Development of T cell help for B cells

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Declaration

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

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Abstract

After encounter with antigen, naïve CD4$^+$ T cells proliferate and differentiate into effector and memory cells. Effector CD4$^+$ T cells provide help to B cells to undergo isotype class switching and germinal centre formation. T cells that have been activated *in vivo* in the absence of B cells are impaired as they are unable to help B cells to class switch *in vitro*. Furthermore, T cells primed in the absence of CD40 are unable to help normal B cells to class switch or to form germinal centres. The mechanisms underlying this differentiation of helper T cells are not well understood. In this study, I have investigated cellular and molecular interactions involved in these processes.

B cells can directly affect CD4$^+$ T cell effector responses through antigen presentation and costimulation, for example via CD40-CD40L and OX40L-OX40 interactions. By creating bone marrow chimeric mice, we could selectively knock out MHC class-II and CD40 on B cells. This allowed us to study the molecular interaction between B and T cells that is required for T cells to develop helper function. In the absence of B cells that express MHC class-II or CD40, antigen induced differentiation of effector T cells that help B cells to class switch, was impaired.

The role played by inducible costimulator (ICOS) in CD4$^+$ T cell responses was also examined using ICOS$^{-/-}$ mice. In the absence of ICOS-ICOSL interactions, CD4$^+$ T cells were unable to help B cell clonal expansion and class switching to IgG$_1$ and IgG$_{2a}$. Thus, ICOS$^{-/-}$ mice have few small germinal centres, markedly reduced levels of class switched immunoglobulins in their serum and significantly less antibody secreting cells in spleen and bone marrow. Using adoptive transfer studies with ICOS-deficient transgenic T cells, I have shown that ICOS is important for the comprehensive activation, clonal expansion and differentiation of CD4$^+$ T cells.

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Abbreviations

aa	amino acid
AHR	airway hyper-reactivity
APC	antigen presenting cell
ASC	antibody secreting cell
BAFF	B cell activating factor
BCR	B cell receptor
BMDC	bone marrow – derived dendritic cell
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
CFSE	5, 6 - carboxyfluoroscein diacetate succinimidyl ester
CSR	class switch recombination
d	day
DC	dendritic cell
DNP	dinitrophenyl
DO11.10	a transgenic TCR specific for the 323-339 peptide epitope of ovalbumin
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FDC	follicular dendritic cell
FITC	fluoroisothiocyanate
FO	follicular B cell
GM-CSF	granulocyte / macrophage colony stimulating factor
GC	germinal centre
h	hour
HEV	high endothelial venule
i.p.	intraperitoneal
i.v.	intravenous
ICOS	inducible costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KJ1.26	a monoclonal antibody specific for the DO11.10 TCR
KLH	keyhole limpet haemocyanin
LCMV	lymphocyte choriomeningitis virus
LN	lymph node

LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MACS	magnetically activated cell sorting
MHC	major histocompatibility complex
min	minutes
MLR	mixed lymphocyte reaction
MZ	marginal zone
NOD	non-obese diabetic
NFκB	nuclear factor-κB
OD	optical density
Pa	*Propionebaterium acnes*
PALS	periarteriolar lymphoid sheath
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PMA	phorbol 12-myristate 13-acetate
PNA	peanut agglutinin
PNPP	p-nitrophenyl phosphate
PRRs	pattern recognition receptors
PS	phophatidylserine
RT-PCR	reverse transcriptase – polymerase chain reaction
s.c.	subcutaneous
SEA	soluble egg antigen from *Schistosoma mansoni*
SCID	severe combined immunodeficiency
SHM	somatic hypermutation
SRBC	sheep red blood cells
TCR	T cell receptor
TD	T dependent
TI	T independent
T_{FH}	follicular B-helper T cell
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptor
TMB	3, 3', 5, 5' - tetramethylbenzidine
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
VSV	vesicular stomatitis virus
7-AAD	7-amino-actinomycin D

1. INTRODUCTION

1.1. Adaptive immune response: an overview

Humoral immunity and cell-mediated immunity, the two types of adaptive immune responses, are mediated by different components of the immune system and function to eliminate different types of microbes. Antibodies, the main weapon of humoral immunity, are made by B lymphocytes and are the principal defense mechanism against extracellular microbes and their toxins. Cell-mediated immunity is mediated by T lymphocytes and acts to destroy intracellular microbes or kill infected cells and thus eliminate the reservoir of infection. There are two distinct types of T cells: cytotoxic T cells (CTL, $CD8^+$) and helper T cells ($CD4^+$). CTLs kill cells that produce foreign antigens, such as cells infected by viruses and other intracellular microbes. $CD4^+$ T cells recognise peptides presented by MHC-II molecules whereas $CD8^+$ T cells recognise peptides 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-covalently associated α and β chains. They are constitutively expressed on professional antigen presenting cells (APC) and are upregulated upon activation. They can also be expressed on many other cell types upon stimulation by inflammatory signals. $CD4^+$ T cells direct the immune response by regulating the proliferation and differentiation of various cells of the immune system and they achieve this mainly through the secretion of small soluble messengers called cytokines. In order to carry out these functions, the T cells must be activated and this occurs in the secondary lymphoid organs.

1.1.1. Secondary lymphoid organs

Lymphoid tissues are classified as primary lymphoid organs (bone marrow and thymus), where lymphocytes first express receptors that recognise specific antigen and attain phenotypic and functional maturity, and as secondary or peripheral lymphoid organs (spleen, lymph nodes, Peyer's patches etc.) where lymphocyte responses to foreign antigens are initiated. The secondary lymphoid organs are highly organised structures that facilitate the encounter of antigen-specific T cells and APC that express antigen. The main cell types found in secondary lymphoid organs are B and T lymphocytes, dendritic cells (DCs), macrophages, non-haematopoietic stromal cells other than red blood cells. The naïve T cells continually recirculate through lymphoid organs until they encounter specific antigen.

The spleen is the major site of immune responses to blood-borne antigens. It is divided into red pulp, containing mostly erythrocytes, and white pulp comprised of lymphocytes and other cells. The T cell zones are termed periarteriolar lymphoid sheaths (PALS) because cuffs of T cells surround small arterioles. B cells reside in follicles, which are the spheroid-shaped zones located in the periphery of the PALS. A network of reticular cells, predominantly containing resident IgM^+IgD^- B lymphocytes and marginal zone macrophages surrounds the white pulp, and is called the marginal zone (MZ) [1]. Lymphocytes enter the spleen through the splenic artery that opens in the MZ and B and T cells then migrate to their respective areas in the white pulp. The spleen is also important for the filtration of blood and phagocytosis of the opsonised microbes.

The adaptive immune responses to lymph-borne antigens are initiated in lymph nodes. These are small nodular aggregates of lymphocyte-rich tissue situated along the lymphatic channels throughout the body. A lymph node consists of an outermost cortex, the B cell area, which surrounds the T cell area called the paracortex. The medulla is the innermost area comprising of macrophages and plasma cells along with large blood

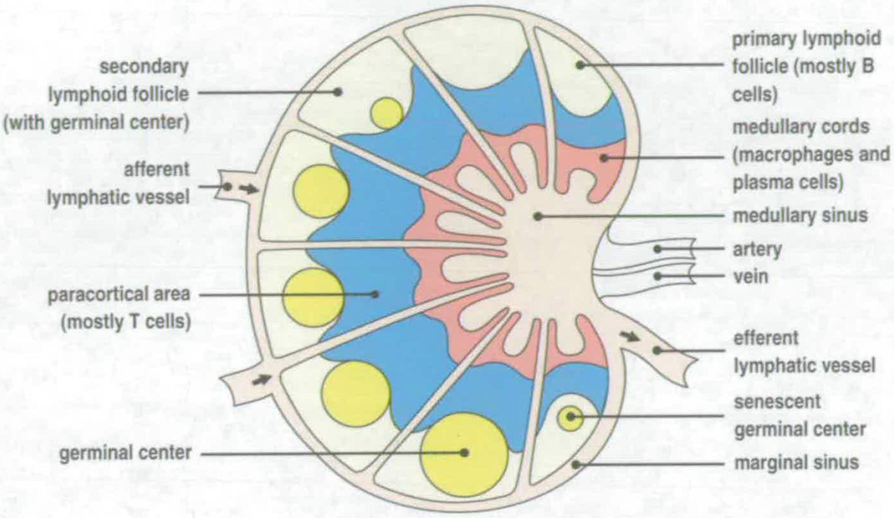


Figure 1.1 Diagrammatic representation of a lymph node. T cells and B cells are segregated into paracortex and follicles, respectively. Adapted from "Immunobiology: the immune system in health and disease", Charles A. Janeway *et. al*, 2005.

vessels (figure 1.1). Entry of the lymphocytes into the lymph nodes occurs through high endothelial venules (HEV). Naïve T and B cells in the lymph use specific receptors (CD62L and CCR7) to recognise ligands on the specialised HEVs (peripheral lymph node addressin (PNAd) and SLC (secondary lymphoid-organ chemokine)) and then pass between these cells to enter the central part of the lymph node [2,3]. Recently, Matloubian *et al.* have found that T cell exit from lymph nodes is also an active process requiring the sphingosine-1-phosphate (S1P) receptor 1 [4]. Thus, naïve T cell migration is a controlled process, delivering T cells to the sites at which they are most likely to meet antigen brought by DCs or through the lymph from the periphery.

1.2. Interaction between T and B cells

The initial priming and activation of naïve T cells occurs on DCs [5]. Within days of the start of an immune response, however, the dominant APC is no longer a DC but a B cell. This happens because DCs have a relatively short lifespan within the lymphnode after entry [6]. On the other hand, the number of B cells that can act as APC increases as the response matures, because antigen-specific B cells undergo clonal expansion. Moreover, owing to their surface antigen receptor, antigen-specific B cells can present the antigen to T cells with very high efficiency [7]. Despite this we know very little about the behaviour of B cells *in vivo* as APC and as programmers of T cell differentiation. Following priming, the B cells first proliferate in T zones, the same place where T cells are stimulated by DCs to divide [8]. Later some of the T cells migrate into the B cell follicles [9]. These two migratory events are possibly an opportunity for the CD4 T cells to provide help to B cells. However, the interactions are cognate and have the potential to deliver late costimuli (e.g. via ICOS, OX40) and/or cytokines to the T cells as well [10,11].

1.2.1. B cell subsets

B-lymphocytes can be divided into B-1 and B-2 cells [12]. B-1 B cells are long-lived self-renewing B cells, which are maintained by multivalent self-antigens [13]. B-1a B cells are of foetal liver origin and are abundant in the peritoneal cavity. They are primarily involved in the generation of T-independent responses to antigens encountered at mucosal sites. Phenotypically, they are identified as IgM^{hi}IgD$^{lo/-}$CD5^{+}Mac-1^{+} B cells and produce natural antibodies. B-1b cells develop from bone marrow derived stem cells and do not express CD5. They are typically self-reactive and need cognate self-antigens to be present for their development [13].

B-2 B cells are the major population of B cells and are further subdivided into follicular (FO) B cells and marginal zone (MZ) B cells. Mature FO B cells located in lymphoid follicles of secondary lymphoid organs are defined as IgD^{hi}IgM^{lo}CD23^{+}CD21^{int} and are relatively short-lived cells with a half-life of around 2-3 months [14]. They contribute to most T cell-dependent GC-based responses. The MZ is a distinct anatomical compartment that in mice is found only in spleen and surrounds the B cell follicles and the periarteriolar T cell areas. The MZ B cells are self-renewing cells with partially activated phenotype IgM^{hi}IgD^{lo}CD23^{-}CD21^{hi} [15]. Due to their specialised location near the marginal sinus and their association with various other cell types including endothelial cells, DCs and macrophages, MZ B cells are designated for exposure to blood-borne antigens [12,15]. MZ B cells are presumed to be critical for TI responses. It is possible that they contribute to TD responses by presenting blood-borne antigens to naïve T cells since they express high levels of CD80 and CD86 [16]. In addition, Pyk2$^{-/-}$ mice lack MZ B cells and also show defective IgM responses to protein antigens [17]. Recently, Notch2 and the Notch ligand Delta-like 1 have been shown to be important for the generation of MZ B cells at the branch point of MZ and FO B cells in the spleen [18,19].

1.2.2. T-independent and T-dependent antibody responses

The production of antibodies by B cells, capable of neutralising and eliminating foreign organisms, is a central role of the immune system. Antibodies can block toxins from certain bacteria or bind them to aid the uptake of this antigen by cells of innate immune system. Immunity to many viruses and parasites requires the presence of specific antibodies: this is particularly well illustrated in μMT mice, which lack B cells, and which readily succumb to infections [20-24]. There are different classes of antibodies that have slightly different functions. Naïve B cells express only IgM and IgD but may switch to IgG, IgA or IgE once activated.

The mechanisms by which the B cells are triggered to produce antibodies depend on the nature of the antigen. Congenitally athymic *Nude* mice, which have no T cells, are unable to mount antibody responses to certain antigens called T-dependent (TD) antigens, but they are able to produce antibodies against other T-independent (TI) antigens. Xid mice, which carry a mutation resulting in deficient B cell maturation, only generate antibodies when immunised with a subset of TI antigens – TI type 1 antigens. The remaining TI antigens are termed TI type 2 antigens.

TI-1 antigens are directly mitogenic to B cells. They are thus able to polyclonally activate large number of B cells to produce antibodies, most of which are not antigen specific. Most TI-1 antigens are derived from components of bacterial cell walls, such as lipopolysaccharide (LPS). TI-2 antigens comprise of repeating polymers, which are able to cross-link large numbers of antigen receptors on the surface of B cells, resulting in production of antigen specific antibodies. The most common forms of TI-2 antigens are bacterial polysaccharides.

TD antigens are much more varied but are all proteins, the most widespread type of antigen encountered by the immune system. They are unable to activate B cells directly, and require the help of T cells. In some cases, for example sheep red blood cells (SRBC), which have a limited ability to cross-link antigen receptors on B cells, soluble molecules produced by T cells are sufficient to allow B cell activation *in vitro*. On the other hand, soluble protein antigens require cell-cell contact between B cells and T cells in order for *in vitro* activation to occur.

The outcome of an antibody response also depends on the type of immunising antigen: TI antigens elicit only low affinity antibodies of the IgM isotype and do not generate lasting immunity. TD antigens, however, are able to generate high affinity antibody of all isotypes (IgM, IgG, IgA, IgE). Furthermore, immunisation results in long-lived immunity; a secondary challenge with the antigen results in a stronger and more rapid immune response.

It is unclear how the antigen-specific B cells acquire antigen in the secondary lymphoid organs. Antigen could either enter through the blood or lymph in soluble form [25,26] or as unprocessed antigen on the surface of APC [27]. Once B cells take up antigen through their BCR, they internalise it and present it in the context of MHC-II on their surface and therefore can act as APC, possibly helping T cell priming. This cognate interaction between B and T cells is crucial for the B cells to receive the CD40 and cytokine receptor signals that are required for their proliferation, antibody secretion and memory cell formation [28].

Duration of the interaction between antigen-specific T cell and APC depends on the number of peptide-MHC-II complexes on the surface of APC. Jenkins and colleagues have speculated that antigen-specific B cells produce even more peptide-MHC-II ligands than the tissue-derived DCs resulting in prolonged interactions between cognate B and T cells [29]. Thus, expression of an antigen-specific surface Ig receptor (BCR) gives a B cell an enormous advantage over other B cells with respect to antigen uptake and presentation [7].

1.2.3. B cell trafficking

Discovery of the chemokines and their receptors in last few years has shed light on the mechanisms of lymphocyte migration. Naïve follicular B cells express low amounts of CCR7, which binds to CCL19 (ELC, EBI-1-ligand chemokine) [30] and CCL21 (SLC, secondary lymphoid tissue chemokine) [31] produced by stromal cells in the T cell area, and high amounts of CXCR5, which binds to CXCL13 produced in the follicles possibly by FDCs [32-34]. This expression pattern allows naïve B cells to reside in follicles. Once the BCR binds the antigen, it transduces signals that change the chemokine receptor expression. The B cells increase the expression of CCR7 and reduce expression of CXCR5, as a result, movement of B cells is restricted to the outer edge of the follicle near the T cell area, referred to as "follicular exclusion" [35,36]. By immunohistochemical studies, the majority of antigen-specific B cells have been shown to interact with antigen-specific T cells at T-B border 48h after antigen injection [37]. This increases the chances of cognate interactions between T and B cells because of random crossing of this area by T cells, which become more numerous as time passes, due to prior cell division.

By uptake of Bromo-deoxy-uridine (BrdU), Toellner *et al.* have shown that B cells start proliferation in the T zones [38]. Following activation, antigen-specific B cells either migrate to the edge of T zones and form foci of proliferation, or form GCs in the centre of the follicles. Progeny of a single activated B cell can give rise both to extrafollicular foci and GCs [39]. Proliferation of B cells within foci continues until 4-8 days after immunisation, by which stage foci no longer contain any T cells. Proliferation then halts, and the B cells differentiate into plasma cells [8,38].

Various molecules play a role in formation of synapse between B and T cells, a critical event for efficient antibody responses. In addition to CD28-CD80/CD86 interactions, ICOS-ICOSL interactions play important role in promoting T-B cell contact (see section 1.4). Cytokine production by T cells clearly influences B cell development, but Schultze *et al.* have reported cytokine production by antigen experienced human tonsillar B cells that may create positive feedback loop to influence helper T cell development [40]. IL-12 and IL-6 produced by B cells in cognate contact with Th1 or Th2 cells, respectively, could maintain or even amplify the ongoing T cell response initiated by DCs *in vivo*. Moreover, B cell activating factor (BAFF), a member of TNF family, expressed on DC and activated T cells, and its counter-receptor BAFFR on activated B cells may also play a role in effector T cell differentiation [41,42].

1.3. Cell mediated immunity

A major focus of this project has been to understand the cellular and molecular requirements for the activation and differentiation of the CD4 T cells. The CD4 T cell response is initiated with the recognition of peptide-MHC-II complexes on the APC by the T cell receptor (TCR). Other interactions, specifically those between costimulatory molecules are required for a productive T cell response. Once the T cell recognises its antigen, these interactions lead to the activation, proliferation and differentiation of the antigen-specific T cells.

1.3.1. T cell priming on DCs

DCs are heterogeneous population comprising of many subsets. The two main subsets being $CD11b^+$ myeloid DCs, which are primarily located in peripheral tissues, and the $CD8^+$ lymphoid DCs, which are found in the T cell areas of spleen and lymph nodes [43]. DCs are possibly the most efficient APCs for the naïve $CD4^+$ T cells [5]. The reason for this remains unclear, although it may be due to high levels of peptide-MHC-II complexes, adhesion and costimulatory molecules on their cell surface. Immature DCs constantly sample 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 [44]. T cells then promote further maturation of the DCs through costimulatory molecules and cytokines. In APCs, the MHC-I and MHC-II molecules are present in different intracellular vesicles resulting in MHC-II molecules being loaded with exogenous antigens in the endocytic pathway and the MHC-I molecules being loaded with endogenous antigens in the endoplasmic reticulum. Recently however, this compartmentalisation in the DC has been shown to be somewhat lenient, giving rise to the phenomenon of cross-presentation [45,46].

It had previously been suggested that antigen-primed DCs from the periphery migrate to the T cell zones of the secondary lymphoid organs where they can present the antigen to naïve antigen-specific T cells. However, by injecting fluorescently labelled soluble antigen, Jenkins and colleagues visualised antigen entering the lymph node via afferent lymph within 15min after injection. The question of whether this antigen 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 peptide-MHC complexes on DCs early after s.c. injection of antigen. This timing is too short to be due to migration of DCs from peripheral tissues, which has been shown to take around 6h [47,48]. Thus, T cells first encounter antigen presented by resident DCs and the migrating DCs arrive later to give additional stimulation [25]. The initial interactions with T cells however are for shorter duration due to small number of peptide-MHC-II complexes displayed by resident DCs. Post-migration DCs from the injection site have been shown to engage T cells for around 24h due to large number of peptide-MHC-II complexes on their surface [29]. In lymph nodes, migrating DCs home to the HEV region after capturing antigen from peripheral tissues. Since T cells also enter the lymph nodes via HEV, this enhances the ability of DCs to scan T cells [49].

Understanding of the initial interaction between T and APC has been enhanced greatly by the recent advent of 2-photon laser scanning microscopy. It allows 3-dimensional visualisation of the cells within intact lymph node and has proved invaluable to our understanding of cellular interactions and migration. DCs scan at least 500 different T cells per hour in the absence of antigen. Antigen bearing DCs are highly efficient in recruiting peptide-specific T cells and can engage more than ten T cells simultaneously [50,51]. Several studies have found that the initial interaction between antigen-specific T cells and DC is transient but that more stable interactions, lasting for hours rather than minutes, take place between 24-48 hours after antigen recognition [52,53]. These stable interactions may enable the formation of an immunological synapse, a tight interaction between two cells that may be required for T cell activation and for the directed release of soluble molecules [54]. Although all naïve T cells are thought to be equally able to differentiate into a spectrum of effector cells, the quality of the TCR-peptide/MHC interaction can influence the functional commitment of T cells.

1.3.2. Costimulation of T cells

Apart from signalling through the TCR as a result of recognition of peptide/MHC on APCs (signal 1), additional signals in form of costimulation (signal 2) are required for effective T cell activation. The interplay between costimulatory pathways is only partially understood. In the absence of costimulation, T cells can become anergic or undergo apoptosis [55]. Costimulatory molecules can be divided into two main groups: immunoglobulin superfamily (CD28, ICOS, PD-1) and tumour-necrosis factor receptor (TNFR) superfamily (CD40, OX40, 4-1BB), although it has been shown that members of both families can engage signalling intermediates common to each other and TCR. The expression of the costimulatory molecules is varied with some molecules being expressed on a few cell types while others widely expressed. Furthermore, some molecules are constitutively expressed e.g. CD28, ICOSL, whereas others are induced upon activation e.g. ICOS, OX40L.

The best-characterised costimulatory pathway involves CD28, expressed by all naïve T cells, which binds to CD80 and CD86 upregulated on activated APCs [56,57]. CD28 can enhance T cell proliferation [58], transcription of IL-2 [59] and expression of anti-apoptotic proteins such as Bcl-xL [60]. Programmed death-1 (PD-1) and CTLA-4, also belonging to Ig superfamily, mediate an inhibitory signal to T cells [61]. ICOS, expressed on T cells following activation, binds to ICOSL on B cells and other APCs. It has been shown to be critical for mature B cell responses (detailed in section 1.4).

CD40 is a member of TNFR family of proteins and is expressed on APCs such as DCs, B cells and macrophages. Since only activated T cells express CD40L, this interaction may play a role in sustaining or enhancing T cell responses rather than initiating them. CD40 is found at low levels on resting APC, and is upregulated after activation [62,63] whereas CD40L is transiently induced on naïve T cells after TCR stimulation [64]. Splenic CD4 T cells express CD40L 4h after *in vitro* anti-CD3 stimulation, which peaks between 6-8h, and then returns to resting levels between 24-48h [64]. CD40L binding to CD40 results in oligomerization of CD40 molecules and this induces the association of cytoplasmic proteins called TNF receptor-associated factors (TRAFs) to the cytoplasmic domain of CD40. This recruitment initiates the enzyme cascades leading to activation and nuclear translocation of transcription factors, including NFκB and AP-1. Hence, CD40-CD40L interaction primarily signals to the APC and sustained activation of NFκB transcription factor regulates the DC differentiation and cytokine production [65]. CD40 signalling also enhances DC survival through upregulation of the survival molecule Bcl-2 [66]. Engagement of CD40 leads to enhanced expression of CD80 and CD86 on B cells, causing more T cell activation. CD40 ligation on DC is a crucial trigger for IL-12 release and priming for a Th1 response [67], and CD40-CD40L interaction synergises with IL-12 in selectively enhancing IFNγ production by T cells [68]. In the absence of IL-12 release, a default Th2 response is observed [69].

CD40-CD40L costimulation is critical for B cell help by T cells and in its absence B cells cannot undergo isotype class switching, somatic hypermutation, germinal centre formation and memory development [70,71]. Early reports suggested that CD40L$^{-/-}$ T cells fail to prime *in vivo* [72], but more recently, it was shown that CD40L$^{-/-}$ T cells become activated, but are unable to sustain a response *in vivo*, and are defective in Th1 development *in vitro* [73]. CD40-ligation delays clonal deletion of antigen-specific T cells and enhanced T cell clonal expansion in response to super-antigen [74]. Recent reports suggest the expression of CD40 by activated T cells and CD40L by B cells indicating the complexity of CD40-CD40L interaction between B and T cells [75-77].

The OX40-OX40L interaction is crucial for T cell activation and survival. OX40 expression by T cells is induced by TCR stimulation and peaks around 48 hours following antigen recognition [78]. Similarly, OX40L expression by APCs is inducible following CD40 or LPS stimulation, and peaks 2-3 days after *in vitro* stimulation [79-82]. Studies using mice deficient in either OX40 or its ligand suggest the importance of this interaction in T cell responses including priming, migration, differentiation, B cell help and memory cell development [78,80,82-85]. Furthermore, OX40-OX40L interaction maintains high levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL and suppresses apoptosis by preventing caspase activation in the absence of cell division [55].

The initial stage of antigen-specific T cell development is highly influenced by DC expression pattern of cytokines (IL-12, IL-6) and costimulatory molecules (CD80, CD86). The antigen-primed T cells can then deliver signals to the DC via cell contact (CD40L) and perhaps early cytokine production (TNFα). This is followed by IL-2 dependent proliferation of the antigen-specific T cells, a process known as clonal expansion. Also associated with this phase is the migration of antigen-specific T cells towards the border of T-B areas *in vivo* to initiate the critical synaptic interaction between antigen-primed T cells and the antigen-primed B cells [37].

1.4. Inducible costimulator (ICOS)

Studying the role played by ICOS in immune response was a major focus of this project, therefore has been introduced in detail in this section. ICOS (also known as H4 and AILIM) is the latest member of the family comprised of CD28 and CTLA-4. It is a glycosylated disulphide linked homodimer expressed on the cell surface of activated T cells. The ICOS gene maps to the CD28/CTLA-4 locus on chromosome-1, suggesting that they arose from a common ancestral gene [86]. Its ligand, ICOSL, also called B7h [87], LICOS [88], B7RP-1 [89], B7H-2 [90] and GL-50 [91], is expressed constitutively on B cells, splenic and peritoneal macrophages [89] and DCs [92] and its expression is upregulated by TNFα and LPS [87]. ICOS-ICOSL is a distinct receptor-ligand pair as interaction between them is specific and separate from that between CD28 and CD80/CD86 [89].

ICOS has an important role in enhancing T cell-dependent B cell help. Transgenic mice producing a secreted form of ICOSL-Ig develop lymphoid hyperplasia, plasmacytosis and high serum levels of IgG [89]. ICOS$^{-/-}$ mice exhibit profound deficits in Ig isotype class switching and germinal centre formation after immunisation [93-96]. However, McAdam *et al.* could restore class switching to IgG$_1$ and IgG$_{2a}$ in ICOS$^{-/-}$ mice by giving stimulatory anti-CD40 antibody [93]. This suggests that ICOS may promote the collaboration between T and B cell through the CD40-CD40L pathway. Using a B and T cell double adoptive transfer system, Smith *et al.* have demonstrated that T cells depend on ICOS-ICOSL interactions to clonally expand as well as to support B cell clonal expansion [97]. The authors transferred OVA-specific DO11.10 T cells along with HEL-specific MD4 B cells into recipient mice in the presence or absence of non-depleting anti-mouse ICOS mAb. After immunisation with OVA-HEL, the treated mice had half the number of KJ1.26$^+$ T cells and 3 fold less antigen-specific B cells in comparison to untreated group.

