Cytotoxic T cell costimulation

Biology and potential for generating long-lasting immunity

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ABSTRACT

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The process by which naïve T cells are programmed to develop into short lived cytotoxic effector cells, and long lived memory cells is a complex one. The highly heterogenous T cell receptor provides T cells with their specificity, which allows them to recognise a myriad of target antigens. Signalling through the T cell receptor alone cannot trigger effector and memory differentiation however; a second signal, know as costimulation, is required. The tumour necrosis family receptor superfamily contains several cell surface molecules that are involved in T cell costimulation. This thesis focuses in detail on two of these molecules – CD70 and 4-1BB. By using a combination of stimulatory antibodies and soluble ligands, the role of these two molecules in T cell costimulation was studied. By using expression microarrays, the genes affected by CD70 costimulation were examined; various genes were specifically affected by CD70 signalling, including the pro-apoptotic Bcl2 member Bim, known to be important in T cell apoptosis, the jun dimerization partner Batf3, recently shown to be involved in dendritic cell generation and the cytoskeletal protein lim-nebulette. CD70 significantly upregulated genes involved with cell cycling and related functional pathways. When compared with CD70, 4-1BB had a stronger effect on the generation of $CD8^+$ T cell memory, an effect that was partly dependent on Bim, and occurred during T cell priming. Both molecules are effective in the generation of anti-tumour T cell immunity, and represent attractive therapeutic targets.

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"You look at where you're going and where you are and it never makes sense, but then you look back at where you've been and a pattern seems to emerge"

Robert M. Pirsig - Zen and the Art of Motorcycle Maintenance: An Inquiry Into Values

List of abbreviations

aa	amino acids
AICD	activation induced cell death
APC	antigen presenting cell
APC	Antigen Processing Cell
ASC	antigen specific cells
CD28sa	CD28 super agonist
CDR	Complimentarity Determining Region
CRD	Cysteine repeat domain
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
DAG	Diacylglycerol
DC	dendritic cell
DC	Dendritic cell
DNA	deoxyribonucleic acid
DP	double positive (referring to CD8+/CD4+ thymocytes)
EAE	experimental autoimmune encephalitis
ER	endoplasmic reticulum
ER	Endoplasmic Reticulum
FCM	Flow cytometry
GITR	Glucocorticoid induced TNF related protein
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
GMEM	Glasgow Minimum Essential Medium
HiGg	Human IgG
hpT	T cells generated by homeostatic profiferation
IFNγ	interferon gamma
IFNγ	Interferon gamma
IL-12	interleukin 12
IL-12	interleukin 12
IL-2	interleukin 2
IL-7	interleukin 7
IL-7rα	interleukin receptor 7 α chain
IP3	Inositol triphosphate
ip	intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motifs
JNK	Jun-N-terminal kinase
kDa	Kilodaltons
Klrd1	Killer cell lectin-like receptor subfamily D, member 1
KLRG1	inhibitory killer cell lectin−like receptor G1
Ko	Knockout

LCMV	lymphocytic choriomeningitis virus
Lif	leukaemia inhibitory factor
Lm-OVA	lysteria monocytogenes expressing ovalbumin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
MHC	major histocompatibility complex
MIIC	MHC class II compartment
Myb	Myeloblastosis Oncogene
NK	Natural Killer (cell)
OX40L	OX40 ligand
pDC	plasmacytoid DC
Poly-ic	Polyinosinic:polycytidylic acid
RNA	ribonucleic acid
RSS	recombination signal sequence
s4-1BBL	soluble 4-1BB Ligand
SEM	Standard Error of the Mean
TAP	transporters associated with antigen processing
TAP	Transporters associated with Antigen Processing
T_{CM}	Central memory CD8+ T cell
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
T_{EM}	Effector memory CD8+ T cell
TLR	toll like receptor
TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
VSV	vesicular stomatitis virus

Chapter 1. Introduction

1.1. General Properties of the Immune System

The immune system can be broadly separated into two parts; the innate and the adaptive [1]. The innate system includes simple physical and chemical barriers such as skin or the acidic conditions in the stomach, as well as immune cells that recognise infection-associated signals, such as neutrophils, eosinophils, basophils and natural killer cells. These cells recognise generic molecules that are associated with infection (Pathogen Associated Molecular Patterns – PAMPs), and are the only response to infection in phylogenetically ancient species. Higher order animals have developed an additional type of immune response, the 'adaptive' immune system; the adaptive response relies on the generation of a repertoire of cells, known as lymphocytes, which carry unique receptors capable of recognising specific antigens derived from infecting organisms. The immune system develops these antigen-specific receptors without generating responses to 'self'. The differentiation of 'self' from 'non-self' is a key function of the adaptive immune system, and many regulatory pathways exist to control the process, ensuring that responses remain robust to invading pathogens, whilst preventing auto-immunity. By manipulating these pathways we may hope to develop therapeutic agents that could either encourage immune responses where none are present, for example to infectious or malignant antigens, or ablate immune responses where they are inappropriate, for example in autoimmune diseases such as rheumatoid arthritis.

The lymphocytes of the adaptive immune system are divided into two distinct groups: B lymphocytes, which produce soluble antibodies, which can bind to antigens in their native form, and T lymphocytes, which recognise antigens presented in peptide form on specialized cell surface molecules.
T lymphocytes are further sub-divided into two key groups; $CD4^+$ T lymphocytes, which act as key regulators of immune responses through their interactions with B lymphocytes and dendritic cells (DCs); and $CD8^+$ T

lymphocytes, which can leave the immune system and directly kill infected cells. This thesis is concerned with T lymphocytes, particularly $CD8^+$ T lymphocytes, and the molecules which can trigger them to mount an effective immune response.

1.2. Generation of a T lymphocyte response

T lymphocytes are termed ‘naïve’ before they have encountered their cognate antigen. Naïve T lymphocytes are confined to the lymphatic system, and therefore cannot automatically respond to, and destroy, virally infected cells. Before they can leave the lymphatics, and seek out virally infected cells in peripheral tissues, they must first be ‘primed’ by specialist antigen presentation cells (APCs), known as dendritic cells (DCs):

1.2.1. Dendritic cells

These bone marrow derived cells act as a bridge between the phylogenetically ancient innate immune system, and the more modern adaptive immune system. DCs are antigen presenting cells capable of generating primary adaptive immune responses to foreign antigens [2]. DCs do not always trigger an immune response; in fact, DCs have a second key function, which is the prevention of autoimmunity, via the induction of T cell tolerance.

DC development is complex, and there seems to be some plasticity in the system, with both common myeloid and lymphoid progenitors able to reconstitute DCs [3]. Lymphoid DCs are recognised by their expression of CD8α homodimer [4]. Recent evidence points to a lymphoid progenitor for thymic DCs, with the finding of D-J rearrangements in the immunoglobulin heavy chain locus of these cells. In contrast, during periods of inflammation, monocytes, a myeloid phagocytic cell in peripheral blood, can differentiate into DCs [5]. In general however, DCs develop from a common myeloid progenitor, the MDP (macrophage/DC precursor).

DCs have several functions: the uptake of antigens – both those endogenously derived and from invading pathogens; migration to lymph nodes after antigen uptake; processing of antigens on major histocompatibility molecules and recognition of inflammatory/infectious states with subsequent production of cytokines and provision of costimulation. These DC functions will be considered in order:

1.2.2. Dendritic cell antigen uptake

DCs are present in peripheral tissues in an immature state, where they sample the environment using a variety of endocytic processes, and are characterized by low expression of costimulatory molecules and major histocompatibility molecules. Immature DCs are very effective at taking up and processing antigens. Several pathways are employed –

- ***Macropinocytosis:*** this is an actin dependent process that takes up large quantities of extracellular fluid. Important experimentally, in that it is this pathway that takes up soluble antigens (such as those employed in some experimental vaccines).

- ***Receptor mediated endocytosis:*** including C-type lectin receptor mediated uptake via the mannose receptor (DEC-205) and F_C-receptor uptake of antibody opsonised particles. This method of uptake allows for marked concentration of antigen (compared to non-specific methods of antigen uptake).

- ***Phagocytosis*** of viral/bacterial/parasitic/apoptotic particles

- ***Extracellular loading:*** the simplest form of antigen presentation is for peptides to directly bind to MHC on the cell surface; how important this is in vivo is not certain, but it is the method by which peptide vaccines work.

Once a DC has taken up antigen, several events must subsequently occur: antigen must be processed for presentation to lymphocytes; DCs must receive activation signals which will determine whether an immune response will be stimulated; finally, once activated, DCs must migrate to lymph nodes where they can interact with naïve lymphocytes.

1.2.3. Antigen Processing

APCs, and in particular DCs, must not only take up antigen, but must process it into a form recognisable to the adaptive immune system. The T cell receptor (TCR) does not recognise infectious molecules in their native state; instead it recognises molecules that have been digested into peptides within a cell, and subsequently presented on the cell surface bound to presentation molecules known collectively as the Major Histocompatibility Complex (MHC) [1]. MHC presentation of intracellular molecules allows the immune system to detect and destroy pathogens that exist within cells such as viruses and some bacteria. There are two major classes of MHC: MHC Class I, which is recognised by $CD8^+$ T cells, and MHC Class II which is recognised by $CD4^+$ T cells.

1.2.4. MHC class I

DCs are involved in the presentation of antigen to, and subsequent activation of, $CD8^+$ T cells, via MHC class I. The MHC class I molecule is made up of two polypeptide chains, an α chain and a β-2 microglobulin chain [6]. The α chain is composed of three domains – α1, α2 and α3. The membrane-proximal α3 and β2microglobulin units contain immunoglobulin domains; the α1 and α2 domains are polymorphic (giving rise to histocompatibility) and contain a cleft in which peptide is presented. A majority of cells express MHC class I to a certain extent, and therefore are constantly presenting intracellular antigens [1].

Peptides bound in MHC class I are fixed at the terminal amino and carboxyl groups, and are between 8 and 10 amino acids long. Peptides freed from MHC class I tend to show some recurring motifs – this despite the highly polymorphic nature of MHC class I: they often have a hydrophobic anchor residue at the carboxy terminus; further ‘side’ anchor, or ‘secondary’ anchor residues are found, and altering these will usually prevent a peptide from binding to MHC class I.

Within the ER, MHC class I encounters peptide, which has been transported into the ER by the heterodimer composed of TAP1 and TAP2 (Transporters

Associated with antigen Processing). TAP is inducible by interferons, logically increasing peptide transportation during viral infection. TAP is biased towards binding peptides likely to bind to MHC class I.

Peptides bound to MHC class I molecules are derived from proteins – both endogenous non-pathogenic, and from infecting viruses [6]. Proteins are degraded by a protein complex called the proteosome, which preferentially digests ubiquitinylated proteins, and it is through this pathway that peptide is processed for presentation with MHC class I. Once within the ER, MHC class I folding and binding to peptide come under the control of several enzymes (including calnexin, calreticulin, erp57 and tapasin), the detailed functions of which are outside the scope of this project: it is however important to note that MHC class I cannot be expressed on the cell surface until it has successfully bound peptide within the ER. There exists within the ER an excess of peptide-free MHC class I, providing a useful reservoir to permit immediate presentation of viral peptides should an infection occur. Several viruses have evolved mechanisms that inhibit MHC class I function; for example, herpes viruses encode a protein that inhibits TAP and adenoviruses inhibit MHC class I release from the ER. In the majority of cells MHC class I restricted peptides are derived from proteosomal degradation of endogenous proteins, but in DCs exogenous proteins (ie those not synthesized by the DC) can be redirected to the MHC class I presentation pathway. This process is termed 'cross-presentation'. MHC class II presentation utilises the usual pathway of endosomal uptake of exogenous antigen; for MHC class I presentation a separate mechanism is required; antigen presentation on MHC class I might occur if the DC is itself infected, although there is evidence that infected DCs are dysfunctional [7]. More commonly antigen is taken up by the endosomal pathway and then enters the class I presentation pathway; the pathways by which this occurs are currently elusive, but include endosomal 'leakage', endosomal transfer to the ER, and ER involvement in endocytosis [8]. Cross-presentation is a feature of the $CD8^+$ subset of DCs (see below), and is involved in generating $CD8^+$ T cell immunity to pathogens as well as $CD8^+$ T cell tolerance to non-pathogenic antigens (where it is known as 'cross-tolerance').

1.2.5. MHC class II

MHC class II is involved in the presentation of peptides from endocytic vesicles, taken up by pinocytosis, phagocytosis, or receptor mediated endocytosis [9]. The MHC class II molecule is a heterodimer formed from a related α and β chain. Like MHC class I, there exist immunoglobulin-like motifs in the membrane-proximal (α2 and β2) units, although with two membrane-spanning regions. The peptide binding cleft is more open than in the class I molecule, and the peptides are bound more loosely. MHC class II expression is restricted to antigen presenting cells. Peptides binding to MHC class II are at least 13 amino acids long, and can be much longer. The ends of the peptide are not bound, and binding is more permissive of variations in peptides than MHC class I. This makes prediction of which peptides will preferentially bind to MHC class II more difficult than with MHC class I.

As with all cell-surface glycoproteins, MHC class II must enter the endoplasmic reticulum. To prevent inappropriate peptide binding therein, MHC class II associates with the Invariant Chain (Ii). This binds three MHC class II molecules, forming trimers, and fills the peptide binding groove, preventing peptide binding. Once outside the ER, Ii binds to the surface of endosomes where it is digested to leave only a small fragment within the peptide binding groove – CLIP (class II associated invariant chain peptide) – which is subsequently exchanged for peptide in late endosomal compartments called the MIIC (the MHC class II compartment) (Figure 1). This process is termed *direct presentation*, and is responsible for the presentation of antigen to $CD4^+$ T lymphocytes.

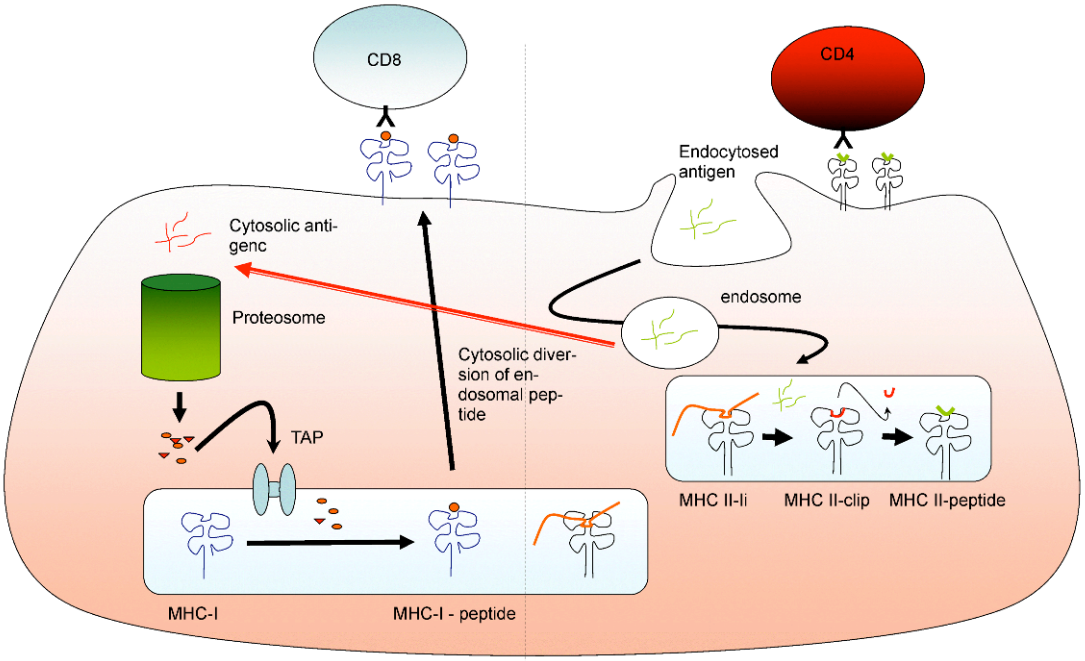

Figure 1 MHC class I and II presentation pathways

Endogenous antigens are processed via the proteosome into peptides, and then loaded within the endoplasmic reticulum onto MHC class I before being presented on the cell surface for recognition by $CD8^+$ lymphocytes. Exogenous peptides are endocytosed and loaded onto MHC class II within the MIIC, replacing CLIP from the peptide groove, before being presented on the cell surface for recognition by $CD4^+$ T cells. In 'cross-presentation', endocytosed antigens are processed as endogenous antigens (red arrow) in a TAP-dependent fashion.

1.2.6. Dendritic cell subsets

DCs can broadly be divided into two categories – plasmacytoid and conventional (previously myeloid and lymphoid) [9]:

1.2.6.1. Conventional DCs

Conventional DCs are comprised of a migratory subset, which enters secondary lymphoid tissue from the periphery, and a resident subset, which is further divided into groups by the presence or absence of surface CD8, which is present as a homodimer, CD8αα, rather than the αβ heterodimer found on $CD8^+$ T cells. Resident splenic DCs are differentiated from their migratory equivalents by their immature phenotype, expressing relatively low levels of MHC class II and costimulatory molecules. When compared with $CD8^-$ DCs, $CD8^+$ DCs are more efficient at cross-presentation through MHC class I, but less efficient at MHC class II presentation [10]. $CD8^{\alpha\alpha+}$ DCs express higher levels of DEC-205 and CD1d. They may have a specialized role in the uptake of apoptotic cells, and are preferentially found in the T zones of the spleen, although are also present within lymph nodes, and are the dominant subtype within the thymus [11]. $CD8^+$ DCs produce IL-12 on activation, and favour a Th1 response (see below) [12], whereas their $CD8^-$ counterparts produce IL10 and IL4, favouring a Th2 response.

$CD4^+$ DCs in contrast are more specialized in MHC class II direct presentation of antigen to $CD4^+$ T cells.

The migratory subset of DCs can be subdivided according to expression of the integrin CD103; the $CD103^-$ subset have recently been shown to be capable of cross-presentation of antigen in an influenza model [13].

The relative contributions of migratory and resident DCs in fighting infection are not completely elucidated; resident DCs may take up and express antigen acquired through the blood stream, or they may take up apoptotic migratory

DCs, and thus become involved in the presentation of tissue-acquired antigens [9].

1.2.7. Plasmacytoid DCs

Initially described in humans, these DCs have morphology similar to that of plasma cells (antibody secreting cells), are $CD4^+$, $CD11c^-$, can be found in blood and enter lymph nodes via an L-selectin dependent mechanism. Evidence of a lymphoid precursor includes the lymphoid-like phenotype, and the finding of mRNA transcripts for immunoglobulin light chains and the pre-T cell receptor-α. Their ability to produce type I interferons has led to them being named interferon-producing-cells; they mature in response to toll-like receptor signals (see below), acquiring a mature DC phenotype [14].

1.2.8. Dendritic cell migration

Whilst some antigens may migrate directly in the lymph to lymph nodes as soluble antigen, DCs must also be able to acquire antigen in the peripheries and migrate to lymph nodes for presentation to T cells within lymph nodes. On activation in the periphery, DCs upregulate CCR7, the chemokine required for DC trafficking to lymph nodes [15]. Once within the lymph node, there is little DC trafficking out of the lymph node via the efferent lymphatic; this reduces the chance of microbial spread to other organs, as well as increasing the concentration of antigen presented within the lymph node. Although a small number of activated DCs may escape the lymph node, and subsequently reach the thymus, the majority of thymic DCs are not migratory, and have developed from intrathymic precursors, and will die within the thymus [16].

1.2.9. Dendritic cell maturation and Toll-like Receptors

DC Maturation involves a change of role of DCs, from antigen capture to antigen presentation; phagocytic receptors are lost, costimulatory molecules are expressed, and cellular motility increases [2]. MHC class I and II are upregulated by maturation signals.

DCs can truly be said to have matured when they become immunogenic rather than tolerogenic, however many studies define DC maturity according to phenotype, or by T cell proliferation, both of which may in fact be associated with tolerogenicity [17, 18].

Several molecules can trigger DCs to mature such as CD40, TNF-R, and IL-1 R. Particularly important are a group of molecules initially identified in Drosophilia *spp* called Toll-like receptors: the importance of toll-like receptors (TLRs) to the innate immune system was first realised when a clone of mice was discovered that were resistant to septic shock and adjuvant effects of lipopolysaccharide (LPS), a bacterial cell wall constituent. The mice, a C3H/HeJ strain, were found to have developed a spontaneous mutation in the gene encoding TLR4. TLR4 also has a role in the detection of the glycoprotein envelopes of several viruses.

TLRs are all single spanning transmembrane proteins with ectodomains composed largely of leucine-rich repeats [19]. Some TLRs are intracellular, present on the endosome of APCs (eg TLR 2, 3, 7, 8 and 9, all of which detect nucleic acids); others are present on the cell surface.

TLR mRNA is detected in monocytes, macrophages and DCs [20], as well as mast cells and various epithelial cells. TLR signalling on DCs leads to DC maturation, with upregulation of costimulatory molecules and production of IL-12 and type I interferons (α/β) [21]. Different TLRs may drive Th1 or Th2 responses. Mice deficient in TLR signalling molecules are susceptible to a variety of infections, and do not respond to TLR-based immune-adjuvants, although they will respond to vaccines based on apoptotic cells, which represent a separate, TLR-independent danger signal.

Once DCs have been matured, and have migrated to lymphoid tissue, they can interact with naïve T cells to provide a link between the adaptive and the innate immune response.

1.3. T lymphocytes

T lymphocytes are divided into two main classes; $CD8^+$ T cells are lymphocytes that can recognise antigen bound to MHC class I. Their predominant role is in the killing of cells infected with intracellular organisms such as viruses. They can also recognise and kill malignant cells under certain circumstances.

$CD4^+$ T cells recognise antigen bound to MHC class II on the surface of APCs. Both $CD4^+$ and $CD8^+$ T cells bind to MHC-restricted antigens with their T cell receptor (TCR).

1.3.1. $CD4^+$ T helper cells

$CD4^+$ T cells have a variety of regulatory immune functions. Upon activation through the TCR, $CD4^+$ T cells can differentiate into type 1 (Th1) or type 2 (Th2) helper cells [22]. Th1 cells produce interferon gamma (IFNγ), stimulate macrophages, mediate delayed-type hypersensitivity, and are involved in the clearance of intracellular pathogens. They secrete IL-12 and IFNγ, and conversely, IL-12 and IFNγ are key cytokines that drive Th1 differentiation.

Th2 cells produce IL-4, and are involved in the development of humoral responses to extracellular antigens, in particular stimulating the production of IgE. Th2 cells differentiation is driven by IL-4. The cytokines that drive Th1/Th2 differentiation are thought to come from a variety of cell types, such as mast cells, macrophages and natural killer cells. Antigen dose and the form of antigen employed have both been shown to have an effect on Th1/Th2 differentiation; when using experimental infectious antigens such as *Leishmania major,* high infectious loads promote a Th2 response and low infectious loads promote a Th1 response (with improved protection from infection) [23]. In contrast, soluble antigens (such as are employed for much of the experimental work of this thesis) show a reversed effect, with low doses of antigen leading to a Th2 response, and vice versa. Why this should be is unclear; differences in cytokine production, particularly IL-4, may play a part, as may costimulation (see relevant section). It is likely however that the terms

Th1 and Th2 are an oversimplification, and that the in vivo situation is more complex [24].

$CD4^+$ T cell help is required for the generation of B lymphocyte responses, in particular in the switching of immunoglobulin subclass from IgM to IgG or IgA. $CD4^+$ help is also important in the generation of $CD8^+$ T cell memory responses (see below).

In addition to Th1 and Th2, other $CD4^+$ T cell subsets exist; Th17 cells are $CD4^+$ T cells which produce IL-17, and have strong pro-inflammatory effects; Th17 cells provide an explanation for the development of paradoxical enhanced autoimmunity in IFN-γ ko mice [25, 26]. Studies on the mouse model of multiple sclerosis, experimental autoimmune encephalitis (EAE), had previously shown a strong role of IFNγ producing Th1 cells in the development of the disease, but the finding that the disease could actually be worsened in the absence of IFNγ or the p35 subunit of the IL-12 receptor (a key Th1 cytokine) raised the possibility of an alternative $CD4^+$ T cell subset being involved. This led to the discovery of a novel cytokine, IL-23, which shared a subunit of the IL-12 receptor; this cytokine was found to stimulate $CD4^+$ T cells to produce IL-17; Th17 cells are generated by IL-6 and TGF-β, and also show autocrine reliance on, and production of, IL-21. Th17 cells are strongly associated with autoimmune conditions in humans such as rheumatoid arthritis and systemic lupus erythematosis [27].

Some $CD4^+$ T cells have an immunosuppressive function, and the study of these 'regulatory T cells' (T_{Reg}) is an active area of research in immunology; T cells which could suppress allogeneic reactions in an antigen-specific manner [28] were initially described in 1971 [29]. Due to variable results in differing systems, the true existence of these cells was in doubt until the subset of $CD25^{hi}$ $CD4^+$ T cells that were that were capable of suppressing auto-immunity were elucidated. Further studies identified additional markers for these regulatory T cells: the transcription factor FOX P3, CLTA-4 and the Glucocorticoid Induced TNFR Related protein (GITR). T_{reg} act in a cell-cell dependent manner, as well as secreting the immuno-inhibitory cytokines IL-10 and Transforming Growth Factor Beta (TGF-β).

T_{reg} have been shown to have remarkable immunosuppressive effects in a variety of animal and human settings, for example they can prevent $CD8^+$ T cell mediated anti-tumour immunity in murine models of fibrosarcoma and melanoma [30].

Tregs are found amongst tumour infiltrating cells in a variety of murine and human tumours; their depletion even at the late stages of disease, can lead to reversal of tumour-specific immune tolerance, and subsequent tumour clearance [31].

1.4. Cytotoxic $CD8^+$T cells

1.4.1. The CD8 molecule

The CD8 molecule recognises and binds to the MHC class I molecule. CD8 is a disulphide-linked heterodimer consisting of an α and β chain, each containing an immunoglobulin-like domain. The CD8 molecule binds to the α2 domain of the MHC class I molecule, at the membrane end of the molecule, leaving the upper surface free to bind to the TCR. The presence of the CD8 molecule increases the sensitivity of the TCR to bind antigen 100 fold. Recently, the CD8 molecule has been shown to be involved in a negative feedback loop that controls TCR signalling in homeostatic $CD8^+$ T cells, where high levels of resting TCR signal are modulated by reduced $CD8^+$ expression, and vice versa [32], leading to a controlled homeostatic rate of baseline TCR signalling.

1.4.2. The T cell receptor

T cells recognise antigen presented as peptide within the MHC via the immunoglobulin-like T cell receptor (TCR). The TCR was discovered by the use of antibodies that recognised certain T cell clonal cell lines, but not others [33], and the ability of these clonotypic antibodies to inhibit antigen recognition by the specific T cells. The T cell receptor consists of an α and β chain joined by a disulphide bond; there is a small subset of T cells whose receptor is made of two similar chains – the γ/δ chains: these γ/δ T cells may have a role in dampening down T cell responses to infections at epithelial surfaces [34].

1.4.3. Structure of the T cell receptor

The T cell receptor is a heterodimer, composed of an α and β chain each with distinct variable (αc and αv, βc and βv) regions. The majority of the heterodimer is present on the extracellular side of the cell membrane, with a hydrophobic region located towards the C-terminus which makes a single passage across the cell membrane and has a notably short cytoplasmic tail comprised of 2-7 amino acids [35].

The TCR has three hypervariable regions which give it its unique specificity; these regions are known as complementarity determining regions (CDR). CDR1 and 2 are thought to primarily interact with the MHC molecule itself, with CDR3 being the dominant area binding to the bound peptide; this has been borne out by several mutagenesis studies [36]. Predictably, being consigned to such a well defined target, α and β chain variability is more constrained in terms of length when compared to the CDR3 of immunoglobulins [37]. Interestingly, γ/δ CDR3 regions are not so constrained.

1.4.4. Structure of the human T cell receptor gene

The α chain gene is composed of 70-80 variable (V) segments, 61 joining (J) segments, and a constant gene. The β chain also contains additional D (diversity) segments.

1.4.5. Rearrangement of VDJ genes to generate functional TCR

The aim of rearrangement of the α and β loci is to create a gene with a single V, J, and in the case of the β chain, D, gene. The β chain is rearranged first; initially Dβ joining to Jβ, then Vβ joins to the DβJβ segment. Since a second Dβ and Jβ cluster exists upstream, cells can be rescued if their initial β chain rearrangement is non-functional. The cell will now undergo several rounds of proliferation, so that one successful β chain rearrangement may pair with many different α chain rearrangements [1]. α chain rearrangement is usually successful; if a non-productive chain is produced, successive attempts are tried with the unused segments, which 'leapfrog' the failed segments. This continues even after a successful α chain is produced, allowing for more than one TCR to be present on the cell surface; the process ceases once positive selection has occurred within the thymus (see below).

The TCR has considerable binding promiscuity, able to bind with varying affinities in excess of 10^5 different MHC peptide complexes [38]. This is, in part, explained by the fact that the majority of the binding affinity of a TCR comes from TCR:MHC class Interactions rather than TCR:peptide interactions.

These features – the multitude of possible V, D and J genes, along with the mutations introduced by Tdt and associated exonucleases, leads to TCR diversity, giving an estimated 10^{18} possible different TCRs.

1.4.6. TCR signalling pathways

The TCR itself has a relatively short intracellular tail and does not contribute significantly to cell signalling. Like many cell surface signalling molecules, the TCR requires dimerization [39] for signalling to commence, using accessory molecules to perform signal transduction to within the cell [40]. How this works in practice is not clear, since to dimerize, two TCRs should meet two identical MHC molecules presenting identical peptides; given that any antigen

presenting cell presents thousands of different peptides at any given time, how TCR dimerization is brought about remains unexplained. TCR signalling by monoclonal antibodies to the TCR is effective, but the Fab fragments of the same antibodies do not lead to TCR signalling, offering further support for a need for TCR dimerization. Mathematical models of TCR downregulation have also been used to provide evidence for TCR dimerization [41]. Alternative explanations for TCR signalling, other than simple TCR dimerization, have been proposed, including pseudo-dimerization and kinetic segregation, and the reality of TCR signalling may involve several co-operating mechanisms [42].

Each TCR is associated with up to 6 CD3 polypeptides including CD3εγ and CD3εδ heterodimers, and CD3ζ (Figure 2). Each CD3 subunit contains recurring signalling motifs – the ITAMS (immunoreceptor tyrosine-based activation motifs). ITAMs have a signature pattern of two tyrosines flanking a series of amino acids containing recurring leucine/isoleucine motifs. The ten ITAMs provide the signal transduction following TCR binding [43]: they are phosphorylated by Fyn and Lck, both members of the Src family of protein kinases, upon TCR cross-linking by antigen. These are in turn kept in an inactivated state by Csk (C terminal Src kinase), which is constitutively expressed. Csk is in turn inhibited by CD45 (the common leucocyte antigen); the balance between the two depends on which isoform of CD45 is expressed. The level of Src-family kinases is controlled in part by ubiquitin, which targets proteins for degradation by the proteosome; ubiquitinylation of Src-family kinases is controlled by Cbl, a regulatory protein (see section on anergy (p50) for more on the function of cbl).

ZAP70 (zeta-associated protein) phosphorylation is a key mediator of TCR signalling; cells deficient in ZAP70 show severe defects in TCR signalling in terms of intracellular calcium flux and IL-2 production, and this can be corrected by recovering ZAP70 production [44]. On ITAM phosphorylation ZAP 70 is bound and activated by Lck, and the cascade continues via LAT (linker of activation in T cells).. Activation of this proximal signalling complex

leads to activation of phospholipase C γ1 (PLC) which leads to activation of the key signal transduction messengers, IP_3 and Diacyl Glycerol (DAG):

DAG leads to two separate signal transduction pathways:

- Ras activation, MAP kinase pathway activation, and activation of the AP1 transcription factor
- Protein kinase C θ leading to NFκB activation (see below).

IP_3, the other key product of PLC activation, is involved in Ca^{2+} production. Ca^{2+} ions are universal signalling compounds in eukaryotic cells, and the increase in intracellular Ca^{2+} mediated by TCR signalling leads to the activation of various Ca^{2+} dependent transcription factors, and lead to activation of the Nuclear Factor of Activated T cells (NFAT). Ca^{2+} signalling can be blocked via calcineurin (a calcium phosphatase) inhibitors such as Ciclosporin, which is a powerful immunosuppressant [45].

Role of Nuclear Factor κ B

Many transcription factors are themselves, under control of other transcription factors. Those transcription factors that are freely available for activation within the cell are known as primary transcription factors. For a signal at the surface of the cell to be transduced into changes in gene transcription, it is necessary that the signal cascade should activate one or several primary

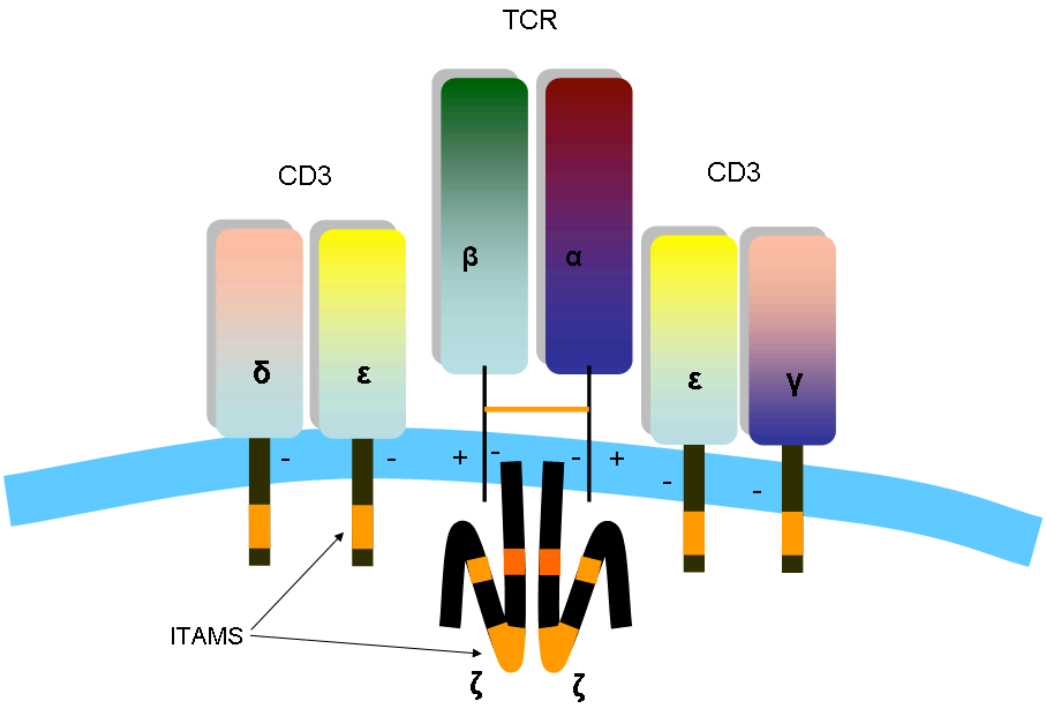

Figure 2 The T cell receptor complex

Note that the ζ chain has three iTAMS (immunoreceptor tyrosine-based activation motif), and that all the signalling chains contain negatively charged residues within the transmembrane portion – this aids co-localisation with the positively charged equivalent in the TCR α and β chains (adapted from [1]).

transcription factors, of which Nuclear Factor κ B (NF-κB) is a classical example. First described in B lymphocytes, it was shown to have DNA binding affinity for the transcriptional enhancer region of the κ light chain [46]

NF-κB is ubiquitously expressed, and is not restricted to the immune system; neither is it required, contrary to its name, for light chain expression in B cells [47]. NF-κB is, in fact, a group of transcription factors important to both immunology and cancer research [48]. Also known as the REL family, NF- κB consists of dimers of p50, p52, REL, REL-A and REL-B. These can all (with the exception of REL-B) dimerize to form functional NF- κB dimers. In the resting state NF-κB is inhibited by one of three small cytoplasmic molecules; Iκα, Iκβ or Iκε. IκB kinase (IKK), which has two isoforms, IKK1 and 2, marks these inhibitors for degradation in the proteosome; this frees NFκB dimers to translocate to the nucleus where they can bind their cognate DNA-binding sites. NF-κB binds with high affinity to known decameric DNA sequences. Two pathways of NF-κB activation exist:

1.4.7. Canonical NF-κB pathway

Pro-inflammatory signals through Toll-like receptors, or TNF receptors, rapidly induce NFκB. This is mediated by IKK2, which targets IκB for proteosomal degradation releasing the p50:Rel-A isoform of NF-κB to enter into the nucleus. Enhanced NF-κB activity is measurable within 10 minutes of cell stimulation

1.4.8. Non-canonical NF-κB pathway

This pathway is active in areas of lymphoid organogenesis, and is stimulated through TNF family members such as lymphotoxin β and BAFF. The activation of NF-κB by this pathway is much slower than that of the canonical pathway, proceding over hours and days.

Studies of various murine knockout models of the NF-κB pathway have confirmed its role in survival and proliferation of lymphocytes; depending on which subunit is deficient, mice show a variety of phenotypes; some are lethal (eg Rel-A), and others show proliferation defects in lymphocytes (Rel-B).

NF-κB activation has a prominent function in the regulation of inflammation. Inflammatory stimuli are potent inducers of genes, which are nearly all NF-κB dependent [49].

Various subunits of TCR signalling are involved in NF-κB activation, such as PKCθ, ZAP70 and Fyn. NF-κB has also been shown to be activated by CD28, and there is evidence that TRAF2 (see below), which is involved in CD27 signalling, can also lead to NF-κB activation, although this has only been shown for B cells [50]. Thus it can be seen that NF-κB is involved in the downstream effects of several components of T cell activation (Figure 3).

Subtle differences in the kinetics of activation of these various transcription factors can lead to markedly differing T cell fates; for example, NFAT and AP-1 activation tends to lead to a functional, proliferative T cell response, with the production of the key T cell cytokine interleukin 2 (IL-2); in contrast, NFAT activation in the absence of AP-1 activation can lead to T cell anergy [42].

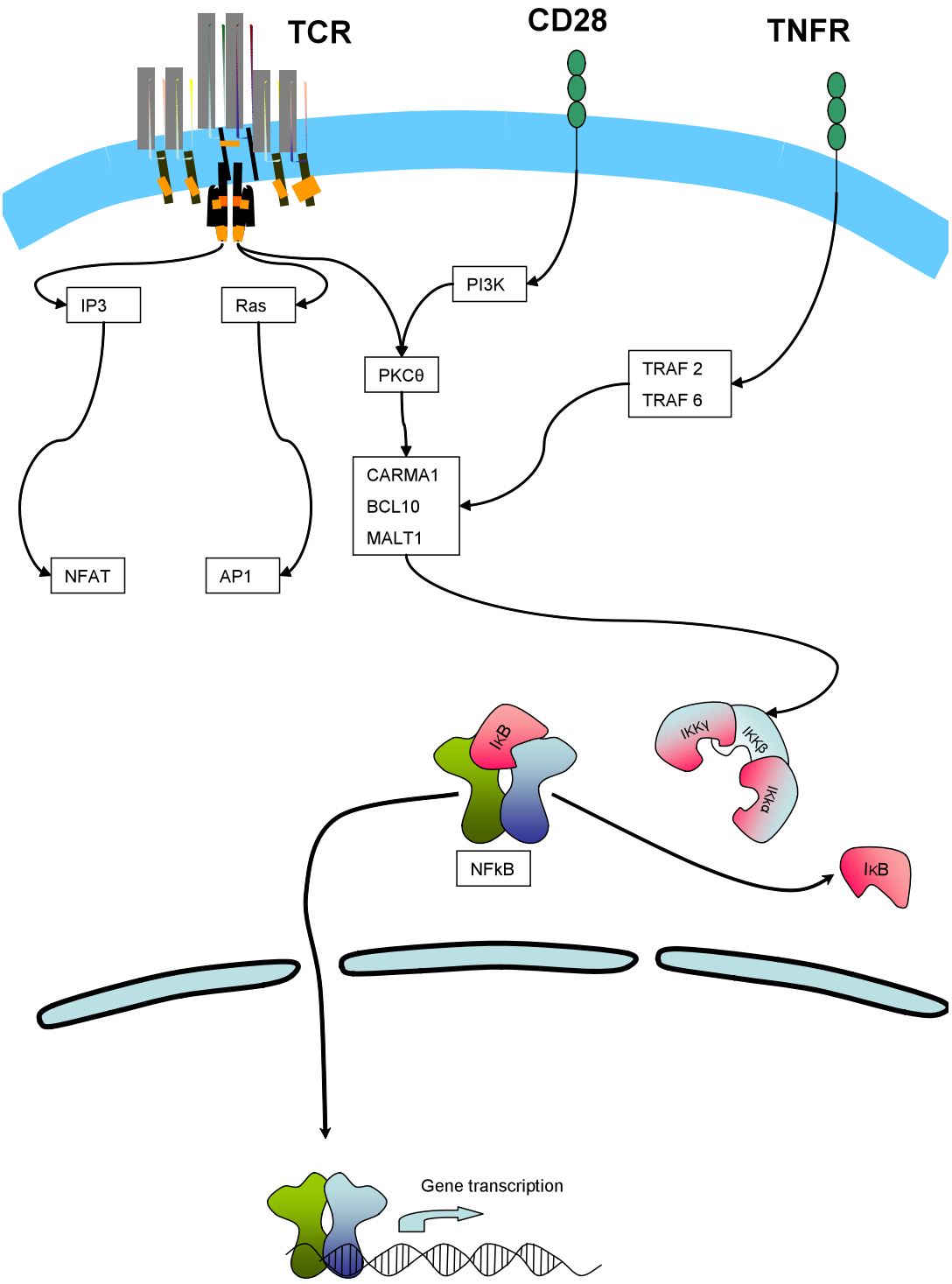

Figure 3 NF-κB activation via TCR/CD28/TRAF activation

In addition to AP-1 and NFAT, TCR signalling can lead to NF-kB activation via PKCθ. This leads to phosphorylation of the CARMA1/MALT1/BCL10 complex which activates the IKK complex; this leads to release of IkB from NF-kB, allowing it to translocate to the nucleus and bind to DNA (adapted from [50])

1.4.9. TCR signalling and the actin cytoskeleton

On TCR ligation by its cognate MHC-peptide ligand, TCR signalling leads to polymerization of the key cytoskeletal protein actin; the T cell undergoes polarization around the synapse between the T cell and the interacting APC. The immunological synapse, and associated cytoskeletal changes, are discussed in more detail in the first results chapter.

1.4.10. T cell positive thymic selection

Although T lymphocytes originate in the bone marrow, all their development takes place within the thymus [1]. The thymus is situated in the anterior thorax, and consists of an outer thymic cortex and an inner thymic medulla. Thymocytes are attracted to the thymus where they become committed to T cell development. They start to acquire T cell-specific cell surface markers, but are initially CD3/CD4/CD8 negative [51]. From this point they may develop into γδ T cells, or natural killer T cells, but the majority will enter the pathway towards αβ T cell development. The aim of T cell development within the thymus is to develop T cells that have an affinity for self-MHC that is not so strong as to lead to auto-immunity.

1.4.11. αβ development

Immature lymphocyte precursors enter the thymus near the cortico-medullary Junction. Initially 'double negative' for CD4 and CD8, they undergo TCR gene rearrangement; initially the β chain is rearranged in thymocytes whilst in the subcapsular region of the cortex of the thymus; if a successful β chain is formed (both β chains are tried) it is initially paired with a pre-T-cell-alpha chain (pTα) that allows a partial TCR to be expressed at the T cell surface along with the formation of the CD3 co-receptor. This signals to the cell to cease β chain rearrangement, to undergo several steps of proliferation, and to become double positive (DP) $CD4^+CD8^+$ thymocytes. Subsequently, α chain rearrangement occurs until a functional αβ TCR is produced. These small double positive cells move to the inner cortex to undergo positive and negative selection:

1.4.12. Positive selection

Once T lymphocytes express a functional αβ receptor on their cell surface they undergo a process of positive selection which allows only those T lymphocytes which can recognise MHC class I or II to survive. Developing T cells encounter MHC bound peptides presented on the surface of cortical thymic epithelial cells. Cells whose TCR recognises MHC class I will develop into $CD8^+$ cytotoxic lymphocytes, and those whose TCR recognises MHC class II will develop into $CD4^+$ lymphocytes. How lineage choice is controlled is not fully elucidated, especially as CD4 and CD8 molecules both signal through Lck (see below) [52]; one explanation is that the stronger signalling through Lck by CD4 drives lineage differentiation; others have proposed a 'kinetic signalling' mechanism, where temporary loss of CD8 expression in DP thymocytes drives the differentiation choice.

Thymocytes undergoing positive selection need to recognise MHC:peptide complexes. Which peptides drive positive selection is an area of active research, and it would seem that a small repertoire of self peptides capable of driving positive selection exists for each TCR; conversely, these peptides will actively antagonise mature T cell responses outside of the thymus [53].

1.4.13. T cell negative thymic selection

Those T cells that receive a strong TCR signal during thymic development are at risk of causing auto-reactivity, and must be purged by negative selection. Double-positive (DP) thymocytes that have survived positive selection enter the thymic medulla where they become single positive (SP) (ie either $CD4^+$ or $CD8^+$). This process involves medullary thymic epithelial cells, but also bone marrow derived dendritic cells [54].

Medullary thymic epithelial cells are able to synthesize many peripheral tissue-specific antigens, and thus provide a reservoir of self antigens capable of purging auto-reactive T cells. This is under control of the Autoimmune Regulator gene AIRE [55]. AIRE knockout mice develop lymphocyte infiltrates in multiple organs, associated with autoantibodies. By performing gene arrays on RNA from purified thymic epithelial cells it was shown that AIRE upregulates expression of several hundred genes, consistent with its role in presenting autoantigens. Despite this, thymic deletion of autoreactive T cells is not sufficient: firstly, not all peripheral autoantigens are expressed at the necessary levels, nor are the various non-pathogenic dietary and environmental antigens expressed within the thymus; secondly, only auto-reactive T cells with strong affinity are purged – those with weak affinity remain, and the threshold at which autoreactive T cells are deleted is lower in the periphery than in the thymus, ie the thymus favours the survival of autoreactive T cells [56]. Therefore there is a need for mechanisms of peripheral tolerance (see below). Cells similar to thymic epithelial cells, including the expression of AIRE and the presentation of tissue-specific

antigens, have recently been discovered in the peripheral lymph nodes of the mouse [57], which may have a role in peripheral tolerance.

1.5. Peripheral tolerance of T lymphocytes

1.5.1. The role of DCs in peripheral tolerance

As tissue DCs acquire autoantigens from apoptotic cells, they migrate to draining lymph nodes where they can cross present autoantigens to naïve $CD8^+$ T cells [58]: these immature DCs express low levels of costimulatory molecules B7.1 and B7.2, and in the absence of CD28 signalling, T cells recognising cross-presented antigens on naïve DCs undergo a brief period of proliferation followed by tolerance; tolerance occurs either by deletion or the development of anergy. Only when DCs have been activated by the presence of innate signals, or via $CD4^+$ T cells cells expressing high levels of CD40 ligand do they upregulate the requisite costimulatory molecules that trigger a $CD8^+$ T cell response. DCs can also be 'back-stimulated' through B7 receptors by activated $CD8^+$ T cells – this explains how large numbers of $CD8^+$ T cells can provide their own help [59, 60].

1.5.2. Role of antigen persistence in peripheral tolerance

Experiments using TCR transgenic mice have shown that small amounts of antigen can produce an effective $CD8^+$ T cell response with subsequent clearance of the antigen, whereas a large antigen level leads to $CD8^+$ T cell deletion or anergy [61]. This should mean that autologous antigens, given the continuous nature of their presentation, will lead to deletion of the majority of autoreactive $CD8^+$ T cells.

1.5.3. Anergy versus Deletion

Anergic T cells are T cells which fail to proliferate and produce IL-2 in response to TCR engagement. Anergy is induced in T cells by providing a suboptimal signal – either a strong TCR signal with no costimulation, or a weak TCR signal with adequate costimulation [62]. Whether suboptimally stimulated T cells undergo apoptosis, and are therefore deleted, or survive in

an anergic state, is dependent on several factors. Anergy is a continuous process, dependent on the persistence of antigen to maintain $CD8^+$ T cells in an anergic state [62]. It seems that TCR avidity may in part determine whether $CD8^+$ T cells are deleted or anergised, with high antigen doses given continuously leading to anergy, and low antigen doses leading to clonal deletion [63]. Cells anergised by high levels of antigen show reduced phosphorylation of TCR signalling molecules, and this effect is lost as the levels of antigen fall. Anergy is also associated with downregulation of CD8 and the TCR, and many downstream TCR signalling molecules.

The E3 ubiquitin ligase Cbl-b has been shown to mediate the anergic effect of TCR signalling in the absence of costimulation [64]. Cbl-b deficiency corrected the T cell anergy seen in CD28-/- mice. Cbl-b lowers the level of calcium mobilisation in response to TCR signalling, and Cbl-b mice are prone to autoimmunity [65]. Cbl-b works by ubiquitinylation of TCR signaling proteins such as PLCγ1, leading to reduced TCR signaling.

The strength and timing of the TCR signal is clearly important in determining the fate of a lymphocyte, but many more cell surface molecules are involved, providing a second signal to a lymphocyte at the same time as TCR engagement; this second signal is provided by many different receptor/ligand pairs, and is collectively termed *costimulation*, and is discussed in the next section.

1.6. Costimulation

For the majority of their responses, $CD8^+$ T cells cannot respond directly to antigen presented on the surface of infected/malignant cells; rather they first require priming by appropriately activated DCs [2]. Therefore it is not enough that the TCR meets its cognate, MHC restricted antigen – a second signal must be transmitted independent of the TCR, and this signal must come from the DC (or similar APC). Evidence for a second signal came from experiments with MHC-peptide complexes fixed on artificial lipid bilayers – instead of inducing T cell activation, anergy was induced [66]; this suggested that a second signal in addition to the TCR was required for T cell activation. Further evidence in support of a second signal came by showing that peptide pulsed splenocytes treated with a chemical cross-linker could not activate T cells, but that activation could be brought about by adding in non-peptide-pulsed APCs. This also confirmed that the second signal was provided in *trans* to the TCR/MHC signal. This signal is known as costimulation, and multiple costimulatory pathways exist:

1.6.1. Immunoglobulin superfamily of costimulatory molecules

This group of costimulatory molecules is defined by receptors on lymphocytes containing a single immunoglobulin variable (IgV) domain and ligands on antigen presenting cells containing an IgV and an immunoglobulin constant (IgC) domain. The principal functions and distributions of these molecules are summarised in Figure 4.

1.6.2. CD28/B7 costimulation

CD28 is present on the majority of murine T cells and binds to CD80/86 (B7.1/7.2) on activated DCs and other APCs [67]. CD28 -/- mice show reduced immune responses to some pathogens, alloantigens and autoantigens [68]. It is important to note that, depending on experimental

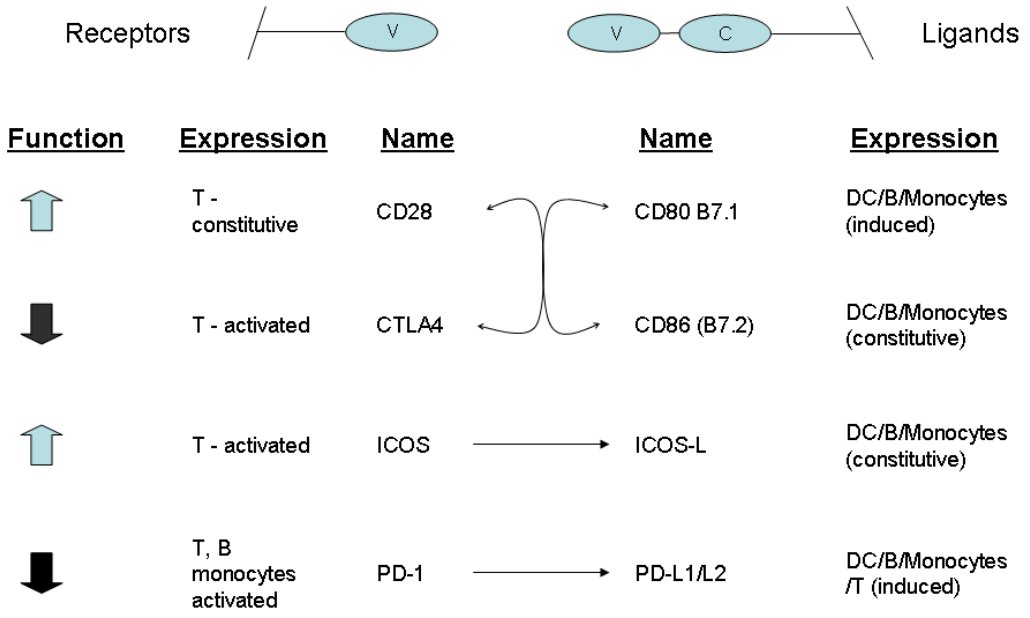

Figure 4 Summary of the B7 family of ligands and their receptors.

The conserved structure of a single IgV extracellular domain and IgV and IgC extracellular domains for ligands is depicted at the top. Solid arrows depict whether overall effects on T cells are co-stimulatory (up) or suppressive (down) (adapted from [69].

conditions, effective $CD8^+$ T cell responses can be generated in CD28 -/- mice, suggesting that other costimulatory pathways are present. Signalling through CD28 enhances $CD8^+$ T cell proliferation, survival and trafficking to non-lymphoid sites [70].

CD28 activation results in phosphorylation of its cytoplasmic tail, with resulting attraction of the signalling enzymes Lck, Fyn and phosphatidylinositol-3 kinase (PI3K); these pathways converge with those involved in TCR signal transduction, and thus CD28 and TCR signalling is a synergistic process [71].

The ligands for CD28 are CD80 and CD86; CD80 is virtually absent from resting DCs; CD86 is present at low levels. Their functions appear to be in part overlapping, with knockout mice of either molecule showing similar responses.

CD80/86 can also bind to cytotoxic T lymphocyte antigen 4 (CTLA-4), exerting a negative effect on T cell survival. CTLA-4 is upregulated on T cell activation, and acts to control the positive effect of CD28 costimulation. CTLA-4 binds to CD80/86 with an avidity 100-1000 times that of CD28; it's effects are predominantly found in $CD4^+$ T cells, but effects on $CD8^+$ T cells are also demonstrable; more recently, conditional knockout models of CTLA-4 under the FOXP3 promoter has recently been confirmed as a key molecule in the function of regulatory T cells [72].

CTLA-4 knockout mice develop a severe lymphoproliferative disorder, which is fatal around 3-4 weeks of life [73]. Whilst the CTLA-4 cytoplasmic domain is 100% conserved across many species, not all CTLA-4 function is mediated through its intracellular tail; mice engineered to express a transgenic CTLA-4 that lack the cytoplasmic tail develop lymphadenopathy but have a normal life span, with no abnormal tissue infiltration [74], suggesting a dual role for the CTLA-4 molecule, with the intra- and extra-cellular domains conferring separate but important functions.

CD28/CTLA-4 interactions may play a part in determining Th1/Th2 differentiation; CD28 stimulation on $CD4^+$ T cells favours Th2 differentiation, and can be blocked by the use of soluble CTLA-4 constructs; CD28 -/- mice

show a defect in Th2 differentiation [23]. Recent data suggests that CD80 and CD86 may differ in their priming of $CD4^+$ T cells, with CD86 favouring the formation of Th2 cells.

1.6.3. CD28 targeting for therapeutic purposes

CD28 costimulation has been employed *ex vivo* for the expansion of $CD8^+$ T cells for immunotherapy, although the effects of CD28 costimulation are more pronounced on $CD4^+$ T cells; in addition, CD28 stimulation of $CD8^+$ human T cells *ex vivo* seems to produce numbers of double positive lymphocytes, of uncertain function [75].

The finding that CD28 "super-agonists" (CD28sa) caused preferential expansion of T_{reg}, led to interest in the use of CD28sa in the treatment of human autoimmune diseases. A disastrous phase I clinical trial in healthy adult volunteers brought such investigation to a halt; all six volunteers became acutely unwell due to a syndrome known as 'cytokine storm' that occurred rapidly following infusion of the investigational product, TGN1412. Why the effects were so dramatic in humans is not clear; pre-clinical studies in primates did not demonstrate undue toxicity and the extracellular region of CD28 is conserved between humans and the tested primates. Possible explanations include the absence of 'Siglecs' in humans (membrane based inhibitory proteins), Fc receptor differences (which would be involved in binding to, and dimerization of, any bound antibody) or the differences between adult humans and primates kept under relatively sterile animal house conditions. As a result of the TGN1412 study, initial doses of investigational agents are now much lower, and are calculated according to measurable variables such as receptor occupancy, rather than estimated from non-human dose finding studies [76].

1.6.4. ICOS/ICOS-L pathway

ICOS (Inducible Costimulator) is expressed on activated, but not resting, T cells, and has 38% homology with CD28. Its expression on $CD4^+$ T cells is partly dependent on CD28 signalling, suggesting that it is functionally distal to CD28 in the T cell response [77].

The ligand for ICOS, ICOS-L (also known as B7h, B7RP-1, GL50 and LICOS) is 20% homologous to CD80 and CD86.

Stimulation through ICOS enhances IL-10 production by $CD4^+$ T cells, and to a lesser extent IL-2, augmenting both Th1 and Th2 responses. $CD8^+$ T cell responses are also augmented, enhancing tumour rejection and IFNγ and IL-2 production.

1.6.5. PD-1/PD-L pathway

PD-1 is expressed on activated $CD8^+$ and $CD4^+$ T cells. It has two ligands, PD-L1 and PD-L2, both expressed on a wide variety of tissues such as placenta, as well as on activated DCs. This negative regulator of $CD8^+$ T cell activation is upregulated during chronic infection, including in patients infected with the human immunodeficiency virus (HIV) [78]. Conversely PD-1 is down regulated on memory $CD8^+$ T cells. Blockade of PD-1 can aid viral clearance in models of chronic infection [79]; surprisingly, CTLA-4 blockade in the same system had little or no effect.

1.6.6. Tumour Necrosis Superfamily

First described 30 years ago, this large group of proteins is involved in modulating immune responses, where their rapid and powerful signalling effects can drive proliferation and functional changes of pathogen reactive cells [80].

Tumour necrosis factor receptors (TNFR) are type I transmembrane proteins containing repeated cysteine rich domains (CRDs). These 40 amino acid pseudorepeats typically contain 3 intra-chain disulphides, formed from 6 highly conserved cytseine residues. The ligands are type 2 proteins that are active as self assembling trimers with 25-30% amino acid similarities between family members. It is thought that binding of the ligand to the receptor results in trimerization of the receptor – this has been shown for LTα/TNFR1 [81] and TRAIL/DR5 interactions [82].

TNFRs can be divided into three groups: death domain (DD) containing, decoy receptors and TNF receptor-associated factor (TRAF) binding receptors. DD-containing TNFRS include FAS, TNFRI and DR3, and can activate caspase cascades, leading to apoptosis.

The ligands for TNFRs are type II transmembrane proteins with structural homology to tumour necrosis factor in their extracellular domains [83].

1.6.7. TNFR signalling

TNFRs lack intrinsic enzymatic activity within their cytoplasmic tails [84], and require adaptor proteins to mediate signal transduction – TRAFs. TRAFs have a conserved TRAF domain at their C-terminus, and link receptor binding to downstream events, such as JNK and NF-κB activation. TRAF binding TNFRs lead to cellular activation, differentiation and survival signalling. All TNFR can recruit TRAF to their signalling complexes; of the six identified mammalian TRAF proteins, TRAF 2 can be recruited by all, although with differing affinities [85]. When over-expressed, TRAF 2 and 5 activate NF-κB, a family of transcription factors that upregulate genes involved in cell survival such as Cellular Inhibitor of Apoptosis (cIAP1 & 2), BCL_{XL} and c-flip.

The TRAF binding TNFR ligand-receptor pairs involved in T cell costimulation are summarised in Figure 5.

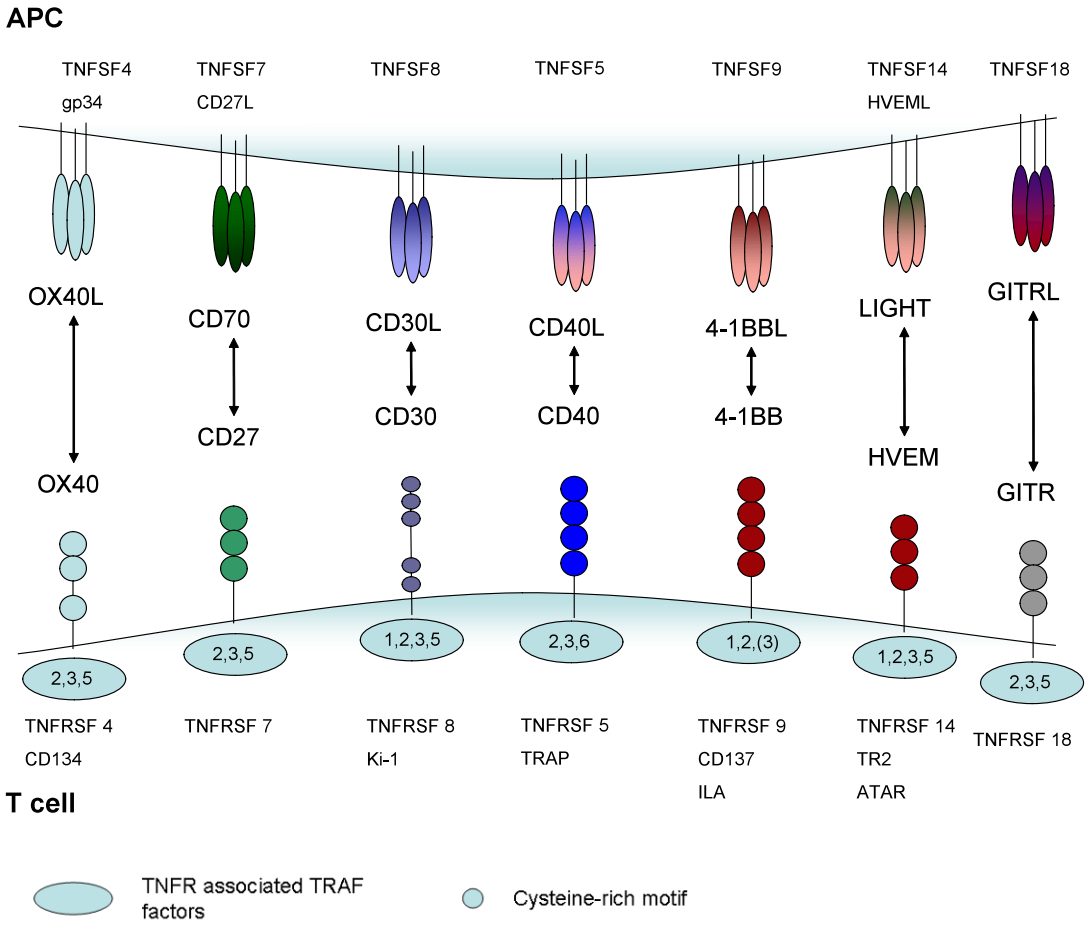

Figure 5 Summary of TNFR superfamily (adapted from [85])

ligands (above) and receptors (below) are shown, with their alternate names; the receptors are type 1 transmembrane proteins containing multiple cysteine-rich motifs in their extracellular domains (indicated by circles); the TRAF proteins that interact with each receptor are shown in blue.

1.6.8. CD40/154

1.6.9. Distribution

CD40 is a 48 kDa transmembrane glycoprotein that shares significant sequence homology with other members of the tumour necrosis factor receptor family. Initially discovered on B cells, CD40 is also found on activated T cells, DCs, macrophages and epithelial cells; its function is effected primarily through its expression on APCs [86]. CD40 ligand, CD154, is found primarily on activated $CD4^+$ T cells, but also can be detected on B cells, NK cells, basophils and platelets [87].

Most studies of CD40 have examined its function on APCs, but CD40 is also present on $CD8^+$ T cells, and vascular endothelial cells, where it promotes cytokine release as well as changes in various adhesion molecules; the results from studies looking at the effects of CD40 need to be interpreted with this wide distribution in mind.

1.6.10. Function of CD40

CD154 on activated CD4 cells binds to CD40 on naïve B cells, promoting cell proliferation and maturation; these B cells upregulate costimulatory molecules such as CD80 and 86, and undergo class switching. The importance of this interaction is demonstrated in the X-linked hyper-IgM syndrome; in this condition patients lack CD154 and have high levels of IgM, with low levels of IgA/G/E, and have impaired resistance to pyogenic infections. Like other members of the TNFR family, CD40 signalling leads to the binding of TRAFs to the CD40 cytoplasmic tail resulting in a variety of downstream signalling events including NF-κB activation.

CD40 ligation on DCs by CD154 on activated $CD4^+$ T cells leads to maturation of the DC, with upregulation of costimulatory molecules, such as CD70. These DCs are then 'licenced' to prime $CD8^+$ T cell responses [88]. Although initially it was thought that CD40 signalling was enough to trigger full DC maturation, more recently it has been shown that additional signals, especially through TLRs, may be required for full maturation including IL-12 production.

Nevertheless, CD40/CD154 interactions have a profound effect on whether DCs present antigen in a tolerizing or a stimulating fashion; for example blockade of this interaction can allow bone marrow transplantation across major MHC mismatches [89].

The activation of CD40 on DCs by activated $CD4^+$ T cells expressing CD40L provides an explanation for how $CD4^+$ T cells can 'help' $CD8^+$ T cell responses develop: however, $CD8^+$ T cell responses can develop in the absence of $CD4^+$ T cell help under certain conditions; a possible mechanistic pathway for such CD4 'independent' responses has recently been published; Johnson *et al* [90] demonstrated the upregulation of CD40L on DCs activated by TLR agonists and showed that this could directly prime $CD8^+$ T cells through CD40 (Figure 6).

1.6.11. CD40 targeting for therapeutic purposes

CD40 targeting to generate immune responses is attractive, since this approach, when coupled with vaccination, might be expected to enhance both cellular and humoral responses: nevertheless, caveats apply; CD40 signalling, despite its effect on $CD8^+$ priming, has recently been shown to ameliorate $CD8^+$ memory responses, possibly to below a pre-vaccination level [91], and may also cause autoimmunity.

