

# **The Role of B cells in Influencing T cell Responses**

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Thesis submitted for the Degree of Doctor of Philosophy

University of Edinburgh University

December 2004



## **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.

Alison Crawford

December 2004

“The covers of this book are too far apart.”

**Ambrose Bierce**

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“I’ve had great times in my life. This wasn’t one of them.”

**Groucho Marx**

## **Acknowledgements**

I would like to thank my supervisor, David Gray, for all the help and advice throughout my PhD. Thanks also to the others who read my thesis for corrections: Megan, Tom, Georgia and Meera.

The T cell memory experiments would have been impossible without the MHC-II tetramers produced and provided by Ton Schumacher, Koen Schepers and Mireille Toebe so I am deeply indebted to them.

Thanks to everyone in the lab, past and present, especially Megan, who have helped me during my PhD in various ways.

Thanks also to Meera for the much-needed coffee breaks and gossip sessions. Also, to Georgia for the chats and especially for fetching me chips on evenings where the FACs machine was becoming my best friend. Thanks to the Tuesday evening pub group (Megan, Mandy, Leigh and Kristin) for the much-needed alcohol and nachos and also Andrew and Steve for the chats, scientific and otherwise.

Finally, thanks to my family for their support.

This work was supported by the Wellcome Trust.

## Abstract

The role B cells play in influencing T cell priming and differentiation has been strongly debated for a number of years. Although studies using B cell deficient mice have been useful in understanding the importance of B cells under different conditions, it is difficult to then dissect exactly how B cells could be regulating T cell responses. By transferring OT-II transgenic T cells into either B cell deficient ( $\mu$ MT) or C57BL/6 mice, expansion and contraction of T cells can be tracked *ex vivo*. Expansion of OT-II T cells is reduced in  $\mu$ MT mice compared to C57BL/6 mice. Thus, B cells are required for efficient T cell expansion. In addition to cognate interactions with T cells, B cells can provide costimulatory signals, secrete cytokines and influence the lymphoid microarchitecture. To dissect which B cell factor(s) are involved in enhancing OT-II T cell expansion, a model system was used where one molecule on the B cells is depleted at one time. This was achieved by creating bone-marrow chimeras using a combination of  $\mu$ MT bone-marrow with wildtype or deficient bone-marrow. Thus, all the B cells are either wildtype or deficient for a particular molecule. The molecules examined were MHC-II, which is required for antigen presentation, CD40, due to its costimulatory role, and lymphotoxin-alpha, for its role in maintenance of splenic architecture. Using the OT-II adoptive transfer system, we have shown a requirement for MHC-II but not CD40 on B cells for efficient T cell expansion. In light of these observations, the role of B cell-derived MHC-II for T cell memory generation was examined. To do this, I used MHC-II tetramers to track a polyclonal population of T cells in the host. Using this technique, I have shown that T cell memory is also diminished when the B cells do not express MHC-II. Thus, a cognate interaction with B cells is required for both efficient expansion and memory generation of CD4<sup>+</sup> T cells.

## Abbreviations

|       |  |
|-------|--|
| Ab    | Antibody   |
| Ag    | Antigen  |
| APC   | Antigen presenting cell                              |
| BCR   | B cell receptor                                      |
| BM    | Bone-marrow  |
| BSA   | Bovine serum albumin                                 |
| CD    | Cluster of differentiation                           |
| CFA   | Complete Freund's adjuvant                           |
| CFSE  | 5,6 carboxyfluorescein diacetate, succinimidyl ester |
| DC    | Dendritic cell                                       |
| DNP   | Dinitrophenyl  |
| ELISA | Enzyme-linked immunosorbent assay                    |
| FACs  | Fluorescence-activated cell sorting                  |
| FCS   | Foetal calf serum                                    |
| FDC   | Follicular dendritic cell                            |
| FITC  | Fluorescein isothiocyanate                           |
| GC    | Germinal centre                                      |
| IC    | Immune complex                                       |
| IFN   | Interferon   |
| Ig    | Immunoglobulin                                       |
| IL    | Interleukin  |
| i.p.  | Intraperitoneally                                    |
| i.v.  | Intravenously  |
| KLH   | Keyhole limpet haemocyanin                           |
| LN    | Lymph node   |
| LPS   | Lipopolysaccharide                                   |
| LT    | Lymphotoxin  |
| MHC   | Major histocompatibility complex                     |
| MM    | Metallophilic macrophages                            |
| MOG   | myelin oligodendrocyte glycoprotein                  |
| MZM   | Marginal zone macrophages                            |

|         |                                  |
|---------|----------------------------------|
| OD      | Optical density                  |
| OVA     | Ovalbumin                        |
| PE      | Phycoerythrin                    |
| PBS     | Phosphate buffered saline        |
| PNA     | Peanut agglutinin                |
| PNPP    | P-nitrophenyl phosphate          |
| s.c.    | Subcutaneously                   |
| SRBC    | Sheep red blood cells            |
| TCR     | T cell receptor                  |
| Tg      | Transgenic                       |
| Th1/Th2 | T helper type 1/ T helper type 2 |
| TLR     | Toll-like receptor               |
| TNF     | Tumour necrosis factor           |

“Theft from a single author is plagiarism – from three or more is research.”

**Mark Twain**



## Introduction

### **Overview**

The initiation of an immune response requires the complex interaction of various cell types. The requirements for the different cellular interactions and the molecules involved are only beginning to be unravelled and will almost certainly differ depending on the pathogen or immunisation strategy. CD4<sup>+</sup> T cells are key regulators of the immune response and therefore understanding the cellular and molecular requirements for the expansion, differentiation, and the formation of memory of these cells is crucial to understanding how immune responses can be directed.

### ***The initiation of adaptive immune responses***

The immune system is comprised of two parts: the innate and the adaptive compartments. The innate immune response relies on the detection of evolutionarily distant biochemical products that are produced by pathogens, but not by their hosts. These are recognised by pattern recognition receptors (PRRs). Cells of the innate immune system form the first arm of defence against an invading pathogen; they are present in peripheral tissues and have the crucial job of picking up antigen (Ag) and travelling to lymphoid tissues to present this Ag to T cells.

The adaptive immune response, on the other hand, is Ag-specific and has the ability to carry out a faster response upon re-exposure to the same Ag, termed immunological memory. It is made up of lymphocytes, of which B and T cells are the two major types. T cells can also be further divided into two main groups: CD4<sup>+</sup> and CD8<sup>+</sup> T cells. CD4<sup>+</sup> T cells recognise peptides presented by MHC-II molecules whereas CD8<sup>+</sup> T cells recognise peptide on MHC-I molecules. MHC-II molecules (HLA-DR, -DP, and -DQ in humans and I-A and I-E in mice) are heterodimers

formed by non-covalent association of the  $\alpha$  and  $\beta$  chains. They are constitutively expressed on professional antigen presenting cells and are upregulated upon activation. They can also be expressed on many other cell types upon inflammatory signals.

The CD4<sup>+</sup> T cell response begins when the T cells recognise peptide/MHC-II complexes on antigen-presenting cells (APCs) and receive APC-derived costimulatory signals. These interactions lead to the activation, proliferation and differentiation of Ag-specific T cells.

An adjuvant is an agent that increases immunological responses. Most infectious microorganisms contain built-in adjuvants such as lipopolysaccharide (LPS) or unmethylated CpG DNA. These are recognized by pattern recognition receptors, e.g. toll-like receptors (TLRs). Experimentally, there are a number of adjuvants used to enhance responses to antigen. The two main mechanisms of adjuvants are to form a depot of antigen or to modulate the cells of the immune system. Depot adjuvants, for example alum, give a slower release of the antigen. In theory, the slow release should increase the length of time the Ag is available to the immune system for processing. Complete Freund's adjuvant (CFA) is a depot adjuvant but also possesses immunostimulatory potential through the presence of killed mycobacteria. It is a very potent adjuvant but has the disadvantage that it can result in granulomas forming. Adjuvants are used in vaccines to increase immunological memory.

### **Architecture of secondary lymphoid tissue**

Ag presentation to T cells occurs in secondary lymphoid tissues, including the spleen, lymph nodes (LNs) and Peyer's patches. These are highly ordered structures containing lymphocytes (B and T cells), myeloid cells, e.g. dendritic cells, and non-haematopoietic stromal cells. These tissues are crucial for the initiation of immune responses since they increase the probability of the low number of naïve T cells coming into contact with an APC expressing their specific peptide. By continually

recirculating throughout the different lymphoid organs, T cells are able to survey the many different tissues for their specific Ag in a matter of days.

The spleen is the largest of the secondary lymphoid organs and is important for the filtration of blood. It is divided into the red pulp, containing mostly red blood cells, and the white pulp, where lymphocytes are found (figure 1.1 provides a diagrammatic representation of splenic architecture). The T cell-rich compartment, called the periarteriolar lymphoid sheath (PALS), is focused around the central arteriole. The B cells are organised into areas called follicles. The T and B cell areas are bordered from the red pulp by the marginal sinus and marginal zone. The marginal sinus, where metallophilic macrophages are found, is embedded in a layer of marginal zone composed of predominantly marginal zone macrophages (MZMs) and resident  $\text{IgM}^+$ ,  $\text{IgD}^{\text{low}}$  B lymphocytes.

DCs are present in various regions of the spleen.  $\text{CD11c}^+$  DCs are found concentrated near the marginal zone at breaks in the macrophage sheaths and in the T area of the white pulp (Pulendran et al., 1997). The DCs in the T cell area are termed interdigitating dendritic cells and are thought to serve as important antigen-presenting cells early in the immune response.

In contrast to the spleen, lymph nodes (LNs) collect antigen from lymph, a fluid that moves from the extracellular spaces of tissues to lymphatic vessels and is returned to the blood via the thoracic duct. The T cell area of LNs is composed of one central area called the paracortex. The B cell area, termed the cortex, surrounds the T cell area. The medulla is the innermost area and is made up of large blood vessels and the medullary sinus and medullary chords (made up of macrophages and plasma cells).

The migration of lymphocytes into the spleen and LNs is very different. For the spleen, entry is non-specific and all cells enter through the splenic artery into the marginal zone where the T and B cells then migrate into the white pulp. Entry into

the LNs, in contrast, is very specific and involves entry via high endothelial venules (HEVs) using ligands on the HEV (e.g. peripheral lymph node addressin (PNAd) and CCL21/ SLC (secondary lymphoid-organ chemokine) and receptors on the lymphocytes (CD62L and CCR7) (Berg et al., 1991; Potech et al., 1999).

## **T cell activation**

The T cell response to Ag can be divided into several phases: expansion, contraction and memory. Once T cells become activated, they undergo IL-2-dependent proliferation and differentiate into effector cells that secrete various cytokines. The expansion phase reaches a peak and is followed by the death of around 95% of the effectors. The few cells that persist after the contraction phase are termed memory cells; though it still is not clear whether they are derived from early activated cells or differentiated effectors.

The first step towards the activation of T cells *in vivo* is the capturing of Ag by APCs and their movement to secondary lymphoid tissues where they can present this Ag to T cells. Dendritic cells (DCs) are possibly the only cells able to activate naïve T cells. The reason why this is so remains unclear, though it may be due to the high levels of peptide–MHC complexes present on the cell surface, high levels of adhesion molecules, chemokine production, and a large variety of costimulatory signals.

DCs are a heterogeneous cell population, which reside in most tissues. The two main subsets of DCs are the CD11c<sup>+</sup>CD8α<sup>-</sup> myeloid DCs, which are situated in peripheral tissues and the CD11c<sup>+</sup>CD8<sup>+</sup> lymphoid DCs that are found in the T cell area of the spleen and lymph nodes (LNs). Immature DCs are constantly sampling the environment around them, taking up antigen and presenting it. Once exposed to bacterial and viral products or inflammatory cytokines, they undergo maturation, increase expression of costimulatory molecules and traffic to secondary lymphoid organs where they interact with antigen-specific T cells. This movement to

secondary lymphoid tissues is the result of changes in chemokine receptor and adhesion molecule expression (Sallusto et al., 1998). T cells then promote further maturation of the DCs through costimulatory molecules and cytokines. In APCs, the MHC-I and -II molecules are present in different intracellular vesicles resulting in MHC-II molecules being loaded with exogenous Ags in the endocytic pathway and the MHC-I molecules being loaded with endogenous Ag in the endoplasmic reticulum (ER). Recently however, this compartmentalisation in the DC has been shown to be somewhat lenient, giving rise to so called cross-presentation (Albert et al., 1998; Bevan, 1976).

Understanding the initial interaction between T cells and DCs has been enhanced greatly with the application of confocal microscopy and two-photon laser-scanning microscopy (TPSCM). TPSCM allows the 3D visualisation of living cells within intact LNs and is proving invaluable to our understanding of cellular interactions. It has additional benefits over confocal microscopy in that the longer wavelengths are less damaging and they can penetrate more deeply into tissues (Cahalan et al., 2002).

It had previously been suggested that T cells first encounter Ag as peptide presented on maturing DCs that have migrated to the spleen and LNs from the periphery. However, by injecting a fluorescently labelled soluble Ag, Jenkins and colleagues visualised Ag entering the LN via the afferent lymph within 15 minutes after injection. The question of whether this Ag can be picked up and presented by resident DCs was answered using antibodies specific for peptide MHC complexes. Itano *et al*, as well as Manickasingham and Reis e Sousa, have shown pMHC complexes on DCs early after s.c. injection of Ag. The timing is too short to be due to migration of DCs from peripheral tissues, which has been shown to take around 6 hours (Itano et al., 2003; Manickasingham and Reis e Sousa, 2000). Thus, T cells first encounter Ag presented by resident DCs and the migrating DCs arrive later to give additional stimulation. These migrating DCs home to the LNs via the afferent lymphatics. This is where T cells enter the LN from the blood and therefore this

might enhance the ability of the DC to scan T cells entering the LN (Bajenoff et al., 2003).

Each DC can cluster with as many as 10–20 lymphocytes at a time (Bouso and Robey, 2003; Hommel and Kyewski, 2003). T cells interacting with DCs can do so either for a prolonged time (several hours) due to antigen recognition leading to synapse formation or through a transient interaction (Miller et al., 2002; Stoll et al., 2002).

Possibly, different Ags are presented in different ways. It is unclear how non-soluble antigen first gets to the spleen and LNs. Also, Ag presentation by resident DCs may depend on the molecular mass of the antigen. Nolte *et al* have recently described the presence of a conduit system in the spleen. Conduits are networks of collagen fibres encased by fibroblastic reticular cells. Since only low molecular mass molecules are able to move out of conduits (Nolte et al., 2003), perhaps resident DCs are more likely to present these molecules compared to large molecular mass molecules.

Since TPSCM allows us to look at living cells, it has been very useful in furthering our understanding of how the T cells move throughout the LNs. Although previous studies have shown that T cells move along chemokine gradients (Cyster, 1999), recent work using TPSCM has visualised T cells moving by what appears to be a random walk throughout the LNs (Miller et al., 2003). This area is still controversial with new theories suggesting that although it looks like the T cells are moving randomly, they are actually moving along a reticular network (Matsumoto et al., 1997).

The speed at which the T cells move is also unclear at this stage. It is possible that whether the T cells move randomly or along gradients and what speed they move at depends on the area of the LN that the T cell is present in. For example, Ron Germain and colleagues have recently shown T cells moving more slowly in the

cortex area of the LN compared to deeper areas (Stoll et al., 2002). Moreover, by injecting fluorescent dextrans i.v. it has been shown that many of the poorly motile cells are displayed along the vessel walls (Huang et al., 2004).

## Costimulation

In order to become activated, T cells receive signals through the T cell receptor (TCR) when they recognise of peptide-MHC complexes on APCs; however, they also require additional signals provided by costimulatory molecules. Figure 1.2 demonstrates the interaction between a T cell and an APC. Many of the costimulatory molecules belong either to the TNF/TNF receptor family or the CD28 and B7 families and can either amplify or oppose the TCR signals. The costimulatory molecules vary greatly in their expression; some are only expressed on a few cell types whereas others are widely expressed. Furthermore, some are constitutively expressed, e.g. CD28 whereas others are induced, e.g. inducible costimulator (ICOS) and OX40.

There are two ways costimulatory molecules can enhance T cell responses. They can increase the activation of the T cell thus promoting more division, or they can enhance the survival of the T cells. B7:CD28 interactions are critical for activation of naïve cells. CD28 amplifies the TCR signal and is also crucial for efficient IL-2 production by activated T cells (Diehn et al., 2002; Jenkins et al., 1991).

CD40 is a member of the TNF receptor family of proteins and is expressed on APCs, e.g. DCs, B cells and macrophages. It enhances APC maturation and increases expression of late costimulatory molecules, e.g. OX40L. CD40:CD40L interactions also have an essential role in enabling T cell help for B cells. Lack of CD40 on B cells results in the absence of germinal centres (GCs), immunoglobulin (Ig) class switching, somatic hypermutation and B cell memory formation (Kawabe et al., 1994). The understanding of the CD40:CD40L interaction between B and T cells has become more complex since B cells have now been shown to express CD40L on

their surface as well as produce a soluble form (Wykes et al., 1998b) and activated T cells can express CD40 (Bourgeois et al., 2002). The expression of CD40 by CD8<sup>+</sup> T cells has been shown to have a positive role in memory formation (Bourgeois et al., 2002) though others have disputed this finding through the use of mixed bone-marrow chimeras (Lee et al., 2003a).

Recently, it was shown that CD40L is expressed by Tg T cells in two waves; it is expressed very early after activation then is internalised after encounter with CD40 on the APC before being upregulated on the surface again (Lee et al., 2002). Interestingly, CD40L is expressed more by Th1 cells compared to Th2 cells, probably due to the fact that IL-4 abolishes the second wave of CD40L expression (Lee et al., 2002). Thus, CD40L expression may be one way Th1 and Th2 T cells regulate responses differently.

OX40 is a member of the TNF family and is expressed one to two days after T cell activation. It is important for the survival of activated T cells (al-Shamkhani et al., 1996) as it inhibits the peripheral deletion of CD4<sup>+</sup> T cells *in vivo* after expansion in response to Ag (Gramaglia et al., 2000). This is achieved through maintaining the levels of the anti-apoptotic molecules Bcl-2 and Bcl-xL (Rogers et al., 2001).

The requirements for the different costimulatory molecules in the activation of naïve T cells compared to memory T cells seems to differ. Memory T cells appear to be less dependent on costimulatory molecules compared to naïve cells (London et al., 2000). Different requirements for costimulatory molecules can lead to requirements for different cells for T cell activation. Whereas naïve T cells are activated by DCs and possibly activated B cells, memory T cells can also be stimulated by resting B cells and macrophages, although to a lesser extent than with activated DCs and B cells (Croft et al., 1994).



## Th1/Th2 paradigm

Once T cells have become activated, they direct the ongoing response through the secretion of various cytokines. Their differentiation places them in subsets according to the cytokines they produce. Th1 cells secrete IL-2, tumour necrosis factor-alpha (TNF- $\alpha$ ), lymphotoxin, and  $\gamma$ -interferon ( $\gamma$ -IFN). They support the activation of CD8 T cells and macrophages and promote immunoglobulin class switching to the IgG2a isotype. Th2 cells, on the other hand, express IL-4 and IL-5 and facilitate B-cell activation and the production of IgG1 antibody (Ab) (Whitmire et al., 1998). *In vivo*, progressive polarisation of the cytokine response occurs in response to chronic antigenic stimulation due to the self-augmentation and negative cross-regulation inherent in T cell differentiation (Abbas et al., 1996). Some pathogens, such as *Leishmania major*, can elicit clearly polarised CD4<sup>+</sup> T cell responses *in situ* (Scott, 1989). Th1 and Th2 responses are critical for the eradication of different pathogens; Th1 responses eradicate intracellular pathogens such as *Listeria monocytogenes* whereas Th2 responses eradicate extracellular organisms such as nematodes.

The differentiation pathway of naïve cells, which only secrete IL-2, into polarised T helper cells, remains unclear. The instructive model implies that all naïve T cells can differentiate down any of the various pathways, with commitment to one pathway being based upon the cytokines present. In the selective model, daughter cells adopt differentiation states in a random (stochastic) manner and a pure population can arise due to the selective outgrowth of one cell type (Coffman and Reiner, 1999). Thus, in the instructive model, cytokines cause commitment to Th1 or Th2 whereas in the selective model, cytokines merely induce the outgrowth of already committed cells. There are many exceptions to the Th1/Th2 paradigm, however, with coexpression of Th1 or Th2 cytokines being confined to a small minority of cells with most cells expressing just one or two cytokines (Karulin et al., 2000).

T cell polarisation involves the expression of specific transcription factors, as well as epigenetic changes in cytokine genes that increase their accessibility. The IFN- $\gamma$  gene is demethylated in T cells expressing IFN- $\gamma$  whereas methylation is accompanied by

lack of expression (Fitzpatrick et al., 1999; Fitzpatrick et al., 1998). The transcription factor T-bet is rapidly induced in developing Th1 cells but not Th2 cells. T-bet is controlled by TCR signalling and IFN- $\gamma$ R signalling (Lighvani et al., 2001). Another transcription factor involved in Th1 differentiation is STAT4. It is a component of the IL-12 signalling pathway and its role is to augment the level of IFN- $\gamma$  produced by cells (Yap et al., 2000). A transcription factor important for Th2 differentiation is GATA-3. In addition to increasing the expression of Th-2 specific cytokines, it downmodulates the expression of IL-12R $\beta_2$  (Ouyang et al., 2000).

Factors that determine the subset of Th cells that predominate in a response include: the form of the antigen, costimulation, hormones, cytokines, and the genetic background (Conboy et al., 1997; Gorham et al., 1996). Due to their key role in activation of naïve T cells, DCs are thought to be involved in the decision of a T cell to become Th1 or Th2. Reports in the literature are consistent with three models through which DC may control T cell polarization: 1) subclasses of DC; 2) the nature of the stimuli that activate DC; and 3) the kinetics of DC activation (Langenkamp et al., 2000; Moser and Murphy, 2000). It is likely, however, that these theories are not mutually exclusive.

Several groups have shown that CD4<sup>+</sup> T cell populations activated *in vitro* progressively lose the ability to change their cytokine profile when transferred from type 1 to type 2 polarising conditions or vice versa (Assenmacher et al., 1998; Murphy et al., 1996; Sornasse et al., 1996). This suggests that activated T cells ultimately become irreversibly committed to the expression of a particular cytokine profile *in vitro* (Doyle et al., 1999). The loss of multipotentiality of activated T cells is in agreement with the differential expression of cytokine receptors in polarised cells as well as demethylation of cytokine promoters, changes in chromatin structure, and induction of type-specific transcription factor expression (Agarwal and Rao, 1998; Fitzpatrick et al., 1998). Indeed, studies looking at the progeny of differentiated cells demonstrate that changes in chromatin configuration are inherited

by subsequent generations of differentiated cells (Kass et al., 1997), becoming independent of continued IL-4 stimulation or STAT6 activation.

This leads to the question of whether memory T cells are pre-programmed to express Th1 or Th2 cytokines. There are obvious advantages to a memory response that retains the information from the primary response. Being able to respond immediately without the need for the decision of if and how to react would enable a much faster response. However, the response made during the primary reaction may not be appropriate the second time the host meets this organism. There is also the increased risk of autoimmunity if memory cells are fairly independent of further instructions. The memory cell population has been shown to be able to express a polarised phenotype. When looking *in vivo*, it was demonstrated that the response induced by the primary CFA or IFA immunisation largely maintains its Th1 or Th2 cytokine recall and Ab isotype profile after the mice are re-injected with the opposite type of adjuvant (Yip et al., 1999). This finding is consistent with the notion that, unlike naive T cells, memory cells are committed to their Th1 or Th2 lineages (Swain, 1994). It is likely that any committed memory T cells will be a subset of the memory compartment and there will also be non-committed memory cells.

### **Migration of T cells to Peripheral Tissues**

Once activated, T cells leave the secondary lymphoid tissue and migrate to peripheral tissues. It is believed that the APCs can direct the T cells to migrate to the tissue that the APC originated from. For example, CD8<sup>+</sup> T cells stimulated *in vitro* by DCs derived from Peyer's patches selectively upregulated the gut-homing integrin  $\alpha_4\beta_7$  whereas CD8s stimulated with DCs from LNs or spleen did not (Mora et al., 2003). Also, intranasal immunisation with virus led to short-term cytotoxic T lymphocyte (CTL) memory in the spleen but long-term in mesenteric LN whereas the opposite was seen with intraperitoneal injection (Sprent, 1997). However, recent research has suggested that activated T cells may migrate into all peripheral tissues. By infecting with a tissue-specific virus, Sendai virus, Masopust *et al* detected viral specific cells in a variety of tissues (Masopust et al., 2004). Also, tracking of CD8 memory cells

has recently been studied using parabiosis, the surgical joining of mice. Resting memory T cells tracked to both lymphoid and non-lymphoid tissues although entry into certain tissues, e.g. the intestinal mucosa was more restricted (Klonowski et al., 2004).

## Peripheral Deletion

The contraction phase of the T cell response is required to maintain the homeostasis of the T cell compartment. Death of the T cells can occur through death by neglect, due to lack of growth factors and survival factors, or activation induced cell death (AICD). AICD of CD4<sup>+</sup> T cells occurs mainly by Fas/FasL interaction where FasL engages Fas on the same cell (suicide) or neighbouring cell (fratricide) (Lenardo et al., 1999; Mogil et al., 1995). Fas is expressed on a wide variety of cell types whereas the expression of FasL is more restricted, being expressed on CD4<sup>+</sup> and CD8<sup>+</sup> T cells only after their activation.

When cells are deprived of growth and survival factors, cytochrome c is released from the mitochondria resulting in caspase activation and apoptosis. In AICD, oligomerisation of Fas or TNFR leads to the recruitment of the Fas-adaptor protein FADD or TRADD, respectively. FADD contains death effector domains (DEDs), which recruit FLICE/caspase 8 resulting in activation of FLICE and the onset of apoptosis (Muzio et al., 1996). Since the apoptotic pathways differ between passive cell death and AICD, the inhibitory molecules also differ. Passive cell death is inhibited by Bcl-2 and Bcl-x<sub>L</sub> whereas AICD is inhibited by FLICE inhibitory protein (FLIP).

It is unclear to what degree the different cell death mechanisms contribute to the CD4<sup>+</sup> clonal deletion phase. It is possible that Fas–FasL interaction promotes cell death early in AICD whereas TNFRII–TNF functions at a later stage (Lenardo et al., 1999). Another theory is that the mechanism of cell death depends on the duration of

Ag presentation; transient presentation may result in death by growth factor removal whereas chronic presentation may induce AICD (Van Parijs et al., 1998).

### ***Immunological memory***

Immunisation with antigen under inflammatory conditions induces immunological memory. An example of immunological memory is the discovery that those living on the Faroe Islands were resistant to a measles outbreak if they had survived infection during the first outbreak 65 years before. Memory is described as the ability of the immune system to respond more efficiently to an antigen upon re-exposure. The memory response is both quantitatively and qualitatively different to the primary response; the number of antigen-specific memory T cells is up to several hundred-fold higher than naïve cells and memory cells respond to lower concentrations of Ag and release effector cytokines faster than naïve cells do (Pihlgren et al., 1996; Swain, 1994). Another important trait of memory cells is that they can be present in peripheral tissues, where a pathogen is first encountered.

One of the main difficulties in examining T cell memory is the lack of reliable phenotypic markers that distinguish memory CD4<sup>+</sup> T cells from effector cells. In mice, effector and memory CD4<sup>+</sup> T cells have a similar surface phenotype including CD45Rb<sup>lo</sup>, CD44<sup>hi</sup>, LFA-1<sup>hi</sup> and CD62L<sup>lo</sup> although CD45Rb and CD62L can vary. Paralleling the gradual transition of activated T cells into resting memory cells, the phenotype of long-lived memory cells shows a partial reversion towards a naïve phenotype (Bunce and Bell, 1997). Late memory CD4<sup>+</sup> cells are relatively quiescent and show only limited expression of typical acute activation markers such as CD69, CD25 and CD40L (Swain et al., 1996).

### **Development of immunological memory**

The differentiation of naïve T cells into memory cells is still not fully understood. The linear differentiation model predicts that cells go through an effector stage before escaping death and becoming memory cells. The branched differentiation

model predicts that T cells become activated and either differentiate into effector cells or form memory cells. These pathways are outlined in figure 1.3. Substantial evidence suggests that CD8<sup>+</sup> T cells go through an effector phase before becoming memory cells. For example, memory CD8 cells represent the descendants of proliferating cells, which, at least transiently, express effector molecules such as perforin (Opferman et al., 1999) and granzyme B (Jacob and Baltimore, 1999). Further support for memory cells differentiating from effectors comes from the finding that memory cell development can take several weeks (Kaech et al., 2002). However, it is possible that there may be several ways for memory cells to form since Manjunath *et al* has also reported that memory CD8<sup>+</sup> cells can form without going through an effector phase. They used the T-GFP Tg mouse, where all the naïve T cells express GFP but fully differentiated cells lose GFP expression. Cells differentiated under certain conditions did not develop effector function but exhibited characteristics of memory cells (Manjunath et al., 2001).

As for CD4<sup>+</sup> T cell memory generation, CD4 effectors have been shown to differentiate into memory cells without further division, as shown by transfer of rested *in vitro* generated CD4<sup>+</sup> effectors into MHC-II<sup>-/-</sup> hosts (Hu et al., 2001). Thus, effector cells can form memory cells at least under certain conditions. Whether all effector cells have the potential to develop into memory cells or just a subpopulation has not been elucidated. In support of the opinion that memory cells differentiate from effectors, there is a strong correlation between the number of effector cells generated and the number of memory cells that result for the CD8<sup>+</sup> compartment (Sprent and Tough) and this may also be the case for CD4<sup>+</sup> T cells (Evans et al., 2001).

If memory CD4<sup>+</sup> T cells are generated from cells that have not gone through an effector stage then the question remains why some cells are activated and differentiate into effectors whereas others become activated and form memory cells. One hypothesis is that activation at a late stage of the immune response and, thus, encounters of low doses of antigen, instructs T cells to become memory precursors.

This hypothesis is supported by experiments showing that exhausting infections do not lead to memory formation (Moskophidis et al., 1993). When mice were infected with a high dose of LCMV, the CTLs disappeared within fifteen days (Moskophidis et al., 1993).

The mechanism by which some T cells manage to evade AICD and become memory cells is still unknown. An important question is whether memory cell development is a default pathway that occurs in absence of cell death-inducing signals or if the cells require positive signals to become memory cells. IL-7 has recently been proposed as a potential positive signal (Kaech et al., 2003; Kondrack et al., 2003).

### Subsets of memory T cells

Long-lived memory CD4 and CD8 T cells exhibit phenotypic heterogeneity based on the surface expression of various molecules including CD62L (Lalvani et al., 1997) and CCR7 (Sallusto et al., 1999). Sallusto and colleagues have defined two types of memory CD4<sup>+</sup> T cells in humans based on CCR7 expression. Human T cells expressing high levels of the chemokine receptor CCR7 and low levels of CD45RA mostly express IL-2 but not IFN- $\gamma$ , IL-4, or IL-5 when restimulated. In contrast, CCR7<sup>-</sup>CD45RA<sup>low</sup> T cells express all four cytokines. CCR7<sup>-</sup> memory cells also express high levels of  $\beta$ 1 and  $\beta$ 2 integrins, which are required for homing to inflamed tissues (Sallusto et al., 1999). Thus, CCR7<sup>+</sup> memory (central memory) T cells are found in lymph nodes whereas CCR7<sup>-</sup> (effector memory) cells are found in non-lymphoid tissues.

Whether central and effector memory populations occur in mice is a contentious issue. In support of this idea, Bjorkdahl *et al* created an antibody against mouse CCR7 and found that CCR7<sup>low</sup>CD44<sup>high</sup> cells were considerably more numerous in the blood and spleen than in lymph node (Bjorkdahl et al., 2003). In addition, IL-2 production was detected from the CCR7<sup>high</sup> population whereas IFN- $\gamma$  was mainly

produced by the CCR7<sup>low</sup> population. However, in a different study, when Tg T cells against an LCMV epitope were examined, IFN- $\gamma$  production was seen in both CCR7<sup>+</sup> and CCR7<sup>-</sup> T cells (Unsoeld et al., 2002). In addition, although Ahmed and colleagues identified memory CD8<sup>+</sup> T cells that fit the central and effector memory definition according to CCR7 expression, these cell types were found to exhibit similar effector functions *ex vivo* (Wherry et al., 2003).

Due to the heterogeneity among memory cells, it is possible that some memory cells are derived from activated cells whereas others originate from effectors. Figure 1.3 shows the possible pathways in memory cell development.

### **Maintenance of memory T cells**

Since memory T cells generate a faster, stronger response, it is essential for the production of efficient vaccines that we understand how memory T cells are maintained. There are a number of extrinsic factors that may play a role in maintaining memory cell numbers. The two main factors that have been described are antigen and cytokines. Given that antigen can be retained on the surface of FDCs for long periods of time, it was suggested that T cells may see small amounts of antigen long after the primary response has waned and this is required for their maintenance (Gray and Matzinger, 1991). This view has been questioned, however, since both CD4<sup>+</sup> and CD8<sup>+</sup> memory T cells seem to survive in the apparent absence of antigen as shown by the persistence of memory cells in the MHC<sup>-/-</sup> mice (Murali-Krishna et al., 1999; Swain et al., 1999). However, merely examining memory cell numbers is not enough: the migration and function of these T cells are essential to their ability to mount a strong response against a pathogen. As shown by Kassiotis *et al.*, although MHC-II does not appear to be required to maintain memory cell numbers, the CD4<sup>+</sup> memory cells from MHC-II<sup>-/-</sup> mice did not have the ability to respond to naïve B cells, unlike those from wildtype mice. Thus, the numbers of memory cells are maintained but the function of these cells is defective (Kassiotis et al., 2002).



The role cytokines play in maintaining memory T cells has been receiving increasing interest recently. For CD8 cells, IL-15 is required for the maintenance of memory T cells (Becker et al., 2002). Becker *et al* described normal memory CD8 T cell generation in response to LCMV in IL-15<sup>-/-</sup> mice but these memory CD8 T cells were not maintained (Becker et al., 2002). Whether CD4<sup>+</sup> T cells also require cytokines for maintenance as memory cells is unclear. It is generally agreed that CD4<sup>+</sup> memory T cells do not need IL-15 for generation or maintenance (Zhang et al., 1998). Lantz *et al* demonstrated activation of CD4<sup>+</sup> T cells and memory recall responses of  $\gamma_c$  chain<sup>-/-</sup> TCR Tg cells (Lantz et al., 2000) suggesting none of the  $\gamma_c$  chain cytokines are required for memory CD4<sup>+</sup> T cells, although others have shown that resting Tg memory cells are not maintained after transfer into IL-7<sup>-/-</sup> mice (Kondrack et al., 2003).

### **B cell responses**

B cells play an important role in protection from various pathogens. This is thought to be largely due to the production of antibodies that bind to the pathogen and lead to its destruction. Antibodies can block toxins from certain bacteria or bind them to aid the uptake of Ag by cells of the innate immune system. There are different classes of Ig and these have slightly different functions. For example, both IgM and IgG can activate complement, however, only IgG can be transferred across the placenta. Naïve B cells express only IgM and IgD but may switch to IgG, IgA or IgE upon activation. However, B cells are likely to have other roles in the immune response to pathogens. Once B cells take up Ag through their BCR, they internalise it and present it in the context of MHC-II on their surface and can therefore act as Ag presenting cells, possibly helping T cell priming.

### **B cell subsets**

Not all B cells act in exactly the same way. B-cells can be subdivided into B1 B cells, marginal zone (MZ) B cells and follicular B cells with the different subsets having slightly different functions. B1 B cells are found mainly in the peritoneal and

pleural cavities and recognise bacterial antigens and a proportion express CD5 (Martin and Kearney, 2001). They produce 'natural' antibodies and their responses are mostly T cell-independent. MZ B cells ( $\text{IgM}^{\text{bright}}\text{IgD}^{\text{dull}}\text{CD21}^{\text{high}}$ ), on the other hand, have a partially activated phenotype (Martin and Kearney, 2002). They are non-recirculating and reside in the MZ of the spleen (Gray et al., 1982). Exposure to bacterial products or Ag initiates movement of the MZ B cells to the white pulp (Gray et al., 1984). It has recently been shown that they require sphingosine 1-phosphate receptor 1 ( $\text{S1P}_1$ ) for localisation in the MZ (Cinamon et al., 2004).  $\text{S1P}_1$  acts to counteract the CXCL13 signal, which guides B cells to follicles and therefore downregulation of  $\text{S1P}_1$  may be the mechanism by which MZ B cells move to the white pulp (Cinamon et al., 2004).

Although MZ B cells were originally believed to be involved in T-independent responses, they may also regulate T-dependent responses. T-independent responses are against mitogenic stimuli (TI-1) or to repeating polymers (TI-2), which cross-link BCRs, whereas T-dependent responses are against protein Ags, requiring a cognate interaction with T cells to provide help for B cells to respond efficiently. MZ B cells have recently been shown to express high levels of B7.1 and B7.2 (Oliver et al., 1999) and therefore may be able to present Ag to naïve T cells. In addition, mice that lack MZ B cells ( $\text{Pyk2}^{-/}$  mice) also showed a defect in IgM responses to TD antigens (Guinamard et al., 2000).

