbing onto a plastic Petri dish for 90mins. Non-adherent cells were removed and counted using a haemocytometer. The samples were spun at 1500g for 5 mins and resuspended in FACS buffer at a concentration of 1x 10^6 cells/ml. The Cells were incubated in the dark for 20 mins with PE labelled tetramer, A2Kb chimeric GILGFVFTL

(Proimmune, Oxford, UK). 10µl of PBS/ antibody solution was then added. Each 10µl of solution contained 2µl of APC-labelled anti-CD8α (Pharmingen, Oxford, UK) and 2µl of FITC-labelled I-A/I-E MHC II antibody (Pharmingen, Oxford, UK). The plate was then incubated at 4°C for 30 mins. Each well was then washed with 150µl of PBS and spun at 1450g for 4min. The supernatant was removed and the cells were then washed with 200µl PBS for 4min at 1450g. The cells were resupended and analysed on a FACScaliber.

3.2.18 Lung harvest and lung virus titre measurement

Freshly removed lungs were homogenized in 1.0ml of PBS containing 100 U/ml penicillin and 100µg/ml streptomycin. Aliquots (0.1 ml) of serial 10-fold dilutions of lung homogenates were inoculated into the allantoic cavity of 10-day-old embryonated eggs. After incubation at 35° for 48 hours, allantoic fluid from each egg was sampled and assayed for the presence of haemagglutinating activity using 0.5% chicken erythrocytes in a volume of 50µl. Titres of virus (EID_{50}) were expressed as the reciprocal of the highest dilution of the original homogenate that gave haemagglutination in 50% of eggs.

3.2.19 GILGFVFTL tetramer staining

10 ml of Puregene RBC lysis buffer was added to each 1ml blood sample. The sample was incubated at room temperature for 10 mins. It was inverted prior to incubation, after 5 mins and at the end of the incubation. After a 5 min spin at 1450g, the supernatant was removed and the sample was washed in 15ml PBS. After a further spin at 1450g for 5 mins the liquid was removed and the pellet resuspended in 150µl PBS /2% normal mouse serum and transferred to a 96 well U-bottomed plate. After blocking for 15 mins, the sample was spun at 1450g for 5 mins. The supernatant was discarded and the cells were washed with 200µl PBS. After another spin at 1450g the pellet was resuspended in 39µl PBS /0.2% tetramer solution. The tetramers used were the test tetramer, A2Kb chimeric GILGFVFTL (Proimmune, Oxford, UK), and a control tetramer A2Kb chimeric NLVPMVATV (Proimmune, Oxford, UK). The plate was incubated in the dark for 20 mins after which 10µl of PBS/ antibody solution was added. Each 10µl of solution contained 2µl of APC-labelled anti-CD8α (Pharmingen, Oxford, UK) and 2µl of FITC-labelled I-A/I-E MHC II antibody (Pharmingen, Oxford, UK). The plate was then incubated at 4°C for 30 mins. Each well was then washed with 150µl of PBS and spun at 1450g for 4min. The supernatant was removed and the cells were then washed with 200µl PBS for 4min at

1450g. Finally, the cells were fixed with 150µl PBS/ 1% formaldehyde solution until they were analysed on a FACScaliber.

3.2.20 Detection of IFN-γ secretion by ELISPOT analysis

T cells were assessed for the secretion of IFN-γ by enzyme-linked immunospot (ELISPOT) assay according to the manufacturer's instructions (BD Pharmingen) with some modification. Sterile 96-well ELISPOT plates were coated with 100µl/well of 5µg/ml purified anti-mouse IFN-γ. Sixteen to twenty-four hours later plates were blocked with complete medium for 2 hours at ambient temperature (37°C) and 5% CO_2. Plates were washed with sterile PBS and splenocytes (either 4 x 10^5 or 1x10^5) were added to each well along with peptides at the concentrations indicated. All sample wells were set up in triplicate and every plate included at least 3 wells containing splenocytes cultured in the absence of peptide to determine background levels of IFN-γ secretion.

Twenty-four hours later cells were lysed by two 5 min washes in dH_2O and plates were further washed 3 times with 0.5% PBS-tween prior to incubation with 1-2µg/ml biotinylated anti-mouse IFN-γ supplied in 10% PBS-FCS (foetal calf serum). After 2 hours at room temperature or following an overnight incubation at 4°C, plates were washed 3 times with 0.5% PBS-tween and incubated for a further hour with a 1 in 500 dilution of streptavidin-alkaline phosphatase (Mabtech AB, Nacka Strand, Sweden) in 10% PBS-FCS. Spots were visualised following 4 washes with 0.5% PBS-tween and 2 washes in PBS using a BCIP (5-Bromo-4-Chloro-3-Indolyl phosphate)/NBT (nitroblue tetrazolium salt) substrate according to manufacturers' instructions (Zymed, San Francisco, CA). The reaction was terminated within 10-15 min with dH_2O when spots became visible. Plates were allowed to air-dry prior to reading on an AID ELISpot reader (Autoimmun Diagnostika, GmbH, Strassberg, Germany). The mean number of spots detected in each of the 3 triplicate wells was calculated and used to determine the number of spots (corresponding to individual peptide-specific cells) obtained per million splenocytes.

3.3 Results

3.3.1 Validation of vaccine design

The DNA vaccines used in this study were pDOM.FLU and pDOM. The integrity of these vaccines was established by DNA sequencing, restriction digestion and in vitro transcription and translation (IVTT). The sequences were shown to be correct. In addition, the digests and the IVTT assays yielded products of the expected sizes.

3.3.2 The DNA vaccine pDOM.GILGFVFTL induces specific cytotoxicity

The ability of the DNA vaccine pDOM.FLU to induce specific cytotoxicity was initially demonstrated in a standard ^{51}Cr release assay (Figure 53). Mice which were transgenic for the human HLA-A2 molecule were vaccinated with either the pDOM.GILGFVFTL (n=6) or pDOM (n=6) DNA vaccine as previously described. The mice were sacrificed 17 days later. Splenocytes from pooled animals were stimulated with IL-2 and the peptide GILGFVFTL at 1μM for 6 days. Vaccination with pDOM.GILGFVFTL induced cells which caused cytolysis of T2 cells loaded with the peptide GILGFVFTL. There was 72% lysis of peptide loaded T2 cells at an effector to target ratio of 100:1. T2 cells which had not been pulsed with GILGFVFTL were not lysed. Furthermore, vaccination of mice with the control vaccine "pDOM" did not induce cells cytotoxic for GILGFVFTL loaded T2 cells (Figure 53).

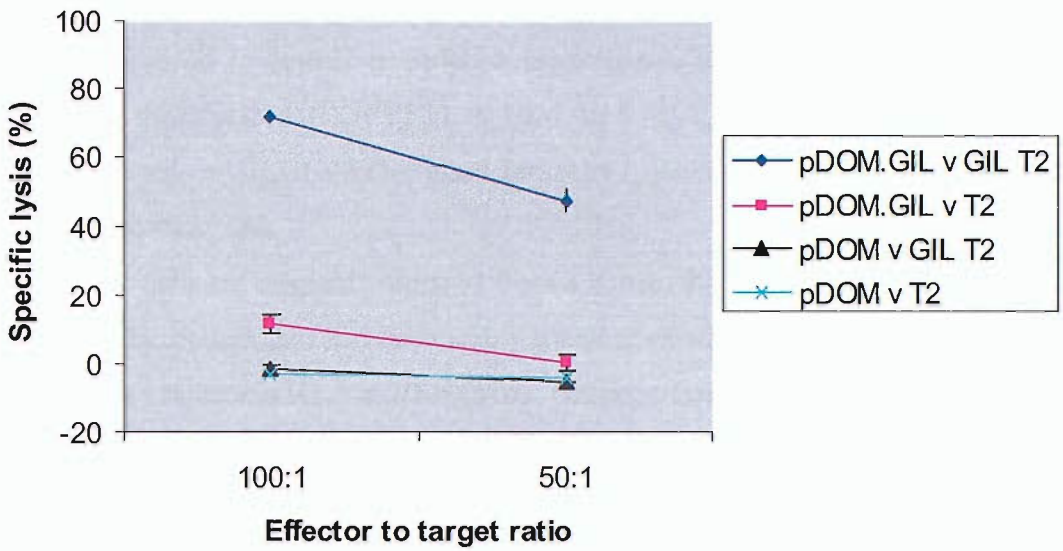


Figure 53. Splenocytes from mice vaccinated with pDOM.GILGFVFTL (pDOM.GIL) caused specific lysis of GILGFVFTL loaded target cells (GIL T2) but of not unloaded target cells (T2).
Splenocytes from mice vaccinated with pDOM (pDOM) were unable to cause lysis of GILGFVFTL loaded or unloaded target cells.

3.3.3 pDOM.GILGFVFTL induces CTLs which lyse influenza infected cells

Mice which were transgenic for the human HLA-A2 molecule were vaccinated with either the pDOM.GILGFVFTL (n=4) or pDOM (n=4) DNA vaccine as previously described. The mice were sacrificed 16 days later. Splenocytes from pooled animals were stimulated with IL-2 and with the peptide GILGFVFTL at 1μM for 6 days. The ability of these cells to lyse influenza infected or GILGFVFTL pulsed Jurkat or L-929 cells was then assessed in a standard cytotoxicity test.

Jurkat cells are originally derived from a human T-lymphoblast clone. The Jurkat cells used in this experiment had been stably transfected with a plasmid containing the chimeric class I molecule HLA-A2/Kb ($\alpha 1 \alpha 2$, domains from HLA-A2, $\alpha 3$ domain from murine Kb). L929 cells are originally derived from a murine connective tissue tumour. The L929 cells used in this experiment had been transfected retrovirally with HLA-A2.

Vaccination with pDOM.GILGFVFTL induced cells which caused 34% lysis of influenza infected Jurkat cells at an effector to target ratio of 75:1 (FIGURE 54A). Peptide loaded Jurkat cells experienced 57% lysis at the same effector to target ratio. Influenza infected L-929 cells were not killed but there was 41% lysis of GILGFVFTL peptide loaded L-929 cells at an effector to target ratio of 150:1 (FIGURE 55A). CTL from mice vaccinated with the control vaccine pDOM were unable to lyse either influenza infected or peptide loaded Jurkat or L-929 cells (FIGURE 54B and 55B).