Several phase I clinical studies have been conducted using agents that target the CD40 pathway; these include agonistic antibodies, antagonistic antibodies and soluble CD40 ligand. Trials in various malignancies have achieved a handful of partial remissions, but there are reports of cytokine release syndromes and liver toxicity [92]: for example, recently a novel agonistic anti-CD40 antibody was reported to induce partial remissions in 27% of patients with melanoma [93] although the numbers were small and the period of follow up was short. Responses have also been reported in patients with non-hodgkin's lymphoma [94], although concerns have been raised that CD40 signalling may in fact provide a positive growth signal in this disease [95]. The recent development of a new class of molecule that mimics the trimeric structure of CD40L that can lead to CD40 signalling raises the possibility of a whole new class of agents targeting this pathway [96]. A third possible pathway, important in CD4 independent reponses, is via CD40L upregulation on DCs activated through certain TLRs leading to direct activation of the CD8+ T cell via CD40

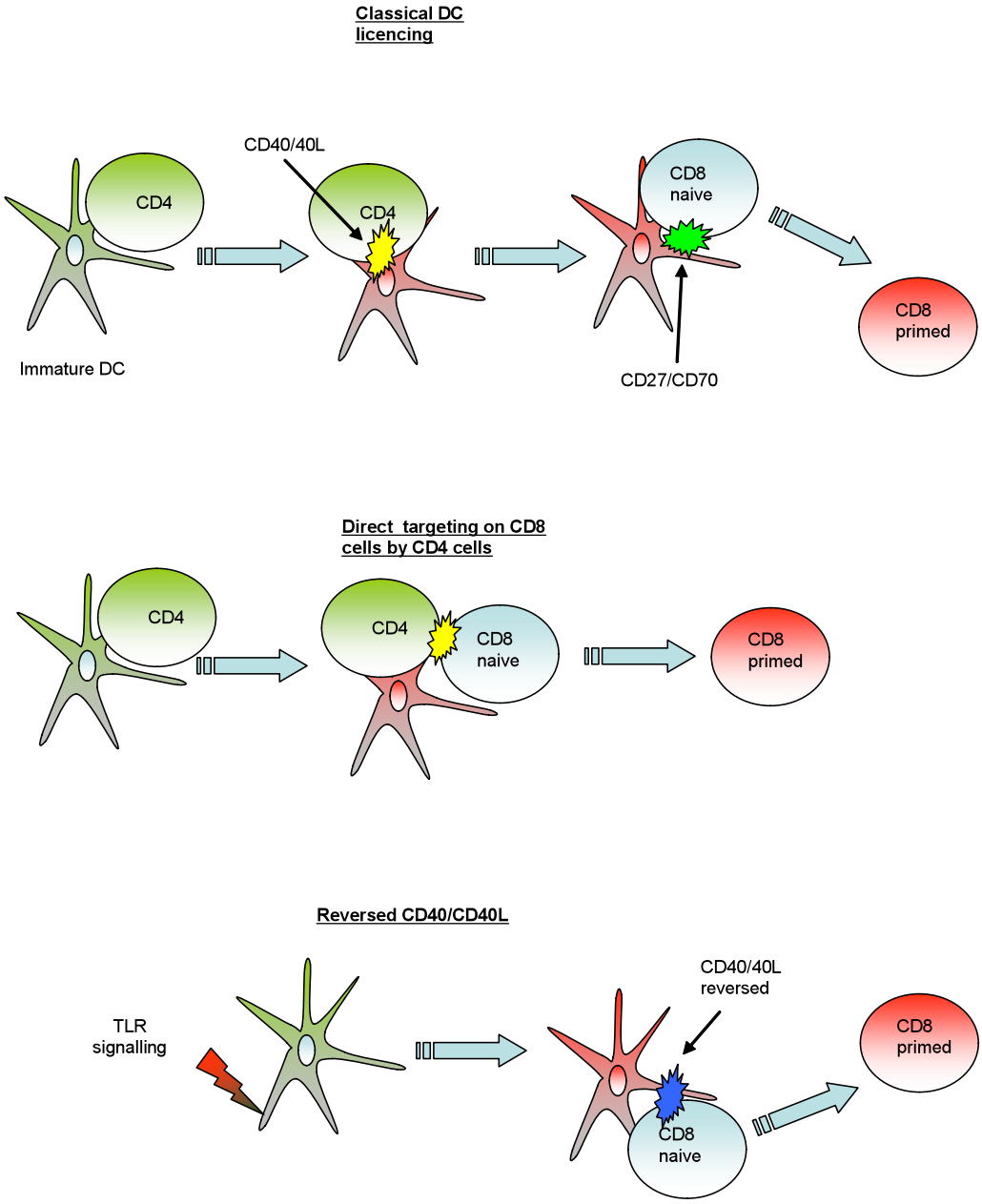

Figure 6 Pathways leading to CD8$^+$ T cell priming

Various pathways are possible explanations for the licencing of DCs to prime CD8$^+$ T cell responses: the 'classical' pathway is that CD4$^+$ T cells recognising their cognate MHC class II restricted antigen on a DC and upregulate CD40L; this in turn leads to DC maturation and 'licencing' to prime CD8 responses, possibly mediated by upregulation of costimulatory molecules on the DC surface such as CD70 or 4-1BBL.

An alternative explanation is that direct CD4-CD8 interactions lead to CD8 priming, possibly by CD40/CD40L or CD27/CD70 interactions.

1.6.12. CD27/CD70

CD27 (tumour necrosis factor receptor superfamily 7/tnfrsf7) was discovered in 1988 [97] by immunising mice with cryopreserved cells from a patient with Sezary syndrome. A monoclonal antibody was developed which bound to $CD4^+$ and $CD8^+$ T cells, the intensity of binding increasing with activation by phytohaemogluttinin. Two dimensional gel-electropheresis showed the molecular target of the antibody to be a 120kDa disulphide linked homodimer. Thus CD27 is consitutively expressed on T lymphocytes and its expression is increased after lymphocyte activation; in addition, activation of T cells leads to production of a small 32 kDa soluble form of CD27, which can serve as a marker of immune activation [98]. CD27 is evolutionarily conserved between mouse and man (65% conserved [99] and has 29% homology to another TNFR, 4-1BB).

1.6.12.1. Distribution of CD27

CD27 is found on naïve $CD4^+$ and $CD8^+$ T cells, most NK cells, and memory B cells [98, 100]. CD27 is found on central memory, but not effector memory, T lymphocytes [101]; this is similar to CD28 expression, which is also downregulated in CD8 effector memory cells. CD27 is also found on the majority of murine haematopoietic progenitor cells, although it is the smaller $CD27^{-ve}$ population of progenitor cells that has been shown to provide the best engraftment in irradiated mice [51]. CD27 is not found on human haematopoietic progenitor cells and the role of this pathway in haematopoietic development remains obscure.

1.6.12.2. CD70

The natural ligand for CD27 is CD70. CD70 was discovered by screening an EBV transformed cell line with a fusion protein containing the extracellular domain of CD27 [102]: this led to the discovery of a 193 amino acid type II transmembrane protein with significant homology to TNF. The gene for CD70

was mapped by fluorescent in-situ hybridization to 19p13 in man and chromosome 17 in the mouse [103].

1.6.13. Distribution of CD70

Newly synthesized CD70 is trafficked to late endosomal compartments where it is delivered along with MHC class II to the DC surface where it forms the immunological synapse with $CD4^+$ T cells [104]. CD70 is transiently expressed on T and B lymphocytes after stimulation; more recently CD70 has been shown to be expressed on activated murine DCs; CD70 expression is detectable on DCs activated with LPS or αCD40 mAb in vitro [105] and in vivo [106]. A unique population of cells from the lamina propria has been shown to constitutively express CD70 [75]; these DCs were $CD11c^{-ve}$ $DEC\text{-}205^{+ve}$ $B220^{+ve}$, and had antigen presenting and phagocytic activity.

1.6.14. Structure of CD70/CD27

CD27 forms homodimers linked by a single disulphide bond in the extracellular domain. CD70 is predicted to form homo-trimers, possibly interacting with three CD27 homodimers [107].

1.6.15. Gene targeted mouse models of CD27/CD70

For a summary of TNFR superfamily gene targeted mouse models see Table 1-1.

Over-expression of CD70 in B cells

Mice transgenic for CD70 under control of the CD19 promoter generate B lymphocytes with strong over-expression of CD70 [108]. These CD70-CD19 transgenic mice show normal T cell development, with reduced expression of CD27 on mature T cells; in addition, lymphadenopathy, with increased $CD4^+$ and $CD8^+$ T cell numbers, develops by 8 weeks of age. These lymphocytes acquire an effector/memory phenotype ($CD44^{hi}CD62^{lo}$), with increased IFNγ production, but are no different from wildtype in terms of TNF or IL-2 production. Conversely, a decrease in B lymphocytes is seen, with corresponding reduction in IgG2a, IgG2b and IgG3. These mice show

enhanced responses to influenza virus, as well as enhanced clearance of tumours. Despite this initial over activity of the immune response, CD70 transgenic mice succumb to opportunistic infections by six months of age, attributed to the gradual acquisition of effector function within the T cell pool, and the concomitant reduction in the naïve T cell pool.

CD27 complete knockout mouse

CD27 -/- mice develop normal levels of B lymphocytes and $CD4^+$ and $CD8^+$ T cells [109], suggesting that CD27 is not required for the antigen independent development of precursor lymphocytes. CD27 -/- mice show reduced proliferation in response to TCR signalling, that is not corrected by addition of CD28 costimulation or IL-2, suggesting a non-redundant role for CD27.

When challenged with intranasal influenza, CD27-/- mice generate lower numbers of lung-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes, although the percentage of virus-specific $CD8^+$ T cells out of total infiltrating $CD8^+$ T cells was not different from wild-type. A similar reduction in virus-specific $CD8^+$ T cell total number was seen when mice were rechallenged six weeks later. Ex vivo analysis of immunized mice showed no role for CD27 signalling in effector development.

Antibody responses to influenza in CD27 -/- mice were no different from wild type, which is in agreement with other studies which show that CD27 has only a limited role in B cell function. This may partly explain why there was no difference in survival between CD27 -/- and wild type mice infected with influenza, since humoral protection is important in resistance to influenza.

In summary, CD27 -/- mice showed normal lymphoid development, but reduced $CD4^+$ and $CD8^+$ T lymphocyte infiltration in a viral lung infection.

CD2 promoter driven CD70 transgenic mouse

T cell activation leads to upregulation of CD70 on T cells; thus some CD27/CD70 interactions may be autocrine T cell – on –T cell interactions, rather than DC/T cell interactions. For example, CD70 constitutive expression on T cells is seen in HIV infection and some autoimmune diseases [110]. To study the effects of constitutive CD70 expression limited to T cells, Van Guisbergen *et al* generated a transgenic mouse that expressed CD70 under the CD2 promoter. These mice showed enhanced development of effector memory $CD8^+$ T lymphocytes, with enhanced effector responses to influenza, but with subsequent waning of the memory $CD8^+$ response, similar to what is seen in chronic infection [111].

CD11c promoter driven CD70 transgenic mouse

To study the effect of CD70 on conventional DCs, Keller et al [112] generated mice that overexpressed CD70 under control of the CD11c promoter. These mice were maintained on a CD27 -/- background to prevent the immune pathology seen in the CD19 promoter CD70 transgenic mouse (see above). These $CD70^+$ DCs were able to overcome tolerance *in vivo* in two separate models; CD70 transgenic DCs overcame tolerance in $CD8^+$ T cells, but CD70 overexpression on B cells did not; this provides direct evidence that it is CD70 on the APC, rather than on activated B cells, that is key in priming $CD8^+$ T cell responses. Subsequent studies using CD11c CD70 tg DCs showed that it is the $CD8^+$ subset of DCs that prime $CD8^+$ T cell responses [113].

1.6.16. CD27/70 Function

T cell priming

CD70 is upregulated on CD40 stimulated bone marrow derived DCs [88], the effect being greatest when combined with TLR stimulation [114]. Therefore it is postulated that CD70 expression acts downstream of CD40 licencing of DCs to prime $CD8^+$ T cell responses: evidence to support this includes the

fact that CD70 blockade abrogates CD40 enhanced $CD8^+$ T cell primary responses in both TCR transgenic and endogenous models. In addition, the capacity of CD40 signalling to bypass $CD4^+$ T cell help is partially inhibited by CD70 blockade [115].

Despite this evidence, it is possible that at least some of the effects of CD27 signalling are mediated in an auto/paracrine fashion by CD70 expression on lymphocytes rather than on DCs (similar to the pathway examples shown in Figure 6 for CD40/CD40L interactions), although this seems less likely given recent published evidence (see section on CD70 transgenic mice and [112]). Further convincing evidence of the importance of CD70 expression on DCs (rather than on T lymphocytes) comes from Ballesteros-Tato *et al* [13], who demonstrated CD70 expression on migratory $CD103^-$ $CD11b^{hi}$ DCs, and showed that CD70 blockade significantly attenuated the ability of these DCs to prime $CD8^+$ T cell responses [13] to influenza.

A similar role for CD27 signalling in helper dependent $CD8^+$ T cell responses has also been shown; blockade of CD70 dramatically abrogates $CD8^+$ T cell responses to cross-presented cell-associated antigen, even in the presence of excessive $CD4^+$ T cell help [106]. The need for CD27/CD70 interaction is partially bypassed when TLR3 agonists are co-administered, an effect that seems to be due to α-interferon.

Xiao et al [116] examined the role of CD27 signalling in $CD4^+$ T cell responses and the subsequent effect on $CD8^+$ responses; posing the question; what happens when CD27 deficient $CD4^+$ help is given to CD27 replete $CD8^+$ T cells? By using CD27 -/- OT-II dual transgenic mice (which carry transgenic $CD4^+$ T cells specific for an MHC class II restricted peptide from chicken ovalbumin) they found that the wild type $CD8^+$ responses were diminished when the $CD4^+$ T cell help was deficient in CD27. This reduction in the primary response was mirrored in the secondary response. Gene expression microarrays from WT and CD27 -/- $CD4^+$ T cells showed that CD27 signalling significantly upregulated genes downstream from IFNγ suggesting that CD27 signalling is involved in driving Th1 differentiation of $CD4^+$ T cells. A particular gene involved in Th1 differentiation was downregulated in the absence of

CD27 signalling – MS4A4B - also known as Chandra. MS4A4B is a transmembrane molecule from the same family of molecules as the B cell marker CD20. Over-expression of MS4A4B has previously been shown to increase IFNγ and IL-2 levels [117, 118]. This gene was also, extraordinarily, the only gene found to be differentially expressed by microarray when $CD8^+$ T cells were examined that had been primed with CD27 -/- vs WT $CD4^+$ T cell help; it has yet to be shown that Chandra expression is affected at the protein level by CD27 signalling.

Effect on regulatory T cells

A model of T cell tolerance has been used to study CD27/CD70 interactions: intratracheal administration of allogeneic splenocytes leads to tolerance, allowing subsequent allogeneic cardiac transplantation in mice; co-administration of mAb to CD70 during tolerance induction has been shown to markedly increase the rejection of cardiac grafts [119]. Paradoxically, CD70 blockade at the time of non-tolerant cardiac transplantation led to tolerance. Similar effects were seen for OX40/OX40L and CD30/CD30L blockade, but not for 4-1BB/4-1BBL blockade. A possible effect on CD27 enhancement of regulatory T cells was postulated as an explanation, and this has been demonstrated *in vitro* with CD70 expressing B cell non-Hodgkin's lymphomas, although the effect was small [120].

Effect on B cell response

In humans CD27 signalling enhances immunoglobulin production, in part due to enhanced plasma cell differentiation [121]: however, CD27 -/- mice show normal somatic hypermutation and normal immunoglobulin responses. Some indirect evidence for a role of CD27 signalling in B cell responses exists: CD27/28 double knockout mice fail to form germinal centres, and this defect was partially reversed with adoptive transfer of CD27 positive T cells [122]. Nevertheless, the predominant effect of CD27 signalling seems to be on T lymphocytes.

1.6.17. CD27 signalling

When coupled with TCR signalling, CD27 signalling rapidly phosphorylates Jun-N-terminal kinase (JNK), mediated by Traf-2 [123]. In addition, CD27 signalling also activates NF-κB via Traf-2 and Traf-5 [124]. This has been confirmed using Traf-5 -/- mice[125].

Consistent with its effect on cell survival, there is evidence that CD27 signalling upregulates anti-apoptotic proteins such as Bcl-2 and Bcl-XL [126], although this evidence comes from human B cell lymphoma lines. Studies of human Burkitt's lymphoma cell lines have shown that CD27 signalling can lead to apoptosis, and that this was mediated by CD27 binding to the pro-apoptotic molecule Siva, which contains a death domain similar to other pro-apoptotic TNFR interacting molecules such as FADD and RIP [127]. How important this is physiologically is uncertain, given the mainly pro-survival/proliferation effects of CD27 signalling.

1.6.18. CD27 in humans

The gene for CD27 in humans is located on chromosome 12, at the 12p13 band [128] – an area classically deleted in various human malignancies such as acute myeloid leukaemia [129]; in addition, CD27/CD70 interactions may play a role in myeloma [130] and acute lymphoblastic leukaemia [131]. CD27 is present on the majority of B cell lymphomas [132].

In humans CD27 is acquired on B cells after B cell receptor triggering, and is maintained long term (contrary to the situation in the mouse, where only a minority of memory B cells express CD27 [122]). It is considered a marker of memory B cells [133], and $CD27^+$ B cells are found in germinal centres and marginal zones. Naïve human $CD8^+$ T cells express CD27 [134]; this expression is lost on effector memory cells.

1.6.19. CD27/CD70 interactions in human disease

The CD27/CD70 pathway may be a suitable target for immunotherapy, possibly by stimulating T cells ex vivo along with a suitable antigen, and then transferring the activated T cells into suitable recipients: however; when cultured in vitro with IL-2, human $CD8^+$ T cells upregulate CD70 in a dose response manner; and a reciprocal reduction in CD27 occurs. This effect has been observed *in vivo* (in patients receiving IL-2 for treatment of melanoma), and on tumour infiltrating lymphocytes [135]. Thus IL-2 stimulation of $CD8^+$ T cells may lead to upregulation of CD70, and subsequent autocrine stimulation of neighbouring $CD8^+$ T cells through CD27.

Another possible area of interest is in the blocking of CD27/CD70 interactions to bring about a therapeutic effect. As discussed above, it is not clear how physiologically important lymphocyte expression of CD70 is for CD27 signalling; CD27 signalling is only thought to act in the presence of TCR signalling, and it remains most likely that the key CD27/CD70 interaction takes place between DCs and T lymphocytes. In lymphoid malignancies however CD27/CD70 interactions on malignant lymphocytes may provide an autocrine growth signal; indeed, blockade of CD70 has been shown to have a therapeutic effect in a mouse model of Waldenstroms Macroglobulinaemia, a form of low grade B cell non-hodkgin's lymphoma [136]. Other groups have proposed CD70 as a potential tumour suppressor gene, and shown it to undergo epigenetic silencing in some *in vitro* models of cancer progression [137].

In summary, CD27 is a powerful stimulator of $CD8^+$ and $CD4^+$ primary and secondary responses. It interacts with its ligand, CD70, which is predominantly expressed on mature DCs. Its main difference from the other TNFRs involved in T lymphocyte responses is its constitutive presence on naïve T lymphocytes. As such it represents an attractive therapeutic target for anti-cancer vaccine development.

1.6.20. 4-1BB/4-1BBL

1.6.21. Distribution

4-1BB (CD137) is a member of the tumour necrosis receptor superfamily [138] which binds a high affinity ligand (4-1BBL) present on APCs. 4-1BB expression peaks at around 48 hours post T lymphocyte activation, declining over the subsequent four to five days. Although expressed on both $CD4^+$ and $CD8^+$ T cells, expression occurs more rapidly in $CD8^+$ T cells. 4-1BB is also found on a subset of splenic DCs, where it is downregulated by CD40 signalling; it is also found on human monocytes, follicular dendritic cells, cytokine activated NK cells and microglia. After expression, 4-1BB is shed from the cell surface following proteolytic cleavage and can be detected in culture supernatant [139].

4-1BB ligand (4-1BBL) is expressed on activated macrophages, DCs and B cells. 4-1BBL expression on DCs and B cells is mediated by CD40. At sites of inflammation, 4-1BB can be detected on diverse cell types, including cardiac myocytes, human neurons and aortic tissue.

1.6.22. Function

4-1BB can stimulate $CD4^+$, as well as $CD8^+$ responses, enhancing cell division, survival and effector function [140]. 4-1BB can replace CD28 signalling in some instances, although this effect is reduced at limiting levels of antigen. 4-1BB not only stimulates $CD8^+$ T cell responses, but broadens the response to include non-dominant epitopes [141].

4-1BB stimulation of murine T lymphocytes leads to production of IL-2, Il-4 and IFNγ, but in humans IFNγ alone. 4-1BB has shown to prime responses to peptide vaccines, implying a role in sub-optimal conditions [142], and is important in the resulting recall responses. Although the 4-1BB and CD28 pathways are active at different times during $CD8^+$ T cell priming, their function seems to overlap. Both 4-1BB and CD28 signalling upregulate anti-apoptotic molecules c-$FLIP_{short}$ and BCL_{XL}, mediated via the PI3K pathway [143], which protects T cells from AICD.

4-1BBL engagement can lead to 'reverse' effects downstream of 4-1BBL [144]; this seems to be independent of 4-1BB; rather, 4-1BBL-TLR4 interactions lead to tumour necrosis factor production by macrophages; thus 4-1BBL knockout mice are resistant to lipopolysaccaride toxicity.

1.6.23. Role of 4-1BB in $CD8^+$ T cell memory

4-1BB stimulation via monoclonal antibody has a direct, antigen-independent effect on resting memory cells [145]. It causes increased proliferation of $CD8^+$ and $CD4^+$ T cell resting memory cells (as defined by CD44 positivity) and increased $CD44^{hi}$ intrahepatic memory cell numbers .

CD28 -/- mice have attenuated recall responses to influenza virus, and this can be corrected by giving stimulating-anti-4-1BB antibody [142]. Of note, the recall response to influenza is $CD4^+$ T cell dependent; $CD4^+$ T cell responses are reduced in CD28-/- mice, and there was only a weak effect on $CD4^+$ T cell activation when costimulation through 4-1BB was provided; ie, either only a small amount of $CD4^+$ T cell help is required to restore influenza-$CD8^+$ T cell responses, or 4-1BB bypasses the need for $CD4^+$ T cell help in this model.

In some circumstances, 4-1BB costimulation of $CD8^+$ T cell responses can negate the requirement for $CD4^+$ T cell help: Miller et al [146] used an agonistic anti-4-1BB antibody that was capable of enhancing tumour clearance in several tumour models: this was unaffected by $CD4^+$ T cell depletion (or NK depletion) given during the primary response; but when mice that had successfully cleared tumours were rechallenged with tumours 3 months later, $CD4^+$ T cell depletion during the primary response resulted in a poorer response to rechallenge; ie $CD4^+$ T cell help was not required during the primary response, but its absence during the primary response adversely affected the recall response (see section on T cell memory below), an effect that was also seen, to a lesser extent, when MHC class II -/- mice were used instead of antibody mediated $CD4^+$ T cell depletion. The memory response was not affected in IL-15 -/- mice, a cytokine with a major function in $CD8^+$ T cell memory responses. 4-1BB stimulation coupled with $CD4^+$ T cell depletion has also been shown to enhance tumour protection, an effect that was

mediated through enhanced $CD8^+$ T cell proliferation and effector differentiation [147] . Thus 4-1BB/4-1BBL interactions may remove the need for $CD4^+$ T cell help in the generation of $CD8^+$ T cell memory, to the point that $CD4^+$ T cells may in fact be detrimental to the $CD8^+$ T cell memory generated.

1.6.24. 4-1BB in humans

The cytoplasmic tail of human 4-1BB has notable differences from its mouse counterpart, with altered lck binding sites, although both bind to the TNFR-associated factor 2 (Traf 2). 4-1BB maps to chromosome 1p36, an area involved in several malignancies. Like its murine counterpart, human 4-1BB is upregulated by TCR signalling. 4-1BBL stimulates $CD8^+$ and $CD4^+$ T cell expansion and differentiation *in vitro* [148]. 4-1BB stimulation can stimulate CMV specific antigen-experienced $CD8^+$ T cells in vitro to proliferate (where CD28 stimulation has no effect) [149]; other groups have confirmed the skewing of 4-1BB over CD28 in stimulating human $CD8^+$ T cells *in vitro* [150] – this would fit with the murine experiments outlined above, with a prominent role for 4-1BB in costimulation of memory responses. Conversely, it would seem that 4-1BB is relatively poor at stimulating naïve $CD8^+$ T cells to proliferate. Nevertheless, 4-1BB stimulation is an attractive approach for generating ex vivo $CD8^+$ T cells for adoptive immunotherapy [151].

4-1BB signalling in human lymphocytes enhances proliferation in response to antigen, and leads to increased levels of perforin, granzyme A and cytokines. Of note, $CD28^{-ve}$ lymphocytes are found increasingly with age, and these $CD28^{-ve}$ lymphocytes can be stimulated through 4-1BB to proliferate and acquire effector function – thus there is a potential therapeutic role for 4-1BB signalling to enhance vaccine efficacy in the elderly.

1.6.25. 4-1BB gene targeted mice models

Lck promoter 4-1BB transgenic mouse

Physiological 4-1BB is only expressed on T lymphocytes after activation; 4-1BB forced expression on T lymphocytes by using the lck promoter yields mice that express 4-1BB on the majority of resting T lymphocytes [152]. Lymphocyte development appears normal in these mice, other than higher levels of thymocyte apoptosis than in wild type controls. *In vitro* proliferation in response to anti-CD3 stimulatory antibody showed enhanced proliferation in $CD4^+$ and $CD8^+$ T lymphocytes, as well as increased contact hypersensitivity (which is mediated by $CD4^+$ T cells) and *ex vivo* recall responses to keyhole limpet haemocyanin. IgG responses were slightly increased. This data adds evidence to the role of 4-1BB in stimulating $CD4^+$ and $CD8^+$ responses; it also argues that earlier provision of costimulation in a T cell response will augment that response.

MHC class II I-E_α promoter 4-1BBL transgenic mouse

4-1BBL transgenic expression under the MHC class II I-E_α promoter generates DCs that constitutively express 4-1BBL [153]; the main effect seen was a gradual but profound depletion of B lymphocytes and antibody production. Studies on allogeneic T lymphocyte responses were inconclusive. No clear conclusions regarding 4-1BB biology have been drawn from this model.

4-1BBL knockout mouse

4-1BBL -/- mice show reduced $CD8^+$ T cell expansion and memory formation during acute infections [114]. A surprise finding in these mice is the high incidence of B cell lymphoma, with 60% of mice affected by twelve months of age [154]; this is despite an apparently normal B cell response in 4-1BBL -/- mice. No mechanism for this phenomenon has yet been proposed.

4-1BB knockout mouse

4-1BB -/- mice show reduced $CD8^+$ T cell responses to certain viral infections; there are reports of paradoxically enhanced $CD4^+$ T cell responses which are discussed in more detail in the second results chapter.

In general 4-1BB -/- or 4-1BBL -/- mice exhibit attenuated $CD8^+$ T cell responses, with a much smaller effect on $CD4^+$ T cell responses [155]. This attenuation of the $CD8^+$ T cell response is most marked with weak antigens; conversely the response to rapidly dividing antigens eg lysteria or LCMV, is only slightly affected. When a normal primary $CD8^+$ T cell response is generated in a 4-1BB deficient background, the resting memory population and the recall response is reduced.

4-1BB signalling

4-1BB signalling is mediated through TRAFs 1 and 2 [156] which links 4-1BB to downstream JNK, MAP kinase and NF-κB [157] TRAF-1 has recently been shown to mediate ERK phosphorylation, which in turn has downstream effects on pro-survival BCL_{XL} and the pro-apoptotic BH3 protein Bim (discussed in subsequent chapters).

1.6.26. Other TNFR superfamily members involved in T cell responses

A detailed discussion of the other TNFR superfamily members is outside the scope of this thesis, however, the salient features are listed here and in Table 1-1:

1.6.27. CD30/CD30L

CD30 was initially discovered as a cell surface marker in Hodgkin's lymphoma, where it contributes to ligand independent NF-κB activation. CD30 is found on activated T cells, particularly the Th2 $CD4^+$ T lymphocyte subset; further evidence for Th2 bias in CD30 signalling comes from the observation that CD30 signalling can trigger IL-13 production, a cytokine involved in parasite clearance and lung eosinophilia. The CD30 knockout models have a mild phenotype, with little reduction in viral immunity [85].

1.6.28. OX40/OX40L

OX40 is preferentially expressed on activated $CD4^+$ T cells, particularly the Th2 subset. Like other TNFR members, OX40 signalling can produce T lymphocyte proliferation, and induces the expression of anti-apoptotic Bcl-2 family members. OX40 knockout mice seem to confirm the requirement for OX40 in $CD4^+$ T cell responses.

1.6.29. HVEM/LIGHT

So called because of its use by herpes simplex 1 (HSV1) virus entry into cells, HVEM (Herpes Virus Entry Mediator) shares with CD27 a constitutive presence on T cells. Unlike CD27, HVEM is downregulated on T cell activation. The ligand for HVEM, LIGHT, is found on immature, but not mature, DCs, the two molecules sharing a reciprocal expression pattern, with only a narrow window where both molecules are present and thus able to contribute to the T cell response [85]. LIGHT/HVEM interactions cause T cell proliferation, with a preference for $CD8^+$ T cell responses.

1.6.30. GITR/GITRL

The Glucocorticoid Induced TNF Receptor, GITR, provides a proliferative signal to $CD4^+$ and $CD8^+$ T lymphocytes. Normally expressed after T cell activation, GITR is constitutively expressed on T_{regs} and may play a role in the survival and activity of this cell type.

TNFRsf	Distribution	Transgenic	Effect
CD27	Resting T cells, increasing with activation	Full KO [109]	Diminished primary and secondary $CD8^+$ responses to influenza
CD70	Activated DCs and T and B lymphocytes	B cell Tg [108]	CD4 & CD8 effector function ↑ Virus and anti-tumour
		DC Tg [112]	CD4 & CD8 effector function ↑ Lack of CD8 deletional
		T cell Tg [111]	CD8 effector function ↑ CD8 memory maintenance ↓
4-1BB	Activated T cells, B cells, NK and DCs	Full KO	CD8 response to VSV ↓ T cell response to protein vaccine ↑
		T cell tg	T cell proliferation ↑ Contact hypersensitivity ↑

TNFRsf	Distribution	Transgenic	Effect
4-1BBL	B cells, DCs, macrophages	Full KO	CD8 primary and memory ↓
		B cell tg[i]	Normal T cell response Reduced IgG
CD30	T cells	Full KO	CD4 expansion to mycobacterium ↓
		T cell Tg	Enhanced thymocyte apoptosis
CD30 L	T and B cells, macrophages	Full KO	CD4 (effector) & CD8 (memory) ↓
HVEM	T and B cells, DCs, macrophages	Full KO	T cell activation & cytokine formation ↑

TNFRsf	Distribution	Transgenic	Effect
LIGHT	T cells, DCs	Full KO	CD4 & CD8 effector function ↓
		T cell Tg	CD4 & CD8 effector ↑
OX40	T & B cells, DCs	Full KO	CD4 proliferation and effector to influenza & LCMV ↓
			CD8 effector memory ↓ to
OX40L	T & B cells, DCs, macrophages	Full KO	CD4 responses to protein vaccine ↓
			CD8 responses to allogeneic
		DC Tg	Enhanced CD4 accumulation in B cell follicles
		T cell Tg	Enhanced CD4 responses

Table 1-1 Summary of TNFR superfamily gene targeted mouse models

1.7. The Cytotoxic T cell response

Commencing with a newly formed naïve $CD8^+$ T cell leaving the thymus, the $CD8^+$ T cell response can be broadly divided into five phases:

- Naïve $CD8^+$ homeostasis
- Effector phase
- Contraction phase
- Memory phase
- Rapid recall phase

1.7.1. $CD8^+$ T cell homeostasis

$CD8^+$ T cell numbers are closely controlled; for naïve $CD8^+$ T cells this depends on continuous contact with self-peptide/MHC [158]; this was shown by adoptive transfer of $CD8^+$ T cells that recognise male Y chromosome antigens in an MHC restricted fashion: naïve $CD8^+$ T cells only survived and proliferated in hosts that expressed the male antigen presented by the appropriate MHC. Memory $CD8^+$ T cells survived in hosts in the absence of antigen, but required MHC class I. This holds true for memory and naïve $CD8^+$ T cell homeostatic proliferation in lymphopaenic hosts, although the proliferative rate is much slower for naïve cells [159]. The key cytokine involved in naïve $CD8^+$ T cells homeostatic proliferation is IL-7, and this was confirmed when homeostatic proliferation was unaffected in IL-15 or IL-4 knockout hosts, but was dramatically decreased when IL-7 knockout hosts were used, and was corrected by the exogenous administration of IL-7. The loss of proliferation in IL-7 hosts could not be corrected by transferring bcl-2 transgenic $CD8^+$ T cells (IL-7 is known to upregulate bcl2 expression), and this can in part reverse the effects of IL-7 deficiency on thymic development [159].

1.7.2. Effector phase

Prior to infection the $CD8^+$ T cell precursor frequency for any given antigen is around 1 in 100,000. Following TCR stimulation accompanied by the appropriate costimulation these precursors undergo dramatic proliferation, increasing the numbers by around several thousand fold. The T cells generated during this effector phase are very sensitive to apoptosis, with around 95% dying following the primary response. This leaves behind a number of long-lived memory $CD8^+$ T cells. These can respond more rapidly to restimulation than naïve $CD8^+$ T cells, resulting in improved defence to subsequent exposure by the same infectious organism. These memory cells are still detectable in humans up to 75 years following primary exposure, thus conferring, in some cases, life-long protection [160].

1.7.3. Cytotoxic activity

Once activated through TCR and costimulatory signals, $CD8^+$ T cells are primed to recognise and destroy target cells. Within a day of activation granules begin to be produced containing cytotoxic proteins - perforin and granzymes [161]. On recognition of a target cell a tight Junction is formed between the T cell and the target cell, and cytotoxic granules are transported to the T cell surface; there, perforin polymerizes in the presence of Ca^{++}, and provides a channel in the target cell membrane for the entry of various granzymes, which are potent activators of pro-apoptotic caspases within the target cell. Whether the channel produced by peforin is physically large enough to allow large molecules through is currently under question, but it is clear that perforin is required for cytolytic activity. Interestingly, the action of perforin on the target cell membrane may require the presence of a receptor, with some evidence pointing to platelet activating factor (PAF) receptors. In support of the concept of a perforin-specific receptor, there is a correlation, in leukaemic cells, between perforin binding activity and susceptibility to cell mediated lysis – ie those leukaemic cells which demonstrated reduced perforin binding were resistant to NK cell mediated cytotoxicity.

Once perforin has compromised the outer membrane of the target cell, various granzymes are released. Granzyme B is the best documented of the granzymes, and is able to cleave a variety of pro-caspases, as well as directly cleaving DNA, resulting in irreversible apoptosis of the target cell.

1.7.4. Timing of antigenic exposure

It is thought that the effector phase in $CD8^+$ T cells results from quite short-lived antigenic exposure [162] . Shortening the initial antigenic exposure (by infecting mice with listeria spp then clearing the infection with antibiotics) does not affect the primary response, but significantly accelerates the contraction phase, resulting in a lower memory population. The mechanism of this may be that in this model $CD4^+$ T cell responses were dependent on the timing of antigenic exposure; thus when antigen exposure was short the primary $CD4^+$ T cell response was attenuated (see section on $CD4^+$ T cell for $CD8^+$ T cell memory generation). The size and potency of the memory response, as well as the differentiation to effector function, or the development of tolerance, all depend on the nature of this initial antigenic stimulation [163]. *In vivo* it seems that the majority of antigenic exposure takes place in the first 2-3 days of infection, with the bulk of $CD8^+$ T cell expansion taking place after this [164]. Two-photon microscopy has shown that DC/$CD8^+$ T cell interactions have three distinct phases; an early phase lasting 8 hours of brief DC/CD8 T cell contact; in the second phase DCs and $CD8^+$ T cells form a close, stable interaction that lasts up to 48 hours, followed by a period of $CD8^+$ T cell expansion with much reduced DC interaction [165].

1.7.5. Requirement for CD4 help during the effector phase

$CD4^+$T cells 'licence' DCs to prime $CD8^+$ T cell responses, as outlined above. This was initially suggested by the need for both MHC class I and II epitopes to be present on the same DC for priming to occur, implying a requirement for $CD4^+$ T cell help [166]. Subsequently it was shown that this could be bypassed by signalling through CD40, and conversely that CD40 blockade abrogated the effect of $CD4^+$T cell help.

The current model of $CD8^+$T cell licencing describes DCs activated by $CD4^+$ T cells via CD40/CD40L interactions; $CD8^+$ T cells do, however, express CD40, and some groups have suggested that $CD4^+$ T cells may directly activate $CD8^+$ T cells rather than via DCs [167]. The basic possible pathways of $CD8^+$ T cell licensing are shown in Figure 6.

There are many in vivo systems that demonstrate $CD8^+$ primary responses in the absence of $CD4^+$ T cell help: possibly because of DC licensing via alternate, innate, pathways eg binding of CpG DNA to toll-like receptors (TLR). DC activation via TLRs can lead to DC maturation, with upregulation of costimulatory molecules (including CD70), migration to secondary lymphoid tissues, and cross-priming of $CD8^+$ T cell responses [168]. Thus, whilst $CD4^+$ T cell help is required in some experimental systems to prime $CD8^+$ T cell responses, it seems dispensible in others, being replacable by other innate, TLR mediated pathways to DC maturation.

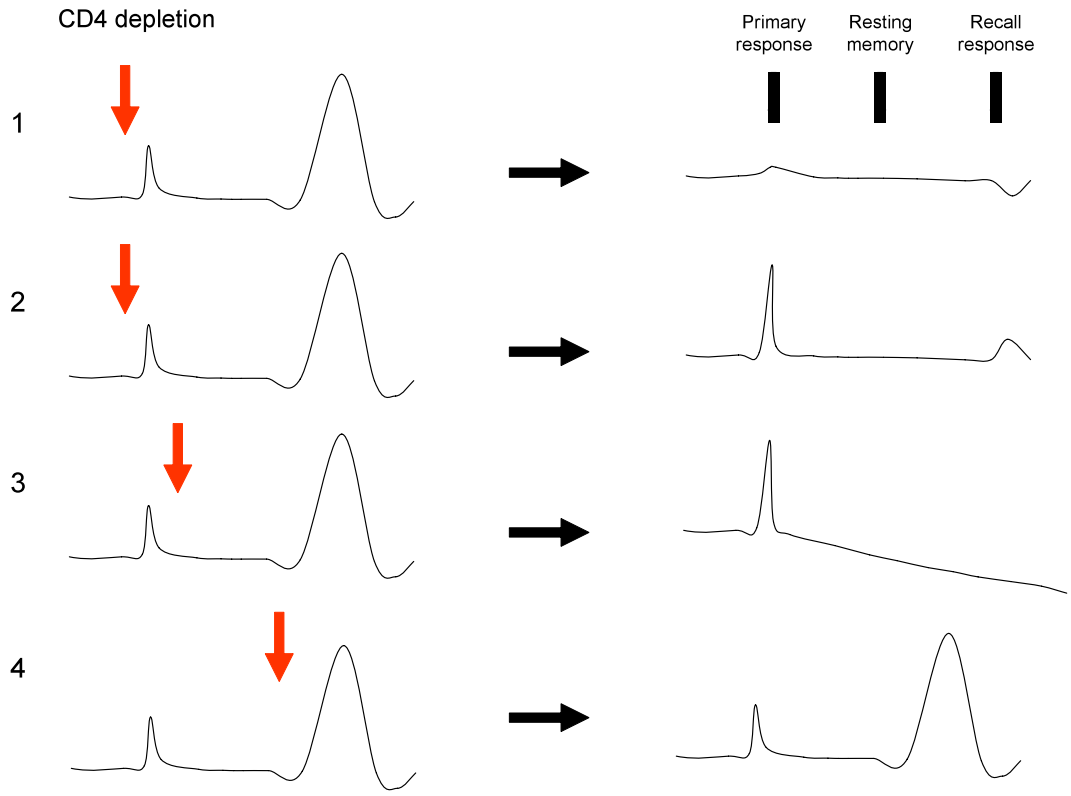

Figure 7 CD8$^+$ T cell requirement for CD4 help

The effects of CD4 help at different timepoints of the CD8$^+$ T cell response are shown – on the left a normal CD8$^+$ T cell primary and secondary response are shown by the black line - the red arrow indicates the timing of CD4$^+$ T cell depletion, and on the right the effect on the primary/secondary CD8$^+$ T cell response:

1. Absence of CD4$^+$ T cell help during priming in non-inflammatory conditions results in failure to generate a CD8$^+$ T cell primary response
2. Loss of CD4$^+$ T cell help during priming under inflammatory conditions results in an appropriate primary response, but the secondary response is impaired.
3. Absence of CD4$^+$ T cells during the resting memory phase causes a gradual decline in resting CD8$^+$ T cells numbers.
4. Absence of CD4$^+$ T cell help at the time of rechallenge does not affect the recall response

1.7.6. Requirement for Signal 2 and Signal 3 during $CD8^+$ T cell priming

The $CD8^+$ T cell receives several signals to initiate an effector response, broadly grouped into:

- Signal 1 – the binding of the TCR to MHC class I bound peptide – providing the specificity of the response
- Signal 2 – involving the various costimulatory pathways, this determines whether the outcome will be (broadly) tolerance or activation and is reviewed in the section on costimulation.
- Signal 3 – soluble cytokine signals

Signal 3 is required for a fully-fledged $CD8^+$ T cell primary and memory response. Interleukin 12 (IL-12) production by activated DCs and phagocytes binds to the heterodimeric IL-12 receptor on activated $CD8^+$ T cells, and enhances IFN-γ production. IL-12 boosts IFN- γ production and $CD8^+$ T cells primed in the absence of IL-12 are tolerogenic [169].

IL-12 is a heterodimer formed from a 35 kDa light chain (p35/IL-12α) and a 40kDa heavy chain (p40/IL-12β) [170]. Phagocytes and DCs are the prime producers of IL-12. The IL-12 receptor is also a heterodimer (IL-12Rβ1/β2) that activates the JAK-STAT pathway of signal transduction [170]. IL-12R expression is mainly restricted to NK and activated T cells, but has also been described on DCs and B-cell lines. IL-12 production by DCs is primarily mediated by innate signals, although it can be enhanced by stimulation through CD40L.

Signal 3 can also be mediated via type I interferons (IFN), as shown by the poor primary and recall responses in IFN receptor knockout mice, although it is not clear how important this is in the physiological setting [171].

If the effector phase fails to clear completely the antigen, as is the case in some infections eg mycobacterium tuberculosis, or some herpes viruses, $CD8^+$ T cells gradually lose the ability to secrete cytokines, and are tolerized

or deleted. The degree to which this happens seems to be linked to the level of antigenic persistence; where large levels of antigen persist deletion is likely to occur; conversely during low levels of antigen persistence – eg during Epstein Barr virus infection – effective effector function can persist in the EBV-specific $CD8^+$ T cell population [172]. The costimulatory molecule PD-1 is involved in this response to chronic antigenic exposure (see section on immunoglobulin superfamily costimulatory molecules).

1.7.7. The Contraction Phase of the $CD8^+$ T cell response

Following the primary/effector phase of $CD8^+$ T cell proliferation (which may involve up to 50% of total $CD8^+$ T cells), the majority of the antigen specific cells undergo apoptosis. This is necessary to avoid massive accumulation of $CD8^+$ T cells, as well as autoimmunity [173]. Two key apoptotic pathways are utilized to bring about the contraction phase – the intrinsic and extrinsic apoptotic pathways [174]. Which pathway predominates may depend on whether antigen clearance is complete or persistent (as may occur in chronic infections); when an activated T cell continues to receive signals through its TCR it undergoes apoptosis; this activation-induced cell death (AICD) is dependent on ligation of Fas, a member of the tumour necrosis superfamily, by Fas-ligand (FasL) [175]. When antigen clearance is complete however, the intrinsic apoptotic pathway may predominate [173].

1.7.8. Memory Generation

Following the first encounter with an antigen, $CD8^+$ T cells divide extensively into cytotoxic effector cells, resulting (usually) in clearance of the antigen, followed by apoptosis of the majority of effector cells, leaving a pool of memory cells. Re-exposure to antigen is dealt with by these memory cells, which have a higher capacity for proliferation and more rapid cytokine production [176], allowing for rapid clearance of repeated infections.

During the primary $CD8^+$ T cell response, proliferating cells downregulate surface expression of CD127, the IL-7 receptor α chain: however, on a small subset, CD127 expression is maintained, and it is this subset that is destined to survive the primary response as long term memory $CD8^+$ T cells [163]. The IL-7rα population have higher levels of survival factors such as Bcl-2 and Bcl-xl, have higher levels of CD27, and show stronger homeostatic proliferation and secondary responses when compared to the IL-7rα-ve population. CD127 is probably only a marker of memory formation however, since there is little evidence that IL-7 is itself important for memory generation: for example, IL-7 deficient mice still show the characteristic downregulation of IL-7rα during the primary response. In contrast, the IL-7rα-ve sub-population of $CD8^+$ T cells present during the effector phase have an effector phenotype, with higher levels of inhibitory killer cell lectin−like receptor G1 (KLRG1), a marker of effector cells [177], and lower levels of CD27.

1.7.9. The Role of $CD4^+$ T cell Help in generating $CD8^+$ T cell memory responses

Whilst the $CD4^+$ T cell help during $CD8^+$ T cell priming may be dispensible, or at least replacable by alternative pathways to DC maturation, lack of $CD4^+$T cell help during $CD8^+$ T cell priming has repeatedly been shown to affect subsequent $CD8^+$ T cell recall responses: MHC class II -/- mice primed with recombinant lysteria monocytogenes show similar clearance of the bacteria when compared to wild-type: however, on rechallenge 60 days later, MHC class II mice are significantly less able to clear bacteria [178]. Of note, $CD4^+$ T cell depletion near to the time of restimulation does not reduce the memory response, implying that it is the presence of $CD4^+$ T cell help at the time of priming that programs the subsequent memory response. Although this implies a qualitative difference in $CD8^+$ memory T cells generated with or without $CD4^+$ T cell help during priming, there was also a quantitative difference between the two in these experiments, with higher resting memory cell numbers present in the wild-type animals ie it may be simply that $CD4^+$ T cell help during priming leads to higher numbers of resting memory

$CD8^+$ memory T cells, and that it is this numerical advantage, rather than any imprinted qualitative difference, that leads to the stronger memory responses. However, other groups have confirmed the presence of qualitative differences between 'helped' and 'helpless' $CD8^+$ memory T cells; 'Helpless' $CD8^+$ memory T cells (ie those generated in the absence of $CD4^+$ T cell help) fail to show the characteristic enhanced proliferation in response to repeat stimulation with antigen [179] [180]; $CD8^+$ memory T cells generated in the absence of CD4 help show defective IFNγ and IL-2 production, with associated epigenetic changes in the promoter regions of these genes [181].

$CD8^+$ T cell responses generated in the absence of $CD4^+$ T cell help have been variously reported to result in deletion [59], tolerance [182], or persistence [183] of $CD8^+$ T cells. These differences reflect the very different experimental approaches employed. The effects of $CD4^+$ T cell depletion at different points in a $CD8^+$ T cell response are shown in Figure 7.

In the absence of $CD4^+$ T cell help during priming, $CD8^+$ T cells are more prone to AICD: these cells express TNF-related apoptosis-inducing-ligand (TRAIL) on restimulation with antigen, a cell surface molecule, signalling through which can mediate AICD; because they also express the TRAIL receptor, they signal their own apoptosis [184].

Memory $CD8^+$ T cells can be adoptively transferred into lymphopaenic hosts, where they will undergo homeostatic proliferation; however, in the absence of $CD4^+$ T cell help these cells still fail to mount an adequate memory response; this can be corrected by the co-administration of $CD4^+$ T cells, or by using TRAIL knock-out $CD8^+$ T cells [185]. It is not known what the mechanism is by which these bystander $CD4^+$ T cells maintain memory $CD8^+$ T cell functionality, but it suggests a role for $CD4^+$ T cell in the general maintenance of the memory $CD8^+$ T cell population. The effect is not entirely TRAIL dependent, since $CD8^+$ memory T cells will gradually decline in a $CD4^+$ T cell deficient host even if they are TRAIL deficient [186], losing expression of IL-7rα, and showing reduced ability to produce TNF or IL-2 in response to peptide.

The effect of $CD4^+$ T cell help on generating $CD8^+$ T cell memory seems to be independent of CD40 interactions: memory responses to LCMV in vivo were reduced in MHC class II -/- mice but not in CD40 -/- mice [187], although it is not clear if this would have been true if $CD4^+$ T cell help had only been missing during the primary response.

If the role of $CD4^+$ T cell help is, indeed, critical during $CD8^+$ T cell priming, then various groups have postulated that antigen specific $CD4^+$ and $CD8^+$ T cells must both interact with the same DC; recent evidence has confirmed that this does happen *in vivo* [188]. Whether the $CD4^+$ and $CD8^+$ T cells must bind the APC simultaneously, or whether the $CD4^+$ T cell can leave its 'mark' is an area of active research. Any solution must explain how three rare cell types – the antigen specific $CD4^+$ and $CD8^+$ T cells, and the antigen bearing DC, can all come together in a timely manner. Recent evidence suggests that activated $CD4^+$ T cell/DC clusters can attract $CD8^+$ T cells, through the excretion of chemokines CCL3 and CCL4, which bind to CCR5 on activated $CD8^+$ T cells [189].

Thus in summary, $CD4^+$ T cell help is dispensable for primary $CD8^+$ T cell responses, but is likely to be required for the generation of $CD8^+$ T cell memory. The evidence for the critical timing of $CD4^+$ T cell help is conflicting, but points to an antigen specific requirement early during $CD8^+$ T cell priming, and a possible late, polyclonal effect, in maintaining the $CD8^+$ T cell memory pool.

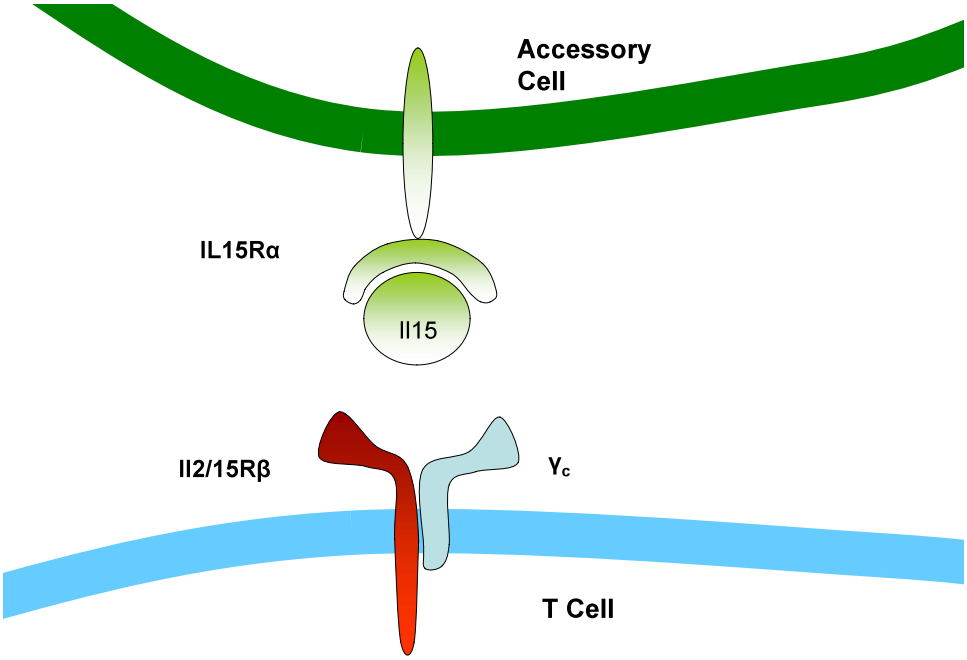

Figure 8 The IL-15 receptor acts in *trans*

The IL-15 receptor works in trans ie a heterodimer of IL-2/15β chain/γc chain on the T cell surface interacts with IL-15 bound to IL-15rα (green) on the surface of an accessory cell. This explains why the absence of IL15rα on the surface of CD8+ T cells does not affect their response to exogenous IL-15.

1.7.10. CD8 memory homeostasis

Both naïve and memory CD8s require IL-7 for survival. Compared to naïve cells, memory $CD8^+$ T cells have a reduced requirement for MHC class I interactions, and an ability to slowly divide in the absence of antigen. The cytokine IL-15 is important in $CD8^+$ memory cell homeostasis: the IL-15 receptor subunit IL-15rα is present on most resting $CD8^+$ T cells at low levels, and is upregulated early in the primary immune response. IL-15 -/- mice show a reduction in memory $CD8^+$ T cells, possibly due to a reduction in proliferation (in contrast to IL-7 which seems to promote memory $CD8^+$ T cell through enhanced survival) [190]. IL-15rα, despite binding IL-15 with high affinity, does not confer improved survival of $CD8^+$ T cells, yet IL-15-/- mice fail to generate $CD8^+$ T cell memory. The explanation for this paradox is that IL-15/IL-15rα complexes are presented on accessory cells (probably DCs [191]) to the γc/IL-2/15Rβ complex in trans (see Figure 8). From a practical point of view this means that IL-15Rα cannot be used to identify the memory population's IL-15 receptor status, and CD122 (the β chain) has to be used as a surrogate marker.

One effect of IL-15 is to upregulate 4-1BB expression by $CD8^+$T cells; since memory $CD8^+$ T cells show attenuated survival in 4-1BB ligand knockout mice, this suggests one possible mechanism by which IL-15 is involved in maintaining the memory population. 4-1BB stimulation has been shown to confer an antigen-independent proliferative signal to resting memory $CD8^+$ T cells. Recently it was shown that $CD8^+$ memory T cells deprived of DC interactions underwent a partial conversion back to a naïve phenotype, as defined by proliferation and requirement for costimulation [192]. This effect was mediated by CD27 and 4-1BB interactions. Thus both 4-1BB and CD27 may have a novel, antigen independent role, in the maintenance of $CD8^+$ T cell memory.

In summary, the cytotoxic $CD8^+$ T cell response is a complex process, involving the generation of naïve T cells through positive and negative selection in the thymus, followed by the stimulation of naïve $CD8^+$ T cells by activated DCs within the lymph nodes, which, when adequate costimulation

and cytokine stimulation are present, leads to a fully fledged $CD8^+$ T cell response; this consists of an initial primary phase predominantly consisting of cytolytic effector cells capable of inducing target cell lysis via the perforin/granzyme pathways, a contraction phase, and a memory phase. This elaborate system has evolved in order to combat the myriad of intracellular infective organisms that multicellular organisms are susceptible to; it is the purpose of this thesis to examine how costimulation can be manipulated to commandeer $CD8^+$ T cells to directly lyse malignant tumour cells and this requires a knowledge of tumour immunology.

1.8. Tumour Immunology

Paul Ehrlich first postulated in 1909 that the immune system may protect against malignancy [193] by detecting and eliminating malignant cells, a process described as 'immunosurveillance'. It was many years later, following several unsuccessful attempts to demonstrate immunosurveillance of tumours, that it was shown that suppression of IFN-γ, a key cytokine regulator of immunity, could lead to tumour development [194]. This was backed up by similar findings in mice lacking either the IFN-γ receptor, or the transcription factor mediating IFN-γ activity (STAT1) [195]. Further evidence is abundant, including the high spontaneous tumour development in RAG knockout mice (RAG = recombinase activating gene, required for all adaptive immune cell development). A role for cellular cytotoxicity in tumour surveillance was demonstrated by showing enhanced tumour growth in mice lacking perforin, a major functional component of cytotoxic T cells and NK cells [196]. The role of $CD8^+$ α/β T cells has also been confirmed in TCR β knockout mice, which are more susceptible to carcinogen induced tumours [197].

1.8.1. Immunosurveillance in humans

In humans the evidence for functional immunosurveillance is inconsistent; individuals with congenital immunodeficiencies are known to experience higher rates of virally-induced tumours such as Kaposi's sarcoma (caused by human herpes virus 6), but the evidence for spontaneous malignancies being controlled by immunosurveillance is weaker: cancer has a high incidence, causing around a third of deaths in humans, despite the presence of a functional immune system; in addition, there is apparently no increase in non-virally induced cancers in patients with congenital immunodeficiencies [198]. This does not mean that the immune system does not protect against the development of cancer – most cancers occur in the elderly, when there is some deterioration in cellular immune function [199], and patients with immunodeficiency may have a worse outcome when diagnosed with malignancy [200].

Some of the strongest evidence for immunosurveillance in humans comes from the detection of anti-tumour humoural and cytotoxic immune responses in patients with malignancy. Initially discovered by comparing immune responses to autologous tumour compared to autologous fibroblasts, the individual antigens recognised have been identified using techniques where the cDNA library of a tumour is screened against the patient's serum/T cells [201]. The antigens targeted by these immune responses vary in terms of their specificity; in an ideal world the antigens targeted would be present uniquely on malignant cells (to avoid autoimmune destruction of non-malignant cells), be vital for tumour survival (so that the tumour could not escape immune attack by downregulating the antigen), and be presented by MHC class I (to allow for recognition by $CD8^+$ T cells). In reality, cancer antigens rarely meet all these criteria; they can be separated into four broad categories:

1.8.2. Differentiation antigens

Differentiation antigens are expressed in the malignancy, and in the normal equivalent tissue, for example tyrosinase, which is expressed in normal melanocytes and in malignant melanoma. Generation of an immune response to a differentiation antigen may cause auto-immunity affecting the tissue of origin. For example, an immune response to tyrosinase may result in vitiligo.

1.8.3. Mutational antigens

Mutational antigens are those that come about from a mutation of a normal protein within the malignant cells. These can make ideal targets for immunotherapy as any immune response generated should be specific for the tumour; an example would be a response raised to the antibody idiotype of a myeloma or B cell lymphoma; any response generated should be entirely restricted to malignant cells. The problem with targeting mutational antigens is that they are, in general, unique to individual patients, although some broad targets do exist, such as BCR-ABL in chronic myeloid leukaemia [202].

1.8.4. Over-expressed antigens

Over-expresed antigens have a wide range of physiological expression, but this is at a much lower level than is present within the malignancy. Generating responses to over-expressed antigens always runs the risk of causing significant auto-immunity.

1.8.5. Viral antigens

Viral antigens are relevant immune targets in the subset of malignancies that are caused by viral infection, such as Epstein Barr virus (in endemic Burkitt's lymphoma) or human papilloma virus (in cervical cancer). The main drawback of these targets is that they are present in only a minority of human malignancies. Their potential in cervical cancer is enormous, and they are more likely to be immunogenic since they are derived from exogenous antigens.

1.8.6. Cancer-testis antigens

The cancer-testis antigens, as their name implies, are antigens expressed predominantly in the testis, and by certain cancers. Importantly the non-malignant cells expressing cancer-testis antigens express low levels ofMHC class I, and thus may be unaffected by any cytotoxic T cell response generated [203]. Initially described in a patient with melanoma, up to 20 such antigens have been described in humans. NY-ESO-1 is an example of a cancer-testis antigen: first described in a patient with oesophageal cancer, it has also been found to be expressed in a variety of other tumours: NY-ESO-1 antibodies are found in patients with NY-ESO-1 expressing tumours, with a high degree of specificity, and may confer some protective immunity, although clearly the naturally-occurring immune responses to this, and other cancer-testis antigens, are not capable of completely eradicating the tumour.

Another example of an immune response to a tumour antigen that is physiologically expressed in an immune-privileged site is to be found in

patients with paraneoplastic disorders. The paraneoplastic neurological disorders are a heterogenous group of neurological disorders that can complicate various malignancies; they are associated with autoantibodies and CTLs autoreactive for neuronal antigens, and may represent a cross-reactivity between tumour antigens and normal neurological antigens. The presence of antibodies to neuronal antigens has been associated with improved outcomes in some instances [204].

Other evidence for effective immunosurveillance in humans comes from studies examining the immune cells infiltrating tumours. Various studies have shown a correlation between outcome and the extent of tumour infiltrating lymphocytes (TIL). This has been shown in melanoma [205], ovarian cancer [206] and follicular lymphoma. The presence of $CD8^+$ T cells infiltrating colorectal cancer has an association with positive outcomes [207]. More recently, functional $CD8^+$ T cells that recognise the malignant plasma cells of multiple myeloma, have been identified [208],

In summary, there is a wide body of circumstantial evidence for the presence of anti-tumour immunity in patients with cancer, and it may be that this is protective against disease progression. It is less clear how much a functional immune system prevents the occurrence of malignancy.

1.8.7. Immunoediting

Despite all the evidence for a protective role of the immune system against cancer, immunocompetent patients can, and frequently do, develop cancer. It is logical to postulate that a functioning immune system provides an environment in which only those cancers which can successfully evade immune recognition can survive. Evidence for this theory comes from experiments showing that tumours grown in immunodeficient mice are more immunogenic than those grown in immunocompetent hosts [209]. Thus, the role of the immune system in malignancy represents a double edged sword; whilst its presence may provide some protection, it will also serve as a selective pressure, driving tumours to evade the immune system. For example, there is evidence that myeloma is controlled at an early stage by

$CD8^+$ T cells, but the malignant cells escape this immunosurveillance through downregulation of various proteasome subunits involved in antigen presentation [210]. This effect on cancer development by the immune system is known as immunoediting. A similar process 'in reverse' has been shown, with $CD8^+$ T cells specific for BCR:ABL, the mutant tyrosine kinase implicated in chronic myeloid leukaemia: these T cells can arise when the disease is controlled at a low level, being undetectable when high levels of disease are present [210].

The immune response to cancer has been artificially divided into three separate phases:

- *Elimination* – the successful orchestration of an immune response to a de-novo cancer, resulting in its complete destruction
- *Equilibrium* – a phase where the balance between tumour cells and the immune system results in a stable or slow growing tumour.
- *Escape* – where a tumour develops despite the presence of a competent immune system.

Greater understanding and manipulation of the processes that govern these separate stages will inform future strategies for tumour immunotherapy.

1.8.8. Cancer Vaccines

The aim of a successful cancer vaccine is to generate a sustained immune response to an antigen that is specific to the malignant cells. This task is a complex one, and malignant cells can adapt to avoid immune responses by a variety of mechanisms [211]: since immune responses are triggered by the innate immune system, and this, in turn, requires inflammatory or similar signals, tumours can suppress the generation of an effective immune response by inhibiting these initial triggers. Tumours can secrete immunosuppressive cytokines such as transforming growth factor β. This may be overcome by cancer vaccines designed to present the antigen in such a way as to generate an immune response.

When a successful tumour-specific immune response is generated, tumour cells may adapt by downregulating expression of the antigen targeted by the immune response. This has been described in patients with melanoma, with both gp-100 and MART-1 antigens undergoing specific downregulation [212]. Antigen downregulation may be overcome by targeting multiple epitopes simultaneously in a polyvalent vaccine; this clearly depends on having an appropriate number of targets. Although not strictly antigen-loss, certain antigens may not be present on all the tumour cells; an ideal tumour antigen would be present on all tumour cells rather than just those which have metastasised or differentiated.

Auto-reactive T cells should be deleted during thymic selection (see above). For those auto-reactive T cells meeting their cognate antigens in the periphery, the absence of appropriate costimulation should cause deletion/or anergy (see section on peripheral tolerance). Thus, vaccines targeting autologous antigens face an uphill struggle in overcoming the multiple mechanisms present to prevent autoimmunity. These mechanisms may be overcome for antigens that are weakly expressed, or expressed only in immunologically protected sites. Ideal vaccination strategies would target mutated antigens present only in the tumour itself, which would not have been subject to central tolerance mechanisms.

$CD8^+$ T cells require that antigen is processed, loaded onto MHC class I, and then presented on the cell surface. Therefore downregulation of MHC class I by tumour cells should lead to evasion of $CD8^+$ T cells; however NK cells recognise and kill cells which have downregulated MHC class I. Some murine tumours downregulate MHC class I, such as the B16 melanoma; some residual expression remains however, since these tumours can be eradicated by $CD8^+$ T cell response generated by dendritic cell vaccines [213]. MHC class I downregulation is frequently seen in human tumours, circumstantially giving credence to the role of $CD8^+$ T cells in controlling tumour growth. The majority of human studies rely on immunohistochemical staining, which are not sufficiently sensitive to differentiate between complete and partial MHC class I downregulation. If the murine studies are correct, then residual MHC

class I expression may remain at a low level; this is necessary if $CD8^+$ targeted immunotherapy is to have an effect. There are descriptions of complete loss of β2 microglobulin expression in melanomas [214] implying that tumour cells can completely avoid $CD8^+$ cytotoxicity by MHC class I downregulation whilst simultaneously negating NK cell cytotoxicity.

The evidence from murine studies suggests that although $CD8^+$ primary responses may occur in the absence of $CD4^+$ T cell help under appropriately inflammatory conditions (see section on $CD8^+$ memory), $CD8^+$ memory responses require $CD4^+$ help to be present during the priming phase. Whether T cell help is critical outside of controlled murine experiments remains to be shown, but ideally cancer vaccines would be designed to provide T cell help, given the evidence from murine studies.

Tumour reactive $CD8^+$ T cells are rare, and difficult to isolate from patients suffering with cancer. One way of overcoming this hurdle is to introduce tumour-reactive TCRs into endogenous $CD8^+$ T cells. This approach has been translated to clinical use, with lymphocytes transduced with TCRs recognising the MART-1 melanoma antigen being administered to patients, with two patients showing subsequent tumour regression [215]. A concern regarding this approach is that the transgenic α and β chain may pair with the endogenous α or β chain, yielding a potentially auto-reactive novel TCR [216]. Other approaches include transducingT cells with TCR endo-domain/scFv ectodomain chimaeras (which would have the advantage of not being HLA-restricted), with or without the addition of costimulatory signalling domains. Another interesting approach is the transduction of T cells with siRNA (inhibitory RNA) to FAS/CD95; this renders adoptively transferred T cells resistant to apoptosis mediated by FAS-ligand expressed on tumour cells. These approaches are attractive, but remain in their infancy, with many technical hurdles to overcome. In particular, getting adoptively transferred T cells to remain in the memory pool is proving difficult.

Despite all the barriers to raising anti-tumour immune responses, generating or enhancing anti-tumour immunity is an active area of research world wide, and it is the aim of this project to add to the understanding in this field. Much

of this project relies on the use of monoclonal antibodies, particularly those designed to stimulate costimulatory receptor present on various cells of the immune system.

1.8.9. Monoclonal antibody therapy

The development of a technique that allowed for the reproducible production of monoclonal antibodies (mAb) in 1975 opened the floodgates for a host of novel therapies [217]. By vaccinating mice with the target of interest, lymphocytes can be fused with a mouse myeloma cell line, and a long term cell line capable of producing the monoclonal antibody of interest can be generated. This technique potentially allows for any cell surface molecule to be specifically targeted. Although antibodies raised in mice or rats can be used therapeutically in humans, problems of anti-rat or anti-mouse antibody development can reduce subsequent efficacy of any therapeutic antibody, this hurdle can be overcome by 'humanizing' the antibody produced, so that only the murine CDR regions are retained; despite this, human anti-chimera antibodies can still develop (in up to 12% of patients) [218]. Various other modifications can be made to the basic antibody construct, including generating mAbs capable of recognising more than one target (Figure 9). Monoclonal antibodies can have a variety of effects; monoclonal antibody use as a cancer therapy can be divided into two broad categories; antibodies that directly target the tumour, and those that seek to attack the tumour via indirect routes.

1.8.10. Monoclonal antibodies directed to tumour surface antigens

Targeting antibodies to tumour cell surface antigens can bring about the death of the tumour cell through a variety of mechanisms (Figure 10); antibody binding can directly induce apoptosis, cause complement dependent cytotoxicity (CDC) or antibody dependent cellular cytoxicity (ADCC). A key element of this aspect of monoclonal antibody therapy lies in their ability to bind not only to target antigens via their variable regions, but also to recruit effector cells through interactions between the Fc portion of the antibody and Fc receptors on effector cells. The importance of this particular mode of action of mAbs is seen in the recent observation that the response to rituximab correlates with polymorphism in genes encoding Fc receptors [219] [220]. In addition, tumours known to be sensitive to therapeutic antibodies,

when grown in mice lacking the requisite Fcγ receptor, become resistant to Rituximab and Trastuzumab [221].

