Tracking memory CD4 T cells *in vivo*: requirements for the generation, survival and re-activation of memory cells

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

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Abstract

How memory cells form and how they survive are pertinent, unanswered questions in the field of immunology. T cell memory develops following an adaptive immune response, which is initiated in secondary lymphoid organs. This response involves T cell proliferation, effector cell differentiation and the generation of memory cells. It has proved difficult to study CD4 T cell responses due to the problems of tracking the small number of antigen specific T cells. MHC class II tetramers, which bind to the T cell receptor of responding T cells, enable the identification of antigen specific CD4 T cells and, therefore, can be used to track memory cells. The importance of this system lies in the physiological number of precursor cells, providing a more realistic system to study CD4 T cell immunology than T cell receptor transgenic studies.

In this PhD, MHC class II tetramers have been used to track endogenously generated, antigen specific CD4 T cells *ex vivo*. This has enabled the tracking of a CD4 T cell response from early activation, into memory, and during recall responses. Long-term studies have been carried out to determine the survival of memory cells in both the absence and presence of antigen, and to describe the phenotype of these cells.

Furthermore, this system has been used to answer questions concerning the requirements of both naïve and memory cells for the costimulatory signals necessary for activation and proliferation. Specifically, the requirements for the interactions between the costimulatory molecules, CD40L-CD40 and ICOS-B7h, have been examined by comparing T cell priming, memory cell generation and recall responses in wild-type and knockout mice.

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Abbreviations

ACAD	activated T cell autonomous cell death
AICD	activation induced cell death
APC	antigen presenting cell
BCG	bacillus Calmette-Guerin
BH	Bcl-2 homology
BLC	B lymphocyte chemokine
CFA	complete Freund's adjuvant
CFSE	caroxyfluoroscein diacetate succinimidyl ester
CLA	cutaneous lymphocyte antigen
DC	dendritic cell
DC-i.v.	dendritic cell injected intravenously
DC-s.c.	dendritic cell subcutaneously
DLN	draining lymph node(s)
ERK	Extracellular signal-regulated kinase
FACS	fluorescent activated cell sorter/ used to signify flow cytometric analysis of fluorescently stained cells
FADD	Fas associated death domain
FDC	follicular dendritic cells
GM-CSF	granulocyte/ macrophage colony stimulating factor
GFP	green fluorescent protein
IL-/IL-R	interleukin-/receptor
HEL	hen egg lysozyme
HEV	high endothelial venule
IFN-α,β	interferon alpha,beta
IFN-γ	interferon gamma
i.p.	intrapertoneal
i.v.	intravenous
ICOS	inducinble costimulatory
KO	knockout
LCMV	lymphocytic choriomeningitis virus
LM	*Listeria monocytogenes*

LN	lymph node(s)
LPS	lipolysaccharide
MACS	magnetically activated cell sorting
MHC	major histocompatibility complex
MMLV	Moloney murine leukemia virus
mRNA	messenger RNA
NES	*Nippostrongylus brasiliensi*
NK	natural killer cell
OVA	ovalbumin
PCC	pigeon cytochrome c
Peptide-DC	peptide pulsed, LPS activated dendritic cells
PI(3)K	Phosphatidylinositaol -3-kinase
PLP	proteolipid protein
PMA	Phorbol 12-myristate 13-actectae
Rag	recombinase activating gene
SLC	secondary lymphoid tissue chemokine
SEM	standard error of the mean
T_{cm}	central memory T cell
T_{em}	effector memory T cell
TCR	T cell receptor
Th1/2	T helper cell type 1/2
TNF/R	tumour necrosis factor/ receptor
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
VSV	vesicular stomatitis virus
WT	wild-type

1. Introduction

1.1. Overview

How memory cells form and how they survive are pertinent, unanswered questions in the field of immunology. Both B and T cell memory develop following an adaptive immune response that is initiated in secondary lymphoid organs (1), (2, 3). Dendritic cells (DC), carrying antigen acquired from the periphery, migrate to this site and interact with antigen-specific T cells, resulting in T cell activation and proliferation (4). This interaction involves recognition by the T cell receptor (TCR) of antigen presented as peptide on major histocompatibility complex (MHC) molecules and the plethora of co-stimulatory molecules, such as CD28-B7.1 and B7.2, and CD40-CD40-Ligand (5) (6). B cells can be activated by binding antigen which filters into the lymph node via the lymph, or may also be able to capture antigen from incoming DC cells (7). Once activated, B cells can interact with primed T cells and this provides the signals required by B cells to proliferate and produce antibodies.

Activated specific CD4 T cells orchestrate the ensuing response by releasing small soluble signals called cytokines or via expression of cell surface molecules. Following clearance of the antigen the response contracts as the activated T and B cells begin to die. This is essential to maintain the homeostasis of T and B cell numbers. Although over 90% of the activated cells die, a small number survive as memory cells (1), (8), (2).

These surviving cells, when re-exposed to antigen, form the basis of the rapid recall response. The memory response is rapid and effective because memory cells respond at lower concentration of antigen, quickly release effector cytokines and are present at higher frequencies than naïve cells (9), (10), (11), (12). Moreover, memory T cells

are thought to re-circulate through the tissues as well as secondary lymphoid organs, providing immediate protection from subsequent challenge (13), (14).

Immunological memory has proved to be a controversial research area. Memory cells clearly exist and can be found many years after exposure to antigen. For example, a recent human study has found smallpox-specific memory B and CD4 T cells 40-50 years after immunisation (15). Over the past decades, with our increasing understanding of the immune system, certain aspects have become clear. For example, it is now thought that rather than existing as long-lived cells, memory cells divide periodically to maintain numbers at a fairly constant level in the face of cell death, (16), (17), (18), (19). The requirements for memory cell survival, however, are still contentious. How do these few cells survive the massive death that ensues during the contraction phase of the immune response? What signals (if any) do memory cells require to survive in the long-term? And, finally, are there competitive factors within the T cell memory pool that result in differential survival kinetics for different T cell clones?

1.2. T cell activation

1.2.1. Initial events in T cell activation

In recent years our understanding of the initial interactions between antigen presenting cells (APC) and naïve T cells has advanced greatly. This is partly as a result of pioneering experiments tracking live individual T cells *in vivo* using sophisticated microscopy. Confocal and 2-photon microscopy has been used to follow cells in whole lymphoid organs providing extended movies detailing these initial contacts (20), (21).

Naïve CD4 T cells are thought to be activated by DC that present antigen in the form of peptides in MHC class II molecules. Recent experiments from Marc Jenkins and colleagues has challenged the dogma that the initial DC that activate T cells migrate from the infection or challenge site to the draining lymph node (DLN) or spleen (22). Rather, soluble antigen can itself leak through to lymphoid organs where it can be picked up by resident DC, which can then interact with specific T cells. A second wave of DC, migrating from the injection site, provides the further stimulation that is required for full T cell activation.

Direct visualisation of the T cell-DC interaction using 2-photon microscopy suggests that T cells make a number of short-term contacts with DC carrying specific antigen (23), (24). After about eight hours, these T cells then slow down and form stable interactions with DC; these contacts can last 12 hours and T cells only divide on the second day after immunisation (24). These stable interactions may enable the formation of an immunological synapse, a tight interaction between two cells that may be required for T cell activation and for the directed release of soluble molecules (25).

T cell activation requires at least two distinct signals between the DC and the T cell (3), (4), as illustrated in figure 1.1. Signal one is the interaction between TCR and peptide-MHC and defines the specificity of the immune response. Signal two, or costimulation, influences the outcome of the ensuing immune response. In the absence of costimulation, T cell activation results in an abortive immune response, leading to tolerance to the specific antigen (26), (27). However, when antigen is presented in the context of costimulation, T cell activation and sustained proliferation occurs. This requires maturation of the presenting DC. This is induced by microbial molecules that bind pattern recognition receptors, such as Toll-like receptors, on the DC (28) and results in an increase in the expression of costimulatory molecules such as B7.1,2 and MHC class II on the DC (28), (29).

There are myriad costimulatory molecules that are categorised into two main molecular families. The immunoglobulin superfamily includes CD28, which is found on all naïve T and binds to B7.1 and B7.2 expressed on activated DC and B cells (5). The CD28 family includes ICOS, which binds to B7h (also known as ICOSL) on APC and is required for the maturation of the B cell response (5). PD-1 is also a member, but the interaction between it and its ligand, PD-L1, is thought to have a negative effect on T cell activation (5). A second major costimulatory family is the tumour necrosis factor/receptor (TNF/TNFR) family that includes the molecular pairs CD40L/CD40 and OX40L/OX40 expressed on APC and T cells respectively (30). These molecular interactions can be important in initial T cell activation and in sustaining the T cell response (6).

These costimulatory molecules act in a positive feedback loop. CD28-B7.1/2 signals induce the upregulation of ICOS and OX40 expression on the T cell, and in turn ICOS signalling can upregulate CD40L expression on T cells (31). For the DC, CD40 signals are critical for full DC maturation, inducing the upregulation of costimulatory molecules, such as OX40L, and cytokine production (32), (33), (32), (34), (35).

Costimulatory signals can act in two non-mutually exclusive ways: 1. by amplifying the signals and gene expression that TCR signals initiate; or 2. activating a different set of genes. CD28 acts as an amplifier of TCR signals as most of the gene transcription affected by CD28 signalling is also altered by TCR signals (30).

However, other costimulatory molecules do have distinct roles in the immune response. For example, CD28 is found on naïve T cells and is involved in the initial phase of T cell activation (5). CD40L and ICOS, however, are expressed on activated T cells, and thus have a role in sustaining the ensuing T cell response and in inducing T cell effector functions (5), (6).

1.2.2. Effector cell differentiation

The type of the effector cytokine that is produced by the T cell depends on the differentiation pathway that the T cell takes. Classically, differentiation is defined by two major pathways: T helper 1 (Th1) and T helper 2 (Th2) (36). Th2 cells secrete cytokines such as interleukin (IL-)4 and IL-5 that can aid B cell activation and plasma cell differentiation (37). Th1 cells, on the other hand, secrete cytokines such as interferon-gamma (IFN-γ) and TNF-α that can activate macrophages to kill engulfed bacteria (38).

The signals that influence Th1 and Th2 differentiation are complex and debatable (39), (37). The main differentiation signals are cytokines that are released by the presenting DC and also by other cells such as NK cells. IL-12 production by DC induces Th1 development, while early IL-4 production, from an as yet unidentified source, leads to a Th2 response (40). In turn, the production of these cytokines and upregulation of costimulatory molecules is influenced by the microbial stimuli in the

antigen and/or adjuvant, which can bind Toll-like receptors on the surface of innate cells (28), (41).

Particular costimulatory molecules may be more important in Th1 or Th2 responses. For example, there are reports that ICOS and OX40 costimulation are important in the differentiation of Th2 cells (42), (43), (44). However, defects in Th1 responses have been described in the absence of ICOS and OX40 costimulation was found to be required for both Th1 and Th2 responses in allergic asthma (45), (46), (47).

1.2.3. T cell migration

T cell activation occurs in secondary lymphoid organs but activated cells must travel to the site of inflammation or infection in order to execute the effector arm of the immune response. Small soluble molecules called chemokines dictate this migration (48), (49).

Chemokines direct the migration pattern of both naïve, activated and memory T cells (49), (50). Naïve T cells are mainly found in the blood and in secondary lymphoid organs (the spleen and LN) (51), (52). T cells enter LN from the blood via high endothelial venules (HEV). This entry is an active process requiring G-protein signalling by the chemokine receptor, CCR7 (53), (49). CCR7 binds to secondary lymphoid tissue chemokine (SLC) secreted by cells in LN and HEV and enables T cell to slow down and form contacts with adhesion molecules on the endothelial cells. This allows CD62L (also known as L-selectin) to bind to its ligand, GlyCAM-1, on HEV, and thereby enter the LN (54), (52), (55).

Recently, Matloubian *et al* have found that T cell exit from lymph nodes is also an active process requiring sphingosine-1-phosphate (S1P) receptor 1 (56). Thus, naïve

T cell migration is a controlled process, delivering T cells to the sites at which they are most likely to meet antigen that has been delivered either in the lymph, or carried there by DC from the periphery.

T cell activation involves changes in the expression of chemokine receptors and adhesion molecules. CCR7 expression is down-regulated and other chemokine receptors, such as CXCR3 and CCR5 that bind chemokines released by inflamed tissues, upregulated (57). Activated T cells can also express CXCR5, allowing them to migrate to B cell follicles in response to B lymphocyte chemokine (BLC) and provide the necessary help for the B cell response (58), (59), (60), (61).

CD44 binds to hyaluronate and this is also important in migration of T cells to inflamed sites (62), (63). It is upregulated on activated cells and remains at high levels on memory cells. Activated T cells downregulate CD62L and upregulate carbohydrate ligands for CD62P and CD62E (64), (65). These two selectins are expressed on inflamed endothelial cells ((66), reviewed in (65)) and enable the activated T cell to transmigrate into the inflamed tissue.

There are reports of tissue specific migration of T cells. For example, T cells activated by DC from Peyer's Patches from the gut upregulate the gut tissue specific adhesion molecules, $\alpha_4\beta_7$, and T cells activated in skin DLN express cutaneous lymphocyte antigen (CLA), which allows them to home specifically to the skin (67), (68). Thus, the site of T cell activation can control the subsequent migration pattern of the T cell (69).

However, elegant studies tracking CD4 and CD8 T cell migration from the groups of Marc Jenkins (14) and Leo Lefrançois (13) respectively, have found that activated and memory antigen-specific cells can be found throughout the body. Thus, whether there are 'tissue specific' memory cells is an area of debate. Ley and Kansas suggest that subtle differences in the pattern of expression of adhesion molecules controls

where T cells migrate and that currently these distinctions are not fully appreciated (65).

Migration of effector cells brings the response to the site of infection or challenge where, for example, effector cytokines are required to activate innate cells, or kill infected cells. This leads to a decline in the antigen load and this in turn results in the resolution of the immune response.

1.3. Memory Cell Generation

1.3.1. Cell death

The immune response terminates with the majority of activated cells undergoing cell death by apoptosis (2). Memory cells must, by definition, avoid this fate. To understand how they achieve this, it is necessary to understand the process of cell death. There are two main ways in which T cells can die by apoptosis: activation induced cell death (AICD), and as a result of cytokine withdrawal, which Marrack, Kappler and colleagues refer to as "activated T cell autonomous death" (ACAD) (70), (71).

It had been thought that T cell death at the end of the immune response was mainly a consequence of AICD brought about by the ligation of death receptors such as Fas-FasL or TNFR-TNF at the cell surface (72), (73), (74). Naïve cells are protected from Fas induced death by the expression of a molecule called FLIP (75). This binds to the intracellular portion of Fas, preventing the downstream signalling molecules from binding and initiating death (see below). T cell activation leads to a decrease in FLIP and, this, with concurrent increases in Fas expression induced by IL-2, makes the T cell more receptive to death (76), (77).

Ligation of FasL to Fas results in a cascade of signalling events that leads to the activation of enzymes that bring about cell death, see figure 1.2. First, adaptor molecules, FADD or TRADD, bind to the intracellular domain of Fas or TNFR respectively. These adaptor molecules contain death domains that induce a cascade of enzyme activation of molecules called caspases. Activation of effector caspases results in the inactivation of proteins required to maintain the structure of the cell (78), (71), for example, the cytoskeletal proteins, fodrin and gelsolin are cleaved by

caspases 3 (79). The caspases also activate enzymes that contribute to the destruction of the cell, such as DNAse that cleaves the cellular DNA (79).

Cell death is also controlled by a separate set of molecules: the Bcl-2 family molecules that regulate cell death induced by the withdrawal of growth factors. The main growth factors for T cells are cytokines, in particular cytokines in the common γ-chain receptor family (IL-2, 4, 7, 9, 15, and 21) (80). Despite using this common receptor, the cytokines have distinct functions, for example, IL-2 is important for T cell proliferation while IL-7 is required for T cell survival (81). The specificity of the effect of the cytokine is conferred by binding to additional receptor chains in combination with the common γ-chain, for example, IL-7 binds to IL-7Rα and the common γ-chain and this can promote the expression of Bcl-2 (82), (83), (84).

There are three groups of molecules in the Bcl-2 family that share Bcl-2 homology (BH) regions: the anti-apoptotic molecules such as Bcl-2 itself, $Bclx_L$ and Bcl-3; the pro-apoptotic molecules such as Bax, Bak and Bad; and the BH3 molecules that contain only one of the BH regions (85), (70). The exact molecular mechanism of this form of cell death has not been fully elucidated and is an area of debate, but it is clear that the mitochondria, the power-house of the cell, is centrally involved (reviewed in (85) and (86)). The process is also illustrated in figure 1.2.

Bcl-2 is thought to preserves the integrity of mitochondria by preventing the release of cytochrome *c* (78), which, when released can amplify cell death (see below). However, if growth factors become limiting, pro-apoptotic molecules such as Bax and BH3 molecules (e.g. Bmf and Bim) can migrate to the mitochondria and this results in the release of cytochrome *c* (87), (88), (89).

The exact molecular mechanism for this action is unclear. The BH3 molecules may act upstream of pro-apoptotic Bcl-2 family members such as Bax, binding and activating them and this then results in the inactivation of Bcl-2 (90). However, Marsden and Stasser argue that the binding of BH3 molecules to Bax or Bak has not

been clearly demonstrated and the BH3 molecules may only act indirectly to induce apoptosis via the pro-apoptotic molecules (85). Some results suggest that Bax and Bak can form pores in the mitochondrial membrane and thus facilitate the release of cytochrome *c* (86). Alternatively, as caspase-2 can induce the release of cytochrome *c*, the mitcochondrial pathway may be a down-stream consequence of caspase activation (91), (85).

Bcl-2 can also be inactivated via the death receptor pathway as caspase 8 activates Bid, another Bcl-2 family molecule, which can then migrate to the mitochondria and inactivate Bcl-2 (87). This illustrates that cellular apoptosis act as an amplification process: once the programme has been initiated there are positive feedback mechanisms that contribute towards cell death. Moreover, the caspase cascade is also activated as a result of the release of cytochrome *c* from the mitochondria. Cytochrome *c* associates with an adaptor molecule called Apaf-1 and with caspase 9 (86). These molecules form the apoptosome that can then induce the caspase cascade.

Some recent evidence suggests that there is no role for the caspase enzymes in T cell death at the end of immune response. Nussbaum and Whitton found that pan-caspase inhibitors did not block cell death *in vivo* and thus did not alter the transition of activated cells into the memory pool (92). However, other investigators have found that T cells that undergo caspase independent death appear more necrotic than apoptotic (93), (94).

Despite the molecular convergence of the two mechanisms of cell death, they are also distinct processes, for example, increases in Bcl-2 will not prevent Fas induced cell death by AICD (72). *In vitro* activated cells have been found to die via Fas/FasL induced signalling (95) however, the mechanisms that control cell death *in vivo* may be more complex. Hildeman *et al* (70) argue that most of the *in vivo* evidence for the control of cell death by Fas-FasL is based on experiments using repeated exposure to

antigen (72), (73), (74). This repeated exposure is fairly non-physiological and leaves the activated cells sensitive to cell death upon re-exposure to antigen (75). Furthermore, superantigen activated *in vivo* T cells have been found to die in the absence of Fas or TNF signalling, demonstrating that these death-inducing molecules are not absolutely required for T cell apoptosis (96), (72).

There is evidence for the involvement of Bcl-2 family members in *in vivo* T cell death. The contraction phase of the immune response can be prevented by artificially increasing Bcl-2 levels, (72). Moreover, experiments involving immunisation with the superantigen, staphylococcal enterotoxin B (SEB), found reduced death in T cells deficient in the pro-apoptotic molecules, Bim, Bax and Bak (96), and mice that are deficient in both Bax and Bak develop splenomegaly as a result of the accumulation of memory T cell that were not deleted following immune responses (97). Furthermore, Mitchell *et al* found increased levels of the anti-apoptotic molecule, Bcl-3, improved the survival of activated T cells (98). However, there are also reports that neither Bcl-2 nor $Bclx_L$ offer any protection from cell death at the conclusion of the immune response (99), (100).

These findings prompt a variety of questions about the generation of memory cells. Costimulation, for example via CD28, is known to increase the levels of Bcl-2, providing some protection to activated cells early in the response (101). The decrease in anti-apoptotic and the increase in pro-apoptotic molecules occur at the peak of T cell activation (96), (102). This is perhaps a result of the limitation of IL-2 family cytokines as the immune response progresses, or an increase in toxic reactive oxygen species that cause intracellular and mitochondrial damage (103). This does not explain why the future memory cells survive. Do they increase levels of a protective molecule, or decrease expression of a pro-apoptotic molecule?

1.3.2. The transition phase: how activated cells become memory cells

To investigate these questions, the transition phase of the immune response must be examined, tracking antigen specific cells as they journey from activation into the memory pool. This is exactly the approach Kaech *et al* have taken, examining the gene expression of CD8 lymphocytic choriomeningitis virus (LCMV)-specific transgenic cells at various time-points after activation (104).

By studying the expression level of a wide range of genes, Kaech *et al* found that 60% of the genes expressed by activated cells at the peak of the response (day 8) were maintained in the day 40 memory cells (104). This activated population was found to contain the precursors of the memory cells, however, three to four weeks were required for these precursors to express the extra genes associated with long-lived memory cells. For example, the authors describe that Bcl-2 levels were not high in the cells that survived past the peak of the response, but increased gradually over-time.

By transferring the transgenic cells after various time points post-immunisation, the authors measured the ability of the populations to protect naive recipients from LCMV infection. Day 8 and day 15 cells were poor at controlling the virus, day 22 cells protected better, but only cells primed 40 days or more prior to transfer could offer full protection. Thus, even at 22 days post-infection, the cells had not fully generated into protective memory cells. The authors found that this defect was a result of reduced and less sustained ERK (Extracellular signal-Regulated kinase) phosphorylation in the day 8 and day 15 T cells after reactivation *in vivo*. As ERK phosphorylation is required for T cell proliferation (105) these cells were unable to proliferate extensively.

These data suggests that memory cell formation is a gradual process occurring after the flux of the active immune response. Whether this gradual formation requires signals from other cells or is an autonomous cellular process is not known. An argument for the latter may be that CD8 T cell activation is an autonomous process as, once activated, CD8 T cells will proliferate and differentiate into effectors in the absence of continual activation (106), (107), (108). Moreover, Huster *et al* (109) and Wong *et al* (110) argue that CD8 memory T cells are generated early in the response to *Listeria monocytogenes* (LM) infection. The differences observed by these investigators in comparison to Kaech *et al* (104) may be due to the different infection models used. Wong *et al* argue that as LCMV infection induces a huge antigen-specific response, the effect of the memory cells would be masked by the presence of large numbers of effector cells that would rapidly eliminate any antigen with which the immune mice were challenged.

Badvinac, Harty and colleagues have also investigated the transition phase. They found that IFN-γ regulated the contraction of the CD8 T cell response to LM (111), (112). By reducing the early IFN-γ response by pre-treating mice with ampicillin, the authors found that the contraction phase of the immune response did not occur (112), leading them to argue that early events in the immune response programme both the contraction phase and memory cell generation.

1.3.3. A role for cytokines in memory cell generation

There is growing evidence that the cells that become memory cells are marked from an early stage during activation. This population has been identified by a number of groups using a variety of markers. Jacob and Baltimore created an elegant system in which activated, granzyme expressing CD8 cells are induced to express a stable marker (113). They found that only a proportion of activated antigen-specific cells expressed the marker, but that the majority of memory cells did, implying that a selected group of activated cells become memory cells.

More recent work has defined a number of natural markers of "future" memory cells. For CD8 T cells, the cells that become memory cells were found to express increased levels of IL-15R and/or IL-7Rα (109), (114), (112) and the expression of CD8αα has also been found on this population of activated CD8 T cells (115).

A functional role for IL-7 in memory cell generation has been described by a number of groups. Li *et al* (116) and Kondrack *et al* (117) found that in mice treated with a blocking antibody to IL- 7Rα, transferred CD4 transgenic cells did not form memory cells and that memory T cells transferred into IL-7 knockout mice were unable to survive. A similar role for IL-7 in the generation of memory CD8 cells from activated CD8 cells has been described, demonstrating that expression (or re-expression) of IL-7Rα is functionaly important at this stage (114), (83).

Schluns and Lefrançois question whether cytokine receptor expression is maintained on these cells or is re-expressed either by cells that have received a particular signal or stochastically by a proportion of the activated cells (80). Indeed cells that receive signals either from the IL-2 family cytokines (118), (119), or through the TCR (120) have been found to down-regulate IL-7Rα. Although Xue *et al* have identified that the transcription factor, GABP, a member of the Ets family, is responsible for IL-7Rα expression (121), this does not identify the upstream factors required to either retain receptor expression, or induce its re-expression.

The maintenance of IL-7Rα signalling could provide the enhanced survival signals necessary to protect the "future" memory cells from the massive cell death that marks the conclusion of the immune response. For example, Schluns *et al* found that wild-type transgenic CD8 T cells upregulated Bcl-2 after the peak of the immune response, but IL-7Rα deficient T cells did not and few memory cells were generated from these cells (83). The signals involved in IL-7R signalling that upregulate Bcl-2 levels have been studied *in vitro*, but with conflicting conclusions. Although it is

clear that the IL-7R signals via Jaks 1 and 3 and STATS 3 and 5 (122), the downstream response is less well defined. Barata *et al* found that phosphatidylinositol-3-kinase (PI(3)K) activation was required and acted via Act phosphorylation (123), while Rathmell *et al* report that IL-7 induced Bcl-2 upregulation and maintenance of cell viability was independent of PI(3)K activity (84).

1.4. Factors controlling the size and quality of the memory pool

The marking of the cells that become memory cells will make it easier to identify the signals that influence cell survival (112). However, past and current research has already identified an array of factors that can influence the number and, perhaps quality, of cells that enter the memory pool. These factors include the amount and type of antigenic signal and the costimulatory molecules that the T cell encounters during activation.

T cells can undergo exhaustion in response to very large antigenic signals and it appears that the continued presence of high doses of antigen can induce death in most, if not all, of the responding cells (2). The precise role of antigen in memory cell generation is contentious. Harbertson *et al* suggest that the continuing presence of antigen is detrimental to memory cell generation as it induces AICD (124). However, Badovinac *et al* found that LCMV-specific CD8 T cells contracted with similar kinetics in mice with a persistent infection and those that had cleared the virus (125).

Removing the infection at an early stage does not appear to have a negative affect on memory cell formation. Mercado *et al* removed Listeria monocytogenes (LM) infection from mice using ampicillin on various days following infection. Despite this loss of antigenic signal, an equal number of memory cells were found to form (108). John Harty and colleagues have reported similar findings (125), (126). The authors found that both CD8 (125) and CD4 T cells (126) responding to LM infection expanded to a similar extent in mice treated with ampicillin one to two days after infection as in untreated mice. Thus, continued antigen presentation is not absolutely required for the generation of memory T cells, suggesting that T cell activation and memory cell development may be autonomous processes.

However, although the infection had been removed, antigen may remain on APC and follicular dendritic cells (FDC). FDC are found in B cell follicles and capture antigen via various receptors (127). This antigen depot forms the basis of B cell affinity maturation in germinal centres during the immune response (128). FDC appear to maintain this antigen for some time, prompting suggestions that it may have a role in memory cell maintenance (129), (1). It is, therefore difficult to ascertain whether antigen is completely removed from the host and under what circumstances T cells have access to it.

The kinetics of T cell contraction have recently been studied in detail for CD8 T cells in a LM model by Badovinac *et al* (125). The authors have found that although different doses of infection result in different levels of expansion of specific T cells (with a higher dose resulting in increased expansion), the contraction phase of the response is almost identical, with approximately 75% of the activated cells dying. Thus, following a high dose of antigen more memory cells are formed. This confirms earlier findings that approximately 5% of the cells in the primary response survive to become memory cells (130), (131).

Similar to the amplified expansion observed with increased antigen dose, it has been found that enhancing the costimulation signal results in increased primary expansion and a consequential enlargement in memory. For example, adding agonistic anti-OX40 and/or lipopolysaccharide (LPS) to ovalbumin (OVA) peptide leads to increased proliferation of transferred naïve OVA specific TCR transgenic T cells, and a consequential increase in the number of cells which survive over time (9), (132), (133). Similarly, an increase in IL-12 (134) or TLR signalling (135) can increase the number of memory cells that are generated.

Comparably, the availability of APC can affect the size of the initial expansion. Linton *et al* found decreased primary proliferation of transferred OVA specific T

cells in μMT mice (which have no B cells) and that this was reflected in a reduced memory pool (136). This suggests that as the immune response progresses and activated DC start to die, B cells may play an important role in maintaining the response. However, van Essen *et al* suggest that the initial T cell clonal burst is unaffected by the lack of B cells and that decreased T cell memory in μMT mice is a result of reduced maintenance of memory T cells (137). In contrast London *et al* found no differences in T cell memory in μMT mice (138). There could be a number of possible explanations for these differences, for example in the strains of mice used, the dose and type of antigen, or in the experimental procedures.

One issue arising from these studies is whether these factors (dose, co-stimulation, type of APC) only result in an increased pool of cells from which memory cells develop or whether they provide a different signal that improves cell survival. The TNF/TNFR family of molecules enhance survival by increasing the expression of the anti-apoptotic Bcl-2 family of molecules (2) and both OX40 and CD27 (both members of the TNF family) signals have been found to be important in the generation of memory T cells (139), (140), (141) (142). OX40 deficient T cells were found to undergo fewer divisions than wild-type T cells and were also more prone to die as levels of Bcl-xL and Bcl-2 were greatly reduced (139). CD27 signalling has been found to enhance the survival of primed antigen specific CD8 T cells in response to influenza infection and thereby could enhance the number of cells that enter the memory pool (142).

The formation of memory cells requires T cell activation and is enhanced following a strong response, although too much activation can be detrimental. Whether memory cells receive a specific signal or cellular interaction which improves their chance of survival, or whether memory cell generation is a much more stochastic event, is still open to question. It makes teleological sense that an infection that requires a strong primary response (either because it is present at a high dose or appears very

dangerous) results in the development of a large cohort of memory cells that can act promptly on secondary challenge.

1.5. Heterogeneity within the memory pool

There has been much debate in the memory field as to whether memory T cells must first go through an effector cell stage before passing into the memory pool (143), (144), (145), (146), (147). The description of the various signals that can influence T cell memory generation, suggests that there is not one particular route into survival. There are, however, two classical models of T memory cell generation: the linear differentiation pathway and the branching or non-linear pathway (145), (144), (147), see figure 1.3 In the linear pathway of differentiation, memory T cells develop from differentiated effector cells, while in the branching pathway, memory cells are generated from activated but not fully differentiated cells. There is evidence for both models. Some investigators have found that resting memory T cells can be formed from effector cells (148), (149), while others describe memory cell generation in the absence of differentiation of effector cells (150), (151), (110).

To investigate the different pathways of development, Manjunath *et al* developed a transgenic mouse in which naïve and activated T cells express GFP (green fluorescent protein) (147). GFP expression, however, is lost upon complete differentiation of the CD8 T cells. This has allowed them to track *in vivo* transferred effector cells and cells that have been activated, but not fully differentiated, *in vitro*. Cells activated in the presence of high IL-2 differentiated completely losing GFP expression, while culture with IL-15 induced cells that were activated but remained GFP^+. Both sub-types were found to survive as memory cells for at least 10 weeks following transfer *in vivo*, demonstrating that memory cells may develop along both pathways.

The survival of these two memory cell sub-types reflects the division of memory T cells into T central memory cells (T_{cm}) and T effector memory cells (T_{em}) (146). T_{cm} are found in secondary lymphoid organs as they express the chemokine receptor,

CCR7, which binds SLC released by cells in LN (48). T_{cm} cells are similar to naïve cells in that they are thought to secrete only IL-2 and re-circulate through the lymphoid organs (80). They do express the memory marker $CD45RB^{lo}$, however, and they are more sensitive to stimulation than naïve cells. T_{em} are generally found in tertiary tissues, such as the liver and lungs, do not express CCR7, and secrete effector cytokines such as IL-4 or IFN-γ (14), (13). Thus, immediate protection may be provided by T_{em} patrolling the tissues while T_{cm} cells act as a back up, quickly generating effector cells should a secondary infection persist and induce a full blown immune response. This division, which was initially based on human peripheral blood T cells (146), has also been described in mice (147), (14), (13), although Unsoeld *et al* found no differences in cytokine production by $CCR7^+$ and $CCR7^-$ memory T cells (152). Furthermore, Roberts and Woodland found that despite the presence of two phenotypically different populations, both T_{cm} and T_{em} cells were capable of proliferating in response to re-exposure to antigen (153).

The description of two sub-groups could explain why memory T cells have been reported to develop from both effectors and non-differentiated cells. Lefrançois and Masopust propose a number of possible developmental pathways for differentiation of the two populations (154). T_{cm} and T_{em} could develop separately from activated and differentiated cells respectively, or T_{cm} may become T_{em} upon terminal differentiation. Alternatively, a molecule in the tissues, such as a chemokine, could induce differentiation into T_{em} upon entry of the activated T cell into that tissue. They also suggest that both sub-groups could divide and differentiate into each other to maintain numbers at a fairly constant level.

In support of this, Wherry *et al* describe the transition of naïve CD8 T cells into T_{em} cells that can then differentiate into T_{cm} cells (143). Upon transfer to new hosts, it was found that the protection to challenge with LCMV was largely contained in the T_{cm} phenotype cells. The authors suggest that the T_{em} phenotype is a transient state with cells becoming "true" memory cells upon differentiation into T_{cm}. In a similar

vein, Wu *et al* found that, after transfer to naïve hosts, T_{em} phenotype cells quickly disappeared but that T_{cm} cells survived for at least three weeks (155).

Lanzavecchia and colleagues have suggested an inclusive model in which the level of activation signal (via the TCR and costimulatory molecules) determines the fate of the activated cell (156). A strong signal leads to full differentiation and subsequent death while a lower signal results in differentiation and formation of T_{em} cells, and a lower signal still leads to T_{cm} development. This corresponds with Sprent and Tough hypothesis that T cells that arrive late in a response, and therefore receive a lower signal as a result of decreased antigen and reduced IL-2 levels, are more likely to become memory cells than die by AICD (2).

There has been no real conclusion to the debate about whether memory cells are generated from a linar or branching pathway. Rather a middle-ground has been reached: memory cells can be generated from either effector cells or non-differentiated cells, suggesting that memory cell generation is not a narrow path and generally occurs following T cell activation that takes place in the context of costimulation. This conclusion does not, however, take into account recent findings in the CD8 T cell memory field.

In some infection models, CD4 T cell help is not required for CD8 T cell activation, proliferation and differentiation (157), (158), (109), (159). However, recall responses in these mice are defective (160). Thus, activation does not always correlate with efficient memory cell generation. There is some debate as to when and how CD4 T cells act to help the memory CD8 T cells. Shedlock and Shen found that CD4 T cells were required during the primary response (157). Bourgeois *et al* (158) and Huster *et al* (109) suggest that CD4 T cells provide help via binding of CD40L to CD40 expressed on the activated CD8 T cell. However, Sun *et al* found that CD8 T cells primed in wild-type mice then transferred to CD4 deficient hosts lost their ability to

respond rapidly and make effector cytokines (159). Thus, in this system, CD4 cells were required during the maintenance of the memory cells.

That a normal primary expansion and effector cell differentiation can occur in the absence of the generation of memory CD8 T cells is intriguing. It implies that the generation of functional memory cells is a separate process to cell activation and differentiation, perhaps requiring a different set of signals and/or cellular interactions. Moreover, if CD4 T cells are required to maintain CD8 memory T cells this indicates that an important cellular interaction is required for the long-term functional survival of memory T cells.

1.6. Memory Cell Survival

1.6.1. Factors controlling memory cell survival

The enhancement of the recall response, compared to the primary response, is a consequence of a number of factors. First, there is an increase in the precursor number of antigen-specific cells, and second memory cells are functionally distinct from naïve cells, able to make effector cytokines without several rounds of proliferation and respond to lower doses of antigen (145) or reduced levels of costimulation (161), (9), (12), (145).

Therefore, memory cells represent a separate population to naïve cells and this suggests that the two populations will require different survival signals (162), (163). It is evident that naïve T cells require periodic contact with MHC molecules (18) and IL-7 to survive (164), (165), (166). The signals required for the survival of memory cells are, however, less clear.

It had been thought that persisting antigen provided low levels of TCR stimulation, and thereby maintained memory cells (1), (167), (168). However, memory cells can survive in the absence of antigen in a number of different systems (169), (170), (171), (172), (138), (173). Antigen may be required for the maintenance of functional memory cells at least in Leishmania infections: Stobie *et al* found that mice cleared of this persistent infection were no longer protected from re-challenge (174).

It has also been suggested that memory cells may recognise cross reactive self or environmental antigen which could provide low level TCR signalling (2). Studies involving the transfer of memory T cells into MHC class I (175) or class II knockout mice (172), suggest that CD8 and CD4 T cells respectively, do not require any TCR-

peptide-MHC contact, although some CD8 T cell cells may require contact with MHC molecules to survive (18). However, in these experiments the transferred cells do not have to compete with endogenous CD8 or CD4 T cells due to absence of cells in MHC deficient hosts. It may not be surprising, therefore, that the cells persisted.

Furthermore, although T cells can survive in MHC knockout mice, it has been proposed that they may be functionally impaired. Kassiotis *et al* found that T cells maintained in the absence of MHC signals could not provide help to B cells or initiate skin graft rejection (176). This highlights a major concern in memory T cell studies that use either antibodies or tetramers to measure the number of surviving cell, but do not investigate the functional response of these cells. Additionally, the appropriateness of using *in vitro* activated cells (which are removed from the complex micro-environment of the host), as a correlate to *in vivo* cells can be questioned. For example, Veiga-Fernandes *et al* found that *in vitro* activated cells divided less but differentiated more than *in vivo* activated memory cells (12).

Although TCR signals do not appear to be necessary for memory T cell survival, other factors, such as soluble molecules, may play important roles. A role for IL-15 in the maintenance of memory CD8 T cells has been widely accepted and IL-7 may also contribute (80), (83), (82). IL-15 (177) and IL-15Rα (178) knockout mice are deficient in memory phenotype CD8 T cells and IL-15 induces the division of antigen-specific memory cells *in vitro* and *in vivo* (179), (180), (181), (182).

Surprisingly it has been found that expression of the high affinity IL-15Rα chain by activated and memory CD8 T cells is not required for their survival (17). This may be because the parts of the receptor involved in signal transduction are the common γ-chain and IL-15Rβ (183), (184). Although there are reports that IL-15Rα binds to TRAF-2 and that this contributes to T cell survival by blocking TNFR induced apoptosis (185). IL-15Rα does have to be expressed, however, by as yet unidentified,

accessory cells that present IL-15 to memory CD8 cells (186), (187). This demonstrates that memory CD8 cells require specific cellular interactions in order to survive.

IL-15 does not appear to affect CD4 memory T cells, but a similar role for IL-7 has been described. Seddon *et al* found that contrary to previous reports (188), (164), IL-7 was required for the homeostatic proliferation of CD4 T cells in lymphopaenic hosts, but only in the absence of TCR signals. This result prompted a re-evaluation of the requirement for IL-7 for the survival of memory CD4 T cells. Previously, it had been thought that anti-IL-7 antibodies did not diminish the memory CD4 T cell pool (189) and that memory CD4 T cells could persist in the absence of the common γ-chain which forms part of the IL-7 receptor (164). More recently, Lenz *et al* found that anti-IL-7Rα blocked the basal proliferation of antigen specific CD4 memory cells and that *in vitro* exposure to IL-7 results in an increase of Bcl-2 levels in these cells (82).

These data mean that naïve and memory CD4 and CD8 T cells all depend to some extent on IL-7 for their survival. It had been thought that the naïve and memory T cells pools were regulated separately (8), (81), (190). However, it now appears that IL-7 is a master regulator of T cells: critical for development of thymocytes (164) and in determining the size of the naïve (164) and memory T cell pools (83), (82), (82); it is perhaps not surprising, therefore, that memory CD4 T cells may require IL-7 for long-term survival. However, there are additional factors that control the size of the separate pools. Specifically, naïve CD4 and CD8 T cells require contact with self-MHC class II (191) and class I respectively (18), (192), and IL-15 plays a key role in the long-term survival of CD8 memory T cells (181), (179). Whether there is an additional factor that contributes to CD4 memory cell maintenance is a question that remains to be answered.

1.6.2. Survival of memory cells in competitive environments

Many of the studies outlined above have been conducted in either lymphopenic mice or in mice that are not challenged with other infections. The immune system has to contend with a constant onslaught of micro-organisms and the memory pool must either stretch to accommodate new entrants or somehow reduce its numbers.

The fate of memory cells in a competitive environment has been studied in viral and bacterial models of infection. Both studies have focussed on the CD8 T cell response, but also briefly examine the CD4 response. Welsh, Selin and colleagues have used a long-term study employing several different viruses to investigate memory T cells (193), (194), (195), (196), (197). Following the finding that some viruses share cross reactive epitopes, they have shown that the hierarchy of T clones responding to LCMV can be altered by infecting the mice with a range of other viruses. LCMV specific memory CD8 T cells which do not respond to the cross reactive epitopes are found to decrease, while those that receive a boost following infection with a different virus, remain at a stable level.

Similarly, Smith *et al* found that immunising mice with bacillus Calmette-Guerin (BCG) reduced their ability to reject a tumour to which they were previously immune (198). This attrition in the face of new infections prevents the CD8 T cell pool from becoming saturated, especially as it has been shown that 50% of the pool can be specific for a particular viral epitope (131), (199).

These two studies of multiple infections found distinct effects on the survival of antigen specific CD4 T cells. In the viral model the number of CD4 T cells was found to decrease only slightly following the subsequent infections (195). The authors suggest that this may be due to more pressures on the CD8 T cell memory pool as so many cells are activated during the primary response. However, in the

bacterial model, the numbers of antigen-specific CD4 T cells were found to be dramatically reduced following BCG infection (198).

In contrast, in a single infection model using LCMV, Homann *et al* found stable levels of CD8 T cells but decreasing numbers of CD4 T cells over the one to two years examined following infection (19). This was accompanied by a decrease in Bcl-2 expression in the CD4 T cells that was not observed in the memory CD8 T cells. Despite this loss in CD4 T cells, the mice were still protected from secondary infection one and a half years after priming. Therefore, it appears the CD8 memory pool alters in response to new infections, providing space for the cells that have proved most useful, while the CD4 memory pool may decline slowly overtime, even in the absence of external pressures, and thereby provide space for new memory cells.

However, the attrition observed in the models described above may not be a direct consequence of competition between memory CD8 T cells for space within the memory pool. In follow-up papers, both groups have found that cytokines released during the subsequent response induce the death of the original memory population. Welsh's group found that memory cells decline as early as two days after the new infection (200) and this may be a result of increased levels of type I IFNs (201). Similarly, Chapdelaine *et al* described that an increase in IL-15 caused the observed attrition of memory CD8 T cells in the BCG model (202). As type I IFN induce the expression of IL-15 (180), the mechanisms described by these two groups may be the same.