Follicular B cells are found in secondary lymphoid tissue and respond to T-dependent antigens. They are  $\text{IgM}^{\text{dull}}\text{IgD}^{\text{bright}}\text{CD21}^{\text{low}}\text{CD23}^+$ . These B cells go on to form germinal centres giving rise to memory B cells.

### **Formation of the Germinal Centre**

It is unclear how follicular B cells first see antigen. Antigen could either enter through the blood or lymph in soluble form (Itano and Jenkins, 2003) or as unprocessed antigen on the surface of APCs (Wykes et al., 1998a). Within a few

hours of B cells recognising their specific antigen, they increase the expression of CCR7 and migrate into the PALS where they proliferate in the presence of cognate T cell help (Reif et al., 2002). T and B cells form stable interactions at this area between 24 and 48 hours after s.c. injection of antigen (Garside et al., 1998). The majority of activated B cells form plasma cells that initially secrete IgM but later switch to other isotypes. Some members of the expanded B and T cell populations migrate into follicles where they commence dividing and go on to form GCs. The GC is a specialised microenvironment where B cells can undergo proliferation, maturation and selection. While the B cells that stay and proliferate in the T cell area form short-lived plasma cells, cells arising from the germinal centre form either long-lived plasma cells or memory cells. Molecules that are known to support the development of GCs include CD40 and CD40L (Foy et al., 1994a), CD28 (Ferguson et al., 1996), and B7-2 (Borriello et al., 1997). These molecules have the potential to act directly at the level of the B cell/T cell interactions that are required for maturation of B cells into GC cells (Fu and Chaplin, 1999). Figure 1.4 demonstrates the movement of the various cell types involved in the initiation of an immune response.

GCs tend to peak in number around fourteen days after immunisation and persist for around 30 days (MacLennan et al., 1997). The GC initially contains only dividing B cell blasts (centroblasts) but shortly resolves into dark and light zones with the light zone being the area rich in the FDCs (described below). While B cells proliferate in the dark zone, a hypermutation mechanism is activated which diversifies the rearranged variable region genes (Jacob et al., 1991; Liu et al., 1992). The cells are then selected for high affinity antibodies, possibly via immune complexes on FDC (Camacho et al., 1998). Those that do not produce high affinity antibodies by hypermutation can be rescued through another round of mutation and selection.

Follicular dendritic cells (FDCs) are found in the B cell follicles of secondary lymphoid tissue and are therefore likely to play an important role in regulating B cell responses. The origin of these cells is still controversial with some studies suggesting

that FDC develop locally from the surrounding reticular network (Dijkstra et al., 1984) and others indicating that FDC migrate into the peripheral lymphoid organs (Kapasi et al., 1998). It may not be the case of one or the other. FDC in the spleen may develop from the surrounding reticular meshwork and FDC in the LN may develop from migrating cells, which enter via the subcapsular sinus (Ettinger et al., 1998).

Antigen is deposited on FDC, which retain antigen in its native state complexed with antibody and complement. These immune complexes (ICs) can be held on the surface of FDC for months if not years (Nossal et al., 1968). In addition to being a source of antigen long after the infection has cleared, FDC also provide contact-dependent antigen-nonspecific costimulatory signals that facilitate chemotaxis and proliferation of B cells (Cyster et al., 2000).

FDCs are thought to play a key role in GC responses. Although it was originally thought that FDCs were crucial for the initiation of germinal centres, studies showing GC formation in the absence of FDCs has questioned this role (Koni and Flavell, 1999). However, although GCs did form in the absence of FDCs, they were not sustained suggesting that FDCs are necessary to maintain GCs. Once the GC B cells exit cell cycle and become centrocytes they are programmed to die unless they receive a positive signal. If their BCR engages Ag on the FDCs, and they receive a CD40 signal from T cells, they obtain these survival signals and can exit the germinal centre (Casamayor-Palleja et al., 1996).

### **The B cell memory phenotype**

The B cell memory response is faster than the primary response with higher antibody titres and antibody of higher affinity. Memory B cells display several differences to naive B cells: they have a lower threshold of activation and ability to directly present antigen to helper T cells. Memory B cells can either join the recirculating pool of lymphocytes or home to antigen draining sites such as the marginal zone of the

spleen (Liu et al., 1996). They can be identified by lack of expression of sIgD and CD38 or by the expression of switched Ig isotypes IgG and IgA (although identification by this method excludes non-switched memory B cells).

### **Migration of lymphocytes throughout the secondary lymphoid tissue**

Given that the initiation of immune responses requires the interaction of various cell types, it is essential that the cells are able to migrate throughout the secondary lymphoid tissues to interact with various cells. Chemokines, cytokines and adhesion molecules all play a role in maintaining the ordered structure of lymphoid tissues. Chemokines are soluble low-molecular weight molecules that have leukocyte activation and/or chemotactic activity. Several chemokines have been identified which direct the positioning of B and T cells within the splenic white pulp, e.g. CXCL13 (BLC) (Gunn et al., 1998a; Legler et al., 1998), CCL19 (ELC) (Ngo et al., 1998) and CCL21 (SLC) (Gunn et al., 1998b). Different cell types produce different chemokines; DCs and stromal cells in the PALS produce CCL19 and CCL21, which maintain T cells in the PALS whereas FDCs produce CXCL13 and attract B cells as well as some T cells to B cell follicles. CCL19 and CCL21 bind CCR7, expressed by naïve T cells and a subset of memory T cells whereas CXCL13 binds CXCR5 expressed on B cells and a subset of T cells.

### ***Maintenance of secondary lymphoid architecture***

Members of the tumour necrosis factor (TNF) family, including TNF, lymphotoxin- $\alpha_3$  (LT $\alpha_3$ ) and LT $\beta$  (LT $\alpha_1\beta_2$ ), have been implicated in both the development and the maintenance of B cell follicles, FDC clustering and GC formation in mice (Alimzhanov et al., 1997; Koni et al., 1997).

### **Lymphotoxin**

TNF- $\alpha$  and LT- $\alpha$  (TNF- $\beta$ ) are structurally related homotrimeric proteins, which share the receptors TNFR-I/p55 and TNFR-II/p75. LT also exists in a membrane-

associated form in combination with the  $\beta$  chain, a transmembrane protein, forming both  $LT\alpha_1\beta_2$  and  $LT\alpha_2\beta_1$ . The  $LT\alpha_1\beta_2$  heterotrimer is designated lymphotoxin- $\beta$  ( $LT\beta$ ) (Browning et al., 1991) and binds with high affinity to  $LT\beta R$  (Crowe et al., 1994) on stromal cells in various lymphoid tissues (Ware et al., 1995). The  $LT\alpha_2\beta_1$  heterotrimer can interact with  $TNFR-I$  and  $TNFR-II$ , but its ability to interact with and activate the  $LT\beta R$  is not fully defined or indeed even if the  $LT\alpha_2\beta_1$  heterotrimer is present in biologically meaningful quantities *in vivo* (Fu and Chaplin, 1999).  $LT\alpha$  and  $LT\beta$  are produced by activated T and B cells.

### **Lymphotoxin in organogenesis**

The role of the lymphotoxin has been studied using various knockout mice. Mice lacking  $LT\alpha$  (and therefore deficient in both  $LT\alpha$  and  $LT\beta$ ) have virtually no LN or Peyer's patches (PP). A certain number of the mice do contain mesenteric lymph nodes (mLN) but the number varies from 1%-20% depending on the investigator (Futterer et al., 1998) and in some mice they were reduced to one node (Fu and Chaplin, 1999).  $LT\alpha^{-/-}$  mice are also deficient in MZMs and FDC and fail to achieve normal B/T segregation in the white pulp of the spleen (De Togni et al., 1994; Matsumoto et al., 1996).  $LT\beta^{-/-}$  mice exhibit a similar phenotype to  $LT\alpha^{-/-}$  mice with the exception that some LNs are retained (mesenteric and cervical) and the splenic architecture is less disturbed (Ngo et al., 1999). This suggests signalling through both  $LT\beta R$  and the TNF receptors play a role in LN organogenesis and splenic architecture.

The different roles of LT and TNF are highlighted when comparing the phenotype of  $LT\beta^{-/-}$  mice with TNF or  $TNFR^{-/-}$  mice. Mice deficient in TNF or TNF receptors develop a complete set of LNs (Fu and Chaplin, 1999), the splenic architecture is less disturbed, with T and B cell zones segregated (Le Hir et al., 1996; Pasparakis et al., 1996), and the marginal zone, albeit altered, is present in the spleen of these mice. This suggests that the disrupted LN formation, T/B segregation and marginal zones

in  $LT\alpha^{-/-}$  mice is mediated through a failure of signalling through the  $LT\beta/LT\beta R$  arm of the family. The phenotypes of the mice are outlined in table 1.1.

| Genotype                | T/B segregation | MZM | FDCs     | References                |
|-------------------------|-----------------|-----|----------|---------------------------|
| $TNF-\alpha^{-/-}$      | +               | +/- | -        | (Pasparakis et al., 1996) |
| $TNFR-1^{-/-}$          | +               | +/- | -        | (Pasparakis et al., 1997) |
| $TNFR-2^{-/-}$          | +               | +   | +/-      | (Erickson et al., 1994)   |
| $LT\alpha^{-/-}$        | -               | -   | -        | (De Togni et al., 1994)   |
| $LT\beta^{-/-}$         | +/-             | -   | -        | (Alimzhanov et al., 1997) |
| B cell- $LT\beta^{-/-}$ | Mostly +        | +/- | Mostly - | (Tumanov et al., 2002)    |

Table 1.1 shows the phenotypes of the various TNF or LT knockout mice.

Knockout mice are important for revealing the role of various molecules, however, they do not allow us to differentiate between a role for that molecule during development of the organism and an ongoing requirement for maintenance. Fusion proteins that block signalling are invaluable for uncovering an ongoing requirement for a particular molecule as they can be given at various times after birth. For example, treatment with the  $LT\beta R$ -Ig fusion protein led to the disappearance of FDCs in the LN within twenty-four hours (Mackay and Browning, 1998). In the

spleen, FDC were also absent and the marginal zones were disrupted (Mackay et al., 1997). Thus, FDC require the continual presence of LT $\beta$ .

### **B cell control of splenic architecture**

B cells have a critical role in organising the splenic architecture. Spleens of  $\mu$ MT mice are around six-fold smaller than WT mice (Asano and Ahmed, 1996). In addition, Crowley *et al* showed that in mice lacking B cells, marginal zone DCs were no longer found at specific DC areas bordering the T cell area (Crowley et al., 1999). Moreover, there were almost no marginal zone macrophages and metallophilic macrophages, although the white and red pulp remained well-defined. There is a possibility that DC populations are different in  $\mu$ MT mice. Similar proportions of subsets has been described (Bradley et al., 2002; Moulin et al., 2000) with similar numbers of DCs (Linton et al., 2000). However, others have described decreased numbers of CD11c<sup>+</sup> DCs (Crowley et al., 1999; Ngo et al., 2001).

Wang *et al* have shown that either T- or B-cell-derived TNF in the context of B-cell-derived LT is sufficient to form FDC and GC (Wang et al., 2001). Given that LT $\beta$ R and TNFR-I are both expressed on FDC themselves (Ryffel and Mihatsch, 1993) it is reasonable to assume that the B cell delivers its LT signal directly to the FDC precursor.

### **Role of TNF and LT in immune responses**

LT $\alpha$ <sup>-/-</sup> mice do not form GC though rare aggregates of PNA-binding cells can be detected around central arterioles when mice are immunised with sheep red blood cells (SRBCs) (Alimzhanov et al., 1997). The defect is less severe in LT $\beta$ <sup>-/-</sup> mice, with clusters of PNA positive cells present in the spleen and mLN although the size and number is reduced (Alimzhanov et al., 1997).



The presence of GC in the LNs of  $LT\beta^{-/-}$  mice but not in the spleen led to the suggestion that the lack of splenic GCs may be the result of disorganisation of splenic architecture (Koni and Flavell, 1999). Fu *et al* examined whether the reduced Ag-specific IgG response in  $LT\alpha^{-/-}$  mice was due to the absence of  $LT\alpha$  on the lymphocytes or the disrupted splenic architecture. When irradiated  $LT\alpha^{-/-}$  mice were reconstituted with wildtype splenocytes and immunised immediately with SRBC, the splenic microarchitecture remained disturbed and there was no IgG response. In contrast, when irradiated wildtype mice were reconstituted with  $LT\alpha^{-/-}$  splenocytes, a strong IgG response resulted (Fu and Chaplin, 1999). Thus,  $LT\alpha^{-/-}$  lymphocytes had no intrinsic defect in the ability to form an IgG response. Whether GC form when splenic architecture is disturbed may depend on the immunisation protocol used, as others have found that disrupted splenic architecture does not prevent GC formation. Using a transgene that expresses LT in the pancreas, kidney, and skin, this limited ectopic expression of LT restored LNs in  $LT\alpha^{-/-}$  mice but did not restore splenic architecture. Despite this, GC responses were restored in both the spleen and the LNs when immunising with OVA emulsified in CFA, a much stronger immunisation protocol (Sacca *et al.*, 1997).

Along with a defect in GC formation,  $LT\alpha^{-/-}$ ,  $LT\beta^{-/-}$  and  $LT\beta R^{-/-}$  mice have impaired high affinity isotype switched Ig responses following primary and secondary immunisation with T cell dependent antigens without adjuvant (Futterer *et al.*, 1998; Koni *et al.*, 1997; Matsumoto *et al.*, 1997).

The requirement for  $LT\beta$  in GC responses appears to be continuous. Following treatment with  $LT\beta R$ -Ig, no splenic GCs were formed upon immunisation with SRBCs. GCs were detected in the mLN of these mice though they were more diffuse and their number and size varied between experiments (Mackay *et al.*, 1997). Memory B cell responses are also reduced in  $LT\alpha^{-/-}$  mice (Fu *et al.*, 2000).

The role of LT $\alpha$  and LT $\beta$  in T cell responses is less well understood. Fu *et al* reported that the microenvironment of LT $\alpha$ <sup>-/-</sup> mice could support the generation of memory T cells but not of memory B cells suggesting that T cell responses are less affected than B cell responses in these mice (Fu et al., 2000).

### **B cell regulation of T cell responses**

Whether B cells are involved in regulating T cell responses is a contentious issue. The very first experiments looking at the role of B cells involved injecting mice with anti-mouse  $\mu$ -chain antibodies resulting in an almost complete loss of B cells (Janeway et al., 1987; Ron and Sprent, 1987). These studies showed that T cell priming to protein Ags does not occur in the absence of B cells. However, when mice with a genetic deficiency in B cells were produced, conflicting results arose.

B cell deficient mice were created either by disrupting the gene encoding the membrane-spanning exon of the  $\mu$  heavy chain ( $\mu$ MT mice) (Kitamura et al., 1991) or by disrupting the JH variable region of the Ig heavy chain (JHD mice) (Chen et al., 1993). Both types of mice have a block at the pre-B cell stage. Using these mice, B cells have been shown to be either essential or dispensable for every stage of the CD4<sup>+</sup> T cell response (Epstein et al., 1995; Linton et al., 2000; Macaulay et al., 1998; Rivera et al., 2001; van Essen et al., 2000). There are several reasons why such conflicting results may have arisen. In addition to the different types of B cell deficient mice and the different genetic backgrounds the mice are maintained on, a diverse range of antigen dose, immunisation routes and *in vitro* read-outs have been used to examine T cell responses.

Nevertheless, even when the same antigen was used, conflicting results arose. A common antigen used to study T cell responses is the protein keyhole limpet haemocyanin (KLH). Immunisation with KLH has been reported to result in both reduced recall responses from  $\mu$ MT mice (Linton et al., 2000; Rivera et al., 2001)

and normal T cell responses (Constant et al., 1995; Epstein et al., 1995). Linton *et al* and Rivera *et al* both used *in vitro* recall assays to examine T cell responses. Epstein *et al* used a variety of tests including *in vitro* restimulations and also skin grafts and granuloma formation to schistosome eggs. Although Constant *et al* showed no difference between C57BL/6 mice and  $\mu$ MT mice in response to KLH, they did see reduced responses in  $\mu$ MT mice in response to ovalbumin (OVA) and pigeon cytochrome c (PCC).

In addition to responses to nominal antigens, the requirement for B cells has also been studied in various autoimmune diseases; for example,  $\mu$ MT NOD mice do not develop diabetes (Serreze et al., 1996). B cells are also required for the induction of some autoimmune diseases such as some models of EAE (Myers et al., 1992) but, confusingly, not for others (Hjelmstrom et al., 1998). The difference seems to be the method of EAE induction; Whereas induction of EAE with human MOG requires B cells, murine MOG does not (Oliver et al., 2003). In additions, B cells have also been shown to be important for the regulation of myelin oligodendrocyte glycoprotein (MOG)-induced EAE (Fillatreau et al., 2002).

By transferring activated B cells into  $\mu$ MT mice, several groups have shown that T cell priming can be restored. While some have found that using activated B cells that are specific for any antigen is sufficient (Linton et al., 2000), others have shown that the B cells must recognize the same antigen as the T cells and are therefore likely to be required for Ag presentation to T cells. For example, Rivera *et al* demonstrated that HEL-specific B cells did not rescue T cell responses to KLH whereas polyclonal B cells did (Rivera et al., 2001). Abbas and colleagues also demonstrated a requirement for Ag-specific B cells *in vivo* by transferring Ag-specific B cells into anti- $\mu$  treated mice (Kurt-Jones et al., 1988). Thus, whether B cells are required for Ag-presentation or some other B cell-derived factor remains unclear.

B cells can influence not only the expansion of T cells but also their differentiation, too. B cells may play a role in helping T cells to differentiate into Th2 cells. When OT-II T cells were transferred to  $\mu$ MT or C57BL/6 mice, the OT-II T cells from  $\mu$ MT mice produced less IL-4 (Linton et al., 2003). Since some cells were able to produce IL-4 in this study, B cells were not absolutely required for Th2 differentiation but the number of IL-4 secretors was greatly reduced. B cells may not always be required to enhance Th2 responses, though, as Epstein *et al* showed equivalent IL-4 production in  $\mu$ MT mice when they were infected with *Schistosoma mansoni* eggs (Epstein et al., 1995). This may be due to the fact that schistosomes are such potent Th2 inducers.

One mechanism by which B cells could promote IL-4 production is through costimulation. The costimulatory molecule OX40L appears to be involved in Th2 differentiation. Indeed, when CD4<sup>+</sup> T cells were stimulated *in vitro* with anti-CD3 and anti-CD28, they produced significantly more IL-4 in the presence of an OX40L transfectant compared to a control cell line (Flynn et al., 1998). A role for B cell-derived OX40L for *in vivo* IL-4 production by CD4<sup>+</sup> T cells has also been shown. B cells from C57BL/6 mice could reconstitute IL-4 production by OT-II T cells in  $\mu$ MT mice but OX40L<sup>-/-</sup> B cells could not (Linton et al., 2003).

A reason why B cells may preferentially promote Th2 over Th1 responses is that Th2 cells may preferentially migrate into B cells follicles to provide help to B cells (randolph et al., 1999). However, Th1 cells can also enter follicles to provide help to B cells (Smith et al., 2004). This is not completely surprising considering that Th1 responses promote production of high affinity IgG2a antibodies.

The case for B cell involvement in CD8 responses is equally controversial. While several investigators have found that the CD8 response to influenza is normal in  $\mu$ MT mice, others have shown that CD8<sup>+</sup> T cell responses are decreased (Bergmann et al., 2001; Kopf et al., 2002). However, despite the conflicting reports, there is a

general trend that B cells are not required for acute infections such as influenza but may be required for persistent infections such as chronic LCMV infection (Thomsen et al., 1996; Topham et al., 1996).

Understanding the importance of B cells in modulating CD8<sup>+</sup> T cell responses has the additional complication that CD4<sup>+</sup> T cells may also regulate CD8 responses in some cases. Thus, it has to be considered whether B cells have a direct role in affecting CD8 responses or an indirect role through the regulation of CD4 responses. Shen *et al* examined CD8 responses to recombinant *Listeria monocytogenes* (*L. monocytogenes*) expressing a peptide for LCMV (gp33) (Shen et al., 2003). Both CD4 and CD8 expansion was similar in  $\mu$ MT and wildtype mice but the contraction phase of CD8<sup>+</sup> T cells was greatly enhanced in  $\mu$ MT mice resulting in fewer memory cells. The enhanced contraction phase was only partly due to CD4<sup>+</sup> T cells, since it was only slightly enhanced in CD4<sup>-/-</sup> mice. Thus, B cells appear to directly modulate the CD8<sup>+</sup> clonal deletion phase in the response to *L. monocytogenes*. This similar expansion but greater decline was also witnessed in  $\mu$ MT mice in response to LCMV infection (Asano and Ahmed, 1996).

Several studies have shown that CD4<sup>+</sup> T cells may provide help for CD8 responses (Matloubian et al., 1994; Shedlock and Shen, 2003; Sun and Bevan, 2004). The mechanism suggested some time ago was through CD4 T cells further activating APCs and thus allowing the APC to efficiently activate the CD8 T cells. This was termed “licensing” (Lanzavecchia, 1998) and is thought to be achieved through CD40:CD40L interactions. The importance of CD40 was revealed by the finding that some CD8 responses are being reduced in CD40<sup>-/-</sup> mice (Whitmire et al., 1999). Also, expansion of OT-I CD8 T cells can occur by immunising with soluble OVA in the presence of an agonistic anti-CD40 mAb (Lefrancois et al., 2000).

The discovery that CD40 is also expressed on CD8<sup>+</sup> T cells led to the hypothesis that perhaps CD4<sup>+</sup> T cells could directly interact with CD8<sup>+</sup> T cells through CD40 on the

CD8 T cell and CD40L on the CD4<sup>+</sup> T cell (Bourgeois et al., 2002), though this possibility has been challenged by others (Lee et al., 2003a; Schoenberger et al., 1998).

A difficulty in understanding the role of B cells in modulating T cell responses is the many and various ways B cells could affect T cell responses. In addition to MHC-dependent cognate interaction, B cells can provide costimulatory signals (Linton et al., 2003), secrete cytokines, such as IL-10 (O'Garra et al., 1990) and LT $\beta$  (Tumanov et al., 2002), produce antibodies, and affect the lymphoid microarchitecture (Tumanov et al., 2002). Therefore, to fully understand what role B cells play in regulating T cell responses, individual molecules on B cells must be examined. The use of bone-marrow chimeras allows us to dissect how the B cells are helping T cells by allowing us to deplete one molecule at a time.

### ***Tracking antigen-specific T cells***

In a mouse, as few as one in one hundred thousand T cells are specific for a single Ag. In the past, many of the assays used to detect T cell responses *in vivo* relied on functional tests, e.g. limiting dilution assays (LDAs). LDAs give an indication of how many cells are able to perform a particular function in response to antigen. However, not all cells will produce a particular cytokine or are cytotoxic so the number of antigen-specific cells present is often underestimated. To overcome this problem, TCR transgenic mice were produced where all the T cells were specific for a single antigen. Due to the large number of antigen-specific cells and the absence of T cell memory in these mice (Croft et al., 1994) an elegant solution was to transfer TCR transgenic cells into a wildtype recipient mouse allowing the number of antigen-specific cells to be present at a more physiological number (Kearney et al., 1995). The antigen-specific cells can then be tracked *ex vivo* using an anti-idiotypic antibody or antibodies to a congenic marker. The advantage to transferring antigen-specific cells is that the increased number of Ag-specific cells allows them to be tracked even before immunisation.

The OT-II Tg mouse is on the C57BL/6 background and recognises the OVA peptide 323-339. Unlike the DO.11.10 Tg cells, which can be tracked using an antibody to the TCR (anti-idiotypic antibody), the OT-II cells do not have an anti-idiotypic antibody. Instead, they are tracked using the V $\alpha$  and V $\beta$  chains of the TCR. Since there are a certain number of T cells in a C57BL/6 mouse that also use this combination of TCR genes, around 0.5% of CD4<sup>+</sup> T cells stain for V $\alpha$ <sub>2</sub>V $\beta$ <sub>5</sub> even in a C57BL/6 mouse. To overcome this problem, many have now crossed these mice with mice expressing a congenic marker, e.g. Ly5.1 mice, allowing the cells to be tracked with antibodies to this marker.

Transgenic mice do possess a certain number of T cells with an additional V $\alpha$  chain as a result of incomplete allelic exclusion and therefore around 5-10% of T cells show a memory phenotype in these mice. The transgenic mice can be crossed with RAG<sup>-/-</sup> mice to prevent this incomplete allelic exclusion.

Altman and co-workers devised the revolutionary concept of using multimers of MHC molecules with peptide to detect antigen-specific cells (Altman et al., 1996). MHC tetramers allow the detection of a polyclonal T cell response in an unmanipulated mouse. This is especially important since the different transgenic cells used may be biased towards particular responses due to their affinity for the peptide-MHC complex or the genetic background. The limitation of MHC tetramers comes with the low number of T cells present before immunisation; clonal expansion must occur before the cells are readily detectable, hampering the ability to study the initial expansion of the T cells.

## **Conclusion**

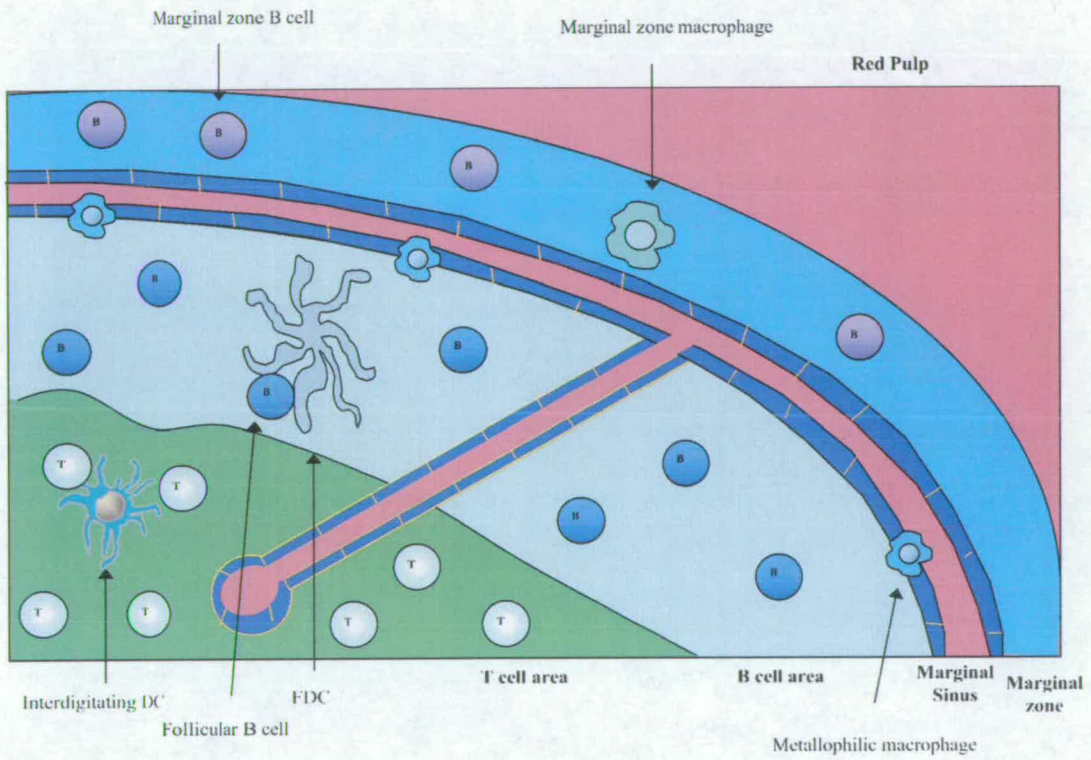
T cells are critical for secreting cytokines at the site of infections and also for directing B cell responses. The factors that T cells require to mount efficient

responses remain undefined. Although it is generally agreed that T cells are first activated via interactions with DCs in the T cell areas of secondary lymphoid tissues, other cells may play an important role in regulating T cell responses. In support of this, many T cells responses are reduced in the absence of B cells, thus, B cells or a B cell-derived factor play a role in modulating the T cell response. Whether the requirement for B cells is due to a requirement for Ag presentation, costimulation or some other factor, for example cytokines, is not clear.

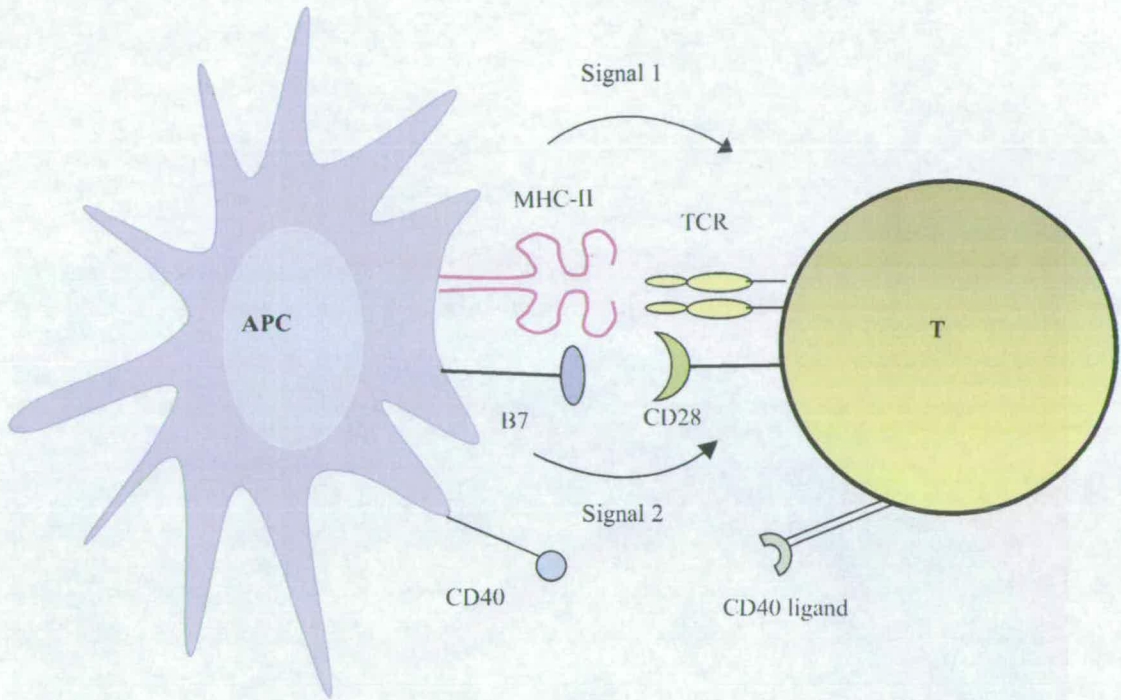
### ***Aims of PhD***

In order to understand how B cells can regulate T cell responses, a number of potential factors have been examined in this project. Possibly the most important way B cells could regulate a T cell response is through the presentation of Ag. However, B cells may also provide costimulatory molecules, such as CD40. Another possibility is that B cells affect T cell responses indirectly by affecting the microarchitecture of the secondary lymphoid tissues. Therefore, T cell responses in the absence of B cell-derived MHC-II, CD40 or LT $\alpha$  has been determined.

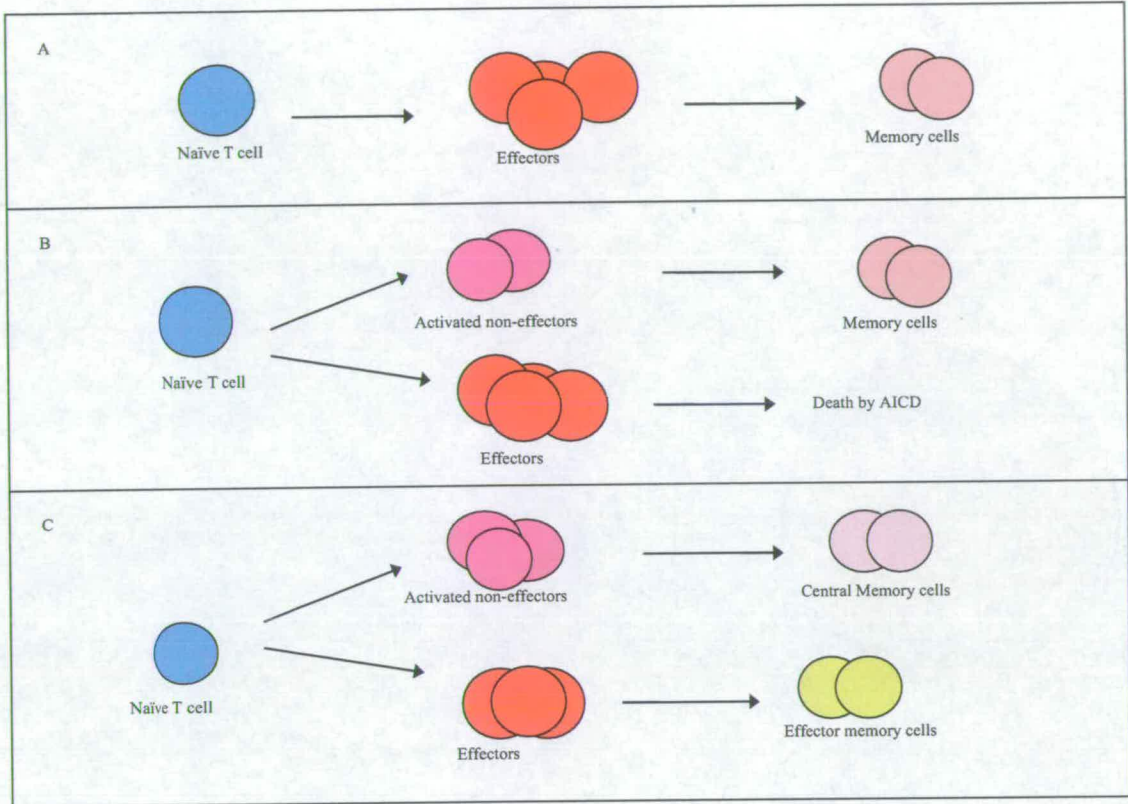




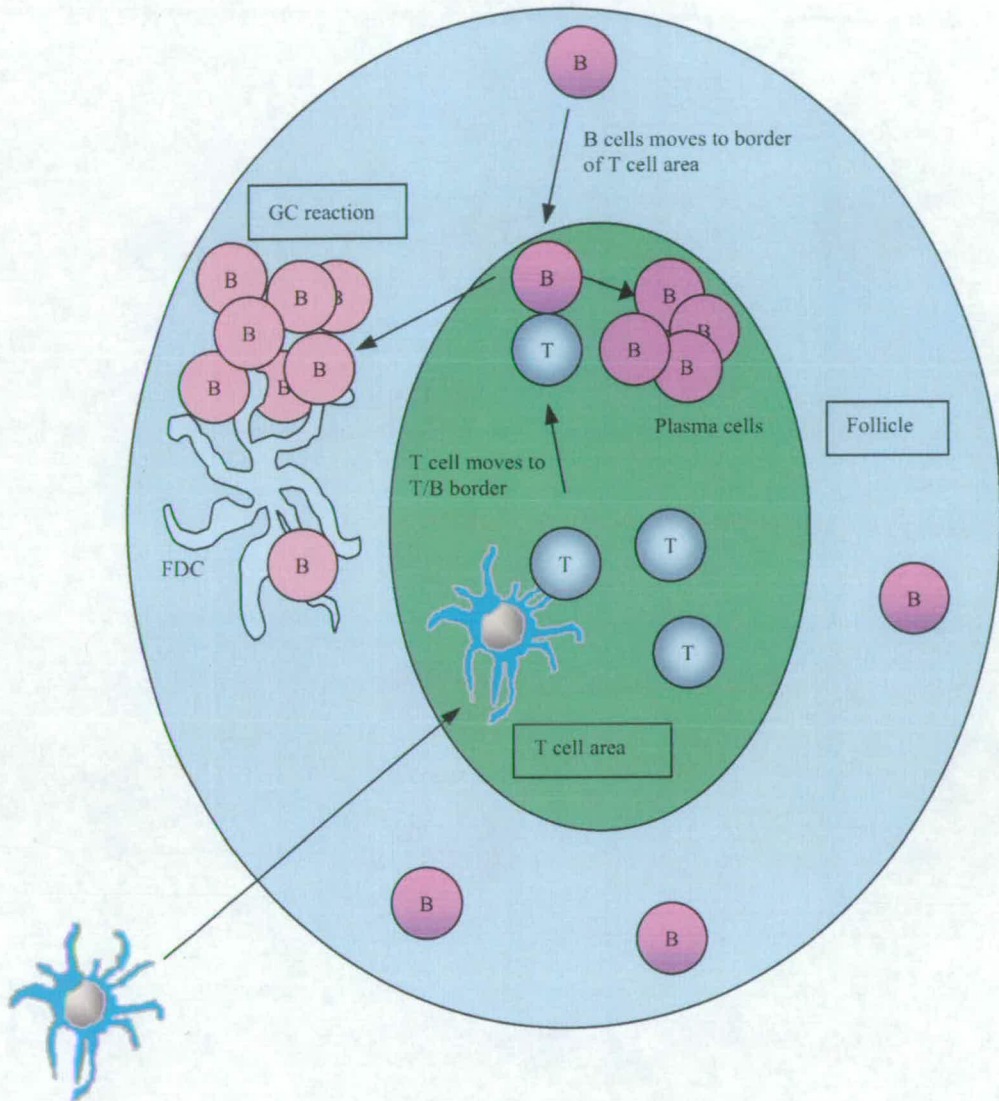
**Figure 1.1.** Diagrammatic representation of the splenic architecture. Different cell types are compartmentalised into different areas of the spleen. The T cell area is surrounded by the B cells area and both are encompassed by the marginal zone and marginal sinus.



