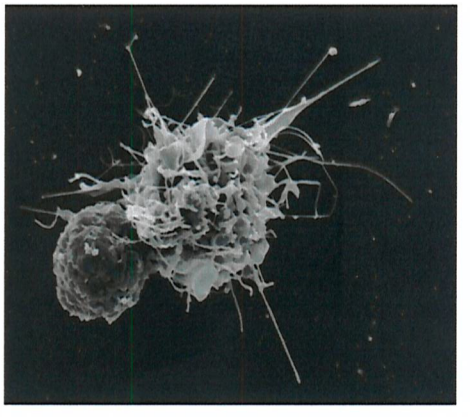
UNIVERSITY OF SOUTHAMPTON

FUNCTIONAL ANALYSIS OF THE CO-STIMULATORY MOLECULES CD27 AND CD137 (4-1BB) DURING T CELL-MEDIATED IMMUNE RESPONSES

By Tania Francesca Rowley



DOCTOR OF PHILOSOPHY

CANCER SCIENCES DIVISION
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
SCHOOL OF MEDICINE

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ABSTRACT

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FUNCTIONAL ANALYSIS OF THE CO-STIMULATORY MOLECULES CD27 AND CD137 (4-1BB) DURING T CELL-MEDIATED IMMUNE RESPONSES

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Identification of the signals required for optimal differentiation of naive T cells into effector and memory cells is critical for the design of effective vaccines for use in immunotherapy. This thesis describes the generation and characterisation of soluble recombinant ligands for two members of the TNFR superfamily of cell surface receptors, CD27 and CD137 (4-1BB). These receptors provide co-stimulatory signals, which act in concert with antigen-driven T cell receptor signals to promote optimal T cell activation. The recombinant fusion proteins, which encompassed the extracellular domains of murine CD27 ligand (CD70) or 4-1BB ligand (4-1BBL) and the Fc domain of human IgG1, formed large multimeric complexes that proved to have potent signalling capacity. Stimulation of CD27 by soluble recombinant CD70 was found to enhance both the magnitude and quality of $CD8^+$ T cell responses. Triggering CD27 in the presence of antigen significantly enhanced the division and survival of $CD8^+$ T cells *in vitro*, as well as their ability to produce interleukin-2 and interferon-γ and up regulate expression of 4-1BB and CD25 (interleukin-2 receptor α-chain). In an *in vivo* model of T cell activation, administration of peptide antigen and soluble CD70 resulted in a massive (> 300-fold) expansion of antigen-specific $CD8^+$ T cells, due to the enhanced division and survival promoted by CD27 co-stimulation. In mice that received antigen and soluble CD70, $CD8^+$ T cells developed into effectors with direct *ex vivo* cytotoxicity and the capacity to eradicate syngeneic tumour cells expressing antigen *in vivo*. Furthermore, unlike immunization with peptide antigen alone, which resulted in a diminished secondary response after rechallenge, CD27 co-stimulation during primary T cell activation led to a strong secondary response being evoked upon rechallenge with the antigenic peptide. Thus, in addition to increasing the frequency of primed antigen-specific T cells, CD27 signalling during the primary response instils a programme of differentiation that allows $CD8^+$ T cells to maintain their responsiveness. Ligation of 4-1BB using soluble 4-1BBL exhibited similar co-stimulatory effects to those of CD27 when administered with a peptide antigen, causing enhanced primary $CD8^+$ T cell expansion and affecting the quality of the T cell response so that a population of reactive T cells was maintained after the primary response. However, closer comparison of the effects of CD27 versus 4-1BB co-stimulation using their soluble ligands demonstrated that while CD27 predominantly enhanced the initial burst of T cell expansion, 4-1BB altered the kinetics of the primary response so that the activated T cells survived for longer and the contraction phase was slowed. Combining these stimuli had an additive effect on the magnitude of cytotoxic effector T cells generated, signifying that these co-stimulatory molecules have non-redundant roles in $CD8^+$ T cell responses. Furthermore, in a murine model of B cell lymphoma, administration of soluble CD70 or 4-1BBL enhanced anti-tumour immune responses and prolonged survival times. The provision of co-stimulation through CD27 and 4-1BB is a powerful means of enhancing $CD8^+$ T cell effector and memory responses and recombinant forms of their co-stimulatory ligands may have potential as anti-tumour immunotherapeutic agents.

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Abbreviations

Ab	Antibody
ACAD	Activated T cell autonomous death
AINR	Activation-induced non-responsiveness
AICD	Activation-induced cell death
AP1	Activator protein 1
APC	Antigen-presenting cell
ASK-1	Apoptosis signal-regulating kinase-1
BSA	Bovine serum albumin
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDR	Complementarity-determining region
CHO K1	Chinese hamster ovary K1 cells
CLIP	Class II invariant chain peptide
CFSE	5,(6)-carboxyfluorescein diacetate succinimidyl ester
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DD	Death domain
DP	Double positive
ECD	Extracellular domain
ELISA	Enzyme-Linked Immunsorbant Assay
ER	Endoplasmic reticulum
FCM	Flow cytometry
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
FSC	Forward scatter
GITR	Glucocorticoid-induced TNFR family receptor
GS	Glutamine synthetase
h	Hours
HEV	High endothelial venule
hFc	Human Fc
hIgG	Human IgG
HRP	Horse-radish peroxidase
HVEM	Herpes-virus entry mediator
ICAM	Intercellular adhesion molecule
ICOS	Inducible co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IKK	IκBα kinase
IL	Interleukin
i.p.	Intra-peritoneal
IS	Immunological synapse
ITAM	Immune tyrosine-based activation motif
i.v.	Intra-venous
JNK	c-jun N-terminal kinase

L	Ligand
LCMV	Lymphocytic chorio-meningitus virus
LFA-1	Lymphocyte function-associated antigen-1
LMP1	EBV latent membrane protein 1
LPS	Lipopolysaccaride
LT-α	Lymphotoxin
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MSX	Methionine sulfoximine
M_r	Molecular mass
MØ	Macrophage
NF-AT	Nuclear factor for activated T cells
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
NK	Natural killer cell
OVA $_{257-264}$	Ovalbumin peptide 257-264
PAGE	Polyacryamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern molecule
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-hydroxykinase
PKB	Protein kinase B
PLAD	Pre-ligand-binding assembly domain
PRR	Pattern-recognition receptor
PTK	Protein tyrosine kinase
P38 MAPK	p38 mitogen-activated protein kinase
RAG	Recombination activating gene
RANK	Receptor activator of NF-κB
s.c.	Sub-cutaneous
sCD70	Soluble CD70
SD	Standard deviation
SEM	Standard error of the mean
SSC	Side-scatter
s4-1BBL	Soluble 4-1BB ligand
TAA	Tumour-associated antigen
TAP	Transporter associated with antigen processing
TBS	Tris-buffered saline
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_H	T helper cell
TIL	Tumour infiltrating lymphocyte
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor

TRAF	TNFR-associated factor
T_{reg}	Regulatory T cell

CHAPTER 1

Introduction

1.1 Overview of the immune system

Multicellular organisms have developed a range of strategies to protect themselves from invasion by pathogenic agents. The evolutionary ancient innate immune system is present from higher plants to mammals and acts as a first line of defence against infection. This non-specific innate system provides anatomic and physiological barriers to the growth of microorganisms and facilitates their removal from the body by phagocytosis and the induction of inflammation. In addition to the ubiquitous innate immune system, vertebrates have evolved adaptive immune recognition of pathogens. The adaptive immune system comprises of the T cell-mediated cellular response and the B cell-mediated humoral response. These two arms of the adaptive response complement one another and allow vertebrates to respond specifically to a wide range of infectious pathogens. Adaptive immune recognition relies on the generation of a random and highly diverse repertoire of antigen receptors on T lymphocytes and B lymphocytes, which allows selection and expansion of those cells with relevant specificities. This selection process allows the generation of immunological memory, a unique property of the adaptive immune system that leads to a rapid, more efficient, immune response the second time a pathogen is encountered. However, the adaptive immune system is still reliant on the innate system, which by virtue of its ability to detect the presence and nature of infection, can control the initiation of adaptive immune responses and their subsequent effector class [1,2].

1.2 T cell immunity

1.2.1 Overview

1.2.1.1 Structure of the lymphoid system

B and T lymphocytes arise from lymphoid progenitor cells, which have themselves differentiated from pluripotent haematopoietic stem cells. The organs in which B and T cells mature and acquire the capability to elicit an immune response are called primary lymphoid organs. In humans and mice, the bone marrow and thymus are the primary lymphoid organs that control the maturation and selection of B cells and T cells, respectively. After emigration from the primary lymphoid organs, mature naïve lymphocytes circulate in the blood and lymphatic system and after activation in secondary lymphoid organs acquire the ability to migrate to peripheral tissues [1].

The most highly organised secondary lymphoid organs are the lymph nodes and spleen, which have distinct regions for the antigen-specific activation of T and B cells. In addition, a less structured form of secondary lymphoid tissue is found at mucosal sites for pathogen entry and is collectively known as mucosal-associated lymphoid tissue [1]. Lymph nodes recruit naïve T lymphocytes from the blood through high endothelial venules (HEVs). Furthermore, they are strategically placed to collect antigen-presenting cells (APCs) and antigen arriving from peripheral tissues in the lymph that percolates through the node. These T cells and APCs interact in the paracortex region of the lymph node [3] (Figure 1.1). Lymph nodes therefore provide an environment for T cell-APC cellular interactions that lead to either productive primary and secondary effector responses or antigen-specific tolerance. Lymph nodes can also direct activated T cells to the site of their cognate antigen, for example effector T cells that home to the skin are preferentially generated in the skin-draining lymph nodes [3]. The spleen is the site of priming for systemic infections, as it is able to filter blood and trap blood-borne antigens. The white pulp that surrounds branches of the splenic artery forms a periarteriolar lymphoid sheath, which is the site of activation of T cells by antigen expressed on interdigitating dendritic cells (DCs) [1].

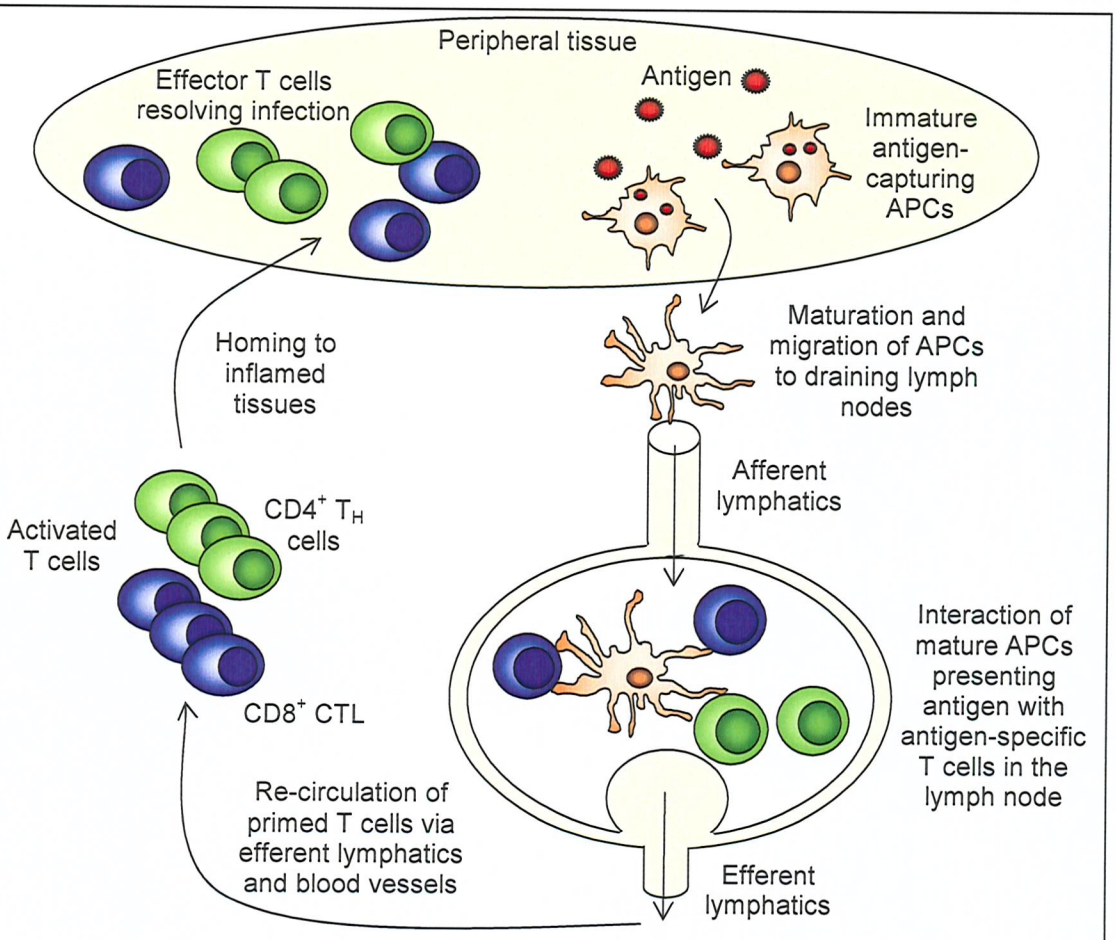


Figure 1.1 Overview of T cell priming by antigen presenting cells in the lymph nodes. Antigens are captured in peripheral tissues by immature antigen-presenting cells and processed for presentation by major histocompatibility molecules (MHC). Antigen-bearing APCs begin to mature and migrate to secondary lymphoid organs such as the draining lymph nodes. Mature APCs expressing high levels of antigenic peptide-MHC and co-stimulatory molecules interact in the paracortex with T cells arriving from the blood via HEVs. After activation, T cell blasts exit the lymph nodes, expand, develop effector functions and become capable of homing to sites of inflammation to resolve the infection.

1.2.1.2 T cell subsets

T cells can be divided into two subsets based on expression of the T cell receptor (TCR) co-receptors CD4 and CD8, which are expressed on mature T cells in a mutually exclusive fashion. $CD4^+$ and $CD8^+$ T cells undergo distinct programmes of differentiation, which gives them a helper T cell (T_H), regulatory T cell (T_{reg}) or a cytotoxic T lymphocyte (CTL) phenotype. $CD8^+$ T cells typically become CTL, which play an important role in killing tumourigenic and virally infected cells expressing

antigen. They mediate their effector functions through the production of cytokines such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α, and cytolytic mechanisms [4]. $CD4^+$ T cells typically differentiate into T_H cells, which have two polarised effector subsets, T_H1 and T_H2, defined by the cytokines that they produce. T_H1 responses are characterised by IFN-γ production and T_H2 responses are characterised by the production of interleukin (IL)-4 [5]. T_H1 responses are essential for protection against a variety of intracellular infections, whereas T_H2 responses can be protective against extracellular infections. This is achieved by T_H $CD4^+$ cells providing cytokine and/or cell-mediated help for the generation of $CD8^+$ CTLs and for antibody production by B cells. Additionally, activated $CD4^+$ T_H cells modulate antigen-presenting cells via cell-cell interactions, such as through CD40-CD40 ligand interactions, which consequently facilitate the induction of CTLs. $CD4^+$ T cells may also develop into T_{reg} cells, which pay a critical role in suppressing autoimmunity and excessive inflammatory responses. T_{reg} are usually characterised as $CD4^+$ $CD25^+$ T cells and exert their effects via cell bound negative regulators such as cytotoxic T lymphocyte antigen-4 (CTLA-4), or immunosuppressive cytokines such as IL-10 and TGF-β [6].

These T cell differentiation subsets not only have diverse effector roles but may also respond to antigen stimulation with different kinetics and efficiency of proliferation. Furthermore, they may require alternate co-stimulatory signals to be provided for optimal expansion and survival [4]. Although $CD4^+$ T cells are generally described as T_H, and $CD8^+$ T cells as cytotoxic, in some cases they may overlap in function. For example, $CD4^+$ T cells may exhibit Fas-mediated cytotoxicity [7] and $CD8^+$ T cells can help promote T_H1 responses by modulating APCs [8].

1.2.2 Generation of the T cell repertoire

1.2.2.1 T cell development and thymic selection

T cells develop in the thymus from lymphoid progenitors, which have migrated out of the bone marrow after differentiation from haematopoietic precursor cells. During T cell development, clonal diversity in the TCR gene sequence is generated by several mechanisms including V (D) J recombination of non-contiguous gene segments,

imprecise joining of nicked segments, addition of non-germline nucleotides by DNA repair machinery and by the random chain pairing of the α and β-chains [9]. Early T lineage cells lack expression of a TCR, CD4 and CD8 and are termed double negative. These cells initially express the pre-TCR α-chain that combines with a somatically rearranged β-chain to form the pre-TCR. The pre-TCR α-chain is then replaced by a rearranged α-chain and in addition to this unique antigen receptor the thymocytes become double positive (DP) for the co-receptors CD4 and CD8.

The DP thymocytes consequently undergo a selection process through the interaction of their TCR complex with self-peptides presented on major histocompatibility complex (MHC) molecules. This selection allows only the subset of T cells that are likely to be functional in the host environment to mature and exit the thymus. The majority (~ 90 %) of DP thymocytes express T cell receptors that bind so weakly to the MHC-peptide complexes expressed on the cortical epithelial cells that the necessary viability signals are not generated, resulting in delayed apoptosis (death by neglect). Thymocytes that have productively rearranged T cell receptors capable of recognising either MHC class I or class II antigen complexes are positively selected and become committed to either the CD4 or CD8 lineage, respectively. However, a proportion of these thymocytes will have such high affinity for self-antigens presented by the epithelial cells and dendritic cells of the thymic medulla that they might cause autoimmune pathology if allowed to escape to the periphery. These are eliminated by high intensity signalling through the TCR, which leads to acute apoptosis (negative selection). The deletion in the thymus of these potentially auto-reactive developing T cells is known as central tolerance. T cells that receive the optimal intermediate amount of TCR signalling are allowed to mature, become single positive for the co-receptors CD4 and CD8 and exit to the periphery [10].

It has been estimated that after undergoing positive and negative selection, the theoretical maximal diversity of the TCR repertoire is 1×10^{13}. However, this is an overestimate because the total number of T cells in the body is limited in mice to 10^8 and in humans to 10^{12}. Experimental estimates of the actual peripheral TCR diversity have recently been made by extrapolation of molecular measurements. This has led to minimal estimates of the mouse TCR repertoire at 2×10^6, and the human TCR

repertoire at 2.5 x 10^7. When combined with the capability for any one TCR sequence to cross-react with multiple peptide-self MHC surfaces, this leads to the T cell repertoire having the capacity to mount an adaptive immune response against a plethora of pathogens [9].

1.2.2.2 Structure of the T cell receptor

The T cell receptor is a type I integral membrane protein of the immunoglobulin family and is structurally related to the antigen-binding fragment of an antibody molecule (Fab_2 fragment). The TCR is typically an αβ heterodimer, but T cells with γδ TCRs are also found. Each polypeptide chain contains one membrane proximal constant domain and one membrane distal variable domain [11]. The variable domains of each chain contain three complementarity-determining regions (CDR) that interact with antigenic peptides in the context of either class I or class II MHC molecules (see Figure 1.2). CDR 3 contacts peptide side chains, and this hypervariable region is therefore particularly important for conferring each TCR its antigen specificity [11].

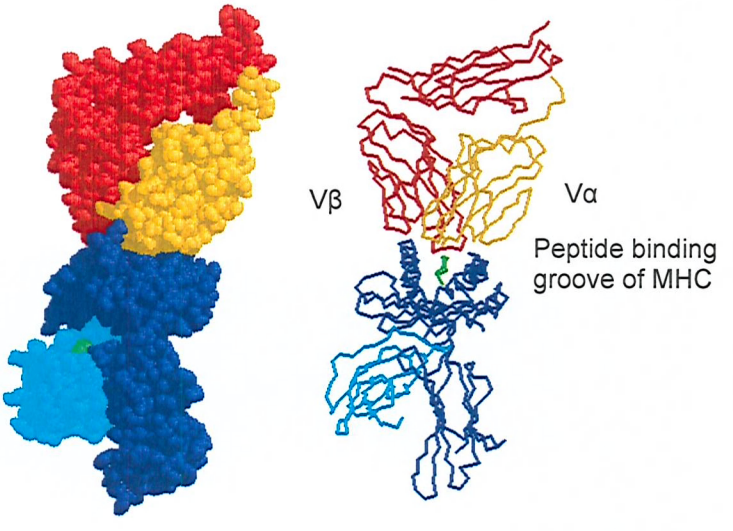


Figure 1.2 Structure of an αβ T cell receptor complexed with MHC class I. The α-chain of the T cell receptor is shown in yellow and the β-chain in red. The T cell receptor constant region domains are incomplete. The three extracellular domains of the MHC α-chain are shown in dark blue and the associated beta-2-microglobulin in light blue. A space-filling model is shown on the left and a model showing just the protein backbones is shown on the right. The groove is viewed end on and the peptide can be seen in green in the model on the right. The complex shown is derived from the Brook Haven Protein Data Bank entry 1A07.pdb, which is of a human MHC Class I, HLA-A 0201 molecule, with a peptide from Tax protein of human T-lymphotropic virus type 1, complexed with the terminal domains of an α/β TCR.

The cytoplasmic domains of αβ TCR chains are short and lack any signalling capacity. This is circumvented by the αβ dimer forming an octameric complex with the γ, δ, ε and ζ chains of CD3, which contain immune tyrosine-based activation motifs (ITAM) and can propagate downstream signalling pathways [11]. These signalling components are arranged in dimers of CD3γε, CD3δε, and CD3ζζ and may form a unit containing two TCRαβ heterodimers [12]. The TCR also associates with the co-receptors CD8 or CD4, which bind to non-polymorphic surfaces of MHC class I or class II molecules, respectively [13].

1.2.2.3 T cell receptor signalling pathways

TCR triggering generates a variety of protein tyrosine kinase (PTK) signalling cascades that result in gene transcription. The initial point in TCR signal transduction is phosphorylation of the two tyrosine residues within the ITAM motif in each CD3 signalling module. This is mediated by the Src family PTK Lck, which associates with the cytoplasmic tail of the co-receptors CD4 and CD8, and Fyn. Phosphorylated ITAMs form a binding site for the Syk family kinase ZAP-70, through SH2 domains. ZAP-70 is then activated by phosphorylation, and in turn phosphorylates the linker proteins SLP-76 and LAT that act as scaffolds for further downstream signalling complexes. Early second messengers such as Ca^{2+}, and changes in inositol phospholipid metabolism, subsequently initiate pathways that lead to the activation and mobilisation of the transcription factors nuclear factor for activated T cells (NF-AT) and nuclear factor-κB (NF-κB). TCR triggering also activates the small G protein Ras, which instigates mitogen activated protein kinase (MAPK) cascades leading to the formation of AP1 heterodimers that promote the transcription of genes involved in IL-2 production and proliferation. In addition, TCR signalling can regulate polarisation of the actin cytoskeleton [11,14]. So how does the binding of the TCR to peptide-MHC ligand trigger the activation of PTKs? It was thought until recently that the tyrosine phosphorylation of CD3ζ ITAMs, brought about by TCR aggregation and mediated by Lck, was sufficient to initiate the signalling cascade. However, recent work has indicated a tandem mechanism at work whereby a conformational change in CD3ε allows recruitment of the adaptor protein Nck [15]. These signals probably converge downstream, possibly at the point of SLP-76, and both mechanisms appear to be necessary for the full activation of T cells [12].

1.2.3 Antigen presentation

1.2.3.1 Antigen presenting cells

The majority of nucleated cells express MHC class I molecules, which allows them to present intracellular antigens to $CD8^+$ T cells and also be potential CTL targets. However, only a few cell types also constitutively express MHC class II and can thereby initiate effective immune responses by presenting antigen to both $CD4^+$ and $CD8^+$ T cells. These cell types are called antigen presenting cells and include B cells, macrophages and dendritic cells [16]. DCs have been denoted as professional antigen presenting cells as, unlike B cells and macrophages, they constitutively express co-stimulatory molecules that are required in conjunction with peptide-MHC complexes for the priming of naïve T cells [17]. DCs are also the ideal candidates for controlling naïve T cell responses for a variety of other reasons. For example, DCs are located at portals of antigen entry, they are efficient at antigen uptake and processing for presentation, and they migrate to the T cell areas of secondary lymphoid organs [17]. In contrast B cells and macrophages are not found in the T cell areas, express lower levels of peptide-MHC complexes and have less potent co-stimulatory capabilities [16,17]. However, these cells may act as APCs for T cells which have previously been primed by DCs [16]. Direct *in vivo* experimental evidence that dendritic cells are responsible for priming naïve T cell responses is now available for both $CD4^+$ and $CD8^+$ T cells. It was initially observed by Ingulli *et al* that naïve ovalbumin-specific $CD4^+$ T cells formed small clusters around peptide-pulsed DCs, or around endogenous DCs in draining lymph nodes 24 hours after subcutaneous injection of whole antigen [18]. More recently, Norbury *et al* observed antigen-specific clustering of naïve $CD8^+$ T cells around recombinant fluorescent vaccinia virus-infected cells in the lymph node. The APCs at the centre of these clusters were determined to be dendritic cells by morphology and phenotypic staining. In contrast, no clusters were seen around infected macrophages, despite these cells making up the majority of infected cells resident in the lymph node [19].

1.2.3.2 Presentation of antigen on MHC Class I/II molecules

The major histocompatibility class I and class II molecules responsible for presenting peptide antigens to T cells are polymorphic members of the immunoglobulin family.

MHC class I molecules consist of an α chain which is stabilised by non-covalent association with the related molecule β_2-microglobulin. The α-chain α1 and α2 domains of MHC class I form a cleft which is large enough to bind peptides of 8-11 amino acids in length (see Figure 1.2 for MHC-peptide-TCR structure). Invariant sites in the cleft of MHC-class I molecules form stabilizing contacts with peptides via their amino and carboxy terminals. In addition, MHC-class I molecules bind anchor residues within optimal peptides, the position of which varies depending on the MHC allele. MHC class II molecules are heterodimers of an α and a β chain which interact non-covalently. These form a peptide binding cleft which is open at both ends and can therefore bind larger peptides of 13-18 amino acids. MHC molecules can bind promiscuously to numerous different peptides and although polymorphic do not have the specificity of TCRs [1].

T cells expressing the CD8 co-receptor along with their TCR can exclusively interact with MHC class I molecules presenting antigenic peptides, and T cells expressing CD4 can exclusively interact with MHC class II-peptide complexes. This preference for different classes of MHC molecules relates to a demarcation in the antigen processing pathways that supply peptides. MHC class II molecules present peptides derived from exogenous antigens that enter the cell via the endocytic route, whereas MHC class I molecules capture peptides derived from the breakdown of antigens synthesised within the cell. This separation allows $CD4^+$ T cells to respond predominantly to antigens from extracellular pathogens such as bacteria, fungus and parasites, while $CD8^+$ T cells will generate responses to viral antigens and may respond to altered-self molecules such as those expressed by tumours. Endogenously synthesised proteins are first degraded into peptides in the cytosol mainly by the 26S proteasome, a multisubunit complex consisting of a catalytic core 20S proteasome and 19S regulator. During inflammatory immune responses, cytokines such as IFN-γ alter proteasome activity quantitatively by incorporation of three alternate immunosubunits into the 20S core proteasome generating the immunoproteosome [20]. The proteasome produces peptides with the correct peptide carboxyl terminal that may subsequently be trimmed by additional aminopeptidases. These peptides then access the MHC class I peptide loading complex, an endoplasmic reticulum (ER)-based oligomeric complex containing dimers of MHC class I heavy chain and β_2-microglobulin, the transporter

associated with antigen processing (TAP), calreticulin, the thiol oxioreductase ERp57 and tapasin. The chaperone protein calnexin associates with partially folded MHC class-I α chains in the ER until β_2-microglobulin can bind and the peptide loading complex is formed. The TAP1-TAP2 heterodimer transports peptides from the cytosol into the lumen of the ER where they may be trimmed by ER aminopeptidases, such as ERAP 1. Tapasin links TAP to the MHC Class I–β_2M dimer and may act as a 'peptide editor' allowing MHC class I molecules to exchange peptides and encouraging the binding of higher affinity peptides [21]. Stable MHC class I - peptide complexes are then trafficked to the cell surface for interaction with $CD8^+$ T cells. Exogenous pathogens and proteins are endocytosed and then degraded in endocytic vesicles by a variety of proteases with broad specificity. These peptide products are then loaded onto MHC class II molecules, which have been prevented from picking up endogenous peptide by their association with the invariant chain. Removal of the invariant chain is accomplished by an ordered proteolytic reaction leading to the generation of a fragment called class II invariant chain peptide (CLIP) lodged in the peptide-binding groove. The DM chaperone protein then catalyses the dissociation of CLIP, stabilises the empty MHC class II molecule and assists in peptide selection. Class II molecules loaded with peptide then exit to the cell surface via late endosomes and lysosomes, and present their antigen to $CD4^+$ T cells [22].

In addition to these two distinct antigen presentation pathways, it has been demonstrated that in some cases exogenous antigen may be diverted to the class I pathway for presentation to $CD8^+$ T cells [23,24]. This 'cross presentation' was originally described by Bevan in the 1970s [25], and is thought to be important for generating $CD8^+$ T cell responses against cell-associated antigens, or viruses which do not infect APCs. Additionally, this mechanism may help promote self-tolerance by the cross-presentation of peripheral tissue antigens under tolerigenic conditions. This specialist function of cross presentation *in vivo* appears to be limited to the $CD8^+$ subset of dendritic cells in mice [26], although it is unclear which is the corresponding DC subset in humans. Particulate and soluble exogenous antigens undergoing capture by phagocytosis or pinocytosis, respectively, may be cross-presented by different mechanisms. In DCs and macrophages the ER functions as a membrane donor during phagocytosis, creating phagosomes that contain ER-based proteins such as the TAP-

associated loading complex and the translocon Sec61, known to retrotransport ER proteins to the cytoplasm for degradation by the proteasome. These phagosomes constitute a unique peripheral organelle which is competent to load peptides derived from particulate exogenous antigens onto MHC class I molecules, allowing their cross-presentation [27,28]. Exogenous soluble antigens that are taken up by pinocytosis can also be cross-presented by DCs, but with lower efficiency than particulate antigens. Soluble proteins which escape proteolysis have been shown to enter the lumen of the perinuclear ER, and presumably can therefore access the retrotranslocation machinery in the ER and be processed for MHC class I presentation on the DC [29]. However, soluble proteins were shown to be unable to access the ER of macrophages and B cells, consistent with previous findings demonstrating that DCs are the only APC capable of cross presentation *in vivo.*

1.2.4 Induction of T cell immunity versus tolerance

During T cell development, selection in the thymus does not deplete all T cells that are capable of responding to self-antigens. Therefore, central tolerance is not sufficient for preventing autoimmunity. Because randomly generated antigen receptors are unable to determine the source or the biological context of the antigen for which they are specific, APCs are required to make the distinction between inducing tolerance to the antigen and provoking an immune response. Dendritic cells have been shown to confer instructive signals for T cell tolerance or immunity, depending on what environmental signals for maturation they have received [30].

1.2.4.1 Factors which lead to the generation of T cell immunity

Microbial infections and related danger signals from tissue damage cause the maturation of DCs from a phagocytic/endocytic phenotype to one capable of efficient antigen presentation. In addition, these signals increase DC migration from peripheral tissues into the draining lymph nodes enabling a rendezvous with naïve T cells [30,31]. Microbial molecules that can promote strong immune responses through DC maturation include lipopolysaccharide (LPS) [32], unmethylated bacterial DNA CpG motifs [33,34] and double stranded RNA found in viruses [35] amongst others. These molecular signatures are conserved between microorganisms of a given class and fulfil the criteria for the pathogen associated molecular pattern molecules (PAMPs)

originally postulated by Janeway to control activation of the innate immune system [36]. In the past five years it has been discovered that the immune system has specific receptors for these PAMPs known as pattern recognition receptors (PRRs) [2]. One of the best characterised of these PRRs is the evolutionarily conserved Toll-like receptor (TLR) family which comprises of a series of homologous molecules with specificity for different PAMPs. For example, mammalian TLR4 in conjunction with several accessory molecules recognises LPS and TLR9 recognises the CpG motifs in bacterial and viral genomes [2]. TLR signalling pathways lead to the induction of various genes that function in host defence including inflammatory cytokines such as type I interferons, chemokines, MHC and co-stimulatory molecules. For example, all TLR agonists tested to date can lead to increased expression of the co-stimulatory molecules CD40, B7-1 and B7-2 on at least one DC subset [37]. Pathogen driven signals may also bias the T cell helper class that is generated e.g. certain TLRs may induce the secretion of cytokines such as IL-12 by DCs which promotes T_H1 responses [2]. In addition to microbe-derived signals, APCs can be activated and mature in response to cellular stress signals such as heat shock proteins [38,39] and uric acid [40]. These 'danger' signals are released during necrosis, but are not generated by cells that die normally via apoptosis [41,42]. This allows the immune system to react more strongly to pathogenic microbes that are causing tissue damage. DCs are also activated by inflammatory cellular signals such as IFN-α, an anti-viral cytokine produced by many types of virally infected cells [41].

For effective $CD8^+$ CTL responses to be generated, APCs may require additional 'licensing' signals from activated $CD4^+$ T_H cells. Interactions such as CD40-CD40L between APCs and antigen-activated $CD4^+$ T cells act as an amplification step to further licence DCs for CTL responses, by up-regulating their co-stimulatory molecules [43-45] and enhancing the production of T cell-polarising cytokines induced by microbial signals [46]. The importance of this form of T cell help has recently been demonstrated. Some anti-viral CTL responses have previously been denoted as $CD4^+$ T cell independent, presumably because viral activation of APCs can be sufficient to induce efficient primary responses. However, these $CD8^+$ T cell responses have now been shown to require $CD4^+$ T cell help during the priming stage in order for a responsive pool of memory $CD8^+$ T cells to be generated [47]. Thus, antigen-presenting

cells integrate danger signals received from foreign pathogens, damaged tissues and T_H cells and signal this to naïve T cells via enhanced antigen presentation and provision of co-stimulation (Figure 1.3).

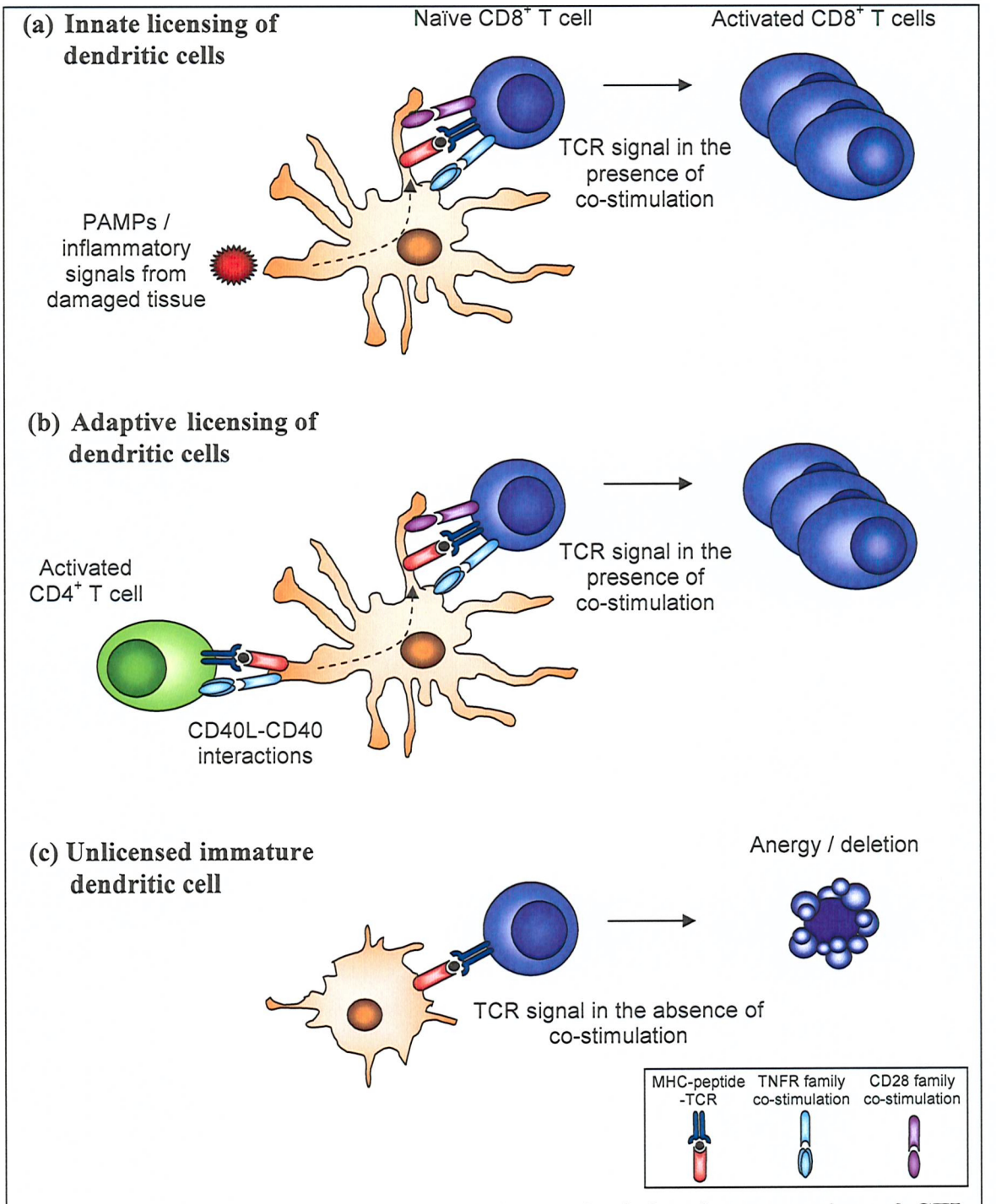


Figure 1.3 Licensing of dendritic cells is required for the generation of CTL immunity. To prime naïve $CD8^+$ T cells, DCs require a licensing signal from (**a**) the innate system via PAMPs or tissue inflammation, or from (**b**) the adaptive immune system, for example via CD40L. (**c**) In the absence of licensing signals, DCs do not up regulate co-stimulatory molecules and cannot effectively prime naïve T cells.

1.2.4.2 Maintenance of peripheral tolerance

In the absence of infection, the maintenance of peripheral T cell tolerance is controlled by several mechanisms. These include induction of a state of non-responsiveness known as anergy, the generation of regulatory T cells and ignorance of antigens expressed at low levels. The full activation of naïve T cells is controlled by the strength of the TCR interaction with MHC-antigenic peptide complexes in conjunction with a mandatory second signal through the binding of co-stimulatory molecules to their ligands on APCs. Quiescent DCs may help maintain peripheral tolerance by steady state migration into lymph nodes and presentation of self-antigens to T cells [30,31]. As these DCs have not been activated by microbial or danger signals they will have a tolerogenic phenotype of low MHC expression and low levels of co-stimulatory molecules. In addition, peripheral tissues expressing self-antigens will tend to promote T cell tolerance because they don't express co-stimulatory molecules. This is in line with growing evidence suggesting that T cell anergy may be a consequence of TCR signalling in the absence of co-stimulation [48] (Figure 1.3). T cell clonal anergy is defined as a hyporesponsive state in which the T cell remains alive for an extended period of time after antigen stimulation but is unable to proliferate or produce IL-2 upon re-encounter with antigen [48,49]. Co-stimulatory signals may prevent the induction of unresponsiveness by synergising with TCR signalling pathways, sustaining cell division and cytokine production and enhancing T cell survival [50]. Thus, it appears that co-stimulatory molecules play a key role in switching the default state of T cell tolerance to protective immunity. This will be discussed in more detail in Section 1.3.

1.2.5 $CD8^+$ T cell responses

1.2.5.1 Programming and kinetics of the $CD8^+$ T cell response

The $CD8^+$ T cell response to antigenic stimuli occurs in three defined phases. The first phase is the expansion of naïve T cells from a very low precursor frequency, which is initiated in the lymphoid organs. During this period of expansion, $CD8^+$ T cells divide every 6-8 hours and can quickly develop effector functions. This burst of expansion is followed by a contraction phase during which ~ 90 % of activated T cells die. The surviving T cells are memory precursors, and during the final phase form a stable pool of memory $CD8^+$ T cells which provide long-term protection against reinfection [51].

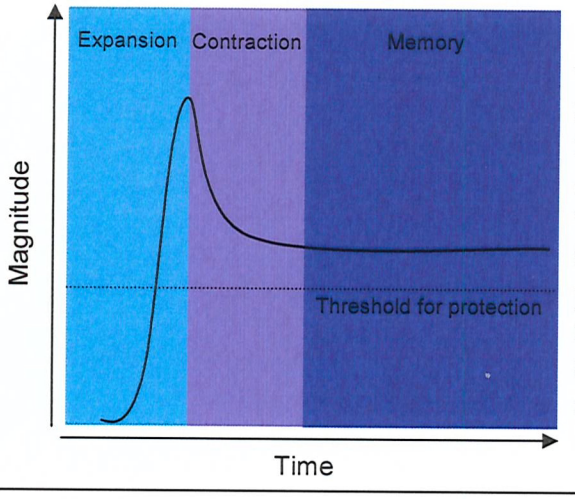


Figure 1.4 CD8$^+$ T cell responses. The three phases, expansion, contraction and memory, of the CD8$^+$ T cell response are indicated. Alteration of any phase can affect the size of the memory pool and therefore whether protective immunity is achieved. The conditions of the priming phase, e.g. duration and intensity of TCR triggering and the presence of co-stimulation, can programme the magnitude of the subsequent response.

The commitment of naïve CD8$^+$ T cells to undergo clonal expansion, contraction, and memory cell formation can be programmed during a transient exposure to antigen [52-54]. A report by Ahmed's group demonstrated that a single period of *in vitro* stimulation was enough to put CD8$^+$ T cells into a developmental programme of a least 7 cell divisions [53]. Furthermore, this initial 24-hour exposure to antigen could induce differentiation into memory cells even after the T cells were transferred to antigen-free hosts [53]. In agreement with this data, Schoenberger's group have showed that naïve CD8$^+$ T cells require as little as 2 hours of *in vitro* stimulation with APCs presenting antigen and co-stimulation in order to divide extensively and develop effector function [52]. Therefore, the commitment to autonomous division occurs before the first round of T cell division, and without the need for further antigenic simulation of daughter cells. This pre-programming allows a quantitatively larger response to be generated than if each daughter cell has to be stimulated with antigen, and allows T cells to migrate away from the site of antigen presentation to the site of infection [51]. The original idea of an instructive developmental programme set in motion by transient exposure to antigen has recently been expanded into a model of progressive differentiation. This suggests that the ability of T cells to survive and complete differentiation into responsive memory cells can be predetermined by the strength of the initial antigen signal. Signal strength is governed by the concentration of peptide-MHC complexes (determines the rate of TCR triggering), the concentration of co-stimulatory molecules (determines the extent of signal amplification) and the duration of the T cell-APC interaction (determines how long signal accumulates for) [55]. Evidence supporting this

hypothesis comes from two recent reports [56,57]. $CD8^+$ T cells exposed to a 20 hour stimulus were capable of proliferating extensively *in vivo* and mediating peripheral tissue destruction, whereas cells given only a 4 hour stimulus underwent abortive clonal expansion [57]. Data from Lanzavecchia's group demonstrated that T cells which were stimulated for extended periods of time, or in the presence of co-stimulation/activated DCs, acquired the ability to respond to homeostatic cytokines and resist death by neglect *in vivo*. However, T cells given weaker stimuli did not up regulate sufficient cytokine receptors and anti-apoptotic molecules to become 'fit' and declined in number *in vivo* [56]. This data suggests that stochastic interactions of naïve $CD8^+$ T cells with DCs will lead to a heterogeneous T cell response in terms of effector functions and survival ability.

1.2.5.2 Naïve $CD8^+$ T cell priming

Recent advances in imaging technology have allowed the visualisation of naïve T cell interactions with DCs in intact lymph nodes. Using intravital two-photon imaging, Memple *et al* have observed that T cell priming occurs in three successive phases. Transient serial encounters between T cells and APCs occur during the first activation phase, this is followed by a second phase of stable antigen-dependent contacts cumulating in cytokine production, which makes a transition into a third phase of high T cell motility and rapid proliferation [58]. The first phase of activity may represent a scanning function, as after entry to the lymph node through high endothelial venules, naïve T cells appear to migrate rapidly in random directions [58]. Differential fluorescent labelling of T cells and DCs has demonstrated that in the absence of antigen naïve T cells scan multiple DCs. Bousso *et al* have predicted that DCs may interact with as many as 500 $CD8^+$ T cells per hour in the lymph nodes, and be in contact with more than 10 simultaneously [59]. This activity may serve to increase the chance of an antigen-bearing DC locating a rare antigen-specific T cell. It has also been suggested that self-recognition promotes enhanced TCR sensitivity [60]. Moreover, in the presence of antigen T cells take part in multiple interactions with DCs during the first few hours after entry to the lymph node, which are sufficient to up regulate the early activation markers CD44 and CD69 [58]. However, these short interactions don't appear to be sufficient for full activation of $CD8^+$ T cells. In the study by Memple *et al*, the second phase from ~ 6 to 10 hours after entry to the lymph node saw T cell-DC antigen-

dependent conjugates forming which were stable in the order of hours [58]. Comparable observations of stable $CD8^+$ T cell-DC interactions have been made in excised lymph nodes [59], and $CD4^+$ T cells have been observed interacting with a single DC for up to 15 hours [61]. In agreement with the progressive differentiation model, these prolonged conjugations appear to be necessary for the full effector differentiation of naïve T cells. For example, use of IL-2 promoter/Green fluorescent protein transgenic T cells has shown that only T cells which have established prolonged contacts with mature DCs are committed to produce IL-2 [62]. Therefore, this 'molecular conversation' dictates the fate of the stimulated cell and ensures that T cell differentiation is controlled by the activation state of the DC presenting antigen for which it is specific. After this stage of stable conjugation, T cells regain rapid motility, perhaps in an effort to leave the lymph node. It is also at this point of 1.5 - 2 days after lymph node entry that cell division is initiated in T cells that have been sufficiently stimulated [58,61].

Transient adhesion interactions between the C-type lectin DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) and intercellular adhesion molecule (ICAM)-3 are critical for initial DC - T cell clustering [63]. DC-SIGN binding to glycosylated ICAM-3 establishes the first molecular interaction between DCs and resting T cells. This cellular interaction facilitates the formation of low-avidity lymphocyte function-associated antigen (LFA)-1 - ICAM-1 interactions and scanning of the antigen-MHC repertoire [63]. When a productive TCR engagement is obtained TCR signalling increases the avidity of LFA-1 and CD2, thereby stabilising the interaction between DC and T cell via multiple adhesive contacts. The membrane contact zone between T cells and APCs that forms during stable interactions has been observed to form an organised interface termed the immunological synapse (IS). Within this structure, receptors and intracellular proteins involved in T cell activation are segregated into discrete areas [64]. This redistribution was first observed by Monks *et al* occurring between $CD4^+$ T cells and B cells *in vitro*. They reported that in the presence of appropriate antigen, TCRs segregate into a central cluster (termed the central supramolecular activation cluster) whereas the adhesion molecule LFA-1 is excluded into a peripheral ring [65]. The formation of an IS has also been observed in $CD8^+$ T cells, and examination of $CD4^+$ T cell-DC couples in excised lymph nodes has shown exclusion of CD43 from the contact zone, suggesting synapse formation also occurs

under near physiological conditions [61]. *In vitro* data from previously activated $CD4^+$ T cells suggests that > 10 hours of continuous synaptic signalling are required to provoke full proliferative and cytokine responses. In this system, blocking TCR-MHC interactions rapidly terminated TCR signalling pathways and caused dissolution of the synapse structure and proportional reductions in IL-2 production and proliferation [66]. This suggests that TCR signalling is cumulative and must reach a threshold level before full responses are initiated. The formation of a synapse may serve to stabilise signalling until the activation threshold can be reached, and potentially could enhance signalling by facilitating the clustering of engaged TCRs with co-receptor and co-stimulator molecules. An additional function of the IS may be to direct helper signals and thus prevent spurious bystander activation [64].

1.2.5.3 Effector functions of $CD8^+$ T cells

Effector $CD8^+$ T cells have an enhanced ability to migrate into tissues, as compared to naïve T cells. Their reduced potential for homing to lymph nodes arises from decreased expression of lymph node homing receptors such as CC-chemokine receptor 7 (CCR7) and L-selectin (CD62L), but they have a greater capacity to migrate to inflamed tissues as a result of increased expression of chemokine receptors such as CCR2 and CCR5 [51]. $CD8^+$ T cells mediate their effector function through the secretion of cytokines such as IFN-γ and TNF-α, and cytolytic mechanisms [51]. CTLs form a membrane contact zone with a target cell presenting antigen on class I MHC molecules, which resembles the immunological synapse formed between naïve T cells and APCs during priming [67,68]. Cytotoxic granules released by exocytosis into the immunological synapse induce apoptosis in the target. These cytotoxic granules contain amongst others the membrane perturbing protein perforin, the anti-microbial granulysin and the serine proteases granzyme A, B and C. Perforin has been shown to be required to deliver granzymes into the cytosol of the target cell, but the mechanism is not well understood [67]. The serine proteases granzyme A B and C trigger rapid apoptosis in target cells through several distinct pathways. Granzyme B triggers apoptosis by caspase cleavage, leading to rapid death of the target cell. Granzyme A can mediate caspase-independent cell death by causing nicks in single-stranded DNA and thus forcing the cell into apoptosis [67,69].

1.2.5.4 Contraction of the effector pool

Memory $CD8^+$ T cells are thought to be derived from the effector cell population by linear differentiation [4,70]. Because the memory T cell compartment is formed from ~ 10 % of the effector cell population, the original burst size and extent of death during the contraction phase directly regulates the size of the memory pool. This indicates that T cell intrinsic and extrinsic survival factors are important for memory formation [71]. During the contraction phase, effector T cells undergo apoptosis as a result of what has been termed 'cytokine withdrawal' [72]. This type of cell death is controlled by the balance of pro- and anti-apoptotic members of the Bcl_2 family of homologous proteins. These molecules regulate T cell death by promoting or inhibiting the mitochondrial pathway of apoptosis [72]. Expression levels and mobilisation of the Bcl_2 family molecules can be regulated by a variety of signals including co-stimulation through the CD28 or tumour necrosis factor receptor (TNFR) superfamilies [73], and the common cytokine-receptor γ-chain family cytokines (e.g. IL-2, IL-7 and IL-15) [74]. In fact it has been demonstrated that increased expression of the IL-7 receptor on effector $CD8^+$ T cells may identify memory precursors [75].

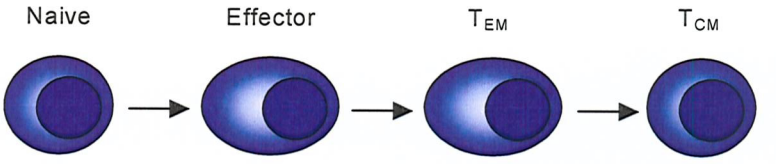
1.2.5.5 Characteristics of memory $CD8^+$ T cells

The formation of a memory population after the primary immune response results in a 1000-fold increase in antigen-specific precursor frequency in immune animals compared to naïve animals [51]. This enlarged precursor frequency allows a quantitatively greater response the second time a pathogen is encountered. Memory T cells are also qualitatively different from naïve T cells in that they have heightened recall responses [51]. For example, the lag time from antigen encounter to the first cell division in memory $CD8^+$ T cells has been shown to be only 12 hours, as opposed to a 27-hour delay for naïve cells [76,77]. Memory $CD8^+$ T cells may therefore be better poised to augment downstream TCR signals. This property has been attributed to memory cells having more extensive lipid rafts with higher phosphoprotein content, and thus more efficient phosphorylation of downstream kinases [78,79]. Furthermore, it has recently been demonstrated by Veiga-Fernandes *et al*, that high cyclin-dependent kinase (CDK)-6 activity and low expression of $p27^{Kip1}$ (a cell cycle inhibitor) in memory $CD8^+$ T cells allows rapid division by maintaining the memory cells in a state of preactivation [80]. Hence, the threshold of activation is reached more quickly in

memory cells than naïve cells. Memory cells may also acquire effector functions more rapidly than naïve cells; for example, resting memory $CD8^+$ T cells can kill targets *in vivo* almost as efficiently as peak effector cells [81,82]. Another salient trait of $CD8^+$ memory cells is their ability to undergo a basal level of homeostatic proliferation in response to cytokines such as IL-7 and IL-15. This attribute allows a stable pool of memory T cells to be maintained for long periods, at least in the absence of further infections [4]. Because memory T cells exhibit altered patterns of cell adhesion and chemotaxis proteins, they can survey peripheral tissues and immediately control re-infection [51].

1.2.5.6 Memory $CD8^+$ T cell subsets

Temporal analysis of gene expression patterns and memory $CD8^+$ T cell qualities (such as homeostatic proliferation, response to secondary antigen and IL-2 production) has indicated that these properties continue to change for several weeks after the peak of the primary response. This progressive differentiation from effectors to memory cells leads to the memory pool becoming fully responsive to secondary challenge only at late time points after antigen has been cleared [83]. Memory $CD8^+$ T cells reside in both lymphoid and non-lymphoid compartments, and as such are optimally placed to mediate recall responses if the antigen returns. Two subsets of memory T cells have been described based on their anatomical localisation and expression of the lymph node homing molecules CCR7 and CD62L, central memory (T_{CM}) and effector memory (T_{EM}) cells [84]. T_{CM} are $CCR7^+$ and $CD62L^+$ and are therefore competent to transmigrate through high endothelial venules to the lymph nodes and thus share some migratory routes with naïve T cells. However, they are also competent to home to sites of inflammation, which naïve T cells cannot [85]. T_{EM} lack expression of CCR7 and only have low expression of CD62L, similar to effector T cells. Thus, this subset of memory cells homes avidly to sites of inflammation but is excluded from the lymph nodes [85] (Figure 1.5). Isolation of these subsets and their transfer to antigen-free hosts has demonstrated that T_{CM} are derived from the T_{EM} population. However, after re-infection T_{CM} convert back to an effector phenotype [86]. These fully differentiated T_{CM} have the best proliferative capacity after secondary stimulation and are capable of self-renewal in response to homeostatic cytokines [86]. Unexpectedly, T_{CM} have also been shown to kill target cells as effectively as T_{EM} *in vivo* [81] (see Figure 1.5).



	Naive	Effector	T_{EM}	T_{CM}
	$CD62L^{hi}$ $CCR7^{+}$	$CD62L^{lo}$ $CCR7^{+/-}$	$CD62L^{lo}$ $CCR7^{-}$	$CD62L^{hi}$ $CCR7^{+}$
Homing capacity				
-2° lymphoid organs	++++	+/-	+/-	+++
Effector function				
-IFNγ, TNFα	-	++++	+++	+++
-CTL	-	++++	++	++
Proliferative capacity				
-antigen specific	++++	++	++	++++
-homeostatic	-	+/-	++	++++

Figure 1.5 Lineage differentiation of memory $CD8^{+}$ T cells. $CD8^{+}$ T cells quickly develop into effector cells after activation. After the contraction phase of the response, $CD8^{+}$ T cells proceed to T_{EM} and then T_{CM} cells. T_{EM} and T_{CM} have comparable cytolytic and cytokine producing ability, but the T_{CM} population has enhanced proliferative capacity. Adapted from: Seder, R. A. & Ahmed, R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol* 4, 835-42 (2003).

Many of the above conclusions about memory T cell differentiation have been made by studying the responses of murine P14 TCR-transgenic T cells to acute lymphocytic chorio-meningitus virus (LCMV) infection [81,83,86]. Some apparently contrary results have been obtained upon examination of human virus-specific memory T cell subsets. For example, the repertoires of human T_{CM} and T_{EM} subsets were found to be largely distinct, suggesting that they have undergone alternate lines of differentiation [87]. Additionally, antigen-experienced human T cells specific for different chronic viral infections can have distinct phenotypes, some of which appear to be a terminally differentiated effector state [88]. This may be attributable to chronic viral infections driving the production of effector cells; and in the continued presence of antigen, T cells are caught in transition between effector cells and T_{EM}. Alternatively, the proportion of each subset produced and their specific phenotypes may be dependent on the type and strength of the viral infection. Therefore, discrepancies noted in the differentiation of memory subsets in mice and humans may be a result of differences in the stimulation of T cells during acute versus chronic viral infection.

1.3 T cell co-stimulation

1.3.1 The importance of co-stimulation

The two-signal model of T cell activation was first proposed in 1970 by Bretscher and Cohn [89], who hypothesised that the interaction of antigen with a receptor on a precursor cell resulted in the generation of signal 1 (i.e. the TCR signal) that was inactivating when delivered alone. The antigen-mediated interaction of a precursor cell with another antigen-specific cell, such as an effector T helper cell, was postulated to result in the generation of another signal called signal 2 (i.e. the co-stimulatory signal) and to the subsequent activation of the precursor cell [90]. Initially, soluble factors such as cytokines, which can enhance the activation of T cells and polarise their functions, were hypothesised to transmit signal two. However, it has now become apparent that interactions between co-stimulatory receptor-ligand pairs on T cells and antigen presenting cells represent a critical event in the activation process. This was first demonstrated by Jenkins and Schwartz, who observed that chemically crosslinked APCs induced unresponsiveness rather than activation in T cells specific for the peptide-MHC complex being presented. This unresponsiveness was dependent on the TCR-peptide-MHC interaction and thus it was proposed that these chemically fixed APCs can provide the antigen signal, but that the lack of a second co-stimulatory signal which would normally be provided by the APC leads to T cell tolerance [91]. This accessory cell-derived co-stimulatory signal was further characterised as being capable of inducing a parallel but separate intracellular signal from the TCR, and both signals were required for IL-2 production and proliferation [92]. CD28 and B7 were then identified as one receptor-ligand pair responsible for the APC-derived co-stimulatory signal involved in antigen-specific IL-2 production by $CD4^+$ T cells [93].

Co-stimulatory molecules are generally defined as receptors which trigger signalling cascades that have no physiological effects in isolation, but can synergise with TCR signals to induce optimal T cell responses. As previously discussed in Section 1.2.4 and Figure 1.3, naïve T cells that are stimulated through the antigen receptor alone fail to produce cytokines, are unable to sustain proliferation and often undergo apoptosis or become non-responsive (anergic) to subsequent stimulation [48].

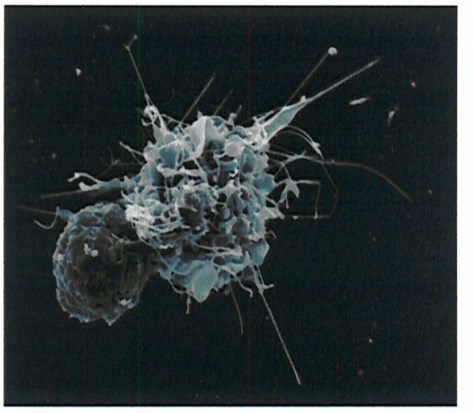
In situations where a full immune response is appropriate, one way the innate immune system signals the presence of pathogens or stressed cells to the adaptive T cell response is by up-regulating co-stimulatory molecules on antigen presenting cells. *In vivo*, administration of LPS up-regulates the expression of the CD28 ligands B7-1 (CD80) and B7-2 (CD86) on splenic dendritic cells within 6 hours [32]. It has further been shown that addition of LPS to cultured DCs up regulates the expression of the TNF family co-stimulatory ligands CD70 and 4-1BBL [94,95]. Treatment with DNA containing unmethylated CpG motifs also up-regulates the expression of B7-2 on murine and human DCs [33,34], as can double stranded RNA [35], the heat shock protein gp96 [38] and necrotic cells [41,42]. Thus, controlled up regulation of co-stimulatory molecules acts as a danger checkpoint for T cell responses.