In contrast to CD28, ICOS is not expressed constitutively on naïve T cells. After activation, it is detected at 12h, peaks at 48h and then gradually declines. ICOS expression is influenced by both TCR and CD28 signals [86,98-100]. CD28 enhances ICOS expression, and ICOS upregulation is markedly reduced in the absence of CD80 and CD86 [100,101] indicating that some of the functions ascribed to CD28 might be due in part to ICOS signalling. However, ICOS expression is not entirely dependent on

CD28 signals, and T cell responses have been modulated in CD28$^{-/-}$ mice with ICOS-Ig fusion protein [102]. Furthermore, Suh *et al.* generated double knockout mice lacking CD28 as well as ICOS and reported that partially impaired humoral responses by CD28$^{-/-}$ mice were abrogated when ICOS is eliminated as well [103]. ICOS is upregulated on both Th1 and Th2 cells during the initial phase of differentiation, but levels remain high on Th2 and decrease on Th1 cells [99,100]. The expression of ICOS on T cells in the GCs and surrounding T cell zones [86,98] corroborates its role in T cell help for B cells.

1.4.1. Role of ICOS in T cell proliferation and differentiation

A role for ICOS in T cell proliferation has not been clearly assigned. Early work showed that cross-linking of ICOS on human or mouse T cells *in vitro* can stimulate T cell proliferation in the presence of suboptimal concentrations of anti-CD3 antibody [86,89,99,100,104]. But *in vitro* blocking studies using ICOS-Ig fusion protein suggested suppression [105] or no effect at all [100] on the proliferation of naïve T cells in response to peptide. Studies with ICOS$^{-/-}$ mice have shown only modest defects in T cell proliferation [93,94].

Although ICOS was identified initially from a subtractive library from activated Th2 clone [99] and was considered an important costimulatory molecule for Th2 responses, enough evidence is now available to suggest its critical role in Th1 responses (Table 1.1).

1.4.2. ICOS in Th1 and Th2 effector responses

Until recently, the prevailing hypothesis was that ICOS signalling is important in Th2 response. This has been confirmed to some extent by *in vivo* findings. Mak *et al.* describe reduced allergic asthma response, a classic Th2 disease, in ICOSL$^{-/-}$ mice [106]. The authors suggest this resulted from the reduced production of Th2 cytokines such as IL-4 and IL-13. Similarly, ICOS blockade by ICOS-Ig has been shown to ameliorate development of airway hyper-reactivity in a Th2 dependent model of allergic airway

Table 1.1 Role of ICOS-ICOSL interactions in cytokine responses

Tools used	Immune response examined	Results	References
BALB/c, ICOS-Ig fusion protein	Bacterial superantigen *Staphylococcus aureus* enterotoxin B (SEB)	Reduced TNFα and IFNγ No effect on IL-2 and IL-4	[105]
DO11.10 mice, ICOS-Ig fusion protein	OVA$_{323\text{-}339}$+ Mitomycin-C treated splenocytes	Reduced IL-2, IL-4, IL-5 and IFNγ from activated CD4 Cells. Reduced IL-5 and IL-13 from effector Th2 cells, no effect on IFNγ from Th1 cells	[99]
Human peripheral CD4 T cells, ICOS-Ig fusion protein	SEB	Reduced IFNγ, IL-4, IL-5, IL-13 and IL-10. No effect on IL-2, TNFα and GM-CSF	[109]
BALB/c mice, B7h-Ig fusion protein. DO11.10 CD4 T cells, ICOS-Ig fusion protein in primary culture	Anti-CD3 stimulation Cytokine production upon *in vitro* secondary culture with pOVA	Upregulation of IFNγ, IL-4 and IL-10 but not IL-2. Increased IFNγ and decreased IL-4 and IL-10	[100]
BALB/c, ICOS-Ig fusion protein	*N. brasiliensis* infection, cytokine production upon restimulation	Reduced IL-4, IL-10, IFNγ	[102]
C57Bl/6 mice, B7RP-1-Fc fusion protein	Anti-CD3 stimulation	Induced IFNγ but not IL-2	[89]
Human peripheral CD4 T cells, Anti-ICOS mAb F44	Anti-CD3 stimulation	Upregulation of IL-4, IL-5, IFNγ, TNFα, GM-CSF and IL-10	[104]
ICOS$^{-/-}$ mice	TNP-KLH-CFA, cytokine production upon *in vitro* restimulation with KLH	Similar levels of IL-2 and increased IFNγ production	[93]
ICOS$^{-/-}$ on 129/Ola x C57Bl/6 background	KLH-IFA, frequency of cytokine producing CD4 T cells upon *in vitro* restimulation with KLH	Low IL-4$^+$ and IL-10$^+$ cells High IFNγ$^+$ CD4 cells	[103]
ICOS$^{-/-}$ on C57Bl/6 background	KLH-CFA, cytokine production upon *in vitro* restimulation with KLH	Reduced levels of IL-4 but normal IFNγ in culture supernatants. Reduced frequency of IL-4$^+$ but normal IFNγ$^+$ cells upon ICCS	[94]
ICOS$^{-/-}$ F6 on C57Bl/6 background	Anti-CD3 stimulation. Real-Time PCR for cytokine mRNA	Reduced IL-4, normal IFNγ	[110]
ICOS$^{-/-}$ F2 129 x C57Bl/6 background	Anti-CD3 stimulation	Reduced IL-2 and IL-4. No effect on IL-10 and IFNγ	[95]
BALB/c naïve mice	Correlation between ICOS cell surface density and cytokine secreted	ICOS^{low} CD4 cells make TNFα, IL-2, IFNγ. ICOS^{intermediate} cells secrete IL-4, IL-5 and IL-13. ICOS^{high} cells secrete IL-10	[111]
ICOSL$^{-/-}$ F2-F3 129 x C57Bl/6 background	KLH-CFA, cytokine production upon *in vitro* restimulation with KLH	Reduced IL-4, IL-13 and IL-10 but normal IFNγ in culture supernatants. Reduced frequency of IL-4$^+$, IL-10$^+$ but normal IFNγ$^+$ cells upon ICCS	[106]

disease [99,107,108], although ICOS$^{-/-}$ mice exhibited infiltration of eosinophils and lymphocytes in bronchio-alveolar lavage fluid comparable to wildtype mice as observed by Dong *et al.* [95]. Furthermore, Miyahira *et al.* found that ICOS$^{-/-}$ mice on the BALB/c background were less susceptible to Leishmania. Normally, BALB/c mice mount a Th2 response to this parasite, preventing the protective Th1 response from acting. The ICOS$^{-/-}$ mice made a reduced Th2 response thus allowing the Th1 response to dominate [112]. Blocking ICOS has also been shown to prevent the development of Th2 mediated chronic GVHD by decreasing both IL-4 and antibody production [113].

ICOS also regulates Th1 effector responses. In a Th1-mediated cardiac allograft rejection model, ICOS blockade prolonged acute cardiac allograft survival [114]. ICOS has been reported to play a critical role in the protective Th1 response required against IFNγ-dependent diseases like Toxoplasmosis [115] and *Leishmania mexicana* [116].

1.4.3. ICOS in CD8$^+$ T cell responses

ICOSL costimulation of CD8$^+$ T cells enhances IL-2 and IFNγ production predominantly in recall responses compared with naïve responses [117]. Also, ICOSL expression in an immunogenic, MHC class-I$^+$ tumour led to enhanced tumour rejection in mice [118]. On the contrary, Kopf *et al.* have reported that ICOSL does not enhance CTL effector function, nor does inhibition of ICOS pathway impair CTL responses to LCMV or VSV infections in mice [102]. Thus, the role of ICOS in CD8 T cell responses is somewhat confused and awaits clarification.

1.4.4. ICOS and immune regulation

Immune responses to foreign antigens decline with time after immunisation. This is mainly because of apoptosis of activated lymphocytes that are deprived of survival stimuli as the antigen is eliminated and innate immunity wanes. Various mechanisms of lymphocyte inhibition function to terminate the immune response, regulatory T cells

(T_{regs}) being one of them. Regulatory T cells can inhibit immune responses by secreting immunosuppressive cytokines such as IL-10 and TGF-β.

Of late, ICOS has been assigned a regulatory role in the immune response. Lohning *et al.* observed a tight link between high cell surface ICOS expression and IL-10 production by T cells [111]. Besides, the role of ICOS in T-regulatory function has been reported in various models. Herman *et al.* have recently described that $CD4^{+}CD25^{+}CD69^{-}$ T_{reg} cells in pancreatic tissue of BDC2.5/NOD TCR transgenic mice (diabetogenic, β-cell specific) express high levels of IL-10 and ICOS alongwith GITR [119]. Blockade of ICOS disrupted the balance between T_{reg} and T effector cells converting insulitis to diabetes. Furthermore, Kohyama *et al.* demonstrated the development of hyporesponsive T_{reg} cells producing IL-10 in DO11.10 mice expressing high levels of pOVA in pancreatic β-islet cells as self-antigen. Since blockade of ICOS signalling by B7h-specific mAb reduced the IL-10 production as well as the *in vitro* suppressive function of T_{reg} cells, the authors suggested a critical role of ICOS in maintaining self-tolerance by IL-10 producing T_{reg} cells [120]. Similarly in a human study, ICOS was induced on a non-proliferating subset of T cells upon stimulation with CD40/80/86 deficient APCs in MLR with PBMCs. This subset when added to secondary MLRs did not secrete IFNγ, IL-5 and IL-13, but produced IL-10 and suppressed the proliferative response when added to secondary MLRs [121].

ICOS-ICOSL pathway has been demonstrated by Akbari *et al.* to be critically important in immune regulation and respiratory tolerance in asthma [92]. The authors showed that ICOS costimulates the induction of T_{reg} cells that inhibit the function of antigen specific T cells via IL-10 and thereby inhibit the development of airway hyper-reactivity (AHR). Interestingly, this costimulation pathway is involved in both Th2 driven inflammation and T_{reg} cell mediated tolerance in AHR. This might be due to production of relatively different amounts of IL-10 and IL-4 in these responses. Undetectable amounts of IL-13 production by T_{reg} cells compared to large quantities secreted by Th2 cells was suggested for the distinct function of T_{reg} and Th2 cells.

1.4.5. ICOS and autoimmune diseases

The severity of the Th1-mediated disease, experimental autoimmune encephalomyelitis (EAE, an animal model for multiple sclerosis) is exacerbated in $ICOS^{-/-}$ mice and in wildtype mice when ICOS is blocked during priming which correlates to decreased IL-4 and increased IFNγ production [122-124]. Such Th1 polarisation of the response might be due to blockade of ICOS-mediated costimulation of Th2 cells and hence reduced IL-4, IL-13 and/or IL-10 production. However, ICOS blockade after EAE induction inhibits the progression of the disease, indicating that ICOS has a key role in sustaining effector Th1 cells [123,124].

In a murine model of collagen-induced arthritis (CIA), administration of neutralising anti-B7h mAb significantly ameliorated inflammatory arthritis by inhibiting T cell proliferative response to type-II collagen and production of IL-10 and IFNγ. Even a delayed treatment after the onset of arthritis is also effective in preventing the disease progression [125]. $ICOS^{-/-}$ mice have been reported to be completely resistant to CIA along with reduced anti-collagen IgM and IgG_{2a} responses [122,126]. ICOS-ICOSL interactions are also required for the T helper cell-mediated humoral immune response to acetylcholine receptor leading to experimental autoimmune myasthenia gravis, as $ICOS^{-/-}$ mice do not develop this disease [127].

1.5. Delivery of T cell help to B cells

T cell help for B cells is provided by two mechanisms: delivery of contact-dependent signals, and secretion of cytokines.

Early studies showed that *in vitro* T cell help for B cell anti-SRBC antibody production could be replaced by supernatant from activated T cell cultures. Likewise, purified B cells could be induced to proliferate *in vitro* in response to anti-Ig if T cell-derived supernatant is added to the culture [128,129]. However, the same effect was not seen in the B cell response to soluble protein antigens. In this case, B cell-T cell contact must occur for delivery of help. Fixed resting T cells cannot provide help to B cells, but T cells preactivated before fixation can. Furthermore, the help is not given if RNA or protein synthesis is blocked during T cell activation [130]. Hence, contact-mediated T cell help requires *de novo* gene expression.

1.5.1. Contact-dependent T cell help

CD40-CD40L interactions are crucial for the delivery of contact-dependent help by T cells. Antibodies against CD40L or soluble forms of CD40 which bind to CD40L, block contact-mediated T cell help *in vitro* [131-133]. Also, mice that lack CD40 or CD40L exhibit severely impaired TD antibody responses: they produce no isotype-switched antibodies, and they cannot form GCs [70,71]. However, they are able to produce IgM antibodies to TD antigens. Moreover, human patients with hyper-IgM immunodeficiency, in whom CD40L expression is deficient, usually have normal or elevated IgM levels [134] indicating the existence of a CD40-independent pathway of T cell help. Although the absence of IgG, IgA and IgE antibodies in CD40 and CD40L-deficient mice suggest the importance of these interactions for class switching, signalling via CD40 is insufficient to cause B cells to isotype switch *in vitro*. Instead, the principal function of CD40-ligation appears to be to induce responsiveness to T cell-derived cytokines, which then direct class switching and further differentiation.

How does the immune system avoid activating 'bystander' B cells via CD40L expressed on activated T cells? B cells that receive a signal through CD40 are induced to express Fas (CD95). This surface receptor transmits a death signal to the cell when it is engaged by its counter-receptor, Fas ligand (FasL) expressed on activated T cells [135]. However, this death signal is blocked in B cells stimulated by their surface antigen receptor [136]. In this way, only antigen-specific B cells are able to benefit from T cell help through CD40; B cells that receive the CD40 signal but which do not have bound antigen are susceptible to apoptosis. Also, the upregulation of CD40L is transient and is only directed towards the synapse with the interacting B cell.

Another receptor-ligand known to deliver differentiative signals to B cells is OX40-OX40L. Cross-linking of OX40L on *in vitro* activated B cells has been shown to enhance proliferation as well as secretion of antibodies [137]. Stuber and Strober showed that *in vivo* blockade of OX40-OX40L interactions by injection of anti-OX40 antibodies abolished the T-dependent IgG production and prevented the formation of extrafollicular B cell foci in the spleen [85]. Furthermore, since this blockade did not affect the cytokine producing T cells, physical contact between B and T cells via OX40L-OX40 seems critical for the delivery of help.

1.5.2. T cell cytokine production

As discussed earlier, one of the earliest cytokines produced by T cells after activation is IL-2, which is required for their subsequent proliferation. A number of other cytokines are also produced by helper T cells, which are able to promote B cell differentiation.

Mosmann *et al.* proposed the concept of helper T cell subsets nearly two decades ago [138]. They examined a panel of antigen-specific mouse T cell clones and found that T cells could be split into two categories according to the cytokines they secreted: T helper type 1 (Th1) and T helper type 2 (Th2) effector subsets. These two types of cells produce distinct profiles of cytokines and regulate different immune responses [139]. Th1 cells produce proinflammatory cytokines such as IFNγ and LTα, and mediate cellular immunity by activating macrophages and CD8 T cells; Th2 cells, in contrast, produce cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) that regulate B cell activation and plasma cell differentiation [140]. One of the principal effects of cytokines on B cells *in vitro* is to direct isotype switching and secretion of high levels of immunoglobulins. For example, Th2 cytokines especially IL-4 mediates switching to and secretion of IgG_1 and IgE isotypes, but this is counteracted by IFNγ (Th1), which enhances production of

IgG_{2a} instead [139,141]. Thus, both Th1 and Th2 cells are able to provide help to B cells, although the nature of this help differs. In IL-4 deficient mice, production of IgG_1 is severely impaired and IgE is totally absent [142,143].

In terms of the control of parasites, Th1 immune responses protect against intracellular protozoa e.g. *Toxoplasma, Leishmania,* whereas Th2 immunity is associated with protection against intestinal helminths. Both subsets have been implicated in pathological responses as well. Inappropriate Th1 responses can result in autoimmune organ destruction, whereas atopic reactions, including asthma and allergy, are associated with unwarranted Th2 responses.

Th1 and Th2 populations are mutually antagonistic: IFNγ, produced by Th1 cells, inhibits the growth of Th2 cells, however, production of IFNγ is blocked by the Th2 cytokine IL-4. In primary *in vitro* T cell stimulations, the presence of IFNγ assists in Th1 development, and IL-4 promotes development of Th2 responses. It therefore appears that cytokine production early in the T cell response dictates the subsequent direction of T cell effector differentiation.

There are many signals responsible for this differentiation process namely cytokines, costimulatory molecules, antigen dose and certain genetic modifiers, but it is still unknown how these signals are integrated [140,144]. DC-derived IL-12 induces Th1 development via transcription factor Stat4, while IL-4 causes Th2 induction through Stat6 [140]. It is not yet clear where the initial IL-4 is produced. Two other transcription factors expressed selectively in Th2 cells are GATA-3 and c-maf, which primarily regulate IL-4 gene expression [145,146]. T-bet, a T-box factor, is a Th1 restricted transcription factor and is expressed by Th1 clones and differentiating T cells [147]. T-bet and GATA-3 operate intrinsically to suppress opposing cytokines. GATA-3 is extinguished in Th1 cells by IL-12/Stat4-mediated signalling; conversely, T-bet is extinguished in Th2 cells by IL-4/Stat6-mediated signalling. With the loss of one of these master regulators, the capacity for downstream cytokine gene expression is lost, and the T cell terminally differentiates in response to the remaining regulator [148].

Among different costimulatory signals involved in T helper cell differentiation, CD28 signal appears important for Th2 differentiation *in vitro* and *in vivo* by augmenting the expression of GATA-3 [149,150]. Furthermore, Th2 cytokine expression and differentiation requires more cell division and an increased threshold of activation [151]. ICOS, expressed on activated T cells, depends largely on CD28 for its induction [103]. Although ICOS has been largely implicated in Th2 induction [99,107,108], recently it has been shown to be important for Th1 function as well [114-116,152]. Similarly, OX40 has also been shown to be involved in the development of both Th1 and Th2 responses [82,153-156].

Antigen dose, as shown by Hosken *et al.*, can also determine the T cell differentiation *in vitro* [157]. Using naïve T cells from TCR-transgenic mice, they found that stimulation on APCs pulsed with high concentrations of specific peptide favoured generation of IL-4, while low concentrations generally favoured IFNγ production.

1.5.3. T_{FH}: T cell subset specialised to help B cells

T cell help for antibody production first occurs in T cell areas and later in B cell follicles. T cell help to B cells had been thought to be solely attributable to Th2 cells [141,158,159], first, because IL-4, a Th2 cytokine, stimulates B cell proliferation and class switching and induces upregulation of costimulatory molecules such as CD40; and secondly, Th2 cells may preferentially migrate into B cells follicles to provide help to B cells [160]. However, B cell help still occurs in the absence of IL-4, because IL-4- and Stat6-deficient mice still make antibodies [161,162]. Moreover, Smith *et al.* have recently demonstrated that *in vivo* generated Th1 cells also enter follicles and provide help to B cells [163]. This is not surprising given the production of high affinity IgG_{2a} antibodies.

Recently, another subset of T cells "Follicular B-helper T cells" (T_{FH}) has been identified through expression of the chemokine receptor CXCR5 [33,164,165]. CXCR5 is also expressed by B cells, and is required for the development of B cell follicles in secondary lymphoid tissues. Mice lacking CXCR5 or its ligand CXCL13 (B lymphocyte chemoattractant) display major aberrations in splenic follicular architecture and reduced numbers of lymph nodes and Peyer's patches [166,167]. The expression of CXCR5 by T_{FH} and B cells allows the co-localisation of these cells to sites of CXCL13 production (i.e. B cell follicles), thus enabling productive T-B cell interactions. T_{FH} cells are nonpolarized with respect to Th1/Th2 cytokine production. Despite the importance of T_{FH} cells, relatively little is known about how they are generated and how they mediate their function.

Expression of CXCR5 by T cells does not necessarily define T cells capable of providing B cell help. Kim *et al.* have shown in a human study that only a small subset of CXCR5$^+$ T cells that co-expressed CD57 and localised to GCs was capable of effector function [33]. However, using Affymetrix microarrays, Chtanova *et al.* did not observe any relevant difference in the pattern of gene expression in the CXCR5$^+$CD57$^+$ and CXCR5$^+$CD57$^-$ subsets, suggesting the need for other markers to define the effector Th subset within CXCR5$^+$ T cells [159].

The genes differentially expressed by T_{FH} cells were CD84 and IL-21 [159]. The preferential expression of CD84, a member of CD2 subgroup of Ig-superfamily receptors, on B and T_{FH} cells indicated its importance in facilitating T cell help for B cells. CD84 signals through SAP, an Src homology domain 2 (SH2) protein that has been shown to be critical for long-term humoral immunity [168]. SAP$^{-/-}$ mice generate strong acute IgG antibody response in response to LCMV despite 10-fold less GCs, and lack memory B cells and long lived plasma cells. Interestingly, by adoptive transfer studies, expression of SAP by CD4$^+$ T cells was found to be essential for T cells to provide B cell help and establish long-term humoral immunity. Presumably, signalling through SAP-associating receptors expressed by T cells leads to expression of cytokines and cell surface molecules important for effector function of T_{FH}.

The cytokine IL-21 is produced by activated CD4 T cells and its receptor, expressed by T, B and NK cells, shares the common γ-chain with IL-2, IL-4, IL-7, IL-9 and IL-15. Although IL-21 promotes Th1 responses by enhancing IFNγ production [169], Ozaki *et al.* have demonstrated markedly diminished levels of IgG_1 in IL-21R$^{-/-}$ mice [170] and increased surface IgG_1^+ B cells in the spleen of IL-21 transgenic mice [171]. IL-21 cooperates with IL-4 in regulating the B cell function *in vivo* since mice lacking both IL-21R and IL-4 exhibit dysgammaglobulinemia and severely impaired IgG and IgE responses [170]. Increased number of post-switch B cells and induction of plasma cell differentiation by IL-21 substantiates its expression on T_{FH} cells [171].

1.6. GC reaction

The overall effectiveness of humoral immunity against pathogen challenges depends not only on the amount of antibody synthesized, but also on its affinity. It is the dynamic microenvironment of GC that caters to this need of the immune response. GCs are formed in response to protein antigens, and this process is dependent on T cell help. GCs do not form in B cell responses to TI antigens, and *nude* mice (which lack T cells) and MHC class-II deficient mice (which lack CD4 T cells) do not form GCs [172]. The GC is a specialised microenvironment where B cells undergo proliferation, maturation and selection. Cells arising from GC form either long-lived plasma cells or memory cells.

The majority of the activated B cells become short-lived IgM secreting plasma cells and may switch to other isotypes depending on the quality of interaction between B and T cells. Some of the expanded B and T cells migrate into the follicles where they rapidly expand and form the GC. Initially GC comprises of rapidly dividing IgD$^-$ B cells, called centroblasts, but 7-10 days after initial priming, dark and light zones become evident. B cells proliferate and diversify their antigen receptors through somatic hypermutation

(SHM) in the GC dark zone [173,174]. Affinity maturation in the GC reaction is achieved through positive selection of centrocytes expressing high affinity variants of their original antigen receptor possibly via immune complexes on FDCs in the light zone [175]. FDCs are thought to play a key role in affinity-based selection of memory B cells in the GCs. They are the immune complex trapping cells and retain the antigen in its native form bound to antibody and complement on their surface. In addition to being a source of antigen long after the infection has cleared, FDC also provide contact-dependent antigen-nonspecific costimulatory signals to B cells for their proliferation and chemotaxis [176]. Although GCs can form without FDC networks, they regress rapidly with reduced affinity maturation [42]. Members of TNF family, including TNFα, LTα (TNFβ) and LTβ ($LT\alpha_1\beta_2$), have been implicated in both the development and maintenance of B cell follicles, FDC clustering and GC formation in mice [177-180]. Once GC B cells exit the cell cycle and become centrocytes, they are programmed to undergo apoptosis unless they recognise the antigen on FDCs.

Class switch recombination (CSR) is another phenomenon supported by GCs in which the rearranged VDJ gene segment recombines with a downstream C (constant) region gene and the intervening DNA is deleted. This results in switching of IgM antibodies to other isotypes namely IgG, IgA and IgE, adding diverse effector functions to Ig with a given antigen specificity. However, Toellner *et al.* have demonstrated that the peak of production of γ1-switch transcripts in the spleen, a marker for actively class switching B cells, happens before GC formation had begun. This suggested that the major site of class switching is in the T zones [38].

Recently, an activated B cell-specific enzyme, activation-induced cytidine deaminase (AID), has been shown to be essential for CSR as well as SHM [181]. AID is a single-stranded DNA deaminase that converts deoxycytidine residues to deoxyuridine on target DNA followed by DNA cleavage by base excision repair enzymes. For CSR, AID is required for DNA cleavage of S (switch) regions of the C_H locus. AID-deficient mice exhibit complete loss of class switching and accumulation of IgM in sera and faeces. Human patients with an autosomal recessive hereditary disease called hyper-IgM syndrome type 2 (HIGM2) have severe defects in class switching, and absence of SHM due to mutations in AID gene [182].

Molecules that are known to support GC development include CD40-CD40L [183], CD28 [184], CD86 [185], CD19 [186] and ICOS [93,94,96]. In support of the importance of TCR-peptide/MHC interactions, nearly complete absence of GC has been reported in the absence of Cathepsin-S, which blocks invariant chain degradation and delays peptide loading into class-II MHC molecules [187]. Various cytokines and chemokines have also been implicated in controlling GC formation. Signalling via the B cell homing chemokine CXCR5 seems to direct GC founder cells into follicles [166,167]. Although CXCR5$^{-/-}$ mice have unimpaired primary antibody production, and contain many PNA^{+} B cells, GCs are lacking (PNA recognises a sialic acid modification upregulated only by GC B cells). Furthermore, CXCR5$^{-/-}$ B cells adoptively transferred into CXCR5^{+} mice were unable to enter follicles, and remained in the T zones [166].

1.7. B cell differentiation into antibody secreting cells (ASC)

T cell cytokines and CD40-mediated signals enhance the transcription of Ig genes and therefore antibody synthesis in response to protein antigens by B cells. Cytokines such as IL-2, IL-4 and IL-6 have been shown to stimulate antibody synthesis and secretion by activated B lymphocytes [188]. The differentiation of B cells from antigen-recognising cells that express membrane Ig receptors for antigens into effector cells that actively secrete antibodies involves a change in Ig expression from the membrane to secreted form, which lacks the hydrophobic amino acids required for anchor in the cell membrane. As differentiation proceeds, more of the Ig mRNA is of the form encoding secreted Ig. Numerous B cell-specific surface proteins are downregulated upon plasma cell differentiation, including MHC-II, B220, CD19, CD21 and CD22. The proteoglycan syndecan-1 (CD138), which recognises extracellular matrix and growth factors, is induced on ASCs and is often used as marker of plasma cells [189].

Within lymphoid organs, ASC are found mainly in extrafollicular sites, such as red pulp of the spleen and the medulla of the lymph nodes. Many of the antibody-secreting B cells change into morphologically distinct plasma cells committed to abundant antibody production. Decreased expression of CXCR5 and CCR7 on ASCs reduces responsiveness to B and T cell zone chemokines CXCL13, CCL19 and CCL21. In contrast, expression of chemokine receptor CXCR4, which recognises CXCL12 present in splenic red pulp, lymph node medullary cords and in bone marrow remains high [190]. These changes mediate the movement of plasma cells from the follicles to other locations, including the bone marrow. At 2-3 weeks after immunisation, bone marrow may be the major site of antibody production. The longevity of the plasma cells however, is unclear, with reports suggesting weeks to even years available [191,192]. Secreted antibodies enter the circulation and mucosal secretions, but antibody-producing cells do not circulate actively.

1.8. Role of B cells in regulation of T cell responses

Interacting B and T cells are involved in a vibrant "conversation" in response to a protein antigen [130]. But, how much influence B cells can have in regulating T cell responses has been a contentious issue. B cells can modulate T cell responses by affecting priming via MHC-II-TCR interactions, provision of costimulation and by secreting antibodies; they can regulate differentiation of T cells by cytokines and other B cell derived factors; and by maintaining the organisation of the secondary lymphoid organs.