As described above, IL-15 and type I IFNs can induce the division of memory cells *in vitro* and *in vivo* (179), (180), (181), (182). However, Welsh's group did not find that the memory CD8 T cells had divided prior to their death, although they may have been activated by bystander activation. Bystander activation can occur when cytokines such as IFN-γ, IL-12 and IL-15, made by innate cells such as NK cells,

induce memory CD8 T cells to make IFN-γ (203), (180). This early and rapid increase in IFN-γ may be important in defence against infection, for example by activating macrophages increasing their phagocytic and killing ability. Less work has been carried out into bystander activation of CD4 memory cells (204), (180), (203). Although Ebrel *et al* found that synthetic double stranded RNA induced bystander proliferation of both CD4 and CD8 memory cells (203), Zhang *et al* found proliferation only in memory CD8 T cells (180). Recently, Jiang *et al* have described that LM infection induced bystander activation followed by apoptosis of both CD4 and CD8 T cells (205). In the absence of a TCR signal these cells die, which Jiang *et al* argue, provides space for the ensuing antigen-specific response (205).

These results lead to questions about how IL-15 and type I IFNs can simultaneously maintain the memory CD8 T cell pool but also cause the death of memory cells. The answer to this may lie in the context of exposure to the cytokines: during an immune response, IL-15, perhaps in concurrently with other signals, brings about the apoptosis of T cells which do not receive a TCR signal, while in the steady-state, IL-15, which may have to be presented by accessory cells to CD8 memory T cells (17), (186), acts as a survival signal.

1.7. Maturation of the T cell Immune response

The maturation of the immune response has been studied in great detail for B cells. The processes of somatic hypermutation and selective survival of high affinity B cells occurs in germinal centres during the immune response (127), (206), (128). It has been suggested that the T cell response also undergoes affinity maturation as an increase in speed and efficiency is observed in the secondary response (138), (207). Although the internal signalling molecules of memory cells have been found to differ from naïve cells, perhaps allowing them to respond at lower concentrations of antigen (145), it appears that higher affinity clones are found to dominate the secondary response (208), (209), (210). This selection of high affinity cells could occur via several mechanisms: 1. by selective preservation of high affinity cells into the memory pool; 2. selective survival of high affinity clones during the memory phase (indicating a role for antigen in memory maintenance); 3. selective activation of high affinity clones in the secondary response; or 4. a combination of all three (210). However, as it is difficult to measure directly the affinity of the TCR for MHC-peptide, the role of other molecules, for example adhesion molecules, in increasing the cell's overall avidity must be considered (207).

Several investigations into affinity maturation have been conducted which seem to favour mechanism 3. Rees *et al* immunised mice with varying doses of antigen and found that high affinity clones were only selected when antigen was limiting, but this effect was only apparent in the recall response (211). This suggests that when limited antigen was available, high affinity clones were preferentially activated in the secondary response, providing an extra survival boost for these cells.

Similarly, Busch and Pamer describe the selection of high affinity clones in the recall response and suggest that maturation results from this discriminating expansion (208). However, they did not examine the repertoire of the memory cells following

the primary response and so cannot rule out mechanisms one and two outlined above. In contrast to these reports, Fasso *et al* suggest that selection of high affinity clones occurs during the primary response, but also that there is a role for outgrowth of a small population of clones in the recall response (209).

A mechanism for this selection of higher affinity cells in the secondary response has been proposed by Kedl *et al* (212). The authors found that high affinity CD8 OT-I OVA specific TCR transgenic cells prevented the host OVA specific response by inducing the down-regulation of MHC-peptide complexes on APC. Thus, only cells with high affinity TCR could interact with MHC-peptide molecules and proliferate in secondary responses.

The increase in affinity of responding T cells may enhance the speed of the secondary and future responses, but it could result in a narrowing of the T cell clones that recognise a particular micro-organism, leaving the host open to challenge by a mutated version of the pathogen. Such a narrowing of the repertoire has not been reported, rather the TCR of cells in the primary and recall responses have been found to be the same (213), (214). Moreover, Slifka and Whitton argue that there may be no functional advantage in increasing the affinity of the TCR for peptide-MHC and that high affinity interactions may prevent serial triggering of the TCR, which may be important in T cell activation (207), (215). Therefore, whether T cell affinity maturation takes place or not is open to debate.

1.8. Immunological Memory and Infectious Disease

Recall responses are faster and more effective than primary responses. Thus, when a host is re-exposed to a pathogen they are either less likely to become ill or will the resolve the infection more quickly. This phenomenon underpins the effectiveness of vaccines that "prime" the host with either an attenuated form, or portion of the pathogen to generate antigen-specific memory cells. Vaccines can, therefore, only be effective if they induce memory cells that are both long-lived and protective thus understanding how memory cells are generated and survive is important for the development of effective vaccines.

The type of memory cell that a vaccine should induce is a topic of debate (144). Some investigators have found that T_{em} are not long-lived cells and, therefore suggest that vaccines should aim to induce T_{cm} cells (143), (155). However, T_{cm} are found in the secondary lymphoid organs and are not poised at the peripheral sites that pathogens mainly invade the host. Thus, T_{em} cells may be required for full protection. For example, Zinkernagel and colleagues have found that, although antigen-specific memory CD8 cells can persist in the absence of antigen, these cells are unable to protect the host from a re-challenge with the same virus (168), (216), (217). They argue, therefore, that antigen is required to maintain T_{em} memory cells in an activate state allowing them to respond promptly to re-infection.

This effect has been described by a number of groups investigating mouse parasite infection models. For example, Belkaid *et al* found that IL-10 knockout mice completely clear Leishmania infection and in doing so are no longer protected from re-challenge (218). This suggests that long-lived memory cells had not been generated. In wild-type mice, the infection is not cleared as regulatory T cells (acting via IL-10) prevent effector CD4 T cells from eliminating the parasite. This results in long-term exposure to antigen and this appears to maintain T_{em} cells. Zaph *et al*, however, dispute the claim that no "true" memory cells are formed in Leishmania

infected mice (219). The authors found that while T_{em} cells could not survive in the absence of the persistent infection, T_{cm} cells not only survived but also provided some level of protection to the mice.

Similarly, true protective memory cells do not appear to either be generated or survive for long periods of time following malaria infection. Anecdotally, it has been observed that people who leave areas of endemic malaria lose any protection they have, and can be severely infected on return to these areas. This complex multi-stage parasitic infection does induce an immune response (220), however, protection in rats induced by immunisation with irradiated sporozoites is lost following treatment to remove all the remaining parasites (221). This perhaps suggests that antigen is required to maintain the potentially short-lived T_{em} cells that protect from re-infection by these parasitic infections. These studies suggest that long-lived immunity to infections may require several administration of a vaccine.

In some cases the persistence of antigen can result in exhaustion of the responding CD8 T cells. For example, some strains of LCMV that cause persistent infections in mice result in the clonal exhaustion of antigen-specific CD8 T cells (222). However, in other viral infections that cause persistent infections, such as herpes virus, CD8 memory cells are constantly exposed to antigen and yet remain functional. Khanna *et al* found that in mice infected with herpes simplex virus, antigen-specific CD8 memory cells were retained at the infection site for at least 80 days and these cells may prevent reactivation of the virus (223). The cells had an activated phenotype with most cells expressing the early activation marker CD69 indicating recent exposure to antigen.

In a study of the response of CD8 T cells to murine cytomegalovirus, Karrer *et al* also found that a proportion of antigen-specific cells were activated in LN during the latent phase of the infection (224). The authors found that the number of antigen-

specific memory cells actually increased after day 40 post-infection; by one year after the infection 20% of all CD8 cells were specific for one viral epitope. These cells had an active phenotype and could make IFN-γ six hours after exposure to antigen *ex vivo*. Therefore, as in the study by Khanna *et al,* these T cells may be actively involved in preventing re-activation of the virus. Similarly, Sendai virus-specific memory CD4 cells described by Cauley *et al* in the lungs of infected mice had a very activate phenotype in terms of CD25 and CD69 expression (225). The authors suggest that only most differentiated cells were able to migrate to the lung, however, these memory cells do not persist beyond 100 days after infection.

This phenotype of viral-specific CD4 and CD8 cells is more reminiscent of activated cells than memory cells and calls into question the definition of memory cells. Can cells that are actively involved in an immune response in a particular tissue be considered part of the re-circulatory pool that forms a rapid response to re-challenge? Moreover, can cells that require this contact with antigen for survival be classified as memory cells?

These questions have been addressed in a recent paper from Rafi Ahmed's group (226). The authors studied the memory cells generated following infection with two different strains of LCMV, one that induces an acute immune response that completely clears the virus and one that results in a chronic infection. CD8 memory cells generated by the acute infection survive in the absence of antigen and proliferate in response to IL-15 and IL-7 *in vivo*. In contrast, cells from mice with chronic infections could not survive in the absence of antigen after transfer to naïve hosts. The authors suggest that this is because the memory cells do not express IL-15Rα or IL-7Rα and thus cannot receive survival signals from these cytokines.

The authors argue that memory cells from hosts with persistent infection did not differentiate into classic long-lived memory cells based on the ability of memory

cells to proliferate in response to IL-15, changes in IL-2 production and the re-expression of the lymphoid homing molecules, CD62L and CCR7. These differences suggest that there may be two pools of memory CD8 cells: one that is maintained by exposure to IL-15 but survives independently of antigen, and the other that requires antigen but not IL-15. Schluns *et al* did indeed find that some memory CD8 cells are not dependent on IL-15 for survival (227). These cells, therefore, exist in separate niches within the CD8 memory pool and thus do not compete with one another for survival resources. The existence of these distinct pools may, therefore, increase the number of pathogens for which the host has protective memory.

The data described above suggest that the pathogen which the immune system is exposed to can determine the type of memory cells generated. For example, in persistent infections, the cells that survive the primary response may retain an effector phenotype as they are exposed to antigen and are involved in protecting the host from re-activation of the "dormant" pathogen. In this sense then, these cells are memory cells: protecting the host from full-blown infection by responding rapidly to presentation of specific antigen.

1.9. Systems for studying T cell memory

Tracking the cells involved in immune responses provides an essential opportunity to improve our understanding of immunology. Memory T cells can be tracked in a number of different ways. Markers of antigen experience, such as high expression of CD44 and reduced expression of CD45Rb, can be used to follow the "memory" population in either unmanipulated or immune mice (228), (229), (230), (161). The major shortcomings with this approach is that the analysed cells are undefined in terms of antigen specificity and, or contain a heterogeneous mix of cells, including T regulatory cells which are defined by the same phenotypic markers. Moreover, in the majority of these systems mice that have not been intentionally immunised with antigen are studied, thus responding cells cannot be tracked during activation and into the memory phase.

Antigen-specific memory cells have proved difficult to study due to the small number of cells specific for a particular antigen, and because the markers which have been described, such as CD45RB^{lo}, L-selectin^{lo}, CD44^{hi}, are not stably or consistently expressed (229). Antigen specific cells can be tracked, and markers of memory used to indicate the phenotype of the cells. There are two commonly used ways of doing this: 1. by immunising mice with superantigens which activate families of T cells (211), (98), (132); and 2. by transferring a population of antigen specific TCR transgenic cells into wild-type mice (14), (231), (9), (132), (136).

Superantigens, for example staphylococcal enterotoxins, bind directly to MHC molecules and to the variable region of the β-chain of the TCR. This bypasses the processing of antigen by the APC and presentation of peptide in the class II groove. The superantigen binds to T cells expressing the same beta chain, which can be 2-20% of T cells (232). These activated cells can then be tracked using a monoclonal antibody to this region of TCR.

This system enables antigen-specific cells to be identified and tracked during and after an immune response to an antigen from a pathogenic micro-organism. The mechanism of T cell activation is, however, distinct from that in most immune responses and thus the results from studies using superantigens may not be wholly representative of T cell activation.

TCR transgenic cells have provided a wealth of information about how the immune response functions, particularly during the early events of T cell activation (22), (233), (24). About 5-10% of the transgenic cells express additional V-α chains as a result of incomplete allelic exclusion. This gives rise to cells with additional specificities such that these transgenic cells can recognise other antigens. This can lead to questions about the survival of memory cells in the absence of cognate antigen, as the cells may be able to recognise a self or environmental antigen. This problem can be overcome by crossing the transgenic mice to Rag knockout mice in which, due to the disruption of the Rag gene responsible for gene recombination, the α-chains cannot be expressed.

The TCR transgenic cells can be transferred into wild-type mice. This provides a small population of antigen-specific cells that can be activated and tracked during the immune response and the memory phase. The cells can either be tracked by monoclonal antibodies that recognise the TCR or by antibodies that recognise congenic markers such as Ly5.1 or Thy1.1.

Although transgenic cells have proved invaluable as a research tool in T cell immunology, there are several concerns about using them to study T cell memory. First, even though in most studies a small population of transgenic cells are transferred into wild-type mice, the precursor frequency is still much greater than in

endogenous responses. Thus, the ensuing T cell response may have different kinetics and require different signals to an endogenous response.

A further problem is that most TCR transgenic cells recognise peptides from model antigens such as OVA or pigeon cytochrome c (PCC). In recent years several strategies have been used to overcome this problem. To study the CD8 T cell response to viral or bacterial infections, the OVA peptide that OT-I TCR transgenic T cells recognise, has been genetically incorporated into infectious micro-organisms such as vesicular stomatitis virus (83) or LM (234). This allows the response to a virus or bacteria to be tracked with established tools.

A second approach has been to develop TCR transgenic mice with T cells that directly respond to bacterial antigens. The SM1 mice developed by Stephen McSorley and colleagues have T cells that recognise a peptide from the flagellum of *Salmonella typhimurium* (235). In this example however, the transgenic cells cannot be used to track the T cell response to Salmonella infection as they are competed out by the host response to other antigens from the bacteria (236).

This obstacle illustrates an additional problem with transgenic cells. In a primary immune response a number of T cells with different specificities will be activated by the different epitopes within the antigen. These cells may compete for antigen and costimulation and this will induce a population of memory cells containing a range of TCR specificities. This phenomenon has been widely studied in the CD8 response to virus such as LCMV and influenza and is known as epitope dominance (193), (237). Transferring a population of monospecific cells into wild-type mice can skew the response to this one epitope and TCR specificity. Thus, the resulting memory pool does not reflect that in the endogenous response in wild-type mice.

The same arguments hold for experiments in which transgenic cells are activated *in vitro* prior to transfer (231), (124). This provides a large source of antigen-experienced cells that can easily be manipulated. However, these cells cannot represent memory cells generated in the complex *in vivo* microenvironment. Similarly, memory cells generated after the transfer of transgenic cells into Rag knockout mice that are then immunised, cannot represent memory cells from wild-type mice. Rag knockout mice contain neither T nor B cells and memory cells generated in Rag deficient mice exist in a very different environment to those in wild-type hosts.

Given these deficiencies in the experimental protocols for the study of T cell memory it is necessary to study the activation, generation and survival of memory cells *in vivo* under physiological conditions. To achieve this, and to overcome the problems of studying monoclonal populations of transgenic cells, endogenously activated antigen-specific T cells must be studied. To do this, molecules that bind the TCR of antigen-specific cells are required: in other words MHC-peptide molecules. As the TCR interacts with the MHC-peptide complex with low affinity, the MHC complex must be multimerised to increase affinity by binding biotinylated MHC molecules with steptavidin to form tetramers. MHC class II tetramers are probably composed of a range of multimers due to heterogeneous cross-linking of streptavidin-PE in commercial preparations (238). However, these molecules will be referred to as tetramers throughout this thesis.

MHC tetramers enable the accurate and quantitative measurement of antigen specific cells responding to an immune challenge (239), (240). Class I MHC tetramers were first used by Altman *et al* to identify human influenza specific CD8 T cells *ex vivo* (241) and have been widely used to study the dynamics of $CD8^+$ T cells responses *in vivo* (242), (131), (240). For example, it has been shown that the percentage of CD8 T cells responding to LCMV infection contains a greater percentage of antigen-

specific cells than was previously calculated using other methods such as limiting dilution assays (131).

MHC class II tetramers have also been produced (243), (244) and have been used in a number of studies, for example to identify T cell epitopes (245) or to investigate the activation of T cells (246). However, the use of class II tetramers to study antigen-specific CD4 T cell responses has been more limited. This is probably for two main reasons: 1. class II tetramers have proved more difficult to produce and 2. the responding CD4 populations are much smaller than CD8 populations (247) , (240), (248), (249). To get round the problem of the low number of antigen specific cells, Day *et al* enriched the tetramer positive cells by using anti-fluorescent beads followed by magnetic purification. However, this also selects for high affinity cells and may not provide a full picture of the cells present (249).

There are other "tricks" to improve the generation of and staining by class II tetramers. Cunliffe *et al* added a flexible linker peptide between the class II peptide and MHC chain that improved the binding of the peptide to the class II groove (250). Reddy *et al* have optimised the time, temperature and pH of the staining procedure and also describe how pre-treatment of the cells with neuraminidase, which cleaves sialic acid from the cell surface, can enhance tetramer staining (251), although this latter "trick" also increases the level of non-specific binding by the tetramer (252).

Cameron *et al* have highlighted a potential problem with using class II tetramers (238). The authors found that tetramer binding required endocytosis and cytoskeletal rearrangements. Therefore, the activation state of the cell may affect the level of tetramer binding and *in vitro* anergised cells could not bind the tetramer. However, Reddy *et al* have stained putative anergic T regulatory $CD4^+CD25^+$ self-reactive T cells with class II tetramers (253). Moreover, staining by tetramers may be dependent

on the integrity of lipids rafts in the cell membrane (254) or may be affected by persistent exposure to antigen, which can downregulate the TCR (255).

Mallet-Designe *et al* have described an alternative approach to identifying antigen specific cells (247). They have incorporated MHC-peptide molecules into a lipid bilayer and found that these liposomes bound OVA specific T cells with much greater avidity than class II tetramers composed of the same MHC-peptide molecules. The authors suggest that only two MHC molecules within the tetramer could bind any one T cell while the linear display of MHC molecules on the liposome increased the number of MHC molecules that could interact with TCR to between 60-200.

Flow cytometry has been the tool of choice for most of these studies, enabling the accurate quantification of responding cells. However, microscopy offers an alternative view, enabling the identification of the location of antigen specific cells and providing information about the interactions between different cell types. For example, Garside *et al* tracked OVA-specific transgenic T cell and HEL-specific transgenic B cells in wild-type mice immunised with HEL conjugated to OVA. By identifying the two transgenic cells, the authors were able to show when and where the cells interacted during the immune response. To study the distribution of transgenic memory cells, Reinhardt *et al* stained whole mouse sections with antibodies that recognise the transferred cells and could locate the cells in many of the mouse's organs (14). The precise quantification of this sort of experiment is now possible using laser scanning cytometry (256).

MHC tetramers have been use to identify antigen specific cells in tissue sections (257), (258), (223) (259). Class I tetramers that recognise a peptide from herpes simplex virus type 1, have been used to demonstrate the presence and position of antigen specific CD8 T cells in the sensory neurons where the virus has been found

to persist (223). Similarly, class II tetramers have been used to identify CD4 T cells that recognise a component of the myelin sheath, proteolipid protein, in the central nervous system (257). Combining this technology with laser scanning cytometry would provide an opportunity to track the exact location of antigen-specific endogenous cells and provide information about their cellular interactions. However, the technical challenges of identifying the small number of antigen specific cells will certainly prove challenging.

1.10. Conclusion

Memory T cells have proved a difficult and controversial area of study. Problems with identifying and tracking these cells *in vivo* has meant that various non-physiological systems, such as transfers into immunodeficient mice, are relied upon to provide the basis of our knowledge. With the increasing use of transgenic cells and tools such as tetramers, a greater understanding of memory will be achieved, and with this, better vaccines could be developed.

The current picture in memory T cell immunology is still, however, a confused one. There are many questions still to be asked and answered. For example, what allows some cells to survive while those around them are dying: is it an ability to escape cell death, either by up-regulating survival factors such as the anti-apoptotic Bcl-2 family molecules, or by expressing death inducing receptors such as Fas at reduced levels? Or is there a specific survival signal which some cells receive? Does this lead to the increase in IL-7Rα and IL-15Rα expression? Or are cells that avoid (or arrive too late to receive) repeated antigenic stimuli allowed to survive?

Moreover, what of memory cell maintenance? The many experiments carried out in artificial systems (whether because cells are transferred into immunodeficient mice,

or the cells themselves are activated *in vitro* without being exposed to the complex array of signals which occur during a response) do not provide an accurate evaluation of what actually occurs *in vivo*. The basal proliferation of memory cells provides an answer to the ability of these cells to persist; but whether this division occurs by default or requires certain signals, remains open to question.

Thus, the signals required (or not required) to maintain memory cells remain unknown and the competitive (or not) environment of the memory T cell pool remains a mystery. What is required is for the composite parts of memory cell development and maintenance to be broken down and examined individually *in vivo*.

1.11. Aims

The aims of this PhD have been to track antigen-specific memory cells and to ask questions about the requirements for memory cell generation and survival. Specifically, class II tetramers have been used to follow CD4 T cells through the activation, contraction and memory phases of the immune response and to identify the requirements for the costimulatory interactions between CD40L-CD40 and ICOS-B7h in these three phases. The survival of either TCR transgenic or polyclonal memory cells has also been studied in mice that have a competitive memory pool to attempt to establish factors that may influence memory T cells.

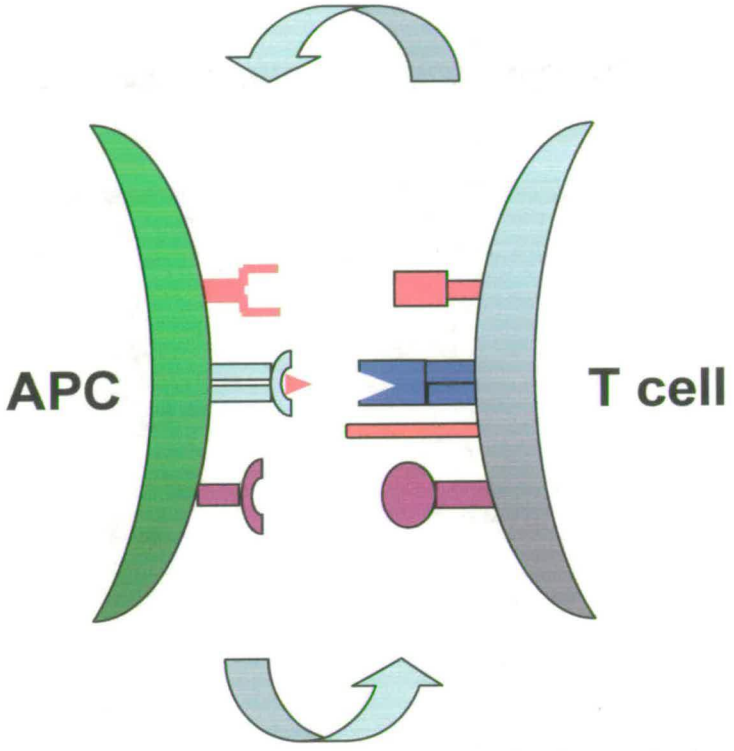


Figure 1.1 T cells are activated by interacting with APC: T cells express TCRs (dark blue) and co-receptors (CD4 or CD8, red) that bind to MHC-peptide molecules (light blue) on APC. Activation also requires interactions between costimulatory molecules, providing signals for both the T cell and the APC. For example, CD28 expressed by all T cells binds to B7.1 and B7.2 on APC and enhances the TCR signal; and CD40L on activated T cells binds to CD40 on DC, providing signals to activate the DC.

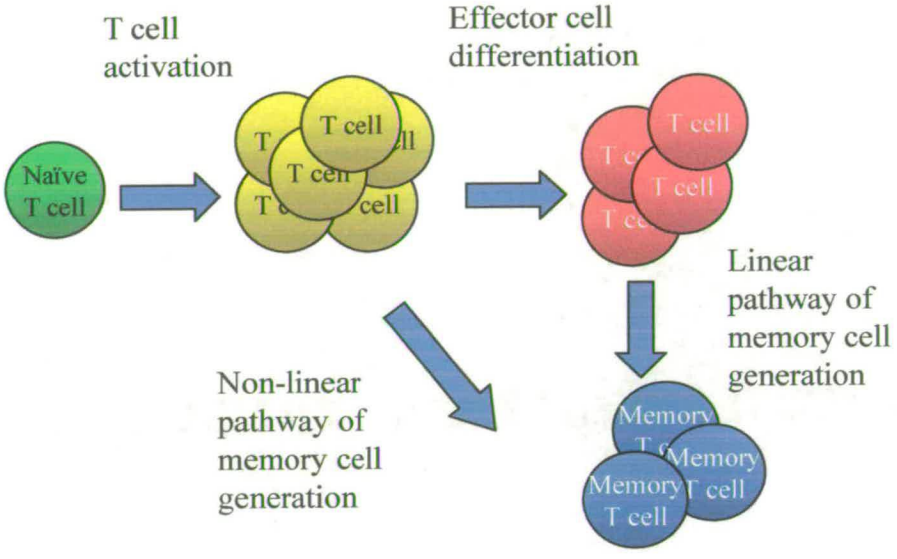


Figure 1.3 Pathways of memory cell generation: Once activated, naïve T cells proliferate and can form effector T cells (red). Memory cells may either be generated from effector T cells (linear pathway) or memory T cells may be generated from activated, but non-differentiated cells.

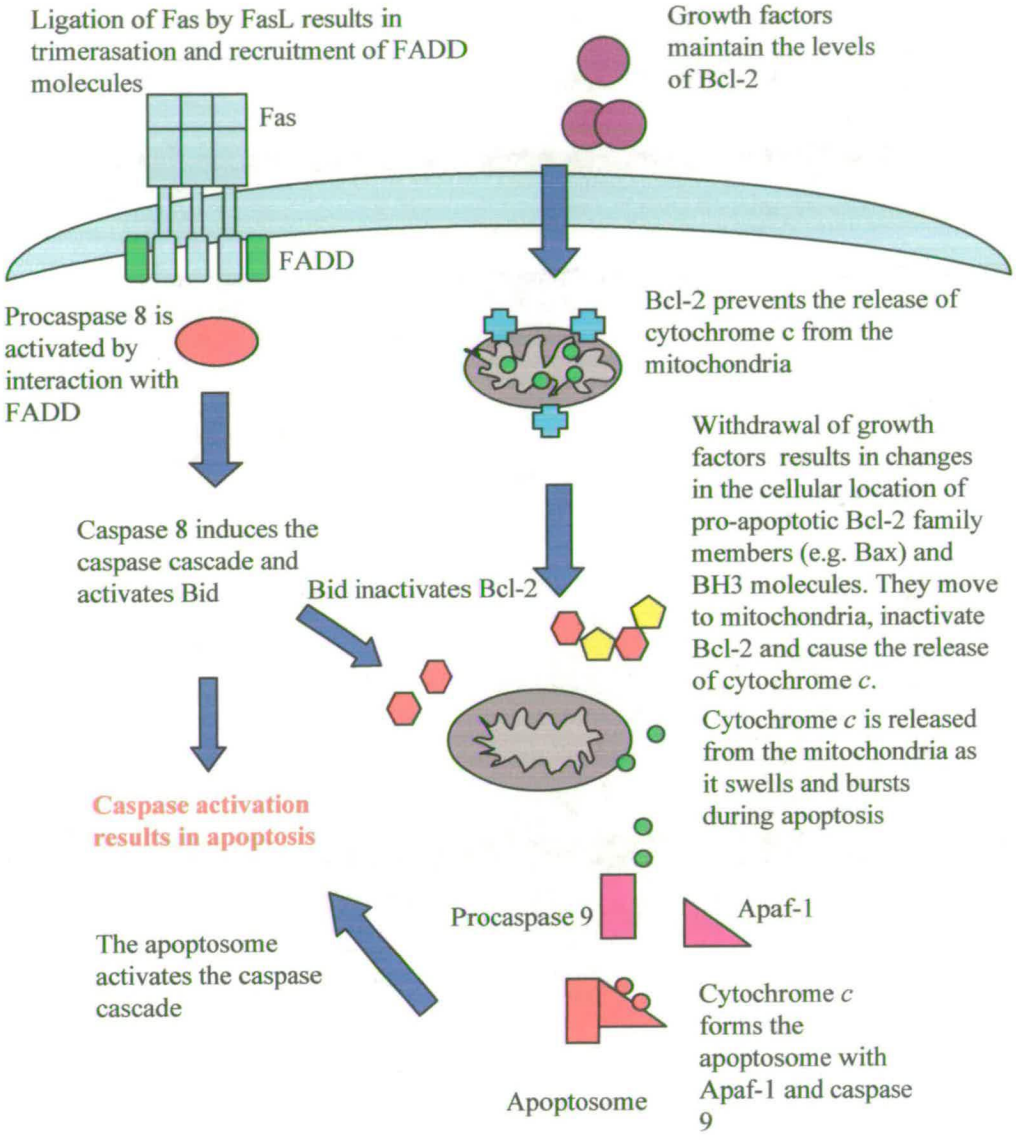


Figure 1.2 Pathways of T cell apoptosis: T cells can be induced to undergo apoptosis either by ligation of death receptors such as Fas or by the withdrawal of growth factors. Ligation of Fas by FasL results in trimerasation and recruitment of FADD. This results in the activation of the proenzyme caspase 8. Caspase 8 activates the rest of the caspase cascade and also the Bcl-2 family member Bid. Bid inactivates Bcl-2 thereby amplifying apoptosis. The withdrawal of growth factors results in the movement of pro-apoptotic Bcl-2 and BH3 molecules to mitochondria where they inactivate Bcl-2. This leads to the release of cytochrome *c*, which forms the apoptosome with Apaf-1 and caspase 9. The apopotsome then activates the caspase cascade. Adapted from references 71 and 78.

2. Materials and Methods

2.1. Mice

All mice were bred and maintained in specific pathogen free conditions at the Animal Facility at the University of Edinburgh. Mice were used at 6-12 weeks of age; sex and age matched mice were used within experiments. All experiments were performed under home office regulations.

The mice used and the conditions they were kept in are detailed below in table 2.1. Some of the mice were screened to check for the presence of transgenes or absence of molecules and details are given in table 2.1.

Strain	Screening	Conditions maintained in	Reference
BALB/c	None	Normal cages with water	
C57BL/6	None	Normal cages with water	
CD40 KO	Stain for CD40 expression by FACS	Normal cages with Borgal	(260)
CD40L KO	PCR for CD40L and neomycin genes	Normal cages with Borgal	(261)
ICOS KO	PCR for ICOS and neomycin genes	Normal cages with water	Made by Ana Cervera in David Gray's lab
DO11.10	Stain for expression of KJ1.26 by FACS	Normal cages with water	(262)
OT-II	Stain for expression of Va_2 and Vb_5 by FACS	Normal cages with water	(263)
(C57BL/6xBALB/c)F1	None	Normal cages with water	
μMT	Stain for B220 or CD19 by FACS	Filter topped cages with Borgal	(264)

Table 2.1: Summary of mice used, the conditions they were maintained in and screening requirements.

2.2. Media

Ex vivo cells were prepared and washed in Iscove's Modified Dubleco's Medium (IMDM) (Sigma-Aldrich, Poole, UK) containing 5% foetal calf serum (FCS) (Bioest, Nuaillé, France) supplemented with 2mM L-Glutamine (Gibco BRL, Life Technologies, Paisley, UK), 100U/ml penicillin, 100μg/ml streptomycin (Gibco) and 50μM 2-mercaptoethanol (BDH Merck, Poole, UK). This media is referred to as 5% IMDM

In vitro activation of H19env-specific cells and transgenic cells was done in 5% IMDM.

Tetramer staining was done in 10% IMDM (IMDM containing 10% FCS) supplemented with 2mM L-Glutamine,100U/ml penicillin,100μg/ml streptomycin (Gibco) and 50μM 2-mercaptoethanol (BDH). Transgenic cells and DC were stained in FACS buffer (PBS containing 2% FCS and 0.05% sodium azide).

Phosphate buffered saline (PBS) for FACS staining was made using PBS tablets (Sigma) and Dubleco's PBS used for cell transfers (Sigma).

DC were grown in RPMI (Sigma) supplemented with 2mM L-Glutamine (Gibco), 100U/ml penicillin,100μg/ml streptomycin (Gibco) with either 10% FCS or 0.5-1% mouse serum (Harlan Sera Labs, Loughborough, UK or Cedarlane, Hornby, Ontario, Canada).

Cell separation using magnetic beads was done in MACS buffer: HANKS (Sigma) with 2% FCS and 100U/ml penicillin and 100μg/ml streptomycin (Gibco).

2.3. Immunisations.

For class II tetramer studies, mice were immunised with 100μg of H19env (N-EPLTSLTPRCNTAWNRLKL-COOH) or ovalbumin peptide (OVA) (N-ISQAVHAAHAEINEAGR-COOH) (both from ABC, Imperial College, London, amino acid codes given in the one letter code) emulsified in Complete Freund's Adjuvant (CFA) (Sigma). 50μl were injected subcutaneously (s.c.) into each hind-leg unless otherwise stated. The CFA emulsification was done using a sonicator.

For bone marrow derived DC immunisations, mice were immunised with $1x10^6$ DC or as indicated in the figure legend, either intravenously (i.v.) in the tail vein in 200μl or s.c. in the hind-legs (50μl in each leg).

In the multiple immunisation experiment, mice were immunised with 100μg KLH intraperitioneally (i.p.) (Calbiochem, La Jolla CA, USA); 100μg OVA protein (Sigma) or OVA-DNP (kindly provided by Alison Crawford) both precipitated in alum and injected i.p.; and 10μg of secreted proteins from *Nippostrongylus brasiliensis*, kindly provided by Adam Balic was injected i.p. (265).

In DO11.10 and OT-II studies, adoptively transferred mice were immunised one day after cell transfer with OVA-peptide. The peptide was precipitated in 9% aluminium potassium sulfate dodecahydrate (Sigma) solution and washed three times in PBS prior to use. The (C57BL/6xBALB/c)F1 mice were immunised with 100μg of alum precipitated OVA-peptide i.p..

In the pre-immunisation studies, BALB/c mice were immunised with 100μg OVA protein precipitated in alum i.p. with 10 days in-between each injection and the final injection given 10 days prior to the DO11.10 cell transfer.

2.4. Bone marrow derived DC.

DC were derived from bone-marrow progenitor cells according to the procedure developed by Inaba *et al* (266) and the protocol followed (e.g. concentration of LPS used for activation and concentration of peptide used) had been optimised by Dr. Georgia Perona-Wright during her PhD in David Gray's laboratory.

The bone marrow was flushed from the tibias, femurs and humeruses using medium and gentle pressure from a 5ml syringe and a 25 gauge needle. Cell clusters were dispersed by passage through an18 gauge and then a 26 gauge needle. Red blood cells were removed using lysis buffer (Sigma) and the remaining cells were seeded into 24 well plates at $3.75x10^5$ in 1ml in RPMI (Sigma) containing 10% FCS, 5% GMCSF (supernatant from GM-CSF producing cell line supernatant (26.7)) supplemented with 2mM L-Glutamine (Gibco) and 100U/ml penicillin and 100μg/ml streptomycin (Gibco). Cells were grown for 7 days and washed on days 3 and 6 to remove non-adherent granulocytes and lymphocytes. Washing involved swirling the plates before removing 1ml from each well and replacing it with fresh medium that contained 0.5-1% mouse serum (Harlan or Cedarlane, Canada) instead of FCS. This was done to reduce unwanted immune responses to FCS antigens absorbed onto the DC surface.

Loosely adherent DC were harvested on day 7 by more vigorous washing, leaving firmly adherent macrophages on the plate. The DC were then counted and replated at

$1x10^6$ cells per well with 0.1μg/ml LPS (Sigma) and incubated for 12-13 hours at 37°C, 5% CO_2. After they had been re-harvested, the DC were incubated with H19env or OVA peptide at 50μg/ml for 90 minutes at 37°C, 5% CO_2. The DC were then washed extensively in PBS before transfer into mice.

The purity of the DC was determined by staining the cells with anti-CD11c-Allophycocyanin (APC) (BD-Pharmingin, San Diego, CA, USA), anti-MHC class II-FITC (M5114, in house) and the level of activation determined by staining with anti-B7.1- Phycoerythrin (PE), anti-B7.2-PE, anti-CD40-PE and anti-OX-40-biotin with SA-PE(all from BD-Pharmingin) and also anti-B7h-PE (eBioscience, San Diego, CA, USA). The antibodies used for FACS staining are summarised in table 2.2. Before addition of antibodies, DC were incubated at 4°C for 15 minutes with anti-FcRecptor (2.4G2, in house) to prevent non-specific binding of antibodies. After washing with FACS buffer, the DC were incubated with the diluted primary antibodies for 15 minutes, the cells were washed and secondary staining done if necessary. After further washes, the samples were acquired on a FACS Caliber (Becton Dickinson, Mountain View, CA, USA) and analysed by FlowJo software (TreeStar, California, USA). The purity of the cells was between 70-95% CD11c+ve, MHC class II +ve, figure 2.1. The activation state of the DC following LPS treatment is also shown in figure 2.1. The cells expresses increased levels of CD40 and B7.2 (and also B7.1, data not shown) and also express low levels of OX40L. LPS stimulation did not alter the expression of B7h.

2.5. Class II Tetramer staining.

Single cell suspensions were prepared from spleens and LN by mashing between two pieces of gauze (Wm Ritchie, London, UK) and erythrocytes were lysed using Red Blood Cell Lysis Solution (Sigma). Approximately 1-2 x10^6 cells were plated in round bottom 96 well plates and washed in 10% IMDM (Sigma) before the addition of PE-labelled class II tetramers diluted in 10% IMDM. Tetramers were made as described (268). See chapter three and figures 3.1 and 3.2 for details of the constructs used to make the tetramers and an outline of how they are made. A single batch of MHC class II monomers was used throughout this PhD although these were tetramerised with steptavidin-phycoerythrin (SA-PE) at different times. New batches of tetramer were tested before use and compared to the previous batch; comparable staining was always obtained. The staining protocol had been standardised in Ton Schumacher's lab.

The cells were incubated at 37°C, 5% CO_2 for 3 hours with gentle agitation every 20-30 minutes to prevent clumping of cells. APC labelled anti-CD4 (BD-Pharmingen), R. Phycoerythrin (RPE)-Cy5 labelled anti-F4/80 (Serotec, Kidlington, Oxford, UK) and FITC-labelled anti-CD44 (142.5, in house) or FITC-labelled anti-CD62L (MEL-14, in house) or FITC labelled anti-IL-7R α (CD127, eBioscience) were added and incubated for 10-15 minutes at room temperature. Cells were washed 3 times in FACS buffer. Propidium iodide (PI) (BD-Pharmingen) was added prior to acquisition. 200 000 live events were collected on a FACS Calibur Flow Cytometer (Becton Dickinson) and analysed using FlowJo software (TreeStar). Tetramer positive cells were identified by gating on CD4 positive and live lymphocytes, excluding macrophages (see figure 3.4).

Experiment	Staining Reagent	Clone	Source	Dilution
Tetramer +ve cells	Class II tetramer		Schumacher lab	1/10
			Pharmingen	
	CD4-APC		In house	1/400
	CD44-FITC	142.5	In house	1/100
	CD62L-FITC	Mel-14	Serotec	1/100
	F480-RPE-Cy5		Pharmingen	1/400
	Ly5.1		eBioscience	1/200
	IL-7Rα		Pharmingen	1/100
	PI			1/400
DO11.10 cells	TCR-specific-biotin	KJ1.26	In house	1/100
OT-II cells	Va_2-FITC or PE		Pharmingen	1/200
	Vb_5-biotin		Pharmingen	1/200
	SA-PE		Calbiochem	1/1500
	SA-quantum red		Sigma	1/100
DC markers	CD11c-APC		Pharmingen	1/200
	MHC class II-FITC	M5114	In house	1/100
	B7.2-PE		Pharmingen	
	CD40-PE		Pharmingen	1/100
	OX40-biotin		Pharmingen	1/100
	B7h-PE		eBioscience	1/100
				1/100
Chimera reconstruction	B220-PerCP		Pharmingen	1/300
	CD40-biotin		Pharmingen	1/100
Intracellular cytokines	IL-2		Pharmingen	1/100
	IFN-γ		Pharmingen	1/100
	IL-10		Pharmingen	1/100

Table 2.2: Summary of FACS staining reagents: The staining reagents were either used at standard dilutions or were tested when they were first used. Antibodies made in house were titrated before use and diluted for use at 1/100.

2.6. *In vitro* activation.

For reactivation of H19env-specific cells, single cell suspensions, prepared as above, were plated in flat-bottom 96 well plates at $1x10^6$ cells per well and incubated for 3 days at 37°C and 5% CO_2 with H19env peptide at 1μg/ml in 5% IMDM.

For *in vitro* activation of DO11.10 cells prior to transfer, single cells suspensions of the LN (popliteal, inguinal, auxillary, brachial, superficial cervical, iliac and mesenteric) and spleens from DO11.10 mice were prepared. Red blood cells were removed from spleens using lysis buffer (Sigma). $1-2x10^6$ cells were incubated in 1ml in 24 well plates with $2-3x10^6$ irradiated BALB/c splenocytes with 0.5μg/ml OVA peptide at 37°C, 5% CO_2 in 5% IMDM.

Three days later, the cells were harvested and dead cells removed by layering the cells in 1ml of medium onto 1ml of lympholyte (Cedarlane). The cells were spun at 2000 rpm for 20 minutes with the brake off. The live cell layer was then recovered and the cells washed extensively to remove lympholyte and FCS prior to transfer. In some experiments the cells were labelled with CFSE.

For the activation of DO11.10 and OT-II cells, single cells suspensions of the lymph nodes (popliteal, inguinal, auxillary, brachial, superficial cervical, iliac and mesenteric) and spleens from DO11.10 and OT-II mice were prepared. Red blood cells were removed from spleens using lysis buffer (Sigma). A CD4 purification was then carried out either by positive or negative selection using magnetic beads (see below). In some experiments the CD4 cells were labelled with CFSE.

CD4 purified lymph node and spleen cells were plated in 96-well flat bottom plates at $4-5x10^4$ cells with $4-5x10^5$ irradiated APC from either BALB/c or C57BL/6 mice.

In the mixed cultures, the total number of cells was maintained, giving: 2-2.5x10^4 DO11.10 and OT-II cells and 2-2.5x10^5 of each APC type. Cells were either activated over a range of doses of OVA peptide (0.05ng/ml-0.5μg/ml) or with 0.005μg/ml which had been determined to be the optimal concentration for these experiments (see figure 4.5). The division of the transgenic cells was either measured by thymidine incorporation (see below) or by dilution of CFSE measured by flow cytometry in conjuncture with markers for the transgenic cells.

2.7. Preparation of cell suspensions for Adoptive Transfer

Lymph nodes (popliteal, inguinal, auxillary, brachial, superficial cervical, iliac and mesenteric) from DO11.10 or OT-II mice were taken and a single cell suspension prepared in 5% IMDM. Cells were washed in PBS (Sigma), resuspended at the correct concentrations and passed through parachute silk before transfer. In DO11.10/OT-II co-adoptive transfer experiments, the percentage of transgenic cells was confirmed by FACS and equal numbers of transgenic cells transferred into recipient mice. A total of 4-5x10^6 cells were transferred into each mice as this had been determined the optimal number in DO11.10 transfers into BALB/c mice by Dr Georgia Perona-Wright during her PhD in David Gray's laboratory. In some experiments cells were labelled with CFSE prior to transfer.