1.3.2 Diversity of co-stimulatory molecules

1.3.2.1 Candidate co-stimulatory molecules

The best-characterised pathway of T cell co-stimulation is the CD28/B7 system. The CD28 glycoprotein is constitutively expressed on the surface of 80 % of human T cells and on virtually 100 % of murine T cells. Its ligands, B7-1 and B7-2, are members of the immunoglobulin superfamily and are expressed on APCs. The interaction of CD28 with its ligands promotes T cell division, IL-2 production and up regulates anti-apoptotic molecules [96]. However, the demonstration that productive antigen recognition by T cells can still occur in mice deficient for CD28 indicates that other co-stimulatory signals can replace CD28 as signal 2 [97,98]. A recently identified candidate is inducible co-stimulator (ICOS), which is related to CD28 structurally and functionally. Unlike the constitutively expressed CD28, ICOS has to be *de novo* induced on the T cell surface and instead of inducing IL-2 production, it up regulates the synthesis of effector cytokines such as IFN-γ, TNF-α, IL-4, IL-5 and IL-10 [99]. This suggests that its co-stimulatory role is more important for effector and memory cells. Related members of this family, such as CTLA-4 and PD-1 have an inhibitory function on T cell activation. CTLA-4 can act as a decoy receptor for B7-1 and B7-2 due to its higher affinity than CD28, subsequently preventing CD28 signalling. Furthermore, signals downstream of CTLA-4 can antagonise TCR signalling [100]. PD-1 binds two B7 related ligands and can inhibit T cell proliferation and cytokine production [101]. The

balance between these co-inhibitors and co-stimulators may set thresholds for T cell activation [50].



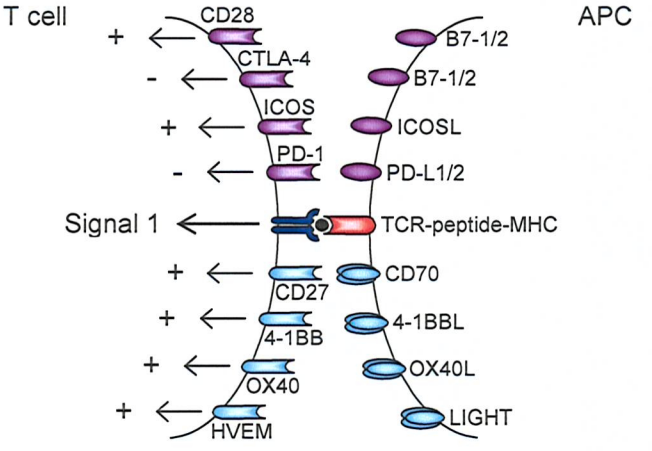


Figure 1.6 The expanding families of co-stimulatory molecules. Shown is an electron micrograph of a T cell interacting with a dendritic cell (from www.med.sc.edu:85/WBOWERS.HTM). The array of co-stimulatory / co-inhibitory interactions identified to date that may occur during this T cell-APC interaction, along with the TCR-mediated signal 1, are illustrated on the schematic diagram below. Members of the B7/CD28 family are shown above the TCR-peptide-MHC interaction, members of the TNF/TNFR superfamily are shown below. (⟵ +/- Signal 2)

Another family of cell surface glycoproteins that are emerging as critical co-stimulators are members of the TNFR superfamily. These include the T cell co-stimulatory molecules CD27, OX40 (CD134) and 4-1BB (CD137), and the DC and B cell co-stimulatory molecule CD40. This superfamily of molecules will be described in more detail in Section 1.4. Some of the key co-stimulatory molecules from the CD28 and TNFR classes are compared in Figure 1.6 and Table 1.1.

Receptor	Receptor expression on T cells	Ligand expression on APCs	T cell activation / inhibition	Primary functions
CD28 Family				
CD28	Constitutively expressed on naïve T cells	B7-2 constitutively expressed at low levels on B cells, DC and MØ. B7-1 and B7-2 up regulated after activation.	Activation	Activation of naïve T cells and induction of IL-2 production, proliferation and survival
ICOS	Up regulated after activation	ICOSL, activated B cells, DC and MØ	Activation	Promotes effector cell function such as production of IL-10 and B cell help
CTLA-4	Rapidly up regulated after activation	Shares ligands with CD28	Inhibition	Inhibits T cell proliferation and cytokine production. Controls $CD4^+$ T cell peripheral tolerance
PD-1	Rapidly up regulated after activation	PD-L1, activated B cells, DC and MØ. PD-L2, activated DC.	Inhibition	Inhibits T cell proliferation and cytokine production
TNFR Family				
4-1BB	Up regulated after activation	4-1BBL, activated B cells, DC and MØ	Activation	Enhances proliferation and survival of predominantly the $CD8^+$ T cell subset, particularly at late time points
OX40	Up regulated after activation	OX40L, activated B cells, DC and MØ	Activation	Enhances proliferation and survival of predominantly the $CD4^+$ T cell subset, particularly at late time points
CD27	Constitutively expressed on naïve T cells, up regulated after activation	CD70, activated B cells, DC and MØ	Activation	Enhances T cell proliferation and survival
HVEM	Constitutively expressed on naïve T cells, down regulated after activation	LIGHT, resting DC	Activation	Early role in T cell activation and expansion.

Table 1.1 Comparison of T cell co-stimulatory molecules of the CD28 and TNFR families. The table shows representative members of the CD28 and TNFR families, their expression pattern on T cells, the expression of their ligands on APCs, whether they promote activation or inhibition and their main functions identified to date [73,102]. MØ, macrophage.

Other proposed co-stimulatory molecules are adhesion molecules such as the integrin family member LFA-1, and CD2 that associates with the TCR-CD3 complex. These adhesion molecules may be necessary to facilitate TCR triggering, but cannot always prevent the induction of T cell unresponsiveness and therefore may not provide a complete signal 2 [103]. For example, a comparative study using CD28- or LFA-1-deficient T cells demonstrated that while LFA-1 facilitated the functional triggering of

TCRs by promoting T cell-APC adhesion, it could not prevent the induction of T cell unresponsiveness. In contrast, CD28 reduced the minimum number of TCRs that needed to be triggered for T cell activation and prevented the induction of T cell anergy [104].

1.3.2.2 Spatial and temporal segregation of co-stimulatory molecules

Analysis of mice deficient for various co-stimulatory molecules or their ligands has indicated that distinct accessory molecules regulate the $CD4^+$ and $CD8^+$ T cell subsets separately (see Table 1.2). $CD28^{-/-}$, $OX40^{-/-}$ and $CD40L^{-/-}$ mice have a profound defect in $CD4^+$ T cell anti-viral responses, however they still exhibit $CD8^+$ T cell activity suggesting that these molecules are not always critical for $CD8^+$ T cell activation. Conversely, 4-1BBL-deficient mice have no deficit in their $CD4^+$ anti-viral responses, but do display reduced $CD8^+$ T cell responses. The recent generation of $CD27^{-/-}$ mice has demonstrated that this molecule is required for full $CD8^+$ and $CD4^+$ T cell responses; at least in the influenza model investigated so far. Thus, the response of $CD4^+$ and $CD8^+$ T cells to antigen can be controlled separately by co-stimulation.

Cell Type	Wild-type	$CD28^{-/-}$	$CD40L^{-/-}$	$4\text{-}1BBL^{-/-}$	$OX40^{-/-}$	$CD27^{-/-}$
$CD4^+$ T cell	+++	-	-	+++	-	+/-
$CD8^+$ T cell	+++	+/-	+++	+/-	+++	+/-

Table 1.2 T cell responses in the absence of co-stimulatory molecules. The table shows data for antigen-specific T cell responses in co-stimulation deficient mice during the expansion phase of infection with LCMV, VSV or influenza virus, taken from references [97,98,105-109]. +++, normal T cell response; +/-, moderately reduced T cell response; -, severely reduced T cell response. Adapted from Kaech, S. M., Wherry, E. J. & Ahmed, R. Effector and Memory T-cell Differentiation: Implications for Vaccine Development. *Nature Rev. Immunol.* **2**, 251-262 (2002).

The influence of differing co-stimulators may also be temporally segregated, depending on the timing of the receptor-ligand interaction. For example, comparison of the roles of CD28 and ICOS in T_H cell-mediated lung mucosal inflammation has shown that while CD28 signalling is essential for priming it becomes dispensable later on during the effector response when ICOS becomes critical [110]. Furthermore, reactivation of memory $CD4^+$ T cells can occur independently of the CD28 ligands B7-1 and B7-2 [111]. This suggests that CD28 co-stimulation is key for the initiation of naïve

T cell responses, but a subsequent switch in the co-stimulatory requirement occurs over time and alternate molecules become vital for maintaining the response. Comparison of $CD8^+$ T cell responses to influenza infection in $CD28^{-/-}$ and 4-1BB ligand $(4\text{-}1BBL)^{-/-}$ mice indicated that whilst CD28 knockouts have severely impaired early primary T cell expansion there is little reduction in T cell numbers in $4\text{-}1BBL^{-/-}$ mice. However, $4\text{-}1BBL^{-/-}$ mice show a decrease in specific T cells late in the primary response and also have reduced secondary responses [112,113]. Similarly, OX40 has been proposed to act sequentially after CD28 to regulate late $CD4^+$ T cell turnover at the peak of the expansion phase and to regulate the subsequent survival of T cells when antigen becomes limiting [114]. Therefore, receptors with apparently overlapping functions may have non-redundant roles during different stages of the immune response. Co-stimulatory molecules can send and receive reciprocal signals between different cell types. e.g. CD28 signalling can up regulate CD40 ligand (CD154) expression on the T cell, which in turn signals back to the APC through CD40 to further up regulate the CD28 ligands B7-1 and B7-2 [50]. Thus a positive feedback loop occurs which prolongs the response.

1.3.3 Co-stimulation can prevent the induction of T cell anergy

T cell anergy is a tolerance mechanism by which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period in a hyporesponsive state. Anergy is a cell-autonomous state, which distinguishes it from other immunoregulatory mechanisms such as suppression by T_{reg}. T cell anergy has been observed in a variety of *in vitro* and *in vivo* systems. *In vitro* it can be induced following incomplete T cell activation, whereas in more complex *in vivo* systems T cell anergy is observed in the presence of low levels of co-stimulation or high levels of co-inhibition and may require the presence of persistent antigen for the anergic state to be maintained [49].

In vitro $CD4^+$ T cells can become anergic following a strong TCR signal in the absence of co-stimulation or by stimulation with a low affinity peptide-MHC ligand in the presence of co-stimulation. Both these sets of conditions result in weak or incomplete activation of the T cell but give a sufficient signal to induce factors that control the anergic state. The CD28/B7 pathway of co-stimulation appears to be critical for the

prevention of anergy induction in $CD4^+$ T cells. CD28 signalling may act to prevent anergy by directly inhibiting the production of anergy factors or indirectly via mediation of cell-cycle progression and IL-2 production [49]. The presence of IL-2 can reverse the anergic state in some cases, leading to a regain of T cell responsiveness [115]. Furthermore, OX40 co-stimulation of anergised $CD4^+$ T cells has been shown to reverse the anergic state [116]. Clonal anergy of $CD8^+$ T cells has also been demonstrated, known as activation induced non-responsiveness (AINR). This was first demonstrated by Otten and Germain [117] in $CD8^+$ clones stimulated with APCs lacking co-stimulatory molecules. The resultant anergised $CD8^+$ T cells had a phenotype of inhibition of IL-2 production and proliferation with little effect on IFN-γ production or CTL activity, a phenomenon known as split anergy. More recently, work by Mescher and colleagues has shown that AINR of $CD8^+$ T cells can be induced even in the presence of CD28 co-stimulation [118]. Thus, other molecules may be required to prevent unresponsiveness developing in $CD8^+$ T cells, which is in agreement with the less dramatic effect on $CD8^+$ T cell responses in $CD28^{-/-}$ mice (Table 1.2).

In vivo induction of T cell anergy was first clearly shown for transgenic $CD8^+$ T cells specific for the male H-Y antigen. Following transfer to male athymic syngeneic mice the transgenic T cells expanded and then contracted, leaving a population of anergic cells which was resistant to restimulation *in vitro* [119]. Similarly, $CD4^+$ transgenic T cells transferred into normal mice could be rendered anergic by intravenous administration of soluble antigenic peptide [120]. In some models studied, persistence of the antigen appears to be required for maintenance of the anergic state. This suggests that T cell anergy may have evolved to prevent autoimmunity in self-specific T cells which have escaped deletion in the thymus [49]. However, signalling through co-stimulatory molecules can prevent the induction of anergy *in vivo* as well as *in vitro*. For example, triggering 4-1BB using agonistic monoclonal antibodies can convert a tolerogenic tumour peptide vaccine into a formulation capable of efficient CTL priming and generation of secondary responses [121]. Moreover, signalling through OX40 has also been demonstrated to break established peripheral T cell tolerance induced by a peptide antigen administered in the absence of adjuvant. [116]. Thus, this two-signal system may be critical for the maintenance of self-tolerance and the prevention of autoimmunity [122].

1.3.4 Mechanisms of co-stimulation

Under physiological conditions, very few MHC molecules on the APC are loaded with any one antigenic peptide for which a TCR may be specific. Consequently, because of its low affinity for peptide-MHC complexes, the TCR may not undergo sufficient interaction with peptide-MHC to reach the signalling threshold for initiating an immune response. Thus, the TCR signal requires additional support by adhesion receptors and co-stimulatory signals. Co-stimulatory signals may promote T cell activation through multiple mechanisms, including enhancement of TCR engagement, modulation of TCR signalling and directly switching on the transcription of genes that would otherwise be only weakly initiated by the TCR signal (Figure 1.7).

1.3.4.1 Enhancement of TCR engagement

Studies on CD28 have led to a model by which co-stimulators enhance TCR engagement with peptide-MHC complexes and prolong signalling, by inducing cytoskeletal rearrangements that promote the formation of an immunological synapse [122]. Initially, Viola and Lanzavecchia showed that CD28 co-stimulation reduced the threshold number of TCRs required to be triggered for activation from 8000 to ~1500, and hypothesised that this was due to synergy between signalling pathways [123]. Subsequent work by the same group indicated that CD28 co-stimulation promoted reorganisation and clustering of membrane domains at the site of TCR engagement, which allowed more persistent global tyrosine phosphorylation to occur [124]. A complementary study by Wülfing and Davis demonstrated that co-stimulation leads to accumulation of molecules at the T cell-APC interface by triggering myosin-dependent cytoskeletal movement [125]. More recently, it has been shown that co-stimulation via CD28 actually drives formation of a mature IS [126,127]. This suggests that increased density/stability of TCR-CD3 at the interface is a major component of the co-stimulatory effect of CD28.

1.3.4.2 Modulation of TCR signalling

CD28 may affect the earliest biochemical event during T cell activation; the phosphorylation of the ITAM sequences in the TCR by the Src family kinase Lck. CD28 co-operates with CD4 to induce sustained autophosphorylation of Lck and thus by enhancing Lck activity can enhance TCR sensitivity to weak antigens. This effect

may be mediated by the interaction of proline residues in the cytoplasmic domain of CD28 with the SH2 domain of Lck, which can facilitate kinase activity [128]. CD28 can also amplify TCR signal transduction by boosting tyrosine phosphorylation cascades further downstream. Signalling through CD28 in conjunction with the TCR has been shown to enhance the phosphorylation of SLP-76 and PLCγ1, which in turn amplifies Ca^{2+} flux and activation of the transcription factor NF-AT. This response is controlled by CD28-mediated activation of the protein tyrosine kinase Itk [129]. Co-stimulatory molecules such as CD28 may therefore facilitate TCR signal transduction by providing activated signalling components, and this in turn may lower the number of TCR that are required to be triggered in order to reach the T cell activation threshold [123,129].

1.3.4.3 Signalling pathways that integrate with those from the TCR

It is currently unclear if co-stimulatory members of the TNFR family have any role in promotion of IS formation, or if they can directly link into TCR tyrosine phosphorylation cascades. However, both the TNFR and CD28 families of co-stimulatory receptors can initiate signalling pathways that are distinct from the TCR but which lead to the activation of common targets, such as the up-regulation of anti-apoptotic members of the Bcl_2 family and modulation of cell cycle control proteins [114,130,131]. Co-stimulatory TNFR molecules can activate NF-κB transcription factors that are important for pro-inflammatory and anti-apoptotic responses, and c-jun N-terminal kinase (JNK), which regulates the activator protein 1 (AP1) transcription complex (formed from the Fos/Jun family proteins) implicated in promoting cytokine expression and proliferation. These transcription factors are also targets of co-signals transduced through CD28 [73]. Another central target is activation of the serine/threonine kinase protein kinase B (PKB, also known as Akt). It has been shown recently that maintenance of high PKB activity is an essential downstream signal of OX40, a TNFR that controls the survival of $CD4^+$ T cells [132]. This prolonged survival was achieved by the up-regulation of anti-apoptotic Bcl_2 family members [114,132]. Furthermore, PKB is critically implicated in CD28-mediated control of cell cycle progression [131].

Co-stimulatory molecule signals converge in the nucleus with TCR signals at the transcription factor level, and allow the translation of genes required for full T cell activation. For instance, activation of unequal proportions of NF-AT to AP1 by TCR

signalling in the absence of co-stimulation has been shown to lead to the transcription of genes encoding anergy factors, rather than those involved in cytokine production and proliferation [133]. Because of the exponential nature of T cell division, small increases in activation threshold, cellular survival or cell cycling provoked by co-stimulation may have a dramatic effect on the total numbers of effector cells produced during an immune response [134].

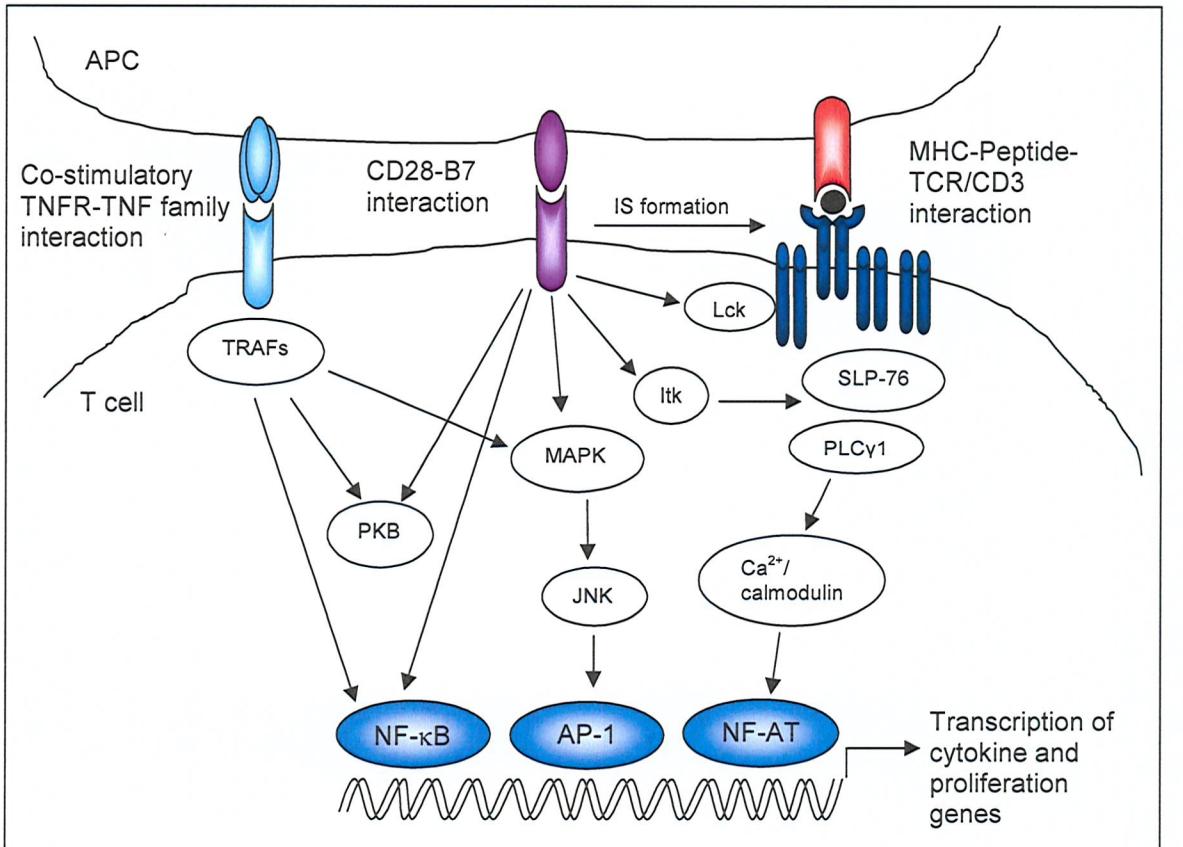


Figure 1.7 Potential mechanisms of T cell co-stimulation. Shown is a simplified diagram indicating some of the signalling pathways affected by co-stimulation. TNFR and CD28 family co-stimulatory signals appear to overlap in the activation of MAP kinase cascades, PKB, and activation of the transcription factor NF-κB. CD28 can also promote the formation of a stable IS and provide activated PTKs such as Lck and Itk that contribute to TCR signalling cascades. It is unclear if co-stimulation through TNFR family has any effect on IS formation or directly influences TCR signalling pathways. Concomitant activation of the transcription factors NF-κB, AP-1 and NF-AT may be critical for the transcription of genes that promote T cell activation.

1.3.5 Application of co-stimulation to tumour immunotherapy

1.3.5.1 The immune response to tumours

Recent reports of a role for IFN-γ and lymphocytes in the prevention of primary tumour development have led to the resurgence of the idea of cancer immunosurveillance. Mice deficient for IFN-γ signalling and/or T and B lymphocytes, were shown to have increased incidences of both spontaneous and carcinogen-induced tumours [135,136]. Furthermore, it was demonstrated that the immune system could influence the immunogenicity of a tumour by selection of less immunogenic variants. Tumours transplanted from recombination activating gene 2-deficient ($RAG2^{-/-}$, lymphocyte deficient) mice were rejected by wild type mice. However, tumours transplanted from mice with intact immune systems into other wild-type mice continued to grow, indicating that in the presence of a fully functional immune system tumours which are less immunogenic may be selected [135]. This evidence, along with reports that tumour-infiltrating lymphocytes (TILs) isolated from patients or experimental animals contain tumour-antigen specific CTLs, indicates that the immune system is capable of responding to spontaneous self-tumours [137].

A large number of tumour-associated antigens (TAAs) have now been identified by serological screening of phage-display libraries from tumours, and also by using tumour-reactive T cells from patients to screen tumour libraries. The antigens described to date can be divided into four categories: unique tumour-specific antigens that are the products of mutation, viral antigens in virus-associated cancers (e.g. human papilloma virus 16 E6 and E7 antigens), tissue-specific differentiation antigens (e.g. melanoma antigens which are melanocyte-specific such as gp 100 and MART1) and tumour-selective antigens (normal genes which are up regulated in tumours due to epigenetic effects) [138]. However, despite the presence of potential rejection antigens natural anti-tumour lymphocyte responses are often inefficient and can lead to the selection of non-immunogenic derivatives.

1.3.5.2 Initiation of anti-tumour immunity through provision of co-stimulation

The induction of tolerance upon T cell encounter with signal 1 alone may have implications for the generation of immunity against malignant cells. The initiation of

an effective anti-tumour adaptive immune response may require the capture and presentation or cross-presentation of TAAs by activated APCs [139]. This is because tumour cells generally cannot act as efficient APCs themselves, as they express no co-stimulatory molecules. Furthermore, at early stages in their development they do not usually die necrotically, or cause damage to surrounding tissues, and thus don't emit danger signals that could activate professional APCs. Presentation of tumour-derived antigens by non-activated APCs can lead to the induction of tolerance. One way to overcome the lack of host responses is by provision of effective T cell co-stimulation [39,140]. Table 1.3 outlines some of the results of manipulating various co-stimulatory pathways in murine cancer models. In many cases, providing co-stimulation during tumour challenge in the form of soluble antibodies or ligands, or by transfection of tumour cells with co-stimulatory ligands, can lead to complete eradication of the tumour and the formation of specific, protective anti-tumour immunity.

One approach involves transducing tumour cells with co-stimulatory ligands such as B7 for use as vaccines. Its potential was first investigated by the laboratories of Allison and Chen [141,142]. They showed that transfection of murine melanoma cells with B7 led to rejection of the tumour *in vivo*. Furthermore, exposure to the B7-expressing tumour induced T cell immunity which resulted in regression of co-transferred B7-negative tumours [142], or could protect against subsequent challenge with B7-negative parental tumours [141]. The success of this approach depends on the concept that tumours can directly stimulate the immune system if they express sufficient co-stimulatory molecules. In opposition to this idea, another study has shown that this direct pathway for anti-tumour T cell priming may be minor compared to indirect presentation of tumour antigens by dendritic cells [143]. In this case, expression of B7-1 on tumour cells was postulated to have promoted rejection by activating natural killer (NK) cells. Furthermore, in another study it was shown that the expression of B7 on tumour cells could prolong anti-tumour T cell responses but was not sufficient to directly prime them [144]. Disparities between these studies of B7-expressing tumours could be a result of different levels of B7 expression, differential immunogenicity of the tumour antigens expressed and routes of administration. Further work by Allison's group has focused on inducing anti-tumour immunity through blockade of the counter-stimulatory receptor for B7-1 and B7-2, CTLA-4. Treatment of mice with an anti-

CTLA-4 blocking antibody can promote the rejection of B7-1$^+$ and B7-1$^-$ colon carcinoma cells [145]. Therefore, removing the inhibitory effects of CTLA-4 can allow effective immunity to be generated against tumour cells.

Stimulation of anti-tumour T cell immunity has also been achieved by manipulation of TNFR superfamily co-stimulatory molecules. For example, administration of an agonistic antibody against CD40 can eradicate a range of tumours by evoking a rapid expansion of cytotoxic T cells [146-148]. Agonistic antibodies against the T cell co-stimulator OX40, or soluble recombinant OX40 ligand, can promote anti-tumour responses against several tumour types, depending on their initial immunogenicity [149]. Stimulation of the recently identified TNFR superfamily member herpes-virus entry mediator (HVEM) by intra-tumoural gene-transfer of its ligand LIGHT in a DNA vaccine induces therapeutic immunity against established murine tumours [150]. Furthermore, anti-tumour immunotherapy has been reported after triggering the receptors CD27 and 4-1BB [151-154]. These molecules are the focus of this thesis and their associated therapeutic data will be discussed in detail in Sections 1.4.4 and 1.4.5.

Co-stimulatory receptor	Tumour type and method	Result	Reference
CD28	Murine melanoma, transfection of tumour with ligand for CD28, B7.	Rejection of transfected melanoma mediated by $CD8^+$ T cells and protection against re-challenge by parental strain.	141
	Murine melanoma expressing a human papillomavirus antigen, transfection of tumour with ligand for CD28, B7.	Rejection of transfected melanoma mediated by $CD8^+$ T cells and treatment of established metastatic parental tumours.	142
CTLA-4	Murine colon carcinoma, blockade of CTLA-4 using mAbs.	Rejection of B7 negative tumours, resulting in immunologic memory.	145
4-1BB (CD137)	Murine colon carcinoma and lymphoma, treatment with anti-4-1BB mAb.	Complete rejection of tumours mediated by both $CD4^+$ and $CD8^+$ T cells and protection from re-challenge.	151
	Murine sarcoma and mastocytoma, treatment with anti-4-1BB mAb.	Eradication of established tumours mediated by $CD4^+$ and $CD8^+$ T cells, induction of tumour-specific immunity.	153
	Murine transformed epithelial line, lung cancer and melanoma, combined immunisation with anti-4-1BB mAb and tumour-specific peptide antigen.	Regression of established poorly immunogenic tumours by breaking immunological ignorance.	154
CD27	Murine fibrosarcoma and mammary adenocarcinoma, vaccination using irradiated tumours transfected with CD70 (CD27 ligand).	Vaccination promoted long-lasting anti-tumour immunity, synergistic therapeutic effect when combined with B7.1 (CD80) transfected tumours.	152
OX40 (CD134)	Murine melanoma, sarcoma, breast cancer and colon carcinoma, treatment with OX40 ligand or anti-OX40 mAb.	Enhanced anti-tumour immunity, therapeutic effect correlated with immunogenicity of tumour.	149
CD40	Murine lymphoma, treatment with anti-CD40 mAb.	T helper independent $CD8^+$ CTL response, which eradicates lymphoma and provides protection from re-challenge.	146
HVEM	Murine mastocytoma, intra-tumoural LIGHT-encoding (ligand for HVEM) DNA vaccination.	Tumour rejection accompanied by tumour-specific CTL activity, dependent on $CD8^+$ and $CD4^+$ T cells. Protection from re-challenge.	150

Table 1.3 Anti-tumour immunity can be induced by co-stimulatory molecules. Examples of how *in vivo* manipulation of members of the CD28 or TNFR families can promote anti-tumour immunity.

1.4 The tumour necrosis factor receptor superfamily

1.4.1 Overview

Lymphocyte homeostasis requires a precise balance between the rates of cellular proliferation and apoptosis. The TNFR superfamily of cell surface protein receptors, in conjunction with their TNF superfamily ligands, undertake a diversity of pivotal roles in the adaptive immune system which maintain this equilibrium, whilst promoting effective immune responses to pathogens. The first members of the TNF family to be described were TNF-α and lymphotoxin (LT-α, also known as TNF-β), which were originally identified as products of lymphocytes and macrophages that caused tumour cell lysis. Since then, knowledge of this evolutionary conserved family has been expanded to encompass, at present, 19 TNF-homologous ligands that mediate their cellular response through 29 receptors of the TNFR superfamily [155].

1.4.2 Structure and modes of signalling

The TNF/TNF-receptor superfamily ligand-receptor pairs consist of type I transmembrane (N-terminal extracellular) glycoprotein receptors and type II transmembrane (C-terminal extracellular) glycoprotein ligands, some of which (e.g. TNF-α) may also have a soluble cytokine form [156]. Individual polypeptide chains of the TNF-like ligands exhibit a "jellyroll" β-sandwich structure, and form active trimeric complexes via non-covalent interactions between chains [157]. The extracellular region of the receptor molecules forms an extended structure, which contains between two and six hallmark cysteine-rich domains that form the ligand-binding site [158]. The crystal structures of TNFR-I and DR5 complexed with their ligands show that homotrimeric ligands interact with three receptors on the cell surface [156,157]. It was originally thought that trimeric ligands were responsible for recruiting monomeric receptors together to form the active signalling complex, but recent evidence has suggested that TNFRs contain pre-ligand binding assembly domains (PLADs), which are sufficient and necessary to mediate formation of ligand-independent complexes [159-161]. This suggests that ligand engagement induces a conformational change in the pre-formed trimeric receptor complex, thereby allowing it to transduce signals by efficient recruitment of downstream signalling molecules [162]. Alternatively, ligand engagement may initiate the formation of higher order receptor "superclusters" which provide

effective signalling platforms [163] (see Figure 1.8 for a schematic diagram of representative TNFR family members).

The cytoplasmic domains of the TNFRs share little homology, but can be used to split the TNFRs into two groups based on the presence or absence of a 70-80 amino acid motif designated the death domain (DD). Upon ligand binding, the DD serves as a docking site for DD-containing adaptor proteins such as TRADD and FADD, via homotypic DD interactions. These adaptors can link death receptors to pro-apoptotic intracellular signalling pathways such as caspase activation [156,158].

TNFRs that lack a death-domain mediate their signal through tumour necrosis factor receptor-associated factors (TRAFs), which function as scaffold and docking proteins. This family of molecules are capable of both negatively regulating apoptosis, and inducing the expression of cell survival and proliferation genes. These adaptor proteins interact with TNFR cytoplasmic domains during receptor oligomerisation, possibly through specific binding sites such as the Pro-Xaa-Gln-Xaa-(Thr/Ser) motif present in CD40, CD30, CD27 and EBV latent membrane protein 1 (LMP1) [164]. As discussed in Section 1.3.4.3 and illustrated in Figure 1.7 these co-stimulatory TRAF-TNFR complexes initiate signal transduction pathways in the cell that cumulate in the activation of transcription factors such as AP-1 and NF-κB, which in turn may switch on genes required for cell survival, proliferation and differentiation. TRAF proteins trigger MAP kinase cascades at a very proximal step, which leads to activation of the JNK/c-Jun pathway. In addition, most members of the TNFR superfamily can activate NF-κB through the ubiquitin-mediated degradation if its inhibitor IκBα. This process is initiated by phosphorylation of IκB by IκB kinase (IKK) complex, which in turn is activated by MAPKs such as NF-κB inducing kinase (NIK). NIK is part of the signalling complex assembled upon multimerisation of TRAFs [164]. Furthermore, TRAFs can also directly bind inhibitors of apoptosis, such as A20 and c-IAP, thereby preventing caspase activation [164,165].

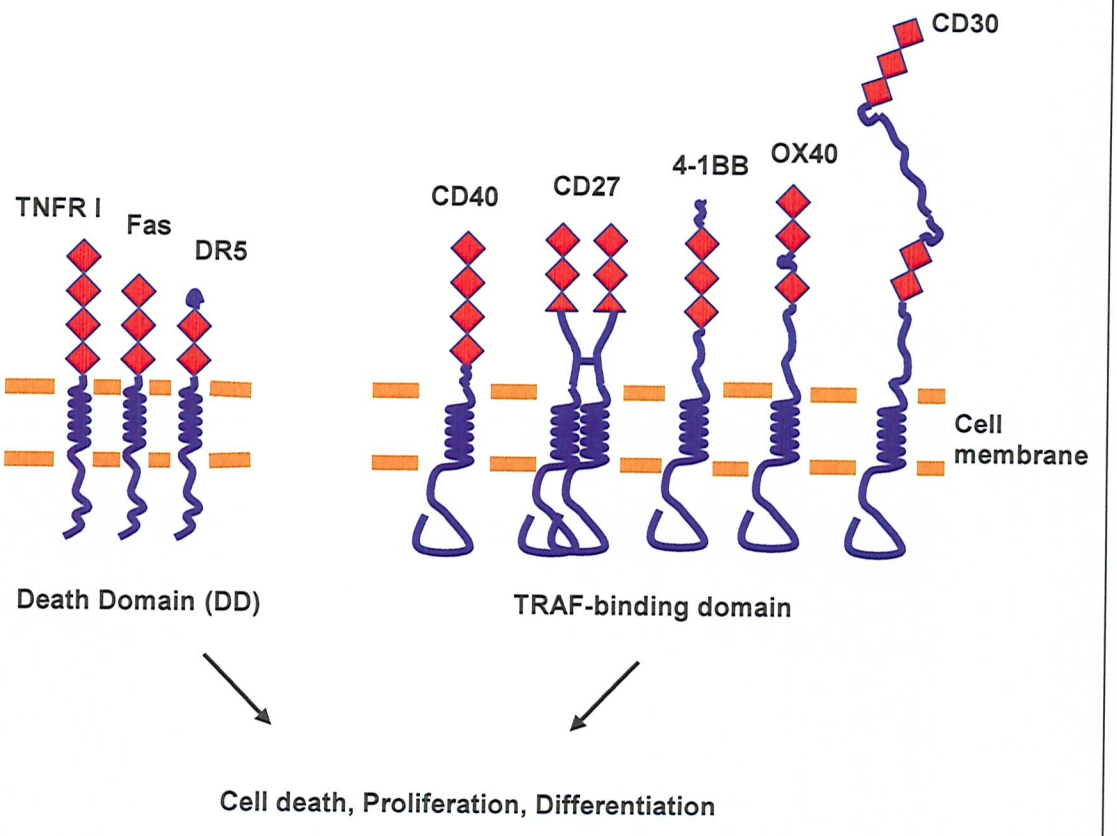


Figure 1.8 Structures of representative TNFR superfamily members. Red diamonds represent cysteine-rich TNFR motifs in the extracellular domains.

Despite being apparently divided into two subsets based on the presence of a death domain, the functions of DD and TRAF-linked TNFR superfamily members are not always as clearly delineated. Many TNFRs can induce context-dependent pleiotrophic effects, which may be dependent on the bioenergetic or differentiation state of the cell [166]. For example, TNFR-II can induce either activation or death of T lymphocytes, depending on whether the signal is transduced during initial T cell activation, or later on after differentiation. This switch is controlled by the Ser/Thr kinase RIP. Early in T cell activation the expression of RIP is low and binding of TRAF2 to TNFR-II induces NF-κB activation and consequent cellular activation. Following exposure to IL-2 during differentiation, RIP expression increases and via TRAF2 bridges TNFR-II to FADD/MORT1 and the apoptosis machinery [167].

1.4.3 Involvement of TNFRs in T cell responses

Members of the TNFR superfamily have been found to regulate T cell responses from naïve T cell priming through expansion, contraction of the effector pool and the survival of memory cells (see Figure 1.9). For example, CD40 is expressed on dendritic cells indicating it has a role in the initiation of T cell responses. The interaction of CD40 with its ligand, expressed on activated T_H cells, has been found to provide vital signals for CTL priming by "licensing" the dendritic cell. CD40 up-regulates CTL helper factors such as IL-12, and induces the expression of co-stimulatory ligands such as B7-1/B7-2 and TNF family members, allowing efficient CTL activation [43-45]. A newly identified member of the TNFR superfamily is receptor activator of NF-κB (RANK). This receptor shows high homology with CD40 and its expression on dendritic cells indicates that it may also modulate the function of dendritic cells during T cell priming [168].

The ligands for 4-1BB, CD27 and OX40 are expressed on the surface of activated dendritic cells, B cells and T cells and the ligand for HVEM, LIGHT, is expressed on immature dendritic cells but conversely is down regulated with activation [73]. Interaction of these ligands with their TRAF-linked receptors on the surface of T cells provides co-stimulatory signals, which enhance T cell activation, proliferation and cytokine production during antigen priming. These receptors may be expressed on different T cell subsets and act as co-stimulators during different stages of T cell activation and differentiation [165]. Another TRAF-linked receptor with homology to 4-1BB and CD27 is Glucocorticoid-induced TNFR family receptor (GITR). GITR has at present unknown functions but appears to be localised on the T_{reg} subset of T cells, which are involved in maintaining peripheral tolerance [168,169].

Receptors such as Fas and TNFR-II are crucial in lymphocyte homeostasis during late phases of the immune response. They regulate activated lymphocyte numbers via the negative feedback system of activation-induced cell death, termed propriocidal death [170-172]. The importance of these TNFR family members is demonstrated by *lpr* and *gld* mice, and human Autoimmune Lymphoproliferative Syndrome patients, which contain mutations in either their Fas or Fas ligand genes and manifest lymphoproliferative disorders and autoimmunity [161].

TNFR superfamily co-stimulatory interactions may function in secondary responses in an analogous fashion to that in primary responses. However, there is a paucity of data that directly examines their roles during memory responses. CD27-deficient and 4-1BB deficient mice have been shown to have reduced memory recall responses [109,173]; but it is unclear if this is due to reduction of numbers of T cells surviving the primary response, or attributable to these molecules being important for directly stimulating memory cells. However, OX40 has been shown to be critical for directly co-stimulating antigen-specific memory T_H2 cells that mediate allergic lung inflammation [174].

This thesis focuses on two members of the TRAF-linked TNFR superfamily, CD27 and 4-1BB, which are thought to provide important T cell co-stimulation or modulation during the immune response to antigen, and may be key targets for anti-tumour immunotherapy.

DC activation	Naïve T cell priming, expansion and survival	Contraction phase, apoptosis of activated T cells	Memory T cell formation and re-activation
CD40, RANK, 4-1BB	CD27, 4-1BB, OX40, HVEM	Fas, TNFR-II	OX40, CD27?, 4-1BB?

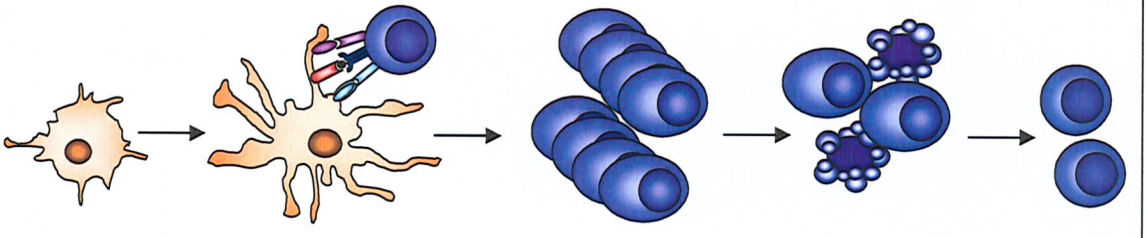


Figure 1.9 Involvement of TNFRs in T cell responses. Examples of regulation of the T cell response by the TNFR superfamily. Receptors are grouped according to the stage in the response at which they exert their effects.

1.4.4 CD27 (TNFRSF7)

1.4.4.1 Structure and expression pattern of CD27 and its ligand, CD70 (TNFSF7)

CD27 is a type I transmembrane glycoprotein which, in contrast with other members of the TNFR superfamily, forms a disulphide-linked homodimer with subunits of ~ 50-55 kDa. The extracellular ligand binding section of CD27 contains two full cysteine-rich domains and one partial (see Figure 1.8 for schematic diagram). A soluble 33 kDa form of the extracellular domain of CD27 is released from activated T cells, probably via proteolytic cleavage at a site close to the transmembrane region [175]. CD27 is evolutionary conserved, with murine CD27 showing 65 % identity overall with human CD27 and the putative ligand binding domain and carboxy-terminal of the cytoplasmic tail both being approximately 80 % identical between mouse and man [176]. CD27 is exclusively expressed on cells of the lymphoid lineage. It is constitutively expressed at low levels on naïve murine and human T lymphocytes, and is rapidly up regulated after TCR stimulation. In mice, CD27 surface expression is maintained on memory $CD8^+$ T cell subsets, with the resting T_{CM} subset having high levels of CD27 and the T_{EM} subset having low to intermediate levels of CD27 [86]. In humans, CD27 expression is maintained on some subsets of memory/antigen experienced T cells, whilst it appears to be lost on effector T cells at terminal stages of differentiation, particularly in the context of persistent viral infection [88,177,178]. In contrast, CD27 expression on B cells is highly restricted, and appears to be only found on memory B cells that have undergone somatic hypermutation [179]. It has also been observed that NK cells, which are part of the innate immune system, constitutively express CD27 on their surface [180]. Microarray analysis of murine haematopoietic stem cells has uncovered functionally distinct $CD27^+$ and $CD27^-$ subpopulations in these primitive bone marrow cells. CD27 is a marker for a population enriched for cells with short-term haematopoietic properties, whereas the $CD27^-$ population is more effective in clonal long-term transplantation [181].

The CD27 ligand, CD70, is a 29 kDa type II transmembrane glycoprotein of the TNF family. Murine and human CD70 share 62 % homology at the protein level. CD70 has a restricted expression pattern within the lymphoid system. It is present on 5 - 15 % of T cells within human peripheral blood and correlates with cellular activation markers

[182,183]. It is also found on activated B lymphocytes post-ligation of the B cell antigen receptor and/or CD40 [94]. CD70 is expressed on thymic DCs in humans, and on lymph node DCs isolated from mice infected with *Leishmania major* [109,184]. CD70 can also be highly up regulated on the DC line D1 and *in vitro* generated murine DCs upon maturation with GM-CSF, LPS or anti-CD40 mAb [94,95]. The expression of CD70 on T cells may be regulated by cytokines; pro-inflammatory cytokines like TNF and IL-12 potently enhance its expression, whereas anti-inflammatory cytokines such as IL-4 and IL-10 down-regulate CD70 expression [185].

1.4.4.2 Signalling cascades induced by CD27

Several studies looking at the intracellular signalling components downstream of CD27 have identified TRAF2 and TRAF5 as binding to its cytoplasmic tail. These two TRAFs were shown to be responsible for activating NF-κB and JNK via NIK, during CD27 signalling. C-terminal deletion mutants have determined the PIQEDYR motif, located in the cytoplasmic tail, as being essential for TRAF2 and TRAF5 binding and consequential NF-κB activation. This motif is similar to the PXQXT sequence known to be necessary for the interaction of TRAFs with other TNFR superfamily members [186,187]. CD27 has also been shown to bind TRAF3 in co-immunoprecipitation experiments. TRAF3 may work as a negative regulator of CD27, as it can inhibit NF-κB activation in response to CD27 signalling [188]. In contrast with the pro-survival functions of TRAFs, CD27 has been reported to bind a novel protein called Siva that contains death domains homologous to those found in RIP and FADD and can induce apoptosis. However, this interaction has not been demonstrated under physiological conditions [189].

1.4.4.3 Function of CD27/CD70 interactions in T cell responses

On T cells, the ligation of CD27 induces potent co-stimulatory responses. For example, stimulatory antibodies against murine or human CD27 have been shown to enhance T cell proliferation to mitogenic concanavalin A [190] or CD3 stimulus [191], respectively. Studies of murine CTL generation in mixed lymphocyte reactions (MLR) indicated that CD27-CD70 interactions primarily enhance the proliferation of $CD8^+$ cells responding to class I MHC alloantigens, and have little effect on $CD4^+$ T cells during MLRs [192]. The appearance of CD70 on activated T cells has led to the

hypothesis that CD27 co-stimulation may be important in T cell-T cell contacts, during the clonal expansion phase of the immune response and it has been shown that CTL can be directly co-stimulated by helper T cells expressing CD70 [193]. CD27-CD70 interactions have also been demonstrated to comprise part of the CD28-independent co-stimulation of polyclonal T cells by activated B cells, along with OX40-OX40L [194].

The generation of CD27 knockout mice by Hendriks *et al* illustrated that while CD27 is not essential for production and maintenance of T and B cell populations, it is required for generation of an effective T cell memory compartment. This paper demonstrated that the numbers of $CD4^+$ and virus-specific $CD8^+$ T cells were reduced during the primary response in $CD27^{-/-}$ mice, and that the T cell memory response after secondary infection was impaired. However, it is not clear if the effects on T cell memory observed in this paper were a result of the lack of CD27 during the primary or the secondary responses. Surprisingly, the B cell response in $CD27^{-/-}$ mice appears to be normal, in that their serum titres of virus-specific antibodies after primary infection are equivalent to those found in wild-type mice [109]. Converse experiments by the same group, where constitutive CD27-CD70 interactions were induced by over-expressing CD70 on B cells, led to similar conclusions. Mice over-expressing CD70 had higher peripheral T cell numbers, and these T cells showed increased differentiation towards a memory/effector phenotype of $CD44^{hi}$ $CD62L^{neg}$ as a result of CD70 amplifying T cell responses to environmental antigens. Moreover, a greater proportion of T cells from CD70 transgenic mice were positive for IFN-γ, again indicating a role for CD27/CD70 interactions in effector cell differentiation [185]. However, the chronic un-physiological stimulation of CD27 in these mice eventually led to T cell exhaustion and susceptibility to infection [195].

1.4.4.4 Function of CD27/CD70 interactions in B cell responses

CD27 is classed as a memory B cell marker; its ligation has been demonstrated to promote the differentiation of memory B cells into CD27-negative plasma cells, and induce immunoglobulin production. This interaction may occur during germinal centre B cell development, where activated T cells expressing CD70 can interact with CD27-expressing memory B cells which have been previously primed through CD40-CD154 interactions [179].

1.4.4.5 Role of CD27/CD70 in the innate immune system

Murine and human NK cells constitutively express high levels of CD27, which increases upon IL-2 stimulation. Cross-linking CD27 via antibodies or CD70-transfected cells induces proliferation and IFN-γ production, but does not directly enhance the cytolytic capabilities of NK cells [180]. A recent study by Kelly *et al* showed that the expression of CD70 on MHC class I-negative tumours could promote their rejection by NK cells, most probably by expanding the numbers of perforin expressing NK cells *in vivo*. Furthermore, the CD70-CD27-mediated rejection of tumours by NK cells led to the formation of specific, protective, T cell memory against the parental MHC class I-positive tumour. Thus, they concluded that CD27–CD70 interactions were a link between the innate and adaptive immune system [196].

1.4.4.6 Potential of CD27/CD70 as immunotherapeutic targets

CD27 and CD70 may be important targets for immunotherapy; studies have shown that blocking their interaction protects against the induction of experimental autoimmune encephalomyelitis [197], and several murine cancer models have demonstrated that expression of recombinant CD70 on tumour cells has restricted tumour growth and elicited anti-tumour immunity [152,198-201]. Thus, transfection of murine fibrosarcoma and mammary adenocarcinoma tumour lines with CD70 slowed their growth *in vivo*. In addition, vaccination of mice using irradiated CD70-transfected tumours promoted long-lasting anti-tumour immunity and had a synergistic therapeutic effect when combined with B7-1 transfected tumours, indicating independent effects of these two co-stimulatory ligands [152]. In similar experiments, the growth of a murine colon adenocarcinoma was inhibited completely when the tumour cells were infected with a recombinant vaccinia virus encoding human CD70, and gave partial protection from re-challenge with parental tumour [201]. However, these studies used murine tumours transfected with human CD70, thus the immunogenicity of these tumours may have been enhanced by the presence of a xenogeneic molecule, which contributed to their rejection. Furthermore, this approach of vaccination with live modified tumour cells is not directly applicable to human immunotherapy, thus further investigation into the requirement for CD70 expression on the target cells and alternate methods of using CD70 to enhance immunisation against tumour antigens is required.

1.4.5 4-1BB (CD137, TNFRSF9)

1.4.5.1 Structure and expression pattern of 4-1BB and its ligand, 4-1BBL (TNFSF9)

4-1BB is a type I transmembrane glycoprotein of the TNFR family. It was first identified in the mouse, and the murine protein shares 60 % homology with the human form. Its extracellular domain contains four cysteine-rich TNFR motifs, with the first one being partial (see Figure 1.8 for a schematic diagram). Its cytoplasmic tail does not contain a death domain, but rather possesses two TRAF-binding consensus sequences. 4-1BB is expressed on activated T cells, NK cells, and recently it has been reported that it is also expressed on the surface of dendritic cells [202]. Signalling through the TCR induces T cell surface expression of 4-1BB, which peaks approximately 48 hours post-activation [151]. 4-1BBL is a type II transmembrane member of the TNF family, which is inducible on T cells and is found on a variety of APC such as activated B cells, macrophages and DCs [73,203].

1.4.5.2 Signalling cascades induced by 4-1BB

The oligomerisation of 4-1BB results in TRAF1, TRAF2 and TRAF3 recruitment to its cytoplasmic domain. TRAF2 is required for activation of the transcription factors NF-κB and AP1. It is suggested that TRAF2 mediates the production of IL-2 via activation of JNK (and therefore activation of AP-1), which is induced by apoptosis signal-regulating kinase-1 (ASK-1) [204]. 4-1BB signalling via TRAF2 and ASK-1 can also activate p38 mitogen-activated protein kinase (p38 MAPK), which is critical for 4-1BB–dependent cytokine production by primary T cells and the development of T_H1 and T_H2 subsets [205]. 4-1BB signalling in $CD8^+$ T cells can increase the expression of the anti-apoptotic genes *bcl-x_L* and *bfl-1* [206]. The up regulation of these Bcl2 family members may be responsible for the effect of 4-1BB on T cell survival (see below). 4-1BB also regulates cell cycle progression of $CD8^+$ T cells by increasing cyclin D2, D3 and E expression and co-ordinately down regulating the expression of the CDK-inhibitor $p27^{kip1}$ [207].

1.4.5.3 Function of 4-1BB/4-1BBL interactions in immune responses

4-1BB and 4-1BBL appear to function as a bi-directional signalling pair, providing simultaneous co-stimulation to T cells via the receptor, and stimulus for APC

activation and proliferation via the ligand [204,208]. *In vitro* co-stimulation of anti-CD3 mAb activated T cells, through 4-1BB, has a more potent proliferative and cytokine-inducing effect on the $CD8^+$ cells compared to the $CD4^+$ subset, although they have similar surface expression of this molecule [151,209]. 4-1BB co-stimulation *in vitro* is independent of CD28 signalling, as studies have shown that CD28 negative T lymphocytes can still respond to B cell lymphomas expressing 4-1BBL [210]. One function of 4-1BB appears to be to provide a long-term survival signal to activated T cells. An example of this is the rescue of *in vivo* superantigen-activated CD8 T cells from rapid deletion, by injection of an agonistic anti-4-1BB mAb [211]. This property of 4-1BB co-stimulation may be responsible for its effects on long-term immunity. Experiments examining anti-viral immunity in 4-1BBL knockout mice showed that 4-$1BBL^{-/-}$ mice had reduced peptide-specific primary and memory $CD8^+$ T cell responses to LCMV peptide $NP_{396-404}$. This resulted in higher viral titres in immunised $4\text{-}1BBL^{-/-}$ mice, as compared to wild type, when they were challenged with LCMV. Thus, indicating an impaired immune response in the knockout mice [173]. In a separate study, administration of anti-4-1BB mAb during influenza A viral infection was found to preferentially expand $CD8^+$ T cells that recognised non-dominant epitopes, and could restore $CD8^+$ responses in the absence of CD28 co-stimulation. This demonstrates that 4-1BB can replace CD28 co-stimulation and by lowering the threshold of T cell activation can enhance responses to weak agonist epitopes [212].

4-1BB, like CD40 and RANK, is expressed on the surface of DCs and acts as an activating molecule. Signalling via 4-1BB on DCs results in the production of IL-6 and IL-12 cytokines and up regulation of the co-stimulatory ligands B7-1 and B7-2 [95,202]. Thus, 4-1BB-4-1BBL interactions can shape T cell responses by acting at a number of regulatory stages.

1.4.5.4 Potential of 4-1BB/4-1BBL as immunotherapeutic targets

4-1BB also plays an important role in the regulation of cytotoxic T cells in cellular responses to alloantigens, such as in transplant models, or to syngeneic tumours. In murine graft versus host disease models, the administration of anti-4-1BB mAbs amplified the generation of specific cytotoxic T cells against host alloantigens [209]. Reciprocally, experiments using $4\text{-}1BB^{-/-}$ splenocytes for graft transplant showed that

4-1BB$^{-/-}$ T cells had reduced cytotoxic responses against the host and thereby increased host survival [213]. Thus, 4-1BB-4-1BBL interactions could be targeted in human transplants. Evidence that agonistic monoclonal antibodies against 4-1BB can eradicate established large tumours in mice, including the poorly immunogenic AG104A sarcoma and the highly tumourigenic P815 mastocytoma, was first shown by Melero *et al* [153]. Recently, a combined immunisation protocol using anti-4-1BB mAb and a tumour antigen peptide has been shown to induce regression of established poorly immunogenic tumours that were refractory to treatment with anti-4-1BB mAb alone. This dual delivery of antigen and co-stimulation breaks immunological ignorance and may be applicable to human cancer immunotherapy [154]. Studies by our group have shown both solid tumours and lymphomas can be completely rejected upon treatment with anti-4-1BB mAb. This effect appears to be mediated by both CD4$^+$ and CD8$^+$ T cells and provides protection from a second tumour challenge in the vast majority of cases [151].

1.5 Aims of the project

The TNFR superfamily molecules are now becoming recognised as critical modulators of T cell responses. In this context, detailed scrutiny of their functions will provide information relevant to a variety of immune-based therapies. CD27 is a relatively poorly characterised member of this family, which potentially may play a powerful role in T cell responses. Its co-stimulatory effects on *in vivo* antigen-specific responses have only just begun to be explored. Therefore, a major aim of this project was to attempt to define more clearly the role of CD27 in T cell mediated responses. More specifically, this study addresses whether CD27 acts predominantly during primary or secondary T cell responses, its role on $CD4^+$ and $CD8^+$ T cells and the influence CD27 exerts on T cell homeostasis. For example, it is not clear if CD27 acts only as a survival factor or if it can lower the threshold of TCR signalling required for activation and cell division, as has been shown for CD28. In order to analyse CD27 co-stimulation during *in vitro* and *in vivo* antigenic T cell responses a soluble recombinant form of the ligand for murine CD27, CD70, with effective signalling capabilities was generated. In addition, a similar soluble recombinant form of 4-1BBL was produced and characterised for use in further investigating the function of 4-1BB. Moreover, this fusion protein was used to directly compare the co-stimulatory effects of 4-1BB with CD27 using their native ligands.

A further aim of this project, considering the potent anti-tumour effects obtained with agonistic monoclonal antibodies against 4-1BB, is to assess whether delivery of co-stimulatory signals via soluble forms of 4-1BBL or CD70 are capable of vaccinating against, or eradicating, tumours. If they prove to be effective, soluble recombinant ligands may have several potential advantages over monoclonal antibodies. These include lack of immunogenicity; a recombinant ligand can easily be made fully species-specific without losing affinity for the receptor, which can be a problem during the chimerisation of monoclonal antibodies. A soluble ligand may also signal more effectively than monoclonal antibodies directed at the receptor. For example, the anti-CD27 antibodies generated so far require secondary cross-linking in order to transduce stimulatory signals, complicating their potential as *in vivo* reagents. As mentioned previously, a number of TNF superfamily members have been shown to require

trimerisation or higher order clustering in order to transduce signals through their receptors. Thus, soluble multimeric ligands may be more potent therapeutic reagents than divalent monoclonal antibodies.

CHAPTER 2

Materials and methods

2.1 Reagents, cells and antibodies

2.1.1 Reagents

Ovalbumin peptide 257-264 (OVA $_{257-264}$) with the sequence SIINFEKL was obtained from Peptide Protein Research Ltd (Fareham, U.K.). The lyophilised peptide (> 95 % purity) was dissolved in phosphate buffered saline (PBS; 120 mM NaCl, 24 mM Na_2HPO_4, 5.8 mM KH_2PO_4) and its concentration measured by BCA assay (Pierce, Rockford, USA). Aliquots were stored at – 20 °C until use. PE-labelled H-2K^b OVA $_{257-264}$ tetramers were obtained from Proimmune (Oxford, U.K.). LPS from *Salmonella minnesota* was obtained from Sigma (Poole, UK).

2.1.2 Cells

2.1.2.1 Cell lines

The *in vitro* cell lines CHO K1, COS-7, NS1 (used for hybridoma production), the C57BL/6 T cell thymoma line EL4 and E.G7, a derivative of EL4 which has been transfected with chicken ovalbumin cDNA and the neomycin resistance gene [214], were obtained from the American Type Culture Collection (ATCC; Manassa, VA, USA).