1.8.1. T cell Priming

The earliest studies to assess the role of B cells in T cell activation were done in mice rendered B cell-deficient by injecting anti-μ antibodies from birth. T cell priming in these anti-μ suppressed mice (μsm) was defective in response to protein antigens [193-195]. Later, B cell deficient mice were created by disrupting the gene encoding the membrane spanning exon of the μ heavy chain (μMT mice) or by disrupting the JH variable region of the IgM locus (JHD mice) [196,197]. Both types of mice do not have peripheral B cells. Various groups reported conflicting results, varying from complete deficiency to near normal T cell responses in these mice [198-205]. However, differences in genetic backgrounds of mice used, immunisation regimes including antigen dose and route of immunisation, and *in vitro* readouts might account for these results. Although many questions remain, a role of B cells in T cell responses has been generally accepted.

Transfer of wildtype B cells in the μMT mice could restore T cell priming [193,199,203], however, whether these B cells were required to present the antigen to T cells is unclear. Kurt-Jones *et al.* and Rivera *et al.* found that the transferred B cells have to be antigen-specific in order to restore the T cell priming [193,199]. On the other hand, Bradley *et al.* could successfully prime T cells in μMT mice after adoptive transfer of splenic DCs, suggesting an APC deficit rather than a unique role of B cells [203]. Furthermore, in relB$^{-/-}$ mice which are deficient in myeloid and functional lymphoid DCs, CD4 T cells can be primed in response to soluble protein antigen, suggesting that B cells play a role in T cell priming in the absence of DC functions [206].

That B cells can affect T cell responses by providing costimulatory molecules, was demonstrated by Linton *et al.* [82]. They showed restoration of expansion of transgenic CD4 T cells in μMT mice by transferred activated OX40L^{+} B cells and not OX40L$^{-/-}$ B cells. Furthermore, Th2 cytokine response was restored in OX40L$^{-/-}$ mice that received wildtype B cells.

1.8.2. Effector T cell differentiation

Although several studies have identified a link between B cell antigen presentation and the induction of IL-4 secretion by CD4 T cells [207-209], this may not always be required. Epstein *et al.* observed similar levels of IL-4 secretion in μMT and wildtype mice in response to KLH as well as the Th2 inducing soluble egg antigen from *Schistosoma mansoni* (SEA) [205]. A role for B cell-derived OX40L for *in vivo* IL-4 production by $CD4^+$ T cells has been shown by Linton *et al.* [82]. B cells from C57BL/6 mice could reconstitute IL-4 production by OT-II T cells in μMT mice but $OX40L^{-/-}$ B cells could not. Harris *et al.* demonstrated for the first time that B cells are able to regulate polarisation of T cells through differential expression of cytokines [210]. They proposed subsets of B cells, Be1 and Be2, which could support the differentiation of naïve T cells into effector Th1 and Th2 cells, respectively. B cell modulation of immune response through cytokine secretion has been described as context-dependent in humans by Duddy *et al.* [211]. They proposed that B cells when activated appropriately through the BCR and CD40, produce high levels of IL-6, TNFα and lymphotoxin (LT), a combination that promotes GC development and amplifies T cell responses. In contrast, bystander B cells activated through CD40 without antigen-BCR engagement produce negligible amounts of proinflammatory cytokines, but secrete significant levels of IL-10, which would act to suppress the local inflammatory response.

Skok *et al.* have shown that B cells are able to secrete cytokines such as IL-10, IL-6, TGFβ, TNFα and IFNγ. They found that DC-derived IL-12 was required for induction of Th2 response by a negative feedback loop involving IL-10 secreted by B cells [11]. B cell-derived IL-6 can direct the differentiation of Th2 cells by inducing IL-4 transcription [11,212]. These reports indicate that B cells can influence T cell differentiation either directly (IL-6) or by modulating DC function (IL-10).

It has long been realised that delivery of help by T cells require cognate recognition of antigen presented by B cells, however the mechanisms by which this is achieved are unclear. Although initial activation of naïve cells occurs on DCs, it seems unlikely that T cells primed in this way should immediately express all the functions of a competent helper cell, as this would allow help to be given to all the B cells, regardless of the antigen specificity. Instead, there may be further signals which are required for the T cells to mature as helper cells, or which trigger delivery of help. This could be delivered during secondary interactions between recently primed T cells and B cells.

Whether T cells primed in B cell deficient mice can deliver help to B cells for antibody production is also unclear. Several groups have reported provision of little or no help to B cells by primed JHD T cells for antibody production [202,204]. Macaulay *et al.* suggested reduced upregulation of IL-4 synthesis by JHD T cells as the probable reason [204]. In support, Rivera *et al.* observed greatly reduced DTH response in μMT.B6 as well as μMT.BALB/c in comparison to control mice [199]. In contrast, they found that T cells from these mice were equally capable of helping B cells in an *in vivo* adoptive transfer system when primed with a high dose of SRBC. They argued that efficient B cell antigen presentation is restricted to antigen-specific B cells and hence dependence on B cells when low antigen dose is used.

B cells may regulate their own help by directly influencing T_{FH} cell phenotype, proliferation and function. *In vivo* studies in mice demonstrate that effective help for Ig production requires T-B interactions [204,208]. Ebert *et al.* in a human study showed that $CXCR5^+$ T_{FH} cells when cultured with B cells, express CD70, which interacts with CD27 and allows efficient differentiation of the expanded B cells to Ig-secreting plasma cells [134,213,214]. The authors have also shown that these B cells promoted ICOS-dependent IL-10 secretion by T_{FH} allowing their own differentiation to plasma cells [213].

B cells have been shown to play a critical role in the development of respiratory tolerance. Effective induction of T cell unresponsiveness to aeroallergen in BCR-transgenic but not JHD mice, was observed by Tsitoura *et al.* [215]. Moreover, T cell tolerance developed in JHD mice reconstituted with B cells. Fillatreau *et al.* demonstrated a key role of B cells in the recovery from EAE, a Th1-driven autoimmune disease [216]. The authors showed that μMT mice failed to resolve EAE and that IL-10 produced by B cells is required for the recovery from the disease. Also, NOD-μMT mice do not develop diabetes [217].

Impaired CD4 T cell memory in the absence of B cells has been shown by van Essen *et al.* [218]. Intermittent stimulation by persisting antigen has been shown to be necessary for the survival of memory cells, and this may happen in form of immune complexes on the surface of FDCs [219]. Given the mice which lack B cells can form neither immune complexes nor support the development of FDCs, this is not surprising [220,221].

B cells have been implicated in CD8 T cell responses as well, although contradictory literature is available. Christensen *et al.* have shown that $CD8^+$ T cell response to LCMV initially is qualitatively similar in wildtype and $B^{-/-}$ mice but CD8 T cells loose functional capacity in the absence of B cells [198]. Increased contraction of antigen-specific CD8 T cells has been witnessed in B cell deficient mice despite normal activation and antigen-driven expansion following *Listeria monocytogenes* infection [222]. On the contrary, normal CD8 responses have been reported in mice lacking B cells in response to acute infections with LCMV or Influenza virus [222,223].

Last but not the least, B cells have been implicated in maintaining the architecture of secondary lymphoid organs [224]. B cell-derived LTβ has been reported to be critical for splenic microarchitecture but not for lymph nodes and Peyer's patches [177]. Mice with gene deletion for LTβ from B cells had significantly reduced populations of MZ B cells, metallophilic and MZ macrophages, and FDC networks [177]. B cell-derived LT was demonstrated by Gonzalez *et al.* to be playing critical role in FDC maturation and organisation of B cell follicles [178]. The authors showed that FDCs are lacking in SCID mice, but are induced when reconstituted with spleen cells. However, this induction was dependent on LTβ-R signalling by B cells since $LT\alpha^{-/-}$ B cells were incapable of inducing FDC development in SCID mice, even upon $LT\alpha^{+/+}$ T cell co-transfer.

1.9. Tools to study immune responses

1.9.1. T cell help for antibody responses

Much of our knowledge of T-dependent antibody responses comes from the studies of "hapten-carrier" antigens. Haptens are small organic molecules, to which antibodies are able to bind, but which are unable to elicit specific antibody production when used alone to immunise an animal. This inability may be overcome if the hapten is covalently attached to a larger molecule, called the carrier, which is immunogenic in its own right. When immunised with the conjugated hapten-carrier, not only antibodies specific for carrier, but also antibodies specific for the hapten alone are generated [225]. If the carrier is a TD antigen, production of antibodies against the hapten is also T-dependent. Mitchison [226] demonstrated that different cells are specific for the hapten and for the carrier. They immunised mice with a hapten-carrier conjugate, and showed that splenocytes from these mice could produce hapten specific antibodies *in vitro* when stimulated by same hapten-carrier conjugate. In contrast, no antibody production was seen if the same hapten conjugated to another carrier was used for the *in vitro* stimulation. However, antibody production could be restored if splenocytes from a second, syngeneic mouse, immunised with the other carrier, were added at the same time. Thus, carrier-specific cells from one mouse were able to help the hapten-specific B cells from the other.

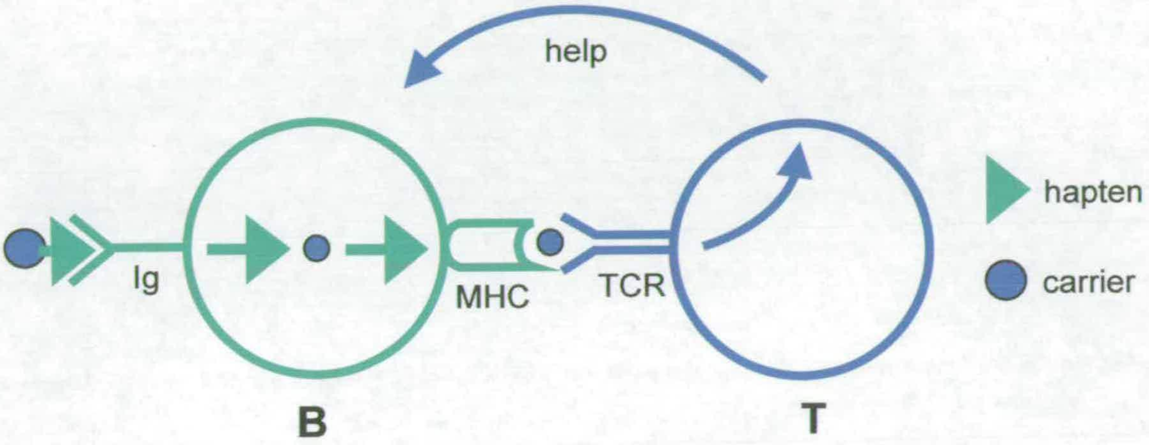


Figure 1.2 Hapten-carrier system. Hapten-specific B cells bind antigen and present the carrier on MHC class-II molecules. Carrier-specific T cells recognise this, and provide help to the B cells.

The identity of the helper cells as T cells was revealed by Raff and co-workers by showing that the carrier-specific help was lost when T cells were depleted *in vitro* using an anti-Thy-1 antibody and complement [227,228]. Furthermore, the ability of T cells to help B cells is restricted by the MHC expression of the B cells [229-231]. Hence, T cells help B cells only when the latter present specific antigen in association with MHC molecules recognised as self by the T cells. The ability of B cells to present antigen to T cells was shown by Chestnut and Grey [232]. They immunised mice with rabbit IgG, and then measured the proliferation of T cells from these mice *in vitro*, using B cells or macrophages to present the antigen. Macrophages were able to stimulate T cells using either normal rabbit γ-globulins or rabbit anti-mouse IgG as antigen, but B cells could elicit T cell responses only when rabbit anti-mouse IgG was used. Thus, B cells can act as APC, only when they are able to bind the antigen with their surface antigen receptor.

Together, these findings provide an elegant explanation of the hapten-carrier effect: B cells specific for the hapten are able to bind it and present the attached carrier to carrier-specific T cells. These T cells then deliver the help required for antibody production (figure 1.2).

1.9.2. Tracking antigen-specific T cells

The frequency of antigen-specific CD4 T cell in a naive individual can be as low as 1 in 100,000 and can go up to 1 in 10,000 after immunisation [233]. Such low frequencies are below the detection limits of the usual detection methods of flow cytometry and immunohistology, and difficult to be assessed accurately by techniques like limiting dilution assay (LDA) which rely on functional capacity of the cells. Moreover, the only way to distinguish between antigen-specific and non-specific T cells is by virtue of their TCRs.

The production of TCR transgenic mice revolutionised the study of antigen-specific T cells. Most of the T cells in such mice are specific for a defined peptide-MHC complex. Clonal competition between the inordinately abundant TCR transgenic T cells for peptide/MHC bearing APCs results in inefficient T cell activation in these mice. An elegant solution to this problem developed by Marc Jenkin's group was to transfer a small cohort of TCR transgenic T cells into syngeneic recipient mice, which was small enough to behave in a physiological manner following immunisation. The antigen-specific transgenic T cells can be tracked by flow cytometry following staining with anti-clonotypic antibody or antibody specific for congenic markers like Ly5 and Thy etc [233]. The DO11.10 transgenic mice have TCR specific for chicken ovalbumin peptide 323-339 bound to I-A^d MHC class-II molecule that can be detected with KJ1.26 mAb. The OT-II transgenic mice are on I-A^b background and also recognise chicken ovalbumin peptide 323-339, but they do not have a clonotypic antibody. Instead, they can be detected by antibodies specific for Vα and Vβ chains of their TCR ($V\alpha_2$, $V\beta_5$).

Another technique that has been recently developed to track antigen-specific CD4 T cells is MHC-II tetramers [234]. This allows detection of antigen-specific T cells in an un-manipulated mouse in more physiological settings. Due to exceedingly small number of antigen-specific cells, however, they have to undergo clonal expansion for detection by tetramers. This limits the use of this technique to track T cells following the initial phase of expansion.

1.10. Aims

Despite the rarity of antigen-specific T and B lymphocytes and the random movement they undergo in the secondary lymphoid organs, amazingly, they are able to find each other [29]. My PhD has focussed on studying the importance of T-B cell interactions not just for the B cells but for the T cell responses as well.

Despite the plethora of costimulatory molecules and the intriguing mass of conflicting knowledge available, there still are questions to answer. In an attempt to address the issue of "costimulation" in T-B interactions, I have tried to answer the following questions:

- Is CD40 dispensable for T cell proliferation?

  (Chapter 3)
- How the ICOS-ICOSL interaction affects the immune response?

  (Chapter 4)
- How can B cells affect the T cell acquisition of helper function?

  (Chapter 5)

This thesis presents some novel insight into the collaboration between various cells of the immune system via costimulatory molecules.

2. Materials and Methods

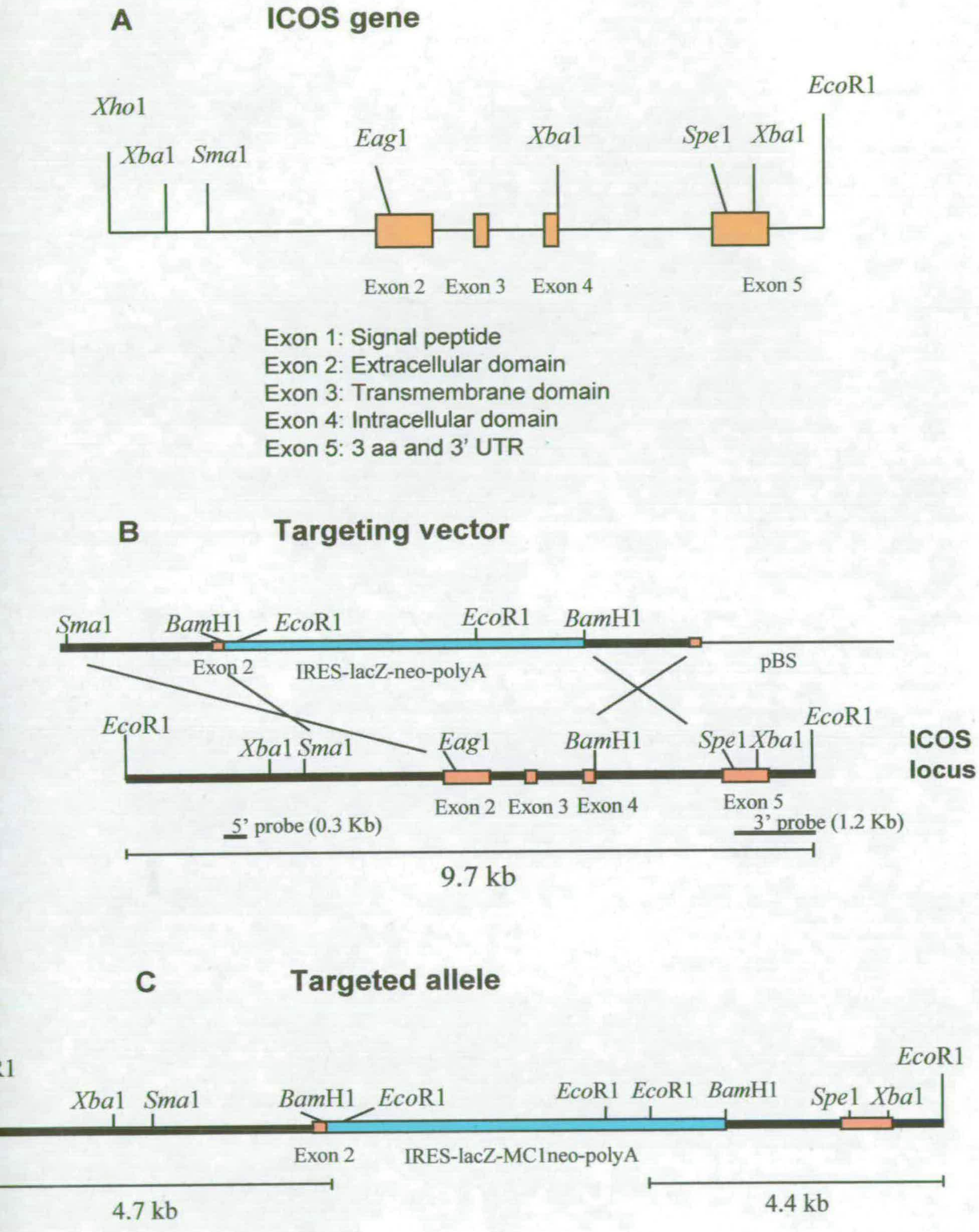
2.1. Animals

All the mice were maintained under specific pathogen free conditions in the animal facilities of the Faculty of Science and Engineering at the University of Edinburgh. Strains of mice used in this study were: BALB/c, C57Bl/6, CD40$^{-/-}$(H-2^{d} & H-2^{b}) [71], μMT [196], DO11.10 [235], DO11.10 CD40$^{-/-}$, OT-II [236], OT-II CD40$^{-/-}$, OT-II Ly5.1$^{+/-}$, (BALB/c x C57Bl/6) F1, OX40L$^{-/-}$ [154], I-A$^{\beta-/-}$ [172], ICOS$^{-/-}$ (section 2.1.1), OT-II ICOS$^{-/-}$.

Mice were used at 6-12 weeks of age, and were sex and age matched within experiments as closely as possible. All strains of mice were bred in house. All experiments were performed under guidance from the appropriate Home Office personal and project licenses.

2.1.1. Generation of ICOS$^{-/-}$ mice

The ICOS$^{-/-}$ mice used in this study were generated by gene targeting in this lab by Dr. Ana Cervera, in collaboration with Dr. Andrew Smith (ICSR, Edinburgh). The targeting vector replaced the exons 3, 4 and parts of exons 2 and 5 of the ICOS locus with the (IRES-lacZ-MC1neo-polyA) neomycin resistance gene (fig 2.1). The germ-line transmission of the ICOS disruption was confirmed by Southern blotting, figure 2.2 (by Dr. Ana Cervera and Stephen Meek). ICOS$^{-/-}$ mice were generated by inter-crossing heterozygous offsprings. Since the knockout mice were generated on 129/Sv background, they were backcrossed onto C57Bl/6 (H2^{b}) background for seven



igure 2.1 Gene targeting for the generation of ICOS$^{-/-}$ mice. The targeting vector replaced the exons 3, 4 and rts of exons 2 and 5 of the ICOS locus with the (IRES-lacZ-MC1neo-polyA) neomycin resistance gene.

generations. Unless otherwise stated, the experiments were done while the backcrossing was being carried out, in which case the control group in experiments were either ICOS$^{+/+}$ or ICOS$^{+/-}$ littermates. It was not feasible to differentiate between the ICOS$^{+/+}$ and ICOS$^{+/-}$ mice due to some problems arising with the optimisation of the Neo PCR used for the screening of these mice, during the period of study. Wherever backcrossed mice (F7) were used in experiments, the control group comprised of C57Bl/6 mice.

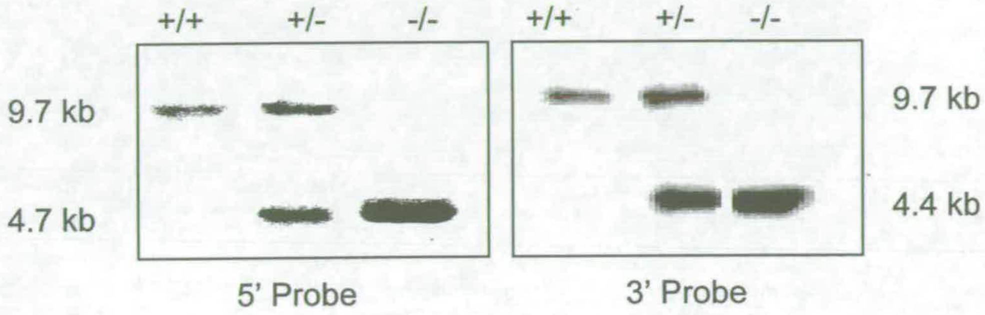


Figure 2.2 Confirmation of ICOS$^{-/-}$ genotype. The germline transmission of the ICOS disruption was confirmed by Southern blotting (by Dr. Ana Cervera and Stephen Meek). ICOS$^{-/-}$ mice were generated by inter-crossing heterozygous offsprings.

2.2. Reagents

2.2.1. Media

Unless otherwise stated, medium was Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 5% foetal calf serum (FCS) (Labtech International, Andover, MA, USA), 2mM L-Glutamine (Gibco), 50μM β-mercaptoethanol (2-ME) (BDH Merck, Poole, UK), 50U/ml Penicillin and 50μg/ml Streptomycin (Gibco).

Tetramer staining was done in IMDM with 10% FCS and supplements as above. During MACS purifications, cells were prepared and washed in Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, Poole, UK) with 2% FCS. Dendritic cells were grown in RPMI 1640 (Sigma) supplemented with 2mM L-Glutamine and antibiotics as above. For acquisition by FACS, cells were stained in FACS buffer (PBS containing 2% FCS).

For injections into mice, cells were resuspended in Dulbecco's PBS (Sigma). For all other purposes, PBS was made in house by dissolving 137mM NaCl, 8.2mM $NaH_2PO_4.2H_2O$, 2.7mM KCl and 1.5mM KH_2PO_4 in Millipore water. PBS was autoclaved to sterilise.

2.2.2. Antigens

Phycoerythrin (PE, Prozyme, CA, USA), Ovalbumin (Sigma), keyhole limpet haemocyanin (KLH, Calbiochem, La Jolla, CA, USA) were used a protein antigens. Ovalbumin and KLH were coupled to dinitrophenyl (DNP) by reacting with 32μl and 14μl, respectively, of dinitrofluorobenzene (DNFB, Sigma) solution (in oil) in 0.1M Borate buffer, pH 8.4, per mg of protein. Conjugated proteins were extensively dialysed against PBS to remove free hapten.

To study clonal expansion of T and B cells, ovalbumin and PE were alum precipitated by mixing equal volumes of antigen and 9% aluminium potassium sulfate dodecahydrate (Sigma). The precipitated antigens were washed extensively after the pH was adjusted to 7.0 using 5M NaOH (Sigma). Ovalbumin was also emulsified in Freund's complete adjuvant (CFA, Sigma). Wherever stated, killed *Bordetella pertussis* (LEE laboratories, Grayson, USA) bacteria were used as adjuvant, 10^9/mouse.

For tetramer experiments, the peptide H19env (EPLTSLTPRCNTAWNRLKL; ABC, Imperial College, London, UK), the epitope of moloney murine leukaemia virus, was emulsified in CFA. Ovalbumin peptide $OVA_{323-339}$ (ISQAVHAAHAEINEAGR; ABC, Imperial College, London, UK) was used in *in vitro* proliferation assays.

Schistosoma mansoni eggs were injected subcutaneously into the footpad of the hindlegs of mice, 1500 eggs/footpad. Soluble egg antigen (SEA) prepared from isolated *Schistosoma mansoni* eggs, was used to restimulate lymph node cells *in vitro*. Heat-killed *Propionebacterium acnes* (Pa), 50µg/footpad, was given s.c. in the hind footpads. *Schistosoma mansoni* eggs, SEA and Pa were all kindly provided by Dr. Andrew S. MacDonald.

2.3. *In vivo* antigen administration

2.3.1. Intraperitoneal immunisations

For help assays, mice were injected either with 200µg alum-precipitated DNP-KLH along with 10^9 killed *Bordetella pertussis* bacteria or 150µg alum-precipitated ovalbumin with 10^9 pertussis bacteria. Studies for clonal expansion of T and B cells involved injections with 200µg ovalbumin-alum and 100µg PE-alum, respectively.

2.3.2. Subcutaneous immunisations

$ICOS^{-/-}$ and $ICOS^{+}$ mice were challenged *in vivo* with Moloney Virus peptide H19env emulsified in CFA for tetramer studies. 1mg/ml peptide was combined with an equal volume of CFA containing heat killed *Mycobacterium tuberculosis* H37RA (Sigma), vortexed and sonicated. 50µl was injected subcutaneously (s.c.) into each hind leg. Ovalbumin 50µg emulsified in CFA was injected s.c. in each hind leg of $ICOS^{-/-}$ and wildtype mice 24h after adoptive transfer of OVA-specific transgenic cells for clonal expansion studies. *Schistosoma mansoni* eggs (1500 eggs/footpad) or *P. acnes* 50µg/footpad were injected s.c. in the hind footpads of ICOS$^{-/-}$ and wildtype mice to study the differentiation of effector CD4 T cells in ICOS$^{-/-}$ mice.

2.4. Cell purification

2.4.1. DC preparation

Splenic DCs by plastic adherence

BALB/c and CD40$^{-/-}$ (H-2^{d}) spleens were removed and a single cell suspension prepared. Cells were resuspended in 10ml medium per spleen and distributed into 60mm, tissue culture grade petridishes (Nalge Nunc International, Roskilde, Denmark) using 5ml cell suspension per plate. These were incubated at 37°C for 90min to enable macrophages and DCs to adhere. The plates were rinsed, using pre-warmed medium and gentle pipetting, until it could be seen that the majority of the non-adherent contaminants had been removed. The plates were given 5ml fresh culture medium, without GM-CSF, and returned to the incubator overnight. This long incubation allowed transiently adherent DCs to detach and they were harvested the following morning by more vigorous washing. Strongly adherent macrophages were left on the plates. Yields at this stage averaged 2-3x10^{6} cells per spleen and were 30-40% CD11c^{+}MHC-II^{+} DCs, as assessed by flow cytometry. The majority of the contaminants were CD19^{+} B cells.

To further enrich the DCs, cells collected after overnight adherence, were depleted of B cells and T cells using Dynabead technology (Dynal, Oslo, Norway). Cells were resuspended at 2x10^{7}/ml and incubated with unconjugated antibodies against CD19 (10µg/ml) and Thy1.1 (5µg/ml) (all from Pharmingen, San Diego, CA, USA) for 30min at 4°C on a rotating mixer. Excess antibody was removed by washing in cold culture medium. Dynabeads were prepared for use by washing to remove the azide preservative and finally resuspended at 1×10^7 beads/ml. Antibody-labelled cells were added to the bead suspension to give 6 beads per labelled cell and 4×10^8 beads/ml, and the mixture incubated, rotating, for 30min at 4°C. Beads were then removed using a strong magnet (Dynal) and the unbound cells collected from the supernatant.