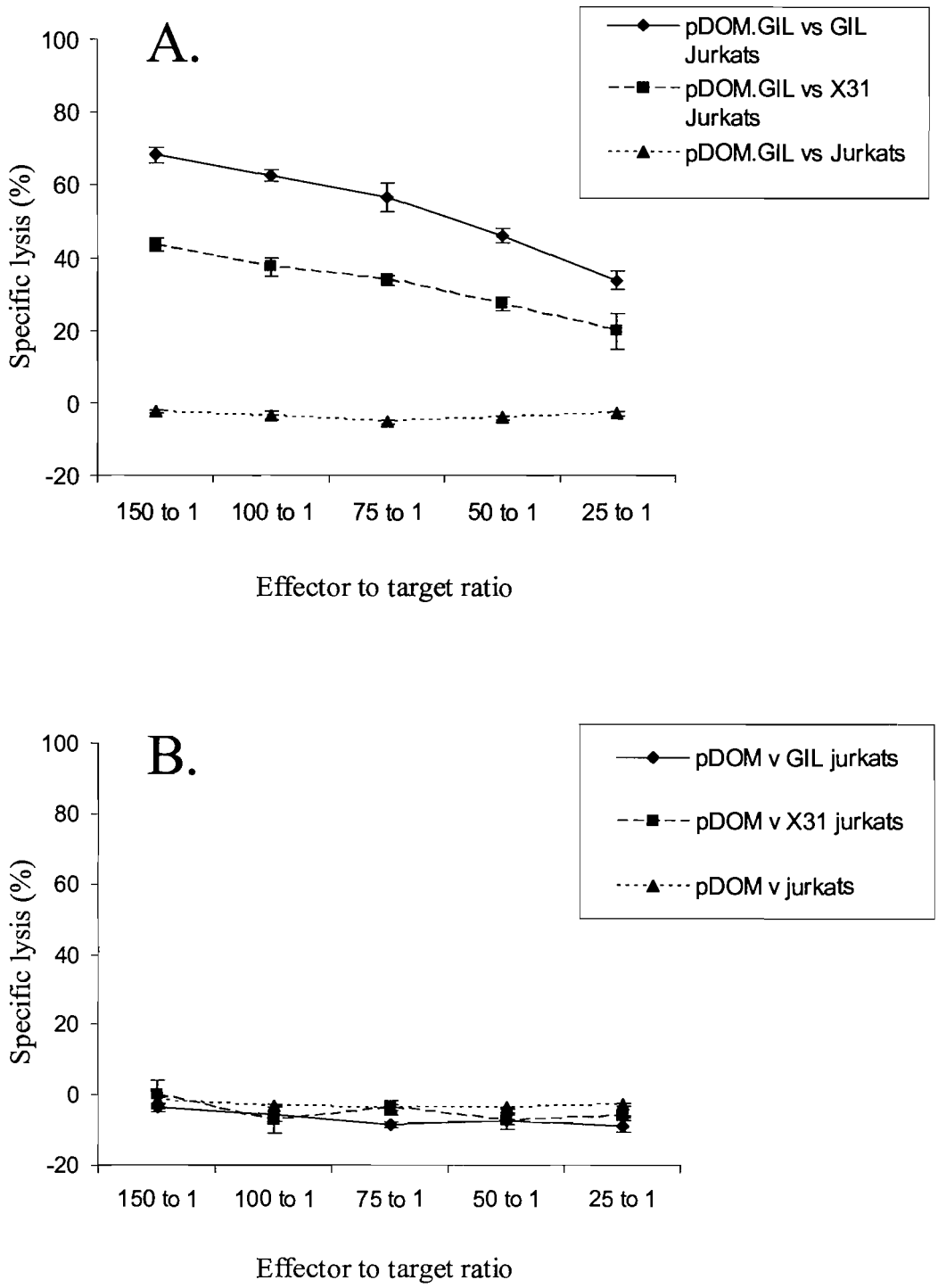


Figure 54. (A) Vaccination of HLA-A2/Kb transgenic mice with pDOM.FLU.GIL (pDOM.GIL) induced CTLs which caused specific lysis of GILGFVFTL pulsed Jurkat cells (GIL Jurkats) and X31 influenza infected Jurkat cells (X31 Jurkats). (B) No lysis of target cells was caused by mice vaccinated with the control vaccine pDOM.

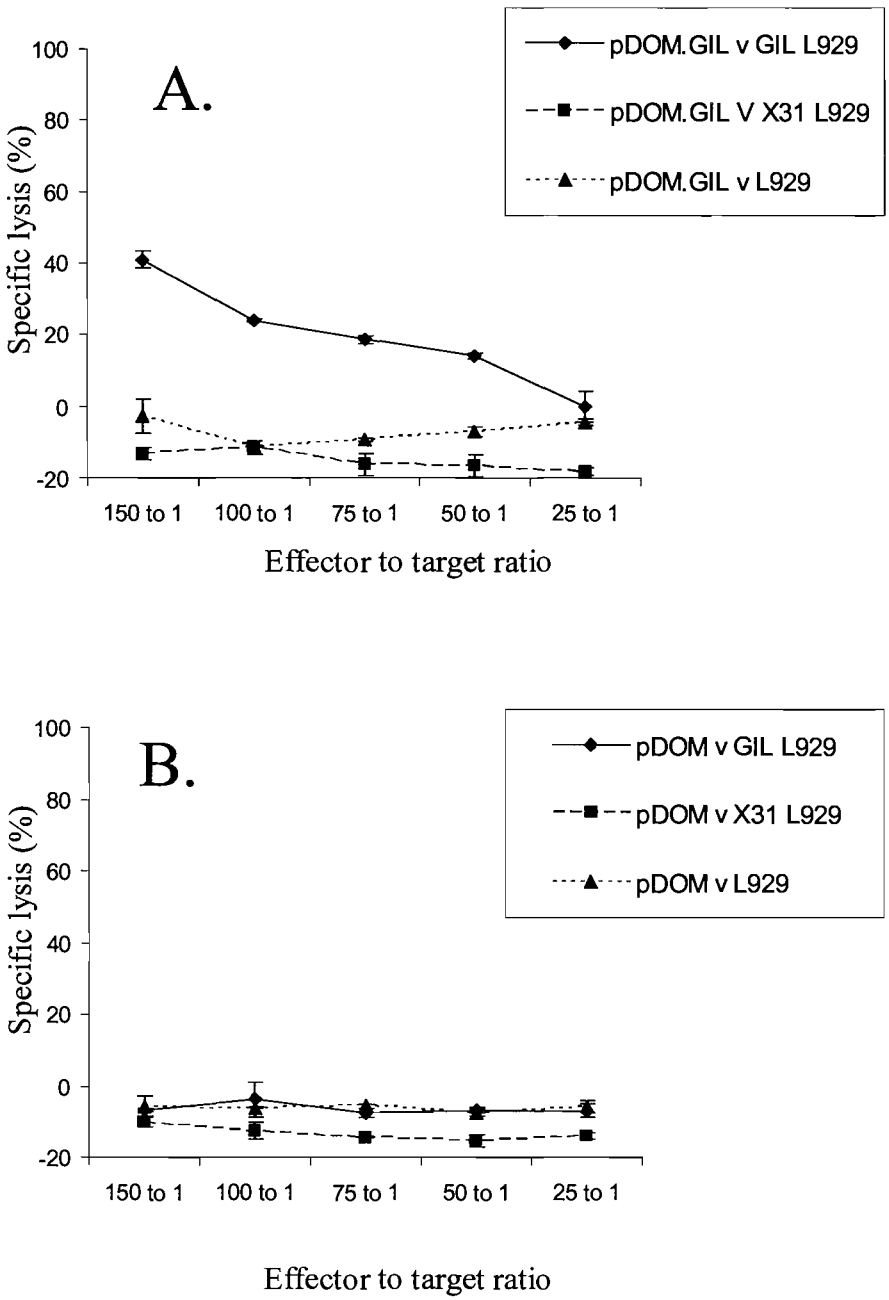


Figure 55. (A) Vaccination of HLA-A2/Kb transgenic mice with pDOM.FLU.GIL (pDOM.GIL) induced CTLS which caused specific lysis of GILGFVFTL pulsed L929 cells (GIL L929) but not X31 influenza infected L929 cells (X31 L929). (B) No lysis of target cells was caused by mice vaccinated with the control vaccine pDOM.

3.3.4 A high proportion of HHD transgenic mice respond to vaccination with pDOM.GILGFVFTL

Our next goal was to test the effect of DNA vaccination with pDOM.GILGFVFTL on the course of influenza infection in mice. It was important to ascertain the proportion of pDOM.GILGFVFTL vaccinated mice that produced GILGFVFTL specific CTLs as previous experiments had been performed with splenocytes pooled from several mice. Previous experiments in our laboratory indicated that only 25-40% of A2/Kb transgenic mice responded to vaccination due to preferential usage by mouse CTLs of H-2 class I molecules for antigen recognition.

HHD transgenic mice became available during these experiments. These HLA-A2 transgenic $H2K^{b-/-}$ $\beta2m^{-/-}$double knockout mice express a chimeric MHC class I molecule in which the $\alpha1\,\alpha2$ domains are derived from HLA-A2.1 whilst the $\alpha3$ transmembrane domain is derived from murine $H\text{-}2D^{b}$. They were created by transfecting $H\text{-}2D^{b}$ and mouse $\beta2$ microglobulin double knockouts with a HHD (**H**uman $\beta2$m-**H**LA-A2.1 ($\alpha1\,\alpha2$)-$H\text{-}2\mathbf{D}^{b}$($\alpha3$ transmembrane cytoplasmic)) monochain construct [281]. This forces the mouse $CD8^{+}$ repertoire to make use of the transgenic HLA class I molecules and restores a sizeable peripheral $CD8^{+}$ T-cell repertoire educated on the transgenic human molecule [281]. This is therefore a more useful animal model for the study of HLA-A2.1 restricted CTL responses.

Six HHD mice were vaccinated with pDOM.GILGFVFTL and 2 control HHD mice were vaccinated with pDOM. Mice were sacrificed on day 17 and splenocytes from each mouse were then were tested for responses to GILGFVFTL by two separate methods. Firstly, interferon production by splenocytes in response to overnight incubation with GILGFVFTL peptide was assessed by an ex-vivo Elispot assay. Secondly, the ability of residual splenocytes (after culture for one week with IL-2 and the peptide GILGFVFTL at 1μM) to lyse GILGFVFTL loaded T2 target cells was assessed in a standard ^{51}Cr release assay.

Splenocytes from all six individual HHD mice vaccinated with pDOM.GILGFVFTL produced interferon γ in response to incubation with GILGFVFTL at 1μM, 0.1μM and 0.01μM. The median number of spots per 10^{6} splenocytes was 207 following incubation with GILGFVFTL at 1μM, 208 at 0.1μM and 142 at 0.01μM (FIGURE 56). Control mice vaccinated with pDOM showed no response to GILGFVFTL at any of the concentrations listed above.

Splenocytes from all six pDOM.GILGFVFTL vaccinated mice caused cytolysis of GILGFVFTL loaded target cells. At an effector to target ratio of 70:1, lysis of target cells ranged from 75% by HHD mouse number 5 to 30% by mouse number 3. There was minimal lysis of control target cells loaded with the irrelevant peptide NLVPMVATV by HHD mice vaccinated with pDOM.GILGFVFTL (FIGURE 57). One of the 2 mice vaccinated with pDOM exhibited a similar degree of lysis of GILGFVFTL loaded target cells as the weakest response seen in one of the group of mice vaccinated with pDOM.GILGFVFTL. A possible explanation for this was LAK cell activity. Alternatively, it was possible that a GILGFVFTL specific response may have been induced by a cross reactive region present in the pDOM vaccine.

3.3.5 Vaccination with pDOM.GILGFVFTL induces CTLs which specifically kill GILGFVFTL pulsed target cells whilst vaccination with pDOM does not

The observation that vaccination with pDOM seemed to induce cells capable of causing specific lysis, albeit at a low level, of GILGFVFTL loaded target cells was further investigated in a subsequent experiment. Four HHD mice were vaccinated with pDOM.GILGFVFTL, six mice with pDOM and six control mice received no vaccine. Mice were sacrificed on day 17 and their spleens were harvested. On this occasion, splenocytes from 2 mice within each group were pooled. Cells were then incubated for one week with IL-2 and with GILGFVFTL at concentrations of 1μM, 0.1μM or 0.01μM. In addition, some splenocytes from each group were incubated with irradiated autologous feeder cells which had previously been pulsed with GILGFVFTL at 10μM for 1 hour. The ability of all groups of splenocytes to lyse target cells pulsed with