2.1.2.2 Cell culture

Untransfected CHO K1 cells, COS-7 and EL4 were cultured in RPMI 1640 medium (Invitrogen Ltd, Paisley, UK), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM pyruvate (Invitrogen) and 10 % (v/v) Foetal Calf Serum (FCS; Myoclone Plus, Invitrogen). E.G7 were cultured in 10 % RPMI (as above) supplemented with 400 µg/ml G418 sulphate (Invitrogen). Where indicated E.G7 cells were exposed to 200 Gy γ-irradiation to inhibit cell growth and allow their use as stimulator cells. NS1 cells were cultured prior to fusion in DMEM medium (Invitrogen) supplemented with 100 U/ml

penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 10 % (v/v) FCS. All cell lines were re-cultured every 2-3 days as required and maintained at 37 °C in a 5 % CO_2 humidified incubator. CHO K1 and COS-7 are adherent cells and therefore were detached from culture flasks by incubation with Trypsin-EDTA (Invitrogen).

2.1.2.3 Cell quantitation

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.

2.1.3 Antibodies and soluble ligands

2.1.3.1 Antibodies and soluble ligands for in vitro assays and flow cytometry

The following monoclonal antibodies used for *in vitro* assays and flow cytometry (FCM) were produced and purified in-house from hybridoma lines: anti-rat CD4 (OX68), anti-human Fc (hFc) (SB2H2), anti-CD3 (145.2C11), anti-Bcl1 idiotype (Mc3916 and Mc106A5), anti-4-1BB (LOB12 and LOB12.3), anti-OX40 (OX86), anti-B7-1 (GL-1), anti-B7-2 (1610A1), anti-CD28 (37.51), anti-CD25 (3C7), anti-FcγRII and III mAb (2.4G2) and anti-CD8 (YTS169). The hybridomas Mc3916, Mc106A5, LOB12, LOB12.3 and SB2H2 were generated in-house. The hybridomas OX86 and OX68 were a gift from N. Barclay (Sir William Dunn School of Pathology, Oxford, UK). The hybridoma YTS169 was a gift from S. Cobbold (Sir William Dunn School of Pathology, Oxford, UK). Hybridomas GL-1, 1610A1 and 2.4G2 were obtained from the ATCC (Manassa, VA, USA). mAbs used for FCM were fluorescein (FITC) or phycoerythrin (PE)-conjugated in-house as noted in the figure legends. Anti-mouse IL-2, allophycocyanin-labelled anti-CD8α (53-6.7), allophycocyanin-labelled anti-CD4 (RM4-5), biotinylated anti-CD70 mAb (FR70), anti-CD27 (LG.3A10) and FITC-labelled anti-CD45RB (16A) were obtained from BD Pharmingen (San Diego, CA, USA). FITC-labelled anti-CD44 and anti-CD49d (MCA123 OF), and PE-labelled anti-CD19 were obtained from Serotec Ltd (Oxford, UK). FITC-labelled anti-CD62L (L-selectin) was obtained from Caltag Laboratories (Burlingame, CA, USA). PE-

labelled anti-Rat IgG was obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). PE-conjugated streptavadin was obtained from Sigma (Poole, Dorset, UK). Normal human IgG (hIgG) was prepared in-house. The soluble ligands sCD70, s4-1BBL, OX40L.rCD4, CD153.Fc and CD40L.Fc were generated in-house (see Section 2.2 for generation of sCD70 and s4-1BBL). Other antibodies used in immunoassays are specified in their relevant methods sections.

2.1.3.2 Generation of an anti-CD70 mAb (TAN1-6)

The anti-CD70 mAb TAN1-6 was generated by standard hybridoma technology. In brief, a Lou rat (obtained from Harlan, UK) was immunised sub-cutaneously (s.c.) with 50 μg of sCD70 in complete Freunds adjuvant (Difco Laboratories, Detroit, USA). 21 days later the rat was re-immunised intra-peritoneally (i.p.) with 50 μg of sCD70 in incomplete Freunds adjuvant (Difco Laboratories), followed 32 days later by a boost of 25 μg sCD70 in PBS intra-venous (i.v.) and i.p.. 3 days later splenocytes from the immunised rat were fused with the myeloma cell line NS1. A single cell suspension of splenocytes was prepared aseptically and resuspended in serum-free DMEM media (DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate). NS1 cells in log phase growth were added to the splenocytes at a ratio of 1 : 2 (NS1 : splenocytes), and the cell mixture pelleted by centrifugation. The supernatant was removed and the fusion performed by addition of 1 ml PEG solution (480 mg PEG4000 and 500 μl EMEM media (Invitrogen), autoclaved) at 37 °C for 90 seconds with agitation. Cells were washed gently with DMEM, resuspended in 100 ml DMEM-HAT media (DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate, 15 % FCS, 1 x HAT (Invitrogen)) and incubated for 1 hr at 37 °C. Fused cells were plated out on 96-well plates and the cell supernatant screened for antibody production after ~10 days. Screening was done by sandwich ELISA (as standard sandwich ELISA protocol detailed in Section 2.3.3) using sCD70 or a similar negative control fusion protein (CD153.Fc) as a capture reagent (coated at 2 μg/ml), and anti-rat IgG HRP (Serotec AAR 10P; 1 in 2000 dilution) as a detection reagent. Hybridomas positive for sCD70 but negative for the control fusion protein were cloned and expanded.

2.1.4 LPS testing

Certain reagents were tested for LPS contamination (European Endotoxin Testing Service, Cambrex Bioscience, Verviers, Belgium) and were found to contain < 0.05 ng of LPS per 20 nmoles of OVA $_{257-264}$ peptide or 200μg of sCD70.

2.2 Molecular biology

2.2.1 Generation of the sCD70 cDNA construct

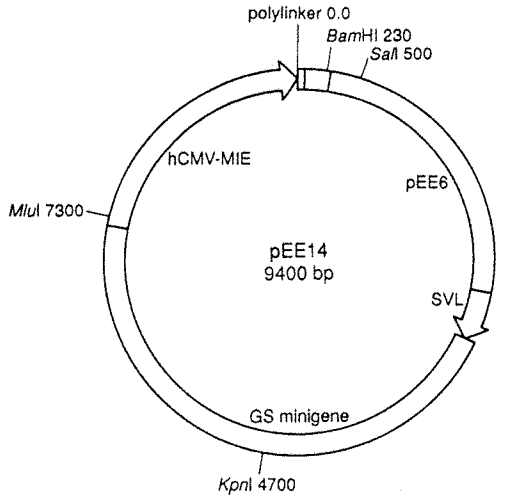
2.2.1.1 Cloning the full-length cDNA of murine CD70

The full-length cDNA of CD70 was isolated by PCR (Pfu polymerase) from a first-strand cDNA template prepared from concanavalin A-activated mouse splenocytes, using primers based on its published sequence [183]. The forward primer 5' GTCTAGATGCTTCCGTGCAGGGATG 3' incorporates an *Xba*I restriction enzyme site (underlined). The reverse primer 5' GGGATCCTCAAGGGCATATCCACTGAAC 3' incorporates a *Bam*HI restriction site (underlined). The resultant PCR product was cloned into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning kit according to the manufacturer's instructions (Invitrogen) and sequenced to check for errors.

2.2.1.2 Sub-cloning the extracellular domain of murine CD70 into a human IgG1 Fc vector

The region of CD70 cDNA that corresponds to the extracellular domain of its translated protein (amino acid residues 41-195) was amplified by PCR using the following primers. The forward primer 5'AATGCATCCAAAACTCACACAGCCCCA CCGGCCCCAAGCGGACTACTCAGTAAG 3' incorporates an *Nsi*I restriction site (underlined) which has an overhang sequence compatible with *Pst*I, allowing ligation into the *Pst*I site in the modified hinge region of the hIgG1 Fc construct (see schematic Figure 3.1). The reverse primer 5' GTCTAGAGTCAAGGGCATATCCACTG 3' incorporates an *Xba*I site (underlined). These restriction sites were used for ligation of the CD70 extracellular domain cDNA into the pOX38 vector (a derivative of pCR 3-Uni; Xenova Pharmaceuticals, Slough, UK) in-frame downstream of the modified human IgG1 Fc region. The entire hFc/CD70 DNA fusion construct was sequenced to check sequence integrity. The full Fc.CD70 DNA construct was then excised via the *Hind*III and *Xba*I sites, and ligated into the multilinker cloning site of the pEE14 vector (Celltech, Slough, UK). 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, high levels of

recombinant protein can be expressed in mammalian cell lines using the glutamine synthetase minigene present in pEE14 for selection and amplification (Figure 2.1) [215].



polylinker

HindIII	*XbaI*	*XmaI SmaI*	*EcoRI*	*BclI*
AAGCTTGGGCTGCAGGTCGATCGACTCTAGAGGATCG		ATCCCCGGGCGAGCTCGAATTCATTGATCA		

Figure 2.1 Map of the pEE14 GS expression vector. pEE14 contains a glutamine synthetase (GS) minigene as the selectable marker which has a single intron and GS polyadenylation signals and is driven from an SV40 late promoter. The hCMV-MIE promoter-enhancer and 5' untranslated region are used to express the gene of interest and the remainder of the plasmid contains an ampicillin-resistance gene and replication origin for replication in *E.coli.* The polylinker sequence is shown below. Adapted from Bebbington, C. R. & Rolfe, M. R. in *Current Protocols in Molecular Biology* Supplement 18: Protein Expression (1991).

2.2.2 Generation of the s4-1BBL cDNA construct

2.2.2.1 Cloning the full-length cDNA of murine 4-1BBL

The full-length cDNA of 4-1BBL was isolated by PCR (Pfu polymerase) from a first-strand cDNA template prepared from con A-activated mouse splenocytes, using primers based on its published sequence [203]. The forward primer $^{5'}$GTCTAGATGACCGACCGTGGTAATG$^{3'}$ incorporates an *Xba*I restriction enzyme site (underlined). The reverse primer $^{5'}$GGGATCCTCATTCCCATGGGTTGTC$^{3'}$ incorporates a *Bam*HI restriction site (underlined). The resultant PCR product was

cloned into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning kit according to the manufacturer's instructions (Invitrogen) and sequenced to check for errors.

2.2.2.2 Sub-cloning the extracellular domain of murine 4-1BBL into a human IgG1 Fc vector

The region of 4-1BBL cDNA that corresponds to the extracellular domain of its translated protein (amino acid residues 108-309) was amplified by PCR. The forward primer $^{5'}$TCTGCAGACAAAACTCACACAGCCCCACCGGCCCCACGGCCAGCG CTCACAATC$^{3'}$ incorporates an *Pst*I restriction site (underlined), allowing ligation into the *Pst*I site in the modified hinge region of the hIgG1 Fc construct (see schematic Figure 6.1). The reverse primer $^{5'}$GTCTAGACTCATTCCCATGGGTTGTCGG$^{3'}$ incorporates an *Xba*I site (underlined). These restriction sites were used for ligation of the 4-1BBL extracellular domain cDNA into the pOX38 vector in-frame downstream of the modified human IgG1 Fc region. The entire hFc/4-1BBL cDNA was sequenced to check that the sequence integrity and frame was maintained. The full Fc.4-1BBL DNA construct was then excised via the *Hind*III and *Xba*I sites, and ligated into the pEE14 vector (Figure 2.1).

2.2.3 Molecular Biology techniques

2.2.3.1 Mini and Maxi preparation of DNA

DNA was prepared from bacterial cultures by QIAprep spin miniprep kit or high speed plasmid maxi kits (Qiagen), according to the manufacturer's instructions.

2.2.3.2 Restriction Enzyme Digests

Restriction enzyme digests were performed by incubation of the required restriction enzyme (Promega) at ~ 10 U/μg DNA in the presence of the relevant restriction enzyme buffer (Promega) at 1 x final concentration, made up with deionised water. Digests were incubated for 2 hours at 37 °C, before analysis by agarose gel electrophoresis.

2.2.3.3 Agarose gel electrophoresis

DNA fragments were analysed on 0.7 % agarose gels made in TBE buffer (89 mM tris, 89 mM boric acid, 2mM EDTA, 0.5 μg/ml ethidium bromide), and compared to standard size markers. DNA samples were mixed 10:1 with 10 x loading buffer (20 % (w/v) Ficoll 400, 0.1 M EDTA, 0.25 % (w/v) bromophenol blue) before loading. Gels were run in TBE buffer at 70 mA in a BioRad electrophoresis system for the appropriate time and visualised on a UV illuminator.

2.2.3.4 Polymerase chain reaction (PCR)

DNA amplification for cloning was performed using Pfu polymerase (Stratagene), which has a proofreading capacity. Each reaction contained 10 ng template DNA, 0.5 μM forward and reverse oligonucleotide primers (Invitrogen), 0.2 mM dNTPs (Promega) and 1 x Pfu reaction buffer (Stratagene), made up with sterile deionised water. 2.5 U Pfu polymerase was only added after the initial denaturing stage of the PCR program in order to reduce non-specific amplification. Amplification was performed in a thermocycler (PTC-100, MJ Research Inc.), using a standard PCR program incorporating the lowest partial annealing temperature (i.e. complementary region annealing temperature) of the primers during the first 5 cycles, and the lowest annealing temperature of the full length primers for a further 21 cycles. Annealing temperatures were calculated as being 4 °C below the melting temperature for the primer-template pair, which was predicted using the following formula: $T_m = 64.9 + 0.41$ (% GC) - 500/L, where % GC is the percentage of G and C in the oligonucleotide and L is the length of the oligonucleotide in bases.

2.2.3.5 DNA ligation

The required vector and insert DNA fragments from restriction digests were isolated using agarose gel electrophoresis and purified from the gel using a QIAquick gel extraction kit (Qiagen) according to the manufactures instructions. Vector and insert were ligated at 1:1 or 1:3 ratios with a total of 10 ng DNA in the presence of 1.5 U T4 DNA ligase (Promega) and 1 x T4 ligase buffer (Promega), for 3 hrs at 25 °C.

2.2.3.6 Bacterial transformation and amplification

For bacterial transformation, 2 ng of DNA was mixed with 50 µl of Epicurian Coli XL-1 Blue supercompetent cells (Stratagene) and incubated on ice for 30 minutes, before heat shocking at 42 °C for 2 minutes. Cells were then returned to ice for 2 minutes before addition of 350 µl 2 x Ty media (1.6 % (w/v) tryptone (Sigma), 1 % (w/v) yeast extract (Sigma), 0.5 % NaCl), and incubation at 37 °C in an orbital incubator for 1 hour. Cultures were spread on agarose plates (1.5 % (w/v) Bacto-Agar (Difco), 10 % (w/v) tryptone (Sigma), 5 % yeast extract (Sigma), 5 % NaCl) containing the appropriate selection antibiotic (ampicillin 100 µg/ml or kanamycin 50 µg/ml) and incubated at 37 °C overnight. Individual colonies were selected and amplified by overnight culture in 10 mls L-Broth (10 % (w/v) tryptone (Sigma), 5 % yeast extract (Sigma), 5 % NaCl, plus selection antibiotic), before DNA preparation.

2.2.3.7 DNA Sequencing

Sequencing reactions were set up containing 500 ng of the DNA of interest, 4 µl of BigDye terminator ready reaction mix (ABI-PRISM BigDye Terminator Cycle Sequencing Kit, PE Applied Biosystems) and 1.6 pmoles of oligonucleotide primer, made up to 10 µl final volume with sterile deionised water in 0.2 ml thin-walled PCR tubes (PE Applied Biosystems). Reactions were run on a thermocycler (GeneAmp 9700) for 25 cycles of: 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 2 min. DNA was precipitated on ice for 10 min by addition of 10 % (v/v) 3 M sodium acetate and 3 volumes of cold ethanol and pelleted in a microfuge at 13000 RPM for 30 minutes. The DNA was rinsed with 70 % (v/v) cold ethanol, re-pelleted briefly and air-dried. DNA was resuspended in 1.5 µl loading buffer (20 % (v/v) dextran blue loading buffer (PE Applied Biosystems), 80 % (v/v) formamide) and denatured at 95 °C for 2 minutes. Samples were run on a 5.25 % denaturing polyacrilamide gel on an ABI-PRISM 3000 sequencer (PE Applied Biosystems) and analysed using DNAstar software.

2.3 Transfection and recombinant protein purification techniques

2.3.1 Stable transfection of CHO K1 cells by calcium phosphate precipitation

Recombinant DNA constructs encoding sCD70 or s4-1BBL in the mammalian expression vector PEE14 were stably transfected into CHO K1 cells via a calcium phosphate precipitate. Selection of stable cell clones was achieved via the glutamine synthetase system. Glutamine is an important metabolite synthesised by cells from glutamate and ammonia using the enzyme glutamine synthetase. Inhibition of this enzyme using methionine sulfoximine (MSX), in the absence of exogenous glutamine, will cause cellular death. However, by transfecting CHO K1 cells with the PEE14 vector, which contains a glutamine synthetase minigene, the cells become resistant to sub-optimal concentrations of MSX because of increased expression of the enzyme [215]. Stably transfected cells can therefore be selected and the expression of the recombinant protein may be amplified by increasing the concentration of MSX.

Briefly, CHO K1 cells were seeded the day before transfection at 1 x 10^6 per 80 cm^2 flask in GMEM-S media (First link, UK) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 10 % (v/v) FCS. A sterile solution containing the 15 µg of the DNA construct and 186 µl of 1 M $CaCl_2$ (BDH) in a total volume of 750 µl (made up with deionised water), was added dropwise with agitation to 750 µl 2 x HBS pH 7.05 (1.636 % (w/v) NaCl, 1.188 % (w/v) HEPES (free acid, Sigma), 0.04 % (w/v) Na_2HPO_4 adjusted to pH 7.05 with 1M NaOH) and incubated for 20 min at room temperature to allow the DNA-calcium phosphate precipitate to form. If the precipitate was too coarse, the pH of the HBS buffer was decreased by addition of 1 M HCl until a fine precipitate was achieved. The precipitate was added to the CHO K1 cells in ~ 20 ml of media and the cells incubated for 6 hours at 37 °C / 5 % CO_2. The CHO K1 cells were then glycerol shocked to improve transfection efficiency. The cells were washed twice with GMEM-S media (no supplements) and then incubated with 2 mls of 15 % (v/v) glycerol (BDH) in 1 x HBS (0.818 % (w/v) NaCl, 0.594 % (w/v) HEPES (free acid, Sigma), 0.02 % (w/v) Na_2HPO_4, pH 7.05) for 2 minutes. CHO K1 cells were washed twice with GMEM-S media (no supplements) and cultured overnight in GMEM-S supplemented with 10 %

(v/v) dialysed FCS (First Link, UK) and 100 U/ml penicillin and 100 μg/ml streptomycin.

The transfected CHO K1 cells were then resuspended to 5 x 10^4/ml in GMEM-S supplemented with 10 % (v/v) dialysed FCS (First Link, UK), 100 U/ml penicillin and 100 μg/ml streptomycin and 25 μM MSX (Sigma) and plated over 96 well flat-bottomed microtitre plates (Nunc) at 200 μl/well. Plates were demi-fed after 7 days and screened by an anti-hFc ELISA (see Section 2.3.3) when colonies started to appear. After expansion into 24-well plates, clones were re-analysed to confirm stable expression of the fusion protein.

2.3.2 Transient transfection of COS-7 cells

COS-7 cells were transiently transfected with the full length cDNA of murine CD70 in the pEF-BOS vector [216], or mock-transfected. Briefly, COS-7 cells in log phase growth were washed x 1 in serum-free RPMI 1640. A transfection cocktail was prepared consisting of RPMI 1640 containing 400 μg/ml DEAE-Dextran and 100 μM chloroquine. 5 μg of the DNA construct was mixed with 5 ml of transfection cocktail and incubated with the COS-7 for 4 h at 37 °C. Cells were washed x 1 with PBS and shocked by incubation for 3 min at room temperature with PBS 10 % DMSO. COS-7 were washed again x 2 and cultured with complete media (RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 10 % FCS) for 2 days before expression analysis. COS-7 were detached from their flasks by washing x 2 with PBS to remove the culture media and then incubating with PBS 0.5 mM EDTA for 25 min at room temperature before analysis by FCM.

2.3.3 Enzyme-Linked Immunsorbant Assay (ELISA) for the detection of soluble human Fc Fusion proteins

To detect the presence of sCD70 or s4-1BBL in cell supernatant an ELISA was performed according to the following standard protocol. Primary rabbit anti-hFc mAb was diluted in coating buffer (15 mM Na_2C0_3, 28.5 mM $NaHC0_3$, pH 9.6) to 1 μg/ml and 100 μl/well added to treated 96 well plates (Maxisorb, Nunc) and incubated overnight at 4 °C. Unbound antibody was removed by flicking and the non-specific

binding sites were blocked by the addition of blocking solution (1 % (w/v) BSA in PBS) for 1 hour at 37 °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 100 µl/well, with all dilutions being made in media. Following incubation for 90 minutes at 37 °C, the plate was washed again (x 5 in wash solution). Horse-radish peroxidase (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 (o-PD; Sigma) in 100 ml phospo-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 min. 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.

2.3.4 Purification of recombinant proteins by immunoaffinity chromatography

Recombinant hFc fusion proteins were isolated from tissue culture supernatant using an immunoaffinity column containing an anti-hFc mAb (SB2H2) conjugated to Sepharose 4B beads (Amersham Pharmacia). Cell culture supernatant was loaded onto the column using gravity flow at 0.5 ml/min at 4°C. The column was then washed to remove non-specific protein with 5 column volumes of low-Tris buffer (0.028 M 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 column 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 90 µl of 2 M Tris-HCl pH 8.5. Recombinant proteins were dialysed into PBS and stored in small aliquots at –20 °C until use.

2.3.5 Estimation of extinction coefficients and molecular mass

The extinction coefficients at 280 nm, $E_{(0.1\ \%,\ 1\ cm)}$, and molecular mass (M_r) of sCD70 and s4-1BBL were estimated from their amino acid sequences using the ProtParam tool (www.expasy.ch).

Soluble ligand	$E_{(0.1\ \%,\ 1\ cm)}$	M_r (kDa)
sCD70	1.094	44.74
s4-1BBL	1.427	50.227

Table 2.1 Extinction coefficients and M_r of sCD70 and s4-1BBL.

2.4 Protein analysis

2.4.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was performed using a mini-gel system (BioRad). 5 μg of purified protein per lane was analysed on a polyacrylamide 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 base 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'-tetramethylethylene at a final concentration of 0.005 % and 0.01 % for the resolving and stacking gels respectively. The reaction was initiated with a fresh 10 % (w/v in dH_20) 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 solublisation buffer (40 % urea (w/v), 1.6 % SDS (v/v), 0.160 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 °C for 5 min and loaded immediately. Gels were run at a 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 de-stained to the extent desired with 10 % (v/v) acetic acid.

2.4.2 Size-exclusion chromatography

2.4.2.1 Structural analysis of recombinant proteins

The structure of sCD70 and s4-1BBL recombinant fusion proteins was analysed by loading ~ 100 μg onto a Superdex 200 column at 0.4 ml/min, in running buffer (0.01 M HEPES, 0.15 M NaCl, 0.2 mM EDTA, pH 7.4, degassed). Percentage absorbance at 280 nm was measured on a chart recorder and peak position compared to the molecular

mass markers thyroglobin (670 kDa), β-amylase (200 kDa), γ-globin (158 kDa) and ovalbumin (44 kDa).

2.4.2.2 Fractionation of recombinant proteins

~ 17 mg of purified s4-1BBL protein was separated into 2 ml fractions using a Hiload 16/60 Superdex 200 column, at 0.8 ml/min, in PBS using an Äkta Prime system (Amersham Biosciences). Percentage absorbance at 280 nM was measured on a chart recorder and peak position compared to the molecular mass markers IgM (900 kDa), IgG (150 kDa) and Fc (50 kDa).

2.5 Cellular assays

2.5.1 Preparation and culture of murine splenocytes

Spleens 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 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM 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.

2.5.2 Purification of $CD4^+$ and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ T cells were purified from BALB/c splenocytes by negative selection using $CD4^+/CD8^+$ Cellect™ cell enrichment columns (Cedarlane Laboratories Limited, Ontario, Canada), according to the manufacturers instructions. The resulting T cell populations were 70 % pure as assessed by flow cytometry

2.5.3 [^{3}H]-thymidine incorporation assays

Proliferation status of cells was determined by measuring radiolabelled thymidine incorporation. Only replicating S-phase cells will incorporate (radiolabelled) thymidine. Therefore, an increase in the number of dividing cells will be evidenced by a increase in [^{3}H]-thymidine incorporation compared to controls. Purified T cells or splenocytes were typically cultured with various reagents (as detailed in the figure legends) at a concentration of 1 x 10^6/ml in a final volume of 0.2 ml in U-bottomed 96 well plates (Nunc). Methyl-^{3}H-thymidine (0.5 µCi /well; Amersham, Buckingham, UK) was added to the cultures 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 via liquid scintillation counting.

2.5.4 Measurement of surface antigens by flow cytometry

2.5.4.1 Standard protocol for staining cell surface antigens

~ 1 x 10^6 cells were incubated at 4 °C for 30 min with the fluorophore-conjugated (direct) or unlabelled (indirect) antibody or soluble ligand of choice (10 μg/ml final concentration or appropriately titrated). Cells were then washed x 2 in PBS-BSA (PBS, 0.2 % (w/v) Bovine Serum Albumin fraction V (BSA; Wilfred Smith Ltd, Middlesex, UK)) and resuspended. For indirect immunofluorescence, cells were further incubated for 30 min at 4 °C with a fluorophore-conjugated secondary antibody directed against the primary antibody or soluble ligand and washed x 2 in PBS-BSA before resuspension in PBS and subsequent analysis on a FACSCalibre (Becton Dickinson, Mountain View, CA, USA) flow cytometer. Forward scatter (FSC) vs side scatter (SSC) analysis was used to gate viable cells or lymphocyte populations. Ten thousand viable cells were typically collected per sample.

2.5.4.2 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 [217]. The success of this technique lies with the increased avidity of soluble biotinylated MHC-peptide for the peptide-specific TCR upon tetramerisation by avidin, which has four biotin-binding sites. The binding of the tetramer to the TCR can be detected by flow cytometry if a fluorochrome-labelled avidin is used. Blood samples (50 μl), or lymph node / spleen cells (1 x 10^6) were stained in PBS-BSA with titrated amounts of PE-labelled H-$2K^b$ OVA $_{257\text{-}264}$ tetramers and allophycocyanin-labelled anti-CD8α for 30 min at room temperature. The cells were then washed x 1 in PBS-BSA and the RBCs lysed before analysis by FCM. Seventy thousand live lymphocyte events were typically collected per sample.

2.5.4.3 Intracellular expression of the Ki67 proliferation antigen

The proliferation status of antigen-specific T cell populations was determined by surface staining lymphocytes with H-$2K^b$ OVA $_{257\text{-}264}$ tetramers and anti-CD8α, and intracellular staining with an antibody against the proliferation antigen Ki67, using a method adapted from Appay and Rowland-Jones [218]. Peripheral blood lymphocytes

were first stained with H-2K^b OVA $_{257-264}$ tetramers and anti-CD8α as described above. Cells were washed x 1 (PBS, 1 % BSA, 0.1 % sodium azide) and fixed and permeabilised with 300 µl of PBS / 4 % formaldehyde / 0.1 % saponin for 15 min at room temperature in the dark. Cells were washed again x 1 (PBS, 1 % BSA, 0.1 % sodium azide) and resuspended in staining buffer in the presence of saponin (PBS, 1 % BSA, 0.1 % sodium azide, 0.1 % saponin). Anti-Ki67 FITC (B56; BD Pharmingen) or an isotype control antibody (MOPC-21; BD Pharmingen) was added according to the manufacturers recommended titration for 15 min at room temperature. Cells were washed again x 1 (PBS, 1 % BSA, 0.1 % sodium azide) and resuspended in PBS 2 % formaldehyde before 3-colour FCM analysis. Seventy thousand live lymphocyte events were typically collected per sample, and quadrants were positioned so that positive staining with the isotype control was < 0.01 %.

2.5.4.4 CFSE dilution analysis

To look at T cell division directly, splenocytes were labelled with 5,(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands). This acetate ester is uncharged and can therefore permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by non-specific esterases resulting in a charged form that leaks out of cells far more slowly than its parental compound. Therefore, the CFSE fluorescent label remains within the cytoplasm and when the cell divides the intensity of fluorescent label is reduced by a half. Splenocytes at 2-4 x 10^7/ml were incubated for 10 minutes at 37 °C with 5 µM CFSE in serum-free RPMI 1640. Labelled cells were then washed x 2 with RPMI 1640 plus 10 % FCS and the level of labelling checked by FCM. Cultures were set up as indicated in the figure legends.

2.5.4.5 Detection of apoptosis / cell death using propidium iodide and annexin V staining

As cells apoptose, they translocate phosphotidylserine from the inner to the outer layer of the plasma membrane. Annexin V is a phospholipid-binding protein with high affinity for phosphotidylserine. Therefore, binding of annexin V to the external surface of a cell is indicative of apoptosis [219]. Membrane damage was also detected using propidium iodide (PI). PI is a dye that is excluded from viable cells possessing intact

membranes. Entry into cells facilitates a large (1000-fold) increase in fluorescence in the red area of the spectrum. Therefore, incubation with this dye detects only those cells that have diminished membrane integrity. 1 x 10^5 cells were washed in PBS and resuspended in 200 µl of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), containing 1 µg/ml FITC-annexin V (BD Biosciences) and 10 µg/ml PI. Cells were incubated in the dark at room temperature for 10 min and subsequently analysed by 2-colour FCM.

2.5.5 Detection of IL-2 and IFN-γ 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 and IFN-γ were detected by ELISA essentially by the same method as for the detection of soluble human Fc Fusion proteins (see Section 2.3.3), 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. IFN-γ reagents: Capture mAb rat anti-mouse IFN-γ (HB170) at 4 µg/ml, detection mAb biotinylated rat anti-mouse IFN-γ (Serotec) diluted 1 in 400, 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 or IFN-γ. Actual cytokine concentrations in assay supernatant were calculated using GraphPad Prism software (GraphPad Prism version 2.01 for windows, GraphPad software, San Diego, California). In some cases the number of viable T cells present in assay wells was measured on a haemocytometer by observation of trypan blue dye exclusion. This value was used to calculate the amount of cytokine produced per 10^5 T cells.

2.5.6 Western blotting for $Bclx_L$ expression

Cell lysates were prepared by resuspending cells in 40 µl of 1 % NP40 lysis buffer (10 mM Tris, 150 mM NaCl, 2.5 mM EDTA, 1 % NP40, pH 8 and ½ a tablet of complete mini protease inhibitor (Roche) per 10 mls) and incubating on ice for 30 min, followed by 5 min centrifugation in a microfuge to remove the insoluble material. Lysates were mixed 1:1 with 2 x protein solublisation buffer and run on standard 10 % SDS-PAGE

gels as described previously (see Section 2.4.1). 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 tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl)), to prevent subsequent non-specific binding. Following blocking, the blot was washed 1 x 10 minutes in TBS-T (TBS + 0.05 % Tween-20). The primary antibodies against BclxL (rabbit anti-BclxL, 1 in 200 dilution (Santa Cruz Biotechnology)) or β-actin (goat anti-β-actin, 1 in 500 dilution (Santa Cruz Biotechnology)) were then diluted in blocking buffer (with 0.05 % Tween-20) and the blot incubated for 1 hour at room temperature with constant agitation, followed by a wash step (3 x 5 min in TBS-T). Detection of bound primary antibody was performed using the relevant secondary peroxidase-conjugated antibody (donkey anti-rabbit, 1 in 20000 dilution (Amersham Pharmacia) or donkey anti-goat, 1 in 2000 dilution (Santa Cruz Biotechnology)), which was diluted in blocking buffer and allowed to bind for 45 min at room temperature with agitation, followed by a wash step (3 x 5 min in TBS-T, 1 x 5 min in TBS). 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).

2.6 Animals and *in vivo* experiments

2.6.1 Mice

OT-I TCR transgenic mice [220] crossed onto a $RAG1^{-/-}$ background, were obtained from Dr Brigitta Stockinger (NIMR, London) and homozygous OT-I TCR transgenic C57BL/6 mice were obtained from Dr Matthias Merkenschlager (Imperial College, London) and bred in the in-house animal facility (Tenovus). Wild-type C57BL/6 mice were purchased from Harlan (Blackthorn, UK). BALB/c and CBA/H mice were originally obtained from Harlan (Blackthorn, UK) and bred in the in-house animal facility. Splenocytes from IL-2 deficient mice [221] were obtained from Dr Fiona Powrie (Sir William Dunn School of Pathology, Oxford). All mice were used at approximately 8-12 weeks of age. Animal experiments were carried out according to the UK Home Office license guidelines, and were approved by the University of Southampton's ethical committee.

2.6.2 In vivo T cell experiments

For adoptive transfer of OT-I T cells to naïve C57BL/6 recipients, a single cell suspension of lymph node (inguinal, brachial and mesenteric) and spleen cells was prepared from OT-I C57BL/6 mice and the proportion of transgenic T cells determined by K^b OVA $_{257-264}$ tetramer and anti-CD8α staining. OT-I transgenic T cells (1×10^6) were then transferred by i.v. injection into sex-matched C57BL/6 recipients. The transferred transgenic T cells had a naïve phenotype ($CD62L^{hi}$, $CD44^{lo}$, $CD25^-$, see Figure 5.2, Chapter 5). Two or three days later T cells were primed *in vivo* by i.v. administration of OVA $_{257-264}$ in PBS in combination with soluble ligands, antibodies, LPS or hIgG as indicated in the figure legends. Soluble ligands, antibodies or hIgG were administered for a further 2 days as indicated. Secondary stimulation was carried out by i.v. injection of OVA $_{257-264}$ in PBS alone.

2.6.3 CFSE dilution analysis in vivo

To monitor T cell division *in vivo*, OT-I were labelled with CFSE (10 μM) for 10 min in PBS containing 0.1 % BSA. Cells were washed once with RPMI 1640 and 10 % FCS and then twice with PBS. One million cells were administered by i.v. injection to C57BL/6 mice. Twenty-four hours later, mice received $OVA_{257-264}$ (20 nmoles) with

sCD70 (200 μg) or hIgG as a control by i.v. injection. Mice were administered two further injections of sCD70 or hIgG (200 μg). Some mice were left unstimulated. At various times after immunisation spleen cells were stained with K^b OVA $_{257-264}$ tetramers and anti-CD8α as detailed in Section 2.5.4.2. The level of CFSE in $CD8^+$ $tetramer^+$ cells was analysed by FCM.

2.6.4 Cytotoxicity assay

Ex-vivo cytotoxicity of CTLs was measured on day 9 or 10 post-antigen priming by a standard ^{51}Chromium release-assay, which measures membrane perturbation of target cells. EL4 or E.G7 target cells (~ 1 x 10^7) were washed x 2 with serum-free RPMI 1640 supplemented 2 mM L-glutamine and 1 mM pyruvate at 37 °C, and then labelled with 0.1 mCi ^{51}Cr (Amersham Biosciences, Amersham, UK) in 100 μl PBS for 1 h at 37 °C. One aliquot of EL4 cells was simultaneously pulsed with 5 μM OVA $_{257-264}$ peptide. Target cells were then washed x 3 with serum-free RPMI 1640 supplemented with 2 mM L-glutamine and 1 mM pyruvate at 37 °C and resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM pyruvate and 10 % FCS at 37 °C. Effector cells were prepared by pooling splenocytes from the same treatment groups, lysing the RBCs and washing x 3 with serum-free RPMI 1640 supplemented with 2 mM L-glutamine and 1 mM pyruvate and then resuspending in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM pyruvate, 10 % FCS at 37 °C. Targets and effectors were enumerated and resuspended at the appropriate concentrations. 5 x 10^3 target cells were incubated with the indicated ratio of effectors in a total volume of 200 μl in U-bottomed 96 well plates for 4 h at 37 °C. ^{51}Cr release was measured by pelleting cells by centrifugation and removing 100 μl of supernatant for counting on a γ-counter (Wallac, Program 3, 5 min/tube). The percentage of ^{51}Cr released was calculated by the following formula: ^{51}Cr release = [(sample cpm-background cpm) / (total cpm-background cpm)] x 100, where background cpm represents spontaneous ^{51}Cr release from targets in media alone. The value for total cpm was obtained by lysis of labelled cells by the addition of 1 % NP-40 in PBS. EL4 targets which had not been pulsed with OVA $_{257-264}$ peptide were used as a control for non antigen-specific killing.

2.6.5 In vivo tumour growth assays

2.6.5.1 Immunisation against E.G7

OT-I T cells were adoptively transferred into C57BL/6 mice as described above, according to the numbers indicated in the figure legends. Three days later mice were primed by i.v. injection of OVA $_{257-264}$ in the presence or absence of sCD70 (200 μg). sCD70 was administered for a further 2 days (2 x 200 μg i.v.) with control groups receiving an equal amount of normal hIgG. E.G7 tumour cells (5 x 10^5) in exponential growth were administered in PBS s.c. 9 or 10 days post-immunization. Tumour growth was monitored twice daily and animals were sacrificed when tumour size (the product of two perpendicular diameters) reached 150 mm^2.

2.6.5.2 A31 B cell lymphoma immunotherapy

The A31 B cell lymphoma line [222] was maintained by *in vivo* passage in CBA/H mice. A31 lymphoma cells from mice at terminal stage were isolated by density gradient centrifugation of a suspension of splenocytes over Lymphoprep (Apogent Discoveries Ltd) and collection of the lymphocyte interface. A31 lymphoma cells were washed x 2 in PBS, and 5 x 10^7 cells administered i.v. to naïve CBA/H mice on day 0. Mice were treated by i.v. injection of relevant antibodies or soluble ligands as indicated in the figure legends. In some experiments $CD8^+$ T cells were depleted by administration of 500 μg anti-CD8 mAb (YTS169) i.p on days –2, 2, 8 and 13. Survival of animals was monitored twice daily.

2.6.6 Statistical analysis

The statistical significance between OT-I T cell numbers in different groups of treated mice was calculated using an unpaired t test (GraphPad Prism version 2.01 for windows, GraphPad software, San Diego, California). The statistical significance between A31 or E.G7 immunotherapy groups was analysed using the logrank test [223] to compare survival curves (GraphPad Prism version 2.01 for windows, GraphPad software, San Diego, California).

CHAPTER 3

Expression and characterisation of a soluble form of murine CD70

3.1 Introduction

In order to elucidate the function of the TNFR-family member CD27 and investigate its potential as a target for augmenting T cell responses against pathogens and tumours, the aim was to generate a soluble reagent that mimicked its naturally occurring membrane-bound ligand, CD70. Studies of transgenic mice which over-express CD70 on B cells have indicated that constitutive interaction between CD27 and CD70 induces the expansion of effector/memory T cells. However, this degree of chronic co-stimulation eventually leads to lethal T cell immunodeficiency [185,195]. A soluble form of CD70 would allow tightly regulated signalling through CD27 in both *in vitro* and *in vivo* experiments. This strategy would avoid the abnormal phenotype induced by constitutive CD70 expression *in vivo,* and be more representative of the limited expression of the naturally occurring ligand [224]. To generate a soluble form of CD70, the extracellular receptor-binding domain of murine CD70 was fused with the Fc region of human IgG1. This was achieved using a modified Fc construct vector designed specifically to produce soluble type II transmembrane proteins, which has previously been used to generate a soluble form of OX40 ligand. The recombinant OX40 ligand was active *in vivo* and was demonstrated to be capable of inducing anti-tumour immunity [149]. This type of construct was chosen because Fc fusion proteins generally appear to fold correctly, and can be expressed at reasonably high levels in mammalian cells. In addition, an Fc tag may give the advantage of increasing the *in vivo* half-life of the fusion protein via it's ability to bind to the FcRn salvage receptor [225].

3.2 Results

3.2.1 Generation of a soluble form of murine CD70

A recombinant soluble fusion protein of murine CD70 (sCD70) was generated by tagging the extracellular domain (amino acid residues 41-195) with the Fc region of human IgG1. The modified Fc construct encodes a leader sequence followed by the hinge-CH2-CH3-modified hinge regions of human IgG1. This modified hinge lacks its disulphide-bonding cysteine residues, therefore allowing greater flexibility of the attached protein. The extracellular domain cDNA of CD70 was inserted 3' of the Fc domain (see Methods section 2.2.1, Figure 3.1 and 3.2 sequence map). In this way, the Fc domain replaces the cytoplasmic and transmembrane domains of natural CD70, and thus it is unlikely that this orientation of the Fc domains will hinder interaction of sCD70 with CD27. sCD70 was expressed in mammalian CHO K1 cells and purified by immunoaffinity chromatography.

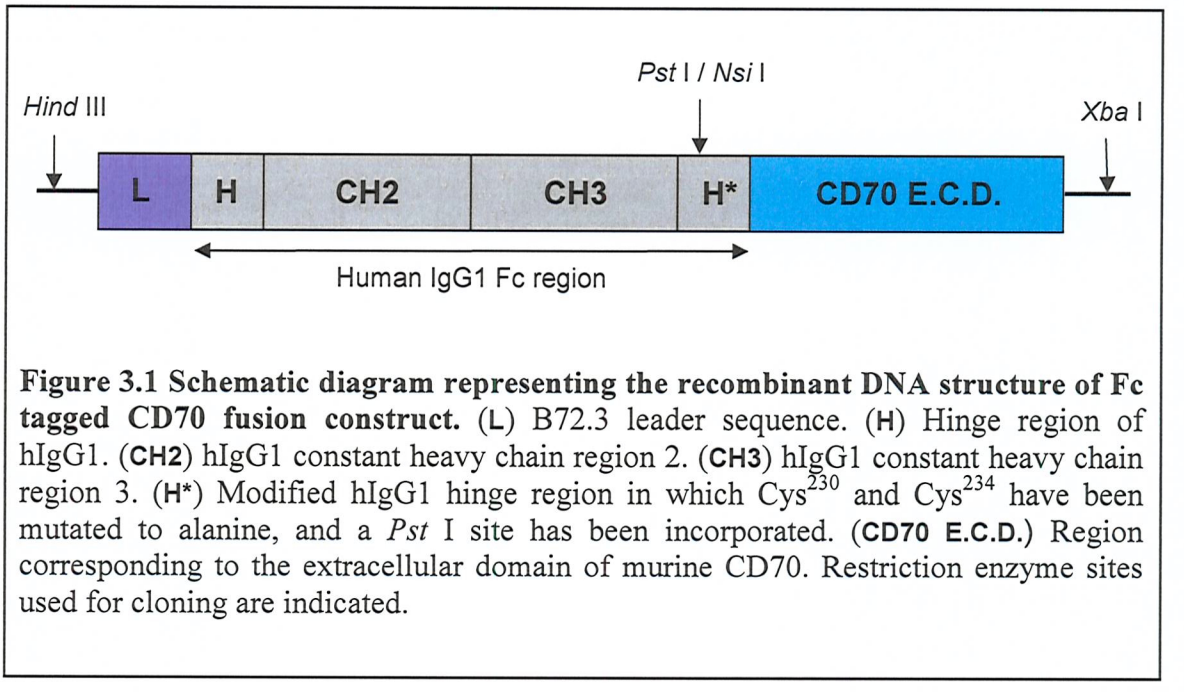
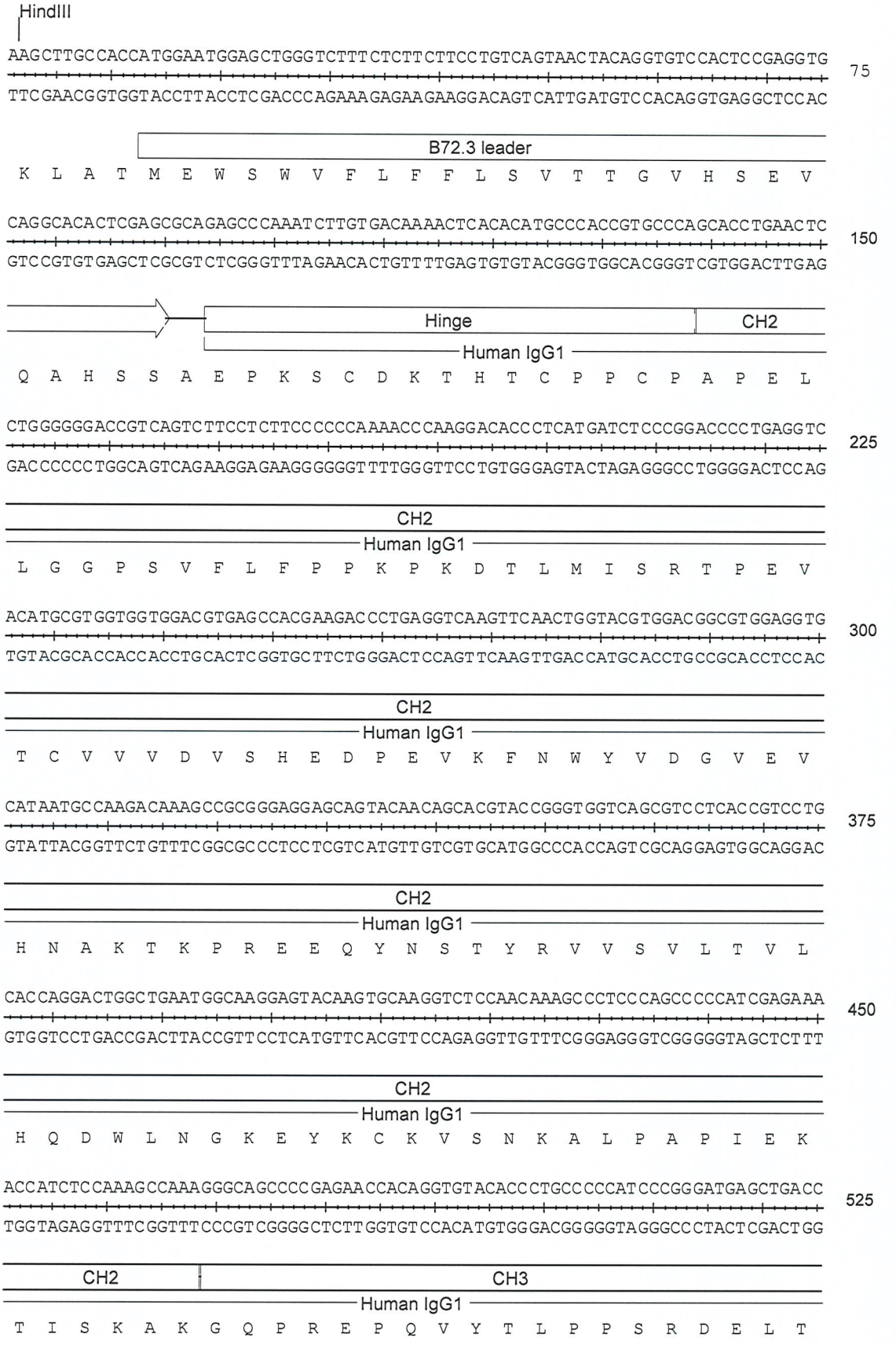


Figure 3.1 Schematic diagram representing the recombinant DNA structure of Fc tagged CD70 fusion construct. (**L**) B72.3 leader sequence. (**H**) Hinge region of hIgG1. (**CH2**) hIgG1 constant heavy chain region 2. (**CH3**) hIgG1 constant heavy chain region 3. (**H***) Modified hIgG1 hinge region in which Cys^{230} and Cys^{234} have been mutated to alanine, and a *Pst* I site has been incorporated. (**CD70 E.C.D.**) Region corresponding to the extracellular domain of murine CD70. Restriction enzyme sites used for cloning are indicated.


HindIII
AAGCTTGCCACCATGGAATGGAGCTGGGTCTTTCTCTTCTTCCTGTCAGTAACTACAGGTGTCCACTCCGAGGTG
TTCGAACGGTGGTACCTTACCTCGACCCAGAAAGAGAAGAAGGACAGTCATTGATGTCCACAGGTGAGGCTCCAC
75
B72.3 leader
K L A T M E W S W V F L F F L S V T T G V H S E V
CAGGCACACTCGAGCGCAGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC
GTCCGTGTGAGCTCGCGTCTCGGGTTTAGAACACTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAG
150
Hinge
CH2
Human IgG1
Q A H S S A E P K S C D K T H T C P P C P A P E L
CTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
GACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAG
225
CH2
Human IgG1
L G G P S V F L F P P K P K D T L M I S R T P E V
ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTG
TGTACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCAC
300
CH2
Human IgG1
T C V V V D V S H E D P E V K F N W Y V D G V E V
CATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGGGTGGTCAGCGTCCTCACCGTCCTG
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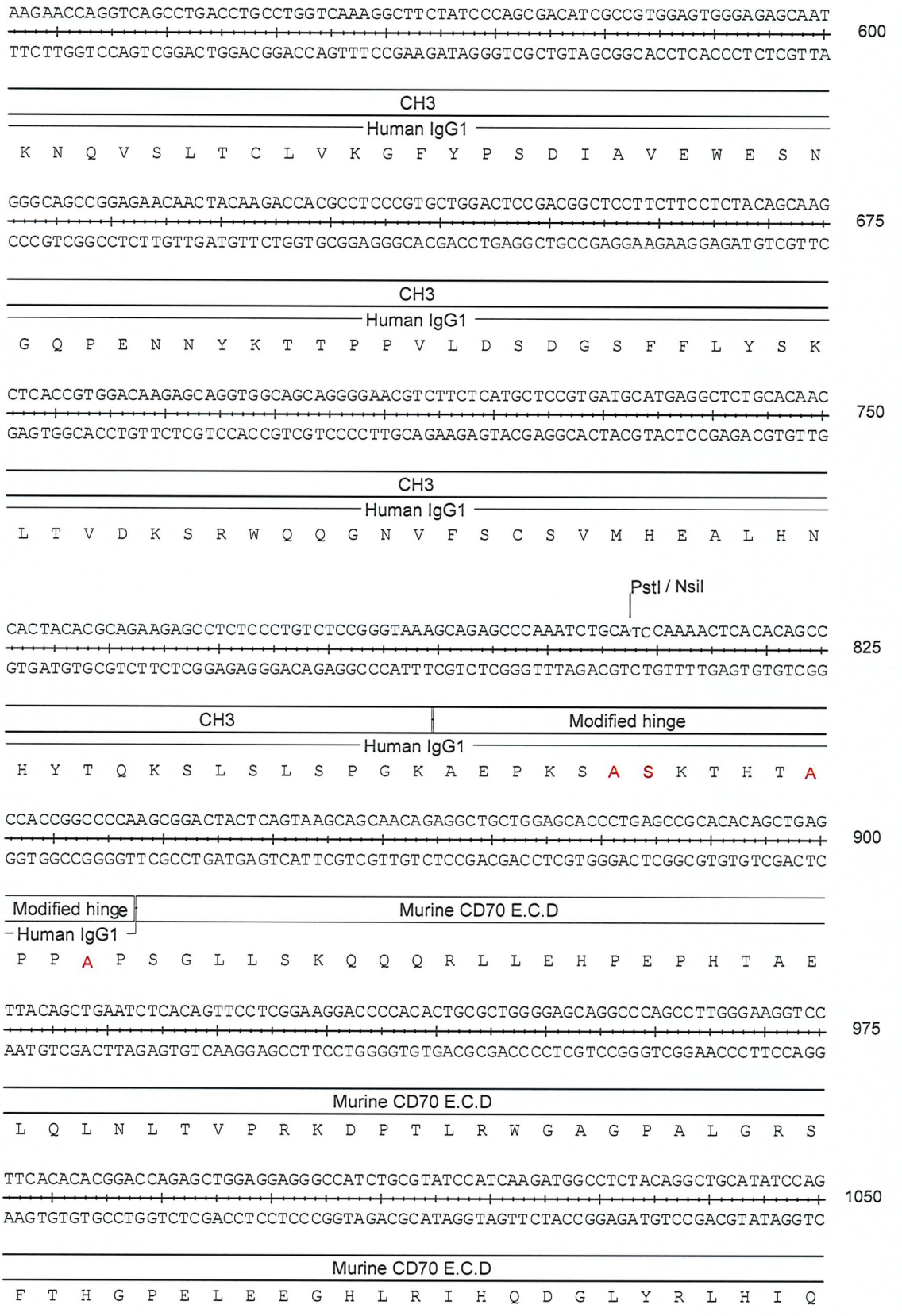
375
CH2
Human IgG1
H N A K T K P R E E Q Y N S T Y R V V S V L T V L
CACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAA
GTGGTCCTGACCGACTTACCGTTCCTCATGTTCACGTTCCAGAGGTTGTTTCGGGAGGGTCGGGGGTAGCTCTTT
450
CH2
Human IgG1
H Q D W L N G K E Y K C K V S N K A L P A P I E K
ACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
TGGTAGAGGTTTCGGTTTCCCGTCGGGGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGG
525
CH2
CH3
Human IgG1
T I S K A K G Q P R E P Q V Y T L P P S R D E L T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TTCTTGGTCCAGTCGGACTGGACGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTA
600
CH3
Human IgG1
K N Q V S L T C L V K G F Y P S D I A V E W E S N
GGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAG
CCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGATGTCGTTC
675
CH3
Human IgG1
G Q P E N N Y K T T P P V L D S D G S F F L Y S K
CTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAAC
GAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTG
750
CH3
Human IgG1
L T V D K S R W Q Q G N V F S C S V M H E A L H N
PstI / NsiI
CACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGCAGAGCCCAAATCTGCATCCAAAACTCACACAGCC
GTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTCGTCTCGGGTTTAGACGTCTGTTTTGAGTGTGTCGG
825
CH3
Modified hinge
Human IgG1
H Y T Q K S L S L S P G K A E P K S A S K T H T A
CCACCGGCCCCAAGCGGACTACTCAGTAAGCAGCAACAGAGGCTGCTGGAGCACCCTGAGCCGCACACAGCTGAG
GGTGGCCGGGGTTCGCCTGATGAGTCATTCGTCGTTGTCTCCGACGACCTCGTGGGACTCGGCGTGTGTCGACTC
900
Modified hinge
Murine CD70 E.C.D
Human IgG1
P P A P S G L L S K Q Q Q R L L E H P E P H T A E
TTACAGCTGAATCTCACAGTTCCTCGGAAGGACCCCACACTGCGCTGGGGAGCAGGCCCAGCCTTGGGAAGGTCC
AATGTCGACTTAGAGTGTCAAGGAGCCTTCCTGGGGTGTGACGCGACCCCTCGTCCGGGTCGGAACCCTTCCAGG
975
Murine CD70 E.C.D
L Q L N L T V P R K D P T L R W G A G P A L G R S
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Murine CD70 E.C.D
F T H G P E L E E G H L R I H Q D G L Y R L H I Q

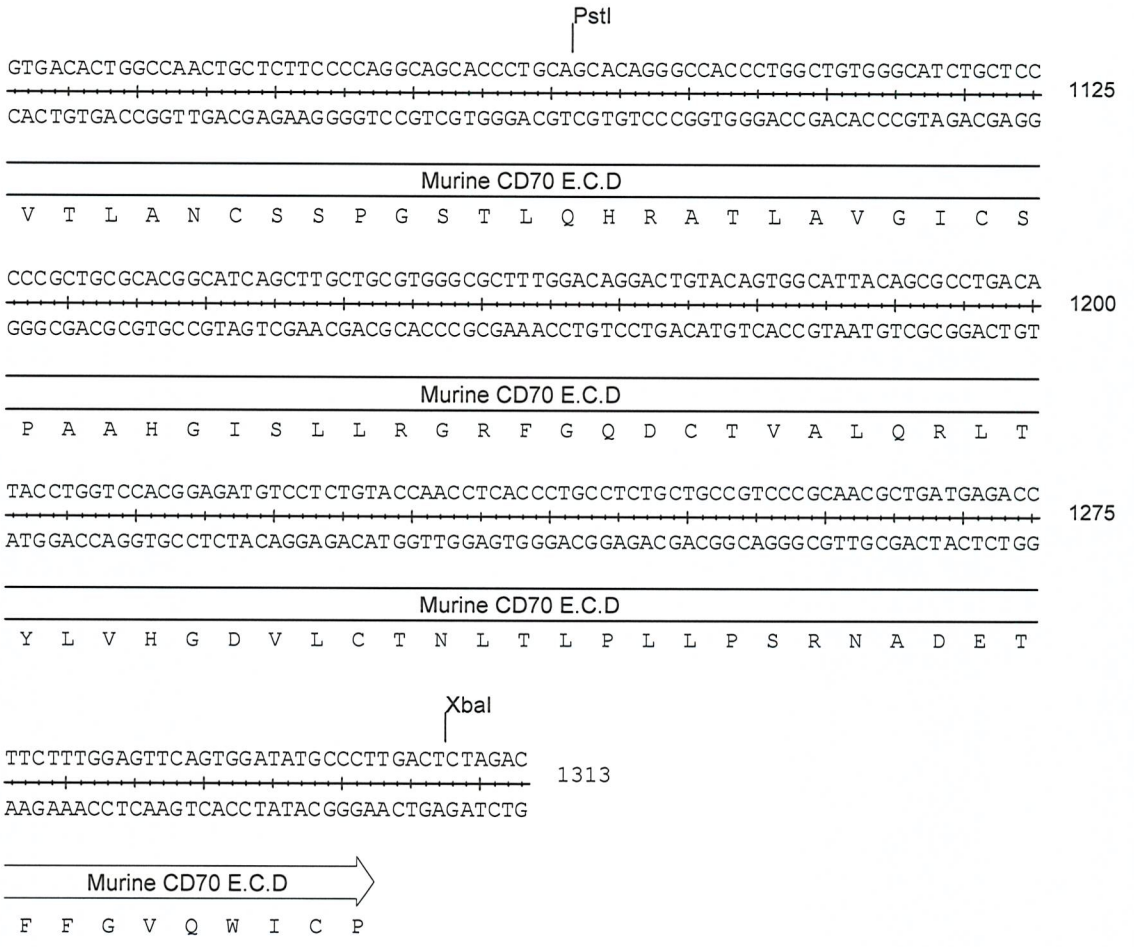


Figure 3.2 Sequence map of the recombinant DNA structure of sCD70. Regions corresponding to the B72.3 leader sequence; the hIgG1 hinge, CH2, CH3, modified hinge; and the extracellular domain of murine CD70 are indicated below the cDNA sequence. Amino acid sequence is also shown. The restriction sites *Hind*III, *Pst*I/*Nsi*I and *Xba*I that were used for cloning are marked. Because of the presence of a *Pst*I site in the extracellular domain of CD70, the sequence was instead cut with the enzyme *Nsi*I, which generates a compatible overhang with *Pst*I allowing ligation in frame with the modified hinge region. The D→S mutation caused by this is indicated in red, as are the C→A mutations that remove the disulphide-bonding cysteines in the modified hinge.

3.2.2 Structural analysis of sCD70 by SDS-PAGE and size-exclusion chromatography

To further characterise the structure of sCD70, it was analysed by denaturing SDS-PAGE (Figure 3.3a). Under reducing conditions sCD70 was resolved as a single band with an apparent molecular mass (M_r) of ~ 50 kDa. This is in agreement with the predicted M_r of 44.47 kDa for a single polypeptide chain of sCD70. Under non-reducing conditions, sCD70 formed multiple bands of ~ 100 kDa, ~ 150 kDa and > 175 kDa. This indicated that the denatured sCD70 polypeptide forms dimers, trimers and higher-order multimers via covalent disulphide bonding between cysteine residues, most probably within the hFc hinge region. The structure of sCD70 in its native form in solution was analysed by size-exclusion chromatography (Figure 3.3b). Two major forms of the protein could be seen, one represented by a broad peak eluting between the 200 kDa and 670 kDa M_r markers, and another with a M_r > 670 kDa. In addition a minor peak of ~ 150 kDa was observed. This suggested that in its native form sCD70 was forming high M_r multimers that may be stabilised by non-covalent interactions between polypeptide chains, in addition to covalent disulphide bonding. However, the exact stoichiometry of the native forms of sCD70 could not be determined because of the tendency of size-exclusion chromatography to overestimate the size of asymmetric proteins [226].