Bone marrow-derived DCs

DCs were grown from early bone marrow progenitors under the influence of GM-CSF, using a protocol based on that of Inaba *et al.* [237]. Bone marrow was flushed from the femurs and tibias of C57Bl/6, BALB/c, $CD40^{-/-}$ (both $H-2^d$ and $H-2^b$) and (C57Bl/6xBALB/c) F1 mice using medium and gentle pressure from a 2.5ml syringe and a 25G needle. Cell clusters were dispersed by passage through a second 25G needle. Red blood cells were removed using lysis buffer (Sigma) and the remaining cells seeded into 24 well tissue culture plates at 3.75×10^5cells/ml and 1ml per well. The culture medium was as normal, but using 10% FCS and without 2-ME. GM-CSF was added in the form of 5% supernatant from the transfected cell line X63-gmcsf [208], providing 10-15ng/ml GM-CSF. Cultures were washed at day 3 and day 6 to remove non-adherent granulocytes and lymphocytes. This involved swirling the plates before removing 1ml from each well and replacing it with fresh medium. On day 7, loosely adherent DCs were harvested by more vigorous washing, leaving firmly adherent macrophages attached to the plate. Preparations were 85-90% $CD11c^+$, $MHC-II^+$ DCs.

2.4.2. $CD4^+$ T cell purification

Positive selection

Cells from spleens and lymph nodes were incubated with L3T4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), 10μl/10^7 cells for 20min on ice, followed by washing in large volume of MACS buffer. Cells were loaded onto LS positive selection column in a quadroMACS magnet (Miltenyi). Cells were routinely >90% CD4$^+$ and yield was typically about 10-15%.

Negative Selection

Cells from spleens and lymph nodes were depleted of B cells, other MHC-II$^+$ cells and CD8$^+$ T cells to purify CD4$^+$ T cells. Following red blood lysis, cell preparations were incubated with biotin-conjugated antibodies against MHC-II (clone M5114; I-A specific; grown in house and used at 1:100), κ-light chain (187.1; 1:100), anti-IgM (Southern Biotechnology Associates, AL, USA) and CD8 (53-6.72; 1:100) for 15 min on ice. The cells were washed and incubated with streptavidin-conjugated microbeads (Miltenyi) 10μl/10^7 cells, for 20min on ice, before being loaded onto a CS MACS column in a varioMACS magnet (Miltenyi). A single column was used for up to 2x10^8 total cells. The column was washed and unbound cells collected. They were routinely >80% CD4$^+$, and yield was typically about 5-10%.

2.4.3. B cells

B cells were purified from the spleens of naïve C57Bl/6 mice for transfer into MHC class-II or CD40-deficient B cell chimeras. This was done by depletion of CD43$^+$ cells from splenocytes. Single cell suspension of splenocytes were treated with red blood cell lysis buffer (Sigma) to get rid of RBCs and after washing twice with MACS buffer, cells were incubated with anti-CD43 microbeads (Miltenyi), 5μl/10^7 cells for 20min on ice. Following a wash in large volume of MACS buffer, unbound B cells were collected using CS depletion columns and varioMACS magnet (Miltenyi). Cells were routinely >90% B220$^+$.

2.4.4. Blood mononuclear cells

To confirm reconstitution of irradiated bone marrow chimaeras (section 2.12), peripheral blood mononuclear cells were harvested and stained for analysis by flow cytometry. 50μl of tail blood was collected into an equal volume of heparin (1000U/ml; CP

Pharmaceuticals, Wrexham, UK) and layered over 500μl Lympholyte^{M} (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada). After centrifugation at 2000rpm for 20min at zero deceleration, mononuclear cells were harvested from the interface and washed before use.

2.5. Adoptive transfer of cells

OT-II (CD40$^{-/-}$, ICOS$^{-/-}$, Ly5.1$^{+/-}$ or wildtype) and DO11.10 (CD40$^{-/-}$ or wildtype) lymph nodes (popliteal, inguinal, brachial, axillary, superficial cervical, periaortic, iliac and mesenteric) were mashed using nytex mesh (Wm Ritchie, London, UK) in IMDM. Cells were then washed in serum free IMDM, a viable cell count performed in 0.05% trypan blue (Sigma) and resuspended in PBS (Sigma). They were then filtered through parachute silk (Wm Ritchie, London, UK) to remove any clumps before being injected i.v. into recipient mice, 4-5x10^{6} cells/mouse in 200μl PBS. The lymph nodes from one donor mouse usually yielded sufficient cells to transfer into 5-6 hosts. Purified CD4 T cells from transgenic mice were adoptively transferred into chimeras during help assays. CD4^{+} T cells were purified as described in 2.4.2. B cells were purified as described in 2.4.3 before transfer into chimeras.

2.6. CFSE labelling

Wherever stated, transgenic T cells were labelled with CFSE (5, 6-carboxyfluoroscein diacetate succinimidyl ester; Molecular Probes, Leiden, The Netherlands) before either transfer into recipient mice or culture *in vitro*. CFSE is a membrane permanent dye that binds covalently to cytoplasmic proteins and is distributed equally to each daughter cell upon division, thus the mean fluorescence halves progressively as the cells divide [238]. Purified T cells were resuspended at 1x10^{7} cells per ml in serum-free HANKS (Sigma) containing 5μM CFSE. After 8min incubation at 37°C, the CFSE was quenched with an equal volume of FCS, and cells washed 2-3 times with serum positive medium before *in* *vitro* cultures. If cells were to be transferred *in vivo*, further washes with PBS were done. Background fluorescence was determined for each sample 16-18h later, using flow cytometry.

2.7. Flow cytometry

Samples were acquired with a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA, USA), using a live cell gate set by forward and side scatter characteristics (see fig 3.13, for example). The analysis was done by FlowJo software (TreeStar, California, USA).

2.7.1. Surface staining

Cells were taken from culture or directly after isolation and placed in a 96-well, V-bottomed plate for staining. 1-2x10^5 cells were used routinely to collect 10 000 events on the flow cytometer. When the target population was small, as in the OT-II adoptive transfer experiments, 1x10^6 cells were stained and 200 000 events collected. The cells were washed and resuspended in 100µl of antibody diluted in PBS supplemented with 2% FCS. Details of the specific antibodies and dilutions are listed in table 2.1. The cells were stained for 20min on ice in the dark, washed and, if necessary, stained with a secondary antibody. Cells were resuspended in 200µl PBS/2% FCS and analysed immediately.

Table 2.1 Antibodies used in flow cytometry.

	Antibody	Clone	Source	Dilution	Isotype
T-cell markers	CD4-FITC	GK1.5	In house	1:100	rIgG2$_b$
	CD4-APC	RM4-5	Pharmingen	1:400	
	DO11.10 TCR-FITC	KJ1.26	In house	1:400	mIgG$_{2a}$
	DO11.10 TCR-biotin	KJ1.26	In house	1:100	mIgG$_{2a}$
	CD69-PE	H1.2F3	Pharmingen	1:100	HsIgG$_1$
	CD25-PE	7D4	Pharmingen	1:100	IgM
	CD62L-PE	Mel-14	In house	1:100	rIgG$_{2a}$
	CD44-PE	IM7	Pharmingen	1:100	
	CD44-biotin	142.5	In house	1:100	
	Ly5.1-biotin	A20	Pharmingen	1:200	
	ICOS-biotin	7E.17G9	Pharmingen	1:100	rIgG$_{2b}$
	Vα_2-R-PE	B20.1	Pharmingen	1:200	rIgG$_{2a}$
	V$\beta_{5.1}$, V$\beta_{5.2}$-FITC/biotin	MR9-4	Pharmingen	1:100	rIgG$_1$
	OX40-biotin	OX-86	Pharmingen	1:10	rIgG$_1$
	MHC-II Tetramer-PE		Schumaker lab		
Other cells	CD8-biotin	53.6.72	In house	1:100	rIgG$_{2a}$
	CD19-FITC	ID3	In house	1:100	rIgG$_{2a}$
	CD40-PE	3/23	Pharmingen	1:100	rIgG$_{2a}$
	Gr1-PE	RB6-8C5	Pharmingen	1:100	rIgG$_{2b}$
	B220-PerCP	RA3-6B2	Pharmingen	1:300	rIgG$_{2a}$
	B220-PE	RA3-6B2	Pharmingen	1:400	rIgG$_{2a}$
	CD11c-APC	HL3	Pharmingen	1:200	HsIgG$_1$
	MHC-II-biotin/FITC	M5114	In house	1:100/200	
	F4/80-RPE-Cy5	CI-A3-1	Serotec	1:400	rIgG$_{2b}$
	B7-1-PE	16-10A1	Pharmingen	1:100	HsIgG2
	B7-2-PE	GL1	Pharmingen	1:100	rIgG$_{2a}$
Cytokines	IFNγ -FITC	XMG1.2	Pharmingen	1:100	rIgG$_1$
	IL-2-PE	JES6-5H4	Pharmingen	1:100	rIgG$_{2b}$
	IL-4-PE	BVD6-24G2	Pharmingen	1:100	rIgG$_1$
	IL-10-PE	JES5-2A5	Pharmingen	1:100	rIgG$_1$
Secondary Reagents	Streptavidin-PE		Calbiochem	1:1500	
	Streptavidin-PerCP/Quantum Red		Pharmingen/ Sigma	1:200/100	
Miscellaneous	Propidium Iodide		Pharmingen	1:400	
	Annexin-V-FITC/PE		Pharmingen	1:20	
	7-AAD		Pharmingen	1:40	

Monoclonal primary antibodies were supplied by Pharmingen, Serotec or grown and conjugated in house (section 2.9). Isotype controls from Pharmingen were used at the same concentration as the test antibody, and in house antibodies were matched with an irrelevant antibody of the same isotype at the same concentration. Abbreviations in isotype column: r, rat; Hs, hamster; m, mouse.

2.7.2. Intracellular cytokine staining

CD4 T cells cultured *in vitro* with DCs or *ex vivo* splenocytes from mice were stimulated in the presence of 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 500ng/ml ionomycin (both from Sigma) for 5h at 37°C. GolgiStop™ (Pharmingen) was included at a dilution of 1:1500, as recommended by the manufacturer. Cells were stained with CD4-APC to detect surface CD4 expression, washed and then fixed and permeabilised by resuspending in Cytofix/Cytoperm™ reagent (Pharmingen) for 20min on ice. Cells were washed in permeabilisation buffer (provided in the Cytofix/Cytoperm™ kit) and stained with PE-labelled antibodies against IL-2, IL-4, IFNγ and IL-10 for 30min on ice. Subsequent washes were again performed in permeabilisation buffer and the cells finally resuspended in PBS/2% FCS for analysis by flow cytometry.

2.7.3. Assessing cell division by CFSE staining

T cell proliferation was visualised using CFSE. CD4 T cells from spleens or lymph nodes taken from mice transferred with CFSE labelled OT-II or OT-II ICOS$^{-/-}$ cells and immunised with ovalbumin were assessed for CFSE expression at various day points. DO11.10 and OT-II T cells from *in vitro* cultures with DCs were also examined for CFSE expression at various days.

2.7.4. Dead cell analysis

To quantify cell death, Annexin-V and 7-amino-actinomycin D (7-AAD) staining of the CD4 T cells was undertaken. One of the earliest signs of apoptosis is the translocation of membrane phospholipid phophatidylserine (PS) from inner membrane to outer plasma membrane. Annexin-V binds to the PS and therefore is a sensitive tool to detect early apoptotic cells. 7-AAD is a nucleic acid dye that stains the nonviable cells.

Cells from *in vitro* cultures or lymph nodes of mice were surface stained for CD4 and the markers for transgenic TCRs (KJ1.26 for DO11.10; $V\alpha_2$ and $V\beta_5$ for OT-II). After washing with cold PBS, cells were resuspended in 100µl Annexin-V binding buffer (supplied as 10x with apoptosis detection kit, Pharmingen). FITC or PE labelled Annexin-V (Pharmingen) was added at a dilution of 1:20, as recommended by the manufacturer and incubated at room temperature in dark for 15min. Additional 400µl binding buffer was added to the samples followed by immediate acquisition by FACS. 7-AAD was added to surface stained cells 10min before acquisition, $5\mu l/10^6$ cells. It is detected on FL3.

2.7.5. Tetramer staining

For detection of polyclonal cell populations, MHC-II tetramers were used. Erythrocytes were lysed in the single cell suspensions prepared from spleens and lymph nodes. Approximately 1-2 $x10^6$ cells were plated in round bottom 96-well plates (Corning Inc, Costar, VWR International Ltd, Poole, UK), washed in 10% IMDM (Sigma), before the addition of PE-labelled class II tetramers diluted in 10% IMDM. Tetramers were made as described in chapter 4 and figure 4.9 [239]. The cells were incubated at 37°C for 3 hours with gentle agitation every 20-30 minutes to prevent clumping of cells. APC-labelled anti-CD4 (Pharmingen), R Phycoerythrin (RPE)-Cy5-labelled anti-F4/80 (Serotec, Kidlington, Oxford, UK) and FITC-labelled anti-CD44 (142.5, in house) were added and incubated for 10-15 minutes at room temperature. Cells were washed 3 times in FACS buffer. Propidium iodide (Pharmingen) was added prior to acquisition. 200 000 live events were collected on FACS. Tetramer-positive cells were identified by gating on $CD4^+$ and live lymphocytes, excluding macrophages (see figure 4.10). The tetramers and the staining protocol used had been developed and standardised in Ton Schumacher's lab.

2.8. Immunofluorescence

Spleens were frozen in OCT-embedding medium (BDH, Dorset, UK) in cryomoulds (BDH, Dorset, England) on top of dry ice and stored at -80°C. Tissue sections (5μm thick) were cut onto multi-well glass slides (Hendley-Essex, London, UK) using a cryostat (model CM1510, Leica) and left overnight to dry before fixation in acetone for 10min at 4°C. Sections were stained with texas-red labelled polyclonal goat anti-mouse IgM (SouthernBiotech) and FITC-conjugated peanut agglutinin (PNA). Stained tissue sections were mounted with the embedding medium moviol (Hoechst, Frankfurt, Germany) and analysed using an Olympus BX50 microsope. Images were captured with a Hamamatsu digital camera and Openlab imaging software (Improvision, Coventry, UK).

2.9. 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_2. The contents of the roller bottles were filtered to remove cells and the immunoglobulin was precipitated with ammonium sulfate (Sigma) using 291g per litre of supernatant. Precipitates were dissolved in PBS and extensively dialysed against PBS. Antibodies were purified by binding to a 1 or 5ml Hitrap protein G column (Amersham Bioscience, Bucks, UK) at pH 7 and eluted at pH2.8 using an AKTA*prime* (Amersham Bioscience). The antibody was dialysed with PBS until the pH was neutral. Antibody concentration was estimated by measuring absorbance at 280nm.

GM-CSF supernatant was prepared by growing the cell line X63-gmcsf in tissue culture flask in 5% IMDM with Geneticin (G418 sulfate; Gibco) at a final concentration of 1mg/ml to select for the GM-CSF producing cells. Before transfer into roller bottles, the cells were washed twice in 5% IMDM to remove the G418. The cells were grown in 500ml of medium in roller bottles for 4-5 days. The cells were then transferred into 50ml

Falcon tubes and the cells separated from the supernatant by centrifugation. The supernatant was pooled and titrated each time a new batch was made.

Purified antibodies were conjugated to biotin by reacting each 1mg of protein with 75µg of succinimidyl-6 (biotinamido) hexanoate (EZ-Link™ NHS-LC-Biotin; Pierce, Rockford, IL, USA) in dimethyl formamide (Sigma). FITC conjugation was performed by reacting each 1mg of antibody with 50ng FITC Isomer I (Sigma) in 0.05M carbonate-bicarbonate buffer at pH9. Conjugated antibodies were then dialysed extensively against PBS to remove excess label. Antibodies were filter sterilised and stored frozen at -20°C.

2.10. *In vitro* assessment of T cell function

2.10.1. *In vitro* proliferation assays

Single cell suspensions from spleens of immunised mice were plated at 5-8x10^5 cells/well of a 96-well, flat-bottom plate in 200µl final volume. Graded doses of pOVA$_{323-339}$ or KLH were added. Proliferation was measured by the addition of 0.5µCi of 35Ci/mmol [240]-thymidine (ICN, Basingstoke, UK) to each well, 16h before the end of culture. Plates were harvested onto filter mats (Printed Filtermat A; Wallac, Turku, Finland) using a 96-well MachIIIM Tomtec harvester (Wallac). The mats were dried on a hot plate and a solid scintillation wax melted into them (Meltilex™ A; Wallac). When the wax had re-solidified, the mats were read using a Trilux 1450 Microbeta liquid scintillation and luminescence counter and software (Trilux, Arnsberg, Germany).

Wherever stated, irradiated splenocytes were used as APCs in T cell proliferation assays. Before single cell suspension was prepared, spleen was placed in a bijou in a small volume of medium and irradiated with a γ-source for 100 seconds.

2.10.2. Help Assay

B cells were purified from the spleens of C57Bl/6 mice immunised intraperitoneally with alum-precipitated DNP-KLH and killed *B. pertussis*. CD4$^+$ T cells were purified from spleens of wildtype C57Bl/6 or knockout mice or bone marrow chimeras with defective B cell compartment, immunised with ovalbumin-alum and killed *B. pertussis*. A minimum of three mice constituted a group and T cells were purified from the pooled splenocyte population. Purified B and T cells were cultured *in vitro* in the presence of soluble DNP-ovalbumin. After 2 days, the medium containing the antigen was removed and replaced with fresh medium. The culture supernatant was harvested after 5 days and DNP-specific immunoglobulin isotypes measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. The experimental protocol is outlined in figure 5.1.

2.10.3. Measuring cytokines by cell-based ELISA

Splenoctyes were plated at 8x10^5 cells per well in 96-well flat-bottom plate and incubated at 37°C in the presence of OVA$_{323-339}$ titrated in log-fold dilutions starting at 100μM. After 48 hours, 100μl of cells was transferred to maxisorb plates coated with antibodies against IL-2, IL-4, IL-10 and IFNγ (all from Pharmingen). Double dilutions of recombinant cytokines (Pharmingen; Peprotech EC Ltd., London, UK) were done for standard curve. The following day, plates were washed and incubated with cytokine-specific biotinylated antibodies. Extravidin-AP was used as secondary antibody followed by addition of p-nitrophenyl phosphate (PNPP) substrate. Amount of cytokine secreted was measured by interpolation of absorbance from the standard curve.

2.10.4. Measurement of cytokine in supernatant by ELISA

Maxisorb plates were coated overnight with antibodies against IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ in 0.1M carbonate/bicarbonate buffer (Sigma) pH9.6 at 4°C. After blocking with 10% FCS-PBS, culture supernatant and standards were added and incubated at 4°C overnight. After washing, cytokine-specific biotin-conjugated antibodies were added (Pharmingen) for 1h at room temperature. Peroxidase-conjugated streptavidin was then added followed by substrate TMB. Absorbance was measured at 450nm and amount of cytokine measured by interpolation from standard curve. For measuring TNFα, TNFα DuoSet kit (R&D) containing all the reagents including coating antibody, standards and detection antibody was used.

2.11. Assessment of B cell function

2.11.1. B cell clonal expansion

Mice were immunised intraperitoneally with 100μg alum-precipitated Phycoerythrin (PE). Spleens were harvested 2 weeks following the immunisation and cells stained with 10μg/ml PE and FITC-labelled anti-CD19 mAb ID3. By the addition of PE, we can detect the PE-binding B cells when analysed by flow cytometry (see figure 4.4A). PE^+ cells were compared between groups either as percentage of $ID3^+$ cells or total number of $ID3^+PE^+$ cells per spleen. Background PE staining in un-immunised mice was minimal.

2.11.2. Measurement of antigen-specific immunoglobulins by ELISA

ELISA was performed on either serum from DNP-KLH immunised animals or the culture supernatants from the help assays. Blood collected from the tail veins of the mice was allowed to clot at 4°C before centrifuging twice at 15000 rpm for 20min and serum was collected. Culture supernatants were harvested after centrifugation of 96-well plates at 1500rpm for 1min, and frozen at -20°C until analysed.

Bovine serum albumin (BSA, Sigma) was conjugated to DNP using DNFB as done for ovalbumin (section 2.2.2). 96-well PVC microtitre plates (Dynex Technologies Inc.) were coated with 50μg/ml DNP-BSA in 0.1M carbonate/bicarbonate buffer, pH 9.4, overnight at 4°C. Plates were washed in PBS and sera added in two fold dilutions in PBS starting from 1/20. The culture supernatants were added without further dilution to the top rows. Plates were incubated for 2h at room temperature. Following washing in PBS 0.05% Tween-20, plates were incubated with alanine phosphatase (AP) conjugated polyclonal goat anti-mouse IgM, IgG, IgG_1, IgG_{2c} and IgG_{2a} (all from Southern Biotechnology Associates, Inc., USA) in PBS 0.05% Tween-20 for 1h at room temperature. Antibody binding was detected by addition of PNPP substrate (SouthernBiotech) dissolved in 0.5M magnesium chloride in 9.8% diethanolamine, buffered at pH9.8 and the optical density (OD) at 405 nm was measured on ELISA reader (Labsystems). Comparative titres were calculated by plotting serum concentration against OD, and reading the dilution at the half-maximal OD, compared with a positive control.

2.11.3. Enumeration of antigen-specific antibody secreting cells (ASC) by ELISPOT

96-well nitrocellulose plates (Millipore) were coated with 10μg/ml DNP-BSA in 0.1M carbonate/bicarbonate buffer, pH 9.4, overnight at 4°C. Plates were washed in PBS and blocked with 10% FCS-PBS for 2 hours at room temperature. Following 3 washes with PBS, 10- or 2-fold dilutions of spleen and bone marrow cell suspensions were added in triplicate, over the range of 10^4-10^6 total cells per well. Plates were incubated overnight at 37°C, 5% CO_2 in humid atmosphere. Plates were washed thoroughly: thrice with PBS, thrice with PBS 0.05% Tween-20, then thrice again with PBS. The secondary antibody, AP-conjugated goat anti-mouse IgM, IgG, IgG_1 or IgG_{2a} were added in PBS-5% BSA, 4h at room temperature, then washed thoroughly as before. Spots were developed by incubation with 1mg/ml of 5-Bromo 4-Chlorophosphate (BCIP, Sigma) in 0.1M Tris (Sigma) pH9 plus 5mM MgCl (Sigma) and 0.1M NaCl (Sigma). Spots were scored by eye using a dissection microscope and the average number of spots per well was used to calculate the frequency of DNP-specific ASCs. Spleen and bone marrow from unimmunised control mice consistently gave no spots for IgG, although in one experiment IgM spots were noticed in control mice.

2.12. Generation of bone marrow chimeras

Recipient C57Bl/6 mice were lethally irradiated by exposure to 1150rad over 47-50min, using a caesium source irradiator (^{137}Cs source). 24h later they were reconstituted with pooled bone marrow cells from μMT (80%) and C57Bl/6 /CD40$^{-/-}$/Aβ$^{-/-}$/OX40L$^{-/-}$ (20%) mice. A single cell suspension of each bone marrow population was prepared and depleted of Thy1^{+} T cells by incubating with CD90 microbeads and subsequent removal on a MACS depletion column (Miltenyi). This aimed to prevent graft-versus-host disease in the recipient mice. T-depleted bone marrow cells were then combined at a ratio of 80:20, filtered through parachute silk and 3.5x10^{6} injected i.v. into each irradiated host. The chimeras were left 8 weeks before use, to allow reconstitution of the immune system. This was confirmed by a normal ratio of B to T cells in the peripheral blood, measured by flow cytometry. The FACS plots showing the reconstitution are shown in figure 5.7B.

2.13. Statistics

Statistics were calculated using GraphPad Prism® (version 3.0, GraphPad software, San Diego, CA, USA, www.graphpad.com), following the recommendations made by this programme. Results were considered significant if *P*<0.05.

3. Is CD40 dispensable for CD4$^+$ T cell proliferation?

Introduction

There has been a great interest in the roles of costimulatory interactions in T cell responses, largely because of the potential for targeting these receptor-ligand pairs for the prevention of graft rejection and the treatment of various immunological diseases [73]. A detailed understanding of the relative contribution of various costimulatory pathways in T cell responses to antigen is critical to optimise clinical protocols that employ costimulator-blocking agents in an effort to modulate immune responses.

CD40 is widely expressed *in vivo* and is detectable on APCs such as DC, B cells and monocyte/macrophages and also on other cells like endothelial cells, epithelial cells, mast cells, fibroblasts and even smooth muscle cells [75,241]. Its ligand, CD40L (CD154), is also widely expressed, notably on T cells, NK, basophils, mast cells, eosinophils, platelets and even B cells [75,241]. Given this broad pattern of distribution of both receptor and ligand, it is not surprising that CD40-CD40L interactions are thought to play a key role in a diverse array of *in vivo* activities during immune response. CD40 ligation on the APCs leads to various responses including proliferation, maturation, increased survival, increased expression of cell surface proteins (CD80, CD86, CD54 etc.), and production of cytokines (IL-1, IL-6, IL-12, IL-10 and TNFα) [75].

The absence of CD40-CD40L interactions during the initiation of T-dependent immune responses *in vivo* has marked effects on these responses. Various studies in CD40 and CD40L knockout mice have provided insights into the importance of these interactions in T cell priming. While several studies have shown reduced CD4 and CD8 T cell priming, others have found a defect only in the CD4 response or no defect at all [72,242-244]. These variations perhaps reflect the various immunisation strategies used and inherent differences between viral and protein based immunisations. Given the complex *in vivo* interactions between cells bearing CD40-CD40L along with plethora of other costimulatory molecules, it is difficult to definitely associate defective T cell priming and survival with CD40-CD40L interactions. To simplify the analysis of T cell-APC interactions to gain additional insight into the role of CD40 in inducing CD4$^+$ T cell proliferative responses, I examined the *in vitro* responses of naïve CD4$^+$ cells from transgenic mice (DO11.10 and OT-II) to antigens presented by CD40-sufficient or -deficient APCs.

To address the issue of TCR-affinity for peptide-MHC-II complex, I have used altered peptide ligands in this study. The term "altered peptide ligand" (APL) was coined to describe analogues of immunogenic peptides in which the TCR contact sites have been manipulated. The first description of APLs, more than a decade ago, marked an important turning point in the understanding of the immune response. The notion of single ligand specificity for any particular T cell was challenged by many studies using analogues of immunogenic peptides demonstrating flexibility in this recognition [245]. In response to antigenic stimulation, T cells mount a continuum of responses, the individual components of which may be effectively uncoupled by introducing subtle changes in the TCR contact sites of their cognate ligand. Interactions with such APLs can result in dramatically different phenotypes of the T cells, ranging from inducing selective stimulatory functions to completely turning off their functional capacities.

Results

3.1. CD40-deficient bone marrow derived DCs (BMDC) stimulate strong primary CD4$^+$ T cell proliferative response

To examine the CD40 dependence of CD4$^+$ T cell proliferative responses *in vitro*, BMDCs from BALB/c (CD40$^{+/+}$) mice versus CD40$^{-/-}$ mice were used. CD4$^+$ T cells were purified (by negative selection) from spleens and pooled lymph nodes of TCR transgenic DO11.10 mice bred onto CD40$^{-/-}$ background (I-A^d). A total of 10^5 CD4 T cells were cultured with a titration of BMDCs in the presence of 0.5μg/ml of peptide OVA$_{323\text{-}339}$ in 96-well plates. T cell proliferation was measured by thymidine incorporation at d3 and d6 of culture. As shown in figure 3.1, CD40$^{-/-}$ DCs induced strong primary proliferative responses that were equivalent to or slightly higher than responses to CD40$^{+/+}$ DCs in various experiments. These findings are representative of 5 experiments and applied regardless of the number of DCs added (fig 3.1A) or concentration of antigen used (fig 3.1B). CD40$^{-/-}$ DCs are thus not intrinsically defective in stimulating naïve CD4$^+$ T cells.