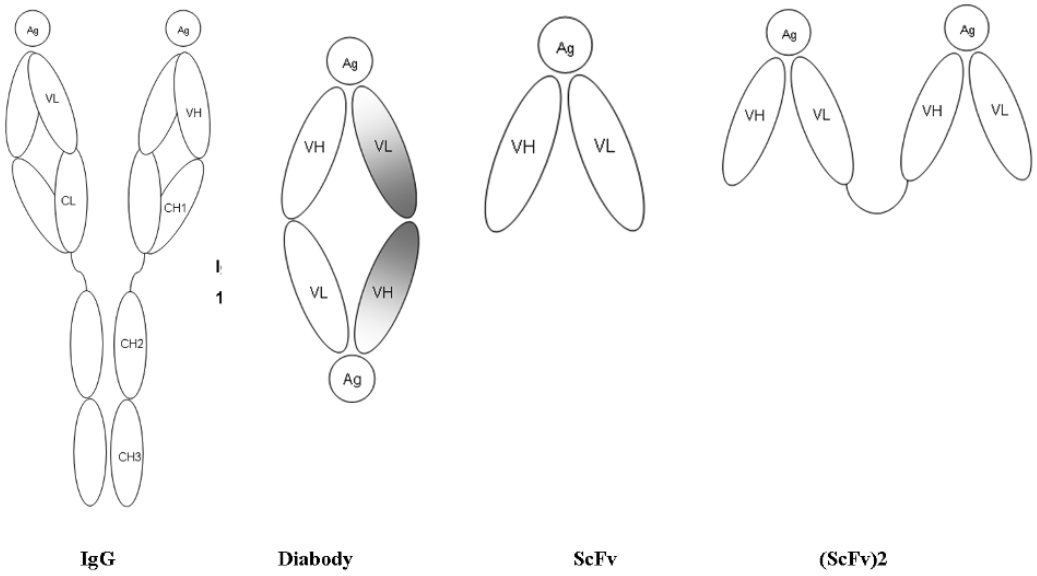

Figure 9 Therapeutic antibody constructs.

The standard IgG molecule is shown on the left. Diabodies are composed of two separate variable light and heavy regions, and can recognise separate targets. ScFv and (ScFv)2 are single chains comprising the variable regions of the light and heavy chain.

MAbs may also target apoptotic tumour cells to an immunogenic rather than tolerogenic pathway, and thus may lead to T cell priming against tumour antigens. The activity of some mAbs has been enhanced by conjugation to radioisotopes [222] or to cellular toxins [223]. A variety of mAbs are in clinical use: Rituximab is a chimaeric mAb which recognises the B cell marker CD20; it has proven clinical efficacy in diffuse large B cell lymphoma [224], and is increasingly used in other B cell malignancies [225], as well as in various autoimmune disorders [226]. Other examples include anti-CD33 antibody (Gemtuzomab Ozogomicin) which has shown efficacy in acute myeloid leukaemia [227] and anti-CD52 (Campath 1H) which has shown promise in a variety of lymphoid neoplasms [228].

1.8.11. Immunostimulatory antibodies for cancer therapy

Mabs can be used in the treatment of cancer without directly targeting the tumour itself. The example most pertinent to this project is the targeting of antibodies to the costimulatory molecules of the immune system (Figure 11); it is hoped that by combining costimulation with vaccination, active anti-tumour responses may be generated. These antibodies bind to their targets, cross-linking them, and inducing intracellular signalling. Different antibodies to the same target can have different effects; an extreme example is that of the recent clinical trial of a mAb to CD28, TGN1412, which, despite showing minimal toxicity in animal subjects, caused life threatening multi-organ failure in healthy human volunteers (see above).

Despite this occurrence, interest in costimulation manipulation to drive anti-tumour responses remains high; there is a clear need for more powerful manipulation of the immune system if anti-cancer vaccines are to work – cancer vaccines led to only 2 complete remissions out of 381 patients treated (summarised in [229]); given that these vaccines were undertaken in patients with tumours whose clinical condition can fluctuate (eg melanoma), these numbers are extremely disappointing. Several antibodies against costimulatory/TNFR superfamily members are in trials, including to CD40,

CTLA4, and CD80 [230] and 4-1BB. Predictably, use of these molecules is associated with some toxicity, including organ-specific auto-immunity; this has, in the case of CTLA-4 blockade in melanoma, been associated with tumour regression; since this side effect is reversible on antibody clearance, it may be a necessary evil. Importantly, CTLA-4 has been shown to synergise with co-administration of vaccines to tumour associated antigens [231], and trials are in progress combining vaccination with CTLA-4 blocking antibody. Recombinant CD40L and anti-CD40 antibodies are also in clinical trials, with some clinical responses reported [94].

1.8.12. $CD8^+$ T cell anti-cancer therapy

The simplest approach to generating $CD8^+$ T cells specific for cancer antigens is via vaccination. Various approaches have been tried, mostly in small phase I studies; peptides, whole proteins and DNA plasmids have all been tried with various degrees of success [232], but none are yet in routine use. The only consistently employed T cell therapy is in bone marrow transplantation, where lymphocyte infusions from the donor can be given to the recipient at the time of disease relapse, and bring about remission; this approach is not specific, and is frequently associated with the development of graft versus host disease [233]. New approaches to refine adoptive therapy of T cells, such as the use of chimaeric antigenic receptors [234] are under active investigation, but face major technical and regulatory hurdles. Other evidence for the potential efficacy of $CD8^+$ T cells in the treatment from cancer exists: Bacillus Calmette-Guerin (BCG) vaccine (commonly given to prevent tuberculosis) given directly into the bladder has a significant effect on the progression of bladder cancer [235] and the mechanism is in part mediated via cytotoxic T cells [236].

In summary, the cellular and humoural immune systems are an ongoing source of novel treatments for malignancy. It is the aim of this thesis to explore how CD8+ T cell costimulation can be employed to generate effective and long lasting immunity, to study the mechanisms by which CD8+ T cell memory is generated, and to examine whether enhanced CD8+ T cell responses can be successfully employed to target tumours therapeutically.

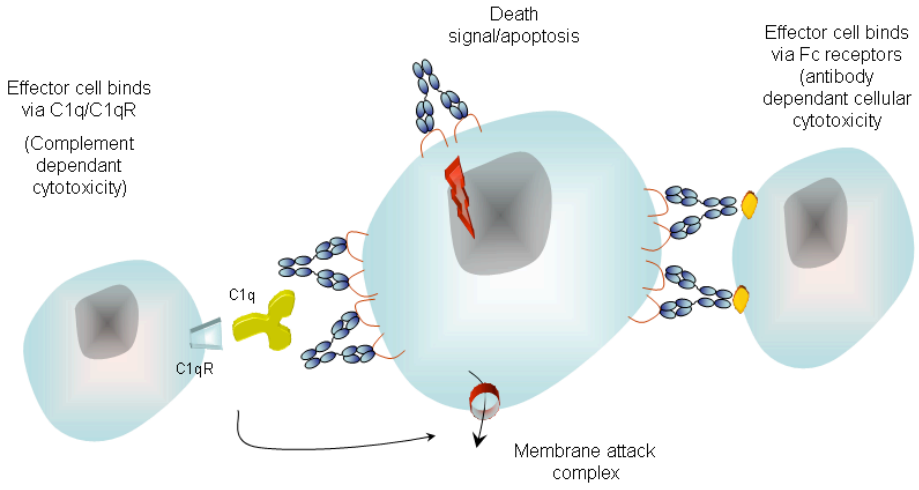

Figure 10 Mechanisms of antibody mediated tumour cell death (adapted from [225]).

mAbs binding a tumour cell (centre) may lead to death of the tumour cell by a variety of mechanisms: cross-linking of the cell surface molecule by mAb may lead directly to apoptosis; mAb binding my trigger complement activation and subsequent death via the compliment membrane attack comples; Fc binding to effector cells, directly, or via C1q, leads to cell dependent cytotoxicity. Another mechanism, not shown, is phagocytosis of the antibody targeted cell and subsequent presentation to the immune system.

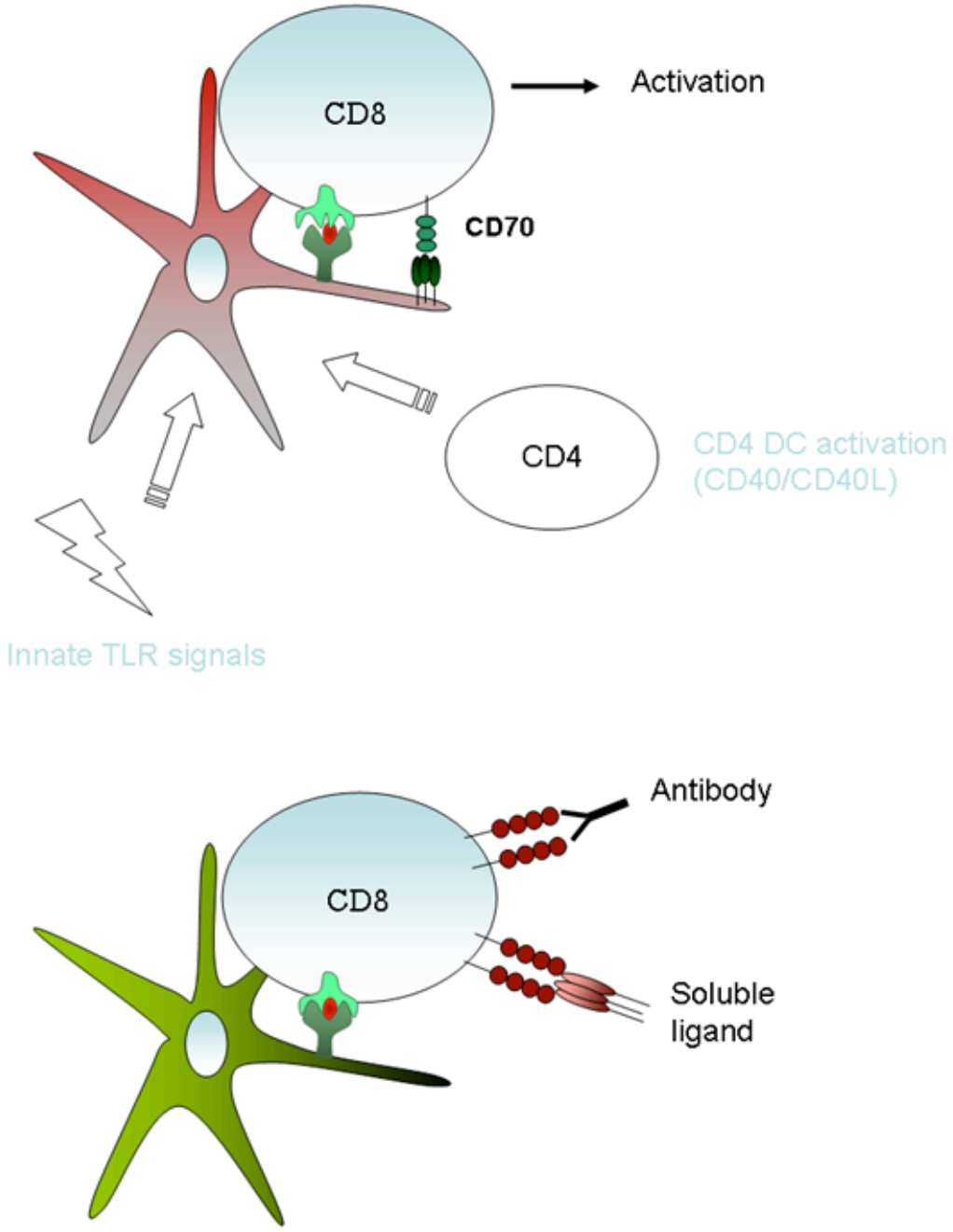

Figure 11 Use of antibodies for therapeutic manipulation of immune responses: The physiological activation of $CD8^+$ T cells by activated DCs is shown above. This is mediated through upregulation of costimulatory lignads, such as CD70, on the DC. $CD8^+$ T cells seeing antigen on inactivated DCs would normally lead to anergy/deletion, but this can be overcome by providing costimulation with soluble ligands or antibodies (below).

Aims

The aim of this thesis is to use soluble ligands for CD27 and 4-1BB to study their biology. By using a soluble form of CD70, the effects of CD27 signalling on gene expression will be studied. Individual genes identified by gene expression array will undergo confirmatory testing by quantitative polymerase chain reaction. Those genes identified will undergo further functional testing.

A secondary aim of the thesis is to compare CD27 with 4-1BB as possible therapeutic anti-tumour agents. Both molecules will be compared in terms of their ability to provoke primary and memory $CD8^+$ T cell responses, anti-tumour responses, and effects on gene expression. Underlying mechanisms will be sought for any differences found.

Chapter 2. Materials and Methods

2.1.1. Reagents

Ovalbumin peptide 257-264 (OVAp) with the sequence SIINFEKL was obtained from Peptide Protein Research Ltd (Fareham, UK). The lyophilised peptide (>95% purity) was dissolved in phosphate buffered saline (PBS; 120mM NaCl, 24mM Na_2HPO_4, 5.8 mM KH_2PO_4) and its concentration measured by BCA assay (Pierce, Rockford, USA). Aliquots were stored at 20^{o}C until use. PE-labelled tetramers were obtained from Proimmune (Oxford, UK).

2.1.2. Cells

2.1.3. Cell Lines

CHO K1 (Chinese Hamster Ovary-epithelial like) cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, and 100Ug/ml streptomycin, at 37^{0}C, in a humidified atmosphere containing 5% $C0_2$. Cells were subcultured when the density reached approximately 2.5 x 106 cells / 75cm2. Cells were re-suspended into medium by adding trypsin and incubating them for 5 min at 37^{0}C.

E.G7 cells ((EL-4 cells (C57BL/6, H-2b, thymoma) transfected with OVA) were grown in RPMI 1640 supplemented with 10% foetal calf serum, 50μM 2-Mercaptoethanol, 2mM L-glutamine, 1mM pyruvate, 100U/ml penicillin, 100μg/ml streptomycin, and 400μg/ml geneticin at 37^{0}C, in a humidified atmosphere containing 5% CO_2.

Human Embryonic Kidney 293-T were grown in DMEM supplemented with 10% foetal calf serum

2.1.4. Cell Culture

Transfected CHO K1 were grown initially in GMEM-S supplemented with 10% (v/v) dialysed FCS (First Link, UK), 100U/ml penicillin and 100µg/ml streptomycin and 25 µM methionine-sulfoximine (Sigma). Cells were re-cultured every 2-3 days as required and maintained at 37^{o}C in a 5% CO_2 humidified incubator. CHO K1 cells are adherent and were detached from culture flasks by incubation with Trypsin-EDTA (Invitrogen). CHO K1 cells were grown to confluence in triple layer flasks and the media harvested every 5-7 days and stored at -20^{o}C prior to purification.

2.1.5. Cell storage

Cell lines were stored in freeze media (90% FCS and 10% dimethylsulphoxide) at -80° C or -196° C (liquid nitrogen). After storage, cells were washed and re-suspended in media as required.

2.1.6. Cell quantitantion

Cell concentrations were determined using a Coulter Industrial D Cell counter (Coulter Electronics, Bedfordshire, UK), or by manual counting using a haemocytometer (Improved Neubauer). Cell viability was measured on the haemocytometer by dilution 1:1 with trypan blue dye (Sigma), and observation of dye exclusion from viable cells.

2.1.7. Antibodies

Anti-human Fc (hFc), anti-CD27 (AT124.1), anti-4-1BB (LOB 12.3), anti-CD40 (3/23) anti-CD3 (145.2C11) was produced and purified in-house from hybridoma lines. Anti-mouse IL-2 and allophycocyanin (APC) -labelled anti-CD8α and APC labelled CD4 were obtained from BD Pharmingen (San Diego, CA, USA). Normal human IgG was prepared in-house.

2.1.8. Protein Analysis

2.1.9. LPS Testing

Reagents were tested for endotoxin contamination using Endosafe-PTS (Charles River Laboratories). Reagents were considered endotoxin low if they contained less than 5 EU/ml of endotoxin.

2.1.10. Enzyme-Linked Immunosorbant Assay (ELISA) for the detection of soluble human Fc Fusion proteins

To detect the presence of sCD70 in cell supernatant an ELISA was performed according to the standard protocol. Primary rabbit anti-hFc mAb was diluted in coating buffer (15mM Na_2CO_3, 28.5 mM Na HCO_3, pH 9.6) to 1µg/ml and 100µl/well added to treated 96 well plates (Maxisorb, Nunc) and incubated overnight at 4^{o}C. Unbound antibody was removed by flicking and the non-specific binding sites were blocked by blocking solution (1% (w/v) BSA in PBS) for 1 hour at 37^{o}C. The plate was then washed twice with wash solution (PBS + 0.05% Tween-20) and the cell supernatant of interest or a standard curve using a similar Fc fusion protein of known concentration was added, in a final volume of 100µl/well, with all dilutions being made in media. Following incubation for 90 minutes at 37^{o}C, the plate was washed again (x5 in wash solution). Horseradish peroxidise (HRP)-conjugated rabbit anti-hIgG antisera (Sigma) was diluted 1 in 40,000 in blocking buffer and added (100 µl/well) for a final 90 minutes. Following washing (x 5 in wash solution), 100 µl of HRP substrate (20 mg o-PhenylDiamine free base (Sigma) in 100ml phosphor-buffered citrate pH 5.0 + 50 µl of 30% (w/v) H_2O_2) was added and incubated in the dark at room temperature for 15 minutes. The reaction was terminated by the addition of 50 µl/well 2.5 M H_2SO_4 and the subsequent colour change quantified by measurement of absorbance at 495 nm on an automatic fluorometer (Dynatec 400, Dynatec). Colour change was proportional to protein concentration and unknowns were determined using standard calibration curves of known concentration.

2.1.11. Purification of recombinant proteins by immunoaffinity chromatography

CHO-K1 cells were already available which had been transfected with constructs encoding the extracellular domain of CD70 or 4-1BBL conjugated to the Fc region of the human IgG1 within the pEEE14 GS expression vector; This plasmid is a mammalian expression vector, with transcription of the inserted gene initiating from the powerful human cytomegalovirus (hCMV-MIE) promoter-enhancer sequence. When transfected, the recombinant protein can be expressed in mammalian cell lines using the glutamine synthetase minigene present in pEE14 for selection and amplificiation. Recombinant sCD70 was isolated from tissue culture supernatant using an immunoaffinity column containing and anti-hFc mAb (SB2H2) conjugated to Sepharose 4B beads (Amersham Pharmacia).

2.1.12. Sepharose Bead Conjugation to anti-human Fc antibody (SB2H2)

Rat anti-human Fc antibody was concentrated to 2mg/ml. 20mg of antibody were injected into a 12ml dialysis cassette and then dialysed with 5 litre 0.2M citrate buffer (0.2M citric acid, 0.58M NaOH, pH6.5), over a period of 48h at 4^0C. The antibody was then diluted to a final concentration of 2mg/ml. 200mlof 10mM HCl was added to 1.2g CNBr activated sepharose 4B beads (Amersham Biosciences) and left at room temperature for 20min. The beads were poured over a sintered glass filter and washed with 500ml 10mM HCl followed by 500ml 0.2M citrate buffer. The beads were placed into a universal tube and 26mg anti-human Fc antibody (SB2H2) was added. The coupling reaction was incubated, rotating for 4 h at room temperature. Antibody binding was confirmed by spectrophotometry at 280nm of the residual citrate buffer. The beads were then poured onto a sintered filter and were then washed with 20ml 1M Ethanoloamine.HCl pH9.5. The beads were rotated for 1 h at room temperature. The beads were again poured onto the sintered

filter and were washed with 1 litre 0.1M Tris, 0.5M NaCl, 5mM Na2EDTA 5mM HCl followed by ammonium thiocyanate (1M KSCN, 0.7M Ammonium), followed by a second wash with 0.1M Tris. Conjugated beads were then packed over glass wool in an appropriately sized syringe, ready for elution.

Cell culture supernatant was loaded onto the column using gravity flow at 0.5 ml/min at 4^{o}C. The column was then washed to remove non-specific protein with 5 column volumes of low-Tris buffer (0.028M Tris-HCl,0.142 M NaCl, 0.0014 M EDTA, pH 7.2) followed by 5 column volumes of high-Tris buffer (0.1 M Tris-HCl, 0.5 M NaCl, 0.005 M EDTA, pH 7.2) and another 5 volumes of low-Tris buffer. Bound protein was eluted with ~1 column volume of 0.1 M glycine-HCl pH 2.5, with protein being collected in 1 ml fractions and the pH being immediately adjusted to pH 7 by addition of 90µl of 2 M Tris-HCl pH 8.5. sCD70 was dialysed into PBS and stored in small aliquots at -20^{o}C until use.

2.1.13. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was performed using a mini-gel system (BioRad). 5µg of purified protein per lane was analysed on a poly Acrylamide resolving gel. In all cases both the resolving and stacking gels were constructed from a 30% (w/v) Acrylamide: 0.8% (w/v) bisacrylamide stock solution (National Diagnostics; Atlanta, Georgia). Resolving gels were made using a 1.5 M tris base pH 8.8 stock solution which contained 0.4% (w/v) sodium dodecyl sulphate (SDS) and were typically 8.5% Acrylamide. Stacking gels were created using a 0.5 M tris HCl pH 6.8 stock solution which contained 0.4 % (w/v) SDS yielding a final Acrylamide concentration of 4 %. The reaction was catalyzed with N N N' N'-tetramethylethylenediamine at a final concentration of 0.0005% and 0.001% for the resolving and stacking gels respectively. The reaction was initiated with a fresh 10 % (w/v) in dH_2O) solution of ammonium persulfate, added at a final concentration of 0.5% in resolving gels and 1.0% in stacking gels. Non-reduced samples were diluted 1:1 in 2 x protein solubilisation buffer (40% urea (w/v), 1.6% SDS (v/v), 0.150 M tris-HCl, 0.08% (w/v) bromophenol blue, pH 8). Reduced samples were diluted in 1:1 in 2 x solublisation buffer +

50 mM DTT. Samples were denatured by heating at 95^{o}C for 5 minutes and loaded immediately. Gels were run at 150 volts in running buffer (0.0125 M tris-HCl, 0.096 M glycine, 0.1% (w/v) SDS) until markers (pre-stained protein markers; P77085, New England BioLabs) had reached their desired position. Gels were fixed by submerging in 25 % isopropanol, 10 % acetic acid for 15 minutes and protein was detected using coomassie brilliant blue stain (0.006% (w/v) coomassie brilliant blue in 10% (v/v) acetic acid). Gels were destained to the extent desired with 10% (v/v) acetic acd.

2.1.14. Cellular assays

2.1.15. Preparation and culture of murine splenocytes

Spleens and lymph nodes were removed aseptically and a single cell suspension prepared by passing through a cell strainer. Red blood cells were lysed by incubation of the suspension for 5 minutes at room temperature in lysis solution (0.83% (w/v) ammonium chloride, 0.1% (w/v) $KHCO_3$ in dH_2O), after which the splenocytes were washed twice in PBS, counted and resuspended in RPMI 1640 medium supplemented with 100 U/ml penicillin and 100µg/ml streptomycin, 2 mM L-glutamine, 1mM pyruvate, 10% (v/v) FCS and 50 µM 2-mercaptoethanol. All assays were set up in this media, with splenocytes typically being at 1 x 10^6/ml.

2.1.16. Purification of $CD4^+$ and $CD8^+$ T lymphocytes

$CD4^+$ and $CD8^+$ T cells were purified from C57 black 6 spleens and lymph nodes by negative selection columns (Cedarlane Laboratories Limited, Ontario, Canada) according to the manufacturer's instructions. The resulting T cell populations were 70% pure as assessed by flow cytometry.

2.1.17. [^{3}H] Thymidine incorporation assays

The level of proliferation in culture cells was measured by the incorporation of radiolabelled Thymidine. Purified T lymphocytes were cultured with various reagents at a concentration of 1 x 10^6/ml in 96 well U-bottomed plates (Nunc). Methyl-^{3}H-Thymidine (1µCi/well; Amersham, Buckingham, UK) was added to each well for the last 16 h of a 72 h culture, after which cells were harvested onto glass fibres (Unifilter GF/B, Perkin Elmer) with an automated harvester (Filtermate harvester, Packard). [^{3}H]-thymidine incorporation was subsequently determined by liquid scintillation counting.

2.1.18. Measurement of surface antigens by flow cytometry

2.1.19. Standard protocol for staining cell surface antigens

~1 x 10^6 cells were incubated at 4^{o}C 30 minutes with the fluorescent antibody of choice suspended in PBS-BSA (PBS 0.2% (w/v) Bovine Serum Albumin fraction V (BSA; Wilfred Smith Ltd, Middlesex, UK) . Cells were washed x 1 in red cell lysis buffer then x 1 in PBS-BSA before being resuspended in PBS. Analysis of antibody-labelled samples was conducted on a FACSCalibur (Beckton Dickinson, Mountain View, CA, USA) flow cytometer. Forward scatter (FSC) vs side scatter (SSC) analysis was used to gate viable cells or lymphocyte populations. 100,000 cells were typically collected per sample.

2.1.20. Tracking antigen-specific T cells using MHC-peptide tetramers

Antigen-specific CD8$^+$ T cells can be visualised by staining with soluble tetrameric forms of the MHC-peptide complex for which they are specific [237]. Endogenous and TCR-transgenic CD8$^+$ T cells can be visualised. Blood samples or spleen/lymph node cells were stained in PBS-BSA with 2µl phycoerythrin-conjugated H-2K^b OVAp tetramers, capable of detecting OVA-specific CD8$^+$ lymphocytes, before being washed with red cell lysis buffer and PBS-BSA as above.

2.1.21. Detection of IL-2 in cell supernatant by ELISA

In vitro culture assays were set up as indicated in the figure legends and T cell supernatant harvested after 48 h. The levels of IL-2 were detected by ELISA essentially by the same method as for the detection of soluble human Fc Fusion proteins (see above), but with two-stage secondary detection using a biotinylated antibody followed by incubation with HRP-conjugated avadin. IL-2 reagents: capture mAb rat anti-mouse IL-2 (PharMingen) at 1 µg/ml, detection mAb biotinylated rat anti-mouse IL-2 (PharMingen) at 1 µg/ml, avadin-HRP (PharMingen) diluted 1 in 1000. Assay supernatants were diluted between 1` in 5 and 1 in 20, and a standard curve was constructed using known concentrations of mouse IL-2. Actual cytokine concentrations in assay supernatant were calculated using GraphPad Prism software (GraphPad Prism version 4.0 for windows, GraphPad software, Sand Diego, California).

2.1.22. Western blotting for Batf3 expression

Purified GST-tagged Batf3 was kindly provided by Dr P Duriez (University of Southampton) and used to raise rabbit polyclonal antibodies at Cancer Research UK (Dr N Oodit). Terminal bleed serum was used at a dilution of 1:1000 to detect Batf3 in western blots. As a positive control, Haemaggluttinin (HA) -tagged Batf3 (a kind gift from Dr V Taraban, University of Southampton) was used to transfect 293T cells using the lipid based transfection reagent Effectene (Qiagen). Cell lysates were prepared by resuspending cells in 40 µl of RIPA buffer (25mM Tris.HCl pH 7.6 150mM NaCl 1% NP-40, 1% Na Deoxycholate, 0.1% SDS) and sonicating for 30 seconds. Lysates were centrifuged at 14000 g for 5 minutes before being diluted 1:1 in protein solubilisation buffer and 30mM dithiothreitol (DTT) and incubated at 95^{o}C for 5 minutes.

Lysates were run on a 14% SDS-PAGE gel (as described above). For western analysis, proteins were transferred from the gel (soaked in transfer buffer) onto PVDF membrane (Immobilon-P, Millipore), which was prepared by immersion in 10% ethanol for 10 seconds, followed by a 10 minute wash in dH_2O and a further 10 minutes in transfer buffer. Proteins were transferred in

transfer buffer (10% (v/v) ethanol, 12.5 mM tris, 96 mM glycine) for 1 hour at 100 volts using a transfer system (Biorad) cooled on ice. Transfer was deemed to have been successful in the presence of visible pre-stained protein markers on the PVDF. The PVDF blot was then blocked overnight at 4^{o}C in blocking buffer (5% dried low-fat milk powder, in western buffer (WB) (10mM Tris, 150mM NaCl, 0.1% Tween20, pH to 7.6-8), to prevent subsequent non-specific binding. Following blocking, the blot was washed three times in WB. Primary antibody was diluted 1:1000 in WB/5% milk and the membrane was incubated with the antibody mix on an agitator for 1 hour at room temperature. The membrane was washed again before being incubated with ECL peroxidase goat anti-rabbit (Amersham) at a dilution of 1:20000 for 1 hour at room temperature. Detection of bound antibodies was performed by chemiluminescence using ECL reagents (Amersham Pharmacia) according to the manufacturer's protocol, with light signals being detected on Hyperfilm ECL (Amersham Pharmacia).

2.1.23. Animals and in vivo experiments

2.1.24. Mice

OT-I TCR transgenic [238] mice obtained from Dr Matthias Merkenschlager (Imperial College, London) and were bred at the in-house animal facility (Tenovus). C57 Black 6 mice were available in-house. All mice were used at approximately 8-12 weeks of age. Animal experiments were carried out according to the UK Home Offic license guidelines, and were approved by the University of Southampton's ethical committee.

BIM knockout mice were screened by PCR; briefly ear tips were incubated for 16 hours in proteinase K and DNA isolation buffer for 15 hours at 55^{o}C. Samples were centrifuged at 13214 rcf (relative centrifugal force/g) for 5minutes.and diluted 1:10 in nuclease free water. Primers were

5' forward primer (PB20) 5´ cat tct cgt aag tcc gag tct 3´

3' reverse primer (PB335(a)) 5´ gtg cta act gaa acc aga tta g 3´

3' reverse primer (PB65) 5´ ctc agt cca ttc atc aac ag 3´The 25µl reaction mix of PCR contained:

1µl DNA

1 x GTaq buffer (promega)

1 x GoTaq polymerase (promega)

0.25µM reverse oligonucleotide primers

0.5µM forward oligonucleotide primer

0.2 mM dNTPs (promega)

Nuclease free water to 25µl total reaction volume

Reaction mixes took place in a DNA free hood. Amplification was performed in a thermocycler (PTC-100, MJ Research Inc) according to the following program:

95^{o}C 10min

94^{o}C 30s

58^{o}C 30s

72^{o}C 45s

72^{o}C 10m

Repeat step 2-4 for 34 cycles

4^{o}C thereafter

Amplified DNA was analyzed on 1.5% agarose gels made in TBE buffer (89mM tris, 89 mM boric acid, 2mM EDTA, 0.5 mg/ml ethidium bromide), and compared to standard size markers. Gels were run in TBE buffer at 70mA in a BioRad electrophoresis system for the appropriate time and visualised on a UV illuminator. An example of screening is shown below (Figure 12).

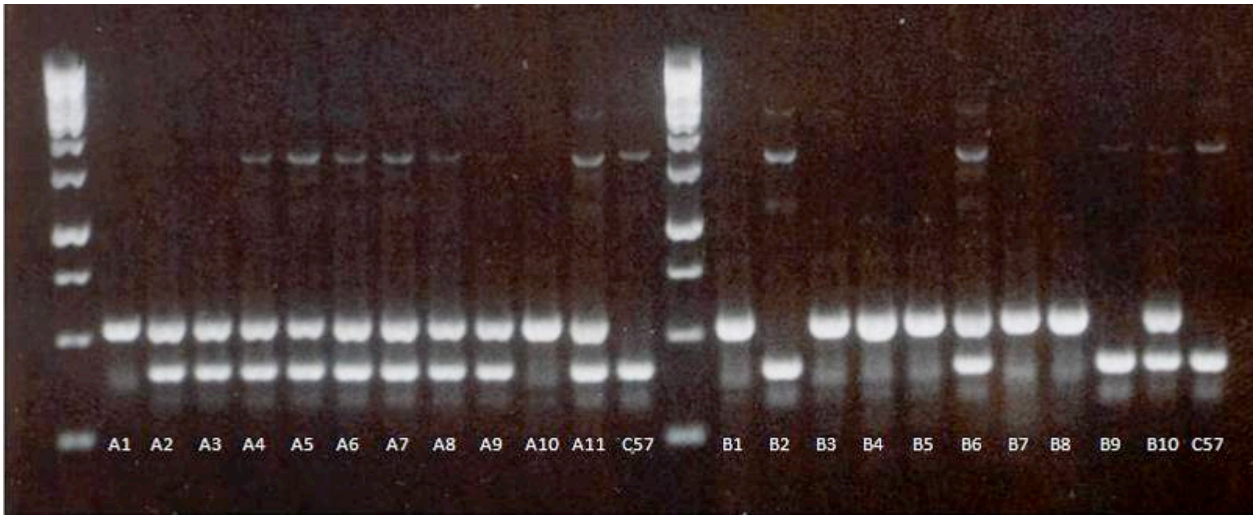

Figure 12

A1, A10 homozygous/A2-9, A11 heterozygous/B1,B3,B4,B5,B7, B8 homozygous/B6, B10 heterozygous/B2, B9 wild type (C57Bl6)

2.1.25. In vivo T cell experiments

Single cell suspensions of spleen and lymph node (inguinal, brachial and mesenteric) were prepared from OT-1 C57BL/6 mice. The proportion of transgenic T cells was determined by tetramer/CD8 FACS analysis. The appropriate number of transgenic T cells was injected intravenously (iv) into sex-matched C57BL/6 recipients. Recovery was confirmed 24 hours later by tail blood analysis. T cells were primed *in vivo* by iv administration of OVAp in PBS in combination with the indicated adjuvants.

2.1.26. In vivo tumour growth assays

2.1.27. Immunisation against EG7

Both endogenous and transgenic TCR models were employed; in the transgenic model OT-I T cells were adoptively transferred into C57BL/6 mice as described above. 24 hours later mice were vaccinated with 5mg ovalbumin whole protein ip and received anti-4-1BB (LOB 12.3) or anti-CD27 (AT124.1) or control antibody (MC106A5) (250µg ip d0,1). Reponses were monitored in peripheral blood to confirm $CD8^+$ T cell responses were representative. 30 days after primary immunisation 10^6 EG7 tumour cells in the exponential growth phase were administered in PBS sc. Tumours were monitored daily and measured on two perpendicular axes using a vernier calliper. Animals were sacrificed when the tumour size reached $150mm^2$.

2.1.28. DNGR-1 targeted tumour vaccines

DK, NK lectin group receptor-1 (DNGR1) targeted antibody (anti-DNGR1) conjugated to biotinolyated SIINFEKL (the H-$2K^b$ restricted immunodominant peptide of ovalbumin), as well as a SIINFEKL conjugated, isotype matched, control antibody, was a kind gift from Dr C E Sousa.

The B16 melanoma cell line, stably transfected with ovalbumin were taken at the exponential growth phase; cells were washed twice in PBS before being filtered through a 100µm nylon filter. 2×10^5 live cells were injected intravenously into the tail vein of recipient mice. 2 days later mice were injected with anti-DNGR-1-SIINFEKL (20 microgrammes subcutaneously) or isotype matched control, along with 10µg poly I:C and the appropriate costimulatory antibodies. 15 days after vaccination, mice were sacrificed, and lung tumours enumerated.

2.1.29. Expression Microarrays

2.1.29.1. RNA Preparation

RNA was prepared from *in vitro* or *in vivo* stimulated cells using RNeasy Mini (Qiagen) according to the manufacturer's instructions. Following sample lysis in denaturing guanidine isothiocyante containing buffer, samples were homogenised by centrifugation in a QIAshredder (Qiagen). Steps were taken to minimise RNAse contamination such as the use of RNase-free pipette tips and plastic-ware, clean gloves and sterilisation of surfaces with Virkon. Around 10-20µg of RNA was prepared from approximately 4 x 10^6 cells and stored at -80^{o}C before concentration/transportation. A minimum of 5µg of purified RNA was required for later hybridisation to microarrays, at a concentration of no less than 600ng/µl. Where purified RNA was at a lower concentration it was purified as below.

2.1.30. Ethanol precipitation of RNA

Samples of a known volume of solution containing RNA were diluted in 0.5 volumes 7.5M Ammonium acetate (Sigma) and 2.5 volumes of ice cold 100% Ethanol (Analar) with 10µg of linear Acrylamide (Ambion) to aid visualisation. After mixing, samples were stored at -20^{o}C overnight to precipitate RNA; they were then centrifuged at 13,000rpm for 30 minutes and washed twice with ice cold 80% ethanol. The resulting pellet was air dried and resuspended in an appropriate volume of RNase-free water (Ambion).

2.1.31. Quantification

RNA concentration was determined by measuring absorbance at 260nm (A_{260}) in a spectrophotometer; an absorbance of 1 unit corresponds to 40µg of RNA per ml. RNA purity was estimated from the ratio of absorbance at A_{260} to A_{280} – values of above 1.8 were considered acceptable.

2.1.32. Interrogation of RNA samples using DNA Microarrays

Samples were removed from storage at -80^{o}C and transported overnight on dry ice to the Cancer Research UK bioinformatics unit. There samples were quantified and converted to cDNA using standard techniques (all protocols used at CRUK are available at http://bioinformatics.picr.man.ac.uk/mbcf/downloads/) before conversion to biotinylated cRNA. This cRNA is then fragmented before undergoing hybridization to an Affymetrix GeneChip Mouse Genome 430 2.0 Array. These microarrays comprise over 45,000 probe sets representing over 34,000 mouse genes. Experimental design was stored according to the Minimal Information About a Microarray Experiment (MIAME) protocol, and is available at http://bioinformatics.picr.man.ac.uk/vice/Mainmenu.vice.

Raw expression data was downloaded from the MIAME-VICE database; it was normalized and analysed with Genespring (Agilent) software to generate lists of significantly altered genes. Various fold changes and significance levels were employed and are indicated in the relevant results chapter. Data analysed using the bioinformatics software R was also used to generate lists of significantly altered genes (courtesy of G Kelly, Cancer Research UK bioinformatics). Both approaches yielded broadly similar lists of genes.

Significantly altered lists of genes were further analysed with the Panther microarray database (http://www.pantherdb.org/tools/uploadFiles.jsp). Significantly altered genes from the various timepoints were uploaded to the database which grouped them into significantly over-represented ontologies or pathways.

2.1.33. Quantitative Polymerase Chain Reaction

2.1.34. Conversion of RNA to cDNA

RNA was prepared as above and either converted immediately or stored at -80oC until use. Conversion to cDNA was performed using First-Strand cDNA synthesis kit (GE Healthcare, Buckinghamshire, UK). 1µg or RNA was used for each 33µl reaction using random hexamers as primers.

2.1.35. Quantitative Polymerase Chain Reaction (QPCR)

QPCR reactions were performed in clear QPCR-specific thin walled tubes (Axygen Scientific, California). Only probes spanning exon Junctions were employed to ensure that no genomic DNA was amplified. Taqman Gene Expression Assays (Applied Biosystems) were used, each containing two unlabeled primers specific to the gene of interest and a 6-FAM dye labelled reporter probe coupled to a quencher. During the PCR reaction the 5'-3' nuclease activity of the DNA polymerase cleaves probe releasing the reporter from the quencher, which is detectable as a fluorescent signal. To prevent contamination Platinum QPCR SuperMix-UDG (Invitrogen) was obtained, which uses the nucleotide dUTP (instead of the naturally occurring dTTP) and Uracil DNA glycosylase (UDG) to remove uracil residue from DNA; this prevents previously amplified material from contaminating QPCR reactions. This mastermix employs a 'hotstart' process by which the Taq DNA polymerase is only activated (by release from a proprietary antibody) when the reaction temperature is reached [239]; this prevents non-specific binding of probes to targets as occurs at low temperatures. Reactions were performed on a PTC 200 Thermal Cycler (MJ Research) according to the manufacturers instructions. CT values were defined as the cycle number where fluorescence intensity increase becomes exponential, and values were determined manually using Opticon Monitor 3.1 (GRI Ltd Gene House, Essex). Relative quantities were calculated using $2^{-\Delta\Delta CT}$ method [240] using Microsoft Excel according to the formula below:

$$\text{Relative Quantity (RQ)} = 2^{-(\Delta\Delta CT)}$$

$$\text{Where } \Delta CT = CT^{(\text{Target Gene})} - CT^{(\text{Reference Gene})}$$

$$\text{And } \Delta\Delta CT = \Delta CT^{(\text{sCD70})} - \Delta CT^{(\text{control})}$$

This method is used where absolute quantitation of gene expression copy number is not required. As this method assumes that target and reference genes have similar amplification it is necessary to validate targets across a range of dilutions; if amplification efficiencies are equal then similar RQs will result from different dilutions. For all experiments hypoxanthine-guanine

phosphoribosyltransferase was used as a control gene which has been recommended for high-sensitivity qPCR [241].

Assay ID	Gene Name	Target	NCBI Gene	Cytogenetic
Mm00434256_m1	interleukin 2	2	NM_008366.2	3 B-C;3
Mm00495182_m1	killer cell lectin-	2	NM_010654.1	6 F3;6
Mm00439631_m1	interleukin 3	2	NM_010556.3	11 B1;11
Mm00476032_m1	activating	2	NM_007498.2	1 H6;1
Mm01318274_m1	RIKEN cDNA	2	NM_030060.1	1 H6
Mm01333921_m1	BCL2-like 11	1	NM_207680.2,	2 F3-G1
Mm01185002_m1	pleiomorphic	2	NM_009538.1	10 A2;10
Mm01302155_m1	immunoglobulin	7	NM_207205.1	3 F2.2;3
Mm00442225_m1	growth arrest and	1	NM_011817.1	13 A5-B
Mm00446968_m1	hypoxanthine	6	NM_013556.2	X A6;X 17.0

Table 1-2

Taqman Gene Expression Assays (Applied Biosystems) used in QPCR reactions

Chapter 3. CD27 signalling effects on gene expression

The aim of this project was to study the downstream effects of CD27 signalling in $CD8^+$ lymphocytes. CD27 is a powerful stimulator of $CD8^+$ and $CD4^+$ T cell responses; it causes proliferative and anti-apoptotic effects, as well as leading to gain of effector function and generation of $CD8^+$ T cell memory; work from our laboratory has already demonstrated a key role for CD27/CD70 interactions acting downstream from CD40 licencing of DCs [88].

To examine the effects of CD27 signalling, a soluble version of its ligand, CD70, was generated. Although monoclonal antibodies can also be used to study the effect of receptor ligation, the effect they bring about may differ significantly from the true physiological effect: they have differing IgG subclasses, epitope binding sites, and affinities, all of which may differ from the physiological setting. Monoclonal antibodies rely on cross-linking of their Fc component by Fc receptors on effector cells to bring about receptor multimerisation. Soluble ligands on the other hand bind physiologically to their receptors; they are less dependent on Fc cross linking via effector cells, relying instead on the formation of higher order multimers to bring about receptor multimerisation; for example, it has been shown that dodecameric CD154 is more effective than trimeric CD154 in triggering CD40 activation [242].

Soluble ligands have previously been successfully utilised in the study of costimulation: a soluble recombinant fusion protein composed of the extracellular domain of CD70 and the Fc fragment of human Immunoglobulin G_1 (Figure 13) (referred to as sCD70) has previously been shown to stimulate primary $CD8^+$ T cell responses [243]. Employing recombinant sCD70 allows for the study of CD27 signalling in a relatively physiological setting, mimicking its brief expression on activated DCs; this is important, as protracted CD27 signalling leads to eventual lethal T cell immunodeficiency [108]. Fc fusion

proteins, such as sCD70, appear to fold correctly and can be expressed at reasonably high levels in mammalian cells. sCD70 was employed to investigate the downstream effects of CD27 signalling.

3.1. RESULTS

3.1.1. Multimeric structure of soluble CD70-Fc fusion protein

sCD70 has a predicted molecular mass (M_r) of 44.5 kDa. Purified sCD70 was examined by SDS-PAGE (Figure 14). Under reducing conditions sCD70 formed a single band with a M_r of ~50kDa. Under non-reducing conditions, sCD70 formed multiple bands of ~ 100kDa, 150kDa and > 175kDa, indicating that sCD70 forms multimers (dimers/trimers/higher order multimers).

These findings were in broad agreement with previous findings from this laboratory; sCD70 has also previously been shown to selectively block anti-CD27 antibody binding to T cells in a specific fashion (Rowley, unpublished observations). sCD70 structure is similar to the predicted structure of membrane bound TNF-family members such as TNFα, TNFβ and CD40L, all of which have been shown to form self-assembling non-covalent trimers [80]. The reducing agent DTT reduces the disulphide bonds of proteins, and it is likely that these are formed between the hinge regions of the IgG1 Fc portions of sCD70 (Figure 15) .

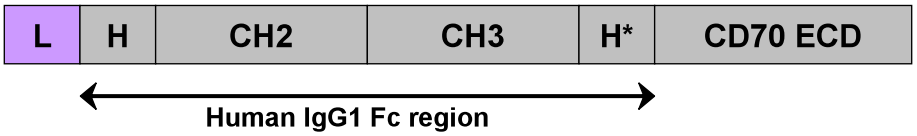

Figure 13 Schematic diagram representing the recombinant DNA structure of Fc Tagged CD70 fusion construct: (**L**) B72.3 leader sequence. (**H**) Hinge region of hIgG1. (**CH2**) hIgG1 constant heavy chain region 2. (**CH3**) hIgG1 constant heavy chain region 3. (**H***) Modified hIgG1 hinge region. (**CD70 ECD**) region corresponding to the extracellular domain of murine CD70.

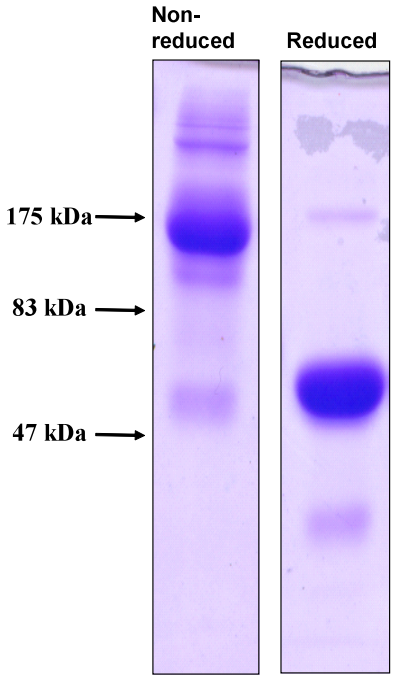

Figure 14 Structural analysis of sCD70 by SDS-PAGE: 5µg of purified sCD70, either non-reduced or reduced with 50mM DTT, was loaded onto an 8.5% polyacrylamide gel. The positions of relevant molecular mass markers are indicated (kDa).

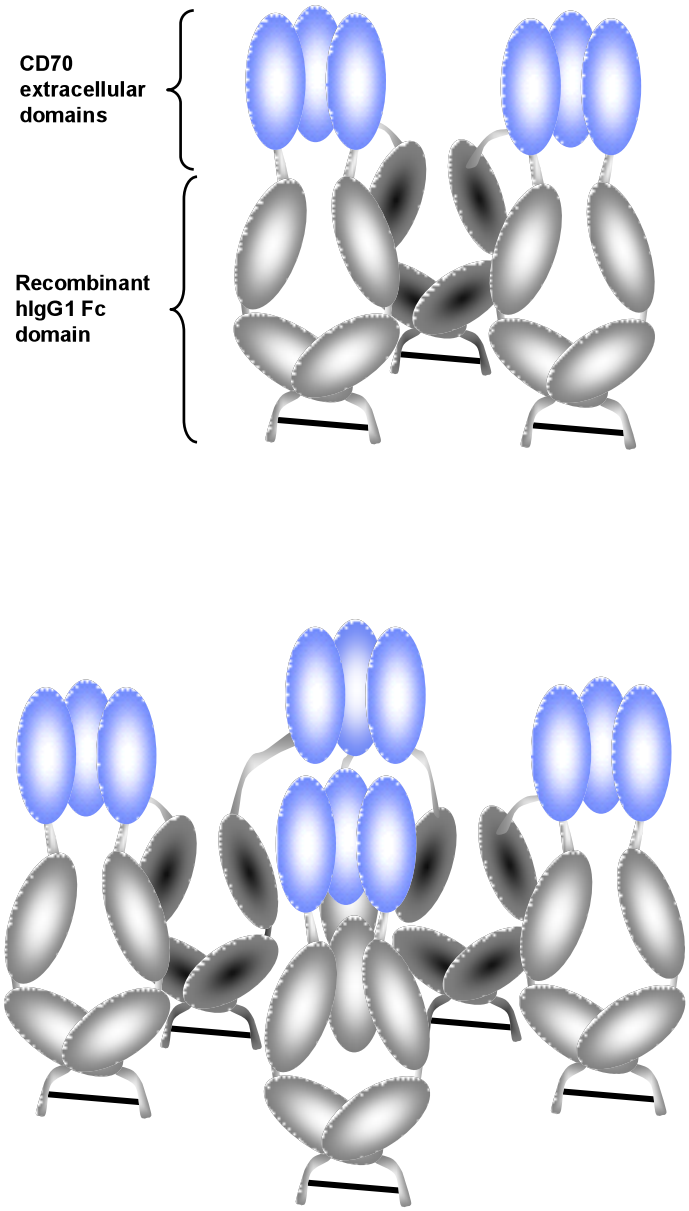

Figure 15 Schematic representations of the major predicted structures of sCD70: hexameric (above) sCD70 corresponds to a M_r ~ 300kDa. Dodecameric (below) sCD70 corresponds to a M_r of ~ 600 kDa. Regions of disulphide bonding within the hIgG1 Fc hinge are represented by black lines.(NB actually 3 disulphide bonds are predicted to be present – only one is shown for clarity)

3.1.2. sCD70 promotes proliferation of CD4+ and CD8+ T cells *in vitro*

Having demonstrated that recombinant sCD70 forms trimers, it was necessary to demonstrate that sCD70 provided a functional signal on T lymphocytes; the effects of signalling through CD27 on $CD8^+$ T cells is of interest because CD27 signalling is reported to play its major role on this subgroup of lymphocytes [106, 109]. In order to generate RNA from $CD8^+$ T cells for microarray analysis an *in vitro* system was developed: spleens and lymph nodes from C57BL/6 mice were purified on $CD8^+$ or $CD4^+$ negative selection columns, and cultured *in vitro* with various concentrations of agonistic anti-CD3 in the presence of sCD70 or human IgG control. Proliferation was determined by [^{3}H] thymidine incorporation, which is a measure of the number of cells in S phase of the cell cycle, or undergoing DNA replication. CD27 costimulation enhanced proliferation significantly across a range of anti-CD3 concentrations (Figure 16) for both $CD4^+$ and $CD8^+$ purified T cells.

An anti-CD3 concentration of 100ng/ml in 24 well plates resulted in modest but reproducible increases in live cell numbers when measured 72h after stimulation. These experimental conditions allowed for the requisite numbers of T cells (minimum of 5×10^6) to be collected for RNA purification; for all experiments where cells were harvested for microarray experiments additional wells were retained to ensure that live cell counts at 72h confirmed higher cell numbers in the sCD70 stimulated arm (Figure 17).

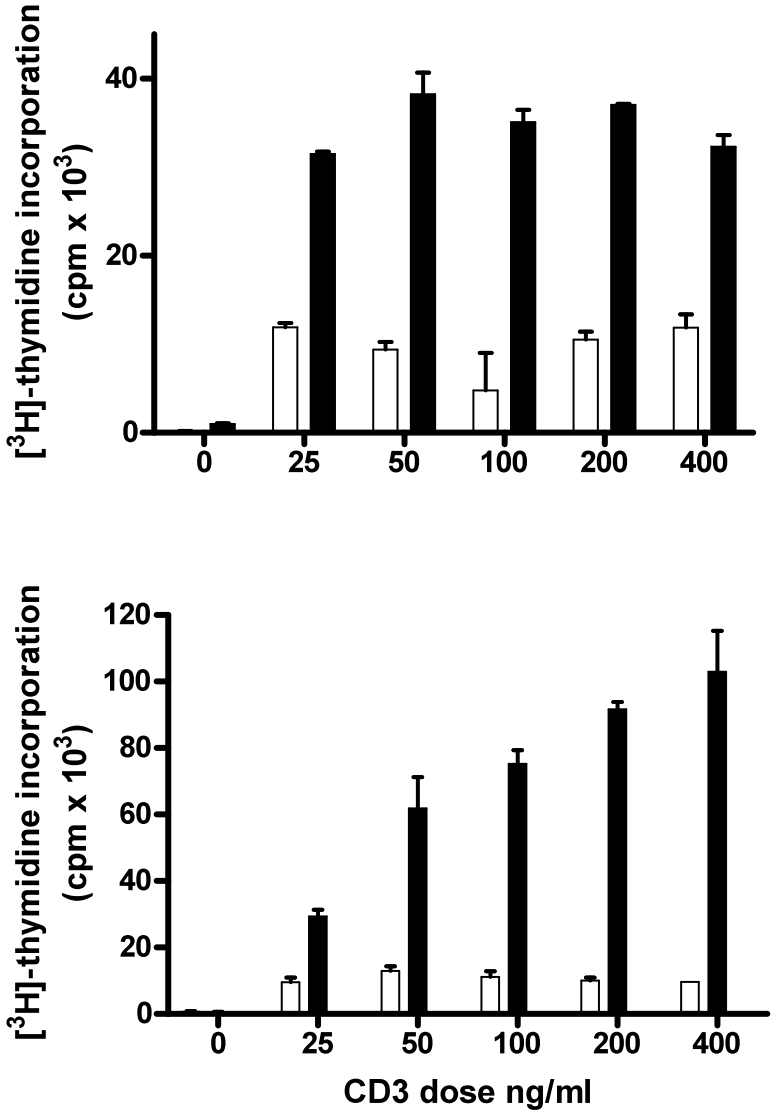

Figure 16 Effect of sCD70 on $CD8^+$ and $CD4^+$ T cell proliferation: $CD8^+$ (above) or $CD4^+$ (below) T cells were purified from C57BL/6 splenocytes and lymph nodes using depletion columns and activated *in vitro* with 100ng/ml soluble anti-CD3 (145.2C11) with sCD70 (solid bars) or control (human IgG (open bars). After 48 hours of culture 1 µCi [^{3}H] thymidine was added and cells were harvested 16 hours later, and incorporation was measured. Graphs represent the average counts from duplicate cultures and error bars represent the SEM.

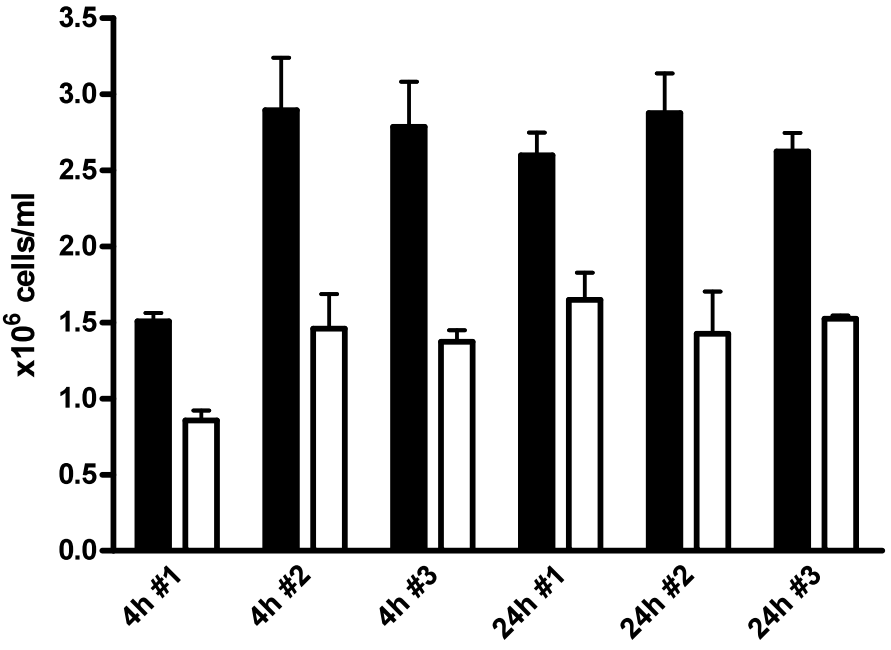

Figure 17 Effect of sCD70 on absolute cell numbers in vitro: $CD8^+$ T cells were purified from C57BL/6 splenocytes and lymph nodes using depletion columns and activated in vitro with 100ng/ml soluble anti-CD3 (145.2C11) with sCD70 (solid bars) or control (human IgG) (open bars). Cells were cultured in 24 well plates at 1.5 x 10^6 live/CD8+ cells/ml. At either 4h or 24h cells were taken and RNA prepared for analysis on gene expression microarrays. For quality control, to ensure that CD27 had resulted in enhanced cell proliferation/survival, at 72 hours cells were resuspended 1:1 with trypan blue and live cells were counted. Graphs represent the average from three wells and error bars represent the SEM. Results from each of three experiments used for microarrays are shown.

3.1.3. Global analysis of gene expression

Gene expression microarrays allow for the simultaneous study of thousands of genes in a single experiment. Microarrays use DNA probes printed onto a silicon chip. The use of this powerful technique comes with several considerations;

Purity – microarrays performed on non-purified cell populations, eg splenocytes, will give data that reflects the cellular distributions, that may be better examined by simpler techniques eg flow cytometry.

RNA quantity - typically 5-10µg of RNA (requiring 10^6-10^7 purified cells) is required for generation of labelled cRNA prior to hybridization to a chip. Although RNA can be amplified, this can introduce errors [244] such as production of smaller cDNA fragments (with knock on effects on quality control) and leads to populations of cDNA that are 3' biased. Experiments designed to compare amplified from non-amplified samples show a 13% difference in gene expression patterns and for this reason non-amplified samples were used [245]

Standardisation – with the increasing use of microarrays there is a problem of comparability and reproducibility between laboratories and experiments – some degree of standardisation of techniques is being attempted – databases such as the Minimal Information About a Microarray Experiment (MIAME) are being used to aid this process. They provide a standard for the reporting of microarray experiments specifying all the information necessary to interpret the results unambiguously [246]. This simple database includes information on: the raw data, the normalized data, essential experimental data eg compound studied/dose etc, experimental design, annotation scheme used and the normalisation scheme employed.

Statistical considerations – because of the large number of genes examined on each microarray, a significant number of differences are predicted to be discovered by chance alone; various statistical techniques

exist to reduce this ‘white noise factor’ although it remains an inevitable problem with any microarray experiment. In fact, it may be that more reliable results can be achieved by using a non-stringent probability cut-off and concentrating on the genes with the highest fold change [247].

Normalisation – this is the process of equalising the global signal intensity across microarrays to allow for comparison between microarrays. This can be different for different microarrays due to differences in quantity of starting RNA, or differing labelling efficiencies [248].

Despite all the difficulties, and the enormous number of data points generated from each microarray experiment, the results from microarray experiments in general show extraordinary consistency across sites, with 89% consistency between lists of genes generated at different sites. For these experiments Affymetrix Genechip 430 2.0 arrays were employed; these microarrays contain 39,000 separate probes.

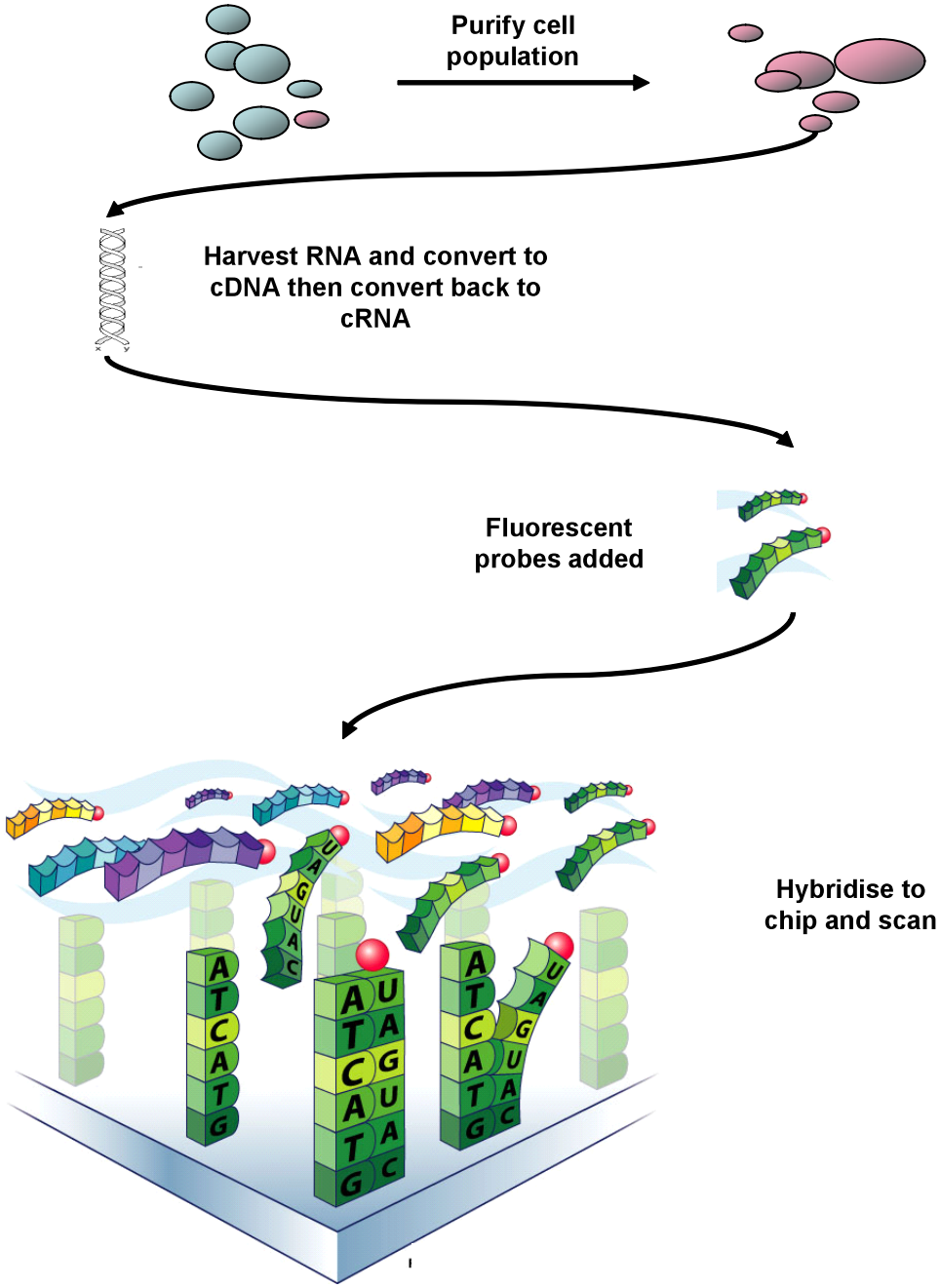

Figure 18 Protocol for preparing samples for microarray: key steps in the microarray process are shown; initially a purified cell sample is prepared, RNA extracted, and converted by reverse transcriptase to cDNA. This is subsequently converted to cRNA using T7 polymerase – this step amplifies the product, but needs to be undertaken with care to avoid differential amplification of targets. A fluorescent tag is then added to the cRNA fragments and these are hybridized to the microarray. Each 'spot' on a microarray is 11µm across and contains millions of identical DNA molecules. Part of this figure was derived from the Affymetrix website.

3.2. CD27 costimulation leads to few transcriptional changes at four hours

3.2.1. Microarray Quality Control

In order to examine the genes involved early in CD27 costimulation, $CD8^+$ T cells were stimulated *in vitro* as described above, RNA was purified from cells at 4 hours following stimulation, and was sent for microarray analysis. Samples were analysed in triplicate. Quality control of arrays was assessed by several factors:

Background – a measure of background fluorescence; this should be similar across chips

Scale – a measure of the number of genes altered between experimental arms – large 'scale' measurements are suggestive of technical problems; ratios of less than 3 are recommended

Genes Present – similar to the 'scale' factor, if the number of genes considered as 'present' differs vastly between chips this is suggestive of a technical problem

3':5' ratios – measurement of gene expression is based on the assumption that the analyzed RNA sample closely mirrors the level of gene expression; however, transcripts can show stability differences of up to two orders of magnitude *in vivo*; the ratio of 3' to 5' expression levels for individual genes is closely correlated with the degree of RNA degradation of a sample, and provides a useful measure of quality control [249]; for GAPDH a ratio of less than 1.25, and for β-actin a ratio of less than 3, are acceptable.

The microarrays analysed were of acceptable quality (Table 1-3), and following normalisation the global signal intensity was approximately equal between all six samples (see appendix (page 293) for normalization box plots, full gene lists and scatter plots of data).

Name	Scale	Background	Present (%)	GAPDH 3'/5'	βActin 3'/5'
CD70	0.55	47.05	55.83	0.72	1.41
CD70	0.54	46.90	55.44	0.81	1.32
CD70	0.77	43.25	50.52	0.71	1.58
Control	0.36	45.16	54.85	0.81	1.24
Control	0.56	59.38	51.13	0.74	1.36
Control	0.65	40.86	52.92	0.69	1.52
QC	<3	Similar	Similar	<1.25	<3

Table 1-3 Quality Control of microarrays from the 4h timepoint experiments: quality control measures from the microarray chips showed low ratios of 3' to 5' signals from control genes GAPDH and beta actin, as well as similar background levels and similar scale factors. The expected values are shown in the bottom row.

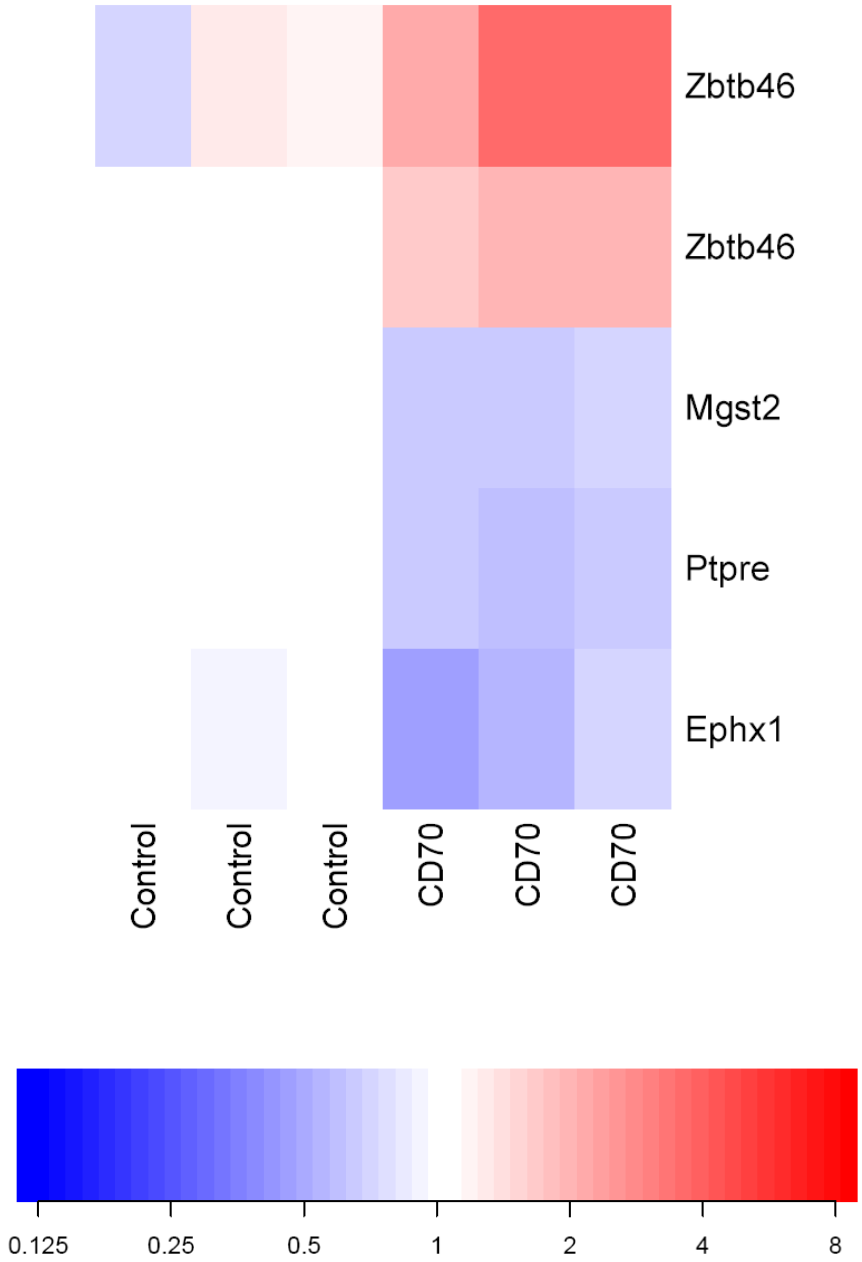

Figure 19 Heatmap showing genes significantly altered by CD27 signalling *in vitro* at the 4h timepoint: experimental setup was as described in Figure 17. Only genes significantly different ($p<0.001$) are shown; colour intensity represents expression relative to the global gene expression. For example a gene coloured deep red is expressed 8 fold higher than the median expression of all 40,000 genes on the chip.