3.2.3 Specificity of sCD70 binding to murine lymphocytes

The ability of sCD70 to bind specifically to the receptor CD27 was investigated by attempting to block the binding of an anti-CD27 mAb to murine splenocytes with an excess of sCD70 (Figure 3.4a). The presence of sCD70 significantly reduced the binding of anti-CD27 mAb to CD27, indicating that sCD70 was competing for receptor binding. However, the binding of anti-CD27 mAb could not be completely inhibited, suggesting that the epitope recognised by this antibody does not fully overlap with the ligand-binding site. The expression pattern of CD27 on naïve murine lymphocytes was analysed by staining with sCD70 followed by detection with a FITC labelled anti-hFc mAb (Figure 3.4b). Double staining with anti-CD8 and anti-CD4 mAb showed that the majority of both the $CD8^+$ and $CD4^+$ T cell subsets expressed CD27. In contrast, double staining with anti-CD19 (a pan B cell marker) demonstrated that CD27 was only expressed on a minor subset of B cells with 1.5 % being stained

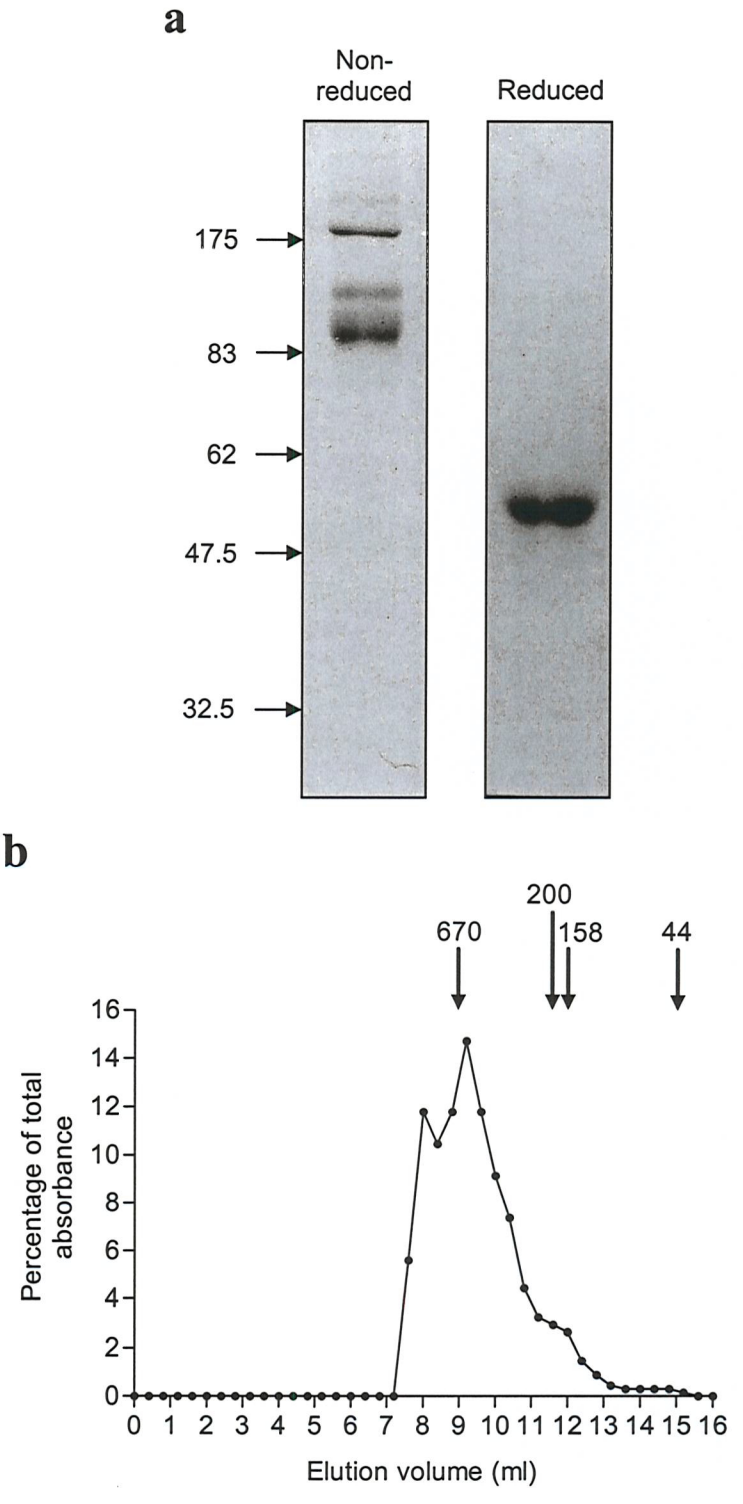


Figure 3.3 Structural analysis of sCD70 by SDS-PAGE and size exclusion chromatography. (**a**) SDS-PAGE. 5 μg of purified sCD70, either non-reduced or reduced with 50 mM DTT, was loaded onto an 8.5 % polyacrylamide gel. The positions of relevant molecular mass markers are indicated (kDa). The data are representative of multiple independent experiments. (**b**) Size-exclusion chromatography. 100 μg of sCD70 was separated on a Superdex 200 column at 0.4 ml/min. The elution positions of relevant molecular mass markers are indicated (Thyroglobin 670 kDa, β-amylase 200 kDa, γ-globin 158 kDa, Ovalbumin 44 kDa). The data are representative of two independent experiments.

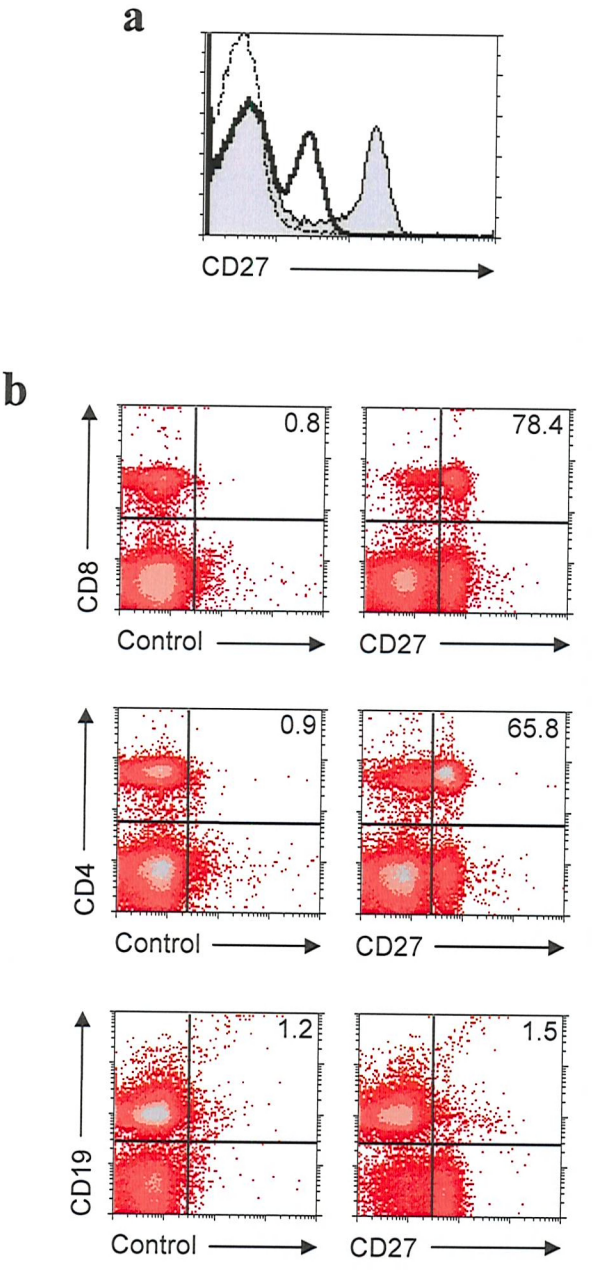


Figure 3.4 Specificity of sCD70 binding to murine lymphocytes. (a) Blocking the binding of an anti-CD27 mAb using sCD70. Naïve BALB/c splenocytes were pre-incubated for 30 min with 50 μg/ml sCD70 or hIgG control and were then stained with anti-CD27-PE in the presence of 50 μg/ml sCD70 (open histogram) or hIgG control (filled histogram), and compared to an isotype control-PE stained in the presence of hIgG (dashed line). Anti-FcR mAb (2.4G2, 10 μg/ml) was present at all stages to block non-specific binding. **(b)** Expression of CD27 on $CD4^+$ T cells, $CD8^+$ T cells and B cells. Naïve BALB/c splenocytes were double stained with anti-CD8-APC, anti-CD4-APC or anti-CD19-PE and either sCD70 or hIgG control followed by anti-hFc-FITC. Anti-FcR mAb (2.4G2, 10 μg/ml) was present at all stages to block non-specific binding. Plots are gated on resting lymphocytes. The percentages of $CD4^+$, $CD8^+$ or $CD19^+$ cells also expressing CD27, or background staining, are indicated. The data are representative of two independent experiments.

with sCD70 compared to 1.2 % non-specific background staining. These results are in agreement with the published expression pattern of CD27, confirming the specificity of sCD70 binding [190].

3.2.4 Anti-CD70 mAb (TAN1-6) blocks the binding of sCD70 to activated T cells

To additionally verify that sCD70 binds to lymphocytes through its CD70 extracellular domain and not its Fc domain, an anti-CD70 mAb, TAN1-6, was used to attempt to inhibit the binding of sCD70 to CD27 expressed on activated T cells (Figure 3.5a). The presence of TAN1-6 completely blocked the binding of sCD70 to activated T cells. The specificity of TAN1-6 mAb was confirmed by staining COS-7 cells which have been transfected either with full length murine CD70, or mock transfected (Figure 3.5b, left hand plot). TAN1-6 bound strongly to the transfected cells, but did not stain the mock-transfected cells. In addition, TAN1-6 detected expression of native membrane-bound CD70 on activated B cells but did not bind to naïve B cells (right hand plots). This pattern concurs with the expected up-regulation of CD70 expression after B cell activation [94]. As positive controls, naïve or activated B cells were stained with a commercially available anti-CD70 mAb or an anti-B7-2 mAb, which confirmed the predicted expression of CD70 and the co-stimulatory ligand B7-2 on activated B cells but not naïve B cells [94].

3.2.5 sCD70 co-stimulates the proliferation of murine T cells

The ability of sCD70 to bind to its receptor is not necessarily an indicator of its capacity to transduce signals. Therefore, the functional outcome of sCD70 binding was investigated by testing its ability to co-stimulate the proliferation of T cells in response to TCR stimulation by an agonistic anti-CD3 mAb (Figure 3.6). sCD70 enhanced the proliferation of splenocytes at all concentrations of anti-CD3 mAb tested, even at a range where the response to anti-CD3 alone was saturating (0.25-0.5 μg/ml). At sub-optimal concentrations of anti-CD3 (e.g. 0.063 μg/ml) the effect of sCD70 was most dramatic, increasing T cell proliferation by 2.8-fold. In the absence of anti-CD3 mAb, sCD70 had no effect on the proliferation of resting T cells. This confirms that the co-stimulatory effect of sCD70 was acting in synergy with TCR signalling and that it had no mitogenic effects in isolation.

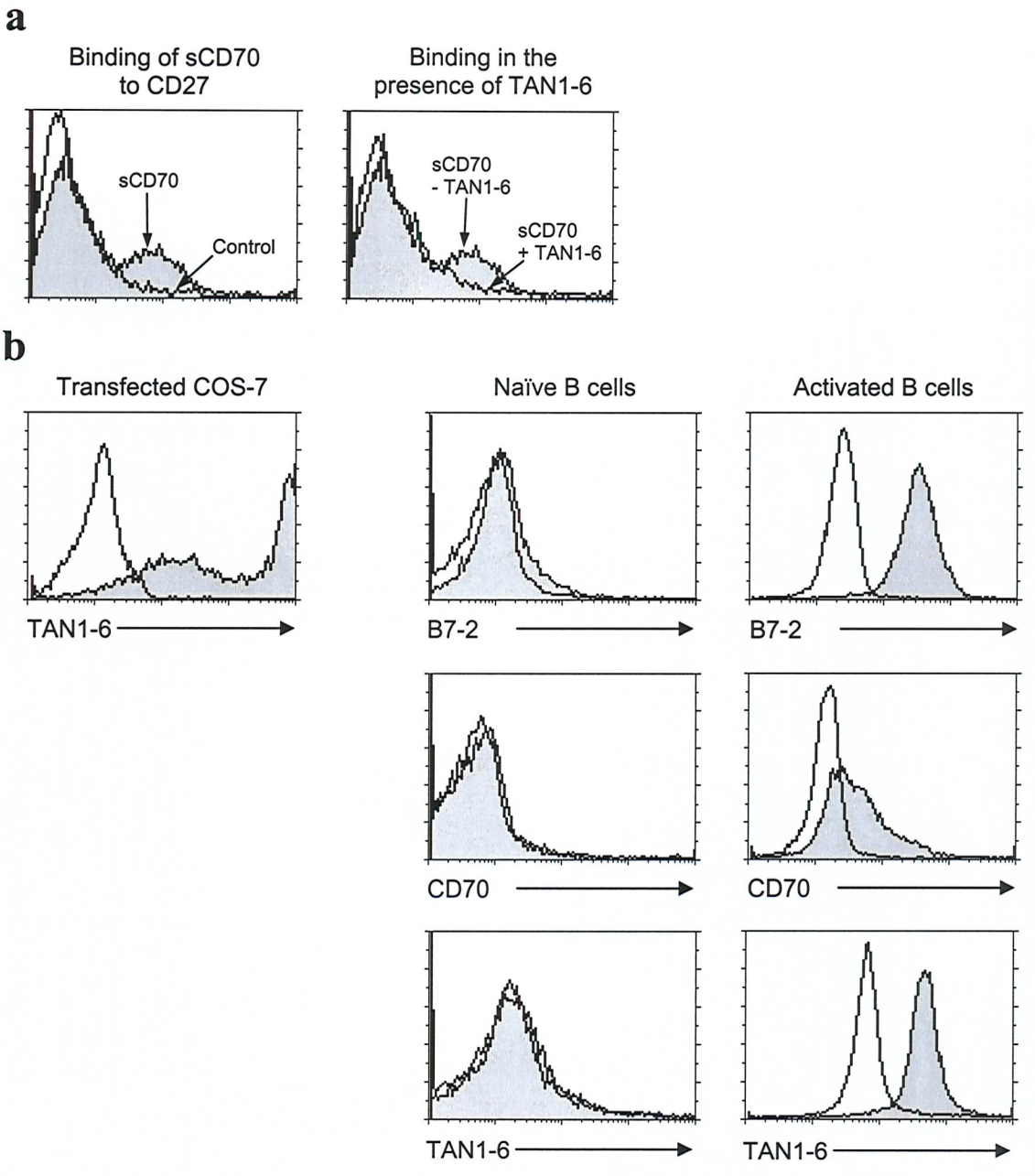


Figure 3.5 Anti-CD70 mAb (TAN1-6) blocks the binding of sCD70 to activated T cells. (**a**) TAN1-6 blocks the binding of sCD70 to activated T cells. Left hand plot, activated T cells (BALB/c splenocytes activated with 1 μg/ml anti-CD3 for 24 h) were stained for CD27 expression using sCD70 (filled histogram), or control stained with hIgG (open histogram) followed by anti-hFc-FITC. Right hand plot, activated T cells were stained for CD27 expression using sCD70 followed by anti-hFc-FITC in the presence (open histogram) or absence (filled histogram) of TAN1-6 hybridoma supernatant. Histograms are gated on live lymphocytes. **(b)** Specificity of TAN1-6 binding. Left hand plot, COS-7 cells transfected with full length murine CD70 (filled histogram), or mock transfected (open histogram), were stained with TAN1-6 anti-CD70 mAb followed by anti-rat FITC. Right hand panel, Naïve or activated B cells (BALB/c splenocytes activated with 10 μg/ml CD40L.Fc, 10 μg/ml LPS for 48 h) were stained with anti-B7-2-FITC, TAN1-6 followed by anti-Rat-PE or a commercially available biotinylated anti-CD70 mAb followed by Streptavadin PE (filled histograms). Control staining is shown (open histograms). Histograms are gated on resting (naïve) or blasted (activated) lymphocytes.

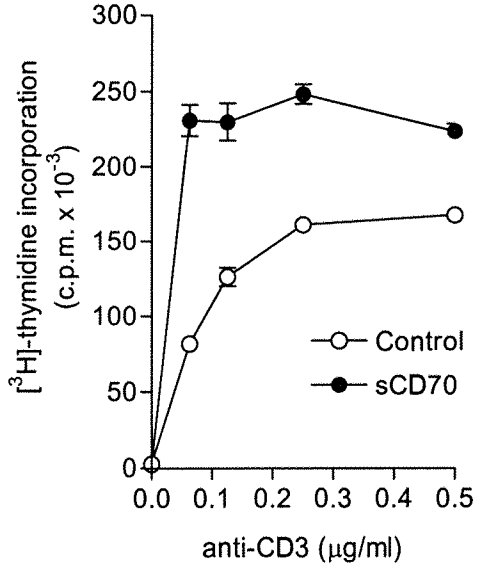


Figure 3.6 sCD70 co-stimulates the proliferation of murine T cells. 2 x 10^5 BALB/c splenocytes were cultured with the indicated titration of anti-CD3 mAb (145.2C11) in the presence of 10 μg/ml sCD70, or a control Ig. 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Error bars represent the SEM of triplicate cultures. The data are representative of three independent experiments.

3.3 Discussion

3.3.1 Structure of sCD70

A soluble recombinant form of the natural ligand for murine CD27 (CD70) has been generated, which can provide potent co-stimulatory signalling to T cells. Characterisation of this protein by SDS-PAGE and size-exclusion chromatography demonstrated that it forms large multimeric structures in solution, mediated by covalent disulphide bonding and non-covalent interactions (Figure 3.3). The crystallographic structures of several TNF-family members including TNFα, TNFβ, CD40L and TRAIL have been determined, and show that their three-dimensional structure is highly conserved despite relatively low sequence homology across the family [227-230]. These ligands all form self-assembling non-covalent trimers, with each monomer folding as a compact 'jellyroll' β-sandwich and interacting with other subunits via hydrophobic core interfaces [157]. The crystallographic structure of CD70 has not been elucidated, but comparative molecular modelling using the known structures of TNFα and TNFβ has predicted that CD70 also forms a homotrimeric complex, as do other members of this family such as FasL and 4-1BBL [231]. The extracellular domain of human CD70 has also been predicted to contain one intra-chain disulphide bond that may stabilise the β-sheet structure of each monomer [232]. The immunoprecipitation of endogenous murine CD70 from a B cell line has demonstrated that it forms a trimeric SDS-stable complex under non-reducing conditions [183]. However, it is unclear from this study whether the precipitated CD70 was heat denatured before SDS-PAGE analysis. Therefore, this SDS-resistant trimeric structure may have been stabilised by the presence of covalent intra-chain bonds. TNF family members have not generally been reported to form inter-chain disulphide bonds [232]. The inter-chain disulphide bonds observed in the denatured but non-reduced Fc-CD70 fusion protein (Figure 3.3a) are therefore most likely to be mediated by cysteine bonds between the hinge regions of the IgG1 Fc domains [233].

The large multimeric forms of native sCD70 seen in solution (Figure 3.3b) are therefore likely to be stabilised by the trimerisation of CD70 extracellular domains in conjunction with disulphide bonding between Fc domains. The predicted multimeric structures generated upon the fusion of dimeric IgG1 Fc domains and trimeric CD70

extracellular domains are represented schematically in Figure 3.7. The ~ 300 kDa hexameric structure is analogous to the structure of a similar Fc-Fas ligand fusion protein observed by electron microscopy [234]. In addition, sCD70 may form a dodecameric structure of ~ 600 kDa that would correspond to the largest peak seen by size-exclusion chromatography.

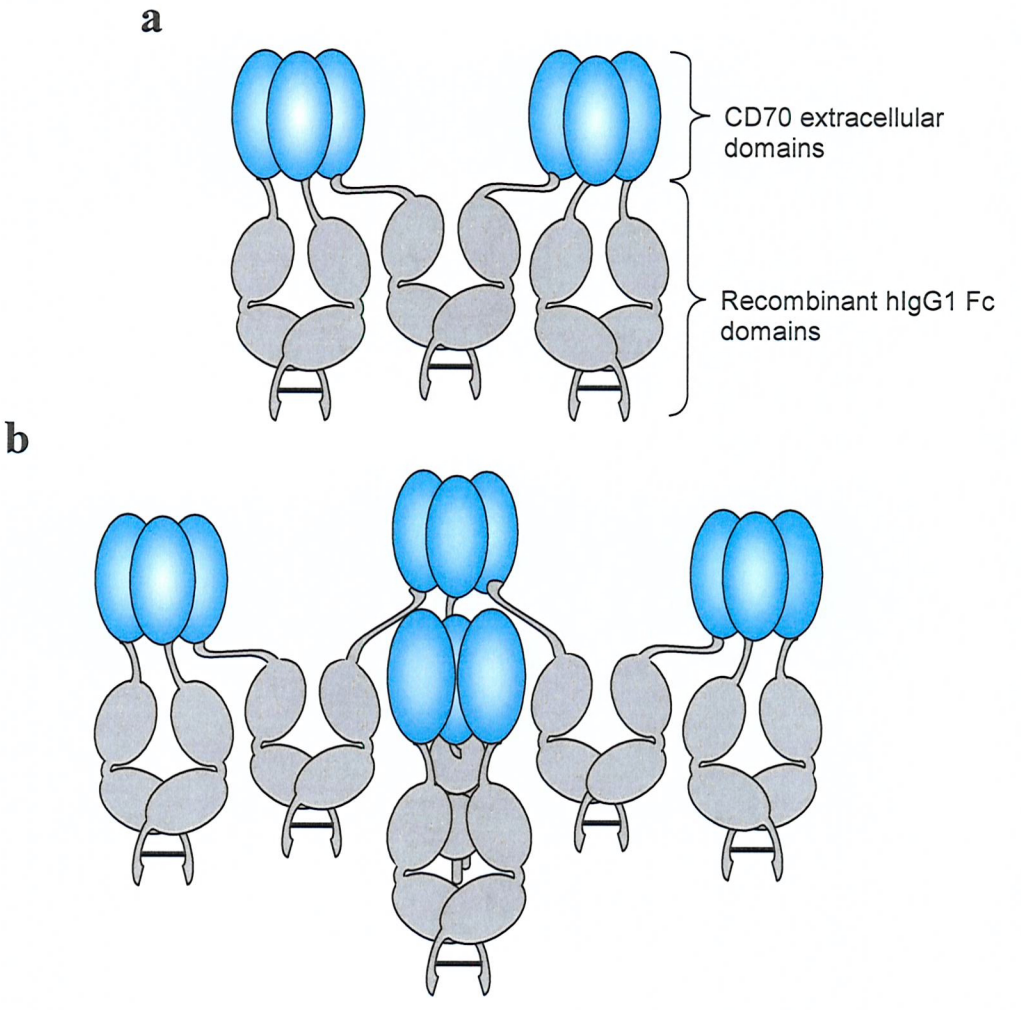


Figure 3.7 Schematic representations of the predicted major structures of sCD70. **(a)** Hexameric sCD70 corresponds to a M_r of ~ 300 kDa. **(b)** Dodecameric sCD70 corresponds to a M_r of ~ 600 kDa. Regions of disulphide bonding within the hIgG1 Fc hinge are represented by black lines.

3.3.2 Specificity and activity of sCD70

The ability of sCD70 to bind to CD27 was examined. sCD70 binds specifically to the receptor CD27, as shown by its ability to block the binding of an anti-CD27 mAb, and for its binding to be blocked by an anti-CD70 mAb (Figures 3.4 and 3.5). Furthermore, staining murine lymphocytes with sCD70 revealed that CD27 is expressed on the majority of $CD4^+$ and $CD8^+$ T cells, but is only expressed on a small minority of B cells. This data is in agreement with other expression studies in mice [176,190] and mirrors the expression of CD27 in humans [235], therefore corroborating the specificity of the sCD70 construct generated. sCD70 dramatically enhanced the proliferation of T cells responding to TCR stimulation via anti-CD3 mAb (Figure 3.6). This demonstrated that this soluble reagent is capable of inducing potent co-stimulatory signalling *in vitro* without the need for cross-linking. In contrast, previous studies with agonistic anti-CD27 mAbs have shown that they require further cross-linking to mediate T cell co-stimulation [190]. The ability of sCD70 to form large multimeric platforms consisting of more than one trimeric ligand may confer its effective receptor signalling ability. It has previously been shown that the extent of receptor clustering directly regulates signalling by other members of the TNFR superfamily. For example, studies with CD40, Fas and TNFR-II have indicated that trimeric ligands are not sufficient to generate signals through these receptors and that high-order oligomeric ligands are required [163,234,236]. This will be discussed in more detail in Chapter 6.

CHAPTER 4

Analysis of the T cell co-stimulatory effects of CD27 *in vitro*

4.1 Introduction

CD27 is a relatively poorly characterised co-stimulatory member of the TNFR superfamily. In order to define more clearly the role of CD27 in T cell mediated responses and the molecular mechanisms responsible for mediating its effects, the soluble form of CD70 generated will be used to investigate the effects of CD27 stimulation on T cells *in vitro* (this chapter) and *in vivo* (Chapter 5). More specifically, it is unclear if CD27 acts predominantly during primary or secondary T cell responses, which subset of T cells it exerts its effects on and what influence CD27 has on T cell homeostasis. CD27 has been reported to preferentially influence the $CD8^+$ T cell subset [192], however $CD27^{-/-}$ mice have deficits in the $CD4^+$ subset as well as in $CD8^+$ T cells and it is unclear whether this is due to direct or indirect effects [109]. CD27 has been implicated as acting only as a survival factor for T cells without effecting cell cycle progression [109]. In contrast, other members of the TNFR family (e.g. OX40 and 4-1BB) have been shown to lower the threshold of TCR signalling required for activation and enhance cell division in a similar fashion to CD28 [73]. Thus, a more in depth examination of the manner by which CD27 promotes T cell expansion is warranted. Detailed analysis of the mechanisms underlying CD27-mediated T cell stimulation should lead to a greater understanding of the role of this co-stimulatory molecule in T cell responses and therefore its potential as an immunotherapeutic target.

4.2 Results

4.2.1 Comparison of the cell-surface expression and co-stimulatory effect of CD27 on CD4$^+$ and CD8$^+$ T cells

In order to determine whether CD27 co-stimulation predominantly affects the CD4$^+$ or the CD8$^+$ T cell compartment, the cell-surface expression and proliferative effects of CD27 on purified murine CD4$^+$ or CD8$^+$ T cells were compared with that of either OX40 or 4-1BB. OX40 and 4-1BB are TNFR-superfamily members that have previously been shown to be important in CD4$^+$ and CD8$^+$ T cell co-stimulation, respectively [151,153,237].

Purified CD4$^+$ and CD8$^+$ T cells were analysed by FCM over a time course of activation *in vitro,* using an anti-CD3 mAb to simulate TCR ligation (Figure 4.1). CD27 was constitutively expressed on the surface of naïve CD4$^+$ T cells and was marginally up regulated after activation by TCR-stimulation, reaching a peak of expression after 48 hours following which its expression started to drop slightly. In contrast, OX40 was not expressed on naïve CD4$^+$ T cells but was up regulated after activation, and reached its peak of expression after 48 hours. CD27 was also constitutively expressed on the surface of naïve CD8$^+$ T cells and was up regulated after activation with parallel kinetics and to a similar degree as on CD4$^+$ T cells. 4-1BB was initially absent from the surface of naïve CD8$^+$ T cells but was up regulated after activation reaching a peak at around 48 hours, after which it was rapidly down regulated.

The proliferation of purified CD4$^+$ and CD8$^+$ T cells 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. T cells were activated by culture with anti-CD3 mAb in the presence or absence of soluble ligands for CD27 (sCD70), 4-1BB (s4-1BBL, see Chapter 6 for characterisation) or OX40 (trimeric OX40L.rCD4 cross-linked with OX86 mAb) (Figure 4.2). CD27 co-stimulation could enhance the proliferation of CD4$^+$ T cells four-fold over that induced by 0.01 μg/ml anti-CD3 alone. This enhancement of proliferation was comparable to that induced by OX40 co-stimulation. CD27 co-stimulation increased the proliferation of CD8$^+$ T cells by up to

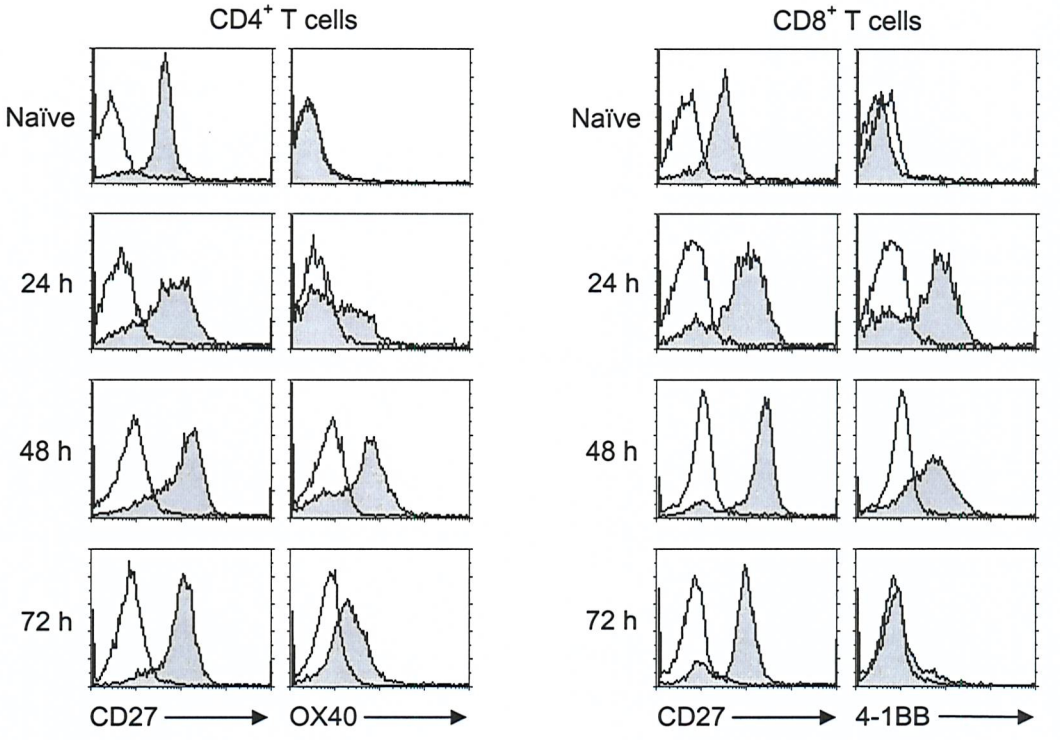


Figure 4.1 Cell-surface expression of CD27 on $CD4^+$ and $CD8^+$ T cells. $CD4^+$ or $CD8^+$ T cells were purified from BALB/c splenocytes using depletion columns and activated *in vitro* by culture in the presence of 1 μg/ml soluble anti-CD3 (145.2C11) for the indicated time periods. T cells were stained for surface expression of CD27 and either OX40 or 4-1BB using sCD70, OX40L.rCD4 or s4-1BBL respectively for primary staining, and FITC-conjugated anti-hFc (SB2H2) or anti-rat CD4 (OX68) for secondary staining (filled histograms). Isotype controls were hIgG primary stain and FITC-conjugated anti-hFc for CD27 and 4-1BB, or FITC-conjugated anti-rat CD4 alone for OX40 (open histograms). 10000 viable cells were analysed by FCM.

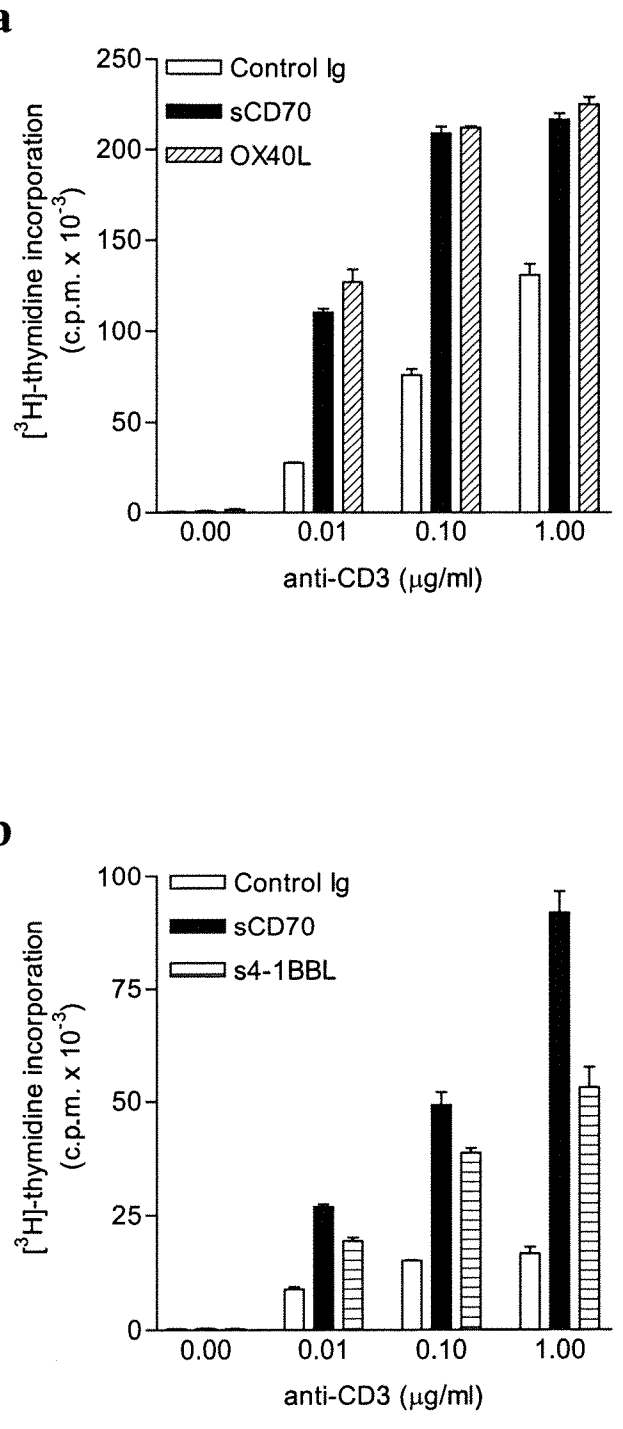


Figure 4.2 CD27 co-stimulation enhances the proliferation of both $CD4^+$ and $CD8^+$ T cells. 2 x 10^5 T cells were purified from BALB/c splenocytes using depletion columns. (**a**) $CD4^+$ or (**b**) $CD8^+$ T cells were activated by culture in the presence of soluble anti-CD3 (145.2C11) plus 10 μg/ml control Ig (Mc3916), sCD70, s4-1BBL or OX40L.rCD4 and anti-rat CD4 cross-linker (OX68). 0.5 μCi [^{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.

5.5 fold, as compared to that induced by 1 μg/ml anti-CD3 alone. This enhancement of $CD8^+$ T cell proliferation was greater than that induced by 4-1BB upon engagement by soluble 4-1BB ligand. These experiments show that stimulation of CD27 using sCD70 can potently co-stimulate both the $CD4^+$ and $CD8^+$ T cell subsets.

4.2.2 CD27 co-stimulation enhances the primary responses of antigen-specific OT-I transgenic $CD8^+$ T cells

In order to investigate the effect of CD27 signalling on antigen-specific T cell responses, OT-I TCR-transgenic mice were utilised as source of $CD8^+$ T cells. These transgenic mice express a single T cell receptor specific for an ovalbumin-derived epitope (OVA $_{257-264}$) with the peptide sequence SIINFEKL, in the context of MHC class I H-$2K^b$ [220]. These mice have been crossed onto a *RAG1* knockout background, and are therefore lacking any other T cells or B cells. The cell-surface expression of CD27 on OT-I $CD8^+$ T cells was compared with that of 4-1BB and OX40 on naïve T cells and after 48 hours of activation, by pulsing splenocytes with a range of OVA $_{257-264}$ concentrations from 7.5 – 500 pM (Figure 4.3). It can be seen that CD27 was constitutively expressed on naïve OT-I T cells and was maximally up regulated by very low concentrations of antigen. In contrast, 4-1BB surface expression was dependent on antigen concentration with both the numbers of cells expressing, and the level of expression, increasing in a concentration dependent manner. The same was true for OX40, except its expression was less sensitive to low levels of antigen, which reflects the lesser role of OX40 in $CD8^+$ T cell co-stimulation [151].

The proliferation of naïve OT-I T cells was observed after either pulsing splenocytes with OVA $_{257-264}$ peptide, or by incubation with irradiated E.G7 cells. E.G7 are a derivative of the thymoma tumour cell line EL4, which has been stably transfected with ovalbumin cDNA. These cells express the epitope OVA $_{257-264}$ on MHC class I H-$2K^b$, and secrete soluble ovalbumin [214]. Co-stimulatory signalling through CD27, 4-1BB or OX40 was provided via soluble ligands (CD27 and 4-1BB) or antibodies (OX40). The proliferation of OT-I T cells responding to pulsed OVA $_{257-264}$, as measured by [^{3}H] thymidine incorporation, could be enhanced 2.7-fold by the presence of sCD70 as compared to 10 pM OVA $_{257-264}$ alone (Figure 4.4a). The proliferation of OT-I T cells in response to irradiated E.G7 was radically increased by sCD70, for

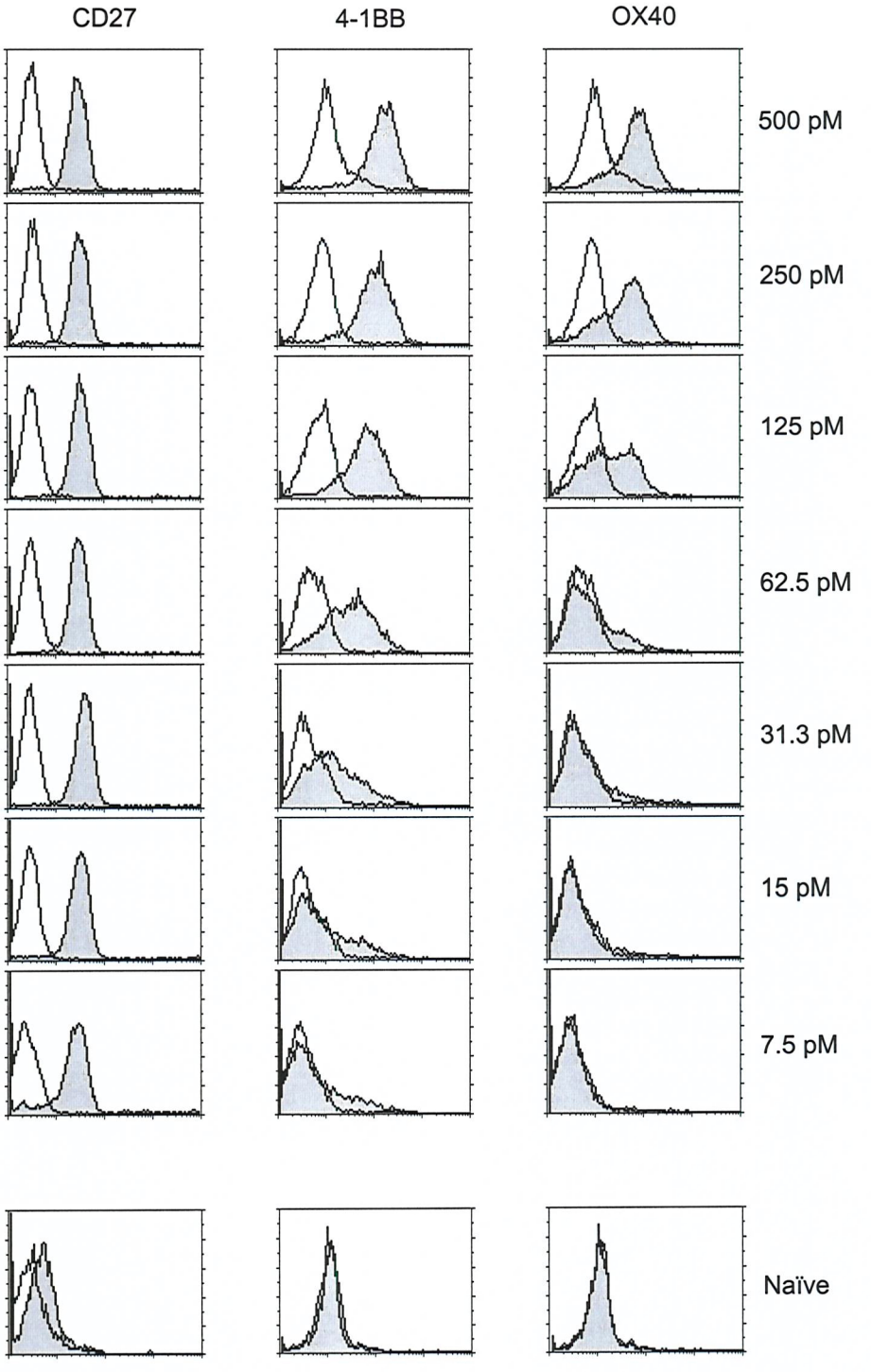


Figure 4.3 Expression of CD27 on CD8$^+$ TCR-transgenic OT-I T cells is sensitive to low levels of antigen. OT-I *RAG1*$^{-/-}$ splenocytes were stimulated with the indicated concentrations of $OVA_{257-264}$ peptide for 48 h. T cells were then stained for surface expression of CD27, 4-1BB or OX40 using sCD70, anti-4-1BB (LOB12) or anti-OX40 (OX86) respectively for primary staining, and FITC-conjugated anti-hFc (SB2H2) or goat anti-rat for secondary staining. Isotype controls were human IgG primary stain and FITC-conjugated anti-hFc for CD27, or irrelevant Ig (Mc106A5) primary stain and FITC-conjugated goat anti-rat for 4-1BB and OX40. ~10000 viable cells were analysed by FCM.

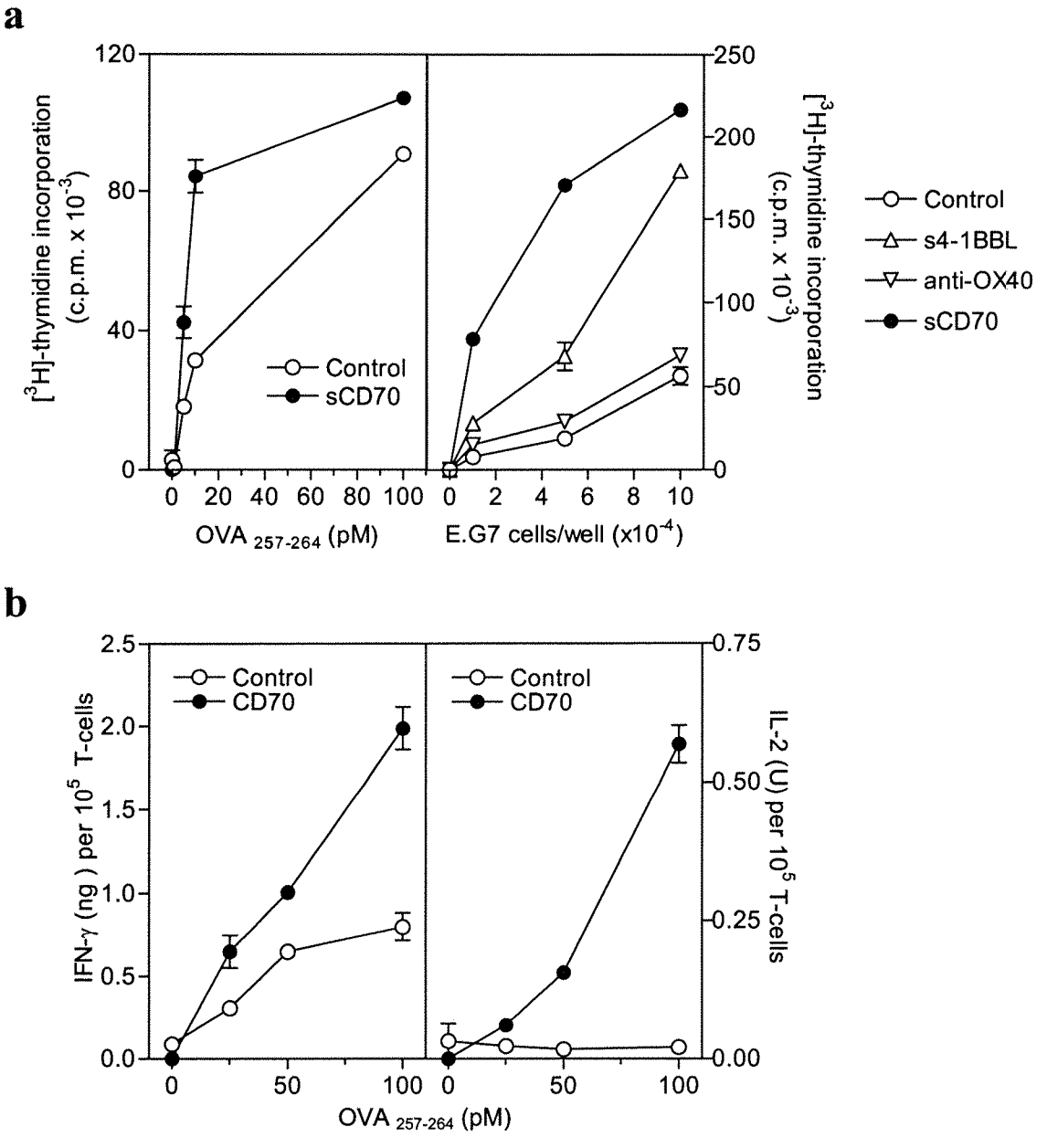


Figure 4.4 CD27 co-stimulation elevates the antigen-specific proliferation and cytokine production of naïve CD8$^+$ OT-I T cells. (**a**) 2 x 10^5 OT-I *RAG1*$^{-/-}$ splenocytes were cultured with the indicated concentrations of $OVA_{257-264}$ plus control Ig (OX68) or sCD70 (left), or cultured with the indicated numbers of irradiated E.G7, plus control Ig (Mc3916), sCD70, s4-1BBL or anti-OX40 (OX86) (right). For both experiments 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM. (**b**) OT-I *RAG1*$^{-/-}$ splenocytes were stimulated with the indicated concentrations of $OVA_{257-264}$ plus control Ig (Mc3916) or sCD70 for 48 h. IFN-γ (left) or IL-2 (right) concentrations in culture supernatant were determined by ELISA. Viable cells were enumerated and this value was used to calculate the amount of cytokine produced per 10^5 T cells. Graphs represent the mean of duplicate cultures with error bars representing one SD. All data are representative of two independent experiments.

example up to ten-fold at a ratio of E.G7-OT-I T cells of 0.05:1 (1 x 10^4 E.G7/well). 4-1BB co-stimulation by s4-1BBL also dramatically enhanced OT-I proliferation in response to E.G7, whereas OX40 had a negligible effect.

Cytokine production is an important effector function of T cells. Thus, it was investigated if CD27 signalling could induce OT-I $CD8^+$ T cell differentiation into cytokine-generating cells. OT-I splenocytes were cultured with OVA $_{257-264}$ in the presence or absence of sCD70. Production of the cytokines IL-2 and IFN-γ in cell supernatant was measured by ELISA after 48 hours of culture, and then calculated on a per cell basis using viable cell counts taken at the same time point (Figure 4.4b). CD27 co-stimulation increased the cellular production of IL-2 by ten-fold, and enhanced the production of IFN-γ by up to two-fold when compared with that produced by the highest concentration of OVA $_{257-264}$ alone.

To examine if sCD70 can stimulate T cells independently of the co-stimulatory molecule CD28, antibodies directed against the CD28 ligands, B7-1 and B7-2, were used to block CD28 signalling. Incubation of OT-I splenocytes with OVA $_{257-264}$ or E.G7 cells resulted in a dose-dependent proliferation of T cells, which was significantly inhibited by the presence of anti-B7-1 and anti-B7-2 mAbs (Figure 4.5). However, sCD70 enhanced T cell proliferation even in the presence of anti-B7-1 and anti-B7-2 blocking mAbs, suggesting that sCD70 triggers signalling independently of CD28. In addition, at high antigen levels (100 pM OVA $_{257-264}$ or 5 x 10^4 E.G7) sCD70 stimulated maximal T cell proliferation in the presence or absence of anti-B7 antibodies. This indicates that CD27 can fully replace the proliferative signal from CD28 under conditions of relatively strong TCR signalling. At low levels of OVA $_{257-264}$ (Figure 4.5a) or low numbers of E.G7 cells (Figure 4.5b), T cells proliferated to a greater extent when sCD70 was present in the culture and the B7-1/B7-2 interaction with CD28 was not blocked. These results suggest that CD27 acts in an additive fashion with CD28 in enhancing the proliferation of $CD8^+$ T cells.

To determine if CD27 signalling can enhance the activation status of antigen-stimulated T cells in the absence of CD28 signalling the cell surface expression of 4-1BB and OX40, whose up-regulation (as shown in Figure 4.3) is determined by the

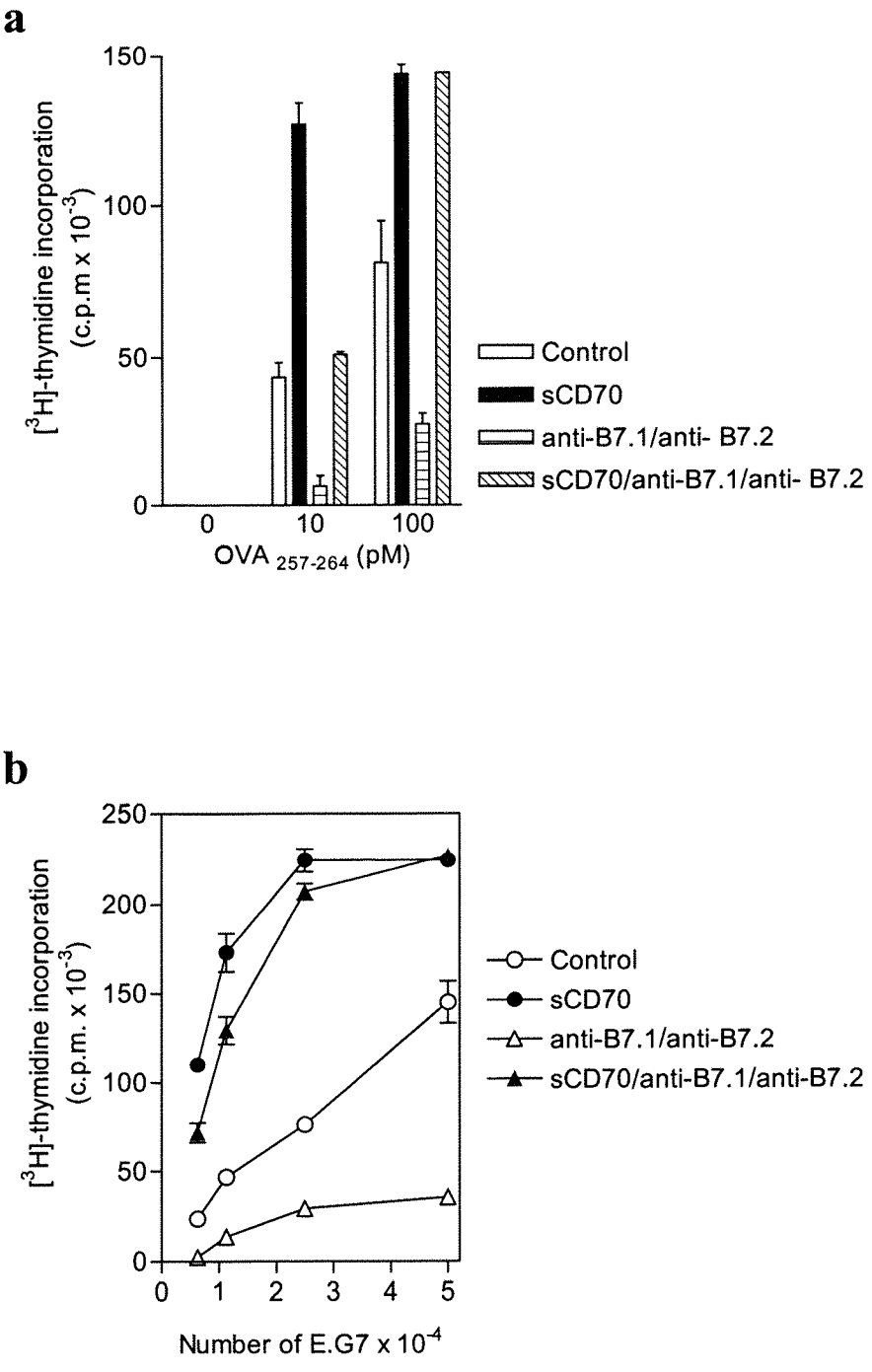


Figure 4.5 CD27 co-stimulation promotes CD28-independent CD8$^+$ T cell proliferation. 2 x 10^5 naïve OT-I *RAG1*$^{-/-}$ splenocytes were cultured with (**a**), the indicated concentrations of $OVA_{257-264}$, or (**b**), with the indicated numbers of irradiated E.G7 cells in the presence or absence of sCD70 and 10 μg/ml anti-B7.1 (GL1) / anti-B7.2 (1610A1) mAbs. For both experiments 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM. The data are representative of two independent experiments.

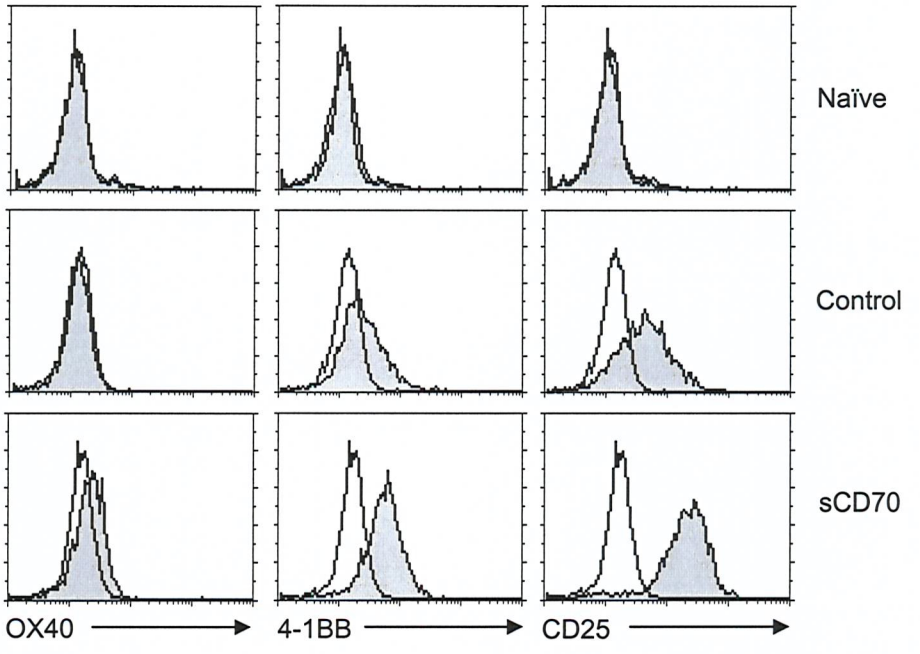


Figure 4.6 CD27 co-stimulation up-regulates CD8$^+$ T cell surface activation markers. Naïve OT-I *RAG1*$^{-/-}$ splenocytes were stimulated with 50 pM OVA $_{257-264}$ in the presence of 40 μg/ml anti-B7.1 (GL1) and anti-B7.2 (1610A1), and 10 μg/ml of either control hIgG or sCD70 for 48 h. Naïve, control or sCD70-activated T cells were stained for surface expression of OX40, 4-1BB or CD25 using FITC-labelled anti-OX40 (OX86), anti-4-1BB (LOB12.3) or anti-CD25 (3C7), respectively (filled histograms), or stained with isotype control mAbs (open histograms). Cells were double labelled with anti-CD8-PE and analysed by FCM. Histograms are gated on viable CD8$^+$ T cells. The data are representative of two independent experiments.

degree of TCR signalling, was examined. The expression of the IL-2 receptor α chain, CD25, which is required for efficient clonal expansion and induction of CTL activity [238] was also observed. OT-I splenocytes were activated *in vitro* for 48 hours by culture with 50 pM OVA $_{257-264}$, in the presence sCD70 or a control Ig. T cells were then stained for surface expression of OX40, 4-1BB and CD25 (Figure 4.6). OX40, 4-1BB and CD25 were absent from the surface of naïve OT-I T cells. No expression of OX40 was seen after stimulation with 50 pM OVA $_{257-264}$ alone, but low levels of OX40 were induced on the cell surface after CD27 co-stimulation. 4-1BB and CD25 were both expressed at low levels on a proportion of OT-I T cells after stimulation with 50 pM OVA $_{257-264}$ alone and this was enhanced by CD27 co-stimulation. These experiments demonstrate that CD27 can potently enhance proliferation and cytokine production by antigen–specific $CD8^+$ T cells during primary antigen responses. CD27 co-stimulation also enhances the activation status of T cells during primary responses, as seen by up-regulation of surface activation markers such as CD25 and TNFR superfamily members, in the absence of CD28 signalling.

4.2.3 CD27 co-stimulation augments the secondary responses of effector OT-I transgenic $CD8^+$ T cells

It was observed that CD27 is still highly expressed on effector OT-I T cells after four days of activation, suggesting that this molecule may also regulate the responses of antigen-experienced T cells. To assess the effect of CD27 co-stimulation during secondary responses, OT-I T cells were activated for 96 hours with 100 pM OVA $_{257-264}$ and 10 U/ml IL-2. These effector cells were then re-stimulated by culture with irradiated E.G7 cells in the presence or absence of sCD70 or s4-1BBL. The proliferation of re-stimulated OT-I T cells was measured after 72 hours by [^{3}H]-thymidine incorporation (Figure 4.7a). CD27 co-stimulation augmented the secondary proliferation response of these effector cells to E.G7. 4-1BB co-stimulation also increased the secondary proliferation of OT-I T cells and, in contrast to the case with primary proliferation, was more effective than CD27. The production of the cytokines IL-2 and IFN-γ by OT-I effector cells was measured after 48 hours re-stimulation with E.G7 (Figure 4.7b). IL-2 production was enhanced to a similar extent by CD27 or 4-1BB co-stimulation, as was IFN-γ production. Thus, CD27 may have a role in secondary T cell responses, as well as being involved during the initial priming phase.