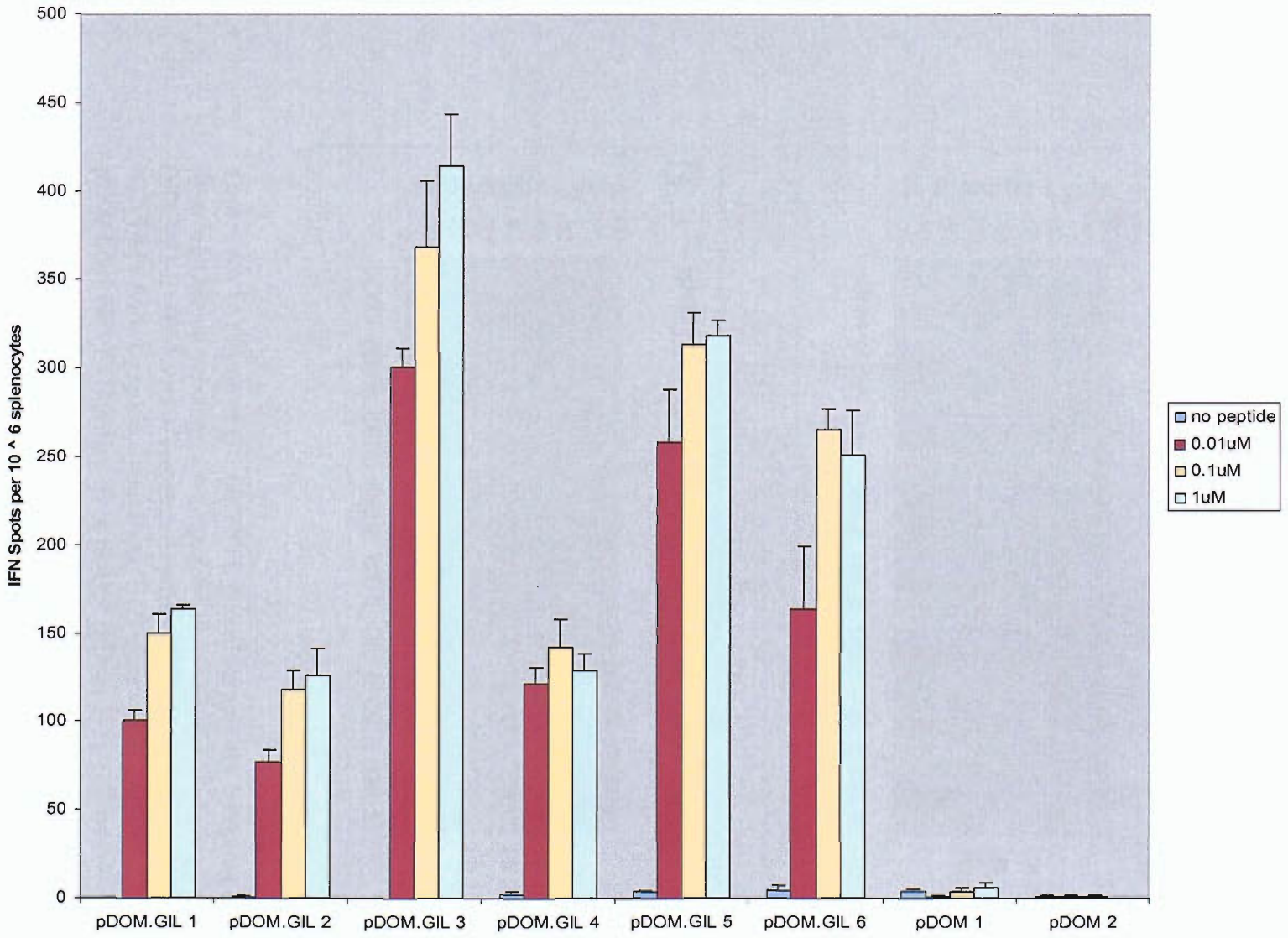


Figure 56. Splenocytes from all six HHD mice vaccinated with pDOM.GILGFVFTL (pDOM.GIL 1-6) produced Interferon-γ after incubation with GILGFVFTL peptide. No spots were produced by mice vaccinated with the control vaccine pDOM.

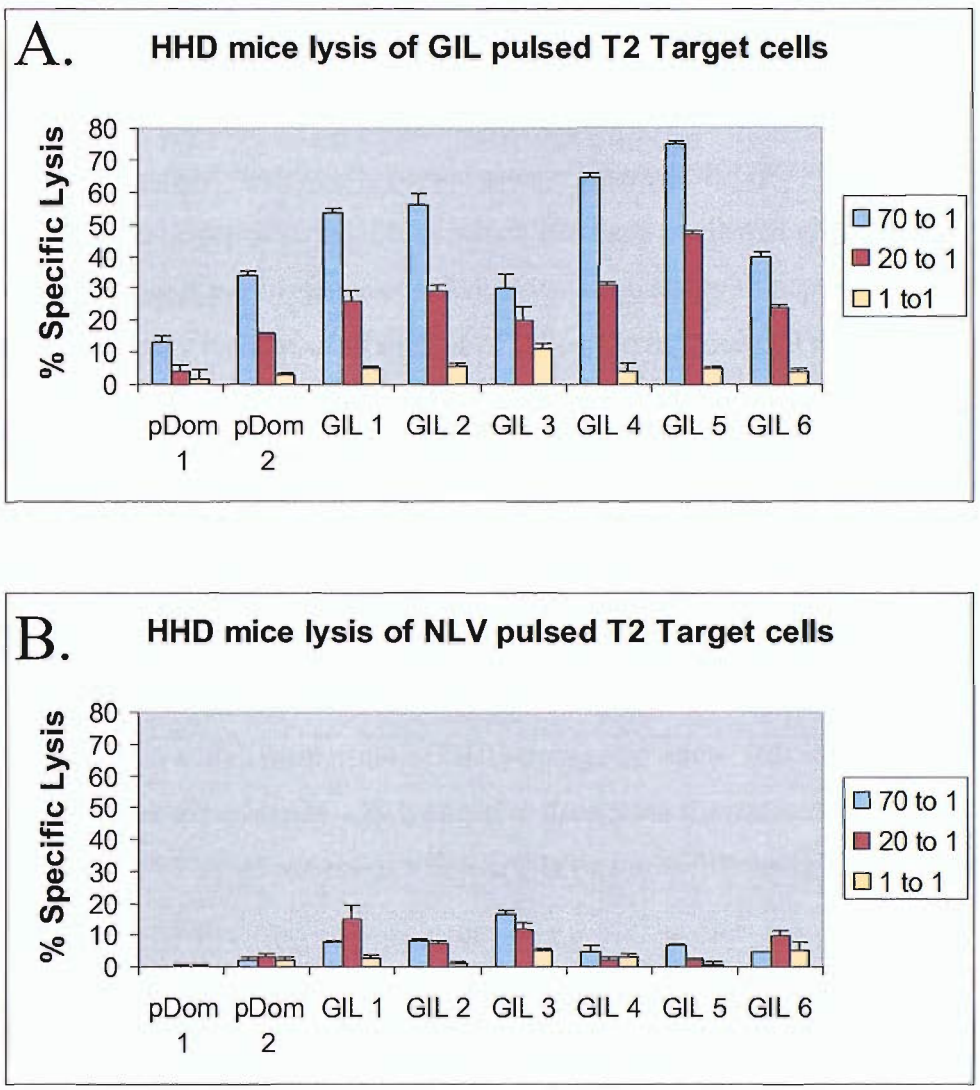


Figure 57. (A) Lysis of GILGFVFTL pulsed target cells by splenocytes from HHD mice vaccinated with pDOM.FLU-GIL (GIL1-6) or pDOM (pDOM1-2) **(B)** Lysis of target cells pulsed with the control peptide NLVPMVATV by splenocytes from HHD mice vaccinated with pDOM.GILGFVFTL (GIL1-6) or pDOM (pDom 1-2). E:T ratio= effector to target ratio.

GILGFVFTL or NLVPMVATV was then assessed in a standard ^{51}Cr release assay (Figure 58).

Splenocytes from either unvaccinated mice (Figure 58C) or those which had been vaccinated with pDOM (Figure 58B) did not cause lysis of either NLVPMVATV or GILGFVFTL pulsed target cells at any effector to target ratio, indicating that no cross reactivity existed. Splenocytes from mice vaccinated with pDOM.GILGFVFTL clearly caused specific lysis of GILGFVFTL pulsed target cells (Figure 58A). Cells which had been incubated with GILGFVFTL at 0.01 μM and those which had been incubated with GILGFVFTL pulsed feeder cells caused greater lysis of GILGFVFTL pulsed target cells at all 4 effector to target ratios than cells which had been incubated with GILGFVFTL at 0.1μM. The poorer performance of splencoytes incubated with peptide at the higher concentration may have been as a result of activation induced cell death of GILGFVFTL specific CTLs.

3.3.6 Influenza viral challenge of pDOM.GILGFVFTL vaccinated mice

Our experiments revealed that pDOM.FLU-GIL vaccination induced CTLs specific for GILGFVFTL in a high proportion of HHD transgenic mice. This information allowed us to consider further experiments which aimed to determine if mice vaccinated with pDOM.GILGFVFTL experienced a favourable outcome following a subsequent encounter with Influenza virus.

Many factors combined to make the design and execution of such experiments challenging. We did not expect that influenza specific CTLs would protect mice against viral infection in the sterile manner exhibited by virus specific haemagglutinin antibodies. Rather, mice with virus specific CTLs mice would still be expected to develop an influenza infection, possibly with some degree of virus induced morbidity, but would then be expected recover from viral infection more quickly than control mice.

Current Home Office regulations do not permit traditional protection experiments to be performed. In such experiments, mice were challenged with a large amount of virus and then observed over a period of days to see if they survived or succumbed. Regulations require that experiments are designed in such a manner as to limit disease induced morbidity and prevent the disease induced mortality of experimental animals. These concerns were of paramount importance in the design of our experiments.

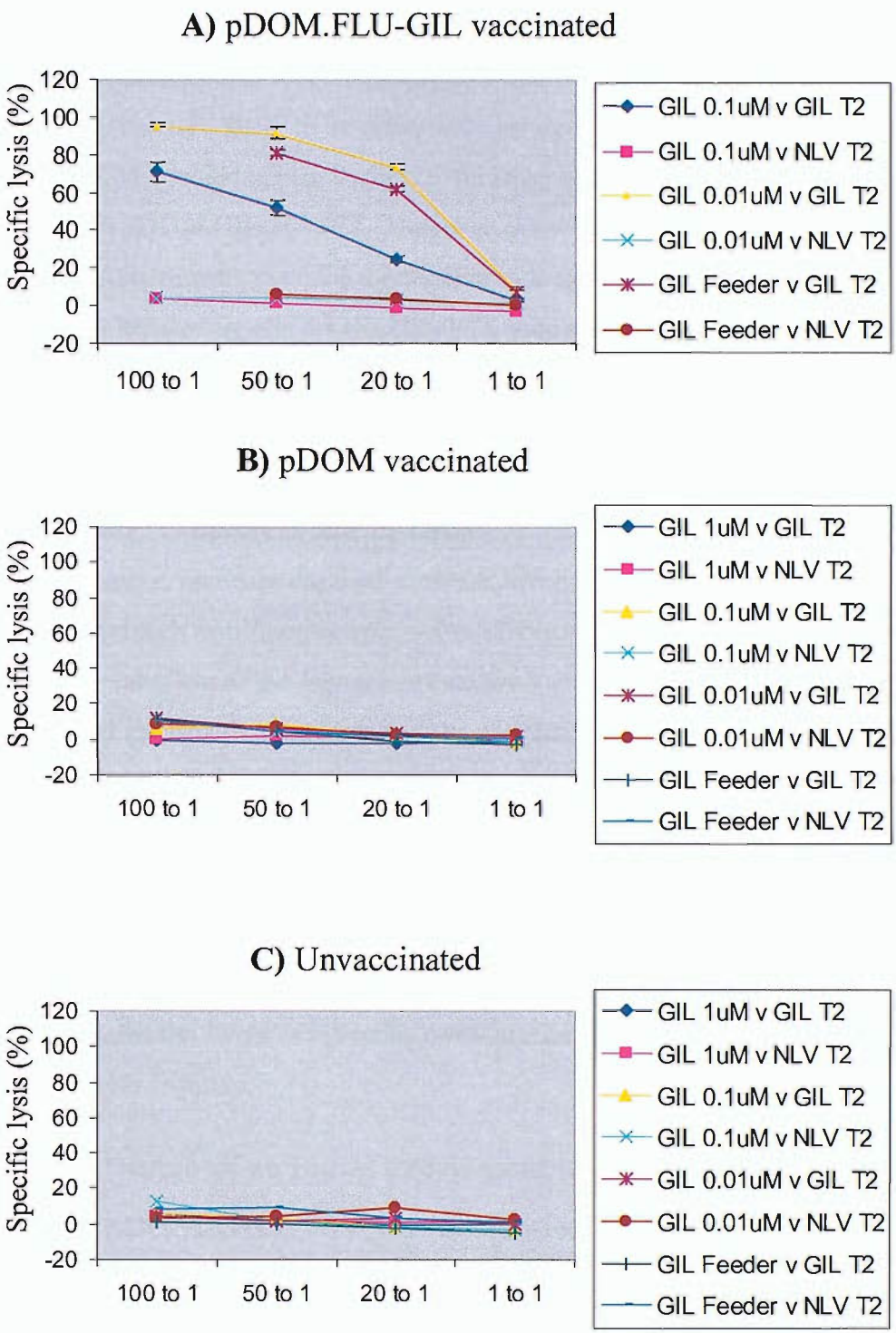


Figure 58. (A) CTLs from HHD mice vaccinated with pDOM.GILGFVFTL killed target cells pulsed with GILGFVFTL (GIL T2) but not NLVPMVATV (NLV T2). CTLs had been stimulated in-vitro for1 week with GILGFVFTL at 1μM, 0.1μM, 0.01μM or by feeder cells which had been pulsed with GILGFVFTL (GIL feeder). CTLs from mice vaccinated with pDOM **(B)** or unvaccinated mice **(C)** did not exhibit specific lysis of GILGFVFTL target cells. E:T ratio = effector to target ratio.