3.2.2. Genes altered by CD27 costimulation at the 4 h time point

Gene expression changes can be detected as early as 4 hours after cell signalling; for example, the cytotoxic agent Bleomycin has been shown to significantly alter expression of over 200 genes at this time point [250]; the effects of Bleomycin are directly felt within the nucleus, but cell surface signalling also results in gene expression changes as early as 3 hours after receptor ligation: adenosine signalling on macrophages showed over a hundred genes with altered expression at this early timepoint. Therefore, it was postulated that CD27 may exert significant effects detectable at this timepoint.

Relatively few genes were affected by CD27 signalling at 4h (Figure 19). Only 2 genes were upregulated by more than a factor of 2 (Table 1-4). The first, Zbtbd4, (upregulated by a factor of 2.14), is a poorly characterised putative zinc-ion-binding transcription factor. Also upregulated was Bhlhb2 (basic helix-loop-helix domain containing B2) (2.19 fold change. Bhbl2 is also known as Clast5/Stra13/Dec1. When Bhbl2 is over-expressed in transgenic mice under control of the immunoglobulin variable heavy chain promoter, lymphogenesis is reduced, as are responses to IL-7 and CD40 ligation [251]. Bhbl2 -/- mice conversely develop autoimmune disease, with T cell infiltration of various organs [252]. Bhbl2 is considered a negative regulator primarily of B cell function [251].

Given the paucity of transcriptional effects found at the 4 h time point, the 24 h time point was subsequently examined.

Symbol	Fold Change	P value	Full name	Description
Bhlhb2	2.19	4.9E-05	basic helix-loop-helix domain containing, class B2	Transcriptional repressor involved in brain development
ZBtbd46	2.14	6.9E-04	BTB (POZ) domain containing 4	Presumed transcription factor
IL-2	1.85	3.2E-04	interleukin 2	Lymphocyte cytokine
Rgs1	1.71	2.0E-04	regulator of G-protein signaling 1	G protein regulatory molecule involved in chemokine signalling
Btbd4	1.71	3.4E-04	BTB (POZ) domain containing 4	
Igsf3	1.68	2.6E-04	immunoglobulin superfamily, member 3	Presumed cell surface molecule – function unknown
Itgae	1.55	8.9E-04	integrin, alpha E, epithelial-associated	CD103 – cell surface integrin expressed in activated $CD8^+$ T cells
Btbd4	1.55	8.2E-04	BTB (POZ) domain containing 4	Putative regulator of transcription
Tsc22d1	-1.61	7.0E-04	TSC22 domain family, member 1	Leucine-zipper transcription factor – early response factor
Tm6sf1	-1.65	8.5E-04	transmembrane 6 superfamily member 1	Transmembrane protein unknown function
Ephx1	-1.73	2.1E-04	epoxide hydrolase 1, microsomal	Enzyme involved in drug metabolism

Table 1-4 List of genes altered by CD27 signalling *in vitro* at the 4h timepoint: RNA was purified from CD27 and control stimulated $CD8^+$ T cells (with the same protocol as Figure 17). Genes up- or downregulated by CD27 signalling are shown (with a fold change of greater than 1.5 or less than -1.5, and a p value of <0.001).

3.2.3. CD27 costimulation leads to several transcriptional changes at 24 hours

In vitro cultures of CD3 - stimulated CD8+ enriched T cells were set up as above, and RNA purified 24 h after stimulation with CD3 with or without sCD70. Quality control was within acceptable limits for all samples and normalisation yielded similar signal intensities across all microarray chips. In contrast to the 4 h time point, multiple genes were up- or down-regulated by CD27 signalling at the 24 h time point (Table 1-5). A heatmap of the most significantly altered genes is shown (Figure-20). Analysis of the types of genes affected by CD27 signalling *in vitro* showed that several families of genes were significantly represented above the level predicted from their prevalence in the entire genome. This analysis was performed by the Panther (**P**rotein **An**alysis **Th**rough **E**volutionary **R**elationships) classification system website; several such databases exist (eg DAVID and Genego); Panther was chosen because it had the most comprehensive list of genes, meaning that relatively few genes were excluded from any analysis (characteristically less than 5%). CD27 signalling significantly upregulated genes from three biological process groups –*immunity and defence, natural killer cell mediated immunity* and *MAPKKK cascade* (Figure 21).

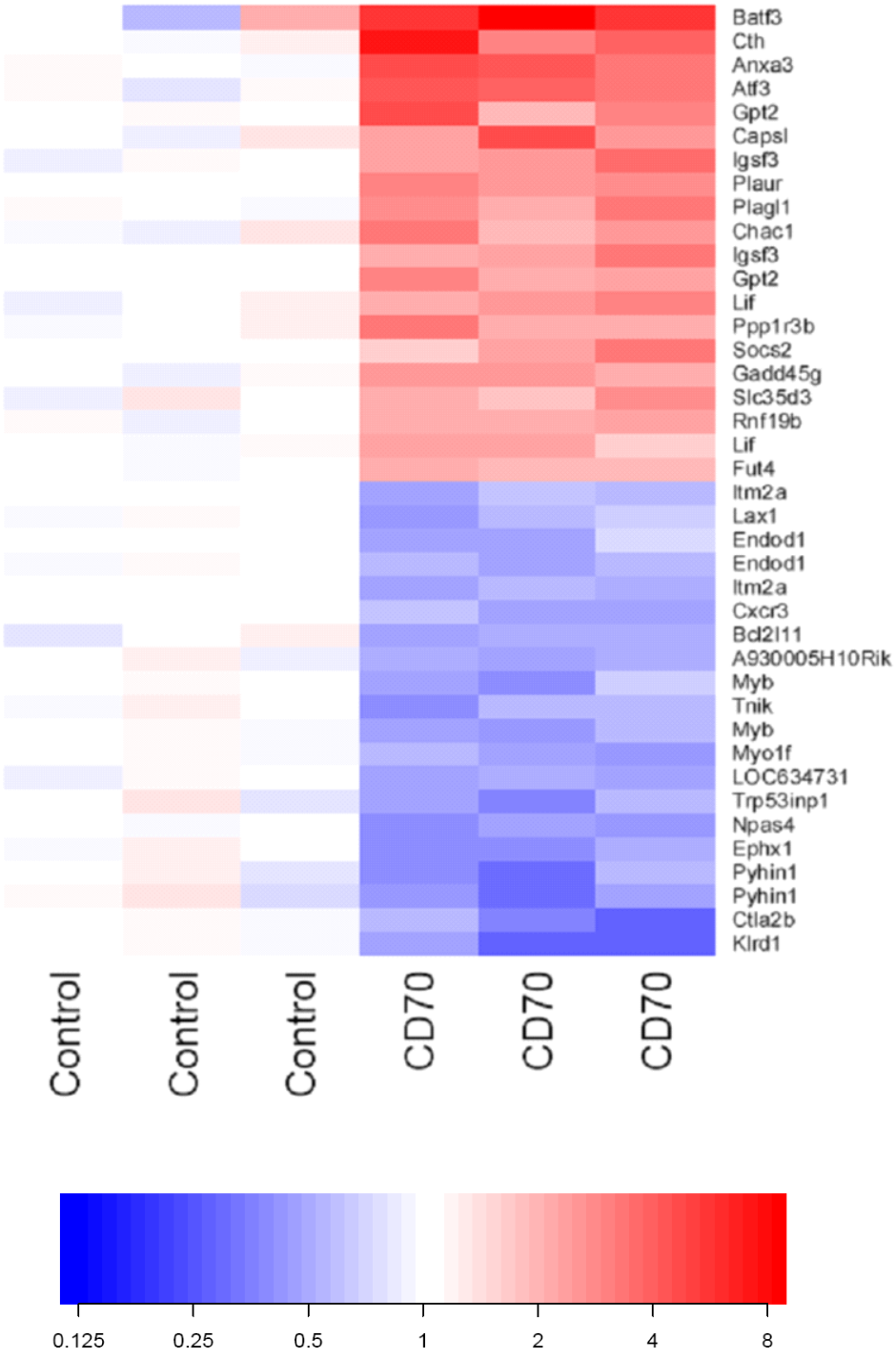

Figure-20 Heatmap showing genes significantly altered by CD27 signalling *in vitro* at the 24h timepoint: RNA was purified from CD27 and control stimulated $CD8^+$ T cells (with the same protocol as Figure 17). Only genes significantly different ($p<0.001$) are shown; colour intensity represents expression relative to the global gene expression. For example a gene coloured deep red is expressed 8 fold higher than the median expression of all 40,000 genes on the chip.

Symbol	Full Name	Fold Change	P value	Description
Batf3	Jun dimerization protein 1/p21^{SNFT}	4.14	2.56E-04	Transcription factor involved in Dendritic cell development
Cth	cystathionase (cystathionine gamma-lyase)	3.89	6.74E-04	Cysteine metabolism
Atf3	activating transcription factor 3	3.71	7.71E-06	Transcription factor involved in stress pathways
Egln3	EGL nine homolog 3 (C. elegans)	3.37	3.56E-03	Hydroxylation of HIF genes
Anxa3	annexin A3	3.31	1.18E-05	Role in neutrophil/monocyte granule membrane fusion
Il24	interleukin 24	3.28	1.39E-03	Cytokine produced by activated T lymphocytes
Ccr4	chemokine (C-C motif) receptor 4	3.19	2.17E-02	T cell chemokine receptor
Egln3	EGL nine homolog 3 (C. elegans)	2.95	1.23E-02	Hydroxylation of HIF genes
Capsl	calcyphosine-like	2.94	3.02E-04	Calcium binding
Trib3	tribbles homolog 3 (Drosophila)	2.85	1.06E-03	Transcription factor – blocks Cyclin D1
A930002I21 Rik	RIKEN cDNA A930002I21 gene	-2.75	5.72E-03	Unknown function
AI447904	expressed sequence AI447904	-2.81	1.66E-03	Unknown function

Symbol	Full Name	Fold Change	P value	Description
Klf2	Kruppel-like factor 2 (lung)	-2.89	3.14E-02	Transcription factor – affects $CD8^+$ T cell trafficking and CD62L expression
Nt5e	5' nucleotidase, ecto	-2.90	2.16E-02	CD73 – expressed on lymphocytes and some tumours
AI447904	expressed sequence AI447904	-2.92	8.01E-04	Unknown function
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	-2.98	5.20E-02	Unknown function
Ctla2b	cytotoxic T lymphocyte-associated protein 2 beta	-3.06	2.97E-04	Cysteine peptidase upregulated by IL12
Pou2af1	POU domain, class 2, associating factor 1	-3.09	3.51E-03	Transcription factor Bob1 involved in IFNγ/IL2
Tmem71	Transmembrane protein 71	-3.19	8.36E-03	Function unknown
Klrd1	killer cell lectin-like receptor, subfamily D, member 1	-3.33	8.20E-05	Inhibitory receptor on NK and $CD8^+$ T cells

Table 1-5 Significantly up- or downregulated genes at the 24 hour timepoint *in vitro*: RNA was purified from CD27 and control stimulated $CD8^+$ T cells (with the same protocol as Figure 17). The genes showing the greatest fold changes are shown ($p<0.002$) – the full list is shown in the appendix (page 293).

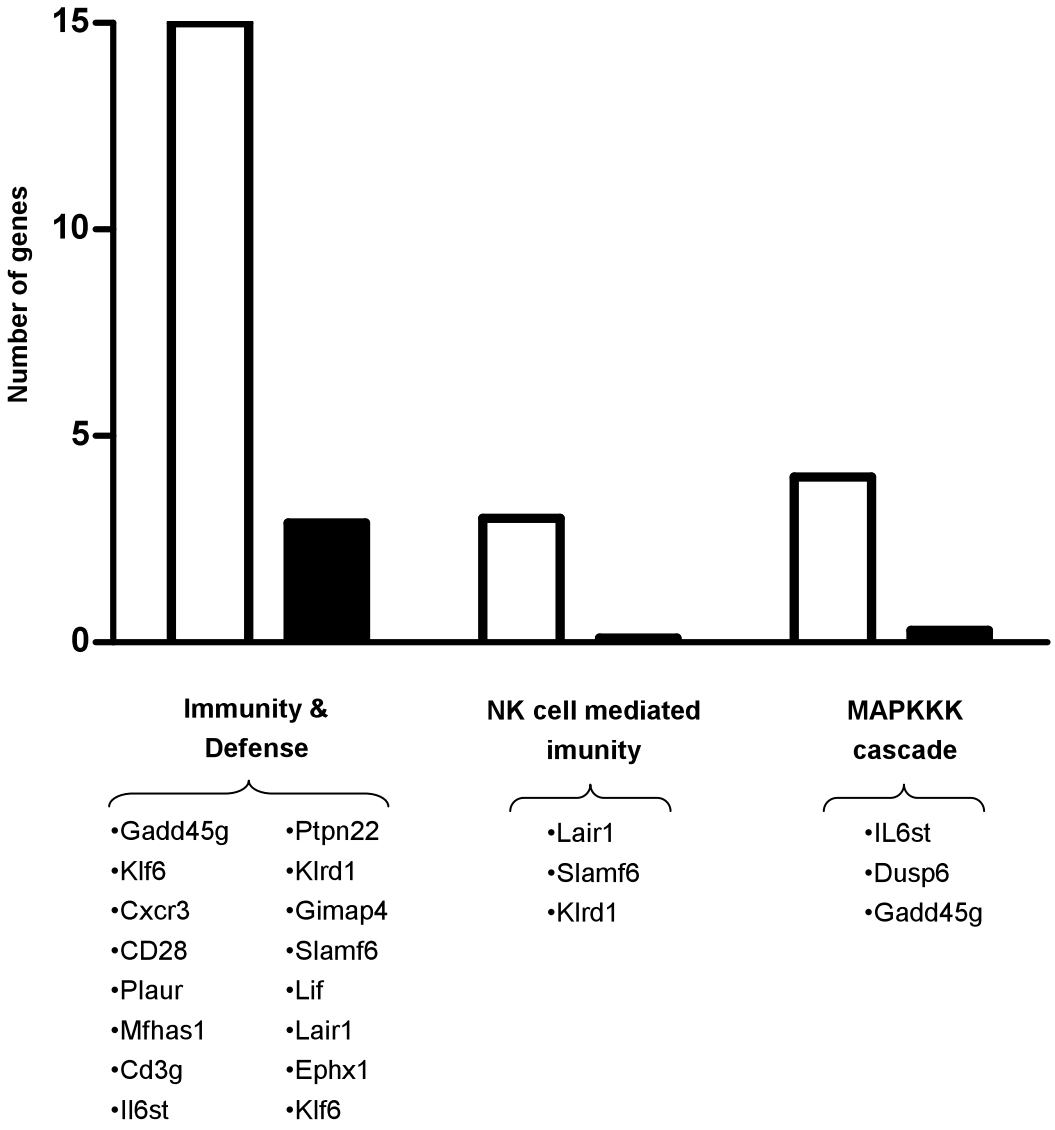

Figure 21 Pathways upregulated by CD27 signalling *in vitro* at 24h: genes significantly upregulated by a fold change of greater than 2 were interrogated by the Panther classification system; no pathways were significantly affected, but three biological processes were significantly over-represented. The observed (open bars) number of genes from each biological process is compared with the number expected (filled bars) and the exact genes involved detailed underneath. NB genes may belong to more than one biological process.

3.3. Confirmation of in vitro gene expressions by RT-qPCR

Multiple genes were significantly up- or down-regulated by CD27 signalling; several genes were selected for further investigation by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) to confirm the microarray findings. Only genes that were increased or decreased by greater than a factor of 2 were examined, (with a p value of <0.001), because of the difficulty of assessing smaller differences by qPCR. Genes were selected because of their high fold change (eg Batf3), involvement in relevant pathways (eg gadd45g) or, following literature searches, their likely involvement in CD27 signalling (eg IL-3). Using the same *in vitro* conditions as were used for the microarray experiments, the expression of various genes was assessed using probes that spanned intron-exon boundaries (and thus would not amplify genomic DNA); the 8, 24 and 48 hour timepoints were all studied. Hypoxanthine-Guanine Phosphoribosyltransferase Reporter (HPRT) was used as a reference gene [241] to which target gene expression was compared. To ensure similar amplification efficiency between reference and target genes, probes were evaluated using differing concentrations of cDNA; similar results were obtained at different dilutions for all probes. qPCR of the listed genes gave similar results to the microarray data (Figure 22).

3.3.1. Immunoglobulin superfamily 3 (Igsf3)

Igsf3 is an uncharacterised member of the immunoglobulin superfamily. It was considered for further study because it is predicted to be expressed at the cell surface, and because of the extensive involvement of proteins from this family in immune system function [253]. Igsf3 expression was found by microarray to be enhanced by a factor of 2.65. This was confirmed by qPCR at the 8, 24, and 48 h time points (Figure 23).

3.3.2. Interleukin 3 (Il3)

Il3 has been associated with the augmentation of $CD8^+$ [254] and $CD4^+$ [255] T cell responses. It has also been shown to promote myeloid development

[256] and may have pro-survival activity in the Reed-Sternberg cells of Hodgkin's Disease [257]. Il3 expression was increased by a factor of 2.7 as assayed by microarray, and this was confirmed by qPCR.

3.3.3. Growth arrest and DNA-damage-inducible 45 gamma (Gadd45g)

Gadd45g is a small nuclear protein involved in negative growth control [258]. Gadd45g interacts directly with cell cycle proteins, inhibiting proliferation [259]. Its expression is known to be induced in $CD4^+$ T cells, where it mediates AICD. Additionally, Gadd45g deficiency is associated with autoimmunity. Gadd45g expression was induced by CD27 signalling *in vitro* as detected by microarray (2.5 fold) or qPCR, with its expression increasing by 48 h.

3.3.4. Activating Transcription Factor 3 (Atf3)

Atf3 (Activating Transcription Factor 3) is a stress-inducible transcription factor [260]. Atf3 can homodimerize, where it acts to suppress transcription, or heterodimerize with Jun and other molecules, resulting in active transcription. Atf3 is involved in cytokine induced apoptosis [261]; it has been shown to be induced by Jun-N-terminal kinase and NF-κB signalling in β pancreatic cells, and its absence can protect β cells from cytokine induced apoptosis.

Atf3 expression was upregulated by CD27 signalling by a factor of 3.7 in microarrays, which was confirmed by qPCR, expression increasing by the 48 h timepoint.

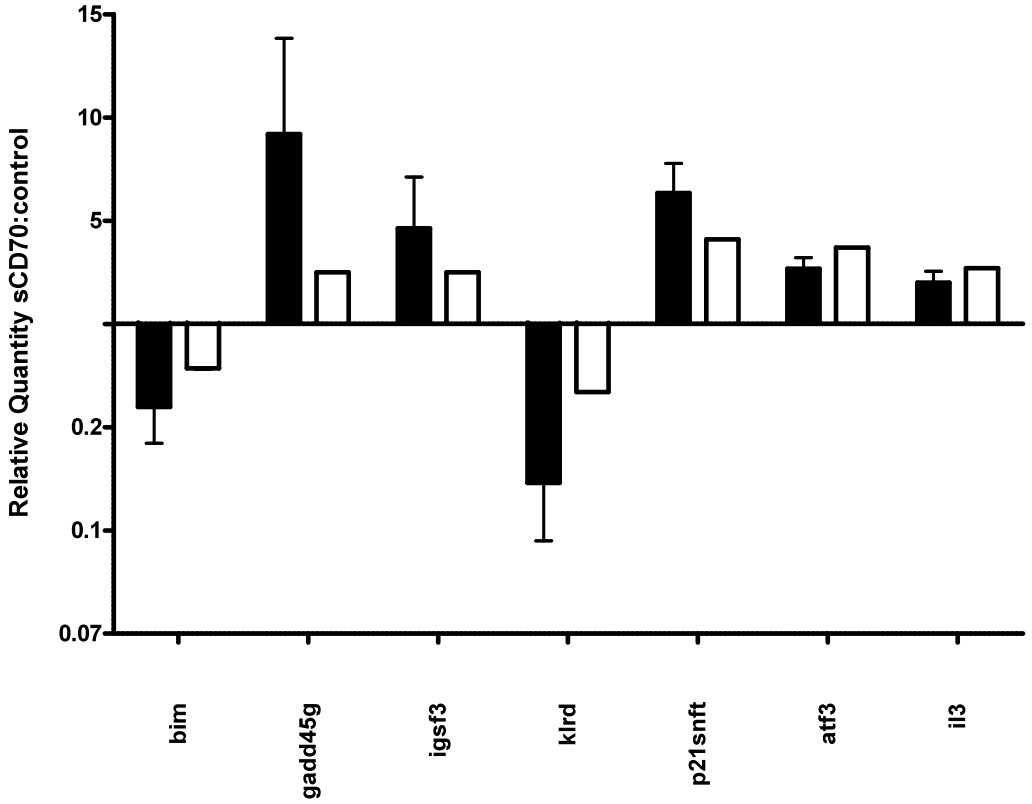

Figure 22 Comparison of microarray and qPCR gene expression yields similar results (*in vitro* experiments): to confirm the integrity of the microarray data, the expression of several genes was measured by quantitative PCR (solid bars) and the results compared to those generated by gene expression microarray (open bars). Both techniques yielded comparable results for gene expression.

3.3.5. Bcl2-like Interacting Mediator of Cell Death (Bim)

Bim is a member of the BH3 group of pro-apoptotic proteins, that induce apoptosis in response to various stresses [262]. It is known to be important in mediating the contraction phase of the $CD8^+$ response and activation induced cell death (AICD) [263]. Bim expression was downregulated, as assessed by microarray, by a factor of 2.1. This was confirmed by qPCR, which showed downregulation of Bim at 8 hours after *in vitro* stimulation; Bim levels remained lower in CD27 stimulated cells at 24 and 48 hours (Figure 23), albeit to a lesser extent.

3.3.6. Interleukin 2 (IL-2)

IL-2 is powerful growth factor for a variety of immune cells. It is expressed within four hours of TCR signalling, shortly followed by expression of its receptor, CD25 [264]. IL-2 was upregulated at the 4 h time point by a factor of 1.85 but at 8h was downregulated (Figure 23).

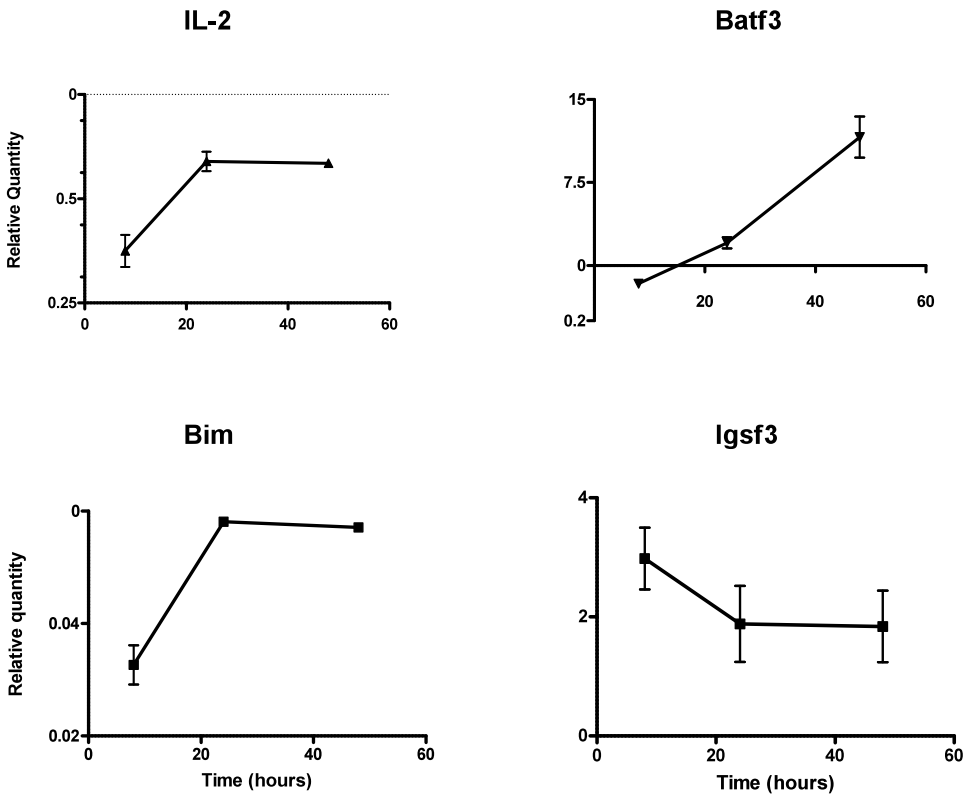

Figure 23 Timecourse of genes detected by microarray *in vitro*: RNA was purified from CD27 and control stimulated $CD8^+$ T cells (with the same protocol as Figure 17). Cells were harvested at 8, 24, and 48 h timepoints and individual gene expression was assayed by qPCR. Relative quantities (ie the fold change) of gene expression mediated by CD27 signalling are shown on the vertical axis – note different scales. Results are representative of two independent experiments.

3.4. Gene expression induced by CD27 co-stimulation in vivo

To determine whether the gene expression changes induced by CD27 *in vitro* were genuine, or due to artefactual effects of the experimental system, an alternative *in vivo* system was used. Instead of using non-specific anti-CD3 TCR stimulation, TCR-transgenic OT1 $CD8^+$ responses were studied. 5×10^6 OT-I $CD8^+$ T cells were adoptively transferred into normal syngeneic C57BL/6 mice. The mice were then challenged iv with $ova_{257-264}$ together with sCD70, or hIgG as a control. CD27 signalling with sCD70 resulted in enhanced $CD8^+$ T cell proliferation at 72h (Figure 24). At 72h or 48h following primary challenge mice were sacrificed and spleens and lymph nodes harvested. Cells were enriched for $CD8^+$ T cells by negative selection columns before being stained with CD8α and MHC tetramer. Live, antigen-specific $CD8^+$ T Cells were then sorted on a Facs-Aria (BD) to > 95% purity. Viability of greater than 95% was confirmed by trypan blue dye exclusion before collection of RNA.

Of the genes changed *in vitro* by CD27 signalling, Bim, IL-2, and Batf3 gave similar results *in vivo*. Conversely Gadd45g, ATF3 and Igsf3 were downregulated *in vivo* (Figure 25). Explanations for the difference between *in vivo* and *in vitro* data may be due to several factors: more stringent cell purification in *in vivo* experiments may have minimised off-target sCD70 effects on non-$CD8^+$ cells and the use of a different time point – examination of the 72 h time point *in vitro* was made difficult by the large number of dead cells present at this time point. There may also exist fundamental differences between OT-I and wild type $CD8^+$ T cells.

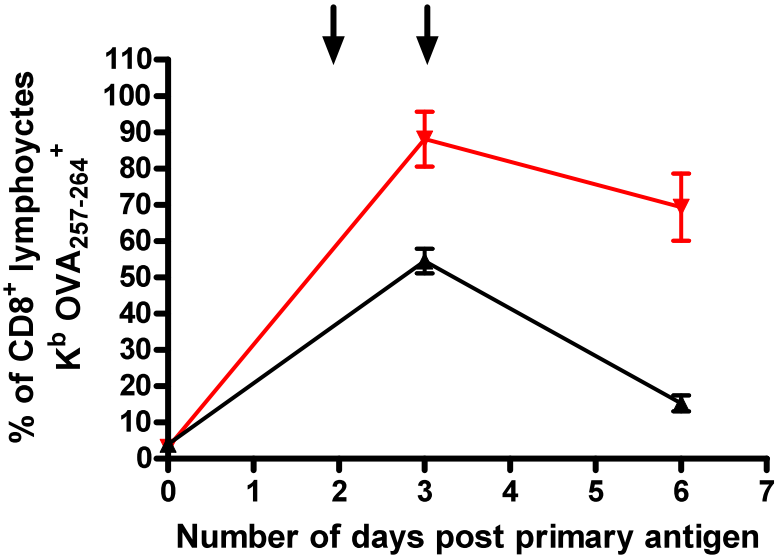

Figure 24 sCD70 amplifies the primary expansion of naïve OT-I CD8$^+$ T cells *in vivo*: 5 x 10^6 naïve OT-I cells were transferred into C57BL/6 recipients and mice were primed by iv injection of $ova_{257-264}$ (30 nanomoles) in the presence of sCD70 (▼) or a control (human IgG)(▲) (3 x 200µg). Percentage of antigen-specific CD8$^+$ T cells in peripheral blood was measured by double staining with K^b $ova_{257-264}$ tetramers and anti-CD8α. CD27 stimulation by sCD70 resulted in significant amplification of the primary response. At 48 and 72 hours (arrows) spleens and lymph nodes were harvested and antigen specific cells were purified on a FACS Aria cell sorter and RNA prepared for interrogation by microarray.

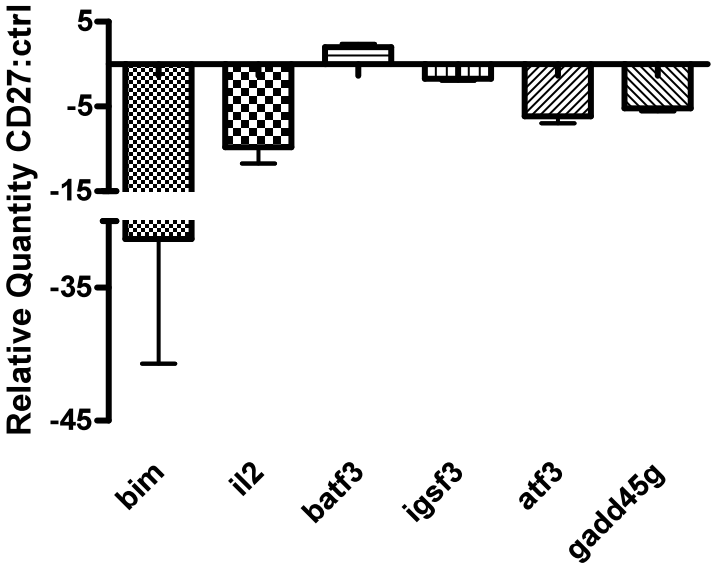

Figure 25 *in vivo* quantitative PCR measurement of genes detected *in vitro* by microarray (72 hour timepoint): expression of Bim, IL-2 and Batf3 *in vivo* mimicked the results obtained *in vitro*. Conversely Igsf3, atf3 and gadd45g were all downregulated by CD27 signalling *in vivo* whereas these genes were upregulated at 24h *in vitro*.

3.4.1. Characterisation of Bim and Batf3 gene expression in resting and activated CD8$^+$ T cells: effect of CD27 costimulation

In vitro and *in vivo* experiments demonstrated that Bim and Batf3 are dowregulated and upregulated respectively in CD8$^+$ T cells after costimulation by CD70. To compare the expression of Bim and Batf3 between activated T cells and resting naïve CD8$^+$ T cells, naïve OT-I cells were purified and expression of Bim, Batf3 and Jun-dimerization-2 (jdp2) was examined. Jdp2 is a related Jun dimerization protein that was not altered by CD27 stimulation, and was used as a negative control for Batf3, which also dimerizes with Jun. Bim, Batf3 and jdp2 were all expressed at low levels in naïve T cells (Figure 26). TCR stimulation increased the expression of Bim, Batf3 and Jdp2 from that found in naïve T cells. Bim levels were increased 40 fold by TCR signalling; this effect was significantly diminished by the presence of CD27 costimulation. For further investigation on the role of Bim in CD27 signalling see the third results chapter.

In an attempt to analyse the expression of Batf3 protein in T cells a recombinant GST-Batf3 was generated and purified (a kind gift from Dr P Duriez) and used to generate a polyclonal rabbit-anti-mouse-Batf3 antibody. This antibody was able to detect both purified GST-Batf3 as well as Batf3-HA expressed 293T cells, but did not detect Batf3 in mock transfected cells, nor OT-I cells activated with $ova_{257-264}$ with or without CD27 costimulation (Figure 27). It is possible that expression of Batf3 was below the detection limit of the western blot assay employed; other groups have confirmed Batf3 expression at the protein level in CD4$^+$ T cells by incubating them in Th1 conditions for seven days followed by an additional 24 hours of CD3 stimulation [265]; therefore it is possible that the method used in Figure 27 was not optimized for Batf3 expression. The human ortholog of Batf3 is expressed at the protein level in the Jurkat T cell line Jurkat [266].

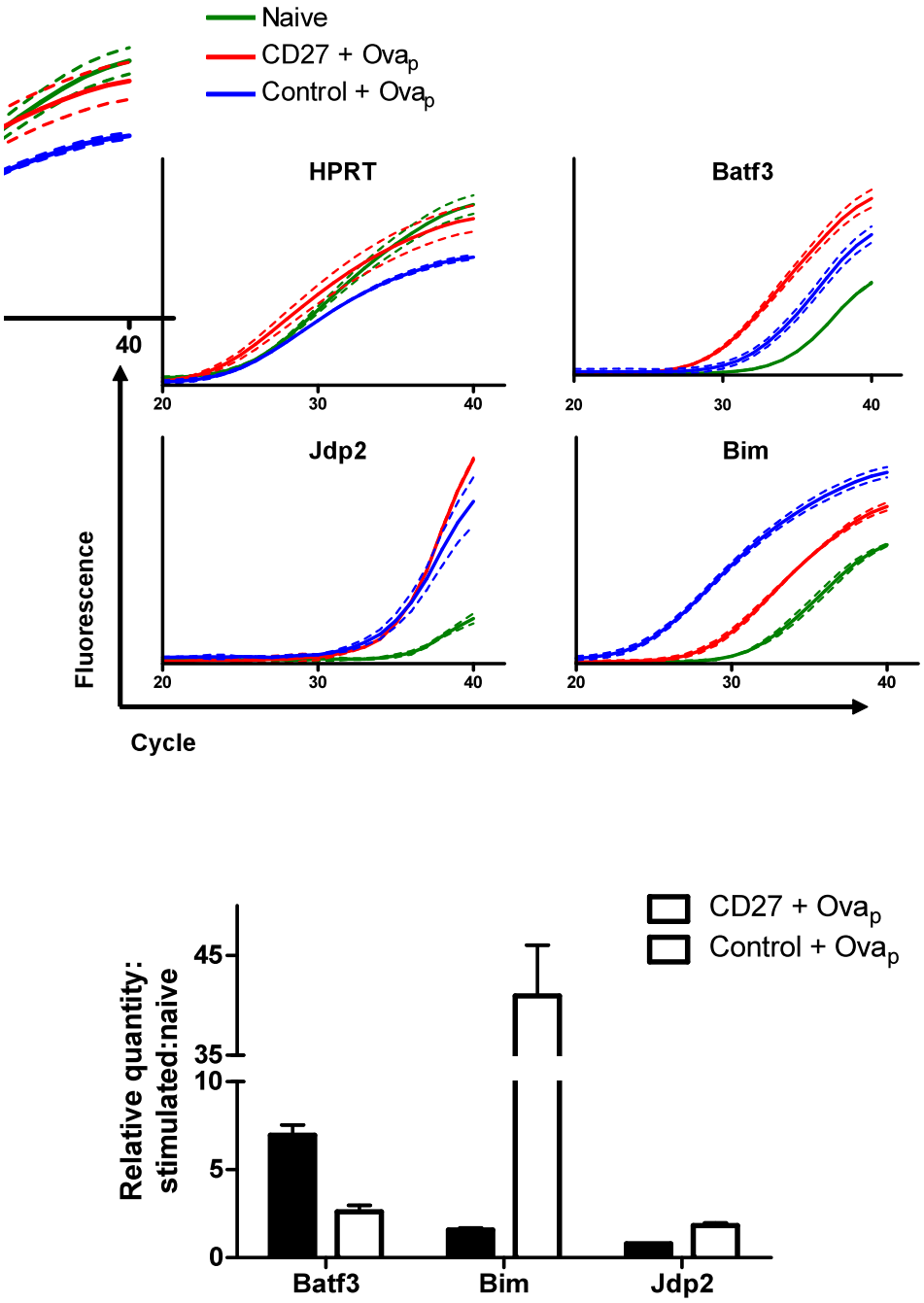

Figure 26 *in vivo* expression of Bim and Batf3 compared to unstimulated cells: $Ova_{257-264}$ specific $CD8^+$ T cells were purified from either naïve OT-I mice (naïve), or 72h following *in vivo* challenge with peptide (control + Ova_p) or sCD70 + peptide (CD27 + Ova_p). Expression of Bim, Batf3, and jdp2 (which is related to Batf3) was measured by quantitative PCR. Fluorescence plots are shown for each gene, as well as the house keeping gene HPRT (above). The relative quantities of each gene's expression for CD27 or control stimulated cells compared to naïve cells is shown below. Error bars represent the SEM. Data is representative of two independent experiments

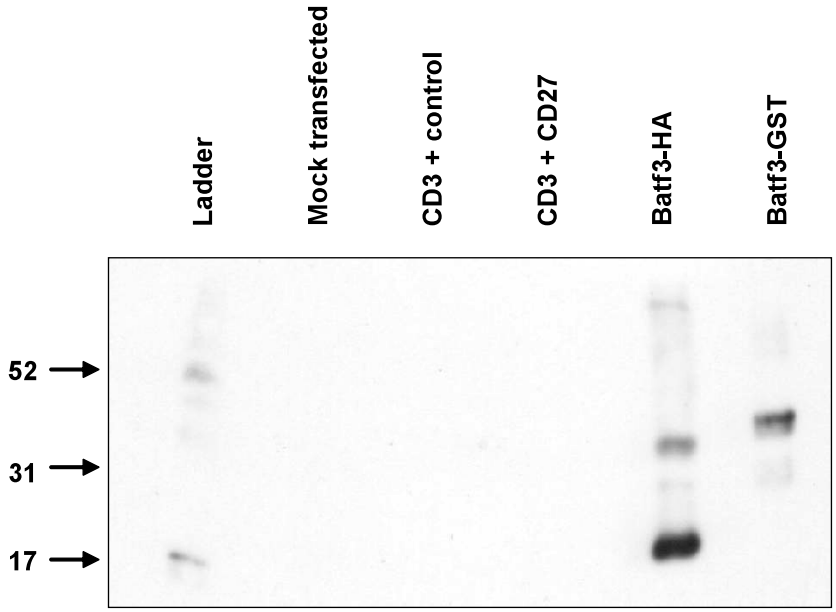

Figure 27 polyclonal anti-Batf3 binds specifically to Batf3 in transfected cells: cell lysates were prepared of $CD8^+$ enriched cultures stimulated with anti-CD3 with or without sCD70 (2×10^7 cells) and harvested at 24h after stimulation. 293T cells (5×10^6) transiently transfected with Batf3-HA construct and purified Batf3-GST (1µg) were used as positive controls. Lysates were run on a 14% SDS PAGE gel and analysed for Batf3 expression using polyclonal rabbit antibody to Batf3. Appropriately sized products consistent with Batf3-HA (around 21kda) and Batf3-GST (50kda) were detected. No Batf3 was detectable in activated cells (results are representative of 3 separate experiments).

3.5. Gene expression changes induced by CD27 signalling in vivo

Since there were several genes upregulated *in vitro* but downregulated *in vivo*, it was postulated that the *in vitro* gene expression data may not accurately reflect the physiological effects of CD27 signalling; to investigate this further, gene expression profiles were undertaken using an *in vivo* approach. Antigen specific $CD8^+$ T cells were purified as described above (Figure 24). RNA from CD27 and control stimulated antigen specific $CD8^+$ T cells was examined by microarray at the 48h and 72h timepoints. Biological triplicates were used for each timepoint. CD27 signalling caused multiple gene expression changes at 48h (Figure 28 and Table 1-7), and at 72h (Figure 29 and Table 1-8). The key findings from the *in vivo* array experiments are listed below, and are discussed in full in the discussion section:

Upregulation of LIM-nebulette - this Actin binding gene was upregulated by CD27 signalling, as detected by two probes, at 48h (fold change 3.57 and 2.93) and at 72h (6.5 and 4.9). LIM-nebulette was confirmed to be upregulated by quantitative PCR (Figure 31).

H2-Q10 – this MHC Class Ib molecule was upregulated by CD27 signalling by a factor of 4. These non-classical MHC molecules are non-polymorphic, with membrane bound and soluble forms; H2-Q10 behaves much like classical MHC class I molecules, binding a classical peptide repertoire [267]. Conversely H2-Ob, a non-classical MHC class II molecule, was significantly downregulated by CD27; H2-Ob is a negative regulator of H2-M, which is involved in antigen presentation [268].

Downregulation of Egr1, Egr2 and Egr3 – Egr 1 was downregulated by a factor of 2.84, Egr2 by a factor of 3 and Egr3 by a factor of 4.59 by CD27 signalling. All three genes are known to be involved in TCR signalling [269].

Downregulation of Fos – Fos is part of the heterodimeric transcription factor AP-1 – it was downregulated by a factor of 5.34 by CD27 signalling at 72h. AP-1, in conjunction with NFAT, drives proliferative and pro-survival signals in T cells [270].

Upregulation of cell-cycle and DNA repair genes – when analyzed by the Panther classification database of biological processes, the genes upregulated by CD27 at 72h were biased towards those involved with cell cycle/DNA repair/DNA metabolism/Mitosis (Figure 30).

Downregulation of Ephx1 – Ephx1 was significantly downregulated by CD27 signalling in all four microarray experiments (ie all four timepoints) (Table 1-6). Ephx1 is a microsomal enzyme involved in metabolism of various toxins in the liver [271].

Timepoint	Fold change	P value
4h in vitro	-1.73	0.00021
24h in vitro	-2.62	0.000037
48h in vivo	-2.45	0.00021
72h in vivo	-4.59	0.0000021

Table 1-6 Downregulation of Ephx1 by CD27 signalling in all microarray experiments

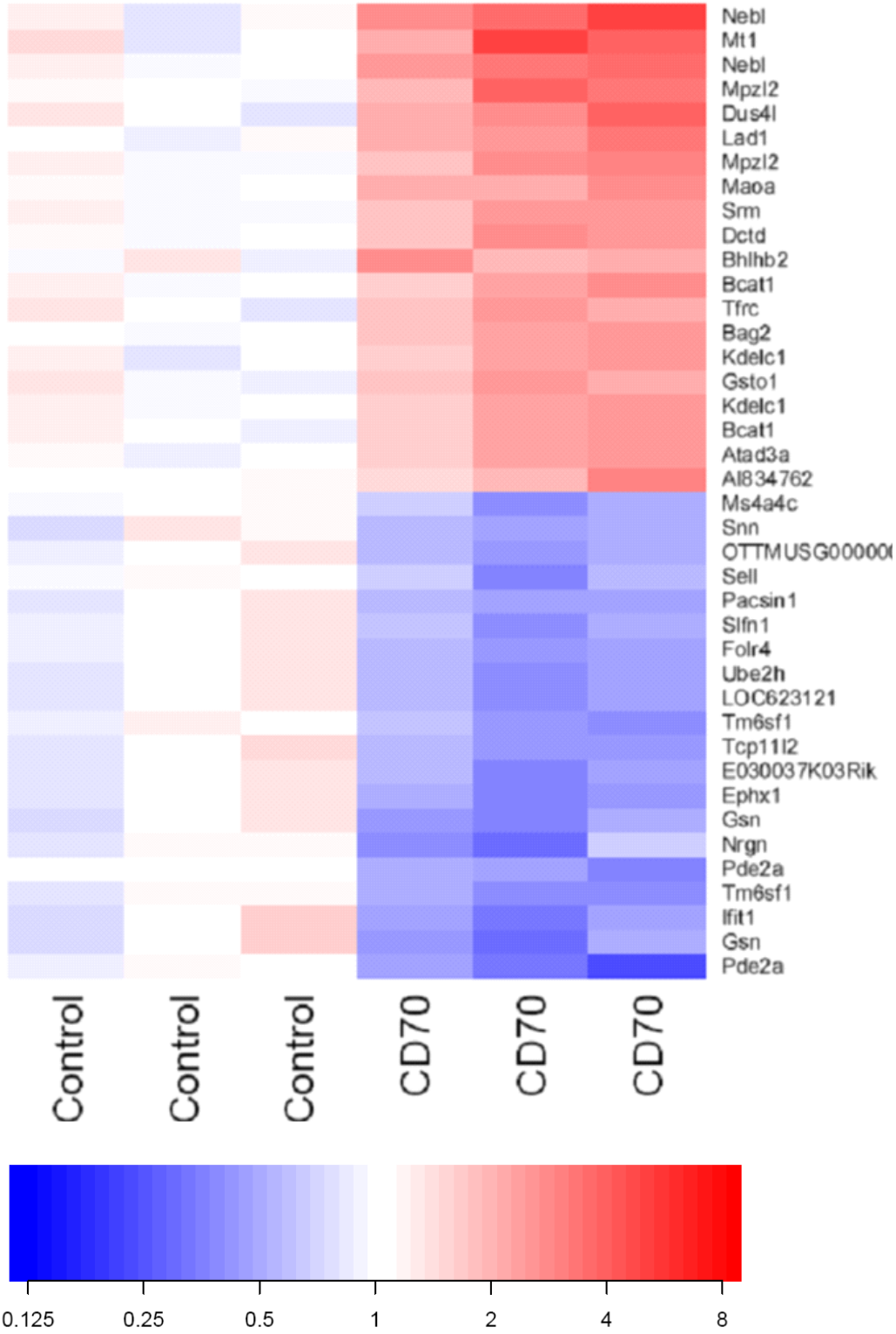

Figure 28 Heatmap showing genes significantly altered by CD27 signalling *in vivo* at the 48h timepoint: experiments were set up as in Figure 24. Only genes significantly different ($p<0.001$) are shown; colour intensity represents expression relative to the global gene expression. For example a gene coloured deep red is expressed 8 fold higher than the median expression of all 40,000 genes on the chip.

Symbol	Name	Fold Change	P value	Description
Nebl	(probe-1) LIM LIM-nebulette	3.57	3.2E-05	Actin binding protein
Nebl	(probe-2) LIM LIM-nebulette	2.93	3.4E-06	
Lad1	ladinin	2.59	3.1E-05	Possible anchoring to basement membrane
Maoa	monoamine oxidase A	2.24	1.5E-05	Monoamine catabolism
Dctd	dCMP deaminase	2.21	4.6E-05	Nucleotide catabolism
Bag2	Bcl2-associated athanogene 2	2.13	2.0E-05	Regulation of apoptosis
Itga6	integrin alpha 6	-2.00	3.7E-05	Leucocyte adhesion molecule
Bach2	BTB and CNC homology 2	-2.02	2.0E-05	Transcription factor affecting BCL2 and IL-2
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-2.48	4.0E-06	Catalytic activity
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-3.23	3.5E-05	

Table 1-7: List of genes upregulated by CD27 signalling *in vivo* at the 48h timepoint: RNA was purified from CD27 and control stimulated CD8$^+$ T cells (with the same protocol as Figure 24). Genes significantly up- or down-regulated as detected by expression microarray are shown. Only those genes experiencing a greater than 2 fold change at a significance level of $p<0.001$ are shown. A more complete list is shown in the appendix (page 295)

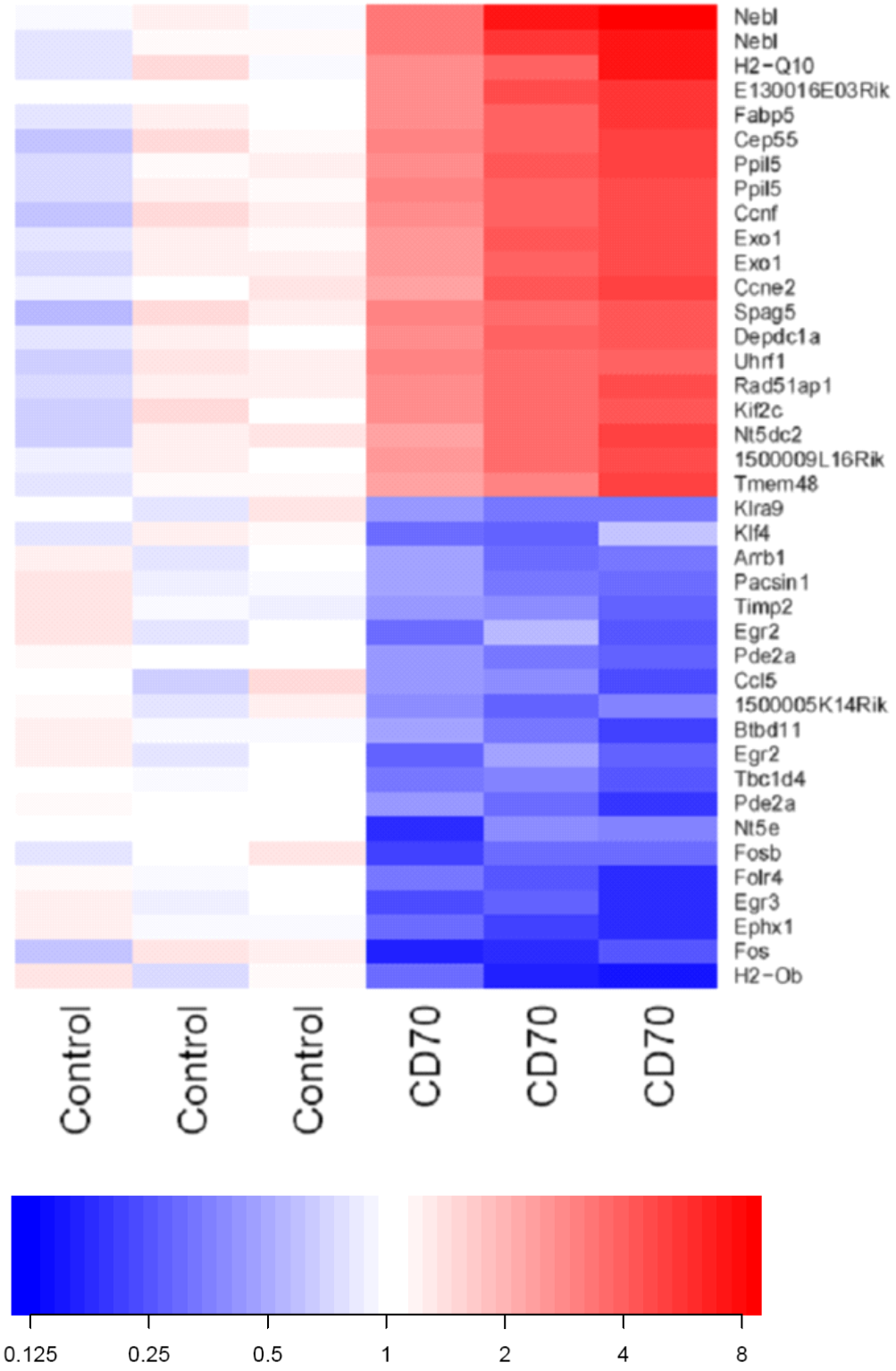

Figure 29 Heatmap showing genes significantly altered by CD27 signalling *in vivo* at the 72h timepoint: experimental setup was as described in Figure 24. Genes significantly different ($p<0.001$) are shown; colour intensity represents expression relative to the global gene expression. For example a gene coloured deep red is expressed 8 fold higher than the median expression of all 40,000 genes on the chip.

Symbol	Description	Fold Change	P value	Description
Nebl	(probe-1) LIM LIM-nebulette	6.49	2.1E-04	Actin binding
Nebl	(probe-2) LIM LIM-nebulette	4.80	4.0E-05	
H2-Q10	histocompatibility 2, Q region locus 10	4.19	4.5E-04	MHC Class Ib molecule
RAD54 homolog B	RAD54 homolog B	3.97	1.8E-05	DNA break repair
Fabp5	fatty acid binding protein 5, epidermal	3.72	8.6E-05	Fatty acid metabolism
Cep55	centrosomal protein 55	3.71	5.3E-04	Microtubule bundling
Ppil5	peptidylprolyl isomerase (cyclophilin) like 5	3.67	1.0E-04	Suppresses TRAF2 signalling
Ccnf	cyclin F	3.58	4.8E-04	Regulation of cell-cycle transition
Exo1	exonuclease 1	3.49	1.1E-04	DNA mismatch repair
Ccne2	cyclin E2	3.47	2.0E-04	Progression to G1 cell cycle phase
Spag5	sperm associated antigen 5	3.44	6.7E-04	Sperm cell surface antigen
Depdc1a	DEP domain containing 1a	3.40	3.2E-05	Function unknown
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	3.40	1.1E-04	Maintenance of DNA methylation
Rad51ap1	RAD51 associated protein 1	3.37	8.3E-05	DNA recombination
Kif2c	kinesin family member 2C	3.37	2.7E-04	Mitosis
Nt5e	5' nucleotidase, ecto	-3.60	6.1E-05	CD73 – expressed on lymphocytes and some tumours

Symbol	Description	Fold Change	P value	Description
Fosb	FBJ osteosarcoma oncogene B	-3.90	1.8E-05	AP-1 transcription factor partner gene
Folr4	folate receptor 4 (delta)	-4.27	1.1E-05	Folate acid receptor – T_{reg} function
Egr3	early growth response 3	-4.54	1.7E-06	Immediate response genes
Ephx1	epoxide hydrolase 1, microsomal	-4.59	2.1E-06	Microsomal enzyme
Fos	FBJ osteosarcoma oncogene	-5.34	4.4E-05	AP-1 transcription factor partner gene
H2-Ob	histocompatibility 2, O region beta locus	-5.47	4.3E-05	Class II MHC like molecule

Table 1-8 Most significantly altered genes by CD27 signaling at the 72h timepoint *in vivo*: experimental setup was as described in Figure 24. Only the genes with the highest fold changes are shown – a full list is in the appendix (page 297).

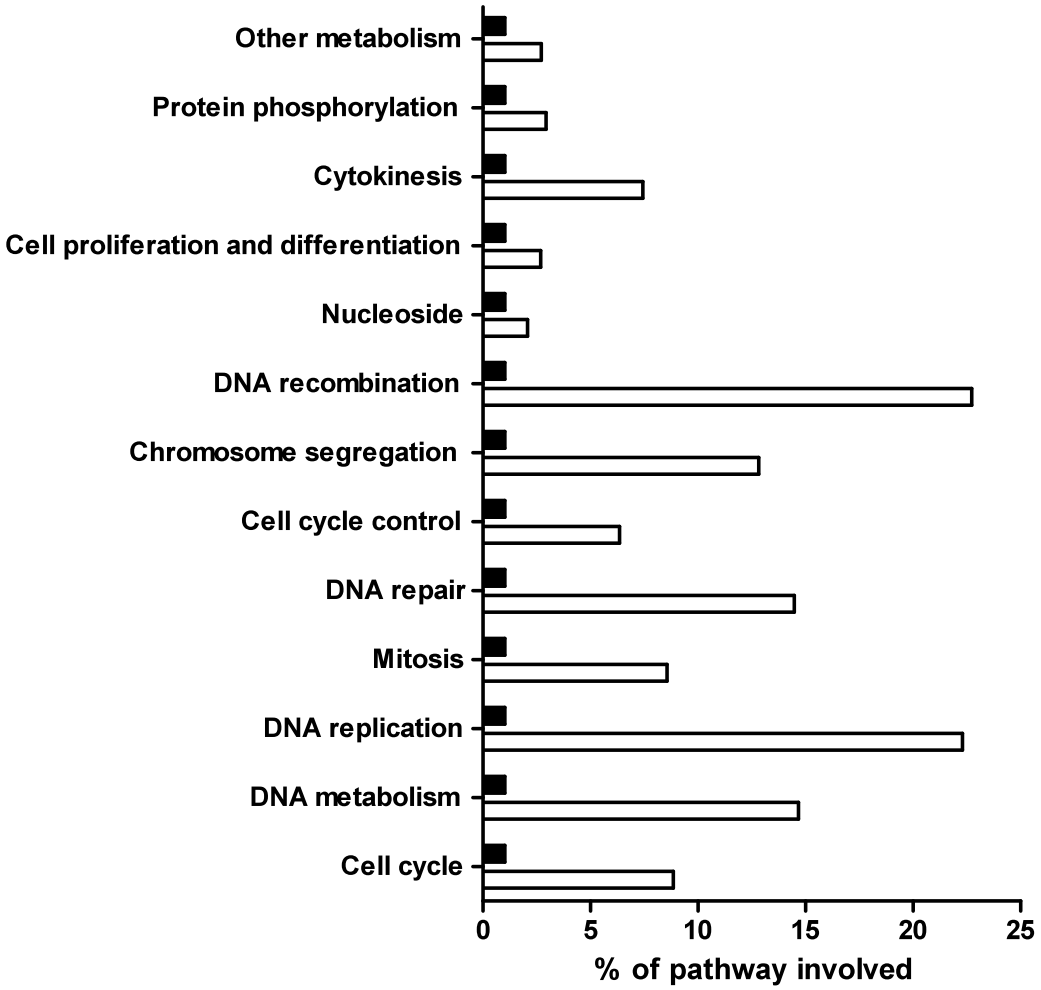

Figure 30 Biological pathways upregulated at the 72h *in vivo* timepoint: gene expression data shown in Figure 30 was analysed: all genes significantly upregulated by a fold change of greater than 2 were interrogated by the Panther database. Genes were grouped according to biological processes and the percentage of genes overexpressed from each pathway is shown (open bars) compared to the expected number from that process (filled bars). Results are shown in increasing order of significance, ie the most significantly over-represented biological process was 'cell cycle' ($p\ 9.31 \times 10^{-55}$)

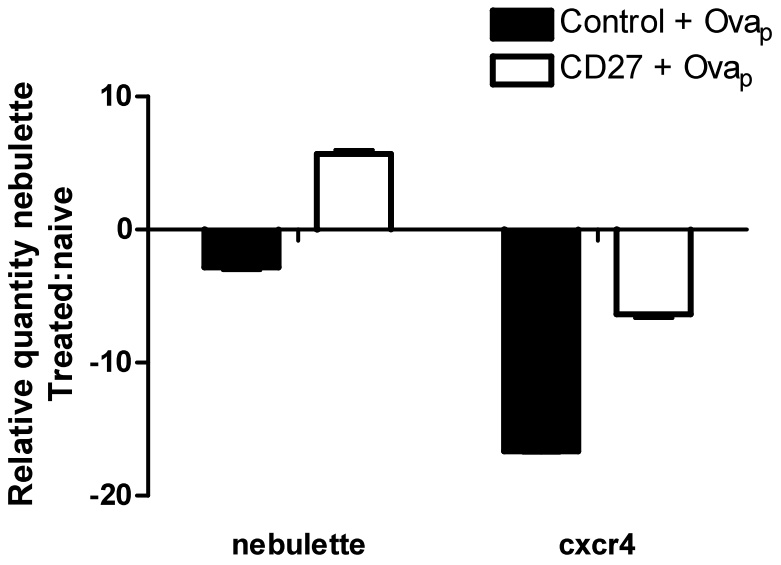

Figure 31 Expression of LIM-nebulette and CXCR4 – 72h timepoint in vivo: RNA purified from in vivo CD27 or control stimulated CD8+ T cells (from experiment described in **Figure 24**) was compared to RNA generated from purified naïve OT-I cells. Quantitative PCR was used to measure the expression of LIM-nebulette and CXCR4 in stimulated cells and naïve cells. CD27 signalling enhanced the expression of LIM-nebulette. TCR signalling resulted in the downregulation of CXCR4 expression, but this was abrogated by the presence of CD27 signalling. Results are representative of two separate experiments.

3.6. Discussion

Consistent with previous findings in this laboratory, a soluble form of CD70 (sCD70), the natural ligand for CD27, was generated. CD70 is predicted to form self assembling non-covalent trimers; this is based on crystallographic structures of other TNF family members; CD40L, TNFα and LTα all form trimers with each monomer folding as a compact 'jellyroll' (β pleated sheets with Greek key topology) [272]. The crystal structure of CD70 has not been elucidated, but purified murine CD70 is trimeric when analyzed under non-reducing conditions [273]. In the data presented in this chapter a soluble form of CD70 was generated by fusing the N-terminus of the extracellular domain of CD70 to the C-terminus of the Fc fraction of human IgG1. The presence of the human Fc dimer drives formation of hexamers and higher order multimers via disulphide and non-covalent interactions.

sCD70 formed dimers, trimers, and higher order multimers when examined by SDS-PAGE; when disulphide bonds were broken by the reducing agent DTT, sCD70 was resolved on an SDS-PAGE gel as a monomer. The disulphide bonds are likely to have formed between the hinge regions of the IgG1 Fc domain, although disulphide bonds have been shown to form within other TNF members [274]. The predicted structures of sCD70 multimers are shown in Figure 15. sCD70 has biological activity without the need for chemical cross linkers; this is likely to be due to the presence of higher order multimers; other TNFR family members have been shown to be unresponsive to trimeric ligands, and that higher order oligomeric ligands are required [242, 275]. sCD70 promoted $CD8^+$ T cell responses *in vitro* and *in vivo*, and this was used to study the effects of CD27 signalling at the genetic level. It is notable that CD27 signalling caused many more gene expression changes late in the T cell stimulation process. 536 genes were altered at 72h compared with 51 at 48h, with similar differences between the 24 and 4h timepoints *in vitro*. This may because the effects of CD27 signalling are delayed, or it may imply that although CD27 is expressed constitutively in resting T cells, its function becomes much more dominant after it is upregulated by TCR engagement.

3.6.1. CD27 costimulation upregulates the Batf3 gene in vivo and in vitro

CD27 signalling upregulated Batf3 expression by a factor of 4.14 *in vitro*. It was also upregulated at 48hours following stimulation *in vitro* and at 48h and 72h *in vivo*. Batf3 is a transcription factor that binds to Jun. The transcription factor Jun (or its isoforms JunB and JunD) binds to various partner proteins, notably Fos, but also members of the ATF family, to form a heterodimer known as AP-1. The term AP-1 collectively describes a number of protein complexes which may be formed from various combinations: homodimers of Jun, or heterodimers of Jun with Fos, ATF or JDP family members [276]. The defining feature of AP-1 is its binding to AP-1 responsive elements within the genome (TRE sequences). Different AP-1 complexes can have dramatically different effects; AP-1 is important in mediating FasL induced cell death, but AP-1 can also provide anti-apoptotic and proliferative signals.

Given the large number of different molecules that can be described by the term 'AP-1' it is not surprising that AP-1 activity can result in varied and often opposing cellular outcomes. Combinatorial transcription factors, ie those composed of several different proteins, greatly increase the total number of transcription factors eg four isoforms of Jun and four isoforms of Fos can generate at least 22 combinations from eight genes; increasing the number of proteins involved (eg AP-1 is known to interact with NFAT) in the transcription complex exponentially increases the number of potential transcription factors; without this system, a large proportion of the genome would be given up to genes for individual transcription factors [277].

CD27 signalling is known to trigger AP-1 activity: CD27 signals via TRAF2 to cause Jun phosphorylation via Jun N-terminal kinase (JNK). [278]. JNKs (of which there are three isoforms, JNK 1, 2 and 3) are part of the mitogen activated protein kinase (MAPK) family (see above). Once activated, JNKs translocate to the nucleus where they can phosphorylate Jun, increasing its transactivation potential and increasing the production of proteins such as IL-2 (Figure 32).

AP-1 is involved in many immunological processes; AP-1, in conjunction with another transcription factor, NFAT, AP-1 is involved in the transcription of IL-2 [279]. JunD suppresses lymphyocyte proliferation, and Jun B deficiency causes a myeloproliferative disease in mice which has similarities to chronic myeloid leukaemia in humans. Batf3 has been shown to bind to Jun in place of Fos, resulting in reduced AP-1 activity [280]. Batf3 can compete with Fos for binding to Jun, resulting in decreased AP-1 activity, with resultant reduced production of IL-2 in Jurkat cells [266]. It is notable that Fos was downregulated by CD27 signalling (-5.34 fold change at 72h *in vivo*), and it is possible that this, in conjunction with Batf3 production acts to attenuate AP-1 activity. JNK activation is known to normally lead to *increased* Fos expression; the data on Fos presented here are from relatively late timepoints (eg 48 and 72 hours *in vivo*); Fos was originally described to be upregulated very early (within minutes) in lymphocyte activation [242],and therefore the downregulation of Fos seen with CD27 may represent part of a negative feedback loop.

Recently Batf3 -/- mice have been generated and show a severe defect in $CD8^+$ DC and resultant reduction in the ability to cross-present to $CD8^+$ T cells, resulting in reduced resistance to viral infection and tumour challenge [265]. This effect was not mediated by any $CD8^+$ T cell dysfunction: Batf3 -/- $CD8^+$ T cells responded normally to viral challenge, and manufactured normal amounts of IL-2 (suggesting other pathways are active in the control of IL-2 production *in vivo*).- but its not known if under these conditions Batf3 is induced.

In summary, AP-1 is a transcription factor complex composed of varying heterodimers, including Jun with Batf3. It is clear that Batf3 gene expression was upregulated by CD27 signalling in two separate systems, one *in vivo* and one *in vitro*, and it is plausible that CD27 may mediate some of its effects through Batf3 but that there is enough redundancy in the system for normal $CD8^+$ T cell function in the absence of Batf3. It is logical that after an initial burst of JNK mediated AP-1 activity a negative feedback is required to prevent run away cellular proliferation and IL-2 production, and this might be the function of Batf3 upregulation at 24, 48 and 72 hours (in conjunction with Fos downregulation). Alternatively, the pro-apoptotic molecule Bim is under the

control of AP-1 [281]; a dominant negative form of c-Jun has been shown to reduce Bim expression in neurons [276]. It is possible that Batf3 may have a similar role in leading to reduced expression of Bim at the peak of a $CD8^+$ T cell response (as was detected in the experiments presented here) which may in turn promote T cell survival.