2.8. FACS staining of transgenic cells

The division and/or presence of DO11.10 and OT-II cells was examined by staining the cells with anti-CD4-APC and antibodies that recognise the specific TCRs: DO11.10: KJ1.26-biotin and SA-PE; OT-II: Va_2-PE or FITC and Vb_5-biotin and SA-quantum red or SA-PE. Cells were transferred from *in vitro* cultures into V-shaped plates, washed with FACS buffer, stained with the primary antibodies at 4°C for 15

minutes, washed twice in FACS buffer and then incubated with the secondary antibodies (SA-PE or SA-quantum red) for a further 15 minutes. After two more washes in FACS buffer, the cells were resuspended then acquired on a FACS Caliber and the data analysed using FlowJo software (TreeStar).

To identify the transgenic cells *ex vivo*, single cells suspensions of splenocytes were prepared and red blood cells removed using lysis buffer (Sigma). 1-2x10^6 cells were plated into V-shaped plates and stained as above.

2.9. Thymidine assays

Proliferation was measured by the addition of 0.5μCi of 35Ci/mmol [^{3}H]-thymidine (ICN, Basingstoke, UK) to each well 16 hours before the end of the three day culture. Plates were harvested on filter mates (Printed Filtermat A; Wallac, Turku, Finland) using a 96 well MachIIIM Tomtec harvester (Walllac). The mats were dried on a hot plate and a solid scintillation wax sheet melted into them (Meltiles^{TM} A; Wallac). When the wax had re-solidified, the mats were read using a Trilux 1450 Microbeta liquid scintillation and luminescence counter and software (Trilux, Arnsberg, Germany).

2.10. Irradiation of spleens

Before single cell suspensions were prepared, spleen were placed in a bijou in a small volume of media and irradiated with a γ-source for 90 seconds.

2.11. Intracellular cytokine staining.

Cells were plated at 4-5x10^6 cells per well in 24 well plates and incubated for 4 hours with Phorbol 12-myristate 13-actectae (PMA) (final concentration of 10ng/ml) and Ionomycin (at 1μg/ml) (both from Sigma) in the presence of 'Golgi Stop' (Becton Dickson) diluted at 1/1500. Cells were transferred into V-shaped 96 well plates, washed with FACS buffer, before staining at 4°C with APC labelled anti-CD4 (BD-Pharmingen) for 15 minutes. After washing, cells were fixed for 20 minutes with Cytofix then washed in Cytoperm (Becton Dickinson). Anti-cytokine antibodies (anti-IL-2-PE, anti-IFN gamma-FITC and anti-IL10-PE all from BD-Pharmingen) diluted in Cytoperm were added for 45 minutes. The cells were washed in cytoperm 3 times, resuspended in PBS and acquired on a FACS Caliber.

2.12. CFSE labelling

CFSE (caroxyfluoroscein diacetate succinimidyl ester) (Molecular Probes, Leiden The Netherlands) is a membrane permanent dye that binds covalently to cytoplasmic proteins and is distributed equally to each daughter cell upon division, thus the mean fluorescence halves progressively as the cells divide (269). CFSE was tested each time a new batch was made up and used within 6 months of being made. Splenocytes and, or lymph nodes cells were resuspended at 1x10^7 cells per ml in warmed, serum free HANKS (Sigma) containing 1μM CFSE. After 7-9 minutes incubation at 37°C, the CFSE was quenched with an equal volume of FCS, and cells washed 2-3 times with serum positive medium. If cells were to be transferred *in vivo*, further washes with PBS were done.

2.13. CD4 cell purification

2.13.1.Positive selection:

Single cell suspensions of splenocytes and or LN cells were incubated with anti-CD4 mirobeads according to the manufacture's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) in MACS buffer (HANKS with 2% FCS) at 4°C for 15-20 minutes with occasional gentle agitation. After washing with MACS buffer, the cells were resuspended in 1ml and loaded onto a LS MACS column prepared and used according to the manufacture's protocol. Briefly, the cells were loaded onto the column, which was then washed with MACS buffer. The column was then removed from the magnet and the remaining cells flushed out in MACS buffer using the supplied plunger. Purities using this method were routinely over 90% CD4 positive, figure 2.2.

2.13.2.Negative Selection:

Cells were prepared as for positive selection but incubated with a cocktail of antibodies: M5114 (anti-class II), 53.6.78 (anti-CD8) and 187.1 (anti-Kappa) all labelled with biotin and prepared in house. The dilution of antibody depended on the batch and had been titrated each time. After incubation at 4°C for 15 minutes, the cells were washed twice in MACS buffer then incubated with steptavidin-conjugated microbeads (Miltenyi) for a further 15 minutes. After washing, the cells were loaded onto a CS MACS column in a varioMACS magnet (Miltenyi). A single column was used for up to $2x10^8$ cells. The column was washed with 20-30ml MACS buffer and unbound cells collected. Purities using this method were routinely over 80%, also shown in figure 2.2.

2.14. Ly5.1 cell transfers

Ly5.1 positive mice were immunised as above with either peptide pulsed DC or peptide in CFA. Spleens and, when mice were immunised with CFA, lymph nodes, were taken 2-4 weeks after priming. A single cell suspension was prepared and a CD4 purification carried out as above. The purified CD4 positive cells were then washed with PBS, resuspended and injected in 200μl PBS into the tail vein of C57BL/6 mice. As described in chapter 3, the number of cells transferred was varied to optimise the system.

The mice were immunised the following day with peptide-CFA, and the percentage of tetramer positive, Ly5.1 positive cells in spleens and draining lymph nodes examined after 5-7 days. The tetramer staining was done as above but the cells were stained with anti-Ly5.1-FITC (BD-Pharmingin).

2.15. Bone Marrow Chimeras.

Recipient mice were lethally irradiated with 1150cGy from a γ caesium source 24 hours prior to bone marrow reconstruction as described (270), (60). Bone marrow was removed from donor mice and depleted of T cells using anti-Thy1 microbeads (Miltenyi) on a MACS magnetic column (Milteny) according to the manufacturers instructions. Between $3x10^6$ and $5x10^6$ cells were injected into the irradiated recipients.

Mixed chimeras were made by reconstituting with 80% μMT bone marrow and 20% CD40 knockout bone marrow (see figure 5.13). Thus, B cells can only develop from the CD40 knockout bone marrow, and were, therefore, CD40 deficient. Control wild-type chimeras were made with 80% μMT bone marrow and 20% C57BL/6 bone marrow. The chimeric hosts were then left for eight weeks to allow full

reconstruction from the donor cells. Reconstitution was confirmed by FACS of splenocytes when the chimeras were sacrificed, see figure 2.3.

2.16. Tracking of Ly5.1 DC *ex vivo*

Bone marrow DC were prepared as above but using cells from Ly5.1 positive mice. The cells were either injected i.v. or s.c. into C57BL/6 mice (Ly5.2). Two or five days after transfer the DLN or spleens were taken and treated as described by Vremec and Shortman (271). The organs were separated into small portions using needles. The small pieces of organ were incubated at 37°C, 5% CO_2 for 30-40 minutes in an enzyme mix containing collagenase IV (Worthington, Lakewood, NJ, USA) at a final concentration of 0.5mg/ml and DNAse 1 (Sigma) at a final concentration of 50μg/ml in HANKS serum free medium as protein can disrupt the activity of collagenase. The pieces of organ were then mashed between two squares of gauze to prepare a single cell suspension and the red blood cells removed from spleens using lysis buffer (Sigma).

CD11c positive cells were then enhanced using anti-CD11c magnetic beads (Miltenyi). This was carried out according to the manufacture's instructions. Briefly, the cells were incubated with the anti-CD11c microbeads for 15 minutes at 4°C, washed and then loaded onto a MS column. The column was washed, removed from the magnet and the remaining cells flushed out. The cells were then stained with anti-CD11c-APC (Pharmagin), anti-MHC class II-FITC (M1554, in house) and anti-Ly5.1-PE (BD-Pharmingin) see figure 5.10.

2.17. Antibody production, biotinylation and FITC conjugation

Hybridomas were grown in culture flasks in 5% IMDM at 37°C, 5% CO_2 until transferred to roller bottles where they were cultured in 2% IMDM and bubbled with 5% CO_2. The contents of the roller bottles were filtered to remove cells and the immunoglobulin was precipitated with ammonium sulfate (Sigma). Precipitates were

dissolved in PBS and dialysed against PBS for several days. Antibodies were purified by binding to a 1 or 5ml Hitrap protein G column (Amersham Bioscience, Bucks, UK) at pH 7 and eluted at pH2.8 using an AKTAprime (Amersham Bioscience). The antibody was dialysed with PBS until the pH was neutral.

GMCSF supernatant was prepared by growing the cell line in tissue culture flask in 5% IMDM with Geneticin (G418 sulfate) (Gibco) at a final concentration of 1mg/ml to select for the GM-CSF producing cells. Before transfer into roller bottles, the cells were washed twice in 5% IMDM to remove the G418. The cells were grown in 500ml of 2% IMDM in roller bottles for 4-5 days in the absence of G418 sulfate. The cells were then transferred into 50ml Falcon tubes and the cells separated from the supernatant by centrifugation and the supernatants pooled. Each batch of GM-CSF was tested and titrated before use.

Purified antibodies were conjugated to biotin by reacting with 204μg EZ-LinkTM NHS-LC-Biotin (Pierce, Cheshire, UK) in demethyl formamide (DMF, Sigma) per mg of antibody. FITC conjugation was carried out by reacting 1mg of antibody with 50ng FITC (Sigma) in 0.05M carbonate-bicarbonate buffer, pH9. In both cases, conjugated antibodies were dialysed against PBS.

2.18. Statistics.

Data are expressed, when appropriate, as mean and standard error of the mean (SEM). Significance was assessed using unpaired t-tests using GraphPad Prism® software (version 3.0, GraphPad software, San Diego, CA, USA). P values below 0.05 were considered significant.

3. Tracking CD4 T cell activation, memory cell survival and recall responses

3.1. Introduction

The first aim of this PhD was to study the generation and maintenance of endogenously generated CD4 memory T cells using class II tetramers. Altman and colleagues first developed MHC class II tetramers in 1993 (243). However, in the succeeding decade there has been only limited use of class II tetramers (272), (273), (274), (249), (275), (276) and scant use of them in mouse CD4 memory studies (257), (209), (211). This reflects the complication in producing the tetramers and the small responding populations (277), (247), (240), (248).

This chapter demonstrates that antigen-specific cells can be enumerated using class II tetramers following immunisation with peptide in adjuvant. Furthermore, the memory population can be tracked overtime and the survival of the cells monitored in both the presence and absence of antigen.

Previously, the CD4 recall response has generally been examined using *in vitro* recall assays (9), (161), (278). This chapter details *in vivo* recall responses, examining how they differ from the primary response. To study memory cell reactivation in more detail, an adoptive transfer system was established to ask questions about the importance of precursor frequency and recruitment of naïve cells into the secondary response. Thus, class II tetramers have been used to provide a complete picture of the CD4 T cell response from early activation, entry into the memory pool and reactivation.

3.2. Results

3.2.1. Class II tetramer can be used to enumerate antigen specific cells

MHC class II tetramers were obtained from Ton Schumacher's research lab at the Netherlands Cancer Institute in Amsterdam (268). The tetramer constructs, as shown in figure 3.1, were designed to enhance the production of the tetramers: the T cell epitope (H19env) was covalently attached to the extracellular domain of the beta chain (antigen link in figure 3.1); heterodimerization in the insect cells was promoted by the addition of leucine zippers to the COOH-terminus of both chains; a His-tag was attached to the α-chain to aid purification of the tetramers; and a biotin-tag added to the beta chain to allow tetramer formation. Figure 3.2 illustrates the generation of the tetramers. They are produced in insect cells transfected with the constructs. The His-tag then allows for purification using affinity-based chromatography. The tetramers were finally generated by the addition of streptavidin-PE, which has four binding sites for biotin. Monomers were removed by gelfiltration chromatography, and the tetramers stored at -20°C in glycerol.

The class II tetramers recognise the immunodominant epitope from the Moloney murine leukemia virus (MMLV). MMLV is an onco-retovirus that induces sarcomas in infected mice. The tumour is infiltrated by large numbers of lymphocytes and granulocytes and control of the tumour is mediated by both CD4 and CD8 T cells (268). Figure 3.3 shows representative staining from a mouse infected with the virus to allow comparison with class II tetramer staining after peptide immunisation. This staining, and the accompanying class I tetramer staining, was done by Koen Schepers at the Netherlands Cancer Institute in Amsterdam. Control staining of cells from the spleen and LN of a naive mouse are also shown. The CD8 response to the immunodominat epitope is much larger than the CD4 T cell response in the spleen, draining lymph nodes (DLN) and the tumour site.

This reflects the importance of CD8 T cells in the immune response against the tumour (268) and demonstrates the difference in the clonal expansion of CD4 and CD8 T cells as has been reported (248), (19).

In order to identify the small population of responding cells, several gates were drawn on the cells acquired on the FACS, as shown in figure 3.4. First, a live lymphocyte gate was drawn using forward and side scatter characteristics. This removed cell debris from the analysis. As the class II tetramers stick to macrophages and dead cells, these two populations were removed from the analysis to reduce background staining. This was achieved by staining macrophages with RPE-Cy5 labelled anti-F4/80, and dead cells with propridium iodide, then gating out any FL3 positive cells. A CD4 gate was drawn on the remaining cells and these are then analysed for tetramer staining in conjuncture with a marker of memory or activation, typically anti-CD44 or anti-CD62L.

In this PhD, the CD4 response to the single immunodominant eptitope in MMLV was studied to remove the complications of the viral immune response. To define whether class II staining after peptide immunisation was achievable, wild-type C57BL/6 mice were immunised s.c. in the hind-leg with H19env peptide emulsified in complete Freund's adjuvant (CFA). CFA was chosen as it is a strong adjuvant that induces good CD4 T cell responses (279), (280). Spleens and the DLN of mice were taken and stained as described in materials and methods.

Samples of tetramer staining on day 9 after immunisation with peptide-CFA are shown in figure 3.5. Background staining was set by the level of tetramer staining in mice immunised with an irrelevant peptide $OVA_{323-339}$, in CFA. Typically this was less than 0.1% of CD4 cells, also shown in figure 3.5. The amount of class II tetramer staining after virus infection and peptide immunisation is broadly

comparable, (compare figures 3.3 and 3.5). Thus, the peptide immunisation system could be used to track antigen-specific CD4 T cells *ex vivo*.

To confirm that the small number of responding cells were antigen-specific, splenocytes from immunised mice were labelled with the intracellular dye CFSE and activated *in vitro* with peptide. CFSE is divided equally between daughter cells and thus the fluorescence is reduced by half with each division (269). Figure 3.6 shows the proliferation of tetramer positive cells after culture with H19env peptide, demonstrating that the small population identified *ex vivo* was real and that the tetramer positive cells were functional. Splenocytes from mice primed with OVA-CFA and cultured with H19env peptide did not contain tetramer positive cells. This demonstrates that naïve H19env-specific cells are not present at a high enough frequency to be detected after activation *in vitro*.

3.2.2. Tracking T cell activation and memory cell survival

A time course of the responding T cells was carried out to determine the peak of the response to CFA and to follow the memory population over time. As in the previous section, mice were immunised with the peptide, H19env, in the potent adjuvant, CFA, which forms an antigen depot at the injection site (280). Unless stated, mice were immunised subcutaneously (s.c.) in the hind leg that primarily drains to the inguinal LN. The spleens and DLN were taken at various time points after immunisation and stained with class II tetramers.

The primary response peaks at between days 8 to 10 post-immunisation at 0.25-0.5% tetramer positive cells, (all percentages of tetramer positive cells are given as a percentage of CD4 cells), (figure 3.7a,b). This is followed by a contraction phase that leaves, in the spleen, about 0.15% tetramer positive cells corresponding to approximately $1x10^4$ cells, (figure 3.8). Tetramer positive cells were visible *ex vivo*

in the DLN at the longest time-point examined, day 229 after immunisation with peptide-CFA, figure 3.8a. However, tetramer staining in the spleen was at background levels at this time-point, figure 3.8b.

3.2.3. Phenotypic analysis of activated and memory tetramer positive cells

The phenotype of the tetramer positive cells was examined at various time-points after immunisation to track activation and memory cell generation. Figure 3.9a shows representative staining of activated (day 7 and day 9) and memory cells (day 176) from the DLN of peptide-CFA immunised mice. In terms of CD44 and CD62L expression the tetramer positive cells at day 7, before the peak of the response, contain a mixed population of cells, perhaps indicating that that the H19env naïve cells were not activated synchronously. By day 9, the peak of the response, all the tetramer positive cells were $CD44^{hi}$ and $CD62L^{lo}$ and the tetramer positive cells maintained this expression at all time-points examined; in memory cells, the low expression of CD62L is indicative of T_{em} cells.

The expression of IL-7Rα was also examined as IL-7 has been shown to have a role both in the generation and long-term survival of CD4 memory cells (117), (116), (82), (228), (114). The activated DLN cells at day 7 and day 9 (figure 3.9a,b) down-regulated the expression of IL-7Rα, although even at day 9 a significant proportion of tetramer positive cells expressed the receptor.

Virtually all the tetramer positive memory cells were IL-7Rα positive, however, as shown in figure 3.9b, it took several weeks for the majority of tetramer positive cells in the DLN to express normal levels of IL-7Rα. At day 14 most of the DLN tetramer positive cells were still $IL\text{-}7R\alpha^{lo}$, while the splenic tetramer positive cells at this time were mainly IL-7Rα positive. Even at day 32, only about 60% of the DLN tetramer positive cells were also IL-7Rα positive, compared to 80% in the spleen. This

distinction may indicate that the cells in the DLN were still exposed to antigen at this time, more than three weeks after immunisation.

3.2.4. Antigen specific T cell response to exogenous peptide-pulsed DC transfer

CFA immunisation forms a depot of antigen that persists for a long period of time (residual CFA was still visible at the last time-point, day 229). To examine the T cell response initiated with an adjuvant that does not persist, mice were immunised with peptide-pulsed, LPS activated bone marrow derived dendritic cells (peptide-DC).

DC were grown as described in materials and methods and provided a homogenous and easily reproducible cell population for immunisation, (see material and methods for phenotypic analysis of the DC). The DC were either transferred into mice intravenously (i.v.) into the lateral tail vein or s.c. into the hind leg.

First, mice were immunised with different numbers of peptide-DC to ascertain the optimal number of DC to immunise with, figure 3.10a. Antigen specific cells could be seen and, as expected, the number of responding cells corresponded to the increase in the number of DC injected. The background staining was set by immunisation with the same DC pulsed with the irrelevant peptide, OVA. Optimal T cell activation was found after the transfer of $1x10^6$ DC and thus this dose was used in subsequent experiments.

A time course of the response was carried out using $1x10^6$ DC per mouse, injected i.v., figure 3.10b. Tetramer positive cells could not be detected after day 20 post-immunisation. Therefore, antigen-specific memory cells could not be tracked *ex vivo* after this time.

3.2.5. The recall response is faster and larger than the primary response

The restimulation of tetramer positive cells *in vitro* enables the identification of antigen-specific cells more readily and provides a way in which the recall response can be examined. However, this does not reproduce the complex microenvironments and interactions that take place *in vivo*. Therefore, in order to study the recall response *in vivo*, mice that had been immunised with peptide-DC were boosted with peptide in CFA.

This response was faster and larger compared to the primary response, as shown in Figure 3.11a. The peak of the primary response to peptide-CFA at day 8 was between 0.25-0.5% of CD4 cells; the memory response at day 5 was usually above 0.7% of CD4 cells. Again, the background staining was set by staining cells from mice immunised with OVA peptide-pulsed DC and boosted with OVA-CFA.

This recall assay can be used to denote the survival and functional capacity of tetramer positive cells. The rapid recall response can be measured up to 160 days post-primary immunisation demonstrating that antigen-specific memory cells had survived for this length of time. However, the size of the recall response was reduced at day 168 compared to day 90, figure 3.11b. Moreover, at day 266, a recall response was only measurable in 2 out of the 6 mice tested, and the response in these mice was only found in the DLN and was much lower than at earlier time-points. These data suggest that the population of tetramer positive memory cells declined over-time.

3.2.6. T cells in the recall response bind class II tetramer at higher intensity than cells in the primary response

Over the course of an immune response B cells are known to increase their affinity for antigen by somatic hypermutation followed by selection of high affinity cells (206), (127), (128). Less, however, is known about the maturation of the T cell response, or even whether it occurs at all (214), (213), (207). This is mainly due the technical difficulties in calculating the affinity of the TCR for peptide-MHC.

It is thought that the intensity of tetramer staining can be used to provide a "crude" measurement of TCR affinity for peptide-MHC (272), (210). Thus, T cells that bind the tetramer with high intensity are thought to have a greater affinity for the peptide-MHC complex than low staining cells. Figure 3.12a illustrates how the tetramer positive population can be divided into high and low staining cells for this analysis.

Tracking tetramer positive cells in mice immunised with peptide-CFA provides an opportunity to test whether affinity maturation occurs after a single exposure to antigen and in the presence of persistent antigen. The intensity of tetramer staining of cells from mice immunised 176 days previously was compared with that of cells from mice immunised 9 days prior to analysis. As shown in figure 3.12b, no difference in the intensity of tetramer staining was found, demonstrating that affinity maturation had not occurred in mice that had received only one immunisation with H19env-CFA.

The same comparison was made with tetramer positive cells from mice immunised with DC then boosted with peptide-CFA. Here (figure 3.12), it can be seen that a greater percentage of the antigen-specific cells from the boosted mice bind the tetramer at high intensity compared to cells from mice killed at day 9 post primary CFA immunisation. This suggests that the observed "affinity maturation" occurs during the recall immune response by a process of clonal selection, with higher affinity cells activated in preference to lower affinity cells.

3.2.7. The transfer of memory cells to naïve hosts can allow a closer examination of the recall response

The memory response is more rapid than the primary response. This is thought to be for two main reasons: 1. because of the increased precursor frequency of antigen-specific cells and 2. a result of qualitative differences between memory and naïve cells that allow the former to be activated more readily (1). To investigate these differences, and to ask whether the recall response is only composed of memory cells or whether naïve cells are also activated, a transfer model was established.

Wild-type Ly5.1 congenic mice were primed with either peptide-pulsed DC or with H19env-CFA. The mice were rested for 2-4 weeks before their spleen and/ or LN were taken. A CD4 enrichment by magnetic cell separation (MACS) of the spleen and LN cells was carried out to increase the frequency of tetramer positive cells in the transferred cell population. The cells were transferred into wild-type C57BL/6 (Ly5.2) mice, which were then immunised with peptide in CFA the following day. Spleens and LN were removed for tetramer staining after 5 to 7 days. This protocol is illustrated in figure 3.13a and an example of the tetramer containing CD4 cells that were transferred is shown in figure 3.13b.

In the first experiment (experiment 1) the Ly5.1 mice had been immunised with peptide-DC and left for 3 weeks. C57BL/6 mice received between $5x10^5$-$5x10^6$ Ly5.1 CD4 cells, which corresponded to between 700 and 7000 tetramer positive cells. After immunisation of the recipient mice, no tetramer positive-Ly5.1 positive cells were detected.

However, by measuring the tetramer positive host response after the transfer of different numbers of CD4 cells, an effect was apparent. In mice that received the greatest number of Ly5.1 cells, the host tetramer positive response was reduced compared to that in mice receiving less or no cells, figure 3.14. Thus, it appeared that

the transferred cells reduced the host response, however, this effect was only apparent in spleen, and not in the DLN.

The inability to detect Ly5.1 positive, tetramer positive cells could have been because so few specific cells had been transferred. Therefore, in a second experiment, Ly5.1 mice were first challenged with H19env-CFA and cells taken at an earlier time point, week 2, to ensure that a higher frequency of antigen-specific cells were transferred into recipient mice.

The recipient C57BL/6 mice received almost 20 000 tetramer specific cells and were immunised with peptide-CFA then sacrificed on day 6. As in the first experiment, no tetramer positive Ly5.1 cells were detected. Disappointingly, no effect on the host response was observed in this experiment.

In a final attempt to find the antigen-specific transferred cells, a further transfer was carried out (experiment 3). There was a possibility that cells from mice immunised 14 days previously may be effector cells and more prone to apoptosis than memory cells (281). Therefore, this time the cells were purified from Ly5.1 mice that had been immunised 4 weeks previously with peptide-CFA. The recipient C57BL/6 mice received 5000 tetramer positive cells. In this experiment tetramer staining was carried out on day 7 after immunisation as it was thought that this may allow time for the smaller number of antigen-specific cells to reach a detectable level.

The results of this last experiment are detailed in figure 3.15. Tetramer binding cells could not be detected in mice that received tetramer positive Ly5.1 cells and were immunised with PBS-CFA. In recipient mice that were immunised with H19env-CFA, Ly5.1 positive, tetramer positive cells were detected in both the DLN and spleen. However, a similar percentage of tetramer positive-Ly5.1 negative (host) cells were detected in these mice. Thus, the host response was equivalent to the recall response by the Ly5.1 tetramer positive cells. Although there was a trend towards an increase in the total tetramer positive response in the draining lymph node of mice

that received Ly5.1 tetramer positive cells compared to mice that received naïve Ly5.1 cells, there was no significant difference here and the response in the spleen was equivalent. Therefore, this result did not display the major characteristics of the recall response detailed earlier in this chapter.

3.3. Discussion

The experiments described in this chapter demonstrate that class II tetramers can be used to follow the generation and subsequent survival of endogenous CD4 memory cells. Tracking these cells *ex vivo* provides an opportunity to test whether antigen-specific memory cells are maintained as a stable population for the life-time of the host, or whether numbers decrease with time. Moreover, the functional response of the memory cells can be examined using the *in vivo* recall assay.

3.3.1. The generation of memory cells

Tetramer positive cells could be enumerated after either immunisation with peptide-pulsed DC or with peptide-CFA. However, there were several differences in the kinetics of the responses to the two immunisation protocols. First, the peak of the response induced by peptide-pulsed DC was earlier than that resulting from immunisation with peptide-CFA. This is probably because immunisation with peptide-CFA means that APC must acquire antigen and migrate to lymphoid organs before T cells can be activated. DC immunisation obviously bypasses the first two stages of this process and thereby hastens the T cell response (170), (282).

The second main difference was that tetramer positive cells in mice that had been immunised with peptide-DC could not be detected after day 20. There could be two explanations for this: 1. the peak of the response after peptide-CFA immunisation was greater than after peptide-DC immunisation; 2. the contraction phase was greater after peptide-DC immunisation. From examining the kinetics of the T cell responses (Figure 3.7 and 3.9), it is apparent that peptide-CFA immunisation induces a greater expansion than that induced by peptide-DC immunisation. Thus, a greater primary expansion results in a larger memory pool as has been previously described (125), (131), (132). In peptide-DC immunised mice, the T cell expansion was not large

enough to generate a population of memory cells above the background level of staining.

3.3.2. The tetramer positive cells have a T_{em} cell phenotype

During T cell activation, the expression of various surface markers alters. The tetramer positive cells upregulated CD44 and downregulated CD62L during activation, such that by day 9 they were all $CD44^{hi}$ and $CD62L^{lo}$, a classic response to activation. The level of expression of IL-7Rα has been shown to reduce as a result TCR and cytokine signals (120), (119), (118). During activation, the majority of tetramer positive cells did downregulate receptor expression, however, even at the peak of the response, about 20% of tetramer positive were IL-7Rα positive. This correlates to findings by several other groups that the activated cells that continue to express IL-7Rα are the cells that go on to become memory cells (109), (114), (112). However, in the DLN, IL-$7R\alpha^{lo}$ tetramer positive cells were still visible at day 32, although the majority of tetramer positive cells in the spleen were IL-7Rα positive by day 14.

This may indicate that the cells in the DLN were still exposed to antigen at this time-point, inducing some of them to downregulate expression of the receptor. Alternatively, there may be two populations of memory cells that survive the initial contraction phase (day 10-15): one population that expresses IL-7Rα and survives in the "longer lived" memory pool; and a shorter lived population of IL-$7R\alpha^{lo}$ tetramer positive cells. Certainly all the memory cells at later time-points were IL-7Rα positive.

In a study of IL-7Rα expression on LCMV specific CD8 cells, Kaech *et al* found a similar gradual increase in antigen-specific cells that were IL-7Rα positive (114). The authors report that only by day 40-50 were all the antigen-specific cells IL-7Rα positive This corresponds to the slow acquisition of memory characteristics describe

by this group (104) and the fact that stable levels of antigen-specific cells were not reached until around day 40 (114). The authors also suggest that the two populations may represent different memory pools or that IL-7Rα^{lo} cells may differentiate into IL-7Rα positive cells. They argue that it is not clear whether a proportion of cells do not downregulate the receptor on activation or whether some activated cells re-expresses it. However, they do suggest that cells that receive reduced levels of activation, and consequently do not divide as much as terminal differentiated cells, may remain IL-7Rα positive and generate the longer lived memory pool.

At later time-points the tetramer positive cells were all CD44^{hi}, CD62L^{lo} and IL-7Rα^{hi}. The homogeneity in marker expression of the memory cells is contrary to studies by Donna Farber and Susan Swain who have described heterogeneity within an antigen-specific memory pool based on CD62L staining (283), (284). Farber argues that this heterogeneity is a result of differential activation of the cells, with full activation inducing a T_{em} phenotype and a reduced signal inducing T_{cm} cells (285). However, in these studies, a fairly large number of transgenic cells are transferred either to wild-type, or to lymphopenic mice. The importance of the system used in this PhD lies in the physiological number of precursor cells, providing a more realistic system to study CD4 T cell memory than TCR transgenic studies. It may be that, as there are only a small number of naïve H19env-specific cells, all these cells are activated to a similar extent in the immunisation protocols used in this study. It would be interesting to prime mice with lower doses of peptide and track the phenotype of these cells and investigate whether the resultant memory cells had a more heterogeneous phenotype.

3.3.3. Antigen-specific memory cells are not long-lived

A detectable decline of the tetramer positive cells was observed such that by day 229 after peptide-CFA immunisation, tetramer positive cells were not visible in the spleen. Similarly, in mice immunised with peptide-pulsed DC, a small response was only found in 2 out of the 6 mice examined 260 days after immunisation. Thus, both in mice that had a source of persistent antigen (peptide-CFA immunised) and those without (peptide-DC immunised), life-long populations of antigen-specific memory cells were not formed. The cells did survive for a considerable time, however, and could be reactivated: demonstrating the two classical characteristics of memory.

The stability of CD4 memory and the signals required to maintain these cells has long been a source of debate (167), (216), (176), (172). Although memory cells can survive in the absence of antigen and MHC II molecules (172) they are dependent on TCR signals via MHC II to maintain their function (176). Zinkernagel and colleagues argue that merely recording the survival of memory cells is not sufficient to demonstrate the persistence of protective memory (216). Rather, only by challenging the putatively immune mice with infections can productive memory be established. From such experiments (168), (216), they claim antigen is required to maintain the ability of the memory cells to migrate to the tissues where infections occur.

The T_{em} phenotype of the tetramer positive cells described in this PhD may explain the decline in survival over-time. Wu *et al* suggest that T_{em} are not long-lived cells (155). The authors transferred CD4 TCR transgenic T_{em} and T_{cm} cells into naïve recipients and tracked the cells. They found that T_{em} cells disappeared within 8-10 days, but T_{cm} cells survived for at least three weeks. This rapid disappearance of the T_{em} cells does no correlate with the survival of tetramer positive cells for at least 229 days in this PhD.

That the CD4 memory cells decline in this study supports findings by Homann *et al* and Cauley *et al* who observed a similar phenomenon (19), (225). Tracking the

immune response to LCMV using both class I and class II tetramers, Homann *et al* found that although the antigen-specific CD8 cells formed a stable memory pool, the number of CD4 memory cells never stabilised and declined from the peak of the response onwards. LCMV infection is cleared by wild-type mice and there is no obvious source of persistent antigen in this model. However, the authors could not rule out that antigen may remain, for example trapped on follicular dendritic cells. Regardless, any survival signals were insufficient to maintain the memory CD4 cells over the lifetime of the host.

There have been reports of stable populations of memory CD4 T cells. London *et al* found memory DO11.10 cells 6 months after the transfer of a large number of *in vitro* activated transgenic cells (138). Furthermore, using limiting dilution assays to enumerate antigen specific CD4 cells, Varga *et al* described the relatively stable persistence of LCMV specific CD4 memory cells for at least a year (286). The differences found in this PhD and by Homann *et al* (19) may be due to the more physiological numbers of cells examined over a longer time period and the more direct measurement of antigen-specific cells, using class II tetramers rather than *in vitro* restimulation assays.

If antigenic signals are not sufficient, or even required, to maintain memory cells, then what signals are important? Recent studies from a number of groups have described an important role for IL-7 in both the generation and survival of memory cells. Lenz *et al* found that giving anti-IL-7Rα to mice immune to LCMV resulted in a decrease of antigen specific cells (82). Moreover, the administration of recombinant IL-7 to immune mice caused an increased rate of turnover of the memory cells *in vivo*; *in vitro* experiments showed that this was accompanied by a rise in the expression of the survival molecule, Bcl-2. The authors suggest that IL-7 plays a homologous role to IL-15 for CD8 memory cells, which maintains memory at a population level by inducing the proliferation of memory cells (181), (182), (230), (227).

Li *et al* (116) and Kondrack *et al* (117) showed TCR transgenic memory cells could not survive in IL-7 deficient hosts, leading them both to argue that IL-7 is required for the transition of effector cells to long-lived memory cells. In contrast to Lenz *et al* (82), these two studies did not find that IL-7 induced proliferation of the memory cells, suggesting that there may be differences between transgenic and endogenously generated memory cells.

This PhD did not assess the requirement for IL-7 in long-term survival; although memory cells were found to express IL-7Rα. This, and the data from Homann *et al,* suggests that even if IL-7 does induce the periodic proliferation of CD4 memory cells, this is insufficient to maintain them for the life-time of the host (19).

This is in contrast to CD8 memory cell studies were the long-term survival of memory cells has been described by a number of groups (173), (287), (288), (19). This is perhaps best exemplified by Homann *et al* who, as stated above, describe the decline in antigen specific CD4 memory cells in mice that have a stable CD8 memory pool (19).

This prompts the question: why should memory CD4 cells be less well maintained than CD8 memory T cells? It may just be a result of the difference in the clonal expansion of responding CD4 and CD8 cells. The CD8 T cell response is generally larger than the CD4 T cell response (248), (19). This is particularly evident in LCMV infection where $1x10^7$ CD8 T cells can respond to the infection (289). Thus, a greater memory pool (5% of the activated cells, about $5x10^5$ cells) is formed that may be large enough to endure the loss of cells over-time. Moreover, there has been considerable evidence demonstrating that IL-15 can prevent this slow decline in CD8 memory cells by inducing their proliferation (181), (182), (230), (227). Whether IL-7 acts in a similar was for CD4 T cells remains open to question.

Teleologically, it is interesting to question the importance of immunological memory. In the case of B cells, the advantage of maintaining long-lived plasma cells

is obvious: antibodies can bind and inactivate viruses and bacteria before they have change of establishing an infection. Similarly, memory CD8 T cells can rapidly kill infected cells and thereby prevent the spread of viruses and intracellular bacteria. Indeed, Homann *et al* found that despite the reduction in LCMV specific CD4 memory cells, immune mice were still protected from re-challenge (19). What role CD4 cells can play in protecting the host from subsequent infection will depend on the type of infection. For example, rapid responses from CD4 T cell may be critical for protection from intracellular bacteria, such as tuberculosis.

3.3.4. Recall responses differ from primary response in terms of speed, size and binding to the class II tetramer

The rapid recall response is one of the fundamental tests of cellular memory. Moreover, it provides an immediate response to re-infection: T_{em} cells patrolling the tissues are strategically placed to make cytokines upon re-infection and T_{cm} can promptly proliferate in secondary lymphoid organs, and thereby back-up the T_{em} response. In this chapter, the recall response has been studied for two main reasons:
1. to demonstrate the differences between the primary and secondary response; and
2. to establish the functional survival of CD4 memory cells after immunisation with peptide-pulsed DC.

Although tetramer positive cells could not be detected *ex vivo* in the peptide-DC immunised mice after 20 days, memory cells were present. This was confirmed by examining the recall response by challenging primed mice with peptide-CFA. Rapid recall responses were evident at least up to 160 days post-immunisation, however, after this time, the recall response was less evident with only 2/6 mice responding at day 266 after priming.

Many *in vivo* experiments tracking the CD8 T cell recall response have been published (290), (153), (291), (288), (292). However, *in vivo* CD4 recall studies are much more scarce. London *et al* demonstrated the rapid kinetics of memory

DO11.10 cells responding in wild-type hosts (9) and Dawicki *et al* have shown that OX40-OX40L interactions are important in CD4 secondary responses (291). However, most CD4 recall studies have been carried out *in vitro* where the conditions of reactivation can be more strictly defined (9), (161), (278). This allows questions to be asked about differences in costimulatory requirements and the type of APC that can activate memory cells in comparison to naïve cells. Although such experiments can increase understanding of memory, *in vivo* recall assays are vital to fully appreciate the kinetics and requirements of CD4 memory.

The recall response was clearly faster and larger than the primary, confirming the classical definition of the memory response. There have been *in vivo* reports, however, that for CD4 memory T cells the magnitude of the secondary response does not exceed that of the primary response. Merica *et al* found that an unidentified substance in the host prevented maximal T cell proliferation of memory DO11.10 cells, although the memory cells did respond faster than naïve cells in terms of both entry into the cell cycle and cytokine production (293). The full proliferative capacity of the memory cells could be restored upon transfer to a naïve recipient. However, this also induced the reversion of the cells to a naïve phenotype based on CD45Rb expression.

In a study of the response to the central nervous system autoantigen, proteolipid protein (PLP), Bischof *et al* found the size of the secondary response to be equivalent to the primary response (257), although, the secondary response was faster than the primary. In both these studies, the precursor frequency was higher than normal, either artificially via the adoptive transfer of a large number of transgenic cells, or because the SJL mice used by Bischof *et al* contain a large number of PLP specific T cells (294). This large precursor frequency may affect the kinetics and magnitude of the primary response and any differences could be carried into the memory pool.

A further difference between the primary and the secondary response in this PhD was the intensity of tetramer staining. Tetramer positive cells activated in the recall

response bound the tetramer at higher intensity than tetramer positive cells either in the primary response or memory cells. The finding that this increase in intensity of staining, which correlates to an increase in TCR affinity for peptide-MHC (272), (210), was only found in the secondary response, supports similar findings in the literature (211), (208). Busch and Pamer argue that this is due to the successful competition of high affinity cells for peptide-MHC on APC (208). These cells proliferate and become the majority population in the recall response. Kedl *et al* suggest that, at least for CD8 T cells, this competition between T cell clones for the same antigen can be partly be explained by high affinity cells inducing the loss of antigen from APC (212).

The increase in the intensity of tetramer staining has also been shown to be due to the localisation of TCR in rafts rather than an actual increase in the affinity of the interaction between TCR and peptide-MHC (207). Regardless, the change in intensity of staining indicates a change in the cells involved in the recall response compared to those in the primary response, a change that may partly explain the increased speed of the recall response.

3.3.5. Transfer of antigen-specific memory cells

These properties of the memory response predict that the recall response is composed of reactivated memory cells, which, due to this increase in affinity, would prevent the activation of naïve cells. From the literature it is known that a large number of antigen-specific cells can block the host response (295), (289). For example, elegant studies by Kedl *et al* describe that the transfer of high affinity specific OT-I cells into naïve hosts reduces the host response to OVA (295). To test this prediction, the transfer experiments depicted in figure 3.13 were carried out.

It was thought that by transferring memory H19env-specific T cells into naïve mice, the reactivated memory cells could be tracked and that these cells would influence the magnitude of the host response. However, the experiments involving the transfer of Ly5.1 tetramer positive cells proved to be inconsistent.

The main deficiency in this set of experiments was the small number of antigen-specific cells transferred into the C57BL/6 hosts especially as only approximately 10% of transferred cells arrive in the spleen (289). Ly5.1 tetramer positive cells were only visible after boosting with H19env-CFA in the third experiment, even though only 5000 tetramer positive cells were transferred in this experiment compared to 20 000 in experiment two. There could be two possible explanations for this: 1. the cells transferred were memory cells rather than activated cells that may be more prone to apoptosis (281); 2. in the final experiment the response was examined 7 days after immunisation, allowing the small number of cells to proliferate above the detection limit of the class II tetramers. At this time-point, the primary response is usually visible, so it may not be surprising that the host response was equivalent to the "recall response" by the Ly5.1 positive cells.

Although the Ly5.1 tetramer positive cells were visible, this response did not resemble the recall response in boosted C57BL/6 in terms of size and speed. It is not possible, therefore, to state whether, in general, recall responses are composed

entirely of memory cells or if naïve cells also participate. As reducing the numbers of antigen-specific cells removes the recall phenotype, this result does suggest that the increase in precursor frequency after an immune response is vital to the rapidity of the recall response. Given more time, a complete kinetic analysis of the Ly5.1 transfer system could have been carried out. Once an optimal protocol was established, other experiments could be done. For instance, it would be interesting to examine whether the "secondary" memory pool is composed of the reactivated memory cells or the newly activated naïve cells, or a mixture of the two.

3.3.6. Conclusion

CD4 memory cells have remained elusive: the small population has proved difficult to enumerate without using TCR transgenic cells or carrying out experiments in lymphopenic mice. Tracking endogenously generated CD4 memory cells in intact hosts is vital to advance these studies; to envision what actually happens to CD4 memory T cells.

The results described in this chapter demonstrate that this is possible. The activation and survival of antigen-specific cells can be examined *ex vivo*. These tools and protocols can be used to ask questions about the generation and survival of memory cells. In the following chapter the ability of both polyclonal and transgenic memory cells to survive in competitive memory pools is investigated. In the final results chapter, the individual molecules involved in the various stages of the T cell response: activation; memory cell generation; survival; and recall responses are dissected using the class II tetramers.