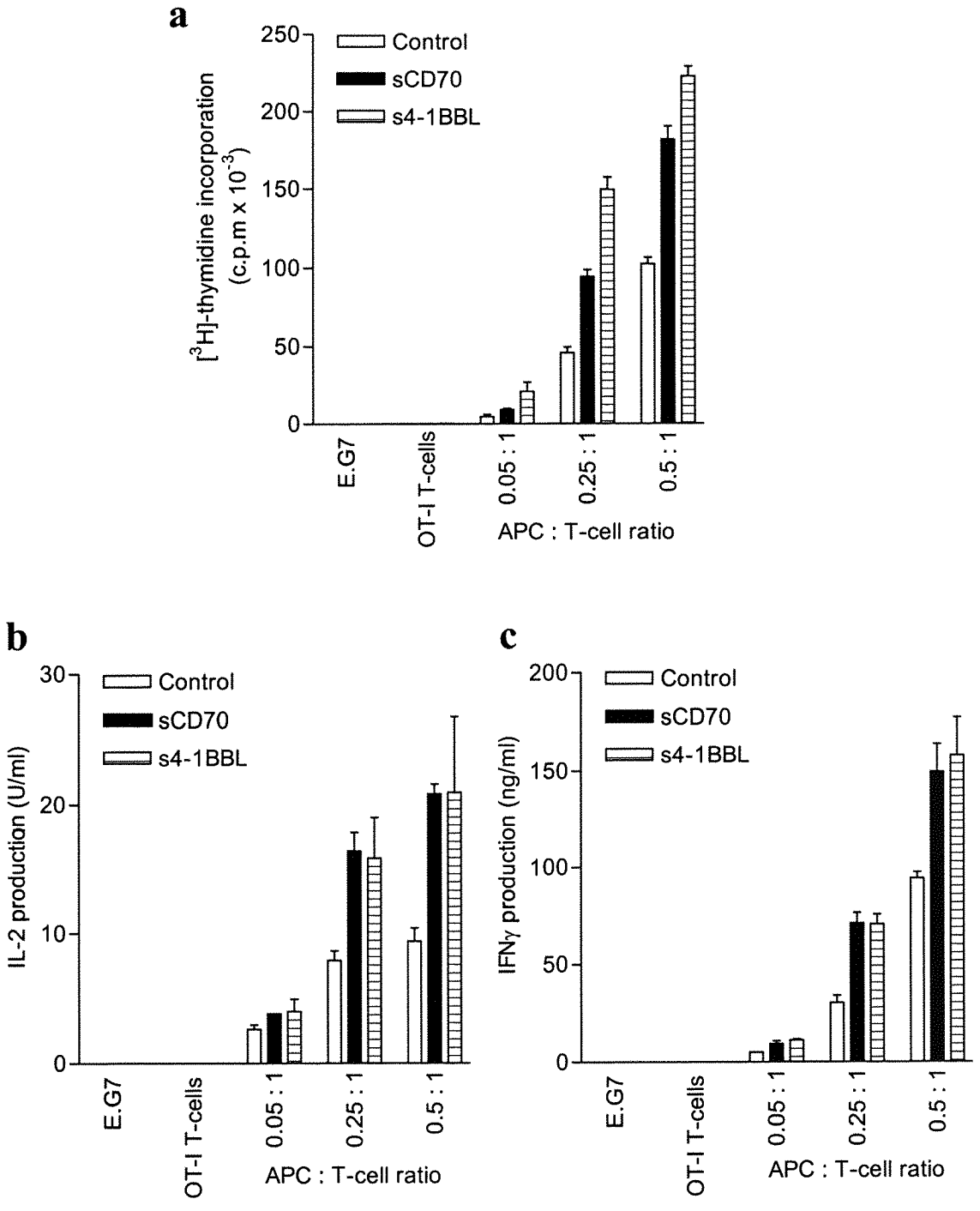


Figure 4.7 CD27 co-stimulation elevates proliferation and cytokine production by re-stimulated CD8$^+$ T cells. OT-I *RAG1*$^{-/-}$ splenocytes were stimulated *in vitro* with 100 pM of $OVA_{257-264}$ plus 10 U/ml IL-2 for 96 hours. 2 x 10^5 antigen experienced T cells were then re-stimulated *in vitro* by incubation with irradiated E.G7 cells at the indicated ratios, plus control Ig (hIgG), sCD70 or m4-1BBL. (**a**) 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM. Cell supernatants were analysed after 48 h for (**b**) IL-2, and (**c**) IFN-γ, by ELISA. Graphs represent the mean of duplicate cultures with error bars representing one SD. The data are representative of two independent experiments

4.2.4 CD27 signalling induces T cell division

The proliferation assays shown previously use the incorporation of [^{3}H]-thymidine as a measure of DNA replication. In these assays, an increase in proliferation may indicate that the cells are undergoing more cycles of division. Alternatively, an increase in incorporation could indicate that there are greater numbers of viable cells in the culture, which are undergoing the same rate of division. In order to distinguish between the possibilities of CD27 signalling inducing either an increase in cell division, or an increase in cell survival, T cells were CFSE (5,(6)-carboxyfluorescein diacetate succinimidyl ester) labelled to enable cell division to be tracked specifically. CFSE is a fluorescent acetate ester dye, which when loaded into cells remains within the cytoplasm. Consequently, when a labelled cell divides the fluorescence intensity of the dye is reduced by half. This allows the number of divisions a cell has undergone to be tracked using flow cytometry.

OT-I splenocytes were labelled with CFSE and cultured with OVA $_{257-264}$ in the presence of sCD70 or an anti-CD28 mAb to compare the effects of CD27 and CD28, respectively, on OT-I T cell division. CD28 was used for comparison as signalling via this molecule has previously been shown to enhance T cell division [239]. Blocking antibodies against B7-1 and B7-2, the natural ligands for CD28, were added in the culture in order to investigate the CD28-independent effects of CD27 signalling. With a high level of antigen present, (100 pM OVA $_{257-264}$, Figure 4.8a) initially a similar rate of division was seen under all conditions, with no cell division after 24 hours (not shown) and T cells having undergone up to three cell divisions by 48 hours. However, after 72 hours activation OT-I T cells had undergone up to six cell divisions, with the majority of those stimulated through either CD27 or CD28 having undergone four to six divisions. In comparison, a relatively greater proportion of the control stimulated T cells were still in division two or three. At 96 hours the control T cells had not undergone significantly more division than at 72 hours, whereas T cells stimulated through CD27 or CD28 were continuing to divide. Under sub-optimal antigen conditions (10 pM OVA $_{257-264}$, Figure 4.8b), the majority of the control OT-I T cells did not undergo any cell division, even after 96 hours. However, with CD27 co-stimulation a large proportion of cells underwent one or two divisions, with some cells undergoing three to six divisions. CD28 co-stimulation enhanced division even more

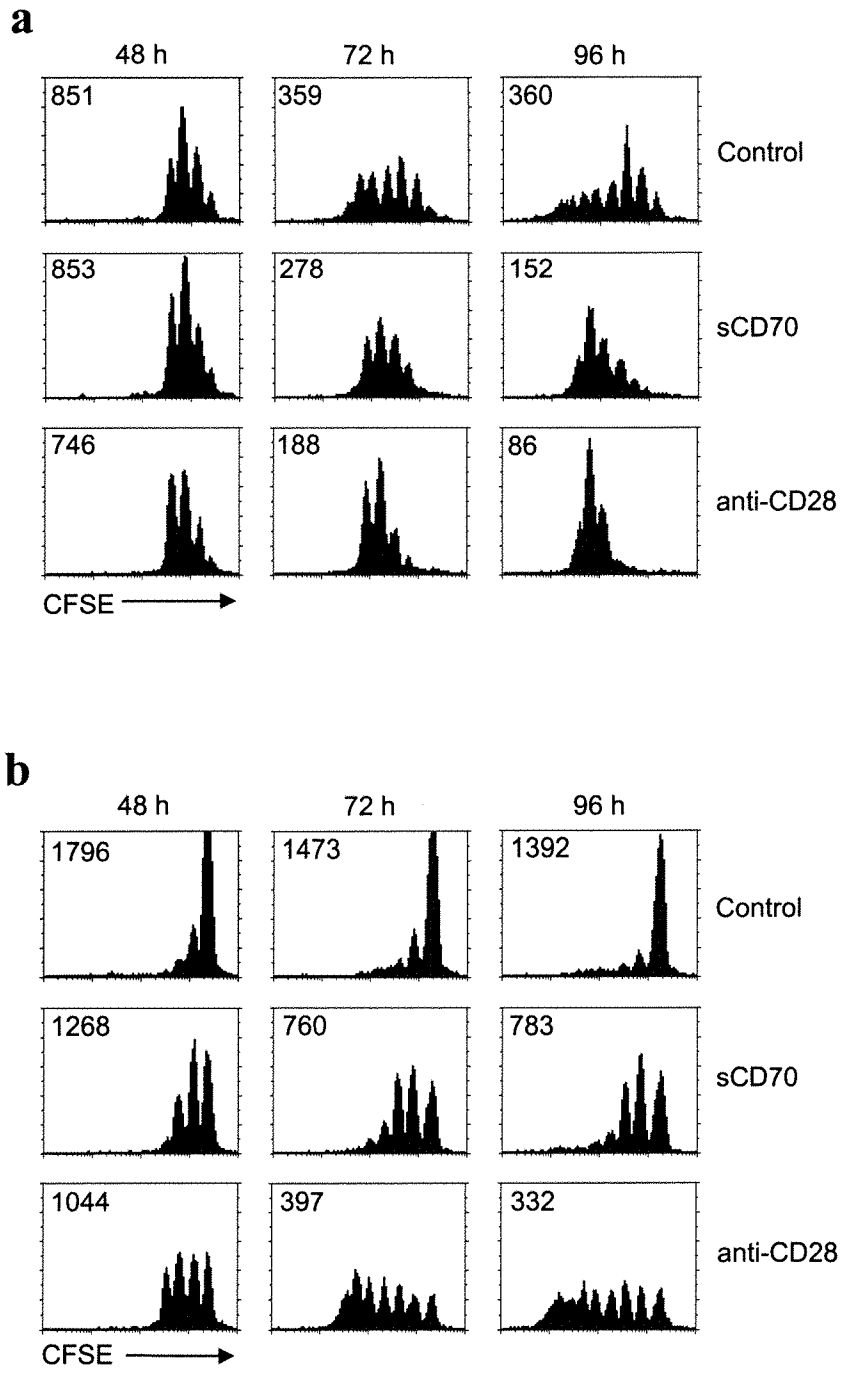


Figure 4.8 CD27 signalling induces CD8$^+$ T cell division. 2 x 10^5 CFSE labelled OT-I *RAG1*$^{-/-}$ splenocytes were stimulated with either (**a**) 100 pM or (**b**) 10 pM $OVA_{257-264}$ in the presence of 40 µg/ml anti-B7.1 (GL1) and anti-B7.2 (1610A1) plus either 10 µg/ml sCD70 or anti-CD28 (37.51). Cells were stained at the indicated time points with anti-CD8-APC and propidium iodide. 5000 viable CD8$^+$ T cells were analysed by FCM. Values represent the average MFI of duplicate samples. The data are representative of three independent experiments.

potently, with a major fraction of T cells undergoing three to six divisions by 96 hours. This experiment demonstrates that CD27 co-stimulation can affect the cell cycle, by increasing the proportion of T cells initially induced to divide and increasing the rate of cell division.

As shown previously (Figures 4.4 and 4.6), CD27 co-stimulation enhances the production of IL-2 by T cells and up-regulates the IL-2 receptor α chain, CD25. IL-2 signalling through its receptor is known to induce T cell division [239] and it was therefore interesting to discern if the T cell division induced by CD27 co-stimulation was dependent on IL-2. To address this, OT-I T cells were activated by pulsing OT-I splenocytes with OVA $_{257-264}$ in the presence of sCD70 or anti-CD28, and an anti-IL-2 blocking mAb was added to prevent IL-2 signalling. To test if this mAb was blocking IL-2 efficiently, up to 200 U/ml IL-2 were added to control wells with or without anti-IL-2 mAb. This concentration of IL-2 is greater than ten-fold the levels of IL-2 produced by CD27-stimulated T cells and should therefore be a stringent control. Cell division was measured at 72 hours by CFSE tracking of labelled T cells (Figure 4.9a) and [^{3}H]-thymidine incorporation (Figures 4.9b and 4.9c). CFSE tracking of OT-I T cells activated with 5 pM OVA $_{257-264}$ (Figure 4.9a) showed that T cell division was being induced by co-stimulation through CD27, CD28, or by the addition of 200 U/ml IL-2. Blocking IL-2 only minimally affected the cell division induced by CD27 or CD28, whereas all cell division induced by 200 U/ml IL-2 was inhibited by addition of the blocking antibody. This showed that the IL-2 blocking mAb was working efficiently. [^{3}H]-thymidine incorporation by OT-I T cells after activation with 10 pM OVA $_{257-264}$ (Figure 4.9b) showed that sCD70 enhanced T cell proliferation but this was only partially blocked by addition of anti-IL-2 mAb. CD28 co-stimulation also enhanced proliferation, which could be partially blocked by addition of anti-IL-2. After activation with a lower concentration of OVA $_{257-264}$, 5 pM (Figure 4.9c), CD27 and CD28 co-stimulation both enhanced T cell proliferation and this proliferation was unaffected by the addition of anti–IL-2 mAb. Spiking the cultures with 50 or 200 U/ml IL-2 dramatically enhanced OT-I T cell proliferation in both experiments and this increase in proliferation could be completely blocked by adding anti-IL-2.

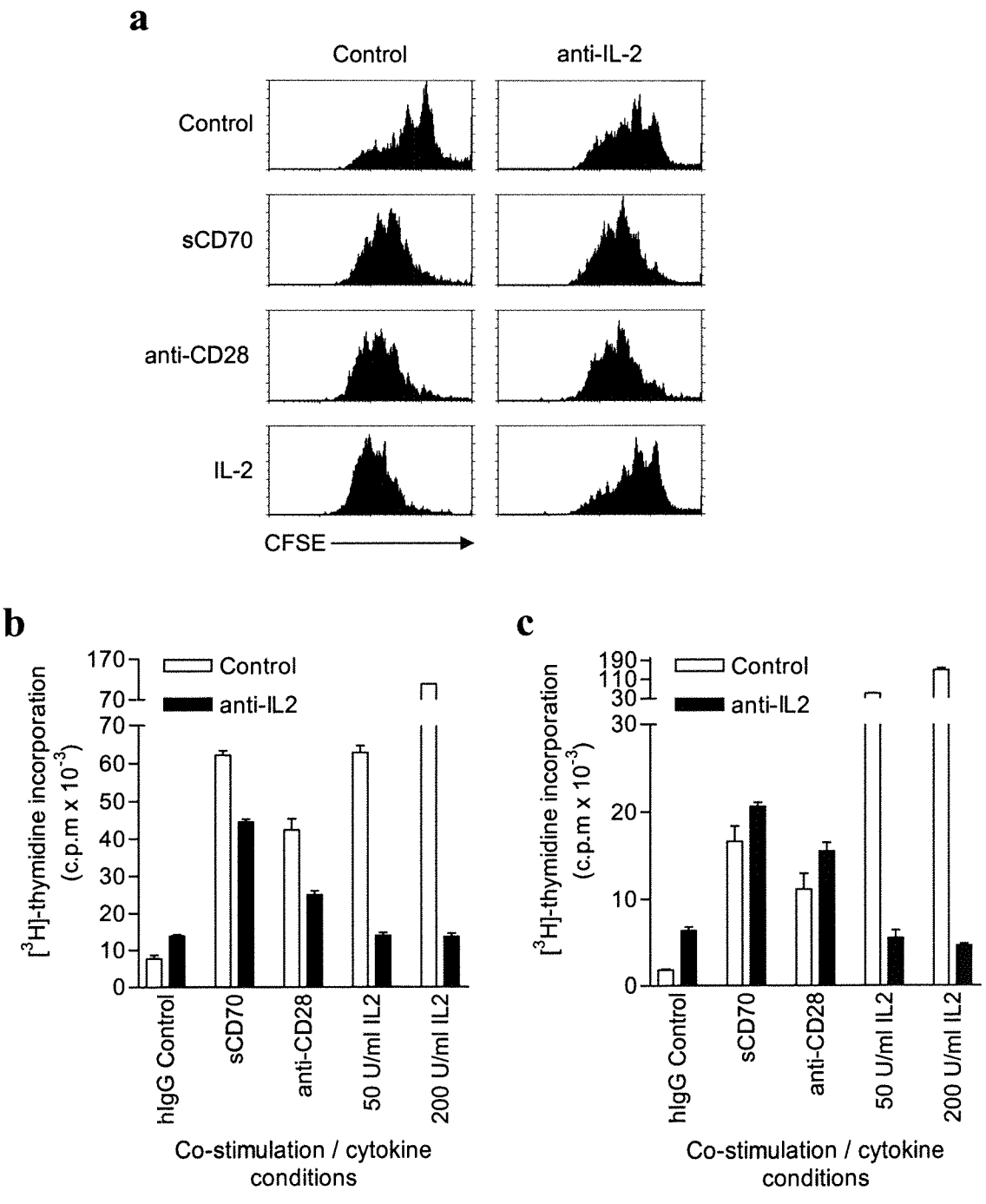


Figure 4.9 CD27-induced CD8$^+$ T cell division is independent of IL-2 production. (**a**) 2 x 10^5 CFSE labelled naïve OT-I *RAG1*$^{-/-}$ splenocytes were activated with 5 pM OVA $_{257-264}$ in the presence or absence of anti-IL-2 (20 μg/ml), plus either sCD70, anti-CD28 (37.51) or 200 U/ml IL-2. After 72 h duplicate cultures were pooled and stained with anti-CD8-APC. 6500 viable CD8$^+$ T cells were analysed per sample by FCM. 2 x 10^5 naïve OT-I *RAG1*$^{-/-}$ splenocytes were activated with (**b**), 10 pM or (**c**), 5 pM OVA $_{257-264}$ in the presence or absence of anti-IL-2 (20 μg/ml), plus either sCD70, anti-CD28 (37.51) or IL-2. 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM. The data are representative of four independent experiments.

To further assess the role of IL-2 in CD27 co-stimulation, the effect of CD27 signalling on T cells from IL-2 knockout C57BL/6 mice [221] was examined (Figure 4.10). Splenocytes from an IL-$2^{-/-}$ mouse were activated *in vitro* with anti-CD3 mAb in the presence or absence of sCD70 or anti-CD28 (Figure 4.10a). The IL-$2^{-/-}$ T cell responses observed were quite abnormal in that some proliferation occurred without anti-CD3 mAb stimulation and the presence of sCD70 enhanced this. In addition, higher levels of anti-CD3 mAb (> 0.1 μg/ml) appeared to reduce T cell proliferation, presumably by causing cell death. CD28 co-stimulation also caused cell death rather than proliferation in this assay. IL-2 deficient mice have been reported to develop inflammatory bowel disease after approximately 8 weeks of age due to an abnormal immune response [240]. As the splenocytes utilised here were from mice in this age group it is possible that the T cells were already activated and subsequent strong stimulation caused them to undergo activation-induced cell death. However, under low levels of TCR stimulus CD27 co-stimulation could enhance the proliferation of IL-2 deficient T cells, as measured by [^{3}H]-thymidine incorporation. The proliferation of normal C57BL/6 splenocytes is shown for comparison (Figure 4.10b).

The production of IL-2 by normal and IL-2 knockout splenocytes was also examined (Figure 4.10c). No IL-2 was produced by the IL-2 knockout splenocytes under any conditions, as expected. The production of IL-2 by normal C57BL/6 splenocytes was enhanced by both CD27 and CD28 co-stimulation. Therefore, despite the abnormal responses observed in IL-2 deficient mice, it appears that CD27 co-stimulation can still induce IL-$2^{-/-}$ T cell proliferation, thus indicating that its co-stimulatory effects are mediated independently of IL-2.

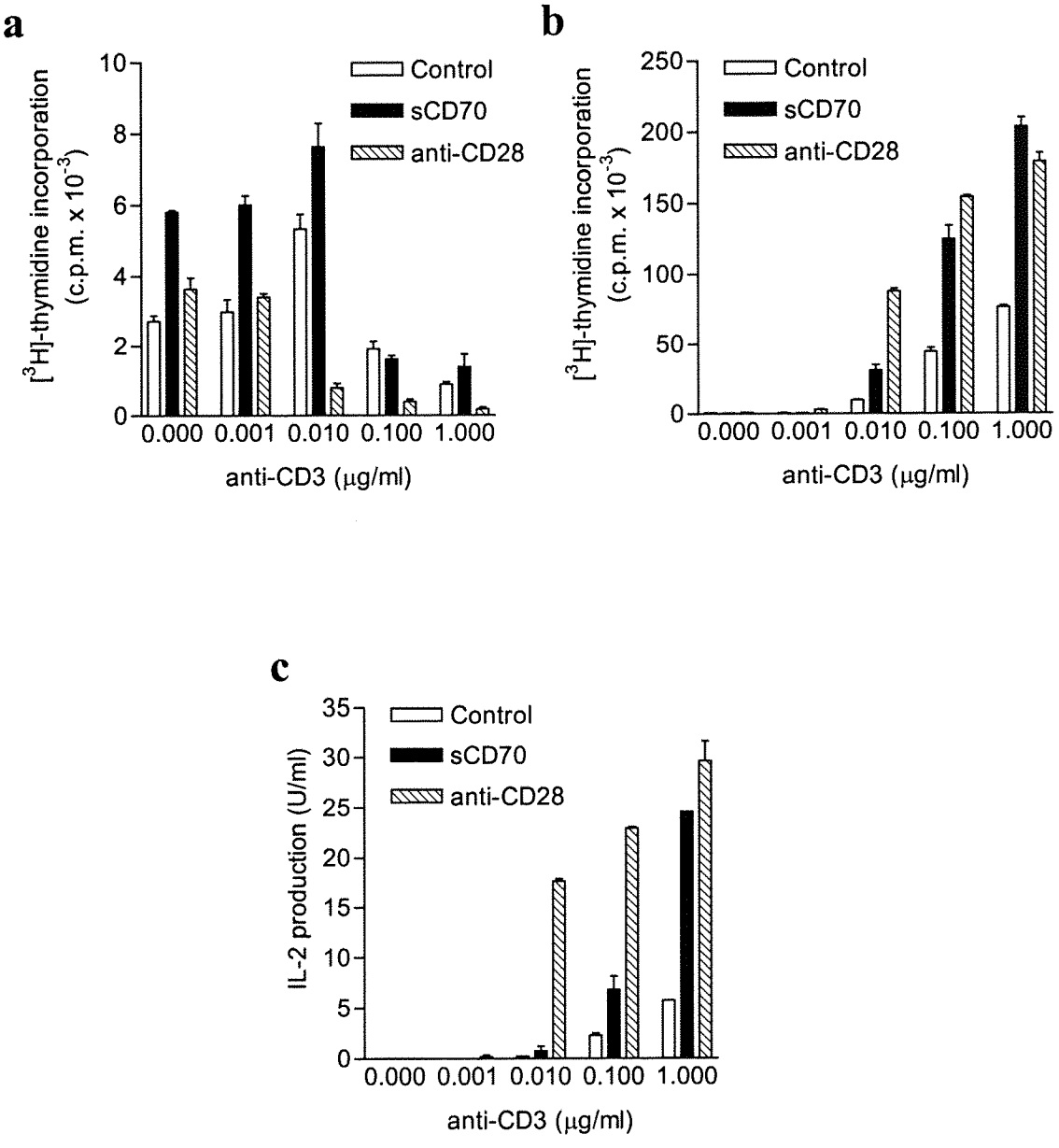


Figure 4.10 CD27 co-stimulation enhances the proliferation of both IL-2 knockout C57BL/6 and normal C57BL/6 T cells. 2 x 10^5 splenocytes from IL-2 knockout (**a**) or normal C57BL/6 mice (**b**) were cultured in the presence of soluble anti-CD3 (145.2C11) plus 10 μg/ml control Ig (Mc3916), sCD70 or anti-CD28 (37.51). 0.5 μCi [^{3}H] thymidine was added for the last 16 h of a 72 h culture after which cells were harvested and incorporation measured. Graphs represent the average counts of triplicate cultures and error bars represent the SEM. Culture supernatant was analysed for IL-2 after 48 hours by ELISA. IL-2 production by C57BL/6 splenocytes is shown (**c**); no IL-2 could be detected in supernatant from IL-2 knockout splenocytes. Graphs represent the mean of duplicate cultures with error bars representing one SD. The data are representative of two independent experiments.

4.2.5 CD27 signalling enhances T cell survival

TRAF-linked members of the TNFR-superfamily, such as OX40 and 4-1BB, have previously been implicated in boosting T cell survival *in vitro* and *in vivo* [114,211]. We therefore sought to investigate if CD27 was also involved in mediating the survival of T lymphocytes during immune responses and if so which survival factors were the targets of CD27 signalling.

As a model system for the induction of apoptosis, activated T cells were subjected to 3 Gy γ-irradiation [130]. As irradiation-induced DNA damage inhibits the cell cycle as well as initiating apoptosis, this protocol allowed cell survival to be distinguished from continued proliferation. OT-I T cells were activated *in vitro* by pulsing splenocytes with OVA $_{257-264}$ for 48 hours, in the presence or absence of sCD70 or s4-1BBL. T cells were exposed to 3 Gy γ-irradiation and equivalent numbers of cells were then re-cultured for 24 hours without further stimulation, before FCM analysis of apoptosis / cell death using propidium iodide and annexin V staining (Figure 4.11a). Annexin V is an early marker of apoptosis (lower right quadrants), with cells later becoming positive for propidium iodide as the integrity of the plasma membrane is lost (upper right quadrants). The percentage of viable non-apoptotic cells (lower left quadrants) from duplicate cultures was plotted as a graph (Figure 4.11b). CD27 co-stimulation increased the numbers of viable cells from less than 10 % to 40 % in cultures which had been stimulated with 50 pM OVA $_{257-264}$. 4-1BB co-stimulation had a minimal effect on survival under these conditions. The viability of T cells which had been activated with 100 pM OVA $_{257-264}$ could be enhanced from 12 % to 40 % with CD27 co-stimulation. 4-1BB co-stimulation in conjunction with 100 pM OVA $_{257-264}$ enhanced the numbers of viable cells to 25 %.

In addition, the effect of CD27 co-stimulation on the survival of OT-I T cells in long term *in vitro* culture was investigated. OT-I T cells were activated by culture with OVA $_{257-264}$ for 48 hours, in the presence or absence of sCD70. T cells were washed and equal numbers of viable cells re-cultured without further stimulation for a further 96 hours, with cell viability being assessed every 24 hours by a trypan-blue exclusion assay (Figure 4.12a). An increase in the percentage of viable cells could be seen in cultures stimulated with sCD70. Co-stimulation of T lymphocytes through CD28, or

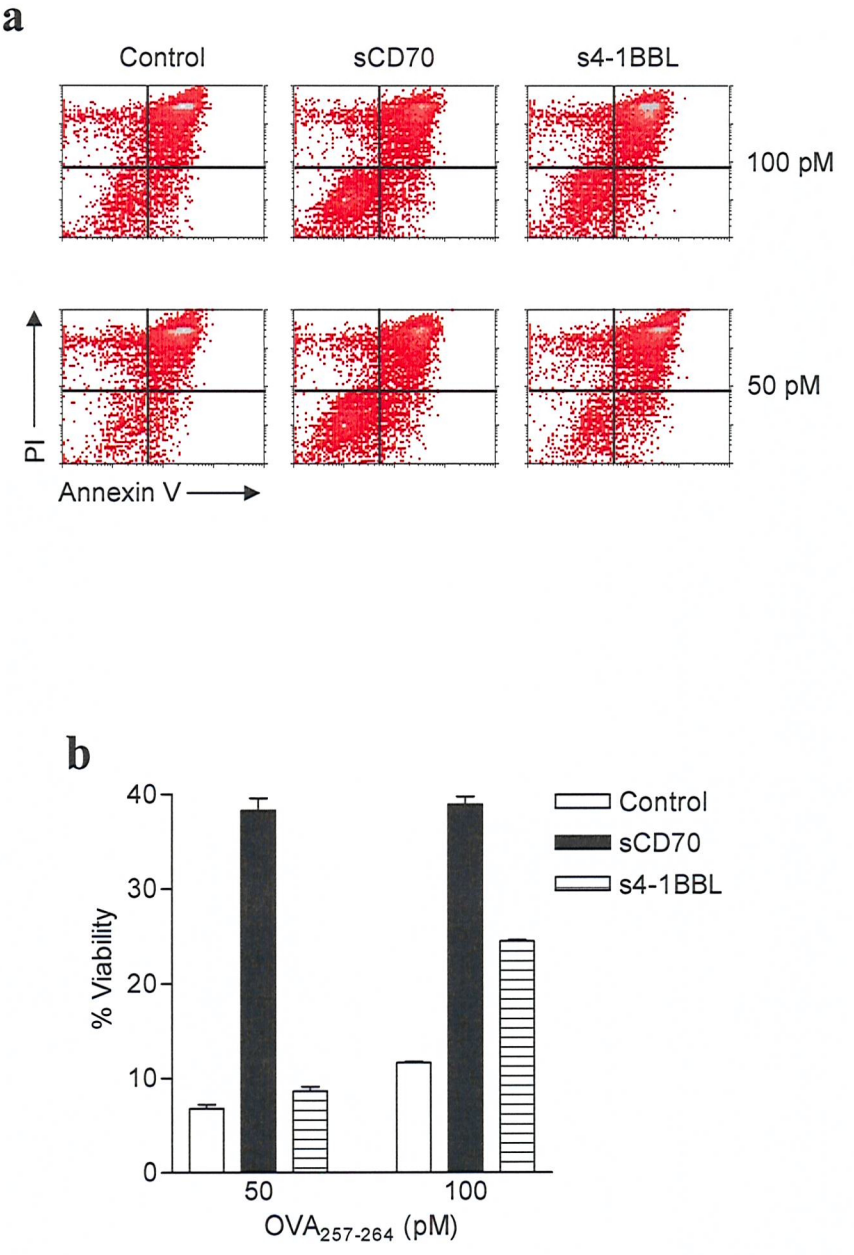


Figure 4.11 CD27 co-stimulation promotes CD8$^+$ T cell resistance to irradiation-induced apoptosis. OT-I *RAG1*$^{-/-}$ splenocytes were activated *in vitro* for 48 h with either 50 or 100 pM $OVA_{257-264}$ plus 10 μg/ml of either control Ig (Mc3916), sCD70 or s4-1BBL after which they were exposed to 3 Gy γ-irradiation. 24 h later apoptosis was assessed by FACS analysis of propidium iodide and annexin V stained cultures. (**a**) Representative density plots with the lower left quadrants being viable non-apoptotic cells. (**b**) The average percentage of viable cells from duplicate cultures was plotted as a graph with error bars representing one SD.

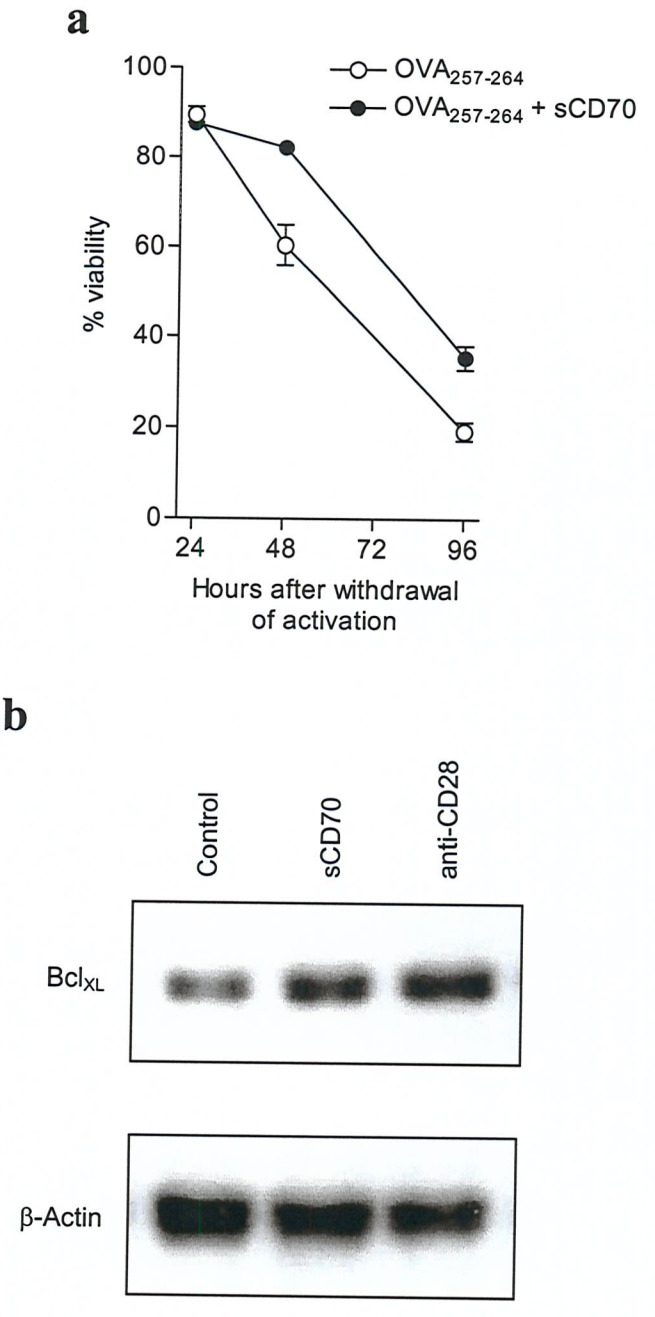


Figure 4.12 CD27 signalling promotes CD8$^+$ T cell survival. (a) Survival in vitro. OT-I *RAG1*$^{-/-}$ splenocytes were cultured for 48 h with 100 pM OVA $_{257\text{-}264}$ plus 10 μg/ml sCD70, after which cells were washed to remove any remaining peptide and re-cultured. 24, 48 and 96 h later cell viability was assessed by a trypan-blue exclusion assay. The average percentage of viable cells from duplicate cultures was plotted as a graph with error bars representing one SD. **(b)** Expression of Bcl_{XL}. OT-I *RAG1*$^{-/-}$ splenocytes were cultured for 48 h with 100 pM OVA $_{257\text{-}264}$ in the presence of 40 μg/ml anti-B7.1 (GL1) and anti-B7.2 (1610A1), plus 10 μg/ml either sCD70 or anti-CD28 (37.51). Cell lysates were prepared and run on a 10 % SDS PAGE gel and analysed for Bcl_{XL} or β-Actin expression by western blotting. The data are representative of two independent experiments.

certain TNFR-superfamily members, can up-regulate the expression of anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and $Bclx_L$ [114,130]. To analyse the levels of $Bclx_L$ in T cells stimulated via CD27, cell lysates were prepared from OT-I T cells activated with OVA $_{257-264}$ and sCD70 in the presence of blocking antibodies against B7-1 and B7-2 (Figure 4.12). Lysates were run on a 10 % SDS-PAGE gel and analysed for $Bclx_L$ and β-Actin protein expression by western blotting. After 48 hours of activation the expression of $Bclx_L$ was elevated relative to the control in T cells stimulated through either CD27 or CD28. However, the expression of $Bclx_L$ after either 24 or 72 hours was comparable under all conditions (not shown).

Taken together, these results indicate that CD27 influences the homeostasis of T cells by prolonging their survival, in conjunction with enhancing cell cycling. One downstream target of CD27 that may mediate its pro-survival effects is $Bclx_L$.

4.3 Discussion

4.3.1 CD27 can co-stimulate both the CD4$^+$ and CD8$^+$ T cell subsets

In agreement with previous studies [235], CD27 appears to be constitutively expressed on the majority of both CD4$^+$ and CD8$^+$ murine T cells (Figure 4.1), unlike 4-1BB and OX40 which are expressed only on activated cells. However, the cell surface expression of CD27 on CD4$^+$ and CD8$^+$ T cells is up regulated by TCR engagement with similar kinetics to 4-1BB or OX40, with the highest levels being reached 48 hours after TCR stimulation. This is followed by a slight drop in cell surface expression at 72 hours. The down regulation of CD27 at the cell surface may be controlled by proteolytic clipping of the receptor, as a soluble form has been shown to be released from activated cells [175]. This soluble form of CD27 is found in body fluids, and can be used as a marker of local and systemic immune activation [235]. The demonstration that the loss of expression of OX40 and 4-1BB from T cells is regulated by metalloproteases suggests that the post-activation shedding of these receptors may represent one mechanism for limiting co-stimulatory signalling [151]. *In vivo* studies of murine memory T cell development after LCMV infection have demonstrated that CD27 is down regulated on the T_{EM} subset, but remains highly expressed on the T_{CM} subset [86]. Similarly, in human chronic viral infections antigen-specific CD8$^+$ T cells, which appear to be terminally differentiated effectors, down regulate CD27 [88]. This suggests that activated effector T cells lose the ability to receive signals through CD27, but may later regain CD27 expression as the T cells convert to resting T_{CM}.

Co-stimulation of purified CD4$^+$ and CD8$^+$ T cells by CD27, using soluble sCD70, establishes that CD27 can potently enhance the proliferation of both CD4$^+$ and CD8$^+$ T cells to levels comparable to that induced by OX40 or 4-1BB, respectively (Figure 4.2). This finding is in contrast with a study on the effect of CD27 co-stimulation during mixed lymphocyte reactions (MLR), which indicated that while CD27 enhanced the proliferation of murine CD8$^+$ T cells it had little effect on the response of CD4$^+$ T cells [192]. One explanation for this difference may be that in the MLR experiments the amount of MHC class I or class II disparate cells was not titrated, so it is possible that the CD4$^+$ T cells were already receiving optimal stimulation and additional co-stimulation would therefore appear to have little effect. *In vivo*, CD27-

deficient mice have reduced $CD4^+$ and $CD8^+$ T cell responses to influenza virus, providing evidence that colludes with the data presented in this chapter to suggest this receptor controls the antigen-specific responses of both T cell subsets [109].

4.3.2 CD27 can enhance T cell proliferation and cytokine production during primary and secondary responses

Naïve OT-I transgenic $CD8^+$ T cells constitutively expressed a low level of CD27 that was up regulated after activation with minimal amounts of antigen. In contrast, expression of the TNFR-family members OX40 and 4-1BB required TCR signalling, and the level of expression was directly regulated by the strength of signal through the T cell receptor (Figure 4.3). The ability of naïve OT-I T cells to proliferate in response to antigen, in the form of agonist peptide or E.G7 tumour cells, and soluble ligands for CD27, 4-1BB or OX40 correlated with the receptor expression pattern; CD27 being the most potent co-stimulator as it was constitutively expressed on T cells (Figure 4.4a). 4-1BB co-stimulation also enhanced primary OT-I T cell proliferation, but OX40 had no effect on the proliferation of these $CD8^+$ T cells to E.G7 cells, presumably because its expression required very high levels of antigen. These observations imply that the effect of OX40 and 4-1BB will be more dependent on the amount of antigen and the degree of additional co-stimulation present during initial T cell priming than CD27. In agreement with this, several studies have shown that while 4-1BB and OX40 can work independently of CD28 *in vitro* this requires strong TCR stimulation [121,151,241]. Additionally, *in vivo* anti-4-1BB mAb-mediated CTL responses to a tolerogenic tumour vaccine were shown to be critically dependent on initial co-stimulation through the CD28 pathway [121]. In this model naive $CD8^+$ T cells were being activated by cross-presentation in the absence of $CD4^+$ T cell help. Therefore, in this poorly immunogenic setting, CD28 signalling was required for the up regulation of 4-1BB on the responding $CD8^+$ T cells. In contrast, administration of agonistic anti-4-1BB mAbs during primary influenza A virus infection could restore $CD8^+$ T cell responses in the absence of CD28 [113,212], presumably because this type of viral infection is pro-inflammatory and generates sufficient antigen to up-regulate 4-1BB expression on T cells without requiring CD28 signalling. The data presented in this chapter demonstrates that CD27 can promote the expansion of $CD8^+$ T cells independently of CD28 signalling even under conditions of low TCR stimulation

(Figure 4.5). Moreover, at sub-optimal levels of antigen there is a synergistic effect between CD27 and CD28, which indicates that their signalling pathways are distinct from one another. Complementary to this, it has been shown that the diminished proliferation of $CD27^{-/-}$ T cells cannot be completely rescued by stimulation through CD28 or by the addition of IL-2, which also suggests that CD27 provides qualitatively different signals [109]. Cellular production of the cytokines IL-2 and IFN-γ was dramatically enhanced by CD27 co-stimulation during primary OT-I T cell responses (Figure 4.4), as was the expression of activation markers such as CD25 and members of the TNFR family (Figure 4.6). CD25 is the α subunit of the IL-2 receptor; therefore, up regulation of this receptor molecule in conjunction with production of IL-2 cytokine will provide a potent signal for maintenance of the T cell response.

Comparison of the effect of CD27 and 4-1BB co-stimulation during secondary stimulation of antigen-experienced OT-I T cells demonstrated that, in contrast with naïve T cell priming, 4-1BB is more potent than CD27 during secondary proliferative responses (Figure 4.7). This is in agreement with data from other groups showing that 4-1BB only influences T cell numbers late in the primary response and can prevent activation-induced cell death of re-stimulated T cells [112,242]. Therefore, CD27, like CD28, may be more important in initial T cell priming, with its triggering up-regulating the expression of 4-1BB or OX40 that then become critical stimulators later in the response. Endogenous membrane-bound CD70 is expressed on mature dendritic cells [224], and these APCs have been shown to be responsible for the priming of naïve $CD8^+$ T cells in draining lymph nodes [19]. Therefore, it is conceivable that CD27 co-stimulation occurs during the first stages of naïve T cell priming upon interaction with antigen-loaded dendritic cells. Additionally, CD70 is up regulated on B and T cells upon activation [224]. CD27 stimulation may therefore also act to maintain T cell responses via T-B and T-T cellular interactions.

4.3.3 CD27 regulates T cell division

Co-stimulation of T cells through CD27 using soluble sCD70 induced T cell cycling, as seen by tracking the division of CFSE-labelled OT-I T cells. CD27 mediated co-stimulation appears to synergise with TCR signals, lowering the threshold of activation required for T cell division (i.e. fewer TCR signals are required in the presence of

CD27 signalling) and thereby allowing a greater proportion of T cells to initiate cycling. It also appears to regulate the speed or continuation of T cell division so that a greater percentage of cells are seen to undergo multiple divisions. These results are in contrast with previous work by Hendriks *et al,* which showed that $CD27^{-/-}$ T cells had no deficit in T cell division *in vivo* in response to influenza infection. *In vitro* experiments by this group comparing an anti-CD28 mAb with co-stimulation using an anti-CD27 mAb also indicated that CD27 did not increase T cell cycling; leading them to postulate that CD27 only affected T cell survival [109,243]. This difference may be due to their use of low concentrations of a soluble anti-CD27 mAb in conjunction with a strong TCR signal using cross-linked anti-CD3 mAb, which could have masked the cell cycle effects. Alternatively, this disparity may be a reflection of differences between signalling induced upon antibody ligation as compared to the natural ligand. Transgenic mice that over express CD70 on B cells exhibit a progressive conversion of naïve T cells to an effector/memory phenotype. Closer analysis of these T cell responses using 5-bromodeoxyuridine labelling and staining for the proliferation marker Ki-67, has demonstrated that these mice contain increased numbers of cycling T cells responding to environmental antigens [195]. Taken together, these results indicate that the effect of CD27 on cell division may be masked by that of the TCR/CD28 under situations of high antigenic stimulation/inflammation, such as viral infection. However, optimal stimulation by CD70 via its over expression *in vivo* or ligation of sCD70 can clearly enhance T cell division.

The cell division induced by CD27 was independent of the proliferative effects of the cytokine IL-2. At high levels of antigen, the division of OT-I T cells responding to CD27 co-stimulation was only partially inhibited by addition of an anti-IL-2 blocking mAb. At lower levels of antigen, where less IL-2 is likely to be being produced, the addition of anti-IL-2 had no significant effect on OT-I T cell division in response to CD27 stimulation. This IL-2-independent proliferation is also seen with CD28 co-stimulation in these experiments, confirming previous findings on primary human T cells [239]. CD28 has been shown to mediate T cell expansion via IL-2-dependent and IL-2-independent regulation of cell cycle progression. IL-2-independent signals via the CD28 pathway were sufficient to allow entry into the G1 phase, activation of cyclin-dependent kinases and progression into the S phase of the cell cycle. These effects

were induced by the down-regulation of the cdk inhibitor $p27^{kip1}$ [239]. This group went on to show that this down regulation of $p27^{kip1}$ by CD28 was mediated through activation of the phosphatidylinositol 3-hydroxykinase (PI3K) – PKB pathway in conjunction with TCR-dependent activation of the mitogen-activated protein kinase kinase (MEK) signalling pathway [131]. Similarly, it has been shown that engagement of 4-1BB increases expression of cyclins D2, D3 and E and down regulates $p27^{kip1}$ protein in T cells, using the same molecular mechanisms as CD28 [207]. $p27^{kip1}$ and other members of this cdk inhibitor family have been implicated in controlling T cell tolerance by acting as anergy factors which inhibit IL-2 transcription and clonal expansion of helper T lymphocytes [244,245]. Thus, the regulation of this molecule may play a critical role in the ability of co-stimulatory molecule signalling to prevent or reverse T cell tolerance [48,116,121].

4.3.4 CD27 promotes T cell survival

Lymphocyte homeostasis is maintained by the induction of T cell apoptosis during the contraction phase of an immune response. One pathway for the induction of apoptosis is via 'death receptors' such as Fas. Fas has been implicated in controlling peripheral T cell tolerance by deleting autoreactive cells by a process known as activation-induced cell death (AICD). Fas ligand expression is induced on T cells after repeated TCR activation, and subsequent cross-linking of Fas leads to the recruitment of intracellular adapters such as FADD that in turn recruit cysteine proteases to initiate the effector caspase cascade which leads to apoptosis [72]. However, studies on Fas or Fas ligand defective T cells have demonstrated that while these cells lack the ability to die in response to chronic stimulation via systemic self-antigen, they display a normal contraction of T cell numbers after an acute immunisation. This 'passive' T cell death after immunisation may be due to the withdrawal of cytokines or growth signals and is controlled by the Bcl-2 family of apoptotic regulators [246,247]. The Bcl-2 family is a large group of homologous proteins that exert either pro- or anti-apoptotic effects by controlling mitochondrial integrity. The anti-apoptotic members include Bcl-2 and Bcl-x_L and contain four homologous domains termed BH1-4. Pro-apoptotic members of the Bcl-2 family can be spilt into two groups, one containing BH1-3 homology domains (e.g. Bax and Bak) and one group containing BH3 only. BH3 only members are responsible for sensing various types of apoptotic stimuli but appear to be reliant

on Bax and Bak to mediate mitochondrial disruption [72,248]. Disruption of the outer mitochondrial membrane leads to release of the mitochondrial factors cytochrome C and Smac, which directly activate apoptosis pathways [249]. Anti-apoptotic members Bcl-2 and Bcl-x_L insert in the outer mitochondrial membrane and appear to help maintain mitochondrial integrity by heterodimerising with, and hence inhibiting, pro-apoptotic members [72].

Activated T cell autonomous death (ACAD), or death by cytokine withdrawal, *in vivo* is mediated by a balance between the pro-apoptotic BH3 only molecule Bim and anti-apoptotic Bcl-2 [250]. This type of death probably corresponds to that seen in our model of survival *in vitro* after withdrawal of activation (Figure 4.12). In addition, γ-irradition (Figure 4.11) induces cell death via the mitochondrial pathway by activating the BH3 only proteins Noxa and Puma [248]. The results described here demonstrate that CD27 can act as a survival signal for antigen-specific $CD8^+$ T cells, promoting resistance to irradiation-induced apoptosis and enhancing survival *in vitro* (Figures 4.11 and 4.12). One potential downstream target of CD27 is Bcl-x_L. Bcl-2 and Bcl-x_L exhibit a reciprocal expression pattern in lymphocytes. Bcl-2 expression is high in mature peripheral T cells whereas Bcl-x_L expression is absent until activation, when it is dramatically up regulated [251]. *In vitro* studies of $OX40^{-/-}$ T cells showed that while OX40 up regulates Bcl-x_L expression after activation it also maintains the levels of Bcl-2 over time, allowing long term T cell survival [114]. More recently, studies on 4-1BB have shown that survival signals are mediated by NF-κB activation leading to increased expression of the antiapoptotic genes *bcl-x_L* and *bfl-1* [206]. The data presented here suggest that Bcl-x_L protein expression is also up regulated following CD27 cross-linking by soluble ligand (Figure 4.12b). It is possible that CD27 will also effect Bcl-2 expression or alternatively act antagonistically on pro-apoptotic Bcl-2 family members such as Bim. The survival of a proportion of lymphocytes during the contraction phase of the T cell response is important for the formation of a responsive memory T cell pool. $CD27^{-/-}$ mice exhibit impaired memory responses and mice over-expressing CD70 have greater numbers of T cells with a memory phenotype [109,185]. Thus, one way CD27 may promote the formation of memory cells is via its anti-apoptotic effects.

The data presented in this chapter demonstrate that CD27 can co-stimulate both the $CD4^+$ and $CD8^+$ T cell subsets, and in an antigen-specific system, CD27 promotes naïve and effector $CD8^+$ T cells to undergo cell division, produce cytokines and survive. In conclusion, the outcome of CD27 co-stimulation is likely to be dependent on the strength, duration and context of the antigenic stimulation. However, the sensitivity of CD27 up regulation in response to TCR stimulation, and its independence from CD28 signalling indicates that CD27 may be a good target for enhancing sub-optimal responses *in vivo*.

CHAPTER 5

In vivo analysis of the effect of CD27 stimulation on the magnitude and quality of CD8$^+$ T cell responses

5.1 Introduction

Strategies that enhance T cell responses are likely to be invaluable for vaccine development against infectious agents and tumours [138,146,252]. As described earlier (Chapter 4), co-stimulation of naïve CD8$^+$ T cells by CD27 enhances sub-optimal responses and leads to increased cell division, survival and cytokine production *in vitro*. CD27, thus, represents a potential target for augmenting T cell responses that are normally ineffective. Previous work on CD27$^{-/-}$ mice has shown a role for this molecule *in vivo* during anti-viral responses [109]. However, it has not been addressed whether stimulation through CD27 can enhance T cell priming in the absence of innate inflammation, and thus whether it has potential as an adjuvant for vaccination against tumour antigens. The transfection of tumour cells with CD70 has previously been demonstrated to enhance their immunogenicity [152], however it has not been investigated if CD70 expression is explicitly required on the target cell or if its CD27 co-stimulation during T cell priming is sufficient to allow responses to be generated that can then eradicate CD70-negative tumours. The lack of CD27 co-stimulation appears to impinge on both CD4$^+$ and CD8$^+$ anti-viral T cell responses *in vivo*. However, full CD8$^+$ responses are often dependent on CD4$^+$ T cell help, thus some of the negative effects on CD8$^+$ effector and memory T cells seen in CD27$^{-/-}$ mice could be attributed to indirect effects on the CD4$^+$ T cell compartment. In this chapter, using sCD70 the role of CD27 signalling in the development of a CD8$^+$ T cell response *in vivo* was determined following immunization with an antigenic peptide. Data presented in this chapter shows that stimulation via CD27 has profound effects on both the magnitude and quality of the T cell response generated. Furthermore, the administration of sCD70 enhances anti-tumour immune responses. These results therefore suggest a novel strategy to boost CD8$^+$ T cell responses.

5.2 Results

5.2.1 CD27 signalling enhances the magnitude of the primary $CD8^+$ T cell response

To examine the effects of sCD70 on the response of $CD8^+$ T cells *in vivo*, 10^6 OT-I $CD8^+$ T cells were adoptively transferred into normal syngeneic C57BL/6 mice. The mice were then challenged i.v. with OVA $_{257-264}$ peptide together with sCD70, or hIgG as a control and the kinetics of the response monitored using K^b OVA $_{257-264}$ tetramers (see Figure 5.1 for experimental time-line). Transfer of small numbers of transgenic T cells into normal mice allows tracking of antigen-specific responses without causing significant skewing of the T cell compartment. In this system, transfer of 10^6 OT-I T cells to C57BL/6 mice seeded the lymphocyte population with ~ 0.1 % antigen-specific T cells (Figure 5.3a).

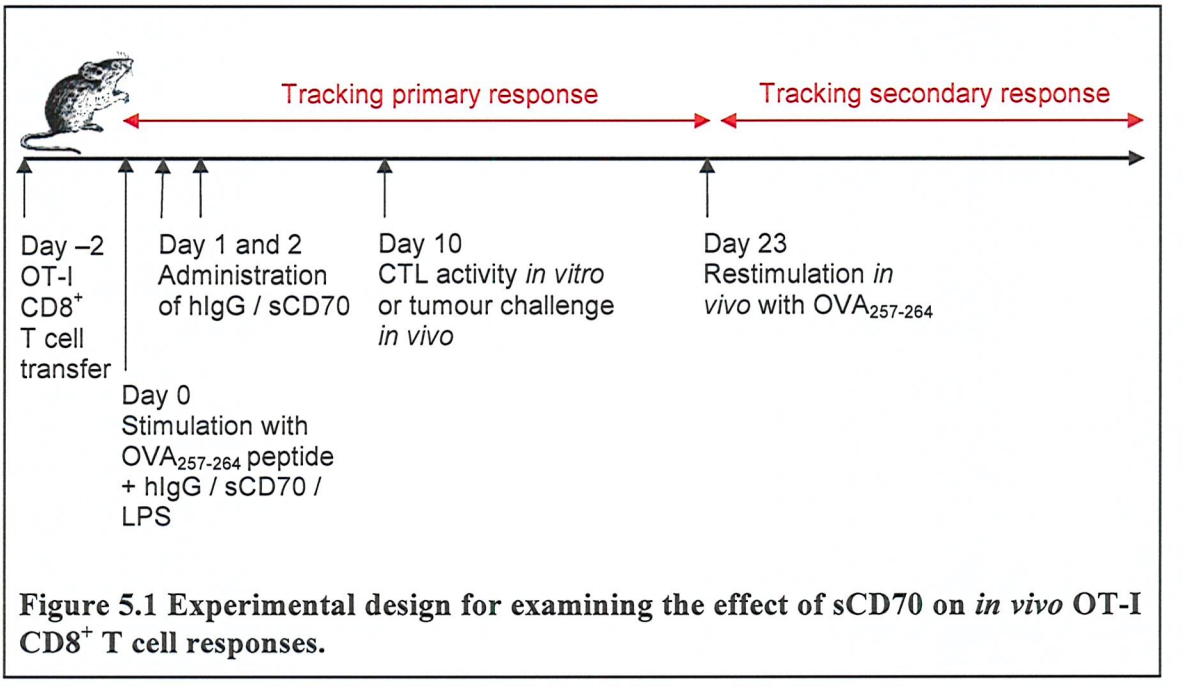


Figure 5.1 Experimental design for examining the effect of sCD70 on *in vivo* OT-I $CD8^+$ T cell responses.

Initially, the expression of the target molecule CD27 on the OT-I T cells to be transferred was investigated. K^b OVA $_{257-264}{}^+$ $CD8^+$ T cells from OT-I C57BL/6 mice constitutively expressed CD27, as expected, and this expression could be up regulated by *in vitro* activation with antigen (Figure 5.2a). In addition, the expression of the cell surface phenotypic markers CD44, CD62L (L-selectin) and CD25 (IL-2 receptor α-chain) on OT-I T cells prior to transfer was investigated. CD44 is an adhesion

molecule involved in lymphocyte migration, which is up regulated after T cell activation and is highly expressed on memory T cells [253]. CD62L is also an adhesion molecule but conversely is highly expressed on naïve T cells. CD62L interacts with peripheral-node adressin expressed on lymph node high endothelial venules, mediating lymphocyte attachment and rolling and therefore allowing naïve T cells to recirculate through peripheral lymph nodes [51]. The IL-2 receptor α-chain, CD25, is not present on the surface resting T cells and its expression is indicative of recent activation [253]. The OT-I T cells to be transferred had a homogeneous, typically naïve phenotype of CD44 low, CD62L high and CD25 negative [253] (Figure 5.2b).

In order to be sure at what stage of the T cell response sCD70 was having its effects, the stability of sCD70 fusion protein *in vivo* was measured. Following three daily injections of 200 μg of sCD70, the amount bound to peripheral blood T cells was measured by staining with a FITC labelled anti-hFc mAb. Two hours after the final injection sCD70 was bound to CD27 on the majority of $CD8^+$ T cells (Figure 5.2c). However, by 72 hours only very low levels remained bound and by 168 hours no sCD70 could be detected (Figure 5.2c) on the surface of $CD8^+$ T cells. This demonstrated that sCD70 had a relatively short half-life *in vivo,* and that by using this protocol (Figure 5.1) sCD70 would only be delivering it's signal during the initial priming phase of the $CD8^+$ T cell response.

After transfer of OT-I T cells to C57BL/6 mice, administration of sCD70 in the absence of antigen had no effect on the homeostasis of the OT-I T cells, with cell numbers remaining stable at ~ 0.1 % (Figure 5.3a). Following challenge with $OVA_{257-264}$ and hIgG, the percentage of OT-I T cells rose significantly reaching a peak by day 4. When $OVA_{257-264}$ was injected together with sCD70 a much greater increase in the percentage of OT-I T cells was observed (Figure 5.3a). When $OVA_{257-264}$ was injected with sCD70 OT-I T cells expanded by over 300-fold, which is in marked contrast with the 18-fold expansion observed in the absence of sCD70 (Table 5.1).