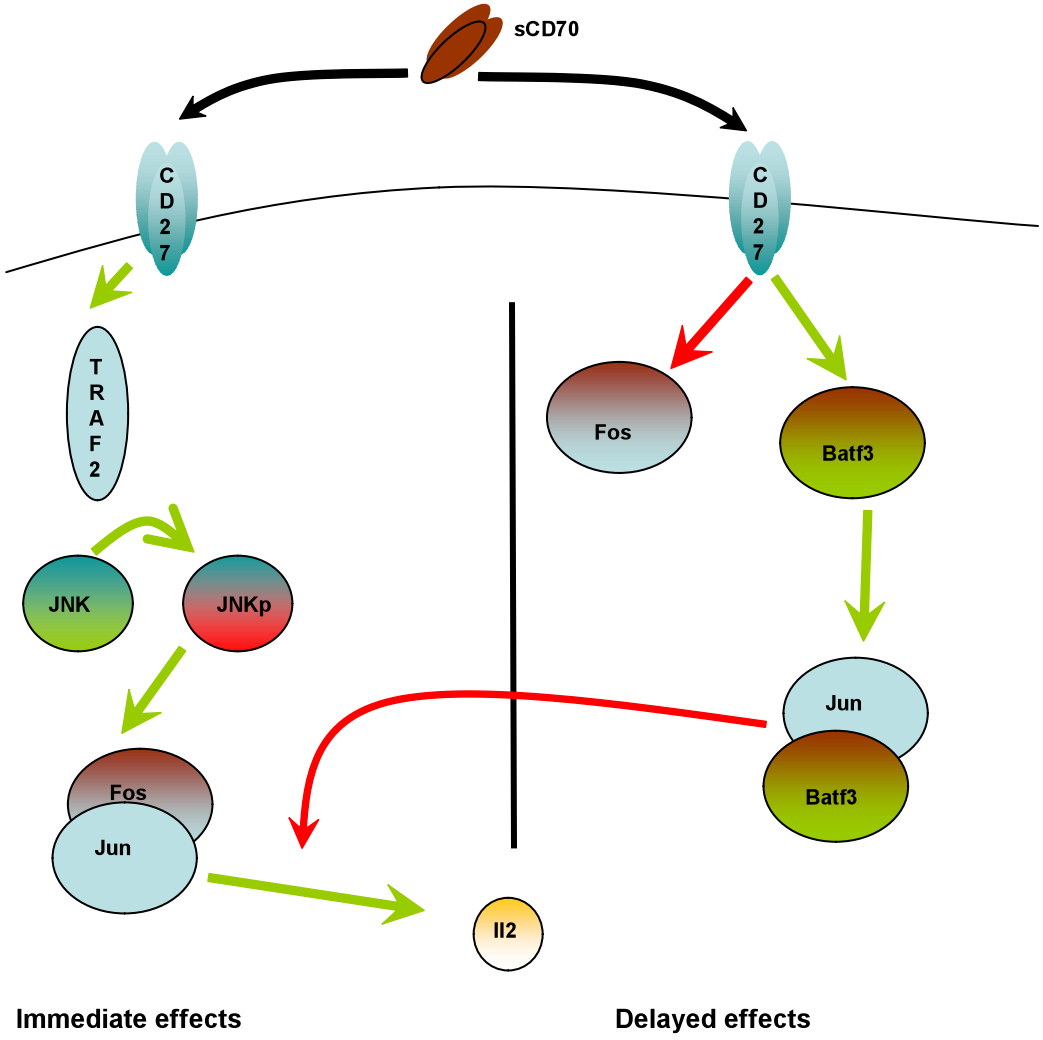

Figure 32 Putative role for *Batf3* in CD27 signalling: it is already known that CD27 signalling leads to Jun phosphorylation via Traf 2 (left side of diagram, 'immediate effects'). We have shown that CD27 downregulates Fos and upregulates expression of Batf3 – this may suppress AP1 activity, with reduced production of proteins known to possess AP1 promoter sites, such as IL2

3.6.2. CD27 signalling downregulates Egr1 Egr2 and Egr3

Fos is one of around 80-100 genes induced very early in lymphocyte activation; studies of lymphocyte activation have repeatedly identified the zinc-finger containing transcription factor Egr1 as upregulated by activation. Various proteins relevant to the immune response contain binding sites for Egr1, such as TNF, IL-2 and CD44; blockade of Egr1 by antisense oligonucleotides *in vitro* blocks TCR and IL-2 mediated T cell proliferation, Other studies have confirmed the importance of Egr1 in IL-2 upregulation, where it contributes between 40 – 80% of the IL-2 promoter activity. Redundancy in the system does however remain, in that Egr1 knockout mice seem to develop normally, and show normal immune responses [282].
Egr2 and Egr3 are transcription factors known to have a role in T cell signalling: all three are induced on TCR engagement; Egr1 upregulates IL-2 and CD154, whilst Egr2 and Egr3 upregulate FasL expression [283]. Egr2 and Egr3 have recently been shown to be upregulated in $CD8^+$ T cells primed to undergo tolerance or deletion [284]. T cells from Egr 3 knockout mice are resistant to anergy induction *in vivo*. It has been postulated that Egr2 and Egr3 act to oppose runaway TCR signalling as part of a negative feedback loop; it can be inferred that CD27 signalling may enhance the positive effects of TCR engagement partly by suppression of transcription of these molecules.

3.6.3. CD27 costimulation upregulates the gene for LIM-nebulette

LIM-nebulette was detected as upregulated by CD27 signalling by two probes at both the 48h and 72h timepoints. LIM-nebulette is a splice variant of a cardiac actin binding protein called Nebulette. Nebulette is an (107kDa) actin-binding protein detectable in myofibrils [285]. It is detectable in humans and mice, with 99% sequence homology. Mutations of nebulette may cause cardiomyopathies in humans [286]. LIM-nebulette is detectable in various non-cardiac tissues [287], where it interacts with Zyxin, and adaptor proteins

involved in focal adhesions: focal adhesions link the extracellular matrix to the cytoskeleton, and are involved in cell adhesion and motility.

It is interesting that CD27 signalling affects expression of an actin-binding gene – actin is important in T lymphocyte function; mutations in genes controlling actin polymerization can cause immunodeficiency in humans (eg Wiskott Aldrich syndrome) [288] and actin polymerization disrupts TCR mediated calcium influx. Actin is a ubiquitous and highly conserved protein involved in muscle contraction, cytoskeleton, cell motility and cellular organelle transport.

T lymphocytes employ changes in the cytoskeleton for many of their functions [289]. Circulating T lymphocytes are covered in microvilli containing parallel actin bundles; these microvilli are involved in T lymphocyte tethering and rolling along the endothelial wall; under the appropriate chemokine signals these microvilli collapse enabling the formation of tight adhesion to the endothelial wall, and T cells can squeeze through tight endothelial junctions; as the T cell migrates through the endothelium it has a leading edge rich in branched actin filaments, which pull the T lymphocyte along; T lymphocytes within tissues are highly motile, reaching speeds of 25μm/min, allowing them to sample multiple APCs [290].

Actin changes are also present at the Junctions formed between the lymphocyte and APCs, and are responsible for formation of the *immunological synapse* (IS). The IS is organized into *supramolecular activation complexes* (SMAC) [291]. Central, peripheral and distal areas of the synapse exist (cSMAC, pSMAC and dSMAC); cSMAC is enriched in TCRs (Figure 33) and the downstream signalling effector protein kinase θ. pSMAC is enriched with cell adhesion molecules such as LFA1/ICAM1. Initially in T cell/APC interactions, as few as ten TCR/MHC-peptide interactions may be enough to generate a TCR signal; following this, a nascent IS is formed aided by costimulatory molecules such as CD28 [292].

Much of the evidence for IS formation comes from the study of $CD4^+$ T cell/APC interactions: studies of $CD8^+$ T cell synapses with target cells (rather than cross-presenting APCs) show evidence of a small area of the cSMAC devoid of TCRs containing cytotoxic granules[293]. The relevance of this is that $CD8^+$ T cells show distinct cytoskeletal changes associated with target

cell killing, and the finding that CD27 signalling leads to increased expression of LIM-nebulette suggests possible CD27 mediated effects on $CD8^+$ T cell motility and immunological synapse formation which warrants further investigation. That costimulatory molecules may have a role in the IS formation is not without precedent, as has been shown for CD28 [294]. Contrary to LIM-nebulette being involved in IS formation, is the fact that the most intense period of $CD8^+$ T cell/DC interaction takes place within the first 24 hours of $CD8^+$ T cell priming [165]. It may be that CD27 signalling could alter the timing of DC/$CD8^+$ T cell interactions, although this remains to be seen.

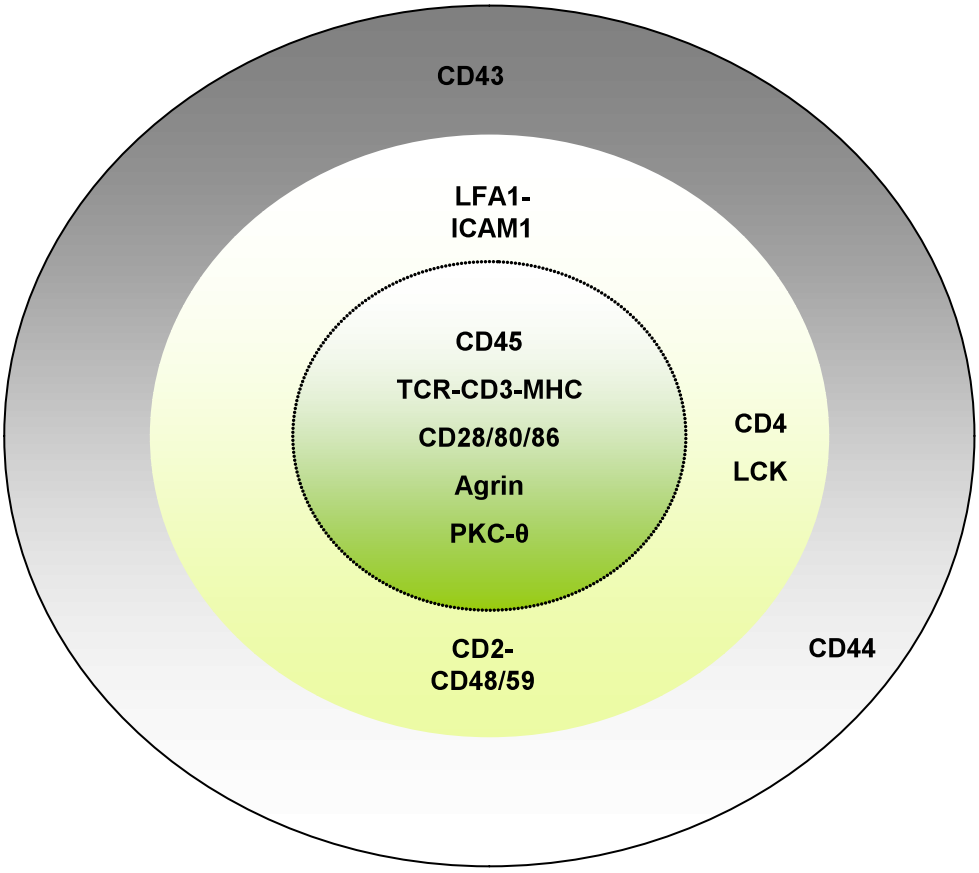

Figure 33 The immunological synapse: organized into supramolecular activation complexes – central (cSMAC) in green, peripheral (pSMAC) in yellow and distal (dSMAC) in grey,

3.6.4. CD27 upregulates genes involved involved in DNA replication

A large number of biological processes were significantly over-represented at the 72h timepoint (Figure 30). Genes involved with DNA replication, cell cycle, and repair, were strongly over-represented, implying that CD27 signalling drives a proliferative response; at this timepoint, only 5 genes from the 'apoptosis' biological process were over-expressed; this would fit with previous observations that suggest that CD27 signalling provides a strong proliferative stimulus to $CD8^+$ T cells [295].

The Panther classification has grouped most of the mouse genome into various biological processes. The term 'biological process' is a standard one in the field of gene ontology, and is defined as "series of events accomplished by one or more ordered assemblies of molecular functions" [296].

The Panther Classification also curates genes into well defined 'pathways' – these are more exacting than 'biological processes', with information included on the order and dependency of each item within a biological pathway. CD27 signalling significantly upregulated the 'DNA replication' pathway – a series of integrated protein-protein and protein-DNA interactions and enzymatic reactions to ensure high accuracy of DNA replication (Figure 34). 6 of the 21 genes from this pathway were upregulated (

Table **1-9**). These findings, at the gene expression level, provide evidence that CD27 exerts many of its effects on $CD8^+$ T cells through driving proliferation.

Gene	Fold change
Topoisomerase 2α	2.14
Polymerase (DNA directed) δ1 catalytic subunit	2.1
Polymerase (DNA directed) δ2 regulatory subunit	2.02
Polymerase (DNA directed) α1 subunit	2.31
DNA primase p49 subunit	2.37
DNA primase p58 subunit	2.3

Table 1-9 Genes from 'DNA replication' pathway upregulated by CD27 signalling *in vivo* at the 72h timepoint

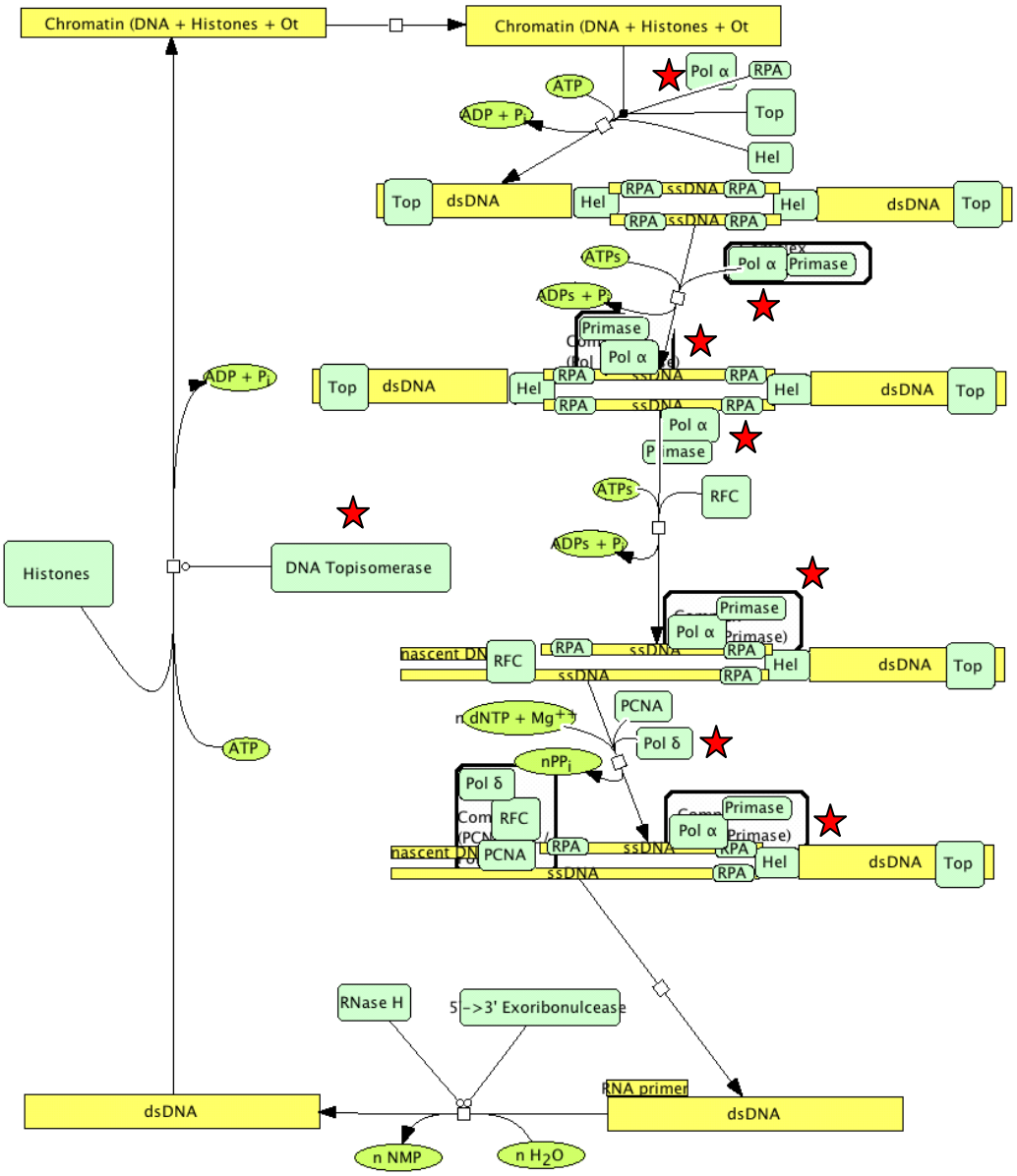

Figure 34 Schematic of the Panther classification database pathway 'DNA Replication': genes that were upregulated by CD27 signalling *in vivo* at the 72h timepoint are starred.

3.6.5. Downregulation of Ephx1

Ephx1 (epoxide hydrolase) is a microsomal enzyme predominantly expressed in the liver. It is thought to play a role in the metabolism of toxins and many pharmaceutical agents. There is no published evidence of its involvement in immune system function, although it was recently detected by microarray as over-expressed in $CD8^+$ T lymphocytes programmed for deletion/tolerance [284]. Ephx1 polymorphisms have been associated with various disease states [297], although this association may have been due to genotyping errors in some cases [298]. From the perspective of the experiments detailed in this chapter it is noteworthy purely because of the regularity with which it was significantly altered by CD27 signalling (Table 1-6)

3.7. Key findings

In summary, CD27 costimulation of $CD8^+$ T cells leads to multiple transcriptional changes. CD27 is consitutively expressed on resting $CD8^+$ T lymphocytes, setting it apart from several similar TNFR molecules, which are only expressed after TCR stimulation; it might be supposed that the effect of CD27 signalling would be greatest early on in T cell activation. In fact, the converse appears to be true, with many more genes affected at 24 h than 4 h *in vitro,* and similarly *in vivo*. By the 72h timepoint, many genes involved cell cycle control and related functions were significantly upregulated by CD27 signalling; this mirrors the effect seen at the cellular level; T cell numbers were similar *in vivo* at 48 hours whether CD27 costimulation was present or not, but by 72h the number of cells was doubled by CD27 signalling (Figure 24).

Key individual genes affected by CD27 signalling include Batf3; significantly upregulated *in vitro* and *in vivo*, Batf3 dimerizes with Jun, possibly acting as part of a negative feedback loop involved in IL-2 expression. Also involved in IL-2 is the early/immediate transcription factor Egr1; this molecule was downregulated by CD27 *in vivo* and would fit with a negative feedback loop involved in IL-2 control.

Consistent with a role in IL-2, the IL-2 receptor (CD25) was significantly upregulated *in vivo* at the 72 hour timepoint, a factor that is associated with effector memory differentiation [299]. This finding has subsequently been confirmed at the protein level, where CD27 primed $CD8^+$ T cells express significantly higher levels of CD25 (A. Al-Shamkhani, personal communication).

The pro-apoptotic molecule Bim was significantly downregulated *in vitro* and *in vivo.* This molecule is known to be important in the contraction phase of the $CD8^+$ T cell response; that CD27 reduces its expression supports a role for CD27 signalling in promoting T cell survival. Analysis of pathways affected by CD27 suggest that, in addition to its effect on survival, CD27 signalling has marked effects on cellular proliferation.

Significantly upregulated by CD27 signalling was LIM-nebulette, a molecule involved in the actin cytoskeleton; this may represent a pathway mediating functional changes in activated $CD8^+$ T cells that may affect the T cell synapse, or ability to migrate and interact with the endothelium.

Thus CD27 signalling leads to multiple and diverse changes in the gene expression profile of $CD8^+$ T lymphocytes.

Chapter 4. Comparison of 4-1BB and CD27 costimulation

4.1. Introduction

The previous chapter examined the gene expression profile of $CD8^+$ T cells activated in the presence and absence of CD27 signalling. This chapter will seek to compare the effects of CD27 signalling to another well described TNFR known to be involved in $CD8^+$ T cell costimulation; 4-1BB (CD137). In contrast to CD27, 4-1BB is not expressed on naïve $CD8^+$ T cells; its expression occurs following TCR ligation. It is present on the cell surface as monomers, dimers and possibly tetramers [300]. 4-1BB signals through TRAFs 1 and 2 (Sabbagh, Pulle et al. 2008) (and Traf 3 in humans). There is evidence that 4-1BB, like CD27, signals through NF-κB and JNK [140]. Thus there are great similarities between CD27 and 4-1BB.

Although both CD27 and 4-1BB have a wide distribution on cells of the immune system, both seem to have their greatest effect on $CD8^+$ T cells. A recent comparison of primary and memory responses to influenza infection in CD27 and 4-1BBL knockout mice showed the greatest reduction in primary responses occurred when CD27 was absent [301]. Resulting memory populations were of similar size, but secondary expansion was lower when 4-1BB signals were absent.

Not all studies show a positive effect of 4-1BB signalling on $CD8^+$ T cell reseponses: 4-1BB agonistic antibodies, whilst clearly enhancing CD8+ T cell responses, can show immunosuppressive effects [302]. 4-1BB costimulation can abrogate humoral responses, reversing autoimmunity in mouse models of lupus, or EAE: 4-1BB -/- mice show paradoxically enhanced CD4+ T cell responses to soluble antigen [303]. More recently 4-1BB has been described on $CD4^+CD25^+$ regulatory T cells, and has been shown to enhance their

proliferation in vitro, and these cells were capable of inhibiting allogeneic islet cell transplantation rejection [304].

Another potentially negative effect of 4-1BB/4-1BBL interactions on immune function has recently been reported [305]: it has been shown that some myeloid progenitor subsets express 4-1BBL, and that myeloid lineage and dendritic cell numbers are increased in 4-1BBL knockout mice.

Taken together these results suggest that 4-1BB targeting to generate immune responses remains an uncertain approach, with the possibility of negative as well as positive immune effects. The purpose of this chapter was to examine if the subtle differences between the two (different expression kinetics and different combinations of Traf adaptor molecules) lead to meaningful functional differences in the physiological $CD8^+$ T cell response.

4.1.1. Primary and memory $CD8^+$ T cell responses differ depending on costimulatory signal

To compare CD27 and 4-1BB costimulation of $CD8^+$ T cells, an established system of soluble antigen immunisation was used. OT-I TCR-transgenic mice were used as a source of $CD8^+$ T cells. OT-I $CD8^+$ T cells are specific for a peptide derived from chicken ovalbumin ($OVA_{257-264}$) with the peptide sequence SIINFEKL, restricted to MHC Class I H-$2K^b$. OT-I $CD8^+$ T cells constitutively express CD27, upregulate 4-1BB after TCR engagement, and have a naïve phenotype ($CD25^{low}$ $CD44^{low}$ $CD62L^{high}$) [243, 306].

To compare the effects of CD27 and 4-1BB costimulation, 10^4 OT-I $CD8^+$ T cells were adoptively transferred into normal syngeneic C57BL/6 mice. The mice were then challenged ip with 5mg ovalbumin (whole protein) and either anti-4-1BB (clone Lob 12.3, IgG1) or anti-CD27 (AT124.1, IgG2a) or control (MC10-6A5 rat IgG2a) antibodies (250µg d0, d1). Antigen specific $CD8^+$ responses were followed up in peripheral blood by flow cytometry (FCM). Samples were stained and gated on live lymphocytes and the percentage of $CD8^+$ Kb-$ova_{257-265}$ positive cells was determined.

Ovalbumin protein alone did not affect antigen specific T cell (ASC) numbers in peripheral blood (Figure 35). Both 4-1BB and CD27 caused ASC proliferation during the primary response; the primary response occurred earlier with CD27 than with 4-1BB, peaking at day 6 and 10 respectively. 53 days following the primary antigenic stimulus all groups were rechallenged with $OVA_{257-264}$ peptide iv and anti-CD40 (3/23 250µg) ip. Costimulation during priming enhanced subsequent $CD8^+$ T cell memory responses, but this effect was much stronger if anti-4-1BB was used compared with anti-CD27. The magnitude of the secondary responses was higher, and developed more rapidly than primary responses, confirming that they were true memory responses. The small memory response seen in the control arm was presumed to be due to contaminating lipopolysaccaride present in the ovalbumin preparation.

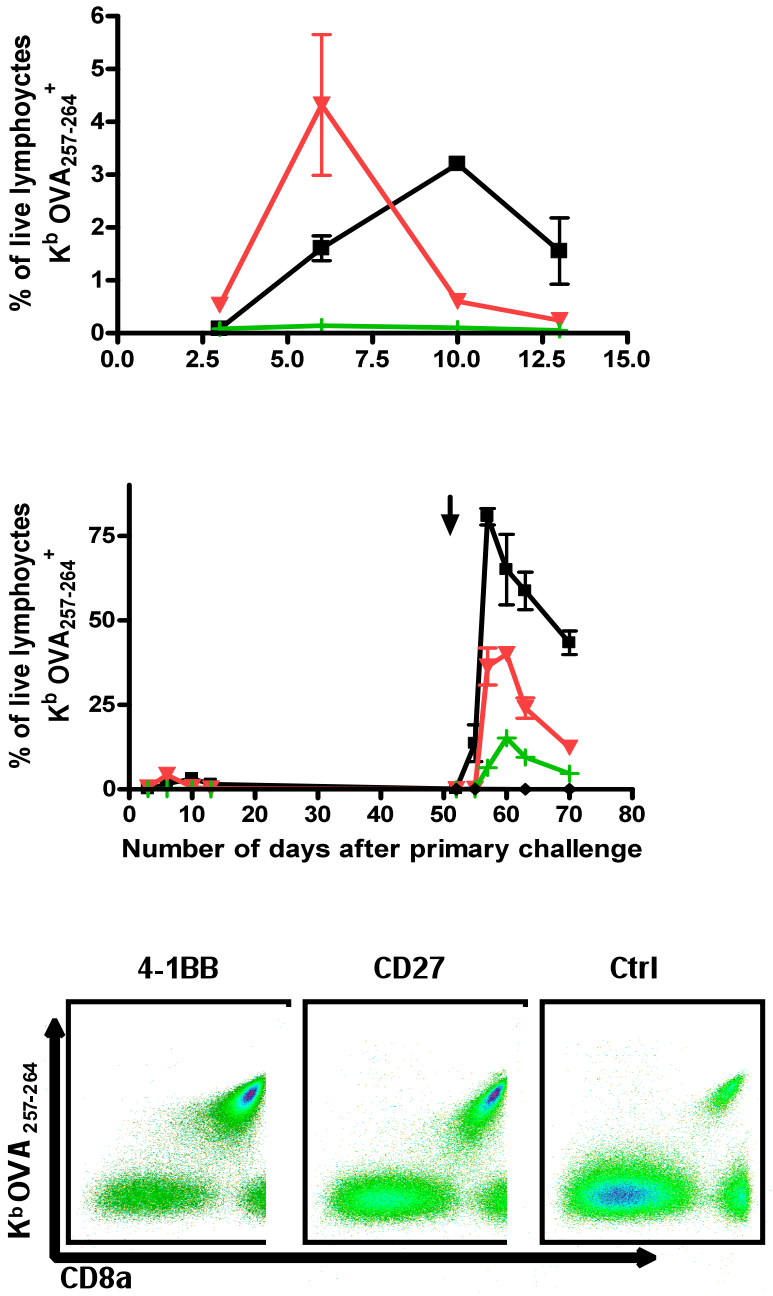

Figure 35 Comparison of 4-1BB and CD27 costimulation: 1 x 10^4 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice (3 per group)were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■) anti-CD27 (▼) or control (MC10-6A5) (+) antibody (250µg ip d0,1). Both 4-1BB and CD27 boosted the primary CD8+ T cell response (above). The peak of the response occurred earlier in the CD27 group, but the decline was faster. The secondary response (middle) to ova peptide ($ova_{257-264}$) with 250µg αCD40 (arrow) was greatest in the 4-1BB group. There was no response in naïve OT-I cells (♦). Representative plots from the peak of the secondary response are shown (below) gated on live lymphocytes. Error bars represent the SEM.

4.1.2. Resting memory $CD8^+$ T cell distribution

The enhanced memory response with 4-1BB costimulation may have been due to the generation of higher numbers of resting memory cells, or alternatively the generation of memory cells with enhanced proliferative capacity. To examine whether 4-1BB costimulation generated higher numbers of resting memory cells, 10^6 OT-I $CD8^+$ T cells were adoptively transferred into C57BL/6 syngeneic recipients. 24 hours later, mice were challenged with ovalbumin protein (5mg ip) and anti-4-1BB or anti-CD27 antibody. ASC were enumerated 65 days later in spleens, liver and bone marrow. ASC numbers were significantly higher when 4-1BB costimulation had been given during priming (Figure 36). This provided evidence that some of the enhanced memory response with 4-1BB costimulation was due to the generation of higher numbers of resting memory cells.

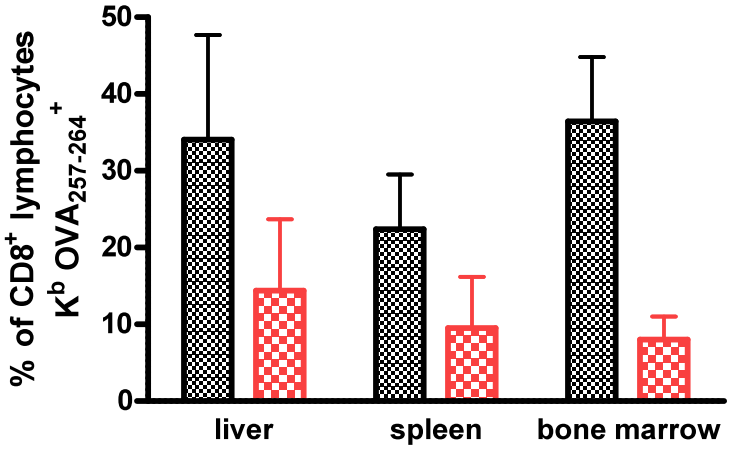

Figure 36 Tissue distribution of memory CD8$^+$ T cells: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and either anti-4-1BB (black) or anti-CD27 (red) antibody (250mcg ip d0,1). 65 days later lymphocytes were harvested from the tissue sites indicated, and the percentage of antigen specific CD8$^+$ T cells measured by flow cytometry. The enhanced CD8$^+$ memory generation with 4-1BB (black) was apparent in spleen, liver and bone marrow. The difference between 4-1BB and CD27 was significantly different ($p=0.0129$, unpaired Student's T test.

4.1.3. Soluble 4-1BBL forms high and low molecular weight fractions

The data above suggested that 4-1BB costimulation generated higher numbers of resting memory cells than CD27 costimulation, and this translated into an enhanced secondary response to rechallenge. This could be a physiological effect, reflecting biological differences between CD27 and 4-1BB costimulation, or it may be due to differences in the immunostimulatory capacities between antibodies due to the different ability of various antibody isotypes to interact with Fc receptors, the different epitopes recognised or differences in affinity [307]. To confirm that the effect on ASC memory generation was physiological, an alternative system was developed using soluble forms of the ligands for 4-1BB and CD27 (4-1BBL and CD70 respectively). The soluble form of CD70 (sCD70) has already been described in the previous chapter. A recombinant soluble form of 4-1BBL consisting of 4-1BBL extracellular domain and the Fc region of human IgG1 (Figure 37) has previously been generated and shown to posses costimulatory activity in preliminary studies (T Rowley, unpublished observations). s4-1BBL was expressed in mammalian Chinese hamster ovary cells and purified by immunoaffinity chromatography in the same way as sCD70 (using anti-human Fc mAb column).

s4-1BBL was fractionated by size-exclusion chromatography (Figure 38). s4-1BBL formed two major peaks, one with a molecular mass (M_r) of approximately 175kDa and one of around 700kDa corresponding to trimers and higher order multimers, respectively. Analysis of unfractionated s-41BBL and the high and low M_r fractions by SDS-PAGE under reducing conditions demonstrated that all fractions formed protein bands with a M_r of 50 kDa, consistent with a single Fc-4-1BBL polypeptide chain. Under non-reducing conditions low M_r s4-1BBL formed a band of ~ 150kDa, consistent with trimer formation and high M_r s4-1BBL formed hexamers and higher order multimers which could not be resolved on the gel.

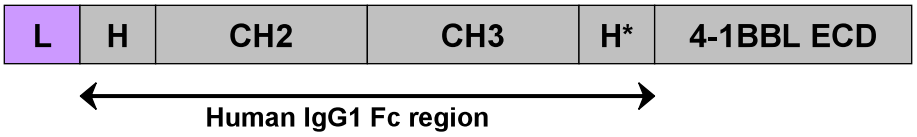

Figure 37 Schematic diagram representing the recombinant DNA structure of Fc tagged 4-1BBL fusion construct. (**L**) B72.3 leader sequence. (**H**) Hinge region of hIgG1. (**CH2**) hIgG1 constant heavy chain region 2. (**CH3**) hIgG1 constant heavy chain region 3. (**H***) Modified hIgG1 hinge region. (**4-1BBL ECD**) region corresponding to the extracellular domain of murine 4-1BBL.

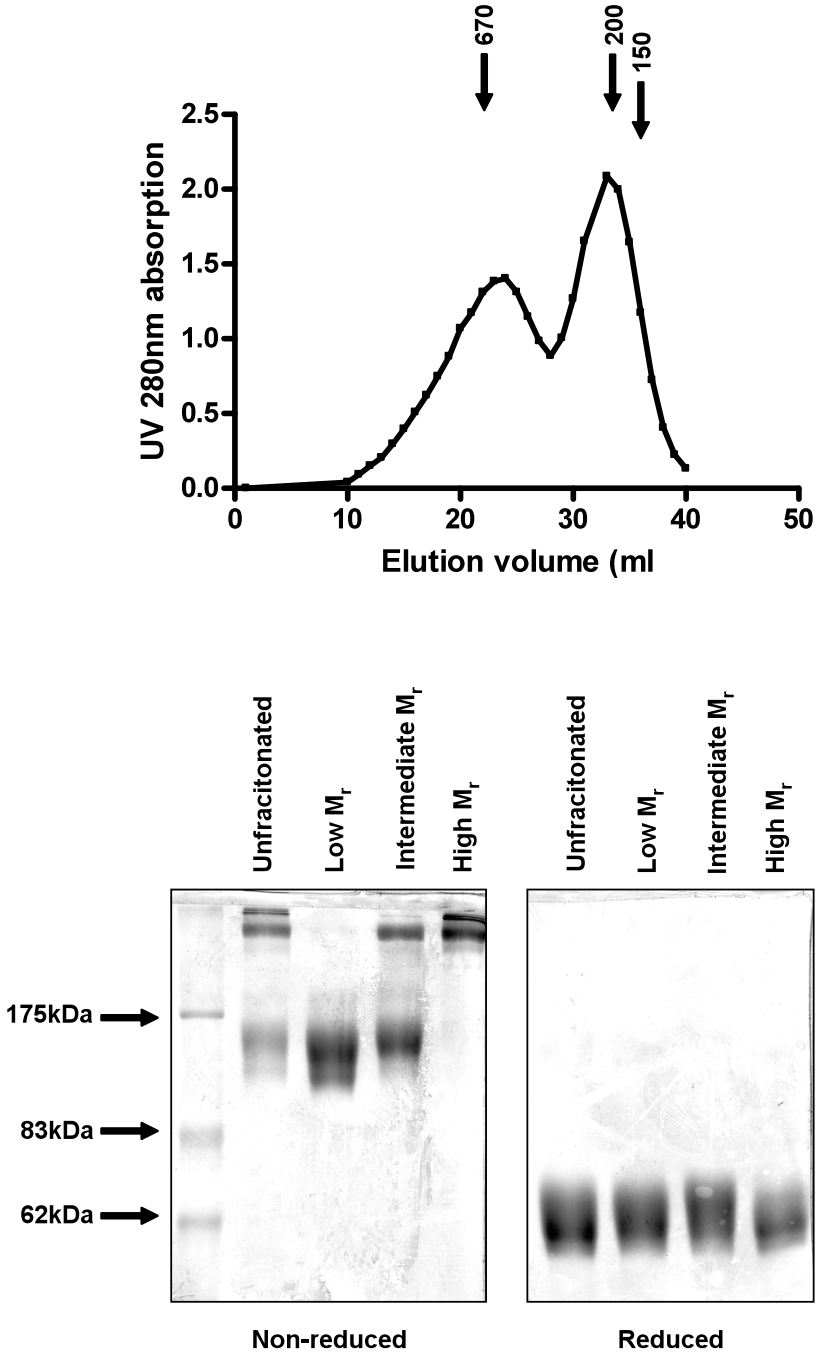

Figure 38 Analysis of s4-1BBL by size exclusion chromatography and SDS PAGE: (top) – fractionation by size exclusion chromatography. 20mg of purified s4-1BBL was separated into 2ml fractions using a Hiload 16/60 Superdex 200 column at 0.8ml/min in PBS. Relative elution positions of M_r markers thyroglobin (670 kDa), β-amylase (200 kDa) and IgG (150kDa). Products were pooled into high and low M_r groups and 5µg of each fractionated product was run on an 8.5% polyacrylamide gel (below), either reduced with 50mM DTT or non-reduced. The positions of the relevant $M_{r\ markers}$ are indicated. The data are representative of two independent experiments.

4.1.4. High M_r s4-1BBL is more effective than low M_r s4-1BBL *in vivo*

The high M_r fraction of s4-1BBL has previously been shown to be more effective in the costimulation of primary $CD8^+$ T cell responses *in vitro*, (T Rowley, unpublished observations) than the low M_r, or unfractionated s4-1BBL, despite similar binding affinities. To confirm this effect *in vivo* the ability of high and low M_r s4-1BBL to enhance the OT-I response to ovalbumin was examined. 1×10^5 OT-I cells were adoptively transferred into syngeneic C57BL/6 hosts and 24 hours later mice were immunised with ovalbumin (5mg) and s4-1BBL (high or low M_r) or human IgG as a control (200µg iv d0,1,2).

Responses were followed in peripheral blood by FCM as described above. Both the high and low M_r fractions of s4-1BBL enhanced the primary response over antigen alone, but the effect was greater with the high M_r fraction as measured at the peak of the primary response (~75% vs 25% of $CD8^+$ live lymphocytes) and in the resting memory population (~7% vs 3% of $CD8^+$ live lymphocytes). Because of its superior effects *in vivo*, and because the low M_r fraction shows little activity *in vitro*, high M_r s4-1BBL was used for subsequent experiments (referred to as s4-1BBL from this point on).

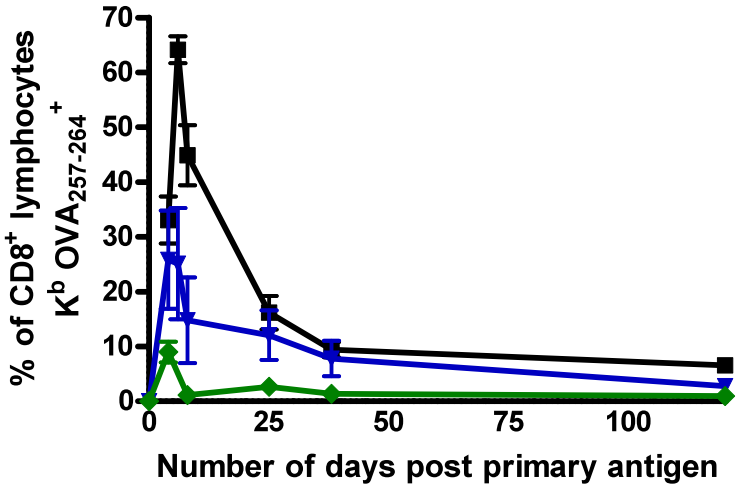

Figure 39 Comparison of *in vivo* activity of high and low M_r fractions of s4-1BBL: 1 x 10^5 OT-I cells were adoptively transferred into C57/Bl6 recipients (3 per group) and 24 hours later were primed with either high M_r (■), low M_r (▼) s4-1BBL or human IgG (♦) (200µg iv d0, 1,2). Responses were followed up in peripheral blood. Both high and low M_r fractions boosted responses, but the primary response and residual memory were both significantly higher with the high M_r fraction.

4.1.5. Costimulation with soluble ligands confirms the results from stimulatory antibodies

To investigate whether the effects of 4-1BB on $CD8^+$ T cell memory were physiological, or artefactual, similar experiments were performed employing soluble ligands in place of stimulatory antibodies. 1×10^5 OT-I $CD8^+$ T cells were adoptively transferred into syngeneic C57BL/6 hosts; 24 hours later mice were immunised with $Ova_{257-264}$ and either sCD70, s4-1BBL or control (200µg iv d0,1,2). Peptide was employed rather than whole ovalbumin protein in these studies for three reasons; it allowed for comparison with previous studies performed at this laboratory; it also excluded the effects of $CD4^+$ T cell help; it also got around the problem of LPS contamination of ovalbumin, since the synthetic peptide was confirmed as endotoxin low.

Primary responses were higher with CD27 costimulation (Figure 39), but despite this, the resting memory generated was higher with 4-1BB than with CD27 (Figure 40), and this translated into a larger memory response when mice were rechallenged with peptide and αCD40. This indicates that the enhanced effect on $CD8^+$ T cell memory formation generated through 4-1BB signalling is a physiological effect, confirming the findings with stimulatory antibodies shown in Figure 35.

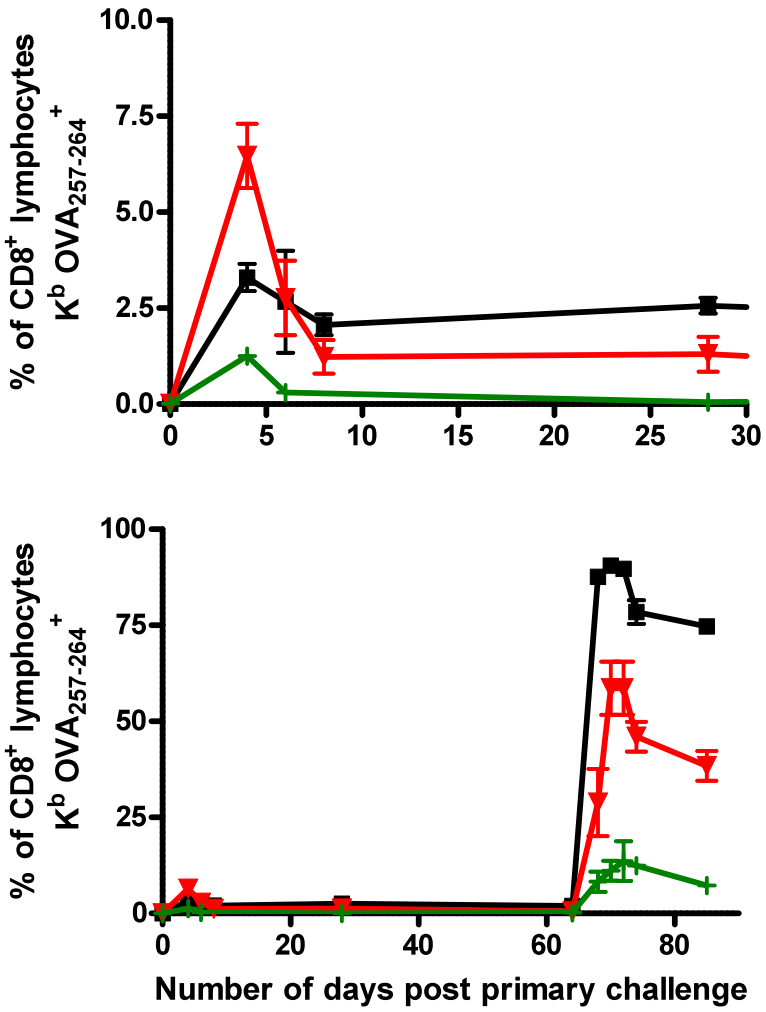

Figure 40 *In vivo* comparison of soluble ligands: 1 x 10^5 OT-I cells were adoptively transferred into C57/Bl6 recipients (3 per group) and 24 hours later were primed with $OVA_{257-265}$ (30 nmols iv) with sCD70 (▼), s4-1BBL (■) or human IgG (+). The primary response (above) confirmed the delayed contraction of the 4-1BB costimulated response, which translated into higher resting memory levels at day 64 (see Figure 41). Rechallenge was performed with $OVA_{257-265}$ (30 nmols iv) and αCD40 (3/23, 500µg ip) on d63; 4-1BB and CD27 both boosted the secondary response, with the greatest effect in the 4-1BB arm. Error bars represent the SEM.

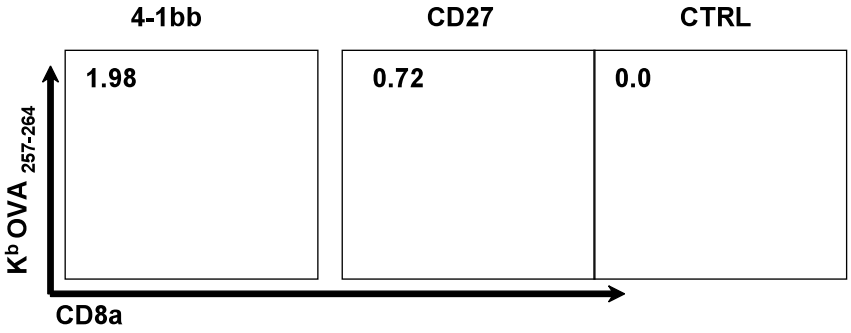

Figure 41 *In vivo* comparison of soluble ligands – resting memory: (from **Figure 40**): 1 x 10^5 OT-I cells were adoptively transferred into C57/Bl6 recipients (3 per group) and 24 hours later were primed with $OVA_{257-265}$ (30 nmols iv) with sCD70 (▼), s4-1BBL (■) or human IgG (+). Summary (above) and representative facs plots (below) are shown. 4-1BB costimulation results in higher resting memory populations when soluble ligands are employed during priming.

4.1.6. The resting memory $CD8^+$ T cells generated by 4-1BB or CD27 costimulation show similar homeostatic proliferation

The experiments documented above demonstrate that the higher resting memory generated by 4-1BB costimulation was associated with larger secondary responses on restimulation. The larger number of memory cells generated may have come about through two mechanisms: the first mechanism is enhanced generation of memory T cells either via enhanced differentiation or protection of progenitor memory $CD8^+$ T cells from apoptosis during the contraction phase of the primary response. The second mechanism could be the generation of memory $CD8^+$ T cells with a higher rate of homeostatic proliferation during the resting phase. To examine the homeostatic proliferation of memory $CD8^+$ T cells generated by CD27 or 4-1BB the following experiment was performed: a high OT-I transfer of 3×10^6 $CD8^+$ T cells was used to ensure adequate numbers of memory cells were present in both experimental groups to allow subsequent purification prior to adoptive transfer: immunisation was performed with stimulatory antibodies as in Figure 35. The $CD8^+$ T cell memory was again significantly higher when 4-1BB costimulation had been used. 65 days following primary immunisation, spleens and lymph nodes were harvested and antigen specific $CD8^+$ memory cells were purified by gating on $CD8^+$ MHC-tetramer$^+$ live lymphocytes, resulting in greater than 95% purity; equivalent cell numbers were adoptively transferred into Rag -deficient recipients (three per group) and the homeostatic proliferation of these cells was followed up in peripheral blood for three weeks. No significant difference in the rate of homeostatic proliferation was observed between $CD8^+$ memory cells generated by 4-1BB or CD27 costimulation (Figure 42). This suggested that the enhanced memory generation relied on an attenuated contraction phase when 4-1BB costimulation is employed.

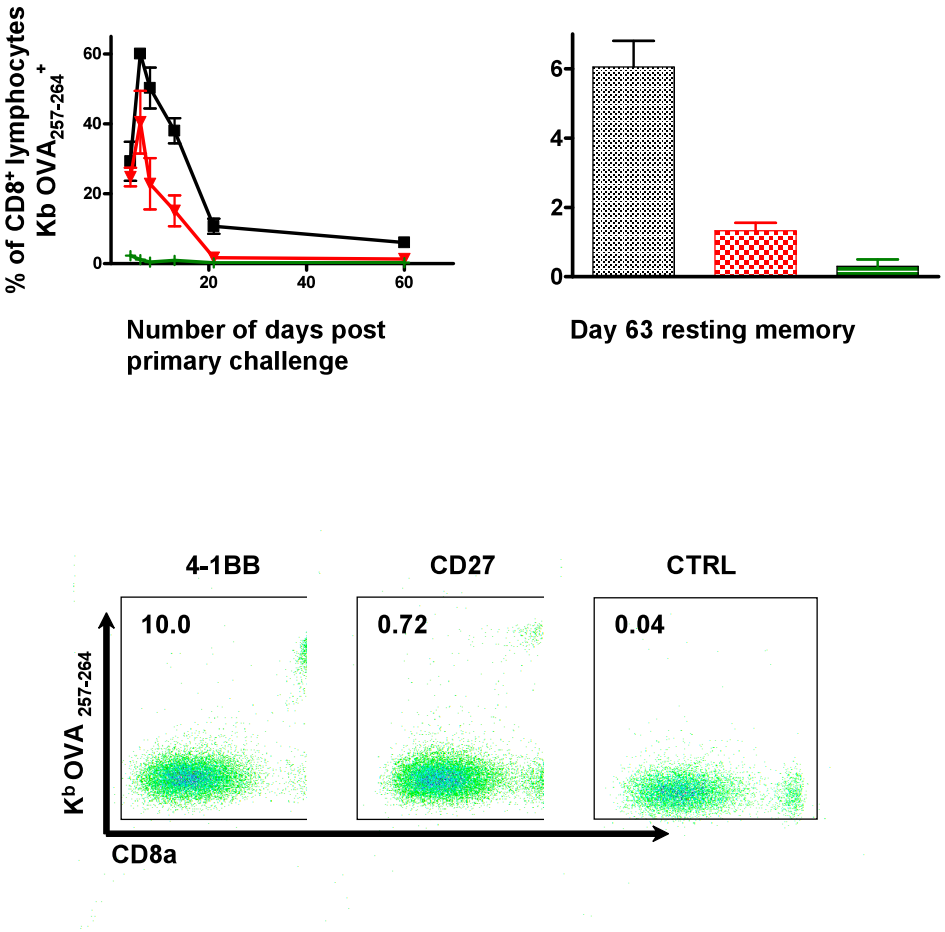

Figure 42 Homeostatic proliferation of memory CD8$^+$ T cells – comparison of 4-1BB and CD27 costimulation: to assess the homeostatic proliferation potential of memory CD8+ T cell generated by 4-1BB or CD27 costimulation 3 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and either anti-4-1BB (■) or anti-CD27 (▼) antibody (250mcg ip d0,1). Responses were measured in peripheral blood (above left). At D65 following the primary challenge 4-1BB costimulation had permitted the greatest survival of memory CD8+ T cells (above right and below). These cells were subsequently examined for their homeostatic proliferative ability (**Figure 43**).

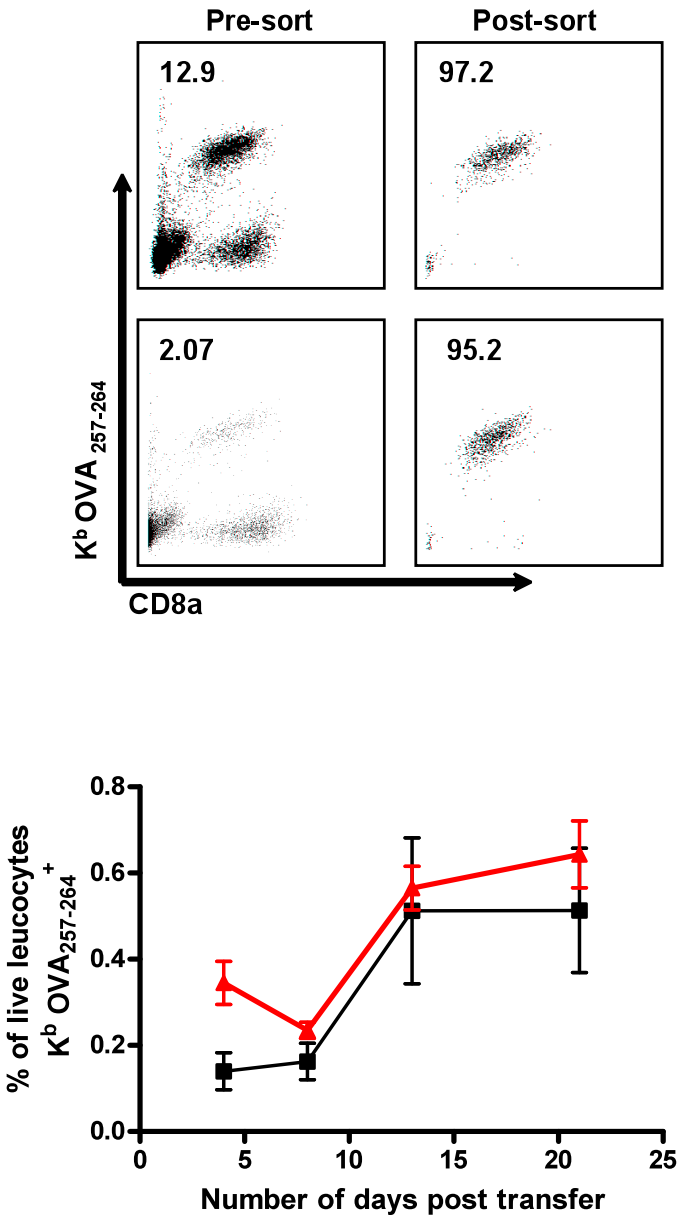

Figure 43 Transfer of memory CD8$^+$ T cells into RAG ko recipients: memory OT-I cells 65 days following primary challenge (see **Figure 42**) were purified to >95% purity on a Facs Aria cell sorter (above); results of purification for 4-1BB (above) and CD27 (below) memory cells are shown. After purification, 3 x 10^5 memory OT-I cells were transferred to RAG -/- recipients. Homeostatic proliferation was monitored in peripheral blood over three weeks. Both 4-1BB (■)and CD27 (▲) stimulated cells proliferated at the same rate. Results are based on the percentage of live lymphocytes due to downregulation of CD8α.

4.1.7. The resting memory $CD8^+$ T cells generated by 4-1BB or CD27 costimulation show similar capacity for secondary expansion

4-1BB and CD27 costimulation generated memory $CD8^+$ T cells with equivalent rates of homeostatic proliferation, and responses to secondary immunisation were higher in the 4-1BB costimulated cells. It was likely that this enhanced secondary response was due to the higher numbers of resting memory cells, but to confirm this it was necessary to examine the response to secondary stimulation when memory T cell numbers were equivalent as it was possible that 4-1BB costimulation generated memory $CD8^+$ T cells with an enhanced ability to respond to restimulation.

To examine this, 1 x 10^6 OT-I $CD8^+$ T cells were adoptively transferred into syngeneic C57BL/6 hosts, and 24 hours later mice were immunised as in Figure 35. The primary response was tracked, and the enhanced effect on $CD8^+$ T cell memory with 4-1BB costimulation was again confirmed. 65 days following primary immunisation, spleens and lymph nodes were harvested and K_b-$ova_{257-264}$ antigen specific memory $CD8^+$ T cells were purifed (as inFigure 42); following purification, equal numbers of ASC were adoptively transferred into syngeneic C57BL/6 recipients. 24 hours later mice were immunised with $ova_{257-264}$ peptide and α-CD40 (323, 500μg ip), and responses were followed up by FCM in peripheral blood. $CD8^+$ memory cells generated with CD27 and 4-1BB costimulation demonstrated equivalent capacity for secondary expansion, confirming that the majority of the difference in secondary expansion seen is due to the higher precursor frequency of memory cells generated by 4-1BB costimulation during priming. There was no detectable response to immunisation in mice that had received adoptive transfer of equivalent numbers of naïve OT-I $CD8^+$ T cells,

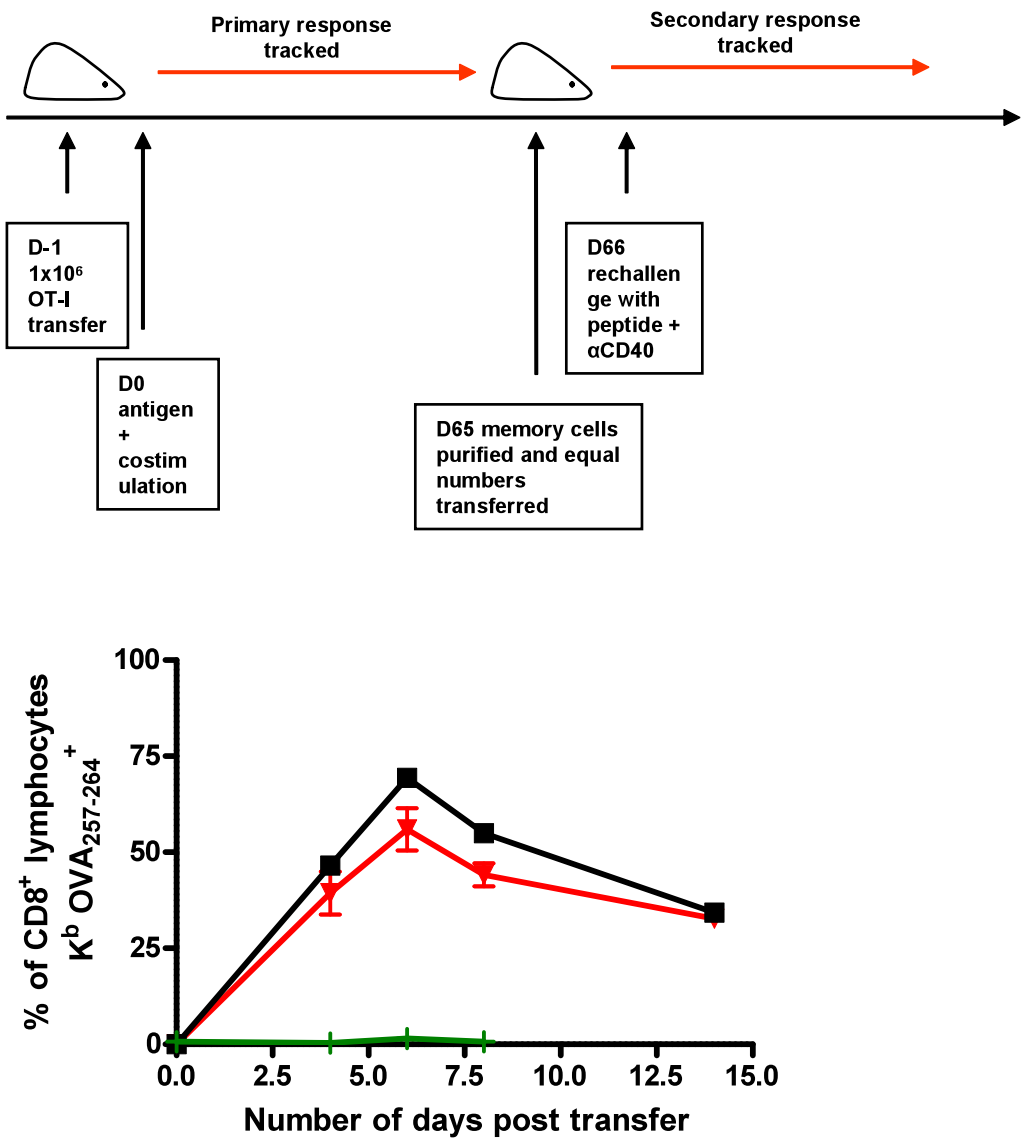

Figure 44 Secondary expansion potential of memory cells: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and either anti-4-1BB (■) or anti-CD27 (▼) antibody (250mcg ip d0,1). 65 days later spleens and lymph nodes were harvested and purified to >95% purity on a Facs Aria cell sorter. 3 x 10^5 memory cells (either 4-1BB or CD27 primed) or 3 x 10^5 naïve OT-I cells (green +) were transferred into naïve C57BL/6 hosts; 24 hours later mice were challenged with $OVA_{257-265}$ (30 nmols iv) and αCD40 (3/23 500μg ip). Naïve cells were used to confirm that the purified memory T cell were 'true bona fide' memory cells. The experimental design is shown above; below, responses measured in peripheral blood show the markedly increased expansion of memory cells compared to naïve cells; 4-1BB or CD27 costimulation during priming resulted in cells of similar expansion potential.

4.1.8. CD27 and 4-1BB endogenous secondary responses confirm findings in transgenic $CD8^+$ T cells

The observations that 4-1BB and CD27 contribute disproportionately to the generation of $CD8^+$ T cell memory were all made using TCR transgenic $CD8^+$ T cells. Although the effect was seen across a broad range of transfer numbers (10^4 to 3 x 10^6), even at the lowest dose this represents a level of antigen-specific precursors 10 to 1000 times more than would be found in the endogenous setting. Transgenic TCR $CD8^+$ T cell doses as low as 500 per mouse will completely suppress the endogenous $CD8^+$ T cell response, and virtually every aspect of the $CD8^+$ response will be affected by the transfer dose [261].

To address the concerns that transgenic T cells may not represent the physiological effects of CD27 and 4-1BB, the effect was examined in an endogenous system: naïve C57BL/6 mice were vaccinated with ovalbumin and α-4-1BB or αCD27 stimulatory antibodies (as in Figure 35), and responses were monitored in peripheral blood by FCM. 55 days later mice were re-immunised with $ova_{257-267}$ (30 nmols iv) and αCD40 (3/23, 500μg ip). Consistent with the findings using transgenic TCR T cells, 4-1BB had a stronger effect on $CD8^+$ T cell memory than CD27 (Figure 45).

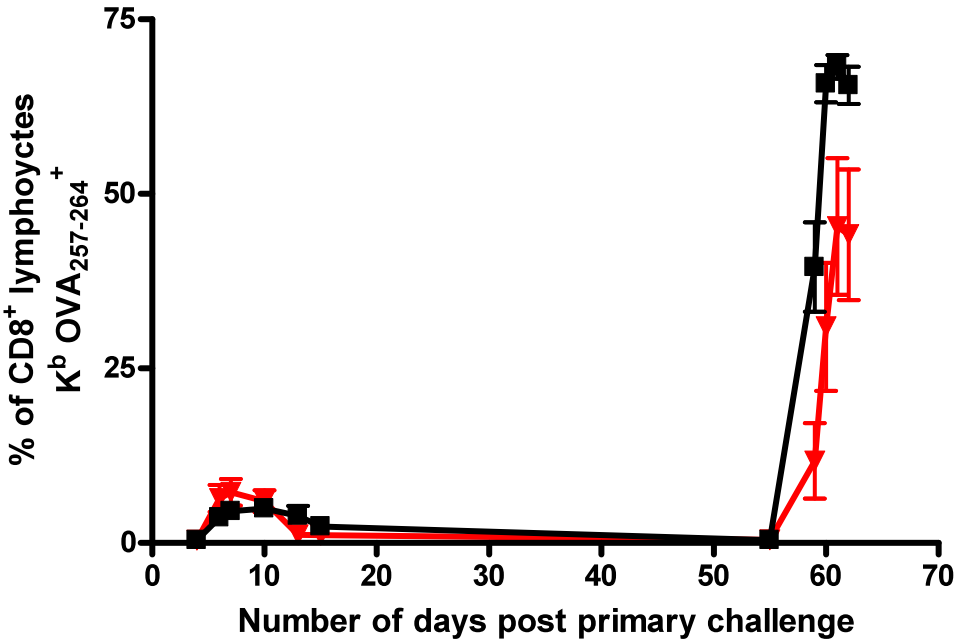

Figure 45 Endogenous memory CD8$^+$ T cell responses: C57BL/6 mice were primed with ip injection of 5mg ovalbumin and either anti-4-1BB (■) anti-CD27 (▼) (250mcg ip d0,1). On day 55 mice were rechallenged with $OVA_{257-265}$ (30 nmols iv) and αCD40 (3/23 500μg ip). Memory responses were higher in the 4-1BB experimental arm.

4.1.9. The enhanced memory generated by 4-1BB costimulation persists in the absence of CD4 T cells

4-1BB and CD27 are both detectable on the cell surface of cells other than $CD8^+$ T cells, including $CD4^+$ helper T cells and $CD4^+CD25^+$regulatory T cells [308]. $CD4^+$ T cells can augment, and may even be required for, $CD8^+$ T cell memory; it is possible that the increased memory seen with 4-1BB costimulation was due, not to the direct effects of 4-1BB on naïve CD8+ T cells, but indirectly, through 4-1BB signalling on CD4+ T cells – although this is unlikely since the effects were observed when the CD8 T cell epitope was used instead of OVA; however this was in the TCR transgenic system which can be helper independent because of high precursor frequency. To establish whether CD4+ costimulation by CD27 or 4-1BB was contributing to the effect on endogenous CD8+T cell memory, 4-1BB and CD27 costimulation were compared in the presence of $CD4^+$ T cell depletion: CD4+ T cells were depleted according to a standard protocol (500µg each of CD4 depleting antibodies YTS 191.1.2 and GK 1.5 d-1 and d+1); this resulted in rapid depletion of $CD4^+$ T cells on day 0 (from ~15% of live lymphocytes to <3% as assessed by CD3/CD8α dual staining of peripheral blood - Figure 46). When mice were rechallenged 60 days following primary immunisation, robust memory responses were seen, and these remained significantly higher where 4-1BB costimulation had been employed. This suggests that the effect on endogenous $CD8^+$ memory generation was more likely to be due to direct 4-1BB and CD27 stimulation of $CD8^+$ T cells.

An unexpected observation was the significant augmentation of the $CD8^+$ primary response by $CD4^+$ depletion (Figure 47). $CD4^+$ T cell help has been reported to be necessary for some primary $CD8^+$ T cell responses [309] and dispensable for others [310]. Augmentation of $CD8^+$ primary responses by $CD4^+$ depletion has not been reported, to our knowledge: however, $CD4^+CD25^+$ regulatory T cells (T_{reg}) are known to interfere with $CD8^+$ T cell responses [311]. To investigate whether the augmentation of $CD8^+$ T cell responses in the presence of $CD4^+$ depletion was due to depletion of T_{reg} the experiment was repeated using CD25 depletion. CD25 depleting antibody

(PC61 500µg d-7) was given, which resulted in a significant reduction in the circulating numbers of $CD25^{+}CD4^{+}$ cells (Figure 48) at levels comparable with published methods [312]. Primary responses were significantly reduced, consistent with the presence of CD25 on activated $CD8^{+}$ T cells and their subsequent depletion. This had little effect on the recall response, which remained higher in the 4-1BB experimental arm. This experiment was not able to answer the question of why CD4 depletion significantly increased $CD8^{+}$ primary responses, but did suggest that the enhanced effect on $CD8^{+}$ memory generation with 4-1BB was not due to 'off target' effects on regulatory $CD4^{+}$ T cells.