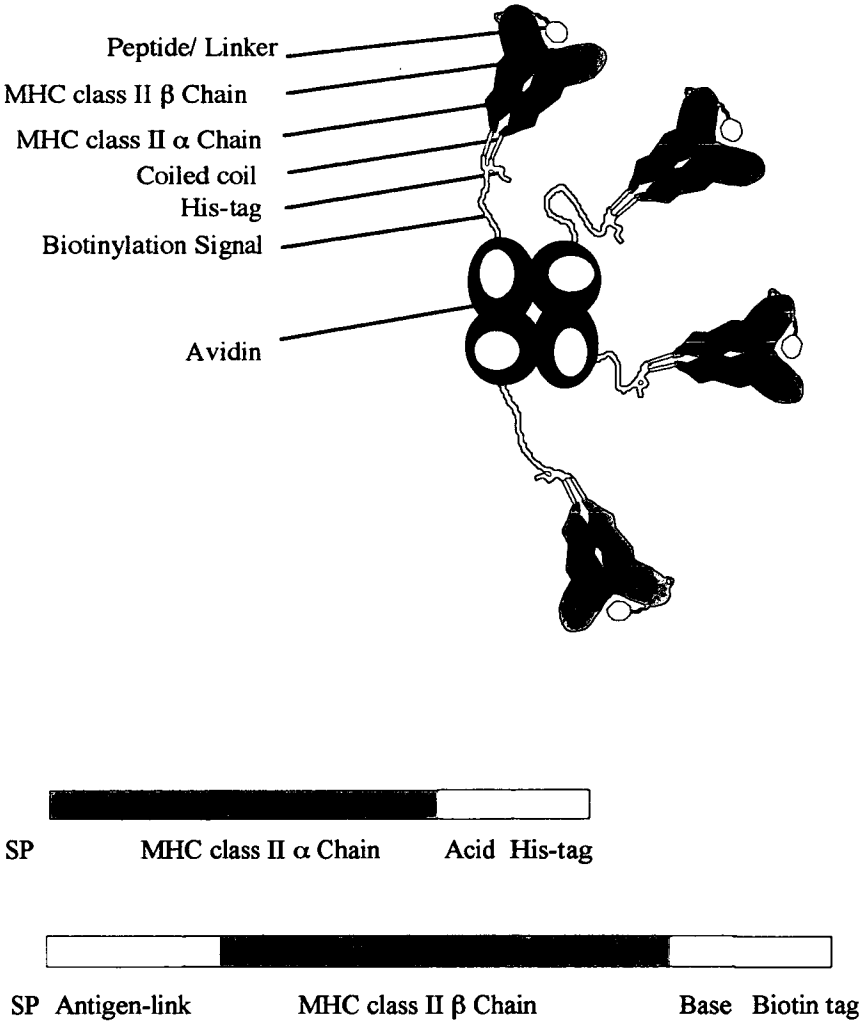


Figure 3.1 Schematic drawing of MHC class II tetramers, and constructs used to generate them: The constructs which form the α and β molecules contain a number of modifications to improve the generation of the class II tetramers, see text and Figure 3.2 for details.

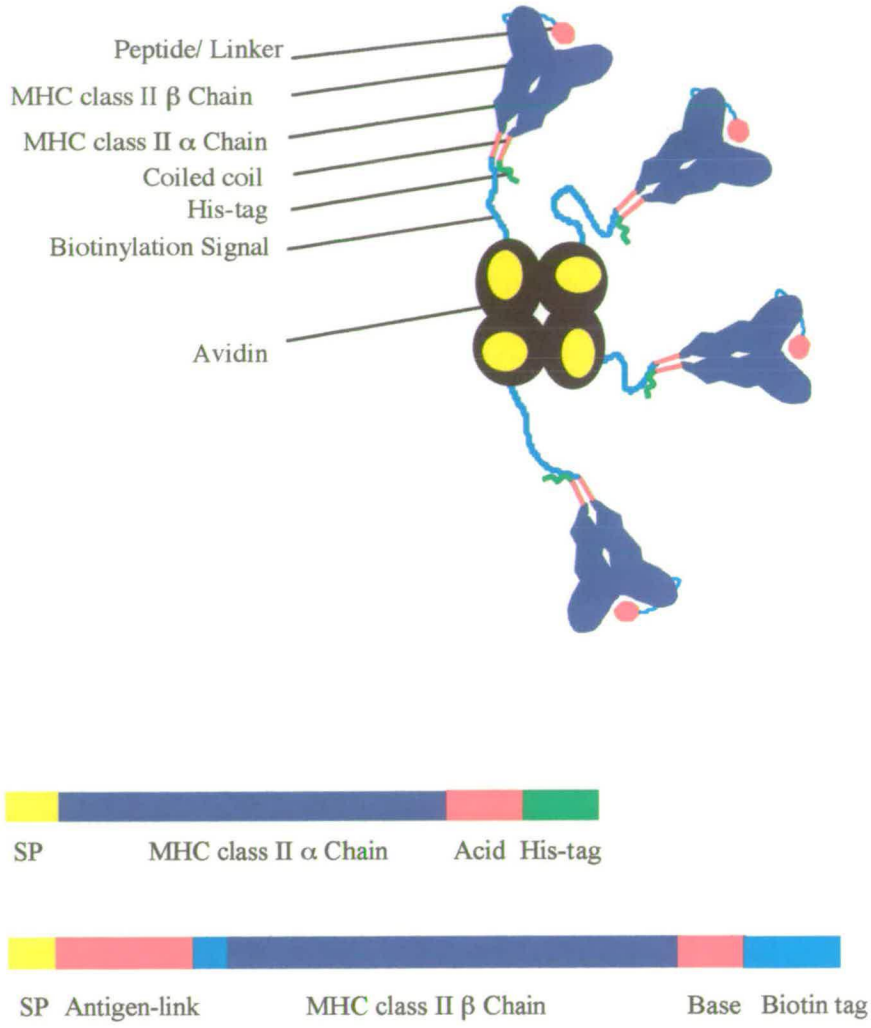


Figure 3.1 Schematic drawing of MHC class II tetramers, and constructs used to generate them: The constructs which form the α and β molecules contain a number of modifications to improve the generation of the class II tetramers, see text and Figure 3.2 for details.

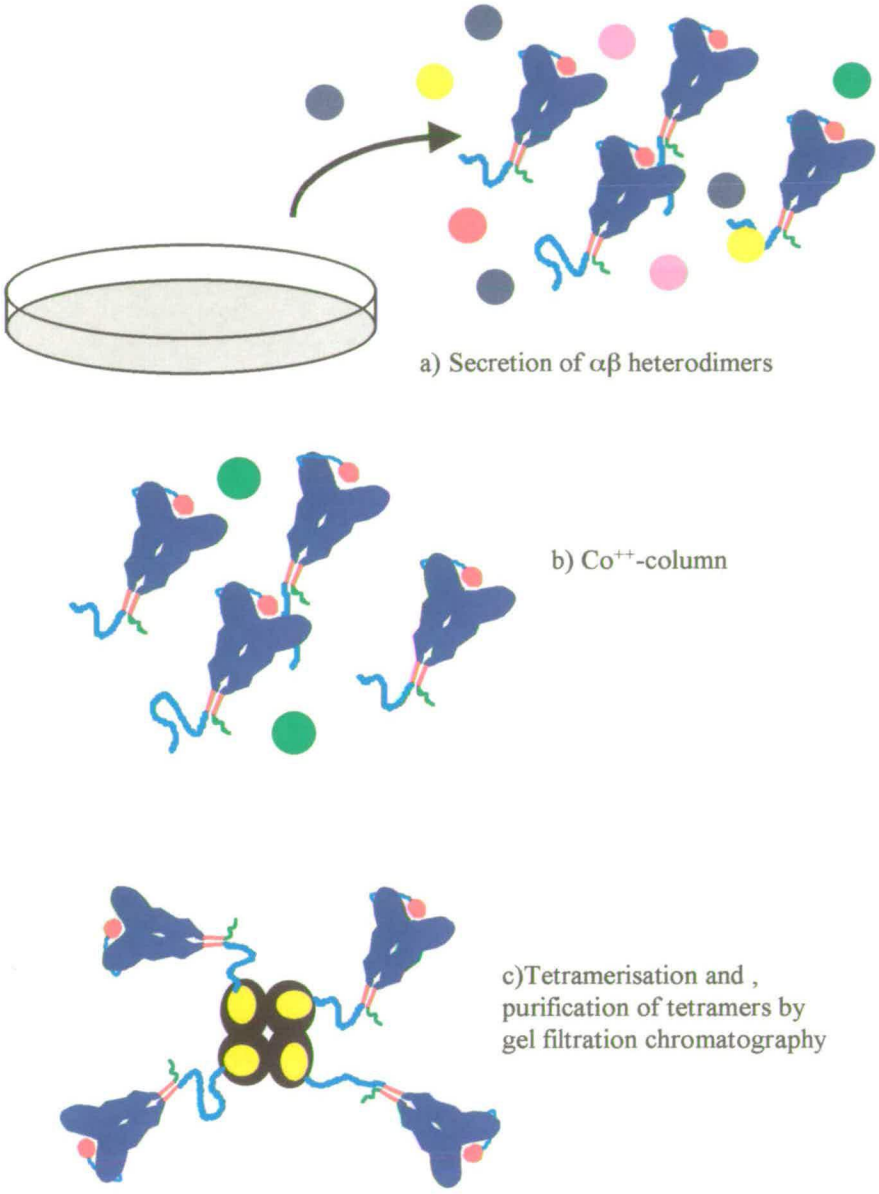


Figure 3.2 Schematic drawing of the generation of MHC class II tetramers: The α and β chains are produced in insect cells which secrete them as monomers containing the peptide, H19env. The His tag on the α chain enables purification by affinity based chromatography and the biotin tag allows for tetramerisation with PE labelled streptavidin. Finally, the tetramers are purified from any remaining monomers by gel filtration chromatography.

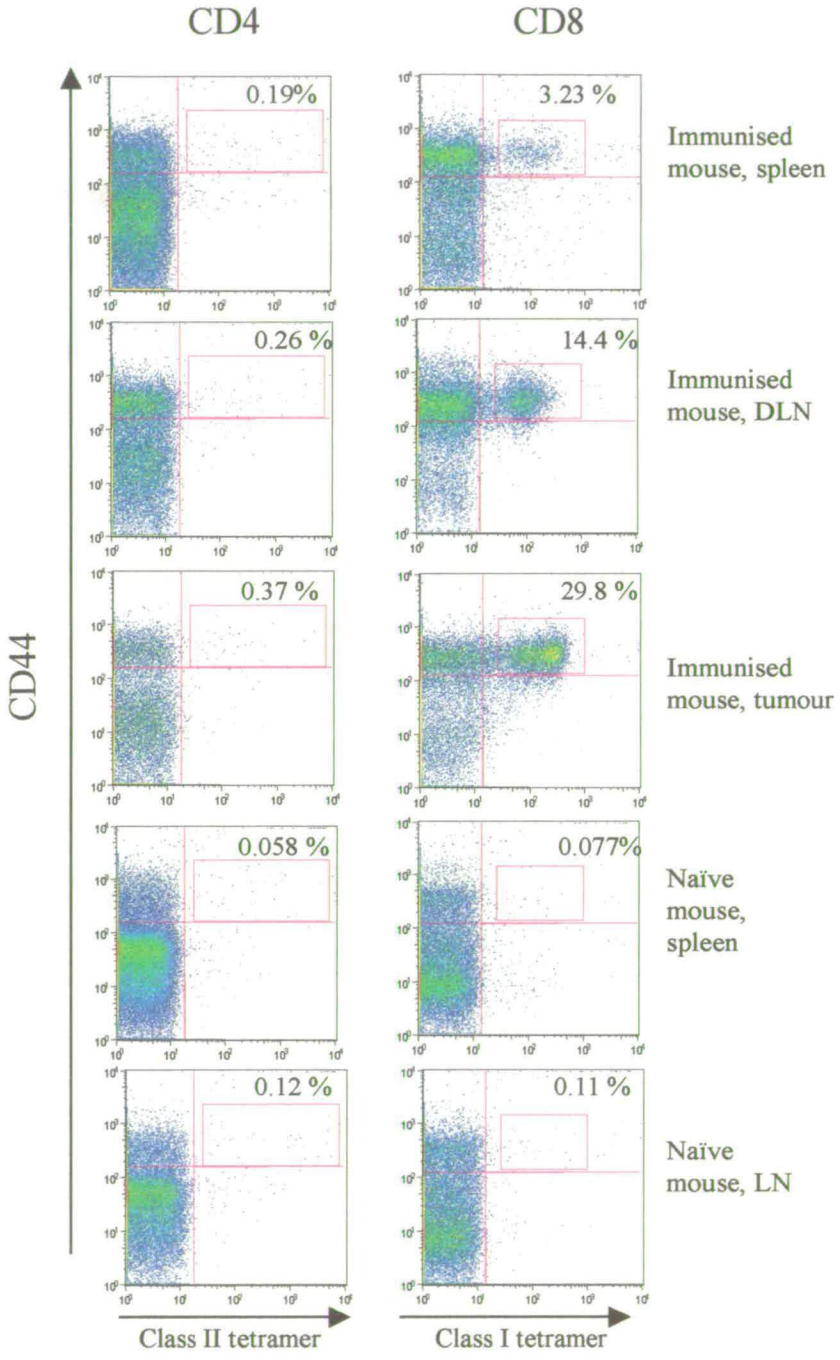


Figure 3.3: Example tetramer staining from mice infected with MMLV: C57BL/6 mice were infected with MMLV. 8 days later, spleens, DLN and the resulting tumour site were taken and stained with class I, class II tetramers and anti-CD44, anti-CD4 or CD8, F4/80 and PI. Cells are gated as in Fig. 3.4. The number shows the percentage of cells in the box.

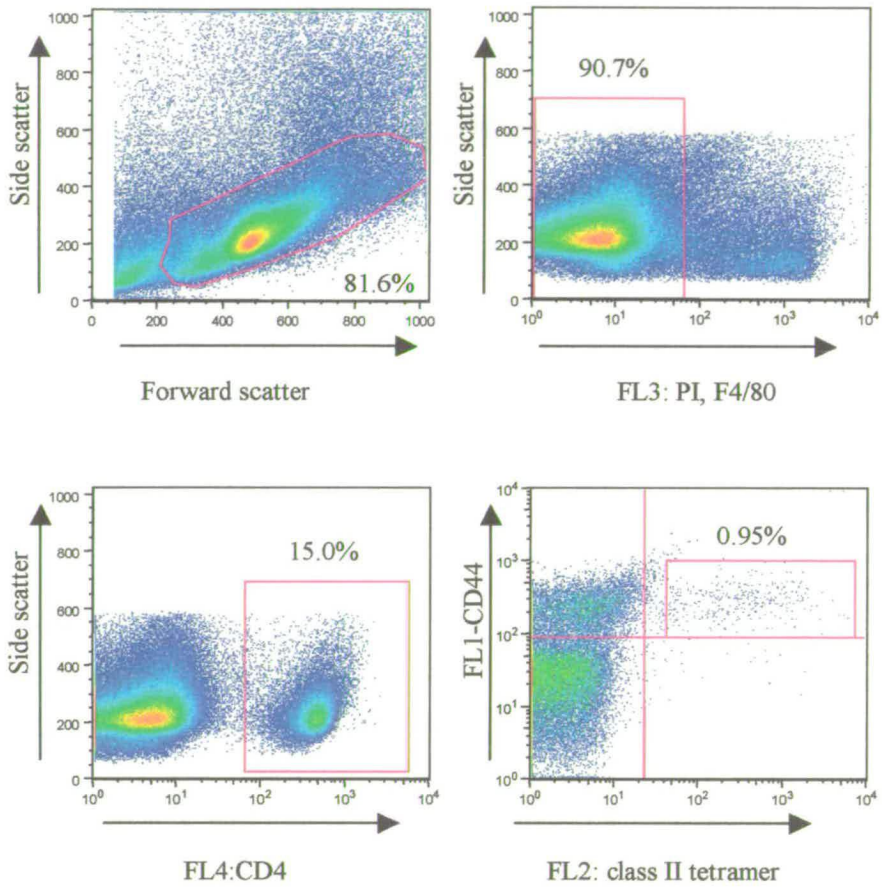


Figure 3.4 Gating analysis for tetramer positive cells: To analyse tetramer positive cells, first a broad lymphocyte gate was drawn. FL3 positive cells (macrophages and dead cells) were gated out. Of the remaining cells, only CD4 positive cells were analysed for tetramer staining in conjuncture with activation marker expression. The number shows the percentage of cells in the gates.

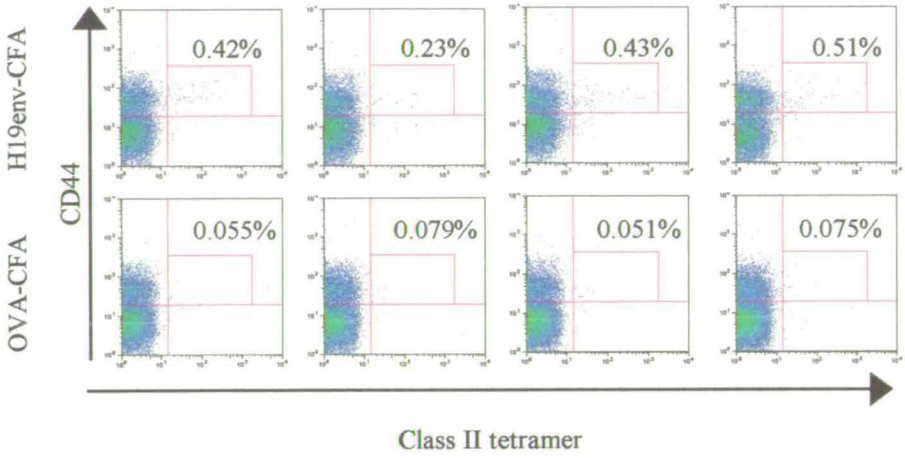


Figure 3.5 Class II tetramers can be used to enumerate antigen specific cells: C57BL/6 mice were immunised with H19env peptide (top row) or OVA peptide (bottom row) emulsified in CFA. DLN were taken after 9 days and stained with class II tetramers and labelled antibodies as described in materials and methods. Cells are gated on CD4 +ve, lymphocytes, excluding PI +ve and F4/80 +ve cells. Numbers show the percentage of tetramer positive cells out of CD4 cells in the rectangle gate. Each plot is from an individual mouse.

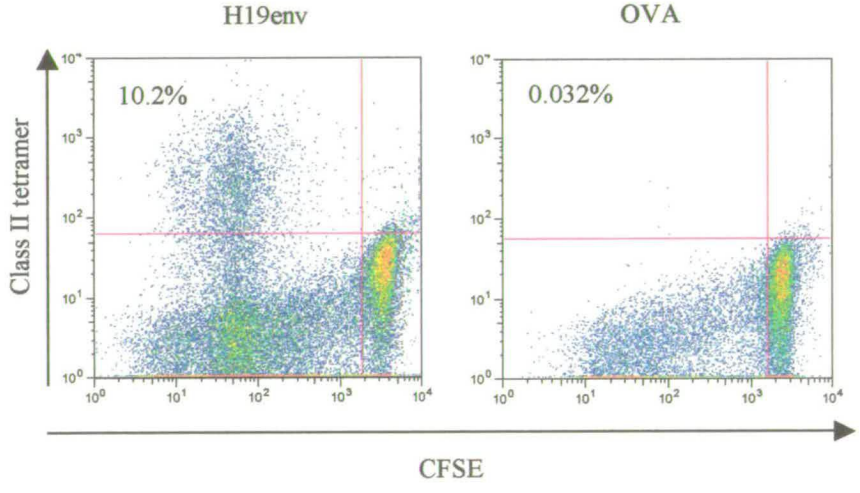
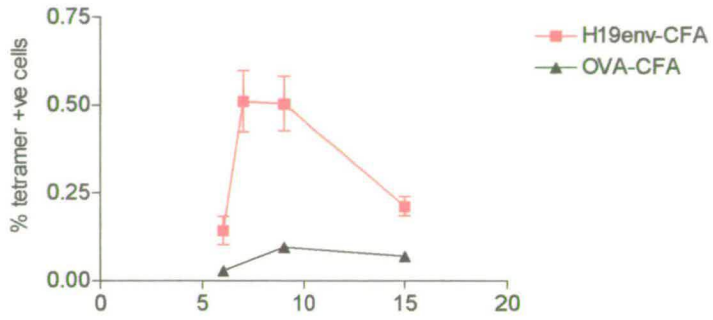


Figure 3.6 Division of tetramer positive cells *in vitro*: CFSE labelled splenocytes from C57BL/6 mice immunised with H19env-CFA or OVA-CFA, were activated *in vitro* with H19env peptide for three days. The cells were then stained with class II tetramers as described in materials and methods. Cells were gated as in Fig. 3.4. The numbers show the percentage of cells in the top left quadrant, tetramer +ve, CFSE low.

a) DLN



b) spleen

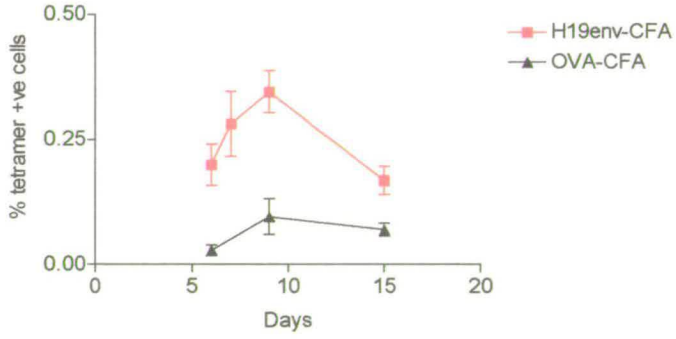
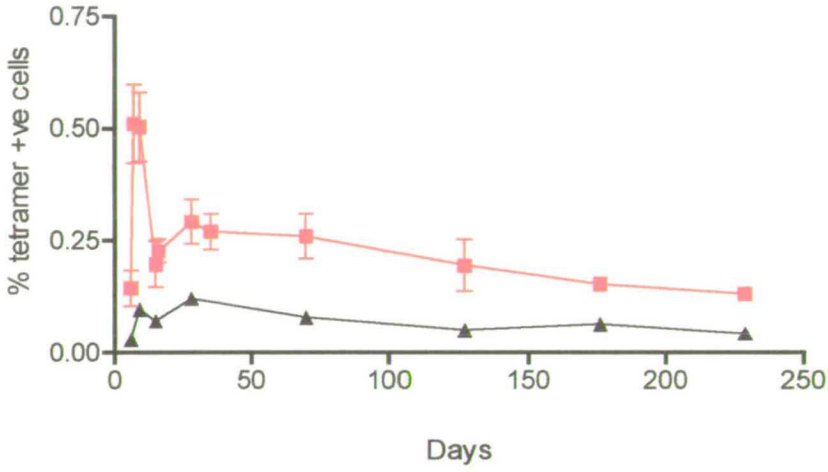


Figure 3.7 Activation and contraction phase of the tetramer positive immune response: C57BL/6 mice were immunised with H19env peptide emulsified in CFA. Mice, three – four per group, were sacrificed at various time points post-immunisation and cells from the draining lymph node were stained with class II tetramers(■.) Control mice were immunised with OVA-CFA (▲). Graph a) DLN, b) spleen. Percentage of tetramer positive cells out of total CD4 cells is shown and the error bars are the SEM.

a) DLN



b) Spleen

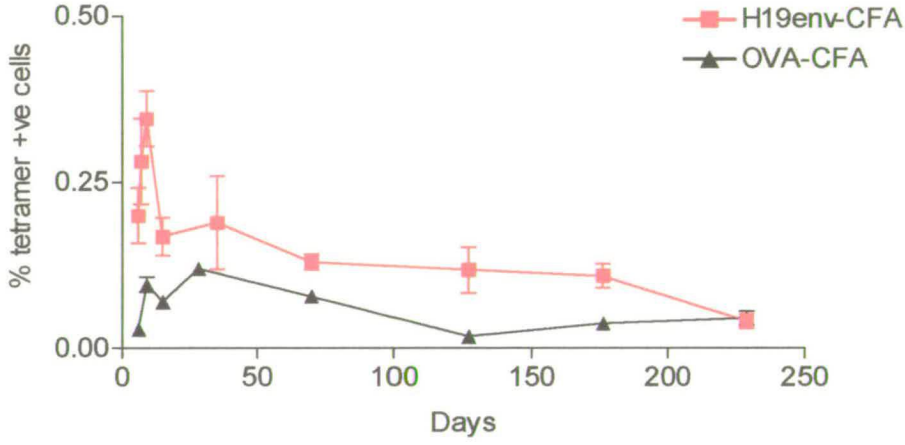


Figure 3.8 Long term survival of tetramer positive cells: C57BL/6 mice were immunised with H19env peptide emulsified in CFA. Mice, three – four per group, were sacrificed at various time points post-immunisation and cells from the draining lymph node were stained with class II tetramers(■) Control mice were immunised with OVA-CFA (▲). Graph a) DLN, b) spleen. Percentage of tetramer positive cells out of total CD4 cells is shown and the error bars are the SEM.

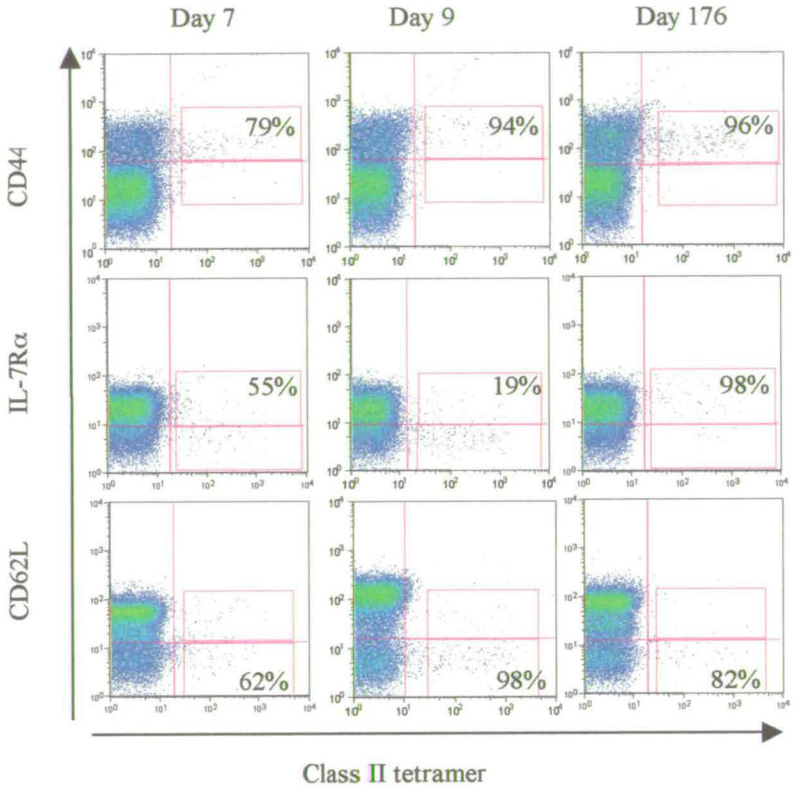


Figure 3.9a Memory cells have a homogeneous phenotype compared to activated cells: C57BL/6 mice were primed with H19env-CFA and sacrificed at various time-points after immunisation. LN cells were stained with class II tetramers and cells gated as in Figure 3.4. The first column shows cells from day 7 (early in the primary response), second column day 9 (the peak of the primary response), and third column staining at day 176 (memory cells). First row shows CD44 staining, middle row IL-7Rα, and bottom row CD62L on the y axis, tetramer staining in all rows on the x axis. Numbers show the percentage of the total tetramer positive cells that are $CD44^{hi}$, IL-7Rα^{+}, or $CD62L^{lo}$

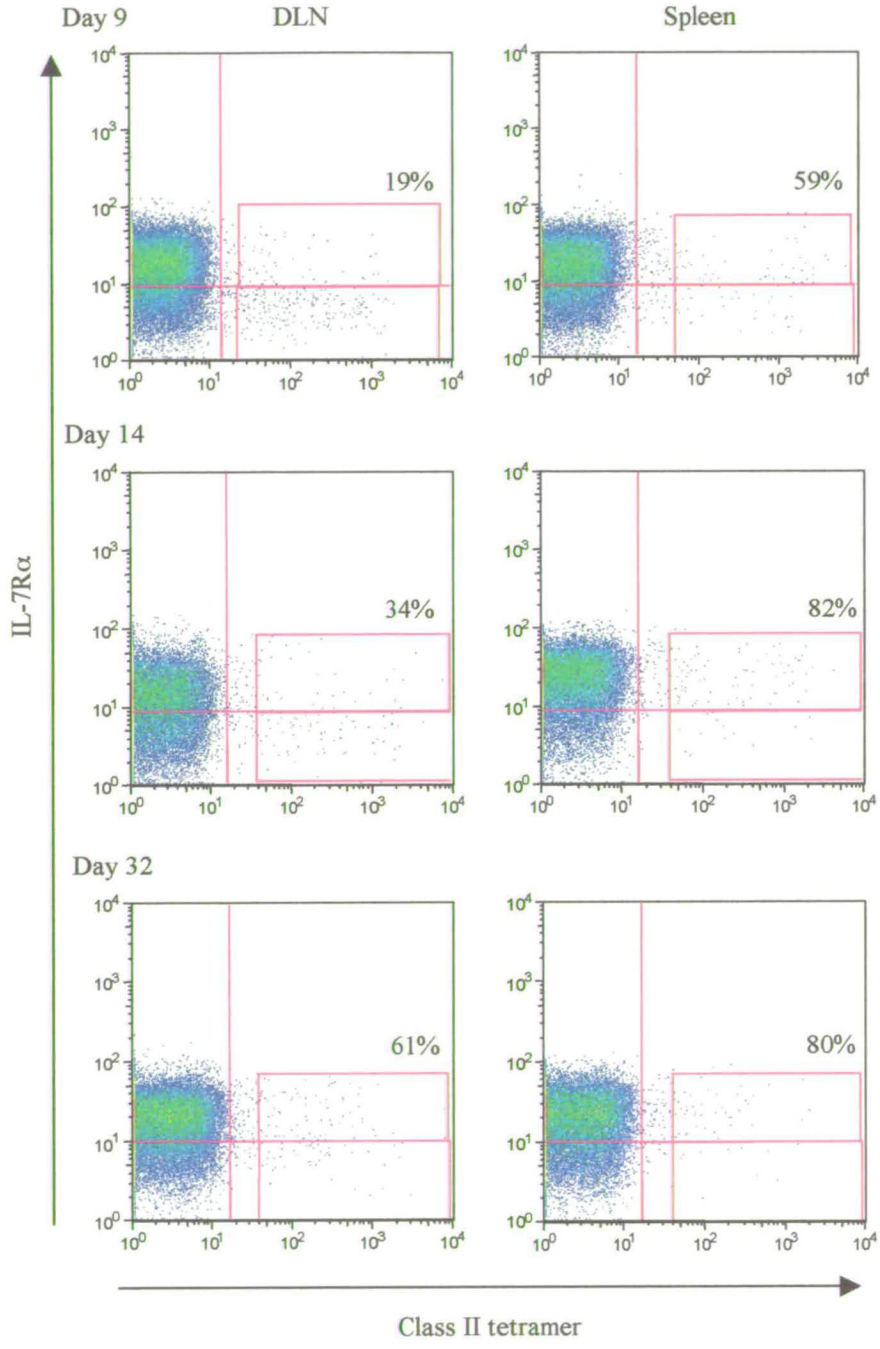


Figure 3.9b Memory cells in the DLN take several weeks to acquire full expression of IL-7Rα: the level of expression of IL-7Rα on tetramer +ve cells in the DLN and spleen was examined 9, 14 and 32 days after immunisation with peptide-CFA. Cells are gated as in figure 3.4. The number shows the percentage of IL-7Rα +ve tetramer +ve cells out of total tetramer +ve cells.

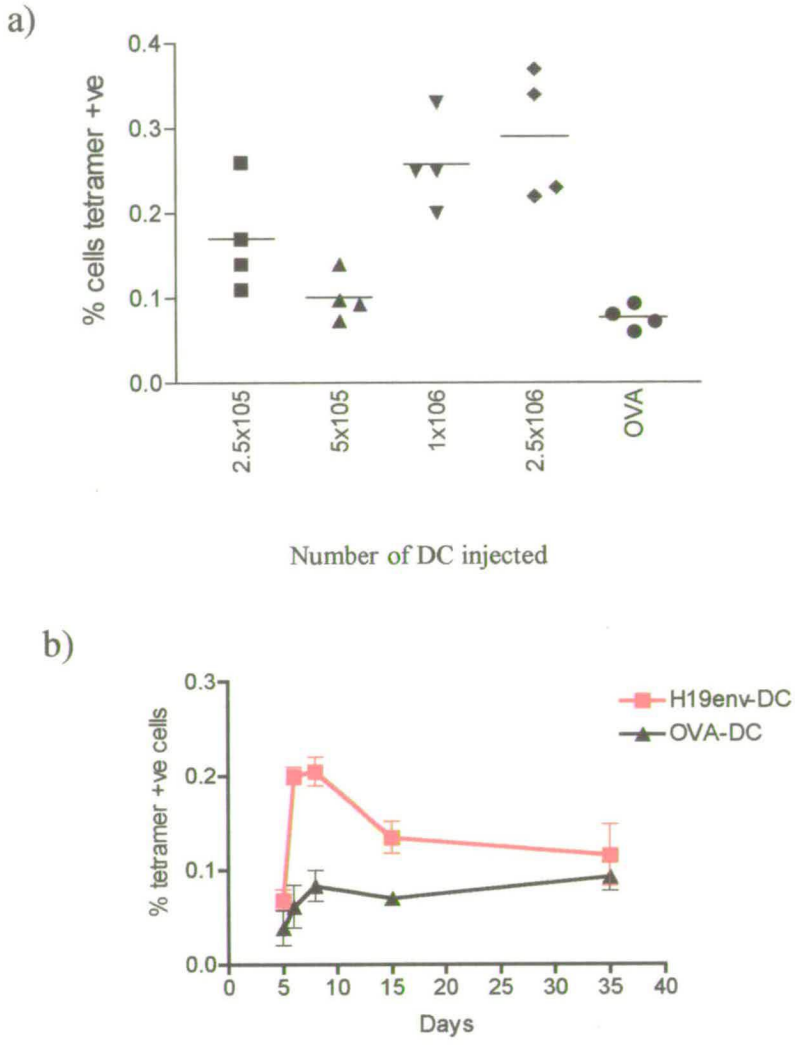


Figure 3.10 The transfer of peptide-DC primes tetramer positive T cells: Bone marrow DC were activated with LPS, pulsed with H19env or OVA peptide and injected i.v. at various doses (a) or $1x10^6$ DC per mouse (b) into C57BL/6 mice. On day 6 (a) or at various days post-transfer (b), spleens were removed and cells stained with class II tetramers and labelled antibodies. Background staining was set by staining cells from mice immunised with OVA pulsed DC. In a) each point represents one mouse and the line shows the mean of each group, in b) each point is the mean of 4 mice and the error bar shows the SEM.

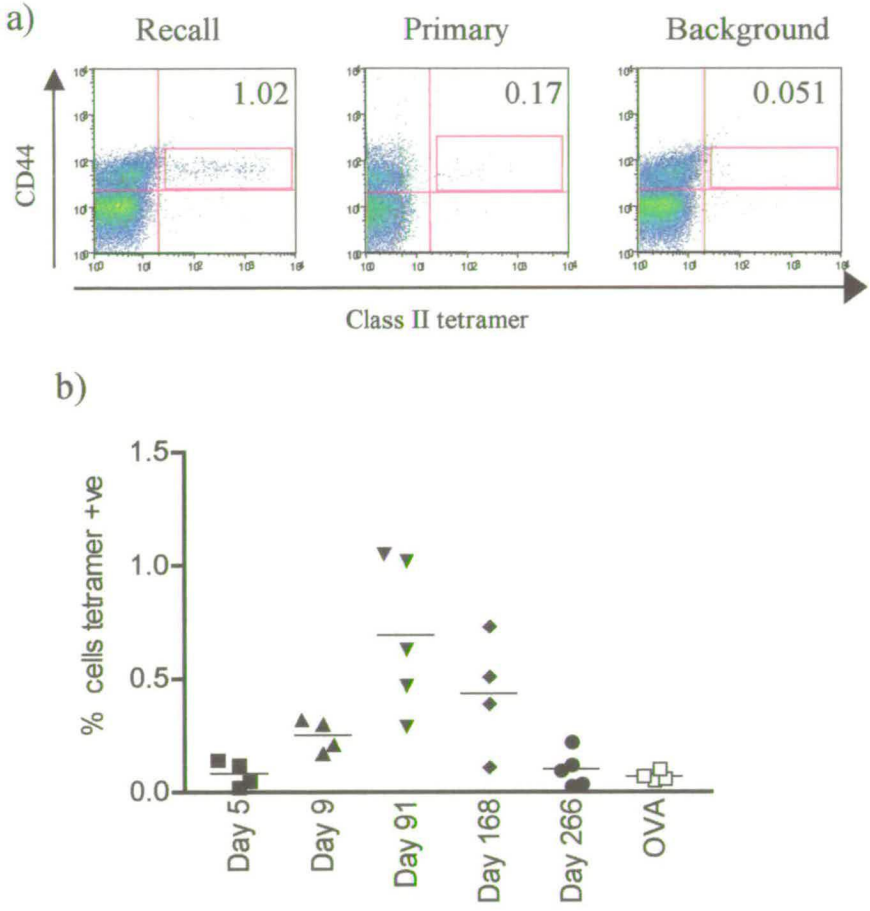


Figure 3.11 The recall response is larger and faster than the primary: C57BL/6 mice were immunised with H19env pulsed DC, rested for 90, 168 or 266 days then boosted with H19env-CFA. Cells from the spleens and DLN were stained with class II tetramers and analysed as in figure 3.4.
a) Example staining of lymph node cells from the recall response (after 91 days), primary response, and background staining at day 5.
b) Percentage of tetramer positive cells, out of CD4 cells, in the DLN of boosted (C57BL/6 mice immunised with DC, rested then boosted with H19env-CFA and sacrificed on day 5); Day 5 (primary response to H19env-CFA), Day 9 (peak of the primary response to H19env-CFA); OVA (background staining from mice primed with OVA pulsed DC, then boosted with OVA-CFA). Each point represents one mouse and the line shows the mean of the group.

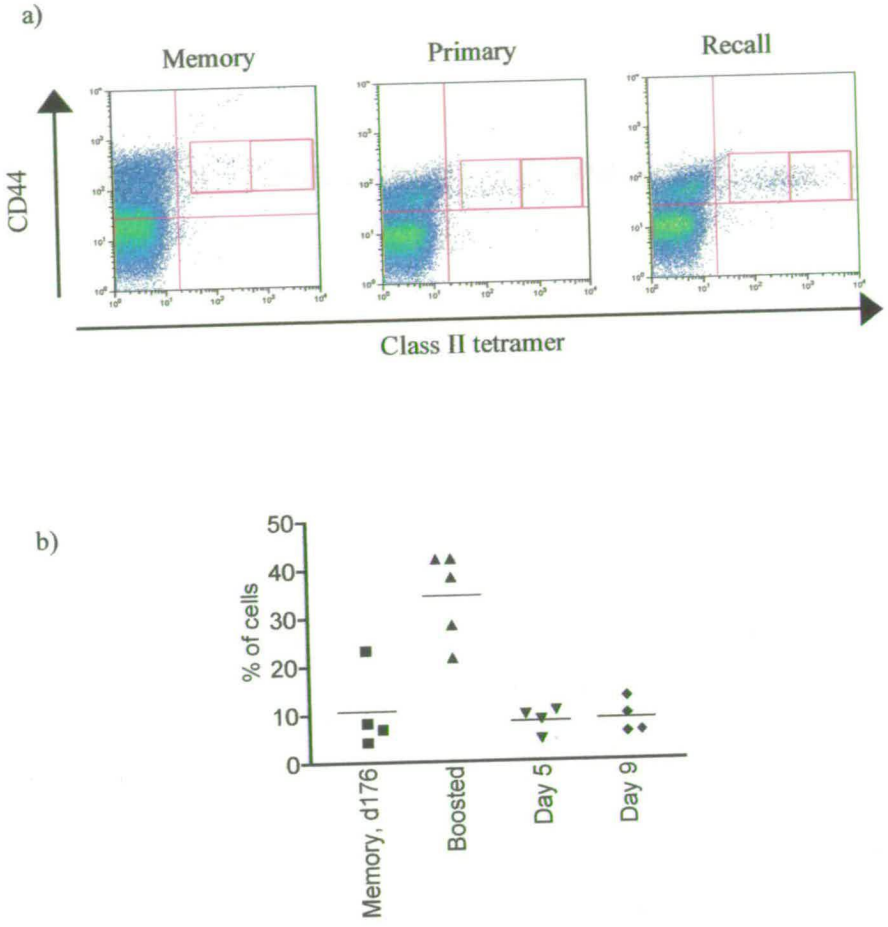


Figure 3.12 Tetramer positive cells in the secondary response bind the tetramer at a higher intensity than tetramer positive cells in the primary response:
a) Example tetramer staining of: memory cells (day 176 after immunisation with H19env-CFA); cells during the primary response (day 9); and cells from a mouse immunised with H19env pulsed DC then boosted with H19env-CFA.
b) Percentage of cells binding the tetramer at high intensity (as shown in a) after immunisation protocols as described in a). Each point represents one mouse and the line shows the mean of each group.

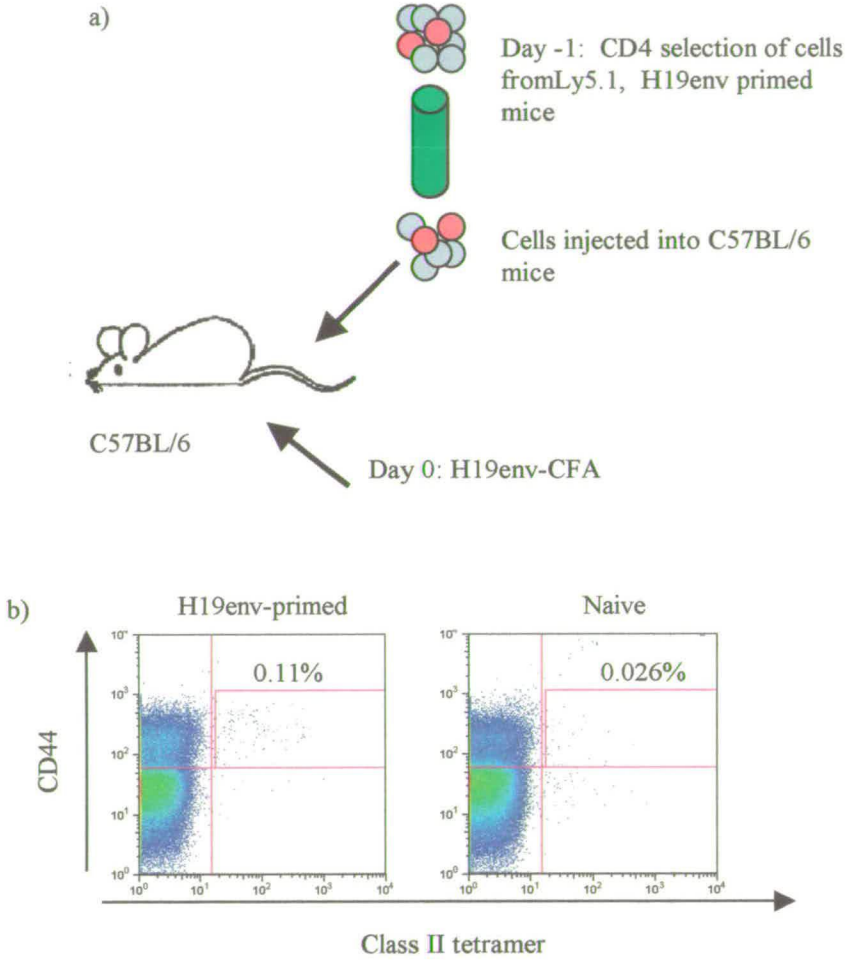
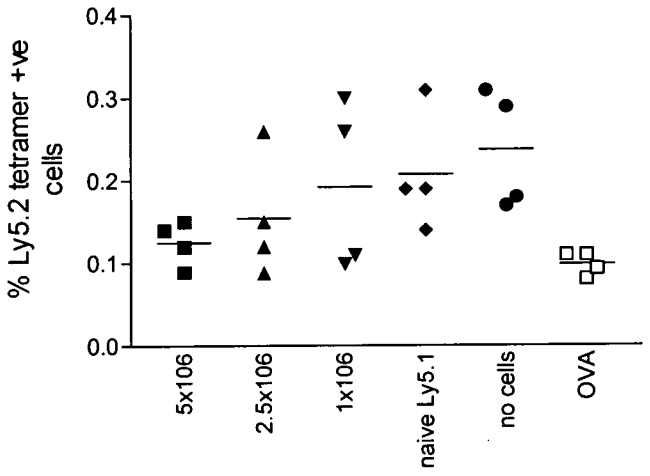


Figure 3.13 Ly5.1 tetramer positive cell transfer into C57BL/6 mice:
a) Protocol: Ly5.1 mice were primed with H19env-pulsed DC or with H19env-CFA. 2-4 weeks later a CD4 selection was carried out on spleen and LN cells, and cells containing tetramer positive cells (red) transferred into C57BL/6 mice.
b) Example tetramer staining prior to transfer of H19env primed or naïve Ly5.1 mice

a) spleen



b) DLN

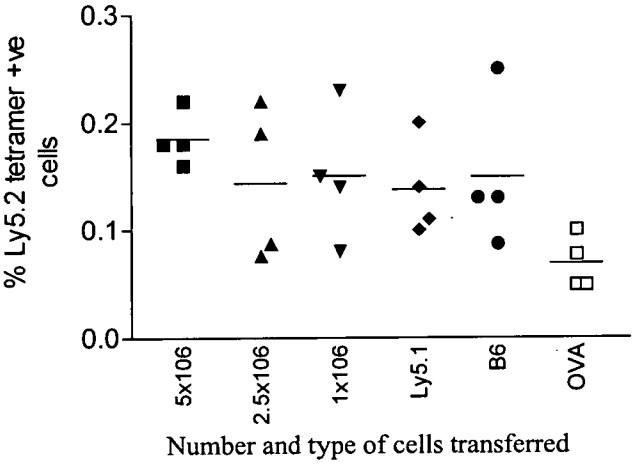


Figure 3.14 Effect on the host response after transfer of tetramer positive Ly5.1 cells:
Experiment 1 of the Ly5.1 transfer system: $1x10^6$-$5x10^6$ Ly5.1 cells containing tetramer +ve cells were transferred into C57BL/6 hosts that were immunised the following day as in Fig. 3.14a. Control groups were either given naïve Ly5.1 cells or no cells and primed with H19env-CFA. Background staining was set by immunisation with OVA-CFA. Percentage of host (Ly5.2) tetramer positive cells in the DLN (a) and spleen (b). Each point represents one mouse and the line shows the mean of each group.