**Figure 1.2.** T cell interacting with an APC. T cells require multiple signals to become fully activated. Signal 1 is the interaction between the TCR and peptide-MHC complexes on the APC. Signal 2 consists of interactions between various costimulatory molecules.



**Figure 1.3.** There are a number of possible pathways for the differentiation of naïve T cells into memory cells. Naïve T cells could fully differentiate into effectors before becoming memory cells (A). Alternatively, naïve T cells may differentiate either into effectors, which then go on to die by AICD, or activated cells that become memory cells (B). A third possibility is that effector memory cells are generated from fully differentiated effectors whereas central memory cells are derived from activated cells that have not differentiated into effectors (C).



DC enter from periphery

**Figure 1.4.** The initiation of the adaptive immune responses requires the interaction of various different cell types. T cells are first activated by DCs and then migrate towards the border of the T cell zone. Meanwhile, B cells are being activated in B cell follicles and are migrating towards the border T cell/B cell border to interact with T cells. Once this interaction occurs, the B cells either differentiate into plasma cells in the T cell area or migrate back to the follicles to initiate GC formation.

“A theory is something nobody believes, except the person who made it. An experiment is something everybody believes, except the person who made it.”

**Albert Einstein**

## Chapter 2 – Materials and Methods

### ***Animals***

All animals were maintained under specific pathogen free conditions at the University of Edinburgh facilities. All mice were bred in-house except  $LT\alpha^{-/-}$  mice, which were obtained from Neil Mabbott at the Institute for Animal Health, Edinburgh. The conditions and the screening protocol are outlined in the table below (Table 2.1). For screening, mice were bled from the tail and 50 $\mu$ l of blood was added to 50 $\mu$ l of heparin at 100U/ml (AAH Pharmaceuticals, West Midlands, UK) to prevent coagulation. Red blood cells were removed using red blood cell lysing solution (Sigma, Dorset, UK), cells were stained with antibody and antibody binding was detected by flow cytometry. All experiments involving animals were performed under guidance from the appropriate Home Office personal and project licenses.

### ***Antigens and antigen administration***

For adoptive transfer experiments, ovalbumin (OVA) (Sigma) was coupled to dinitrophenyl (DNP) (Sigma) by reacting 32 $\mu$ l of 2, 4-dinitrofluorobenzene (DNFB) solution (Sigma) per mg of OVA in 0.1M sodium borate buffer, pH 8.4 (Sigma). The reaction was then dialysed against PBS to remove free hapten. Protein antigens were precipitated in alum with 9% aluminium potassium sulfate dodecahydrate (Sigma) solution and washed three times in PBS. Mice were immunised intraperitoneally (i.p.) with 200 $\mu$ g alum precipitated DNP-OVA in 200 $\mu$ l.

For tetramer experiments, mice were immunised with H19env peptide (EPLTSLTPRCNTAWNRLKL), the epitope of moloney murine leukaemia virus presented by I-A<sup>b</sup>. Before immunisation, the peptide was emulsified in complete Freund's adjuvant (CFA) by sonication of equal amounts of peptide with CFA then adding a little extra CFA. Peptide OVA (pOVA) (ISQAVHAAHAEINEAGR)

emulsified with CFA was used as a negative control. Both H19env and pOVA were obtained from the Advanced Biotechnology Centre (ABC), Imperial College, London, UK.

When immunising with DCs, DCs were pulsed with peptide for 90 minutes before  $10^6$  DCs being transferred s.c. into the hind legs ( $50\mu\text{g}$  each leg) or i.v. ( $200\mu\text{l}$ ).

### ***Preparation of cell suspensions for adoptive transfer***

Peripheral lymph nodes (popliteal, inguinal, axillary, brachial, superficial cervical, and iliac), and mesenteric lymph nodes were mashed using Nytex mesh (Wm Ritchie, London, UK) and forceps in Iscoves modified Dulbecco's medium (IMDM, Sigma) with 2% foetal calf serum (FCS, Labtech International, Andover, MA, USA), 2mM L-glutamine (Gibco™, Invitrogen Ltd, Paisley, UK), 100U/ml penicillin, 100 $\mu\text{g}/\text{ml}$  streptomycin (Gibco™) and 50 $\mu\text{M}$   $\beta$ -mercaptoethanol ( $\beta$ -ME, BDH Ltd, Poole, UK). They were then washed once in IMDM with no added serum and resuspended in phosphate-buffered saline (PBS, Sigma). The cells were passed through parachute silk before being injected intravenously into mice.

### ***T cell purification***

Where stated, LN and spleen preparations were depleted of B cells, other MHC-II<sup>+</sup> cells and CD8 cells before being transferred. Cells were incubated with biotinylated anti-kappa (187.1, in-house), anti-MHC-II (M5114, (Bhattacharya et al., 1981), anti-IgM (Southern Biotechnology Associates, AL, USA) and anti-CD8 (53.6.72, in-house).

### **CFSE labelling**

Cells were washed in PBS then resuspended at  $1 \times 10^7$ /ml in  $5 \mu\text{M}$  CFSE (Molecular Probes, Paisley, UK) in serum-free IMDM for 8 minutes at  $37^\circ\text{C}$ . The reaction was quenched with an equal volume of FCS and the cells washed several times. If cells were used for adoptive transfers, cells were washed in serum-free IMDM and resuspended in PBS (Sigma). If used for culture, cells were washed in complete IMDM.

### **Bone Marrow Chimeras**

Chimeras were made as described in Fillatreau *et al* (Fillatreau and Gray, 2003). Recipient mice were lethally irradiated with  $1150\text{cGy}$   $\gamma$  radiation from a caesium source. Bone marrow cells were removed (femur, tibia and humerus) from donor mice and flushed with IMDM containing 2% FCS. They were then passed through an 18 gauge needle then a 25 gauge needle to generate a single cell suspension. Red blood cells were removed using red blood cell lysing solution (Sigma) and the cells washed in 2% IMDM. Bone marrow cells were depleted of T cells using anti-Thy1 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated on a MACS magnetic column (Miltenyi Biotec) according to the manufacturers instructions. The bone marrow cells were resuspended in PBS and mixed to the desired ratio of 80%  $\mu\text{MT}$  and 20% WT or KO BM. Between  $3 \times 10^6$  and  $5 \times 10^6$  cells were injected into the irradiated recipients. The chimeric hosts were then left for eight weeks to allow loss of radiosensitive cells and for establishment of transferred bone-marrow. Reconstitution was confirmed by staining for T cells and B cells by flow cytometry.

### **Production of Bone-marrow derived DCs**

Femurs and tibias were removed and single cells obtained as described above. Cells were resuspended in RPMI medium (Sigma) with 10% FCS, 2mM L-glutamine (Gibco<sup>TM</sup>), 100U/ml penicillin and 100 $\mu\text{g}/\text{ml}$  streptomycin (Gibco<sup>TM</sup>) and 5% GM-



CSF supernatant (from X-63 transfected cell line (Stockinger et al., 1996)). Cells were plated at  $3.75 \times 10^5$  cells per well in a 24 well plate. Cells were washed on day 3 by swirling plates then removing 1ml of medium and replacing with new media. Cells were also washed on day 6 but this time media was replaced with RPMI containing 1% mouse serum (Harlan Sera-Lab, Leicestershire, UK) instead of 10% FCS. The DCs were harvested on day 7 by pipetting up and down. They were incubated overnight with LPS ( $0.1 \mu\text{g/ml}$ ) (Sigma) at  $1 \times 10^6$  cells per ml then washed and peptide-pulsed ( $50 \mu\text{g/ml}$ ) for 90 minutes before being washed and resuspended in PBS then passed through parachute silk before being transferred into mice.

### ***Flow cytometry***

Single cell suspensions of spleens and LNs were prepared and the RBCs lysed from spleens. Up to  $10^6$  cells were stained in  $50 \mu\text{l}$  FACs buffer (PBS with 2% FCS) for 15 minutes on ice, then washed in FACs buffer. The secondary antibody was incubated for a further 15 minutes on ice then the cells washed twice before transferring to FACs tubes. Details of the antibodies used are outline in table 2.1. Most antibodies were purchased from Pharmingen (San Diego, CA, USA). Stained samples were acquired using a FACScalibur (Becton Dickinson) and Cellquest software. FACS analysis was completed using Flowjo software (Tree Star Inc, CA, USA).

For detection of polyclonal cell populations, MHC-II tetramers were used. These were produced in Ton Schumacher's lab in the Netherlands Cancer Institute, Amsterdam. Splenocytes and LN cells were plated in round bottom 96-well plates (Corning Inc, Costar, VWR International Ltd, Poole, UK) at  $1 \times 10^6$  cells per well and labelled with the class II tetramers in 10% IMDM at  $37^\circ\text{C}$ . Plates were gently agitated every 20-30 minutes for 3 hours then additional stains added. CD4-APC (Pharmingen), F4/80-RPE-Cy5 (Serotec, Oxford, UK) and CD44-FITC (142.5 clone) were added for 15 minutes at room temperature. Cells were then washed in FACs buffer (2% FCS in PBS with 0.05% sodium azide (Sigma)) and added to FACs tubes (BD Falcon, San Diego, CA, USA). Just before acquisition of the FACScalibur,

propidium iodide (PI) (Pharmingen) was added to identify dead cells. Cells were gated for live cells and 200,000 events in this gate were collected.

In some experiments, CFSE-labelled splenocytes were also stimulated with peptide for three days then stained with tetramers. For *in vitro* stimulation, splenocytes were labelled with CFSE and plated with 1µg/ml H19env for three days at 37°C with 5% CO<sub>2</sub>. They were then stained as before except CD44 was not added.

### ***Antibody Production, biotinylation and FITC conjugation***

Hybridomas were grown in culture flasks in 5% IMDM until transferred to roller bottles where they were cultured in 2% IMDM and bubbled with 5% CO<sub>2</sub>. The contents of the roller bottles were filtered to remove cells and immunoglobulin was precipitated with 291g of ammonium sulphate (Sigma) per litre of supernatant. Precipitates were dissolved in PBS and dialysed against PBS. Antibodies were purified by binding to a 5ml Hitrap protein G column (Amersham biosciences, Bucks, UK) at pH 7 and eluted at pH 2.8 using the AKTA<sub>prime</sub> (Amersham biosciences). The antibody was then dialysed against PBS until pH was neutral.

Purified antibodies were conjugated to biotin by reacting with 204µg EZ-Link™ NHS-LC-Biotin (Pierce, Cheshire, UK) in dimethyl formamide (DMF, Sigma) per mg of antibody. FITC conjugation was carried out by reacting 1mg antibody with 50ng FITC in 0.05M carbonate-bicarbonate buffer, pH 9. In both cases, conjugated antibodies were dialysed against PBS.

### ***Digestion of lungs and livers***

Mice were euthanised with CO<sub>2</sub> individually and the rib cage immediately opened up. The hepatic vein was cut and the mouse perfused through the heart with PBS containing 75U/ml heparin. The liver and lungs were then removed and the tissue was passed through cell strainers (BD falcon, CA, USA). Livers were spun at 30g for

3 minutes to remove large parenchymal cells then the supernatant layered onto Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). Lung cells were also layered onto Lympholyte-M and both liver and lung cells were spun at 2000g for 20 minutes. The opaque layer of cells, containing lymphocytes, was removed and washed in complete IMDM.

### ***Serum ELISAs***

Blood was left on ice for 4 hours to allow coagulation and then spun at 15000rpm to collect serum. Microtitre plates were coated with 5µg/ml DNP-BSA overnight at 4°C. After washing with PBS, two-fold dilutions of sera were added to wells starting with a 1 in 5 dilution and left for 2-4 hours at room temperature. After washing again with PBS, alkaline-phosphatase-conjugated secondary antibodies, anti-IgM and anti-IgG were added (Southern Biotechnology Associates, AL, USA) for 2 hours at room temperature. Antibody binding was detected using P-nitrophenyl phosphate (PNPP, Southern Biotechnology Associates, AL, USA) dissolved in 0.5M magnesium chloride in 9.6% diethanolamine. Readings were made at an OD of 405nm.

### ***Cell-based ELISAs***

T cells were plated at  $2 \times 10^5$  cells per well with  $2 \times 10^6$  irradiated APCs in a 96-well plate. The OVA peptide was diluted in log-fold dilutions at a starting concentration of 100µM. After 48 hours, 100µl of cells were transferred to maxisorb plates coated the day before with anti-cytokine antibodies (IFN-γ (clone R4-6A2), IL-2 (clone JESE-1A12) or IL-4 (clone 11B11) (Pharmingen) and cytokine standards added to top rows. The following day, plates were washed and biotinylated antibodies (IFN-γ (clone XMG1-2), IL-2 (clone JES6-5H4) or IL-4 (clone BVD6-24G2)) (Pharmingen) added for two hours at room temperature. Plates were then washed and extravidin-AP (Sigma) added at 1/10,000 for one hour at room temperature. Antibody binding detection was performed as described above.

### ***Proliferation Assays***

Cells were plated as for cell-based ELISAs. After 48 hours, 0.5 $\mu$ Ci of [*methyl*-<sup>3</sup>H]Thymidine (Amersham Biosciences, Buckinghamshire, UK) was added per well. 16 hours later, the cells were harvested onto filter mats (Wallac, Turku, Finland) using a 96well harvester (Wallac). The mats were dried on a hot plate then wax was melted onto them (Wallac) then allowed to re-solidify. The mats were counted using a Micrbeta scintillation counter and software (Trilux, Arnsberg, Germany).

### ***Immunohistochemistry***

Spleens were frozen in OCT-embedding medium (BDH, Dorset, UK) in cryomoulds (BDH, Dorset, England) on dry ice and stored at  $-80^{\circ}\text{C}$ . Tissue sections (5 $\mu\text{m}$  thick) were cut onto glass multi-well slides (Hendley-Essex, London, UK) using a cryostat (model CM1510, Leica, Germany) and left overnight to dry before fixation in acetone for 10 minutes. Sections were stained in the combinations stated in table 2.3. Primary stains were left for 2 hours and secondary stains for one hour. After staining, sections were mounted with the embedding medium moviol (Hoechst, Frankfurt, Germany) and analysed using an Olympus BX50 microscope. Images were captured with a Hamamatsu digital camera and Openlab imaging software (Improvision, Coventry, UK).

### ***PCR for genomic LT $\alpha$***

B cells were purified using CD19 beads and LS magnetic columns from miltenyi biotec according to the manufacturer's instructions. Cells were lysed in 200 $\mu\text{l}$  lysis buffer (100mM Tris-HCl pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100 $\mu\text{g}/\text{ml}$  proteinase K) then proteinase K inactivated by incubating at  $99^{\circ}\text{C}$  for 10 minutes. 1 $\mu\text{l}$  of this was then used in a PCR reaction with the following cycling conditions ( $94^{\circ}\text{C}$  for 3 mins then  $94^{\circ}\text{C}$  for 30 secs,  $62^{\circ}\text{C}$  for 30 secs and  $72^{\circ}\text{C}$  for 1 min for 35 cycles followed by  $72^{\circ}\text{C}$  for 10 mins). Primer sequences were given by Neil Mabbott:

Lymphotoxin alpha Oligos. (band at 550bp)

LTa11 – CTC CAC ATG ACA CTG CTC GG

LTa22 – CTC TCC AGA GCA GTG AGT TC

| Strain                               | Reference               | Conditions kept in   | Screening   |
|--------------------------------------|-------------------------|--|---|
| C57BL/6                              |                         | Normal cages with water  | None  |
| BALB/c                               |                         | Normal cages with water  | None  |
| CD40 <sup>-/-</sup>                  | (Kawabe et al., 1994)   | Filter topped cages with borgal  | Bled occasionally and stained with antibodies (Ab) to CD40    |
| $\mu$ MT-H2 <sup>b</sup>             | (Kitamura et al., 1991) | Breeders kept in isolator, weans removed from isolator and kept in filter topped cages with borgal | Bled occasionally and stained with Ab to B220                 |
| I-A <sup>b-/-</sup>                  | (Cosgrove et al., 1991) | Filter topped cages with borgal  | Bled occasionally and stained with Ab to CD4 and MHC-II       |
| OT-II (H-2A <sup>b</sup> restricted) | (Barnden et al., 1998)  | Normal cages with water  | Bled and stained with Ab to CD4, V $\alpha$ 2 and V $\beta$ 5 |

Table 2.1 Animal strains

| <b>Detection</b>         | <b>Antibody</b>      | <b>Clone</b> | <b>Source</b>        | <b>Dilution</b> |
|--------------------------|----------------------|--------------|----------------------|-----------------|
| OT-II T cell expansion   | CD4-APC              | 142.5        | Pharmingen           | 1/400           |
|                          | V $\alpha$ 2-PE      |              | Pharmingen           | 1/200           |
|                          | V $\beta$ 5-biotin   |              | Pharmingen           | 1/100           |
|                          | CD44-FITC            |              | In house             | 1/100           |
|                          | SA-QR                |              | Sigma                | 1/100           |
| OT-II Annexin-V staining | CD4-APC              |              | Pharmingen           | 1/400           |
|                          | V $\alpha$ 2-FITC    |              | Pharmingen           | 1/200           |
|                          | V $\beta$ 5-biotin   |              | Pharmingen           | 1/100           |
|                          | Annexin V-PE         |              | Pharmingen           | 1/100           |
|                          | SA-QR                |              | Sigma                | 1/100           |
| Chimera reconstitution   | B220-PerCP           | M5114        | Pharmingen           | 1/300           |
|                          | CD8-PE               |              | Pharmingen           | 1/200           |
|                          | MHC-II-FITC          |              | In house             | 1/100           |
|                          | CD40-biotin          |              | Pharmingen           | 1/100           |
|                          | SA-APC               |              | Pharmingen           | 1/200           |
| MAC purification         | CD4-APC              |              | All Pharmingen       | 1/300           |
|                          | B220-PerCP           |              | Pharmingen           | 1/200           |
|                          | CD8-PE               |              |                      | 1/400           |
| Tetramer binding         | CD4-APC              |              | Pharmingen           | 1/400           |
|                          | F480-RPE-Cy5         |              | Serotec              | 1/400           |
|                          | CD44-FITC            |              | In house             | 1/100           |
|                          | PI                   |              | Pharmingen           | 1/400           |
|                          | MHC-II tetramers- PE |              | Ton Schumacher's lab | 1/10            |

Table 2.2 Staining for flow cytometry

| Stain        | 1° Ab              | Source   | Dilution | 2° Ab  | Dilution           | Source   |
|--------------|--------------------|--|----------|--|--------------------|--|
| <b>No. 1</b> | Ant-Thy1<br>FITC   | In-house   | 1/100    |  |                    |  |
|              | Anti-IgM-<br>TRITC | Serotec  | 1/200    |  |                    |  |
|              | FDC-M2<br>biotin   | Immunokontakt  | 1/100    | Alexa<br>fluor 350                                       | 1/100              | Molecular<br>Probes  |
| <b>No. 2</b> | Ant-Thy1<br>FITC   | In-house   | 1/100    |  |                    |  |
|              | Anti-IgM-<br>TRITC | Serotec  | 1/200    |  |                    |  |
|              | ERTR9              | Supernatant<br>from G. Kraal,<br>Dept of Cell<br>Biology and<br>Immunology,<br>Amsterdam,<br>Netherlands |          | Anti-rat<br>IgM biotin<br>then<br><br>Alexa<br>fluor 350 | 1/100<br><br>1/100 | Southern<br>biotechnol<br>ogies<br><br>Molecular<br>Probes |

Table 2.3 Immunohistochemistry stains



“We’ve made great medical progress in the last generation. What used to be merely an itch is now an allergy.”

**Mark Twain**

## CHAPTER 3 – Expansion of OT-II T cells is reduced in B cell deficient mice

### ***Introduction***

CD4<sup>+</sup> T cells help to direct the immune response through the production of various cytokines, which act to regulate many cells of the immune system. For example, IFN- $\gamma$  promotes the activation of macrophages whereas IL-4 promotes B cell activation. Since CD4<sup>+</sup> T cells play an integral role in regulating immune responses, it is crucial for vaccine design to understand the mechanisms involved in T cell activation.

Although DCs are thought to be the only cell capable of activating a naïve T cell, other cells may be crucial for enhancing T cell responses or in controlling the differentiation of T cells. After initial activation by a DC in the T cell area, T cells migrate to the border of the B cell follicle where they interact with B cells (Garside et al., 1998). Whether this interaction is required merely for B cell activation or whether it is also critical for T cell activation or differentiation is unclear.

Several studies have attempted to examine the activation of T cells in the absence of B cells. However, many of these have relied on *in vitro* restimulation assays to measure T cell responses, and this may not provide a complete picture of the events occurring *in vivo*. In addition, many of these *in vitro* assays detect a T cell function, for example, limiting dilution assays can be used to detect effector functions, such as cytokine production. However, the problem with this assay is that not all activated cells will necessarily have this function. Thus, it tends to underestimate the numbers of activated T cells (Doherty, 1998). In this study, the OT-II adoptive transfer system was used to follow CD4<sup>+</sup> T cell expansion *ex vivo* in the presence or absence of B cells.

In addition to T cell expansion, B cells may also play a role in regulating T cell memory generation. For example, memory T cell responses to KLH have been reported to be reduced in the absence of B cells (van Essen et al., 2000). In these experiments, SCID mice, which contain neither T nor B cells, were reconstituted with T cells by transplantation of foetal thymi. The mice were then immunised and a cohort of these given B cells at the same time. The mice that also received B cells had significantly more KLH-specific T cells 10 weeks after immunisation than those that did not (van Essen et al., 2000).

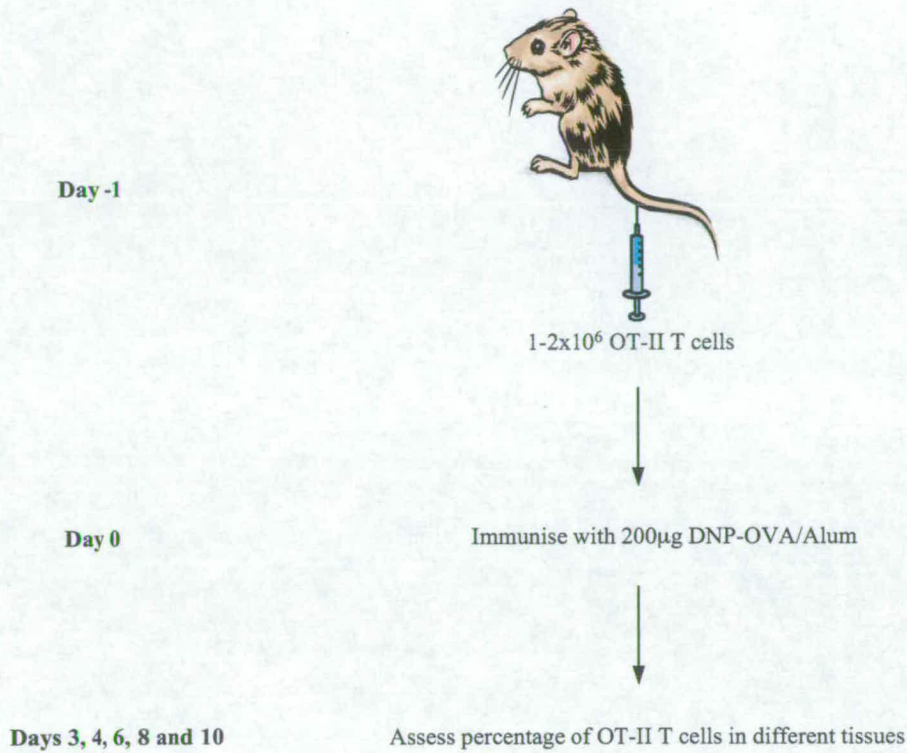
## Results

### The OT-II Adoptive Transfer System

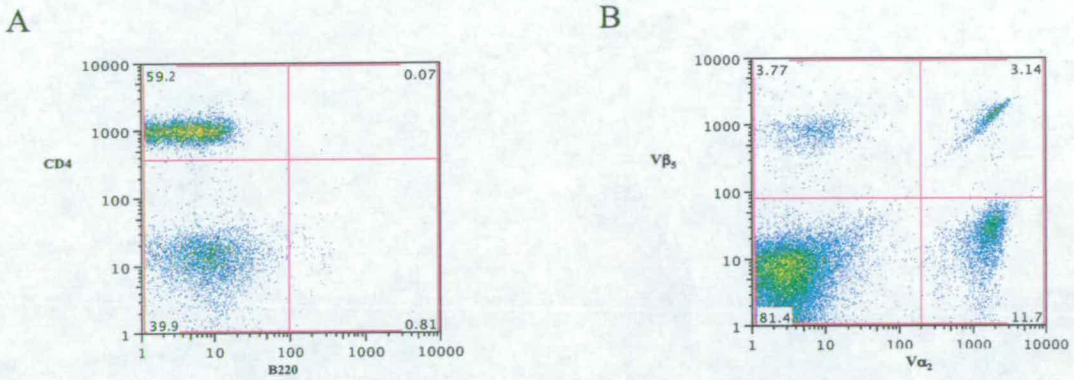
The adoptive transfer system was used to follow T cell responses *ex vivo*. Figure 3.1 shows a diagrammatic representation of the adoptive transfer system used. OT-II TCR Tg mice contain CD4<sup>+</sup> T cells that are specific for a peptide from chicken ovalbumin, 323-339. The spleens and LNs from OT-II TCR Tg mice were removed and APCs, especially B cells, and CD8<sup>+</sup> T cells were depleted. To deplete these cells, splenocytes were incubated with biotinylated antibodies against MHC-II, IgM, Igκ and CD8 then using streptavidin microbeads and running on a magnetic column was used to deplete Ab-bound cells, as described in the MACs protocol. The crucial cell to be depleted is B cells to ensure that no B cells are transferred into the μMT mice. The cells that did not bind the antibodies were transferred into recipient mice by injection into the tail vein. The mice were then immunised in the peritoneum the next day with DNP-OVA/alum and sacrificed at various days after immunisation to examine OT-II T cell expansion. The OT-II T cells use the Vα2 and Vβ5 chains for their TCR therefore antibodies to the Vα2 and the Vβ5 chains were used to identify the transgenic cells *ex vivo* (shown in figure 3.2). The peak of expansion in this system occurs four days after immunisation (figure 3.3).

### The percentage of OT-II T cells in the spleen is lower in μMT mice after immunisation

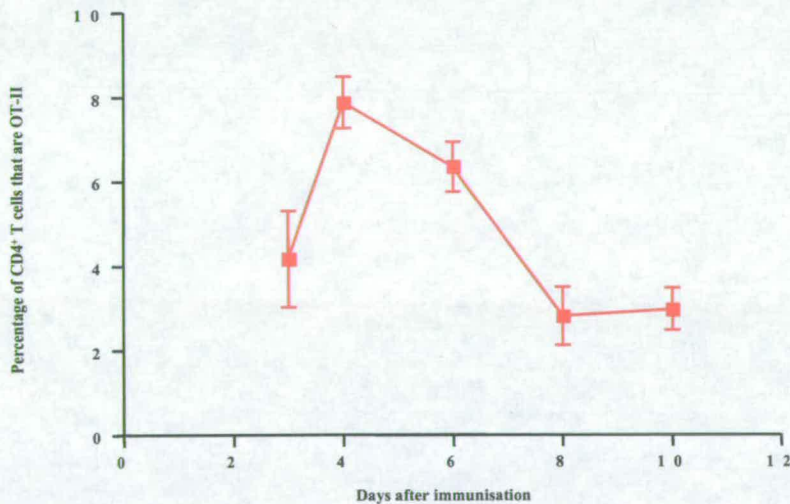
To determine if B cells play a role in regulating T cell expansion, OT-II T cells were transferred into μMT or wildtype (C57BL/6) mice and the mice immunised the following day. On day four after immunisation, the peak of the response, spleens were isolated and the number of OT-II T cells as a percentage of CD4<sup>+</sup> T cells were determined. The percentage of OT-II cells was lower in spleens from μMT mice compared to wildtype mice (figure 3.4). This shows that in the absence of B cells, T cell proliferation is reduced. Therefore, B cells play a role in regulating CD4<sup>+</sup> T cell responses.



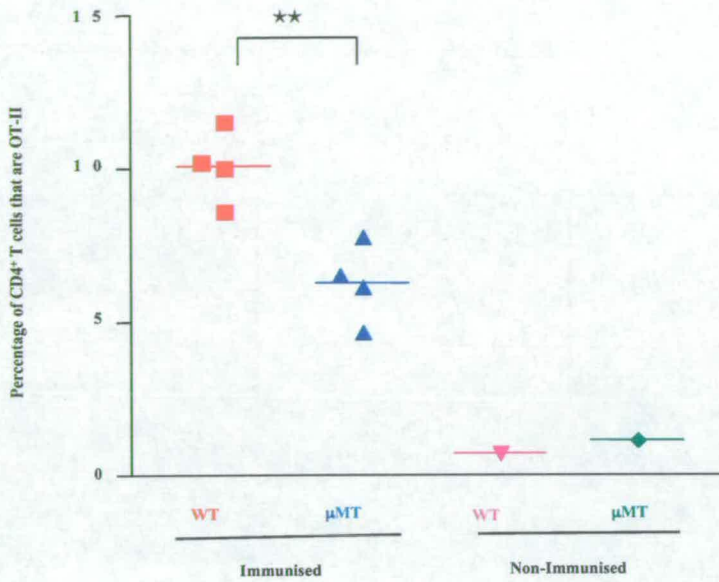
**Figure 3.1.** The adoptive transfer system. To follow antigen-specific T cell responses *ex vivo*, an adoptive transfer system was used. Spleens and lymph nodes from OT-II Tg mice were removed and APCs and CD8<sup>+</sup> T cells depleted using biotinylated antibodies against IgM, Igκ, MHC-II and CD8. Streptavidin beads were then added and cells depleted using a MACs column according to the manufacturer's instructions. Between one and two million T cells were transferred into the tail vein of each mouse and, the following day, mice were immunised with 200µg DNP-OVA/alum. T cell responses were followed on various days after immunisation.



**Figure 3.2.** FACS staining. APCs and CD8 T cells were depleted from spleens and LNs of OT-II mice and the remaining cells were transferred. Less than 1% of cells are positive for B220 and around 60% are T cells (Figure 3.2A). OT-II T cells can be identified by the V $\alpha$  and V $\beta$  chains used to form their TCR. They are double positive for V $\alpha$ 2 and V $\beta$ 5 (figure 3.2B).



**Figure 3.3.** The peak of OT-II T cell expansion occurs four days after immunisation. OT-II T cells were transferred into C57BL/6 mice and immunised with DNP-OVA/alum the following day. Mice were euthanised on days 3, 4, 6, 8 and 10 after immunisation and the percentage of transgenic cells determined. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for both V $\alpha$ 2 and V $\beta$ 5. Symbols show the mean of each group and the error bars show the standard error of the mean (SEM).

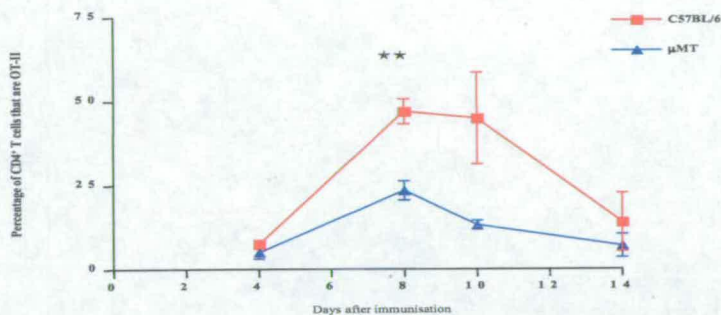


**Figure 3.4.** OT-II T cells undergo less proliferation in B cell deficient mice compared to wildtype mice. CD4<sup>+</sup> T cells from OT-II mice were transferred into μMT or C57BL/6 mice. The mice were then immunised intraperitoneally with 200μg DNP-OVA/alum. Expansion is expressed as the percentage of CD4<sup>+</sup> cells that are positive for Vα2 and Vβ5. Symbols show the result of an individual mouse and lines show the mean of each group. Statistical comparisons were made using the un-paired T-test. \*\*,  $p < 0.01$ . Day four was examined three times with a minimum of three mice per group.

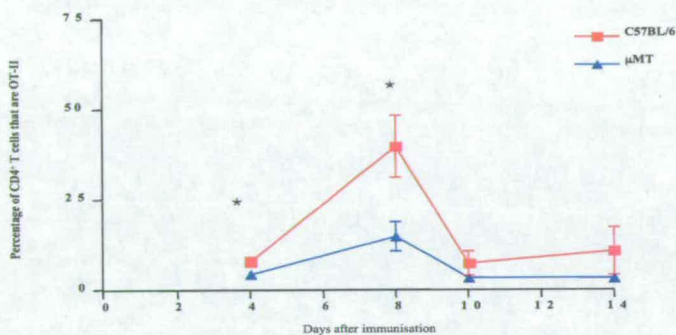
### OT-II T cell numbers are also decreased in peripheral tissues

There are two possible reasons why there are decreased numbers of OT-II T cells in the spleens of μMT mice compared to wildtype mice. Either the cells have proliferated less in μMT mice or, in the absence of B cells, the T cells preferentially exit the spleen and migrate to peripheral tissues. To examine T cell migration, the mice were sacrificed various days after immunisation then immediately perfused and the percentage of OT-II T cells in the lungs and livers of μMT and wildtype mice were examined. The numbers of OT-II T cells in these organs was also reduced in μMT mice compared to wildtype mice (figure 3.5). Therefore, there was no preferential migration of cells to peripheral tissues in μMT mice and the reduced numbers of OT-II T cells in the spleens of μMT mice is due to reduced T cell expansion.

A. LUNG



B. LIVER



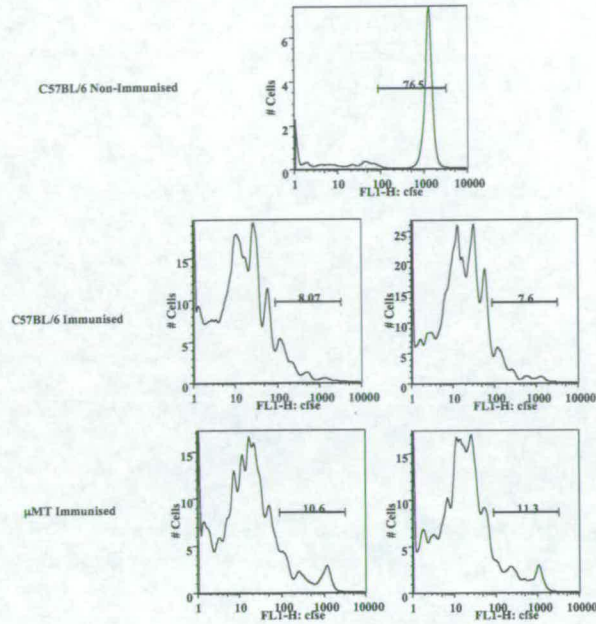
**Figure 3.5.** OT-II T cells do not preferentially migrate to peripheral tissues in the absence of B cells. After transfer of OT-II T cells and immunisation, mice were euthanised and perfused on various days after immunisation and the liver and lung examined for the percentage of OT-II T cells. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for Vα2 and Vβ5. Symbols show the mean of each group and the error bars show the standard error of the mean. Statistical comparisons were calculated using the un-paired T-test. \*, p < 0.05. \*\*, p < 0.01. This experiment was done twice with a minimum of three mice per group.



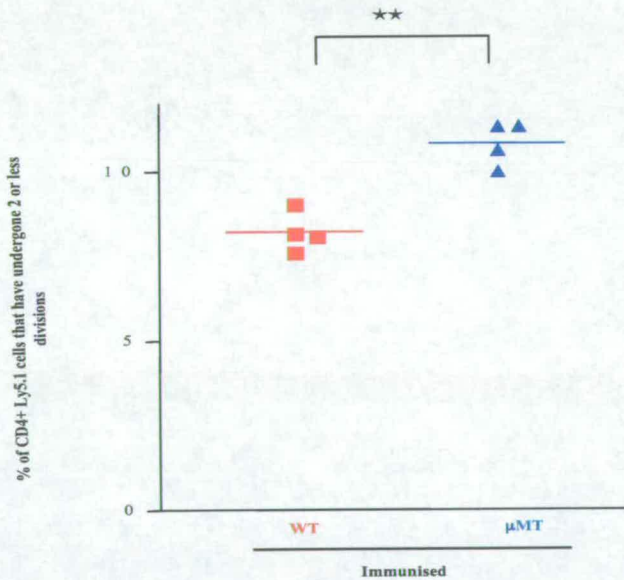
## **The reduced OT-II T cell number in $\mu$ MT mice is due to fewer cells entering division**

To determine whether this reduced expansion of OT-II T cells was due to less T cells entering division or the T cells undergoing fewer divisions, OT-II T cells were labelled with CFSE (carboxyfluorescein succinimidyl ester) before transfer. CFSE brightness is reduced linearly with each division (Lyons and Parish, 1994) allowing the number of divisions a cell has gone through to be tracked. OT-II T cells lose CFSE very quickly so the CFSE loss was examined on day 3 after immunisation. For these experiments, OT-II/Ly5.1 mice were used so that the transgenic cells could be followed using the anti-Ly5.1 antibody instead of antibodies to the TCR. In  $\mu$ MT mice, more T cells appeared to be either undivided or in the first few rounds of division than in wildtype mice but those that did divide could divide to a similar degree as T cells in wildtype mice (figure 3.6A). Indeed, when the numbers of cells that were undivided or had undergone only one or two divisions were examined, there was statistically more cells at this stage in the  $\mu$ MT mice. Thus, in the absence of B cells, the decreased expansion of OT-II T cells is due to less T cells entering cell division rather than cells dividing fewer times.