Although differences in sensitivity exist between various murine strains, naïve mice normally succumb 7-10 days after intra-nasal innoculation Influenza virus at high dose. Effector CTLs are detected in the lungs of naïve mice at 5-7 days after vaccination, although again there are likely to be differences between strains of mice. We expected that virus specific CTLs would appear earlier in the lungs of mice which had successfully been vaccinated with pDOM.GILGFVFTL. However, we were aware that a very narrow window was likely to exist in which a reduction in lung virus titres which resulted from the action of CTLs induced by pDOM.GILGFVFTL vaccination could be observed. In our initial experiment we therefore planned to measure the lung virus titres of test and control mice 5-7 days after challenge with lethal doses of Influenza virus. It was felt that further information would be gained by performing this experiment at two time points after vaccination: early (17 days) and late (60 days).

The isolation facilities required for work involving a highly infectious and pathogenic virus such as Influenza were not available within our institution. We were grateful to Dr. Sam Hou of the Jenner Institute for Vaccine Research at Compton who kindly provided the isolation facilities and the necessary expertise required to challenge mice with influenza. Home office regulations prevented us from vaccinating the mice in Southampton prior to their transport to Compton for viral challenge. Regulations at the Jenner Institute required that only an employee of that institution, in this case Dr. Hou, could vaccinate the mice.

The above considerations were reflected in the design of our initial experiment which investigated the effect of influenza challenge on pDOM.GILGFVFTL vaccinated mice at the Jenner Institute.

3.3.6.1 Design of an initial experiment to assess the effect of pDOM.GILGFVFTL vaccination on the course of influenza infection following challenge with a lethal dose of X31 virus.

Forty-eight HHD mice were transported to the Jenner institute for study. They were divided into 4 groups, each containing 12 mice. On day 0, the first group of mice (X31) received X31 influenza virus intranasally at a dose which would not be expected to cause mortality but would cause an acute episode of influenza. These mice develop CTLs specific for epitopes within the X31 virus (including GILGFVFTL) and recover from the infection. They also develop antibodies against haemagglutinin and neuraminidase on the

surface of the X31 virus which completely protect these mice during a subsequent encounter with this particular stain of influenza.

The second group of mice were vaccinated on day 0 with the DNA vaccine of interest, pDOM.GILGFVFTL (pDOM.GIL). The third group of mice received no intervention on day 0 (Naïve) whilst group 4 were vaccinated with the DNA vaccine pDOM (pDOM).

Three mice from each group were sacrificed on day 17 to determine if they had developed CTL responses against GILGFCFTL.

Three further mice from each group were challenged with a lethal dose of X31 virus on day 17 and euthanased 5 days later. Lung X31 virus titres were measured in these mice to determine if pDOM.GILGFVFTL vaccinated mice cleared the virus more quickly than control mice.

Another three mice from each group were sacrificed at day 60. This was to determine the magnitude of the CTL response to GILGFVFTL at a later time point.

The final three mice in each group were challenged with lethal dose of X31 Influenza virus on day 60 and sacrificed 7 days later. This was to ascertain if pDOM.GILGFVFTL vaccinated mice could clear X31 virus from their lungs more rapidly than control mice following a viral infection several months after vaccination.

3.3.6.2 GILGFVFTL specific CTLs were present in the blood and spleen of HHD mice 17 days after vaccination with pDOM.GILGFVFTL at the Jenner Institute

Three mice from each group were sacrificed on day 17 to determine if the interventions on day 0 had resulted in the production of CTLs specific for GILGFVFTL. Following anaesthesia, mice were exsanguinated from the axilla. Tetramer analysis was performed on the peripheral blood to determine the frequency of GILGFVFTL specific CD8 cells. The spleen was also harvested from each mouse. An Interferon-γ Elispot assay was carried out on splenocytes from each mouse. Residual splenocytes from mice were pooled into one of four groups (pDOM.GIL, pDOM, Naïve and X31). A CTL assay was then performed to determine if CTLs capable of causing specific lysis of GILGFVFTL loaded target cells were present.

CTLs specific for GILGFVFTL detected in the blood and spleen of pDOM.GIL mice 2 and 3 (Figures 59 & 60). GILGFVFTL specific CTLs were detected at a higher frequency in the blood and spleen of pDOM.GIL mouse 2 than in pDOM.GIL mouse 3

(Figures 59 & 60). No CTLs specific for GILGFVFTL were detected by either assay in pDOM.GIL mouse 1 (Figures 59 & 60).

Mice which had previously been inoculated with a low dose of X31 virus (X31 1-3) exhibited GILGFVFTL specific CTLs in the spleen and peripheral blood at a higher frequency than mice successfully vaccinated with pDOM.FLU-GIL (Figures 59 & 60).

No GILGFVFTL specific CTLs were detected by either assay in control mice which had been vaccinated with pDOM (pDOM 1-3) or received no intervention (Naïve 1-3) on day 0 (Figures 59 & 60)

Splenocytes from X31 innoculated mice and from pDOM-GIL vaccinated mice caused specific lysis of GILGFVFTL loaded target cells. (Figure 61) Splenocytes from the X31 group showed greater cytotoxic activity than the pDOM-GIL group (Figure 61). Splenocytes from naïve mice and from mice vaccinated with the pDOM control vaccine did not lyse GILGFVFTL loaded target cells (Figure 61).

These assays demonstrated that mice could be succesfully vaccinated with pDOM.FLU-GIL at the Jenner institute, although response rates were slightly lower than observed in our own laboratory.

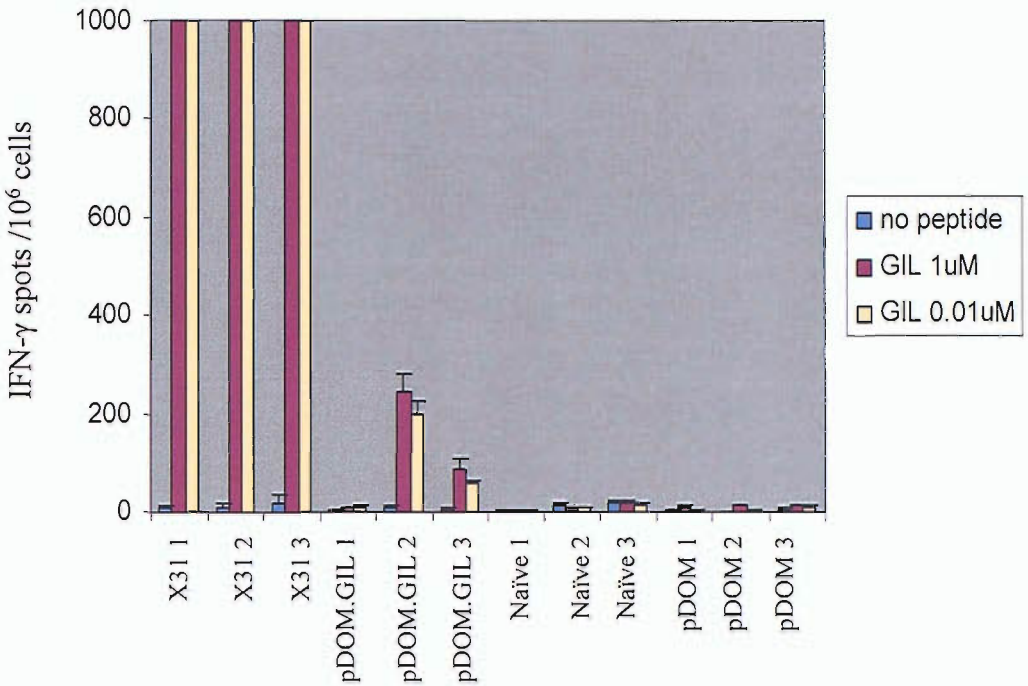


Figure 59. Splenocytes from each mouse were harvested 17 days after vaccination at the Jenner Insitute.. These were analysed in an Interferon-γ Elispot with either A) no peptide(■), B) GILGFVFTL at 1μM (■) or C) 0.01μM (□). On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), no intervention (Naïve) or vaccinated with pDOM (pDOM).

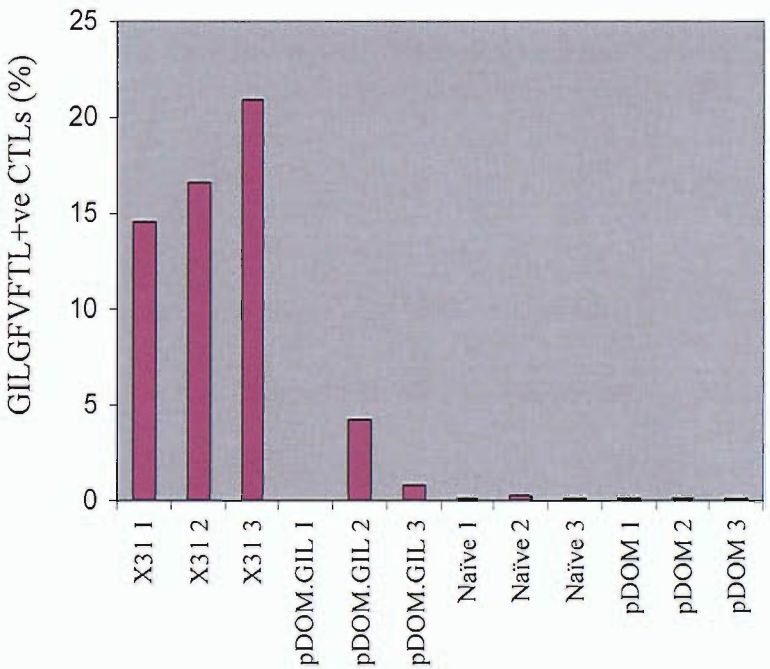


Figure 60. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in peripheral blood of individual mice on day 17. On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM).