To quantitate the dynamics of T cell proliferative responses in the presence or absence of CD40, DO11.10 T cells were labelled with CFSE before culturing with the BMDCs and pOVA. The division of the T cells was followed over various days by examining the loss of CFSE. The rate of division of the transgenic T cells was similar at days 3, 5 and 7 whether CD40 was present on the DCs or not. Figure 3.2 depicts the CFSE overlay at d3.

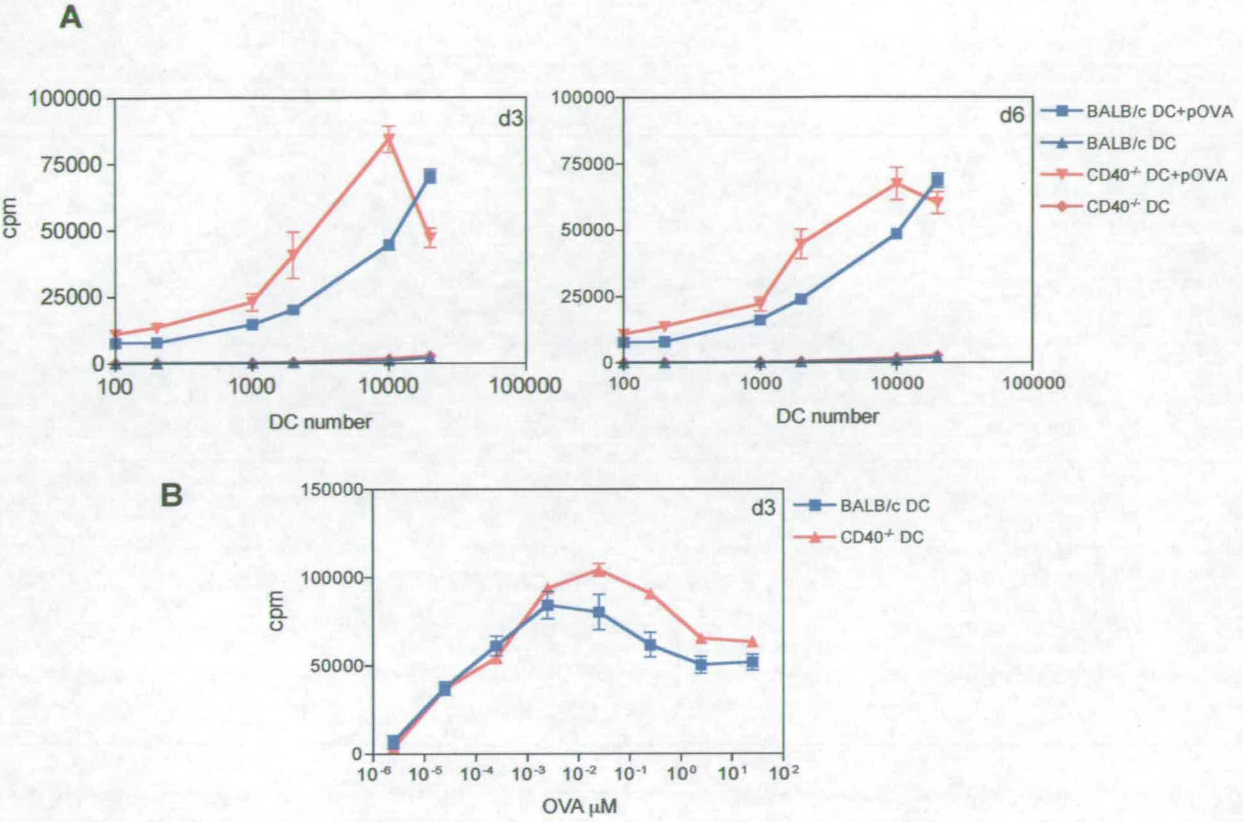


Figure 3.1 *In vitro* proliferation of DO11.10 CD4 T cells in the presence of pOVA$_{323-339}$ and BALB/c or CD40$^{-/-}$ BMDC. DCs were grown from bone marrow precursors in the presence of GM-CSF and harvested at d7 of culture (section 2.4.1). CD4 T cells were purified from spleens and lymph nodes of naïve DO11.10 CD40$^{-/-}$ mice. (A) 10^5 T cells were cultured with a titration of DCs in the presence of 0.5μg/ml pOVA in 96-well plates at 37°C. (B) 10^5 T cells were cultured with 10^4 DCs in the presence of titration of pOVA. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3 or 6 day culture. Data is shown as the mean of triplicate wells ± SEM and is representatative of three independent experiments.

3.2. CD40 does not affect T cell apoptosis

That T cell priming and proliferation *in vitro* can occur in the absence of CD40 on the APCs, was an unexpected finding given that defective T cell priming *in vivo* has been reported in the absence of CD40-CD40L interactions (reviewed in [75]). I next asked the question if there were more T cells in CD40$^{-/-}$ cultures undergoing apoptosis because perhaps the activation was not sustained in the absence of CD40-CD40L interactions. Annexin-V is a sensitive tool to detect early apoptotic cells and 7-amino-actinomycin D (7-AAD), a nucleic acid dye, stains the non-viable cells.

Cultures of 10^6 DO11.10 T cells and 10^5 BMDCs were set up in 24-well plates in the presence of 0.5μg/ml of pOVA$_{323-339}$. At days 3, 5, 7 and 10 of cultures, the cells were harvested and counted using haemocytometer. Cells were stained with anti-CD4, KJ1.26 (clonotypic antibody specific for TCR of DO11.10) and Annexin-V or 7-AAD and analysed for apoptotic cells by flow cytometry.

There was no apparent difference in the cell counts and the number of apoptotic DO11.10 T cells when cultured with either BALB/c or CD40$^{-/-}$ BMDCs (fig 3.3A). Figure 3.3B depicts the histogram overlays of Annexin-V and 7-AAD positive transgenic T cells at different days in culture.

Thus, T cells activated by CD40-deficient APC were not more likely to undergo apoptosis than T cells activated by wildtype APC. Therefore, T cell activation was not curtailed in the absence of CD40 on DC.

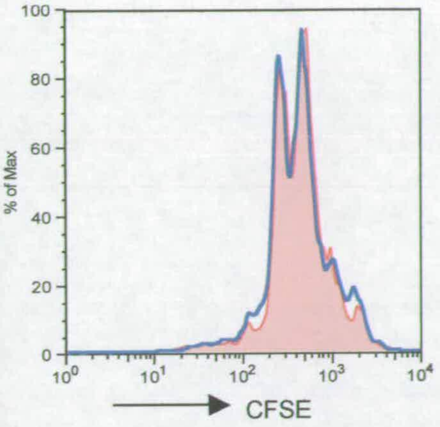


Figure 3.2 Loss of CFSE by DO11.10 CD4 T cells cultured *in vitro* with BALB/c or CD40$^{-/-}$ BMDCs in the presence of pOVA$_{323-339}$. Purified DO11.10 CD4 T cells were labelled with CFSE before culure with DCs. The histogram overlay shows the dilution of CFSE by T cells in culture with BALB/c (blue line) or CD40$^{-/-}$ (red tinted) DCs at d3. Cells gated on CD4$^+$KJ1.26$^+$ cells. Data shown is representatative of three independent experiments.

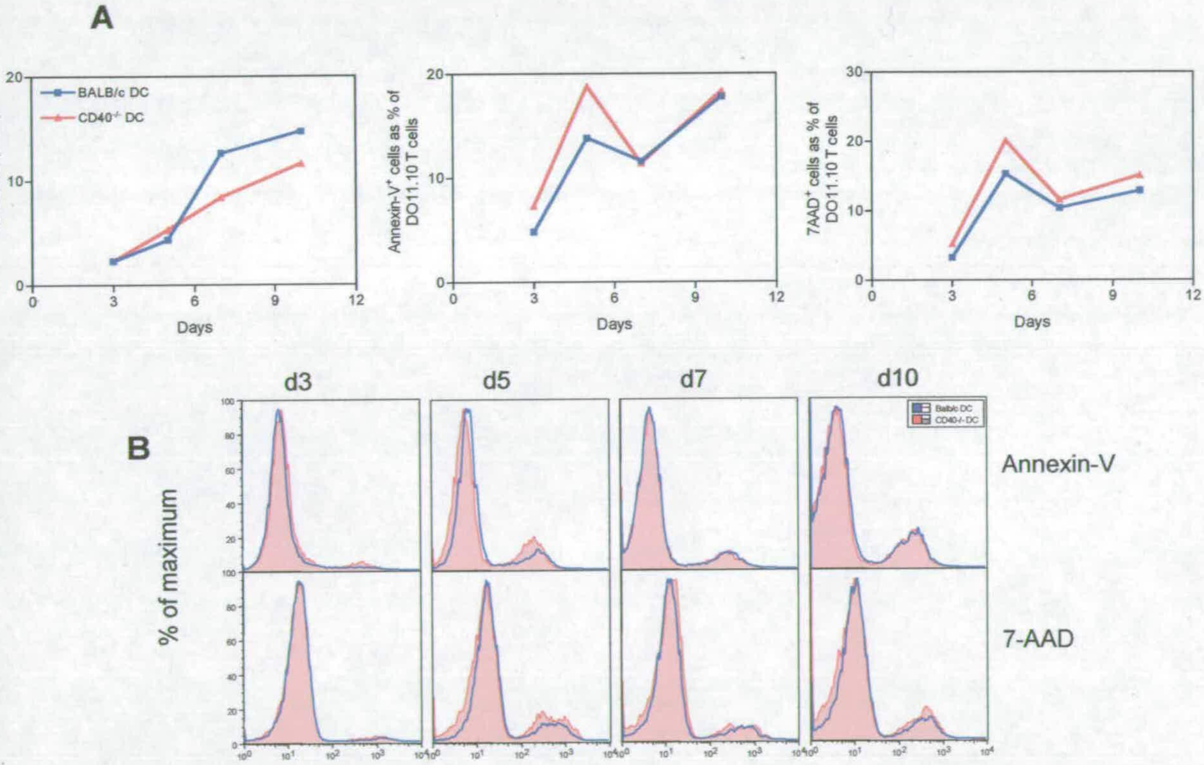


Figure 3.3 CD40 does not affect DO11.10 CD4 T cell apoptosis *in vitro*. 10^6 DO11.10 T cells were cultured with 10^5 BMDCs in the presence of 0.5µg/ml pOVA in 24-well plates at 37°C. At 3, 5, 7 and 10 days of culture, cells were harvested and counted using haemocytometer (A, left). Cells were stained with anti-CD4, KJ1.26 and Annexin-V or 7-AAD, and number of apoptotic cells assessed by flow cytometry. The % of DO11.10 T cells positive for Annexin-V and 7-AAD are shown in A, middle and right plots, respectively. (B) Histogram overlay of Annexin-V and 7-AAD positive DO11.10 T cells cultured with BALB/c (blue line) and CD40$^{-/-}$ (red tinted) DCs at indicated days of culture. Data shown is representatative of three independent experiments.

3.3. DO11.10 T cells proliferate in the absence of CD40 on splenic DC

There are a growing number of reports that have demonstrated that there are different subsets of DCs specialised for priming T cell responses [246]. BMDCs are primarily CD8α^- and DEC-205$^-$ (myeloid DCs) in comparison to DCs freshly isolated from the spleen, which contain both myeloid as well as lymphoid DCs (CD8α^+ and DEC-205$^+$). *In vivo* CD40 plays important role, for example, DC expression of CD40 has been implicated in Th2 responses against schistosome egg antigen [247].

To examine whether expression of CD40 by splenic DCs affect T cell priming and proliferation *in vitro*, 10^5 DO11.10 CD4 T cells were cultured with a titration of splenic DCs purified from the spleens of either BALB/c or CD40$^{-/-}$ mice in the presence of 0.5μg/ml of peptide OVA$_{323\text{-}339}$. The DO11.10 CD4 T cells proliferated equally in the presence of CD40-sufficient or -deficient DCs at d3. At d6 however, there was slightly less proliferation in the absence of CD40, although the kinetics of the response was similar in both groups (fig 3.4A).

When analysed by Annexin-V and 7-AAD staining, the number of viable and apoptotic DO11.10 CD4 T cells were similar in the presence or absence of CD40 on antigen presenting splenic DCs (fig 3.4B).

CFSE-labelled DO11.10 T cells were cultured with wildtype and CD40$^{-/-}$ splenic DCs and pOVA and their division followed over time by examining the loss of CFSE. The rate of division of the transgenic T cells was similar at days 3, 5 and 7 whether CD40 was present on the DCs or not. Figure 3.5 depicts the CFSE overlays at d3 and d5. Thus, the priming, proliferation and survival of DO11.10 T cells *in vitro* are not dependent on CD40-CD40L interactions with the APC.

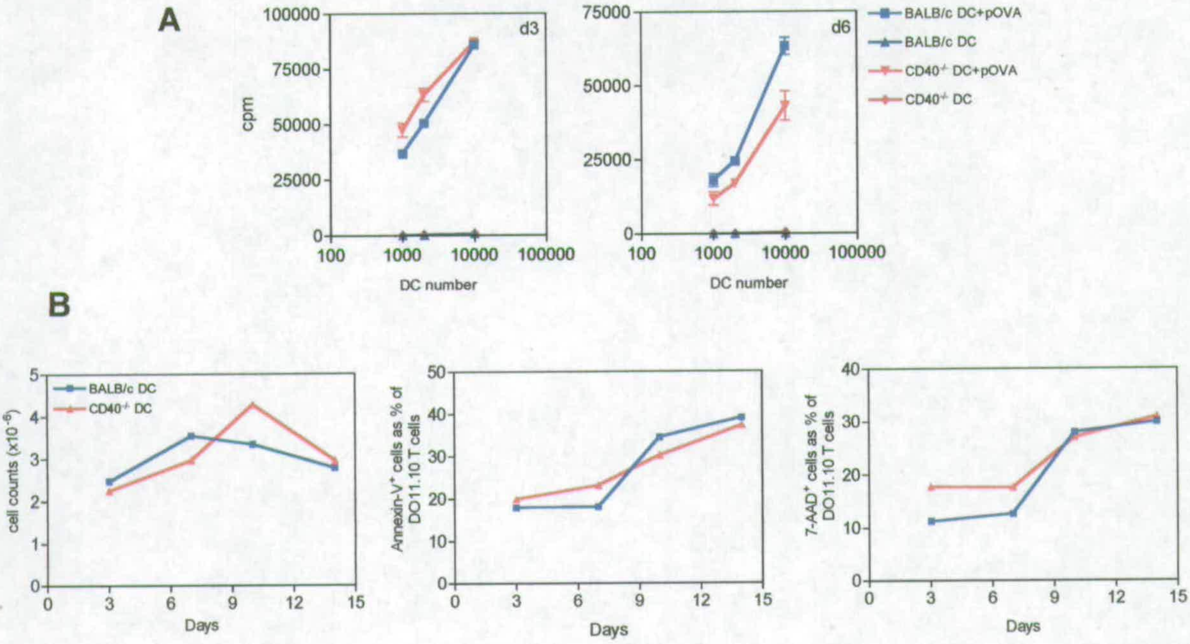


Figure 3.4 *In vitro* proliferation and apoptosis of DO11.10 CD4 T cells in the presence of BALB/c or $CD40^{-/-}$ splenic DCs. DCs were purified from mouse spleen using plastic adherence and depletion of contaminating lymphocytes (section 2.4.1). CD4 T cells were purified from spleens and lymph nodes of naïve DO11.10 $CD40^{-/-}$ mice. (A) 10^5 T cells were cultured with a titration of DCs in the presence of 0.5μg/ml pOVA in 96-well plates at 37°C. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3 or 6 day culture. Data is shown as the mean of triplicate wells ± SEM and is representatative of two independent experiments. (B) 10^6 DO11.10 T cells were cultured with 10^5 splenic DCs in the presence of 0.5μg/ml pOVA in 24-well plates at 37°C. At 3, 7, 10 and 14 days of culture, cells were harvested and counted using haemocytometer (left). Cells were stained with anti-CD4, KJ1.26 and Annexin-V or 7-AAD, and number of apoptotic cells assessed by flow cytometry. The % of DO11.10 T cells positive for Annexin-V and 7-AAD are shown in middle and right plots.

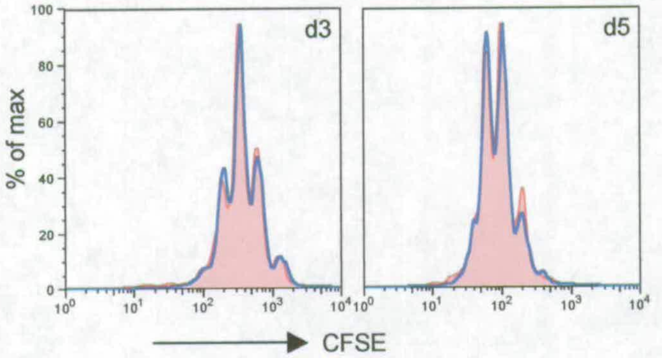


Figure 3.5 Loss of CFSE by DO11.10 CD4 T cells cultured *in vitro* in the presence of $pOVA_{323-339}$ and BALB/c or $CD40^{-/-}$ splenic DCs. Purified DO11.10 CD4 T cells were labelled with CFSE before culure with splenic DCs. The histogram overlay shows the dilution of CFSE by T cells in culture with BALB/c (blue line) or $CD40^{-/-}$ (red tinted) DCs at d3 and d5. Cells gated on $CD4^+KJ1.26^+$ cells. Data shown is representatative of two independent experiments.

3.4. OT-II T cells are dependent on CD40 activation of APC for proliferation and survival *in vitro*

To investigate whether T cell independence of CD40-CD40L interactions for proliferation and survival was a phenomenon specific to DO11.10 TCR transgenic mice, similar studies were conducted on OT-II mice. OT-II mice are TCR transgenic mice that recognise the same $OVA_{323-339}$ peptide as DO11.10 mice but they are I-A^b MHC restricted.

CD4 T cells purified from spleens and pooled lymph nodes of OT-II $CD40^{-/-}$ mice were cultured with BMDCs from C57Bl/6 and $CD40^{-/-}$ (I-A^b) mice. T cell proliferation was measured at days 3 and 5 by thymidine incorporation. T cell proliferation at d3 in $CD40^{-/-}$ cultures was slightly lower compared to C57Bl/6 DCs, when the number of DCs or the amount of pOVA was titrated in the cultures (fig 3.6A & B). This difference, however, became much more evident at d5. At all titrations of DCs except maximal 1DC/5 T cells, the T cells proliferated much less in the absence of CD40 (fig 3.6A). When T cells were stimulated with serial dilutions of pOVA, less proliferation was observed if DCs lacked CD40. At higher concentrations of the peptide, however, T cells in C57Bl/6 cultures started dying due to overgrowth at d5 (fig 3.6B).

3.5. OT-II cells undergo apoptosis in the absence of CD40-CD40L interactions *in vitro*

Cultures of 10^6 OT-II $CD40^{-/-}$ T cells and 10^5 BMDCs were set up in 24-well plates in the presence of 0.5µg/ml of $pOVA_{323-339}$. At days 3, 5, 7 and 10 of cultures, the cells were harvested and counted using haemocytometer. Cells were stained with antibodies against CD4, $V\alpha_2$ and $V\beta_5$ along with Annexin-V or 7-AAD and analysed for apoptotic cells by flow cytometry. In the absence of CD40-CD40L interactions, the OT-II T cells

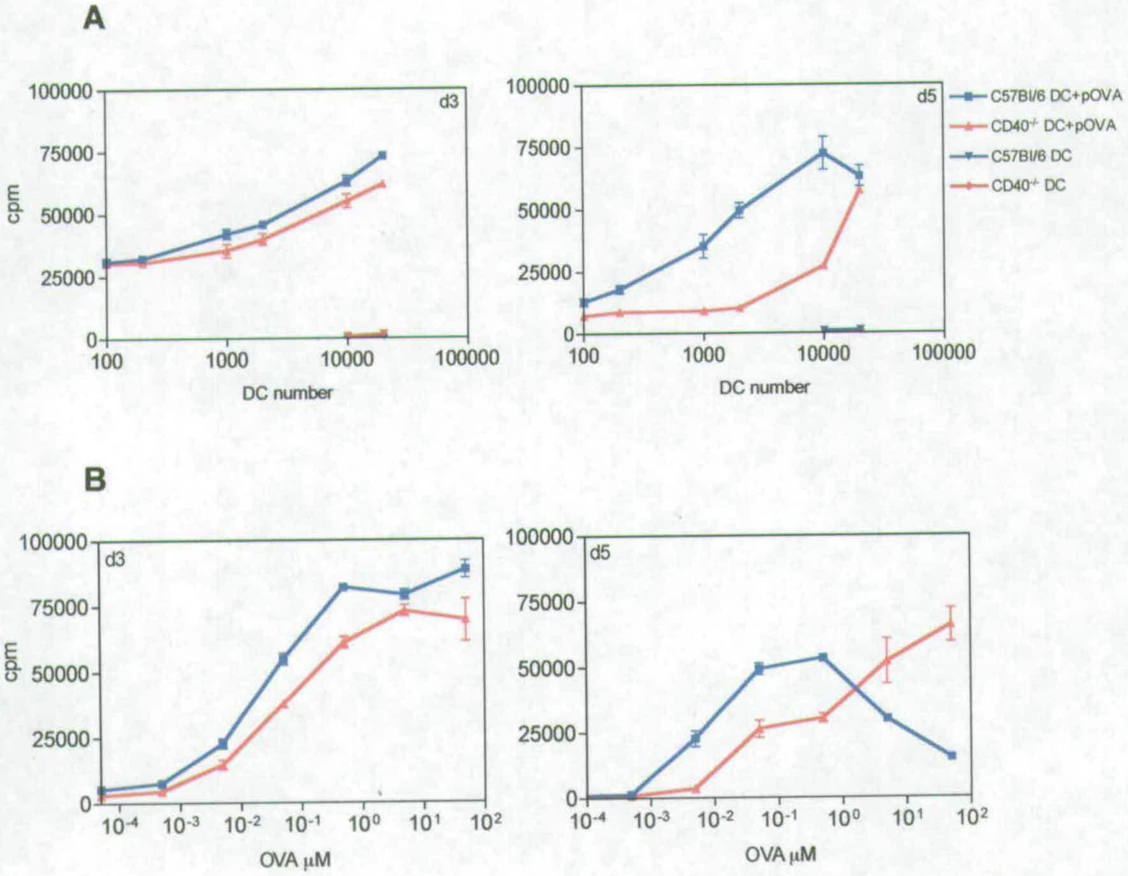


Figure 3.6 *In vitro* proliferation of OT-II CD4 T cells in the presence of pOVA$_{323\text{-}339}$ and C57Bl/6 or CD40$^{-/-}$ BMDC. DCs were grown from bone marrow precursors in the presence of GM-CSF and harvested at d7 of culture. CD4 T cells were purified from spleens and lymph nodes of naïve OT-II CD40$^{-/-}$ mice. (A) 10^5 T cells were cultured with a titration of DCs in the presence of 0.5μg/ml pOVA in 96-well plates at 37°C. (B) 10^5 T cells were cultured with 10^4 DCs in the presence of titration of pOVA. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3 and 5 day culture. Data is shown as the mean of triplicate wells ± SEM and is representatative of two independent experiments.

underwent apoptosis. The CD40$^{-/-}$ cultures had higher proportion of cells positive for Annexin-V and 7-AAD in comparison to wildtype cultures from d5 onwards (fig 3.7A). Figure 3.7B depicts the histogram overlays of Annexin-V and 7-AAD positive OT-II cells in culture with CD40$^{-/-}$ or C57Bl/6 DCs.

3.6. CFSE dilution

OT-II CD40$^{-/-}$ CD4 T cells were labelled with CFSE before culture with BMDCs from C57Bl/6 and CD40$^{-/-}$ mice and their division followed over time. The division of the T cells was equivalent in both cultures on d1, d3 and d5, but at d7 and d10, no further division was noted in CD40$^{-/-}$ cultures, whereas the T cells in C57Bl/6 cultures underwent additional divisions (fig 3.8). This happened because T cells in CD40$^{-/-}$ cultures started to undergo apoptosis.

Thus, OT-II T cells are more dependent on CD40-CD40L interactions than DO11.10 T cells for proliferation and survival *in vitro*.

3.7. DO11.10 versus OT-II

Robertson *et al.* have shown that DO11.10 T cells respond to lower amounts of OVA$_{323-339}$ than OT-II T cells [248]. Various studies have reported that the DO11.10 cells proliferate extensively following transfer into T cell-deficient or irradiated hosts, the phenomenon referred to as homeostatic proliferation [249]. In contrast, the OT-II cells do not divide much after transfer into immunodeficient hosts [250,251]. Since in this system, recognition of self-peptide on MHC molecules is thought to induce the proliferation, it is suggested that DO11.10 cells have greater affinity for self-MHC than OT-II cells do for their respective MHC molecules [252,253].

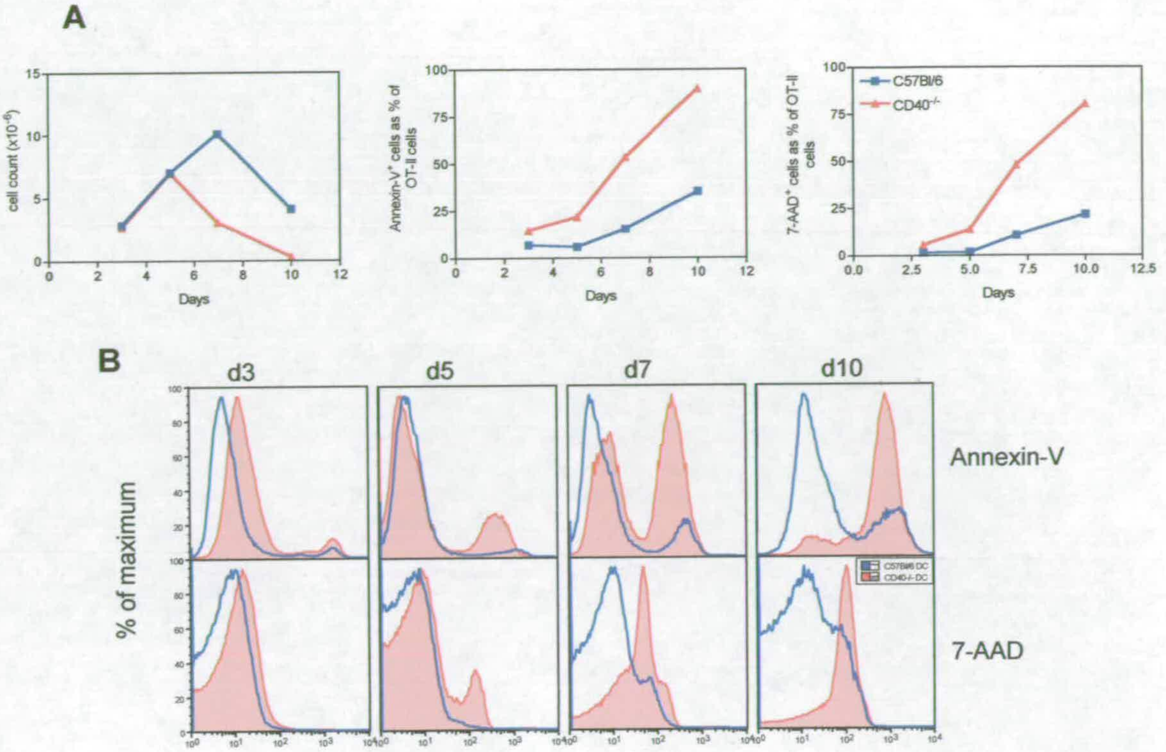


Figure 3.7 Role of CD40 in OT-II CD4 T cell apoptosis *in vitro*. 10^6 OT-II T cells were cultured with 10^5 BMDCs in the presence of 0.5μg/ml pOVA in 24-well plates at 37°C. At 3, 5, 7 and 10 days of culture, cells were harvested and counted using haemocytometer (A, left). Cells were stained with antibodies against CD4, $V\alpha_2$ & $V\beta_5$ and Annexin-V or 7-AAD, and number of apoptotic cells assessed by flow cytometry. The % of OT-II T cells positive for Annexin-V and 7-AAD are shown in A, middle and right plots, respectively. (B) Histogram overlay of Annexin-V and 7-AAD positive OT-II T cells cultured with C57Bl/6 (blue line) and CD40$^{-/-}$ (red tinted) DCs at indicated days of culture. Data shown is representatative of two independent experiments.