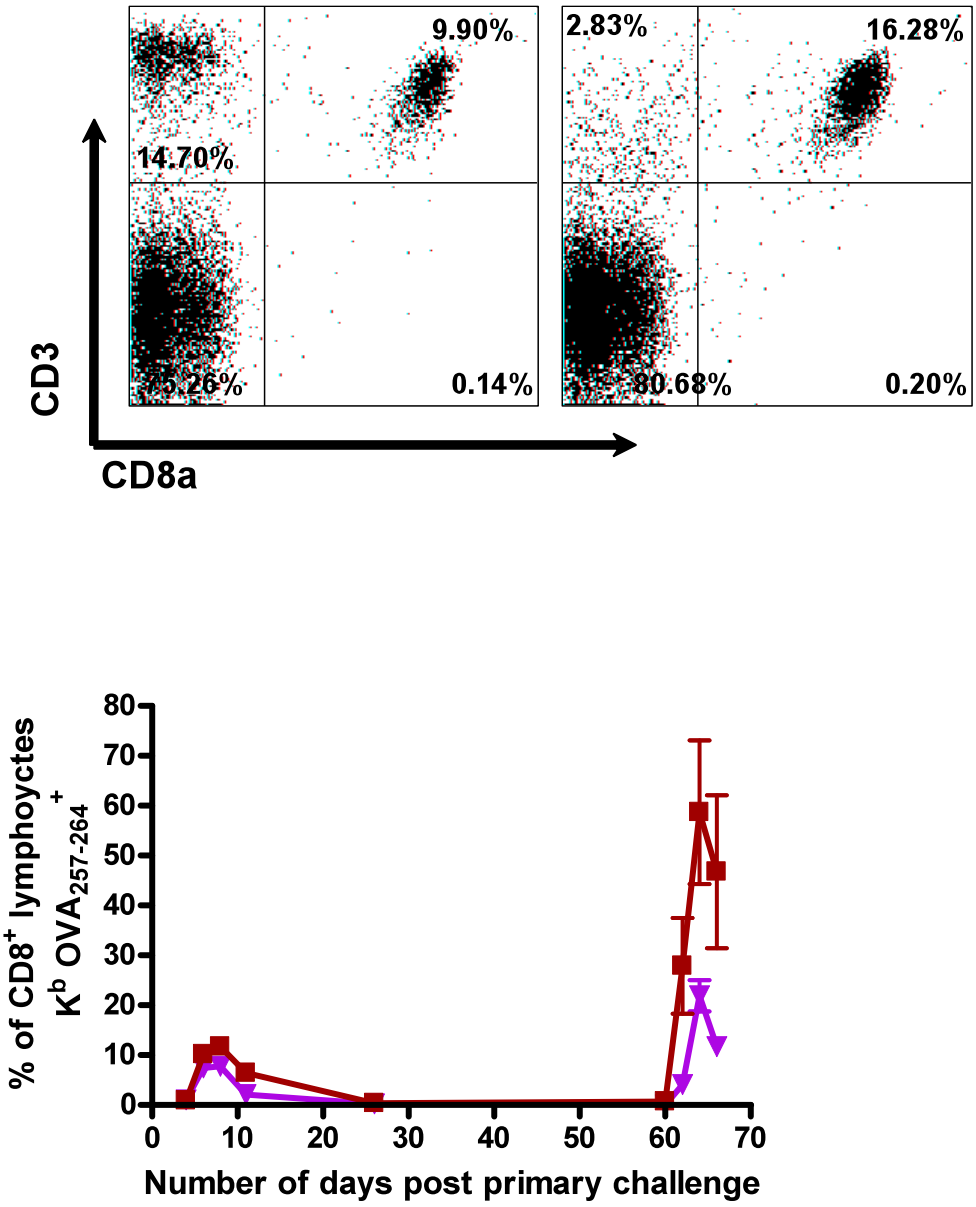

Figure 46 Memory $CD8^+$ T cell responses with $CD4^+$ T cell depletion during priming: C57BL/6 mice were CD4+ depleted (500µg each of YTS 191.1.2 and GK 1.5 d-1, +1) resulting in significant reduction in $CD4^+$ cells (control is shown above left; $CD4^+$ depleted above right).Mice were then primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■) anti-CD27 (▼) (250mcg ip d0,1). At the time of rechallenge $CD4^+$ numbers had returned to normal levels (data not shown). On day 60 mice were rechallenged with $OVA_{257-265}$ (30 nmols iv) and αCD40 (3/23 500µg ip). Even in the absence of $CD4^+$ T cell help during priming, robust memory responses were evident; these were significantly higher in the 4-1BB arm. Error bars represent the SEM. Data is representative of two separate experiments.

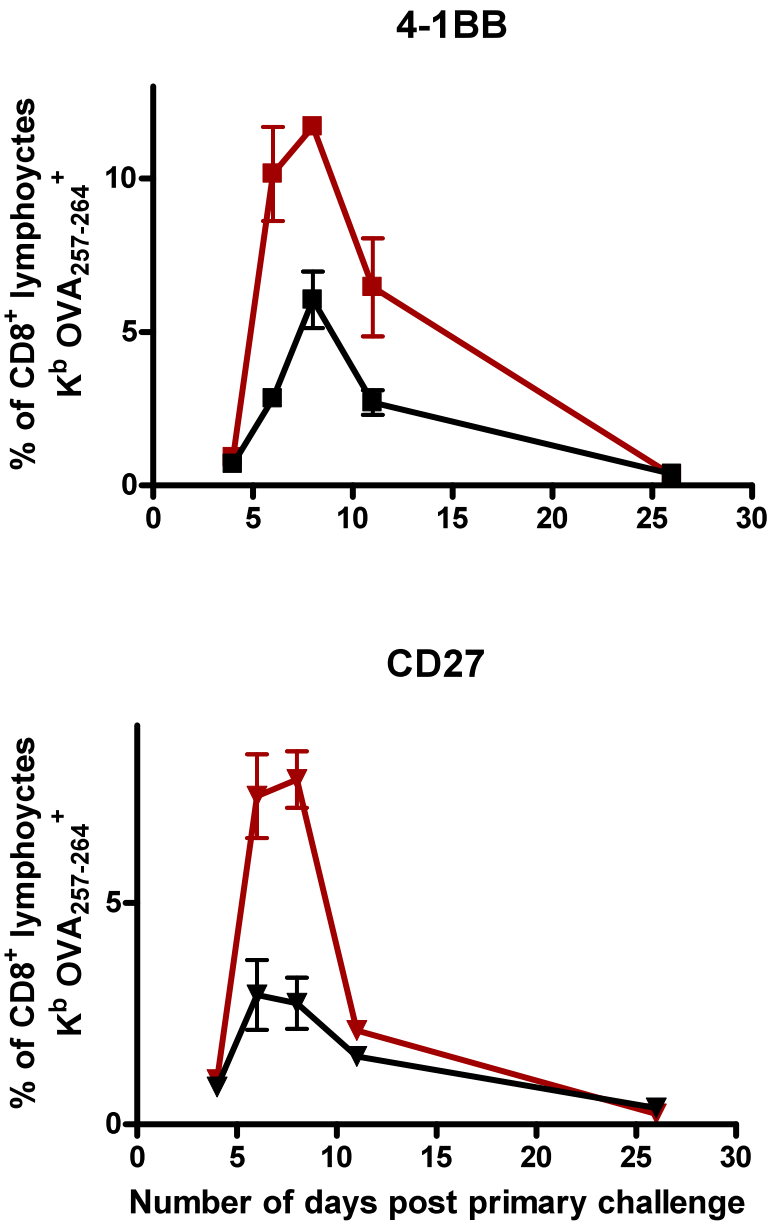

Figure 47 Effect of $CD4^+$ depletion on the size of the primary $CD8^+$ T cell response: Experimental set up as in Figure 46. 4-1BB (■/■) or CD27 (▼/▼) costimulation was compared in the presence (brown) or absence (black) of $CD4^+$ depletion. $CD4^+$ depletion led to significant augmentation of the primary $CD8^+$ response. Error bars represent the SEM. Results are representative of two separate experiments.

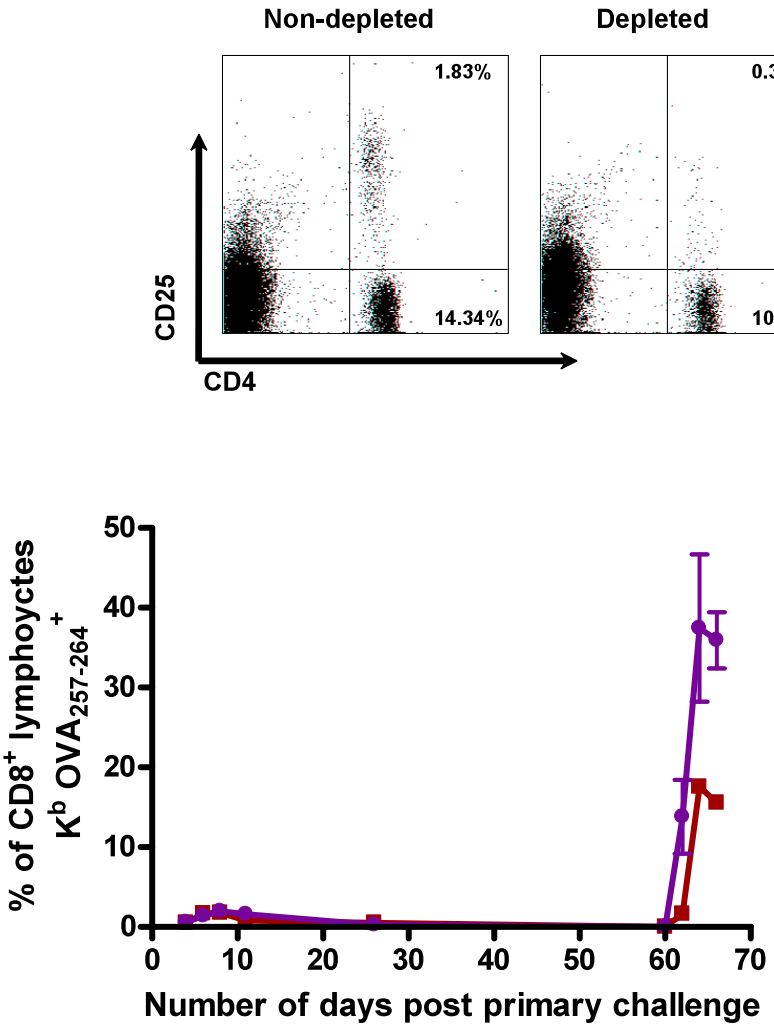

Figure 48 Memory $CD8^+$ T cell responses with $CD25^+$ T cell depletion during priming: C57BL/6 mice were $CD25^+$ depleted (500µg of PC61 αCD25 antibody on D-7) resulting in significant reduction in $CD25^+CD4^+$ cells on D0 (above). Mice were then primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (●) anti-CD27 (■) or isotype control (+) antibody (250mcg ip d0,1). At the time of rechallenge $CD25^+CD4^+$ numbers had returned to normal levels (data not shown). On day 60 mice were rechallenged with $OVA_{257-265}$ (30 nmols iv) and αCD40 (3/23 500µg ip). In the absence of $CD25^+CD4^+$ during priming, robust memory responses were evident; these were significantly higher in the 4-1BB arm. Error bars represent the SEM.

4.1.10. Memory $CD8^+$ T cells do not protect against tumour challenge

The data shown above demonstrate different roles for CD27 and 4-1BB in the generation of $CD8^+$ T cell memory. In order to investigate differences in effector function in the setting of tumour protection, a tumour model was employed. EG7 is a K-2^b mouse thymoma transfected with cDNA of full length ovalbumin. OT-I $CD8^+$ effector cells are able to reject EG7 growth *in vivo* [313]. To establish whether the enhanced memory generated with 4-1BB costimulation translated into better protection from tumour, 10^6 OT-I $CD8^+$ T cells were adoptively transferred into syngeneic C57BL/6 hosts and 24 hours later mice were immunised according to the schedule in Figure 35. Responses in peripheral blood were assessed to ensure that previous observations of enhanced memory with 4-1BB were replicated. 30 days following primary immunisation mice were challenged with 1 x 10^6 EG7 tumour cells subcutaneously, and tumour growth was monitored.

All three groups of memory cells (4-1BB, CD27 and OVA alone) protected against tumour when compared with transferred naïve $CD8^+$ OT-I cells, but there was no detectable difference between 4-1BB vs CD27 vs control costimulation (Figure 50), despite the large detectable effect on circulating memory cells (Figure 49). A similar lack of protection was observed when endogenous memory was generated (data not shown).

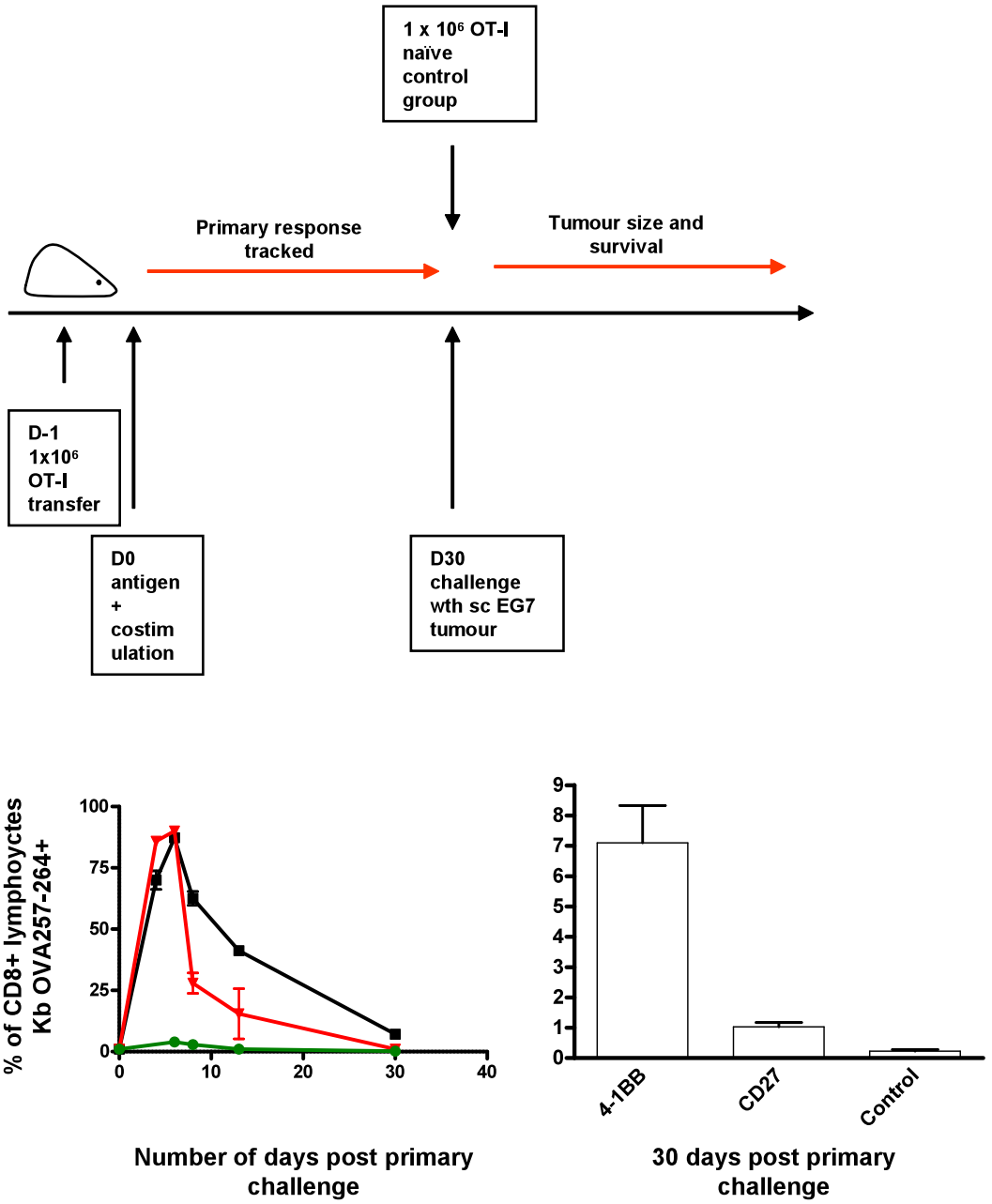

Figure 49 Memory CD8$^+$ T cell protection against E.G7 tumour: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■)or anti-CD27 (▼) or control (●) antibody (250mcg ip d0,1). Responses were measured in peripheral blood to confirm enhanced memory generation of CD8$^+$ T cells (bottom left). Resting memory 30 days after primary immunisation was similar to previous experiments. 30 days later mice were challenged with 1 x 10^6 E.G7 tumour cells. A control group with 1 x 10^6 naïve OT-I given the day before tumour challenge was included. Tumour responses are shown in **Figure 50**

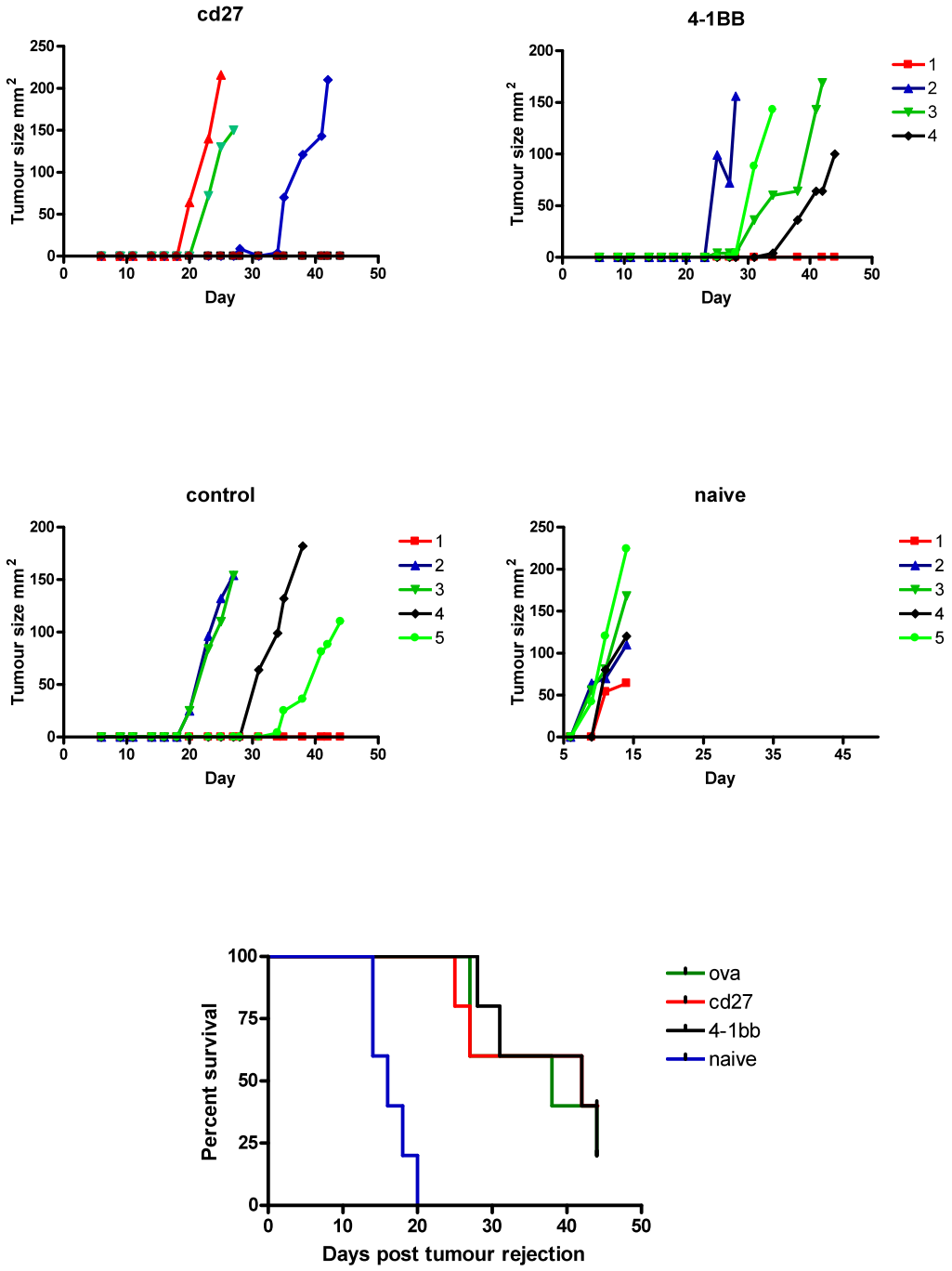

Figure 50 Memory CD8$^+$ T cells do not protect against EG7 tumour challenge: from Figure 49 – memory cells delayed tumour growth, but there was no advantage of 4-1BB or CD27 over cells primed with ovalbumin alone. Individual tumour growth (top four graphs) and Kaplan-meier survival (bottom graph) are shown. This data is representative of two separate experiments.

4.1.11. 4-1BB and CD27 costimulation both protect against tumour challenge during the primary immune response

As the $CD8^+$ T cell memory generated with CD27 and 4-1BB had not translated into a protective effect against tumour challenge, a comparision of the primary responses was made using a novel anti-tumour vaccine model: DNGR-1 is a recently discovered DC cell surface lectin which is restricted to $CD8^+$ DCs, which are specialised in cross-presentation. By targeting antigens to DNGR-1, successful immune responses can be raised, and protection against B16 melanoma expressing ovalbumin (B16-ova) can be demonstrated [213]. This system of tumour protection requires costimulation via CD40; since 4-1BB and CD27 costimulation are thought to act downstream of CD40, this system was employed to see how 4-1BB and CD27 compared to CD40.

2×10^5 B16-Ova in exponential growth phase were administered into C57BL/6 recipients iv. 72 hours later mice were vaccinated with antibody to DNGR-1 (a kind gift from D Sancho) coupled to $Ova_{257-264}$. A control group of irrelevant antibody coupled to $ova_{257-264}$ was employed. All groups received poly-ic 10μg. Anti-CD40 antibody 25μg s/c (the positive control group, from Sancho, Mourao-Sa et al. 2008) was compared to anti-CD27 and anti-4-1BB (200μg iv). Responses were measured in peripheral blood; CD27 costimulation generated a significantly higher primary response than all other groups. 15 days after vaccination mice were sacrificed and lung tumours counted. CD40, 4-1BB and CD27 all gave equivalent protection against tumour. DNGR-1 with poly-ic did not give any protection; neither did CD40 with untargeted $ova_{257-264}$, as expected.

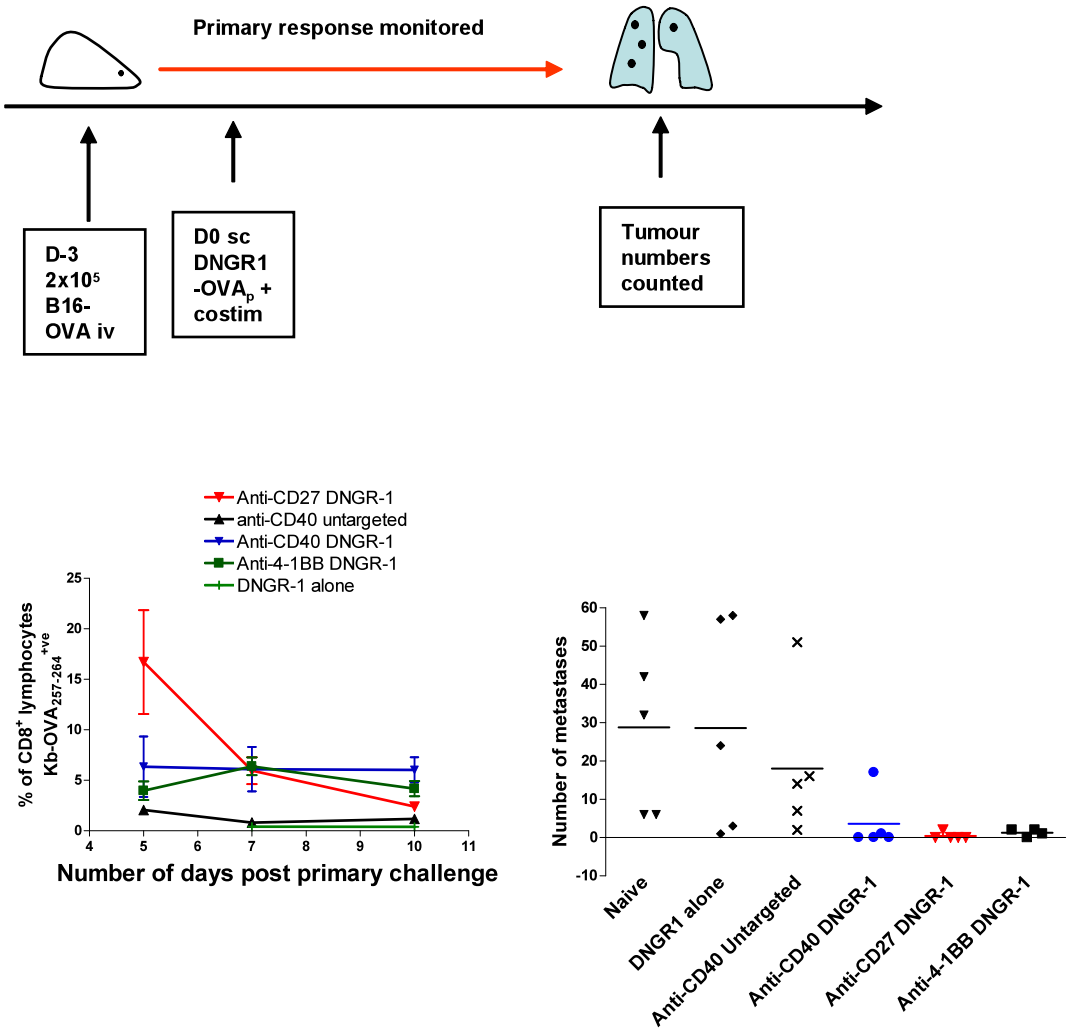

Figure 51 4-1BB and CD27 reduce the number of lung metastases in mice injected with melanoma: 2 x 10^5 B16-OVA melanoma cells were administered iv to naïve C57BL/6 on day -3, followed by treatment on d0 with DNGR-1 targeted $ova_{257-264}$ (20µg sc) with either αCD27, α4-1BB (200µg iv d0,1) or αCD40 (323 50µg sc d0) (five per group) (experimental design shown above). The dose of CD40 was chosen based on published evidence; the dose of CD27 and 4-1BB was the standard dose for our lab. Primary responses were monitored in peripheral blood: primary responses were similar to those seen from previous experiments (below left). On d15 mice were killed and lung metastases counted (below right). DNGR-1-$ova_{257-264}$ or untargeted ova-257-264 with α-CD40 gave no protection against tumour when compared to naïve mice. 4-1BB, CD40 and CD27 costimulation given with DNGR-1-$ova_{257-264}$ all gave marked protection against tumour.

4.2. Discussion

4.2.1. 4-1BB enhances memory generation more than CD27

Costimulation provided via 4-1BB or CD27 boosts primary and secondary $CD8^+$ T cell responses. Both molecules are expressed on several lymphocyte subsets, but their dominant effects appear restricted to $CD8^+$ lymphocytes. The finding that CD27 generates an early peak in the primary $CD8^+$ T cell response (Figure 35) is consistent with the known constitutive expression of CD27 on naïve $CD8^+$ T cells [314] whereas 4-1BB is expressed after TCR ligation. This is, however, an over-simplification: 4-1BB can be upregulated within 6 hours of TCR ligation [148] depending on the system used, yet the peak of the primary response in Figure 35 differed by 2 days. Thus, the difference in kinetics of expression may not fully explain the difference in CD27 and 4-1BB costimulation; different effects on proliferation and protection from apoptosis may also be present.

The discovery that 4-1BB costimulation, compared with CD27 costimulation generated more resting memory is a novel one. In knockout mice CD27 deficiency had the greatest effect on generation of resting memory following influenza infection [301]; this may be because primary responses were affected more in CD27 -/- mice, and the size of the primary response will have knock-on effects on the resulting memory generated. The same study showed that when resting memory numbers were equal it was the absence of 4-1BB signalling during the primary response that had the greatest effect on subsequent recall responses; ie it is suggested that 4-1BB costimulation during the primary response leads to a qualitative difference in the memory cells generated. The data shown here with stimulatory antibodies generated primary responses of comparable size; despite this the resting memory population generated was significantly higher with 4-1BB costimulation.

Naïve $CD8^+$ T cells are restricted to the lymphoreticular system; they circulate between blood, secondary lymphoid tissue, efferent lymph channels, and back into the blood. Only once activated by DCs in lymph nodes can they enter infected tissues and lyse target cells that reside in tissues. After this

effector phase the majority of effector cells undergo apoptosis, leaving a population of memory cells in lymphoid and non-lymphoid tissues. Tissue based memory cells retain effector function better than memory cells confined to lymphoid tissue such as the spleen [315]. The bone marrow is a prominent site for memory cell homeostatic proliferation, where the cells seem particulary sensitive to IL-15 [316]. Memory cell numbers were higher when 4-1BB costimulation was employed during primary immunisation at all tissue sites examined, including liver, spleen and bone marrow (Figure 36).

4.2.2. s4-1BBL forms different multimeric forms with different *in vivo* effects

The initial findings with stimulatory monoclonal antibodies to 4-1BB and CD27 were possibly due to differences in the differing IgG isotypes of the reagents (IgG1 and IgG2a respectively), which have different affinities for Fc receptors; mutations in the IgG heavy chain can alter the effects of therapeutic antibodies [317]. Studies using anti-CD3 mAbs generated on different IgG heavy chains show substantially different therapeutic effects [242]. Additionally, monoclonal antibodies can have different effects according to which epitope they bind to on the target receptor [307]. For these varying reasons it was necessary to confirm that the effects seen on T cell memory were reproducible across experimental systems.

A soluble form of the ligand for 4-1BB, referred to here as s4-1BBL, has been developed in our laboratory and shown previously to effectively costimulate T cells *in vitro*. s4-1BBL forms high and low M_r fractions of around 600 and 150kDa respectively (Figure 38). The low M_r fraction was active *in vivo*, but did not generate as strong an effect on the primary response or the generation of memory in adoptively transferred transgenic $CD8^+$ T cells (Figure 39). This is in agreement with *in vitro* data previously generated in our laboratory (T Rowley, unpublished observations), as well as published data on TNFR signalling: various members of the TNFR superfamily, such as TNFR II, CD40 and Fas, do signal weakly on engagement with trimeric ligands, but require higher order multimers [242, 275]; for example, dodecameric forms of soluble CD40L were more effective than trimeric forms at triggering CD40 signalling, an effect that was independent of binding affinity [242]. The different multimeric forms are likely to be due to different formations of inter-chain disulphide bonds in the hinge regions of the Fc part of the construct (See previous chapter).

The s4-1BBL and sCD70 (see chapter 3) were compared in their costimulation of primary and secondary $CD8^+$ T cell responses; this confirmed the findings seen using antibodies (Figure 40): if the percentage of cells measured at the peak of the primary response is taken as 100%,

approximately 70% of these cells die when CD27 costimulation is employed, compared with a much lower figure (around 40%) when 4-1BB costimulation is used (for an example see Figure 40).

Notably, the size of the primary response did not predict the subsequent numbers of memory $CD8^+$ T cells generated, suggesting that 4-1BB may be preferentially protecting cells already committed to a memory lineage, and that memory generation is not purely a product of the size of the original primary response.

Monoclonal antibodies can persist *in vivo* for several days or weeks. It was possible that the enhanced effect on memory was due to persisting antibody acting on memory cells; 4-1BB signalling on unstimulated memory $CD8^+$ T cells causes them to proliferate [145]. CD27 is expressed on $CD8^+$ memory cells [318] but the effect of CD27 signalling on resting memory has not been investigated. Persistent antibody to 4-1BB or CD27 may have had differing proliferative/survival effects on resting memory cells; however this effect is negated with the use of soluble ligands, which are not detectable *in vivo* a week after administration (A.Al-Shamkhani, personal communication). Therefore the effect on memory is not due to residual regents stimulating the resting memory population, and is likely to be due to increased proliferation or reduced apoptosis during the primary phase (Figure 52).

The findings with soluble ligands suggest that the initial observations with immunostimulatory antibodies were not artefactual findings, but did represent a physiological difference between 4-1BB and CD27. It is probable that the effect on memory generation is due to protection of $CD8^+$ T cells from apoptosis during the contraction phase by 4-1BB signalling, and this would be in line with findings obtained in 4-1BBL deficient mice [319].

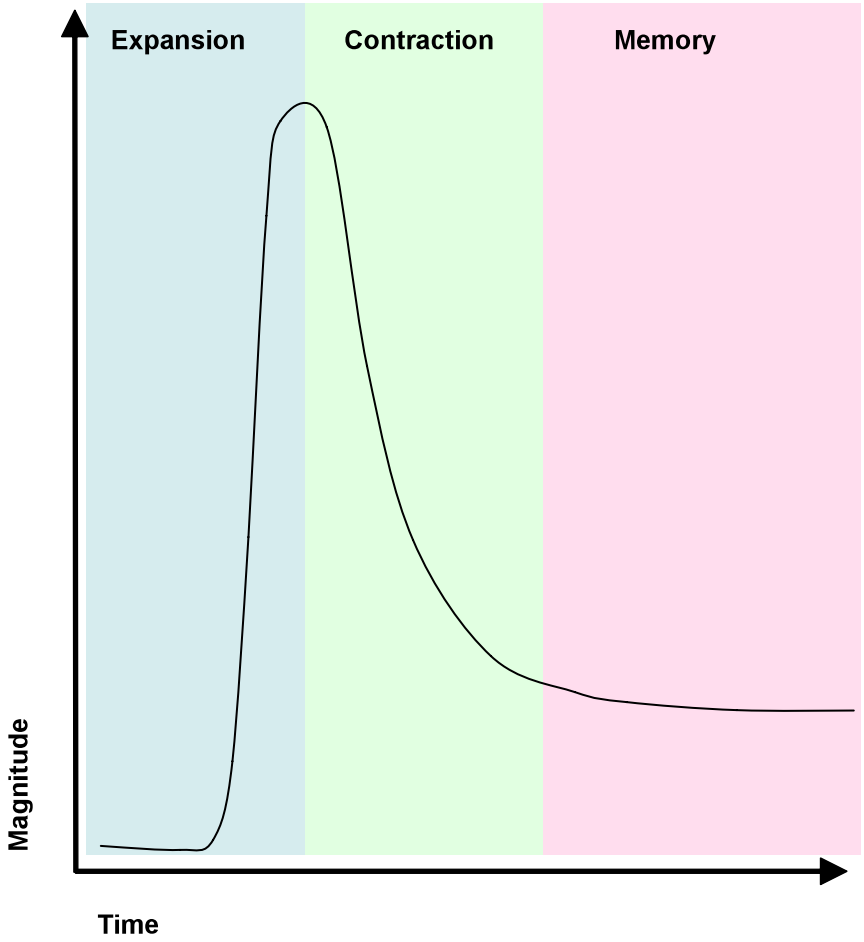

Figure 52 Phases of the primary CD8$^+$ T cell response: increased proliferation in the expansion phase, or decreased apoptosis in the contraction phase can affect the resulting memory generated. Ongoing stimulation during the memory phase can also affect the memory population.

4.2.3. Homeostatic proliferation and secondary expansion is similar in $CD8^+$ memory cells generated with different costimulatory antibodies

$CD8^+$ memory T cells generated with 4-1BB costimulation proliferate at the same rate as those generated with CD27 costimulation (Figure 42); this implies that the effect on memory occurs during T cell priming, rather than by the generation of $CD8^+$ memory T cells with higher rates of homeostatic proliferation. Naïve $CD8^+$ T cells require MHC signals to undergo proliferation for adequate homeostasis. This requirement for MHC interactions seems less for $CD8^+$ memory T cells [320] although this has been disputed [158]. Memory $CD8^+$ T cells also require IL-15 and IL-7 for their homeostasis [321, 322] and undergo low level, antigen independent proliferation. It was important to compare homeostatic proliferation, because 4-1BB signaling has been shown to cause memory cell proliferation (see above), and, conversely, IL-15 has been shown to upregulate 4-1BB expression on resting $CD8^+$ memory T cells [323].

These findings, along with the observation that secondary expansion was also identical when precursor numbers were equalized (Figure 44) confirm that the effect on $CD8^+$ memory generation by 4-1BB occurred during the primary phase, was independent of the size of the primary response, and that CD27 and 4-1BB costimulation generate $CD8^+$ memory T cells with similar homeostatic proliferation and capacity for secondary activation.

4.2.4. Endogenous $CD8^+$ memory responses resemble the findings using transgenic T cells

$CD8^+$ memory generation can be affected by the frequency of antigen specific naïve precursors [324]. High numbers of naïve precursors increase the competition for interaction with DCs, and this results in a skewing of the resultant memory phenotype, reducing the ratio of T_{EM}:T_{CM}, as well as altering phenotype, the kinetics of the primary response, and the number of memory

cells generated. OT-I adoptive transfers of over $3.5x10^2$ (ie 350 cells) per mouse are enough to completely suppress the endogenous response in recipient mice, and transfers of above $5x10^3$ substantially alter kinetics, phenotype, and memory generation [325]. For this reason, it was important to demonstrate that the findings with transgenic T cells presented above could be replicated using endogenous $CD8^+$ T cells (Figure 45).

4.2.5. CD27 and 4-1BB memory responses persist in the absence of $CD4^+$ T cell help during priming

4-1BB and CD27 are both expressed on various lymphocyte subsets, including $CD4^+$ T cells. $CD4^+$ T cell help is important in the generation of $CD8^+$ T cell memory in a variety of systems; the mechanism by which this occurs is not known, but appears to be independent of CD40/CD40L interactions [318]. 4-1BB and CD27 are both able to costimulate $CD4^+$ T cells to proliferate (see previous and following chapters) [109, 326] and it was therefore necessary to assess whether the effect was due to direct signalling by these receptors on the $CD8^+$ T cell rather than indirectly through $CD4^+$ costimulation by 4-1BB/CD27. The method of $CD4^+$ T cell depletion employed is known to be temporary, with $CD4^+$ T cell reconstitution detectable by 42 days. This method of CD4 depletion is also known to block the development of anti-rat antibodies [327].

The observation that $CD4^+$ T cell depletion significantly enhanced $CD8^+$ primary responses when CD27 or 4-1BB costimulation was employed (Figure 13) has not been described in the literature to the best of my knowledge. The increase was seen using a gate on $CD8^+$ T cells – ie it was not due to generalized $CD8^+$ T cell expansion into the 'space' left by $CD4^+$ T cell depletion, and the difference persisted when alternative combinations of CD4 depleting antibodies were employed (data not shown).

This boosting of the primary response by $CD4^+$ T cell depletion may have been due to depletion of regulatory $CD4^+$ T cells, T_{regs}. T_{regs} are capable of suppressing the magnitude of $CD8^+$ T cell primary responses [328]; to

address this possibility, the experiments were repeated with anti-CD25 mAb depletion of T_{regs}. The data presented in Figure 48 showed a reduction in primary responses when T_{reg} were depleted; this is probably due to the expression of CD25 on activated $CD8^+$ T cells. Despite this abrogation of the primary $CD8^+$ T cell response, secondary responses remained robust; the enhanced memory effect was still seen, confirming that the effect was not mediated via 4-1BB or CD27 ligation on T_{regs}.

It is possible that while antigen specific $CD4^+$ help will in general boost $CD8^+$ responses (this particularly the case when antigen is non- or weakly inflammatory), it may be that CD27 and 4-1BB act downstream of the effects of $CD4^+$ T cell help on $CD8^+$ T cell responses; indeed, CD40 signalling upregulates CD70 on DCs [106], and this pathway of $CD8^+$ costimulation is critically dependent on CD27/CD70 interactions. CD40/CD154 interactions are not thought to be the sole pathway of $CD4^+$ T cell licensing of DCs [260], with CD40/CD154 independent DC licensing and direct CD4-CD8 effects described. Whether these alternative pathways involve 4-1BB/4-1BBL or CD27/CD70 interactions remains to be seen, but the robust memory responses seen in the absence of $CD4^+$ T cell help when 4-1BB or CD27 costimulation is present suggests that this an area that warrants further study.

Depletion of regulatory T cells with anti-CD25 antibody (PC61) has been used to enhance $CD8^+$ T cell primary responses [312] and memory responses [329]; PC61 has been shown to act in a $CD4^+$ restricted manner, significantly depleting FoxP3-expressing Tregs,. 4-1BB and CD27 are also both detectable on T_{reg} [330]. 4-1BB has been shown to cause proliferation of T_{reg} [331]. The enhancement of $CD8^+$ memory with 4-1BB costimulation persists in the presence of T_{reg} depletion during primary immunisation, implying that the effect seen is not due to 4-1BB and CD27 effects on T_{reg}.

4.2.6. Memory $CD8^+$ T lymphocytes do not protect against EG7 tumour challenge

The enhanced memory generated with 4-1BB did not confer any benefit of protection against tumour over CD27. EG7 tumour is a modified form of a mouse thymoma (EL4) transfected with the cDNA for ovalbumin; immunity can be raised against this tumour that is $CD8^+$ T cell dependent [332]. The response against EG7 is dependent on effector $CD8^+$ T cells. Studies using memory $CD8^+$ T cells to control EG7 tumours found that central memory $CD8^+$ T cells (T_{cm}) alone were incapable of suppressing tumour growth; this inactivity was reversed when $CD4^+$ T cell help was given at the time of tumour challenge [333]. Conversely, the same group were able to show that effector memory $CD8^+$ T cells were capable of tumour protection without the need for additional $CD4^+$ T cell help. Similarly the approach shown in Figure 50 did not protect against tumour challenge despite the generation of large numbers of memory $CD8^+$ T cells.

The role of $CD8^+$ T cells in the control of tumours in animal models is not straightforward; some models require the ability of $CD8^+$ T cells to kill tumour cells directly via perforin or FasL/Fas mediated mechanisms [334]; in other models it is the production of cytokines such as IFNγ and TNFα by $CD8^+$T cells which is critical for tumour protection [335]. Such cytokine production can lead to upregulation of MHC Class I on tumour cells, or can lead to recruitment of other lymphocyte subsets [336], resulting in subsequent tumour control.

The EG7 tumour model is susceptible to control by activated $CD8^+$ T cells; naïve $CD8^+$ T cells provide no protection (as shown in Figure 50). Recent publications using intravital confocal microscopy show that EG7 control by OT-I cells is due to direct cytotoxicity [337]; this killing is remarkably slow, with individual interactions between cytotoxic $CD8^+$ T cells and tumour cells lasting for an average of 6 hours. In these studies, whilst naïve $CD8^+$ T cells could be primed by tumour directly, they failed to infiltrate the EG7 tumour with the efficiency required to exert any control of tumour growth.

The experiment shown in Figure 50 was not, however, relying on primed or naïve $CD8^+$ T cells; instead resting memory cells were studied. Memory $CD8^+$ T cells are a heterogenous group; T_{cm} are long lived with a high proliferative potential; T_{em} are a more transient population, with less capacity for proliferation, but a retained effector phenotype. The EG7 tumour model is susceptible to T_{em} but not T_{cm} unless concomittent $CD4^+$ T cell help is given; clearly something is missing when T_{cm} alone are given, but what?

It has been known for over a decade that memory T cells have a reduced requirement for costimulation when compared to their naïve counterparts [338]; this does not mean that there is no requirement for costimulation by resting memory cells to become activated. Recent studies have, demonstrated a critical role for dendritic cells in the activation of resting memory T cells (both T_{cm} and T_{em}) [339], in a CD28-dependent manner [339]. Therefore an explanation of the lack of efficacy of memory $CD8^+$ T cells in the control of EG7 tumours may be a lack of DC activation/costimulation.

4.2.7. 4-1BB and CD27 costimulation protect against b16-ova melanoma

In contrast to the use of memory transgenic $CD8^+$ T cells, endogenous effector cells were highly active against B16-ova melanoma (Figure 51). In this system, $ova_{257-265}$ peptide was conjugated to antibody to DNGR-1 – a lectin preferentially expressed on $CD8^+$ DCs, which are specialized in cross-presentation. Use of this vaccine has been shown to protect against b16-ova melanoma when used in conjunction with CD40 stimulation [213]. Vaccination in this system is with the $CD8^+$ immunodominant epitope of ovalbumin ($ova_{257-265}$) and thus no $CD4^+$ help is given in the vaccine, although this may be provided by the tumour itself; of note, peripheral blood $CD8^+$ responses were higher when tumour was given prior to vaccination (data not shown). B16 melanoma is controlled by $CD8^+$ cells in a perforin-independent fashion. Both 4-1BB and CD27 costimulation adequately replaced CD40 costimulation in this system, significantly protecting mice from tumour compared to DNGR-1 alone. In conclusion, both CD27 and 4-1BB costimulation are equally effective, and comparable to CD40 costimulation, in generating effector $CD8^+$ T cells that are capable of eradicating ova expressing tumours.

4.2.8. Key findings

In summary, the key findings from these experiments are as follows:

- 4-1BB and CD27 costimulation both augment the primary, resting memory and secondary responses in $CD8^+$ T cells
- The effect on memory generation is greater with 4-1BB costimulation
- The effect is seen using stimulatory antibodies and soluble ligands
- The memory cells generated using 4-1BB or CD27 exhibit similar homeostatic proliferation and capacity for secondary expansion
- This implies that the effect on memory takes place during the primary $CD8^+$ response
- The effects are independent of $CD4^+$ T cells and $CD4^+CD25^+$ T_{reg} during priming
- $CD4^+$ depletion led to an enhanced $CD8^+$ primary response with 4-1BB and CD27 costimulation
- The $CD8^+$ memory T cells generated did not confer additional protection against EG7 tumour challenge
- The $CD8^+$ primary responsed generated with CD27 and 4-1BB protected against B16-ova melanoma challenge

Chapter 5. Mechanisms of enhanced memory formation with 4-1BB

5.1. Introduction

The previous chapter demonstrated a marked difference between 4-1BB and CD27 costimulation in the generation of $CD8^+$ T cell memory. The effect appeared to be limited to the contraction phase of the primary response; it was independent of the size of the primary response, and did not appear to be due to 'off target' effects on $CD4^+$ helper T cells or on $CD4^+$ $CD25^+$ regulatory T cells. The memory cells generated had comparable homeostatic and secondary proliferative capacity whether 4-1BB or CD27 costimulation was employed during priming. It would seem that 4-1BB signalling protects $CD8^+$ T cells from apoptosis during the contraction phase of the primary response. The aim of this chapter was to examine in detail the possible mechanisms for this difference.

Multiple factors are important in determining $CD8^+$ T cell memory: various cytokines are important in memory development – IL-7 and IL-15 both have overlapping roles: the receptor for IL-7 is CD127 (the IL-7 receptor α chain, IL7rα); CD127 expression during the primary $CD8^+$ response marks the cells programmed to survive beyond the primary response, and their number correlates with the resulting numbers of memory cells [163].

The biology of IL-15 is complex (see discussion). The receptor for IL-15 is a heterotrimer, and the β subunit (IL-2βR, CD122), which is shared with the IL-2 receptor, is used to assess IL-15 receptor expression. IL-15, in contrast to IL-7, does not seem to be important in naïve $CD8^+$ T cell homeostasis; instead, it sustains memory $CD8^+$ T cells, and, in its absence, these undergo a slow attrition [340].

KLRG-1, (killer cell lectin-like receptor G1) is an inhibitory, ITIM containing transmembrane protein present on mouse and human NK and T cells. It is

detectable on memory and effector $CD8^+$ T cells, and interferes with TCR signalling, possibly conferring a negative feedback mechanism for T cell activation [341]. The receptor for KLRG1 is E-cadherin [342]: E-cadherin is expressed on epithelial cells, keratinocytes and langerhans cells, it may have a role in suppressing exaggerated immune responses. KLRG-1 has been reported to be expressed on the short lived effector/memory population

Thus IL-15rβ, IL-7rα and KLRG-1 have all been associated with $CD8^+$ T cell memory generation, and the expression of these three molecules during the primary $CD8^+$ T cell response was examined to see if they could explain the difference in memory generated by 4-1BB and CD27.

5.1.1. 4-1BB and CD27 costimulation yield differing CD127 and KLRG-1 expression during the primary response

To try and define the mechanism by which 4-1BB costimulation yields higher numbers of memory $CD8^+$ T cells when compared with CD27, cell surface markers associated with $CD8^+$ T cell memory generation were studied. To study the expression of these cell surface markers, 1 x 10^6 OT-I cells were adoptively transferred into syngeneic C57BL/6 followed by immunisation with 5mg ovalbumin (d0) and anti-4-1BB (LOB 12.3) or anti-CD27 (AT124.1) (250µg d0,1). Responses were monitored in peripheral blood by FCM, and expression of CD122 (IL15 receptor β), IL-7 receptor α (IL-7rα/CD127) and KLRG-1 was measured on Kb-$ova_{257-265}$ $CD8^+$ positive cells during the primary response.

These three molecules showed differing patterns of expression: CD122 expression was rapidly upregulated on $CD8^+$ T cells after immunisation (Figure 53), with no difference of expression between CD27 or 4-1BB costimulation. CD127 expression was downregulated rapidly during the primary $CD8^+$ T cell response, with the effect more marked in the 4-1BB experimental arm (Figure 54). Conversely, after the peak of the primary response, during the contraction phase, CD127 expression was consistently higher with 4-1BB costimulation. KLRG-1 is expressed on proliferating $CD8^+$ T cells during a primary $CD8^+$ response [343]. KLRG-1 expression was higher during the primary response when 4-1BB costimulation was employed (Figure 55).

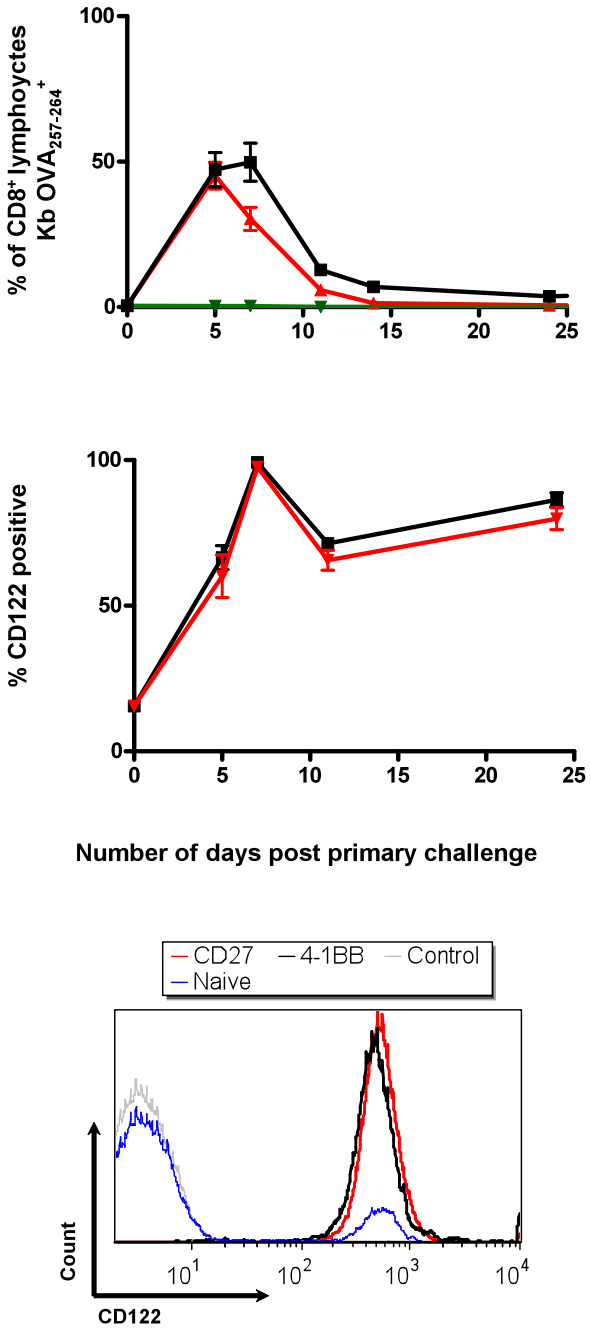

Figure 53 IL15Rβ expression during primary CD8$^+$ T cell response: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■) or anti-CD27 (▼) antibody (250mcg ip d0,1). Responses were measured in peripheral blood (top). Il15Rβ expression on ASC was upregulated early in the response, and was not different between experimental groups. Pooled data during the primary response from three animals per group is shown (middle), as is a representative plot from day 7 (below). Similar results were obtained from two other experiments. Error bars represent the SEM.

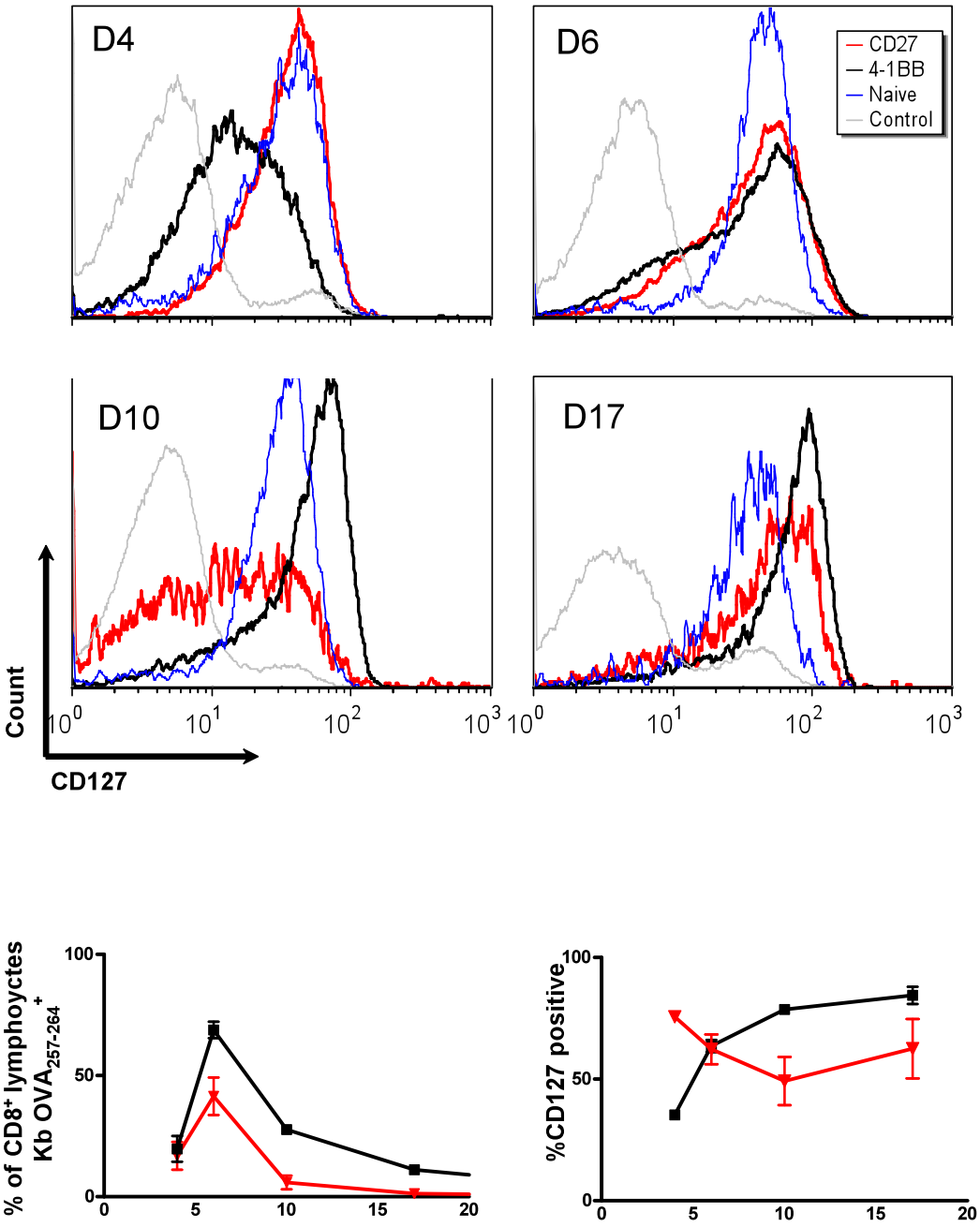

Figure 54 IL7Rα expression during primary CD8$^+$ T cell response: experiment setup was as in **Figure 53**. CD8$^+$ T cells costimulated with 4-1BB (■) downregulated IL-7Rα more rapidly than those costimulated with CD27 (▼) (day 5); this effect was reversed for the remainder of the primary response, where 4-1BB costimulation resulted in higher levels of IL-7Rα for the duration of the contraction phase of the primary response. Representative plots for each day are shown above, with pooled data shown below (red – CD27, black – 4-1BB). This data is representative of three separate experiments. Error bars represent the SEM.

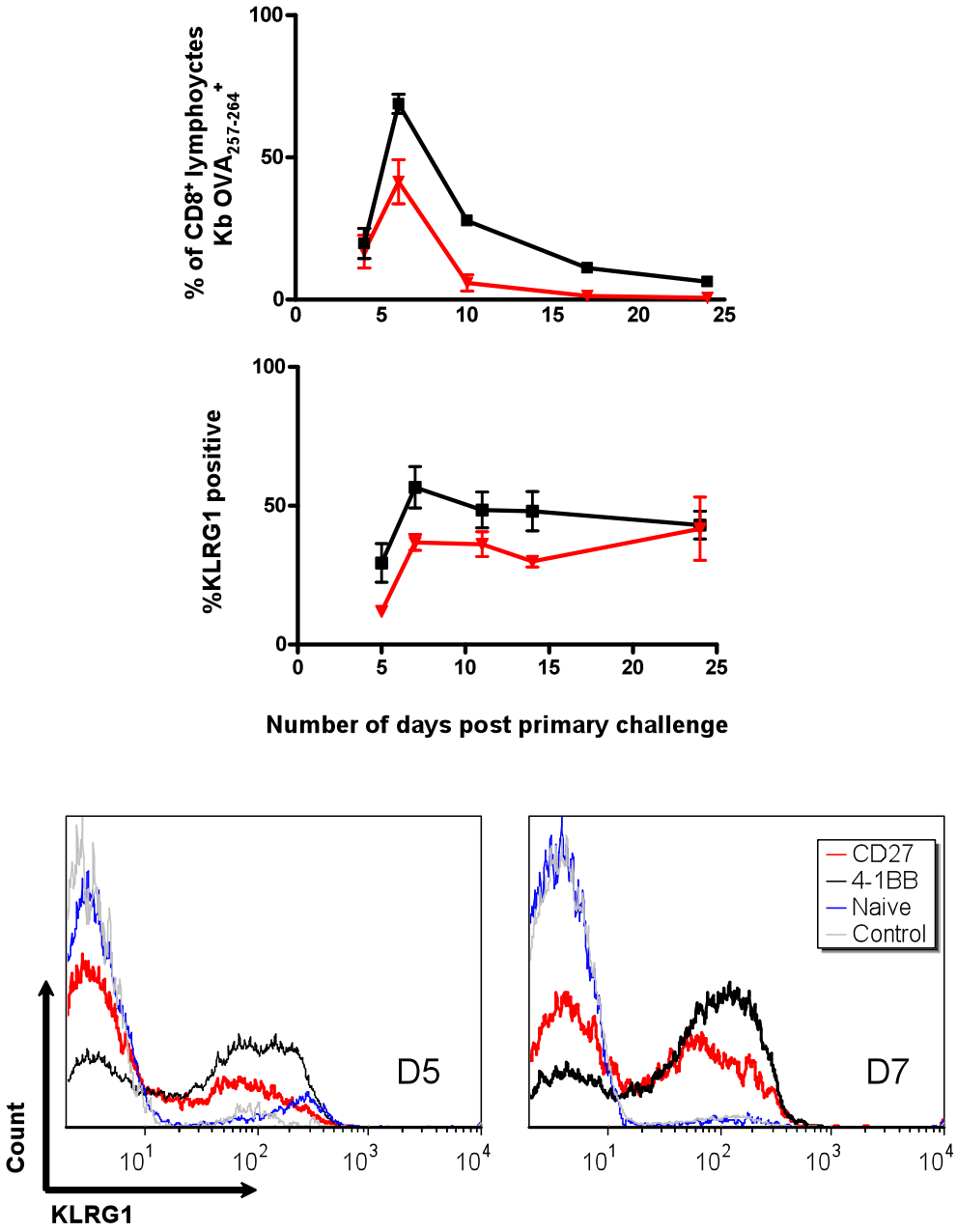

Figure 55 KLRG-1 expression during primary CD8$^+$ T cell response: experimental conditions were as in Figure 53. Responses were measured in peripheral blood (below, left). CD8$^+$ T cells costimulated with 4-1BB (■) rapidly upregulated KLRG-1 to higher levels than CD27 (▼) costimulated cells, and this persisted throughout the primary response. Pooled data during the primary response from three animals per group (below, right) are shown, as is a representative plot from day 7 (below) (black – 4-1BB, red – CD27, grey/silver – istotype staining controls). This data is representative of three separate experiments. Error bars represent the SEM.

5.1.2. CD127 and KLRG-1 expression identify separate subsets of $CD8^+$ T cells at the peak of the primary response

$CD127^{high}$ $CD8^+$ T cells during the primary response have been shown to express less KLRG-1 than their $CD127^{lo}$ counterparts [344]: this suggests two populations of cells during a $CD8^+$ T cell primary response: a $CD127^{high}$ population that is programmed to survive beyond the primary phase and develop into long lasting $CD8^+$ T cell memory, and a short lived, $KLRG\text{-}1^+$ population involved in immediate effector function. It was of interest that 4-1BB costimulation generated $CD8^+$ T cells with higher expression of both of these cell surface molecules (Figure 54/Figure 55), which seemed to conflict with the published evidence. To investigate this further the experiment was repeated, and splenocytes were stained for dual expression of CD127 and KLRG-1 (Figure 56). At this early timepoint, CD127 expression is lower on $CD8^+$ T cells stimulated by 4-1BB, consistent with the findings in peripheral blood. This experiment demonstrated, consistent with the findings of other groups, that KLRG-1 expression was exclusively found on $CD127^{lo}$ cells; conversely $CD127^{high}$ cells did not express KLRG-1. It would seem that in this experimental system it was not CD127 expression at the peak of the primary response that predicted the amount of memory generated: instead, it was CD127 expression during the contraction phase that was higher when 4-1BB costimulation was present, and this was associated with higher numbers of memory cells generated.

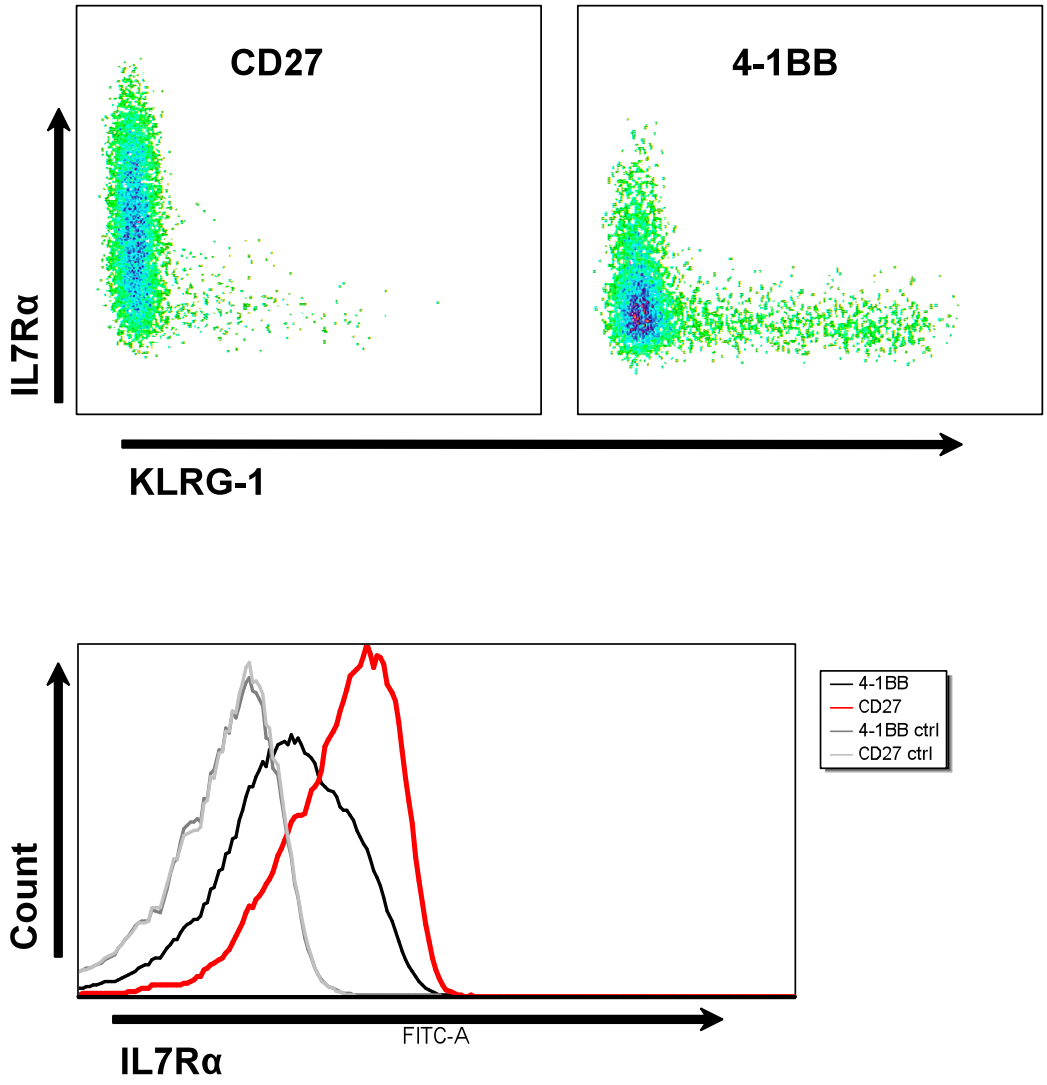

Figure 56 KLRG-1 and IL-7Rα expression on splenocytes during primary responses: to confirm the findings of peripheral blood, and to establish the pattern of KLRG-1/IL-7Rα expression, 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■) or anti-CD27 (▼) antibody (250mcg ip d0,1). 96 hours later spleens were harvested. $CD8^{+ve}$ K_b $OVA_{257-264}^{+ve}$ live T cells were gated to show the expression of KLRG-1 and IL-7Rα (above) simultaneously. Representative plots are shown (above) with a 1D plot of IL-7Rα expression comparing 4-1BB (black) with CD27 (red) and isotype controls (grey/silver). The results are representative of two separate experiments.

5.1.3. The enhanced effect on $CD8^+$ memory with 4-1BB costimulation persists in the presence of IL-7rα blockade

To find out if CD127 expression was the explanation for the enhanced memory generated with 4-1BB costimulation, the experiments in Figure 54 were repeated with or without CD127 blockade (anti-CD127 antibody, clone A7R34, [321]). Blockade of IL-7 signalling with the mAb to IL-7Rα reduces $CD8^+$ T cell memory formation without depleting IL-7Rα expressing cells [345].

IL-7Rα blockade did not prevent the enhanced memory generation with 4-1BB compared with CD27; this was shown with high (Figure 57, Figure 58) and low (Figure 59) OT-I transfers. Different OT-I doses were used to take account of the effect of different OT-I doses on IL-7Rα expression, with higher doses leading to higher IL-7rα expression. It is notable that IL-7Rα blockade reduced the responses in both experimental arms when 1×10^6 OT-I T cells were used, but not when 10^4 OT-I T cells were used; IL-7Rα blockade was complete in these experiments (as assessed by peripheral blood staining for IL-7Rα-FITC staining ; the lack of suppression of the primary response may have been due to less intraclonal competition at lower OT-I doses [325]. In conclusion, the increased $CD8^+$ T cell memory generated with 4-1BB does not seem to be directly due to IL-7Rα signalling during the primary response.