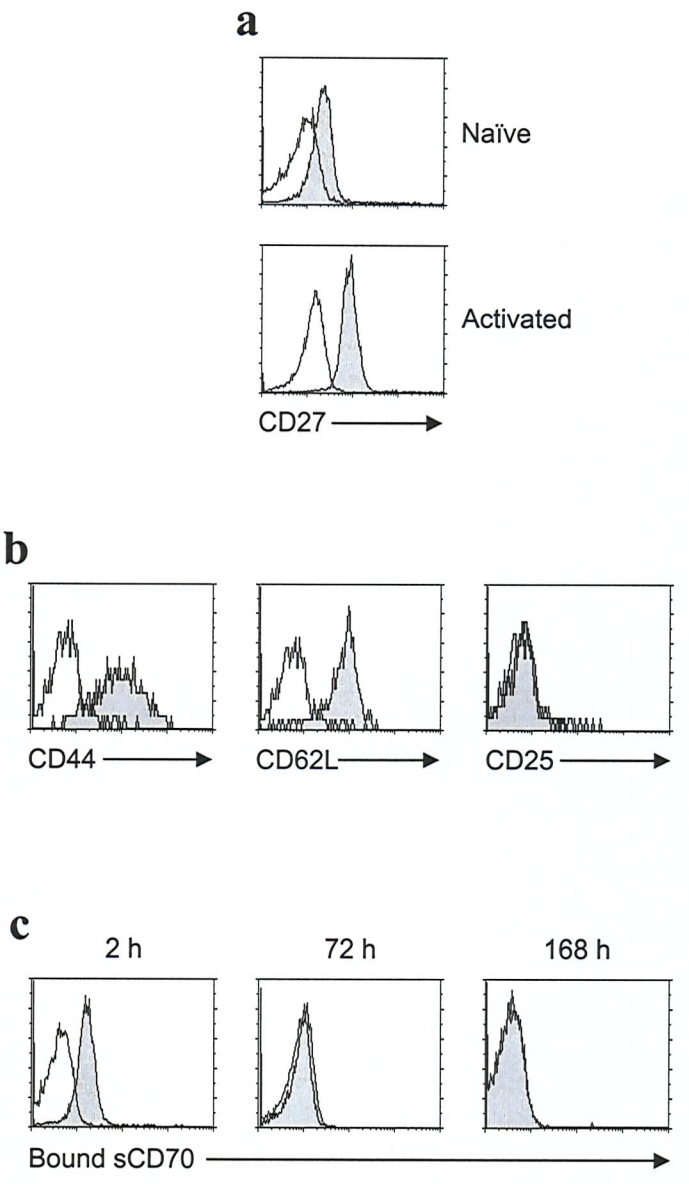


Figure 5.2 OT-I C57BL/6 CD8$^+$ T cells express CD27 and have a naïve phenotype. (**a**) Expression of CD27 on OT-I T-cells. Naïve or *in vitro* activated (72 h with 10 pM OVA $_{257\text{-}264}$) OT-I C57BL/6 splenocytes were stained with sCD70 (filled histograms) or hIgG as a control (open histograms), followed by a FITC labelled anti-human Fc mAb. Histograms are gated on viable, CD8$^+$ and K^b-OVA$_{257\text{-}264}$ tetramer$^+$ cells. (**b**) Naïve phenotype of OT-I T cells. OT-I C57BL/6 splenocytes were stained with FITC-labelled anti-CD44, anti-CD62L or anti-CD25 mAbs (filled histograms), or stained with isotype control mAbs (open histograms). Histograms are gated on viable, CD8$^+$ and K^b-OVA$_{257\text{-}264}$ tetramer$^+$ cells. (**c**) Binding of sCD70 to CD8$^+$ T cells *in vivo*. C57BL/6 mice were administered 200 μg sCD70 or hIgG i.v. per day for 3 days. 2 h, 72 h or 168 h after the final injection peripheral blood T cells were stained with anti-CD8α and FITC labelled anti-human Fc mAb to detect bound sCD70 (filled histograms) or hIgG (open histograms). Histograms are gated on viable CD8$^+$ T cells.

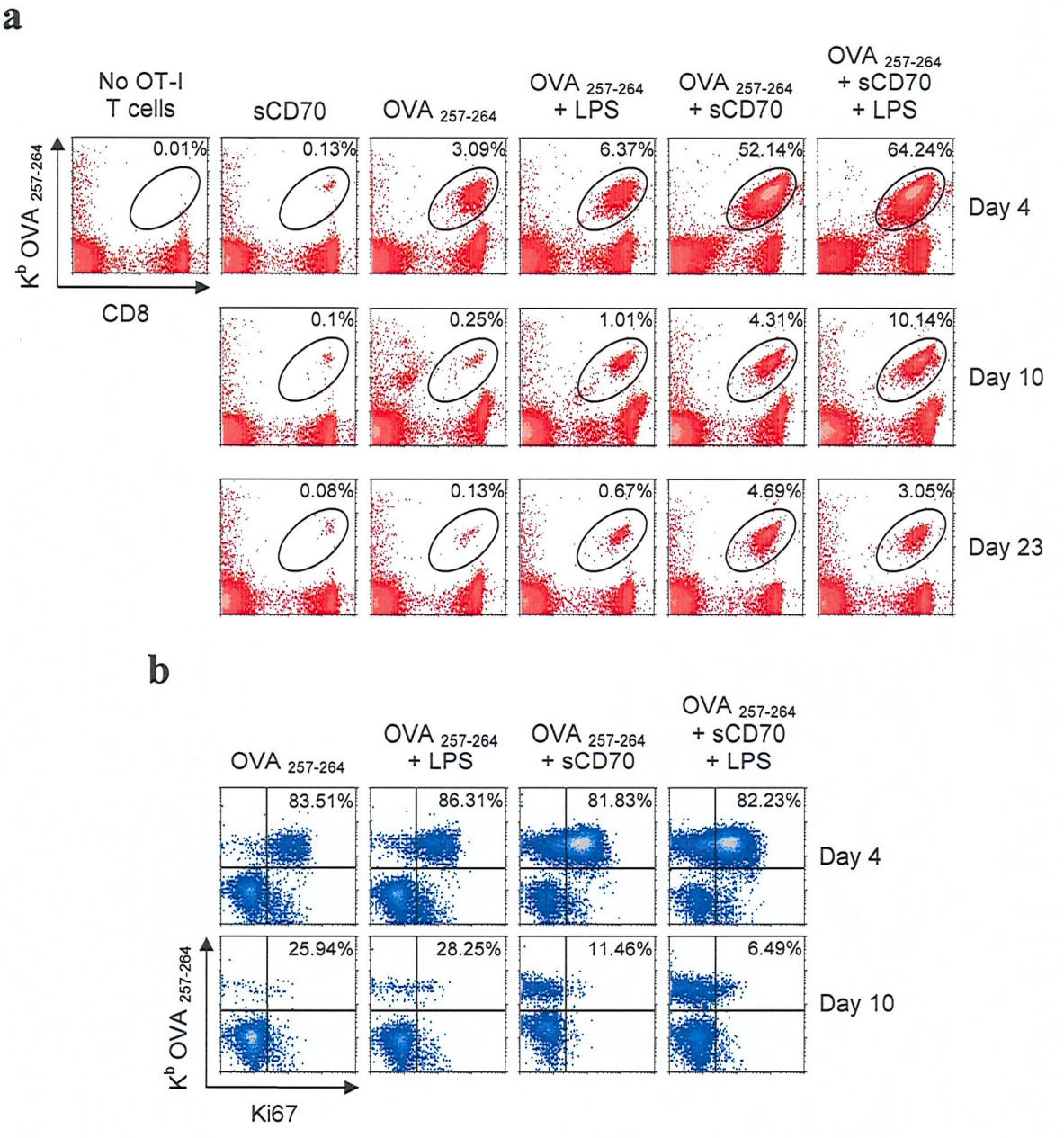


Figure 5.3 Primary expansion of naïve OT-I CD8$^+$ T cells is amplified by CD27 co-stimulation. 1 x 10^6 naïve OT-I C57BL/6 T cells were transferred into C57BL/6 recipients and mice were primed by i.v. injection of OVA $_{257\text{-}264}$ (20 nmoles) in the presence or absence of sCD70 (3 x 200 µg), LPS (10 µg), or a combination of sCD70 and LPS. Groups that did not receive sCD70 received an equal amount of hIgG as a control. (**a**) Percentage of antigen-specific CD8$^+$ T cells in peripheral blood was measured by double staining with K^b OVA$_{257\text{-}264}$ tetramers and anti-CD8α. Representative density plots gated on live lymphocytes are shown, with mean percentages of lymphocytes K^b OVA $_{257\text{-}264}{}^+$ and CD8$^+$ indicated (n = 3 mice per group, except the sCD70 without antigen group where n = 2). The data are representative of three similar experiments. (**b**) Proliferative status of antigen-specific CD8$^+$ T cells in peripheral blood on days 4 and 10 post-antigen was determined by intracellular Ki67 staining in conjunction with K^b OVA $_{257\text{-}264}$ tetramers and anti-CD8. Representative density plots are gated on CD8$^+$ lymphocytes, and the mean percentages of K^b OVA $_{257\text{-}264}{}^+$ cells that are also Ki67$^+$ are indicated (n = 3 mice per group).

Days post-primary antigen	CD70	OVA $_{257-264}$	OVA $_{257-264}$ + LPS	OVA $_{257-264}$ + CD70	OVA $_{257-264}$ + CD70 + LPS
4	0.13 % ± 0.03	3.09 % ± 0.86	6.37 % ± 0.23	52.14 % ± 5.93	64.24 % ± 3.96
6	-	0.68 % ± 0.20	2.02 % ± 0.45	10.63 % ± 1.06	23.48 % ± 0.35
10	0.10 % ± 0.02	0.25 % ± 0.09	1.01 % ± 0.45	4.31 % ± 0.56	10.14 % ± 0.72
23	0.08 % ± 0.01	0.13 % ± 0.04	0.67 % ± 0.1	4.69 % ± 0.82	3.05 % ± 0.68
Maximal fold expansion	**0.8**	**18**	**37**	**307**	**378**

Table 5.1 Primary expansion of naïve OT-I CD8$^+$ T cells is amplified by CD27 co-stimulation. Experimental set up was as described in Figure 5.3. Mean percentages of antigen-specific CD8$^+$ T cells in peripheral blood over time are shown, with the SEM indicated. n = 3 mice per group, except the sCD70 (without antigen) control group where n = 2. Maximal fold expansion was calculated using the day 2 value of 0.17 % (as the background) from mice given sCD70 only. The data are representative of three similar experiments.

Previous studies have demonstrated that the natural adjuvant LPS enhances the expansion and survival of activated T cells *in vivo* [254,255]. Therefore, the effect of sCD70 was compared with that of LPS on the response of OT-I T cells. When compared with injection of OVA $_{257-264}$ alone, administration of LPS together with OVA $_{257-264}$ resulted in a doubling of the percentage of OT-I T cells on day 4 (Figure 5.3a). However, this was only a modest increase (37-fold, Table 5.1) when compared with sCD70 and OVA $_{257-264}$, which triggered a 307-fold expansion. The effects of combining sCD70 with LPS were also examined. When sCD70 and LPS were injected together with OVA $_{257-264}$, the magnitude of the response was similar to that observed after injection of sCD70 and OVA $_{257-264}$ (Figure 5.3a and Table 5.1). The percentage of OT-I T cells at the peak of the response (day 4) in the group receiving the combination of LPS and sCD70 was not significantly different from that of the group receiving sCD70. However, on days 6 and 10 the percentages of OT-I T cells in the mice that received OVA $_{257-264}$ together with LPS and sCD70 were significantly ($P <$ 0.003) higher than those that received OVA $_{257-264}$ and sCD70 (Table 5.1), suggesting that LPS enhances the short-term survival of OT-I T cells. This difference was not maintained overtime since the percentage of OT-I T cells in both groups was similar 23 days after antigenic challenge (Figure 5.3a and Table 5.1). Thus, the long-term survival of CD70-stimulated OT-I cells was not enhanced by LPS. The proliferative status of the OT-I T cells was also investigated at various stages of the response. The

majority of the OT-I T cells (> 80 %) at the peak of the response (day 4) were positive for the proliferation marker Ki67, however, the proliferation was not sustained and by day 10 most cells had stopped dividing (Figure 5.3b). The enhanced numbers of resting T cells remaining at day 10 post-antigen after sCD70 stimulation indicates that CD27 co-stimulation had generated a stable pool of memory precursors, rather than just inducing continuous T cell division.

5.2.2 Effect of CD27 signalling on $CD8^+$ T cell division in vivo

Administration of sCD70 together with OVA $_{257-264}$ resulted in significant expansion of OT-I T cells *in vivo* (Figure 5.3a and Table 5.1). To address the mechanism by which sCD70 mediated its effects, CFSE-labelled OT-I T cells were adoptively transferred into C57BL/6 mice and their ability to divide after administration of OVA $_{257-264}$ with sCD70 or OVA $_{257-264}$ with hIgG was examined. Administration of OVA $_{257-264}$ with sCD70 resulted in extensive division of OT-I T cells as evident by the lower levels of CFSE compared with unstimulated cells (Figure 5.4). The proliferation of OT-I T cells in mice that received OVA $_{257-264}$ with hIgG was also efficient, although the levels of the CFSE label were consistently higher in these cells compared with cells stimulated with OVA $_{257-264}$ and sCD70 (Figure 5.4). The difference in CFSE levels between the two cell populations represents a difference of a single cell division. Thus, OVA $_{257-264}$ plus sCD70-stimulated cells cycle only slightly faster than those stimulated with OVA $_{257-264}$. As the numbers of OT-I T cells were 10-fold (day 3; data not shown) and 17-fold (day 4; Figure 5.3a and Table 5.1) higher in OVA$_{257-264}$ plus sCD70-stimulated mice compared with mice stimulated with OVA $_{257-264}$ and hIgG, these results show that sCD70 enhances T cell expansion only in part by enhancing T cell division. Although OT-I T cells stimulated with OVA $_{257-264}$ and hIgG continued to divide between 52 and 68 h after injection of Ag, they failed to accumulate, most likely due to cell death, which is in contrast with OT-I cells stimulated with OVA $_{257-264}$ and sCD70 (Figure 5.4). Taken together these results suggest that CD27 signalling also promotes the survival of activated $CD8^+$ T cells. Therefore, both enhanced proliferation and increased T cell survival contribute to the ability of sCD70 to promote clonal expansion of OT-I T cells *in vivo*.

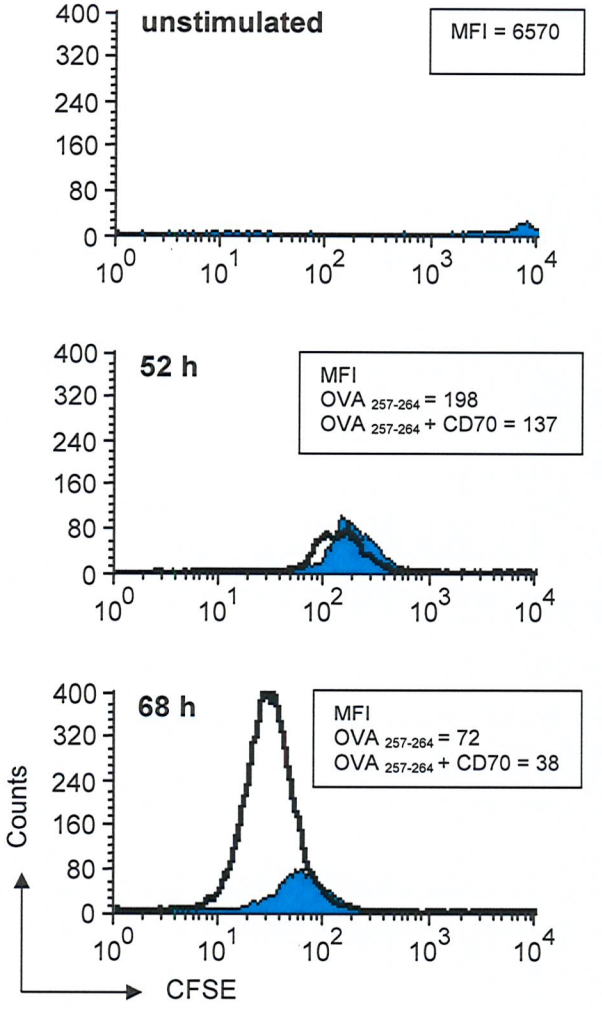


Figure 5.4 The effect of CD27 signalling on T cell division *in vivo*. Representative histogram plots of CFSE-labelled cells gated on $CD8^+$ K^b OVA $_{257-264}$ $tetramer^+$ lymphocytes are shown. 1 x 10^6 CFSE-labelled OT-I T cells were transferred into C57BL/6 recipients, and mice primed 24 h later by i.v. infection of OVA $_{257-264}$ (30 nmoles) plus 3 x 200 µg sCD70 (open histograms) or OVA $_{257-264}$ plus 3 x 200 µg hIgG (filled histograms). 52 and 68 h after antigen administration spleen cells were stained with K^b OVA $_{257-264}$ tetramers and anti-CD8α and were analysed by FCM. Unstimulated mice (top) were analysed 4 days after adoptive transfer. The MFI of the CFSE peaks from unstimulated cells or cells stimulated with OVA $_{257-264}$ plus hIgG (OVA $_{257-264}$) or OVA $_{257-264}$ plus sCD70 (OVA $_{257-264}$ + sCD70) is indicated. The data are representative of two independent experiments.

5.2.3 CD27 signalling promotes the generation of cytotoxic T cells

To examine if CD27 signalling *in vivo* enhances the generation of $CD8^+$ effector T cells, the ability of spleen cells from mice immunized with OVA $_{257-264}$, or OVA $_{257-264}$ and different combinations of LPS and sCD70 to kill target cells was tested. To avoid any potential artefacts caused by *in vitro* re-stimulation, the activity of cytotoxic T cells was measured directly *ex vivo*. The ability of spleen cells to kill EL4 cells pulsed with OVA $_{257-264}$ or E.G7 cells, which express ovalbumin, correlated well with the percentage of antigen-specific T cells present in the spleen 10 days after immunization (Figure 5.5a and 5.5c). Thus, specific cytotoxicity against target cells was easily measurable in spleen cells obtained from animals that were immunized with OVA $_{257-264}$ and sCD70, or OVA $_{257-264}$ with sCD70 and LPS (Figure 5.5a). In contrast, spleen cells obtained from animals that were immunized with OVA $_{257-264}$ alone had no detectable cytotoxic activity, and those from animals immunized with OVA $_{257-264}$ and LPS showed only low levels of cytotoxicity against target cells. When the percentage of cell killing was calculated based on the actual ratio of antigen-specific $CD8^+$ T cells to target cells, no significant difference was observed among cells obtained from mice that received LPS, sCD70 or sCD70 and LPS as indicated by the overlap of the killing curves (Figure 5.5b). Overall, these data suggest that sCD70 promotes the generation of cytotoxic T cells most likely by enhancing the frequency of primed antigen-specific $CD8^+$ T cells rather than directly enhancing their ability to kill.

5.2.4 CD27 signalling enhances secondary responses to antigen

The massive expansion of OT-I T cells following injection of OVA $_{257-264}$ and sCD70 was transient in nature and was followed by a rapid contraction phase. At the end of the contraction phase (day 10) OT-I T cells represented ~ 4 % of the total lymphocytes and this percentage remained the same by day 23 (Table 5.1 and Figure 5.6a). Given that the number of cells that survive after the contraction phase is determined primarily by the magnitude of the primary response [51], it was not surprising that only low numbers of OT-I cells persisted in animals immunized with OVA $_{257-264}$ in the absence of sCD70 (Figure 5.6a and Table 5.1). Since injection of antigenic peptides can lead to T cell unresponsiveness [256-258], it was important to address if the surviving OT-I T cell population was capable of eliciting an effective response following secondary challenge with antigen. Upon re-stimulation with OVA $_{257-264}$ on day 24, mice that

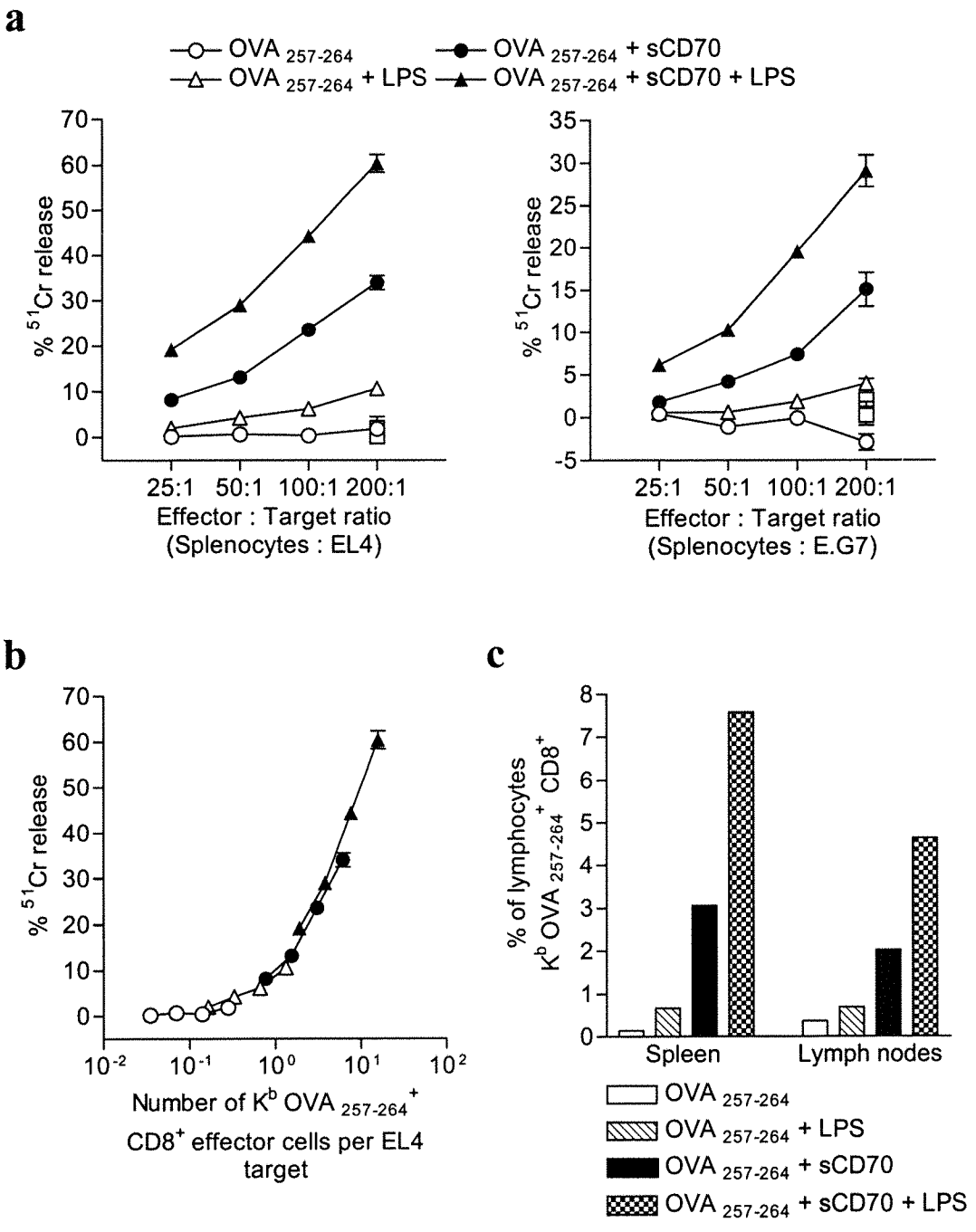


Figure 5.5 CD27 stimulation enhances the number of cytotoxic $CD8^+$ effectors generated during the primary response. Experimental set up was as described in Fig. 5.3. (**a**) *Ex-vivo* cytotoxicity was measured on day 10 post-antigen by ^{51}Cr release from target cells. Pooled splenocytes (n = 3 mice per group) from each group were incubated at the indicated ratios with OVA $_{257\text{-}264}$-pulsed EL4 (left) or E.G7 (right) targets. (□) represents non-specific killing of non-pulsed EL4 targets. Error bars represent the SEM of triplicate cultures. (**b**) The data from (a, left) was replotted as actual number of K^b OVA $_{257\text{-}264}^+$ $CD8^+$ effector T-cells per EL4 target. (**c**) Percentage of antigen-specific K^b OVA $_{257\text{-}264}^+$ $CD8^+$ T-cells in the spleen and lymph nodes (inguinal and brachial) on day 10 post-antigen (n = 3 mice per group). The data are representative of three similar experiments.

were previously immunized with OVA $_{257-264}$ and sCD70 responded with a marked increase in the percentage of OT-I T cells in blood and secondary lymphoid organs (Figure 5.6a and 5.6b). An ~ 8-fold expansion of antigen-specific T cells was observed in the secondary response of mice that were previously immunized with OVA $_{257-264}$ and sCD70 (Figure 5.6c). In contrast, antigen-specific T cells from animals immunized with OVA $_{257-264}$ alone or OVA $_{257-264}$ and LPS showed minimal expansion (< 2-fold) following secondary challenge with OVA $_{257-264}$ (Figure 5.6c). OT-I T cells that had not been previously exposed to antigen were still capable of expanding ~ 9-fold in response to OVA $_{257-264}$ at this time point (Figure 5.6c). The frequency of these naïve antigen-specific T cells (0.08 %) was similar to that of the OVA $_{257-264}$ exposed antigen-specific T cells (0.13 %) prior to restimulation (Figure 5.3a), suggesting that the OT-I T cells which had seen OVA $_{257-264}$ in the absence of sCD70 stimulation had become anergised. Thus, in addition to increasing the frequency of primed antigen-specific T cells, CD27 signalling during the primary response programs naïve T cells to overcome unresponsiveness during secondary exposure to antigen.

The phenotype of the memory cells generated by stimulation of OT-I T cells with OVA $_{257-264}$ and sCD70 was analysed 23 days after primary stimulation. These cells were uniformly high for the memory T cell marker CD44 (Figure 5.7a), which indicates that all cells were exposed to antigen during primary stimulation. The cells exhibited a split phenotype with regard to CD62L, with ~ 50 % being low / negative for this adhesion molecule. CD27 levels were comparable to those on naïve T cells as was the lack of expression of CD25, indicating that the cells were in a resting state. The RB isoform of the glycoprotein CD45 was reduced on these memory cells as compared to naïve T cells, consistent with other reports on murine T cells [253]. In addition the α subunit of the integrin VLA-4 (CD49d) was significantly up regulated on OVA $_{257-264}$ and sCD70 stimulated T cells indicating these cells may have an enhanced ability to home to sites of inflammation [259] (Figure 5.7a).

Tracking the secondary response of OVA $_{257-264}$ and sCD70-induced memory cells over the following 20 days (Figure 5.6a) demonstrated that these T cells had been reprogrammed to undergo an enhanced survival phase after secondary stimulation. The contraction kinetics of the restimulated T cells were much slower than during the

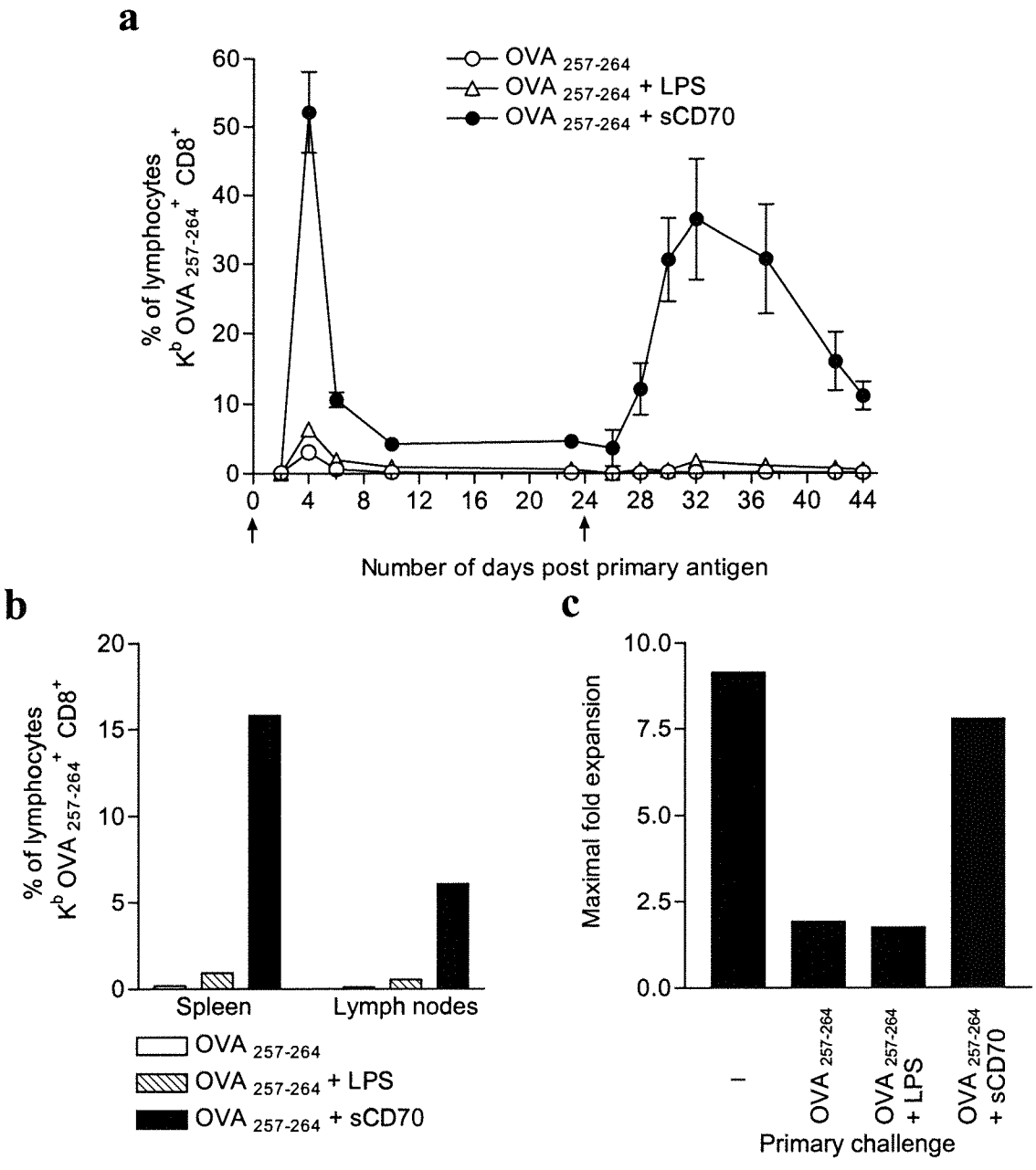


Figure 5.6 CD27 stimulation during the primary response enhances the responsiveness of CD8$^+$ T cells following secondary challenge with antigen. Experimental set up was as described in Figure 5.3. (**a**) Percentage of antigen-specific CD8$^+$ T cells in peripheral blood was tracked over time by double staining with K^b OVA $_{257\text{-}264}$ tetramers and anti-CD8α. 24 days after primary antigen stimulation all groups were given a secondary stimulation of OVA $_{257\text{-}264}$ (20 nmoles) by i.v. injection and antigen specific CD8$^+$ T cells tracked for a further 20 days. (↑) indicates OVA $_{257\text{-}264}$ antigen administration. Error bars represent the SEM, n = 3-6 mice per time-point. (**b**) Percentage of antigen-specific CD8$^+$ T cells in the spleen and lymph nodes (inguinal and brachial) on day 44 (20 days post secondary antigen exposure). n = 3-6 mice. (**c**) Maximal fold expansion of antigen-specific CD8$^+$ T cells during the secondary response was calculated using the mean percentage of antigen-specific CD8$^+$ T cells in peripheral blood on day 23 post primary antigen as the background. The response in mice that were administered sCD70 or hIgG alone during the primary stage of stimulation and are therefore naïve is also shown (-).

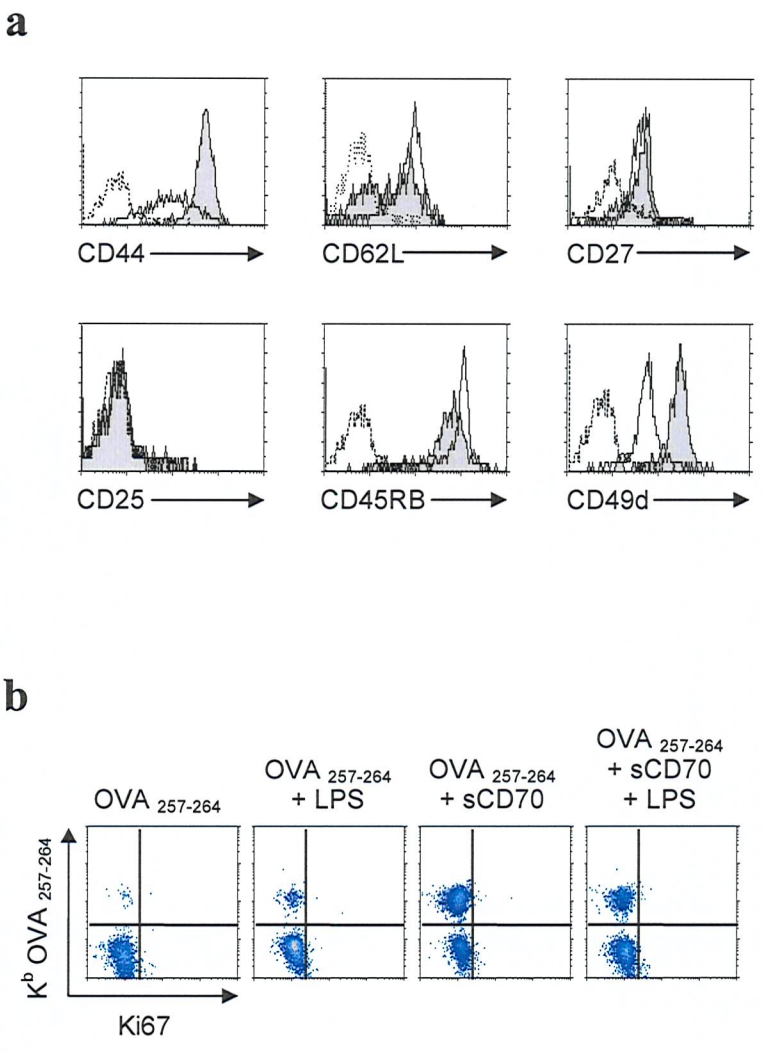


Figure 5.7 OT-I CD8$^+$ T cells remaining 23 days after activation with OVA $_{257-264}$ and sCD70 have a memory phenotype. Experimental set up was as described in Fig. 5.3. (**a**) 23 days post primary antigen stimulation splenocytes from mice given OVA $_{257-264}$ and sCD70 were stained with FITC-labelled anti-CD44, anti-CD62L, anti-CD25 anti-CD45RB or anti-CD49d mAbs, or stained with sCD70 followed by FITC-labelled anti-hFc, (filled histograms). For comparison, naïve OT-I C57BL/6 splenocytes were stained with the same phenotypic markers (open histograms). FITC-labelled isotype control mAb staining of memory cells is shown (dashed line). Histograms are gated on viable, CD8$^+$ and K^b-OVA$_{257-264}$ tetramer$^+$ cells. The data are representative of two independent experiments. (**b**) 24 days after primary antigen stimulation all groups were given a secondary stimulation of OVA $_{257-264}$ (20 nmoles) by i.v. injection and the proliferative status of antigen-specific CD8$^+$ T cells in peripheral blood on day 18 post-secondary antigen was determined by intracellular Ki67 staining in conjunction with K^b OVA $_{257-264}$ tetramers and anti-CD8. Representative density plots are gated on CD8$^+$ lymphocytes. (n = 3 mice per group).

primary response, and this could not be attributed to continued cell division because staining antigen specific T cells for the proliferation marker Ki67 on day 18-post restimulation showed that they were resting cells (Figure 5.7b).

5.2.5 sCD70 enhances the efficacy of a peptide-based anti-tumour vaccine

The data described above strongly suggest that sCD70 could be used to enhance the efficacy of peptide-based vaccines. The ability of sCD70 to promote protective immunity was examined using H-$2K^b$ positive tumour cells (E.G7 cells) that express ovalbumin as a model antigen. In this model, naïve OT-I T cells (10^6 cells) fail to control tumour growth and mice succumb to the tumour eventually (Figure 5.8a). Considering that only ~ 10 % of the transferred T cells engraft or "take" in the host [260], the actual initial OT-I T cell to tumour ratio in these experiments is likely to be ~ 1 to 5. Injection of OVA $_{257\text{-}264}$ provided partial protection against the tumour, with 50 % of the mice surviving beyond 100 days. Interestingly, sCD70 alone gave a similar level of protection to that observed with OVA $_{257\text{-}264}$ (Figure 5.8a). However, optimal anti-tumour immune responses were only observed when OVA $_{257\text{-}264}$ was combined with sCD70; none of the animals that received the combination of OVA $_{257\text{-}264}$ and sCD70 developed tumours (Figure 5.8a). Furthermore, these mice failed to develop tumours following a secondary challenge with E.G7 tumour cells, suggesting that immunization leads to long-term protective immunity (Figure 5.8b). When the numbers of transferred OT-I T cells were titrated down to 10^5 (Figure 5.9a) or 10^4 (Figure 5.9b) the immunisation effects of OVA $_{257\text{-}264}$ or sCD70 alone were largely lost, however the combination of OVA $_{257\text{-}264}$ and sCD70 still generated significant immunity against E.G7 challenge ($P = 0.01$, when compared to OVA $_{257\text{-}264}$ and hIgG with 10^4 OT-I T cells). These results demonstrate that the efficacy of an anti-tumour vaccine can be considerably enhanced by CD27 stimulation *in vivo*.

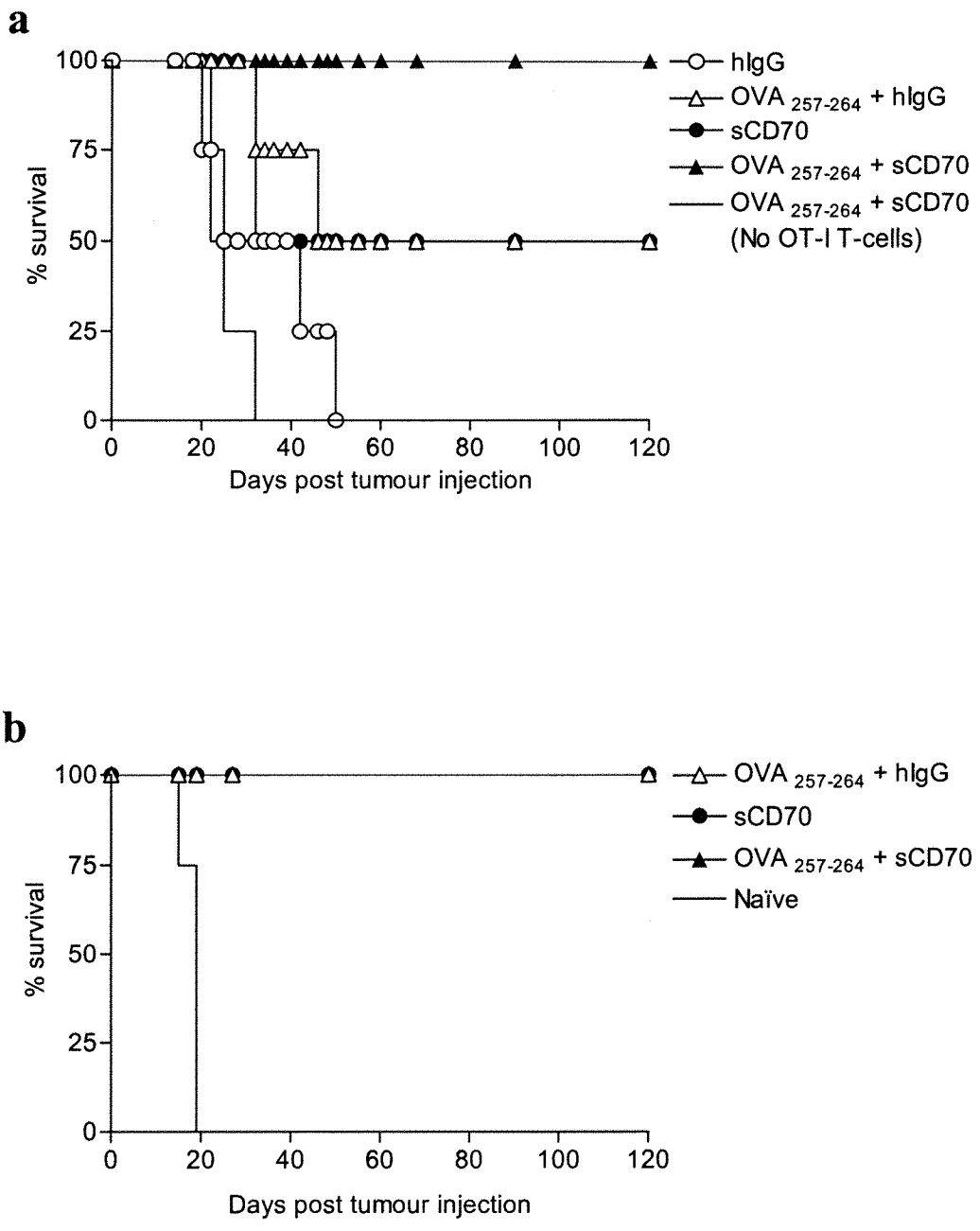


Figure 5.8 sCD70 enhances anti-tumour vaccine efficacy. (**a**) Following adoptive transfer of 10^6 OT-I T cells, naïve C57BL/6 mice were immunized with OVA $_{257-264}$ (20 nmoles) in the presence or absence of sCD70 (200 μg). sCD70 was administered for a further 2 days (600 μg total) with control groups receiving an equal amount of normal hIgG. 5 x 10^5 E.G7 tumour cells were administered s.c. 10 days post-antigen and animals monitored for tumour growth. n = 4 mice per group. (**b**) Surviving mice from (a) were re-challenged with 5 x 10^5 E.G7 tumour cells s.c. on day 147 after primary tumour challenge and monitored for tumour growth. Naïve C57BL/6 mice that had not received OT-I T cells or antigen were also administered E.G7 tumour cells as a control. n = 2-4 mice per group.

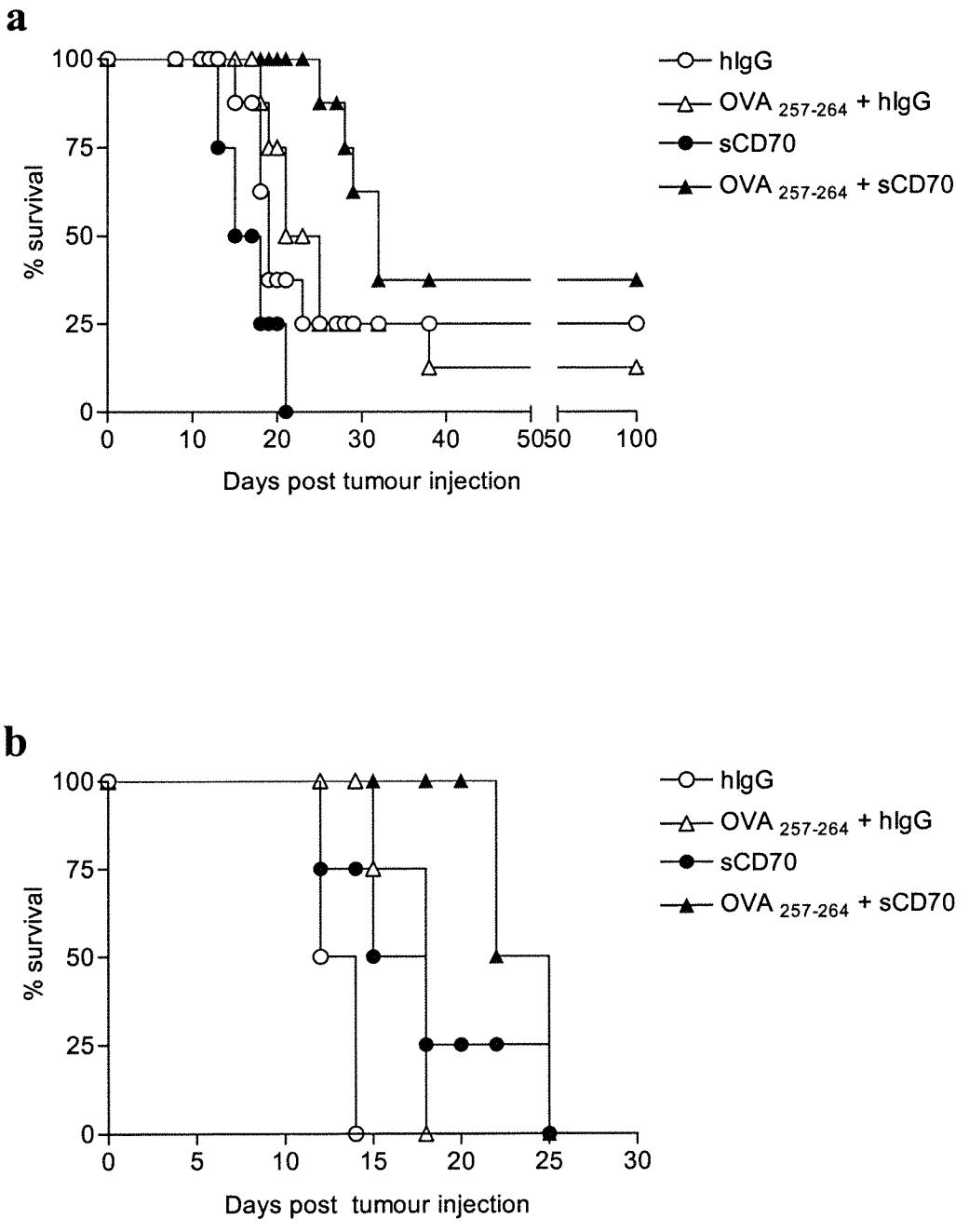


Figure 5.9 sCD70 enhances anti-tumour vaccine efficacy. Following adoptive transfer of 10^5, (**a**), or 10^4, (**b**), OT-I T cells, naïve C57BL/6 mice were immunized with OVA $_{257\text{-}264}$ (20 nmoles) in the presence or absence of sCD70 (200 μg). sCD70 was administered for a further 2 days (600 μg total) with control groups receiving an equal amount of normal hIgG. 5 x 10^5 E.G7 tumour cells were administered s.c. 9 days post-antigen and animals monitored for tumour growth. n = 4-8 mice per group.

5.2.6 sCD70 promotes therapeutic anti-tumour immune responses

In addition to peptide vaccination regimens, the ability of sCD70 to trigger a therapeutic anti-tumour immune response to a syngeneic lymphoma was investigated. A31 is a B cell lymphoma, which grows in the spleen following i.v. injection into CBA mice. This tumour grows progressively in syngeneic mice, however treatment of A31-bearing mice with anti-4-1BB mAbs has previously been shown to generate a T cell response that is capable of controlling tumour growth [151]. Three days after challenge with a high tumour load (5×10^7 A31) mice were treated with either sCD70 or an anti-4-1BB mAb for comparison. In the first experiment (Figure 5.10a), treatment with sCD70 had a dramatic effect on survival times with 60 % of mice surviving for at least 50 days longer than the control treated group. In this experiment, the enhanced survival effectof sCD70 was better than that induced by anti-4-1BB mAb treatment, which could only prolong survival for ~ 30 days longer than the control group. In the second experiment (Figure 5.10b), sCD70 had more modest effects on survival. However, survival times were still significantly enhanced ($P = 0.0025$). In this experiment it was investigated whether $CD8^+$ T cells were required for the therapeutic effect of sCD70. Depletion of $CD8^+$ T cells before tumour challenge completely abrogated sCD70 therapy. Additionally, A31 cells were analysed for cell surface expression of CD27 to rule out sCD70 having any direct effects on the tumour. This B cell lymphoma did not express CD27 (V. Taraban, unpublished observations), therefore it can be concluded that sCD70 mediates its therapeutic effects via activation of anti-tumour immune responses dependent on $CD8^+$ T cells.

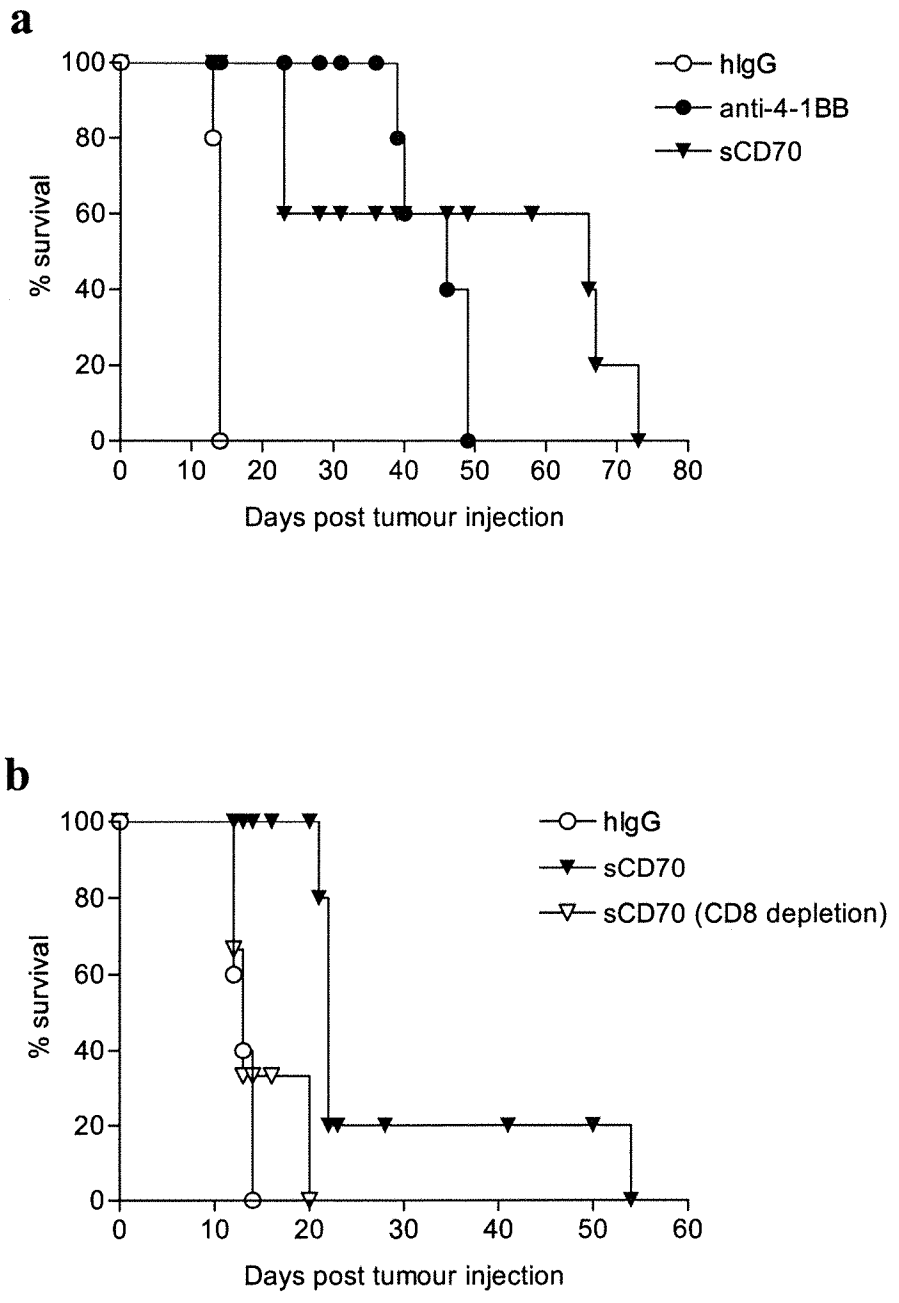


Figure 5.10 Administration of sCD70 prolongs the survival of mice harbouring the A31 lymphoma. (a) 5 x 10^7 A31 lymphoma cells were administered i.v. to naïve CBA mice on day 0, followed by treatment with 200 μg of hIgG, sCD70 or anti-4-1BB (LOB12.3) i.v. on days 3 to 8. n = 5 mice per group. **(b)** 5 x 10^7 A31 lymphoma cells were administered i.v. to naïve CBA mice on day 0, followed by treatment with 250 μg of hIgG or sCD70 i.v. on days 3 to 7. $CD8^+$ T cells were depleted in one group of mice (which were subsequently treated with sCD70) by administration of 500 μg anti-CD8 (YTS169) i.p on days –2, 2, 8 and 13. n = 3-5 mice per group.

5.3 Discussion

The data presented here demonstrate a dual role for the CD27-CD70 interaction in the generation of CD8$^+$ T cell responses *in vivo*. CD27 signalling enhanced both the magnitude of the T cell response, by augmenting the proliferation and survival of CD8$^+$ T cells, and the quality of the response such that CD8$^+$ T cells that respond effectively upon secondary antigen encounter are generated. It has been suggested that the differentiation of naïve CD8$^+$ T cells into effector and memory cells is a progressive process that requires multiple stimulatory signals [56]. Although it is generally accepted that activated APCs such as mature DCs are capable of delivering signals that drive the progressive differentiation of T cells, the molecular nature of these signals is not fully known [56,261]. Activation of naïve CD8$^+$ T cells in the presence of B7-1 and ICAM-1 is not sufficient to drive their complete differentiation. Thus, stimulation of naïve CD8$^+$ T cells via the TCR, CD28 and LFA-1 results in clonal expansion and the acquisition of effector functions, however, these cells become anergic [118,262-264].

5.3.1 Strong primary CD8$^+$ T cell responses induced by CD27 co-stimulation

The data presented in this chapter demonstrates that stimulation via CD27 during the initial activation of CD8$^+$ T cells leads to the effective generation of cytotoxic effector cells that are capable of killing target cells *ex vivo* and more importantly control tumour growth in animals (Figures 5.5, 5.8 and 5.9). This enhanced response, when compared to stimulation by antigen alone or antigen together with LPS as an adjuvant, correlates well with the significant increase in the number of activated antigen-specific CD8$^+$ T cells that results from CD27 stimulation (Table 5.1 and Figures 5.3-5.6). Increased numbers of activated T cells has been previously observed in transgenic mice that constitutively express membrane-bound CD70, presumably as a result of amplification of responses to environmental antigens [185,195]. In these mice T cell proliferation is sustained due to chronic stimulation via CD27 which eventually leads to exhaustion of the T cell pool [195]. In contrast, administration of sCD70, which is cleared relatively quickly from the circulation (Figure 5.2), results in a short period of rapid T cell proliferation that then declines (Figure 5.3b). Thus, the transient proliferative response obtained using sCD70 is likely to be more representative of the

response induced by native membrane-bound CD70, since expression of the latter is tightly controlled under physiological conditions [224].

5.3.2 CD27 programs CD8$^+$ T cell responsiveness to secondary antigen challenge

In addition to augmenting CD8$^+$ T cell immune responses by quantitative means, this data demonstrates that CD27 triggering instils a program of differentiation that allows CD8$^+$ T cells to overcome a state of unresponsiveness. A number of previous studies have shown that injection of soluble antigenic peptides in the absence of adjuvant leads to CD8$^+$ T cell anergy [256-258]. The induction of tolerance after systemic peptide injection as opposed to, for example, T cell immunity after viral infection [256] or subcutaneous vaccination in the presence of CFA [257], may be due to a number of factors. Peptide administration leads to a short timeframe of antigen-exposure and may therefore only cause transient T cell proliferation. In comparison, viral replication or slow release from the subcutaneous depot will lead to more prolonged antigen exposure. The environmental context of antigen presentation is also likely to be critical, with systemic peptide being easily picked up by MHC class I molecules expressed on non-professional APCs which lack MHC class II and co-stimulatory molecule expression. After primary stimulation in this non-inflammatory environment, T cells may become unresponsive even to secondary antigen in the presence of adjuvant [258]. Consistent with this, the results in this chapter show that the secondary response of OT-I CD8$^+$ T cells was diminished in animals that received only OVA $_{257-264}$ systemically during the primary immunization stage. In contrast, animals that received OVA $_{257-264}$ and sCD70 generated effective recall responses with a similar fold-expansion to that of naïve T cells (Figure 5.6). In this *in vivo* model, it appears that CD27-mediated programming of CD8$^+$ T cells occurs during the primary response, since sCD70 was only administered during the initial immunization phase and sCD70 was not detected in the blood prior to secondary immunization with antigen (Figure 5.2c). This data corroborates growing evidence suggesting that anergy may be a consequence of TCR signalling in the absence of co-stimulation [48]. The induction of anergy in CD4$^+$ T cells appears to be predominately controlled by a balance between co-stimulation and counter-stimulation by members of the immunoglobulin superfamily, CD28 and CTLA-4 [265,266]. However, CD4$^+$ and CD8$^+$ T cells have differential requirements for co-stimulation and the factors which control

$CD8^+$ T cell anergy are less well defined [267]. $CD8^+$ T cells have been shown to become non-responsive three to four days following activation in the presence of co-stimulation via CD28 [118,264], and CTLA-$4^{-/-}$ $CD8^+$ T cells can still become anergic *in vivo* after administration of soluble peptide antigen [268]. Thus, other co-stimulatory molecules such as CD27 may have important roles in programming $CD8^+$ T cell reactivity. Previous studies have shown $CD8^+$ T cells that become anergic in the presence of co-stimulatory signals delivered by CD28 display defects in the activation of the mitogen-activated protein kinases (MAP kinases), ERK, JNK and p38 upon secondary stimulation [118,263]. These molecules may be critical for the maintenance of T cell responsiveness, as it has been shown that functional memory $CD8^+$ T cells contain increased levels of phosphorylated components of the MAP kinase cascade upon stimulation [79]. Signalling pathways downstream of CD27 have not yet been clearly delineated, however it is likely that the MAP kinases are one target of the adapter molecules TRAF2 and TRAF5 that interact with the cytoplasmic tail of CD27 [73,186,187]. The results shown here demonstrating that CD27 signalling prevents induction of anergy in $CD8^+$ T cells suggest that the co-stimulatory signals delivered by CD27 may be distinct from those generated by CD28. It is also possible that both CD27 as well as CD28 signalling are required to prevent the induction of anergy in $CD8^+$ T cells.

The inhibitory receptor CTLA-4, which regulates the induction of anergy in $CD4^+$ T cells [269], is not directly involved in the induction of anergy in $CD8^+$ T cells [268]. However, blockade of this receptor on $CD4^+$ T cells prevents the induction of anergy in OT-I T cells, suggesting a role for $CD4^+$ T cells in the regulation of $CD8^+$ T cell anergy [270]. It has recently been established that $CD4^+$ T cells are required for effective secondary responses by $CD8^+$ T cells [47,271-273]. These papers have demonstrated that $CD4^+$ T cells are critically required during the initiation of the naïve $CD8^+$ T cell response, but appear to be dispensable during the maintenance period and recall responses [47,271,272]. This evidence complements the emerging idea that the initial encounter with antigen and co-stimulation sets in motion a developmental program in $CD8^+$ T cells [52,53]. The nature of the help provided by $CD4^+$ T cells is incompletely understood. It is at present unclear if CD4-dependent instruction of $CD8^+$ T cells responsiveness is mediated solely via indirect maturation of APCs or via direct help to $CD8^+$ T cells. Production of the cytokine IL-2 has been shown to constitute at least one

form of CD4-mediated help that could prevent the induction of anergy in $CD8^+$ T cells [264,270]. Observation of OT-I $CD8^+$ T cell responses to intraperitoneal E.G7 tumour has shown they undergo limited expansion, followed by the induction of unresponsiveness and migration away from the tumour site. This unresponsiveness could be overcome by enhancing CD4 help by blocking inhibitory CTLA-4 signalling. IL-2 was found to be necessary and sufficient to mediate the CD4 help and administration of IL-2 alone could reverse tolerance [270]. CD40 ligand, which is expressed on activated $CD4^+$ T cells is also required for the generation of optimal secondary responses by $CD8^+$ T cells [43-45,273]. Activated $CD4^+$ T cells may deliver stimulatory signals through CD40L to CD40 expressed on APCs. These signals 'license' APCs, up regulating their antigen presentation and co-stimulatory ligand array and therefore allowing them to efficiently prime naïve $CD8^+$ T cells [43-45]. The induction of anergy in OT-I T cells in our model system following injection of $OVA_{257-264}$ alone is likely to have resulted from the lack of $CD4^+$ T cell help, since $OVA_{257-264}$ does not activate $CD4^+$ T cells [274,275]. The results shown here suggest that, at least in this model, sCD70 can substitute for the lack of $CD4^+$ T cell help. In addition, endogenous CD70 can be up regulated on APCs following CD40 ligation [224], suggesting that CD70 may constitute one downstream signal of CD4 help for $CD8^+$ T cells. The full mechanism by which CD27 signalling prevents $CD8^+$ T cell unresponsiveness remains to be elucidated. However, it is possible that CD27-mediated enhancement of IL-2 production *in situ* coupled with the up regulation of CD25, the α-chain subunit of the IL-2 receptor (Chapter 4, Figure 4.4b and 4.6), are sufficient to prevent the induction of anergy in $CD8^+$ T cells. Beside IL-2 [264,270] and CD27 stimulation, administration of IL-12 has recently been shown to prevent OT-I T cell anergy induced by injection of $OVA_{257-264}$ [258]. Therefore, it appears that there are multiple signalling pathways capable of preventing the induction of anergy in $CD8^+$ T cells.