A



B



**Figure 3.6.** Less OT-II T cells enter cell division in  $\mu$ MT mice compared to wildtype mice. OT-II/Ly5.1 splenocytes were depleted of APCs and CD8 cells then CFSE-labelled before being transferred into  $\mu$ MT or C57BL/6 mice. The mice were then immunised intraperitoneally with 200 $\mu$ g DNP-OVA/alum and sacrificed three days after immunisation. CD4<sup>+</sup> T cells positive for Ly5.1 were examined for CFSE labelling (A). Using FlowJo, gates were set for each division and the proportion of Tg cells having undergone less than two divisions were examined using the unpaired T-test. \*\*,  $p < 0.01$ . Symbols show the result of an individual mouse and lines show the mean of each group. This experiment was done twice with four mice per group.

## Attempts to get OT-II memory cells

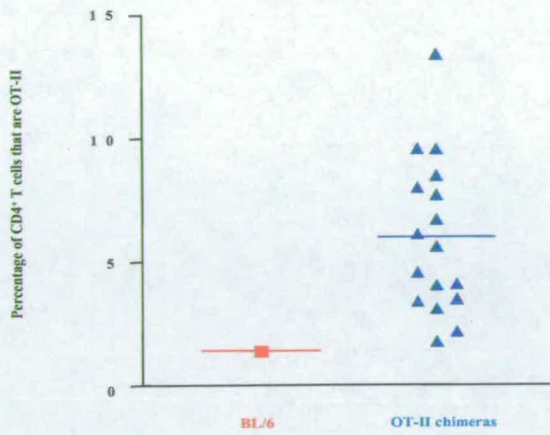
Given that T cell expansion is reduced in  $\mu$ MT mice, I wanted to determine if memory T cell generation and recall responses were similarly reduced. Such experiments were however hindered by an inability to generate memory cells in the OT-II transfer model, as indicated by the percentage of V $\alpha$ 2/V $\beta$ 5 cells present in the spleen. Between ten and twenty days after immunisation, the percentage of V $\alpha$ 2/V $\beta$ 5 T cells in the spleen of the transferred mice had returned to background levels and did not increase in number when the mice were re-immunised. There are several reasons why the OT-II T cells may be disappearing. The OT-II T cells may have an inherent deficiency, perhaps because their TCR is of too low an affinity causing the OT-II T cells to be out competed with host cells. Another possibility is that they are being rejected by host responses to the cells due to minor histo-compatibility differences.

In order to address whether there was minor histo-compatibility differences, I attempted two methods to overcome this potential problem. The first was to backcross the mice an extra four times onto the C57BL/6 background. The second was to create OT-II bone-marrow chimeras by transferring a mixture of OT-II and C57BL/6 bone-marrow into lethally irradiated C57BL/6 mice. This would mean that the OT-II T cells have grown up in the C57BL/6 environment. The theory here was that since there is bone-marrow present from the OT-II mice, the minor histo-compatibility Ags from the OT-II cells will be presented in the thymus and the T cells will therefore be tolerant to these antigens.

OT-II chimeras were made by reconstituting lethally irradiated mice with 90% C57BL/6 and 10% OT-II bone-marrow. Mice were allowed to reconstitute for five weeks before being bled to examine the percentage of OT-II T cells in the blood. Disappointingly, the percentages of OT-II T cells were extremely variable between mice (figure 3.7). To examine expansion, the OT-II chimeras were immunised in the peritoneum with DNP-OVA/alum and the proportions of OT-II cells examined in the

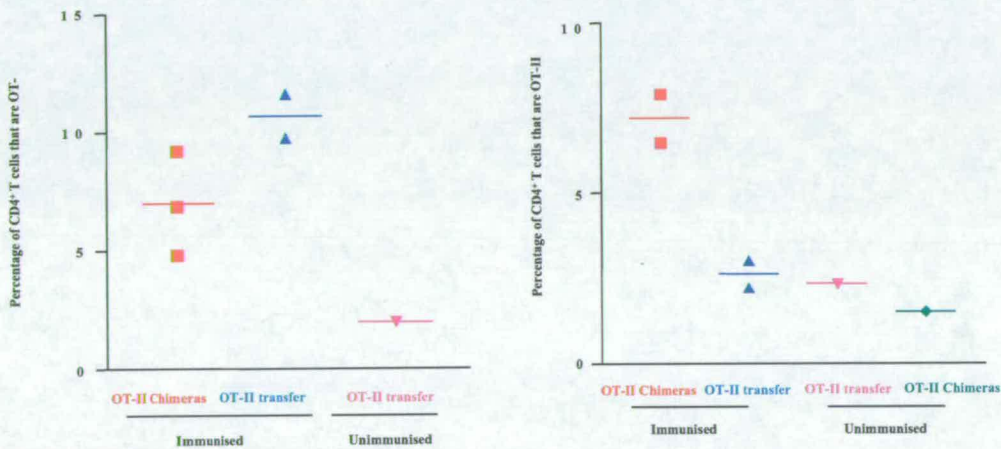
spleen at four and ten days after immunisation. As a comparison to the original system, C57BL/6 mice were transferred with ten million OT-II T cells and similarly immunised. Ten million OT-II T cells were transferred as opposed to the usual number of around one million since the percentages of OT-II T cells were much larger in the OT-II chimeras and the two systems would therefore be more comparable. Both the transferred C57BL/6 mice and the OT-II chimeras showed expansion of OT-II T cells (figure 3.8A). However, the expansion in the OT-II chimeras was lower than I would have expected given that the percentages of OT-II cells in the blood were very high in some of the chimeras. Despite the lower than expected expansion, OT-II T cells were still present in significant numbers ten days after immunisation suggesting that this could be a suitable system to generate memory OT-II cells.

In order to analyse the memory recall response *in vivo*, a cohort of the chimeras were immunised and left for three weeks and examined for the proportions of OT-II T cells remaining and the expansion after reimmunisation. The percentage of OT-II T cells present three weeks after immunisation was very low in both the transferred mice and the chimeras. Although there was an OT-II response three days after reimmunisation, the proportions of OT-II T cells was very variable (figure 3.8B) and was therefore not significantly different. This would make it difficult to compare relatively small groups of chimeras. In order to overcome this difficulty, one possibility would be to bleed the mice before the immunisation to examine proportions of OT-II cells in the blood of the chimeras then mice with similar proportions of OT-II cells could be compared.

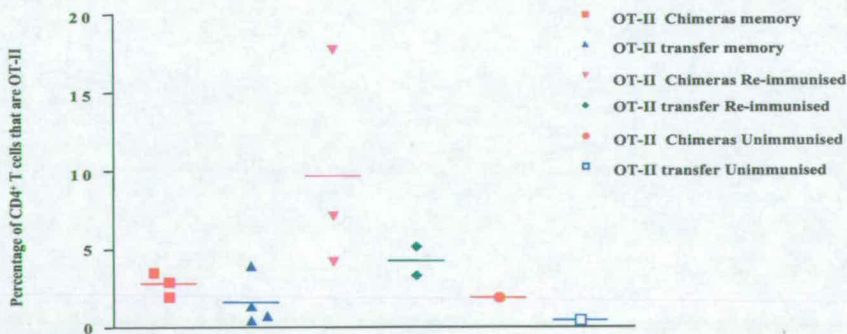


**Figure 3.7.** The proportion of OT-II T cells in the blood of OT-II chimeras was variable. C57BL/6 mice were lethally irradiated and the following day 90% C57BL/6 with 10% OT-II bone-marrow was transferred into the mice. Five weeks later the mice were bled to examine the proportions of OT-II T cells in the blood.

A



B



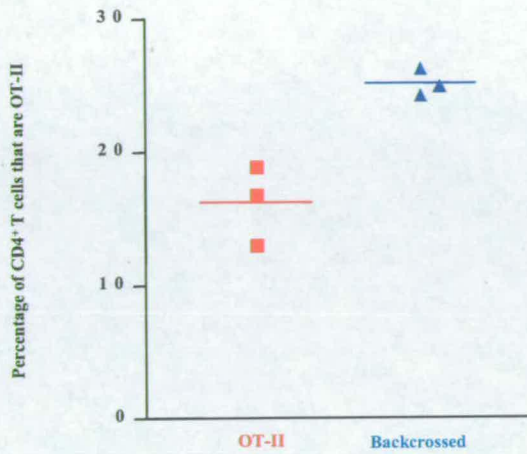
**Figure 3.8.** The responses were variable in OT-II chimeras reflecting the variable numbers of OT-II T cells present. The OT-II chimeras were immunised with DNP-OVA/alum and spleens were analysed for proportions of OT-II T cells four and ten days after immunisation (A). Symbols show the result of an individual mouse and lines show the mean of each group. This experiment was done only once.

## Backcrossing OT-II mice

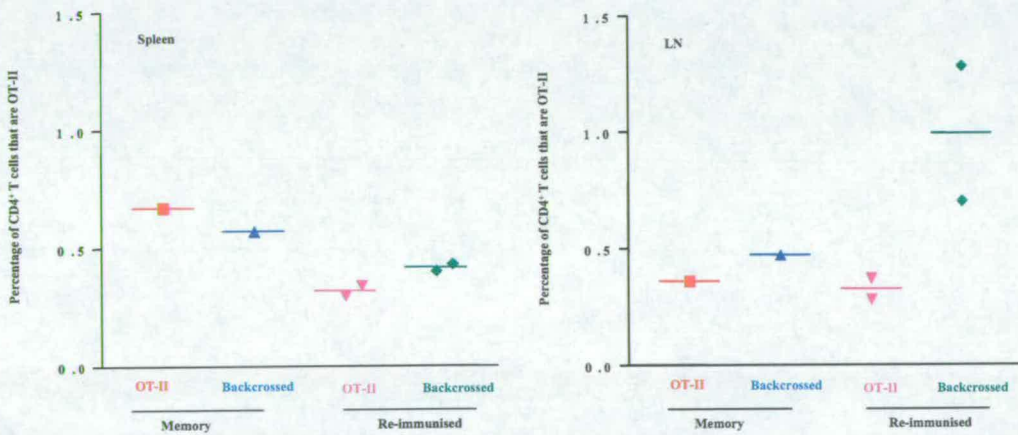
A second approach to achieving a population of OT-II memory cells was to backcross the mice further. Therefore, the OT-II Tg mice were backcrossed a further four times onto the C57BL/6 background. OT-II T cells as before or OT-II T cells from the mice that were backcrossed a further four times (OT-IIBx4 mice) were transferred into C57BL/6 mice. They were then immunised and examined for expansion. Expansion was large in both groups of mice (figure 3.9A).

To examine memory responses, cohort of mice were left for eight weeks before being re-immunised with pOVA/CFA to determine if memory cells were present. In the spleen, neither the mice given OT-II T cells nor those given the OT-IIBx4 cells contained a memory population that was visible after re-immunisation (figure 3.9B). In the LN, however, there was a slight increase in OT-IIBx4 cell numbers after re-immunisation (figure 3.9B). However, since the background of  $V\alpha 2/V\beta 5$  cells can be slightly over 0.5% of CD4s, the numbers of OT-II cells is barely above background levels. Thus, even by backcrossing the mice an extra four times, I could not achieve a sufficiently high number of memory cells to examine memory formation using this system. It is unlikely that more backcrossing of the mice would help given that they had already been backcrossed at least five times before we received them (i.e. a total number of at least 9 backcross generations).

A



B



**Figure 3.9.** Lack of a large memory pool when using OT-II cells. There is a very small memory response in the LN of mice re-immunised with pOVA/CFA. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for V $\alpha$ 2 and VB5. Symbols show the result of an individual mouse and lines show the mean of each group. This experiment was done only once.

## Discussion

From this study, it is clear that B cells or B cell-derived factor(s) are required for efficient CD4<sup>+</sup> T cell expansion. The absence of B cells resulted in decreased CD4<sup>+</sup> T cell expansion by day 3 after immunisation and the number of OT-II T cells remained lower throughout the response. This decrease in OT-II T cell number was seen in both lymphoid and peripheral tissues and was due to fewer T cells entering division rather than increased migration to peripheral tissues.

Although the ability of B cells to stimulate T cells *in vivo* has not been clearly shown, Castiglioni *et al* recently demonstrated that cells other than DCs could activate CD4<sup>+</sup> T cells *in vivo* (Castiglioni *et al.*, 2003). In this study, bone-marrow chimeras were made from *relB*<sup>-/-</sup> mice, which lack functional DCs. When these mice were immunised with plasmid DNA under the control of a B cell-specific promoter, activation of CD4<sup>+</sup> T cells occurred at levels similar to C57BL/6 chimeras. Thus, T cells were activated in the absence of DCs using this stimulatory protocol.

Since the OT-II T cells tended to go back to background levels quite soon after immunisation, attempts were made to generate a memory population in these mice. It is interesting that some groups have achieved memory populations with OT-II T cells suggesting that it is not an inherent defect in the cells that results in defective memory generation (Kondrack *et al.*, 2003; Reinhardt *et al.*, 2001).

Perhaps the OT-II T cells were being rejected due to histo-compatibility differences. Therefore, the OT-II Tg mice were backcrossed a further four times. Unfortunately, a large memory population was still not obtained with OT-II T cells from the further backcrossed mice. When bone-marrow chimeras were made where the C57BL/6 mice were reconstituted with a combination of OT-II and C57BL/6 bone-marrow, OT-II T cells were present in these mice several weeks after immunisation. However, the variability in reconstitution would make it difficult to interpret minor differences. Also, it is difficult to interpret whether the expansion in the OT-II chimeras was due



to memory cells responding or due to newly-developed naïve T cells exiting the thymus. A timecourse of the response and possibly cytokine production is needed to attempt to differentiate between a primary response and a memory response.

Although it is unlikely that the inability to achieve memory cells is due to histocompatibility differences, an experiment that would allow us to exclude this possibility is to lethally irradiate OT-II and C57BL/6 mice and reconstitute with C57BL/6 bone-marrow. After reconstitution, OT-II T cells can be transferred in and the maintenance in the two different sets of chimeras examined. If there were histocompatibility differences, the OT-II T cells would be maintained when OT-II mice were used as hosts but not when C57BL/6 were used as hosts.

As stated earlier, other groups have been able to generate a population of OT-II memory cells. Reinhardt *et al* immunised mice with pOVA and 25-50µg LPS as the adjuvant (Reinhardt et al., 2001). Similar to my results, they reported that most of the cells had disappeared from the secondary lymphoid organs by day eleven after immunisation and that the number of OT-II T cells in non-lymphoid tissues also markedly reduced around this time. However, numbers then stabilised such that similar numbers were present 60 days after immunisation. Kondrack *et al* immunised with OVA/alum and *B. pertussis* and found that the OT-II T cells underwent four- to five-fold expansion and around 50% of these were still present in the mice around one month later (Kondrack et al., 2003).

It is possible that a stronger inflammatory protocol is needed to achieve memory OT-II T cells. For example, 25-50µg LPS, which is a reasonably large amount of LPS to use and giving *B. pertussis* along with alum, gives a stronger response. Therefore, using a stronger adjuvant to increase the response or increasing survival of OT-II T cells could allow us to achieve memory in this system.

The costimulatory molecule OX40 is present on T cells and plays a role in aiding the survival of cells. Adding the anti-OX40 Ab after immunisation has been shown to increase the number of memory cells. Another method of achieving memory is by transferring the OT-II T cells into sublethally irradiated RAG<sup>-/-</sup> mice (shown by Pepi Stamou in the lab). However, it is impossible to look at memory generation in this system. Given more time, it would have been interesting to use MHC-II tetramers to look at memory responses in these mice.

It would have been interesting to look more intensely at T and B cell interactions with immunohistochemistry. For example, to see if I could detect undivided T cells interacting with B cells, especially since Marc Jenkins has suggested that it is such a rare even for a naïve T cell to interact with a B cell, that it is more likely that T cells divide first to increase their numbers before interacting with B cells.

In summary, T cell expansion is reduced in B cell deficient mice. The reduced number of T cells is seen in both lymphoid and peripheral tissues and is the result of fewer cells entering division and more cells in the first few rounds of division. There are several possible reasons why I see this reduced expansion. B cells may be required to present Ag to T cells to help T cells to divide. Another possibility is that B cells are required to provide costimulatory molecules and a third is that it is a B-cell derived factor rather than B cells themselves that is important. B-cell derived factors include antibodies and cytokines. It is unlikely that Ab plays an important role in this system because this is a primary response and B cells require several days to become activated and secrete high amounts of IgG antibody. B cell-derived cytokines are required for maintenance of splenic architecture and as a result of this splenic architecture is disrupted in  $\mu$ MT mice. Perhaps the disrupted architecture is the reason for reduced T cell responses rather than a requirement for interaction with B cells. I went on to examine these possibilities using bone-marrow chimeras allowing me to deplete one molecule on B cells alone and allow other APCs to express this molecule.

“Enough research will tend to support your theory.”

**Mark Twain**

## T cell responses in MHC-II<sup>-/-</sup> B cell chimeras are reduced compared to wildtype chimeras

### **Introduction**

Having shown in the previous chapter that OT-II T cell expansion is decreased in the absence of B cells, I wanted to address whether this requirement for B cells was due to a requirement for B cell Ag presentation to T cells. Although studies carried out *in vitro* have shown that activated B cells can stimulate naïve T cells, whether B cell Ag presentation plays a role *in vivo* is controversial. Croft *et al* reported that activated, but not resting, B cells can stimulate naïve T cells *in vitro* (Croft et al., 1994) and Cassell *et al* demonstrated that activated B cells could stimulate T cells to secrete IL-2 albeit with less efficiency than DCs (Cassell and Schwartz, 1994). This presentation of antigen is greatly enhanced if the B cells possess the surface Ig for that Ag (Sanders et al., 1986) and may be crucial for efficient stimulation of T cells. For example, TNP-specific B cells presented TNP-coupled Ag at 1,000 to 10,000-fold lower concentrations than non-TNP proteins (Abbas et al., 1985; Rock et al., 1984). This increased stimulatory capacity is likely to be due to a number of factors including the upregulation of costimulatory molecules, e.g., B7 as a result of BCR-mediated signalling and the impact the BCR has on Ag processing and presentation (Cheng et al., 1999).

There have been only a limited number of studies demonstrating a potential role for B cell antigen presentation *in vivo* and these studies have tended to use Tg B cells (Constant, 1999; Kurt-Jones et al., 1988). From these investigations it has been shown that B cells can present antigen to T cells very early after immunisation. For example, by immunising Ig transgenic mice then using purified B cells from these mice to stimulate T cells, Constant *et al* demonstrated that B cells could stimulate T cells as early as 4-6 hours after immunisation (Constant, 1999). Moreover, the presentation of peptide-MHC complexes by B cells *in vivo* has been witnessed 4 hours after i.v. injection of hen egg lysosyme (HEL)(Zhong et al., 1997).

Further evidence for B cells as more than just antibody-producing cells comes from studies using mice that possess B cells that cannot secrete antibodies. MRL/lpr mice develop nephritis but when they are crossed onto the B cell deficient JHD mouse, nephritis is attenuated (Chan and Shlomchik, 1998). To examine whether secreted antibodies were involved, mice that can only produce membrane-bound Ig were crossed onto the MRL/lpr background. In this situation, nephritis still developed (Chan et al., 1999). Thus, B cells themselves, in the absence of secreted antibody, are involved in the development of nephritis.

Studies in B cell deficient mice have greatly enhanced our understanding of the role B cells play in regulating T cell responses. However, experiments comparing T cell responses in B cell-deficient mice to those in wildtype mice ignore other deficiencies in these mice, for example, the disrupted architecture of the spleens and LNs. To overcome the problems with comparing B cell deficient mice with wildtype mice, and to assess whether Ag presentation by B cells is important, we created bone-marrow chimeras where the T cells could not interact with B cells in a cognate Ag-specific manner. This was achieved by transferring a combination of  $\mu$ MT and MHC-II<sup>-/-</sup> bone-marrow to irradiated recipients. Thus, all the B cells are derived from the MHC-II<sup>-/-</sup> bone-marrow. This results in MHC-II<sup>-/-</sup> B cell chimeras in which the architecture of the spleen is normal and the B cells are able to provide costimulatory signals and cytokines but they cannot present antigen. Thus, T cells could only recognise Ag on the surface of APCs other than B cells. Transferring OT-II Tg T cells into these mice allowed me to ascertain whether a cognate interaction between the TCR and MHC-II on B cells is required for normal T cell expansion or if it is some other B cell-derived factor that is important.

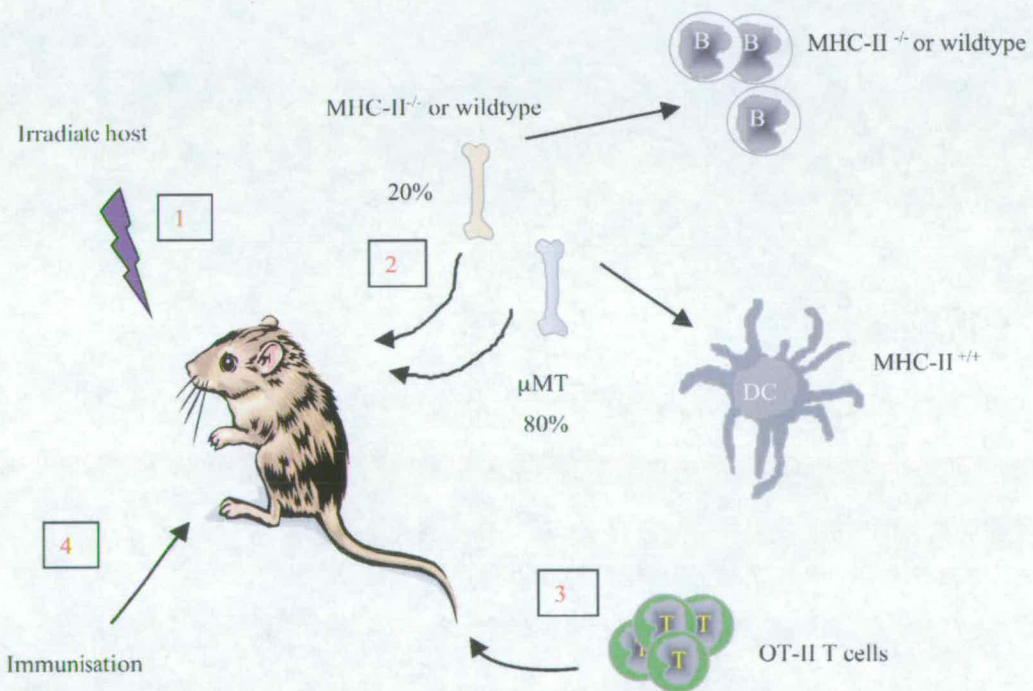
An alternative method of tracking antigen-specific CD4<sup>+</sup> T cells *in vivo* is through the use of MHC-II tetramers. I used MHC-II tetramers in these chimeras to determine whether there is a role for MHC-II on B cells in the primary expansion and T cell memory generation during a polyclonal T cell response as opposed to a

monoclonal response with Tg cells. The tetramers were a tool provided via collaboration with Ton Schumacher in Amsterdam (Schepers et al., 2002).

## Results

### Creating bone-marrow chimeras

To address the question of whether B cell antigen presentation may play a role in enhancing T cell responses, bone-marrow chimeras were created. By combining 80%  $\mu$ MT and 20% MHC-II<sup>-/-</sup> bone-marrow, the resulting chimeras contain B cells that lack MHC-II whereas the majority of other APCs express MHC-II and can therefore present Ag to T cells. As controls, we created bone-marrow chimeras containing a normal B cell compartment by combining 80%  $\mu$ MT and 20% C57BL/6 bone-marrow. Figure 4.1 shows a diagrammatic representation of the bone-marrow chimera procedure.

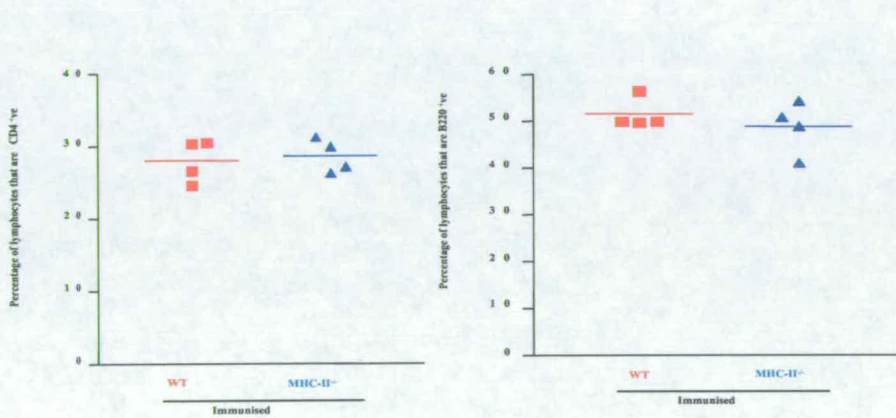


**Figure 4.1.** C57BL/6 mice that were 6 weeks of age were lethally irradiated and reconstituted with  $5 \times 10^6$  bone-marrow cells the following day. Wildtype chimeras were created by combining 80%  $\mu$ MT bone-marrow with 20% C57BL/6 bone-marrow. MHC-II<sup>-/-</sup> chimeras were created by combining 80%  $\mu$ MT bone-marrow with 20% MHC-II<sup>-/-</sup> bone-marrow. The mice were allowed to reconstitute for 8 weeks before use.

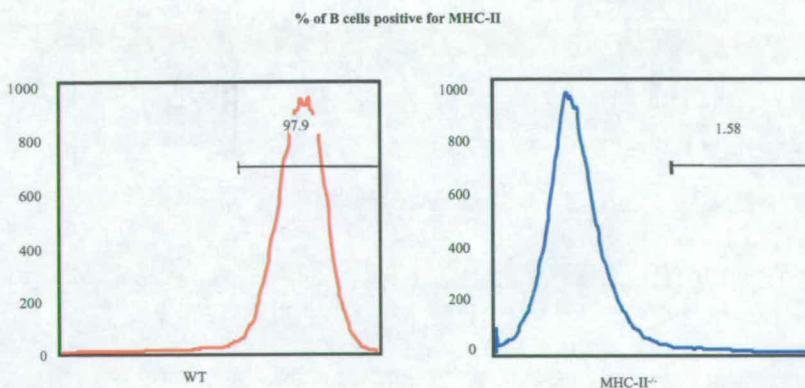
## Bone-marrow chimeras were fully reconstituted before use

The cellular compartments of the wildtype or MHC-II<sup>-/-</sup> chimeras were compared using FACS and immunohistochemistry to ensure full reconstitution of haematopoietic cells and also to ensure that the cell numbers and architecture of the spleen are similar in both types of chimeras. The chimeras contained similar total splenocyte cell numbers and proportions of CD4<sup>+</sup> T cells (figure 4.2A). As expected, in the chimeras created using  $\mu$ MT and MHC-II<sup>-/-</sup> bone-marrow, B cells were negative for MHC-II whereas the control chimeras contained MHC-II-expressing B cells (figure 4.2B). Thus, I have created mice that possess B cells that are deficient in MHC-II to examine the role of Ag presentation by B cells.

### 4.2A



### 4.2B



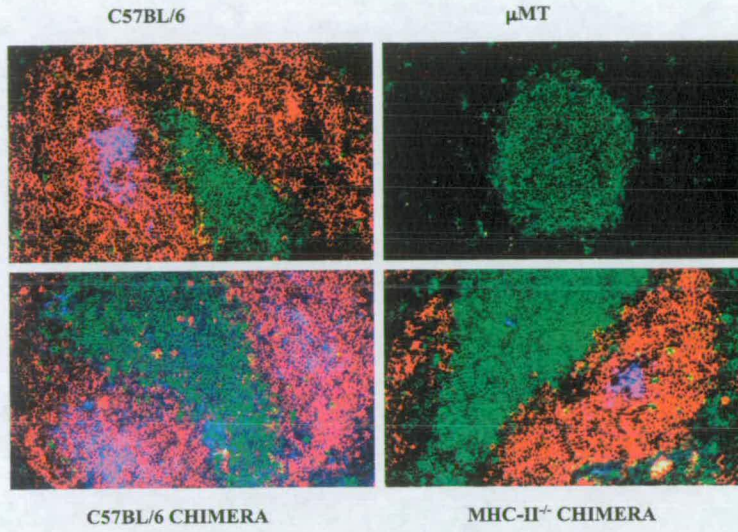
**Figure 4.2.** To ensure complete reconstitution had occurred, mice were sacrificed and splenocytes stained for CD4 and B220. The proportions of CD4<sup>+</sup> and B220<sup>+</sup> cells in the spleen are similar in both types of chimeras (figure 4.2A). Splens from chimeras were stained for B cells and MHC-II. Figure 4.2B shows representative staining.



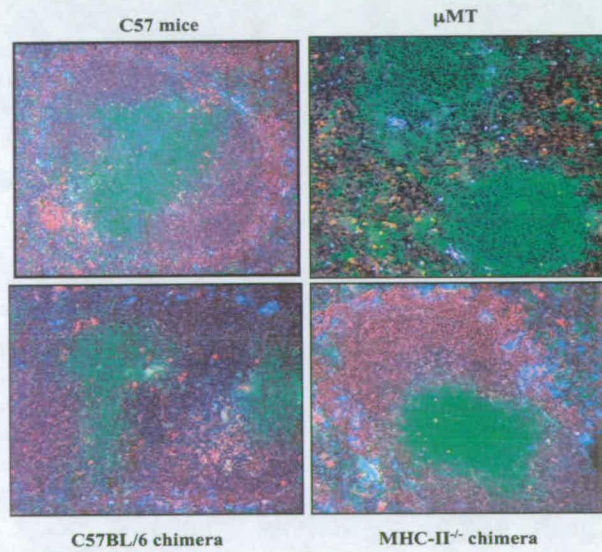
**Splenic architecture is normal in bone-marrow chimeras**

An important difference between  $\mu$ MT mice and wildtype mice is the splenic architecture. In addition to the absence of B cells,  $\mu$ MT mice lack FDCs and marginal zones, making comparisons between the mice difficult. I examined the splenic architecture in our bone-marrow chimeras to ensure that there were no differences between wildtype and deficient B cell chimeras. MHC-II<sup>-/-</sup> and wildtype chimeras contained distinct T and B cell areas, FDCs and marginal zone macrophages (MZMs) to a similar degree. The architecture of the spleen is therefore normal in both types of chimeras (figure 4.3).

A



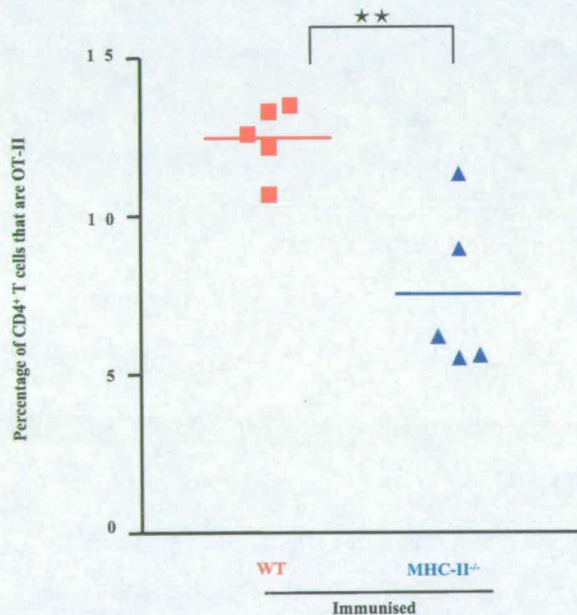
B



**Figure 4.3.** Spleens were removed and frozen in cryo-m-bed before sectioning. Sections were stained for the T cell area in green (anti-Thy1-FITC), B cell area in red (anti-IgM-TXRD) and FDCs in blue (FDC-M2 biotin and SA-AMCA) (A) or T cell area, B cell area and MZMs (ERTR9 spernatant + anti-rat biotin + SA-AMCA) (B).

## OT-II T cell expansion is reduced in MHC-II<sup>-/-</sup> B cell chimeras compared to wildtype chimeras

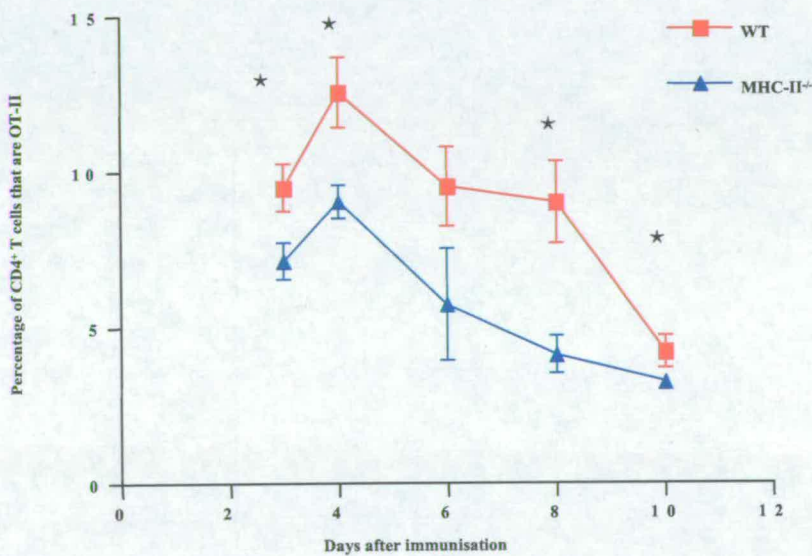
To examine whether an interaction between the TCR and MHC-II on B cells is required for T cell expansion, OT-II T cells were transferred into wildtype or MHC-II<sup>-/-</sup> B cell chimeras and the mice immunised the following day. On day four after immunisation, spleens were isolated and the percentage of OT-II cells determined as in the previous chapter. Although OT-II T cells did expand in both types of chimeras, the number of OT-II cells is lower in MHC-II<sup>-/-</sup> B cell chimeras (▲) compared to wildtype chimeras (■) (figure 4.4). This shows that T cells can expand in the absence of a cognate Ag-specific interaction with B cells but this expansion is reduced demonstrating that B cell Ag-presentation can play a role in T cells expansion *in vivo*.



**Figure 4.4.** OT-II T cell expansion is reduced in the spleen of MHC-II<sup>-/-</sup> B cell chimeras after immunisation. CD4<sup>+</sup> T cells from OT-II mice were transferred into bone-marrow chimeras where the B cells lack MHC-II or control chimeras and the mice immunised the following day. Mice were euthanised on day 4 after immunisation and the percentage of transgenic cells determined. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for V $\alpha$ 2 and V $\beta$ 5. Symbols show the result of an individual mouse and lines show the mean of each group. Statistical comparisons are by un-paired T-test. \*\*,  $p < 0.01$ . Day four was examined three times with a minimum of four mice per group.

### OT-II T cell numbers are reduced throughout the CD4<sup>+</sup> T cell primary response

To determine if Ag-presentation by B cells is required throughout the T cell primary response, mice were sacrificed on various days after immunisation and analysed for proportions of OT-II T cells in the spleen. Figure 4.5 shows the expansion of OT-II T cells at various days after immunisation in the spleen. Although the kinetics of the response are similar, decreased numbers of OT-II cells could be seen in the spleen at day 3 after immunisation and this reduced T cell number was found throughout the primary response. Thus, MHC-II on B cells is required early in T cell expansion and the effect of the absence of B cell MHC-II is seen throughout the T cell response.



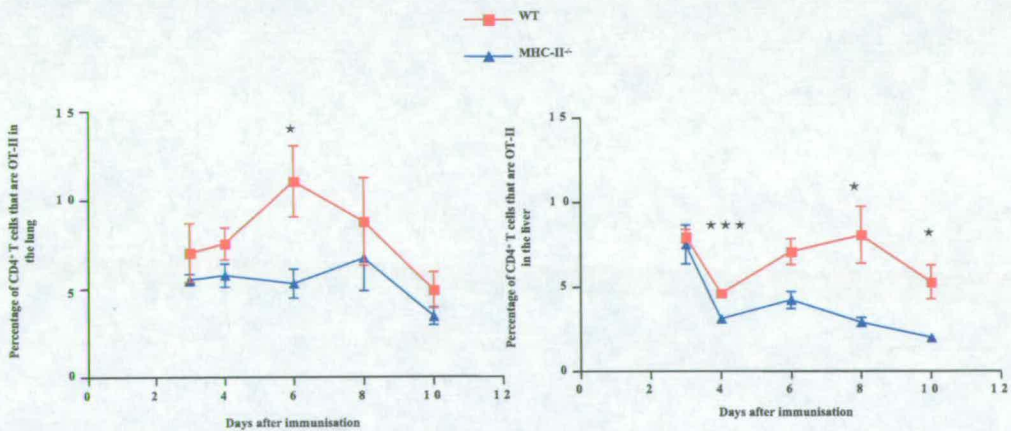
**Figure 4.5.** OT-II T cells undergo less expansion in MHC-II<sup>-/-</sup> B cell chimeras and this decreased cell number is maintained throughout the primary response. OT-II T cells were transferred into bone-marrow chimeras where the B cells lack MHC-II or control chimeras and the following day the mice were immunised. Mice were euthanised on days 3, 4, 6, 8 and 10 after immunisation and the percentage of transgenic cells determined. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for V $\alpha$ 2 and V $\beta$ 5. Symbols show the mean of each group and the error bars show the standard error of the mean. Statistical comparisons were completed by the un-paired T-test. \*,  $p < 0.05$ . All days were examined twice with a minimum of three mice per group.