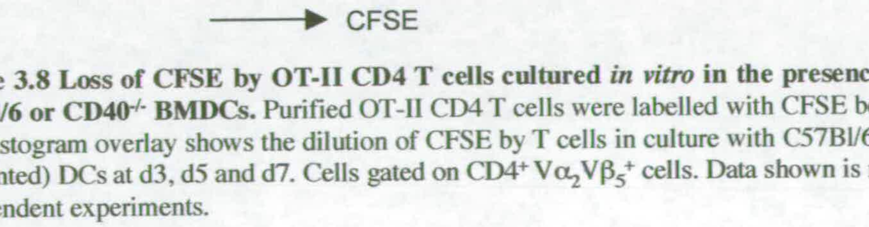


Figure 3.8 Loss of CFSE by OT-II CD4 T cells cultured *in vitro* in the presence of pOVA$_{323-339}$ and C57Bl/6 or CD40$^{-/-}$ BMDCs. Purified OT-II CD4 T cells were labelled with CFSE before culure with DCs. The histogram overlay shows the dilution of CFSE by T cells in culture with C57Bl/6 (blue line) or CD40$^{-/-}$ (red tinted) DCs at d3, d5 and d7. Cells gated on CD4$^+$ $V\alpha_2V\beta_5^+$ cells. Data shown is representatative of two independent experiments.

Requirement for CD40 interactions for T cell proliferation depends on the type of APC used, ligand density and the avidity of interactions among TCR, antigen and MHC molecules [254]. I hypothesized that due to lower affinity for the MHC-peptide, OT-II T cells are dependent on CD40 costimulation more than the DO11.10 T cells for proliferation. Conversely, stimulation of DO11.10 T cells with an altered peptide ligand (APL) might increase their dependence on costimulation for proliferation. To test this hypothesis, the following *in vitro* experiments with APLs were carried out.

3.7.1. APLs

Initial experiments were done to repeat the published results that DO11.10 T cells respond to lower amounts of $OVA_{323-339}$ than OT-II T cells [248]. $5x10^4$ purified CD4 T cells from DO11.10 and OT-II mice were cultured with $5x10^5$ irradiated splenocytes from BALB/c and C57Bl/6 mice as APC. Proliferation was measured by thymidine incorporation during the last 16h of 3d culture. As shown in figure 3.9, proliferation of DO11.10 T cells was much higher when antigen was presented on I-A^d APC as compared to I-A^b APC. In contrast to the findings of Robertson *et al.* [248,249], however, I did not observe any proliferation of OT-II T cells when stimulated with BALB/c APC.

Robertson *et al.* have shown that amino acid (aa) 333 of the OVA peptide is the primary contact residue for both DO11.10 and OT-II TCR transgenic T cells, and the core 9-aa epitope 329-337 recognised by both the cell types is presented by I-A^d as well as I-A^b MHC molecules [248]. Amino acid 331 is an important TCR contact residue for both cell types and substitutions at this residue may yield partial agonist peptides. Based on this report, two APLs were chosen: 331M and 331Q corresponding to methionine and glutamine substitutions at residue 331, respectively.

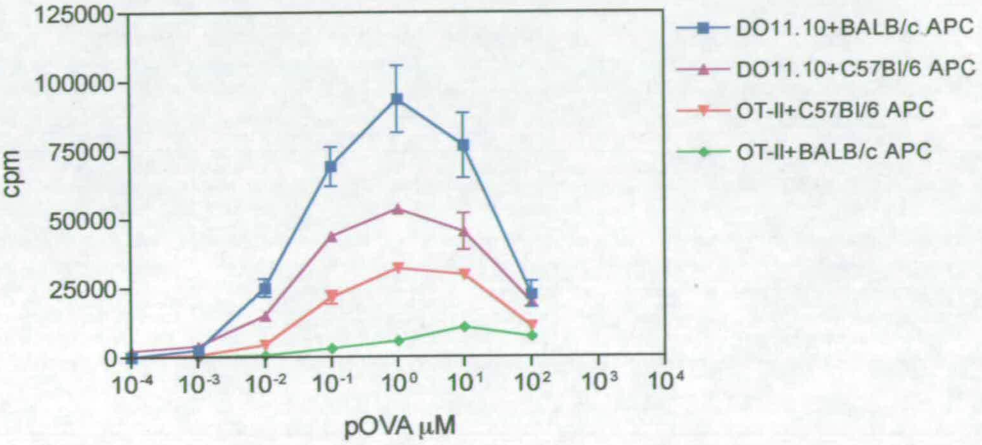


Figure 3.9 ***In vitro*** **proliferation of DO11.10 and OT-II cells.** CD4 T cells purified from spleens of naive DO11.10 or OT-II mice were cultured with titration of pOVA for 3 days with either irradiated BALB/c or C57Bl/6 splenocytes. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of culture. Data is shown as the mean of triplicate wells ± SEM.

3.7.2. Proliferation of CD4 T cells in response to pOVA and APLs

The proliferation of DO11.10 and OT-II T cells in response to pOVA and the APLs - 331M and 331Q was assessed. Since this was a direct comparison between DO11.10 and OT-II T cells, BMDCs derived from (BALB/c x C57Bl/6) F1 mice were used as APCs. 10^5 CD4 T cells were cultured with 10^4 DCs in the presence of titration of all three peptides. By thymidine incorporation at d3 of culture, figure 3.10A, OT-II T cells proliferated much less than the maximal proliferation exhibited by DO11.10 T cells. Moreover, OT-II T cells required 4000 times more pOVA (4µg/ml) in comparison to DO11.10 T (0.001µg/ml) cells to achieve half-maximal proliferation (10^5 cpm). The DO11.10 T cells responded to all the peptides in a dose dependent manner, but used 100x more 331Q and 1000x more 331M than pOVA to proliferate to the same level. Thus, for equal proliferation, DO11.10 T cells need higher concentrations of 331M and 331Q in comparison to pOVA owing to their lower affinity for MHC-APL complex.

Surprisingly, I did not observe any proliferation of OT-II T cells in response to the APLs presented on I-A^{bxd} (F1), thus OT-II cells could only respond to APLs presented by I-A^b at homozygous levels (see section 3.7.4). It is possible that all or most of the peptide was bound to I-A^d MHC molecules, so the OT-II cells could not be activated properly. The CFSE labelling experiments yielded similar data. DO11.10 and OT-II T cells were CFSE labelled before culture with DCs at fixed peptide concentration of 0.5µg/ml for all the antigens. DO11.10 T cells exhibited similar dilution of CFSE at d3 and d5 of culture in the presence of pOVA, 331Q and 331M. On the other hand, OT-II T cells divided and lost CFSE only in response to pOVA (fig 3.10B).

The background proliferation of DO11.10 T cells in response to all the antigens presented by F1 BMDCs was high (fig 3.10A). This may probably be due to allo-response to H-2^b component on the APCs, however, this was not the case when irradiated splenocytes from F1 mice were used as APCs (data not shown). Since the main aim of this experiment was to compare the responses of DO11.10 T cells to pOVA

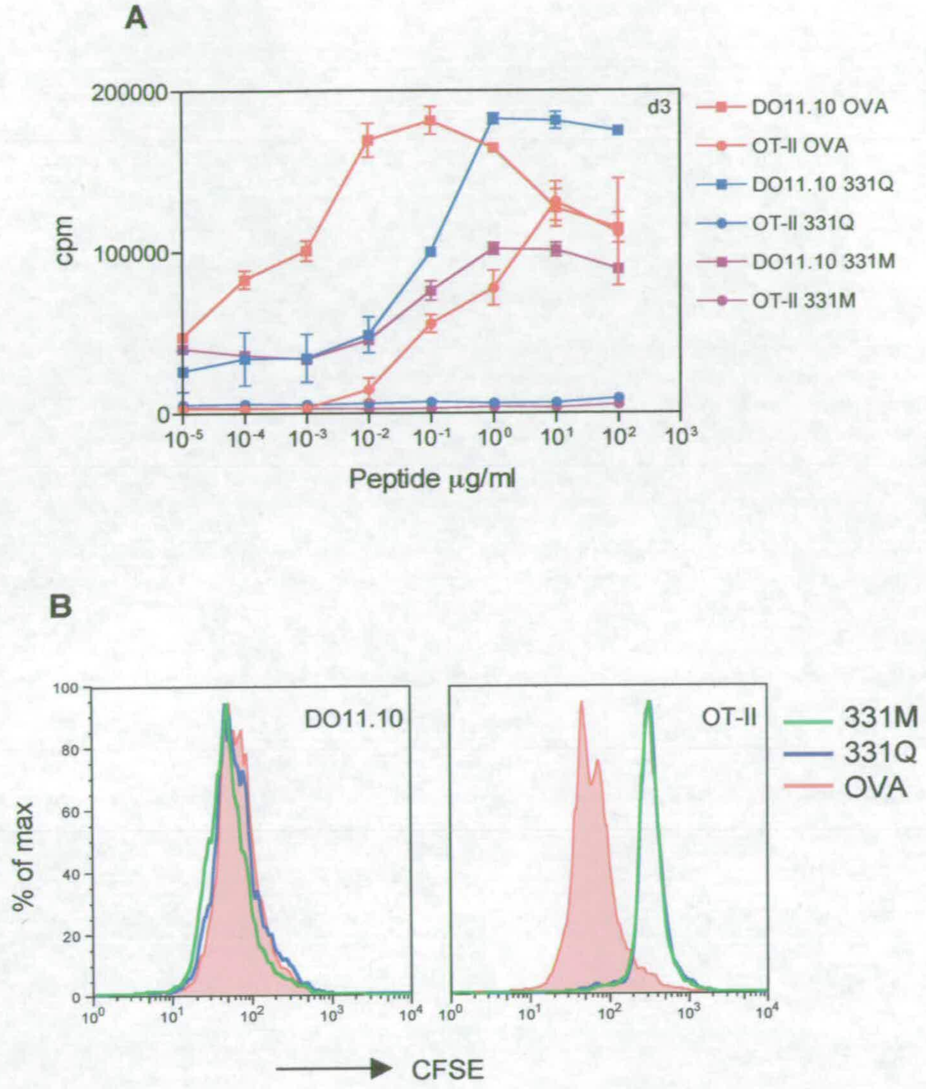


Figure 3.10 *In vitro* proliferation of DO11.10 and OT-II cells in the presence of pOVA and APLs. BMDCs were grown from (BALB/c x C57Bl/6)F1 mice. (A) 10^5 CD4 T cells purified from spleens of naive DO11.10 or OT-II mice were cultured with 10^4 DCs in the presence of a titration of pOVA and APLs 331Q and 331M. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3d culture. Data is shown as the mean of triplicate wells ± SEM. (B) 10^6 CFSE labelled CD4 T cells were cultured with 10^5 DCs in the presence of pOVA (red tinted), 331M (green line) and 331Q (blue line). The histogram overlay shows the dilution of CFSE of T cells at d3. Cells are gated on CD4$^+$KJ1.26$^+$ for DO11.10 cells (left plot) and CD4$^+$Vα_2V$\beta_5$$^+$ for OT-II cells (right plot).

and APLs, which were all presented by same DCs, I felt it was appropriate to use this system to study the role of CD40 in further experiments.

3.7.3. Proliferation of DO11.10 T cells in response to APLs is unaffected in the absence of CD40

5×10^4 DO11.10 T cells were cultured with 5×10^3 BMDCs from BALB/c and CD40$^{-/-}$ mice in the presence of varying concentrations of pOVA and 331M. At d3, proliferation of the T cells was measured by thymidine incorporation. Figure 3.11 shows that in response to either pOVA or 331M, DO11.10 T cells proliferated equally in the presence or absence of CD40 on the APCs.

Thus, lower affinity of the DO11.10 T cells for the APLs in the culture did not increase their requirement for costimulation in terms of CD40.

3.7.4. Proliferation of OT-II T cells in response to APLs presented on I-A^b MHC

5×10^5 irradiated splenocytes from C57Bl/6 and CD40$^{-/-}$ mice were used as APCs to stimulate 5×10^4 OT-II CD40$^{-/-}$ T cells in the presence of varying concentrations of pOVA, 331Q and 331M. OT-II cells responded to all the peptide antigens when presented on I-A^b MHC class-II. Slightly but not significantly lower proliferations were observed to all the antigens in absence of CD40 on APCs (fig 3.12).

3.8. *In vivo* studies: adoptive transfer experiments

As introduced in section 1.9.2, adoptive transfer technique involving transfer of TCR-transgenic cells into the mice followed by immunisation is an elegant way to study the clonal expansion of T cells *ex vivo* [233]. I took advantage of this system to study the role of CD40-CD40L interactions on T cell clonal expansion *in vivo*.

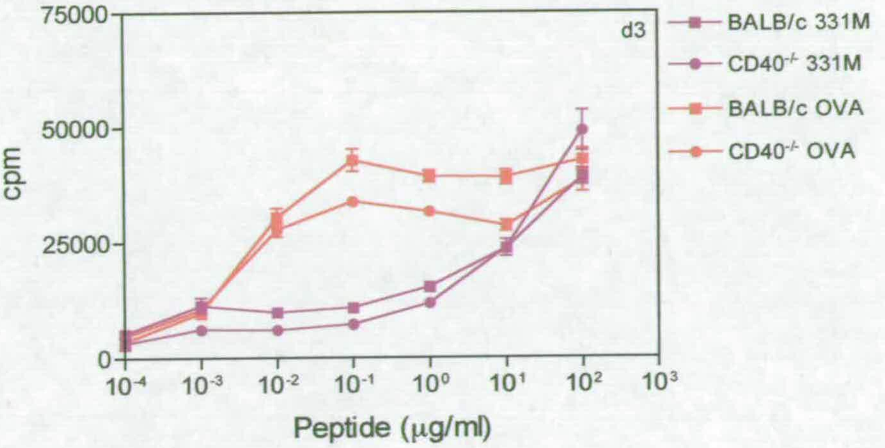


Figure 3.11 ***In vitro* proliferation of DO11.10 cells in the presence of pOVA and 331M.** BMDCs were grown from BALB/c and CD40$^{-/-}$ mice. 5x10^4 CD4 T cells purified from spleens of naive DO11.10 mice were cultured with 5x10^3 DCs in the presence of a titration of pOVA and 331M at 37°C. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3d culture. Data is shown as the mean of triplicate wells ± SEM. Data represents two independent experiments. Proliferation was minimal in the absence of antigen.

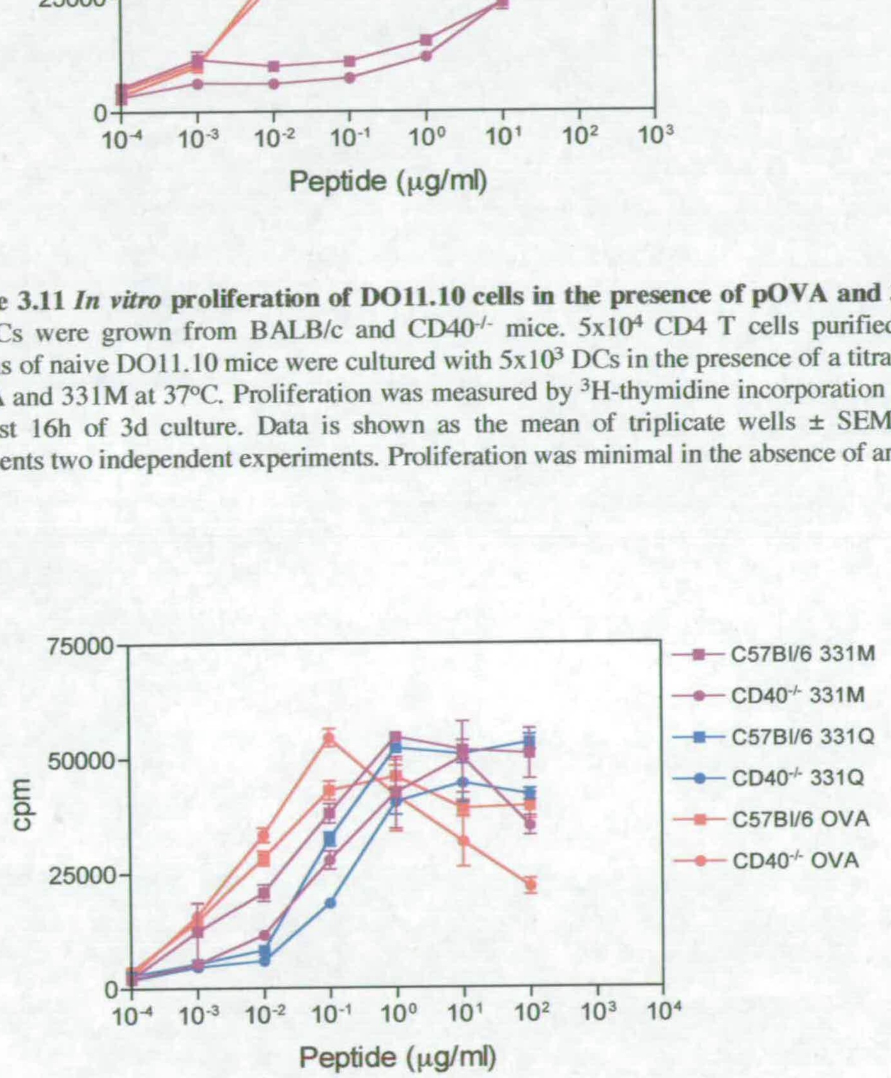


Figure 3.12 ***In vitro* proliferation of OT-II cells in the presence of pOVA, 331Q and 331M.** 5x10^5 irradiated splenocytes from C57Bl/6 and CD40$^{-/-}$ mice were used as APC to stimulate 5x10^4 CD4 T cells purified from spleens of naive OT-II mice in the presence of a titration of pOVA, 331Q and 331M at 37°C. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of 3d culture. Data is shown as the mean of triplicate wells ± SEM. Data represents two independent experiments. Proliferation was minimal in the absence of antigen.

3.8.1. Expansion of DO11.10 T cells

Lymph node cells from DO11.10 CD40$^{-/-}$ mice were injected i.v. in the tail vein of BALB/c and CD40$^{-/-}$ mice, 5x10^{6}/mouse, followed 24h later by i.p. immunisation with alum-precipitated ovalbumin and s.c. immunisation with ovalbumin-CFA in both thighs. The expansion of the DO11.10 T cells in the spleens and draining inguinal lymph nodes of the recipient mice was measured *ex vivo* at d3 and d6 after immunisation by flow cytometry. Cells were stained with anti-CD4 and KJ1.26 and the double positive cells indicated the extent of T cell clonal expansion (fig 3.13).

As shown in figure 3.13, at d3 after immunisation, the percent of KJ1.26^{+} CD4 T cells was similar in CD40$^{-/-}$ and wildtype groups, both in spleen as well as lymph nodes. At d6 however, the expansion in CD40$^{-/-}$ mice was reduced compared to BALB/c mice, although the difference was not significant. From this experiment, I felt that immunisation by single route i.e. either i.p. or s.c., would be a cleaner experimental protocol with results easier to analyse. When this experiment was repeated and mice immunised only i.p., I did not observe any defect in CD4 T cell expansion in the spleens of CD40$^{-/-}$ mice. Rather, the percent of DO11.10^{+} CD4 T cells was higher in CD40$^{-/-}$ mice compared to the wildtype mice (not shown).

Thus, DO11.10 T cells can get activated and undergo proliferation in the absence of CD40-CD40L interactions, but may need these signals to sustain the response, although these results await confirmation.

3.8.2. Expansion of OT-II cells

To see whether OT-II cells are dependent on CD40 for clonal expansion *in vivo*, OT-II CD40$^{-/-}$ lymph node cells were transferred into C57Bl/6 and CD40$^{-/-}$ syngeneic recipient mice followed 24h later by i.p. immunisation with ovalbumin-alum. At d3 and d6, the

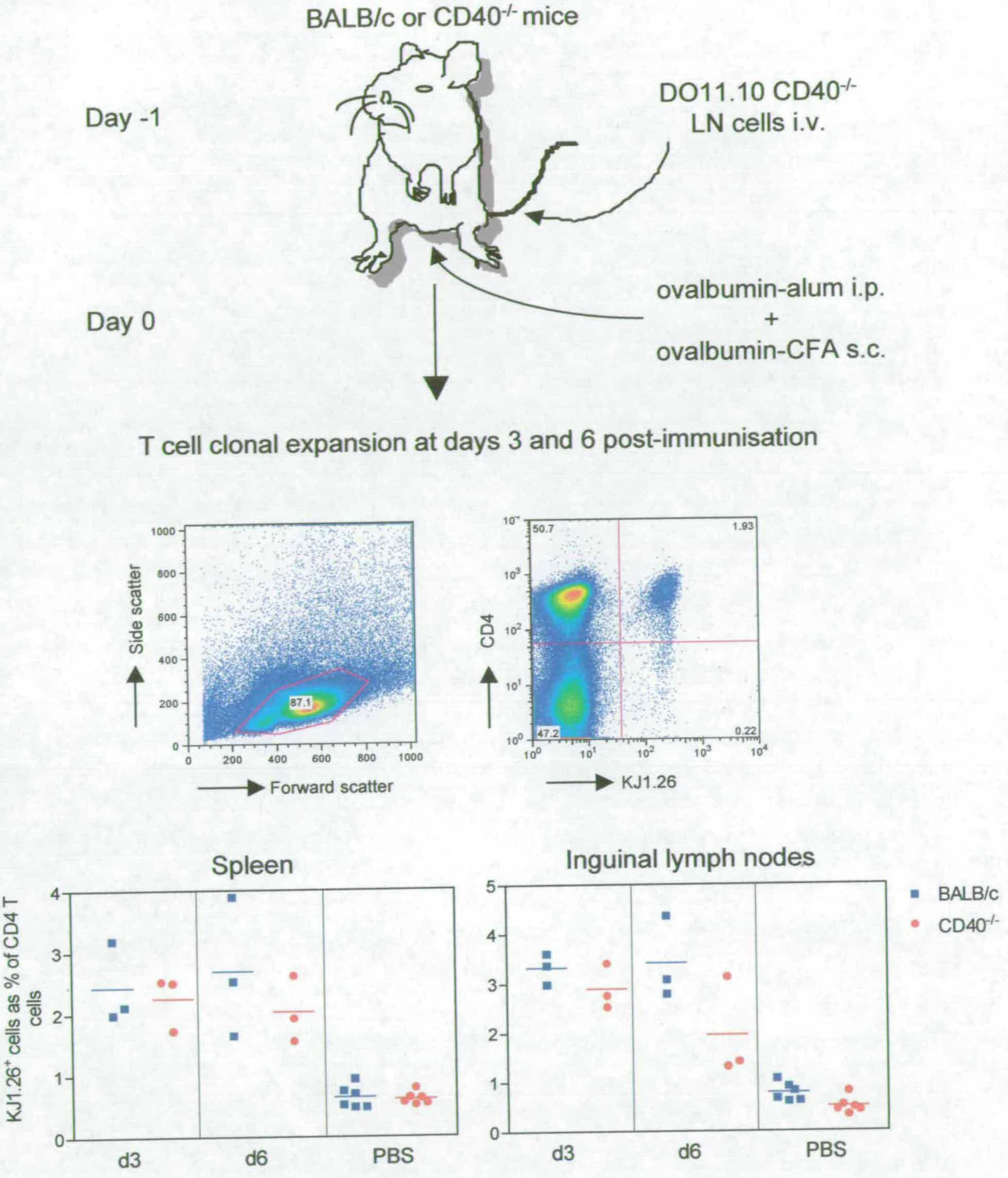


Figure 3.13 Role of CD40 in clonal expansion of DO11.10 T cells *in vivo*: adoptive transfer system. 5×10^6 lymph node cells from DO11.10 CD40$^{-/-}$ mice were transferred into BALB/c and CD40$^{-/-}$ mice followed 24h later by i.p. immunisation with alum-precipitated ovalbumin and s.c. immunisation with ovalbumin-CFA in thighs. The expansion of the DO11.10 T cells in the spleens and draining inguinal lymph nodes of the recipient mice was measured at d3 and d6 after immunisation by flow cytometry. Cells were stained with anti-CD4 and KJ1.26 and the double positive cells indicated the extent of T cell clonal expansion. Graph shows the KJ1.26$^+$ cells as percent of CD4$^+$ T cells. Each point represents an individual mouse. PBS controls harvested as d3 and d6 are shown together. This experiment was done twice.

expansion of transgenic cells was measured by staining the splenocytes with antibodies against CD4, $V\alpha_2$ and $V\beta_5$ and analysis by flow cytometry. At d3, OT-II T cells expanded to similar extent in wildtype and CD40-deficient hosts, however, at d6, the percent of $V\alpha_2V\beta_5^+$ T cells was significantly reduced in $CD40^{-/-}$ hosts in comparison to the wildtype mice (fig 3.14). The data shown suggests that the initial activation and proliferation of OT-II T cells is independent of CD40-CD40L interactions, but it is critical to sustain the proliferative response.

3.9. Discussion

In addition to the confirmed role of CD40-CD40L interaction in B cell responses [183], it has also been suggested that it is critical for T cell responses as well. Impaired T cell expansion of adoptively transferred CD40L-deficient CD4 T cells was demonstrated nearly a decade ago by Grewal *et al.* [72]. The importance of CD40-CD40L interactions in T cell responses was further confirmed in several other reports. Various *in vivo* studies have shown normal initial expansion of T cells in the absence of CD40-CD40L interactions, although this expansion was not sustained resulting in loss of antigen-reactive T cells [73,255,256]. Howland *et al.* found similar proliferation of $CD40L^{-/-}$ and wildtype DO11.10 T cells when cultured *in vitro* in the presence of high antigen concentration and high APC:T cell ratios, although at low concentrations of antigen or APC, $CD40L^{-/-}$ T cells proliferated less than wildtype T cells [73]. Miga *et al.* further argued that lack of CD40 on the DCs impaired their capacity to persist *in vivo*, and hence reduced T cell expansion [256]. In addition, in response to viral infections LCMV and vesicular stomatitis virus (VSV), Oxenius *et al.* observed efficient induction of CD4 T cells as well as T-effector function generation in $CD40^{-/-}$ and $CD40L^{-/-}$ mice [242]. Therefore, the precise role for CD40 costimulation in T cell responses including priming has not been clearly assigned.