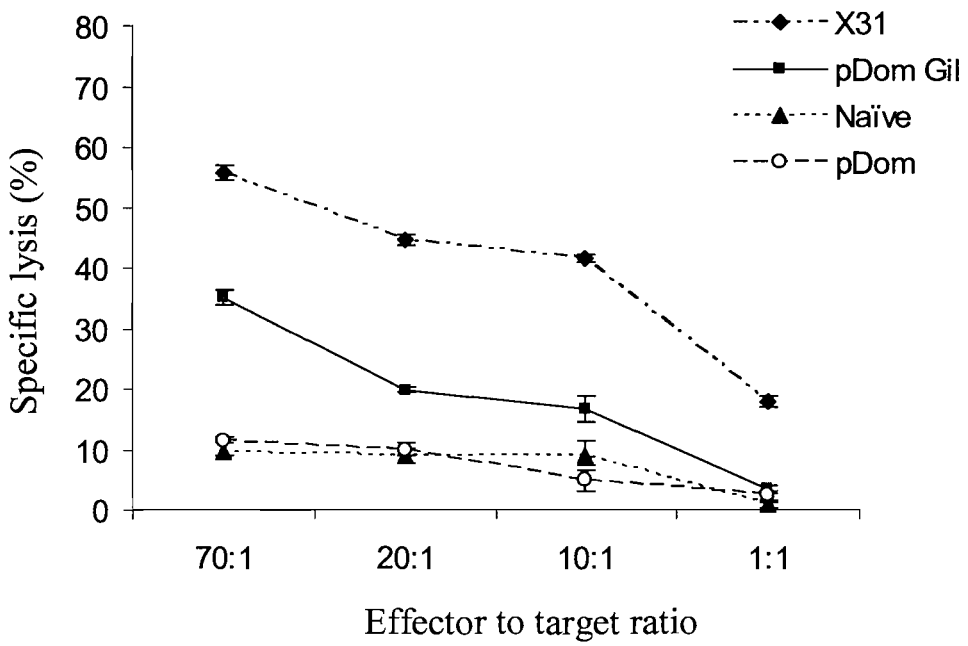


Figure 61. Splenocytes from each mouse were harvested 17 days after vaccination at the Jenner Institute. These were analysed in a killing assay. CTLs from HHD mice which had been vaccinated with pDOM.FLU-GIL (pDOMGIL) or immunised with X31 virus on day 0, caused specific lysis of GILGFVFTL labelled target cells. CTLs from naïve mice or those which had been vaccinated with pDOM did not cause specific killing of target cells.

3.3.6.3 pDOM.GILGFVFTL vaccinated mice did not have GILGFVFTL specific CTLs present at higher levels nor did they have lower X31 lung virus titres than control mice 5 days after viral challenge.

On day 17, three mice from each group were challenged with a lethal dose of influenza virus. Five days later these mice were sacrificed. The % of CTLs specific for GILGFVFTL in the peripheral blood of each mouse was measured by flow cytometry. As expected, mice which had been inoculated with a non-lethal dose of X31 on day 0 had high levels of CTLs specific for GILGFVFTL present in their peripheral blood 5 days after challenge with a lethal dose of X31 virus (Figure 62). Mice vaccinated on day 0 with pDOM.GILGFVFTL did not have higher levels of GILGFVFTL specific CTLs after lethal challenge than naïve or pDOM vaccinated mice (Figure 62)

Lung X31 virus titres were also measured for each mouse 5 days after challenge with X31 virus at a lethal dose. Virus was not detected in the lungs of mice which had previously received a non-lethal dose of X31 virus (Figure 63). Mice which had been vaccinated with pDOM.GILGFVFTL were found to have X31 virus present in their lungs at a similar level to naïve or pDOM vaccinated control mice.

The low level of CTLs specific for GILGFVFTL present in the blood of the pDOM.GILGFVFTL vaccinated mice suggests that these mice had not respond to vaccination and therefore we would not expect them to behave differently to control mice following lethal challenge with X31 virus. We also considered the possibility that day 5 was too early to allow the virus eradicated from the lungs of mice which had been primed against GILGFVFTL. In our next experiment we therefore sacrificed the mice 7 days after lethal challenge with virus.

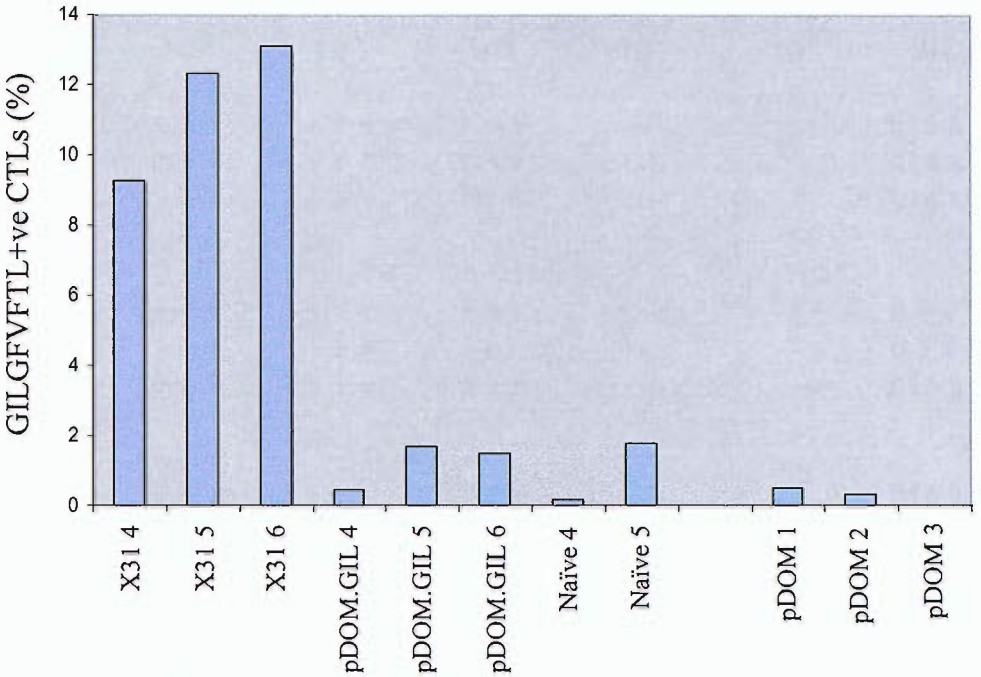


Figure 62. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in peripheral blood of individual mice on day 22 (5 days after challenge with a lethal dose of X31 Influenza virus) On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM).

	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	EID_{50}
pDOM.GIL						
4	+ + +	+ + +	+ + +	- - -		3.16×10^7
5	+ + +	+ + +	+ + +	- - -		3.16×10^7
6	+ + +	+ + +	+ + +	- - -		3.16×10^7
pDOM						
4	+ + +	+ + +	+ + -	+ - +	- - -	6.8×10^7
5	+ + +	+ + +	- - +	- - -	- - -	6.8×10^6
6	+ + +	+ + +	+ + +	- - -	- - -	3.16×10^7
Naive						
4	+ + +	+ + +	+ + +	- - -	- - -	3.16×10^7
5	+ + +	+ + +	+ + +	- - -	- - -	3.16×10^7
X31	Neat	10^{-1}				
4	- - -	- - -				
5	- - -	- - -				
6	- - -	- - -				
+ve control	+ + +					
-ve control	- - -					

Figure 63 X31 Influenza virus titres in lungs of HHD mice sacrificed 5 days after lethal challenge virus (day 22 post vaccination).

X31 Influenza virus titres in lungs of HHD mice sacrificed on day 22 post vaccination. On day 17 mice had been inoculated with a lethal dose of X31 virus. On day 0 mice had been either vaccinated with pDOM.GILGFVFTL (pDOM.GIL), vaccinated with pDOM (pDOM), received no treatment (Naive) or immunised with X31 virus (X31). Aliquots of serial 10-fold dilutions of lung homogenates were inoculated into the allantoic cavity of 10-day-old embryonated eggs. Triplicates of eggs were used for each dilution. Allantoic fluid from each egg was then sampled and assayed for the presence of haemagglutinating activity. + indicates haemagglutination. - indicates no haemagglutination. Lung homogenate dilutions are indicated (10^{-4}, 10^{-5} etc). Titres of virus (EID_{50}) are expressed as the reciprocal of the highest dilution of the original homogenate that gave haemagglutination in 50% of eggs.

3.3.6.4 GILGFVFTL specific CTLs were present in the blood and spleen of HHD mice 60 days after vaccination with pDOM.GILGFVFTL at the Jenner Institute

Two mice from the "X31" and "pDOM.GIL" groups were sacrificed on day 60 alongside three mice from the "naïve" and "pDOM" groups". This was to establish if the interventions on day 0 had resulted in the production of GILGFVFTL specific CTLs at a late time point. High levels of GILGFVFTL specific CTLs were found in the spleen and blood of the mice which had been inoculated with a low dose of X31 virus on day 0 (Figures 64 and 65). Specific CTLs were clearly detected in the spleen and blood of pDOM.GIL mouse 8, although at much lower levels than in the X31 mice (Figures 64 and 65). As expected, CTLs specific for GILGFVFTL were not deteced in the pDOM vaccinated or unvaccinated (naïve) mice (Figures 64 and 65). This confirmed our previous finding that vaccination of mice with pDOM.GILGFVFTL, under the conditions which existed at the Jenner Institute, resulted in the successful induction of CTLs specific for GILGFVFTL. It was encouraging to note that these CTLs could still be detected in ex-vivo assays at the relatively late time point of 60 days after vaccination. Again the proportion of mice responding seemed lower than that observed when vaccination took place at our own institution.

3.3.6.5 No influenza virus was detected in the lungs of a pDOM.GILGFVFTL vaccinated mouse 7 days after challenge with a lethal dose of X31 virus.

On day 60, three mice from each group were challenged with a lethal dose of influenza virus. Seven days later these mice were sacrificed. Lung virus tires were measured. No virus was detected in the lungs of X31 mice (Figure 66). No virus was present in the lungs of 1/3 mice which had been vaccinated with pDOM.GILGFVFTL (Figure 66). Virus was present in the lungs of the naïve and pDOM vaccinated mice (Figure 66).

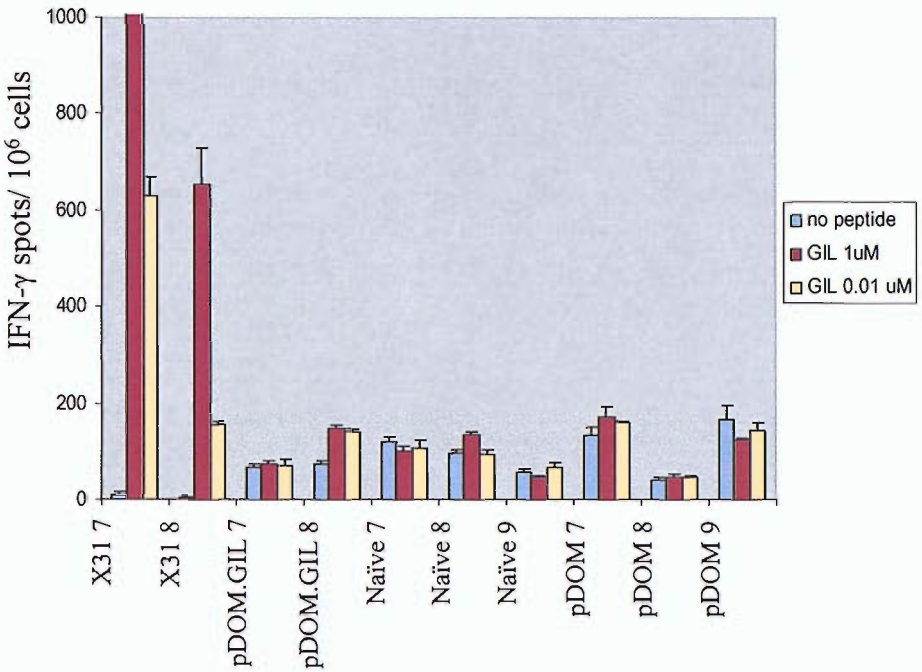


Figure 64. Splenocytes from each mouse were harvested 60 days after vaccination at the Jenner Insitute. These were analysed in an Interferon-γ Elispot with either A) no peptide(□), B) GILGFVFTL at 1μM (■) or C) 0.01μM (□). On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), no intervention (Naïve) or vaccinated with pDOM (pDOM).