## OT-II T cell numbers are also reduced in peripheral tissues of MHC-II<sup>-/-</sup> B cell chimeras

There are two possible explanations for detecting less Ag-specific T cells in the spleen: either the T cells have divided less efficiently; or the T cells have left the spleen and migrated to peripheral tissues. To determine if the T cells had preferentially migrated to peripheral tissues in MHC-II<sup>-/-</sup> B cell chimeras, the mice were perfused to remove blood from the tissues and then the lungs and livers were examined for the presence of OT-II T cells. As shown in figures 4.6 A and B, OT-II T cells were present in equal or lower number in the peripheral tissues of MHC-II<sup>-/-</sup> B cell chimeras. Thus, T cells did not preferentially migrate to peripheral tissues, and the reduced OT-II T cell numbers seen in the spleen is due to less expansion in the absence of MHC-II on B cells.

### A. LUNG

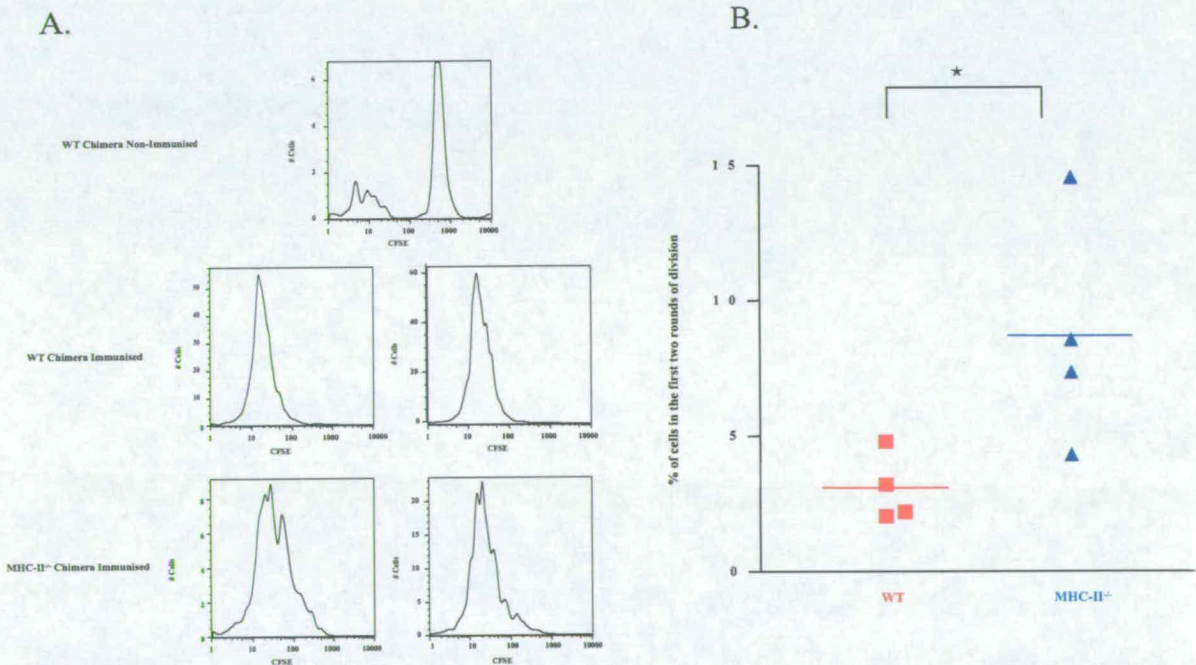
### B. LIVER



**Figure 4.6.** OT-II T cells do not preferentially migrate to peripheral tissues in the absence of MHC-II on B cells. After transfer of OT-II T cells and immunisation, mice were euthanised and perfused on days 3, 4, 6, 8 and 10 after immunisation and the liver and lung examined for the percentage of transgenic cells. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for V $\alpha$ 2 and VB5. Symbols show the mean of each group and the error bars show the standard error of the mean. Statistical comparisons were calculated using the un-paired T-test. \*, p < 0.05. This experiment was done only once with four mice per group.

## The reduced OT-II T cell number in MHC-II<sup>-/-</sup> chimeras is due to less cells entering division

There are several possible explanations for the decreased expansion of T cells. B cells could affect T cell expansion by increasing the number of T cells entering division or increasing the number of divisions they undergo. To determine which of these occurred, OT-II T cells were labelled with CFSE and examined three days after immunisation, as described in the previous chapter. In MHC-II<sup>-/-</sup> chimeras, more T cells were either undivided or in the first few rounds of division than in wildtype chimeras but those T cells that did divide could divide fully (figure 4.7). This suggests that Ag-specific cognate interactions with B cells are important in inducing T cell division.



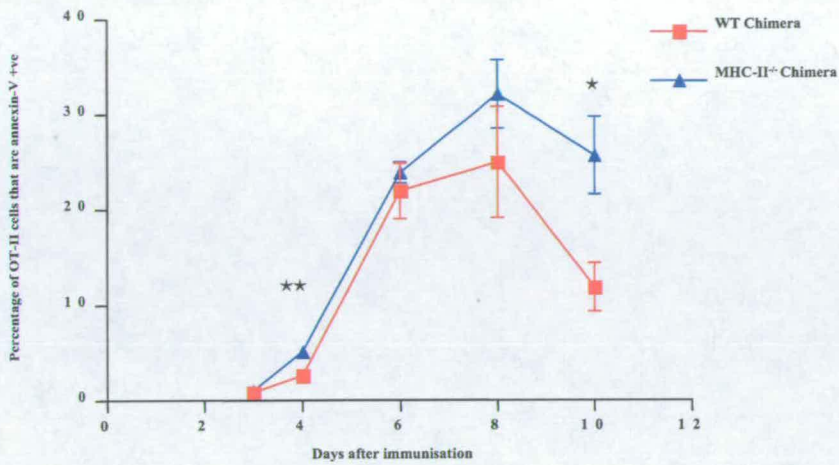
**Figure 4.7.** Less OT-II T cells enter cell division in MHC-II<sup>-/-</sup> B cell chimeras (▲) compared to wildtype chimeras (■). CD4<sup>+</sup> cells from OT-II mice were CFSE-labelled and transferred. The mice were then immunised the following day. CD4<sup>+</sup> T cells positive for Vα2/Vβ5 were examined for CFSE labelling (4.7A). Symbols show the result of an individual mouse and lines show the mean of each group. Statistical comparisons were made using un-paired T-test. \*, p < 0.05. Day four was examined three times with a minimum of four mice per group. This experiment was done twice with a minimum of three mice per group.

**The number of OT-II T cells undergoing apoptosis is similar in both types of chimeras.**

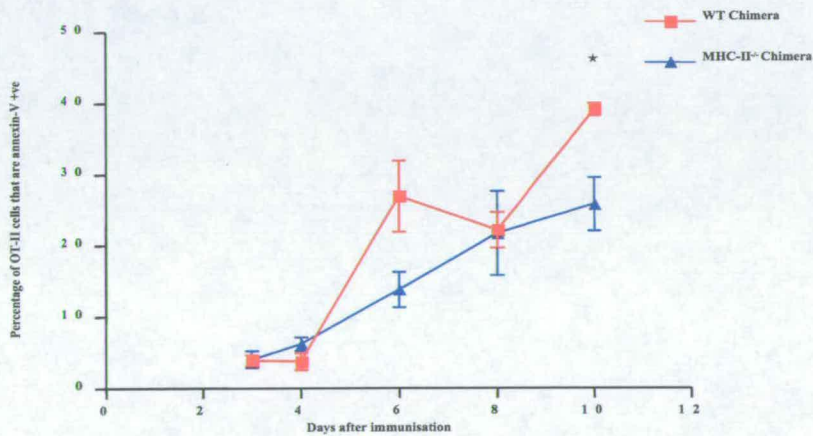
There are several phases of the T cell response: clonal expansion, clonal deletion and memory generation. In addition to clonal expansion, B cells may also be required for clonal deletion or memory generation of T cells. During the clonal deletion phase, the majority of activated T cells undergo apoptosis and the extent of the clonal deletion phase may affect the number of cells entering the memory compartment. To determine if the clonal deletion phase was similar in the presence or absence of a cognate MHC-II interaction with B cells, the proportions of OT-II T cells undergoing apoptosis was determined on various days after immunisation.

It is difficult to detect apoptotic cells *ex vivo* because the cells of the immune system are very efficient at phagocytosing and removing apoptotic cells. Thus, apoptosis has to be detected at a very early stage. One of the earliest signs that a cell is undergoing apoptosis is the movement of the membrane phospholipids phosphatidylserine (PS) from the inner membrane to the outer membrane. Annexin V binds to PS and can therefore be used to detect early apoptotic cells. When OT-II T cells in the spleen were examined for expression of annexin V at various days after immunisation, there was no consistent difference between the numbers of OT-II T cells undergoing apoptosis in MHC-II<sup>-/-</sup> and wildtype chimeras (figure 4.8 A and B). In the first experiment there was more apoptotic T cells in the chimeras lacking MHC-II on B cells at four and ten days after immunisation (figure 4.8A). However, when this was repeated, this effect was not seen. Thus, MHC-II on B cells does not have a clear role in regulating the deletion phase of the OT-II response.

## A. First Experiment



## B. Second Experiment



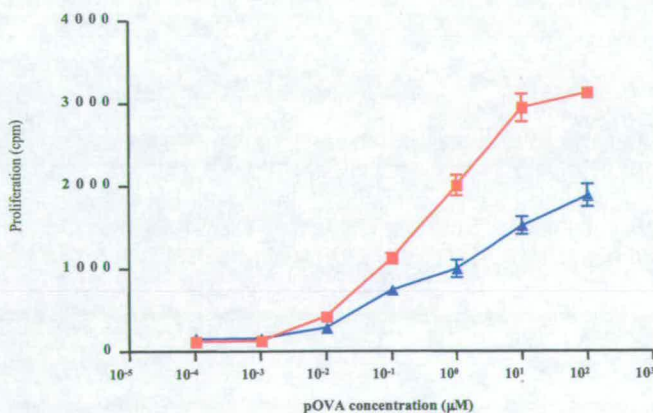
**Figure 4.8.** OT-II T cells do not undergo more apoptosis in the absence of a cognate interaction through MHC-II with B cells. OT-II T cells in the spleen were examined for apoptosis by annexin V staining at various days after immunisation. Symbols show the mean of each group and the error bars show the standard error of the mean. Statistical comparisons were made using the un-paired T-test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . This experiment was completed twice with a minimum of three mice per group.



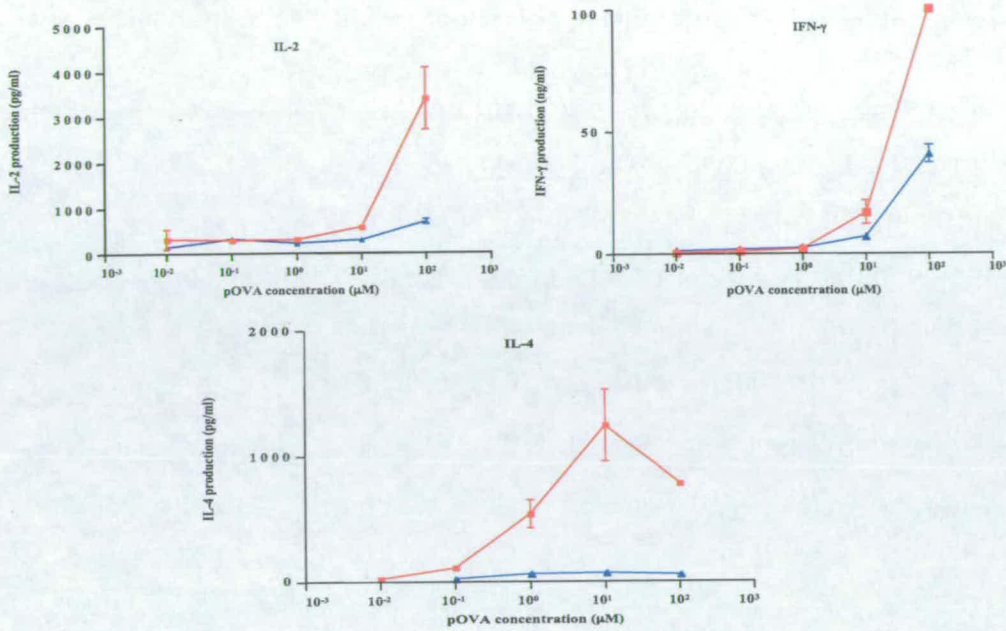
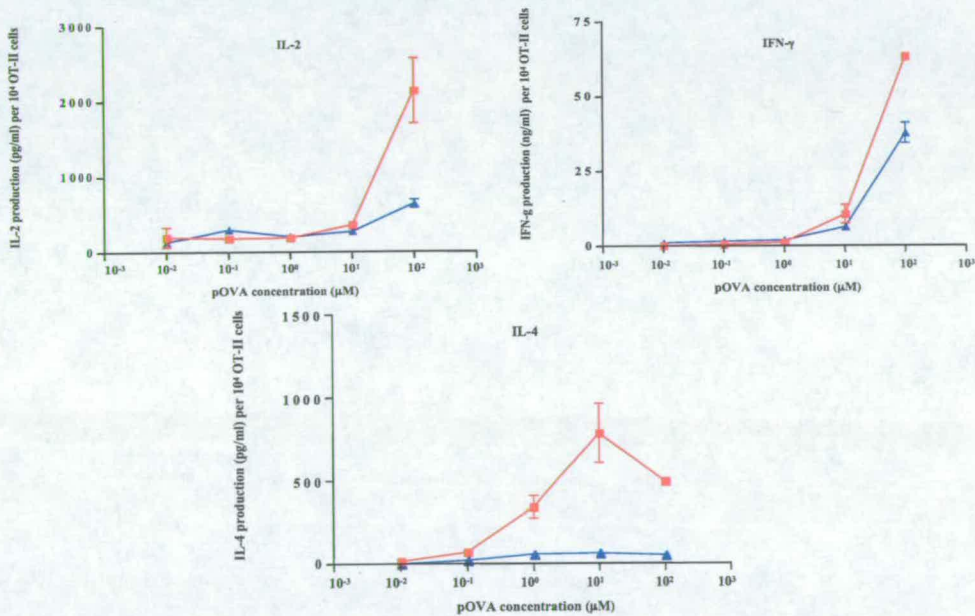
***In vitro* restimulation of T cells to examine proliferation and cytokine production**

To determine whether the absence of MHC-II on B cells influences cytokine production by T cells, the splenocytes from chimeras that received OT-II T cells were harvested at various days after immunisation and examined for *in vitro* proliferation and cytokine production. Figure 4.9A shows the proliferation of T cells from wildtype and MHC-II<sup>-/-</sup> B cell chimeras after three days restimulation *in vitro*. In agreement with *in vivo* data, the T cells from MHC-II<sup>-/-</sup> B cell chimeras proliferate less well than those from wildtype chimeras. Consistent with the reduced proliferation, IL-2 production was reduced in the absence of MHC-II on B cells. The MHC-II<sup>-/-</sup> B cell chimera group also showed less IL-4 and IFN- $\gamma$  production, suggesting an overall reduction in response rather than skewing to a Th1 or Th2 cytokine profile (figure 4.9B). One difficulty in comparing cytokine expression by the two groups is that the starting populations contained different numbers of OT-II T cells. To account for this, the cytokine production was re-analysed for the amount of cytokine produced per 10<sup>4</sup> OT-II T cells. All cytokines were still reduced using this analysis (figure 4.9C).

## 4.9A. Proliferation assay



## 4.9B. Cytokine production

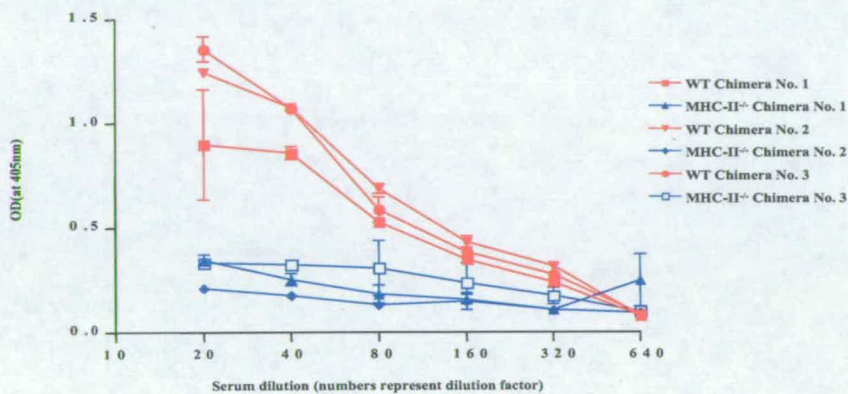
4.9C. Cytokine production per 10<sup>4</sup> OT-II T cells per well

**Figure 4.9.** Proliferation and cytokine production was reduced in T cells from MHC-II<sup>-/-</sup> B cell chimeras (▲) compared to wildtype chimeras (■). Pooled splenocyte populations from both groups were depleted of CD8<sup>+</sup> T cells and MHC-II positive cells. The cells plated at  $2 \times 10^5$  cells per well with  $2 \times 10^6$  irradiated splenocytes with varying concentrations of peptide. Ag-specific proliferation (figure 4.9A) and IL-2, IFN- $\gamma$  and IL-4 production was examined three days after *in vitro* restimulation (figure B and C). The wildtype chimeras produced amounts of IFN- $\gamma$  that were in excess of the limit of detection (100ng/ml) at 100 $\mu$ M pOVA but are shown as 100ng/ml. These graphs show results from six days after immunisation and are representative of all other days examined. The proliferation assays and ELISAs were competed twice for each day.

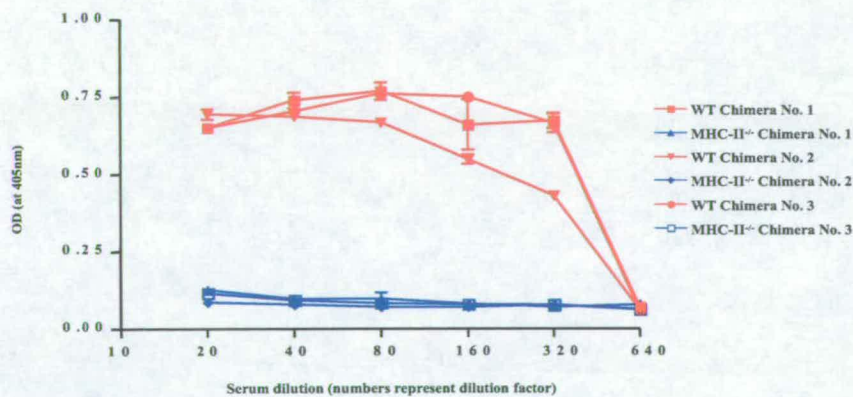
## MHC-II<sup>-/-</sup> B cell chimeras do not form anti-DNP specific antibodies

B cells require a cognate interaction with T cells to form germinal centres and to isotype switch in response to protein antigens. B cells in the MHC-II<sup>-/-</sup> B cell chimeras are unable to form a cognate MHC-TCR interaction and therefore should not produce IgG antibodies in response to DNP-OVA. To determine if this was so, serum was examined from mice immunised 10 days previously with DNP-OVA/alum. MHC-II<sup>-/-</sup> B cell chimeras could not produce any IgG anti-DNP antibodies and produced greatly reduced amounts of IgM anti-DNP antibodies indicating a requirement for cognate MHC-TCR interactions (figure 4.10).

### IgM



### IgG

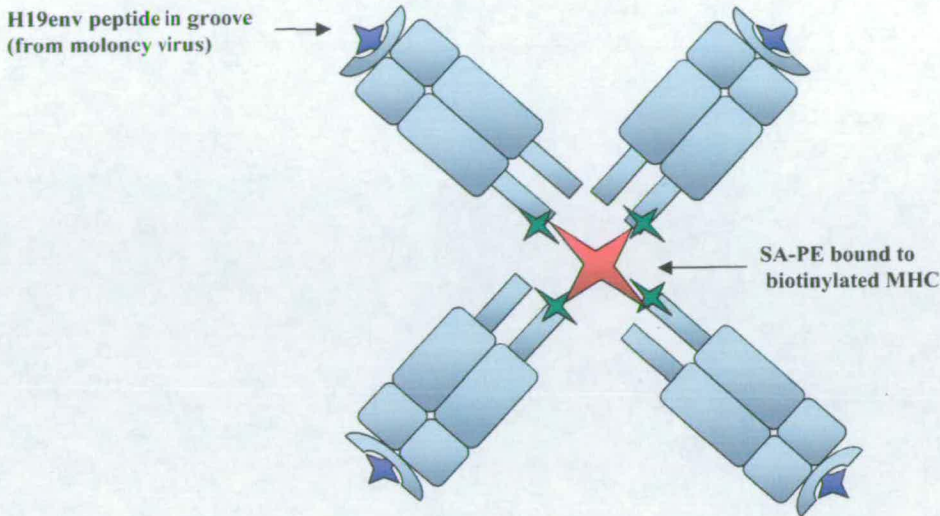


**Figure 4.10.** A cognate interaction with T cells is required for IgM and IgG responses. Mice were immunised with DNP-OVA/alum and ten days later serum was examined by ELISA for antibodies present. Results are expressed as the amount of Ab relative to control serum. Symbols represent individual mice. The error bars show the SEM. Results are representative of two separate experiments.

## **MHC-II Tetramers were used to examine polyclonal T cell responses in MHC-II<sup>-</sup> B cell chimeras**

Given that the transferred OT-II transgenic cells tended to disappear between 10 and 20 days after immunisation, this system could not be used to examine CD4<sup>+</sup> T cell memory generation. Instead, MHC class-II tetramers were used. These were supplied as part of a collaboration with Dr Ton Schumacher in Amsterdam, where the tetramers are produced. The tetramer constructs were created as follows: the alpha chain of the MHC-II was modified by the addition of a Velcro leucine zipper to promote heterodimerisation in insect cells and a His-tag to aid purification. The T cell epitope was covalently attached to the beta chain and the beta chain was modified by the addition of a leucine zipper and a biotinylation signal to allow tetramer formation. Tetramers were formed using streptavidin-PE and monomers were removed by gel filtration chromatography. The recombinant MHC molecules are assembled into multimers to provide multiple ligands for enhanced interaction with Ag-specific TCRs (figure 4.11). It should be noted here that it is possible that multimers other than tetramers are formed but for simplicity I will refer to them as tetramers.

The tetramers contain a peptide from the H19env protein of the retrovirus moloney murine leukaemia virus and therefore responses to either the virus itself or to the H19env peptide can be examined. The response to the H19env peptide was characterised by Megan MacLeod in the lab as part of her PhD project.

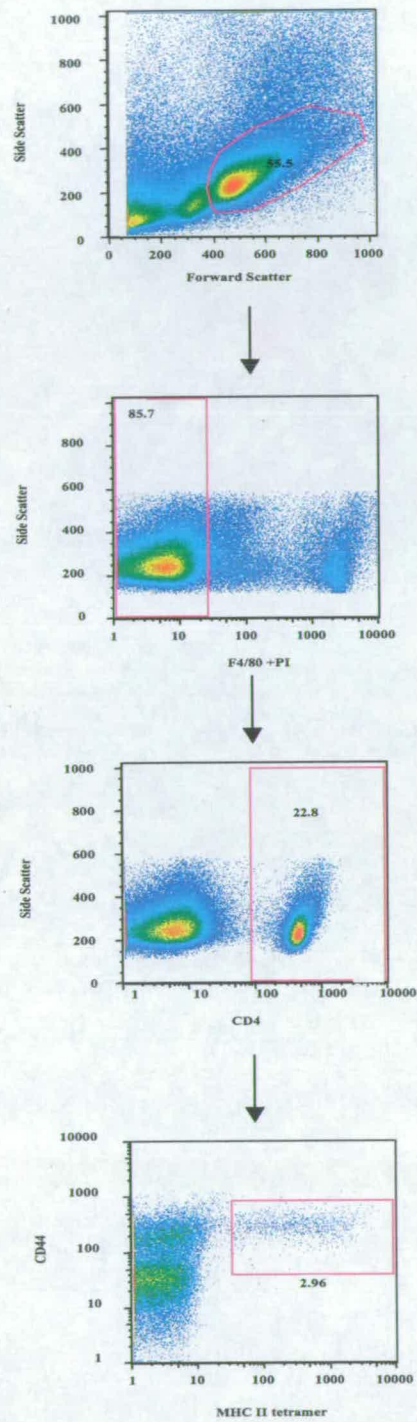


**Figure 4.11.** The MHC-II tetramers present a peptide from moloney murine leukaemia virus. They were multimerised by streptavidin-PE due to the biotin site on the  $\beta$  chain.

### Staining with tetramers

The conditions required for staining with MHC-II tetramers are more stringent than those for MHC-I tetramers. Staining with MHC class II tetramers is highly temperature-dependent and requires prolonged incubation. Cameron *et al* have demonstrated that MHC-II tetramers are internalised, requiring cytoskeletal rearrangements, which is an active process (Cameron *et al.*, 2001).

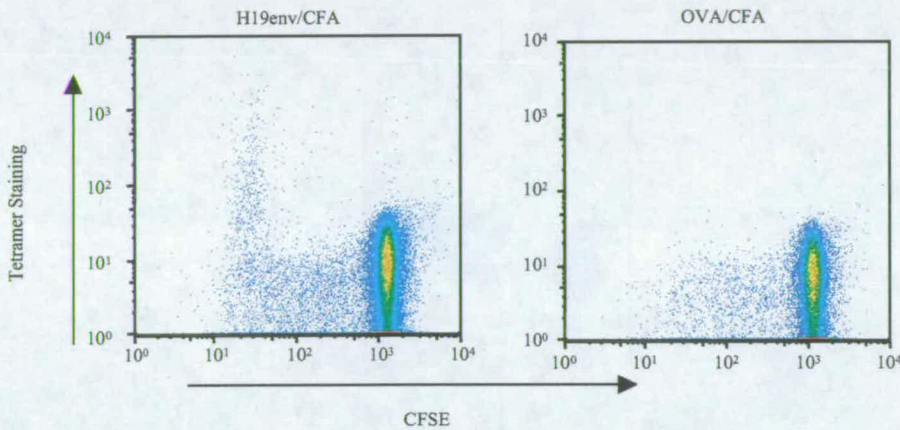
To identify tetramer positive cells, macrophages (F480 positive) and dead cells (PI positive) must be gated out to remove the majority of the background staining, as tetramers stick to them. CD4 cells were then gated and the proportions of cells that were expressing high levels of CD44, showing activation, and were positive for tetramer staining were examined. Figure 4.12 shows the gating procedure. The level of background staining was set using mice immunised with the irrelevant peptide OVA<sub>323-339</sub>. This peptide is from chicken ovalbumin and is a common peptide used to examine T cell responses.



**Figure 4.12.** To examine tetramer positive cells, a lymphocyte gate is first drawn then the macrophages (F4/80 positive) and dead cells (PI positive) are removed. The remaining cells are then gated for CD4<sup>+</sup> cells and the proportions of tetramer positive cells that are CD44<sup>hi</sup> are examined.

### Tetramer staining is specific for cells responding to the H19env peptide

To confirm that the cells binding the tetramers were responding to the peptide, splenocytes from mice immunised with H19env peptide were CFSE-labelled and restimulated for 3 days with peptide. The cells that bound the tetramers had also divided in response to the H19env peptide showing that the tetramers were recognising H19env-specific T cells (figure 4.13).

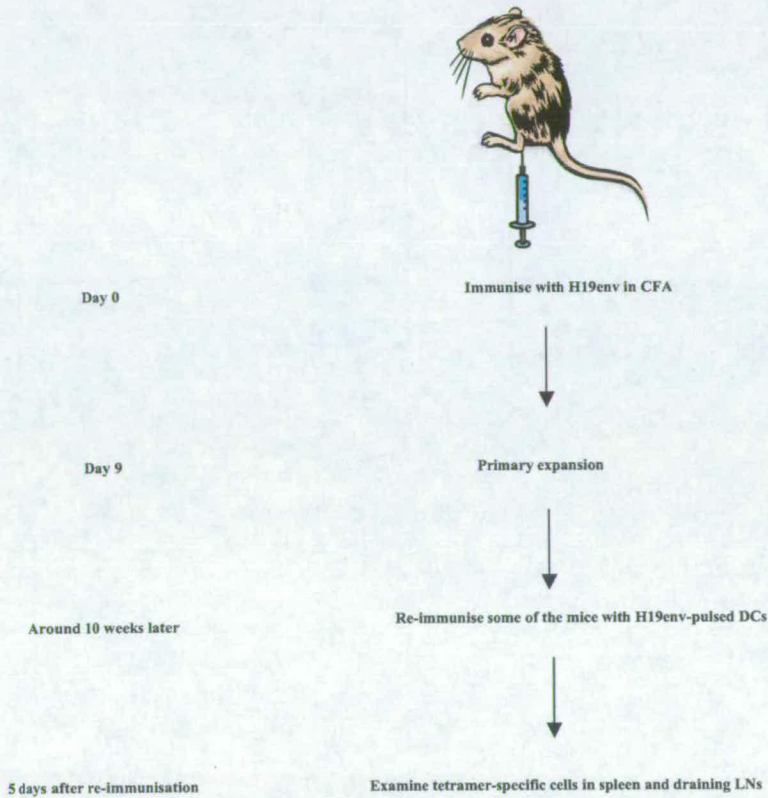


**Figure 4.13.** CD4<sup>+</sup> T cells that respond to H19env peptide also bind the MHC-II tetramers. Splenocytes from immunised mice were red blood cell lysed and labelled with CFSE. They were plated at  $1 \times 10^6$  cells per well in a 96-well plate in the presence of  $10 \mu\text{g/ml}$  H19env peptide and supernatant from ConA-stimulated rat splenocytes for 4 days. They were then examined for tetramer staining and CFSE fluorescence.

### Polyclonal T cell expansion is reduced in MHC-II<sup>-/-</sup> B cell chimeras

To examine whether MHC-II on B cells is also required for T cell memory generation, MHC-II<sup>-/-</sup> B cell chimeras or wildtype chimeras were immunised with H19env in CFA into the hind leg, forming an Ag depot. Mice were examined for primary expansion or were rested and examined for memory responses. For memory response, mice were left for around 10 weeks and either examined for remaining tetramer positive cells or the mice were boosted with H19env-pulsed bone-marrow dendritic cells to examine the memory recall response *in vivo*. Figure 4.14 illustrates the immunisation protocol.

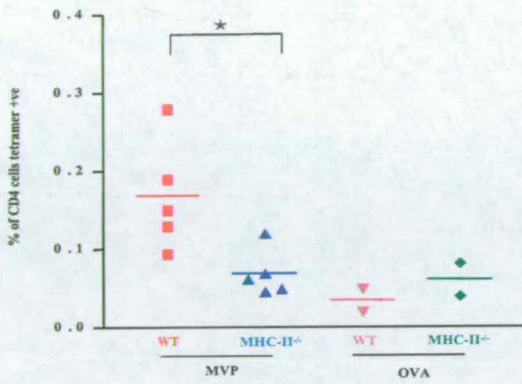
The primary T cell response to H19env in CFA is reduced in MHC-II<sup>-/-</sup> B cell chimeras compared to wildtype chimeras (figure 4.15). Therefore, in both the transgenic system and using MHC-II tetramers, the CD4<sup>+</sup> T cell response is reduced in the absence of MHC-II on B cells.



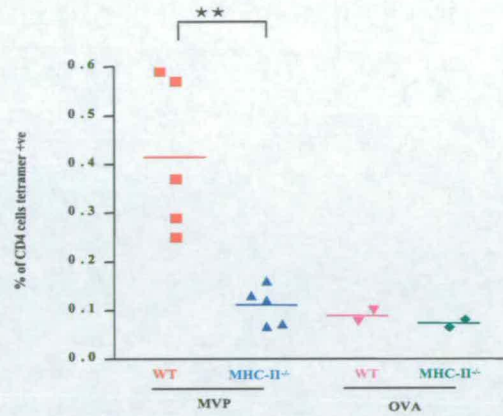
**Figure 4.14.** Experimental protocol for examining polyclonal T cell responses to H19env. Mice were immunised with H19env emulsified with CFA then examined for primary expansion or left for around ten weeks to examine memory cells



Day 9



Day 15



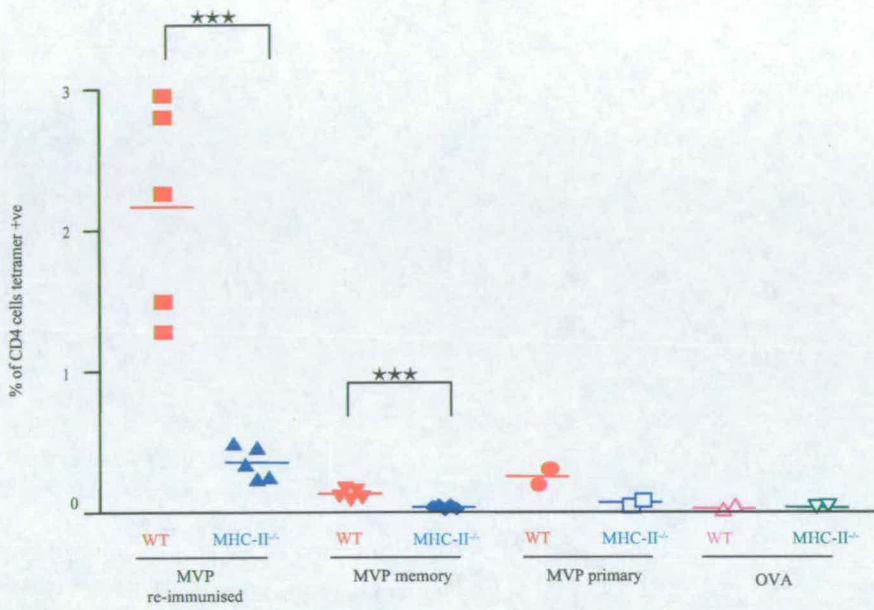
**Figure 4.15.** Wildtype and MHC-II<sup>-/-</sup> B cell chimeras were immunised with H19env/CFA and the number of tetramer positive cells examined on days nine and fifteen after immunisation. Symbols show the result of an individual mouse and lines show the mean of each group. Statistical comparisons were made using the un-paired T-test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . This experiment was done three times with four mice per H19env group. Although the other repeats the difference did not reach significant levels, there was an obvious trend towards reduced response in MHC-II<sup>-/-</sup> B cell chimeras.

### Memory responses are reduced in the absence of MHC-II on B cells

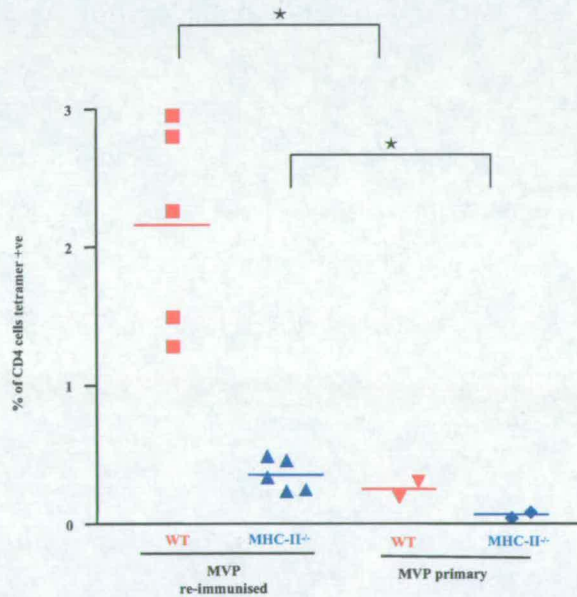
The numbers of tetramer positive memory cells were examined in mice ten weeks after initial immunisation and some were also re-immunised to examine memory recall responses *in vivo*. The number of memory cells remaining in MHC-II<sup>-/-</sup> B cell chimeras was significantly less than those in wildtype chimeras suggesting that either fewer memory cells were generated or fewer memory cells were maintained in the absence of MHC-II on B cells. When the mice were re-immunised, the memory recall response was also lower in these mice (figure 4.16A).

To establish whether the recall response is higher than the primary response, a cohort of the wildtype and MHC-II<sup>-/-</sup> chimeras were also immunised for the first time at this stage. The percentage of tetramer positive cells after re-immunisation was much higher than during the primary response (Figure 4.16B). Thus, a memory response has occurred in these chimeras. Therefore, I have shown that memory cells can be generated using this system in both wildtype and MHC-II<sup>-/-</sup> chimeras, however, the memory cells remaining and the memory recall response is much lower when B cells do not express MHC-II on their surface.