5.3.3 sCD70 has the potential to enhance prophylactic and therapeutic anti-tumour immunity

This chapter clearly shows the advantage of using sCD70 in a vaccination regimen in order to generate optimal $CD8^+$ T cell responses. The effectiveness of this approach was demonstrated by the ability of sCD70 to generate a protective anti-tumour immune response *in vivo*. The administration of sCD70 enhanced the anti-tumour response

generated by immunization with a minimal $CD8^+$ T cell epitope (Figure 5.8 and 5.9). In agreement with earlier studies, OT-I T cells alone were not capable of controlling E.G7 tumour growth despite their relatively high precursor frequency. This most likely was due to the induction of non-responsiveness because of insufficient CD4 help [270,276]. Immunisation with systemic peptide alone could enhance survival after tumour challenge only in the presence of high frequency of antigen-specific cells (10^6 transferred). Surprisingly, immunisation with sCD70 alone also enhanced tumour rejection at this frequency of T cells (Figure 5.8a). This was unexpected because the sCD70 given on days 0-2 was cleared from the system before day 10 when E.G7 cells were administered (Figure 5.2c), and sCD70 had no effect on the homeostasis of OT-I T cells in the absence of antigen (Figure 5.3a). CD70 is known to independently activate NK cells and enhance their proliferation and cytokine production [180,196]. It is therefore conceivable that the presence of cytokines such as IFN-γ from activated NK cells helped prime the CD8 response [277]. However, it is unlikely that NK cells were directly involved in E.G7 tumour clearance because they would be inhibited by the high expression of MHC class I. The administration of peptide antigen in combination with sCD70 had the most significant effect on tumour clearance, which was dependent on the presence of OT-I T cells (Figure 5.8 and 5.9). The data from tracking the kinetics of the primary and secondary OT-I responses indicates that this effect is due to the ability of sCD70 co-stimulation to dramatically enhance the numbers of CTL generated during the primary response and prevent them subsequently becoming unresponsive (Figures 5.3 - 5.6).

In a therapeutic model, treatment with sCD70 prolongs the survival of mice that harbour the A31 B cell lymphoma (Figure 5.10). In this system, administration of a high tumour load provokes a weak endogenous anti-tumour immune response [147]. It has previously been shown that this response can be boosted by agonistic mAb against the co-stimulatory molecules CD40 or 4-1BB to generate protective anti-tumour immunity [146,147,151]. The results reported here show that sCD70 can also augment this ongoing but ineffective anti-tumour immune response without the need for prior vaccination. Whilst a significant survival advantage was seen in both experiments after administration of sCD70, the long-term survival rates were not as reproducible as treatment with anti-4-1BB mAb. One explanation for this may be that the sCD70

fusion protein has a much shorter half-life *in vivo* than the anti-4-1BB mAb. This has been previously observed in our laboratory when comparing the half-life of a similar CD40 ligand–hFc fusion protein with an anti-CD40 mAb (L. Haswell, unpublished observations). A reduced half-life may mean that if stimulation of the T cell response with sCD70 does not completely remove the tumour during the treatment period, the tumour would rapidly grow back unchecked. However, the prolonged half-life seen with antibodies may extend the immune system stimulation until 100 % of the tumour is removed.

CHAPTER 6

Characterisation of a soluble form of 4-1BB ligand and comparison *in vivo* with sCD70

6.1 Introduction

As a comparison with CD27, other potential candidates for bypassing $CD8^+$ T cell anergy were evaluated. Signalling through OX40 has previously been shown to break peripheral T cell tolerance [73,116], however this molecule preferentially activates the $CD4^+$ T cell subset [151]. A homologous TNFR superfamily member that has been shown to amplify CTL responses is 4-1BB [151,209]. Several lines of evidence indicate that 4-1BB may be involved maintaining $CD8^+$ T cell secondary responsiveness. Experiments looking at anti-viral immunity after peptide vaccination in 4-1BBL knockout mice showed that 4-$1BBL^{-/-}$ mice have reduced primary and memory $CD8^+$ T cell responses to LCMV peptide $NP_{396-404}$ [173]. Furthermore, the provision of 4-1BBL in a dendritic cell vaccine expressing a human tumour-associated antigen enhanced the effector and memory CTL responses generated [278]. Investigating molecules which control anergy is likely to be an important aid for the design of immunotherapeutic reagents to induce immune responses to tumours [73,138]. Evidence that agonistic monoclonal antibodies against 4-1BB can eradicate established large tumours in mice, including the poorly immunogenic AG104A sarcoma and the highly tumourigenic P815 mastocytoma, was first shown by Melero *et al* [153]. More recently, a combined immunisation protocol using anti-4-1BB mAb and a tumour antigen peptide has been shown to induce regression of established poorly immunogenic tumours that were refractory to treatment with anti-4-1BB mAb alone [154]. In addition, studies by our group have shown both solid tumours and lymphomas can be completely rejected upon treatment with anti-4-1BB mAb [151].

This study aimed to produce a soluble reagent that mimicked the naturally occurring membrane-bound 4-1BBL and was capable of potently stimulating 4-1BB *in vivo.* Soluble recombinant 4-1BBL was used to investigate if 4-1BB co-stimulation can

prevent the induction of non-responsiveness in $CD8^+$ T cells using a well-characterised system of soluble peptide immunisation to generate T cell anergy [256,257]. The results demonstrate that *in vivo* administration of soluble 4-1BBL in conjunction with antigenic peptide leads to dramatically enhanced expansion and survival of $CD8^+$ T cells during the primary response. This results in an increased number of memory $CD8^+$ T cells being generated, which unlike those stimulated with soluble peptide alone are highly responsive to secondary antigen challenge. Furthermore, combining signals through CD27 and 4-1BB can dramatically potentiate the primary antigen response of $CD8^+$ T cells.

6.2 Results

6.2.1 s4-1BBL enhances the proliferation of naïve CD8$^+$ T cells in vitro

A recombinant soluble fusion protein of murine 4-1BBL (s4-1BBL) was generated by tagging the extracellular domain (amino acid residues 108-309) with the Fc region of human IgG1 (see Methods section 2.2.2, schematic Figure 6.1 and 6.2 sequence map). This was achieved using the same modified Fc construct vector utilised for the sCD70 fusion protein (Chapter 3). s4-1BBL was expressed in mammalian Chinese hamster ovary cells and purified by immunoaffinity chromatography in the same way as sCD70.

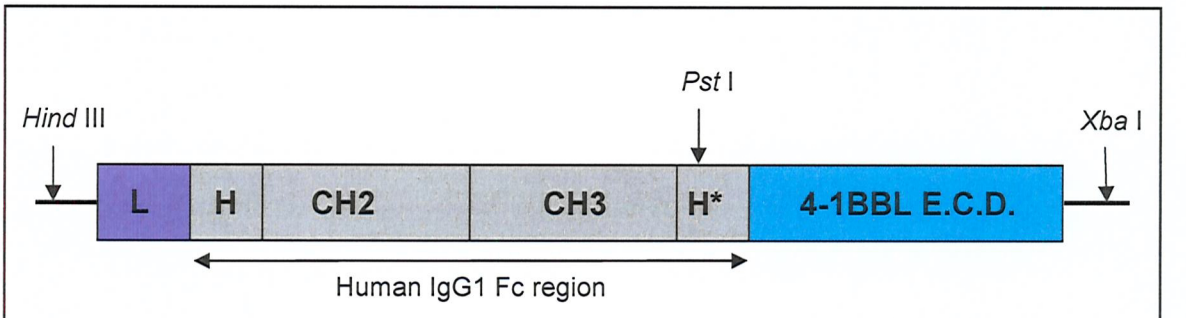
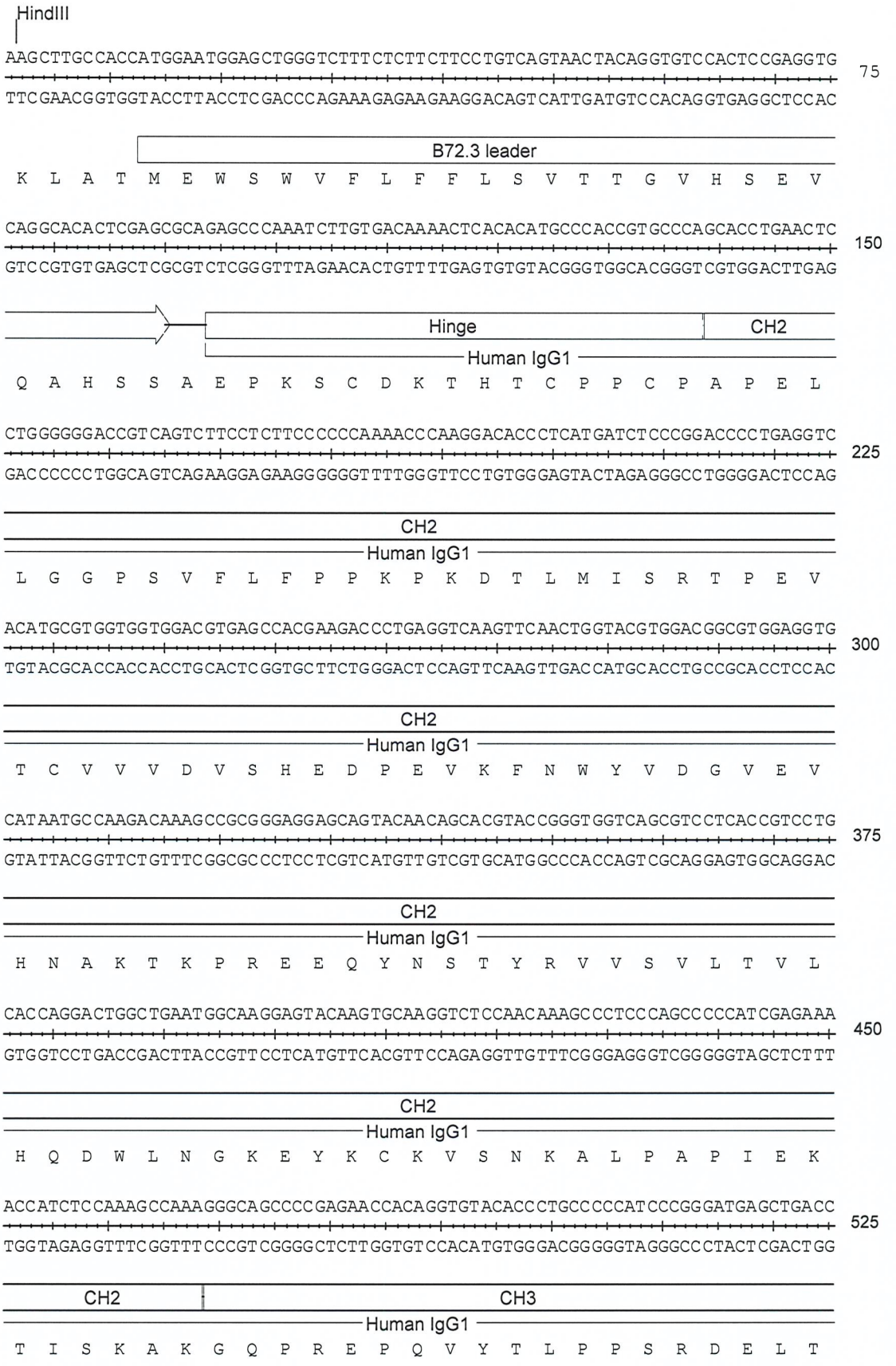


Figure 6.1 Schematic diagram representing the recombinant DNA structure of Fc tagged 4-1BBL fusion construct. (**L**) B72.3 leader sequence. (**H**) Hinge region of hIgG1. (**CH2**) hIgG1 constant heavy chain region 2. (**CH3**) hIgG1 constant heavy chain region 3. (**H***) Modified hIgG1 hinge region in which Cys^{230} and Cys^{234} have been mutated to alanine, and a *Pst* I site has been incorporated. (**4-1BBL E.C.D.**) Region corresponding to the extracellular domain of murine 4-1BBL. Restriction enzyme sites used for cloning are indicated.

Purified s4-1BBL recombinant protein was initially tested for co-stimulatory activity by T cell proliferation. When murine CD8$^+$ T cells were stimulated *in vitro* with an agonistic anti-CD3 mAb to simulate TCR engagement, the presence of s4-1BBL increased T cell proliferation by up to three-fold compared to a control Ig (Figure 6.3a). To examine the effect of s4-1BBL on antigen-specific T cell proliferation naïve OT-I TCR transgenic CD8$^+$ T cells were used. The proliferation of OT-I T cells responding to irradiated syngeneic tumour cells transfected with ovalbumin cDNA (E.G7 cells) was enhanced by s4-1BBL over a range of E.G7 to OT-I ratios, again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75
B72.3 leader
K L A T M E W S W V F L F F L S V T T G V H S E V
CAGGCACACTCGAGCGCAGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC
GTCCGTGTGAGCTCGCGTCTCGGGTTTAGAACACTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAG
150
Hinge
CH2
Human IgG1
Q A H S S A E P K S C D K T H T C P P C P A P E L
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GACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAG
225
CH2
Human IgG1
L G G P S V F L F P P K P K D T L M I S R T P E V
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300
CH2
Human IgG1
T C V V V D V S H E D P E V K F N W Y V D G V E V
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375
CH2
Human IgG1
H N A K T K P R E E Q Y N S T Y R V V S V L T V L
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CH2
Human IgG1
H Q D W L N G K E Y K C K V S N K A L P A P I E K
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CH3
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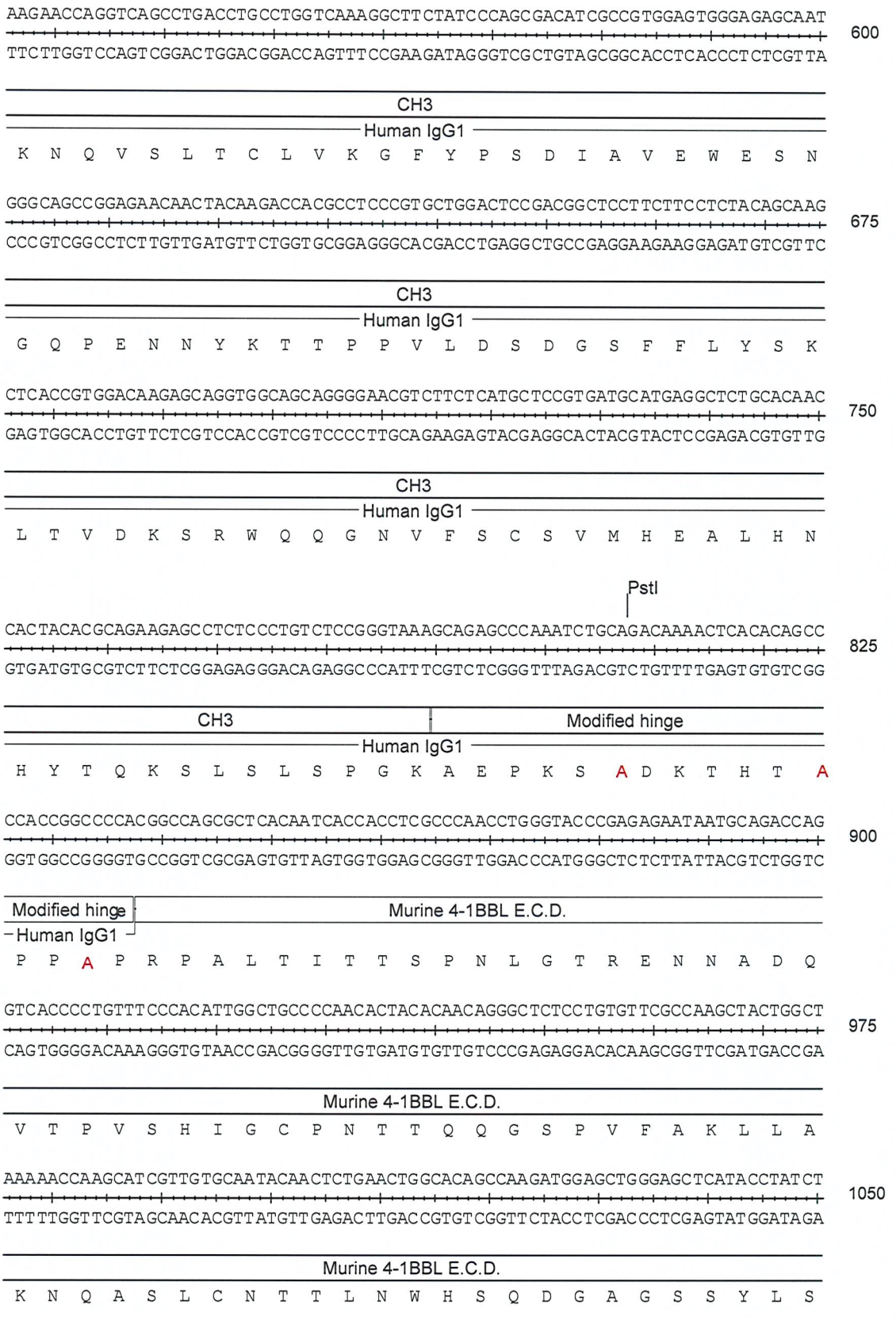
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600
CH3
Human IgG1
K N Q V S L T C L V K G F Y P S D I A V E W E S N
GGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAG
CCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGATGTCGTTC
675
CH3
Human IgG1
G Q P E N N Y K T T P P V L D S D G S F F L Y S K
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750
CH3
Human IgG1
L T V D K S R W Q Q G N V F S C S V M H E A L H N
PstI
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GTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTCGTCTCGGGTTTAGACGTCTGTTTTGAGTGTGTCGG
825
CH3
Modified hinge
Human IgG1
H Y T Q K S L S L S P G K A E P K S A D K T H T A
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GGTGGCCGGGGTGCCGGTCGCGAGTGTTAGTGGTGGAGCGGGTTGGACCCATGGGCTCTCTTATTACGTCTGGTC
900
Modified hinge
Human IgG1
Murine 4-1BBL E.C.D.
P P A P R P A L T I T T S P N L G T R E N N A D Q
GTCACCCCTGTTTCCCACATTGGCTGCCCCAACACTACACAACAGGGCTCTCCTGTGTTCGCCAAGCTACTGGCT
CAGTGGGGACAAAGGGTGTAACCGACGGGGTTGTGATGTGTTGTCCCGAGAGGACACAAGCGGTTCGATGACCGA
975
Murine 4-1BBL E.C.D.
V T P V S H I G C P N T T Q Q G S P V F A K L L A
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TTTTTGGTTCGTAGCAACACGTTATGTTGAGACTTGACCGTGTCGGTTCTACCTCGACCCTCGAGTATGGATAGA
1050
Murine 4-1BBL E.C.D.
K N Q A S L C N T T L N W H S Q D G A G S S Y L S

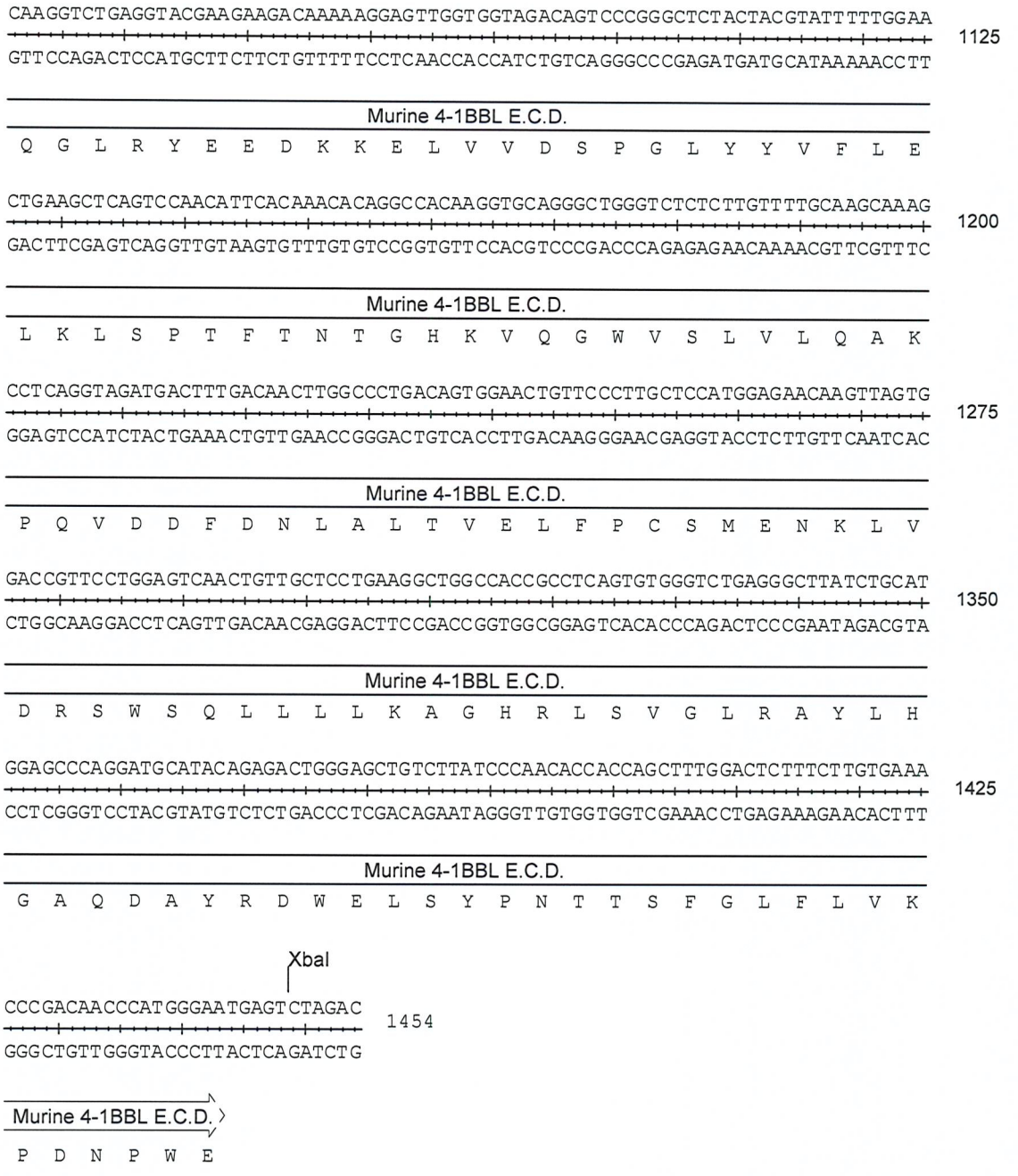

Figure 6.2 Sequence map of the recombinant DNA structure of s4-1BBL. Regions corresponding to the B72.1 leader sequence; the hIgG1 hinge, CH2, CH3, modified hinge; and the extracellular domain of murine 4-1BBL are indicated below the cDNA sequence. Amino acid sequence is also shown. The restriction sites *Hind*III, *Pst*I and *Xba*I that were used for cloning are marked. The C→A mutations that remove the disulphide-bonding cysteines in the modified hinge are indicated in red.

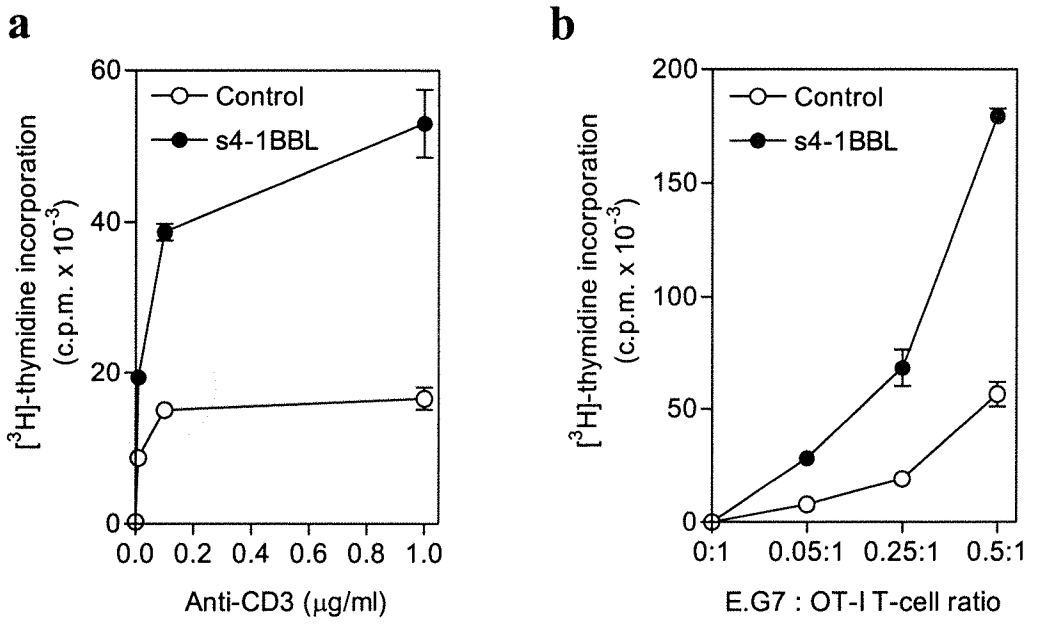

Figure 6.3 s4-1BBL enhances the proliferation of naïve CD8$^+$ T cells *in vitro*. (a) CD8$^+$ T cells were purified from murine BALB/c splenocytes using depletion columns and cultured (2 x 10^5) in the presence of the indicated concentrations of soluble anti-CD3 mAb and either s4-1BBL or a control Ig (10 μg/ml). **(b)** 2 x 10^5 OT-I *RAG1*$^{-/-}$ splenocytes were cultured with the indicated ratios of irradiated E.G7 cells in the presence of either s4-1BBL or a control Ig (10 μg/ml). In both experiments 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Error bars represent the SEM of triplicate cultures.

reaching a three-fold increase with the highest ratio (Figure 6.3b). In the absence of antigen or agonist anti-CD3 mAb, s4-1BBL had no effect on the proliferation of resting $CD8^+$ T cells. This confirms that the co-stimulatory effect of s4-1BBL synergises with TCR signalling and it has no mitogenic effects in isolation.

In both assays, the enhancement of T cell proliferation by s4-1BBL was more pronounced at the higher antigen concentrations. This may be explained by the cell surface expression of 4-1BB requiring TCR signalling for up-regulation [151] and thus greater numbers of receptors being engaged by s4-1BBL at high antigen concentrations. Recombinant s4-1BBL fusion protein therefore appeared to have effective co-stimulatory effects *in vitro* and warranted further investigation as a potential *in vivo* reagent.

6.2.2 Analysis of s4-1BBL by size-exclusion chromatography and SDS-PAGE

To further characterise the structure of s4-1BBL it was fractionated by size-exclusion chromatography (Figure 6.4a). In solution in its native form, s4-1BBL formed two major structures, one with an apparent molecular mass of ~ 150 kDa and one of > 300 and < 900 kDa. The predicted M_r for a single polypeptide chain of s4-1BBL fusion protein is 50.2 kDa; therefore the low M_r peak has the stoichiometry of a trimer. The broad high M_r peak of s4-1BBL corresponds to a hexamer or two groups of trimers, and additional higher M_r multimers. Analysis of unfractionated s4-1BBL and the high or low M_r peak fractions by denaturing SDS-PAGE under reducing conditions, demonstrated that all fractions formed protein bands with a M_r of ~ 50 kDa, which corresponds to a single polypeptide chain (Figure 6.4b, right). Hence, the low M_r peak was not generated by proteolysis. Under denaturing but non-reducing conditions (Figure 6.4b, left), high M_r s4-1BBL formed large covalently-held structures represented by bands greater than 175 kDa. Low M_r s4-1BBL also appeared to be held in its trimeric conformation by covalent bonds, forming a band at ~ 150 kDa. These multimers of s4-1BBL are probably formed by covalent di-sulphide bonding between the hinge regions in the Fc domains of the recombinant protein.

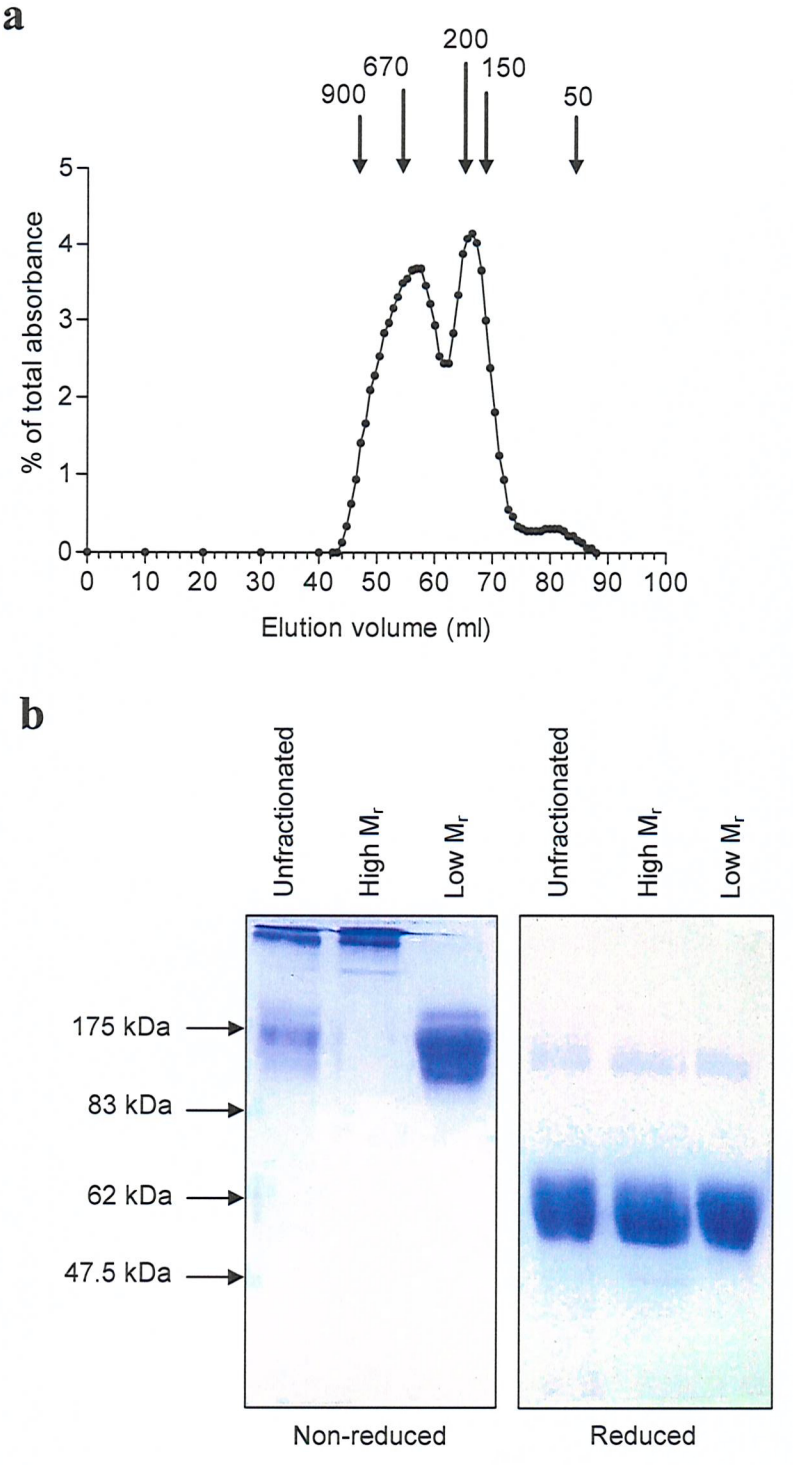

Figure 6.4 Analysis of s4-1BBL by size-exclusion chromatography and SDS PAGE. (**a**) Fractionation by size exclusion chromatography. ~ 17 mg of purified s4-1BBL was separated into 2 ml fractions using a Hiload 16/60 Superdex 200 column at 0.8 ml/min in PBS. Relative elution positions of M_r markers IgM (900 KDa), thyroglobin (670 KDa), β-amylase (200 KDa), IgG (150 KDa) and Fc (50 KDa) are indicated. (**b**) SDS PAGE analysis of fractionated s4-1BBL. 5 μg of unfractionated, high or low M_r peak fractions, either non-reduced (left) or reduced with 50 mM DTT (right), were run on an 8.5 % polyacrylamide gel. The positions of relevant M_r markers are indicated. The data are representative of two independent experiments.

6.2.3 Co-stimulation of OT-I T cells requires the high M_r fraction of s4-1BBL

The two structures of s4-1BBL were consistently produced in equal proportions. However, it is not clear which of these structures is the most effective in stimulating signalling via 4-1BB. Certain members of the TNFR superfamily, such as TNFR-I and TWEAK receptor, appear to signal maximally upon the binding of trimeric ligands [234,279]; however others such as CD40 and Fas require hexameric ligands for efficient signalling [163,234]. This requirement for ligand multimerisation has not yet been established for 4-1BB.

To determine if the differing molecular mass structures formed by s4-1BBL are equally capable of transducing co-stimulatory signals through 4-1BB, the ability of high or low M_r fractions to enhance the proliferation of OT-I $CD8^+$ T cells responding to irradiated E.G7 was compared. Unfractionated, low M_r peak or high M_r peak fractions of s4-1BBL were titrated from 10 to 0.01 μg/ml and compared to hIgG as an isotype control. When 1 x 10^5 OT-I T cells were stimulated with 1 x 10^3 E.G7 (Figure 6.5a) unfractionated s4-1BBL enhanced proliferation, reaching near saturation at 0.1 μg/ml. The high M_r fraction also potently enhanced the proliferation by up to 4.5-fold with a similar saturation curve. However, the low M_r fraction had no effect on OT-I proliferation at any of the concentrations tested. When 1 x 10^5 OT-I T cells were stimulated with 5 x 10^3 E.G7 cells (Figure 6.5b), unfractionated s4-1BBL increased T cell proliferation from 0.1 μg/ml and its effects reached saturation at 1 μg/ml. The high M_r multimeric s4-1BBL was even more potent than the unfractionated at enhancing T cell proliferation at all concentrations tested. In contrast, low M_r trimeric s4-1BBL had no stimulatory effect on OT-I $CD8^+$ T cell proliferation and actually inhibited the response as compared to the hIgG control. One explanation for this is that despite trimeric s4-1BBL being unable to signal through its receptor, it may still bind sufficiently to block interactions with endogenous membrane-bound 4-1BBL. Crosslinking low M_r s4-1BBL using an anti-hFc mAb restored its ability to co-stimulate OT-I T cell proliferation (V. Taraban, unpublished observations), indicating that the multimeric nature of this ligand is critical. Therefore, high-order multimerisation of 4-1BBL appears to be important for inducing co-stimulatory signalling through the receptor, and the removal of inhibitory low M_r fractions may be necessary to generate the most effective agonistic reagent.

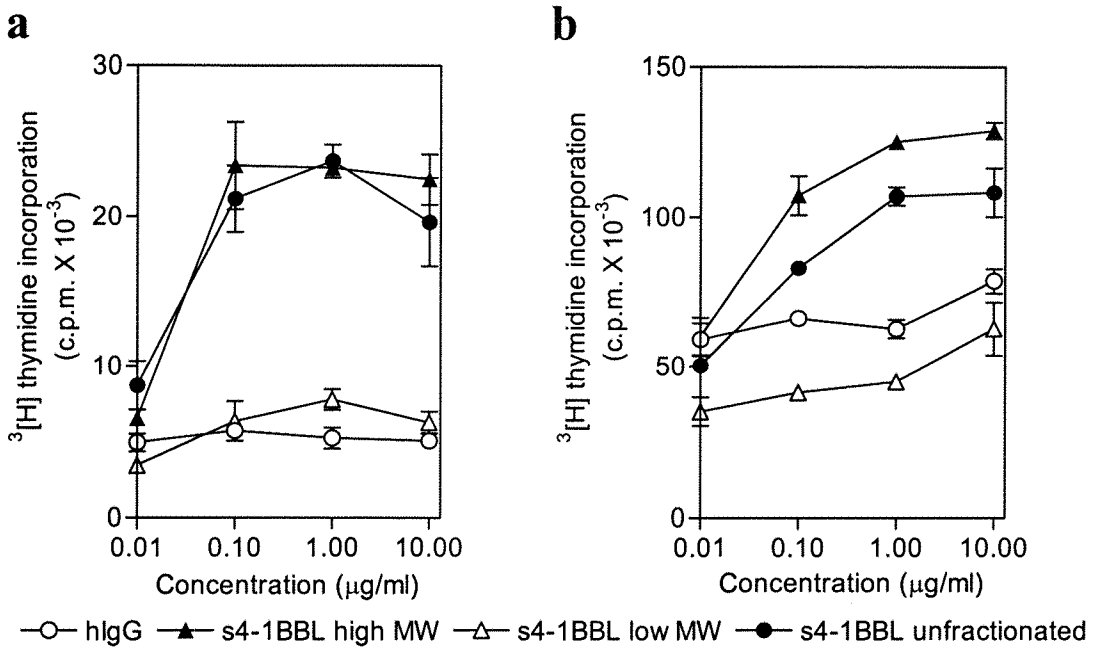

Figure 6.5 Co-stimulation of OT-I T cells requires the high M_r fraction of s4-1BBL. OT-I *RAG1*$^{-/-}$ splenocytes (1 x 10^5) were cultured with either (**a**) 1 x 10^3 or (**b**) 5 x 10^3 irradiated E.G7 cells in the presence of a titration of unfractionated, high or low M_r s4-1BBL, or hIgG as a control. 0.5 μCi [^{3}H]-thymidine was added for the last 16 h of a 72 h culture, after which cells were harvested and incorporation measured. Error bars represent the SEM of triplicate cultures. The data are representative of two independent experiments.

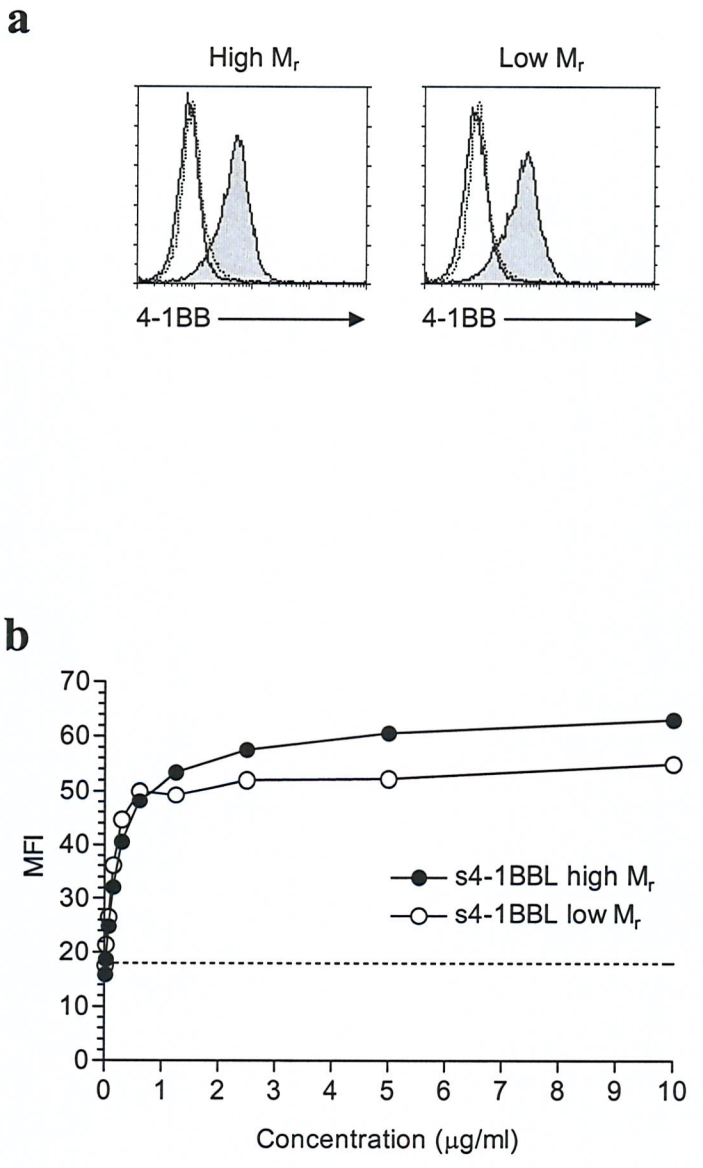

Figure 6.6 High and low M_r fractions of s4-1BBL bind specifically to 4-1BB with comparable affinities. (**a**) Activated OT-I *RAG1-/-* T cells (100 pM OVA $_{257-264}$, 48 hr) were stained with s4-1BBL high or low M_r fractions (10 μg/ml) followed by anti-hFc-FITC (filled histograms) or stained with hIgG followed by anti-hFc-FITC (open histograms). Binding of s4-1BBL was blocked (dashed line) by the presence of anti-4-1BB mAbs (LOB12 and LOB12.3, 100 μg/ml). Histograms are gated on live $CD8^+$ T cells. (**b**) Activated OT-I T cells were stained with a titration of s4-1BBL high or low M_r fractions followed by anti-hFc-FITC and analysed by FCM. Mean fluorescent intensity (MFI, geometric) is plotted versus s4-1BBL concentration. The dashed line represents the MFI of control hIgG binding.

6.2.4 High and low M_r fractions of s4-1BBL bind specifically to 4-1BB with comparable affinities

The lack of activity in the low M_r fraction of s4-1BBL, as compared to the high M_r multimeric fraction, could be explained by it having a low relative affinity for 4-1BB. To address this possibility, the binding of low and high M_r fractions of s4-1BBL to 4-1BB expressed on the surface of activated OT-I $CD8^+$ T cells was compared by FCM (Figure 6.6). Cell surface binding of s4-1BBL was visualised by secondary staining with a FITC-labelled anti-hIgG mAb. Under saturating concentrations, both M_r fractions bound equally to 4-1BB that was expressed on the majority of activated OT-I $CD8^+$ T cells (Figure 6.6a, filled histograms). The cell surface binding of low and high M_r s4-1BBL was specific for 4-1BB as it could be completely inhibited by the presence of an excess of anti-4-1BB Ab (dashed lines). The apparent binding affinities of both fractions were compared by titrating the concentration of s4-1BBL during OT-I T cell staining and analysing the mean fluorescence intensity (Figure 6.6b). High and low M_r fractions of s4-1BBL had comparable affinities for their receptor. This indicated that the difference in signalling ability was not due to differences in apparent affinity.

6.2.5 Administration of high M_r s4-1BBL prevents T cell anergy in vivo

To examine the effects of high M_r s4-1BBL on $CD8^+$ T cell antigen responses *in vivo,* the model of peptide antigen administration that was previously found to induce anergy in OT-I T cells (Chapter 5) was utilised. Naïve OT-I $CD8^+$ T cells (10^6) were adoptively transferred to syngeneic C57BL/6 mice, allowed to equilibrate, and immunised i.v. with soluble OVA $_{257-264}$ peptide in the presence of high M_r s4-1BBL or hIgG as a control. After transfer, naïve OT-I $CD8^+$ T cells stably formed ~ 0.1 % of lymphocytes and this population could be tracked by double staining with anti-CD8 and K^b OVA $_{257-264}$ tetramers. Upon administration of OVA $_{257-264}$ peptide and hIgG OT-I $CD8^+$ T cells expanded ~ 8 fold, peaking by day 4 post antigen at 1 % of peripheral blood lymphocytes (Figure 6.7a, graph and representative density plots). In contrast, upon administration of OVA $_{257-264}$ peptide and s4-1BBL OT-I $CD8^+$ T cells expanded ~ 96 fold and by day 4 made up 12.4 % of peripheral lymphocytes. This percentage was stable until day 7 post antigen and had only declined slightly by day 10. At this time point, OT-I $CD8^+$ T cells in mice that were administered OVA $_{257-264}$

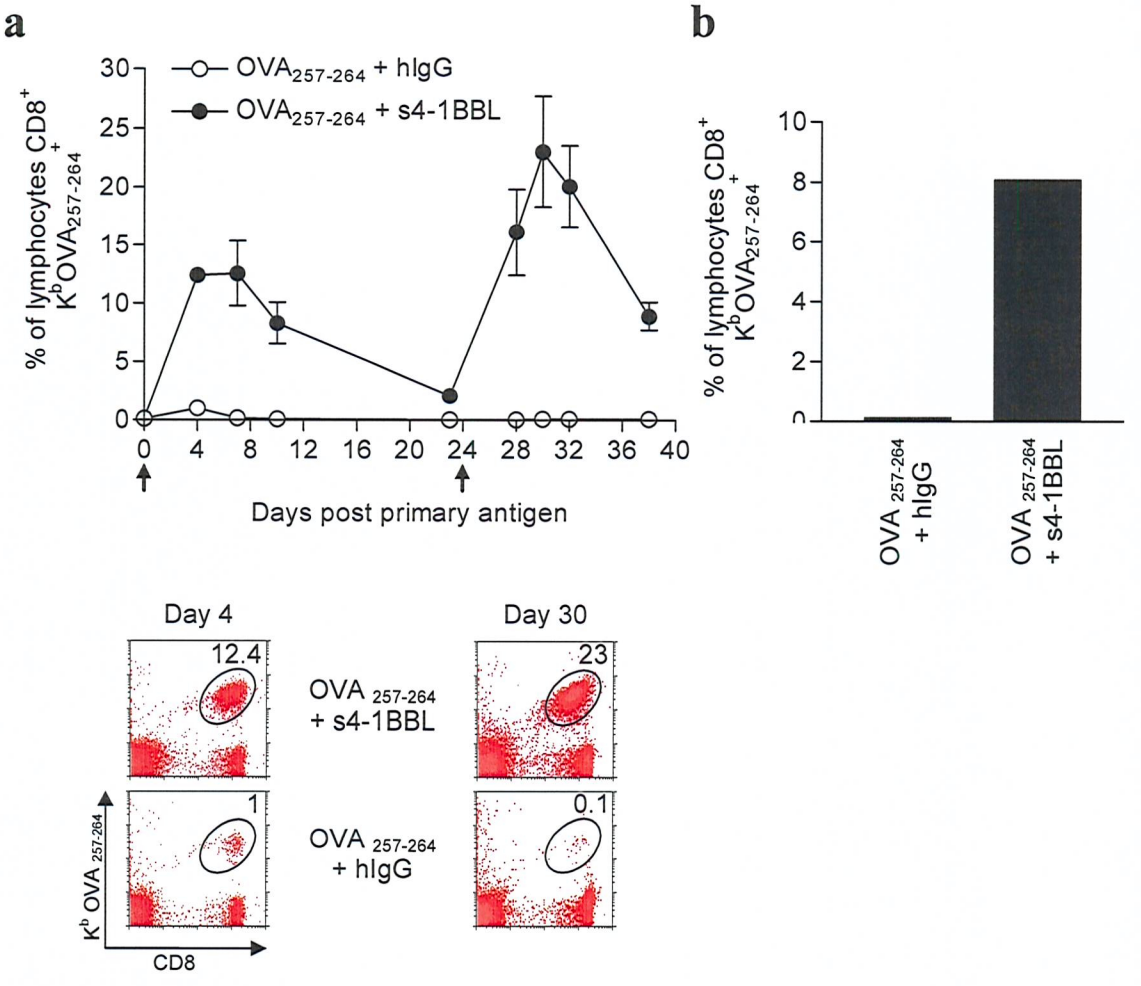

Figure 6.7 Administration of high M_r s4-1BBL prevents T cell anergy *in vivo*. Naïve OT-I C57BL/6 T cells (1 x 10^6) were transferred into C57BL/6 recipients and mice were primed by i.v. injection of OVA $_{257-264}$ (30 nmoles) in the presence of high M_r s4-1BBL or an equal amount of hIgG as a control (3 x 250 μg). (**a**) Percentage of antigen-specific $CD8^+$ T cells in peripheral blood was tracked over time by double staining with K^b OVA $_{257-264}$ tetramers and anti-CD8. Twenty-four days after primary antigen stimulation all groups were given a secondary stimulation of OVA $_{257-264}$ (30 nmoles) by i.v. injection and antigen specific $CD8^+$ T cells tracked for a further 14 days. (↑) indicates OVA $_{257-264}$ antigen administration. Error bars represent the SEM, *n* = 3 mice per time-point. Representative density plots gated on live lymphocytes are shown below, with mean percentages of lymphocytes K^b OVA $_{257-264}{}^+$ and $CD8^+$ indicated. (**b**) Percentage of antigen-specific $CD8^+$ T cells in the spleen on day 39 (15 days post secondary antigen exposure). *n* = 5 mice.

and hIgG had dwindled to pre-expansion background levels (0.1 %). Continued tracking revealed that OT-I $CD8^+$ T cells that had been stimulated with OVA $_{257-264}$ and s4-1BBL still made up 2.1 % of lymphocytes on day 23-post antigen, indicating a significant memory T cell pool had been generated. However, OT-I $CD8^+$ T cells stimulated with OVA $_{257-264}$ and hIgG were very low (0.05 %) by day 23.

In order to test if the remaining T cells were capable of responding to secondary antigen challenge, both groups of mice were administered another dose of OVA $_{257-264}$ in the absence of adjuvant. OT-I $CD8^+$ T cells stimulated with OVA $_{257-264}$ and hIgG during the primary response were unresponsive to secondary antigen exposure, increasing in numbers only very marginally to 0.09 %. Conversely, the OT-I $CD8^+$ T cells remaining after primary stimulation with OVA $_{257-264}$ and s4-1BBL were highly responsive to secondary stimulation, increasing by greater than 10 fold to 23 % of peripheral lymphocytes 6 days after antigen encounter. The percentage of antigen specific $CD8^+$ T cells was still elevated in this group 15 days post secondary stimulation in both peripheral blood (Figure 6.7a) and the spleen (Figure 6.7b). In this model system, the OT-I $CD8^+$ T cells remaining after peptide stimulation developed anergy and showed diminished responses to secondary antigen encounter. Co-stimulation through 4-1BB in addition to soluble peptide appeared to modulate both the primary expansion and survival kinetics of the $CD8^+$ T cells, and allowed the formation of a responsive memory pool.

6.2.6 CD27 and 4-1BB co-stimulation have differential effects on the kinetics of primary $CD8^+$ T cell expansion

The data reported here, along with previous work on CD27 and 4-1BB [73], suggests that these two co-stimulatory molecules work in similar ways to enhance the division and survival of $CD8^+$ T cells via related TRAF-linked signalling pathways. However, their cell surface expression pattern differs and this may lead to them affecting the kinetics of *in vivo* T cell responses differently. To directly compare the effects of CD27 and 4-1BB on $CD8^+$ T cells during an *in vivo* primary response the high M_r soluble ligands sCD70 and s4-1BB were used. These fusion proteins are very similar in structure, except for the receptor-binding region, allowing a straight comparison of the effects of these two molecules in the same experiment (Figure 6.8). As seen previously (Chapter

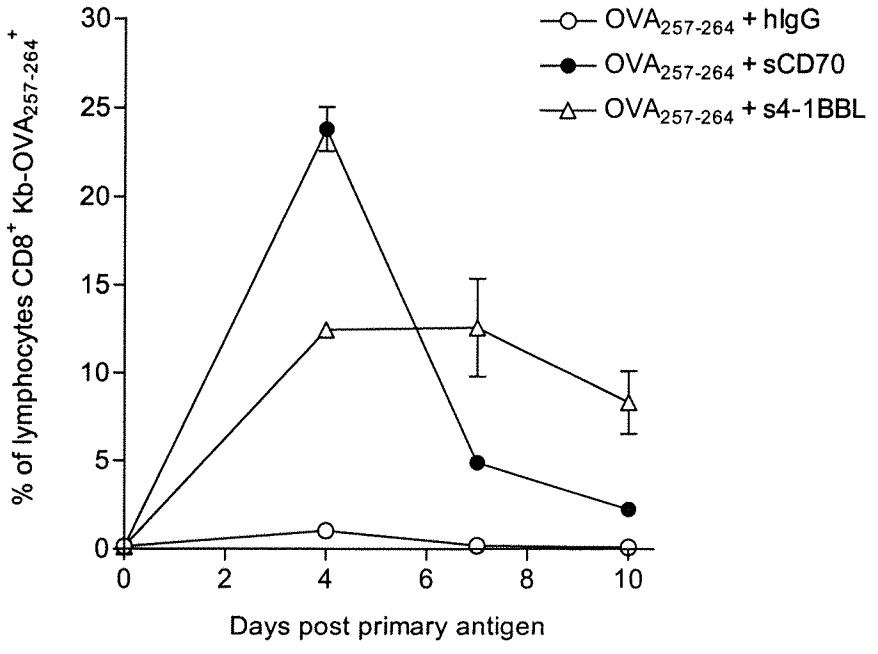

Figure 6.8 CD27 and 4-1BB co-stimulation have differential effects on primary CD8$^+$ T cell expansion kinetics. Naïve OT-I C57BL/6 T cells (1 x 10^6) were transferred into C57BL/6 recipients and mice were primed by i.v. injection of OVA $_{257\text{-}264}$ (30 nmoles) in the presence of high M_r s4-1BBL, sCD70 or an equal amount of hIgG as a control (3 x 250 μg). The percentage of antigen-specific CD8$^+$ T cells in peripheral blood was tracked over time by double staining with K^b OVA $_{257\text{-}264}$ tetramers and anti-CD8. Error bars represent the SEM, n = 3 mice per time-point.

5), after transfer of 10^6 OT-1 T cells administration of OVA $_{257-264}$ and sCD70 led to a sharp increase in OT-1 $CD8^+$ T cell numbers, which peaked at 24 % on day 4 and then contracted quite rapidly but left an enhanced number of surviving cells. In contrast, administration of OVA $_{257-264}$ and s4-1BBL generated a response that was lower in amplitude but which was maintained for much longer after its maximal expansion point (12 %) at day 4. 4-1BB stimulation therefore appeared to have a greater effect on the survival of stimulated $CD8^+$ T cells at late time points, whereas CD27 stimulation primarily enhanced the initial burst size.

6.2.7 Combination of CD27 and 4-1BB co-stimulation has an additive effect on CTL numbers

The differential effects of sCD70 or s4-1BBL on the kinetics of primary OT-I T cell responses suggested that combining these two co-stimulatory signals could maximise the response. To investigate this, antigen was administered along with a combination of sCD70 and anti-4-1BB mAb (3 x 500 μg of mixed reagent) in comparison with anti-4-1BB mAb alone (3 x 500 μg) (Figure 6.9). Anti-4-1BB mAb was used instead of s4-1BBL in this experiment because of lack of availability of the ligand. The same OT-I T cell response kinetics were seen using anti-4-1BB mAb as with s4-1BBL (Figure 6.8), but anti-4-1BB induced a relatively greater response perhaps because more mAb was administered; alternatively the mAb may have an increased half-life *in vivo* compared to the soluble 4-1BB ligand, prolonging signalling through 4-1BB. Combination of sCD70 and anti-4-1BB co-stimulators had a significant additive effect on the magnitude of the primary response in peripheral blood (P = 0.002, day 4) when compared to just anti-4-1BB mAb (Figure 6.9). Moreover, administration of OVA $_{257-264}$ and a combination of sCD70 and anti-4-1BB increased antigen-specific CTL numbers in the spleen and lymph nodes on day 9 (Figure 6.10a) to a greater extent than either reagent alone. This resulted in a concomitant enhancement of ex-vivo cytolytic activity against syngeneic EL4 cells presenting OVA $_{257-264}$ (Figure 6.10b).

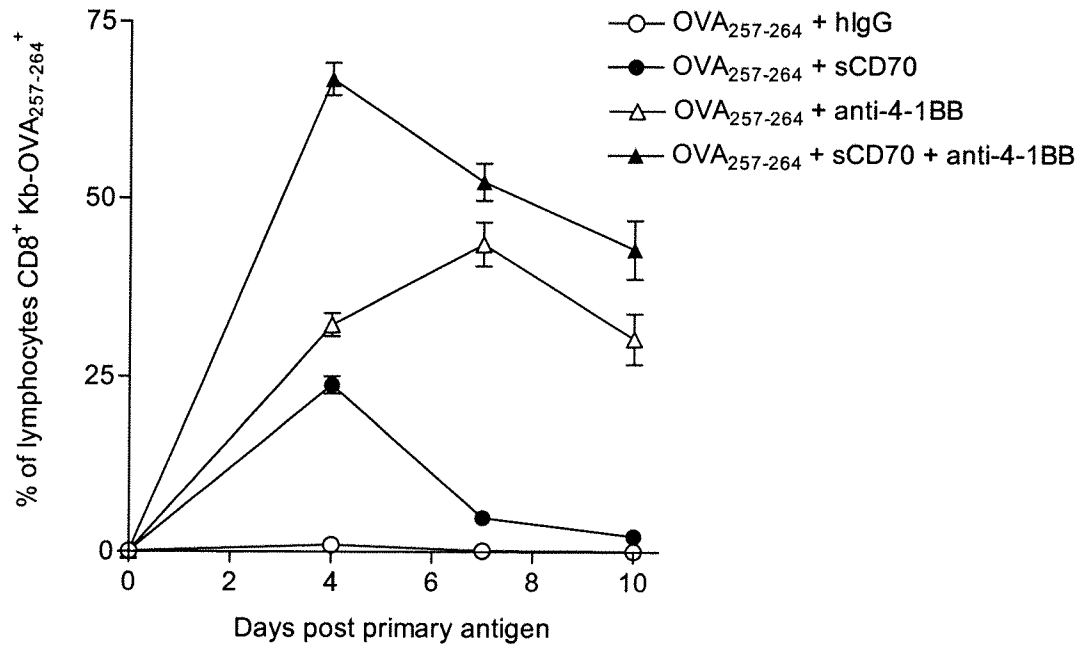

Figure 6.9 CD27 and 4-1BB co-stimulation have an additive effect in combination. Naïve OT-I C57BL/6 T cells (1 x 10^6) were transferred into C57BL/6 recipients and mice were primed by i.v. injection of OVA $_{257-264}$ (30 nmoles) in the presence of sCD70 (3 x 250 μg), hIgG (3 x 250 μg), anti-4-1BB (LOB12.3, 3 x 500 μg) or a combination of sCD70 and anti-4-1BB (3 x 250 μg of each). The percentage of antigen-specific $CD8^+$ T cells in peripheral blood was tracked over time by double staining with K^b OVA $_{257-264}$ tetramers and anti-CD8. Error bars represent the SEM, *n* = 3 mice per time-point.