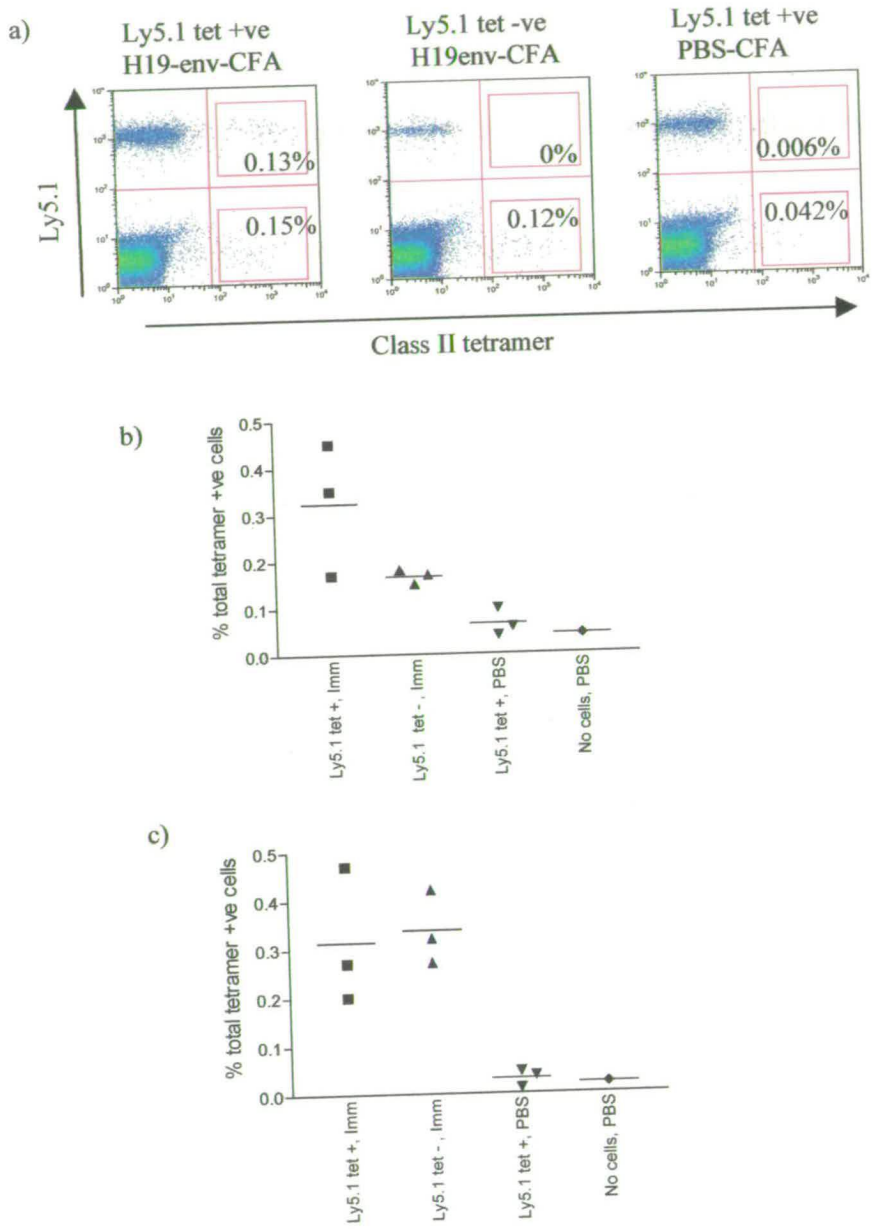


Figure 3.15 Ly5.1 tetramer positive cells can be found after transfer into C57BL/6 mice: Experiment 3 was set up as in 3.13. Spleens and DLN of recipient mice were taken 7 days after immunisation. a) Representative staining of DLN cells. Percentage of total tetramer +ve cells, out of CD4 cells, in the DLN (b) and spleen (c). Each point represents one mouse, and the line shows the mean of each group.

4. Is there competition within the CD4 memory pool?

4.1. Introduction

The factors required for the survival of T cells is an area of much debate (296), (81), (297), (298). Naïve T cells have been found to require signals via self-MHC molecules and IL-7 to survive *in vivo* (18), (164), (165), (166); although, there has been a report that naïve cells can survive in the absence of self-MHC (299). The survival of memory cells is more controversial. Over a decade ago, a series of papers from Gray and colleagues proposed that in the absence of cognate antigen, memory CD8 and CD4 T cells, and also B cells, could not survive when they were transferred into naïve hosts (167), (129). However, more recent studies have described the long-term survival of memory cells in not just the absence of specific antigen, but also in recipients that were deficient in MHC molecules (169), (170), (171), (138), (173). (175), (172). Therefore, memory cells can survive in the absence of any TCR signals.

As discussed in the introduction, such experiments are necessarily carried out in immunodeficient hosts where T cells do not have to compete for survival signals. Such survival signals determine the "space" within the memory pool and could include the availability of MHC molecules presenting self or foreign peptide, or the level of a cytokine such as IL-15 or IL-7. The availability of these signals is finite as both the naïve and memory pools are maintained at a fairly constant level (8), (81). Therefore, the long-term survival of memory cells should be examined in hosts that are challenged with other antigens or infections in order to track the maintenance of memory cells in a competitive environment.

The survival of memory CD8 T cells in competitive environments has been examined by a number of investigators (198), (193), (300). It has been found that

non-cross-reactive memory T cells survive less well after subsequent infections of immune mice, suggesting that antigen can play a role in the survival of memory T cells in competitive environments by providing a TCR specific boost (198), (193). There has only been limited study into the survival of CD4 memory cells in mice intentionally immunised with further antigens, thus, this is a field that requires further investigation.

In this chapter, the survival of memory cells was examined in mice that had been manipulated to have competitive memory pools. In the first two systems, outlined below, TCR transgenic CD4 T cells were used and in the third system, the class II tetramers were used to follow a polyclonal population of antigen-specific memory cells.

4.2. Results

4.2.1. Survival of DO11.10 memory cells in mice with endogenous OVA-specific T cells

The first system examined the survival of DO11.10 cells in mice that had previously been immunised one to three times with OVA. This would induce endogenous OVA-specifc memory cells that may compete with the transferred DO11.10 cells for antigen and for survival (see figure 4.1). BALB/c mice were immunised with OVA protein in alum either once, twice or three times intraperitoneally. Multiple immunisations should lead to an increase in the size of the OVA-specific memory pool and may also increase the affinity of these cells (295). The mice then received $5x10^6$ DO11.10 cells that had been activated *in vitro* and the long-term survival of these cells examined. The DO11.10 cells were activated *in vitro* so that their survival could be compared in naïve mice, which contained no antigen, and in the pre-immunised mice.

Initial experiments were set up to establish whether activated cells survived following transfer into naïve and pre-immunised mice. DO11.10 cells were activated for 3 days with 0.5μg/ml $OVA_{323-339}$ peptide *in vitro* prior to transfer. $5x10^6$ CFSE labelled activated or naive DO11.10 cells were then transferred into mice that had either been immunised 10 days previously with OVA-alum or were naïve. Five days later, spleens and LN were taken and the percentage of DO11.10 cells examined by FACS (using the clonotypic antibody, KJ1.26), and the division of these cells analysed by dilution of CFSE.

Figure 4.2a shows representative staining from the spleens of either pre-immunised or naïve mice that received either activated or naïve cells. The cells were present at

this time and the activated cells had continued to divide after transfer into either naïve or immunised mice, figure 4.2b. Thus, the activated DO11.10 cells survived after transfer into either naïve or immunised mice.

The long-term survival of activated DO11.10 cells was then examined in mice with endogenous OVA-specific T cells. The activated cells were transferred into four groups of mice: naïve mice; and mice immunised with OVA-alum once, twice or three times. Thus, if the endogenous memory pool did compete with the transferred DO11.10 cells this would be more apparent in mice pre-immunised three times compared to mice just pre-immunised once, as depicted in figure 4.1.

The percentage of surviving DO11.10 cells was examined 6 weeks after transfer in the four groups, figure 4.3. The percentage of DO11.10 cells did not differ between the different groups. Therefore, the endogenous memory cells did not affect the survival of the DO11.10 cells. Moreover, as the activated cells survived equally well in naïve mice as they did in immunised mice, the presence of antigen was not required for survival of the memory cells for 6 weeks.

4.2.2. Do memory cells compete for contact with MHC molecules?

One of the problems with the first system described above was that only one population of memory cells could be tracked: the endogenous memory pool could not be directly measured. This, and questions about the survival signals delivered via MHC molecules as opposed to antigenic signals, prompted the development of the following system.

This second set of experiments used both DO11.10 and OT-II cells, thus enabling the tracking of two potentially competing populations of cells. These two transgenic cells recognise the same peptide in OVA: $OVA_{323-339}$, however, DO11.10 mice are on the BALB/c background ($I\text{-}A^d$) and OT-II mice are on the C57BL/6 background

($I\text{-}A^b$). DO11.10 cells respond to lower amounts of $OVA_{323\text{-}339}$ than OT-II cells (301) and are thought to have greater affinity for their self-MHC complex than OT-II cells (296). The latter is based on the finding that DO11.10 cells proliferate extensively following transfer into T cell deficient or irradiated hosts (302). OT-II cells, however, do not divide much after transfer into immunodeficient hosts (296). As the extent of proliferation in this system is thought to be induced by recognition of self-peptide on MHC molecules, it is suggested that DO11.10 cells have greater affinity for self-MHC than OT-II cells do for their respective MHC molecules (303), (304).

If signalling via TCR recognition of MHC, either containing antigenic peptide or self-peptide, is important in memory cell survival, it could be argued that memory DO11.10 cells would survive better than OT-II memory cells. Therefore, experiments were conducted to compare the survival of DO11.10 and OT-II cells in separate mice and in the same host, see figure 4.4.

Initial experiments were carried out *in vitro* to characterise the activation requirements of the two cell populations. CD4 purified DO11.10 and OT-II cells were activated with OVA peptide either with irradiated BALB/c or C57BL/6 APC. In some experiments all four cell populations were combined in the same well. The proliferation of the two cell populations was investigated by both thymidine incorporation and by CFSE dilution measured using flow cytometry.

By thymidine incorporation, it can been seen that DO11.10 cells proliferate at 10-fold lower concentrations of OVA peptide in the presence of BALB/c APC compared to the OT-II response in the presence of C57BL/6 APC, figure 4.5a. Moreover, DO11.10 cells respond to OVA peptide presented on $I\text{-}A^b$ in a dose dependent manner and to lower concentration of OVA peptide compared to the OT-II cell response to OVA peptide presented by $I\text{-}A^b$. However, there was no response of OT-II to OVA in the presence of BALB/c APC, thus OT-II cells could only

recognise $OVA_{323-339}$ presented by $I\text{-}A^b$. Interestingly, in the mixed culture (DO11.10, OT-II T cells, BALB/c and C57BL/6 irradiated APC) the extent of cell proliferation was similar to that found after activation of OT-II to $OVA_{323-339}$ presented by C57BL/6 APC alone. To determine the extent of proliferation of each of the cell types in the mixed culture, the transgenic cells were labelled with the intracellular dye CFSE and their division monitored by flow cytometry.

As shown in figure 4.5b and c, DO11.10 cells proliferated faster than OT-II cells when the cells were cultured separately with their respective APC. This was less clear in cultures in which the two transgenic cells were cultured together with both C57BL/6 and BALB/c APC. In this case, the OT-II cells proliferated to a similar extent as the DO11.10 cells; this effect was observed at all the antigen doses tested (0.005-5μg/ml). The reasons for this are two-fold: 1. the DO11.10 cells proliferated less in this culture than when incubated alone and this is probably because they respond less well to $OVA_{323-339}$ presented by the mixed population of APCs compared to presentation just by BALB/c APC. The second difference was that the OT-II cells proliferated slightly more in the mixed culture than when activated on their own, this may be because the OT-II cells make use of the IL-2 generated by the DO11.10 T cells.

Once (C57BL/6xBALB/c)F1 mice were available, *in vivo* experiments could be carried out. CFSE labelled transgenic cells were transferred into mice that were immunised the following day with $OVA_{323-339}$-alum. Figure 4.6 shows representative staining of splenocytes from a mouse that received both OT-II and DO11.10 cells five days after immunisation, and the CFSE profile of the transgenic cells. Both cell types were found to proliferate, however, OT-II cells did not expand to the same extent as DO11.10 cells.

Experiments to investigate the survival of memory populations of the two transgenic cells in (C57BL/6xBALB/c)F1 were carried out. However, no OT-II cells could be

found in either control mice that only received OT-II cells, or in mice that received both sets of transgenic cells. Other members of the lab, who were tracking OT-II cells in wild-type C57BL/6 mice, encountered a similar problem: OT-II cells could not be identified 15-20 days after transfer. This defect may have been a result of minor histocompatibility differences between the OT-II cells and the host mice resulting in the rejection of the OT-II cells. Experiments using OT-II mice back-crossed an additional four times are currently underway in the lab to investigate this.

4.2.3. Survival of antigen-specific polyclonal memory cells in mice immunised with multiple antigens

The two transgenic systems used for examining competition within the memory pool had not proved fruitful for a number of reasons. Once it had been established that the class II tetramers could be used to track endogenously generated CD4 memory cells over a considerable period of time, experiments were set up to track the survival of H19env-specific T cells in hosts immunised with a number of other antigens. It was thought that subsequent immunisation would result in the generation of memory cells that could compete for survival with the H19env-specific memory cells, for example. by using up a limiting factor such as a cytokine. Thus, in H19env immunised mice subsequently immunised with several different antigens, it was thought that H19env specific cells would survive less well (figure 4.7). Although this meant that only one population of memory cells could be identified, the system involved tracking small populations of endogenous memory cells in the absence of persistent antigen.

Mice were first immunised with H19env pulsed wild-type DC. This immunisation protocol was chosen over CFA immunisation for two main reasons. First, CFA immunisation results in the formation of a depot of antigen and it was thought that, if an effect on long term cell survival was to be observed, there would be more chance of measuring it in the absence of putative survival signals via retained antigen. Second, the read-out of the experiment was by challenging the mice with H19env-

CFA to assay the recall response, as had been done in previous experiments. This demonstrated not just the maintenance of memory cells but also that they were functional.

In the first experiment, three groups of mice were immunised with H19env pulsed DC. The mice were left for 6 weeks to allow the memory cells to form before two of these groups were immunised with OVA protein precipitated in alum. After a further 4 weeks, one group was immunised with KLH. The mice were rested for a further 4 weeks then they were boosted with H19env-CFA to evaluate the recall response by comparing it to the primary response in age-matched naïve mice. Table 4.1 summaries this immunisation protocol.

Group	Immunisation 1	Immunisation 2	Immunisation 3	Immunisation 4
1	H19env-DC			H19env-CFA
2	H19env-DC	OVA-Alum		H19env-CFA
3	H19env-DC	OVA-Alum	KLH	H19env-CFA
4		OVA-Alum		H19env-CFA
5			KLH	H19env-CFA
6				H19env-CFA
7				OVA-CFA

Table 4.1: Immunisation protocol for multiple immunisation experiment.

Groups 4 and 5 were included to rule out any complications with cross-reactivity between H19env-specific cells and the other antigens. Any cross-reactivation would provide extra survival signals for the H19env-specific cells rather than inducing competition. If OVA or KLH had proved to be cross-reactive, the response to the H19env-CFA challenge would have resulted in a response greater than the primary response in group 6. Group 7 is the normal background control for tetramer staining.

The results of this experiment, as detailed in figure 4.8a, suggest that there had been no affect on the H19env-specific memory pool in mice that had been immunised with OVA and KLH. In turn, this implies that, in this system, there had been little or no competition within the CD4 memory pool.

The experiment was repeated with some alterations to the protocol: mice were immunised with more antigens, and they were left for a longer period of time to see if this would provide time for the competition to become apparent. The extra antigen used was secreted proteins from *Nippostrongylus brasiliensis* (NES). This mix of proteins induces a Th2 response in immunised mice (265). Table 4.2 summaries the experimental groups and immunisation timings used in this experiment.

Group	Immunisation 1 13/11/03	Immunisation 2 15/12/03	Immunisation 3 6/2/04	Immunisation 4 14/5/04	Immunisation 5 19/6/04
1	H19env-DC				H19env-CFA
2	H19env-DC	KLH	DNP-OVA		H19env-CFA
3	H19env-DC	KLH	DNP-OVA	NES	H19env-CFA
4		KLH			H19env-CFA
5		NES			H19env-CFA
6					H19env-CFA
7					OVAp-CFA

Table 4.2: Immunisation protocol for second multiple immunisation experiment.

Again, all the groups were challenged with H19env-CFA 5 days prior to tetramer staining. As in the first experiment, no difference were found in the recall response between mice immunised with three different antigens or only primed with H19env pulsed DC, figure 4.8b. This further suggests that no competition had taken place within the CD4 memory pool.

4.3. Discussion

The experiments described in this chapter set out to examine whether CD4 memory cells competed for survival with each other. Using several different experimental systems, based on either transgenic or polyclonal populations, no evidence for competition in the CD4 memory pool was found. However, there were deficiencies in these systems that prevent overall conclusions from being reached.

4.3.1. Survival of memory DO11.10 cells in mice with endogenous OVA-specific memory cells

The experiments using transgenic cells were inconclusive. In the first system, involving the transfer of activated DO11.10 cells into pre-immunised mice, the existence of host OVA-specific memory cells did not affect the survival of the transferred cells. This may suggest that the memory cells do not need to compete for antigen in order to survive. However, there could be other explanations for this result.

DO11.10 CD4 T cells respond to low doses of antigen and are thought to have a high affinity receptor for the peptide-MHC complex, and for self-MHC (301), (305). Therefore, the transgenic cells may recognise their antigen and or self-MHC with greater affinity than the endogenous memory cells, and thereby their maintenance would be unaffected by the presence of the endogenous memory cells.

Furthermore, the "set-up" of this system may not "encourage" competition in the immunised mice. By increasing the number of immunisations, the amount of antigen also increases. The mice were immunised with OVA precipitated in alum, an

adjuvant that is thought to at least partly act by forming a depot at the injection site (280). Thus, in mice immunised three times, there will be more antigen than in mice only primed once. This may have the effect of reducing competition rather than inducing it. Although this problem could be solved by immunising with an adjuvant that does not induce a depot of antigen, this would have removed the potential competition factor. Regardless of the reason why there was no difference in survival of the DO11.10 cells, the result corresponds to findings by various groups (172), (175), (169), (170), (171), (138), (173): memory cells were able to survive in the absence of specific antigen.

4.3.2. Do memory cells compete for MHC molecules for survival?

The second set of experiments, using both DO11.10 and OT-II transgenic cells, did not shed any light on competition in the CD4 memory pool as the OT-II cells disappeared from the adoptive transfer hosts. Nevertheless, the preliminary experiments, mainly carried out *in vitro*, do support findings in the literature showing that DO11.10 cells respond to lower doses of $OVA_{323-339}$ than OT-II T cells (301). This was true whether the peptide was presented to DO11.10 cells by $I\text{-}A^d$ or $I\text{-}A^b$.

A further interesting point is that the difference in proliferation of DO11.10 and OT-II cells was less apparent when the populations were cultured together with both sets of APC (compare the CFSE profiles in figure 4.5b and c). This suggests that one population of activated T cells can affect a different clonotype. What effect such interactions could have *in vivo* has been investigated by other groups. Both Alpan *et al* and Creusot *et al* found that memory or activated T cells could influence the cytokine production by naïve cells when both populations were activated simultaneously (306), (307). Kedl *et al* investigated the competition between CD8 T cells specific for the same antigen but with differing affinities (212). The authors found that high affinity T cells prevented the activation of the lower affinity T cells by down-regulating peptide-MHC complexes on APC. This does not correlate with

the *in vitro* results in this PhD. If this was the case, it might be predicted that DO11.10 T cells would reduce the proliferation of OT-II cells in the same well. Instead the OT-II proliferated slightly more than they did in individual cultures. This may reflect differences between CD8 and CD4 T cells or may be because, in a tissue culture well, the availability of peptide-MHC complexes was not limiting. This may not be the case *in vivo* where OT-II cells proliferated less than DO11.10 cells in the same host at day 5 after immunisation. In this instance, the DO11.10 cells may monopolise the available peptide-MHC complexes.

4.3.3. Survival of polyclonal memory cells in mice immunised with other antigens

Given the deficiencies in the experiments using transgenic cells, a protocol was set up to answer similar questions using the class II tetramers. As described above, this experiment tracked the functional survival of H19env-specific memory cells in mice that had been immunised with a number of different antigens.

The recall response was equivalent in mice immunised with just H19env-pulsed DC and the various groups of mice also immunised with additional antigens. Therefore, the H19env-specific memory cells were able to survive despite the presence of other memory cells. In hindsight, it may have been better to track the survival of H19env-specific cells *ex vivo* as the recall assay may not be subtle enough to distinguish between the differential survival of the memory cells in the various groups. This would have required immunising the mice with H19env-CFA, which, as this adjuvant forms a stable depot of antigen for some time, would have meant that the H19env-specific memory cells had continuous access to antigen and this may have provided occasional TCR signals that could enhance cell survival.

Taking together the results from the first experiment, involving the transfer of DO11.10 cells into pre-immunised mice, and the class II tetramer based experiment, the following two interpretations are possible. 1. that there is no competition within the CD4 memory pool; or 2. the memory pool was not saturated by the experimental systems employed.

The best evidence for competition within the CD8 memory pool comes from the work by Selin and Welsh and colleagues (193), (196), (197), (308), and Smith *et al* (198). In both cases, the decline of antigen-specific memory CD8 cells was found following subsequent infection of immune mice with different pathogens. Both groups also studied CD4 T cell memory in the same systems. Although Selin and colleagues found no decline (195), Smith *et al* found a dramatic reduction in memory CD4 cells after infection with BCG (198).

However, as described in the introduction, this perceived competition may actually be a result of infection-induced apoptosis of memory cells rather than direct competition for a survival factor within the memory pool (309). It is interesting to note that a reduction in antigen specific CD4 memory cells was found after bacterial infection (198) but not after viral infection (193), suggesting that the factors that induce apoptosis in CD4 and CD8 T cells may be distinct, and dependent on the type of infection.

This prompts the question of if and why this attrition could be beneficial for the immune system. Welsh and colleagues and Jiang *et al* suggest that the apoptosis of cells in secondary lymphoid organs, just prior to the expansion phase of the immune response, clears space for this antigen-specific expansion (200), (205). Moreover, the loss of some memory cells may provide space in this pool for newly generated cells, especially considering the large numbers of memory cells following some viral infections in mice (131). The apoptosis induced attrition would, therefore, prevent the saturation of the memory pool.

This attrition does, however, come at a price, and could result in loss of immunity to some pathogens. The memory cells that would have the best chance of survival in this system would be those that received a periodic signal via their TCR. Thus the host would be most likely to maintain memory cells for pathogens to which it was regularly exposed.

McNally *et al* did not observe any alterations in the TCR repertoire following infection-induced apoptosis, suggesting that the immune system is able to maintain a diverse TCR repertoire despite this death (201). This corresponds to findings by Troy and Shen that indicate that the immune system can somehow recognise the extent of diversity within the T cell pool (310). The authors found that TCR transgenic cells could proliferate after transfer into a different TCR transgenic mouse, indicating that there was "space" in this host, but that the cells could not divide if they were transferred into a TCR transgenic host of the same clonotype. How the immune system can recognise the diversity of the T cell repertoire is not yet understood but this knowledge could potentially shed more light on how T cell pools are regulated.

4.3.4. Conclusion

The experiments carried out in this chapter do not conclusively rule out competitive homeostatic deletion within the CD4 memory pool. However, the results are in line with findings in the literature. The mechanism of attrition in the CD8 memory pool has been found to be infection-induced apoptosis rather than direct competition for "space". This lymphopenia induced by infection can also affect CD4 T cells (198), (205). Thus, it would be interesting to track the survival of H19env-specific cells after subsequent infections. Such experiments were not carried out in the PhD for two main reasons. First, because of the practical complications of infecting the H19env immunised mice. Second, and more importantly, in this set of experiments, I first wanted to define whether there was competition in the memory pool for survival, rather than increased death of the memory cells as a result of infection. Had

competition been apparent, this could have led to experiments in which the limiting factor could have been identified.

However, it may be that there is less need for this form attrition in the CD4 memory pool as the peak of CD4 T cells clonal expansion is much smaller than in many CD8 T cell responses (19), (126), (311), (248). There are, therefore, fewer requirements for competition as the CD4 T cell pool would be less likely to be saturated than the CD8 memory pool. Moreover, as described in the previous chapter, antigen-specific memory CD4 cells declined overtime in mice that were not intentionally challenged with other antigens. Therefore, the memory pool would be unlikely to become saturated, unlike the CD8 memory pool in which memory cells survive for a more considerable time and at higher frequencies (173), (287), (288), (19).

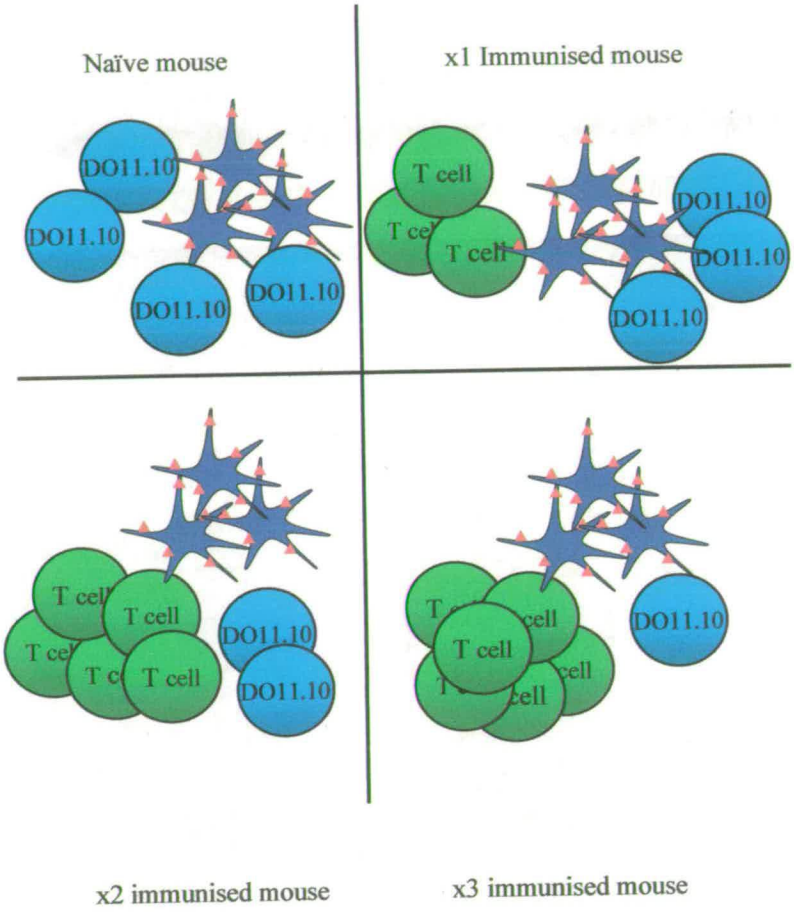


Figure 4.1 Do transferred DO11.10 cells survive less well in mice that contain endogenous OVA-specific memory cells?
By increasing the number of immunisations, the endogenous pool of OVA specific T cells (green) should increase and thereby, potentially, create a more competitive memory pool in which the DO11.10 cells (blue) may have to compete for residual antigen on APC.

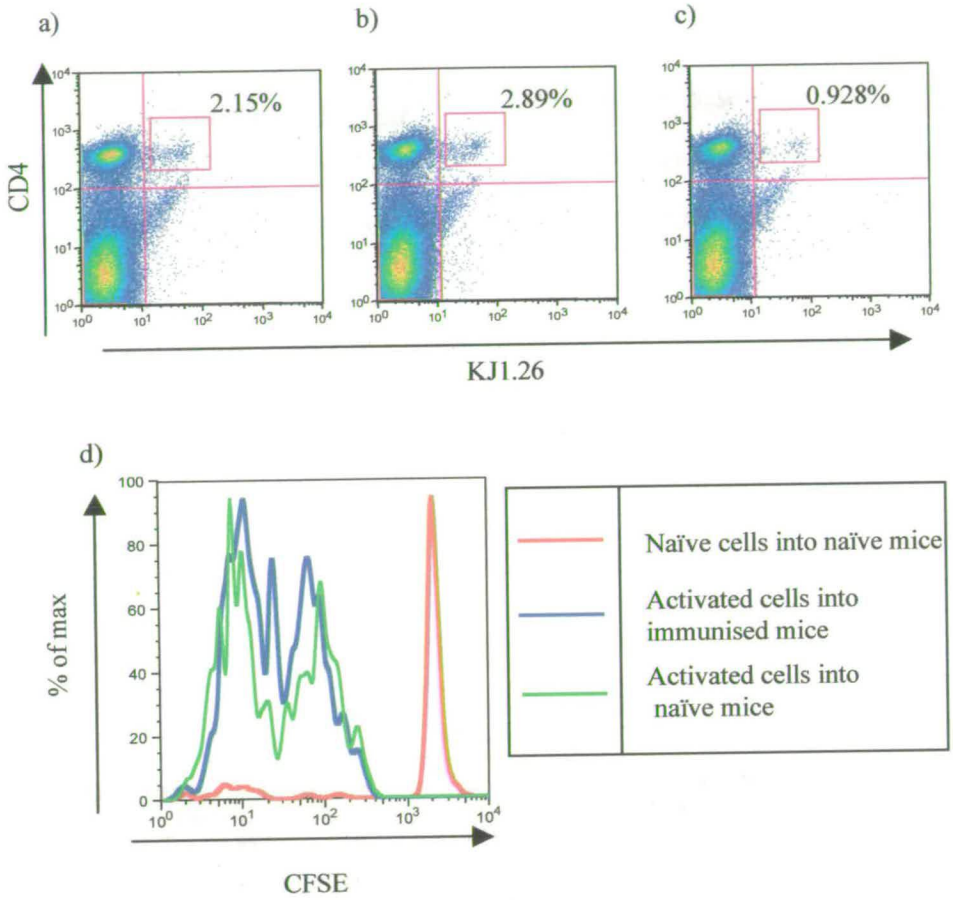


Figure 4.2 Transfer of activated DO11.10 cells into immune mice: $5x10^6$ DO11.10 cells were activated *in vitro* with 0.5μg/ml OVA peptide for 3 days then transferred into BALB/c mice which had been immunised with 100μg of OVA-alum i.p. 10 days previously (a) or naïve mice (b), or naïve cells transferred into naïve mice (c). Percentage of DO11.10 cells, out of CD4 cells, in the spleen are shown, cells gated on live lymphocytes. d) Division of activated KJ1.26 cells in immunised (blue line) or naïve mice (green line), red line: naïve cells in naïve mice.

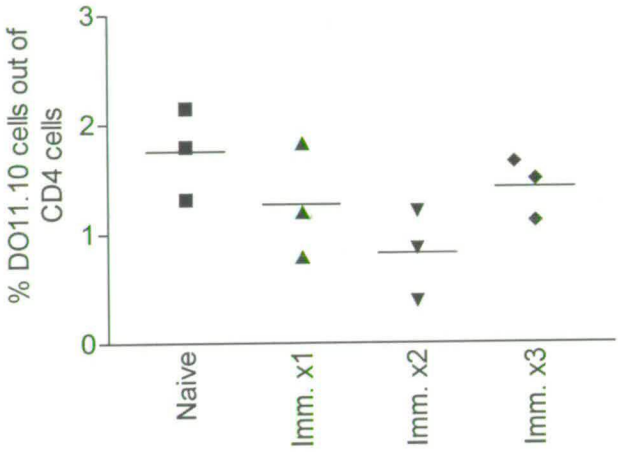


Figure 4.3 Survival of activated DO11.10 cells in naïve of pre-immunised mice: DO11.10 cells were activated *in vitro* for 3 days with 0.5μg/ml OVA peptide before transfer into naïve mice, or into mice pre-immunised 1 to 3 times with OVA-alum i.p. as indicated. Mice were sacrificed after 6 weeks and the percentage of surviving DO11.10 cells in the spleen enumerated by FACS by staining with KJ1.26 and anti-CD4. Each symbol represents one mouse and the line shows the mean of the group. This experiment was only done once.

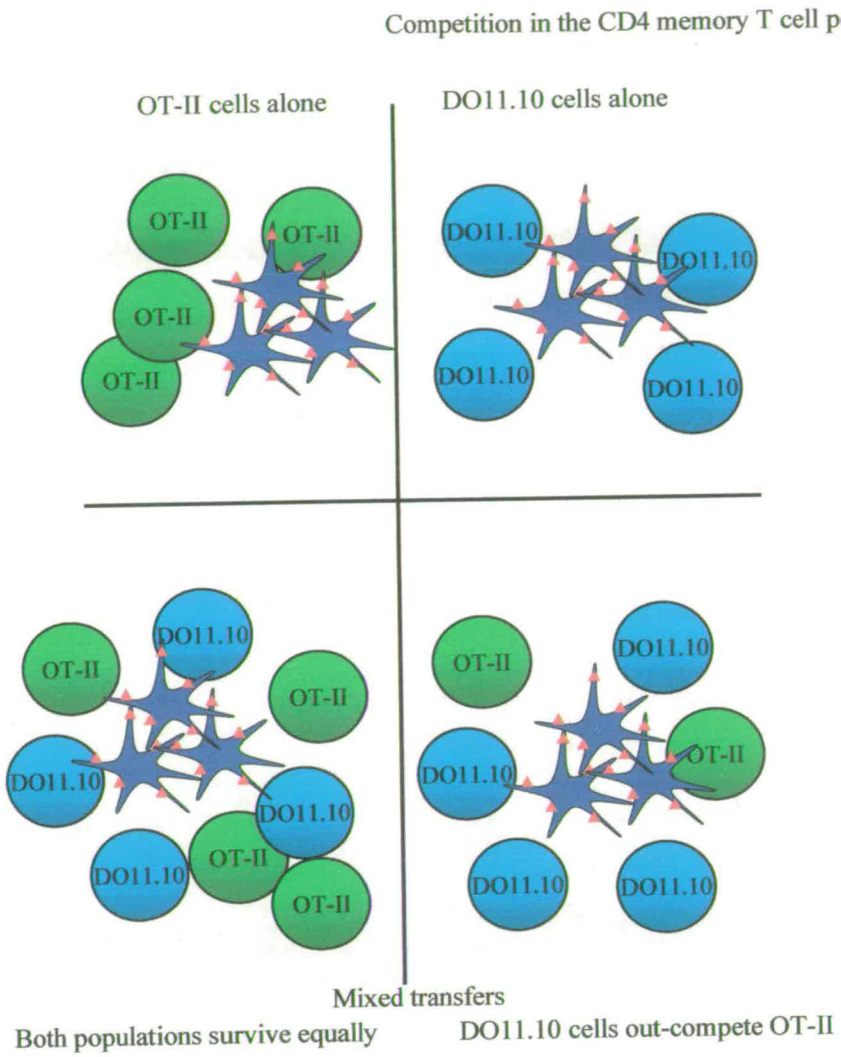


Figure 4.4 Do memory DO11.10 cells survive better than memory OT-II cells? After co-transfer, DO11.10 (blue) and OT-II (green) cells may survive equally well, suggesting that there is no competition for antigen or MHC. Alternatively, DO11.10 cells may survive better, suggesting that antigen or MHC contacts may be important in the survival of memory CD4 T cells.

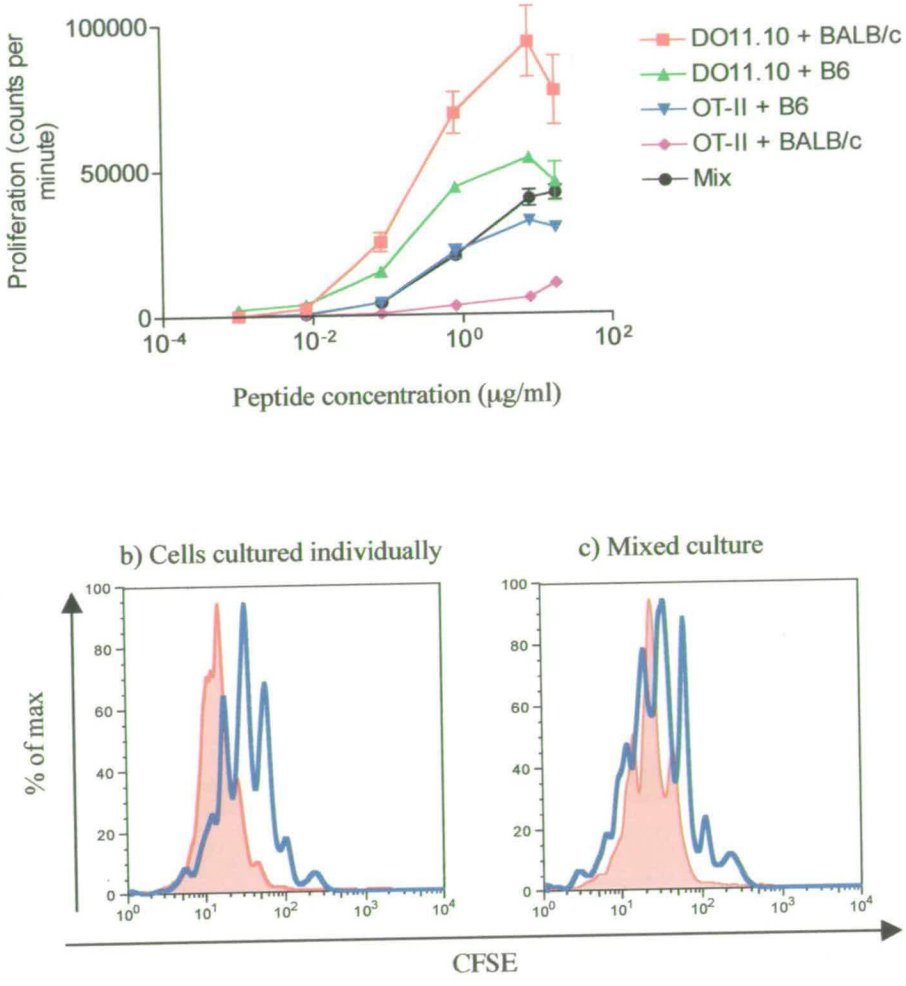


Figure 4.5 *In vitro* division of DO11.10 and OT-II cells: a) CD4 purified DO11.10 or OT-II cells were cultured at various doses of OVA peptide for three days with either irradiated BALB/c or C57BL/6 splenocytes or in a mixed culture of all four cell population as indicated.
b) and c) CD4 purified CFSE labelled DO11.10 (red-fill) and OT-II (blue line) cells were activated individually (b) with respective APC or together with C57BL/6 and BALB/c APC (c), for 4 days *in vitro* with 0.005µg/ml OVA peptide. The cells were then stained with anti-TCR and anti-CD4 antibodies and analysed by FACS.

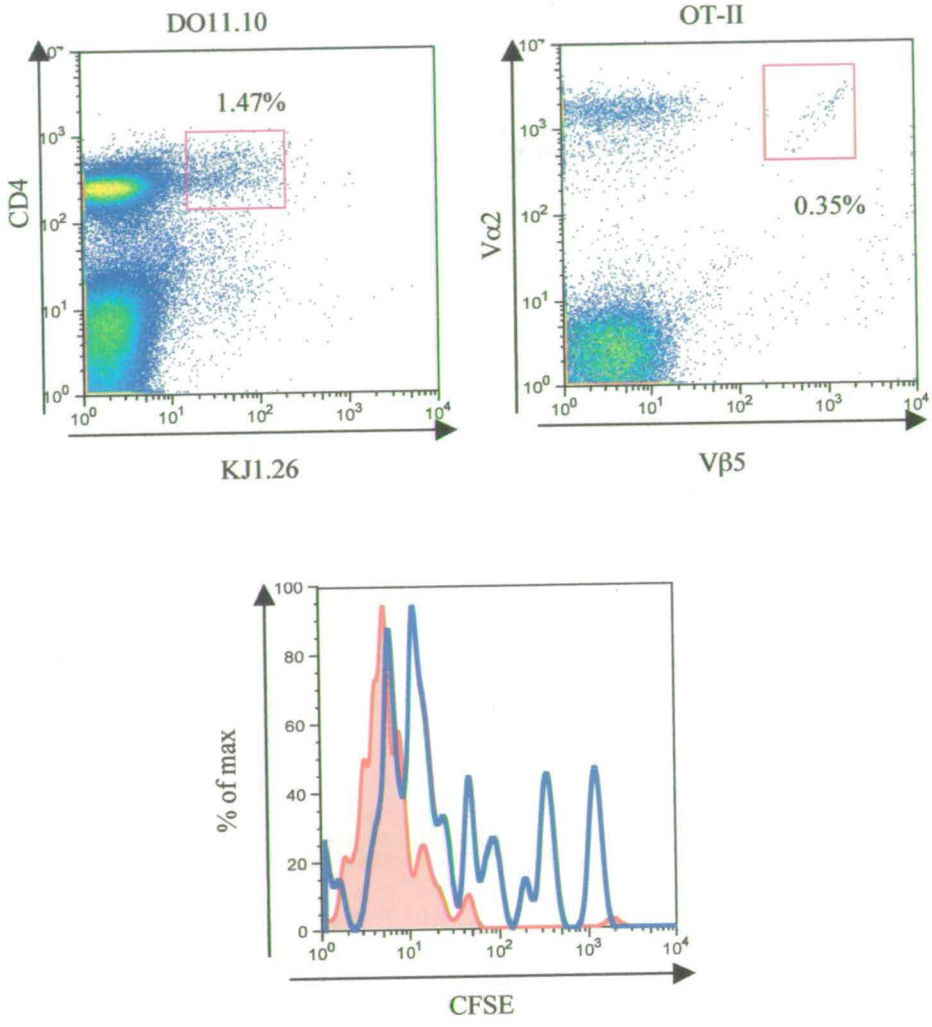


Figure 4.6 *In vivo* response of DO11.10 and OT-II cells: CFSE labelled DO11.10 and OT-II cells were transferred into (C57BL/6xBALB/c)F1 mice which were then immunsied with 100μg OVApeptide-alum i.p.. Splenocytes were stained with anti-TCR and anti-CD4 antibodies on day 5 after immunisation. Cells are gated on live cells (DO11.10) or live CD4+ve cells (OT-II) and the number shows the percentage of transgenic cells out of total CD4 cells. DO11.10 (red-fill) and OT-II (blue line).