## 4.16A



## 4.16B



**Figure 4.16.** Polyclonal memory responses are reduced in MHC-II<sup>-/-</sup> chimeras. Wildtype and MHC-II<sup>-/-</sup> B cell chimeras were immunised with H19env/CFA and left for 10 weeks (MVP memory) then some were re-immunisation with H19env-pulsed BM DCs (MVP re-immunised). Mice were immunised with H19env-pulsed BM DCs for the first time to compare primary and memory responses (MVP primary). Symbols show the result of an individual mouse and lines show the mean of each group. Statistical comparisons are by un-paired T-test. \*,  $p < 0.05$ . This experiment was done three times with four H19env-immunised mice per group.

## Discussion

Bone-marrow chimeras provide a unique system to examine the mechanism by which B cells can regulate T cell responses. By combining  $\mu$ MT bone-marrow with wildtype or MHC-II<sup>-/-</sup> bone-marrow, mice were created which differ only in the expression of MHC-II on B cells. Around 98% of B cells in the wildtype chimeras stain positive for MHC-II whereas less than 2% in MHC-II<sup>-/-</sup> chimeras are positive for MHC-II. These chimeras can be compared to wildtype chimeras created by combining  $\mu$ MT and C57BL/6 bone-marrow. The cellular proportions in the spleen are similar in wildtype and MHC-II<sup>-/-</sup> B cell chimeras as is the splenic architecture.

By transferring transgenic T cells into these chimeras, the expansion of T cells can be tracked *ex vivo*. In these experiments I have shown that when T cells cannot form a cognate interaction with B cells via MHC-II they can expand but this expansion is reduced. The reduced expansion is seen by day three after immunisation, before the peak of expansion, and continues throughout the primary response.

Given that I saw decreased numbers of OT-II T cells in the spleen of MHC-II<sup>-/-</sup> B cell chimeras, it was important to examine whether T cells were more likely to migrate to peripheral tissues when B cells lack MHC-II since this could be the reason for the decreased numbers of OT-II T cell in the spleen. After initial activation with DCs, T cells move to the edge of the B cell follicles and interact there with B cells (Garside et al., 1998). It was possible that without a cognate interaction with B cells, T cells do not remain in the spleen and instead migrate to peripheral tissues. Interestingly, the numbers of OT-II T cell was not increased in the lungs and livers showing that the reduced numbers of T cells in the spleen is the result of decreased expansion rather than preferential migration of the T cells to peripheral tissues. These results show that a cognate interaction with MHC-II on B cells can enhance CD4<sup>+</sup> T cell expansion *in vivo*.

To determine whether this reduced expansion of T cells was due to fewer cells entering division or the cells dividing fewer times, the OT-II T cells were CFSE-labelled before transfer. The CFSE labelling illustrated that the reduced expansion of the OT-II T cells seen in the absence of MHC-II on B cells is the result of less cells entering division rather than the cells undergoing less division. Therefore, B cell MHC-II interactions play a role in regulating the number of T cells entering division and in the first few rounds of division.

It is unclear why less T cells enter division. If T cells move randomly through lymphoid tissues, then perhaps B cells activate the T cells that randomly move near to the B cell follicle region. Since B cells can move to the edge of the follicle within five hours of antigen uptake (Reif et al., 2002), this means they are in a prime location to interact with antigen-specific T cells. This theory is controversial, however, as many believe that the DC is the only cell capable of activating a naïve T cell.

Alternatively, it is possible that T cells may undergo several short interactions with DCs or other APCs instead of one continuous interaction (Miller et al., 2004). Although several groups have witnessed long-term stable interactions of T cells and DCs lasting up to 15 hours (Stoll et al., 2002), Miller *et al* recently demonstrated that in the first few hours after Ag administration T cells made serial contacts with DCs with a mean interaction time of 11.4 mins. Up to 14 hours after Ag administration the T cells remained with DCs for over an hour but the T cells constantly changed their position and some even moved away. Thus, short successive contacts with DC may be enough for T cell activation. Therefore, perhaps some T cells that only undergo a short interaction with a DC have not yet been primed enough to undergo cell division and the second interaction with the B cells enables this to occur. It has been shown by several groups that T cells require several hours of stimulation before they are able to undergo proliferation and that this stimulation can be intermittent (Mempel et al., 2004; Miller et al., 2004).

Having shown that the primary expansion of T cells is decreased in the absence of MHC-II on B cells, the clonal deletion phase was then examined to determine if MHC-II on B cells plays a role here too. Of note, it has been demonstrated that the clonal deletion phase is more extreme in  $\mu$ MT mice when examining the T cell response to *Listeria monocytogenes* (Shen et al., 2003). However, there was no consistent difference in the amount of apoptosis of the OT-II T cells at various days after immunisation. Thus, the absence of MHC-II on B cells did not affect the clonal deletion of the OT-II T cells.

Given that others have demonstrated that B cells may regulate not just T cell expansion but also T cell differentiation (Linton et al., 2003), cytokine production was examined by ELISAs. When stimulated *in vitro*, the OT-II T cells from MHC-II<sup>-/-</sup> chimeras underwent less proliferation and produced less IL-2 as well as less IL-4 and IFN- $\gamma$ . Although there was reduced cytokine production with all three cytokines, the IL-4 production by T cells from MHC-II<sup>-/-</sup> B cell chimeras was more greatly reduced. There it does suggest a possible defect in Th2 differentiation in the absence of a cognate MHC-II interaction with B cells. Even when the data has been normalised for the percentage of OT-II T cells in the wells at the beginning of the assay, the wells with T cells from wildtype chimeras proliferated much more *in vitro* and therefore there are many more of these cells at the end of the three day culture. In light of this, it is difficult to determine whether the reduced effector cytokine production was due to less Ag-specific T cells or the T cells producing less cytokine per cell. ELISPOTs would enable me to determine the amount of cytokine produced per cell and therefore to examine the possible defect in Th2 differentiation more clearly. Alternatively, using the LIGHTcycler to determine the amount of mRNA for various cytokines in the OT-II T cells directly *ex vivo* would allow me to examine cytokine mRNA levels in the absence of *in vitro* restimulations.

To examine the B cell response in these chimeras, serum ELISAs were carried out. As expected, when B cells could not undergo a cognate interaction with T cells through MHC-II, they did not produce significant amounts of anti-DNP IgM and IgG antibodies. Thus, the B cell response was defective in these mice. The absence of IgG antibodies in the MHC-II<sup>-/-</sup> B cell chimeras is unlikely to affect the T cell response because reduced numbers of T cells is seen by day three, before the B cell IgG production is efficiently underway.

MHC-II tetramers were used to study a polyclonal response in these chimeras and to determine if MHC-II on B cells was required for the development and/or maintenance of T cell memory. MHC-II tetramers were chosen for a number of reasons. Although the use of the adoptive transfer system allows T cells to be examined at a near-physiological number compared to transgenic mice, the numbers of Ag-specific T cells are still larger than would occur naturally. Also, the Tg T cells used in this study did not remain at detectable numbers for long periods of time and could not therefore be used to examine T cell memory. When mice were immunised with H19env emulsified in CFA and MHC-II tetramers used to detect expansion, the number of tetramer positive cells in MHC-II<sup>-/-</sup> B cell chimeras was reduced compared to wildtype chimeras. Thus, in both the transgenic system, using a monoclonal population of T cells, and the tetramer system, examining a polyclonal population of T cells, expansion is reduced when B cells cannot interact with T cells using MHC-II.

A relatively stable population of memory cells could be detected after immunisation with peptide in CFA. I have shown that the numbers of memory cells is lower in MHC-II<sup>-/-</sup> chimeras compared to wildtype chimeras and a reduced memory recall response was also seen after re-immunisation. The finding that less Ag-specific T cells are present in the primary response and less memory cells remain supports the hypothesis that the initial clonal burst size affects the size of the memory compartment. This has been shown in a variety of systems with CD8 T cells but has not been shown conclusively for CD4<sup>+</sup> T cells (Sprent and Tough).

From these experiments it is difficult to ascertain whether the reduced memory response is merely a result of the reduced primary response or if MHC-II on B cells is also required for memory recall responses. There are two ways this could be examined: CD4<sup>+</sup> memory T cells could be purified from wildtype chimeras previously immunised with H19env in CFA and transferred to wildtype or MHC-II<sup>-/-</sup> B cell chimeras before re-immunising; alternatively, wildtype B cells could be transferred into wildtype and MHC-II<sup>-/-</sup> B cell chimeras to determine whether they are able to enhance the memory response.

In addition, it is possible that the absence of MHC-II on B cells could affect the ability of the memory cells to respond to further stimulation. Of note, it has been reported that although memory CD4<sup>+</sup> T cells can remain in the absence of MHC-II molecules, they are functionally deficient (Kassiotis et al., 2002). Thus, it would be interesting to examine whether memory T cells generated in wildtype mice but transferred to MHC-II<sup>-/-</sup> B cells chimeras behave differently. It is possible that MHC-II on B cells is the major source of MHC-II for the maintenance of memory cell effector function. To do this, T cells purified from H19env/CFA immunised wildtype chimeras could be purified and transferred into MHC-II<sup>-/-</sup> B cell chimeras or wildtype chimeras as controls and rested before being tested for memory cell function. To test for memory cell function, the T cells could be stimulated *in vitro* with naïve B cells or professional APCs as Kassiotis *et al* did with transgenic memory cells (Kassiotis et al., 2002). By definition, memory cells require less costimulation and so should be able to be activated by naïve B cells whereas naïve T cells would not be activated.

In conclusion, T cells do not undergo optimal T cell expansion when they cannot interact with B cells through MHC-II and this reduced expansion is due to less T cells entering division. When T cell responses were examined using MHC-II tetramers, there was less expansion as well as a lower memory recall response in the MHC-II<sup>-/-</sup> B cell chimeras. Therefore, a cognate interaction with MHC-II on B cells

increases the number of T cells that proliferate in response to Ag *in vivo* and allows a greater memory response.



**“The great tragedy of science – the slaying of a beautiful hypothesis by an ugly fact.”**

**Thomas Huxley**

## CD4<sup>+</sup> T cells require CD40 but this CD40 is not derived from B cells

### ***Introduction***

In addition to the recognition of Ag in the form of peptide on MHC molecules, T cells require costimulatory interactions to become activated and proliferate. There are a number of different costimulatory molecular interactions including B7:CD28, B7h:ICOS and CD40:CD40L. CD40:CD40L interactions have been shown to be important for responses to both immunisations with peptide in adjuvant (DiPaolo and Unanue, 2002) and viral immune responses (Whitmire et al., 1999). CD40L is expressed on activated T cells (Lane et al., 1992) whereas CD40 is expressed by DCs and B cells (Caux et al., 1994). However, the role of CD40 in T cell responses is not completely clear as there has been at least one report that showed normal T cell priming in response to viral infections (lymphocytic choriomeningitis (LCMV) and vesicular stomatitis (VSV)) (Oxenius et al., 1996). This could possibly be explained by the ability of some infectious agents to bypass the need for CD40 due to the strong inflammatory response they induce. Indeed, CD8 responses to these viruses have been shown to be independent of CD4 help suggesting that the licensing of APCs is not required for responses to these viruses (Marzo et al., 2004; Matloubian et al., 1994).

The use of the adoptive transfer system to examine the role of CD40 in T cell expansion has also generated conflicting results. Using CD40L<sup>-/-</sup> Tg T cells, Grewal *et al* reported that these T cells fail to expand *in vivo* (Grewal et al., 1996) while others have shown initial expansion to be similar but lower numbers are seen by the peak of the response (Howland et al., 2000). This initial expansion then abortive response was also observed using anti-CD40L antibodies in wildtype mice and immunisation with CD40<sup>-/-</sup> DCs s.c. (Miga et al., 2001).

It is clear that CD40:CD40L interactions between T and B cells are required for B cells to form GCs, develop into memory cells and class switch (Foy et al., 1994b; Lumsden et al., 2003) but the importance of this interaction for the T cell is unclear. T cells may require an interaction between CD40L and CD40 on B cells for effective responses. For example, CD40 on B cells can enhance T cell proliferation *in vitro*; Ozaki *et al* demonstrated that DO.11.10 Tg T cells stimulated with CD40<sup>-/-</sup> B cells proliferated less well *in vitro* than T cells cultured with wildtype B cells (Ozaki et al., 1999).

*In vivo* evidence that costimulatory molecules on B cells are important for T cell priming arose from experiments showing that either activated B cells or Ag-pulsed DCs restored T cell priming in  $\mu$ MT mice (Linton et al., 2000). Since either cell-type could restore priming, the authors proposed that the primary deficit in these mice was costimulation. Moreover, B cells from OX40L<sup>-/-</sup> mice were unable to restore CD4 T cell responses in  $\mu$ MT mice whereas wildtype B cells could (Linton et al., 2003) showing the importance of OX40L expression by B cells for T cell activation.

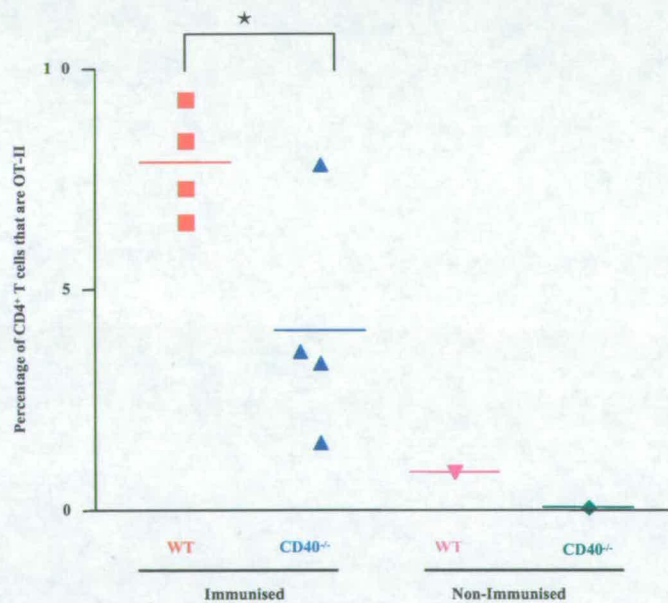
Clonal expansion is only one part of a T cell response. T cells are also required to differentiate into cytokine-secreting effectors. Support for the involvement of CD40 in promoting the differentiation of T cells has been shown by decreased IFN- $\gamma$  production in CD40<sup>-/-</sup> mice using a variety of systems including LCMV infection (Whitmire et al., 1999) and adoptive transfer of Tg T cells (Howland et al., 2000; Miga et al., 2001). It is unclear how CD40 affects IL-4 production, however, as Cunningham *et al* reported IL-4 to be normal in the absence of CD40L (Cunningham et al., 2004) whereas Poudrier *et al* demonstrated that IL-4 production was reduced from LNs of CD40<sup>-/-</sup> mice compared to wildtype mice when immunised with KLH (Poudrier et al., 1998).

To evaluate the requirement for CD40 costimulation by CD4<sup>+</sup> T cells, OT-II CD4<sup>+</sup> T cells were transferred into C57BL/6 or CD40<sup>-/-</sup> mice. This allowed me to follow the response from initial activation and expansion to clonal deletion in both secondary lymphoid tissues and peripheral tissues. Once I had observed a role for CD40 in the clonal expansion of the OT-II T cells, I went on to evaluate whether B cell provision of CD40 could influence T cell responses. To do this, bone-marrow chimeras were created in which the CD40 defect was limited to B cells, allowing other APCs to provide CD40 costimulation to T cells.

## Results

### OT-II T cell numbers are reduced in CD40<sup>-/-</sup> mice after immunisation

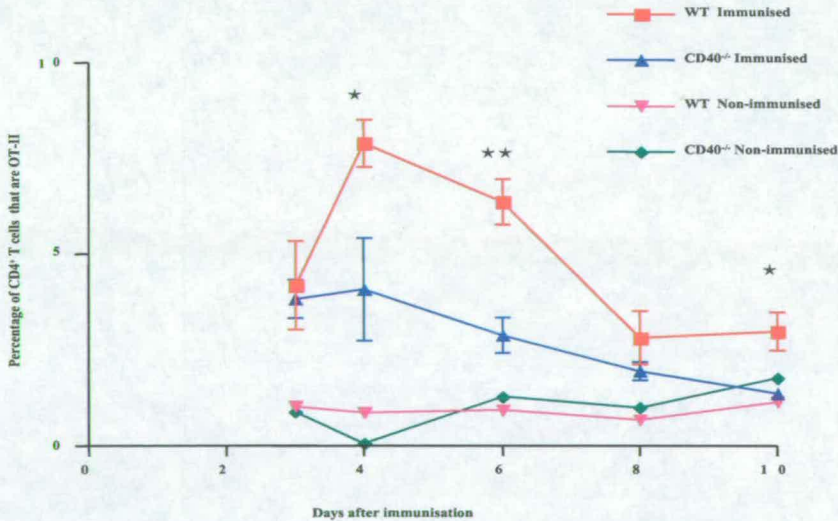
To assess the role of the co-stimulatory interaction CD40:CD40L in T cell priming, OT-II cells were transferred into C57BL/6 mice or CD40<sup>-/-</sup> mice. The percentage of OT-II T cells in the spleen was examined four days after immunisation with DNP-OVA in alum. Although OT-II T cells expanded in both groups of mice, the percentage of OT-II T cells was reduced in CD40<sup>-/-</sup> mice (figure 5.1). Thus, T cells can divide in the absence of CD40:CD40L interactions but this expansion is reduced.



**Figure 5.1.** CD40 signalling enhances CD4<sup>+</sup> T cell expansion. OT-II T cells were transferred into C57BL/6 and CD40<sup>-/-</sup> mice and the mice immunised the following day with DNP-OVA/alum. Mice were euthanised on day 4 after immunisation and the percentage of transgenic cells determined as described in previous chapters. Symbols show the percentage of CD4 cells that are OT-II in individual mice and lines are used to show the mean of each group. Statistical comparisons were made using the un-paired T-test. \*,  $p < 0.05$ . Day 4 was examined twice with at least three mice per group.

## OT-II T cell numbers are reduced throughout the primary response

To determine if CD40:CD40L signals are required throughout the OT-II T cell response, mice that received OT-II T cells were sacrificed at various days after immunisation and analysed for OT-II T cell numbers. Interestingly, three days after immunisation OT-II T cells had expanded to a similar degree in the spleens of C57BL/6 and CD40<sup>-/-</sup> mice (figure 5.2). Yet, by day four after immunisation there was significantly less OT-II T cells and this reduced T cell number was seen throughout the rest of the primary response. Therefore, although CD40 is not required for the initial activation and expansion of OT-II T cells, it is essential for their continued expansion.



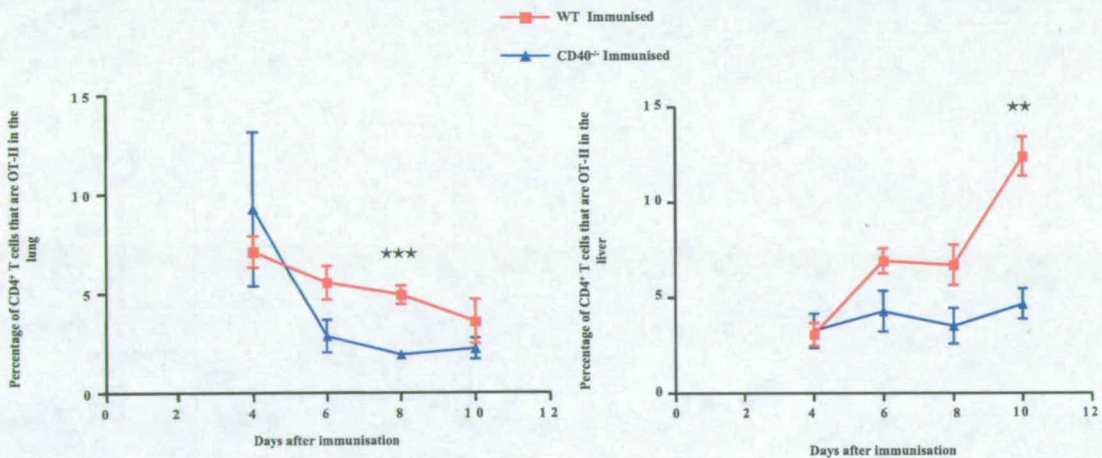
**Figure 5.2.** OT-II T cells initially proliferate in CD40<sup>-/-</sup> mice but the expansion is reduced compared to wildtype mice. OT-II T cells were transferred into C57BL/6 or CD40<sup>-/-</sup> mice and the following day the mice were immunised. Mice were euthanised on days 3, 4, 6, 8 and 10 after immunisation and the percentage of transgenic cells determined as described previously. Symbols show the mean of each group and the error bars show the SEM. Each symbol is representative of at least 3 mice. Statistical comparisons were made using the un-paired T-test. \*,  $p < 0.05$ . \*\*,  $p < 0.01$ . All days were examined twice with three mice per group.

## Reduced OT-II T cells numbers in CD40<sup>-/-</sup> mice was not due to preferential migration to peripheral tissues

Having demonstrated that CD4 T cell expansion is reduced in the absence of CD40, the number of OT-II T cells in the lungs and liver were examined at various times after immunisation. This allowed me to determine if the reduced number of OT-II T cells seen in CD40<sup>-/-</sup> mice was due to the cells preferentially migrating to peripheral tissues. OT-II T cells did not preferentially migrate to peripheral tissues in CD40<sup>-/-</sup> mice suggesting that the decreased numbers of OT-II T cells in the spleens of CD40<sup>-/-</sup> mice was due to decreased expansion (figure 5.3). Thus, T cells expanded less efficiently in the absence of CD40.

### A. LUNG

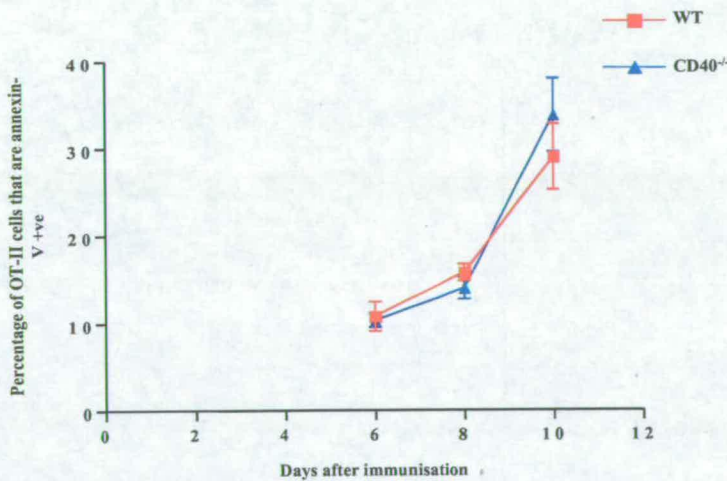
### B. LIVER



**Figure 5.3.** OT-II T cells do not preferentially migrate to peripheral tissues in CD40<sup>-/-</sup> mice. After transfer of OT-II T cells and immunisation, mice were euthanised and perfused on days 3, 4, 6, 8 and 10 after immunisation and the liver and lungs examined for the percentage of transgenic cells. Symbols show the mean of each group and the error bars show the SEM. Statistical comparisons were made using the un-paired T-test. \*\*, p < 0.01. \*\*\*, p < 0.001. All days were examined only once with three mice per group.

## Absence of CD40 does not affect the rate of T cell apoptosis

There have been studies showing that T cells undergo abortive proliferation in CD40<sup>-/-</sup> mice leading to the rapid disappearance of the T cells. This should mean that greater numbers of OT-II T cells undergoing apoptosis should be detected. Although I did not see an obvious difference in the clonal deletion phase in CD40<sup>-/-</sup> mice by tracking OT-II cell numbers, the amount of apoptosis was examined by staining with annexin V. However, when I examined the proportions of OT-II T cells staining positive for annexin V at various days after immunisation, there was no significant difference between the numbers of OT-II T cells undergoing apoptosis in CD40<sup>-/-</sup> mice (▲) compared to C57BL/6 mice (■) (figure 5.4). This shows that the absence of CD40 does not affect the rate of OT-II T cell apoptosis *in vivo*.

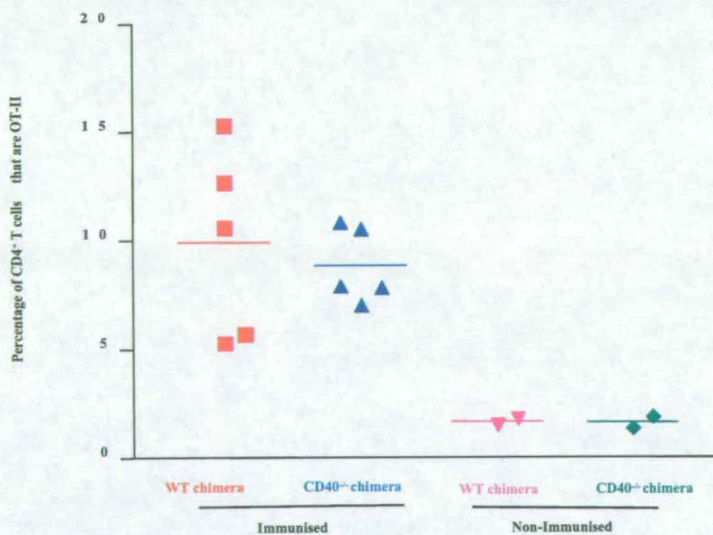


**Figure 5.4.** CD40 does not affect the rate of CD4<sup>+</sup> T cell apoptosis. OT-II T cells in the spleen were examined for apoptosis by annexin V binding at various days after immunisation. Symbols show the mean of each group and the error bars show the SEM. Statistical comparisons were completed by the un-paired T-test. This was completed only once with three mice per group.



## CD40 on B cells is not required for T cell expansion

Having shown that OT-II T cells expand less efficiently in the absence of CD40, I went on to examine whether B cells were the source of this CD40 *in vivo*. To do this, bone-marrow chimeras were made where the B cells are deficient in CD40 but other APCs can provide CD40 costimulation. C57BL/6 mice were lethally irradiated and bone-marrow was transferred. For CD40<sup>-/-</sup> B cell chimeras, 80%  $\mu$ MT bone-marrow and 20% CD40<sup>-/-</sup> bone-marrow was transferred whereas wildtype chimeras were created using 80%  $\mu$ MT and 20% C57BL/6 bone-marrow. After reconstitution, OT-II cells were transferred as described previously and the chimeras immunised with DNP-OVA/alum. At four days after immunisation, there was no difference in expansion of the OT-II T cells in the presence or absence of CD40 on B cells (figure 5.5). Thus, B cells are not required to provide CD40 for T cell expansion.



**Figure 5.5.** T cell expansion is normal in the absence of B cell-derived CD40. OT-II T cells were transferred into wildtype and CD40<sup>-/-</sup> B cell chimeras and the mice immunised the following day. Mice were euthanised on day 4 after immunisation and the percentage of transgenic cells determined. Symbols show the number of OT-II T cells as a percentage of CD4s of an individual mouse and lines are used to show the mean of each group. Statistical comparisons are by un-paired T-test. Day four was examined twice with a minimum of four mice per group.

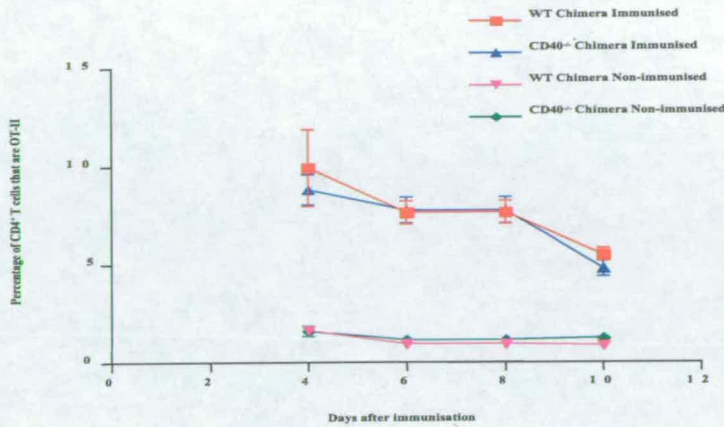
**CD40 on B cells is not required for *in vivo* CD4<sup>+</sup> T cell responses**

To determine if CD40 costimulation by B cells is required at any stage of the OT-II T cell primary response, mice were sacrificed at various days after immunisation and analysed for OT-II T cell numbers. Figure 5.6 shows the expansion of OT-II T cells at various days after immunisation in the spleen, lungs and liver of wildtype and CD40<sup>-/-</sup> B cell chimeras. There was no significant difference in OT-II T cell numbers throughout the primary T cell response in the spleen. Therefore, CD40 expression by B cells is not required to provide T cell costimulation at any stage of the T cell response. In addition, there was no difference in OT-II T cell numbers in the lungs and liver at most days showing that T cell migration was unaffected by the absence of CD40 on B cells. However, significantly higher numbers of OT-II T cells were seen in the liver of CD40<sup>-/-</sup> B cell chimeras at six days after immunisation.

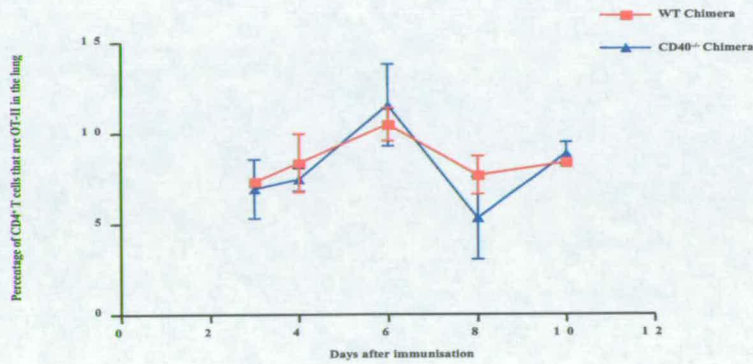
***In vitro* restimulations to examine cytokine production**

CD40 has been shown to play a role in T cell differentiation: CD40<sup>-/-</sup> mice were found to produce less IFN- $\gamma$  using a variety of immunisation protocols (Howland et al., 2000; Miga et al., 2001; Whitmire et al., 1999). To determine whether B cell-derived CD40 affects T cell differentiation, the splenocytes from chimeras that received OT-II T cells were examined for *in vitro* proliferation and cytokine production. Proliferation and IFN- $\gamma$  production of T cells from wildtype and CD40<sup>-/-</sup> B cell chimeras were found to be similar (figure 5.7). Therefore, B cell-derived CD40 does not affect IFN- $\gamma$  production. Unfortunately, the production of IL-4 was consistently below the level of detection in these experiments and so it could not be compared.

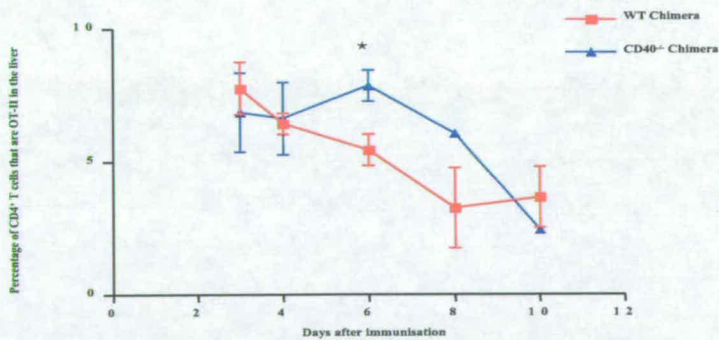
## A. SPLEEN



## B. LUNG

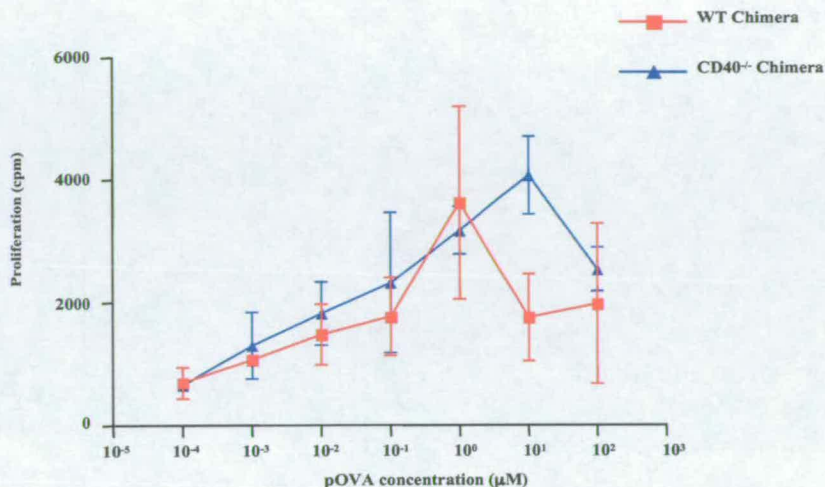


## C. LIVER

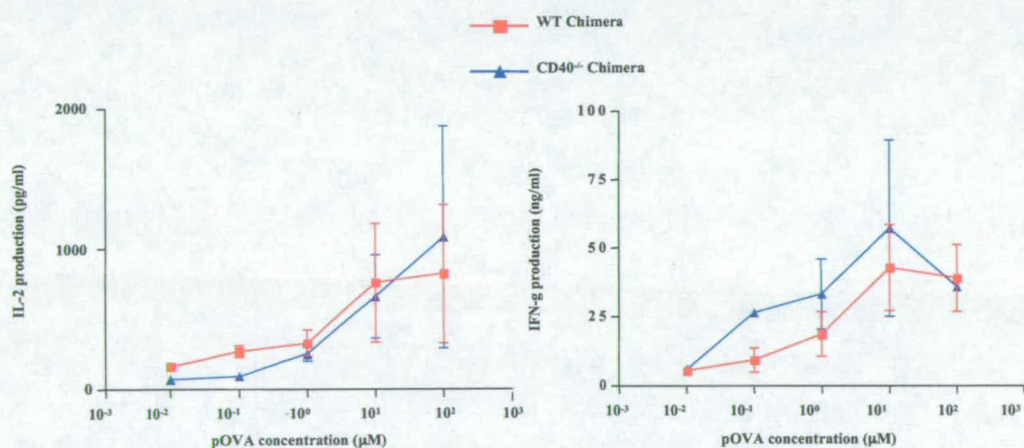


**Figure 5.6.** CD40 on B cells is not required for T cell expansion. After transfer of OT-II T cells and immunisation, mice were euthanised and perfused on days 3, 4, 6, 8 and 10 after immunisation and the spleen, liver and lungs examined for the percentage of transgenic cells. Expansion is expressed as the percentage of CD4<sup>+</sup> cells positive for V $\alpha$ 2 and V $\beta$ 5. Symbols show the mean of each group and the error bars show the SEM. Statistical comparisons were made using the un-paired T-test. \*,  $p < 0.05$ . For the spleen, all days were examined twice with a minimum of three mice per group. For lungs and livers, days three, four and six were examined twice whereas days eight and ten were only examined once.

## A. Proliferation



## B. Cytokine production

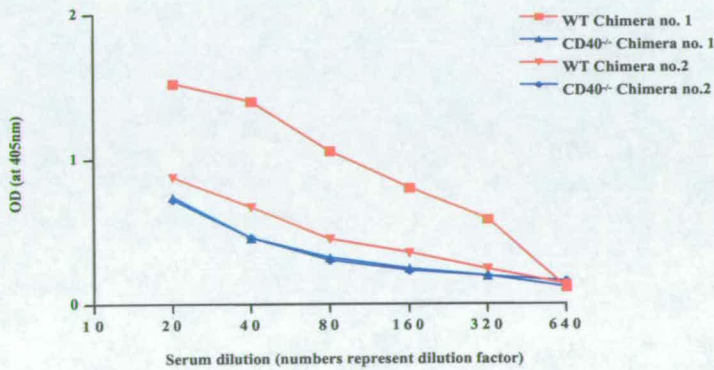


**Figure 5.7.** Proliferation and cytokine production is similar in T cells from CD40<sup>-/-</sup> B cell chimeras (▲) and wildtype chimeras (■). Pooled splenocyte populations from both groups were depleted of CD8<sup>+</sup> T cells and MHC-II positive cells. The cells were analysed for proliferation and cytokine production as described in the previous chapter. These graphs show results from four days after immunisation but the other days showed similar results. The proliferation assays and ELISAs were pooled data from at least three mice per group and were done twice for each day.

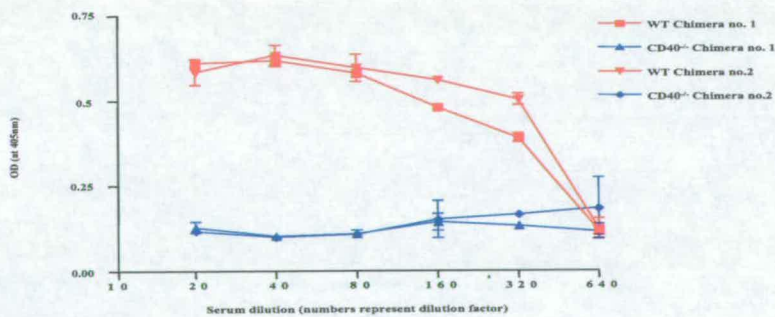
## Antibody responses are reduced in chimeras where the B cells do not express CD40

In the absence of CD40, germinal centres (GCs) and immunoglobulin (Ig) class switching do not occur (Kawabe et al., 1994). Since T cells have recently been shown to express CD40 and B cells to express CD40L, ELISAs were carried out to determine whether the B cells must express CD40 to generate efficient antibody responses. Serum from mice immunised ten days previously with DNP-OVA/alum was examined for anti-DNP IgM and IgG levels. CD40<sup>-/-</sup> B cell chimeras were deficient in both anti-DNP IgM and IgG production (figure 5.8). Therefore, B cells themselves must express CD40 to produce efficient levels of antigen-specific antibodies.