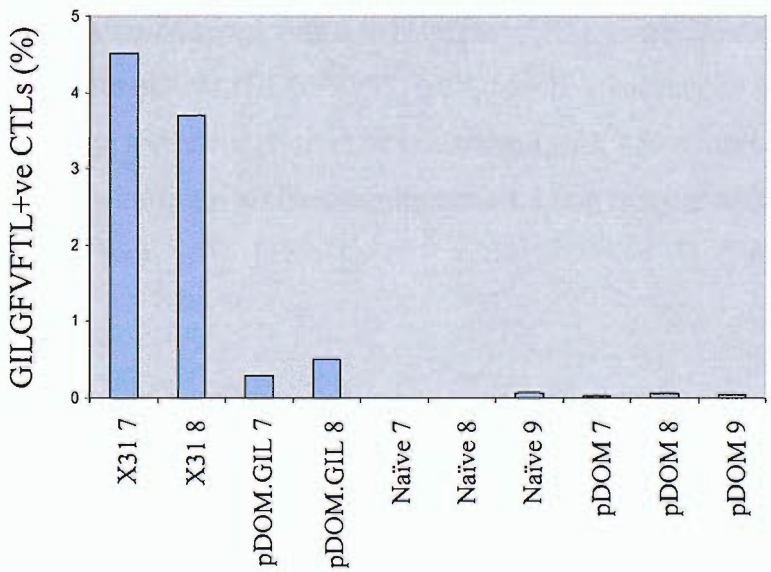


Figure 65. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in peripheral blood of individual mice on day 60. On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM).

	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
pDOM.GIL						
10	+++	+++	--+	---	---	---
11	---	---	---	---	---	---
12	+++	+++	+++	---	---	---
pDOM						
10	+++	+++	---	---	---	---
11	+++	+++	---	---	---	---
12	+++	+++	+++	---	---	---
Naïve						
10	+++	---	---	---	---	---
11	+++	--+	---	---	---	---
12	+++	+++	+++	+-+	---	---
X31	Neat					
10	---					
11	---					
12	---					
+ve control	+++					
-ve control	---					

Figure 66 X31 Influenza virus titres in lungs of HHD mice sacrificed 7 days after challenge with X31 virus at a lethal dose (day 67 post vaccination).

X31 Influenza virus titres in lungs of HHD mice sacrificed on day 67 post vaccination. On day 60 mice had been inoculated with a lethal dose of X31 virus. On day 0 mice had been either vaccinated with pDOM.GILGFVFTL (pDOM.GIL), vaccinated with pDOM (pDOM), received no treatment (Naive) or immunised with X31 virus (X31). + indicates haemagglutination. - indicates no haemagglutination. Lung homogenate dilutions are indicated (10^{-4}, 10^{-5} etc).

3.3.6.6 The pDOM.GILGFVFTL vaccinated mouse which had no X31 virus present in it's lungs 7 days after lethal challenge also had CTLs specific for GILGFVFTL present in the peripheral blood and BAL at a high frequency.

Analysis of the peripheral blood and BAL 7 days after challenge with a lethal dose of X31 virus showed that mouse "pDOM.GIL 11" had GILGFVFTL specific CTLs present at a higher frequency than any of the other mice (Figure 67 and 68). The other 2 mice which had been vaccinated with pDOM.GILGFVFTL had GILGFVFTL specific CTLs present in the blood and BAL at frequencies which were within the range observed in the negative control mice (pDOM and naïve) (Figures 67 and 68).

3.3.6.7 Design of an subsequent experiment to assess the effect of pDOM.GILGFVFTL vaccination on the course of influenza infection following challenge with a lethal dose of X31 virus.

Our initial experiment had shown that CTLs could be successfully induced by vaccination with pDOM.GILGFVFTL at the Jenner Institute. We had also determined that negative control mice survived until at least day 7 after challenge with a lethal dose of X31 virus. Encouragingly, one mouse vaccinated with pDOM.GILGFVFTL had no X31 influenza present in its lungs 7 days after viral challenge. The next experiment was designed ascertain in a larger group of mice if pDOM.GILGFVFTL vaccination enabled mice to survive a challenge with a high dose of X31 influenza virus.

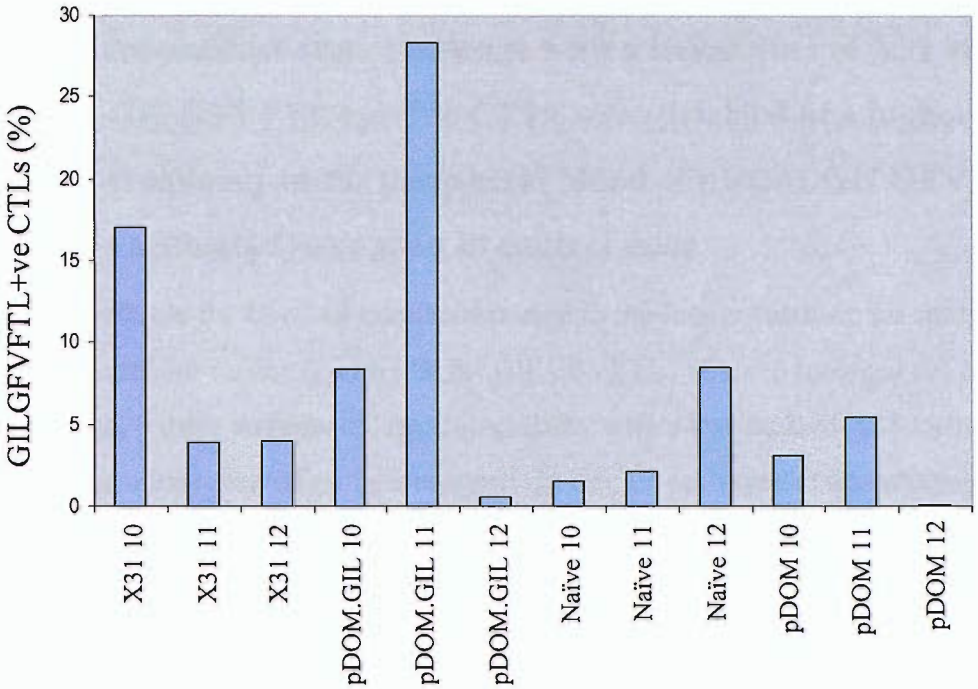


Figure 67. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in peripheral blood of individual mice on day 67 (7 days after challenge with X31 Influenza virus at a lethal dose). On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM).

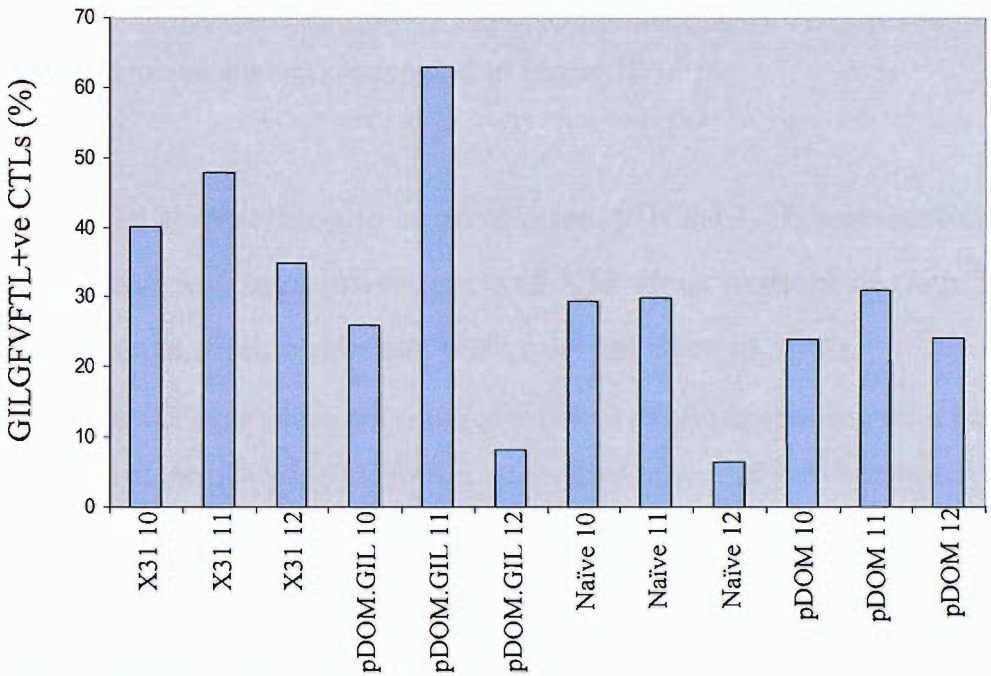


Figure 68. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in bronchoalveolar fluid of individual mice on day 67 (7 days after challenge with X31 Influenza virus at a lethal dose). On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM).

3.3.6.8 Seven days after challenge with a lethal dose of X31 virus, GILGFVFTL specific CTLs were detected at a higher frequency in the peripheral blood of pDOM.GILGFVFTL vaccinated mice than in control mice

Results are available for 41 of 48 mice transported to the Jenner Institute for study. 12 mice were vaccinated on day 0 with pDOM.GILGFVFTL, 11 mice received the control vaccine pDOM, 9 mice were inoculated intranasally with a low dose of X31 virus and the remaining nine mice received no intervention. On day 17 each mouse was challenged with X31 virus at a lethal dose. On day 24 the mice were sacrificed. The lungs were harvested from each mouse and lung virus titres were measured. The frequency of CTLs specific for GILGFVFTL in the peripheral blood of each mouse was measured by flow cytometry.

The group of mice vaccinated with pDOM.GILGFVFTL had CTLs specific for GILGFVFTL present in the peripheral blood at a significantly higher frequency than the naïve mice ($p=0.002$) or the pDOM vaccinated mice ($p= 0.005$) (Figure 69). Although mice which had received a protective dose of X31 on day 0 were found to have CTLs specific for GILGFVFTL present at a lower frequency than the pDOM.GILGFVFTL vaccinated mice and at a higher frequency than either the naïve or pDOM vaccinated mice, the differences did not reach statistically significant levels (Figure 69). The individual results for each mouse studied are depicted in Figure 70.

3.3.6.9 In comparison to control mice, pDOM.GIL vaccinated mice did not have lower levels of X31 virus present in their lungs 7 days after challenge with a lethal dose of virus.

Due to a shortage of eggs which are required for virus measurement, lung virus titres were measured for 9 of the pDOM.GILGFVFTL vaccinated mice, 3 pDOM vaccinated mice and 3 naïve mice. None of the pDOM.GILGFVFTL GIL vaccinated mice had cleared the virus from their lungs on this occasion. The level of residual virus present was similar in each each group (Figure 71).

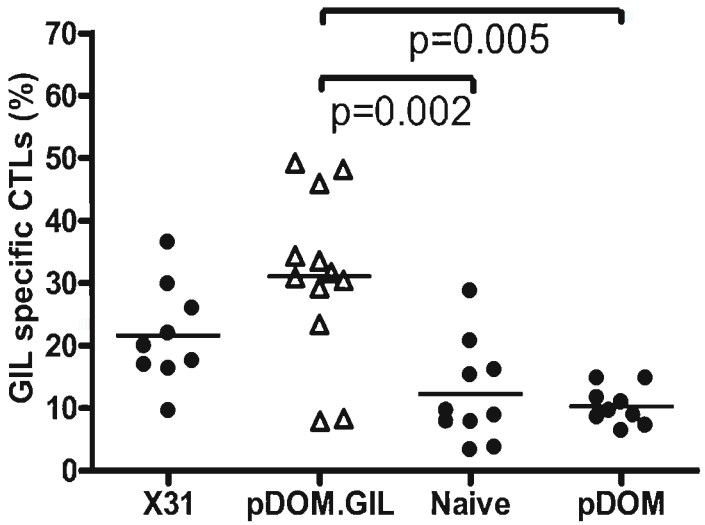


Figure 69. Estimation by FACS of the frequency of GILGFVFTL specific $CD8^+$ T-cells in the peripheral blood mice on day 24 (7 days after challenge with X31 Influenza virus at a lethal dose). On day 0 mice had been either: immunised with X31 virus (X31), vaccinated with pDOM.GILGFVFTL (pDOM.GIL), received no intervention (Naïve) or vaccinated with pDOM (pDOM). Mice vaccinated with pDOM.FLU-GIL had GILGFVFTL specific CTLs present at a significantly higher frequency than Naïve of pDOM vaccinated mice.