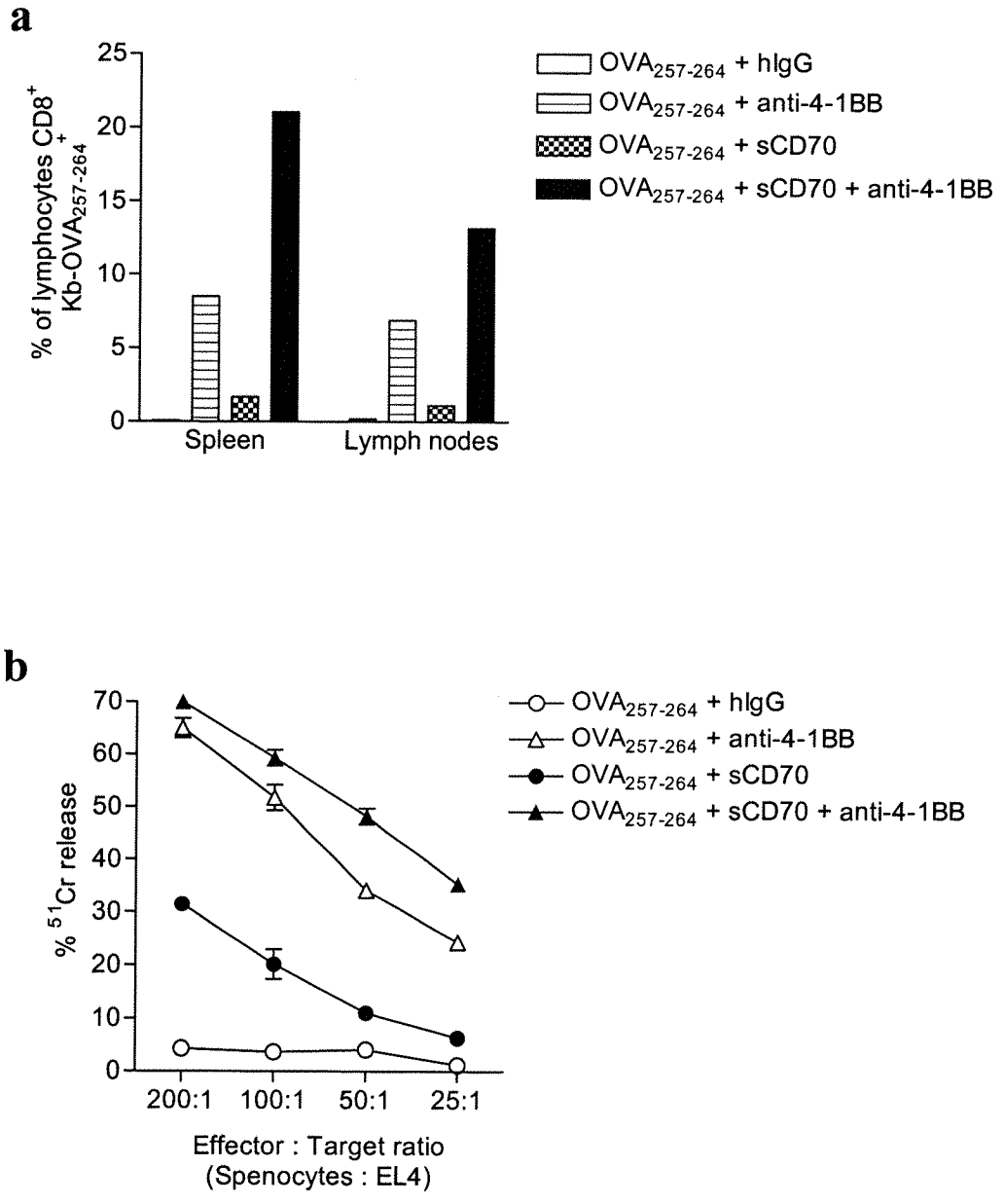

Figure 6.10 Combination of CD27 and 4-1BB co-stimulation has an additive effect on CTL numbers. Naïve OT-I C57BL/6 T cells (1 x 10^6) were transferred into C57BL/6 recipients and mice were primed by i.v. injection of OVA $_{257-264}$ (20 nmoles) in the presence of sCD70 (3 x 200 μg), anti-4-1BB (LOB12.3, 3 x 200 μg), a combination of sCD70 and anti-4-1BB (3 x 200 μg of each) or hIgG as a control (3 x 200 μg). (**a**) Percentage of antigen-specific K^b OVA $_{257-264}^+$ $CD8^+$ T cells in the spleen and lymph nodes (inguinal and brachial) on day 9 post-antigen (n = 3 mice per group). (**b**) *Ex-vivo* cytotoxicity was measured on day 9 post-antigen by ^{51}Cr release from target cells. Pooled splenocytes (n = 3 mice per group) from each group were incubated at the indicated ratios with OVA $_{257-264}$-pulsed EL4. Non-specific killing of non-pulsed EL4 was below background. Error bars represent the SEM of triplicate cultures.

6.2.8 Administration of s4-1BBL prolongs the survival of mice harbouring the A31 lymphoma

The ability of s4-1BBL to trigger a therapeutic anti-tumour immune response against the syngeneic B cell lymphoma A31 was investigated. Treatment of A31-bearing mice with anti-4-1BB mAb has previously been shown to generate a T cell response that is capable of controlling tumour growth [151]. Three days after challenge with a high tumour load (5 x 10^7 A31) mice were treated with either s4-1BBL or an anti-4-1BB mAb for comparison (Figure 6.11). Treatment with s4-1BBL significantly increased survival times (P = 0.0025) when compared to hIgG control treatment. However, the therapeutic effect was not as potent as with anti-4-1BB mAb.

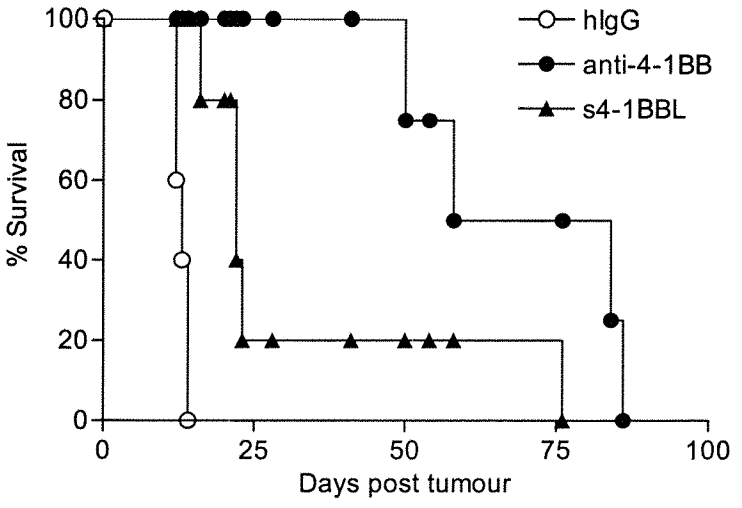

Figure 6.11 Administration of s4-1BBL prolongs the survival of mice harbouring the A31 lymphoma. 5 x 10^7 A31 lymphoma cells were administered i.v. to naïve CBA mice on day 0, followed by treatment with 250 μg of hIgG, high M_r s4-1BBL or anti-4-1BB (LOB12.3) i.v. on days 3 to 7. n = 4 (anti-4-1BB group) or 5 (hIgG and s4-1BBL groups) mice per group.

6.3 Discussion

6.3.1 Signalling through 4-1BB requires a multimeric ligand

The data presented here demonstrate that a soluble multimeric form of 4-1BBL can be a potent *in vivo* reagent for the modulation of $CD8^+$ T cell responses, and that this form of stimulation can prevent the induction of T cell anergy. As previously alluded to, a number of TNFR superfamily members such as TNFR II, CD40 and Fas cannot signal effectively upon engagement by a single trimeric ligand, but require additional receptor clustering [163,234,236]. However, this has not previously been examined in the case of 4-1BB. Experiments using soluble hexameric forms of Fas ligand have demonstrated that two adjacent trimeric ligands are required for Fas signalling, and the resultant induction of apoptosis [234]. Similarly, a dodecameric form of CD40L was far more efficient in inducing B cell activation and proliferation than a trimeric form [163]. The low M_r fraction of s4-1BBL generated here appears to be trimeric as assessed by size-exclusion chromatography and non-reducing SDS-PAGE (Figure 6.4). This is an agreement with numerous studies indicating that TNF family members exist as homotrimers [157]. The high M_r fraction of s4-1BB has an apparent molecular mass of greater than 300 kDa, which suggests its structure is a combination of hexamers and higher order multimers (Figure 6.4, see Figure 6.12 for a schematic representation of the predicted structures of high and low M_r s4-1BBL). A similar human IgG1 Fc–Fas ligand fusion protein has also been suggested to form hexamers in solution [234]. The results comparing the co-stimulatory activity of high and low M_r fractions of s4-1BBL clearly demonstrate that 4-1BB cannot signal after trimeric ligand engagement, but instead requires aggregation of a number of receptors into close proximity with one another (Figure 6.5 and 6.6). This is in agreement with another study, which showed that the interaction of the downstream signalling molecules TRAF1 and TRAF2 with 4-1BB in a T cell hybrid required receptor cross-linking [280]. Structural studies of other members of the TNFR superfamily in complex with TRAFs show that the trimeric ligand-receptor stoichiometry is mirrored in these cytoplasmic adaptor proteins. For example, CD40 has been shown to recruit homotrimers of TRAF3 between three cytoplasmic receptor tails [157,281]. Furthermore, forced oligomerisation of the N-terminal of TRAFs is sufficient for activation of downstream kinases and target gene induction, although the stoichiometry is not known [282]. The recent finding that some TNFR

family members exist as preformed trimeric complexes prior to ligand engagement, has challenged the view that monomeric receptors only transduce their signals after ligand–induced trimerisation [159,160,162]. It is therefore feasible that individual trimeric ligands bridge multiple trimeric receptors, allowing the formation of superclusters which may create a high avidity platform that facilitates a stable interaction with downstream signalling molecules such as TRAFs [163]. Formation of these superclusters during natural cell-cell interactions may be assisted by active clustering of ligands in the membrane, which generates reciprocal receptor clustering in the target cell membrane, as demonstrated for CD40 ligand [283].

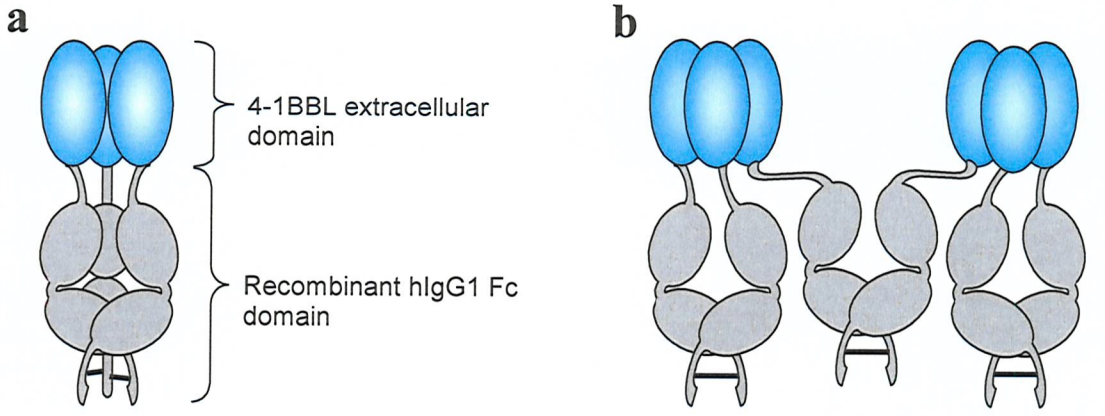

Figure 6.12 Schematic diagram representing the predicted structures of s4-1BBL. **(a)** low M_r trimeric s4-1BBL, **(b)** high M_r hexameric s4-1BBL. Regions of disulphide bonding in the hinge region are represented by black lines.

6.3.2 Soluble multimeric 4-1BBL prevents CD8$^+$ T cell anergy in vivo

Administration of high M_r s4-1BBL during the *in vivo* response of OT-I CD8$^+$ T cells to soluble OVA $_{257-264}$ peptide had a potent adjuvant effect, enhancing the total proliferation and altering the kinetics of the response so that prolonged survival of the activated CD8$^+$ T cells occurred. In contrast, injection of soluble OVA $_{257-264}$ peptide alone induced a modest expansion of OT-I CD8$^+$ T cells that was followed by a rapid contraction phase, leaving very low numbers of surviving OT-I T cells (Figure 6.7). This data corroborates other studies demonstrating enhanced priming of CD8$^+$ T cells

in response to peptide vaccination [121] or viral infection [212], after agonistic anti-4-1BB mAb administration. In addition to boosting the primary responses of naïve OT-I $CD8^+$ T cells, s4-1BBL instilled a programme of differentiation that prevented anergy induction, resulting in the T cells remaining competent to mount secondary responses (Figure 6.7). This evokes the question of how s4-1BBL prevents $CD8^+$ T cell anergy induction *in vivo*. s4-1BBL probably exerts its major effects through direct stimulation of antigen primed $CD8^+$ T cells via 4-1BB expressed on the cell surface. These effects on T cell differentiation may be qualitative and/or quantitative in nature.

Quantitatively, the increased primary burst size and prolonged survival phase induced by s4-1BBL administration leads to the maintenance of a larger pool of memory T cells. The presence of large numbers of clonal memory $CD8^+$ T cells may aid their responsiveness to secondary antigen challenge by increasing levels of autocrine IL-2 production over a critical threshold, thereby reversing the tolerant state. This 4-1BB-mediated increase in $CD8^+$ T cell survival has been attributed to increased expression of the anti-apoptotic genes *bcl-*x_L and *bfl-1* via NF-κB activation [206]. Qualitatively, 4-1BB signalling may alter or prevent the transcription of anergy-associated genes that impose the tolerant state. A model of anergy induction has been proposed, where TCR signalling without co-stimulation leads to higher activation of the Ca^{2+}/calcineurin pathway relative to the PKC/IKK/Ras/MAP kinase pathways. This leads to unbalanced activation of the transcription factor NF-AT relative to its cooperating transcription factor AP-1 (Fos/Jun), which allows NF-AT to promote the transcription of anergy-inducing genes rather than those that lead to a productive response [133]. 4-1BB signalling could interfere with this process by virtue of its downstream adaptor TRAF2 activating p38 MAPK, JNK and IκB signalling cascades, thereby leading to increased nuclear levels of NF-κB and AP-1 [205]. Another mechanism by which 4-1BB could prevent T cell anergy induction is by regulating cell cycle progression. 4-1BB signalling up regulates cyclins D and E, and concomitantly down regulates the cyclin-dependent kinase inhibitor $p27^{kip1}$ [207]. In $CD4^+$ T cells, passage through the cell cycle has been shown to be important for prevention of T cell non-responsiveness [265] and $p27^{kip1}$ has been implicated as an anergy factor [244], however it remains to be shown if this is also the case for $CD8^+$ T cells. In addition to a direct effect on $CD8^+$ T cells, it cannot be excluded that s4-1BBL is having an indirect effect on OT-I $CD8^+$ T cell

activation, via signalling through 4-1BB expressed on other immune cell subsets such as dendritic cells or NK cells. *In vitro*, the ligation of 4-1BB on dendritic cells up regulates B7-1 and B7-2 expression [95,202] and induces production of IL-12, a cytokine that has previously been shown to inhibit tolerance induction in $CD8^+$ T cells [258].

As previously discussed (Chapter 5), the induction of anergy in OT-I T cells following injection of OVA $_{257-264}$ alone is likely to have resulted from the lack of $CD4^+$ T cell help, since OVA $_{257-264}$ does not activate $CD4^+$ T cells [274,275]. The results in this chapter suggest that in this model s4-1BBL can substitute for the lack of $CD4^+$ T cell help. The expression of 4-1BBL on dendritic cells can be induced by the ligation of CD40 by CD40 ligand, which is expressed on activated $CD4^+$ T cells [121]. Therefore, during productive immune responses, the stimulation of $CD8^+$ T cells via 4-1BBL expressed on activated dendritic cells may represent one of the downstream signals involved in CD4 help.

6.3.3 CD27 and 4-1BB T cell co-stimulation have differential outcomes and in combination can have an additive effect on the generation of CTL numbers

Co-stimulation of OT-I $CD8^+$ T cells by CD27 or 4-1BB has differential effects on the kinetics of the ensuing response. CD27 apparently has a more potent effect on the expansion phase during the initial few days of the response, whereas 4-1BB promotes expansion to some extent but also prolongs the numbers of activated T cells at late time points (Figure 6.8). This finding is in agreement with other *in vivo* observations of the action of 4-1BB [112]. Providing concurrent signals via CD27 and 4-1BB had an additive effect on primary OT-I $CD8^+$ T cell antigen responses (Figure 6.9 and 6.10). Thus, these co-stimulatory TNFR superfamily members appear to have non-redundant roles in $CD8^+$ T cell responses. The additive effect seen when combining these signals could be working via several different mechanisms. One explanation is the disparate expression patterns of the receptors. 4-1BB cell surface expression is inducible only after TCR signalling in T cells and it peaks around 48 hours after stimulation (Chapter 4, Figures 4.1 and 4.3). CD27, however, is constitutively expressed on naïve $CD8^+$ T cells, and is therefore better positioned to immediately amplify the response upon provision of ligand. By combining reagents, saturating co-stimulatory signals through CD27 may be prolonged by the continuation of downstream signals when 4-1BB

becomes expressed at a later time point, maximising the response. Signalling through these related molecules may therefore be temporally segregated on $CD8^+$ T cells. Alternatively, CD27 and 4-1BB may work in concert, each receptor stimulating different downstream signalling components and the outcome being the sum of two different sets of signals. However, there is little indication of qualitative differences in signalling between these receptors. Both receptors recruit TRAF2 and TRAF3 to their cytoplasmic domains, and they both activate the transcription factors AP-1 and NF-κB [186,187,205]. Indeed, the signalling target TRAF2 may be crucial to at least some of the signals from all the co-stimulatory TNFR-family members [73]. For example, it has recently been found that OX40-mediated memory T cell development is completely TRAF2 dependent [284]. Another way combining these co-stimulatory reagents may enhance the T cell response is via the ability of CD27 stimulation to up-regulate 4-1BB expression (Chapter 4, Figure 4.6). Thus, signalling through CD27 may potentiate the effect of agonistic 4-1BB binding reagents by increasing the availability of the receptor.

Treatment of mice bearing the A31 B cell lymphoma with s4-1BBL had a significant effect on survival times (Figure 6.11). Nevertheless, its therapeutic effect was not as potent as the anti-4-1BB mAb with which it was compared. As previously suggested (Chapter 5), the most likely reason for this is that the fusion protein has a shorter half-life *in vivo* than the mAb. Investigation of alternate fusion protein structures with greater half-lives, or revised treatment schedules may therefore be necessary to optimise the therapeutic effects of soluble 4-1BBL. Agonistic antibodies against 4-1BB have recently been shown to reverse $CD8^+$ T cell anergy [285], as well as preventing its induction. This suggests that reagents that stimulate 4-1BB would be useful in situations where T cells might already be tolerised, such as in patients harbouring tumours. For example, circulating $CD8^+$ T cell populations specific for melanoma-associated antigens have been found at high frequency in patients, but these cells appear to have been rendered anergic [286]. Methods for reversing this tolerant state and expanding CTL numbers are likely to be critical for the design of effective anti-tumour vaccines. The data presented here suggests that the addition of soluble multimeric 4-1BBL to tumour antigen vaccines maybe one approach which could achieve this.

CHAPTER 7

General discussion

This thesis describes the generation and characterisation of soluble ligands for the T cell co-stimulatory molecules CD27 (sCD70) and 4-1BB (s4-1BBL) and investigates the role of these co-stimulators during T cell responses to antigen. These recombinant fusion proteins, which encompass the extracellular domains of CD70 or 4-1BBL and the Fc domain of hIgG1, formed large multimeric complexes (Chapters 3 and 5). These soluble multimeric ligands proved to be potent *in vitro* and *in vivo* as reagents for investigating the effects of CD27 and 4-1BB signalling on T cells during antigen-priming. In the case of 4-1BB, scrutiny of different M_r fractions of s4-1BBL demonstrated that 4-1BB has a minimal requirement for hexameric ligands for signal transduction, with trimeric ligands being incapable of signalling through the receptor (Chapter 5). This data highlights the importance of receptor aggregation in signalling initiation for some members of the TNFR family of co-stimulators.

In vitro analysis of the functional outcomes of CD27 signalling during T cell priming (Chapter 4) indicated that CD27 could potently co-stimulate the proliferation of both the $CD4^+$ and $CD8^+$ subsets of T cells. Furthermore, in an antigen-specific system, CD27 was shown to promote the proliferation and activation of naïve OT-I $CD8^+$ T cells independently of the archetypal co-stimulator CD28. Signalling through CD27 also provided a potent stimulus for the cellular production of cytokines such as IL-2, which is critical for optimal T cell clonal expansion and prevention of unresponsiveness, and the anti-viral effector molecule IFN-γ that promotes antigen presentation and inflammation. Triggering of CD27 on the surface of antigen-experienced OT-I T cells also enhanced their proliferation and cytokine production, demonstrating that CD27 can regulate secondary stimulation of $CD8^+$ T cells *in vitro*. The powerful effects of CD27 co-stimulation on the overall expansion and accumulation of $CD8^+$ T cells *in vitro* can be attributed to its observed effects on both T cell division and resistance to apoptosis. CFSE dilution analysis clearly showed that stimulation of CD27 by sCD70 during the naïve T cell response to sub-optimal levels of antigen led to increased numbers of T cells becoming committed to divide, and

enhanced the number of cell divisions that they underwent. This increase in cell cycling was shown to be independent of the presence of IL-2, suggesting that CD27 directly induces signals that mediate control of the cell cycle. However, these downstream signals have yet to be characterised for this co-stimulator. CD27-stimulus also improved T cell resistance to apoptosis induced by γ-irradiation or withdrawal of stimulation. Mechanistically, it was shown that CD27 signalling intercedes in the mitochondrial pathway of apoptosis by up-regulation of the anti-apoptotic protein Bcl-$_{XL}$ (Chapter 4).

Transfer of transgenic OT-I CD8$^+$ T cells to syngeneic mice, followed by administration of OVA $_{257-264}$ peptide antigen in the absence of adjuvant led to the activation and expansion of the antigen-specific population; but these cells subsequently became anergic and were unresponsive when challenged for a second time with antigen (Chapter 5). However, co-administration of sCD70 and OVA $_{257-264}$ dramatically enhanced the magnitude of the naïve T cell response which resulted in the formation of a pool of CTL effectors capable of killing target antigen-pulsed cells *ex-vivo* and clearing the syngeneic tumour E.G7 *in vivo*. Moreover, naïve OT-I CD8$^+$ T cell priming in the presence of co-stimulation through CD27 apparently programmed the responding T cells to develop into effective memory precursors. As a result of activation in the presence of sCD70, an enlarged, stable, pool of antigen-specific cells was maintained after contraction of the primary response, which exhibited phenotypic memory T cell markers and which remained highly responsive to secondary antigen challenge (Chapter 5). High M_r s4-1BBL exhibited similar co-stimulatory effects to sCD70 when administered in this system, causing enhanced primary T cell expansion and affecting the quality of the T cell response so that a population of reactive T cells was maintained after the primary response (Chapter 6). However, closer comparison of the effects of CD27 versus 4-1BB co-stimulation using their soluble ligands demonstrated that while CD27 predominantly enhanced the initial burst of T cell expansion, 4-1BB altered the kinetics of the primary response so that the activated T cells survived for longer and the contraction phase was slowed. Combining these stimuli had an additive effect on the magnitude of the T cell response and CTL activity, signifying that these co-stimulatory molecules have non-redundant roles in CD8$^+$ T cell responses (Chapter 6).

In a murine model of B cell lymphoma (A31), therapeutic administration of sCD70 or s4-1BBL significantly increased survival times (Chapters 5 and 6). This is predicted to be due to co-stimulatory enhancement of a weak endogenous T cell response against the tumour. Thus, these co-stimulatory ligands may have potential as anti-tumour immunotherapeutic agents.

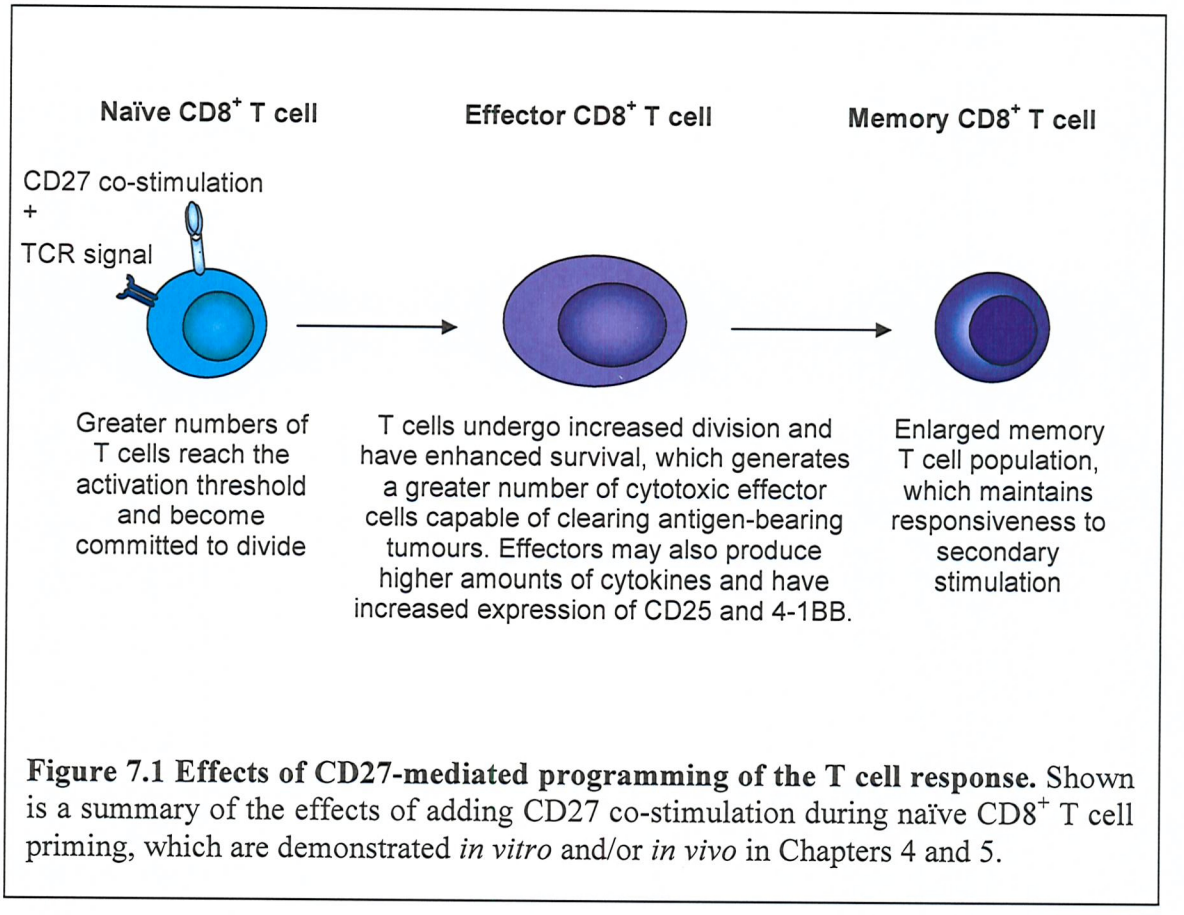

Figure 7.1 Effects of CD27-mediated programming of the T cell response. Shown is a summary of the effects of adding CD27 co-stimulation during naïve $CD8^+$ T cell priming, which are demonstrated *in vitro* and/or *in vivo* in Chapters 4 and 5.

Characterisation of the binding specificity of sCD70 demonstrated that it was able to partially block the binding of an anti-CD27 mAb to the surface of T cells (Chapter 3, Figure 3.4a). This suggests that sCD70 binds CD27 as expected, but that the ligand-binding site does not completely overlap with the epitope recognised by this anti-CD27 antibody. An alternative explanation for this observation could be that CD70 has more than one receptor, as is the case for some other ligands of the TNF superfamily such as TNF, TRAIL and LIGHT [155]. However, cross-reactivity has not been previously observed with this ligand and the lymphocyte-binding pattern of sCD70 mimicked that seen with anti-CD27 antibodies (Chapter 3, Figure 3.4b). Furthermore, the phenotype

of excessive formation of effector-memory cells in CD70 transgenic mice, which eventually leads to lethal T cell immunodeficiency, is completely abolished by crossing with $CD27^{-/-}$ mice [195]. This confirms that CD70 mediates its effects solely through the receptor CD27, and that the functional outcomes of stimulation with sCD70 shown here can be attributed to signalling via CD27. Similarly, 4-1BB ligand is thought to only interact with 4-1BB, and this was confirmed by the ability of anti-4-1BB mAbs to completely inhibit the binding of s4-1BBL to the surface of activated T cells (Chapter 6, Figure 6.6a).

The data described here demonstrate that 4-1BB cannot signal upon engagement of its ligand in a trimeric state. However, s4-1BBL with the stoichiometry of a hexamer can induce strong signal transduction. These differences cannot be explained by differences in avidity or specificity between discrete molecular weight fractions of s4-1BBL, indicating that the extent of 4-1BB receptor clustering directly affects signal transduction (Chapter 6, Figures 6.5 and 6.6). Although it was originally thought that TNFR superfamily members signal upon trimerisation induced by ligand binding, it has now been demonstrated that several members of the TNFR superfamily exist as pre-associated trimers at the cell surface in the absence of their ligands. These include TNFR-I, TNFR-II, Fas, CD40 and TRAIL receptor 1, which all appear to associate via a pre-ligand-binding assembly domain in their extracellular region [159,160]. Of these, TNFR-II, Fas, CD40 and TRAIL receptor 1 have also been shown to require interaction with oligomeric ligands with the stoichiometry of two or more trimers in order for full signal transduction to take place [163,234,236,279]. This suggests that signalling by these TNFR superfamily members is not induced purely by a conformational change in the trimeric receptor complex upon trimeric ligand binding, but perhaps the close association of several groups of trimeric receptors is necessary to initiate downstream signalling cascades. Alternatively, each trimeric ligand could interact with three receptors, which themselves are part of separate trimers, in effect cross-linking several pre-associated trimeric receptors. Thus, it can be envisaged that multimeric ligands could generate the formation of receptor super-clusters in the cell membrane that may provide stable signalling platforms (Figure 7.2).

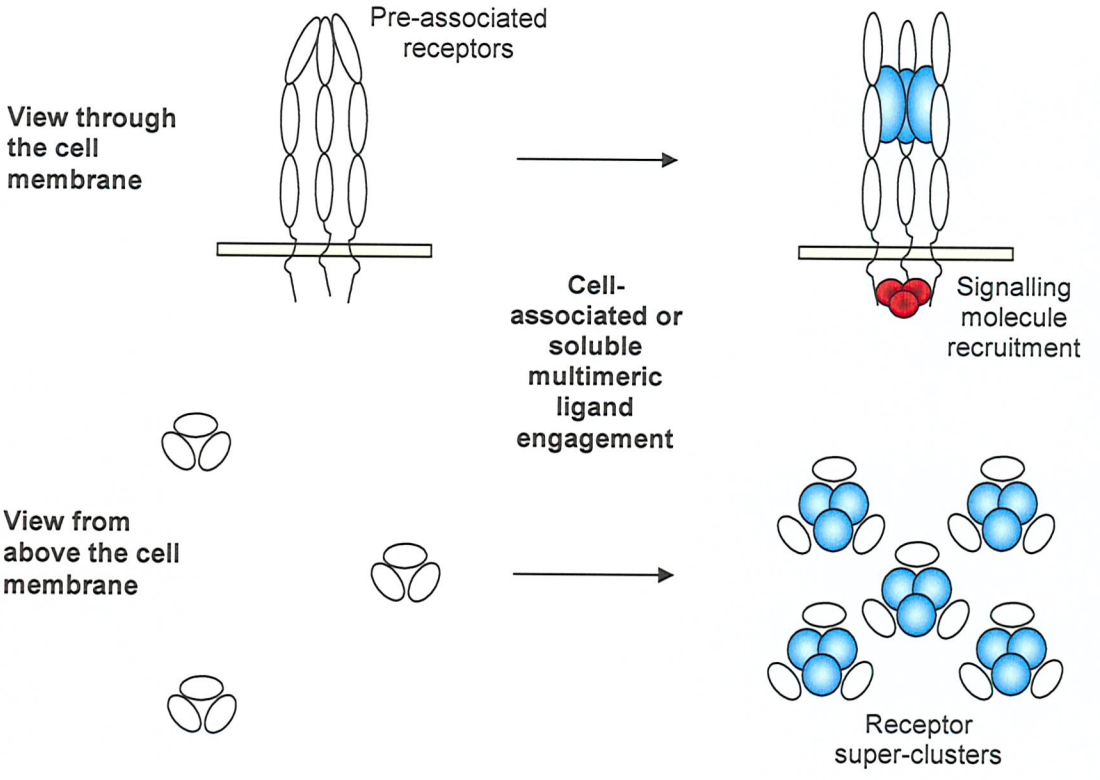

Figure 7.2 Model of signal initiation by TNFR superfamily members. Pre-associated receptors (e.g via a PLAD) undergo a conformational change upon trimeric ligand binding (blue oval cluster), which allows the recruitment of intracellular signalling molecules (red circles). Supermolecular clusters caused by multimeric ligand engagement create stable signalling platforms that allow full signal transduction.

In contrast to all other membrane bound members of the TNFR superfamily CD27 is expressed as a disulphide-linked homodimer, and it is unclear whether additional non-covalent receptor interactions such as through a PLAD are required for the formation of its signalling complexes. The soluble recombinant form of CD70 generated here formed only large multimeric complexes of ~ 300 kDa or greater; thus it could not be determined if this ligand has a tendency to trimerise in the way that 4-1BBL does, or if multimeric forms of the ligand are an absolute requirement for inducing signalling through CD27. However, evidence from comparative molecular modelling and the immunoprecipitation of endogenous CD70 indicates that it does exist in a trimeric complex [183,231]. Furthermore, the requirement for multimeric ligands in members of the TNFR superfamily appears to be related to whether their mode of signalling is via a soluble cytokine form of the ligand (such as TNF-α, which can signal in its trimeric

form), or via cell-cell interactions between membrane bound receptors and ligands (e.g. FasL, CD40L and 4-1BBL, which require higher multimers). In the case of CD40L it has been shown that clustering of ligands in the membrane is an active process that is essential for allowing signal transduction through receptors situated in neighbouring cells [283]. Thus, it can be expected that ligand clustering is also important for signal transduction through CD27 as this ligand-receptor pair appear to be involved only in direct cell-cell interactions. Furthermore, enzymatic cleavage of the ligand from the cell surface may represent a mechanism for rapidly switching off the signal through the receptor, as has been shown previously for Fas ligand. Soluble trimeric FasL released upon cleavage from the cell surface is unable to signal via Fas and consequently is functionally inactive [279,287]. It is probable that this mechanism also controls signalling in other membrane-bound TNF-TNFR pairs that have a prerequisite for tight expression control in order to prevent immune system dysfunction. In summary, higher multimer forms of TNF superfamily ligands formed by fusion of their extracellular domain with hIgG1Fc, as generated here, are effective reagents for mimicking the interactions between natural membrane bound ligands and receptors and lead to efficient signal transduction.

One advantage that these soluble recombinant ligands have over receptor-directed agonistic antibodies is that their hIgGFc domain effector functions are diminished. Work in our laboratory has shown that these types of recombinant ligand that have a reversed hIgG1Fc domain are extremely poor at fixing complement and are not very active in antibody-dependent cellular cytotoxicity mediated by Fc receptors. At least 100-fold more recombinant ligand is required to give the same level of cytotoxicity as a chimeric antibody containing a normal human IgG1Fc domain (L.E. Haswell, unpublished observations). This ensures that the recombinant ligand will not kill cells *in vivo* which are expressing the receptor for which it is specific. However, these forms of recombinant ligands also appear to be more unstable *in vivo* than antibodies, and thus have only a short half life. One explanation for this is that the reversed IgG1Fc domain may be unable to bind to the FcRn salvage receptor [288]. Murine FcRn has been demonstrated to be promiscuous in its binding of IgG for other species and binds to hIgG1 with relatively high affinity [289]. Furthermore, work in our laboratory has shown that a recombinant ligand (Fc-CD40L) similar to those described here has a shorter

half-life than both mouse IgG and a chimeric human IgG1 antibody (L.E. Haswell, unpublished observations). Therefore, the short half-life is not due to cross-species differences in the binding to FcRn. The highly conserved binding site for FcRn has been predicted to be located in the CH2-CH3 interface in the Fc region of IgG [233]. This region is unchanged in the recombinant hIgG1Fc used for the construction of these fusion proteins. However, a global change in structure which inhibits binding to FcRn cannot be excluded because of the reversal of the orientation of the Fc domains coupled with its attachment to the TNF-family domains, which have a fold structure distinct from IgG Fab regions. In addition, it is possible that the highly multimeric nature of the ligands may sterically interfere with the binding of the Fc domain to FcRn. Further investigation of the structures required for these functions may allow the design of soluble ligands with alternate backbone structures that have good *in vivo* half lives but do not have Ig effector functions.

As demonstrated here, CD27 appears to be a potent co-stimulator of naïve T cells but how does it compare with the classical co-stimulatory molecule CD28, which has similar constitutive expression on naïve $CD4^+$ and $CD8^+$ T cells? Recent work by Jannie Borst's group has compared the relative contribution of CD27 and CD28 co-stimulation during T cell responses to influenza infection [243]. Using $CD27^{-/-}$ and $CD28^{-/-}$ mice they demonstrate that CD27 is as important as CD28 for the generation of a virus-specific T cell pool in draining lymph nodes, and is the main determinant for accumulation of these cells at the site of the infection. The authors demonstrate that in this model of virus infection CD27 mediates its effect through promoting the survival of activated T cells, which allows them to accumulate in large numbers in the lung. In accord with this, the data presented in Chapter 4 of this thesis show that CD27-stimulation promotes the survival of $CD8^+$ T cells after antigen withdrawal and up regulates factors that can inhibit the induction of apoptosis after γ-irradiation. One factor identified as a downstream target of CD27 is Bcl-$_{XL}$, which is also up regulated to a similar extent after CD28 co-stimulation (Chapter 4, Figures 4.11 and 4.12). Furthermore, the analysis of $CD8^+$ T cell responses to TCR and CD27 co-stimulation by CFSE dilution reveals that at sub-optimal levels of TCR stimulus CD27 can promote the initiation of cell division and increase the number of divisions that the stimulated T cells undergo (Chapter 4, Figures 4.8 and 4.9). In our *in vivo* model,

administration of OVA $_{257-264}$ peptide in conjunction with sCD70 promotes a massive expansion of antigen-specific cells, 17-fold more than induced by peptide alone (Chapter 5, Figure 5.3a and Table 5.1). Analysis of expression the proliferation antigen Ki67 in these cells at the peak of the response indicated that the majority of OT-I T cells stimulated with either antigen alone, or antigen and sCD70, were actively dividing. To further address the mechanism by which CD27 co-stimulation leads to such a dramatic enhancement of T cell numbers, the division of CFSE-labelled OT-I T cells after *in vivo* activation with antigen, or antigen and sCD70 was examined (Chapter 5, Figure 5.4). These experiments showed that OT-I T cells stimulated with OVA $_{257-264}$ divide extensively but fail to accumulate in number. This is in line with other data which has shown that the ability of $CD8^+$ T cells to proliferate can be dissociated from gain of effector function and prevention of tolerance, a phenomenon known as split anergy [258,290]. OT-I T cells stimulated with OVA $_{257-264}$ and sCD70 cycle faster and undergo at least one more cell division by day 3 than those stimulated with OVA $_{257-264}$ alone, confirming the ability of CD27 to regulate the cell cycle *in vivo*. However, this moderate increase in cell division is not sufficient to account for the dramatic enhancement in T cell numbers observed. Thus it can be concluded that CD27 signalling must also be having a significant effect on the survival of the antigen-activated T cells at this early stage in the response. In this model system of $CD8^+$ T cell activation we have not determined whether CD28 co-stimulation is occurring. However, after administration of peptide in a non-inflammatory environment it would be expected that any APCs presenting antigen would only be expressing low levels of B7-1 and B7-2, which may provide only low intensity signalling through CD28. Furthermore, as the peptide antigen is being administered systemically it may be presented to $CD8^+$ T cells by non-professional APCs that don't express any co-stimulatory ligands. Thus, the effects of CD27 stimulation seen here probably occur in the absence of optimal CD28 signalling.

Despite the apparent overlap between CD27 and CD28 in expression pattern and function, data from knockout mice indicates that their roles are non-redundant and each cannot fully compensate for the loss of the other [243]. This may be due to a differing emphasis in their target signalling pathways. Although many targets of CD28, such as JNK and NF-κB, overlap with those of CD27 and other members of the

TNFR superfamily, one of the main functions demonstrated for CD28 is to potentiate signalling from the TCR and this property may be critical for its dominant effects on T cell division. It is at present unclear if CD27 and other TNFR superfamily co-stimulators purely provide independent signals which synergise with the TCR at the nuclear level, or if they can also directly enhance TCR signalling. Regarding this, it would be interesting to examine if TNFR superfamily members can influence cytoskeletal reorganisation and promote the formation of an immunological synapse as CD28 does [124,127,291].

The downstream signalling pathways of the homologs CD27, 4-1BB and OX40 are much more closely linked, as would be expected. OX40, 4-1BB and CD27 share immediate signalling components such as TRAF2 and 5, and thus may all activate the same distal signalling cascades. However, these molecules still appear to have individual roles in the control of T cell activation. The lack of redundancy exhibited between these molecules may be explained by the temporal and spatial segregation of their signals, and that long-lived T cell responses require a sustained set of signals (Figure 7.3). For example, it has been hypothesised recently by Michael Croft that the ability to activate PKB could be a direct link between co-stimulatory members of the CD28, common γ-chain cytokine receptor and TNFR families [73,132]. Hence, sustained activation of this molecule by sequential co-stimulatory signals may be critical for T cell longevity. PKB itself can up regulate anti-apoptotic members of the Bcl2 family, and can also suppress apoptosis by inactivating its target molecules glycogen synthase kinase-3 and FKHR (a member of the FOXO forkhead transcription factor family), both of which are implicated in control of cellular survival [132]. To date, the ability of the TNFR superfamily to activate PKB has only been shown for OX40; thus, additional work on CD27 and 4-1BB is required to demonstrate if PKB is also critical for relaying their effects on cell survival via up regulation of Bcl2 family molecules.

Non-redundancy between different co-stimulators could also be a function of their endogenous ligand expression patterns. For example, low levels of B7-1 and B7-2 are constitutively expressed on APCs, whereas TNF ligands such as CD70 and 4-1BBL are only expressed after APC activation. Furthermore, the relative pattern of co-stimulatory ligands on APCs after activation with different adjuvants or pathogens *in*

vivo has not been examined in detail. The licensing stimulus could potentially direct the type of ligands subsequently expressed. For example, licensing of DCs by activated T_H $CD4^+$ T cells (e.g. CD40 signalling) may lead to a different co-stimulatory ligand milieu than licensing signals from the innate immune system (e.g. TLR signalling). Further work is required to clarify whether different APC licensing signals can control the subsequent T cell response by the type of co-stimulatory ligands they up-regulate. Recent data obtained from our laboratory suggests that CD70-CD27 interactions act downstream of CD40 signalling, and CD70 blockade inhibits CD40-mediated expansion of $CD8^+$ T cells. This suggests that the up regulation of CD70 on DCs after CD40 ligation may be a critical component of CD4-help for $CD8^+$ T cells. In concurrence with this idea is the novel finding that signalling through CD27 during the primary T cell response prevents the subsequent induction of anergy in $CD8^+$ T cells (Chapter 5, Figure 5.6). Maintenance of secondary $CD8^+$ T cell responsiveness has recently been identified as the salient function of $CD4^+$ T cell help [47,271-273]. Thus, CD70-CD27 interactions fulfil the criteria for a direct $CD8^+$ T cell stimulus downstream of $CD4^+$ T cell help that can programme the cells to remain responsive to secondary stimulation. Similarly, 4-1BB signalling has been shown here, and in other recent work [285], to be able to maintain secondary responsiveness in addition to its effects on T cell survival in the primary response. Thus, signalling through these molecules represents an important part of the programming of naïve T cells to survive and become optimal memory cells that can elicit rapid responses after secondary encounter with antigen.

The molecular mechanisms involved in T cell anergy induction are just beginning to be deduced. One molecular pathway that has been identified is the result of sustained Ca^{2+} - calcineurin signalling, initiated by TCR ligation, which leads to the activation of NF-AT. This transcription factor appears to be crucial for both productive responses and for the induction of tolerance in T cells. In the presence of concomitant activation of the transcription factors AP-1 and NF-κB induced by co-stimulation, genes are transcribed which lead to cytokine production, proliferation and survival. However, in the absence of these other transcription factors NF-AT activation leads to the up regulation of molecules which maintain unresponsiveness [133]. Recent studies have identified the E3 ubiquitin ligases GRAIL, Cbl-b and Itch as being involved in

maintaining the anergic state by controlling the targeted proteolysis of TCR signalling proteins [292,293]. This proteolysis consequently leads to disruption of the immunological synapse and thus a shortened period of TCR signalling occurs upon re-stimulation, which is not sufficient for full T cell activation. It remains to be determined if this type of mechanism is responsible for the induction of anergy in the OT-I $CD8^+$ T cells described here, and what phenotypic changes are occurring in OT-I T cells stimulated via CD27 or 4-1BB that allows them to maintain secondary responsiveness. Candidate target molecules for future investigation that could be affected by co-stimulation include the intracellular ubiquitin ligase anergy factors mentioned above, and cell surface receptors such as those for the cytokines IL-15 and IL-7, which are critically involved in the homeostasis of memory T cells. In fact, the IL-7 receptor has recently been shown to be a selective marker for identifying memory precursor $CD8^+$ T cells [75].

Because the frequency of memory T cells remaining after priming with OVA $_{257-264}$ peptide alone was so low (Chapter 5, Figure 5.3a) it was impossible to directly compare the phenotype of these anergic cells with those memory T cells formed after CD27 co-stimulation. However, before restimulation, the phenotype of $CD8^+$ T cells primed with OVA $_{257-264}$ and CD27 stimulation appeared to be one typical of resting memory T cells, with a mixture of T_{EM} and T_{CM} subsets being contained in the spleen (as determined by the split CD62L low/high population; Chapter 5, Figure 5.7). Furthermore, the memory $CD8^+$ T cells induced by CD27 co-stimulation exhibited secondary responses typical of memory cells, having a significantly enhanced survival phase after re-activation as compared to naïve T cells (Chapter 5, Figure 5.6a and 5.7b). This reduced intrinsic susceptibility to apoptosis of memory versus naïve T cells has been observed previously in anti-viral $CD8^+$ T cell responses [294], and may provide a mechanism for escalating the frequency of antigen-specific memory cells after repeated infections.

Several studies have documented that CD28-mediated co-stimulation is not involved in memory recall responses. For example, OVA-specific transgenic $CD4^+$ memory T cells are less dependent on CD28 co-stimulation than naïve T cells [111]. Similarly, CD28 is critical during T cell priming in T_H2-mediated mucosal inflammation responses, but does not contribute to the recall response [110]. In contrast, OX40 was

shown to be critical for directly co-stimulating antigen-specific memory T_H2 cells that mediate allergic lung inflammation [174]. These results imply that the type of co-stimulator required may change with the transition of naïve to memory $CD4^+$ T cells. Anti-viral $CD8^+$ T cell memory responses are also independent of CD28 co-stimulation. The maintenance of LCMV-specific $CD8^+$ T cell memory has been shown to be unaffected by lack of CD28-B7 interactions, and $CD28^{-/-}$ memory cells are equally capable of conferring protective immunity to secondary LCMV infection as $CD28^{+/+}$ [108]. Furthermore, Bertram *et al* showed that although influenza virus infected mice were reliant on CD28-B7 interactions for their primary anti-influenza $CD8^+$ T cell response, CD28 co-stimulation was not required during secondary responses. This was demonstrated by replacing the primary co-stimulatory signal in $CD28^{-/-}$ mice with co-stimulation via an agonistic anti-4-1BB mAb, which allowed full secondary responses to be generated [113]. In contrast, the addition of anti-4-1BB mAb during priming failed to restore secondary influenza responses in $4\text{-}1BBL^{-/-}$ mice. Thus, it appears that 4-1BB is necessary during memory responses, and that there is a switch in the co-stimulatory requirement of $CD8^+$ T cells from CD28 in the primary response to 4-1BB in the secondary. In addition to aiding the re-activation of normal memory T cells, 4-1BB and OX40 can also break pre-established anergy in $CD8^+$ or $CD4^+$ T cells, respectively. The administration of anti-4-1BB mAb can reverse anergy established by both soluble peptide administration, or by constant exposure of T cells to allogeneic host antigens in the setting of bone marrow transplantation [285]. Likewise, signalling through OX40 has been demonstrated to break established peripheral $CD4^+$ T cell tolerance induced by a soluble peptide antigen [116]. CD27 was expressed at equivalent levels on memory $CD8^+$ T cells as on naïve T cells (Chapter 5, Figure 5.7a). Furthermore, it has been observed that CD27 expression is higher on the T_{CM} than T_{EM} subset, although the functional significance of this is unknown [86]. It has not yet been investigated if CD27 co-stimulation is required during the re-activation of memory $CD8^+$ T cells. Data from Chapter 4 of this thesis indicates that antigen-experienced $CD8^+$ T cells can respond to CD27 co-stimulation *in vitro* (Chapter 4, Figure 4.7). However, the restimulation of short-term *in vitro* activated cells may not be equivalent to that of true memory T cells in an *in vivo* environment. $CD27^{-/-}$ mice show reduced secondary T cell responses to influenza virus [109]. Nevertheless, this reduction in response may be a consequence of lower numbers or reactivity of memory T cells due

to the lack of CD27 signalling during the primary infection. Therefore, further studies are required in which CD27-CD70 interactions are blocked only during secondary responses to unequivocally determine if CD27 co-stimulation is important for activation of memory cells, and if it is capable of breaking pre-established T cell anergy.

It has been demonstrated here that CD27 co-stimulation can prevent the induction of anergy in $CD8^+$ T cells; however, anergy may be regulated differently in $CD4^+$ T cells. For example, signalling by the co-inhibitor CTLA-4 appears to be critical for maintaining $CD4^+$ T cell unresponsiveness *in vivo* [266], but it is not involved in this process in $CD8^+$ T cells [268]. Thus, future work will uncover the significance of CD27 signalling during $CD4^+$ T cell responses and whether it acts to prevent the induction of non-responsiveness in these cells. Furthermore, it is unclear whether the T_{reg} subset of $CD4^+$ T cells can respond to CD27 co-stimulation, and what functional effects this may have on their regulatory phenotype. It has been recently demonstrated that T_{reg} cells are sustained by an environment of low-level basal expression of B7-1 and B7-2, and absence of this stimulation leads to a loss of T_{reg} cells, which can result in enhanced autoimmunity [295]. This effect has been shown to be mediated by co-stimulation through CD28, which promotes the survival and self renewal of T_{regs} by regulating IL-2 production by conventional T cells and CD25 expression on T_{reg} [296]. One co-stimulatory member of the TNFR superfamily, GITR, is constitutively highly expressed on the surface of T_{reg}. Signalling through this molecule has been shown to abrogate the suppressive function of $CD4^+$ $CD25^+$ T_{reg}, and break their anergic state [297,298]. Thus, co-stimulatory interactions 'regulate the regulators' by controlling their number and function, as well as acting on normal effector T cells. It will be important to dissect further whether CD27 and other TNFR superfamily members are required for maintenance of the T_{reg} subset or alternatively if they can reverse the anergic phenotype of these cells and prevent their ability to suppress other T cell responses.

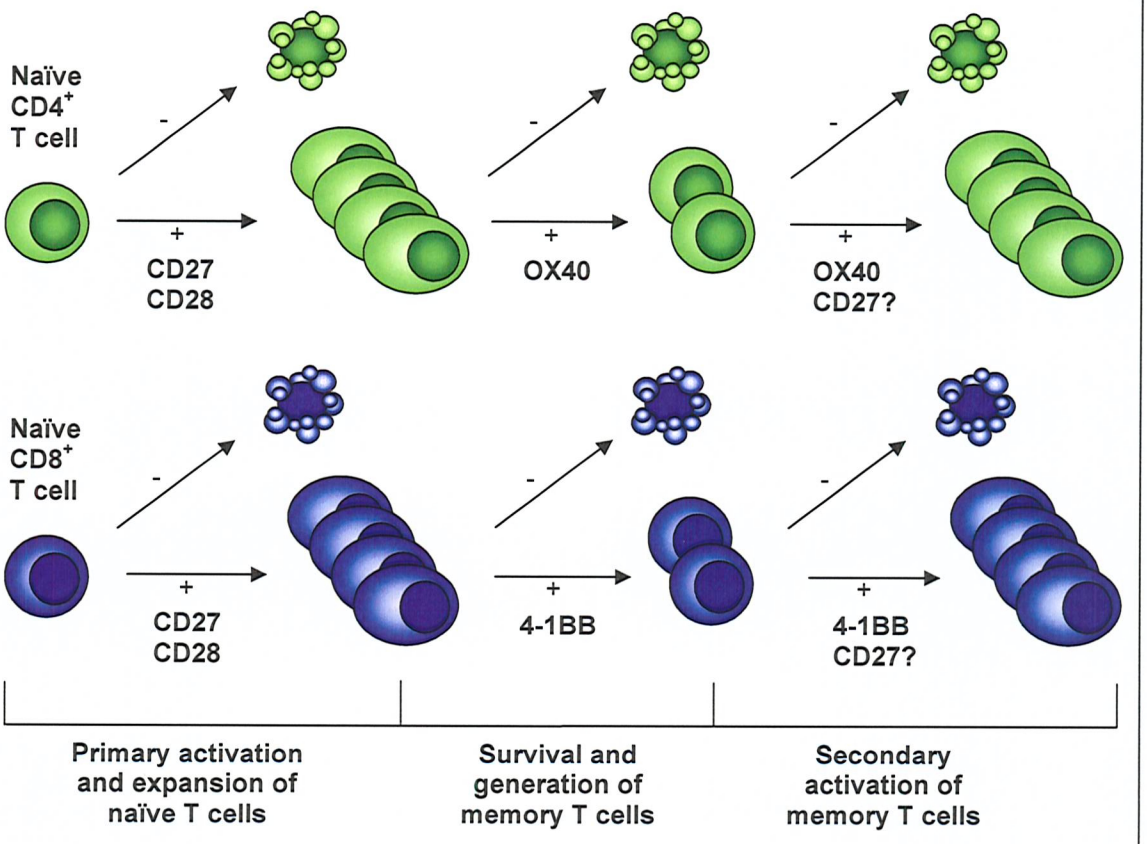

Figure 7.3 Spatio-temporal model of regulation of $CD4^+$ and $CD8^+$ T cell responses by co-stimulation. CD27 and CD28 appear to be critically involved in the initial clonal expansion of naïve $CD4^+$ and $CD8^+$ T cells. OX40 or 4-1BB co-stimulation then becomes important later in the primary response for $CD4^+$ or $CD8^+$ T cells, respectively. These molecules are also required during memory T cell responses. It has yet to be determined if CD27 stimulation is needed at this stage. Co-stimulatory molecules will work in conjunction with other extrinsic immune cell regulators such as cytokines, and the absence of appropriate signals at any stage may lead to T cell anergy or apoptosis.

As discussed above, co-stimulatory molecules are critical regulators of all types of T cell responses. This suggests that appropriate targeting of these molecules will be of therapeutic benefit in a range of human disease states. Analysis of the expression of CD27 and CD28 has been used to identify $CD8^+$ T cell differentiation subsets in human chronic viral infections, such as HIV-1, cytomegalovirus and Epstein-Barr virus. The loss of both these co-stimulatory molecules seems to identify a set of terminally differentiated effector T cells, which have high cytotoxic potential but

shortened telomere length and thus a lack of proliferative capacity [88,299]. It has been proposed that this phenotype may be induced by chronic stimulation of the immune system during persistent viral infection [299]. Transgenic over-expression of CD70 on B cells *in vivo* triggers an analogous T cell phenotype of effector-memory cells that have lost CD27 expression [185]. In these CD70-overexpressing mice, this chronic stimulation depletes the naïve T cell pool over time, eventually leading to T cell immunodeficiency and susceptibility to lethal opportunistic infections [195]. Therefore, blocking CD27-CD70 or other co-stimulatory interactions in chronic infections could potentially avoid the detrimental consequences of persistent immune activation.

In contrast to the situation with chronic viral infections, immune responses against tumours are sub-optimal. The presence of circulating tumour-specific $CD8^+$ T cells has been observed in patients in a number of studies [300]. However, as development and growth of tumours is initially not accompanied by inflammatory immune stimuli, antigens derived from the tumour may be shunted into the same cross-tolerising pathway as peripheral tissue antigens. For example, a study of patients with metastatic melanoma found that a proportion of their $CD8^+$ TAA-specific T cell populations were selectively rendered anergic [286]. Similarly, murine tumour models have demonstrated that the induction of antigen-specific T cell anergy may be an early event in the course of tumour progression [276,301]. This tolerant state may hamper immune intervention schemes that are based on induction or propagation of the T cell immune system in tumour-bearing hosts. However, provision of the appropriate co-stimulatory signals may be able to reverse this tolerant state, and prevent its induction in newly primed tumour-specific T cells. Additionally, in the absence of innate inflammatory signals (as is the case in non-viral induced tumours) CTL responses are T_H cell dependent [252,302]. That is, DCs presenting TAAs need to be first licensed by specific $CD4^+$ T cells before they can go on to trigger CTL responses. Provision of signalling through co-stimulatory molecules such as CD27 and 4-1BB could potentially act as a replacement for $CD4^+$ T cell help that could boost sub-optimal anti-tumour responses. Furthermore, these co-stimulators may have use in a vaccination setting where tumour-specific $CD8^+$ T cell epitopes but not $CD4^+$ T cell epitopes have been identified. Data presented in Chapters 5 and 6 of this thesis suggests that CD27 and 4-1BB have potential as targets for stimulating both prophylactic and therapeutic anti-tumour immune

responses, and that they may have synergistic effects on CTL generation when combined. In addition, it is probable that CD28 co-stimulation in conjunction with CD27 and 4-1BB signalling will be required to prime optimal immune responses to weak tumour antigens. Once the contribution of different co-stimulators to the T cell response has been fully defined it should be possible to build multiple co-stimulatory molecules into vaccines in an appropriate combinatorial fashion. For example, alternate co-stimulators may be more effective during the prime or boost phases of the vaccination protocol, depending on their importance for naïve or memory T cell responses, respectively. Furthermore, different combinations of co-stimulators will be relevant depending on which subset of T cells is likely to have greatest efficacy. Recombinant ligands such as those described here, or adaptations thereof, could be one format by which to provide appropriate co-stimulation when combined with peptide or protein vaccines. Alternatively, the genes encoding these molecules could be incorporated into recombinant DNA or viral vectors for antigen-specific anti-tumour vaccination.

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