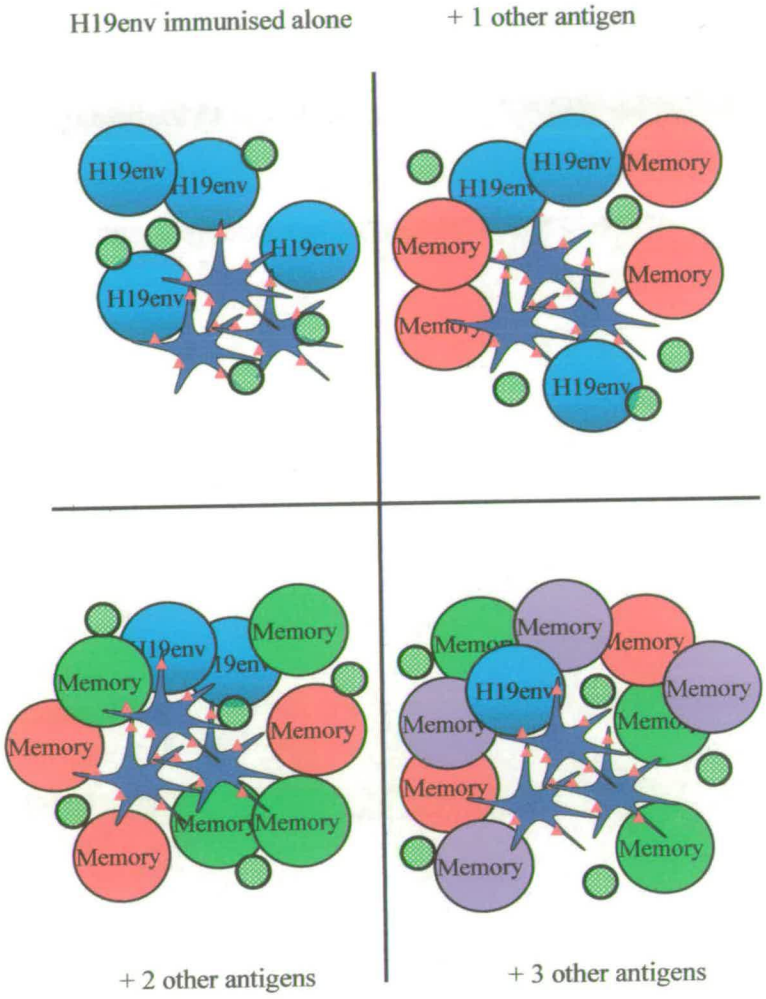


Figure 4.7 Does immunisation with multiple antigens result in a reduction in H19env specific T cells?
After immunisation with other antigens, H19env specific cells (blue) may have to compete with other memory cells (red, green and purple) for survival factors (APC and soluble molecules - small green circles).

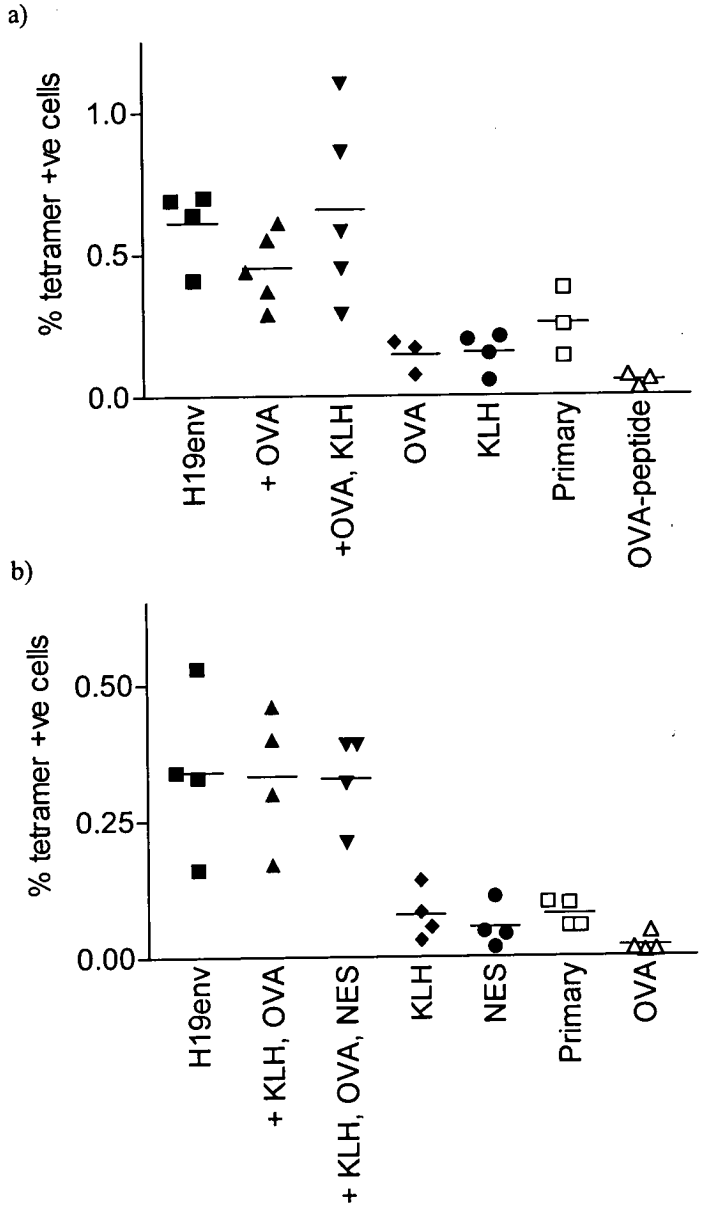


Figure 4.8 Competition in the CD4 memory pool: percentage of tetramer positive cells out of CD4 cells in the DLN of mice immunised as in tables 4.1 (a) and 4.2 (b). Each point represents one mouse, and the line shows the mean of each group.

5. Requirements for costimulation in the generation, survival and recall responses of CD4 T cells

5.1. Introduction

Recent advances in microscopy have offered a new view of T cell activation *in vivo*. Movies detailing the interactions between T cells and APC demonstrate that it is only after sustained interactions between these two cell types that T cells become fully activated and committed to cell division (24), (23). This requires cross-talk between the T cells and APC involving a complex array of costimulatory molecules. The relative importance of a wide range of costimulatory molecules in the initial activation of T cells has been widely studied in various models for both CD4 and CD8 T cells. However, the importance of these molecules in the generation of functional memory cells and the re-activation of these cells has only received limited attention. This is especially true for CD4 recall responses, which have largely been studied *in vitro* (9), (161), (278). Experiments tracking antigen-specific memory cells from activation, through memory and during recall responses *in vivo* are, therefore, required to dissect the precise roles of individual costimulatory molecules during these different phases of the T cell response.

5.1.1. CD40

CD40 is a member of the TNFR family and is expressed on activated DC and B cells (30). It binds to CD40L on activated T cells and forms part of the intricate cross-talk between T cells and APC (312); figure 5.1 illustrates these interactions. While the importance of CD40-CD40L interactions for efficient B cell response is well defined (313), the role this interaction may play in T cell responses is unclear.

The ligation of CD40 on DC by CD40L results in maturation of the DC, promoting further upregulation of the B7 molecules and inducing the expression of other molecules such as OX40L (32), (33), (314), (34). This serves to amplify and sustain the ensuing T cell response. Moreover, this signal is important for the secretion of IL-12 by DC, enabling them to influence the differentiation of the responding T cells (32), (33), (35), (315).

Various studies in CD40 and CD40L knockout mice have provided insights into the role of CD40-CD40L interaction in T cell priming. While some studies have shown reduced CD4 and CD8 T cell priming (316) others have found a defect only in the CD4 response (317) or no defect at all in the T cell response (318). These variations perhaps reflect the different immunisation strategies used and inherent differences between viral infections and protein based immunisations. Moreover, the CD4 T cell response has primarily been examined indirectly using *in vitro* proliferation assays. Studies involving the adoptive transfer of CD40L knockout TCR transgenic cells have also provided contradictory results: Howland *et al* found that the knockout cells could proliferate but that the response was aborted at an early stage (34). In contrast, Grewal *et al,* found that CD40L knockout cytochrome C specific T cells did not proliferate at all in immunised wild-type mice (319).

The importance of the CD40L-CD40 interaction in T cell activation has been demonstrated by tolerance studies. For example, transfer of peptide-pulsed CD40 deficient DC results in tolerance of specific T cells rather than activation (320) and administration of an activating anti-CD40 antibody resulted in priming of tumour specific CD8 T cells, rather than tolerance, following the intravenous injection of peptide (321). Therefore, T cell activation is likely to be limited in the absence of CD40 or CD40L, but the requirement for this interaction in the survival and reactivation of memory cells is a question that remains unanswered.

5.1.2. ICOS

ICOS is a member of the CD28 costimulation family, and, like CD40L, is expressed on activated T cells (322), (323), see figure 5.1. The phenotype of both ICOS and B7h knockout mice has demonstrated the importance of this interaction in the B cell response in both the generation of germinal centres and class switching (31), (324). However, the role and requirement for this interaction in T cell responses is less clear.

Little is known about the importance of the interaction between ICOS on activated T cells and B7h on DC. There have been reports that DC do express B7h, but expression does not appear to be upregulated following activation with a number of stimuli (LPS, TNF-α, IFN-γ, also see materials and methods) (325), (326), (327). Moreover, Witsch *et al* found that DC actually downregulated expression of B7h following culture with activated T cells and that this was dependent on the expression of ICOS by the T cells (326). This interaction may be important, however, for IL-10 production by T cells as blocking ICOS-B7h interactions in *in vitro* cultures of T cells and DC reduced the levels IL-10 made by the T cells (326).

Early work using anti-ICOS antibodies and fusion proteins suggested that ICOS signalling was important in the development of the Th2 response (328), (329). This has been confirmed to some extent by *in vivo* findings (330), (331). For example, Mak *et al* described a reduced allergic asthma response, a classic Th2 disease, in B7h knockout mice and the authors suggested that this resulted from the dimished production of the Th2 cytokines, IL-4 and IL-13 (330).

Despite this role for ICOS in Th2 responses, there have been descriptions of a wider role for ICOS costimulation (30). McAdam *et al* found that stimulating T cells using anti-CD3 and B7h-Ig caused increased production of IFN-γ, IL-4 and IL-10 (42).

This suggested that ICOS costimulation was important in effector responses rather than T cell proliferation. However, Dong *et al* found decreased IL-2 production from ICOS knockout T cells *in vitro* and this resulted in reduced T cell proliferation (332). Similarly, in work with human T cells, Aicher *et al* found that blocking ICOS also resulted in decreased proliferation in both mixed lymphocyte reactions and in responses to tetanus toxoid (327).

Examining T cell proliferation *in vivo* has not resolved this debate. Although Nurieva *et al* and Mak *et al* did not find reduced T cell proliferation in the absence of ICOS signalling (333), (330), Smith *et al* showed that adoptively transferred DO11.10 T cells proliferated less in the presence of a blocking ICOS fusion protein in both Th1 and Th2 inducing environments (45). In support of this role for ICOS signalling in Th1 responses, Mittrucker *et al* found that treating mice with an ICOS fusion protein resulted in increased susceptibility to LM infection, a classic Th1 infection (46).

Therefore, the precise role of ICOS costimulation in T cell responses is far from clear. The different results described above probably reflect the varying protocols used: ICOS fusion proteins, antibodies and different knockout mouse strains. As with CD40-CD40L interactions, T cell responses in the absence of ICOS-B7h interactions need to be examined directly *ex vivo* to establish when, and in what circumstances, T cells require costimulation via ICOS.

As detailed in the previous chapters, class II tetramers can be used to track antigen-specific T cell responses during various stages: priming, memory cell survival and recall responses. This tool provides the opportunity to study the role of specific costimulatory molecules during these different stages. In this chapter the role of CD40L-CD40 and ICOS-B7h costimulation during these stages will be examined by tracking the antigen-specific T cell response in CD40L, CD40 and ICOS knockout mice.

5.2. Results

The role of CD40-CD40L in T cell responses

5.2.1. CD40-CD40L costimulation is required to prime CD4 T cells

To evaluate the role of the costimulatory interaction CD40L-CD40 in T cell priming, wild-type C57BL/6, CD40, and CD40L knockout mice were primed with H19env peptide in CFA. After 8 days, the spleens and draining lymph nodes (DLN) were taken and stained with class II tetramers to determine the extent of CD4 T cell priming. As expected, a population of antigen specific cells were induced in C57BL/6 mice. However, the tetramer staining in CD40 and CD40L knockout mice was not above background (C57BL/6 mice immunised with OVA peptide in CFA), figure 5.2. This suggested that T cell priming had not occurred in the absence of CD40-CD40L costimulation.

It was possible that T cell proliferation was delayed in the absence of this costimulatory signal. Thus, the spleens and draining lymph nodes of mice immunised 15 days previously with peptide-CFA were examined. Again, no antigen specific cells could be detected at this time point, or at the later time points examined (weeks 4 and 6, data not shown).

To test whether priming had occurred at a level below detection by the tetramers, spenocytes from the mice were activated *in vitro* with peptide. The splenocytes were labelled with the intracellular dye CFSE to allow visualisation of the division of the tetramer positive cells. After three days of culture, CFSE low tetramer positive cells were found in the C57BL/6 samples, but the staining in CD40 and CD40L knockout

samples remained at background levels (H19env peptide stimulation of splenocytes from OVA peptide immunised C57BL/6 mice), figure 5.2b and c.

5.2.2. Wild-type dendritic cells restore priming in CD40, but not CD40L knockout mice.

This defect in the initial priming of CD4 T cells in CD40 and CD40L knockout mice meant that the survival and activity of memory cells in the absence of CD40L-CD40 signals could not be evaluated. To overcome this problem, wild-type bone marrow derived DC were used to try to restore priming in the CD40 and CD40L knockout mice.

Bone marrow derived DC were activated with LPS and pulsed with H19env peptide *in vitro*, before they were transferred s.c. (DC-s.c.) into C57BL/6, CD40 and CD40L knockout mice. Six to seven days later, DLN cells and splenocytes were stained with class II tetramer to measure the primary response. The response in both the spleen and DLN was equivalent between C57BL/6 and CD40 knockout mice, figure 5.3. In CD40L knockout mice, in which wild-type DC cannot restore the genetic defect, tetramer staining remained at background levels.

5.2.3. The absence of CD40-CD40L interactions in the recall response does not affect the proliferation of memory T cells

Restoring T cell priming with wild-type DC in the CD40 knockout mice allowed the survival and activity of the memory cells in the absence of CD40 to be evaluated. Exogenous DC do not survive for long after transfer into mice, (see later and refs. (170), (334)) and thus T cells in DC-primed mice would not have contact with antigen, or CD40 in the CD40 knockout mice. The recall response was examined, as in chapter three, by boosting the DC-primed mice with peptide-CFA after 4 to 18

weeks post-DC transfer. Spleens and DNL cells were taken five days after the re-challenge and stained with class II tetramers.

In both the spleen and DLN, the recall response was found to be equivalent between C57BL/6 and CD40 knockout mice, even in the absence of CD40-CD40L costimulation in the recall response in the latter group (figures 5.4 and 5.5). Moreover, in both groups the response was above the primary response in C57BL/6 mice to peptide in CFA, demonstrating that this was a memory response.

Tetramer staining above background levels was detected in the DLN and spleens of CD40L knockout mice that had first been primed with wild-type DC, then boosted with peptide-CFA. However, this response was deficient compared to that in C57BL/6 or similarly treated CD40 knockout mice. Thus, a small number of antigen-specific cells had been primed in the CD40L knockout mice by DC immunisation and could respond to the peptide-CFA boost.

5.2.4. Initial immunisation of CD40 knockout mice with DC intravenously does not restore the secondary response in the DLN

Initially, the recall response had been examined in CD40 knockout mice that had been primed with wild-type DC injected i.v. into the tail-vein (DC-i.v.). In C57BL/6 mice boosted with peptide-CFA, a recall response was found, as above, both in the spleen and in the inguinal LN that drains the CFA injection site. However, in CD40 knockout mice that had been primed with DC-i.v. then boosted with peptide-CFA, a recall response was only measurable in the spleen. In the inguinal LN, tetramer staining remained at background levels, figure 5.6. Thus, when mice were primed with DC-i.v. a memory response in the draining lymph node did not occur in the absence of CD40. The difference in the sites of the recall response following priming with DC- s.c. or -i.v. are summarised in table 5.1:

Strain	Initial DC immunisation	Recall response in spleen	Recall response in DLN
C57BL/6	s.c.	++++ (1.49)	++++ (1.44)
C57BL/6	i.v.	++ (0.24)	+++ (0.42)
CD40 KO	s.c.	++++ (1.36)	++++ (1.54)
CD40 KO	i.v.	++ (0.17)	- (0.055)

Table 5.1: Summary of site of recall response in C57BL/6 and CD40 knockout mice primed with wild-type DC then boosted with peptide-CFA: The relative percentage of tetramer positive cells in the different lymphoid organs have been compared using + symbol with increasing number of + indicating an increase in the percentage of tetramer positive cells present. The means of tetramer positive cells, given as a percentage of CD4 cells, is shown in brackets. This data is from figures 5.5 and 5.6.

There are a number of explanations for this defect in the recall response in the DLN of the CD40 knockout mice:

1. Memory T cells in the CD40 knockout mice were unable to enter LN.
2. Distinct APC populations exist in LN and spleen, the former unable to activate memory cells in the absence of CD40 costimulation.
3. Antigen could reach the spleen, but it was unable to reach the LN from the CFA injection site in the absence of CD40.

Given that CD40 knockout mice primed with DC-s.c. had a recall response in both the spleen and inguinal LN, it seemed unlikely that explanations 2 and 3 could be correct. In experiments in which the antigen-specific response was compared following transfer of DC either s.c. or i.v., similarly percentages of tetramer positive cells were found in the spleens of each group, but tetramer positive cells were only in the LN of mice given DC-s.c., figure 5.7. Therefore, in the recall experiments described above, the site of immunisation, and thus the DLN, in both the primary and recall response was the same. Therefore, it was possible that memory cells primed in

CD40 knockout mice could only migrate to the initial immunisation site: the spleen after DC-i.v. transfer; the inguinal LN and the spleen after DC-s.c injection.

To investigate this, C57BL/6 and CD40 knockout mice were primed with DC-s.c. in the hind-leg. After 7 weeks, the mice were boosted with peptide-CFA injected in the back of the mouse, just below the shoulder blades. This site primarily drains to the axillary LN. 5 days later, tetramer staining was assessed in the spleen, inguinal and axillary LN. As detailed in figure 5.8, tetramer positive cells could be detected in all sites, and the responses were equivalent between C57BL/6 and CD40 knockout mice.

These experiments ruled out explanations 2 and 3. APC in the lymph nodes of CD40 knockout mice were able to present antigen to memory CD4 cells after CFA injection, and therefore, antigen must have reached the LN, either draining there itself in the lymph, or carried by DC from the injection site.

This left the remaining hypothesis: that H19env specific memory T cells in the DC-i.v. primed CD40 knockout mice could not re-circulate through LN. The best way to test this hypothesis would be to transfer the memory cells from DC-primed CD40 knockout mice into C57BL/6 mice and then examine the recall response. However, as detailed in chapter three, such transfer experiments proved to be inconsistent.

Thus, an alternative way in which to track the migration of antigen-specific memory cells in the CD40 knockout mice was devised. As detailed in chapter three, it was not possible to detect tetramer positive cells *ex vivo* in DC immunised mice after day 20. However, by boosting DC-primed mice with peptide-CFA, the resultant memory pool could be tracked *ex vivo*. Therefore, the memory pool after the secondary immunisation was examined to establish both the size and the re-circulatory characteristics of the memory cells in C57BL/6 and CD40 knockout mice.

5.2.5. *The size of the memory pool following the recall response is dependent on the original site of DC immunisation*

C57BL/6 and CD40 knockout mice, which had been immunised either i.v. or s.c. with wild-type DC, were left for 8 weeks, then re-challenged with peptide-CFA. Only after a further 8 weeks was tetramer staining of splenocytes, and the cells in the DLN and non-DLN carried out and the characteristics of this "secondary" memory pool examined.

The results of this experiment are detailed in figure 5.9 and summarised in table 5.2. Tetramer positive cells were found in all test groups, demonstrating that the recall response in CD40 knockout mice resulted in the survival of the activated cells into the "secondary" memory pool. However, there were distinctions between the size and migration characteristics of the memory pools in the different groups.

Strain	Initial DC immunisation route	Tetramer +ve cells in spleen	Tetramer +ve cells in DLN	Tetramer +ve cells in non-DLN
C57BL/6	s.c.	++++ (0.46)	+++++ (0.67)	+++ (0.27)
C57BL/6	i.v.	++ (0.30)	++++ (0.41)	- (0.046)
CD40 KO	s.c.	++ (0.26)	++ (0.21)	+ (0.088)
CD40 KO	i.v.	+ (0.1)	+ (0.1)	- (0.031)
C57BL/6-CFA only	-	++ (0.15)	++ (0.29)	- (0.054)

Table 5.2: Summary of presence of tetramer positive cells in the secondary memory pool in C57BL/6 and CD40 knockout mice immunised with wild-type DC then boosted with peptide-CFA: The relative percentage of tetramer positive cells in the different lymphoid organs have been compared using + symbol with increasing number of + indicating an increase in the percentage of tetramer positive cells present. The means of tetramer positive cells, given as a percentage of CD4 cells, is shown in brackets. This data is from figure 5.9.

C57BL/6 mice that had been immunised s.c. with wild-type DC then boosted with peptide-CFA, had a larger memory pool in the spleen, DLN and non-DLN than mice that were only immunised with peptide in CFA once. This may be because the recall response is larger than the primary response and therefore more cells survive into the subsequent memory pool. This finding corresponds to several reports that the size of the memory pool is determined by the size of T cell expansion during the response (130), (131).

However, C57BL/6 mice that had been initially immunised with DC-i.v., did not have a significantly larger memory pool after secondary immunisation than mice immunised with peptide-CFA alone. This agrees with the less dramatic increase in the secondary response after initial DC-i.v. priming compared to DC-s.c. priming (compare figure 5.5 and 5.6). Similarly, CD40 knockout mice immunised with DC-i.v. had a smaller memory pool than CD40 knockout mice initially primed with DC-s.c. Thus, the initial priming site determines the size of the secondary response and thereby determines the size of the "secondary" memory pool.

5.2.6. CD40 expression determines the size and migratory patterns of memory cells

This experiment also showed that there were differences in the size of the secondary memory pool between C57BL/6 and CD40 knockout mice. Comparing the memory pool in C57BL/6 and CD40 knockout mice that had been initially immunised s.c. with wild-type DC, it can be seen (figure 5.9) that there was a bigger antigen-specific memory pool in the C57BL/6 mice in all lymphoid organs examined. This suggests that even though the recall response at day 5 after the re-challenge was equivalent in C57BL/6 and CD40 knockout mice, either more cells were activated over the course of the recall response or more memory cells survived into the secondary memory pool in the C57BL/6 mice. Similarly, more tetramer positive cells were found in C57BL/6 mice initially immunised with DC-i.v. than in CD40 knockout mice immunised with the same DC-i.v.

There were also differences in the migratory patterns of the antigen-specific memory cells in the different groups of mice. Tetramer positive cells were clearly present in the DLN of both groups of C57BL/6 mice (immunised with DC either i.v. or s.c.) and also in CD40 knockout mice immunised with DC-s.c. However, very few tetramer positive cells were found in the DLN of CD40 knockout mice immunised with DC-i.v. and no tetramer positive cells were found in the non-DLN of these mice. Thus, the migration of the memory cells was deficient in CD40 knockout mice primed with DC-i.v. and boosted with peptide-CFA.

5.2.7. Exogenous DC can be identified after s.c. but not after i.v. injection

This distinction in the response depending on the route of transfer of DC was interesting. To investigate whether there were differences in the migration and survival of the DC following transfer by the two different routes, the injected DC were tracked using the Ly5.1 congenic marker.

Preliminary experiments in C57BL/6 mice found that Ly5.1-DC could easily be detected in the DLN *ex vivo* at 2 days after s.c. transfer, figure 5.10. By day 5, however, very few, if any, DC could be identified even after the selection of CD11c positive cells using labelled magnetic beads (data not shown). No exogenous DC were identified in the spleen at either time point. After i.v. transfer, no exogenous DC could be found in the spleen, again, even after selection for CD11c positive cells, figure 5.10. Given that the DC injected i.v. could not be identified, further experiments tracking them in CD40 knockout mice were not carried out.

5.2.8. IFN-γ production by CD4 cells is reduced in CD40 knockout mice in both the primary and recall response

T cell proliferation is only one component of the immune response. To establish whether the restored T cell proliferation in the CD40 knockout mice was accompanied by an equivalent cytokine response, intracellular cytokine staining was performed. No antigen-specific cytokine (IFN γ, IL-2, TNF-α) could be measured after a peptide-pulse of splenocytes or LN cells *ex vivo* for between 6 to 72 hours. Presumably, this was because the number of specific cells was low.

To provide an indication of the cytokine production in immunised C57BL/6 and CD40 knockout mice, cells were activated with PMA and ionomycin for 4 hours *ex vivo* in the presence of Golgi stop. The cytokine production by total CD4 cells was then examined.

The production of IL-2 and the effector cytokine, IFN-γ, was examined in mice that had received only peptide-DC, or had also been boosted with peptide-CFA. The production of IL-2 by total CD4 cells was equivalent in C57BL/6 and CD40 knockout mice after priming with DC and boosting with CFA, figure 5.11a, but in all cases the percentage of CD4 cells making IL-2 was greater than that from naïve splenocytes activated with PMA and ionomycin.

In the primary response, the percentage of CD4 cells from C57BL/6 primed with peptide-pulsed DC making IFN-γ was greater than that in naïve mice. However, the response in DC-primed CD40 knockout mice was reduced compared to response in the C57BL/6 mice and was not above that in naïve mice, figure 5.9b. This was true if the mice were injected with the DC-s.c. or –i.v.

In the recall response a similar result was found. The percentage of CD4 cells making IFN-γ was much greater in the C57BL/6 mice that had initially been given DC then were boosted with peptide-CFA than that in the primary response (DC alone), figure 5.11b and 5.12. IFN-γ production by CD4 cells from the boosted CD40 knockout mice was slightly above the primary response in CD40 knockout mice and above the level of IFN-γ production in naïve splenocytes. However, the percentage of CD4 cells making IFN-γ in the recall response in the CD40 knockout mice was greatly reduced compared to that in the recall response in C57BL/6 mice.

5.2.9. T cell responses are normal in mice in which the CD40 defect is limited to B cells

The above result suggested that although exogenous wild-type DC could restore T cell priming and memory cell generation in CD40 knockout mice, they were unable to induce full effector T cell differentiation. This suggested a role for endogenous APC in sustaining and amplifying the T cell response. B cells can play a role in T cell responses: some investigators have found either reduced T cell activation or deficient memory cell survival in μMt mice (136), (137). Moreover, as the immune response progresses and DC begin to die, B cells may become the major APC population. B cell may, therefore, be important in the latter stages of the T cell response, including T cell differentiation.

T cell CD40L signals to B cells are essential for productive B cell responses (313), (335). To investigate whether this interaction is also important for the CD4 T cell response, in particular for effector cell differentiation, bone marrow chimeras were made in which the CD40 defect was limited to B cells. These chimeras were made by first lethally irradiating C57BL/6 hosts and then reconstituting them with bone marrow from μMT mice (80%) and CD40 knockout mice (20%). This protocol is illustrated in figure 5.13. Thus, B cells can only develop from the CD40 deficient bone marrow while the majority of other APC, such as DC and macrophages, develop from the μMT bone marrow and thus are CD40 sufficient (270), (60).

Control, wild-type chimeras were made by reconstituting irradiated C57BL/6 mice with 80% μMT bone marrow and 20% C57BL/6 bone marrow. The chimeras were used after 8 weeks to allow for the loss of radiosensitive cells and for the establishment of transferred bone-marrow cells (see materials and methods for confirmation of chimerism). All the chimera experiments were carried out in collaboration with Alison Crawford, another PhD student in David Gray's laboratory.

Initial experiments were carried out to establish whether DC transfer induced a population of tetramer positive cells. CD40 knockout B cell and wild-type control chimeras were immunised with wild-type DC-s.c. at the hind-leg. The primary response in the spleen at day 7 was equivalent in the two sets of chimeras, figure 5.14. However, in the DLN, the response in the CD40 knockout B cell chimeras was greater than that in the control chimeras. This appeared to be because the response in the control chimeras was smaller than expected; it was not statistically above background staining.

Subsequent experiments were unable to substantiate or explain this finding. In the following two experiments, the primary response in both sets of chimeras was very low, not even above background staining. This was true after various immunisation strategies: DC injected i.v. or s.c., or immunisation with peptide-CFA (data not shown). This poor priming was thought to be a consequence of the chimeras being reconstituted with 80% μMT bone marrow. μMT mice have small spleens and LN and this was also true of the chimeras (a typical spleen had approximately $3\text{-}4\text{x}10^7$ cells compared to $6\text{-}8\text{x}10^7$ in a wild-type C57BL/6 mouse). This small number of cells may have meant that the number of tetramer positive cells activated following immunisation was much lower than in C57BL/6 mice and below the level of detection by the class II tetramers.

5.2.10. The recall response is equivalent in B cell CD40 knockout chimeras and wild-type chimeras

Despite the poor primary response in the chimeras, the recall response was examined. As before, DC-primed mice were boosted with peptide-CFA 10 weeks after the initial DC-transfer, and the spleen and DLN stained with class II tetramers 5 days later. The recall challenge established that T cell priming had taken place. As predicted from the experiments in complete CD40 knockout mice primed with DC-s.c. the recall response was equivalent in the wild-type and B cell CD40 knockout chimeras in both the spleen and DLN, figure 5.15. The chimeras were also immunised with DC-i.v. to investigate the site of the recall response as this was defective in complete CD40 knockout mice primed with DC-i.v. Unfortunately, no recall response was apparent in either the spleen or the LN of these chimeras in the two experiments carried out (data not shown).

5.2.11. IFN-γ production by CD4 cells is similar in CD40 knockout B cell and wild-type chimeras

Given the deficiency in IFN-γ production by CD4 cells in the primary and recall response in the CD40 knockout mice, the cytokine response in the CD40 knockout B cell and wild-type chimeras was examined during both the primary and recall responses. The results for the primary immune response are from the one experiment in which tetramer positive cells were found. Splenocytes were activated *ex vivo* for 4 hours with PMA and ionomycin in the presence of Golgi stop. The IL-2 response in the two sets of chimeras was the same in the primary and also in the secondary response, figure 5.16a. The IFN-γ response after priming with wild-type DC was equal in CD40 knockout B cell chimeras and wild-type chimeras, figure 5.16b. Moreover, in CD40 knockout B cell and wild-type chimeras that had been immunised with DC then boosted with H19env-CFA the IFN-γ response was similar, figure 5.16b. This shows that interactions with CD40 sufficient B cells were not required for IFN-γ production by CD4 T cells.

The role of ICOS-B7h interactions in T cell priming

5.2.12.ICOS-B7h interactions are not required for T cell priming, memory cell generation or survival

The ICOS knockout mice used in this PhD were generated in David Gray's laboratory by Ana Cevera in the 129 strain of mice. Most of the experiments were carried out when the mice were in the process of being back-crossed onto the C57BL/6 background and therefore the responses in the knockout mice were compared to those in wild-type littermates. However, some of the DC-priming experiments were carried out using knockout mice that had been back-crossed 7 times onto the C57BL/6 background and thus C57BL/6 mice were used as wild-type controls.

To examine the requirement for ICOS-B7h interactions in CD4 T cell priming, ICOS knockout mice were immunised with peptide-CFA and the tetramer positive cells examined at various times post-immunisation. The percentage of tetramer positive cells in the spleen and DLN of ICOS knockout mice was the same as that in wild-type mice at day 8 and at day 15, figure 5.17a. Thus, T cell priming was not reduced in the absence of ICOS signalling.

To investigate whether the generation and survival of long-lived memory cells was affected by the absence of ICOS signalling, tetramer staining in the spleens and LN from mice that had been immunised 6 or 10 weeks earlier was examined. As shown in figure 5.17b, the survival of antigen specific memory cells 10 weeks after immunisation was unaffected by the lack of ICOS.

5.2.13. The primary immune response is normal, but the secondary response is slightly reduced, in the absence of ICOS after immunisation with wild-type DC

CFA is a potent Th1 inducing adjuvant and it has been suggested that immunisation with CFA can bypass any defects in ICOS knockout mice (332). Thus, as with the CD40 and CD40L knockout mice, ICOS knockout mice were also primed with wild-type peptide-pulsed DC.

The primary response to WT-DC in ICOS knockout mice was equivalent to that in C57BL/6 mice in both the spleen and DLN, figure 5.18. The recall response, which demonstrates the survival and functional response of memory cells, was measured by first priming the ICOS knockout mice with wild-type DC then resting the mice for 10 weeks before boosting with peptide-CFA. This experiment was done twice with similar results. In the first experiment there was no significant difference in the percentage of tetramer positive cells in either the spleen or the DLN of the knockout and littermate controls. However, in both organs there was a trend towards a reduced response in the knockout mice. In the second experiment, carried out in back-crossed mice, this difference was significant in the DLN but not the spleen, although again there was trend towards less tetramer positive cells in the spleen of the knockout mice, figure 5.19. This suggests that ICOS-B7h interactions may play small role in the reactivation of memory CD4 T cells.

5.2.14. Cytokine production in the primary response is unaffected in the absence of ICOS, but is reduced in the recall response

The cytokine response of CD4 cells in ICOS knockout mice either primed with wild-type DC or also boosted with peptide-CFA was examined as before by activating cells *ex vivo* with PMA and ionomycin in the presence of Golgi stop. In the primary response to pepetide-pulsed DC there was no difference in the production of IL-2 or

IFN-γ production by CD4 cells from C57BL/6 and ICOS knockout mice (data not shown). Similarly, after the recall response no differences were found in IL-2 or IFN-γ production. However, in the second experiment, using the backcrossed mice, there was a general trend towards reduced cytokine production (IFN-γ IL-10, IL-2) but this was only significant for IL-2 producing cells in both the spleen and LN and IFN-γ producing cells in the DLN only, figure 5.20.

5.3. Discussion

The ability to track activated and memory cells using class II tetramers provided the opportunity to ask questions about the requirements for costimulatory signals in T cell responses. Using the protocols established in chapter three and costimulation knockout mice, the requirements for CD40L-CD40 and ICOS-B7h interactions were evaluated.

The advantage of using class II tetramers is that priming and recall responses could be examined *ex vivo* rather than relying on indirect restimulation assays. Although experiments using TCR transgenic cells have examined the requirements for various costimulatory molecules *ex vivo,* this PhD has focussed on the requirements for CD40L-CD40 and ICOS-B7h costimulation on the activation of physiological numbers of endogenous antigen-specific T cells. This is important because a large precursor frequency may bypass the need for costimulation and a monoclonal population may not react in the same way as a polyclonal population made up of T cells with various TCRs with different affinities for the antigen.

5.3.1. CD40-CD40L signals are required to prime naïve CD4 T cells

Antigen-specific CD4 T cells could not be detected in either CD40 or CD40L knockout mice immunised with H19env-CFA at any of time points examined, or after *in vitro* restimulation. There are a number of possible explanations for this defect. Moodycliffe *et al* have reported a requirement for a CD40L signal to DC in the skin to induce their migration to DLN (336). If DC are unable to migrate to LN, T cell activation would be much reduced. This explanation, however, is not sufficient to explain the results reported here. First, antigen may itself reach the DLN from immunisation sites (279), (22). Second, in the recall response in CD40 knockout

mice, the memory cells were reactivated, thus antigen presentation must take place in the absence of CD40-CD40L interactions. However, the APC that present antigen to naïve and memory cells may be distinct. Croft *et al* found that macrophages and B cells could present antigen to memory cells *in vitro* (161) and Crowe *et al* demonstrated that macrophages can play an important role as APC in the secondary response to influenza infection *in vivo* (237).

The small recall response in CD40L knockout mice, which had been immunised with wild-type DC then boosted with peptide-CFA, suggests that these signals are not absolutely required for CD4 activation and memory cell generation. However, in the absence of this signal, priming was greatly deficient and very few memory cells were generated. These data may seem to contradict the peptide-CFA immunisation results. However, this difference could be because the injected DC provided increased costimulatory signals compared to endogenous DC in the peptide-CFA immunised CD40L knockout mice.

Moreover, these differences may be because there are other ligands that bind CD40 and thus substitute for CD40L. For example, Lazarevic *et al* (35) and Wang *et al* (337) have found that heat shock protein 70 can bind to CD40 on DC or monocytes and induce them to produce IL-12 or chemokines, respectively. Also, Brodeur *et al* found that C4 binding protein, a regulatory component of the complement system, can bind to CD40 on human B cells and activate them (338).

That a small population of tetramer positive cells were activated in CD40L knockout mice, supports a further explanation for the defect in priming in the knockout mice: that the T cell–DC interaction is curtailed in the absence of this costimulatory interaction. The CD40-CD40L interaction between DC and T cell is not an initial T cell activating event as CD40L is primarily expressed on activated T cells rather than naïve T cells (312). For example, the adoptive transfer of CD40L knockout

transgenic cells into wild-type mice followed by immunisation, results in a brief proliferative response followed by a crash of the transgenic cells (34). Miga *et al* argue that the CD40 signal to the DC is required for full activation and to keep the DC alive in the lymphoid organ and that this is necessary for sustained T cell activation (315). For example, CD40-CD40L signalling results in increased expression of costimulatory molecules such as CD80 and CD86 and also greatly enhance DC IL-12 production (32), (33). Moreover, Straw *et al* found that CD40 signals to DC were required to maintain DC activation during both *Schistosoma mansoni* and *Toxoplasma gondii* infections (339).

In this PhD, using tetramers to examine the T cell response, even this brief proliferative response was not observed. This may be because such a response was below the tetramer detection level. Furthermore, interactions between T cells and DC in the absence of CD40 are thought to lead to tolerance rather than productive T cell activation (320), (321). This may explain why, even if there was a small response in the knockout mice to peptide-CFA immunisation, this would not be measurable after *in vitro* restimulation as the specific cells would be tolerant.

5.3.2. Proliferation of memory CD4 T cells does not require CD40-CD40L interactions

Restoration of the primary response in CD40 knockout mice enabled the examination of the survival of memory cells and the recall response in a CD40 deficient environment. Both of these were examined by boosting the DC-primed mice with peptide-CFA as described in chapter three.

There have been previous reports that CD4 memory cells require less costimulation than naïve cells, however the majority of these experiments have been carried out *in*

vitro (9), (161), (278). The findings reported in this PhD validate these studies, but advanced them as the work shown here was done entirely *in vivo*.

A recall response was measurable in the CD40 knockout mice and was equal to that in similarly treated C57BL/6 mice. This demonstrates a number of points: 1. the cells activated in CD40 knockout mice in response to wild-type DC, developed into memory cells; 2. these memory cells survived in a CD40 deficient environment; 3. the memory cells were functional as they proliferated in response to antigen re-challenge; and 4. the cells divided in the absence of CD40-CD40L signals, demonstrating a qualitative difference between naive and memory CD4 T cells.

The reason why memory cells require less costimulation than naïve T cells may be because they have a lower activation threshold and this may be a consequence of changes at both an intra- and extracellular level. As discussed in chapter three, the memory cell population may have TCRs that interact with peptide-MHC with greater affinity than naïve cells (211), (208). However, Slifka and Whitton found that although activated and memory CD8 cells were more sensitive to activation than naïve cells, the affinity of the TCR for peptide-MHC remained unchanged (207). Rather, they argue, activated and memory cells had increased levels of the signalling kinase Lck, making TCR signalling faster and more efficient.

Farber and colleagues have also reported alteration in the signalling complexes of memory CD4 cells including changes in the overall level of phosphorylation of Zap-70 and CD3ζ-associated proteins and this may allow memory cells to respond differently from naïve cells (340), (145), (341). Other intracellular changes also allow memory cells to respond faster. Veiga-Fernandes *et al* found that memory CD8 T cells are at a different state in the cell cycle to naïve cells, allowing them to enter the cell cycle more rapidly (342). Memory and effector cells can also make effector responses without further division. This is due to the presence of pre-formed

messenger-RNA (mRNA) for effector molecules such as IFN-γ and perforin in the cytosol and changes at the DNA level that allow rapid transcription of mRNA for effector cytokines without further division (279), (343).

5.3.3. The route of injection of DC affects the site of the recall response

The T cell response in CD40 knockout mice immunised with wild-type DC did not, however, entirely correspond to similarly treated C57BL/6 mice. Specifically, the initial immunisation of CD40 knockout mice with wild-type DC-i.v resulted in a defective recall response in the DLN. The recall response in the spleen of these CD40 knockout mice was equal to that in similarly treated C57BL/6 mice and thus functional memory cells were present in these mice. This defect could be overcome by immunising the CD40 knockout mice with wild-type DC-s.c.

This distinction was intriguing. As discussed in the results section, this defect was not a result of the failure of antigen presentation. Rather, it appeared that memory cells in DC-i.v. primed CD40 knockout mice were unable to traffic through LN. The most conclusive test of this would be to transfer memory cells from DC-i.v. primed CD40 knockout mice into wild-type hosts and then examine the site of the recall response in the recipient mice. However, such cell transfer experiments were not possible due to the problems with adoptively transferring tetramer positive cells as described in chapter three.