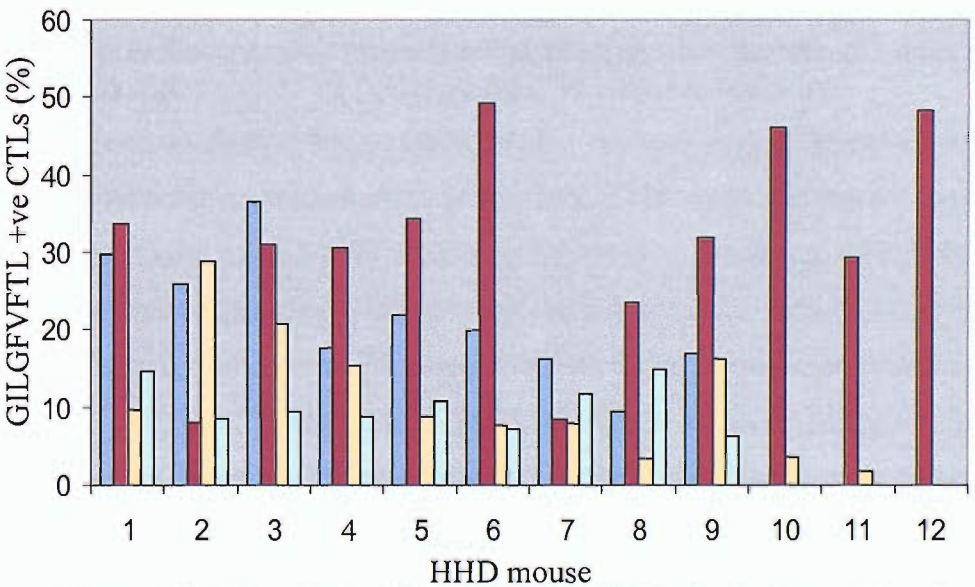


Figure 70. Estimation by FACS % of $CD8^+$ T-cells in the peripheral blood of mice on day 24 that were specific for GILGFVFTL. Mice had been challenged with a lethal dose of X31 influenza virus on day 17. On day 0 mice had been either: immunised with X31 virus (■), vaccinated with pDOM.GILGFVFTL (■), vaccinated with pDOM (□), or received no treatment (■). This is a representation in a different form of the data depicted in Figure 69.

	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	EID_{50}
pDOM.GIL						
1	+ + +	+ + +	+ + -	- - -	- - -	
2	+ + +	+ + +	- + -	- - -	- - -	
3	+ + +	+ + +	+ + -	- - -	- - -	
7	+ + +	+ + +	+ + -	- - -	- - -	1.47×10^5
8	+ + +	+ + +	- - +	- + -	- - -	1.47×10^5
9	+ + +	- - -	- - -	- - -	- - -	3.16×10^3
10	+ + +	+ + +	+ + -	- - -	- - -	1.47×10^5
11	+ + +	+ + +	- - -	- - -	- - -	3.16×10^4
12	+ + +	+ + +	+ + +	- + -	- - -	6.18×10^5
pDOM						
1	+ + +	+ + +	+ + +	+ - -	- - -	
2	+ + +	+ + +	- + -	- - -	- - -	
3	+ + +	+ + +	+ - +	- - -	- - -	
Naive						
1	+ + +	+ + +	+ + +	+ - +	- + -	
2	+ + +	+ + +	+ - +	- - -	- - -	
3	+ + +	+ + +	+ + +	- - -	- - -	
+ve control	+ + +					
-ve control	- - -					

Figure 71 X31 Influenza virus titres in lungs of HHD mice sacrificed 7 days after challenge with X31 virus at a lethal dose (day 24 post vaccination).

X31 Influenza virus titres in lungs of HHD mice sacrificed on day 24 post vaccination. On day 17 mice had been inoculated with a lethal dose of X31 virus. On day 0 mice had been either vaccinated with pDOM.GILGFVFTL (pDOM.GIL), vaccinated with pDOM (pDOM), received no treatment (Naive) or immunised with X31 virus (X31). + indicates haemagglutination. - indicates no haemagglutination. Lung homogenate dilutions are indicated (10^{-4}, 10^{-5} etc). Titres of virus (EID_{50}) are expressed as the reciprocal of the highest dilution of the original homogenate that gave haemagglutination in 50% of eggs.

3.3.7 Experiments with pDOM.GILGFVFTL in human cells

The experiments above indicated that the DNA vaccine pDOM-FLU-GIL could induce specific CTL responses in mice. As we aimed to test this vaccine in humans, we were keen to first establish if the vaccine could be processed and presented by human cells. One way to establish this was to transcribe the vaccine insert into RNA and then transfect dendritic cells which had been grown from a HLA-A2 volunteer. The results of this experiment are listed below.

3.3.7.1 Human dendritic cells transfected with pDOM.GILGFVFTL RNA expand specific CTLs

Dendritic cells were isolated from a healthy HLA-A2 volunteer and incubated with IL-4 and GM-CSF for 5 days as described above. They were divided into two groups. One group was transfected with a RNA derived from pDOM.GILGFVFTL. The other group underwent a mock transfection. The dendritic cells were then matured with Lipopolysaccharide and Interferon-γ for 24 hours. Autologous volunteer CD8+ T cells were co-cultured with these dendritic cells for 7 days in the presence of IL-7 and IL-2. The number of cytotoxic T cells specific for the GILGFVFTL was assessed by tetramer analysis. When compared with the mock transfected DC's, the pDOM.GILGFVFTL transfected DC's expanded three times more GILGFVFTL specific CD8 cells (FIGURE 72).

3.3.7.2 GILGFVFTL specific cytotoxicity of CTLs expanded by pDOM.GILGFVFTL RNA transfected dendritic cells

The ability of CTLs which had been cultured with pDOM.GILGFVFTL transfected autologous dendritic cells to lyse GILGFVFTL pulsed target cells was compared in a standard ^{51}Cr release cytotoxicity assay to that of CTLs which had been cultured with mock transfected DCs. CTLs cultured with transfected DCs exhibited twice as much specific lysis of target cells at effector to target ratios of 90:1, 30:1 and 10:1 than CTLs cultured with mock transfected DCs (FIGURE 73).

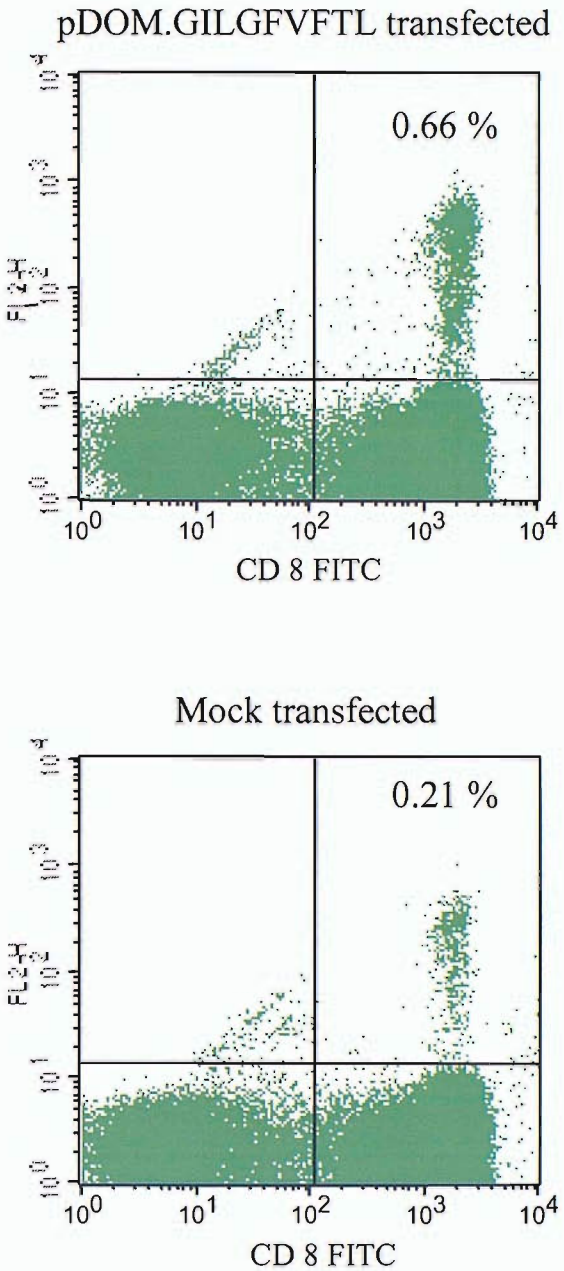


Figure 72. FACS analysis of CTLs following a one week co-incubation with dendritic cells which had been A) transfected with RNA encoding pDOM.GILGFVFTL or B) undergone a mock transfection. A higher frequency of GILGFVFTL specific CTLs were detected following incubation with RNA transfected DCs than with mock transfected DCs. FL2-H = PE labelled GILGFVFTL tetramer.

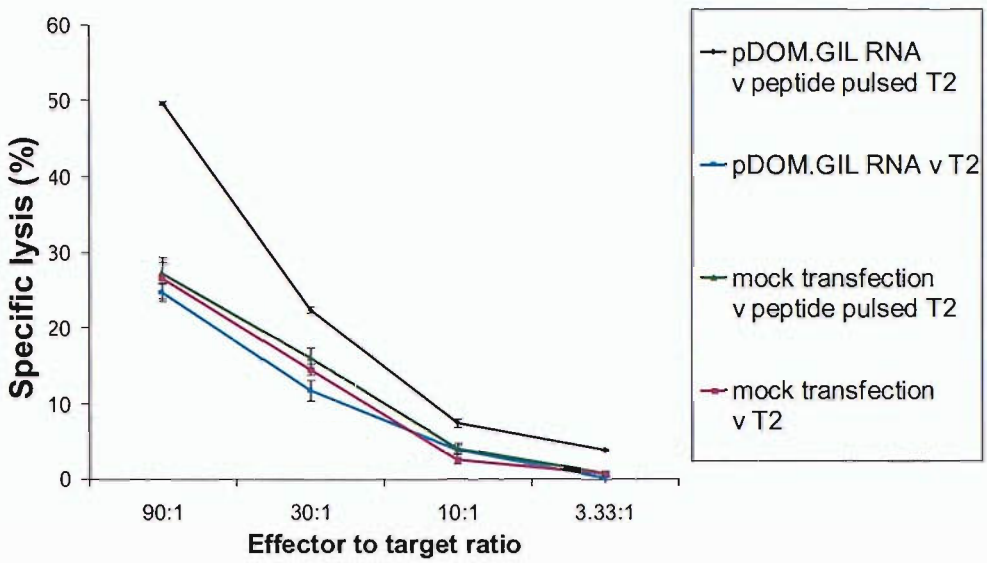


Figure 73. Killing assay which demonstrates that CTLs cultured with pDOM.GILGFVFTL (pDOM.GIL) transfected dendritic cells exhibit a superior ability to lyse GILGFVFTL pulsed target cells than CTLs cultured with "mock" transfected dendritic cells.

3.4 Discussion

The ability of the DNA vaccine pDOM.GILGFVFTL to induce a CTL response against GILGFVFTL was initially tested in HLA-A2/Kb transgenic mice. Vaccination successfully induced CTLs which specifically lysed GILGFVFTL pulsed T2 target cells in a cytotoxicity assay.