### IgM



### IgG



**Figure 5.8.** CD40<sup>-/-</sup> B cell chimeras are deficient in antibody production in response to DNP-OVA/alum immunisation. The amount of anti-DNP antibodies in serum from wildtype and CD40<sup>-/-</sup> chimeras immunised ten days previously was examined by ELISA. Serum was diluted down the plate and amounts of antibody compared to control serum. Two mice from each group were examined.

## Polyclonal T cell expansion and memory responses

Since OT-II T cells disappear within a few weeks after immunisation, MHC-II tetramers (described in the previous chapter) were used to enable us to examine a polyclonal primary T cell response and T cell memory generation in CD40<sup>-/-</sup> B cell chimeras. These experiments were done in collaboration with Megan MacLeod in the lab as part of her PhD.

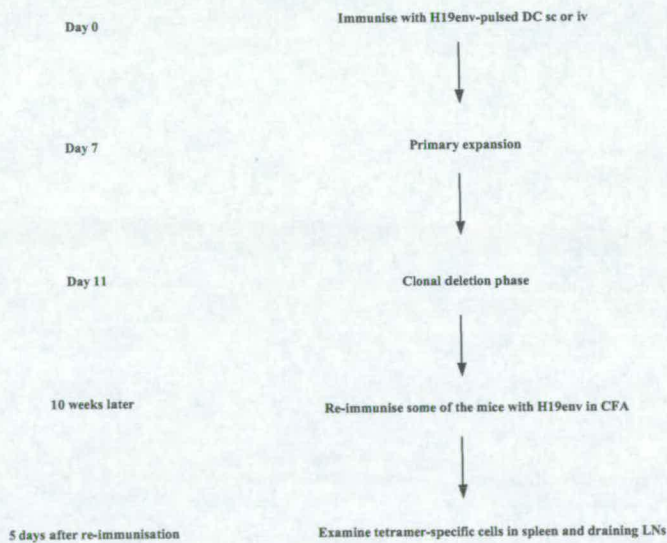
For these experiments, mice were first primed with peptide-pulsed DCs then rested to allow memory cells to form before being re-immunised with H19env in CFA to look at the recall response *in vivo* (figure 5.9). When immunising with peptide pulsed DCs, the response peaks earlier than when immunising with CFA. Therefore, the primary response was examined on day seven after immunisation. Furthermore, after DC immunisation, the mice need to be re-immunised to view the memory response because the tetramer positive cells fall below the level of detection by twenty days after immunisation with DCs. This means that the number of memory cells remaining before re-immunisation cannot be examined. The immunisation protocol was changed from that in the previous chapter to allow us to compare the results in CD40<sup>-/-</sup> B cell chimeras to those in CD40<sup>-/-</sup> mice; work by Megan MacLeod showed that tetramer staining could not be seen in CD40<sup>-/-</sup> mice when they were immunised with peptide in CFA. Wildtype DCs were used to restore the primary immune response in these mice and allow T cell memory to develop (see Appendix 1).

CD40<sup>-/-</sup> B cell chimeras and wildtype chimeras were immunised with DCs s.c. in the hind leg. As expected, the percentage of tetramer positive cells in the spleen was equivalent in both types of chimeras at the peak of expansion, day seven after immunisation and also at day eleven, although there was a trend for the numbers to be slightly higher in the CD40<sup>-/-</sup> B cell chimeras (figure 5.10). This is likely to be due to the response in wildtype chimeras being lower than expected, rather than the CD40<sup>-/-</sup> chimeras responding better than expected. Although the differences in the

spleen were not significant, the tetramer staining in the LN was significantly higher in CD40<sup>-/-</sup> B cell chimeras.

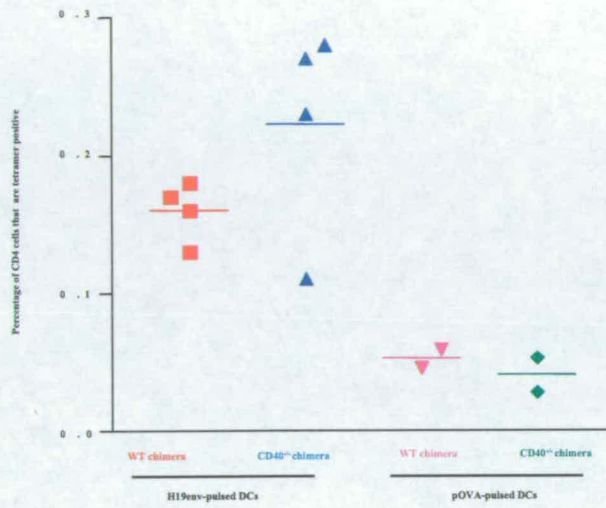
Unfortunately, when these experiments were repeated, poor priming was found in both types of chimeras, so strong conclusions cannot be drawn from these experiments. The main point to make is that the tetramer staining was not reduced in the CD40<sup>-/-</sup> B cell chimeras suggesting that with both the transgenic T cell response and a polyclonal T cell response, B cells do not appear to be required to provide CD40 costimulation.

It is difficult to understand why there was poor priming in two out of three of these experiments. OT-II T cells respond well in bone-marrow chimeras so we did not expect to see poor priming in these mice. We also immunised some of these chimeras with H19env in CFA since responses were seen when immunising the wildtype and MHC-II<sup>-/-</sup> chimeras with this immunisation protocol but the responses were still very poor.

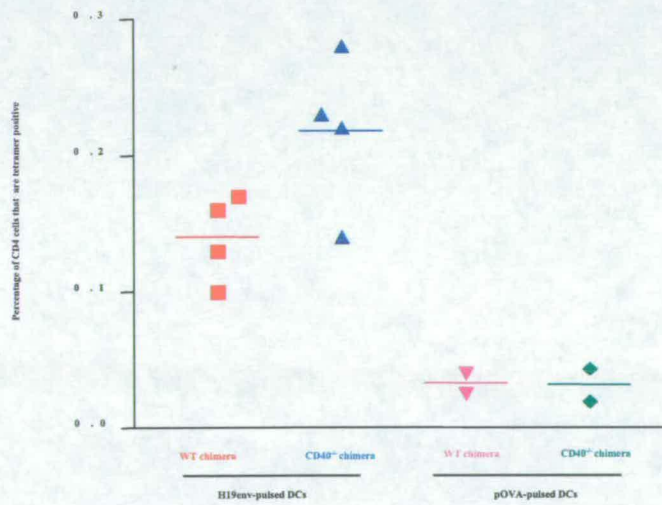


**Figure 5.9.** Diagrammatic representation of the immunisation protocol to examine polyclonal responses in wildtype and CD40<sup>-/-</sup> B cell chimeras. Mice were immunised with peptide-pulsed DCs and examined on days seven and eleven for primary expansion or left for memory to develop and re-immunised with peptide in CFA.

## A. Seven days after immunisation



## B. Eleven days after immunisation

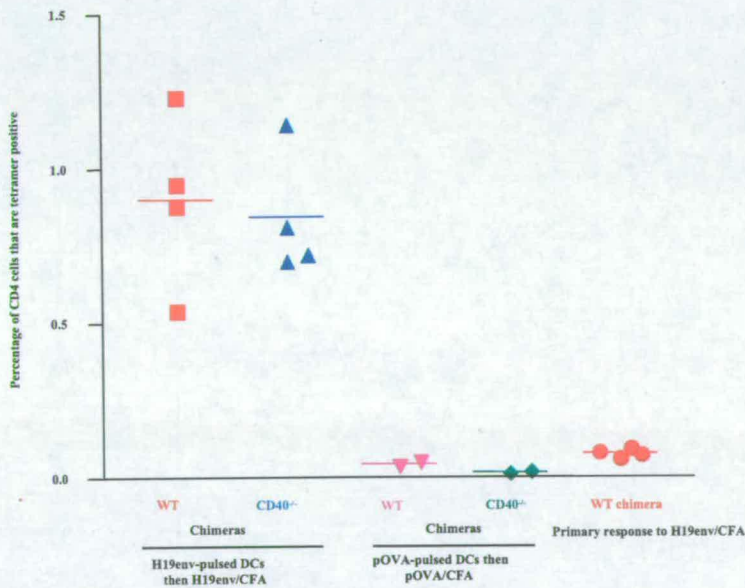


**Figure 5.10.** Polyclonal T cell priming is not reduced in CD40<sup>-/-</sup> B cell chimeras. Wildtype and CD40<sup>-/-</sup> B cell chimeras were immunised with H19env-pulsed DCs and the number of tetramer positive cells examined seven (A) and eleven (B) days after immunisation. Symbols represent individual mice and lines show the mean of each group.



## Memory response in the absence of CD40 on B cells

A cohort of mice were left for ten weeks then re-immunised with H19env in CFA to examine *in vivo* recall responses. The mice were examined for tetramer staining five days after immunisation with CFA (figure 5.11). Despite low primary expansion, a recall response could be seen in these mice and the memory response was similar in both types of chimeras suggesting that CD40 on B cells is not required for T cell memory generation or recall responses *in vivo*.



**Figure 5.11.** Polyclonal memory responses are normal in CD40<sup>-/-</sup> B cell chimeras. Wildtype and CD40<sup>-/-</sup> B cell chimeras were immunised with H19env-pulsed DCs and left for 10 weeks before re-immunisation with H19env/CFA in the hind leg. Mice were immunised with pOVA as controls. Four wildtype chimeras were immunised with H19env/CFA for the first time to compare primary and memory responses. Symbols represent individual mice and lines show the mean of each group. The memory response was examined twice with four mice per group.

## Discussion

### Expansion in CD40<sup>-/-</sup> mice

The OT-II adoptive transfer system was used to determine if CD40 was required for *in vivo* expansion of T cells. Interestingly, the initial expansion of OT-II T cells was normal in CD40<sup>-/-</sup> mice, however, by the peak of the response, there were significantly more OT-II T cells in wildtype mice compared to CD40<sup>-/-</sup> mice. Therefore, CD40 is not required for initial activation and expansion of CD4<sup>+</sup> T cells but it is required for sustained CD4<sup>+</sup> T cell proliferation.

The fact that T cells undergo initial expansion in the absence of CD40 is not surprising since CD40L is only upregulated on activated T cells. It is likely that other costimulatory molecules are required for initial expansion. For example, CD28:B7 interactions are likely to be one of the first costimulatory interactions required by T cells. CD28 is constitutively expressed on T cells and CD80 is expressed on naïve DC and B cells, although it is further upregulated after activation. CD28 signalling results in IL-2 production and T cell proliferation (Jenkins et al., 1991).

The similar early expansion in the absence of CD40 correlates with published data by Miga *et al* and Howland *et al*. However, my results show that the kinetics of the T cell contraction phase is similar, with the OT-II T cells declining slowly at a rate similar to that of wildtype mice. This contrasts with published data showing a dramatic reduction in T cell number after initial expansion (Howland et al., 2000; Miga et al., 2001). Miga *et al* transferred OT-II T cells and DCs with or without the addition of anti-CD154 mAb to block CD40L signalling. When the anti-CD154 mAb was added, they showed initial expansion of T cells but by day five the cells had dramatically decreased in number (Miga et al., 2001). Similarly, Howland *et al* showed that DO.11.10/CD40L<sup>-/-</sup> cells had decreased dramatically in number by seven days after immunisation.

The similar rate of clonal deletion in CD40<sup>-/-</sup> mice compared to wildtype mice in my system was confirmed by examining the number of OT-II T cells that were undergoing apoptosis at different days after immunisation. There was no difference in the proportions of cells staining positive for annexin V showing that clonal deletion was similar in the presence or absence of CD40.

It is unclear why my results are different to those published. A dramatic reduction in T cell numbers would mean that CD40:CD40L interactions are required for the survival of T cells. One reason for a dramatic crash in T cell numbers is due to peripheral deletion leading to tolerance. However, Miga *et al* reported that tolerance was not induced in their system.

There are numerous reasons why CD40 on APCs may be important for T cell responses. CD40 interactions result in signalling through the association of several TNF receptor-associated factor (TRAF) family molecules leading to the activation of NFκB (Ishida *et al.*, 1996a; Ishida *et al.*, 1996b). As a result of CD40 ligation, there is an increase in various molecules on the surface of DCs and B cells including MHC-II (Cella *et al.*, 1996; Clatza *et al.*, 2003), CD80 (Cella *et al.*, 1996; Clatza *et al.*, 2003) and CD86 (Cella *et al.*, 1996; Jenkins, 1994) making them more efficient antigen presenting cells. Another possibility is that CD40 affects migration of APCs to the lymphoid tissues or plays a role in maintaining them once they are there (Miga *et al.*, 2001; Moodycliffe *et al.*, 2000).

### **T cell responses in CD40<sup>-/-</sup> B cell chimeras**

Having confirmed that CD40 is important for OT-II T cell expansion *in vivo* and in light of the MHC-II chimera data, where OT-II T cell expansion is decreased when B cells don't express MHC-II, the hypothesis was that perhaps B cells are also required to provide CD40 costimulation. This is particularly likely given the importance of the CD40:CD40L interaction between T and B cells for the B cell response. Therefore, CD40<sup>-/-</sup> B cell chimeras were created to examine OT-II T cell expansion when the T

cells can only receive CD40 signals from APCs other than B cells. The expansion of OT-II T cells was similar in both wildtype and CD40<sup>-/-</sup> B cell chimeras showing that T cells do not require a CD40 signal from B cells for primary responses for expansion or clonal deletion.

Since OT-II T cell expansion was reduced in the absence of MHC-II on B cells but not when CD40 on B cells was absent, it is tempting to speculate that T cells require further Ag-presentation by B cells for complete expansion but do not need further costimulatory signals. There are a large number costimulatory signals, however. It would be interesting to examine T cell responses when B cells cannot provide key costimulatory molecules such as the B7 molecules or OX40L. The B7.1/B7.2 molecules are very important for T cell responses. Mice that are deficient in both B7.1 and B7.2 are readily available and therefore it would be possible to create chimeras where the B cells are unable to provide B7.1 and B7.2 signals. However, CD40 signalling results in the upregulation of costimulatory molecules, including the B7 molecules (Clatza et al., 2003) therefore it is possible that there will be no difference in T cell expansion when B cells do not express B7.

We chose to examine CD40 because CD40L is expressed on activated T cells. Since T cells are thought to come into contact with B cells only after initial activation with DCs, late costimulatory molecules may be more important in the T and B cell interaction. Another costimulatory molecule, which may be important on B cells, is OX40L. The receptor, OX40, is expressed on activated T cells and its ligand (OX40L) is upregulated on DCs and B cells after CD40 ligation or Ig cross-linking on B cells (Stuber et al., 1995). There are several reasons why OX40 may be important in the interaction between T and B cells. OX40:OX40L interactions have been shown to induce CXCR5 expression on CD4<sup>+</sup> T cells *in vitro* (Flynn et al., 1998). CXCR5 is a chemokine receptor involved in T and B cell migration to the B cell follicles. Additional support for the involvement of OX40L comes from studies reconstituting  $\mu$ MT mice with B cells. When wildtype B cells were used to reconstitute  $\mu$ MT mice, CD4<sup>+</sup> T cell expansion was restored. However, OX40L<sup>-/-</sup> B

cells could not restore T cell priming showing that OX40L on B cells is required for this restoration (Linton et al., 2003). Again, since CD40 signalling is important in the upregulation of OX40L, this may suggest that OX40L is not playing a major role in this system though OX40L may be upregulated by other signalling events.

Given that IFN- $\gamma$  production is reduced in CD40<sup>-/-</sup> mice, I examined the cytokine production when the CD40 deficiency was restricted to B cells alone. I saw no reduction in IFN- $\gamma$  production by the splenocytes suggesting that CD40 on B cells does not affect T cell expansion or differentiation.

Although CD40 is known to be required for germinal centre formation and isotype switching, the recent finding that B cells can also express CD40L and CD4<sup>+</sup> and CD8<sup>+</sup> T cells can express CD40 has opened up the possibility that perhaps CD40L on B cells can interact with CD40 on the T cells (Bourgeois et al., 2002). Therefore, it is possible that chimeras where the B cells are deficient in CD40 may still be able to undergo isotype switching through the interactions of CD40L on the B cells and CD40 on the T cell. In light of these findings, the amount of anti-DNP antibodies was determined from both wildtype and CD40<sup>-/-</sup> B cell chimeras. Mice where the B cells lacked CD40 were inefficient at producing Ag-specific IgM and IgG antibodies. This finding is in agreement with Lee *et al* (Lee et al., 2003b).

It would also be interesting to examine IgA responses in both the MHC-II<sup>-/-</sup> and CD40<sup>-/-</sup> B cell chimeras as Sangster *et al* showed that IgM and IgG antibodies were not produced against influenza but normal IgA production occurred suggesting that IgA production did not require cognate interaction involving MHC-II and CD40 on B cells (Sangster et al., 2003).

## Polyclonal responses in CD40<sup>-/-</sup> B cell chimeras

MHC-II tetramers were used to examine polyclonal T cell responses in CD40<sup>-/-</sup> B cell chimeras, allowing us to compare results using two different systems, Tg T cell transfer and endogenous polyclonal T cell responses.

When immunising with H19env-pulsed DCs or H19env in CFA, T cell priming in the CD40<sup>-/-</sup> B cell chimeras was very low, making it difficult to determine whether primary expansion was affected by the loss of CD40 on B cells. However, in one of the experiments tetramer staining above background levels was measurable and the expansion in CD40<sup>-/-</sup> B cell chimeras was not below that of wildtype chimeras, which correlates with the adoptive transfer results.

Although the primary response was relatively low, memory cells were formed and the recall response in CD40<sup>-/-</sup> B cell chimeras was equivalent to wildtype chimeras. Thus, CD40 on B cells was not required for memory T cell development, maintenance or a memory recall response to antigen. Thus, although CD40 is required for efficient T cell responses *in vivo*, B cells are not required to provide this CD40.

## Conclusions

By examining OT-II Tg T cell responses in CD40<sup>-/-</sup> and wildtype mice, I have shown that CD40 is not involved in the initial activation and expansion of these T cells but becomes required at later stages. Due to my previous findings of reduced OT-II T cell expansion in the absence of MHC-II on B cells and reduced expansion in the absence of CD40, CD40<sup>-/-</sup> B cell chimeras were created to determine if B cells were required to provide CD40. T cell expansion was normal in these mice suggesting that B cells are not the source of CD40. Interestingly, memory responses were also normal in the absence of B cell-derived CD40. Thus, B cells are not required to provide CD40 at any stage of a T cell response. The likeliest cell to provide the

CD40 signal *in vivo* is DCs. CD40 is important for the further enhancing of the APC activation, leading to further upregulation of other costimulatory molecules, which act to promote T cell expansion or survival.

“I don’t have a solution but I admire the problem.”

**Anon**



## B cell-derived $LT\alpha$ is not required during T cell expansion

### *Introduction*

The initiation of an immune response requires the interaction of a naïve T cell with an APC presenting the T cells specific ligand. Since Ag-specific naïve T cells are very rare, the secondary lymphoid tissues play an important role in concentrating the cells of the immune system into a small area thereby increasing the likelihood that these cells interact. The architecture of these organs is formed and maintained by various cytokines and chemokines. The cytokine and chemokine milieu required for the maintenance of the lymphoid organs is a delicate balance kept in check by positive and negative regulation. For example, the FDCs in the B cell follicles attract B cells, which express  $LT\beta$  on their surface inducing maturation of the FDCs and resulting in increased CXCL13 expression (Ngo et al., 2001). B cells have been shown to play a crucial role in maintaining splenic architecture. For example, B cell-deficient mice have a multitude of architectural defects in the spleen including the absence of FDCs, marginal zone macrophages (MZMs) and metallophilic macrophages (MMs) (Crowley et al., 1999) and also decreased expression of chemokines such as CCL21 (MandikNayak et al., 2001).

A growing number of mouse mutants have helped to unravel the various molecules involved in the generation of secondary lymphoid tissues. For example, the cytokines  $LT\alpha$  ( $LT\alpha_3$ , the secreted form) and  $LT\beta$  ( $LT\alpha_1\beta_2$ , the membrane form) are produced by activated T and B cells and play key roles in the organisation of secondary lymphoid tissues (see table 6.1 for expression of the lymphotoxins and their receptors).  $LT\alpha$ -deficient mice (deficient in both the secreted  $LT\alpha$  form and the membrane  $LT\beta$  form) lack LNs and have greatly disrupted splenic architecture. The crucial role of B cell-derived  $LT\alpha$  in lymphoid architecture was shown by the transfer of  $LT\alpha^{+/+}$  T or B cells into SCID mice; only  $LT\alpha^{+/+}$  B cells could restore T/B segregation and FDCs,  $LT\alpha^{+/+}$  T cells could not (Gonzalez et al., 1998).

| Cytokine                                      | Expressed by  | Receptors           | Receptor expression                                   |
|---|---|---------------------|---|
| LT $\alpha_3$ (formally called TNF- $\beta$ ) | Activated T cells<br>Activated B cells<br>NK cells  | TNFR1<br>TNFR2      | Most cell types including lymphocytes                 |
|   |   | HVEM                | Lymphocytes<br>Monocytes                              |
| LT $\alpha_1\beta_2$                          | Activated T cells<br>Activated B cells<br>NK cells<br>CD4 <sup>+</sup> CD3 <sup>-</sup> progenitors | LT $\beta$ R        | Macrophages<br>DCs<br>Stromal cells, e.g. FDCs + HEVs |
| LT $\alpha_2\beta_1$                          | As LT $\alpha_1\beta_2$   | TNFR1<br>TNFR2      | As above  |
|   |   | LT $\beta$ R weakly | As above  |
| LIGHT   | T cells<br>Granulocytes<br>Monocytes<br>Immature DCs  | LT $\beta$ R        | As above  |
|   |   | HVEM                | As above  |
| TNF $\alpha$                                  | Macrophages   | TNFR1<br>TNFR2      | As above  |

**Table 6.1.** Expression of TNF, lymphotoxin, LIGHT and their receptors

Given the importance of B cell-derived  $LT\alpha$ , the role of B cell-derived  $LT\beta$  has been examined by creating mice where only the B cells are deficient in  $LT\beta$  (Tumanov et al., 2002).  $LT\beta$  expression was approximately 60% lower in the spleen of these mice showing that B cells are the major source of  $LT\beta$  in the spleen. These mice had reduced numbers of FDCs, MZMs and MMs. In addition, there was a substantial disruption of the B cell follicles although the T cell areas were not disturbed.

Studies have now focussed on trying to understand what effect disrupted splenic architecture has on T and B cell responses.  $LT\alpha^{-/-}$  mice have been shown to have an impaired ability to clear viral infections, including lymphocytic choriomeningitis virus (LCMV) and they also have delayed responses to infection with influenza (Lund et al., 2002; Muller et al., 2002; Suresh et al., 2002).

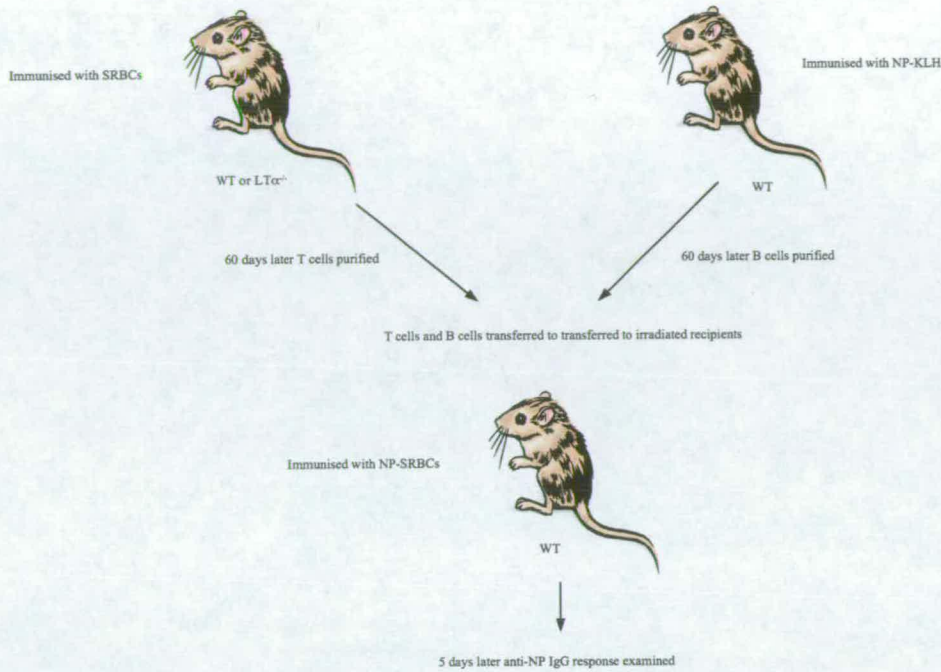
There have been a number of studies examining B cell responses in  $LT\alpha^{-/-}$  and  $LT\beta^{-/-}$  mice. In general, low stimulatory antigen protocols show impaired IgG responses in both  $LT\alpha^{-/-}$  and  $LT\beta^{-/-}$  mice (Banks et al., 1995; Eugster et al., 1996; Fu et al., 1997) but this can be overcome by using high doses of Ag (Matsumoto et al., 1996) or adjuvant (Fu et al., 1997). However, although Fu *et al* witnessed a robust IgG response when  $LT\alpha^{-/-}$  mice were immunised with Ag in adjuvant, when rechallenged three months later, they had a weak B cell memory response (Fu et al., 2000). Therefore, B cell memory appears to be defective in the absence of  $LT\alpha$ , even with high stimulatory immunisation protocols.

When using knockout mice, it is difficult to interpret whether the decreased responses are due to an inherent defect in the cells, or as a secondary result of disorganised splenic architecture. To determine which of these possibilities is true for B cell responses, Fu *et al* sublethally irradiated wildtype mice then transferred  $LT\alpha^{-/-}$  splenocytes at the same time as giving sheep red blood cells (SRBCs) (Fu et al., 1997). Normal IgG responses developed under these circumstances showing that the B cells had no inherent deficiency in responding to this Ag. In contrast, when wildtype splenocytes were transferred into

irradiated  $LT\alpha^{-/-}$  mice with SRBCs, no IgG response occurred. Since the mice were immunised at the same time as cell transfer, the architecture of the  $LT\alpha^{-/-}$  mice was still disrupted. Thus, the microarchitecture of the spleen appears to be the factor preventing normal B cell responses; B cells did not have to express  $LT\alpha$  themselves to become activated and isotype switch.

To determine what role  $LT\alpha$  plays in  $CD8^{+}$  T cell activation during LCMV infection, Suresh *et al* measured CD8 T cell activation in  $LT\alpha^{-/-}$  mice using MHC-I tetramers and by examining  $IFN-\gamma$  production. CD8 T cell activation was reduced in  $LT\alpha^{-/-}$  mice but T cell activation could be restored by transferring  $LT\alpha^{-/-}$  T cells into wildtype mice. Thus, as with B cells, the defective T cell activation was likely to be due to abnormal splenic architecture and not an inherent defect of the cells (Muller *et al.*, 2002; Suresh *et al.*, 2002).

Given that under certain conditions B cells become activated and isotype switch in the absence of  $LT\alpha$  but cannot form memory cells, it is important to examine T cell memory formation as well as T cell activation. Fu *et al* used a hapten carrier system to examine the generation of memory T cells in the absence of  $LT\alpha$  (illustrated in figure 6.1) (Fu *et al.*, 2000). Wildtype or  $LT\alpha^{-/-}$  mice were immunised with SRBCs and sixty days later T cells were purified from the spleen. These were transferred along with B cells from NP-KLH primed mice to irradiated wildtype recipients. T cells from either wildtype and  $LT\alpha^{-/-}$  mice could help B cells to produce NP IgG antibodies in response to immunisation with NP-SRBCs suggesting that memory T cells can form and survive in  $LT\alpha^{-/-}$  mice. Although this technique shows that memory T cells are capable of forming in  $LT\alpha^{-/-}$  mice, it does not allow a detailed enough study of any small differences in expansion, the kinetics of the response, or T cell differentiation.



**Figure 6.1.** Haptent-carrier system to examine memory T cell development in  $LT\alpha^{-/-}$  mice. Wildtype or  $LT\alpha^{-/-}$  mice were immunised and the T cells purified 60 days later. The T cells along with activated B cells were transferred into wildtype mice and the memory T cells tested for the ability to provide help to B cells.

Responses to both infections and SRBCs have been shown to be reduced in  $LT\alpha^{-/-}$  mice and these reduced responses are likely to be due to the disrupted architecture of the mice. However, exactly how this disrupted architecture causes reduced responses is unclear. The disruption of splenic architecture could cause decreased T cell responses by a number of mechanisms: myeloid DCs may be unable to migrate to the T cell area to activate T cells; T and B cell interactions may be reduced; or the trapping of particulate Ag by the marginal zone may be inhibited.

A key role for Ag trapping in the control of certain infections was revealed when decreased trapping of *L. monocytogenes* in the marginal zone led to impaired early control of the infection (Aichele et al., 2003). This was shown by giving mice low-

density clodronate liposomes, which are taken up by macrophages, which then rapidly undergo apoptosis (Naito et al., 1996). Therefore, in this system both MZMs and MMs were depleted. A separate study has also revealed a correlation between control of infection and splenic architecture. Muller *et al* revealed a correlation between reduced amounts of LCMV virus recovered from the spleen and the extent of splenic microarchitecture disruption (Muller et al., 2002). This is thought to be because early virus infection of the splenic marginal zone allows priming of the immune system.

The architecture of secondary lymphoid tissues is dependent on factors required during organogenesis and those required continuously for the maintenance of the architecture. In order to examine if the disrupted architecture in  $LT\alpha^{-/-}$  mice could be rescued by the presence of  $LT\alpha$  after organogenesis has occurred, Fu *et al* reconstituted lethally irradiated  $LT\alpha^{-/-}$  mice with wildtype bone-marrow (Fu et al., 1997). When this was done, FDCs, GCs and strong IgG responses were restored although the follicles remained small and T and B cells were not completely separated. Thus, the deficiency in FDCs and GCs in these mice is not set and can be rescued by the presence of  $LT\alpha$  after development has occurred.

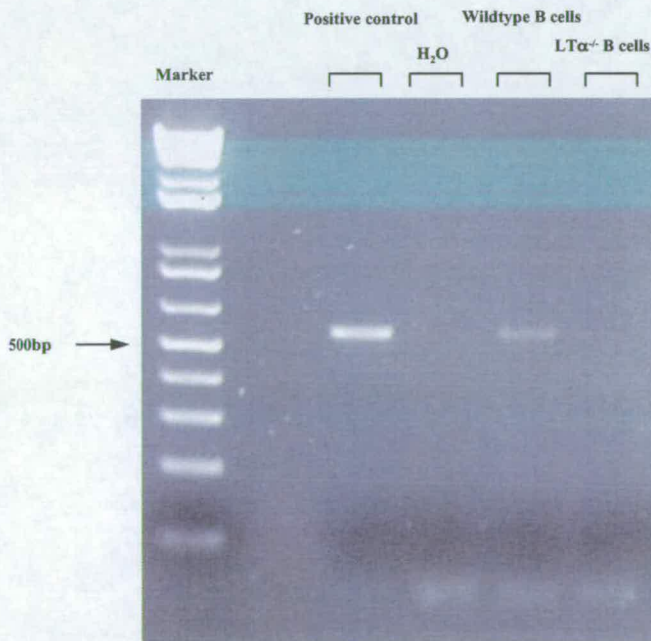
Given the crucial role of B cells in maintaining splenic architecture and, therefore, the disrupted architecture seen in  $\mu$ MT mice, this may be one of the reasons for the reduced expansion of OT-II T cells observed in these mice (shown in chapter three). Since  $LT\alpha$  and  $LT\beta$  play a major role in maintaining the splenic architecture, I created chimeras where the B cells were deficient in  $LT\alpha$  whereas other cell types (e.g. T cells and NK cells) were sufficient. The chimeras were created as described in chapter four but using  $\mu$ MT and  $LT\alpha^{-/-}$  bone-marrow. Since all B cells were derived from the  $LT\alpha^{-/-}$  bone-marrow, the B cells could not produce  $LT\alpha$  or  $LT\beta$ . These bone-marrow chimeras have several advantages to  $LT\alpha^{-/-}$  mice: they exclude the problems of dissecting whether LT is required during organogenesis or continually because the C57BL/6 mice had formed LNs before being irradiated and reconstituted; and the LT deficiency is restricted to one cell

type allowing the importance of one cell type as a source of LT to be examined. In these circumstances, I am examining a requirement for continual LT expression by B cells. As described in chapter three, the OT-II transfer system provides an opportunity to detect CD4<sup>+</sup> T cell proliferation *ex vivo*.

## Results

### The architecture in $LT^{-/}$ B cell chimeras is mildly disrupted

Bone-marrow chimeras were created where the B cells are deficient in  $LT\alpha$  to determine whether B cell-derived LT can affect T cell responses. The absence of  $LT\alpha$  expression by B cells from mice reconstituted with  $\mu$ MT and  $LT\alpha^{-/}$  bone-marrow was verified by purifying splenic B cells using CD19 MACs beads screening for genomic  $LT\alpha$  by PCR amplification using specific primers (figure 6.2). Genomic DNA for  $LT\alpha$  was detected from control chimeras but not  $LT\alpha^{-/}$  B cell chimeras.

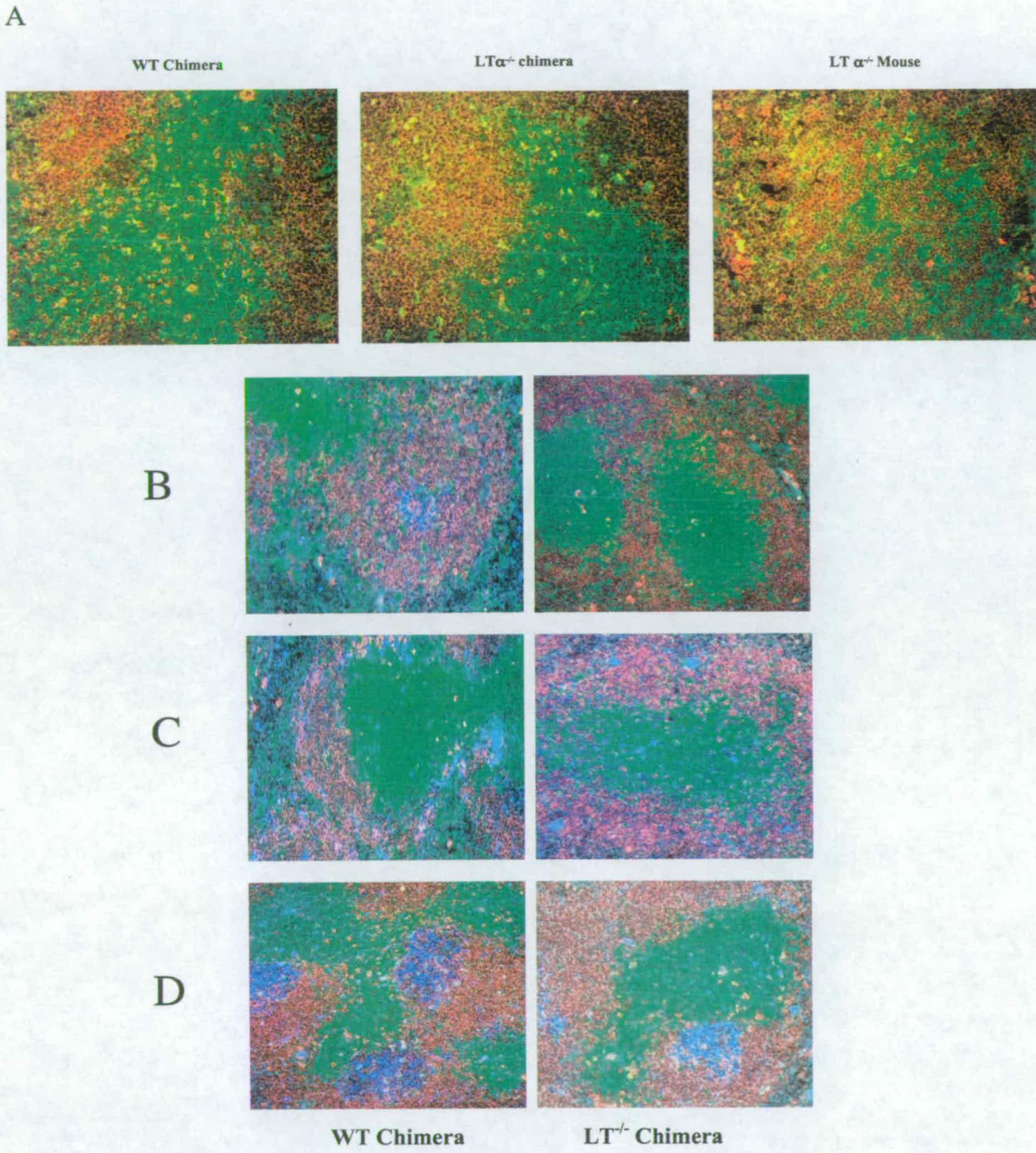


**Figure 6.2.** B cells do not express  $LT\alpha$  in chimeras reconstituted with  $\mu$ MT and  $LT\alpha^{-/}$  bone-marrow. B cells were purified using anti-CD19-beads and the cells lysed then analysed for the presence of  $LT\alpha$  DNA. The results are from 3 pooled mice. This experiment was done once.