5.3.4. Migration of memory cells in DC-i.v. primed CD40 knockout mice is defective

As these experiments could not be carried out, an alternative way in which to track the migration of the memory cells was devised. By boosting the DC-primed mice with peptide-CFA, it was possible to identify the tetramer positive memory cells *ex vivo*, enabling the examination of the migration of this "secondary" memory population.

The results from this experiment substantiate the hypothesis that memory cells in CD40 knockout mice immunised with DC-i.v. were unable to migrate through LN. Very few memory cells were found in the DLN 8 weeks after boosting with H19env-CFA in these mice. However, memory cells were clearly visible in LN of CD40 knockout mice initially immunised with DC-s.c., or in C57BL/6 mice immunised with DC-i.v. or –s.c.

This defective migration is comparable to results described by Mullins *et al* (344). The authors have studied the use of peptide-pulsed DC as vaccines against tumours. After s.c. priming with DC, tumours at s.c. sites were rejected and this was mediated by tumour-specific CD8 T cells. However, if mice were first treated with DC injected i.v., s.c. tumours were not controlled.

The tumour-specific memory cells could be reactivated by a recombinant vaccina virus that expressed the tumour peptide. In mice immunised with DC-s.c. a recall response to the virus was found in various LN and the spleen. However, in mice given DC-i.v., T cell priming was only found in the spleen and the recall response to the virus was also restricted to this organ.

A similar finding was described by Eggert *et al*, however this group also report that in a tumour model in which the antigen was more immunogenic, i.v. injection of peptide-pulsed DC was protective (334). Therefore, perhaps the immunogenicity of the antigenic stimuli determines the pattern of migration of the memory cells. It is of note that Mullins *et al* only transferred 10^5 DC. It would be interesting to know if increasing the number of DC injected i.v., resulted in an increase in the primary response and a subsequent recall response at multiple sites.

Mullins *et al* argue that their results indicated that different subsets of memory cells were generated after i.v. and s.c. injection of DC, such that memory cells primed in the spleen can only migrate back to the spleen (344). The generation of different subsets of memory cells has been an area of much research in recent years and the existence of tissue specific memory cells has also been investigated. For example, T cells primed by DC from Peyer's patches went on to express the gut homing intergrin, $\alpha_4\beta_7$ (67). Campbell and Butcher also found that cells primed in the intestine expressed $\alpha_4\beta_7$ and those primed in the skin draining lymph nodes expressed the skin homing molecule CD62P ligand (68). Therefore, the site of T cell priming can determine the cell's re-circulatory characteristics by influencing which homing molecules are expressed on the activated or memory cell. However, *in vivo* work from the labs of Leo Lefrançois (CD8 memory (13)) and Marc Jenkins (CD4 memory (14)) suggest that memory cells are able to migrate to any tissue.

Lefrançois and colleagues have studied the migratory capacity of memory CD8 T cells either by tracking memory cells *ex vivo* (13), (345), (346). The results from these experiments have found that cells from any tissue were able to migrate to other tissues after transfer to naïve mice. Similarly, Reindhardt *et al* found transgenic memory CD4 cells distributed throughout the body after i.v. injection of antigen with adjuvant (14). Thus, CD4 cells can also enter various tissue sites. In similar experiments by the same group examining the distribution of activated cells following a localised injection of antigen in adjuvant, the transgenic cells were found

primarily in the injection site, although some transgenic cells were also present in the lung and other tissues (347). Activated cells, thus, tend to home back to the site of immunisation, however, as memory time-points were not examined in this study, the migration patterns of memory cells remains an area of debate.

Reindhardt *et al* did determine what signals were required for the migration of activated T cells to the injection site by examining the response in CD62E and CD62P knockout mice (347). CD62E and CD62P are expressed on inflamed endothelial cells and are required for the migration of activated cells into the skin (348), (68), (349), (64). Reindhardt *et al* found the number of transgenic cells at the injection site was reduced in CD62E knockout mice, suggesting that this signal was required to allow the migration of activated T cells into the inflamed injection site. Moreover, the number of transgenic cells was also reduced in the DLN. The transgenic cells in the DLN are made up of two populations: those activated at this site and cells that have returned from the injection site via the afferent lymph. The authors argue the reduced number of transgenic cells in the DLN was not a result of reduced proliferation, but a result of the decreased accumulation of transgenic cells at the injection site and the subsequent diminished drainage back from the injection site in the afferent lymph.

In this PhD, the memory cells found in C57BL/6 and CD40 knockout mice following secondary immunisation were all $CD62L^{lo}$. Thus, these memory cells presumably enter LN via the afferent lymph draining from tissues rather than through HEV (350) and therefore, these cells, would be required to migrate into tissues. The memory cells, therefore, must enter tissue sites and then drain into the afferent lymph. This may require the expression of ligands that bind to CD62E and CD62P.

Why memory cells in DC-i.v. primed CD40 knockout mice were unable to migrate into tissues was not examined in this PhD, however, it is interesting to speculate

what may cause this defect. Tietz *et al* found that the ligands for CD62E and CD62P were only expressed transiently on activated T cells and were not enriched in memory T cells (66). This suggests that the migration patterns of memory cells may not have been determined by the expression of these ligands. However, Lefrançois and Masopust suggest that entry of activated T cells into tissues may be required for the differentiation of memory cells that reside in tertiary tissues (154). Thus, if the ligands for CD62E and CD62P are not upregulated, T cells may be unable to enter tissues and acquire this phenotype.

Therefore, the defect in the CD40 knockout mice primed with DC-i.v. may be due to an inability of the activated and memory T cells to migrate to tertiary tissues rather than a direct deficiency in migration through LN. That tetramer positive memory cells in C57BL/6 mice primed with DC-i.v. were found in LN suggests that the two routes of injection do not necessarily prime different sub-sets of memory cells as argued by Mullins *et al* (344). Rather, the ability of memory cells to migrate out-with the secondary lymphoid system appears to be dependent on both the route of DC injection and the expression of CD40 on endogenous DC.

5.3.5. Size of the memory pool is also determined by the immunisation site

The route of injection of DC also affected the size of both the recall response and the size of the subsequent secondary memory pool. In C57BL/6 mice initially primed with DC-s.c., the recall response and the "secondary" memory pool was greater than that in C57BL/6 mice primed with DC-i.v. (see figures 5.5, 5.6, 5.9).

In experiments in which the number of tetramer positive cells primed in C57BL/6 mice after DC-i.v. or DC-s.c. transfer were directly compared (see figure 5.7), the response in the spleen was comparable. The cells in the spleen of DC-s.c. mice may have either been primed at this site by DC that migrated in the blood from the

injection site to the spleen, or activated T cells from the DLN may themselves migrate to the spleen. Tetramer positive cells were also found in the LN of mice primed s.c., while no tetramer positive cells were found in LN of DC-i.v. primed mice. Thus, in total, more tetramer positive cells were primed in DC-s.c. immunised mice.

Attempts were made to track the injected DC to visualise whether they migrated, and or survived, differently after the two routes of injection and whether this may explain the differences in the levels of T cell priming. Pilot studies in C57BL/6 mice showed that although DC could be tracked after s.c.-transfer, even 2 days after i.v.-transfer, no DC could be detected in the spleen. This was presumably because of the massive size difference between LN and spleens with a typical LN containing 1-3x10^6 cells and a spleen containing 5-8x10^7 cells. It could be argued, therefore, that s.c. immunisation was more potent than i.v. immunisation as the responding T cells had more chance of making multiple contacts with the presenting DC in the LN than in the spleen. Recent studies using 2-photon microscopy to study T cell-DC interactions during the early stages of T cell activation, have indeed found that T cells form multiple contacts with APC (23), (24).

The difference in the size of the secondary memory pool following the different routes of immunisation was probably a result of the increased size of the recall response in mice initially primed with DC-s.c. compared to that in DC-i.v. primed mice (compare figures 5.5 and 5.6). Consequently, the number of cells that survived into the secondary memory pool was greater in mice initially given DC-s.c. compared to mice given DC-i.v. These data (increased total number of tetramer positive cells in the primary response, increased recall response and increased size of the secondary memory pool) indicate that the transfer of DC-s.c. is a much more potent immunisation route than i.v. administration of DC.

5.3.6. CD40 on endogenous APC also affects the size of tetramer positive memory pool

In addition to this difference in the size of the memory pool in C57BL/6 mice primed with DC-i.v. or DC-s.c, and boosted with peptide-CFA, there were differences in the memory pools in C57BL/6 and CD40 knockout mice regardless of the injection route. More tetramer positive cells were found in C57BL/6 mice compared to similarly treated CD40 knockout mice. This differences may be because the recall response in the C57BL/6 mice was more sustained than that in the CD40 knockout mice. This could be demonstrated by a close kinetic examination of the recall response, in particular of the contraction phase.

There are two non-mutually exclusive reasons why the response may be more sustained in the C57BL/6 mice. First, the memory cells may proliferate more as they have access to more costimulation than memory cells in CD40 knockout mice. Second, naïve H19env-specific T cells could be activated during the recall response in C57BL/6 mice and thereby increase the number of cells in the secondary response and the number of cells that could progress into the subsequent memory pool. Badovinac *et al* have described the activation of both memory and naïve CD8 T cells during re-challenge and, therefore, it is not surprising that both types of T cell could get activated (288).

In CD40 knockout mice, naïve cells would be unlikely to participate in the recall response given that peptide-CFA could not prime naive antigen-specific cells in the absence of CD40-CD40L interactions (figure 5.2). This suggests that memory cells can proliferate in response to re-challenge and then become resting memory cells again. The most conclusive way to demonstrate this would be to transfer Ly5.1 H19env-specific memory cells into C57BL/6 or CD40 knockout mice, boost the

recipient mice, and then examine whether the memory pool contained a mixture of Ly5.1 positive and negative antigen-specific memory cells.

5.3.7. IFN-γ, but not IL-2 production by CD4 cells is reduced in CD40 knockout mice during the primary and recall response

Given that the exogenous DC were unable to fully restore the T cell response in terms of the number and migration capacity of primed cells in the CD40 knockout mice, it was interesting to establish whether full effector cell differentiation occurred. As described in the results section, peptide specific cytokines could not be measured. Moreover, attempts to perform both cytokine and tetramer staining on PMA/Ionomycin stimulated cells were unsuccessful.

Therefore, the cytokine response of CD4 cells as a whole was examined *ex vivo*. IL-2 production was found to be equivalent in C57BL/6 and CD40 knockout mice that had been primed with DC and in mice boosted with peptide-CFA. However, the effector cytokine response, as determined by IFN-γ production, was reduced in primed and boosted CD40 knockout mice. Thus, although similar numbers of antigen-specific cells were found, the effector response in the CD40 knockout mice was deficient.

Although this does not show antigen-specific cytokine responses, there is a clear difference in IFN-γ production after the recall response in C57BL/6 mice compared to the primary response in these mice ($p= 0.008$). The IFN-γ response in the CD40 knockout mice is only slightly increased in the recall response compared to the primary response in CD40 knockout mice ($p=0.032$). Therefore, in C57BL/6 mice, the large proliferative response by the tetramer positive cells is accompanied by a large increase in IFN-γ production by CD4 cells. However, in the CD40 knockout

mice, despite a similar level of proliferation by antigen-specific T cells, the CD4 cell IFN-γ response was greatly reduced compared to that in the C57BL/6 mice.

As discussed in the introduction, the lineage of memory cells in an area of much debate (143), (144), (145), (146), (147). The results in this PhD do not rule out either the linear or the non-linear pathway of memory cell development (see figure 1.2) but imply that memory cells can be generated in the absence of full effector cell differentiation.

Other investigators, who have studied polyclonal CD4 cells responding at similar levels to those described in this PhD, have examined cytokine production by CD4 cells after *in vitro* restimulation with antigen. Reddy *et al* restimulated cells twice and then examined the production of cytokines by total CD4 cells after further stimulation for a few hours with anti-CD3 and anti-CD28 (253). Similarly, Bischof *et al* made short-term T cell lines by restimulating cells multiple times and then examining tetramer and cytokine staining by intracellular staining (257).

PMA activates protein kinase C and ionomycin is a calcium ionophore that causes the mobilisation of intracellular calcium. These signals mimic those induced by TCR signals and the resultant cytokine production is from cells that were previously committed to making that particular cytokine. This *ex vivo* picture of cytokine production was considered to be more relevant for this PhD than the cytokine production of cells activated several times *in vitro*, which can alter their cytokine profile.

An alternative way in which to examine cytokine production by antigen-specific cells has been described by Marc Jenkins and colleagues (14). OVA peptide was injected i.v. into mice containing OVA-specific memory cells and the cytokine production by these memory transgenic cells examined without further stimulation, two hours after

this injection. This, however, may not be a successful approach to determine cytokine production by the tetramer positive cells in this PhD for two reasons. First, even at the peak of the recall response only 1-2% of CD4 cells are tetramer positive and presumably not all of these cells would make cytokines. Thus, IC staining may not be sensitive enough to measure this response. Second, specific activation can cause the transient down-regulation of TCR (351) and thus cytokine production and tetramer staining could not be carried out on cells from the same mouse.

A second approach would be to examine cytokine production using a more sensitive assay. ELISPOT assays enable the identification of the number of cells producing a particular cytokine and this would, therefore, be more useful than an ELISA assay. However, both these assays require an *in vitro* restimulation period and thus do not indicate cytokine production directly *ex vivo*.

It is possible that T cells in CD40 knockout mice differentiated into Th2 cells rather than IFN-γ producing Th1 cells. For example, Stüber *et al* found an increase in IL-4 production following the administration of a blocking anti-CD40L antibody to mice in a murine colitis model (352). Although staining for IL-4 production by CD4 cells was carried out, little or no positive cells were found in any of the experiments. However, as antigen specific cytokines could not be detected, no definite conclusions can be reached.

5.3.8. B cell CD40 is not required for T cell proliferation or cytokine production

The differences in the responses in CD40 knockout and C57BL/6 mice immunised with wild-type DC, suggests that endogenous APC expressing CD40 may play a role in the subsequent immune response and memory cell generation. To investigate which APC may be important for this, experiments were carried out in chimeric mice in which the CD40 defect was limited to B cells.

Unfortunately, the primary immune responses in both wild-type and B cell CD40 knockout chimeric mice were low and thus it is difficult to draw firm conclusions from this data. This low priming may be a consequence of the small lymphoid organs and the primary response may have been below the level of detection by the class II tetramers.

However, as the recall responses were much larger than the primary responses, tetramer positive cells could clearly been seen after re-challenge. Confirming the results in the complete CD40 knockout mice, the recall response in the B cell CD40 knockout chimeras was similar to that in wild-type chimeras.

In experiments in which the chimeras were given DC-i.v., no recall response was found in either the DLN or the spleen and thus, the potential role for endogenous DC in inducing the migratory capacity of memory T cells could not be ascertained. Again, any antigen-specific cells may have been below the level of detection by the tetramers and this result again demonstrates that DC-s.c. administration is a more potent immunisation route than the injection of DC-i.v.

Full IFN-γ production was found in chimeric mice in which the CD40 defect was restricted to B cells alone. Thus, interactions with other APC, presumably

endogenous CD40 positive DC, were sufficient to allow full effector cell differentiation. However, interactions between T and B cells via CD40L:CD40, were not required at any stage of T cell differentiation.

B cells may have played a more essential role in the T cell responses if a different type and form of antigen had been used. For example, B cell may pick up soluble antigen (353), (354) and interact with T cells, or coupling the peptide to a hapten, such as DNP, or using a protein antigen may have "encouraged" a more prominent role for B cells (355), (356).

5.3.9. CD40 conclusion

Taking together the above results (the defective recall response in DC-i.v. primed CD40 knockout mice and the reduced effector response in the complete knockout mice but normal cytokine production in the B cell CD40 knockout chimeras) suggests that there is a role for endogenous DC after immunisation with exogenous DC. The endogenous DC may not necessarily be required for the full expansion of antigen-specific cells as the primary responses in both s.c. and i.v. immunised C57BL/6 and CD40 knockout mice were generally the same. However, there may be a role for endogenous DC in providing sustained T cell activation allowing for full effector cell generation and acquirement of migratory potential. DC may not be the only APC involved, although the chimera experiments ruled out a role for B cells in this system, macrophages can be involved in the T cell responses (161), (237).

IL-12 may responsible for the acquisition of both these phenotypes. In experiments involving the transfer of wild-type or IL-12 deficient DC into wild-type or IL-12 deficient mice, MacDonald and Pearce found that IL-12 production by endogenous APC was more critical for the development of a Th1 response than IL-12 production by the exogenous DC (357). Moreover, Miga *et al* found that recombinant IL-12

restored IFN-γ production by transgenic cells activated with CD40 deficient DC (315) and Stüber et al found that IL-12 treatment reversed the protective effects of a blocking anti-CD40L antibody in a mouse model of murine colitis by increasing IFN-γ production (352).

IL-12 is also important in inducing the expression of ligands for CD62E and CD62P. The expression of these ligands, such as CD62P glycoprotein ligand-1 and CD62E ligand 1, is dependent on their glycosylation state and the enzymes required for this, such as fucosyltransferase-VII, are induced by IL-12 (358), (359). Thus, IL-12 can also determine the capacity of T cells to migrate to tertiary tissues (349), (64), (54).

It would be interesting, therefore, to examine the priming of H19env-specific T cells following immunisation with wild-type DC in mice deficient for IL-12. Alternatively, the DC-primed CD40 knockout mice could be given recombinant IL-12 to investigate whether this resolved the defects describe above.

Figure 5.21 illustrates a composite picture of the results discussed above and describes the potential roles of the exogenous and endogenous DC and the effect of the different routes of DC-transfer. The injected DC are probably the DC that initially activate the antigen-specific T cells. Once activated, the T cells will express CD40L and therefore will be able to interact with endogenous DC that constitutively express low levels of CD40.

Endogenous DC could provide antigen-specific interactions with the activated T cells or just provide enhanced levels of costimulation. Antigen could be transferred to endogenous DC either by exosomes, or by endogenous DC picking up dying exogenous cells (360). Alternatively, as suggested by Reis é Sousa and colleagues, the activated T cells could release cytokines that mature bystander DC that can then participate in the immune response (361). This interaction may be important for the

full differentiation of the T cells in terms of cytokine production and the ability to migrate into tertiary tissues. Indeed, a recent paper from Williams and Bevan has shown that if antigen presentation of LM antigen is curtailed after 24 hours by treating infected mice with ampicillin, the CD4 effector and memory response is reduced (362). The authors argue that sustained antigen presentation is required for effector cell development.

In CD40 knockout mice the activated T cells would be unable to interact with endogenous DC via CD40L and thus these DC will not be fully activated to express increased levels of costimulation molecules or produce high levels of IL-12 (32), (33), (34), (35).

Therefore, T cells primed in CD40 knockout mice by DC injected i.v., would receive the smallest signal from both the exogenous and endogenous DC. This reduced interaction may be sufficient for T cell expansion and memory cell generation, but not for effective effector cell development. In the CD40 knockout mice immunised with DC-s.c., normal migration of the tetramer positive cells was observed. Thus, this phenotype was not dependent on interactions with endogenous CD40 sufficient APC and this again demonstrates that the injection of DC-s.c. was a more potent immunisation route than the injection of DC-i.v.

A number of approaches could be made to substantiate this hypothesis. First, it would important to establish whether the reduced IFN-γ production in primed and boosted CD40 knockout mice was antigen-specific and thus determine whether antigen-specific effector cell development is diminished in these mice. Ways in which to do this were described above.

Second, to determine whether sustained interactions were important in effector cell development, the CD40 knockout mice could be given further injections of wild-type

DC. Furthermore, these DC could be activated via CD40 prior to transfer and, as this will activate the exogenous DC more than LPS alone, may enable effector cell differentiation in the absence of interactions with endogenous DC.

In summary, the experiments described above have shown that T cell priming does not occur in the absence of CD40L-CD40 interactions. However, by restoring a primary immune response by injection of wild-type DC, memory cells were generated in CD40 knockout mice. These memory cells could proliferate in the absence of further CD40L-CD40 interactions and this demonstrates a qualitative difference between naive and memory T cells.

5.3.10.ICOS-B7h interactions are not required for T cell proliferation, memory cell generation or re-activation

The CD4 T cell response in the absence of ICOS-B7h interactions was examined in similar ways to those described above in CD40 and CD40L deficient mice. However, only minor defects were found in the T cell response in ICOS knockout mice. T cell priming, memory cell generation and survival were unaffected in the absence ICOS after immunisation with peptide-CFA.

As mentioned above, Dong *et al* suggest that CFA, which is a very potent adjuvant, can bypass defects in ICOS knockout mice (332). However, after immunisation with DC, no significant defect was found in the primary immune response in ICOS knockout mice suggesting that even in less immunogenic responses, ICOS costimulation is not necessary for T cell activation.

There was a trend towards a reduced recall response in the DC-primed knockout mice, although this was only significant in the DLN in the second of two experiments. Similarly, in this experiment, the IL-2 production of total CD4 cells was reduced in both the spleen and DLN of the ICOS knockout mice and this may explain the slightly reduced proliferation of H19env-specific T cells. The other cytokines examined, IFN-γ and IL-10 were not significantly reduced, but in the second experiments there was a trend towards less cytokine in both the DLN and spleen of the knockout mice and IFN-γ production was significantly reduced in the DLN of the ICOS knockout mice.

It is surprising that there was a defect in IL-2 production, as other investigators have found no defect in IL-2 production in the absence of ICOS (42), (323) (328). However, Dong *et al* found that the reduced *in vitro* proliferation of ICOS knockout T cells was restored by the addition of exogenous IL-2 (332). Thus, ICOS

costimulation may enhance IL-2 production by T cells. The results from the second experiment also hint that the CD4 IFN-γ and IL-10 response may be reduced in the recall response. This corresponds to findings by McAdam *et al* that ICOS costimulation enhances the production of effector cytokines (42).

If the recall response is reduced in the absence of ICOS this may be because less memory cells were generated or because this signal plays a role in the expansion of memory cells. Interestingly, Coyle *et al* found a blocking ICOS fusion protein disrupted antibody production in the secondary response to a greater extent than that in the primary response (328).

The question remains, however, as to what APC provides B7h costimulation. DC have been found to downregulate expression of B7h following interactions with T cells (326) and certainly they do not appear to upregulate B7h after activation with LPS (see materials and methods and refs (325), (326)). Thus, the memory T cell may interact with B cells, which constitutively express B7h during the secondary response and this may provide extra costimulation via ICOS-B7h (327) (329).

There is also an alternative explanation for the reduced response. In the second experiment, the response in the ICOS knockout mice was compared to that in C57BL/6 mice and both were immunised with C57BL/6 DC. In the first experiment, where no statistical difference was found, the DC were made from the bone marrow of littermates of the ICOS knockouts and the response compared to that in the littermates. Thus, the C57BL/6 DC may have been rejected from the ICOS knockout mice, which have only been backcrossed 7 times. This may have resulted in a reduction of memory tetramer positive cells as the response in these mice may have been curtailed. Further experiments in more fully backcrossed mice would resolve this issue.

Despite this slight defect, there was a clear recall response in the ICOS knockout mice, confirming the peptide-CFA immunisation data that memory cells can be generated in the absence of ICOS. This suggests that ICOS-B7h interactions are non-essential for T cell proliferation, memory cell generation, survival and reactivation of memory cells. These results confirm some published studies (330), (333) but differ from others (45) and this may reflect the differences between studying responses in knockout mice compared to using blocking antibodies or fusion proteins.

An additional reason why no major defect was found in the ICOS knockout mice either after DC or CFA immunisation may be because both types of immunisation primed Th1 responses. As outlined in the introduction to this chapter, ICOS costimulation may be more critical for Th2 responses than Th1 responses (328), (330), (331), (329). It would, therefore, be interesting to immunise mice with H19env-peptide and a Th2 inducing adjuvant, for example with bone marrow DC pulsed with H19env and incubated with the schistosome egg extract, SEA, which is known to induce a strong Th2 response (339).

5.3.11. Conclusion

The two costimulatory interactions studied in this chapter have illustrated two of the important features of costimulatory molecules. The experiments in CD40 and CD40L knockout mice show how critical this interaction is between these two molecules in T cell activation, demonstrating that productive T cell responses are dependent on costimulation. On the other hand, the experiments in the ICOS knockout mice where T cells can proliferate, form memory cells and be reactivated, demonstrate that not all costimulatory molecules are essential for T cell activation, indicating a level of redundancy in the system.

Why might these two interactions have such distinct roles? Both CD40L-CD40 and ICOS-B7h interactions provide critical communication links between T and B cells, enabling the generation of germinal centres and B cells isotype switching. However, although T cells can interact with DC via ICOS and B7h, this interaction is not critical for either cell. CD40L-CD40 interactions are vital for DC maturation and this in turn is required to support T cell activation, proliferation and memory cell generation.

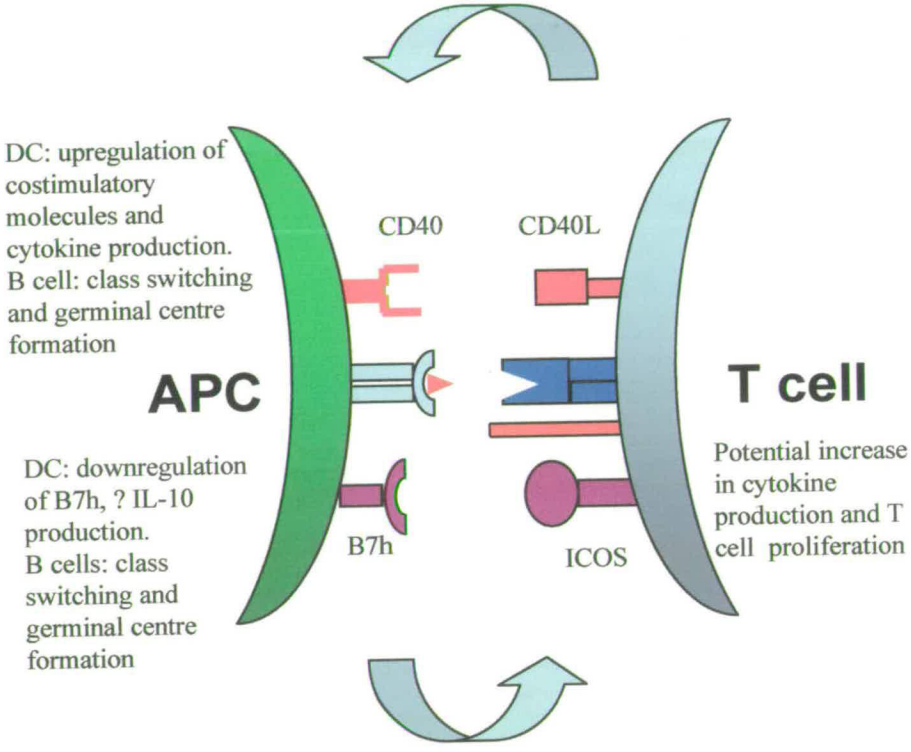


Figure 5.1 Expression and activity of the costimulatory pairs, CD40L-CD40 and ICOS-B7h: Activated T cells can interact with both DC and B cells via TCR and CD4 (dark blue and red) that bind to peptide-MHC complexes (light blue). Activated T cells also express CD40L(dark pink) and ICOS (purple) which bind CD40 and B7h respectively, both expressed by DC and B cells.

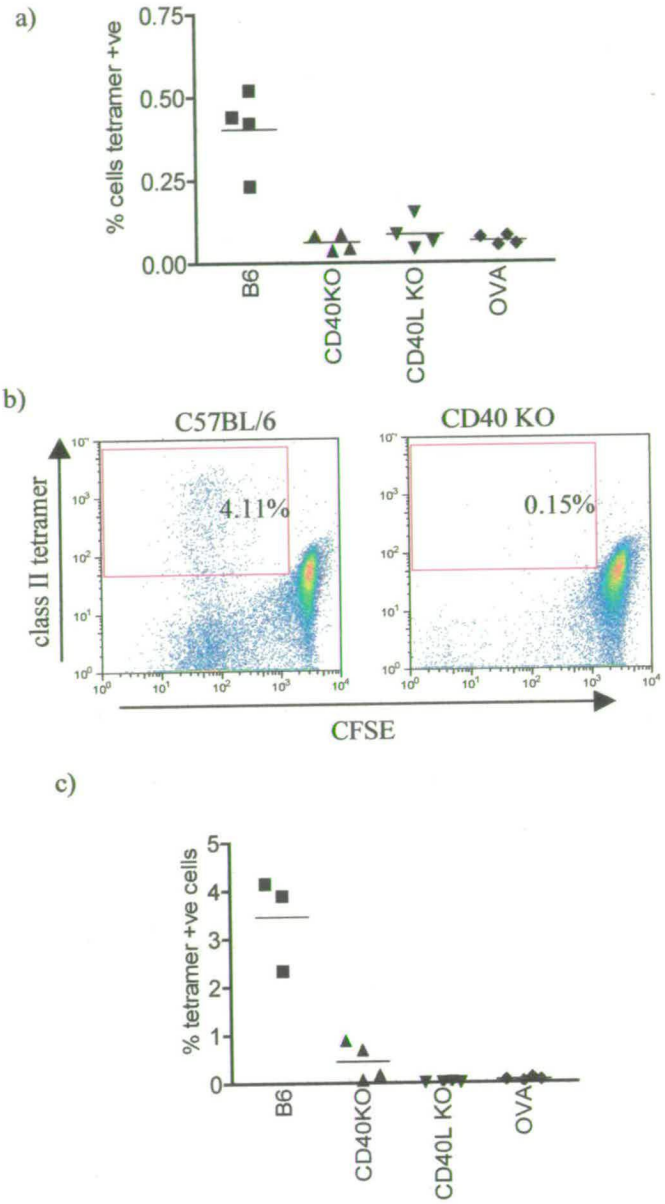
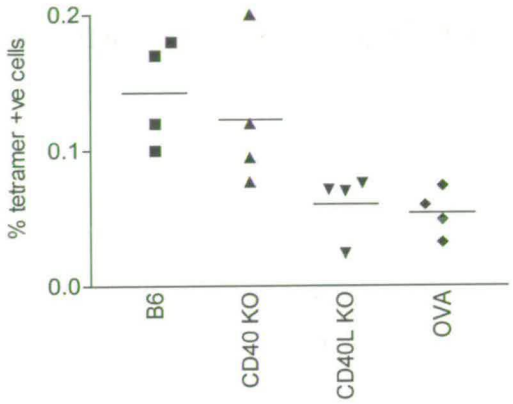


Figure 5.2 CD40-CD40L signals are required for T cell activation a) Percentage of tetramer positive cells, out of CD4 cells, in the spleens of C57BL/6, CD40 and CD40L knockout mice immunised with H19env-CFA 8 days previously. b) Representative FACS staining of CFSE labelled tetramer +ve cells after activation of splenocytes *in vitro* for 72 hours with H19env.OVA: C57BL/6 mice immunised with OVA-CFA, and activated with H19env. The number shows the percentage of tetramer +ve cells, out of CD4 cells, in the top left quadrant. c) Percentage of tetramer +ve cells, out of CD4 cells after *in vitro* activation as in b). Each point represents one mouse and the line shows the mean of the group. Representative of 3 individual experiments.

a) DLN



b) Spleen

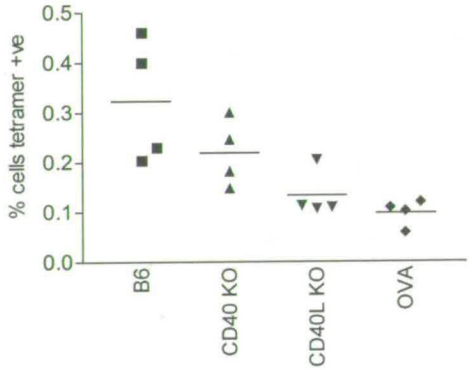


Figure 5.3 Wild-type DC restore priming in CD40 knockout mice but not CD40L knockout: Percentage of tetramer positive cells, out of CD4 cells, in the a) DLN and b) the spleen of C57BL/6, CD40 and CD40L KO mice immunised with wild-type DC s.c. 6 days previously. OVA: C57BL/6 mice immunised with OVA pulsed DC. Each point represents one mouse and the line shows the mean of the group. Representative of 3 experiments.

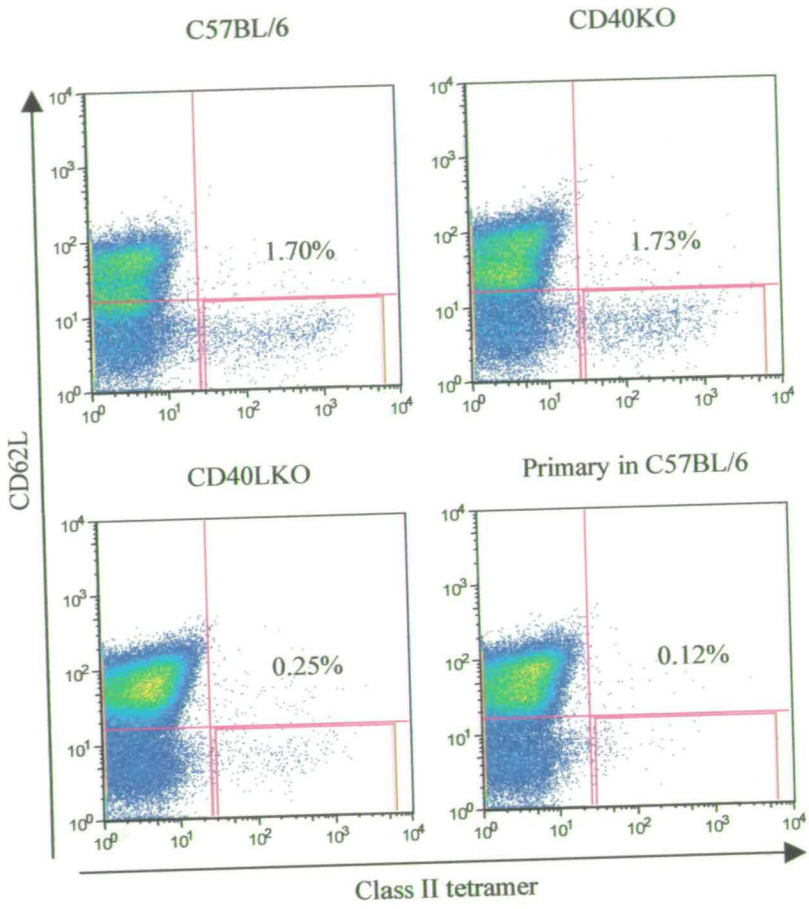


Figure 5.4. The recall response in DC primed CD40 knockout mice and C57BL/6 mice : Percentage of tetramer positive cells, out of CD4 cells, in the DLN of C57BL/6, CD40 KO, CD40L KO mice primed with WT DC sc, rested for 5 weeks then boosted with H19env-CFA 5 days prior to staining. Primary: C57BL/6 mice given H19env-CFA 5 days prior to staining. Cells are gated on CD4 positive live lymphocytes, excluding macrophages. The number express the percentage of tetramer +ve cells, out of CD4 cells, in the rectangle gate.

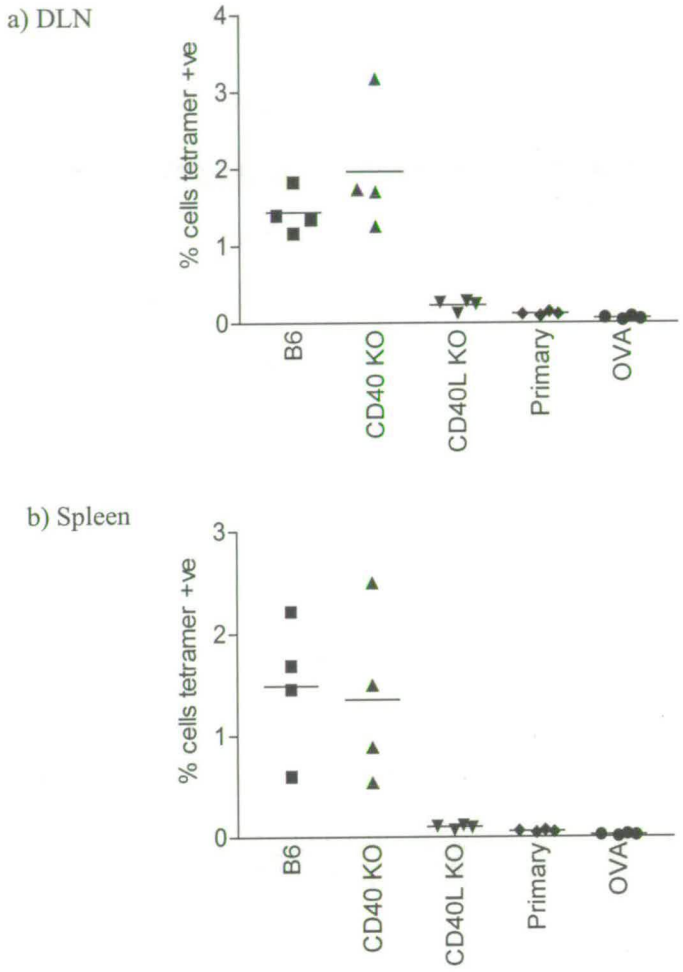
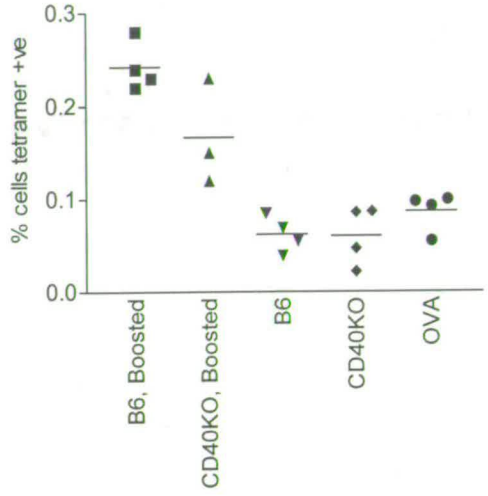


Figure 5.5 The recall responses in CD40 and CD40L knockout mice is equivalent: Percentage of tetramer positive cells, out of CD4 cells, in the DLN (a) and spleen (b) of C57BL/6, CD40 KO, CD40L KO mice primed with WT DC s.c., rested for 5 weeks then boosted with H19env-CFA 5 days prior to staining. Primary: C57BL/6 mice given H19env-CFA 5 days prior to staining. OVA: C57BL/6 mice primed with OVA pulsed DC then boosted with OVA-CFA. Each point represents one mouse and the line shows the mean of the group. Representative of several experiments carried out between 6-8 weeks post DC immunisation.

a) Spleen



b) LN

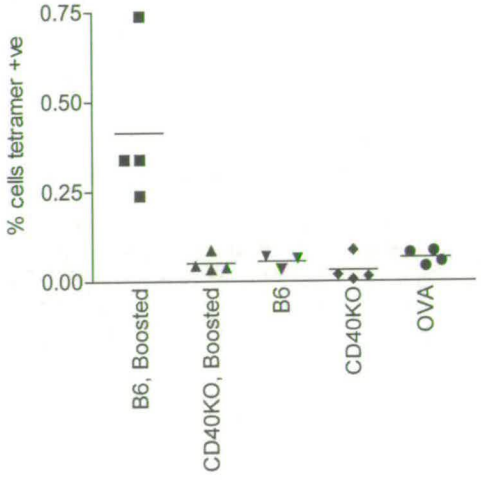
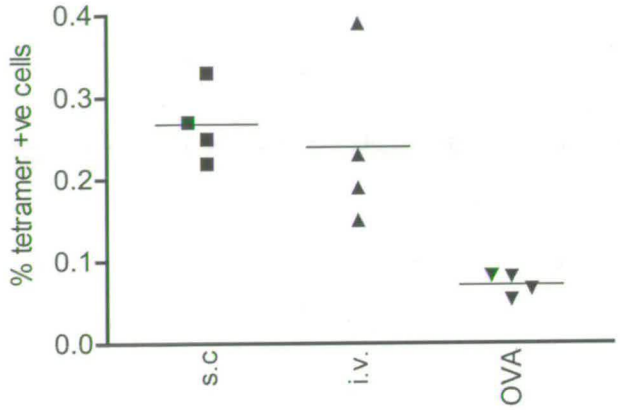


Figure 5.6 Immunisation of CD40 KO mice with DC i.v. does not result in a recall response in the DLN: Percentage of tetramer positive cells, out of CD4 cells, in the spleen (a) and DLN (b) of C57BL/6 and CD40 KO mice primed with WT DC i.v, rested for 6 weeks then either boosted with H19env-CFA 5 days before staining (Boosted) or remaining cells examined *ex vivo*. Primary: C57BL/6 mice given H19env-CFA 5 days prior to staining. OVA: C57BL/6 mice primed with OVA pulsed DC then boosted with OVA-CFA. Each point represents one mouse and the line shows the mean of the group. Representative of 4 experiments carried out between 4-18 weeks post-immunisation with DC.

a) spleen



b) LN

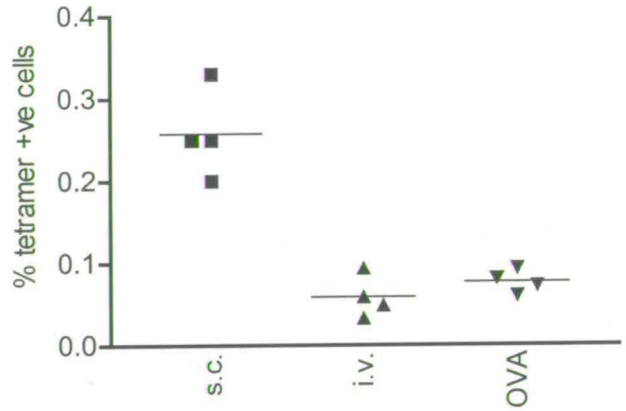


Figure 5.7 T cell priming occurs in the spleen and LN after s.c. transfer of DC but only in the spleen after i..v. transfer of DC: $1x10^6$ wild-type DC were injected either s.c. or i.v. into C57BL/6 mice and the percentage of tetramer positive cells (out of CD4 cells) measured after 6 days. Each point represents an individual mouse and the line shows the mean of the group. OVA: tetramer staining in splenocytes or LN cells from mice immunised with OVA-peptide pulsed DC.