We also demonstrated that pDOM.GILGFVFTL induced CTLs which could kill peptide pulsed Jurkat cells and Jurkat cells which had had been infected with influenza virus. The Jurkat cells had been stably transfected with a chimeric MHC class I molecule in which the $\alpha 1$ and $\alpha 2$ domains were derived from HLA-A2 whilst the $\alpha 3$ domain was of murine H-2 K^b origin. CTLs induced by pDOM.GILGFVFTL were also tested against peptide pulsed and influenza infected L929 cells. These cells expressed the completely human HLA-A2 MHC class I molecule. The inability of CTLs induced by pDOM.GILGFVFTL to kill influenza infected L929 cells illustrates the importance of the CD8/MHC class I $\alpha 3$ domain interaction in the functional CTL response. It has been shown previously that murine CD8 cannot bind to human MHC [282]. The ability of pDOM.GILGFVFTL induced CTLs to kill peptide loaded L929 cells is important as it demonstates specificity.

Our initial experiments were performed on splenocytes pooled from several HLA-A2/Kb transgenic mice. Contrary to initial hopes, the HLA-A2/Kb transgenic murine model has proven to be less than ideal for the study of responses against HLA-A2 epitopes. As a result of preferential use of H-2 class I molecules by mouse CTLs for antigen recognition, A2/Kb transgenic mice posses a limited TCR repertoire for HLA-A2 epitopes [280]. Our group has found that a CTL response against a HLA-A2 epitope is typically induced in only 35% of HLA-A2/Kb mice.

Response rates are greatly improved when HHD are used. These mice are H-2D^b and mouse $\beta 2$ microglobulin double knockouts into which a HHD (**H**uman $\beta 2$m-**H**LA-A2.1 ($\alpha 1 \alpha 2$)- H-2**D**^{b} ($\alpha 3$ transmembrane cytoplasmic)) monochain construct was transfected[281]. This forces the mouse $CD8^+$ repertoire to make use of the transgenic HLA class I molecules and restores a sizeable peripheral $CD8^+$ T-cell repertoire educated on the transgenic human molecule [281]

We initially found that GILGFVFTL specific CTLs were induced in all HHD mice vaccinated with pDOM.GILGFVFTL. These CTLs produced high levels of Interferon-γ in an ex-vivo elispot assay. They also caused specific lysis of GILGFVFTL loaded target cells.

It was interesting to note that the individual HHD mouse which demonstrated the highest response to vaccination by elispot paradoxically showed the lowest response in the killing assay. It is likely that the high avidity CTLs induced by vaccination in the mouse and detected in the elispot suffered a degree of activation-induced cell death following incubation with peptide prior to use in the killing assay. This interpretation is further strengthened by the experiment in which spelenocytes were incubated with GILGFVFTL peptide at 0.1μM, 0.01μM or feeder cells which had been pulsed with GILGFVFTL peptide. Splenocytes which had been incubated with GILGFVFTL at the higher concentration did not lyse targets as effectively as splenocytes which had been incubated with peptide at a lower concentration.

The experiments in the HHD mice allowed us to plan protection studies on the basis that the majority of HHD mice should respond to vaccination with pDOM.GILGFVFTL. As previously discussed, Home Office regulations do not permit traditional protection experiments to be performed. In such experiments, mice were challenged with a large amount of virus and then observed over a period of days to see if they survived or succumbed. Regulations require that experiments are designed in such a manner as to limit disease induced morbidity and prevent the disease induced mortality of experimental animals. The isolation facilities required for work with a highly infectious and pathogenic virus such as influenza were not available within our institution. We are grateful to Dr. Sam Hou of the Jenner Institute for Vaccine Research at Compton who kindly provided the isolation facilities and the necessary expertise required to challenge mice with influenza.

Although differences in sensitivity exist between various murine strains, naïve mice normally succumb 7-10 days after intra-nasal innoculation Influenza virus at high dose. Effector CTLs are detected in the lungs of naïve mice at 5-7 days after vaccination although, again, this varies between different strains of mice. We expected that virus-specific CTLs would appear earlier in the lungs of mice which had successfully been vaccinated with pDOM.GILGFVFTL. However, we were aware that a very narrow window was likely to exist in which a reduction in lung virus titres which resulted from the action of CTLs induced by pDOM.GILGFVFTL vaccination could be observed before the mice would have to be sacrificed under home office regulations.

In our initial experiment we showed that injection with pDOM.GILGFVFTL at the Jenner institute successfully induced GILGFVFTL specific CTLs which could be detected in the peripiheral blood and spleen mice 17 days later. CTLs were present at a higher frequency in the blood and spleen of X31 mice (inoculated with a non-lethal dose of X31 virus on day 0) than in mice which had received the test vaccine pDOM.GILGFVFTL

(Figure 59&60). In the accompanying CTL assay, CTLs from the X31 mice exhibited a greater ability to lyse GILGFVFTL loaded target cells than CTLs from mice vaccinated with pDOM.GILGFVFTL (Figure 61). This was not surprising an infection with Influenza would be expected to evoke a much more vigourous immune response than a DNA vaccine. A natural Influenza infection causes much higher levels of antigen delivery to the mouse. This occurs in association with tissue damage and resulting inflammatory signals which aid the immune response. Nonetheless, we confirmed that our vaccine could induce CTLs specific for GILGFVFTL at high frequency.

In the next part of this experiment, three mice from each group were sacrificed five days after a challenge with X31 Influenza virus at a lethal dose. The frequency of CTLs in the peripheral blood specific for GILGFVFTL was measured by tetramer analysis. The titre of X31 Influneza virus in the lungs of each mouse was also measured. We found that no virus was present in the X31 control mice (Figure 63). The previous inoculation had induced an immune response which had entirely prevented subsequent infection. All of the mice in the other 3 groups had similar levels of X31 virus present in their lungs (Figure 63). We had not expected our vaccine to prevent infection but we did hypothesise that they would clear the virus more quickly than negative control mice. It was possible that we had sacrificed too early, that we had not allowed enough time for the specific CTLs present in pDOM.GILGFVFTL vaccinated mice to eradicate the virus more quickly than unvaccinated mice. For this reason we allowed seven days to elapse between lethal virus challenge and mouse euthanasia. We also noted that the frequency of specific CTLs in the blood of pDOM.GILGFVFTL vaccinated mice was similar to that of the control mice (Figure 62). It was therefore possible that the three mice in the pDOM.GILGFVFTL group had failed to respond to vaccination. Another explanation for this observation was that the pDOM.GILGFVFTL vaccinated mice *did* have higher levels of specific CTLs but that they had exited the blood and relocated to virus infected sites in the tissues. For this reason we chose to measure the frequency of specific CTLs in the broncho-alveolar fluid of mice in our subsequent experiment.

The next experiment was similar in design to the first protection experiment except that incorporated the alterations indicated above. Mice from each group were sacrificed and immune responses to GILGFVFTL were determined in the blood and spleen. As expected, the frequency of specific CTLs in the spleen of X31 mice was lower at day 60 (Figure 63) than had been observed in similar mice sacrificed 17 days after vaccination (Figure 60). Specific CTLs could be detected in the spleen of ½ pDOM.GILGFVFTL vaccinated mice (Figure63). This proportion was slightly less than the figure observed at day 17 when specific CTLs were deteced in 2/3 pDOM.GILGFVFTL vaccinated mice

(Figure 61). When compared to mice sacrificed on day 17, the magnitude of the response exhibited by X31 or pDOM.GILGFVFTL mice sacrificed on day 60 was also lower (Figures 63&64).

In the second part of this experiment, three mice from each group were challenged with a lethal dose of virus on day 60 after vaccination. They were sacrificed seven days later. On this occasion the frequency of specific CTLs was measured in BAL fluid in addition to the peripheral blood. This experiment generated some interesting results. Once again, X31 mice were completely protected against the virus (Figure 66). However, no virus was detected in the lungs of 1/3 pDOM.GILGFVFTL vaccinated mice (Figure 66). Virus was detected at lower levels in the other two pDOM.GILGFVFTL mice than had been seen in equivalent mice five days after viral challenge in the previous protection experiment (Figure 63). The naïve and pDOM control mice also had lower levels of virus present in their lungs, particularly naïve mice 10 and 11 (Figure 66).

The tetramer assays on the peripheral blood and BAL fluid from these mice provide information on the dynamics of the immune response to Influenza.The mouse with the highest frequency of specific CTLs in the blood and BAL fluid was mouse pDOM-GIL 11. This was the also mouse which had cleared the virus from its lungs. Mice pDOM-GIL 10 and 12 both had residual virus present in the lungs. Mouse pDOM.GIL had a higher frequency of specific CTLs in the blood and BAL fluid than mouse pDOM-GIL 12. It also had a lower titre of residual lung virus than pDOM-GIL 12. An insight into the kinetics of the CTL response to influenza can be gained from our naïve mice. Naïve mice 10 and 11 had much higher levels of specific CTLs in the BAL fluid than naïve mouse 12. On the other hand, naïve mouse 12 had a much higher frequency of GILGFVFTL specific CTLs in the peripheral blood than naïve mice 10 and 11. It was interesting to note therefore that naïve mice 10 and 11 had much lower levels of residual lung virus than naïve mouse 12. Although group numbers are small, we appear to have observed CTLs appearing in the blood in response to infection and then moving promptly to infected tissues. This highlights the potential for misleading conclusions to be drawn in human vaccination trials where cellular immune responses can only be measured in peripheral blood samples.

We had previously demonstated that CTLs induced by pDOM.GILGFVFTL had in-vitro activity against peptide loaded or influenza infected cells. This experiment suggested that CTLs induced by pDOM.GILGFVFTL had activity in-vivo also. The numbers of mice in this experiment had been small, so we performed a similar experiment in a larger number of mice were challenged with the virus. On this occasion mice were challenged with a lethal dose of virus on day 17 after vaccination. Seven days later, the mice were sacrificed. Peripheral blood and lungs were harvested. The frequency of

GILGFVFTL specific CTLs was measured in the peripheral blood. Lung virus titres were again determined.

Due to a worldwide egg shortage, lung virus titre results were only available for 9 pDOM-FLU-GIL vaccinated mice and 3 mice from each of the pDOM and naïve groups. On this occasion none of the mice had cleared the virus from their lungs, although 2 pDOM.GILGFVFTL mice had lower levels of residual lung virus than other mice (pDOM-GIL 9 and 11).

As a result of the larger sample numbers, we were able to apply a statistical analysis to the frequency of GILGFVFTL specific CTLs in the peripheral blood of mice from each of the four groups (Figure 69). This indicated that mice which had been vaccinated with pDOM.GILGFVFTL had significantly higher levels of GILGFVFTL specific CTLs than control mice which had also received the same lethal dose of virus. Again this suugests that pDOM.GILGFVFTL induces biologically active and responsive CTLs.

Our influenza challenge experiments have generated some interesting results. However, this system has not provided an ideal setting to evaluate pDOM.GILGFVFTL GIL. Like all experimental systems, optimisation is required. We now plan to repeat the last experiment, this time using a smaller dose of virus to determine the effect of pDOM.GILGFVFTL vaccination in a scenario which may be more biologically relevant.

Murine models have been of enormous value in establishing the principles of DNA vaccination. However successful vaccination of humans still requires the resolution of many issues such as the optimal vaccination schedule and route of administration. The pDOM.GILGFVFTL vaccine may provide a vehicle in which these factors can be successfully studied in the context of a normal immune system. For that reason we were keen to establish if the vaccine could be processed by human cells. This was achieved by transcribing the vaccine insert into RNA and then transfecting dendritic cells which had been grown from a HLA-A2 volunteer. We successfully showed that transfected dendritic cells could expand specific CTLs to a greater degree than dendritic cells which underwent a mock transfection. In addition, CTLs expanded by pDOM.GILGFVFTL transfected dendritic cells demonstrated more efficient killing of GILGFVFTL loaded target cells.

In conclusion we have successfully developed a DNA vaccine which can induce CTLs against the immunodominant epitope of Influenza A virus. This further validates the pDOM vaccine design and may lead to the development of a clinically useful vaccine.

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