To determine whether the absence of LT $\alpha$  on B cells affects the splenic architecture, spleens from LT $\alpha^{-/-}$  B cell chimeras and wildtype chimeras were examined by immunohistochemistry. 5 $\mu$ m thick sections were cut and the sections stained for T cells, B cells, FDC and MZMs. The results show that the T and B cell segregation was not affected by the absence of B cell-derived LT (figure 6.3A). However, FDCs were absent in LT $\alpha^{-/-}$  B cell chimeras and MZMs were reduced (figure 6.3 B + C). Therefore, B cells are the source of LT that is required for the maintenance of FDCs and a complete MZM compartment and this LT is required continuously. However, the segregation of T and B cells areas does not require a continuous supply of LT from B cells to be maintained.

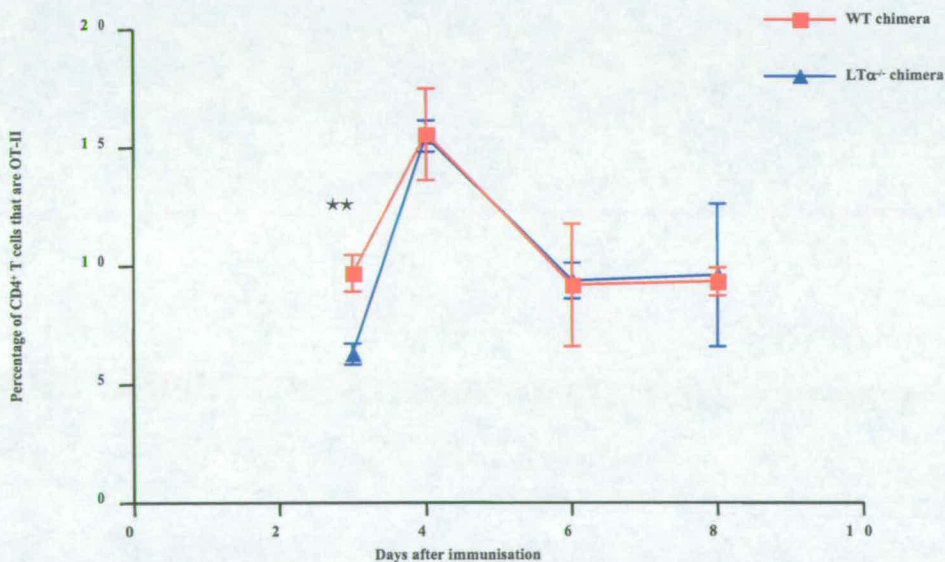
To determine whether GCs can form in the absence of FDCs, mice were immunised with DNP-OVA/alum and the spleens removed ten days later. They were immunised with this Ag because this is the Ag used during the experiments to examine CD4 $^{+}$  T cell expansion. Sections were stained with the lectin peanut agglutinin (PNA), which binds to germinal centre B cells. I observed that GCs could form in LT $\alpha^{-/-}$  B cell chimeras when using this immunisation protocol (fig 6.3D). This confirms other studies showing that germinal centres can occur in the absence of FDCs (Koni and Flavell, 1999) and also that GCs can form in the absence of LT $\alpha$  when a strong immunisation protocol is used (Fu et al., 1997; Matsumoto et al., 1996).



**Figure 6.3.** Immunohistochemistry. Spleens were removed and frozen in cryo-m-bed before sectioning. Sections were stained for the T cell area (green), B cell area (red) (A) The areas in blue are FDCs in (B), MZMs in (C) and Germinal centres in (D).

### OT-II T cell expansion is similar in the absence of B cell-derived LT $\alpha$

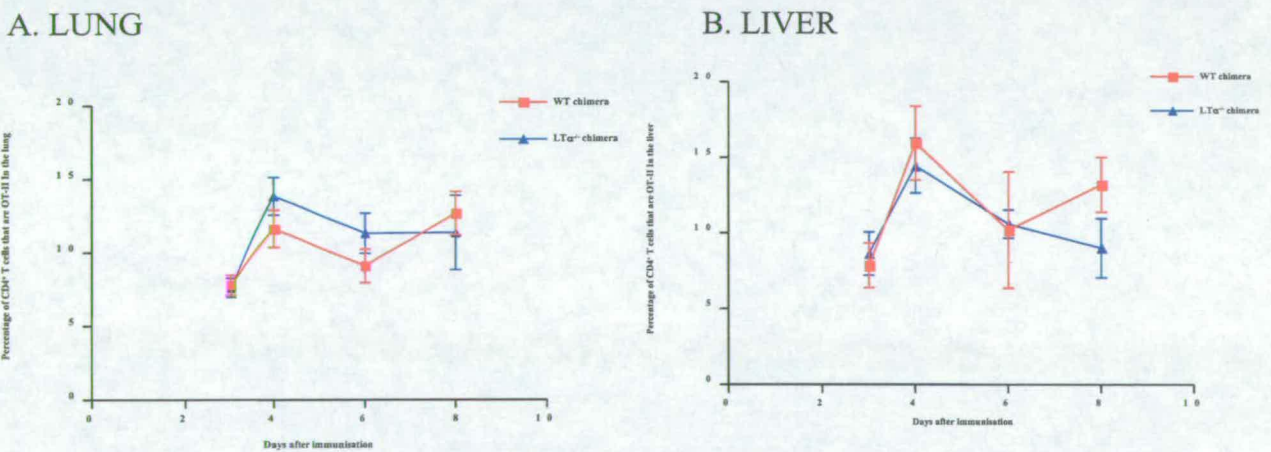
To examine whether LT $\alpha$  production by B cells is necessary for T cell expansion, OT-II T cells were transferred into wildtype or LT $\alpha^{-/-}$  B cell chimeras and the mice immunised the following day. The percentage of OT-II T cells was examined on days three, four, six and eight after immunisation. The initial expansion of OT-II T cells was lower in LT $\alpha^{-/-}$  B cell chimeras compared to wildtype chimeras. However, by the peak of the response there was no significant difference in expansion between the two types of chimeras (figure 6.4). Thus, the expansion of OT-II T cells is initially lower in LT $\alpha^{-/-}$  B cell chimeras but is able to reach normal levels by the peak of expansion.



**Figure 6.4.** OT-II T cell expansion can reach normal levels when the B cells do not produce LT. CD4 $^{+}$  T cells from OT-II mice were transferred into bone-marrow chimeras where the B cells cannot produce LT or control chimeras and the mice immunised the following day. Mice were euthanised on various days after immunisation and the percentage of transgenic cells determined. Expansion is expressed as the percentage of CD4 $^{+}$  cells positive for V $\alpha$ 2 and V $\beta$ 5. Symbols show the mean of each group and the error bars show the standard error of the mean. Statistical comparisons were made using the un-paired T-test.  $\star\star$ ,  $p < 0.01$ . All days were examined twice with a minimum of three mice per group.

## Migration of T cells to peripheral tissues is not affected by the absence of LT from B cells

Given the differences in splenic architecture in  $LT\alpha^{-/-}$  chimeras, the migration of T cells from the spleen after immunisation may be affected in these chimeras. To determine whether the absence of LT expression by B cells led to differences in the migration of OT-II T cells to peripheral tissues, mice were perfused and the lungs and livers examined for the presence of OT-II T cells. In the  $LT\alpha^{-/-}$  B cell chimeras, no differences in T cell migration were seen (figure 6.5 A and B). Therefore, in the absence of proper splenic architecture T cells do not change their migration to peripheral tissues

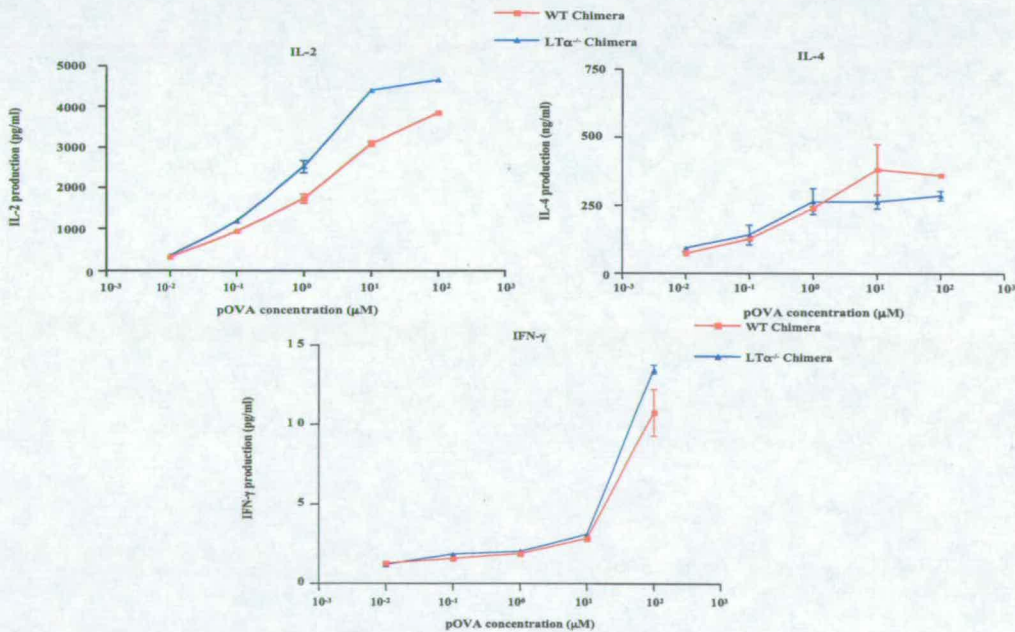


**Figure 6.5.** T cell migration to peripheral tissues is unaffected by the absence of B-cell produced  $LT\alpha$ . After transfer of OT-II T cells and immunisation, mice were euthanised and perfused on days 3, 4, 6 and 8 after immunisation and the liver and lung examined for the percentage of transgenic cells. Expansion is expressed as the percentage of  $CD4^+$  cells positive for  $V\alpha 2$  and  $VB5$ . Symbols show the mean of each group and the error bars show the SEM. Statistical comparisons were made using the un-paired T-test. This experiment was done twice with a minimum of 3 mice per group.

## The absence of B cell-derived LT does not affect T cell differentiation

Lymphotoxin is characterised as a Th1 cytokine due to the fact that  $LT\alpha$  mRNA was only found in clones producing IL-2 and IFN- $\gamma$  but not those producing IL-4 and IL-5 (Cherwinski et al., 1987). When the expression of  $LT\beta$  was examined, it was found that naïve T cells readily express  $LT\beta$  after TCR engagement (Gramaglia et al., 1999). Although  $LT\beta$  expression is not restricted to Th1 cells, IL-4 was shown to downregulate its expression leading to Th2 cells that do not express LT.

To determine whether OT-II T cell cytokine production was affected by the absence of LT production by B cells, cytokine production by OT-II T cells was examined various days after immunisation using ELISAs, as described previously. There was no obvious difference in the production of IL-2, IFN- $\gamma$  or IL-4 by OT-II T cells from wildtype or  $LT\alpha^{-/-}$  chimeras showing that B cell LT did not regulate OT-II T cell cytokine production (figure 6.6).

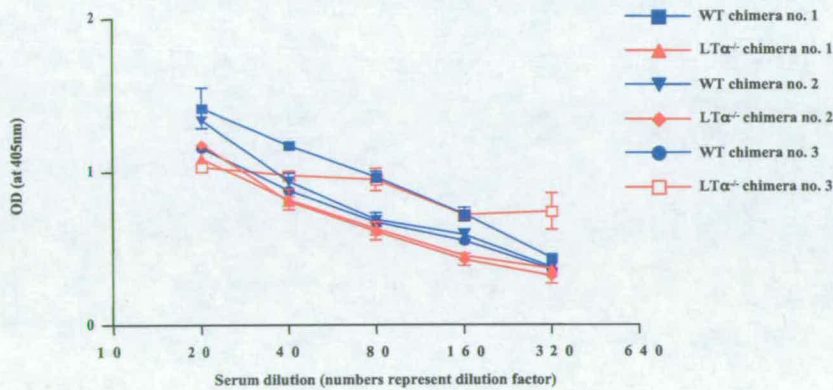


**Figure 6.6.** T cell differentiation is not affected by the absence of B cell-derived LT. Pooled splenocyte populations from wildtype or  $LT\alpha^{-/-}$  B cell chimeras were depleted of  $CD8^+$  T cells and MHC-II positive cells. The cells were analysed for cytokine production as described in chapter four. These graphs show results from four days after immunisation but the other days showed similar results. The ELISAs were pooled data from at least three mice per group and were done twice for each day.

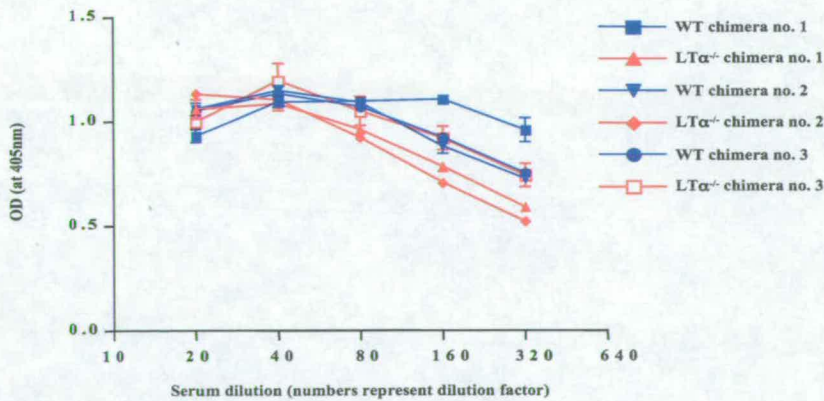
## Antibody production is unaffected by B cell-derived LT $\alpha$

Blood samples were taken from mice sacrificed eight days after immunisation with DNP-OVA/alum to examine serum antibody levels. We determined whether the LT $\alpha^{-/-}$  B cell chimeras were able to produce both IgM and IgG antibodies in response to immunisation with DNP-OVA/alum. As shown in figure 6.7, both anti-DNP IgM and IgG antibody production were unaffected by the absence of B cell LT.

### IgM



### IgG



**Figure 6.7.** LT $\alpha^{-/-}$  B cell chimeras are efficient at antibody production in response to DNP-OVA/alum. Mice that were transferred with OT-II T cells and immunised eight days earlier were bled to determine serum antibody amounts by ELISA. The error bars show the SEM. Results are representative of two experiments.

## Discussion

Given the importance of LT $\alpha$  and LT $\beta$  in maintaining splenic architecture, it is possible that one of the reasons T cells respond less well in  $\mu$ MT mice (shown in first results chapter) is due to the lack of B cell-derived LT and TNF, resulting in disrupted splenic architecture. Therefore, bone-marrow chimeras were created where the B cells were unable to produce LT $\alpha$  or LT $\beta$  but could present Ag and provide cosimulatory molecules.

When the architecture of the LT $\alpha$ <sup>-/-</sup> B cell chimeras was examined, FDCs were absent and MZMs were reduced but the B cell follicles were still distinct and T/B cell segregation was maintained. This showed that, after lymphoid development had occurred, B cell-derived LT was not required for T/B segregation but was essential for the presence of FDCs and normal MZM numbers. The  $\mu$ MT mice also lack FDCs and have greatly reduced MZMs (Crowley et al., 1999).

This crucial role of B cell-derived LT is consistent with cell transfer studies. Fu *et al* restored FDCs in RAG<sup>-/-</sup> mice by transferring B cells alone (Fu et al., 1998). Interestingly, when mice were created where only the B cells were deficient in LT $\beta$ , some FDCs (15-20%) were still present (Tumanov et al., 2002). This suggests that the lack of FDC in LT $\alpha$ <sup>-/-</sup> B cell chimeras is likely to be due to absence of both LT $\alpha$  and LT $\beta$ . Therefore, both cytokines play a role in maintaining FDCs.

Given the complete lack of T and B cell segregation in LT $\alpha$ <sup>-/-</sup> mice and the importance of B cell-derived LT in maintaining FDC and MZMs, it was somewhat surprising that the T and B cells remained segregated in LT $\alpha$ <sup>-/-</sup> B cell chimeras. Evidence that T/B segregation is relatively plastic comes from experiments using the LT $\beta$ R-Ig fusion protein to inhibit the LT $\beta$  pathway. When 6-week-old mice were given a prolonged LT $\beta$ R-Ig treatment, B cell follicular integrity was lost and the T/B border was lost (Mackay et al., 1997). Moreover, when SCID mice were treated with LT $\beta$ R-Ig one week before transfer of

splenocytes, the T and B cells did not segregate properly whereas they did in control-treated mice (Gonzalez et al., 1998). There are two possible reasons for these discrepancies. It is possible that the LT required for T/B segregation is delivered by cells other than splenocytes; when  $LT\alpha^{-/-}$  splenocytes were transferred to SCID mice they were able to segregate (Gonzalez et al., 1998). Another possibility is that the molecule required for T/B segregation is actually LIGHT, a recently discovered ligand for  $LT\beta R$  (Mauri et al., 1998).

In this study, when OT-II T cells were used to examine T cell expansion in  $LT\alpha^{-/-}$  B cell chimeras, the T cell expansion was lower at day 3 but by the peak of the response normal levels of expansion was seen. Thus, although the expansion was slightly slower in  $LT\alpha^{-/-}$  B cell chimeras, OT-II T cells could expand fully in these mice. This shows that the loss of LT from B cells, and therefore FDCs and MZMs, is not required for optimal T cell expansion in this system.

The delayed expansion of T cells may explain the delayed response to influenza infection in  $LT\alpha^{-/-}$  mice seen by others (Lund et al., 2002). Since the mice eventually clear the infection, it shows that, in the absence of  $LT\alpha$  signalling and organised lymphoid tissue, mice can still generate a response to influenza but the response takes longer to occur. It would be interesting to compare OT-II expansion in  $LT\alpha^{-/-}$  mice to wildtype mice and the  $LT\alpha^{-/-}$  B cell chimeras to see if I detect an even slower onset of T cell expansion in the complete absence of  $LT\alpha$ . Any difference between the complete  $LT\alpha^{-/-}$  mice and the  $LT\alpha^{-/-}$  B cell chimeras would either be because another cell type which expresses  $LT\alpha$  plays a role in T cell expansion or because  $LT\alpha$  plays a role in both organogenesis and maintenance of lymphoid tissues.

The proportions of OT-II T cells in the peripheral tissues were examined to determine whether disrupted splenic architecture affected T cell migration. It was particularly relevant to look at this since  $LT\alpha^{-/-}$  and  $LT\beta R^{-/-}$  mice have increased numbers of



lymphocytes in the peripheral tissues including the lungs and livers (Banks et al., 1995; Futterer et al., 1998). Moreover, Lo *et al* showed that there was more robust homing of OT-II cells to the lungs of  $LT\beta R^{-/-}$  mice compared to wildtype mice (Lo et al., 2003). Only naïve T cells were examined in this study, however, so whether effector T cell migration is also affected has not been examined. When the chimeras were examined at various days after immunisation for migration of OT-II T cells to the lungs and livers, there was no statistical difference in T cell migration between the two groups showing that B cell-derived LT does not affect T cell migration. It would be interesting to examine OT-II T cell migration in  $LT\alpha^{-/-}$  mice to determine whether  $LT\alpha$  produced by another cell type can affect T cell migration. To determine whether  $LT\alpha$  on T cell themselves can affect migration, OT-II mice could be crossed with  $LT\alpha^{-/-}$  mice and the OT-II/ $LT\alpha^{-/-}$  T cells transferred to wildtype mice to follow migration of the cells after immunisation.

It was interesting to examine T cell differentiation in these chimeras because LT has been characterised as a Th1 cytokine (Cherwinski et al., 1987). Suresh *et al* examined splenocytes from LCMV-infected mice for  $CD4^{+}$  IFN- $\gamma$  production in response to an MHC-II-restricted epitope. They found less IFN- $\gamma$  production from  $LT\alpha^{-/-}$  splenocytes compared to wildtype splenocytes (Suresh et al., 2002). Unfortunately, from this study it is impossible to determine whether less  $CD4^{+}$  T cells were activated or less T cells differentiated into IFN- $\gamma$  producers. A suggestion that  $CD4$  T cell differentiation could be affected comes from a study examining  $CD8^{+}$  T cells in  $LT\alpha^{-/-}$  mice. They showed that  $CD8^{+}$  T cell expansion was similar to wildtype mice but they were functionally defective, as measured by CTL assays and IFN- $\gamma$  production (Kumaraguru et al., 2001). However, when I examined OT-II T cells for the production of IL-2, IFN- $\gamma$  and IL-4, cytokine production was similar in both wildtype and  $LT\alpha^{-/-}$  B cell chimeras. Therefore, LT from B cells does not affect T cell differentiation. It remains to be determined whether OT-II T cell differentiation would be affected in  $LT\alpha^{-/-}$  mice or when OT-II/ $LT\alpha^{-/-}$  T cells are used.

Antibody production was examined in the  $LT\alpha^{-/-}$  B cell chimeras. Normal levels of IgM and IgG were produced in these mice in response to DNP-OVA/alum showing that isotype switching was not affected in the absence of LT on B cells. This result is most likely due to the fact that a strong immunisation protocol was used. Indeed, when  $LT\alpha^{-/-}$  mice were immunised with Ag in adjuvant they developed robust IgG responses (Fu et al., 1997). Since GC formation and isotype switching do not occur in  $LT\alpha^{-/-}$  mice when Ag without adjuvant is used for immunisation, it is likely that these chimeras will have this similar defect.

It remains to be determined whether memory responses are reduced in these mice. Since OT-II T cell expansion reached normal levels in  $LT\alpha^{-/-}$  B cell chimeras and Fu *et al* found that memory T cells from  $LT\alpha^{-/-}$  mice were able to help B cells make antibody responses efficiently (Fu et al., 2000), it is likely that T cell memory responses would be normal in these chimeras. However, it is possible that B cell memory formation would be defective in these chimeras. B cell memory responses were reduced in  $LT\alpha^{-/-}$  mice even when immunised with Ag in adjuvant, which allowed a robust IgG response (Fu et al., 2000). These chimeras would be an excellent tool for determining if memory B cell responses are still defective when LT is produced by all cells except B cells.

In summary, chimeras where the B cells cannot provide LT lack FDCs and MZMs but maintain T/B segregation. When OT-II T cell responses were followed in these chimeras, the initial expansion was lower compared to wildtype chimeras but the expansion reached normal levels by the peak of the response. Thus, T cells can expand efficiently when B cells are deficient in LT. In addition, T cell differentiation was unaffected by the absence of LT on B cells.

To properly understand the role of B cells in controlling splenic architecture, it would be interesting to make mice where the B cells are genetically deficient in both TNF and LT.

This would be more comparable to  $\mu$ MT mice since both B cell-derived TNF and LT would be absent during organogenesis.

“A conclusion is the place where you got tired of thinking.”

**Steven Wright**

## Discussion and Conclusions

### Summary

The aim of this PhD project was to elucidate the role of B cells in regulating T cell responses. There have been a number of studies examining T cell responses in the absence of B cells; however, many of these have given conflicting results. Therefore, I began by examining T cell responses in  $\mu$ MT mice using the OT-II adoptive transfer system. The advantage to the OT-II system is that T cell expansion can be examined *ex vivo* without the need for *in vitro* restimulations. I showed that the OT-II T cells divided less in  $\mu$ MT mice compared to wildtype mice. Therefore, optimal expansion of T cells requires the presence of B cells.

Having determined that B cells do indeed play a role in enhancing T cell expansion, I then went on to examine how B cells exert this effect. There are a number of possible ways B cells can affect T cells responses including Ag presentation, costimulatory stimulation, cytokine production and antibody production. First, the requirement for Ag presentation by B cells was examined. To do this, bone-marrow chimeras were produced where the B cells were deficient in MHC-II and could not, therefore, present Ag to CD4<sup>+</sup> T cells whereas other APCs, including DCs, which are thought to be essential for the initial activation of T cells, could present Ag. Using both the OT-II adoptive transfer system and the MHC-II tetramer system, the T cells expanded to a lesser degree when B cells were deficient in MHC-II. Moreover, T cell memory responses were also decreased in the MHC-II<sup>-/-</sup> B cell chimeras. Thus, both primary and memory T cell responses are regulated by a cognate interaction with B cells through MHC-II. Importantly, the primary responses were examined using two different methods. When the adoptive transfer system was used, the mice were immunised with a hapten-carrier Ag in alum whereas when the MHC-II tetramers were used to detect polyclonal T cells responses, the immunisation protocols used here were peptide in CFA or peptide-pulsed DCs.

It is likely that the reduced numbers of memory T cells present in MHC-II<sup>-/-</sup> B cell chimeras is due to the suboptimal T cell priming in the MHC-II<sup>-/-</sup> chimeras however a role for B cell MHC-II in the maintenance of memory T cells has not been examined in this system and may also be important. It also remains to be determined whether B cell MHC-II is required for the memory recall response. This is highly likely given that B cells are thought to be more important later in T cell responses. This is for two reasons; Ag-specific B cells are present at very low frequencies in the naïve state; and memory T cells seem to be less stringent in their requirement for activation and can be activated by both activated and resting B cells (Croft et al., 1994).

A second mechanism through which B cells could regulate T cell responses is through the provision of costimulation. In order to address this question, bone-marrow chimeras were created in which the B cells were deficient for CD40, but the other APCs could provide this costimulation. Under these circumstances, OT-II T cells could expand to the same degree as in wildtype chimeras. Thus, CD40 expression by B cells was not required for T cell expansion. This result was somewhat surprising given the integral role of CD40 in maturing B cells. CD40 signalling induces the upregulation of several molecules including CD80 (Yellin et al., 1994) and OX40L (Murata et al., 2000). Thus, although it is possible that other costimulatory molecules are required from B cells for optimal T cell responses, it would be predicted that the upregulation of these (e.g. OX40L) would be impaired in CD40<sup>-/-</sup> B cells.

The architecture of the secondary lymphoid tissues must be highly organised in order to allow the efficient interaction of various cell types. Given that the splenic architecture is disrupted in  $\mu$ MT mice, this could be another possible reason for the decreased T cell responses in these mice. B cell-derived LT $\alpha$  and LT $\beta$  play a crucial role in forming and maintaining the architecture of secondary lymphoid tissues. Therefore, bone-marrow chimeras were created where the B cells could produce neither LT $\alpha$  nor LT $\beta$ . This led to disrupted splenic architecture as shown by the absence of FDCs and MZMs. Although there was less T cell division at three days post-immunisation, by the peak of response, T

cells responded equally in  $LT^{-/-}$  B cell chimeras compared to wildtype chimeras. Thus, the absence of FDCs and a MZM compartment did not result in decreased T cell responses.

Taken together, B cells are essential for optimal T cell primary and memory responses. The principal mechanism appears to be through MHC-II-mediated cognate interactions rather than provision of costimulatory stimulation, antibody production or maintenance of splenic architecture.

### ***Discussion***

Having shown that MHC-II on B cells is required for efficient T cell responses using both the OT-II system and MHC-II tetramers, it is important to consider why MHC-II could be important. The most likely scenario is that MHC-II expression by B cells allows B cell Ag-presentation leading to increased T cell responses, most likely due to the increased numbers of APCs available.

However, given that the MHC-II molecule has also been shown to be involved in signalling, another possibility is that the interaction of the MHC-II molecule with a TCR may give B cells additional signals that result in the upregulation of other costimulatory molecules or increased cytokine production and it is these that regulate T cell responses. MHC signalling has a wide range of effects on B cell function including Ag presentation (Nabavi et al., 1989), proliferation (Cambier and Lehmann, 1989), apoptosis (Catlett et al., 2001) and cytokine release (Guo et al., 1999).

There are several ways in which the role of MHC-II signalling in B cell Ag presentation to T cells could be investigated. Two different signalling pathways are induced by the aggregation of MHC-II on B cells. One requires the cytoplasmic domain of the MHC-II  $\beta$  chain while the other only occurs in B cells previously stimulated with IL-4 or through

BCR aggregation. The signalling that occurs through the  $\beta$  chain cytoplasmic tail is important for Ag presentation and translocation of protein kinase C (PKC) to the nucleus (Nabavi et al., 1989; St-Pierre et al., 1989; Wade et al., 1989). Mice have been produced with MHC-II molecules lacking most of the  $\beta$  chain cytoplasmic domain (Smiley et al., 1995). Unlike MHC-II<sup>-/-</sup> mice, these mice have normal CD4<sup>+</sup> T cell development (Smiley et al., 1995). To determine whether the signalling through the  $\beta$  chain of the MHC-II molecule can affect the ability of B cells to regulate T cell responses, it would be interesting to compare OT-II T cell responses in chimeras with B cells expressing fully functional MHC-II to those with B cells lacking the cytoplasmic domain of the  $\beta$  chain.

The other MHC-II signalling pathway, which occurs only in stimulated B cells, does not require the cytoplasmic domains of MHC-II (Andre et al., 1994). It is the transmembrane sequences of the  $\alpha$  and  $\beta$  chains of MHC-II that are critical for this signalling (Harton et al., 1995). Consequently, creating chimeras with B cells expressing truncated MHC-II will only deplete the PKC signalling pathway but not this pathway. Although this signalling pathway is only beginning to be understood, it has recently been reported that the transmembrane region of the MHC-II molecule can bind the signalling transducers Ig $\alpha$  and Ig $\beta$  (the signalling molecules involved in BCR signalling) (Lang et al., 2001). To study the involvement of both signalling pathways activated by the cross-linking of MHC-II molecules, it would be interesting to compare T cell responses in wildtype chimeras and chimeras with mutant MHC-II molecules that do not bind the Ig $\alpha$  and Ig $\beta$  molecules. B cells expressing mutations in their transmembrane domain would allow this to be examined.

Another difference between the wildtype chimeras and the MHC-II<sup>-/-</sup> B cell chimeras is that the MHC-II<sup>-/-</sup> B cell chimeras do not produce Ag-specific IgG. When interpreting results from these chimeras it is important to take this into account. In these experiments, I cannot rule out that the loss of the Ag-specific IgG response caused the reduced T cell expansion in these mice. IgG could possibly enhance T cell responses by binding to Ag and increasing the Ag uptake by APCs. This is unlikely, however, given that the



decreased T cell expansion was seen as early as day three after immunisation, before an Ag-specific IgG response can occur. Moreover, a DNP-specific IgG response fails to occur in CD40<sup>-/-</sup> B cell chimeras and there was no difference in OT-II T cell expansion in these chimeras. This shows that normal T cell expansion can occur in the absence of Ag-specific IgG.

It would be interesting to examine the expression of costimulatory molecules and cytokines by B cells in the absence of MHC-II or CD40. For example, signalling through MHC-II on several B cell lines has been shown to induce LT $\alpha$  production (Guo et al., 1999). Therefore, it is possible that cytokine production from B cells in the MHC-II<sup>-/-</sup> chimeras differs from that in wildtype chimeras. Both MHC-II and CD40-signalling leads to the upregulation of costimulatory molecules. A microarray system where multiple cytokines and costimulatory molecules can be examined at once would be ideal to identify additional differences between wildtype B cells and those that lack MHC-II or CD40.

Having shown that MHC-II on B cells is required for optimal T cell priming, it would be interesting to examine which subset of B cells is involved in this regulation. There are two main types of B cells in the spleen and the system I have used has not distinguished between them. Follicular B cells are IgM<sup>dull</sup> IgD<sup>bright</sup> and are involved in TD responses whereas MZ B cells are IgM<sup>bright</sup> IgD<sup>dull</sup> and are involved in both TD and TI responses. Although follicular B cells have been given the most attention when examining T and B cell interaction, Kearney and colleagues have recently demonstrated that marginal zone B cells can present protein Ag to CD4<sup>+</sup> T cells resulting in T cell expansion both *in vitro* and *in vivo* (Attanavanich and Kearney, 2004). Therefore, it would be interesting to examine whether MHC-II on follicular B cells versus marginal zone (MZ) B cells are important in this system or whether they both play a role. There are several mice that have been produced that do not possess marginal zones, including protein kinase 2 (Pyk2) deficient mice (Guinamard et al., 2000) and dedicator of cytokinesis 2 (Dock2) deficient mice (Fukui et al., 2001). To determine if MZ B cells play a role in this system, bone-

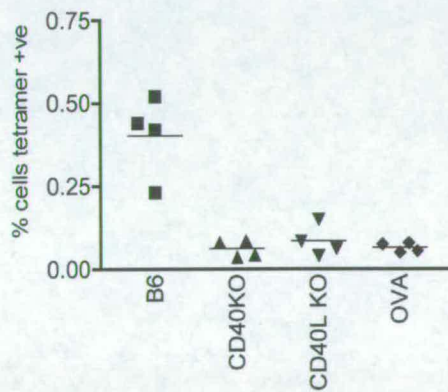
marrow chimeras could be made by combining wildtype or MHC-II<sup>-/-</sup> bone-marrow with bone-marrow from marginal zone deficient mice.

### **Conclusions**

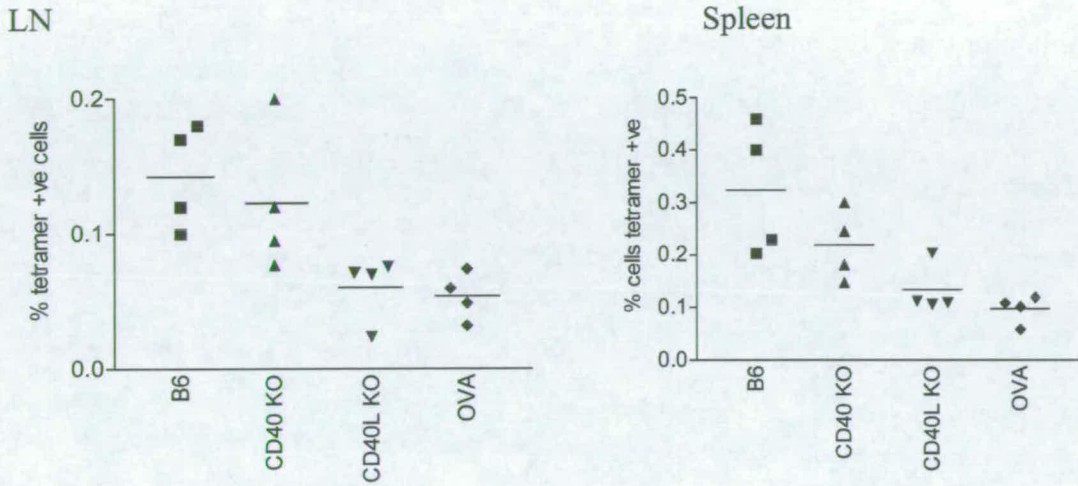
It is critical for vaccine design that a strong immunological response is generated. In order to achieve this, the requirements for the induction of immune responses need to be understood. My results have shown that MHC-II expression by B cells is required for optimal T cell memory generation, therefore, an effective vaccine strategy must take this into account. By understanding the critical role B cells play in promoting T cell expansion and memory development, it becomes possible to exploit these mechanisms to design more efficacious vaccines. The data shown here demonstrate that B cells play a role in enhancing T cell responses, mainly through the provision of MHC-II although costimulatory molecules other than CD40 may also be required. Thus, increasing Ag presentation by B cells may enhance the efficacy of vaccines. Factors that increase Ag presentation by B cells include CD40 signalling (Clatza et al., 2003) and MHC-II signalling (Nabavi et al., 1989). For example, Castiglioni *et al* generated a DNA vaccine that contained a B cell specific promoter to target expression to B cells. When this was used in mice with defective DCs, CD4<sup>+</sup> T cell priming still occurred. This shows that B cells can be an important APC *in vivo*. Thus, given my results, a vaccine strategy that activates B cells as well as DCs will be a more effective vaccine strategy than one that concentrates only on DCs.

## Appendix

As described in chapter five, CD40<sup>-/-</sup> B cell chimeras were immunised with wildtype dendritic cells to compare to results achieved by Megan MacLeod in CD40<sup>-/-</sup> mice. Work completed by Megan MacLeod as part of her PhD showed that the primary response in CD40<sup>-/-</sup> mice could not be seen when CD40<sup>-/-</sup> mice were immunised with peptide in CFA (figure 8.1). Therefore, mice were immunised with wildtype dendritic cells to overcome this defect in priming.



**Figure 8.1.** Tetramer staining cannot be seen in CD40<sup>-/-</sup> mice when immunised with H19env/CFA. Percentage of tetramer positive cells, out of CD4 cells, were examined in the spleens of C57BL/6, CD40 and CD40L knockout mice immunised with H19env-CFA 9 days previously. Graph courtesy of Megan.



**Figure 8.2.** A population of tetramer positive cells can be detected in CD40<sup>-/-</sup> mice when they are immunised with C57BL/6 BM-DCs. DCs were grown from bone-marrow from C57BL/6 mice then activated with PBS and pulsed with H19env or pOVA.  $1 \times 10^6$  DCs were used to immunise each mouse and mice examined for tetramers cells six days after immunisation. Graph courtesy of Megan.

“Success is the ability to go from one failure to another with no loss of enthusiasm.”

**Sir Winston Churchill**

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“How some of the writers I come across get through their books without dying of boredom is beyond me.”

**William Gaddis**