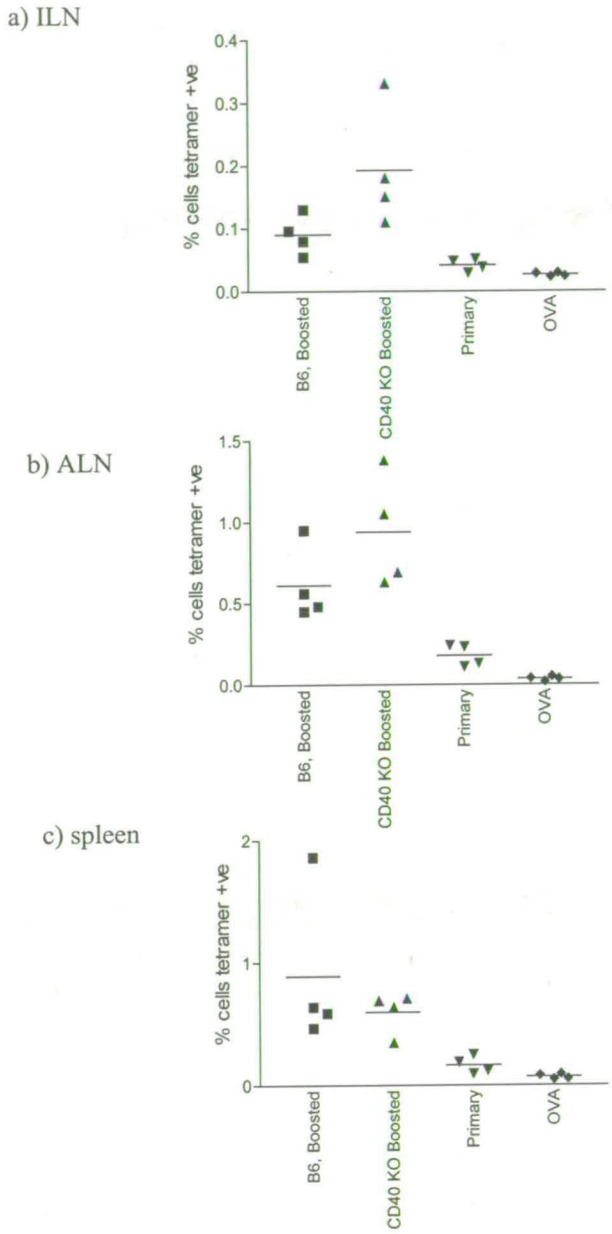
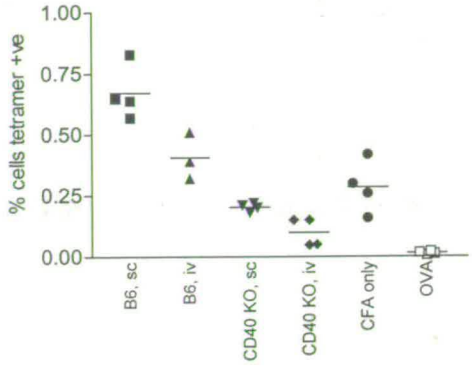
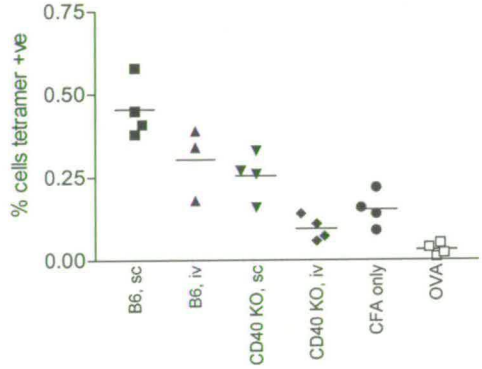


Figure 5.8 The DC immunisation site does not affect the site of the recall response: C57BL/6 and CD40 knockout mice were immunised with wild-type DC s.c. in the hind-leg, 7 weeks later, the mice were boosted with H19env-CFA in the back. 5 days after this, inguinal LN (a) and the axillary LN (b) and spleens (c) were taken for tetramer staining. Percentage of tetramer +ve cells out of CD4 cells is shown. Primary: C57BL/6 mice immunised with H19env-CFA 5 days before staining. OVA: C57BL/6 mice immunised with OVA-pulsed DC then boosted with OVA-CFA. Each point represents one mouse and the line shows the mean of the group. Representative of 2 experiments.

a) DLN



b) spleen



c) non-DLN

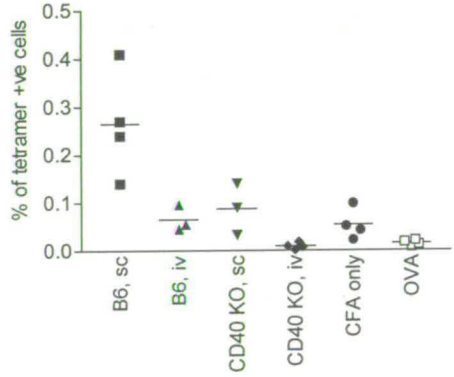


Figure 5.9 The initial immunisation site affects the size and migration patterns of the memory pool: Percentage of tetramer positive cells, out of CD4 cells, in C57BL/6 and CD40 KO mice primed with WT DC either s.c. or i.v., and left for 8 weeks, boosted with H19env-CFA and left for a further 8 weeks. CFA alone: C57BL/6 mice immunised with H19env-CFA at the first 8 week time-point, OVA: C57BL/6 mice immunised with OVA-CFA. Staining in the DLN (a), spleen (b) and non-draining LN (c). Each point represents one mouse and the line shows the mean of the group. This experiment was only done once.

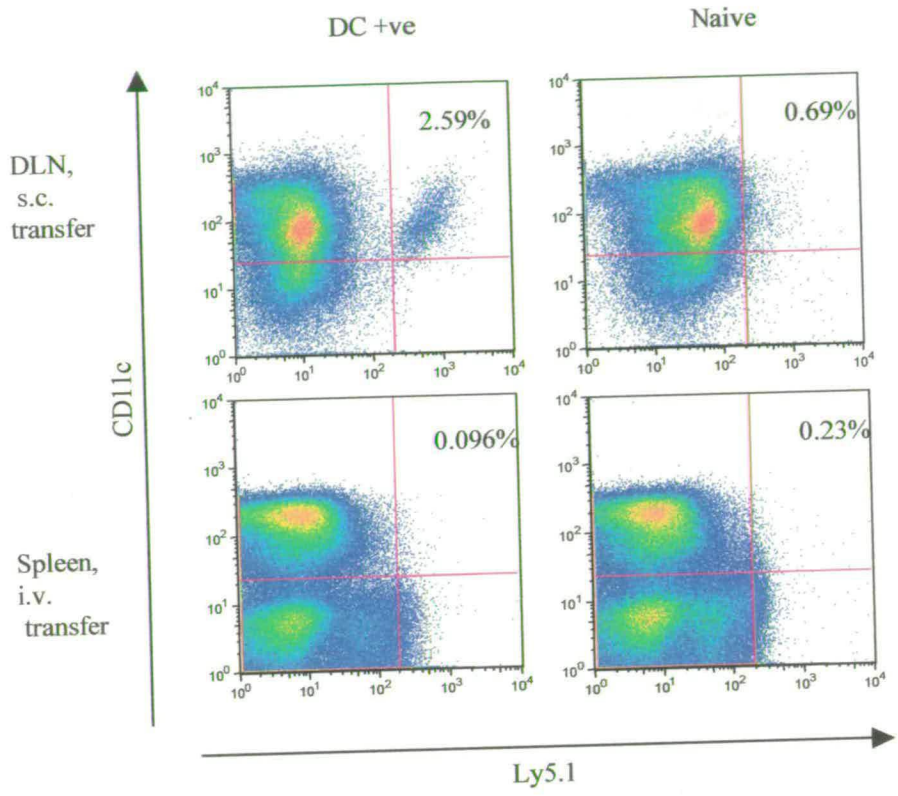
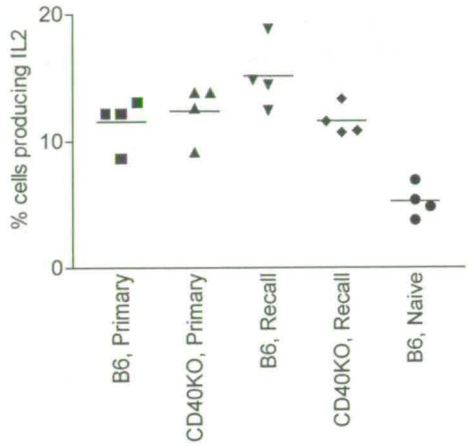


Figure 5.10 Exogenous DC can be tracked after s.c but not i.v injection: Ly5.1 bone marrow derived DC were activated with LPS overnight then transferred into naïve C57BL/6 mice s.c. or i.v. as indicated. 2 days later the DLN and spleens were taken, digested with collagenase as described in materials and methods, and CD11c positive cells enhanced by magnetic cell separation. The combined cells from 3 mice were stained with anti-CD11c, anti-MHC class II and anti-Ly5.1 and analysed by FACS. Cells are gated on live, CD11c and MHC class II +ve cells. The number shows the percentage of CD11c+ve, Ly5.1 +ve cells out of total CD11c cells.

a) IL-2



b) IFN-γ

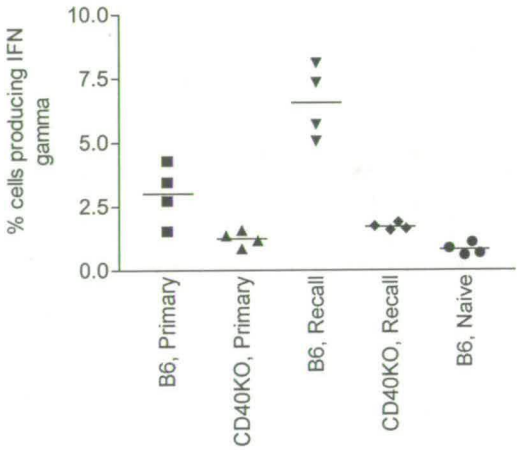


Figure 5.11 IFN-γ production by CD4 cells is reduced in CD40 knockout mice in the primary and secondary response: Percentage of IL-2 (a) or IFN-γ (b) producing CD4 cells in C57BL/6 or CD40 KO mice primed with WT DC or primed with WT DC then boosted with H19env-CFA. Naïve: cytokine production from naïve splenocytes. Each point represents one mouse and the line shows the mean of the group. Representative of 4 experiments.

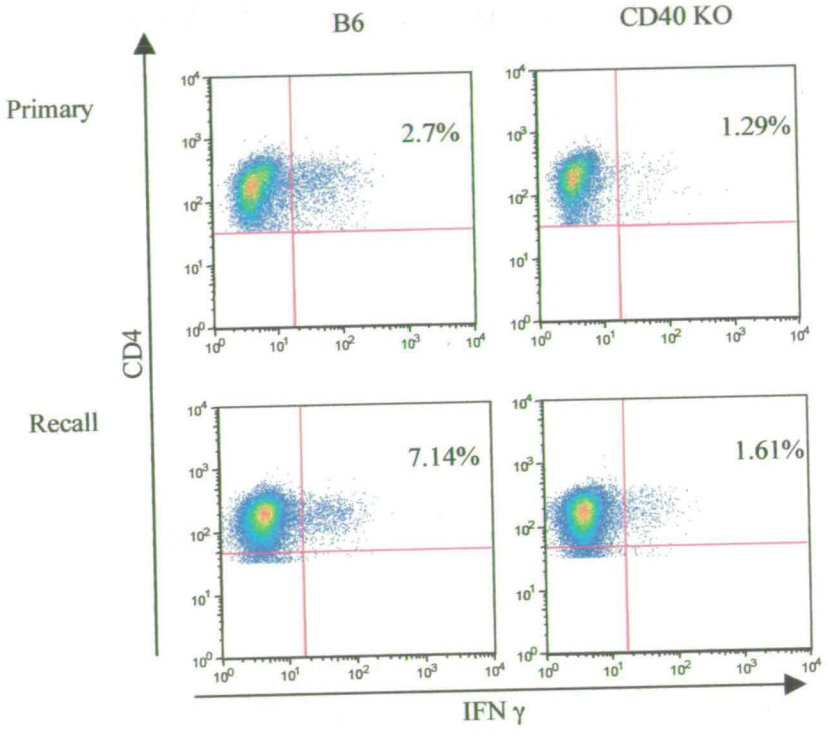


Figure 5.12 IFN-γ production by CD4 cells is reduced in the primary and secondary responses in CD40 knockout mice: Percentage of IFN gamma producing CD4 cells in C57BL/6 or CD40 KO mice primed with WT DC (primary) or primed with WT DC then boosted with H19env-CFA (recall). Cells are gated on CD4 positive live lymphocytes.The number expressed the percentage of cells in the top right quadrant (IFN-γ producing CD4 cells).

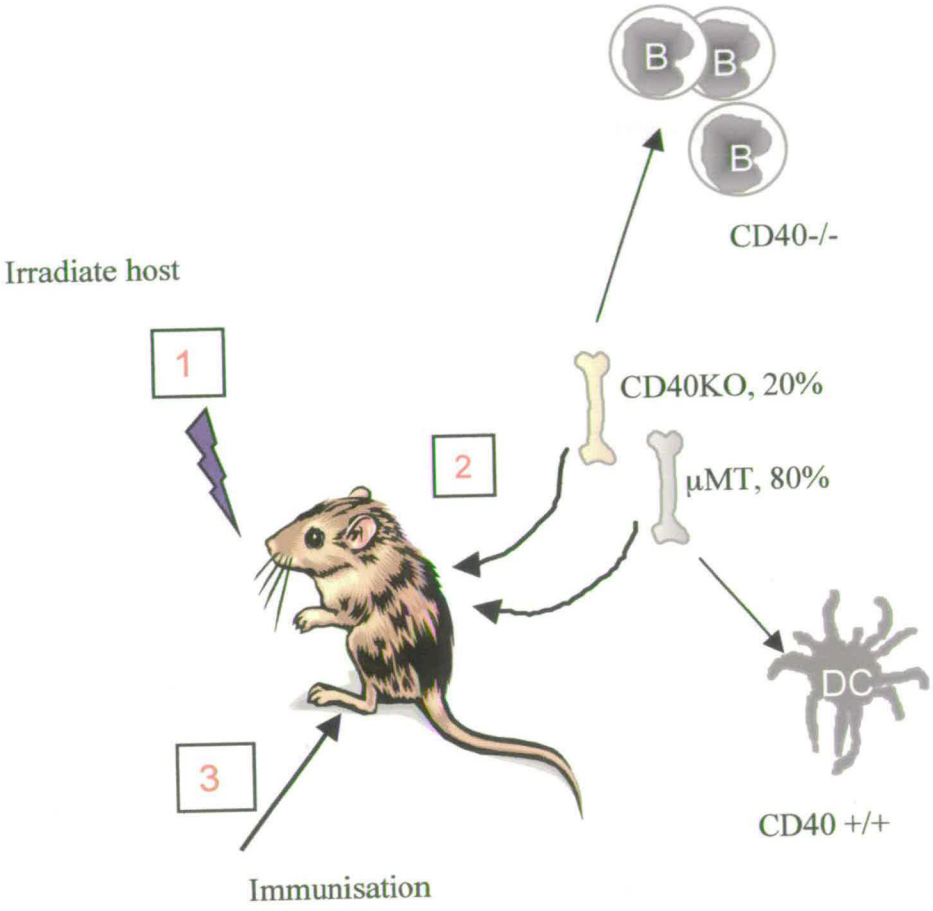
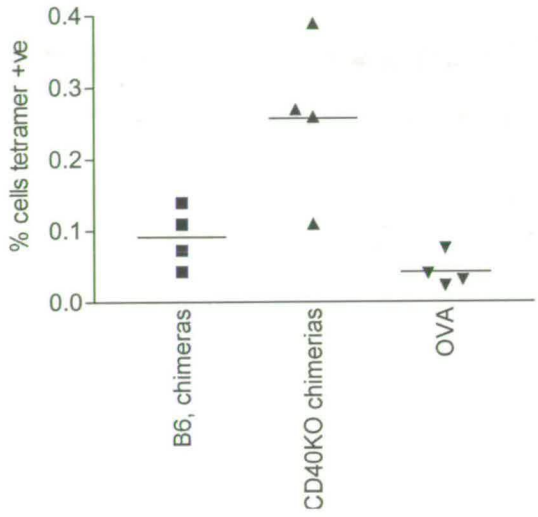


Figure 5.13 CD40 KO B cell chimeras: The chimeras were made by first lethally irradiating C57BL/6 mice (1). The following day, the mice were reconstituted with bone marrow from CD40 KO mice (20%) and μMT mice (80%), (2). After 8 week to allow for reconstitution before the mice are immunised, (3).

a) DLN



b) spleen

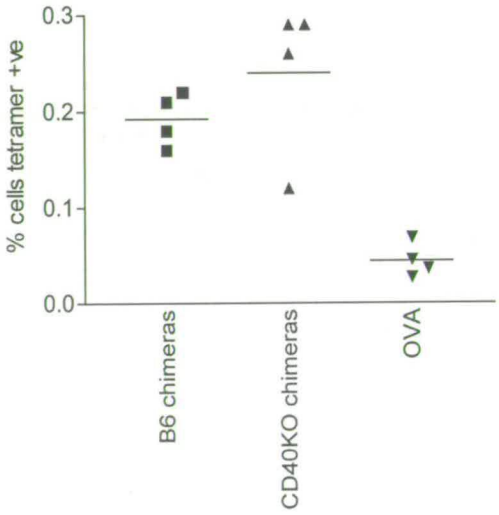
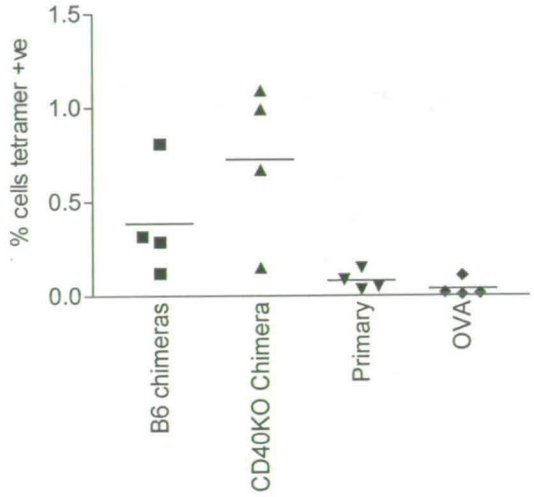


Figure 5.14 The primary response in CD40 KO B cell chimeras is similar to that in wild-type chimeras: percentage of tetramer positive cells, out of CD4, in the DLN (a) and spleen (b) of CD40 B cell KO and WT chimeras immunised with WT DC s.c. 7 days before staining. Each point represents one mouse and the lines show the mean of each group. OVA: WT chimeras given OVA pulsed DC.

a) DLN



b) spleen

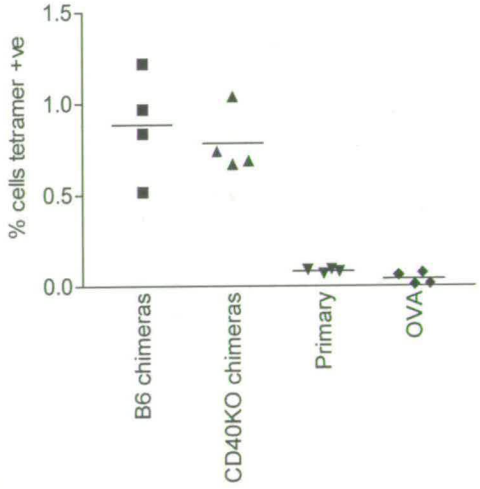
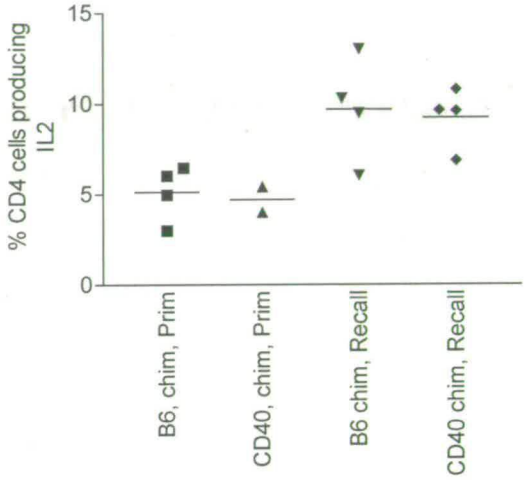


Figure 5.15 B cell CD40 expression is not required for T cell recall responses: Percentage of tetramer positive cells, out of CD4, in the DLN (a) and spleen (b) of CD40 KO B cell chimeras and control chimeras primed with WT DC s.c., rested for 10 weeks then boosted with H19env-CFA 5 days prior to staining. Primary: day 5 response in WT chimeras to H19env-CFA. OVA: response in WT chimeras primed with OVA-DC then boosted with OVA-CFA. Each point represents one mouse and the lines show the mean of the groups. Representative of 2 experiments.

a) IL-2



b) IFN-γ

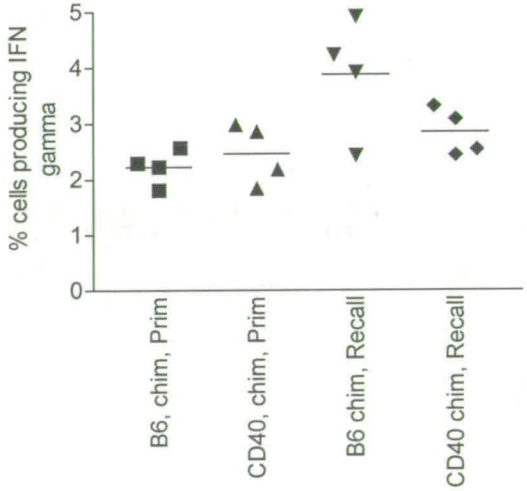
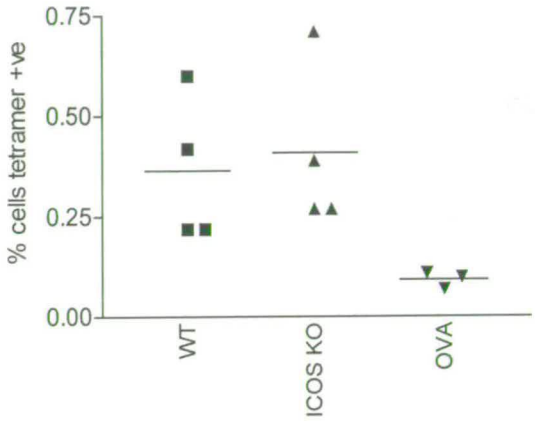


Figure 5.16 Cytokine production is similar in CD40 KO B cell chimeras and WT chimeras: percentage of IL-2 (a) or IFN-γ (b) producing CD4 cells in CD40 KO B cell chimeras and WT chimeras after immunisation with WT DC s.c. (Primary), or primed with WT DC s.c. then boosted 10 weeks later with H19env-CFA 5 days before staining. Each point represents one mouse and the line shows the mean of each group. Representative of 2 experiments.

a) Primary response



b) Memory

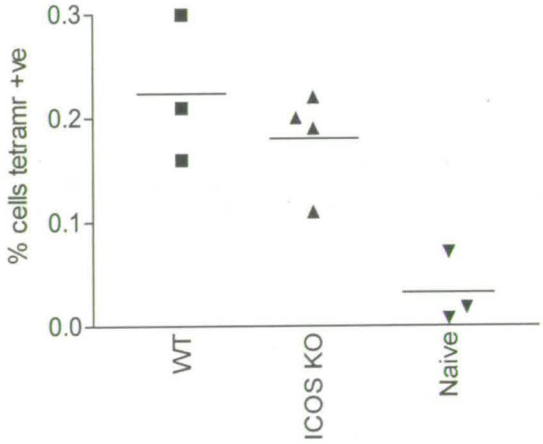
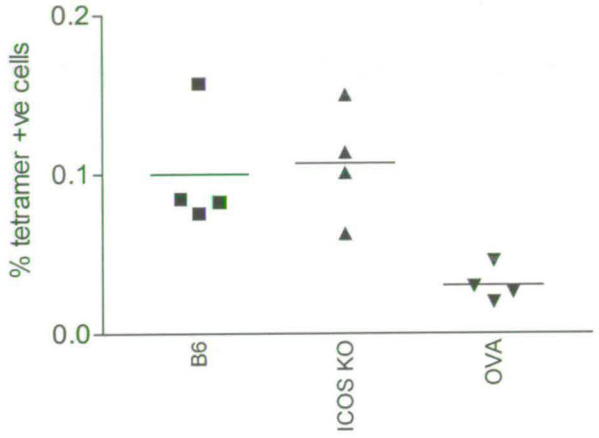


Figure 5.17 T cell priming and memory cell generation is unaffected in the absence of ICOS: percentage of tetramer positive cells, out of CD4 cells, in the spleens of C57BL/6 and ICOS KO mice 8 days (a) or 10 weeks (b) after immunisation with H19env-CFA. Each point represents one mouse and the line shows the mean of the each group. Representative of 2 experiments

a) spleen



b) DLN

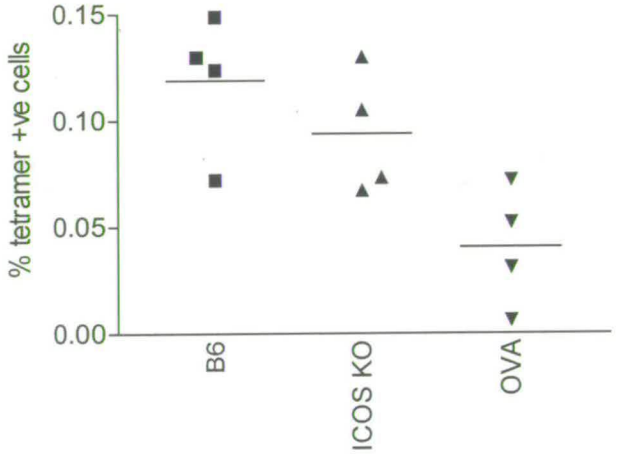
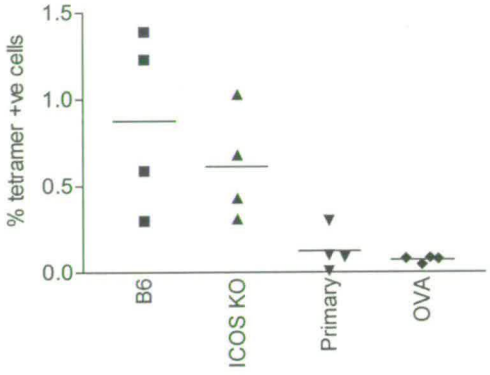


Figure 5.18 The primary response in ICOS KO mice after DC immunisation is similar to that in wild-type mice: ICOS KO and C57BL/6 mice were primed s.c. with WT DC. Spleens (a) and DLN (b) were taken on day 7 and stained with class II tetramers. The percentage of tetramer +ve cells, out of CD4 cells, is shown. OVA: C57BL/6 mice given OVA pulsed DC. Each point represents one mouse and the lines shows the mean of each group.

a) spleen



b) DLN

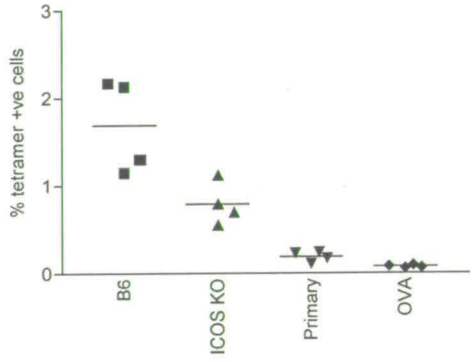
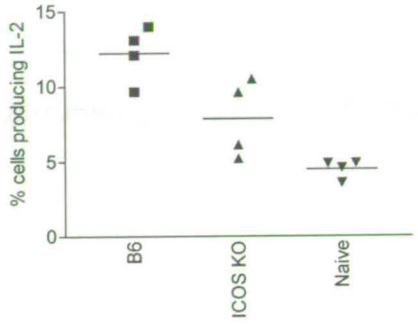
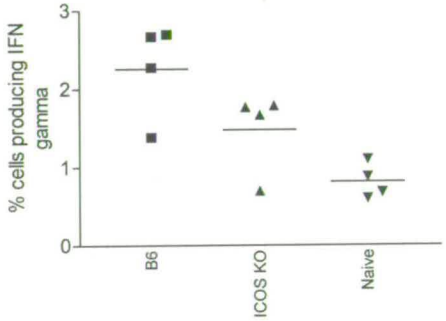


Figure 5.19 The recall response in the DLN of ICOS KO mice is reduced compared to that in WT mice: ICOS and C57BL/6 mice were primed with WT-DC then boosted with peptide-CFA after 10 weeks. The percentage of tetramer +ve cells, out of CD4 cells is shown in the spleen, (a) and DLN, (b).

a) IL-2



b) IFN-γ



c) IL-10

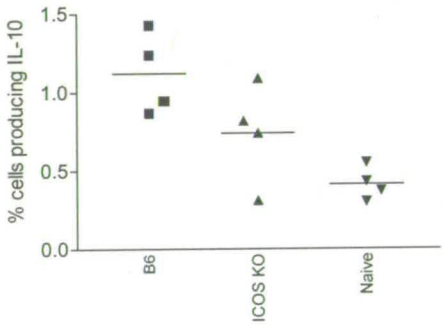


Figure 5.20 The cytokine response in ICOS KO mice is reduced in the recall response: The percentage of CD4 splenocytes producing IL-2 (a), IFN-γ (b) and IL-10 (c) in ICOS KO and C57BL/6 primed with WT-DC then boosted with peptide-CFA after 10 weeks. Naïve: cytokine production after PMA/ionomycin stimulation of cells from naïve mice. Each point represents one mouse and the line shows the mean of the group.

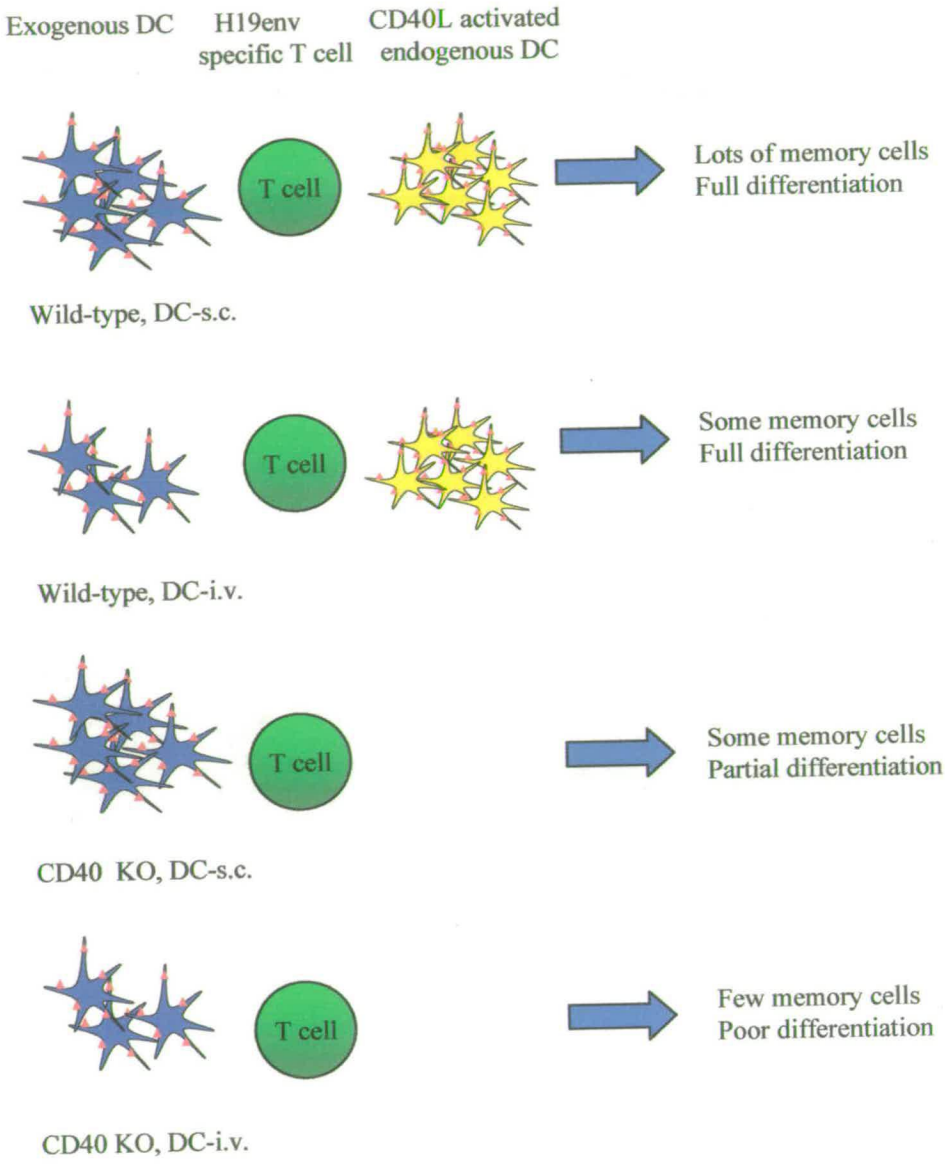


Figure 5.21 Immunisation site and expression of CD40 on endogenous APC determines the effector characteristics of activated and memory T cells: After s.c. immunisation with DC, antigen-specific T cells have more chance of making multiple contacts with the exogenous DC than after DC-i.v. injection in both wild-type and CD40 KO mice. In wild-type mice, the T cells can also interact with endogenous DC and activate them via CD40L, resulting in DC IL-12 production that could contribute to effector cell differentiation.

. Final discussion

The phenomenon of immunological memory has been appreciated for thousands of years: Thucydides described in 430BC that only those who had recovered from the plague could tend to the sick without falling ill themselves. In more recent years this phenomenon has been explained by the survival of antigen-specific memory cells after exposure to infection or immunisation. The understanding of how memory cells form and how they survive underpins how vaccines work and, therefore, it is of critical importance to elucidate the factors that determine the generation and survival of protective memory cells.

This PhD has used a model system to track antigen-specific CD4 T cells through the processes of activation, memory cell generation and into the long-lived memory pool. The system was then utilised to examine the long-term survival of memory cells in the presence or absence of persistent antigen, and in mice exposed to other antigens to induce a competitive memory pool. Furthermore, by tracking the activation and memory characteristics of antigen-specific CD4 T cells in costimulation molecule knockout mice, the importance of CD40L-CD40 and ICOS-B7h interactions were defined. In this final discussion, the importance of some of these results will be discussed in terms of how memory cells can respond with different kinetics to naïve cell and with reference to the production of effective vaccines.

6.1. Memory cell survival

In this PhD, tetramer positive memory cells were found to survive for over 200 days post-immunisation (chapter 3). This was probably not a consequence of the maintenance of long-lived cells. Rather, it is thought that memory cells have stem-cell-like qualities, dividing occasionally to maintain antigen-specific memory at a population, rather than a cellular, level. This basal proliferation may be controlled by cytokines (IL-15 for CD8 T cells and IL-7 for CD4 T cells) rather than antigen (80), (83), (82). These signals were not, however, sufficient to maintain the memory cells studied in this PhD for the life-time of the host.

The decline in memory CD4 cells in this and other studies (19), (225) contrasts with the reports of stable populations of antigen-specific CD8 memory T cell populations (173), (287), (288), (19). However, the stable CD8 memory T cell pool can be disrupted by infections of new micro-organisms (198), (193). It is tempting to speculate, therefore, that the CD8 memory pool is regulated by exposure to new infections that act to prevent over-load of this pool, while the CD4 pool is regulated by the slow reduction of antigen-specific cells.

This prompts the question of whether this slow reduction is an active process or occurs by default. The CD8 memory pool is tightly regulated: despite periodic proliferation of memory cells, the number of specific cells does not increase over-time (144). Thus, the level of proliferation must be matched with an equal level of cell death. McNally *et al* have described low levels of Annexin V staining on a proportion of memory CD8 cells, indicating a small level of continual cell death (201). Whether this death is an active process, for example, induced by a cytokine such as IFN-γ, or a default route, remains

unknown. It would be important to understand this process as it may indicate what, if anything, controls the loss of CD4 memory cells over-time and this knowledge may enable some form of intervention to maintain specific memory cells in the T cell pool.

The CD8 memory pool is also regulated by IL-2 and the mechanism for this regulation appears to involve regulatory $CD4^+CD25^+$ T cells as treatment with anti-IL-2 causes a reduction in the number of these regulatory T cells and an increase in the division rate of CD8 memory T cells (363). Whether this regulatory population also affects the CD4 memory pool is not established, but given that IL-2 does not affect the CD4 memory pool (364), this may not be the case. However, regulatory T cells may be required to maintain antigen, in the form of low-levels of infections such as Leishmania, that is required to retain antigen-specific memory cells (218). This indicates the inter-regulatory nature of the immune system, illustrating that for the design of future vaccines the response of the entire immune system should be considered.

6.2. Why are recall responses different from primary responses?

The gold standard of a vaccine is obviously protection from infection. Protection is either a result of the presence of neutralising antibody that prevents the pathogen from infecting the host, or a strong cellular response that stops the infection from developing. The recall response is an immunological measure of memory. Classically it is defined by two characteristics: an increase in both the size and in the speed of the response in comparison to the primary response to the same immunisation.

The recall responses described in this PhD correspond to this classic description. The explanations for this change in the response are numerous. First, there are more antigen-

specific T cells present at the close of the primary response compared to that in the naïve pool. The importance of this increase in precursor number was demonstrated by the poor response in the Ly5.1 transfer experiments in which the number of antigen-specific cells was small (figure 3.13-15) and also by the reduced size of the recall response in DC-primed mice after day 260 when the memory population had declined (figure 3.11).

A second reason why recall responses have different kinetics to primary responses is that memory cells are thought to respond to lower concentrations of antigen compared to naïve cells. This was not directly tested in this PhD, primarily as it would have been difficult to compare the same number of naïve and memory cells, either *in vitro* or *in vivo,* as the number of naive antigen-specific cells could not be calculated. Such experiments will be possible in the future as a TCR transgenic has recently been made by Gitta Stockinger's group that recognises the H19env peptide. Thus, the activation requirements of these naïve cells could be compared to H19env-specific memory cells.

These differences in the activation requirements of memory cells may be because the proximal signalling events through the TCR/CD3 complex are different in memory and naive cells (340), (145). Thus, the activation threshold of memory cells may be reduced compared to that in naïve cells. Moreover, the presence of messenger RNA for molecules such as IFN-γ and perforin in memory CD8 T cells enables them to make a faster effector response compared to a similar response from naïve T cells (365), (12). It would be very informative to examine the gene expression of activated and memory tetramer positive T cells. Such analysis may provide information about molecules that are important for the survival of activated cells into the memory pool. However, this would require the purification of significant numbers of tetramer positive cells.

The changes in cell signalling that may result in reduced activation requirements could also explain why memory T cells can be activated in the absence of full costimulation. This characteristic of memory cells was demonstrated in chapter five: the recall response in the DC-primed C57BL/6 and CD40 knockout mice was the same, demonstrating that memory cells can proliferate in the absence of full costimulation.

The changes in requirements of memory cells for costimulation may not just be a case of reduced requirements. The slightly reduced recall response in the ICOS knockout mice, which had no defect in the primary response, perhaps suggests that ICOS may be a costimulatory molecule utilised by memory cells more than naïve cells. This in turn may reflect the different APC populations with which memory cells can interact. For example, B cells may play a more significant role as APC in secondary responses (161). Although B cells have been reported to down-regulate B7h upon exposure to antigen, CD40-CD40L interaction can rescue this downregulation and thus activated B cells interacting with T cell could activate memory T cells (329).

Bertram *et al* have found that, although CD8 memory cells were less dependent on costimulation by CD28 interactions with B7.1 and B7.2 than naïve cells, they were more dependent on 4-1BB-4-1BBL interactions than naïve cells (292). Moreover, Dawiicki *et al* found that, although there is a primary CD4 T cell response in the absence of OX40-OX40L interactions, no secondary response takes place in OX40L deficient mice (291). However, this defect may have been due to the decreased survival of activated cells in the absence of this costimulatory signal (139). It would be interesting, therefore, to immunise OX40L knockout mice with wild-type DC, as described for CD40 knockout mice in chapter five. This would restore a "normal" primary response and the recall response in the absence of OX40L could be determined.

The increased size of the recall response may not just be a consequence of the increased capacity of memory cells to proliferate. Badovinac *et al* have shown that naïve and memory cells can respond during the recall response and thereby increase the overall size of the response (288). However, the two populations remain distinct as the authors found that the memory cells contracted much more slowly than the newly activated T cells.

In light of these studies in CD8 memory, it would be interesting to compare the kinetics of activation and contraction of memory and naïve antigen-specific CD4 T cells. In the experiments in which Ly5.1 cells containing tetramer positive cells were transferred into naïve mice, which were then immunised (see chapter three), the tetramer positive population was composed of both the transferred cells and host cells. This suggests that, as for CD8 T cell responses, naïve and memory cells can respond in the recall response. If this transfer system could be optimised, it would provide the opportunity to study the contraction phase and also to determine if the secondary pool was made up of a mixed population of "new" and "old" memory cells, or only contained one of these populations.

The experiment described in chapter five in which DC-primed C57BL/6 and CD40 knockout mice were re-immunised with peptide-CFA and the "secondary" memory pool examined, also provides some insight into these questions. The "secondary" memory pool was greater in the C57BL/6 mice than in the CD40 knockout mice that had been similarly treated. This may have been because naïve cells were activated only in the wild-type mice, and these cells then formed part of the memory pool in conjuncture with the reactivated memory cells. On the other hand, in the CD40 knockout mice, the presence of a "secondary" memory pool demonstrates that the original memory cells can certainly become memory cells again after reactivation. Transfer experiments, for

example, of memory cells from DC-primed wild-type mice into CD40 knockout mice would provide more conclusive evidence that this was the case.

A further difference between naïve and memory T cells is in their migratory patterns. Naïve cells are mainly restricted to secondary lymphoid organs, but memory cells can also be found in tertiary tissues where they can provide immediate protection to pathogens (80), (14), (13). Roberts and Woodland argue that T_{em} in the tissues can proliferate at these sites, perhaps reflecting their ability to interact with APC other than DC (153).

The changes in costimulation requirements may enable proliferation at these sites and this quick, targeted response may prove critical in a recall response to an infection. Thus, the migratory pattern of memory cells is an important consideration in vaccine design. For example, as the experiments in chapter five demonstrated, the route of DC transfer affected both the size of the recall response and the migratory pattern of memory cells in the CD40 knockout mice. As Berzofsky *et al* argue, many pathogens enter hosts at mucosal sites and thus successful vaccines must induce memory T cells that patrol these sites; memory cells confined to the spleen and the blood would provide only limited protection (366), (216).

The type of memory cell that should be induced by vaccines is an important issue. Some, but not all, investigators have found that T_{em} are not long-lived cells and thus vaccines should aim to generate longer-lived T_{cm} cells (155), (143). However, T_{cm} cells may not be present at the entry point of the pathogen and therefore could not provide immediate protection. This dilemma denotes the importance of boosting the immune system, providing a dual defence of long-lived cells and effector cells. Boosting would provide a TCR specific signal for T_{em} cells, increasing their survival or could induce T_{cm} cells to

turn into T_{em} cells. Moreover, this would also increase the overall size of the memory pool.

6.3. Concluding remarks

The development of new and better vaccines requires an intimate knowledge of memory immunology. Understanding how the immune system functions as a whole will provide the means to develop both more effective and safer vaccines. However, increasing the safety of vaccines can mean that they become less immunogenic. Berzofsky *et al* argue that understanding how the immune system functions should help to find a middle ground (366). For example, the addition of costimulatory molecules such as B7.1 into recombinant vaccines would increase the activation of responding cells (367), (368). In addition, combining IL-7 with a vaccine could "encourage" the transition of activated cells into the memory pool, for example the IL-7 gene could be incorporated into DNA vaccines.

By tracking endogenous memory cells, this PhD has aimed to contribute towards the understanding of how memory CD4 T cells are generated, survive and respond to re-challenge. In summary, I have shown that antigen-specific CD4 memory cells can survive for 200 days post-immunisation. This memory pool does decline overtime in both the presence and the absence of persistent antigen, but it is not affected by the entrance of cells of other specificities into the memory pool. These memory cells can, however, rapidly respond to re-challenge, even in the absence of full costimulation, fully demonstrating their memory cell characteristics.

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