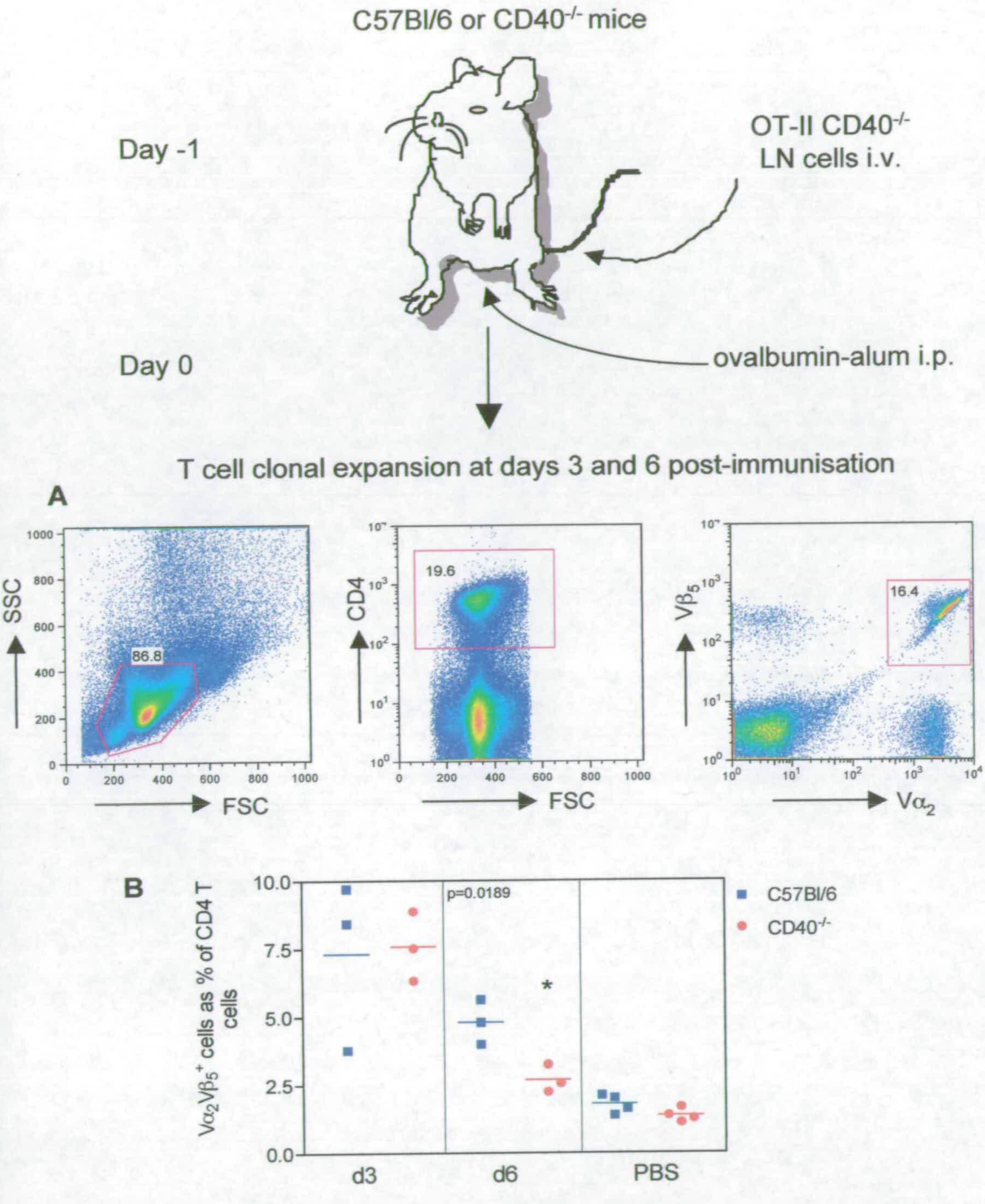


Figure 3.14 Role of CD40 in clonal expansion of OT-II T cells *in vivo*: adoptive transfer system. 5x10^6 OT-II CD$40^{-/-}$ lymph node cells were transferred i.v. into C57Bl/6 and CD$40^{-/-}$ mice followed by i.p. immunisation with alum-precipitated ovalbumin 24h later. At d3 and d6, spleens were harvested and cells stained with antibodies against CD4, Vα_2 and Vβ_5, and analysed by flow cytometry. CD4^+ cells expressing both Vα_2 and Vβ_5 indicate the expansion of transgenic cells. (A) shows the gating procedure to measure the expansion and the numbers show the percentage of cells in the gated box. (B) The Vα_2Vβ_5^+ cells expressed as percent of CD4^+ T cells. The data represents two independent experiments, each using 3-4 mice per group. PBS controls harvested as d3 and d6 are shown together. The groups were compared statistically using unpaired Student's t-test.

In the experiments described here, CD40-deficient DCs on the H-2^d background were not inherently defective in their ability to induce strong CD4 T cell responses. Indeed, responses of DO11.10 T cells to $pOVA_{323-339}$ or the APL 331M were similar when presented by either BALB/c or $CD40^{-/-}$ DCs. Responses induced by $CD40^{-/-}$ BMDCs were higher than those induced by wildtype DCs in approximately half the experiments. Although, I have no explanation for this variability, the key point is that proliferation was never reduced in the absence of CD40 expression on APCs. Similar observations have been reported by Ozaki *et al.* [254]. They found that $CD40^{-/-}$ splenic APCs induced higher proliferative response of DO11.10 T cells in comparison to $CD40^{+/+}$ APCs, however, they could not explain this finding either. By contrast, prominent differences were observed in the proliferation of OT-II T cells. Confirming an important role for CD40-costimulation, CD4 T cells from OT-II mice underwent less peptide-specific proliferation when $CD40^{-/-}$ DCs were used as APC, especially at d5. $CD40^{-/-}$ DCs induced similar proliferation of OT-II cells as C57Bl/6 DCs only when the APC to T cell ratio was high.

OT-II T cells underwent apoptosis in the absence of CD40 on APCs *in vitro*, perhaps, because the activation of APCs is not sustained in the absence of CD40-CD40L interactions. Miga *et al.* found that in addition to the physical persistence of the DC, CD40 triggering of DC also greatly increases the period for which they can productively present antigen to T cells [256]. In addition, the induction of OX40L on the APC is dependent on CD40 crosslinking [9]. The ligation of OX40L with OX40 on T cells promotes the expression of Bcl-xL and Bcl-2 thus prolonging the T cell survival [55].

The *in vivo* adoptive transfer experiments showed that the initial expansion of OT-II T cells was similar in wildtype and $CD40^{-/-}$ mice, but at d6 after immunisation, there were significantly lower numbers of transgenic T cells in $CD40^{-/-}$ mice as compared to the wildtype mice. Similar experiments done with DO11.10 T cells, although await confirmation, suggest that they may require CD40-CD40L interactions to sustain the response. Howland *et al.* have reported similar observations [73]. Considering that CD40L is upregulated on T cells only after activation, it is not surprising that T cells undergo initial expansion in the absence of CD40. The CD28-CD80/CD86 interactions are possibly more critical at the priming stage since CD28$^{-/-}$ T cells show greatly reduced proliferative responses *in vitro* and fail to expand *in vivo* as well [73]. Moreover, CD28 signalling induces the expression of other costimulatory molecules on T cells as well [57]. CD86 is constitutively expressed at low levels on DCs and B cells and is markedly upregulated on activated mature APCs [118]. After immunisation, T cell expansion may be maintained by continuous stimulation by antigen released from its depot. As the quantity of available antigen decreases and the innate immune response to the adjuvant subsides, CD40L-mediated stimulation of APCs becomes increasingly important.

The importance of CD40 on APC in T cell responses is two-fold. Its interaction with CD40L primarily signals to the APC, resulting in sustained activation of NFκB transcription factor, which regulate DC differentiation and cytokine production [65]. Bjorck *et al.* have shown that as a consequence of CD40 ligation, DCs become resistant to Fas-induced apoptosis, possibly as a result of upregulation of bcl-2 protein expression [66]. Furthermore, CD40-CD40L interaction results in signalling to the T cell through various TNF receptor associated factor (TRAF) family molecules eventually activating NFκB [257,258]. Although various studies have shown that CD40L enhances the T cell responses and the likely mechanism is by DC upregulation of costimulatory molecules, chemokines and cytokines [62,259], we and others have observed similar levels of MHC-II, CD80 and CD86 on splenic DCs from either wildtype or CD40$^{-/-}$ mice [255]. Furthermore, Ozaki *et al.* showed that purified B cells but not splenic APC from CD40$^{-/-}$ mice were deficient in inducing T cell proliferation *in vitro*. Although CD40-CD40L interactions upregulate important costimulatory molecules like CD80, CD86, CD44 and ICAM-1 on B cells making them efficient APC, prior upregulation of these molecules by LPS pre-treatment did not increase their efficacy as APCs [254].

Although I have not addressed the role of CD40 on effector T cell differentiation, the literature suggests that this interaction is crucial for induction of Th1 responses. Howland *et al.* found that DO11.10 CD40L$^{-/-}$ T cells had a selective defect in IFNγ production *in vitro* [73]. Moreover, Stüber *et al.* found an increase in IL-4 production following the administration of a blocking anti-CD40L antibody to mice in a murine colitis model [260]. The inability to differentiate into Th1 cells may be due to the fact that in the absence of CD40-CD40L interactions, T cells fail to activate APCs to produce the essential Th1-inducing cytokine, IL-12, since exogenous IL-12 could restore the IFNγ response of CD40L$^{-/-}$ T cells [67,73].

Recent findings that CD4 as well as CD8 T cells can express CD40 [77] and APCs like B cells and monocytes can express CD40L [75,76] have blurred the otherwise sharp demarcations of the expression of these molecules. However, use of T cells from DO11.10 or OT-II mice bred onto CD40$^{-/-}$ background excludes the possibility of any CD40L binding on the T cells in our assays. Moreover, this ensured that any contaminating CD4^{+} DC in the purified T cell preparations would be CD40$^{-/-}$.

Using (C57Bl/6 x BALB/c) F1 DCs in *in vitro* proliferation assays, we showed that DO11.10 T cells respond to lower doses of antigen as compared to OT-II cells. Moreover, DO11.10 T cells exhibited much bigger proliferative response to pOVA presented by F1 DCs. This confirms the previous reports that DO11.10 T cells may have higher affinity receptor for the peptide-MHC complex and for self-MHC [248,249]. Moreover, experiments done in the lab by M. MacLeod showed that the two transgenic cells proliferate differently *in vivo*. CFSE labelled OT-II and DO11.10 transgenic cells were transferred into (C57Bl/6 x BALB/c) F1 mice that were immunised the following day with alum-precipitated pOVA$_{323-339}$. After 5 days, DO11.10 cells were found to proliferate more than OT-II cells.

It was hypothesized that the lower affinity of DO11.10 T cells for analogue peptides 331M and 331Q, might correspondingly increase the requirement of these cells for costimulatory signals to proliferate. However, the proliferation of DO11.10 T cells in response to pOVA or 331M was similar, whether or not CD40 was present on the APCs. Thus, this study did not shed any light on why DO11.10 T cells are independent of CD40 costimulation. Nevertheless, it does suggest that study of one type of TCR transgenic T cells does not do justice to the complex physiology of T cells *in vivo*. Although such T cells provide invaluable models to study T cell responses, it may be inappropriate to draw conclusions based on them, because of variation among different cells in terms of affinity for peptide and/or MHC and other activation or differentiation requirements.

In terms of the affinity for MHC-peptide complex, OT-II T cells may represent the majority of T cells whereas the DO11.10 T cells may lie at the far end of the bell shaped curve with very high affinity. Considering the fact that DO11.10 mice were made using the T cell hybridoma generated in response to chicken ovalbumin [261], these T cells do represent a subset of high affinity T cells that might naturally exist. This illustrates the importance of examining more than one clone of T cells before assigning definitive roles to particular molecular interactions in T cell mediated immune responses.

Collectively, these findings suggest that requirement for CD40 is much more stringent for low affinity TCR-peptide-MHC interactions of OT-II T cells than for high affinity DO11.10 T cells.

4. Role of ICOS-ICOSL interaction in immune responses

Background

For an effective T cell response, recognition of antigen and additional signals from costimulatory receptors are required. Costimulation can promote more efficient engagement of TCR molecules to enhance the initial activation and can also provide additional signals to promote cell division, augment cell survival or induce effector functions. Many receptors have recently been described to be costimulatory, ICOS, a CD28 homologue, being one of them.

It has previously been suggested that ICOS interactions are not involved in the initial response of naïve T cells to antigen, as ICOS is not expressed on resting T cells [104]. This conclusion is supported by *in vitro* studies where naïve transgenic T cell responses to peptide remained intact despite ICOS-ICOSL interactions being blocked by an ICOS-Ig fusion protein [100]. The issue of ICOS involvement in T cell activation and proliferation has been addressed mainly by *in vitro* studies. Among the few *in vivo* studies, although Mak *et al.* did not find reduced T cell proliferation in the absence of ICOS signalling, Smith *et al.* found that adoptively transferred DO11.10 cells proliferated less in the presence of a blocking ICOS fusion protein, in both Th1 and Th2 inducing environments [97,106]. Therefore, the precise role for ICOS costimulation in T cell responses including priming has not been clearly assigned. The different results described probably reflect the varying protocols used: ICOS fusion protein, antibodies and different knockout mouse strains examined. In this chapter I have studied the role of ICOS in the clonal expansion of T cells by *in vivo* adoptive transfer studies using OT-II TCR transgenic T cells. I have also addressed effector T cell differentiation in the context of this interaction.

The phenotype of both ICOS and ICOSL knockout mice has demonstrated the importance of this interaction in the B cell response in both the generation of germinal centres and class switching [93,94]. However, its role in direct T-B collaboration has received limited attention. The B cell responses in ICOS$^{-/-}$ mice have been examined in this chapter by quantitating the clonal expansion in primary and secondary responses. Also, the class switching in terms of serum antibody levels as well as number of antibody secreting cells has been assessed.

Results

While the ICOS$^{-/-}$ mice were being made in our lab, several reports were published characterising such mice made in other labs. The initial experiments were done to confirm the phenotype of the mice generated in this lab. To confirm that gene targeting has abolished ICOS expression, T cells from mice immunised with alum-precipitated ovalbumin, were stained with anti-ICOS antibody and analysed by flow cytometry. ICOS was expressed on the surface of CD4^{+} ICOS$^{+/+}$ T cells, but was undetectable from ICOS$^{-/-}$ T cells (fig 4.1).

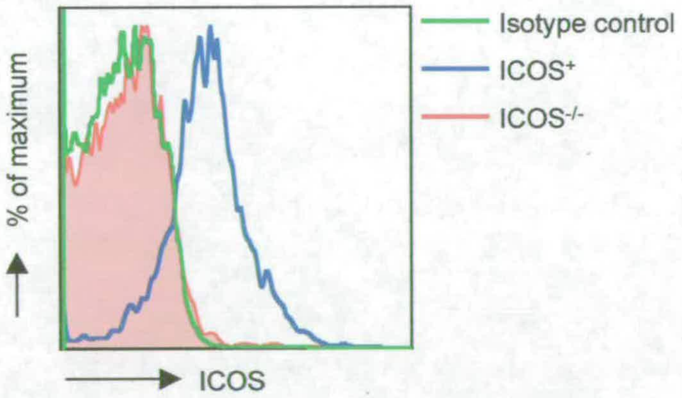


Figure 4.1 Expression of ICOS on the surface of CD4^{+} T cells. ICOS^{+} and ICOS$^{-/-}$ mice were immunised i.p. with alum-precipitated ovalbumin. 48h later, spleens were harvested, splenocytes stained with antibodies against CD4 and ICOS and analysed by flow cytometry. The histogram overlay depicts the expression of ICOS on CD4^{+} cells in the splenocytes of ICOS^{+} and ICOS$^{-/-}$ mice. Data is representative of two separate experiments.

4.1. Role of ICOS in humoral immune responses

4.1.1. Antigen-specific serum Immunoglobulins

Since ICOS has been implicated in T-B cell collaboration, initially we investigated the role of ICOS in humoral immune responses. The role of ICOS in generation of antigen specific antibody response was examined. ICOS$^{-/-}$ and ICOS^{+} mice were immunised with haptenated protein DNP-KLH intraperitoneally. The amount of DNP-specific immunoglobulin isotypes in the sera was measured at days 0, 7 and 13 post-immunisation by ELISA (fig 4.2). DNP-specific IgM was modestly reduced in ICOS$^{-/-}$ mice at d7 although the difference was not as pronounced at d13. A marked defect was observed in the class switched isotypes in the ICOS$^{-/-}$ mice. The anti-DNP IgG response was around 2.5-fold lower in ICOS$^{-/-}$ mice at d7 with a further reduction at d13 when it was around 5-fold lower. Similar reduction in titres of DNP-specific IgG_1 was noticed in the sera of ICOS$^{-/-}$ mice. ICOS$^{-/-}$ mice elicited markedly reduced IgG_{2a} response both at d7 (10-fold lower) and d13 (20-fold lower). Reduction in the titres of IgG_3 and IgG_{2b} was notable but less marked than the other isotypes. Hence, interaction between T and B cells via ICOS-ICOSL is critical for B cells to undergo class switching of antibodies.

4.1.2. Germinal centre reaction

We next evaluated the GC formation in the spleens of ICOS$^{-/-}$ and ICOS^{+} mice after immunisation with DNP-KLH in alum. This was done by staining spleen sections with peanut agglutinin (PNA)-FITC and IgM-Texas Red (see section 2.8). PNA selectively binds to B cells in GCs. At d13 after immunisation, ICOS$^{-/-}$ mice had fewer and smaller GCs than ICOS^{+} mice (fig 4.3).

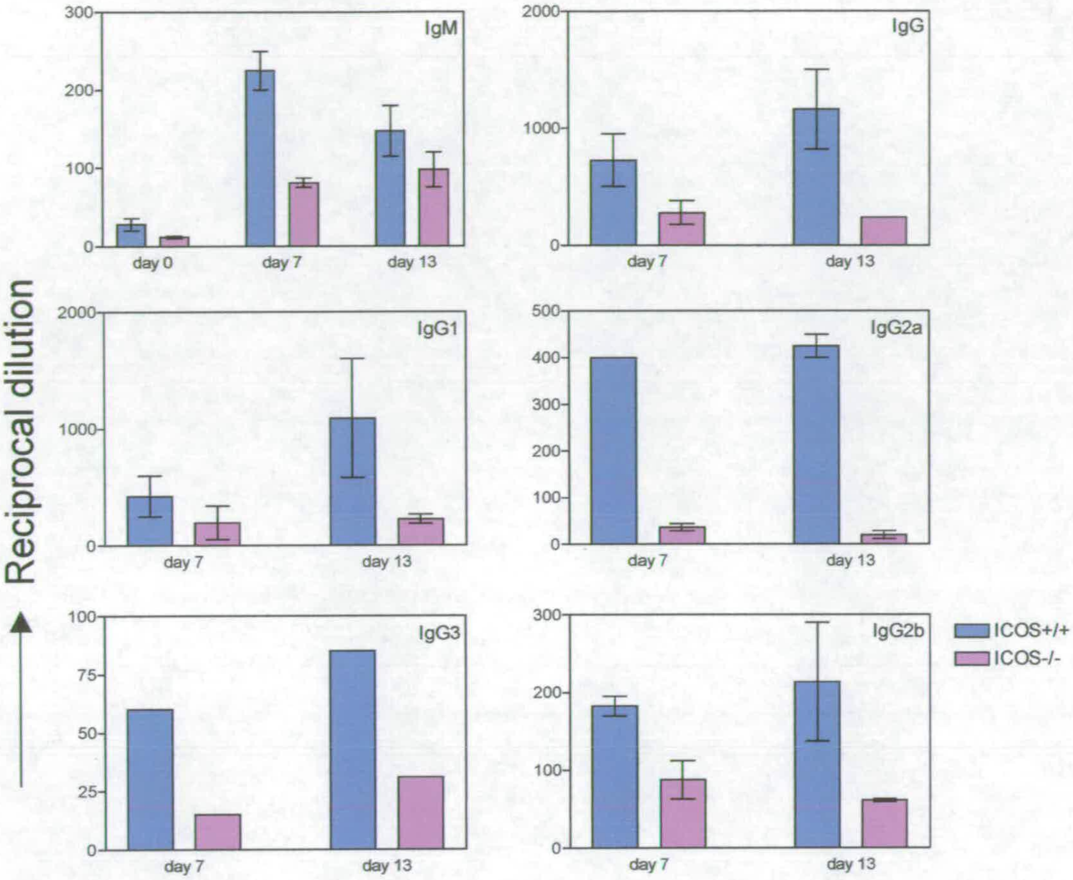


Figure 4.2 Antigen-specific immunoglobulins in the sera of ICOS$^+$ and ICOS$^{-/-}$ mice. Mice were immunised with 100µg alum-precipitated DNP-KLH i.p. once d0 serum samples were taken. Mice were bled 7 and 13 days post-immunisation and serum was separated from blood and stored at -20°C until used. The amount of DNP-specific antibodies of various isotypes was measured by ELISA (section 2.11.2). Comparative titres were calculated by plotting serum concentration against OD, and reading the dilution at the half-maximal OD, compared with a positive control. The class switched antibodies were below the detection limits at d0, hence not shown. Both ICOS$^+$ and ICOS$^{-/-}$ groups consisted of 2 mice each. Data is representative of two independent experiments. The error bars indicate the standard error of mean (SEM).

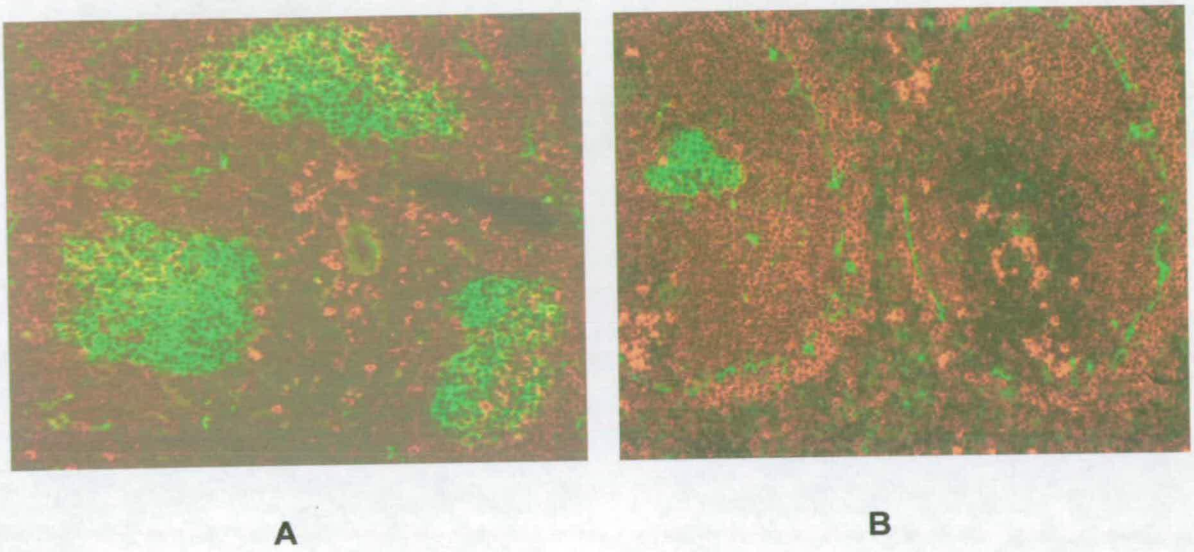


Figure 4.3 Germinal centre formation in the spleens of ICOS knockout and wildtype mice. wildtype (A) and $ICOS^{-/-}$ (B) mice were immunised i.p. with 100μg alum-precipitated DNP-KLH. Two weeks after immunisation, mice were killed and sections from their spleens were examined for the presence of germinal centres (section 2.8). Sections depicted here are stained with FITC-conjugated peanut agglutinin (PNA) and anti-IgM-Texas Red. PNA selectively binds to B cells in germinal centres and IgM is B cell marker. (A) Large PNA^+ GC in a wildtype mouse. (B) Small PNA^+ GC in the spleen of $ICOS^{-/-}$ mouse. PNA also stains the stromal elements that surround the B cell follicle. Original magnification 200x.

4.1.3. B cell clonal expansion

Given that ICOS$^{-/-}$ mice have few small GCs, I next investigated whether interaction with T cells in the absence of ICOS affects the B cell priming and clonal expansion.

Primary response

ICOS$^{-/-}$ and ICOS^{+} mice were immunised intraperitoneally with alum-precipitated Phycoerythrin (PE). Since strong adjuvants can bypass the requirement of costimulation, killed Pertussis bacteria were not used in the immunisation protocol. Spleens were harvested 2 weeks following the immunisation, cells stained with PE and FITC-labelled anti-CD19 mAb ID3 and analysed by flow cytometry. By the addition of PE, we can detect the PE-binding B cells. Background PE staining in un-immunised mice was minimal. As seen in figure 4.4A, a small population of ID3^{+}PE^{+} cells is clearly visible at this time point. At this early time point, these are probably mostly PE-specific, affinity matured, GC B cells, although there will also be some memory cells and antibody secreting cells in the spleen. The percent of PE-specific B cells was markedly reduced in the ICOS$^{-/-}$ mice as compared to the C57Bl/6 mice. When PE^{+} cells/spleen were compared, there was a significant reduction in the number of PE^{+} B cells in the ICOS$^{-/-}$ mice than C57Bl/6 mice (fig 4.4B).

Secondary response

Mice primed with PE were re-challenged 5 weeks later to evaluate if this interaction is required for the B cell secondary response. Spleens were harvested 5 days after the boost and PE^{+} B cells enumerated by FACS. There was a significant reduction in the percent

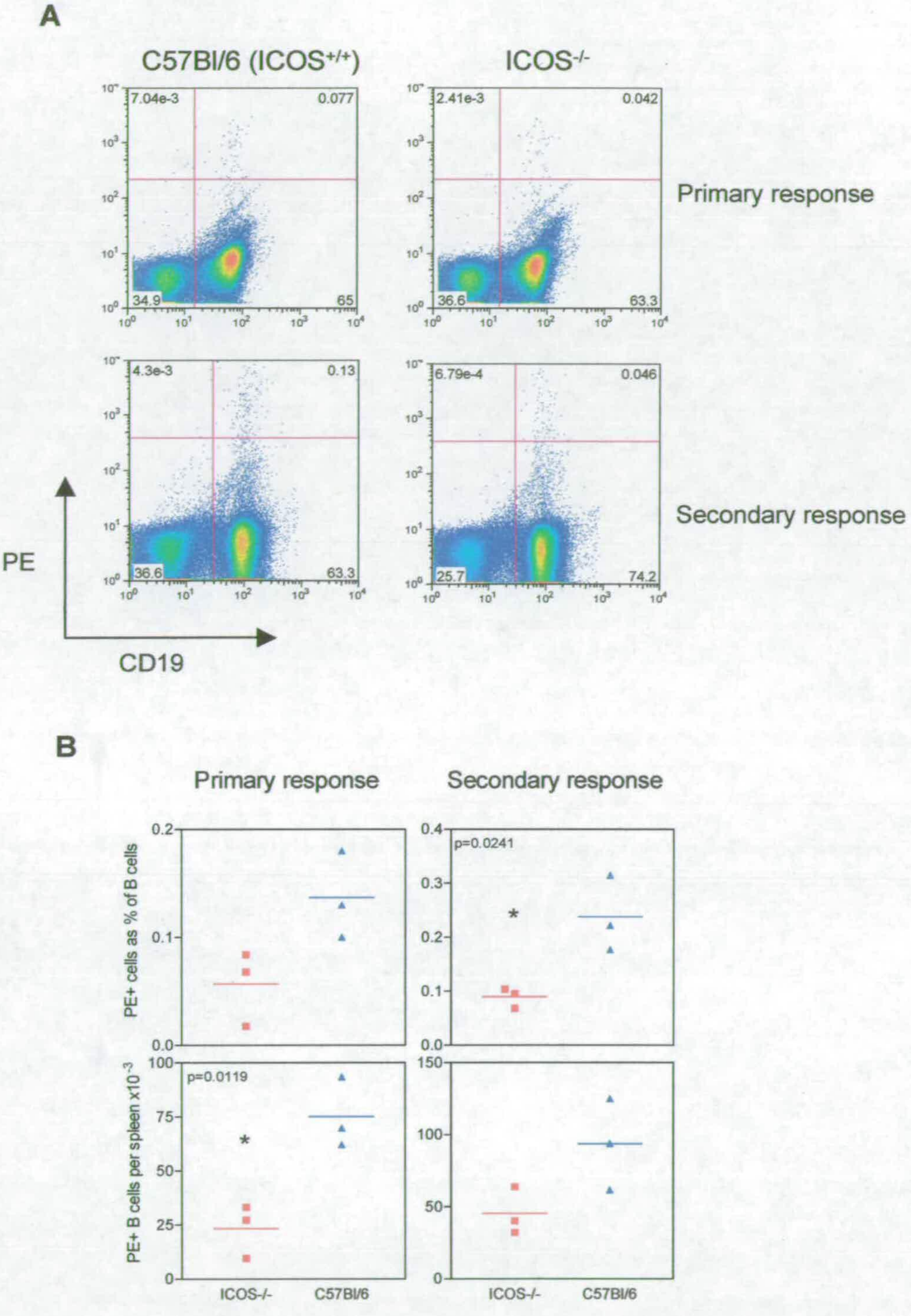


Figure 4.4 B cell clonal expansion during primary and secondary response in ICOS$^+$ and ICOS$^{-/-}$ mice. Mice were immunised i.p. with 100μg alum-precipitated PE. Two weeks later, spleens were harvested and splenocytes stained with anti-CD19 and PE. For secondary response, mice were boosted 5 weeks later with 50μg alum-precipitated PE and spleens harvested 5 days later. (A) FACS plots showing the expansion of antigen-specific B cells. Upper right quadrant of the FACS plots indicate the PE+ B cells. (B) PE+ cells plotted as % of CD19$^+$ B cells in the top graphs. Total PE$^+$ B cells per spleen shown in the bottom graphs. The background staining in the non-immunised mice was minimal. Data is representative of three independent experiments, each using three mice per group. The two groups were compared statistically by unpaired Student's t-test.

of PE$^+$ B cells in ICOS$^{-/-}$ mice as compared to C57Bl/6 mice (fig 4.4B). However, when the total number of PE specific B cells in the spleen was compared between the two groups, the difference was not significant. This was probably due to the variation among C57Bl/6 mice. Thus, ICOS-ICOSL interaction is necessary for the B cells to undergo maximal clonal expansion both during primary and secondary responses.