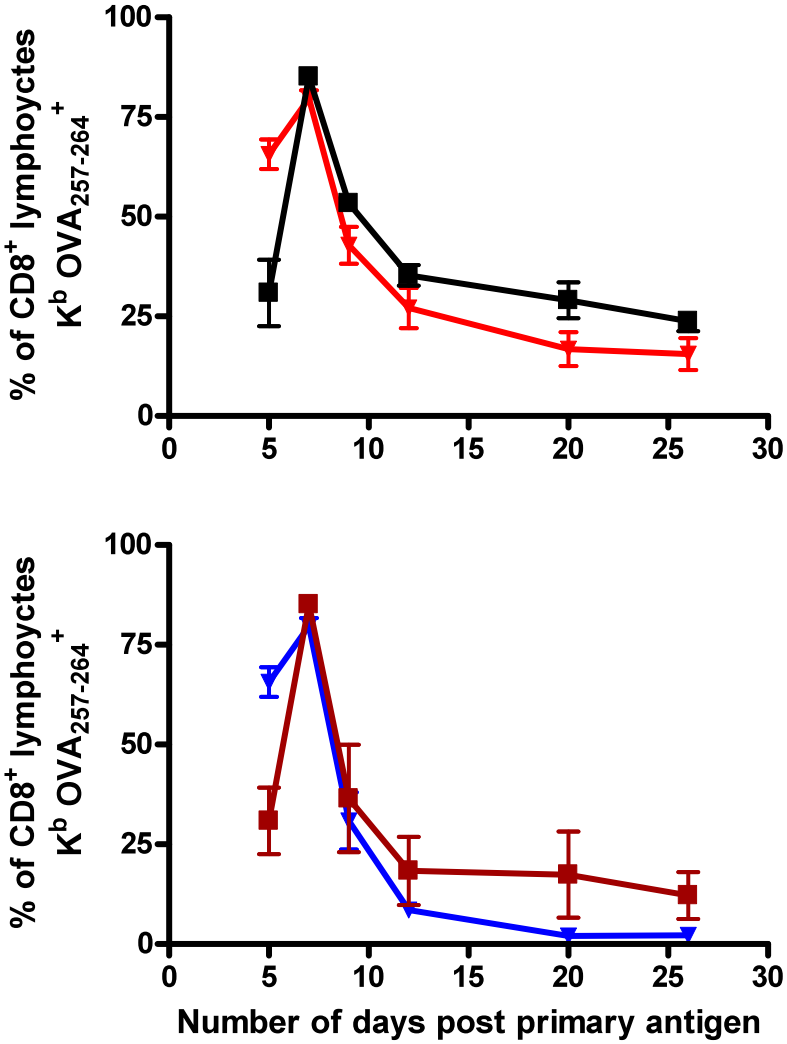

Figure 57 enhanced memory generation by 4-1BB is not mediated by IL-7Rα/CD127 signalling – high OT-I transfer: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■/■)or anti-CD27 (▼/▼) antibody (250mcg ip d0,1) without (above) or with (below) blocking antibody to IL-7Rα (500µg d7, 9, 11, 14). Responses developed earlier with CD27, but a greater number of memory cells survived at day 26 in the 4-1BB stimulated groups; this was unaffected by IL7Rα blockade. Error bars represent the SEM.

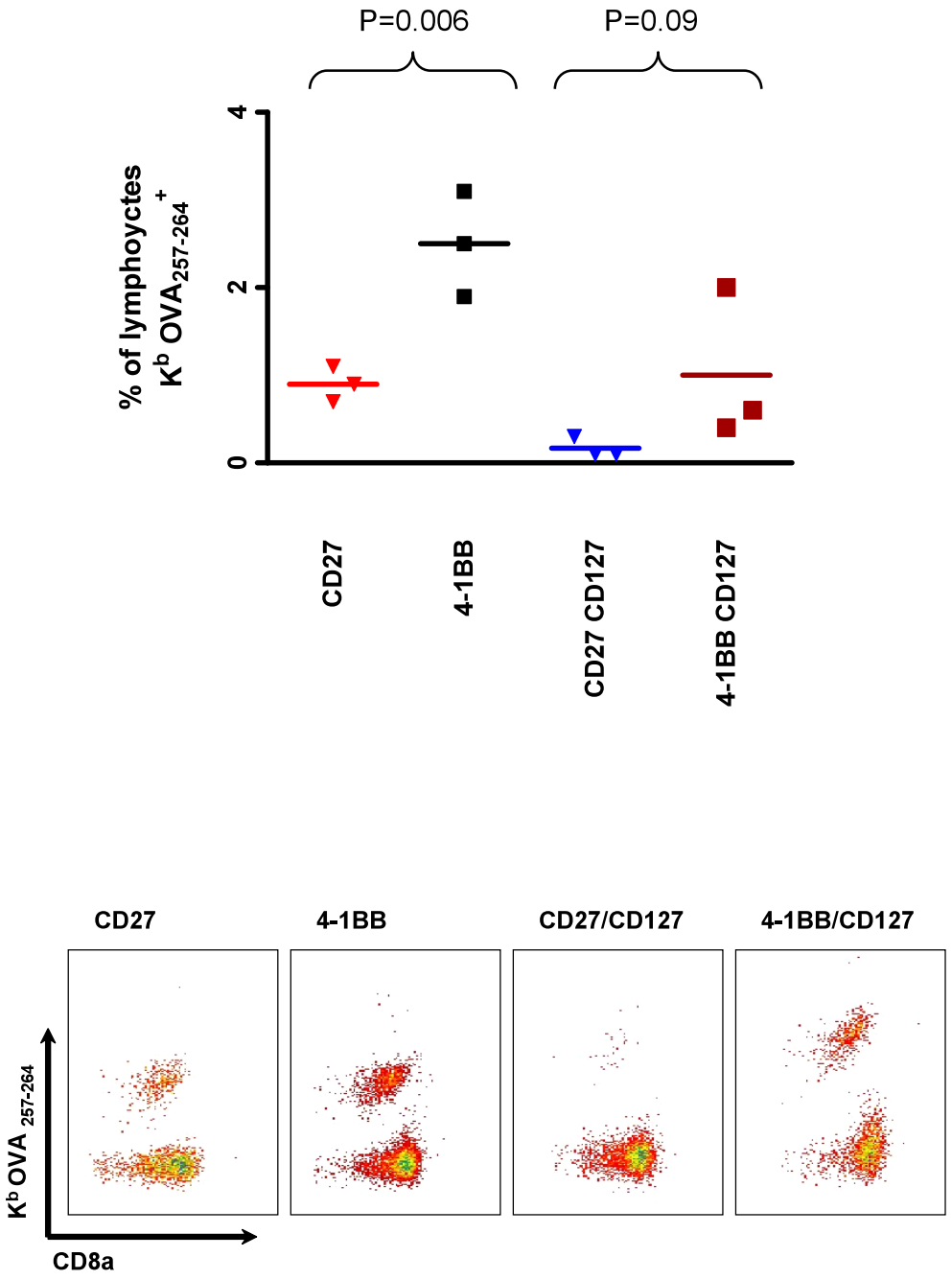

Figure 58 Enhanced memory generation by 4-1BB is not mediated by IL-7Rα/CD127 signalling – high OT-I transfer: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■/■)or anti-CD27 (▼/▼) antibody (250mcg ip d0,1) with or without blocking antibody to IL-7Rα (500µg d7, 9, 11, 14). The resulting memory generated at d22 was significantly higher in the 4-1BB treated subjects (p=0.006 control/p=0.09 IL-7 blocked), regardless of the presence of IL7-Rα blockade; pooled data (above) and representative FACs plots (below) are shown.

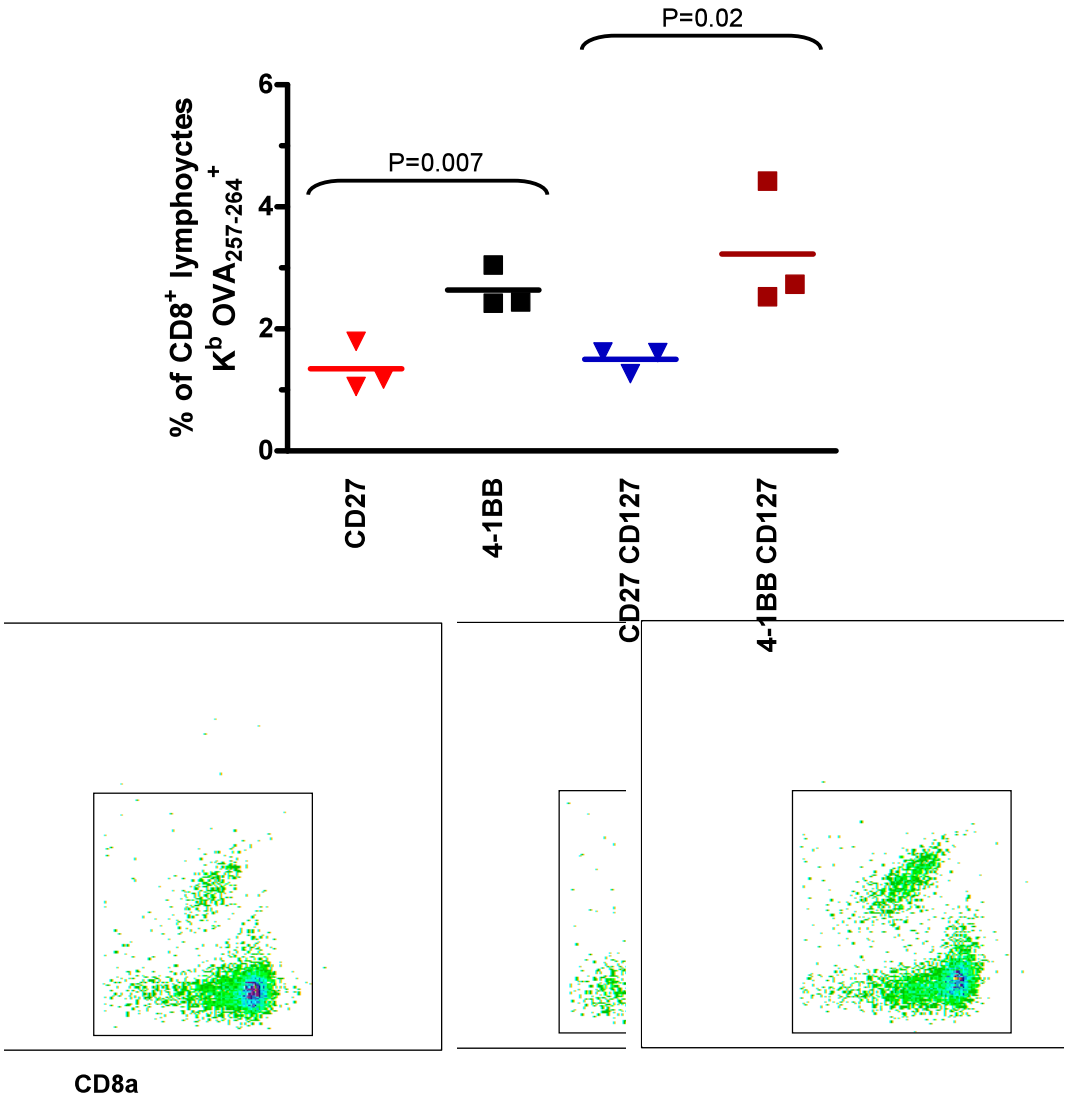

Figure 59: enhanced memory generation by 4-1BB is not mediated by IL-7Rα/CD127 signalling – low OT-I transfer: 1 x 10^4 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■/■)or anti-CD27 (▼/▼) antibody (250mcg ip d0,1) with or without blocking antibody to IL-7Rα (500µg d7, 9, 11, 14). The resulting memory generated at d22 was significantly higher in the 4-1BB treated subjects, regardless of the presence of IL7-Rα blockade; pooled data (above) and representative FACs plots (below) are shown..

5.1.4. Bim is downregulated by CD27 and 4-1BB signalling

It has already been shown in the first results chapter that CD27 signalling downregulates the expression of the pro-apoptotic molecule Bim (chapter 3). In addition 4-1BB signalling has been shown by other groups to downregulate Bim expression [156]. This molecule has been implicated in the contraction phase of the $CD8^+$ T cell response [173]. To compare the expression of Bim, and other molecules known to be involved in $CD8^+$ T cell memory generation, 4-1BB and CD27 were compared as in Figure 56. 96 hours after stimulation, antigen specific cells were sorted to greater than 95% purity as in Figure 25. Gene expression was then measured by qPCR; under these conditions, 4-1BB stimulation led to four fold lower levels of Bim expression (Figure 60). Interestingly, Batf3 showed an inverse relationship with Bim expression, its levels being significantly higher in 4-1BB co-stimulated cells. Bim expression is known to be under control of the AP-1 transcription factor [346], and it may be that Batf3 expression is involved in Bim expression in a negative feedback loop as was discussed in the first results chapter.

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Figure 60: expression of various genes associated with $CD8^+$ T cell memory generation: 1 x 10^6 naïve C57BL/6 OT-I cells were transferred into C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB or anti-CD27 antibody (250mcg ip d0,1). 96h later spleens and lymph nodes were harvested and K^b-$ova_{257-264}^+$ $CD8^+$ lymphocytes were purified on a cell sorter. qPCR of several genes involved in the generation of $CD8^+$ T cell memory was examined (above); Bim expression was higher with CD27 costimulation (4 fold relative quantity); conversely Batf3 expression was higher in the cells that had received 4-1BB costimulation. Individual fluorescence plots are shown (below) for Batf3 and Bim, comparing 4-1BB (black) and CD27 (red).

5.1.5. 4-1BB and CD27 costimulation enhance $CD8^+$ T cell proliferation/survival *in vitro* in the presence or absence of Bim

To examine whether 4-1BB and CD27 costimulation was affected by the absence of Bim, $CD8^+$ T cells from Bim -/- mice were used. Purified $CD8^+$ T cells from wildtype and Bim -/- mice were stimulated with different concentrations of α-CD3 (145.2C11) with or without costimulation (Figure 61) by soluble ligands (sCD70 and s4-1BBL respectively). Both CD27 and 4-1BB costimulation resulted in enhanced proliferation/survival when compared to TCR signalling alone; the effect was retained in the absence of Bim. Commensurate with its role in T cell survival, proliferation/survival were higher in the Bim -/- $CD8^+$ T cells. Of note, 4-1BB signalling was associated with greater proliferation/survival in the presence of Bim, but CD27 and 4-1BB showed equivalent effects on proliferation/survival in the absence of Bim. This raised the possibility that the difference in survival of $CD8^+$ T cells was mediated via effects on the expression of Bim. To examine this further, $CD8^+$ T cell response to soluble antigen were examined *in vivo* in Bim-deficient mice.

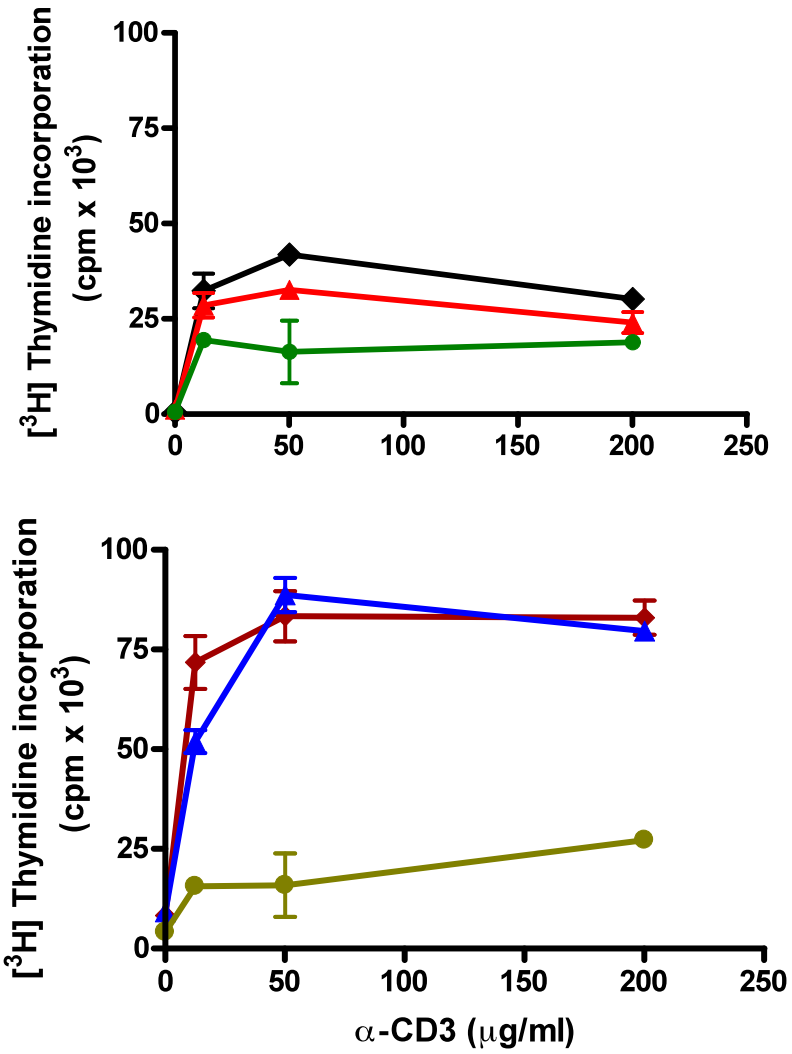

Figure 61 CD70 and 4-1BBL-Fc co-stimulate in wildtype and Bim deficient T cells: 2 x 10^5 C57/Bl6 (above) or Bim -/- (below) $CD8^+$ splenocytes were purified on depletion columns and activated by culture in the presence of soluble α-CD3 (145.2C11) at varying doses plus control Ig (human IgG) (●/●), sCD70 (▼/▼) or s-4-1BBL (■/■). 0.5μCi [^{3}H] thymidine was added for the last 16 hours of a 72 hour culture after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM.

5.1.6. The primary $CD8^+$ T cell response is unaffected by the absence of Bim when 4-1BB and CD27 costimulation are employed

To examine whether the differential effects on Bim expression seen with 4-1BB and CD27 costimulation were replicated *in vivo*, endogenous responses were studied in wildtype and Bim -/- mice. Mice were immunised with ovalbumin and α-4-1BB/α-CD27 (as in Figure 45) and responses were tracked in peripheral blood (Figure 62). The delayed primary response and contraction phase seen in previous experiments was again seen, and seemingly unaffected by the absence of Bim: however, when mice were rechallenged with peptide and α-CD40, the memory responses were significantly different in the wildtype mice, but were equivalent in the Bim -/- mice (Figure 63). It is therefore possible that some of the difference between the contraction phases seen with CD27 and 4-1BB costimulation are due to differences in their effects on Bim expression, with subsequent differences in the quantity of the resulting $CD8^+$ memory population.

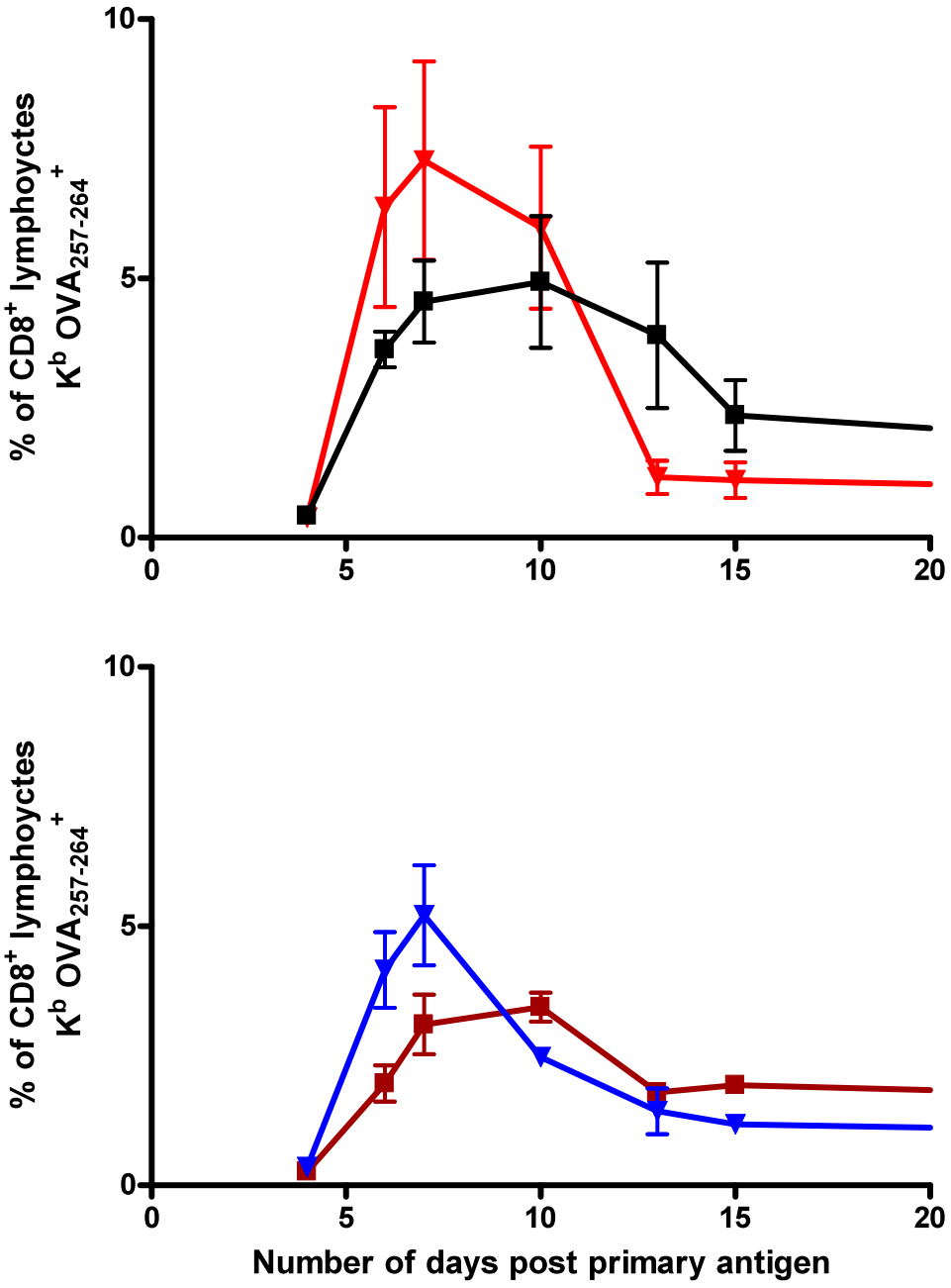

Figure 62 Primary CD8$^+$ T cell responses in Bim -/- and wildtype mice: wildtype (above) and Bim -/- (below) mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-4-1BB (■■) or α-CD27 (▼▼) (LOB 12.3 and AT124.1 respectively, 250μg d0,1). Responses were followed up in peripheral blood. The early peak with CD27, and the delayed contraction with 4-1BB, were unaffected by the presence (above) or absence (below) of Bim.

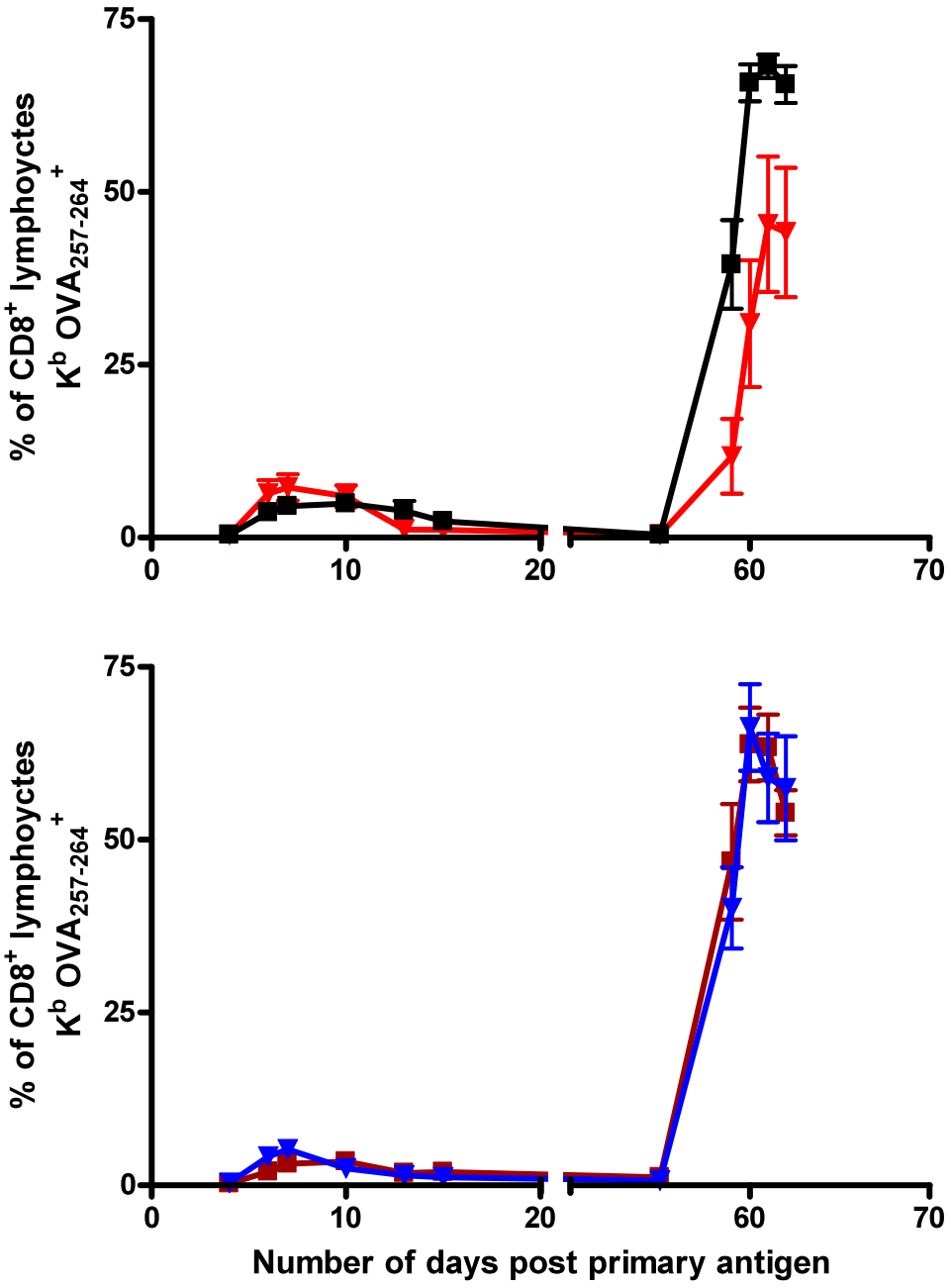

Figure 63 secondary CD8$^+$ T cell responses in Bim -/- and wildtype mice: wildtype and Bim -/- mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-4-1BB (■■) or α-CD27 (▼▼) (LOB 12.3 and AT124.1 respectively, 250μg d0,1). Responses were followed up in peripheral blood. The greater secondary response with 4-1BB seen in WT animals (above) was lost in Bim -/- animals (below).

5.1.7. $CD8^+$ T cell responses mediated by 4-1BB or CD27 undergo apoptotic contraction in a Bim-independent manner

As Bim is a pro-apoptotic molecule, important in $CD8^+$ T cell contraction, it was surprising that $CD8^+$ T cell primary responses were identical in Bim -/- and wildtype mice. To examine this further the experiment was repeated to see if the total number of antigen specific cells differed between Bim -/- and WT animals. To ascertain total antigen specific cell numbers, spleens were taken at various timepoints during the primary response to compare total antigen specific cell numbers, in addition to the previously measured percentages. Commensurate with its role in lymphocyte survival, total antigen specific cell numbers were higher in Bim -/- mice (Figure 64): this is predictable, as Bim-deficient mice are known to have an expanded leucocyte pool, with spleens containing double the number of lymphocytes than are found in wildtype animals (personal observation and [347]).

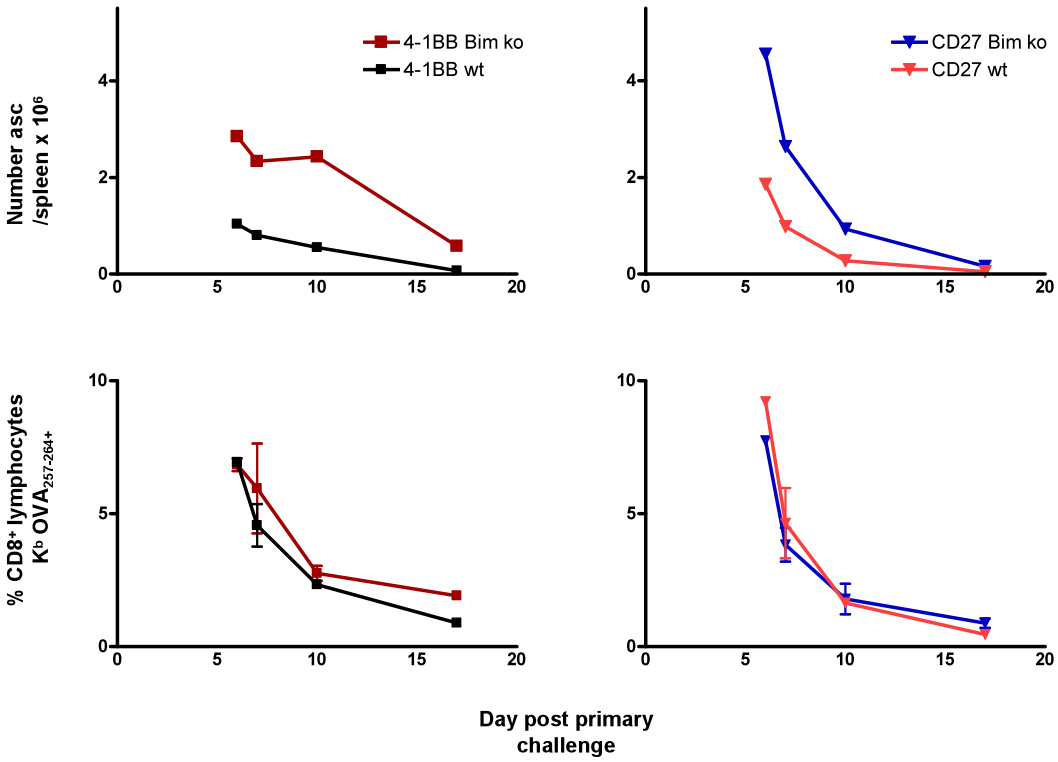

Figure 64: primary CD8$^+$ T cell responses in Bim -/- and wildtype mice – total numbers: wildtype and Bim -/- mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-4-1BB (■/■) or α-CD27 (▼/▼)(LOB 12.3 and AT124.1 respectively, 250μg d0,1). Spleens were taken (2/group/time point) at each time point and the total numbers (above) and relative percentage (below) of antigen specific cells recorded. Total cell numbers were higher in the absence of Bim; the delayed rate of decline seen in peripheral blood with 4-1BB costimulation was confirmed in spleens, and was unaffected by the absence of Bim.

5.1.8. $CD8^+$ T cell responses to soluble antigen with CD40 costimulation are reduced in the absence of Bim

CD27/CD70 interactions have been shown to be critical mediators of CD40 licenced DC priming of $CD8^+$ T cell responses; blockade of CD70 significantly reduces CD40 mediated $CD8^+$ priming . To study whether the downregulation of Bim by CD27 signalling was a critical mediator of its effects, CD40 with or without CD70 blockade was employed. Bim -/- mice have been used in the study of $CD8^+$ T cell responses to viruses [173, 348]; it is assumed that viral models provide 'complete' DC maturation and subsequently provide all the costimulatory and cytokine signals to prime $CD8^+$ T cell responses. CD40 stimulation of DCs is thought to bring about complete, or near-complete, DC maturation and the resulting $CD8^+$ T cell response is thought to mimic closely the physiological $CD8^+$ T cell response to infection. Initially wildtype and Bim -/- mice were vaccinated with 5mg ovalbumin and α-CD40 stimulatory antibody; responses were tracked in peripheral blood (Figure 65). Surprisingly, despite the pro-apoptotic effect of Bim, primary $CD8^+$ T cell responses (as a percentage of total $CD8^+$ T cells) were significantly higher in wildtype mice then in Bim deficient mice, although the total number of cells was higher in Bim deficient mice, which have higher numbers of total leucocytes.

Conversely, when CD27 costimulation was employed, $CD8^+$ T cell primary responses were identical between Bim -/- and wildtype hosts (Figure 66). By studying the contraction phase as a percentage of the peak of the primary response, a measure of $CD8^+$ T cell contraction could be gleaned (Figure 67); this suggested that $CD8^+$ T cell contraction was attenuated by the absence of Bim (as would be expected from the viral models) when CD40 stimulation was employed, but not when CD27 was used; these results were reproduced in subsequent experiments. These experiments suggested two independent findings; firstly, that CD27 signalling on $CD8^+$ T cells was not dependent on downregulation of Bim to mediate its effects; secondly, the possibility is raised that DCs are dependent on Bim, or alternately that DC maturation causes

additional signals to $CD8^+$ T cells that are Bim dependent for example through other costimulatory or cytokine pathways.

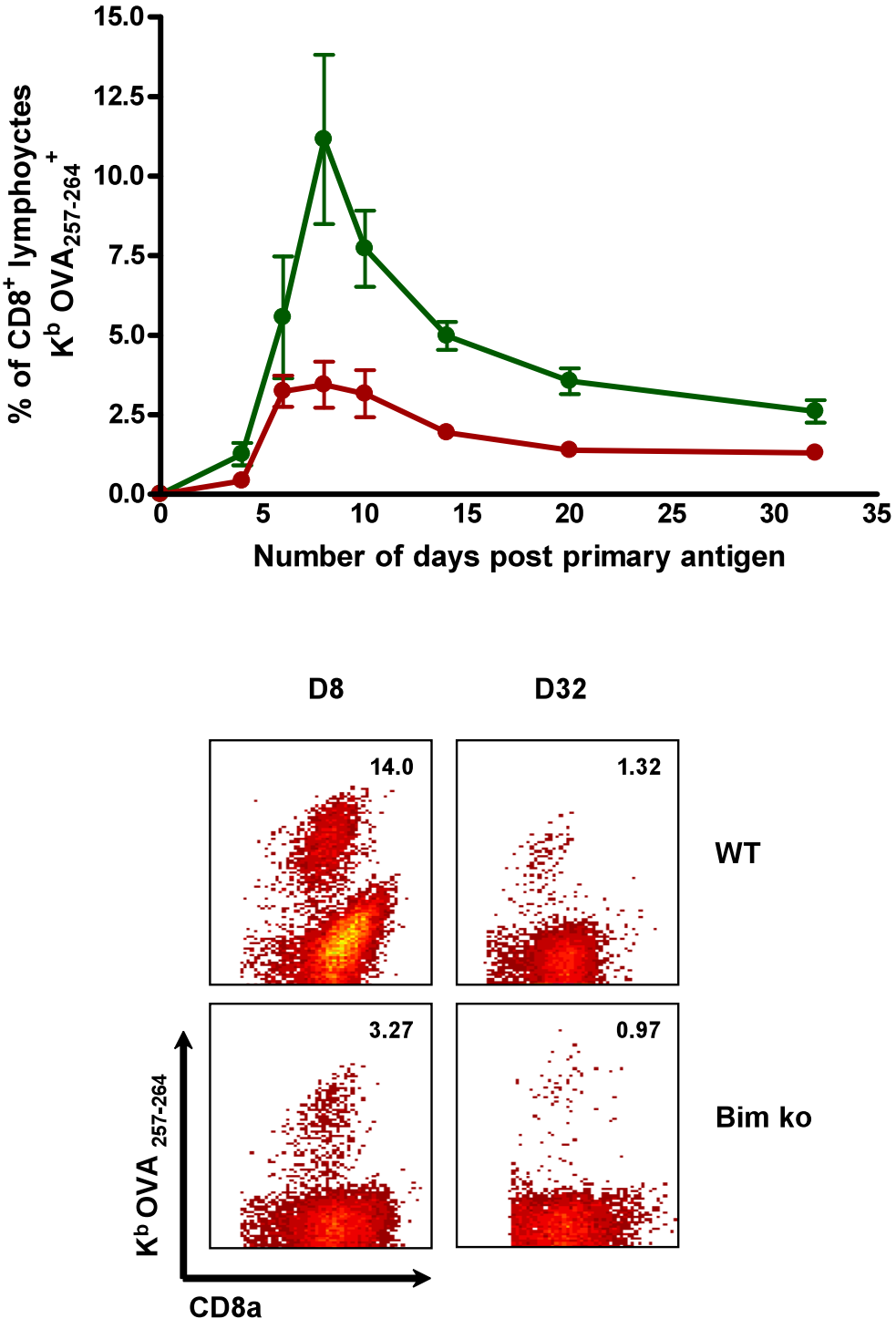

Figure 65 contraction of CD8$^+$ T cell primary responses in wildtype and Bim -/- mice: Wildtype and Bim -/- mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-CD40 (3/23, 500μg ip d0). Responses were followed up in peripheral blood. The peak of the primary response was lower, and the rate of contraction lower, in Bim -/- mice (●) compared with Wildtype (●). Pooled results (above) and representative plots from day 8 and d32 (below) are shown.

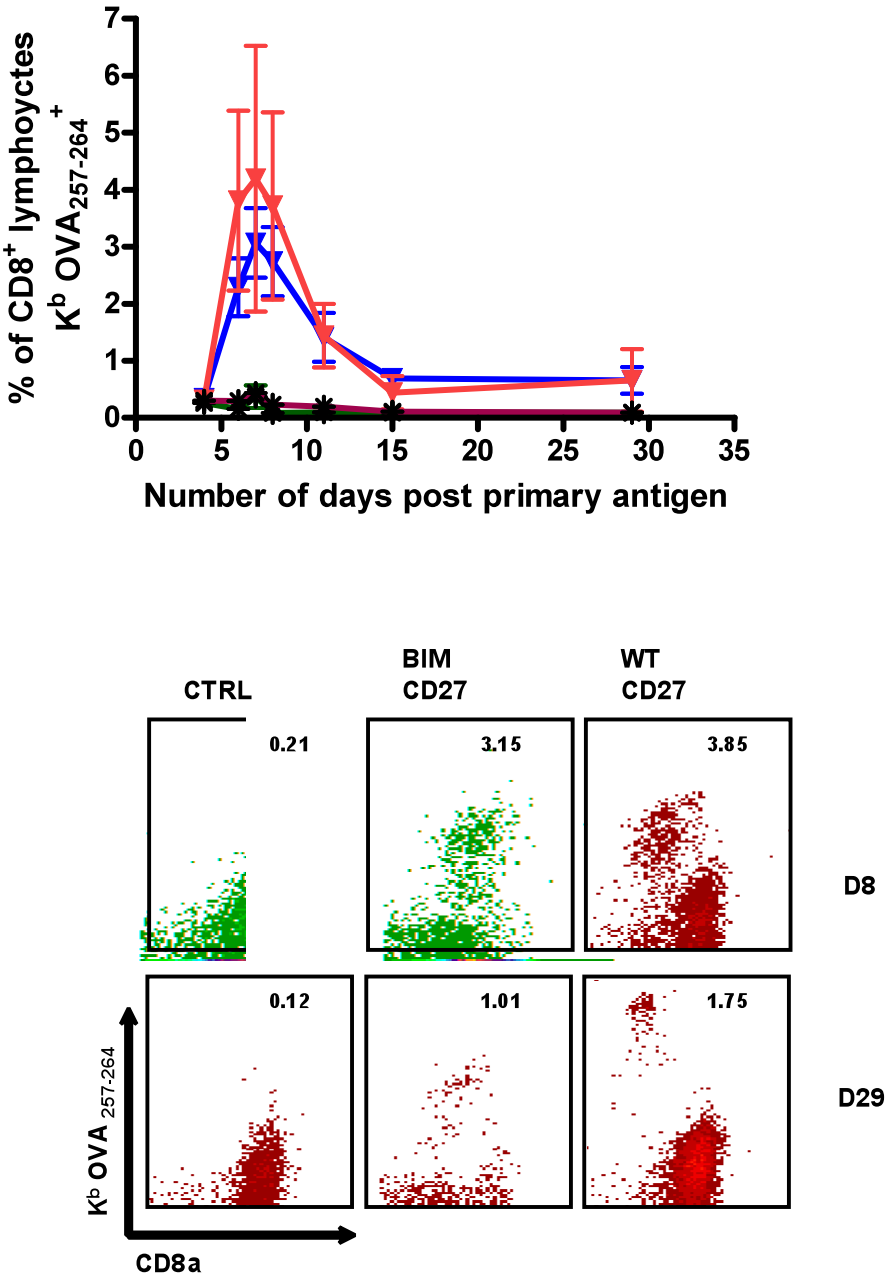

Figure 66 contraction of CD8$^+$ T cell primary responses in WILDTYPE and Bim -/- mice in response to CD27 signalling: Wildtype and Bim -/- mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-CD27 (AT124 250μg ip d0,1) or control (Mc106-A5 250μg ip d0,1). Responses were followed up in peripheral blood. Responses, both at the peak, and during contraction, were broadly equivalent between Bim -/- (▼) and wildtype mice(▼). Control mice (*) did not mount a detectable response. Pooled results (above) and representative plots from day 8 and d29 (below) are shown.

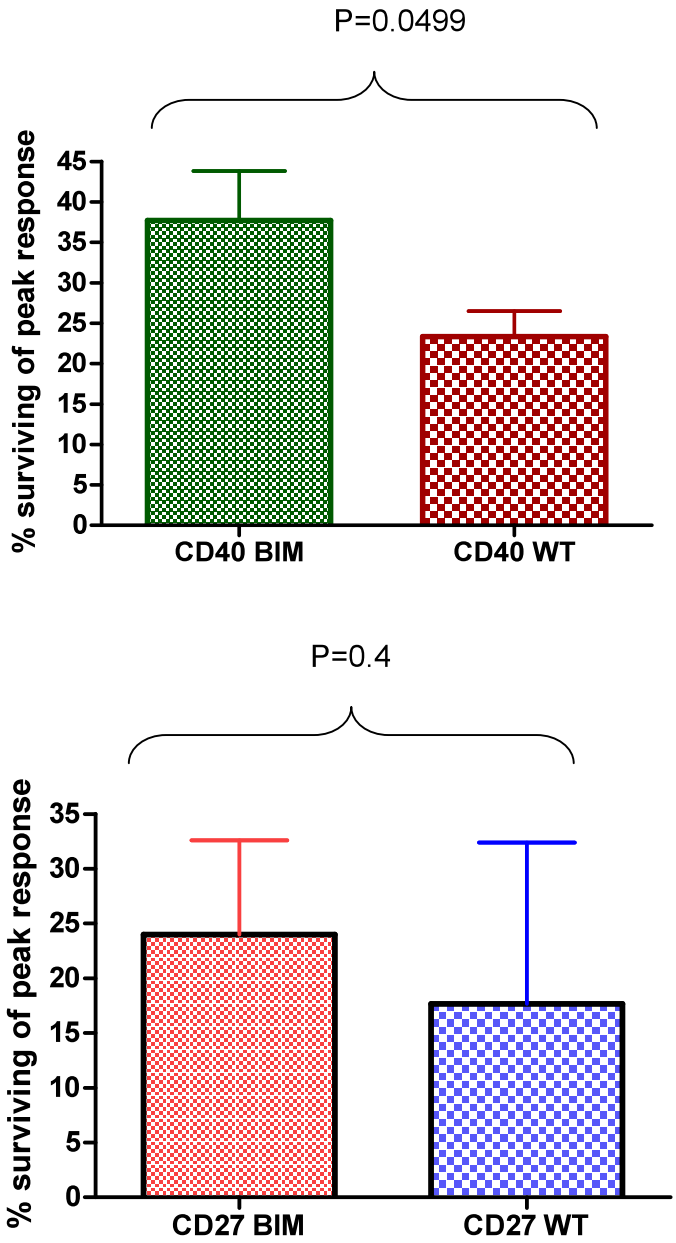

Figure 67 the contraction of CD8$^+$ T cell primary responses in Bim -/- and WILDTYPE mice differs according to costimulation received: wildtype and Bim -/- mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-CD27 (AT124 250μg ip d0,1) or agonistic α-CD40 (3/23 500μg ip d0). The percentage of antigen specific CD8$^+$ T cells surviving after the peak of the primary response is shown: there was no difference in the CD27 stimulated groups, but contraction was impaired in the CD40 stimulated groups.

5.1.9. CD27 costimulation is a critical component of CD40 licenced DC priming of $CD8^+$ T cells, and this is independent of Bim

The experiments shown above suggest that CD27 signalling is not dependent on the presence of Bim for its effects; more accurately, if the main effect of CD27 on primary $CD8^+$ T cell responses was mediated via Bim downregulation, it would be predicted that CD27 signalling would add little in the Bim deficient environment; however, CD27 signalling significantly enhances $CD8^+$ T cell responses in Bim -/- and wild-type hosts. To confirm these findings, the experiment was performed in the 'reverse' scenario; CD70 blockade was employed in the presence of CD40 costimulation (Figure 68). This experimental design assumes that all costimulation is present (4-1BB/OX-40/CD28 etc) *apart* from CD27/CD70 interactions, and has previously shown the critical role that these molecules have in priming $CD8^+$ T cell responses [88]. CD70 blockade significantly abrogated $CD8^+$ primary responses in wildtype and Bim -/- hosts. Since CD70 blockade resulted in abrogation of the $CD8^+$ primary response even in the absence of Bim, it can be concluded that the effects of CD27 signalling on $CD8^+$ T cell primary responses are largely independent of its effects on the expression of Bim.

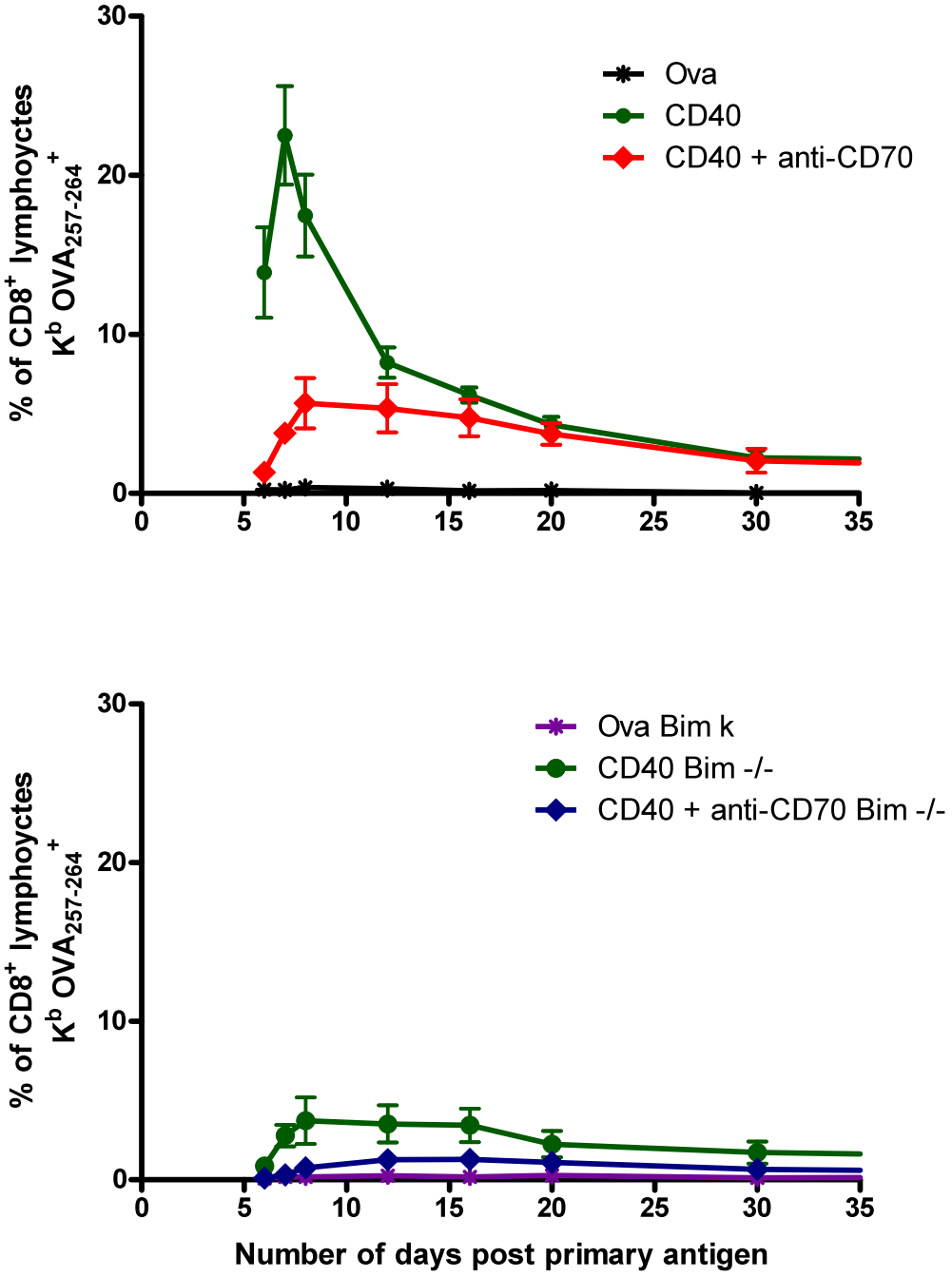

Figure 68: effect of blocking CD27 on CD8$^+$ T cell responses in Bim -/- and wildtype mice: wildtype (above) and Bim -/- (below) mice (3 per group) were vaccinated with 5mg ovalbumin protein and agonistic α-CD40 (3/23, 500μg ip d0) with or without blocking antibody to CD70 (Tan 1.7, 250μg d0,1). CD70 blockade markedly reduced primary responses in the presence or absence of Bim. Responses to CD40 stimulation were significantly reduced in the absence of Bim.

5.1.10. Wildtype CD8$^+$ T cells contract at similar rates in Bim deficient and wildtype hosts

In the experiments shown above, the CD8$^+$ T cell contraction was altered in the absence of Bim when CD40 costimulation was employed, but not when CD27 costimulation was employed. Assuming that CD40 costimulation is acting directly on DCs, and CD27 is acting downstream of CD40, directly on CD8$^+$ T cells, then it is possible that the effect of Bim deficiency was due to the absence of Bim in DCs rather than CD8$^+$ T cells. To study this in more detail, wildtype CD8$^+$ OT-I T cells were purified and adoptively transferred into wildtype or Bim -/- hosts. 24 hours later, mice were immunised with 5mg ovalbumin and α-CD40, and responses were tracked in peripheral blood. The number of antigen specific cells was lower in the Bim -/- recipients, commensurate with the higher numbers of endogenous lymphocytes present in these animals. The contraction rate of primed CD8+ T cells was similar in Bim -/- and wildtype recipients (**Figure 69**). This result suggests that Bim deficiency in DCs was not the reason for the attenuated responses to CD40 costimulation documented above.

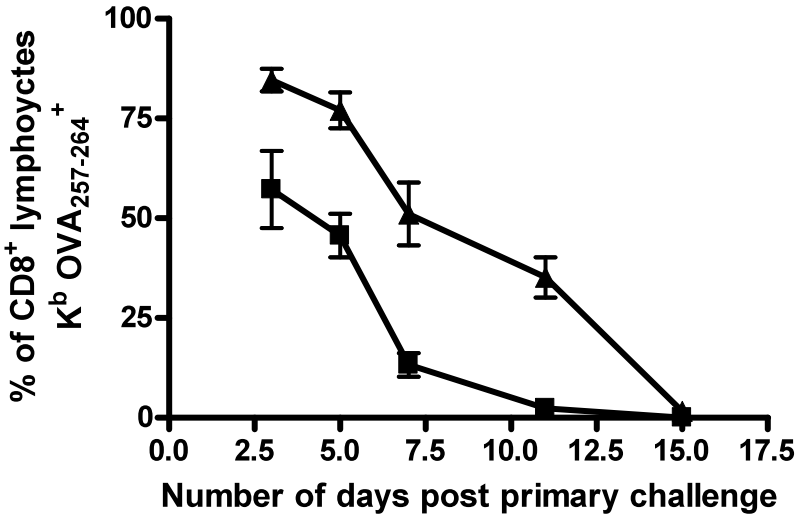

Figure 69: contraction of $CD8^+$ T cells in wildtype and Bim -/- hosts: $CD8^+$ T OT-I cells were purified on negative selection columns to >90% purity and 5 x 10^5 live cells were transferred into wildtype (▲) and Bim -/- (■) hosts. 24 hours later mice were vaccinated with 5mg ovalbumin and anti-CD40 (3/23 500µg ip). Antigen specific responses were measured in peripheral blood. Error bars represent the SEM. Responses were lower in Bim -/- mice, presumably because of the higher numbers of endogenous cells in these animals. The rate of decline was identical in wildtype and Bim -/- environments, suggesting that Bim expression in DCs does not impact on the contraction phase of the $CD8^+$ T cell response.

5.1.11. The effect of 4-1BB costimulation on CD8$^+$ T cell memory persists in the presence of Fas/FasL blockade

The experiments above demonstrate normal CD8$^+$ T cell contraction in the absence of Bim. This is at odds with published studies which show that Bim deficiency inhibits the contraction phase of CD8$^+$ T cells in response to viral immunisation, which is more consistent with the findings using CD40 stimulation. CD40 might be expected to mimic viral infection, since both are predicted to bring about similar levels of DC maturation. This implies that CD8$^+$ T cells generated by 4-1BB or CD27 costimulation die through a Bim-independent pathway. Such an alternative major pathway by which CD8$^+$ T cells die following primary expansion is via Fas/FasL interactions (this pathway may be particularly relevant in chronic antigenic stimulation). To examine whether Fas/FasL pathways were important in the CD8$^+$ T cell contraction following CD27/4-1BB costimulation the experiment in Figure 53 was repeated, in the presence or absence of Fas blockade – an approach that has previously been used to inhibit FasL function *in vivo* [349]). Both the CD8$^+$ contraction phase (Figure 70) and the resulting memory generated (Figure 71) were unaffected by Fas/FasL blockade. This implies that the differences seen between CD27 and 4-1BB costimulation were not due to differences in susceptibility to Fas mediated apoptosis.

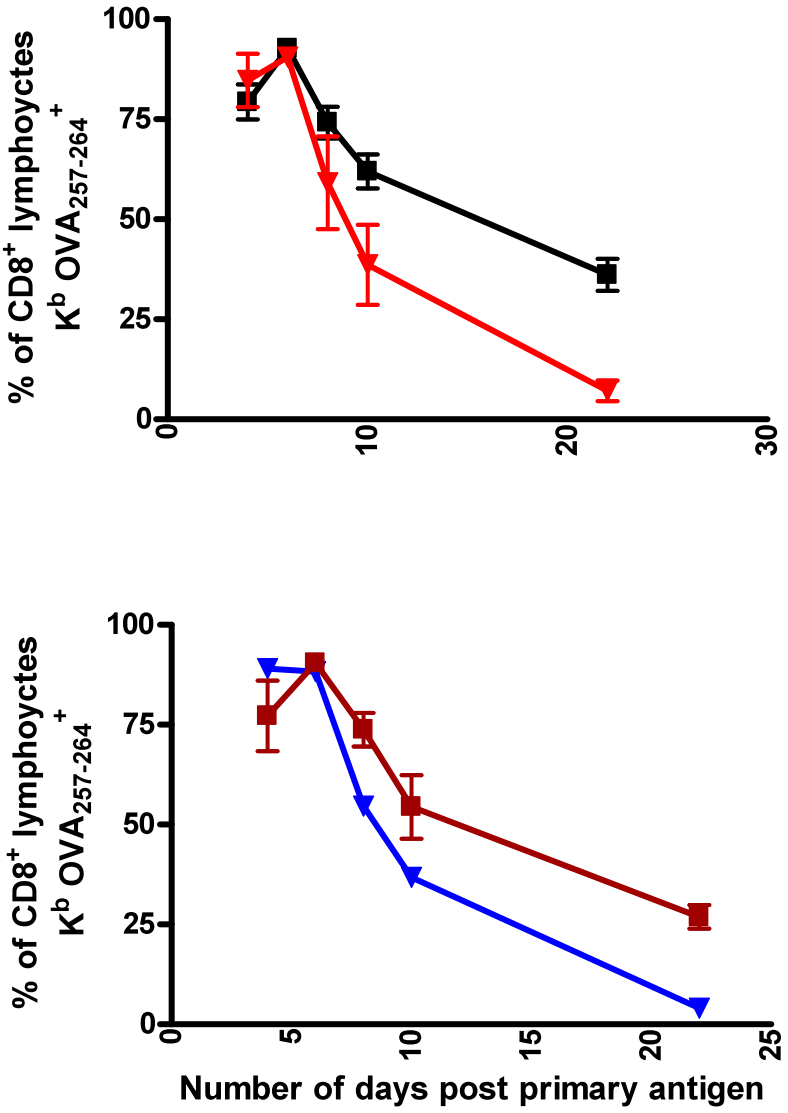

Figure 70: enhanced memory generation by 4-1BB is not mediated by FAS/FAS-L interactions during the contraction phase: 1 x 10^6 naïve C57BL/6 OT-I/Thy 1.1 cells were transferred into congenic (Thy 1.2) C57BL6 hosts and mice were primed with ip injection of 5mg ovalbumin and with either anti-4-1BB (■/■) or anti-CD27 (▼/▼) antibody (250mcg ip d0,1) with (below) or without (above) blocking antibody to CD95L (300µg d4, 6,8). The resulting memory generated at d22 was significantly higher in the 4-1BB treated mice; this remained true when CD95L blockade was used.

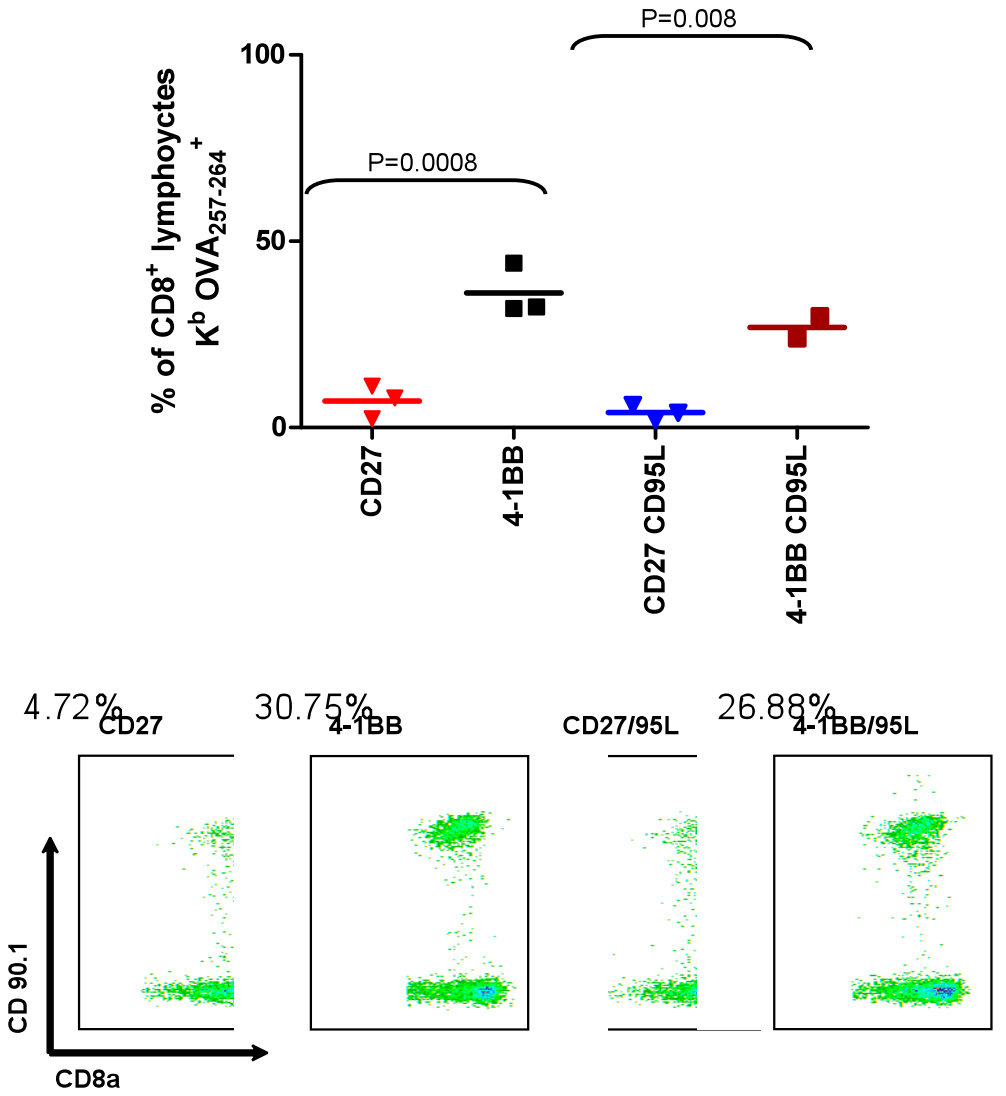

Figure 71 Enhanced memory generation by 4-1BB is not mediated by FAS/FAS-L interactions during the contraction phase – resting memory at day 22:

Same experiment as (Figure 71**)**: the resulting memory generated at d22 was significantly higher in the 4-1BB treated mice; this remained true when CD95L blockade was used. Pooled results from three mice are shown (above), with representative individual plots are gated on $CD8^+$ live lymphocytes (below).

5.2. Discussion

5.2.1. 4-1BB and CD27 lead to an equal and rapid upregulation of the IL-15 receptor

The generation of $CD8^+$ T cell memory can be divided into three distinct phases (Figure 72). 4-1BB and CD27 costimulation both seem to be important during the primary phase, with 4-1BB costimulation giving a stronger signal in favour of memory generation. The subsequent population of memory cells is remarkably stable in these experiments, and is maintained for at least 8 months (see second results chapter). The maintenance of immunological memory is complex; immunity to *vaccinia* in humans gradually declines over decades, possibly in part as space needs to be made for other, more recently generated memory populations; mathematical models point to a need for separate homeostatic mechanisms for naïve and memory compartments [350].

The cytokine IL-15 has preferential effects on the homeostasis of $CD8^+$ memory cells [345]; IL15 -/- mice show a selected deficiency of $CD8^+$ memory T cells, with relatively normal naïve subsets. Multiple additional lines of evidence support the involvement of IL-15 in the maintenance of the $CD8^+$ T cell memory pool, with a particular role on proliferation. The results from this chapter show that the receptor for IL-15, CD122 (the β subunit of the IL-15 receptor), was rapidly upregulated during the primary response when CD27 or 4-1BB costimulation was employed (Figure 53). CD27 and 4-1BB costimulation had comparable effects on CD122 expression during the primary response. From this it can be stated that the difference in memory generated was not due to differences in expression of CD122, although differences in downstream signalling, as well as differences in IL15 production, cannot be discounted.

Another cytokine, IL-7, also has clear roles in the generation and maintenance of $CD8^+$ T cell memory. The IL-7 receptor is a heterodimer, consisting of an alpha chain (IL-7rα) and the common gamma chain (γ_c/CD132). The IL-7R intracellular domain is bound to the janus-kinases, JAK1 and JAK3; on IL-7R binding to IL-7 these kinases mutually cross-phosphorylate each other, leading to downstream phosphorylation of STAT5 and subsequent transcriptional effects on pro-survival molecules such as Bcl-2 [351]. Unlike IL-15 the IL-7 receptor (IL7rα/CD127) is present on naïve $CD8^+$ T cells, and contributes to their homeostasis; IL7 -/- mice are severely lymphopaenic, emphasising the role of IL-7 in T cell generation and naïve T cell homeostasis; but IL-7 also has a role in $CD8^+$ T cell memory; during the primary response, IL7rα is downregulated on the majority of responding $CD8^+$ T cells; it is however the $IL7r\alpha^{high}$ minority that go on to form the resulting memory population [191].

Additionally, CD8+ memory T cells survive poorly in IL-7 -/- hosts. Whilst the expression of IL7rα is a dynamic process, the production of IL-7 appears to be constant, and is maintained at a constitutional level, unaffected by extrinsic feedback, and only altered by consumption by lymphocytes, forming a negative feedback loop, leading to close homeostatic control of T lymphocyte numbers. Therefore, because of its effects on lymphocyte survival and memory generation, IL-7rα expression was studied to see if it could explain the differences in memory generated by 4-1BB and CD27 costimulation. The expression of IL-7rα during the $CD8^+$ T cell response is shown in Figure 72.

In Figure 54 it is shown that IL7rα was initially downregulated on $CD8^+$ T cells, but during the contraction phase the IL7rα expression was higher in the $CD8^+$ T cells which had received 4-1BB costimulation. This result raised the possibility that the delayed contraction/enhanced memory generated with 4-1BB was due to higher levels of expression of IL7rα: however, when the experiment was repeated in the presence of a blocking antibody to IL7rα, the enhanced effect on memory remained (Figure 57

Figure 58). Although IL7rα blockade did reduce the memory generation in the high OT-I transfer in both 4-1BB and CD27 experimental arms, this effect was

not seen in the low OT-I arm, possibly reflecting enhanced scavenging of this cytokine when large numbers of lymphocytes are present. Other researchers have found that IL-7rα blockade during the primary response has little effect on the contraction phase; in addition, exogenous IL-7 does not enhance the memory generated from primary responses. IL-7 and IL-15 affect different memory cell populations: IL-15 works on the KLRG-1$^+$ effector subset, and IL-7 works on the CD127$^+$ central memory subset ([345]). Thus, although IL-7rα is expressed on proliferating CD8$^+$ cells destined to become memory cells, it does not seem to be physiologically vital for their generation, and manipulation of IL-7 did not affect the generation of CD8$^+$ T cell memory in the experiments presented here, and thus does not explain the difference in the effect on CD8$^+$ T cell memory generation.

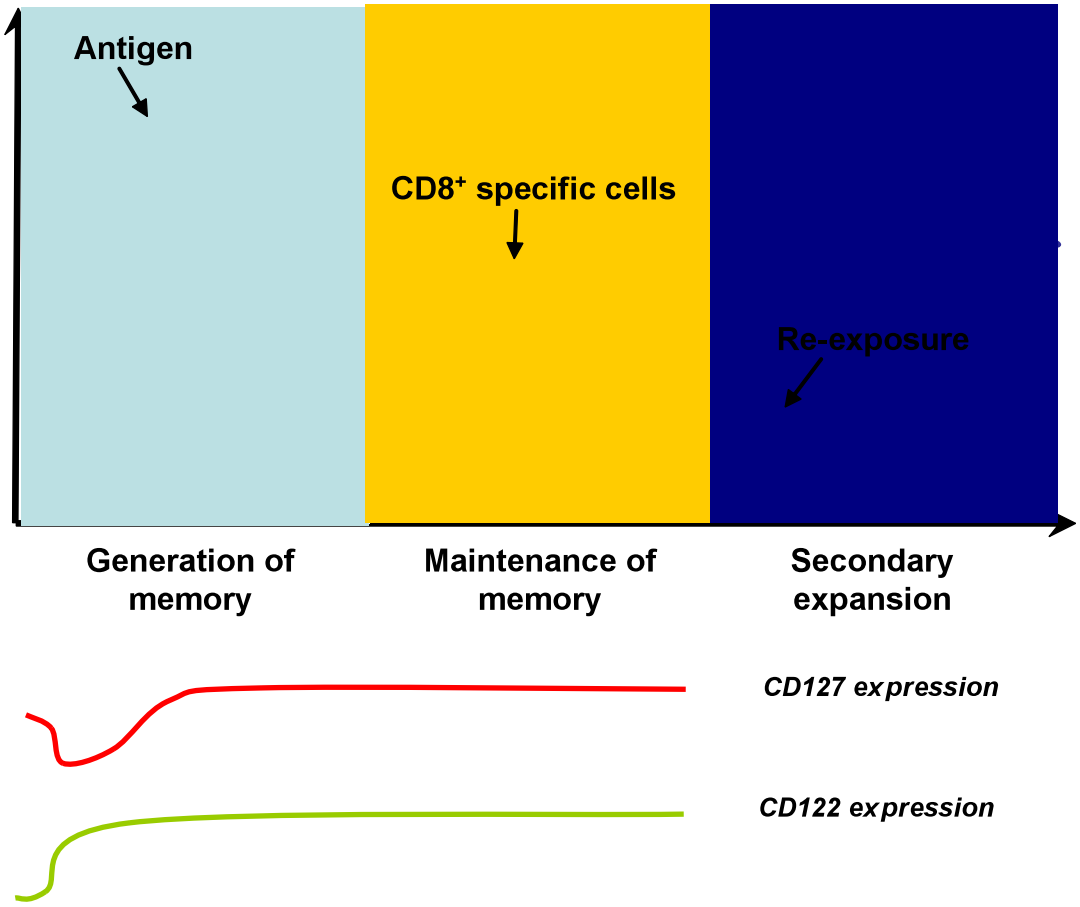

Figure 72 the distinct phases of $CD8^+$ memory responses and association with CD127 and CD122 expression: primary exposure to an antigen generates a characteristic primary $CD8^+$ response, which generates a variable amount of memory. This resting memory population is maintained over years and months, ready to respond to subsequent re-exposure to antigen. Each stage has its own distinct physiological requirements. The relative expression of the IL-7Rα (CD127) and the IL-2rβ (CD122, corresponding to IL-15 receptor status) are shown beneath; this correlates with the relative contribution of these cytokines to the maintenance of the $CD8^+$ T cell population at each time point.

5.2.2. 4-1BB costimulation increases the expression of KLRG-1 on IL7rα^{lo} CD8^{+} T cells

4-1BB signalling generated CD8^{+} T cells with higher expression of the inhibitory KLRG-1 receptor (Figure 55, Figure 56). This is a surprising result, since KLRG-1 expression has been shown to identify a subset of CD8^{+} T cells during the primary response earmarked for short term effector function rather than long term memory generation; KLRG-1^{+} cells are terminally differentiated, and programmed to die after the primary CD8^{+} T cell response; the number of KLRG-1^{+} CD8^{+} T cells is positively correlated with duration of the antigenic stimulus, possibly reflecting its role as a mediator of negative feedback [344]. Why the expression of KLRG-1 was lower with CD27 costimulation is unclear, but it does suggest that KLRG-1 positivity is not universally correlated (inversely) with the generation of CD8^{+} T cell memory.

KLRG-1 expression is mediated by IL-12, which leads to expression of the transcription factor T_{bet} (Figure 73); in T_{bet} -/- mice KLRG-1 expression on CD8^{+} T cells is markedly reduced, but the generation of CD8^{+} T cell memory is unaffected. Whilst it would be interesting to examine the relative effects of 4-1BB and CD27 on T_{bet} expression, T_{bet} expression itself strongly downregulates CD27 expression (or possibly CD27^{+} T cells are especially dependent on T_{bet}). T_{bet} has also been shown to downregulate IL-7rα, with a resulting decrease in the generation of central memory CD8^{+} T cells [352].

Whilst it seems that all KLRG-1^{high} cells are effector cells, not all effector cells are KLRG-1^{high}. It should be noted that KLRG-1^{high} and KLRG-1^{lo} subsets of CD8^{+} T cells are equally capable of lysing target cells, and thus, not all effector cells are KLRG-1^{+}. In summary, KLRG-1 expression only has a loose association with CD8^{+} T cell function. Nevertheless, the enhanced expression of KLRG-1 with 4-1BB costimulation is noteworthy; as KLRG-1^{+} and IL7rα^{+} CD8^{+} subsets were mutually exclusive (Figure 56), and as it is the IL7rα^{+} subset that has been shown to be associated with the generation of memory, KLRG-1 was not investigated further.

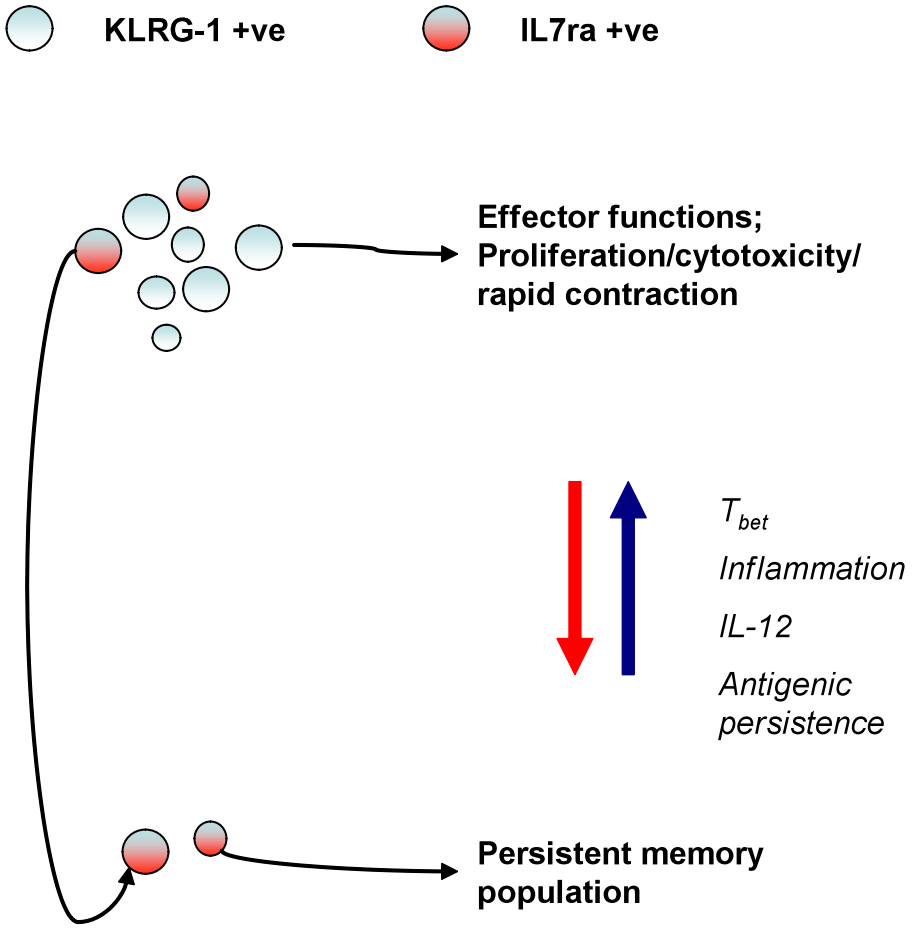

Figure 73 KLRG-1$^+$ and IL7rα$^+$ subsets during CD8$^+$ T cell primary responses: during a primary CD8$^+$ T cell response two subsets are generated: an IL7rα$^+$ subset destined to become a memory population, and a KLRG-1$^+$ subset destined for short-lived survival. The generation of the KLRG-1 subset is increased by various linked factors – IL-12/inflammation/persistent antigenic stimulation as indicated.

5.2.3. The role of Bim in CD27 and 4-1BB costimulation

The pro-apoptotic molecule Bim was identified in the first results chapter as potentially important in mediating the effects of CD27 signalling on $CD8^+$ T cells. Bim is a member of the BH3 family of pro-apoptotic proteins, so named because of their homology to a domain of the pro-survival protein Bcl2.

The involvement of Bim in CD27 costimulation was identified by expression microarray (see chapter 3). TCR activation *in vivo* led to a 40 fold increase in the expression of Bim; this effect was markedly reduced when CD27 costimulation was present, when Bim expression was only increased above that seen in naïve cells by a factor of 3.5 (Figure 26). The effect of 4-1BB costimulation on Bim and Batf3 was studied; 4-1BB costimulation resulted in even lower expression of Bim during T cell activation when compared with CD27 costimulation (four fold) (Figure 60). Conversely, Batf3 expression was increased when 4-1BB costimulation was employed, when compared to CD27. These findings are particularly notable when it is considered that other genes known to have a prominent role in the development of $CD8^+$ T cell memory (bcl6/blimp1/bcl6b) showed similar expression with 4-1BB or CD27 costimulation, suggesting that Bim expression may be a key factor on the generation of $CD8^+$ T cell memory, or at least that it may be involved in the different memory generated by 4-1BB and CD27.

When Bim deficient $CD8^+$ T cells were studied *in vitro* (Figure 61) the [^{3}H] thymidine incorporation was significantly increased when compared to wild type $CD8^+$ T cells, consistent with the predicted reduction in apoptosis in Bim deficient cells: notably, thymidine incorporation was higher with 4-1BB costimulation in wildtype $CD8^+$ T cells, but was identical in Bim deficient cells; this suggested that differences in the ability to downregulated Bim may explain the difference seen between 4-1BB and CD27.

To look at this in more detail, $CD8^+$ T cell responses *in vivo* were studied; during initial examination of costimulatory reagents in Bim deficient mice it was noted that the antigen specific $CD8^+$ T cell primary response primed with anti-CD40 antibody was lower in the absence of Bim (Figure 65), and that the

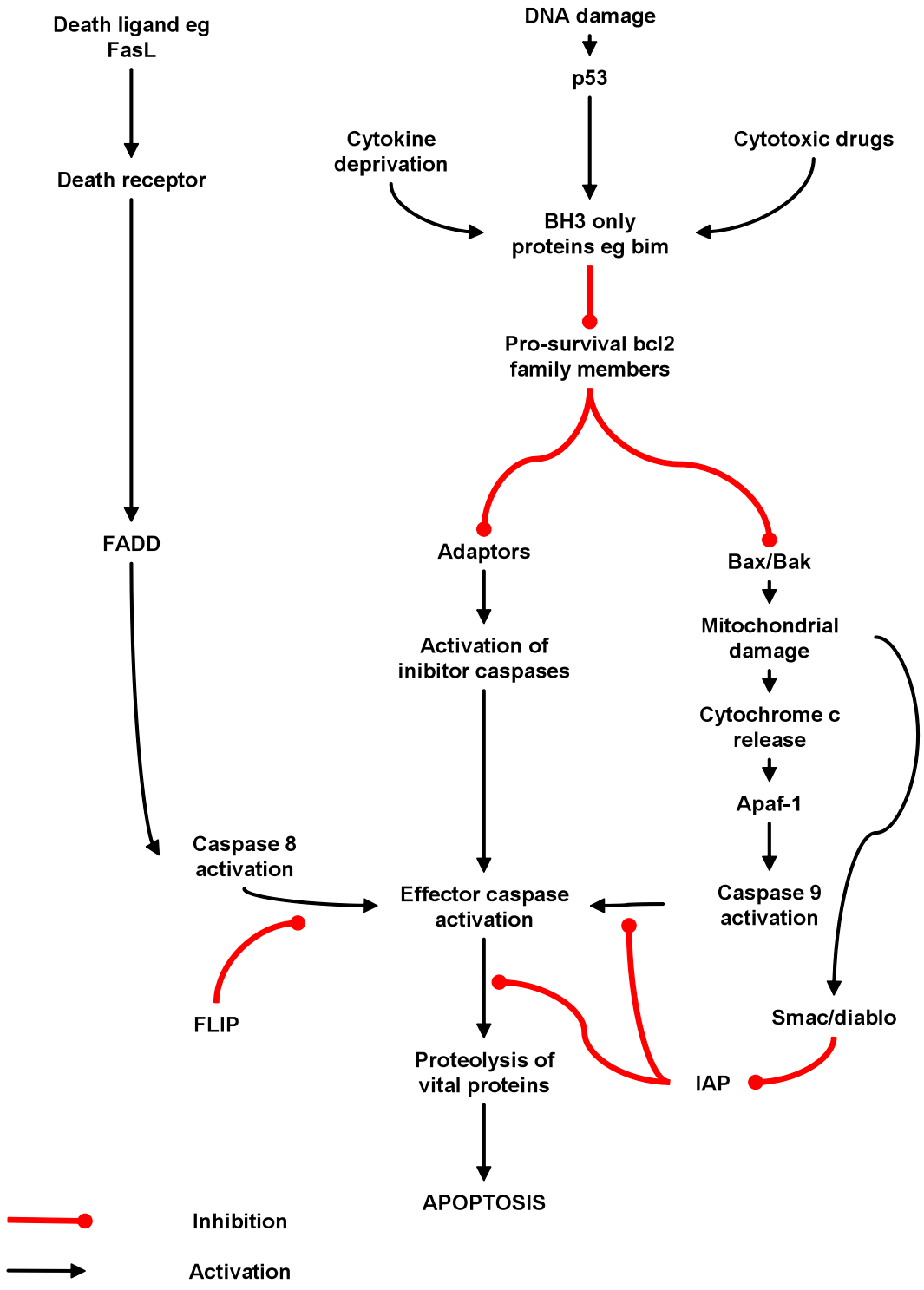

Figure 74 Intrinsic and extrinsic apoptotic pathways: (as adapted from [353])

decline following the peak of the response was reduced, with around 40% of cells surviving the contraction phase in the absence of Bim compared to 25% in the wild-type mice; in contrast, responses with α-CD27 antibody looked comparable in Bim -/- and wild-type groups. Assuming that CD40 indirectly targets CD8+ T cells via DC maturation, and that CD27 directly targets CD8+ T cells (which may be an over-simplification as discussed previously), this raised the possibility that the absence of Bim was more relevant in DCs than in CD8+ T cells. Stimulation of DCs through TLRs is associated with enhanced survival and upregulation of Bcl-2, therefore there is a rationale that Bim may also affect DC survival, since Bim and Bcl-2 are both key molecules involved in the intrinsic apoptotic pathway (Figure 74), with possible knock-on effects on CD8+ T cell priming: however, further investigation did not confirm any involvement of Bim deficiency in DCs, with similar wild-type $CD8^+$ T cell contraction seen in Bim -/- and wild-type recipients (Figure 69).

Quantitative PCR had shown that Bim expression was lower in 4-1BB than CD27 costimulation, and *in vitro* data had suggested that the difference in [^{3}H] thymidine incorporation seen in wild-type cells may be corrected in the absence of Bim (Figure 61). The *In vivo* data was somewhat conflicting; Bim deficiency did not seem to affect the delayed contraction phase seen with 4-1BB costimulation (Figure 62), but it did correct the difference in the secondary response (Figure 63). One possible explanation for this may be that the effector and the memory subpopulations of $CD8^+$ T cells may exhibit different requirements for Bim.

Apoptosis occurs broadly via two separate mechanisms – the extrinsic pathway via specific cell death receptor signals, and the intrinsic pathway (Figure 74). The extrinsic pathway involves various receptors – TRAIL, TNFRs and Fas, which can trigger apoptosis. Binding of the appropriate ligands to cell death receptors leads to caspase 8 activiation and subsequent apoptosis. This is mediated by a complex of proteins (FADD and caspase 8) forming the Death Induced Signalling Complex (DISC).

The intrinsic pathway is triggered by a variety of diverse stimuli that lead to permeabilization of mitochondrial membranes, leading to release of cytochrome c and the formation of a DISC like 'apoptosome'. Subsequent caspase activation leads to irreversible cell death.

Caspases are intracellular enzymes inherent to the apoptotic pathway, with initiator and effector subgroups; the initiator caspase 8 is required for all apoptotic pathways, as is the effector caspase 3. More than 100 substrates have been found for effector caspases including cell cycle, structural, and DNA repair proteins. Caspase-8 cleaves the Bcl-2, (BH3 subfamily) protein Bid, which links the extrinsic and intrinsic apoptotic pathways. The potent apoptotic activity of caspases in controlled by various proteins, the most potent of which are the Inhibitor of Apoptosis (IAP) proteins

Bim is pro-apoptotic, working upstream from Bax and Bak. Bim is normally sequestered on microtubules; Bim activation causes Bax and Bak to oligomerize and insert into the outer mitochondrial membrane, leading to its permeabilization, and subsequent release of pro-apoptotic molecules such as cytochrome c and smac (which inactivates IAPs).

In the experiments presented in this chapter, $CD8^+$ T cell contraction appeared identical in Bim deficient and wild-type mice; an explanation may be that Bim is not important in the $CD8^+$ T cell contraction phase, despite what others have found [173]. More recently, some groups have found that there is some redundancy in the system, and that where there exists a defect in the intrinsic apoptotic pathway (such as in Bim deficiency) the extrinsic apoptotic pathway can mediate $CD8^+$ T cell contraction; it is only when both the intrinsic

and extrinsic pathways are inhibited, such as in Bim and Fas double knockout mice are examined, that the $CD8^+$ contraction phase is completely inhibited [173]. It may be that Bim and Fas mediated pathways are important, with Bim playing a predominant role in acute infections where antigen is cleared completely, and in contrast Fas may play its greatest role in chronic infections where antigen persists in the long term [173].

Fas is a cell death receptor, and when bound by its ligand FasL, it mediates apoptotic cell death through the extrinsic apoptotic pathway. Fas is involved in cytotoxic T cell killing of target cells and deletion of activated T cells after a primary immune response – so called Activation Induced Cell Death. Some tumours express FasL, which may protect them from immune-mediated attack. Fas and FasL deficiencies lead to lymphoproliferative disorders in both mice and humans, confirming this pathway's importance in modulation of the normal immune response.

For this reason, CD27 and 4-1BB costimulation were studied in the absence of Fas signalling (Figure 70, Figure 71). FasL blockade did not equalise the formation of $CD8^+$ T cell memory between 4-1BB and CD27. Therefore neither an absence of Bim, nor blockade of Fas/FasL interactions led to complete equalisation of $CD8^+$ T cell contraction phase and subsequent memory generation between these two pathways of costimulation. It may be that the two pathways are redundant, with each one acting when the other is deficient, or it is possible that other pro-apoptotic pathways are important in this system.

Thus, in summary, several experiments presented here suggest that Bim may have a key role in explaining the difference in memory generated by 4-1BB when compared to CD27 costimulation; nevertheless, additional mechanisms appear to be important in mediating the delayed contraction phase (which was unaffected in the absence of Bim). Further experiments are planned looking at combined blockade of FasL with Bim deficiency as well as the effect of absence or over-expression of other members of the apoptotic pathways such as Bcl2.

5.2.4. Key findings

Compared with CD27, 4-1BB costimulation leads to higher IL7rα expression on $CD8^+$ T cells during the contraction phase

IL7rα blockade during the contraction phase does not lead to an equalisation of the amount of $CD8^+$ T cell memory generated between CD27 and 4-1BB

4-1BB and CD27 costimulation both suppress the expression of Bim during T cell activation; the effect is greater with 4-1BB

CD27 and 4-1BB costimulation are enhanced *in vitro* in the absence of Bim

The secondary $CD8^+$ T cell response is higher when 4-1BB costimulation is used during priming; this difference is lost when priming takes place in the absence of Bim (but the contraction phase remains delayed with 4-1BB)

CD40 costimulation is markedly abrogated in the absence of Bim, but this difference does not seem to be due to Bim expression in DCs.

Chapter 6. General Discussion

6.1. Effects of CD27 costimulation on gene expression

This thesis documents the effects of CD27 stimulation on $CD8^+$ T cells. CD27 signalling was triggered by using a multimeric recombinant fusion protein comprising the extracellular domain of CD70 and the Fc domain of hIgG1. When coupled with TCR triggering, CD27 costimulation resulted in a significantly enhanced primary $CD8^+$ T cell response (Chapter 3). By purifying antigen specific cells at the peak of this primary response it was possible to examine the various changes in gene expression that were brought about by CD27 costimulation during T cell activation, compared with TCR triggering alone.

The activation of a T lymphocyte is a complex process. T cell receptor (TCR) engagement by its cognate antigenic peptide presented on an MHC molecule (pMHC) is the first step in T cell activation, and is termed 'signal one'. How the TCR/pMHC interaction leads to TCR activation is unclear, but may involve various mechanisms such as TCR aggregation, CD3 conformational changes and displacement of inhibitory molecules. Explaining how the relatively small number of pMHC molecules on an APC can lead to TCR aggregation is difficult, but solutions may involve the formation of pseudodimers or heterodimers composed of pMHC and MHC molecules presenting endogenous peptides [42]. TCR activation then leads to several subsequent pathways of signal transduction including the phosphoinositide 3 kinase (PI3K) pathway (via phospholipase C), the MAP kinase pathway (via diaclyglycerol (DAG)/Ras) and calcium signalling. Thus TCR signalling activates several transcriptional pathways – AP-1, NFAT, STAT3 and NF-κB.

CD27 signalling has also been shown to affect several of these pathways, including activation of JNK (a member of the MAP kinase class of proteins), which has multiple downstream effects via AP-1 and other transcription factors [278]. In Chapter 3 it was shown that CD27 signalling results in

upregulation of Batf3 and downregulation of fos, both of which are likely to reduce AP-1 signalling, possibly as part of a negative feedback loop.

TCR activation also leads to various effects on the cytoskeleton; filamentous actin accumulates at the site of activation; the myosin motor protein is deactivated, leading to a reduction in motility; the plasma membrane is freed from the actin cytoskeleton leading to greater plasma membrane fluidity and the microtubular organising centre moves to the TCR contact site, leading to formation of the immunological synapse. Despite the importance of actin in all these processes, the role of actin in TCR signalling is complex: inhibition of actin polymerization has been shown to inhibit TCR adherence and cytotoxicity [354] as well as IFNγ production [355]. CD27 signalling results in a significant increase in the expression of Lim-nebulette. Lim-nebulette is a splice variant of the sarcomeric protein nebulette. Lim-nebulette has been shown to interact with Zyxin; Zyxin is involved in the formation of focal adhesions – these are essential cellular structures that are known to link the extracellular matrix to the cytoskeleton [287]; both Lim-nebulette and Zyxin co-localise in subcellular areas. Lim-nebulette therefore represents a possible link between CD27 signalling and effects on the actin cytoskeleton, which are so critical in the formation of the immunological synapse and T cell migration [289], although such an association remains speculative at this time; however, there is evidence that another costimulatory molecule, CD28, directly affects actin polymerization through the phosphorylation of cofilin, and actin-remodeling protein [356]. The term 'Lim' refers to cysteine rich motifs with two zinc finger-like structures that mediate specific protein-protein interactions; although Lim-nebulette has yet to be proven to be involved in T cell activation, other Lim containing molecules are also known to phosphorylate Cofilin, which is important in TCR signalling [357].

Thus there are several points at which TCR and CD27 signalling converge, in chapter 3 several of the genes whose expression were altered by CD27 signalling are known to be involved in these pathways (Figure 75).

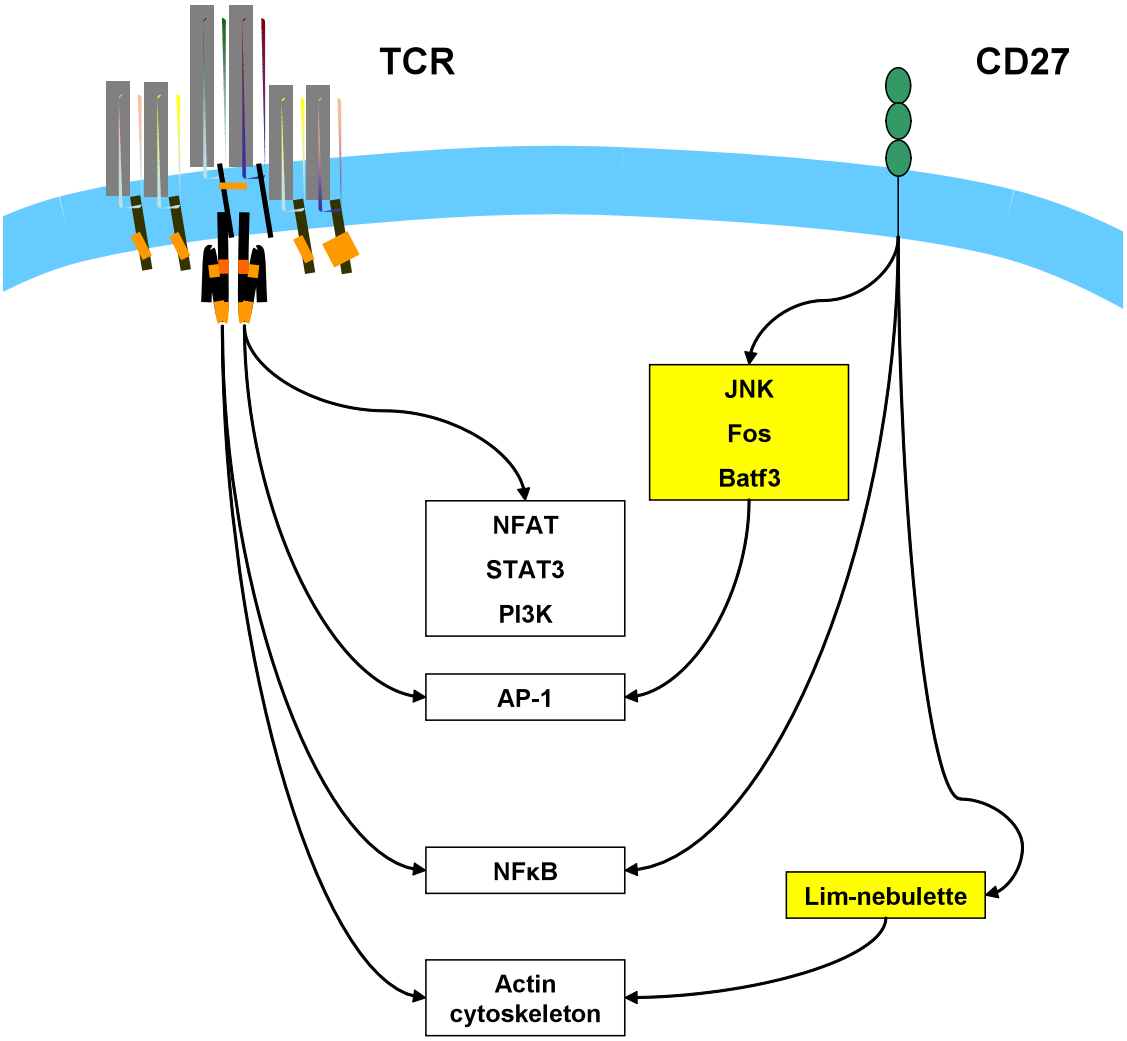

Figure 75 Overlapping effects of TCR signalling and CD27 signalling

Genes detected as significantly altered by CD27 in chapter 3 are highlighted in yellow

This thesis also sought to compare the effects of CD27 with its close family member 4-1BB. These two molecules share significant sequence homology, but their expression, and the expression of their ligands, differ significantly; in particular, CD27 is present on resting $CD8^+$ T cells, whereas 4-1BB only appears after T cell activation. The two molecules also employ different members of the Traf family for signal transduction.

Investigations from chapter 4 consistently demonstrated that whilst the initial primary response occurred earlier with CD27 costimulation, the resulting contraction phase resulted in greater formation of a resting memory population when 4-1BB was employed, and this translated into stronger recall responses. These findings held true whether soluble ligands or stimulatory antibodies were used, and whether endogenous or transgenic experimental systems were employed. The effects were independent of the presence of T_{reg} or $CD4^+$ T cells. Adoptive transfer experiments demonstrated that the resting memory $CD8^+$ T cells were functionally similar in terms of homeostatic proliferation and in their ability to proliferate. Chapter 4 also led to several notable observations; firstly the resting memory population generated was remarkably constant, remaining virtually unchanged for up to 8 months. Secondly, recall responses were unaffected by an absence of $CD4^+$ T cell help when CD27 or 4-1BB costimulation was present during $CD8^+$ T cell priming: this raises the possibility that these TNFR members are involved in the pathway of $CD4^+$ T cell help in the generation of $CD8^+$ T cell memory. Experiments using viral immunity have repeatedly shown the need for $CD4^+$ T cell help to be present at this critical timepoint if $CD8^+$ T cell memory proliferation in response to secondary challenge is to occur [179]; secondary expansion was dramatic in the experiments shown in chapter four, even in the absence of $CD4^+$ T cell help during priming, suggesting a role for CD27/4-1BB in this pathway. Consistent with this theory, previous findings from this laboratory have shown CD70 upregulation on DCs activated by CD40 [88]; conversely $CD4^+$ T cell licensing of DCs has also been shown to be dependent on CD27/CD70 interactions. Future work should concentrate on attempting to confirm or refute the role of 4-1BB and CD27 in the pathway of $CD4^+$ T cell mediated licencing of DCs for $CD8^+$ T cell priming.

As it was the generation of a larger resting memory pool by 4-1BB that led to the enhanced memory effects, in chapter five various mechanisms for this were examined, concentrating initially on differences during the primary response. Since 2003, expression of the interleukin 7 receptor, IL7rα, has identified a subset of $CD8^+$ T cells programmed to survive the initial primary response and form a long-lived memory population [163]. Superficially this is logical: IL7rα signalling results in increased expression of survival molecules such as Bcl2 and Bcl_{XL}. In chapter five, expression of IL7rα was in fact lower at the peak of the primary response when 4-1BB costimulation was used, but conversely expression was higher after the peak, during the contraction phase. Recent publications have enhanced our understanding on the complexity of IL7's involvement in T cell priming; firstly, TCR engagement leads to impaired IL7rα downstream signalling, without affecting IL7rα expression – ie, simple cell surface expression of IL7rα does not necessarily lead to enhanced IL7rα signalling [163]. Secondly, many of the findings relating to IL7rα expression utilise transgenic TCR systems; recent data has demonstrated how the number of transgenic cell used in these experiments can dramatically alter the findings of various measures of $CD8^+$ physiology, including IL7rα expression, which is lower when initial precursor cell frequency approaches endogenous levels [325]. Thus IL7rα expression may be a phenotypic marker, but may not necessarily be the mechanism by which $CD8^+$ T cell memory is generated, and may be heavily affected by experimental conditions. These factors may, in part, explain why blockade of IL7rα did not equalise the amount of memory generated by 4-1BB and CD27 costimulation.

The microarray data from the first results chapter did confirm that the IL-2 receptor α chain, CD25, was upregulated by CD27 signalling. More recent data from our laboratory has confirmed that CD25 is upregulated more by CD27 than by 4-1BB (A. Al-Shamkhani, personal communication); this is consistent with the idea that 4-1BB is biased towards the generation of memory, whilst CD27 is biased more towards effector differentiation. CD25 and IL-2 seem key to the process of effector cell generation [358], with IL15 important in central memory differentiation [299].

Since IL7-Rα expression was not correlated with the generation of T cell memory, alternative explanations were sought. The gene expression data from chapter 3 was examined for candidate molecules known to be involved in memory generation. One of the key molecules affected by CD27 signalling identified was the pro-apoptotic protein Bim. Bim interacts with, and is inhibited by, the anti-apoptotic molecule Bcl2 [359]. Bim is important in apoptosis caused by withdrawal of cytokines and seems to be especially critical in T cell apoptosis when antigen levels are low (for example when an infection is eradicated successfully) [173]. Bim levels are carefully controlled; JNK, MAPK and PI3K pathways are known to impact on Bim expression [360].

In chapter 3, Bim was shown to be significantly upregulated by TCR engagement, but this effect was markedly abrogated by the presence of CD27 costimulation. This suggested that CD27 costimulation protects activated $CD8^+$ T cells from apoptosis at the peak of the primary response by downregulating Bim. This is a logical finding, since most cells present at this time point are scheduled to die as a result of programmed cell death. Protection from this would allow for the formation of a larger memory pool. In addition, Bim expression seemed to be expressed at an even lower level in $CD8^+$ T cells with 4-1BB costimulation. In addition, *in vitro* proliferation of $CD8^+$ T cells was higher with 4-1BB costimulation than with CD27, and difference that was lost in the absence of Bim. Despite these findings, the *in vivo* effects of Bim deficiency were conflicting; primary responses were unaffected by the absence of Bim but the difference in secondary responses disappeared in the absence of Bim.

Bim is a key component of the intrinsic apoptotic pathway; extrinsic apoptotic pathways are also known to be important in T cell contraction. In particular Fas/FasL interactions are involved in Activation Induced Cell Death. Blockade of Fas/FasL interactions did not delay $CD8^+$ T cell contraction (chapter 5) and did not equalize the amount of $CD8^+$ T cell memory generated by 4-1BB and CD27 costimulation respectively.

It is notable that $CD8^+$ T cell contraction continued unaffected in the absence of Bim and in the presence of Fas/FasL blockade. Future experiments will examine the effect of simultaneous interference of both these pathways simultaneously, since loss of both apoptotic systems is known to be additive [173]. It was not possible to explain why Bim deficiency had such a profound effect on CD40 mediated costimulation, whilst showing very little effect on CD27 and 4-1BB costimulation; the effect did not seem to be mediated via Bim deficiency in DCs, and may simply represent differences between $CD8^+$ T cells directly primed via CD27/4-1BB, and indirectly primed by DC maturation via CD40. Nevertheless, primary T cell responses generated using either CD27 or 4-1BB costimulation underwent a contraction phase that was unaltered by the absence of Bim, or the presence of FasL blockade, suggesting either that these two apoptotic pathways act redundantly of each other, or that neither pathway is critical in this experimental system.

Which molecule, 4-1BB or CD27, represents the most attractive target for therapeutic manipulation in the field of anti-tumour immunity? CD27 is attractive due to its constitutional presence on resting T cells, and the rapid generation of effector T cells may be useful in the clearance of tumours. Thus far it also seems that CD27 has a more limited distribution across tissues than 4-1BB, possibly limiting any off target effects. Where a cancer is in remission however, it is generation of a long lasting anti-tumour response that is therapeutically desirable, and in this situation 4-1BB targeting may be preferable, with its predilection for the generation of memory. Neither molecule can be said to have ‘won’: instead, it is to be hoped that such knowledge about individual molecules will lead in the future to therapeutic strategies tailor-made to individual clinical situations.

Chapter 7. Appendix

7.1. Full lists of genes detected by microarray

7.1.1. 24 hour in vitro microarray

Symbol	Description	Fold Change	P value
Atf3	activating transcription factor 3	3.7	7.71E-06
Anxa3	annexin A3	3.3	1.18E-05
Plagl1	pleiomorphic adenoma gene-like 1	2.8	3.19E-05
Lif	leukemia inhibitory factor	2.7	5.21E-05
Igsf3	immunoglobulin superfamily, member 3	2.5	0.000137
Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	2.5	2.61E-05
Plaur	plasminogen activator, urokinase receptor	2.3	0.000104
Ibrdc3	IBR domain containing 3	2.2	0.000116
Hemk1	HemK methyltransferase family member 1	2.0	6.68E-06
RP23-273O7.4	NA	1.9	0.000117
BC087945	cDNA sequence BC087945	1.9	6.95E-05
Ibrdc3	IBR domain containing 3	1.8	9.51E-06
Clic4	chloride intracellular channel 4 (mitochondrial)	1.8	0.0002
Klf6	Kruppel-like factor 6	1.8	0.000136
Btbd4	BTB (POZ) domain containing 4	1.7	0.000177
Clic4	chloride intracellular channel 4 (mitochondrial)	1.7	0.000112
Smpdl3b	sphingomyelin phosphodiesterase, acid-like 3B	1.6	5.54E-05
Pim3	proviral integration site 3	1.6	0.000111
Arid5a	AT rich interactive domain 5A (Mrf1 like)	1.5	0.000139
Mfhas1	malignant fibrous histiocytoma amplified sequence 1	1.5	0.000102
Cobll1	Cobl-like 1	-1.5	8.88E-05
Gimap4	GTPase, IMAP family member 4	-1.5	0.000169
9930117H01Rik	RIKEN cDNA 9930117H01 gene	-1.6	0.000118
Cd3g	CD3 antigen, gamma polypeptide	-1.6	0.000116
2810457I06Rik	RIKEN cDNA 2810457I06 gene	-1.6	0.000208
Il6st	interleukin 6 signal transducer	-1.6	0.000101
Cd28	CD28 antigen	-1.6	0.000198
Rasgrp2	RAS, guanyl releasing protein 2	-1.7	5.06E-05
Zbtb20	zinc finger and BTB domain containing 20	-1.7	7.43E-05
Cd160	CD160 antigen	-1.7	5.97E-05
Evi2b	ecotropic viral integration site 2b	-1.7	2.71E-05
Ptpn22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	-1.7	0.000192
Lair1	leukocyte-associated Ig-like receptor 1	-1.8	2.07E-05
Cd160	CD160 antigen	-1.8	1.59E-05
Dusp6	dual specificity phosphatase 6	-1.8	7.94E-05
Spin2	spindlin family, member 2	-1.8	2.71E-05
Ssbp2	single-stranded DNA binding protein 2	-1.8	0.00013
Slamf6	SLAM family member 6	-1.9	5.50E-05

Pecam1	platelet/endothelial cell adhesion molecule 1	-1.9	0.000107
Itm2a	integral membrane protein 2A	-1.9	6.01E-05
Endod1	endonuclease domain containing 1	-2.0	5.92E-05
Itm2a	integral membrane protein 2A	-2.1	2.69E-05
Npas4	neuronal PAS domain protein 4	-2.1	2.05E-05
Bcl2l11 (bim)	Pro-apoptotic bcl2 member	-2.2	8.20E-04
Cxcr3	chemokine (C-X-C motif) receptor 3	-2.2	6.89E-05
A530080P10	NA	-2.2	3.64E-05
Myb	myeloblastosis oncogene	-2.3	0.000111
Myo1f	myosin IF	-2.3	7.43E-06
Ephx1	epoxide hydrolase 1, microsomal	-2.6	3.70E-05
Klrd1	killer cell lectin-like receptor, subfamily D, member 1	-3.3	8.20E-05

Table 1-10 Significantly up- or down regulated genes at the 24 hour timepoint *in vitro* (fold change greater than +/- 1.5, p< 0.00015)

7.1.2. 48 hour *in vivo* microarray

Symbol	Description	Fold Change	P value
Nebl	nebulette	3.57	3.2E-05
Nebl	nebulette	2.93	3.4E-06
Lad1	ladinin	2.59	3.1E-05
Maoa	monoamine oxidase A	2.24	1.5E-05
Dctd	dCMP deaminase	2.21	4.6E-05
Bag2	Bcl2-associated athanogene 2	2.13	2.0E-05
Smyd2	SET and MYND domain containing 2	1.97	7.7E-06
Dusp14	dual specificity phosphatase 14	1.94	3.9E-05
Nebl	nebulette	1.93	2.5E-05
Shmt1	serine hydroxymethyltransferase 1 (soluble)	1.93	5.0E-05
Cd83	CD83 antigen	1.90	1.2E-06
Fabp5	fatty acid binding protein 5, epidermal	1.84	4.0E-05
Mybbp1a	MYB binding protein (P160) 1a	1.81	2.1E-05
Hspa9	heat shock protein 9	1.81	2.7E-05
Acsl1	acyl-CoA synthetase long-chain family member 1	1.78	2.5E-05
P2ry12	purinergic receptor P2Y, G-protein coupled 12	1.71	2.7E-05
Isg20l1	interferon stimulated exonuclease gene 20-like 1	1.69	4.6E-05
Xrcc6bp1	XRCC6 binding protein 1	1.68	5.3E-05
Pdxk	pyridoxal (pyridoxine, vitamin B6) kinase	1.68	4.2E-05
Tsr2	TSR2, 20S rRNA accumulation, homolog (S. cerevisiae)	1.68	4.6E-05
Nefh	neurofilament, heavy polypeptide	1.67	5.3E-05
Pi4k2b	phosphatidylinositol 4-kinase type 2 beta	1.67	3.4E-05
1300001I01Rik	RIKEN cDNA 1300001I01 gene	1.67	4.5E-05
Heatr3	HEAT repeat containing 3	1.65	1.8E-05
Fgf2	fibroblast growth factor 2	1.65	1.3E-05
Sqle	squalene epoxidase	1.64	5.0E-05
Nedd4	neural precursor cell expressed, developmentally down-regulated gene 4	1.64	1.0E-05
Lman1	lectin, mannose-binding, 1	1.60	3.5E-05
Lrp8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	1.60	3.9E-05
Dhrs13	dehydrogenase/reductase (SDR family) member 13	1.58	4.3E-05
Ltv1	LTV1 homolog (S. cerevisiae)	1.54	4.2E-05
Rev1	REV1 homolog (S. cerevisiae)	1.54	5.1E-05
Ipo4	importin 4	1.52	5.4E-05
Mrps5	mitochondrial ribosomal protein S5	1.48	4.6E-05
Fads2	fatty acid desaturase 2	1.47	4.3E-05
Il6ra	interleukin 6 receptor, alpha	-1.54	2.3E-05
Mylip	myosin regulatory light chain interacting protein	-1.68	2.4E-05
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-1.70	2.0E-05
Trp53inp1	transformation related protein 53 inducible nuclear protein 1	-1.72	4.2E-05
Il6st	interleukin 6 signal transducer	-1.74	5.6E-05
Art2b	ADP-ribosyltransferase 2b	-1.86	4.0E-05
Bach2	BTB and CNC homology 2	-1.88	7.7E-06
Cd55	CD55 antigen	-1.92	5.8E-05
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	-1.94	5.9E-05
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-1.94	3.2E-05

Lats2	large tumor suppressor 2	-1.97	2.8E-05
Itga6	integrin alpha 6	-2.00	3.7E-05
Bach2	BTB and CNC homology 2	-2.02	2.0E-05
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-2.48	4.0E-06
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-3.23	3.5E-05

Table 1-11 Significantly up- or down regulated genes at the 48 hour timepoint *in vivo* (fold change greater than +/- 1.5, p< 0.0001)

7.1.3. 72 hour *in vivo* microarray

Symbol	Gene Desription	Fold Change	P value
Nebl	nebulette	6.49	2.1E-04
Nebl	nebulette	4.80	4.0E-05
H2-Q10	histocompatibility 2, Q region locus 10	4.19	4.5E-04
E130016E03Rik	RIKEN cDNA E130016E03 gene	3.97	1.8E-05
Fabp5	fatty acid binding protein 5, epidermal	3.72	8.6E-05
Cep55	centrosomal protein 55	3.71	5.3E-04
Ppil5	peptidylprolyl isomerase (cyclophilin) like 5	3.67	1.0E-04
Ppil5	peptidylprolyl isomerase (cyclophilin) like 5	3.67	3.5E-05
Ccnf	cyclin F	3.58	4.8E-04
Exo1	exonuclease 1	3.49	1.1E-04
Exo1	exonuclease 1	3.49	1.9E-04
Ccne2	cyclin E2	3.47	2.0E-04
Spag5	sperm associated antigen 5	3.44	6.7E-04
Depdc1a	DEP domain containing 1a	3.40	3.2E-05
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	3.40	1.1E-04
Rad51ap1	RAD51 associated protein 1	3.37	8.3E-05
Kif2c	kinesin family member 2C	3.37	2.7E-04
Nt5dc2	5'-nucleotidase domain containing 2	3.35	6.1E-04
1500009L16Rik	RIKEN cDNA 1500009L16 gene	3.34	8.4E-05
Tmem48	transmembrane protein 48	3.21	2.5E-04
Havcr2	hepatitis A virus cellular receptor 2	3.18	6.4E-06
F630043A04Rik	RIKEN cDNA F630043A04 gene	3.18	7.2E-05
Rad51c	Rad51 homolog c (S. cerevisiae)	3.17	1.1E-04
Ncapg	on-SMC condensin I complex, subunit G	3.17	8.1E-04
Trip13	thyroid hormone receptor interactor 13	3.17	1.1E-04
Il2ra	interleukin 2 receptor, alpha chain	3.16	6.7E-06
Brca1	breast cancer 1	3.15	7.7E-05
Apitd1	apoptosis-inducing, TAF9-like domain 1	3.15	2.1E-05
Cxcr4	chemokine (C-X-C motif) receptor 4	3.14	1.2E-04
Ccnf	cyclin F	3.13	3.5E-05
Hmgb2	high mobility group box 2	3.11	2.2E-04
Skp2	S-phase kinase-associated protein 2 (p45)	3.11	1.5E-05
Brip1	BRCA1 interacting protein C-terminal helicase 1	3.11	1.6E-04
Kif23	kinesin family member 23	3.09	1.7E-04
Melk	maternal embryonic leucine zipper kinase	3.08	3.0E-04
Zdhhc2	zinc finger, DHHC domain containing 2	3.08	7.3E-06
2610002D18Rik	RIKEN cDNA 2610002D18 gene	3.05	1.7E-04
Trip13	thyroid hormone receptor interactor 13	3.04	2.2E-04
Aurkb	aurora kinase B	3.04	2.2E-04
2810417H13Rik	RIKEN cDNA 2810417H13 gene	3.04	9.0E-04
Dtl	denticleless homolog (Drosophila)	3.04	6.8E-04
Chek1	checkpoint kinase 1 homolog (S. pombe)	3.03	9.3E-06
2810433K01Rik	RIKEN cDNA 2810433K01 gene	3.02	2.7E-04
Rrm2	ribonucleotide reductase M2	3.01	8.6E-04
Chek1	checkpoint kinase 1 homolog (S. pombe)	3.01	2.3E-05
Fabp5	fatty acid binding protein 5, epidermal	3.00	1.2E-04
Rfc3	replication factor C (activator 1) 3	2.98	1.8E-05
2700049P18Rik	RIKEN cDNA 2700049P18 gene	2.98	2.4E-04

4930547N16Rik	RIKEN cDNA 4930547N16 gene	2.97	2.6E-04
Tpx2	TPX2, microtubule-associated protein homolog (Xenopus laevis)	2.96	6.2E-05
Bcat1	branched chain aminotransferase 1, cytosolic	2.96	4.9E-04
Lrrc41	leucine rich repeat containing 41	2.95	2.2E-04
Mcm8	minichromosome maintenance deficient 8 (S. cerevisiae)	2.95	3.4E-05
P2ry12	purinergic receptor P2Y, G-protein coupled 12	2.94	4.9E-06
Gins2	GINS complex subunit 2 (Psf2 homolog)	2.93	5.4E-05
Dna2l	DNA2 DNA replication helicase 2-like (yeast)	2.93	7.2E-05
Ccdc99	coiled-coil domain containing 99	2.92	4.7E-05
Ccnb1-rs1	cyclin B1, related sequence 1	2.91	8.1E-04
Rad51	RAD51 homolog (S. cerevisiae)	2.90	2.8E-05
Tube1	epsilon-tubulin 1	2.90	2.7E-05
Cenpi	centromere protein I	2.90	2.2E-04
Rfc3	replication factor C (activator 1) 3	2.88	3.9E-04
BC048355	cDNA sequence BC048355	2.88	7.2E-04
Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2	2.88	7.6E-05
Nuf2	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.87	2.1E-04
Zdhhc2	zinc finger, DHHC domain containing 2	2.87	1.3E-06
2600005O03Rik	RIKEN cDNA 2600005O03 gene	2.87	1.4E-04
Aurkb	aurora kinase B	2.87	9.3E-05
Skp2	S-phase kinase-associated protein 2 (p45)	2.87	1.5E-05
Kif20a	kinesin family member 20A	2.87	2.4E-04
Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2	2.87	2.5E-04
Hmmr	hyaluronan mediated motility receptor (RHAMM)	2.85	4.0E-04
Mastl	microtubule associated serine/threonine kinase-like	2.85	2.4E-04
E2f8	E2F transcription factor 8	2.84	7.4E-04
Mybl2	myeloblastosis oncogene-like 2	2.83	1.2E-05
Cenph	centromere protein H	2.83	4.0E-04
Arhgap19	Rho GTPase activating protein 19	2.83	8.2E-04
Bub1	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	2.82	6.5E-05
	NA	2.82	7.0E-04
Zwilch	Zwilch, kinetochore associated, homolog (Drosophila)	2.82	2.5E-04
Fignl1	fidgetin-like 1	2.82	9.8E-05
Cdc6	cell division cycle 6 homolog (S. cerevisiae)	2.82	6.7E-04
Prdx4	peroxiredoxin 4	2.81	1.5E-04
Zdhhc2	zinc finger, DHHC domain containing 2	2.81	1.5E-04
Aurka	aurora kinase A	2.80	2.2E-04
Pbk	PDZ binding kinase	2.80	6.3E-05
Tmem97	transmembrane protein 97	2.80	8.4E-05
BC055324	cDNA sequence BC055324	2.80	9.2E-05
Ect2	ect2 oncogene	2.80	1.4E-04
Mxd3	Max dimerization protein 3	2.80	5.1E-05
Prr11	proline rich 11	2.79	1.9E-04
C79407	expressed sequence C79407	2.78	3.8E-05
Tk1	thymidine kinase 1	2.77	2.5E-04
Sgol1	shugoshin-like 1 (S. pombe)	2.76	2.7E-05
	NA	2.76	1.3E-04
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	2.76	3.6E-05

Ckap2l	cytoskeleton associated protein 2-like	2.75	6.8E-04
Ncapg	on-SMC condensin I complex, subunit G	2.75	6.2E-05
Plk1	polo-like kinase 1 (Drosophila)	2.75	2.0E-04
4632434I11Rik	RIKEN cDNA 4632434I11 gene	2.75	2.7E-04
Kif2c	kinesin family member 2C	2.75	2.0E-04
E2f7	E2F transcription factor 7	2.74	2.2E-04
Dlg7	discs, large homolog 7 (Drosophila)	2.74	2.5E-04
Suv39h2	suppressor of variegation 3-9 homolog 2 (Drosophila)	2.73	2.5E-04
Chek1	checkpoint kinase 1 homolog (S. pombe)	2.72	1.2E-04
Rrm2	ribonucleotide reductase M2	2.72	2.1E-04
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	2.71	2.9E-06
BC055324	cDNA sequence BC055324	2.71	2.6E-05
Zdhhc2	zinc finger, DHHC domain containing 2	2.71	3.1E-04
Cdca5	cell division cycle associated 5	2.71	3.5E-04
Rad51ap1	RAD51 associated protein 1	2.71	8.3E-05
Kif11	kinesin family member 11	2.70	2.9E-04
Nup133	nucleoporin 133	2.69	6.9E-05
Kif11	kinesin family member 11	2.68	9.6E-05
Brca1	breast cancer 1	2.68	1.3E-05
Tacc3	transforming, acidic coiled-coil containing protein 3	2.68	7.4E-05
Dusp14	dual specificity phosphatase 14	2.68	2.7E-04
Anln	anillin, actin binding protein (scraps homolog, Drosophila)	2.68	5.0E-05
Myo5a	myosin Va	2.68	1.7E-05
Kif22	kinesin family member 22	2.68	2.0E-04
Lmnb2	lamin B2	2.67	8.2E-06
Tpx2	TPX2, microtubule-associated protein homolog (Xenopus laevis)	2.66	1.9E-04
Cdc45l	cell division cycle 45 homolog (S. cerevisiae)-like	2.65	4.5E-05
Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)	2.65	1.5E-04
Shcbp1	Shc SH2-domain binding protein 1	2.65	1.7E-04
Mcm10	minichromosome maintenance deficient 10 (S. cerevisiae)	2.65	5.9E-05
C330027C09Rik	RIKEN cDNA C330027C09 gene	2.65	2.9E-04
Tmem48	transmembrane protein 48	2.64	2.1E-05
Hirip3	HIRA interacting protein 3	2.64	7.0E-05
Rad51l1	RAD51-like 1 (S. cerevisiae)	2.64	4.9E-04
Gcat	glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase)	2.64	1.3E-04
	NA	2.64	5.7E-04
Traip	TRAF-interacting protein	2.64	2.9E-04
Cdc2a	cell division cycle 2 homolog A (S. pombe)	2.64	2.8E-04
Spag5	sperm associated antigen 5	2.63	6.1E-05
Ttk	Ttk protein kinase	2.63	3.5E-04
4930547N16Rik	RIKEN cDNA 4930547N16 gene	2.62	8.3E-04
Lrrc41	leucine rich repeat containing 41	2.61	5.0E-04
Plk4	polo-like kinase 4 (Drosophila)	2.61	1.0E-04
Brca1	breast cancer 1	2.61	1.1E-05
Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9	2.60	2.4E-04
Cenpk	centromere protein K	2.60	1.7E-04
Cdca5	cell division cycle associated 5	2.60	2.2E-04
Espl1	extra spindle poles-like 1 (S. cerevisiae)	2.60	3.0E-05

Tyms	thymidylate synthase	2.59	1.3E-04
Gemin6	gem (nuclear organelle) associated protein 6	2.59	3.7E-04
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	2.59	4.8E-06
Psmc3ip	proteasome (prosome, macropain) 26S subunit, ATPase 3, interacting protein	2.59	1.3E-04
2610016C23Rik	RIKEN cDNA 2610016C23 gene	2.58	2.2E-04
E2f8	E2F transcription factor 8	2.58	1.6E-04
Shmt1	serine hydroxymethyltransferase 1 (soluble)	2.58	1.2E-04
Orc1l	origin recognition complex, subunit 1-like (S.cereviaiae)	2.58	2.2E-04
Cphx	cytoplasmic polyadenylated homeobox	2.58	1.4E-05
Spag5	sperm associated antigen 5	2.57	1.3E-04
Lrrc41	leucine rich repeat containing 41	2.57	4.1E-05
Ndc80	NDC80 homolog, kinetochore complex component (S. cerevisiae)	2.57	3.0E-04
Stil	Scl/Tal1 interrupting locus	2.57	9.5E-05
Kif2c	kinesin family member 2C	2.57	7.8E-04
Rfc4	replication factor C (activator 1) 4	2.57	8.0E-05
Kif22	kinesin family member 22	2.57	1.4E-04
Asf1b	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	2.56	1.8E-05
Hmmr	hyaluronan mediated motility receptor (RHAMM)	2.56	6.4E-05
Cep55	centrosomal protein 55	2.56	6.2E-05
Eme1	essential meiotic endonuclease 1 homolog 1 (S. pombe)	2.56	9.9E-05
E130306D19Rik	RIKEN cDNA E130306D19 gene	2.55	8.1E-05
Spc25	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.55	3.7E-04
Hells	helicase, lymphoid specific	2.55	1.6E-05
Rfc4	replication factor C (activator 1) 4	2.55	1.2E-04
Kntc1	kinetochore associated 1	2.54	1.2E-04
Aspm	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	2.53	1.6E-04
Arhgap19	Rho GTPase activating protein 19	2.53	4.0E-05
Fbxo5	F-box protein 5	2.53	3.5E-04
Myo5a	myosin Va	2.53	3.7E-05
Dusp4	dual specificity phosphatase 4	2.53	1.3E-04
Sgol2	shugoshin-like 2 (S. pombe)	2.52	1.8E-04
Mnd1	meiotic nuclear divisions 1 homolog (S. cerevisiae)	2.52	3.3E-04
Cdca8	cell division cycle associated 8	2.52	6.0E-05
Prc1	protein regulator of cytokinesis 1	2.52	4.4E-04
Top2a	topoisomerase (DNA) II alpha	2.52	1.5E-06
Gins1	GINS complex subunit 1 (Psf1 homolog)	2.52	5.9E-04
Cenpn	centromere protein N	2.51	2.6E-04
Ncaph	non-SMC condensin I complex, subunit H	2.50	9.6E-06
Ercc6l	excision repair cross-complementing rodent repair deficiency complementation group 6 - like	2.50	8.5E-05
Pscd3	pleckstrin homology, Sec7 and coiled-coil domains 3	-2.50	2.4E-04
BB001228	expressed sequence BB001228	-2.52	6.5E-04
Slc28a2	solute carrier family 28 (sodium-coupled nucleoside transporter), member 2	-2.52	2.3E-04
Tmem108	transmembrane protein 108	-2.52	7.4E-04
Lycat	lysocardiolipin acyltransferase	-2.53	8.4E-06
Tm6sf1	transmembrane 6 superfamily member 1	-2.54	6.4E-05
Zbtb20	zinc finger and BTB domain containing 20	-2.56	1.4E-04

Myd116	myeloid differentiation primary response gene 116	-2.58	7.0E-04
Ramp2	receptor (calcitonin) activity modifying protein 2	-2.58	2.4E-05
Jarid1b	jumonji, AT rich interactive domain 1B (Rbp2 like)	-2.59	1.9E-04
Zbtb4	zinc finger and BTB domain containing 4	-2.61	7.4E-05
Cxxc5	CXXC finger 5	-2.61	4.6E-05
Blr1	Burkitt lymphoma receptor 1	-2.66	1.2E-04
	NA	-2.67	8.8E-04
Tm6sf1	transmembrane 6 superfamily member 1	-2.68	3.8E-05
Rgs2	regulator of G-protein signaling 2	-2.72	2.0E-04
Nr4a2	nuclear receptor subfamily 4, group A, member 2	-2.73	9.2E-04
Btbd11	BTB (POZ) domain containing 11	-2.74	7.0E-04
Jun	Jun oncogene	-2.74	3.5E-04
Cxxc5	CXXC finger 5	-2.76	2.6E-05
Tcp11l2	t-complex 11 (mouse) like 2	-2.76	5.0E-04
Zbtb4	zinc finger and BTB domain containing 4	-2.78	6.8E-04
Pacsin1	protein kinase C and casein kinase substrate in neurons 1	-2.78	2.8E-05
1500005K14Rik	RIKEN cDNA 1500005K14 gene	-2.78	2.1E-04
Nr4a2	nuclear receptor subfamily 4, group A, member 2	-2.79	4.8E-05
Ccl4	chemokine (C-C motif) ligand 4	-2.83	1.9E-06
Mxd4	Max dimerization protein 4	-2.83	9.9E-04
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-2.84	5.9E-04
Egr1	early growth response 1	-2.84	2.2E-05
Ccl3	chemokine (C-C motif) ligand 3	-2.84	8.5E-05
Klra3	killer cell lectin-like receptor, subfamily A, member 3	-2.84	1.0E-04
Klf4	Kruppel-like factor 4 (gut)	-2.86	8.0E-04
Arrb1	arrestin, beta 1	-2.91	9.6E-05
Pacsin1	protein kinase C and casein kinase substrate in neurons 1	-2.94	1.0E-04
Timp2	tissue inhibitor of metalloproteinase 2	-2.96	9.5E-05
Egr2	early growth response 2	-2.99	4.7E-04
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-3.04	9.9E-06
Ccl5	chemokine (C-C motif) ligand 5	-3.09	7.2E-04
1500005K14Rik	RIKEN cDNA 1500005K14 gene	-3.10	4.1E-05
Btbd11	BTB (POZ) domain containing 11	-3.19	1.4E-04
Egr2	early growth response 2	-3.27	6.9E-05
Tbc1d4	TBC1 domain family, member 4	-3.41	2.2E-06
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-3.56	5.0E-05
Nt5e	5' nucleotidase, ecto	-3.60	6.1E-05
Fosb	FBJ osteosarcoma oncogene B	-3.90	1.8E-05
Folr4	folate receptor 4 (delta)	-4.27	1.1E-05
Egr3	early growth response 3	-4.54	1.7E-06
Ephx1	epoxide hydrolase 1, microsomal	-4.59	2.1E-06
Fos	FBJ osteosarcoma oncogene	-5.34	4.4E-05
H2-Ob	histocompatibility 2, O region beta locus	-5.47	4.3E-05

Table 1-12 Significantly up- or down regulated genes at the 72 hour timepoint *in vivo* (fold change greater than +/- 1.5, p< 0.001)

Normalisation plots

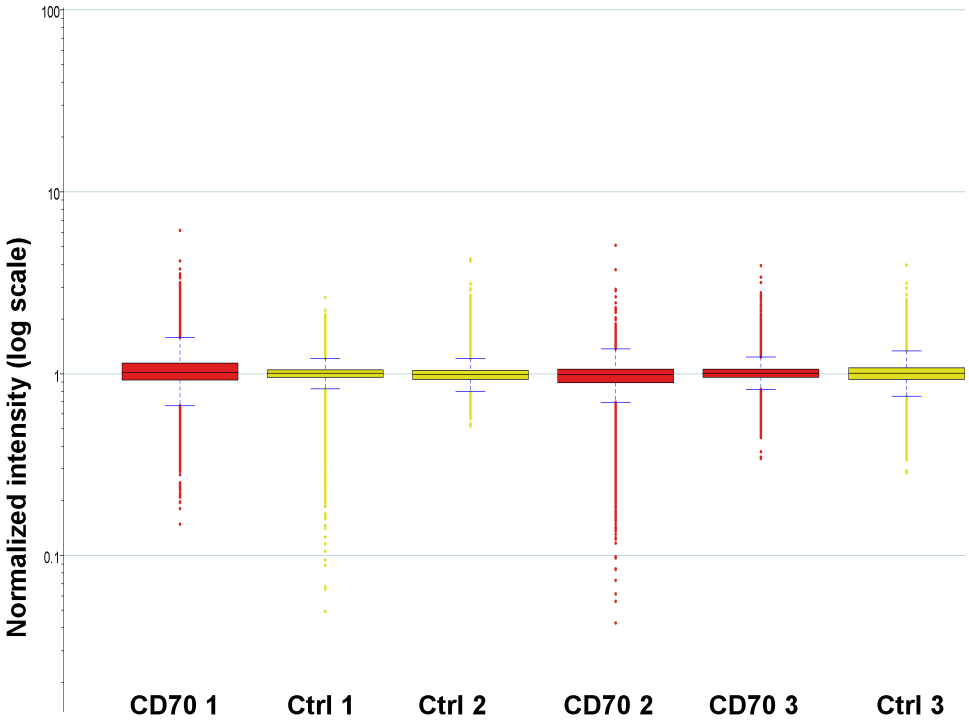

Figure 76 Normalisation of 4 h triplicate samples:

Box-whisker plots of signal intensity across entire chip for four hour triplicates; similar means and standard variations are taken as a mark of good quality control and normalisation.

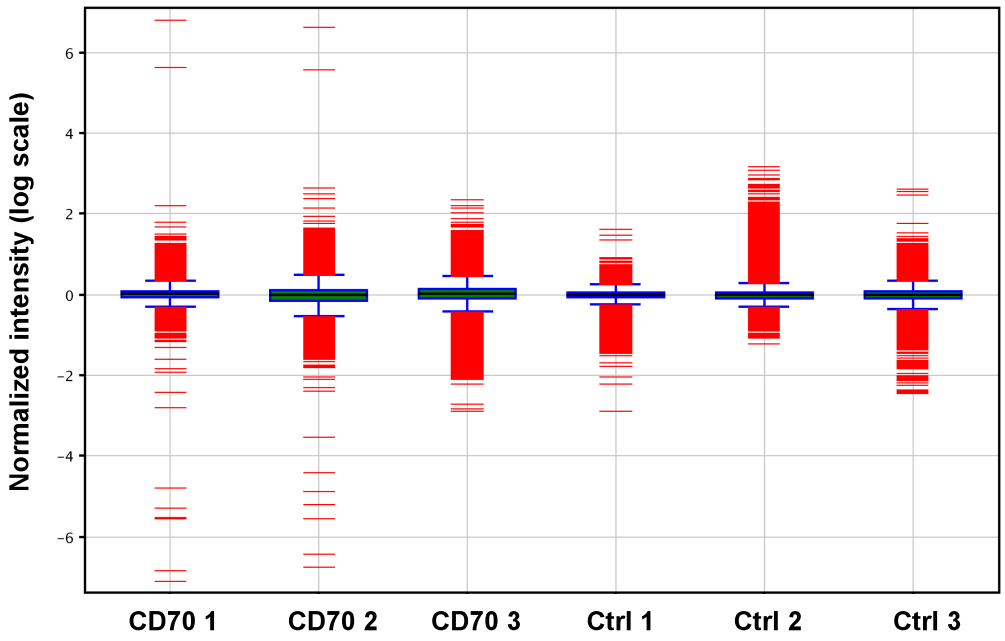

Figure 77 Normalisation of 48h triplicate samples: box-whisker plots of signal intensity across entire chip for four hour triplicates; similar means and standard variations are taken as a mark of good quality control and normalisation.

7.2. Scatter plots

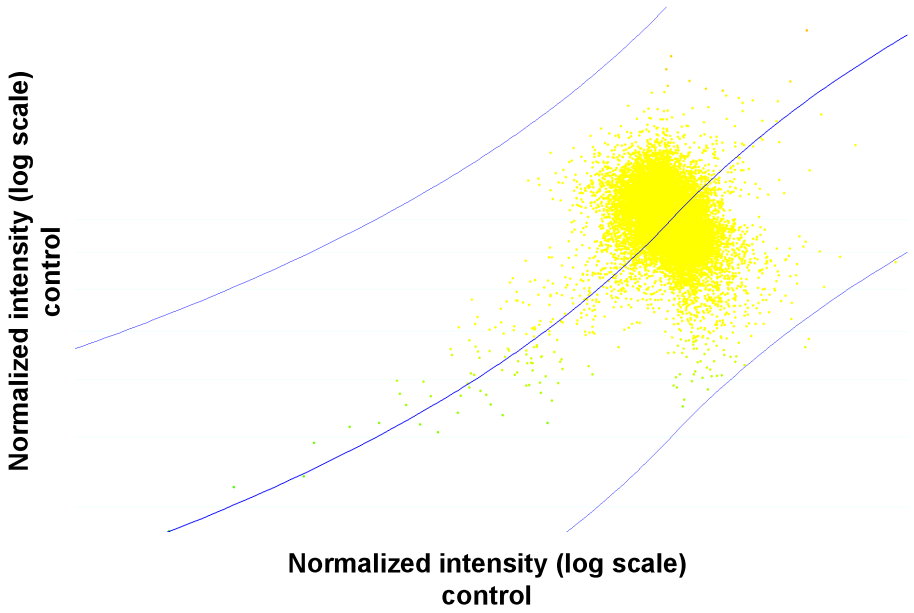

Figure 78 Scatter plot of unfiltered aggregated data from three experiments from the 4 hour *in vitro* timepoint: the central blue line indicates the 'zero difference' line, with gene expression identical between sCD70 and control arms; the outer blue lines indicate genes with a fold change of greater than 2; two genes show greater than two fold variation between the control and sCD70 experimental arms.

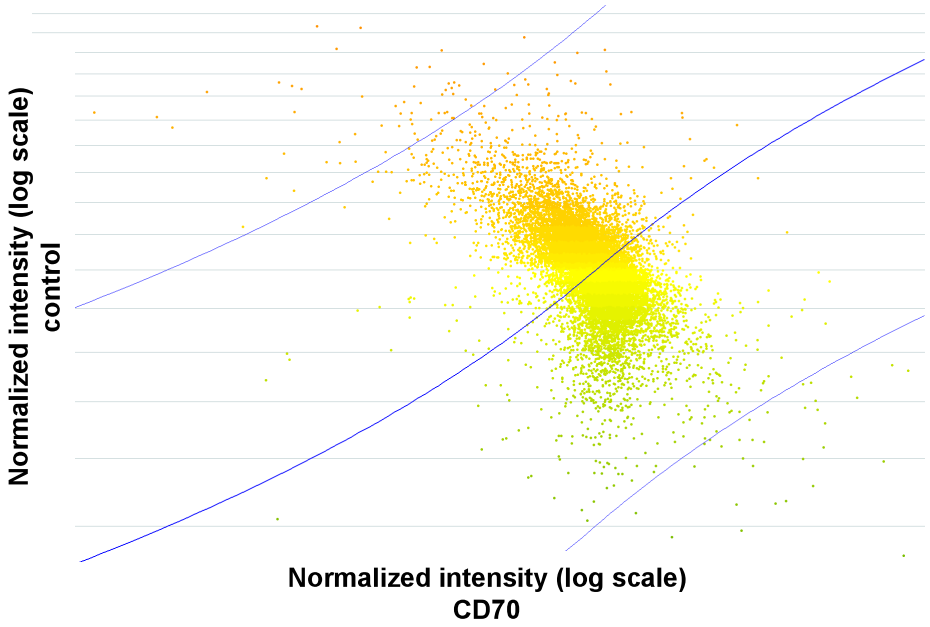

Figure 79 Scatter plot of all genes from microarrays at the 24 h time point

Normalized fold changes of control (y axis) vs sCD70 (x axis) at the 24 hour time point from *in vitro* cultures. Blue lines indicate 2 x fold, zero, and -2 x fold changes. Multiple genes are up- and down-regulated by a factor of greater than 2.

Chapter 8. References

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