4.1.4. Enumeration of the Antibody Secreting Cells (ASC)

Given that B cell clonal expansion as well as isotype class switching is defective in the absence of ICOS, we expected the number of antibody secreting cells in ICOS-deficient mice to be affected as well. This was confirmed by ELISPOT assay.

Primary response

ICOS$^{-/-}$ and control mice were immunised i.p. with alum-precipitated DNP-KLH (200μg/mouse). Frequency of DNP-specific antibody secreting cells in the spleen and the bone marrow was measured 3 weeks after immunisation by ELISPOT assay. Figure 4.5 shows that the number of ASCs producing class switched antibodies (IgG and IgG_1) was lower in the ICOS$^{-/-}$ mice in comparison to ICOS$^+$ mice. However, the number of IgM$^+$ ASCs in the spleen was higher in ICOS$^{-/-}$ as compared to control mice. Similar trend was noticed in the bone marrow. Splenocytes from un-immunised ICOS$^{-/-}$ as well as ICOS$^+$ mice were also plated as controls. Negligible number of class switched ASCs were detected, if any, in wells from these mice. However, some variability in the background levels of anti-DNP IgM$^+$ ASCs in unimmunised mice was noticed, with one mouse having fairly high numbers in the bone marrow. This may be due to cross-reactivity between DNP and a ubiquitous environmental antigen.

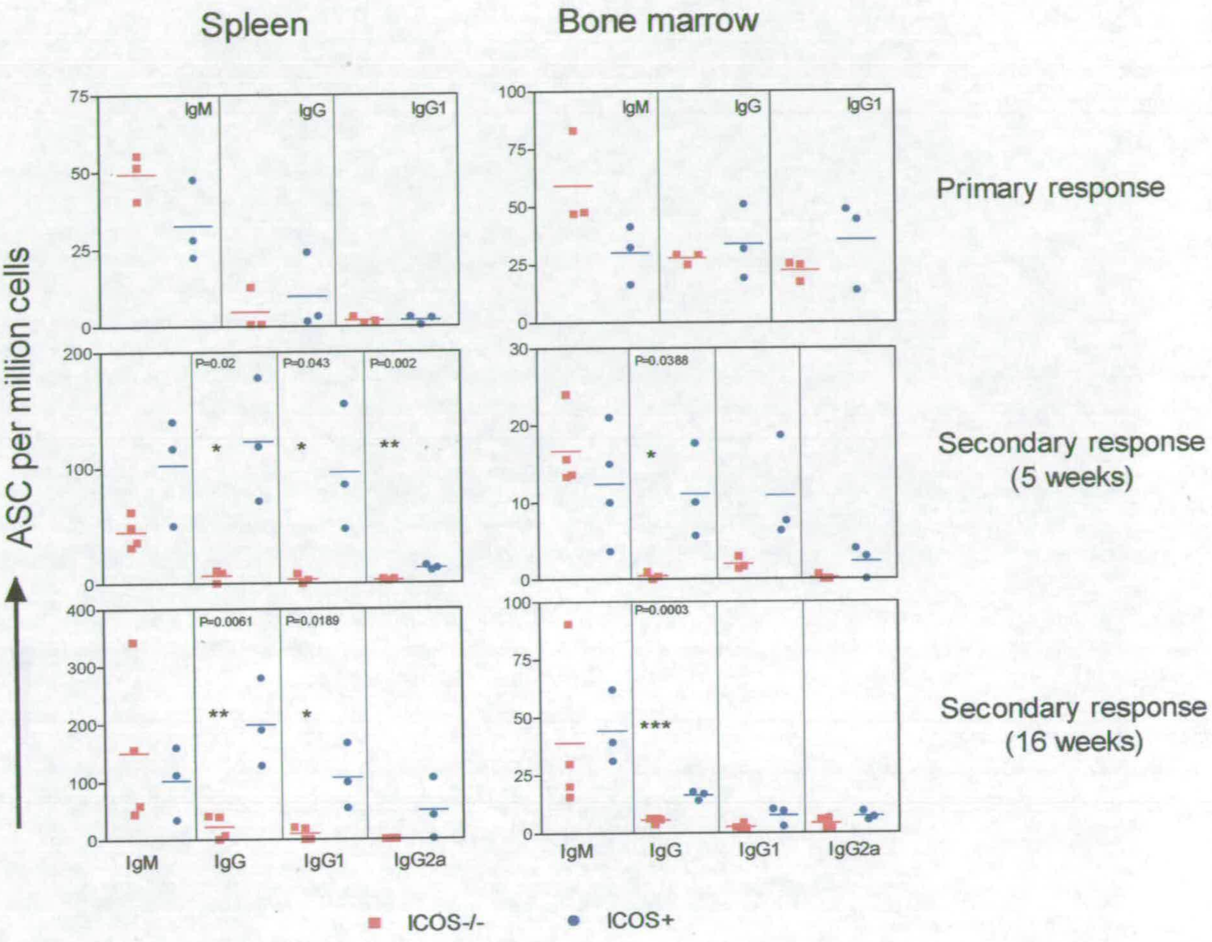


Figure 4.5 Number of antibody secreting cells during primary and secondary response in ICOS$^+$ and ICOS$^{-/-}$ mice. Mice were immunised with 200µg alum-precipitated DNP-KLH i.p. and three weeks later, spleen and bone marrow (from femurs) were harvested. ELISPOT was performed to eneumerate the ASC (section 2.11.3). The spots were counted manually using a dissection microscope. The class switched ASC in non-immunised control mice were minimal, Some variability was observed in IgM ASC in control mice. Both ICOS$^+$ and ICOS$^{-/-}$ groups consisted of 3-4 mice each. Each point in the graph represents an individual mouse. Data is representative of two independent experiments except 16 weeks time point which was done once. The two groups were compared statistically by unpaired Student's t-test.

Secondary response

5 weeks after the primary immunisation, mice were boosted with 100µg DNP-KLH-alum, and spleens and bone marrow harvested 5 days later. The number of ASCs was higher in the spleens as expected for B cell recall responses, however, this was not the case for bone marrow cells. Probably a 5-day period after the boost was not long enough for the ASC to expand in the bone marrow.

Effect of the absence of ICOS was much more pronounced in the secondary response, as there was significant reduction in the number of ASC producing the class switched antibodies (IgG, IgG_1 and IgG_{2a}) in the spleen. A similar trend was noticed in the bone marrow, although the difference was not significant in terms of IgG_1 and IgG_{2a}, which was probably due to the variation among mice within $ICOS^+$ group. IgM producing ASCs in the spleen of $ICOS^{-/-}$ mice were notably reduced as well (fig 4.5).

On the whole, DNP-specific ASCs especially IgG_{2a}, were fewer in the bone marrow in the secondary response at 5 weeks. Probably this time was not long enough for maximal plasma cells migration into the bone marrow. To address this issue, mice were immunised and re-challenged 16 weeks later. 5 days after the boost, spleen and bone marrow were harvested and ASCs enumerated. The number of ASCs in the bone marrow was higher compared to the 5-week time point. Generally, the number of isotype switched ASCs was same as at 5 weeks with the exception of one mouse in the $ICOS^{-/-}$ group that gave an unusual very high IgM response both in the spleen and the bone marrow (fig 4.5).

From the data shown so far, ICOS-ICOSL interactions play a crucial role in B cell responses. The secondary B cell responses are particularly deficient in the absence of these interactions suggesting a role of ICOS in the generation or reactivation of antigen-specific memory B cells.

4.1.5. Help Assay

From the results so far, it is clear that ICOS-ICOSL interaction is involved in the T cell help for B cells to produce class switched antibodies. We wanted to validate this hypothesis using carrier-hapten based *in vitro* help assay.

To investigate whether ICOS-deficient T cells can help primed wildtype B cells to class switch, we set up an *in vitro* culture with DNP-primed purified C57Bl/6 B cells and ovalbumin-primed ICOS deficient or sufficient, purified $CD4^+$ T cells in the presence of soluble DNP-OVA (refer materials and methods, section 2.10.2). The medium containing the antigen was removed after 2 days and replaced by fresh medium. Culture supernatants were harvested 5 days later and DNP-specific IgM, IgG, IgG_1 and IgG_{2a} measured by ELISA. As shown in figure 4.6, the IgM response was unaffected by the absence of ICOS. However, T cells lacking ICOS were unable to help primed B cells to class switch. The amounts of IgG, IgG_1 and IgG_{2a} were significantly lower in the supernatants of cultures with $ICOS^{-/-}$ T cells as compared to the $ICOS^+$ T cells.

Reduced serum levels of DNP-specific IgG_1 and IgG_{2a} in $ICOS^{-/-}$ mice and inability to help DNP-primed B cells to produce IgG_1 and IgG_{2a} by $ICOS^{-/-}$ T cells strongly suggest that it is not just Th2 response that is reduced in $ICOS^{-/-}$ mice; these T cells were equally defective in supporting B cell switching to IgG_{2a}, which is a Th1 isotype.

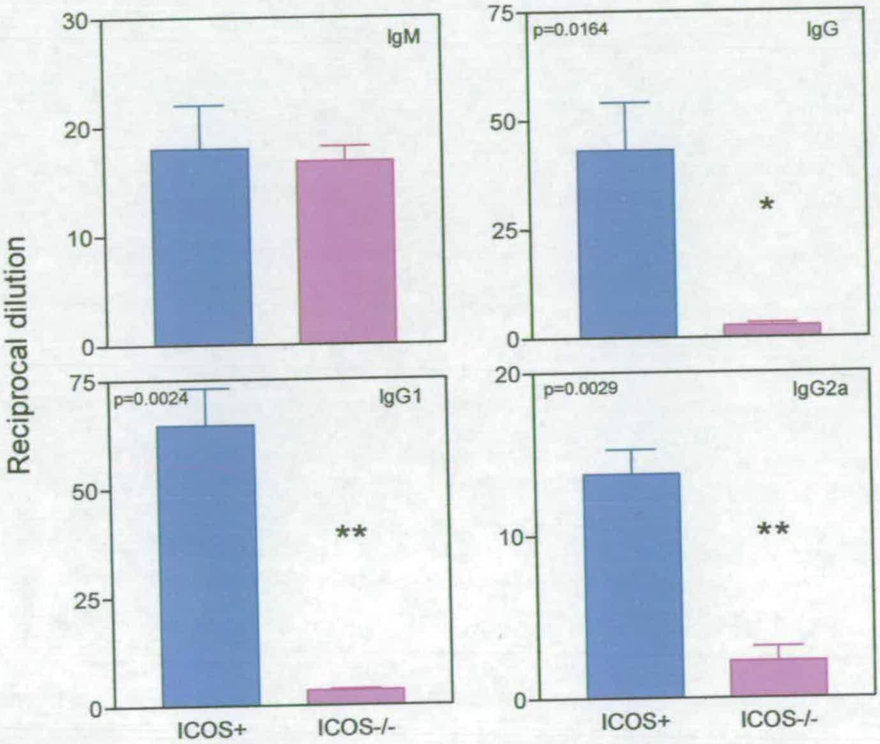


Figure 4.6 ICOS$^{-/-}$ T cells are unable to help wildtype B cells to class switch *in vitro*. B cells were purified from the spleens of C57Bl/6 mice immunised i.p. with alum-precipitated DNP-KLH 200µg/mouse two weeks before the assay. T cells were purified from the spleens of ICOS^{+} and ICOS$^{-/-}$ mice that were immunised i.p. with alum-precipitated ovalbumin 150µg/mouse a week before the assay. Purified B and T cells were cultured in the presence of soluble DNP-ovalbumin at 37°C. Medium containing the antigen was replaced by fresh medium after 2 days. The culture supernatant was harvested 5 days later and the amount of DNP-specific antibodies measured by ELISA. Comparative titres were calculated by plotting the concentration against OD, and reading the dilution at the half-maximal OD, compared with a positive control. Data is representative of three independent experiments, each using 3 mice per group. The error bars indicate the SEM. The two groups were compared statistically by unpaired Student's t-test.

4.2. Role of ICOS in T cell responses

4.2.1. Role of ICOS in T cell proliferation *in vitro*

To examine if ICOS is required for the proliferation of CD4 T cells *in vitro*, ICOS$^{-/-}$ and ICOS$^{+/+}$ mice were immunised with alum-precipitated DNP-KLH intraperitoneally. After 7 and 13 days, *in vitro* proliferation assays were set up with splenocytes in the presence of varying concentrations of KLH. Tritiated-thymidine was added 48h after the culture, and proliferation measured 16h later.

Similar proliferation was noted irrespective of the presence or absence of ICOS on T cells from mice immunised either 7 or 13 days earlier (fig 4.7).

4.2.2. Role of ICOS in T cell activation: activation markers

Activated T cells are often identified by the expression of newly synthesized surface proteins, called activation markers. CD44 is expressed on variety of cell types including mature T cells, thymocytes, B cells and macrophages. Recently activated and memory T cells express higher levels of CD44 than do naïve T cells. Its ability to bind hyaluronate, a matrix molecule, is responsible for the retention of antigen-stimulated T cell in lymphoid organs and at peripheral sites of infection and antigen persistence. CD62L, or L-selectin, expressed on lymphocytes and other leucocytes, serves as homing receptor to lymph node high endothelial venules. It is expressed at high levels on naïve T cells; activated T cells downregulate CD62L and upregulate carbohydrate ligands for CD62P and CD62E. These two selectins are expressed on inflamed endothelial cells and enable the activated T cell to transmigrate into the inflamed tissue [262]. CD69 is not expressed on resting lymphocytes but is induced within 2 hours of activation on lymphocytes.

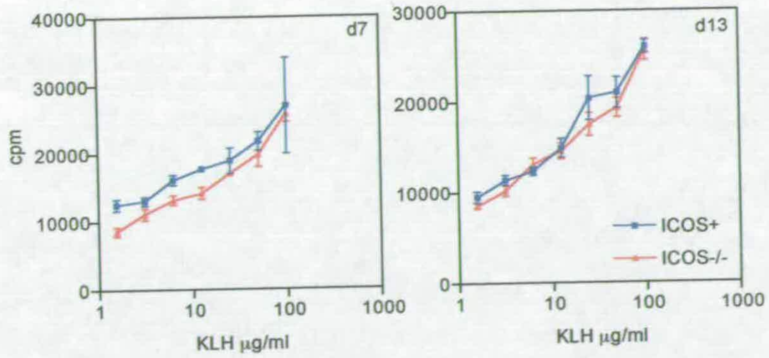


Figure 4.7 Role of ICOS in T cell proliferation *in vitro*. ICOS$^+$ and ICOS$^{-/-}$ mice were immunised i.p. with 100μg alum-precipitated DNP-KLH. 7 and 13 days later, spleens were harvested and *in vitro* proliferation assays were set up with splenocytes in the presence of titration of KLH. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM and is representatative of three independent experiments. Proliferation was less than 2500cpm in the absence of antigen.

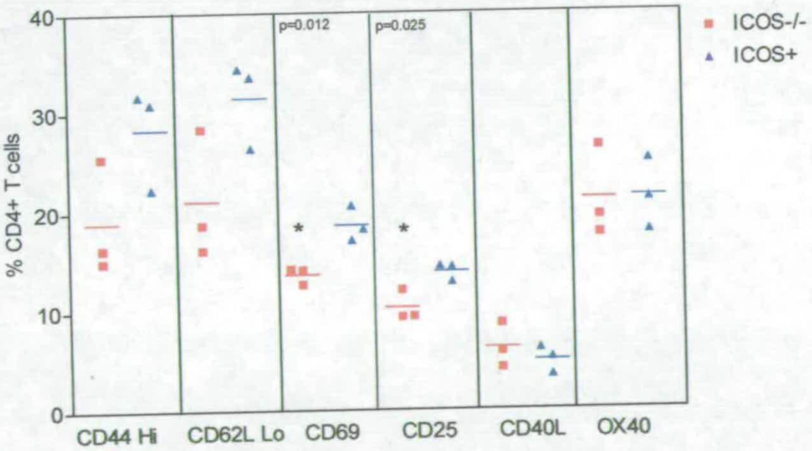


Figure 4.8 Activation markers on CD4$^+$ T cells. ICOS$^+$ and ICOS$^{-/-}$ mice were immunised i.p. with 200μg alum-precipitated ovalbumin. Ten days later, splenocytes were stained with antibodies against CD4 and various activation markers and analysed by flow cytometry. Data is shown as the percentage of CD4$^+$ T cells expressing the indicated marker. Both groups consisted of three mice each, represented by individual points in the graph. Unpaired Student's t-test was used to compare the groups statistically.

CD25 is the α-chain for IL-2 receptor and is upregulated on lymphocytes upon activation. CD43 is present on most of the cells except resting B cells. It has been suggested to have a positive regulatory role in costimulation and negative regulatory role in down-modulation of the immune response [263].

To see if ICOS has a role in activation of T cells, ICOS sufficient or deficient mice were immunised i.p. with alum-precipitated ovalbumin and splenocytes examined for various activation markers on the $CD4^+$ T cells 10 days post-immunisation. As depicted in figure 4.8, fewer $ICOS^{-/-}$ $CD4^+$ T cells exhibited activated phenotype in terms of CD44 high (18.93±3.3) and CD62L low (21.1±3.72) in comparison to $ICOS^+$ mice (28.3±3.0, CD44 high; 31.47±2.54, CD62L low). This indicates that fewer CD4 cells get activated in the absence of ICOS.

Cells expressing CD69 were significantly reduced in $ICOS^{-/-}$ mice (13.68±0.49) in comparison to $ICOS^+$ mice (18.64±1.02). Even though CD69 is an early activation marker, since alum was used as adjuvant during immunisation, antigen persistence lead to longer T cell activation period. Significant difference was noticed in the number of $CD4^+$ T cells expressing CD25 with 10.28±0.89 $ICOS^{-/-}$ and 13.9±0.82 $ICOS^+$ T cells expressing the marker. CD43 was expressed by lower number of $CD4^+$ T cells in $ICOS^{-/-}$ mice (89.42±1.07) as compared to $ICOS^+$ mice (93.44±1.43). Number of cells expressing costimulatory molecules like CD40L and OX40 did not differ between the $ICOS^+$ and $ICOS^{-/-}$ mice (fig 4.8). Thus, ICOS seems to be important for comprehensive activation of the CD4 T cells.

4.2.3. Role of ICOS in antigen-specific T cell priming and clonal expansion *in vivo*

The role of ICOS in antigen-specific CD4 T cell priming and clonal expansion *in vivo* was studied by two different techniques: MHC-II tetramers and adoptive transfer of TCR transgenic cells. Since for adoptive transfer technique, the OT-II mice needed to be crossed onto ICOS$^{-/-}$ background, we initially addressed this issue by MHC-II tetramer technique.

4.2.4. MHC class-II Tetramers

"MHC-II tetramers" are a recently developed tool for tracking antigen-specific CD4 T cells *in vivo* [234]. We used MHC-II tetramers in ICOS$^{-/-}$ and ICOS^{+} mice to determine whether there is a role of ICOS in primary T cell activation and expansion during a polyclonal T cell response.

The MHC class II tetramers were obtained from Ton Schumacher's research lab at the Netherlands Cancer Institute in Amsterdam [239]. The tetramer constructs, as shown in figure 4.9, were designed to enhance the production of the tetramers: the T cell epitope (H19env) was covalently attached to the extracellular domain of the beta chain (antigen link in figure); heterodimerization in the insect cells was promoted by the addition of leucine zippers to the COOH-terminus of both chains; a His-tag was attached to the alpha chain to aid purification of the tetramers and a biotin-tag added to the beta chain to allow tetramer formation. The tetramers were finally generated by the addition of streptavidin-PE, which has 4 binding sites for biotin. Monomers were removed by gel filtration chromatography, and the tetramers stored at -20°C in glycerol.

The tetramers are specific for H19env, a peptide from an envelope protein of Moloney Murine Leukaemia virus, so the mice were immunised with H19env peptide emulsified

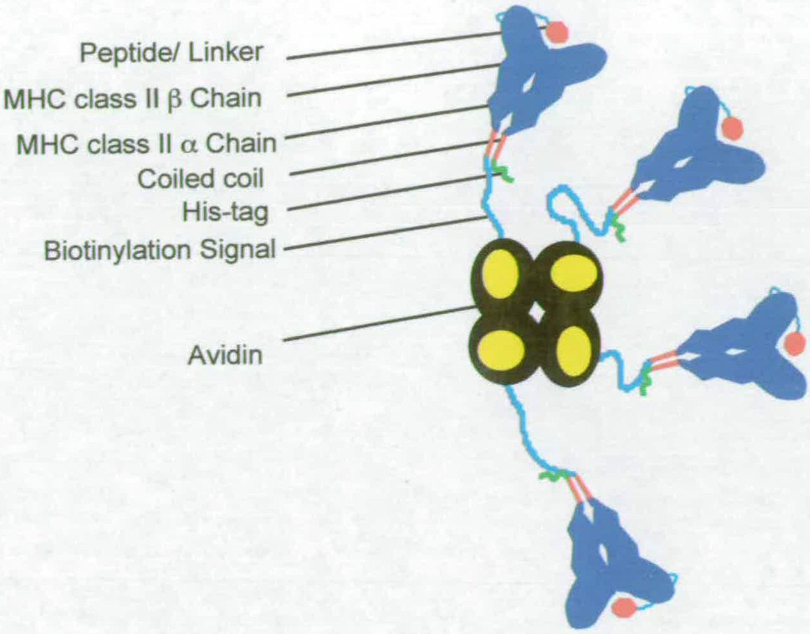


Figure 4.9 Schematic drawing of MHC class II tetramers.

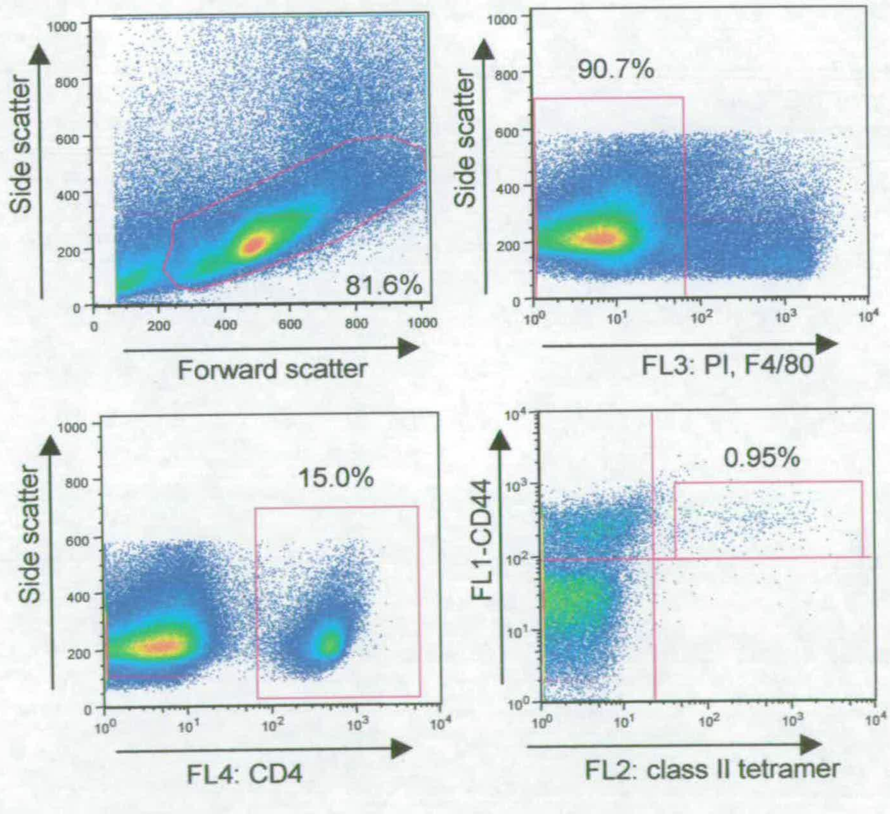


Figure 4.10 Gating analysis for tetramer positive cells: To analyse tetramer positive cells, first a broad lymphocyte gate was drawn. FL3 positive cells (macrophages and dead cells) were gated out. Of the remaining cells, only CD4 positive cells were analysed for tetramer staining in conjuncture with CD44 expression. The number shows the percentage of cells in the gated box.

in CFA, a potent adjuvant, which forms an antigen depot at the injection site [264]. Mice were immunised subcutaneously in the hind leg, which primarily drains to the inguinal lymph nodes. The draining lymph nodes and spleens were taken at various time points after immunisation and stained with class II tetramers. The tetramer staining in response to H19env was characterised by Megan MacLeod in the lab.

To identify tetramer positive cells, a live lymphocyte gate was drawn from which macrophages and dead cells were excluded by gating out cells positive for F4/80 and propidium iodide. This is essential to remove the background staining since the antigen-specific tetramer positive population is so small. The CD4 cells were then gated and the proportion of cells positive for tetramers and expressing high levels of CD44 was measured. Figure 4.10 shows the gating procedure. Cells from mice immunised with $pOVA_{323-339}$, an irrelevant peptide for the tetramers, were used as background staining control.

As shown in figure 4.11, the percent of tetramer positive CD4 T cells in the spleen as well as the draining lymph node at day 8 was equivalent in $ICOS^{-/-}$ and wildtype mice. At day 15 however, fewer tetramer positive T cells were observed in the lymph node of $ICOS^{-/-}$ mice, although this difference was not significant. Using this method, T cell priming was not reduced in the absence of ICOS signalling.

4.2.5. Adoptive transfer system

The adoptive transfer system involves transferring T cells from OT-II OVA-specific TCR transgenic mice into syngeneic host mice. Immunisation with ovalbumin 24h after adoptive transfer induces proliferation of transgenic OVA-specific T cells. The T cell expansion can be measured *ex vivo* by staining for variable chains $V\alpha_2$ and $V\beta_5$ of the TCR, as there is no clonotypic antibody available to stain these transgenic cells. The

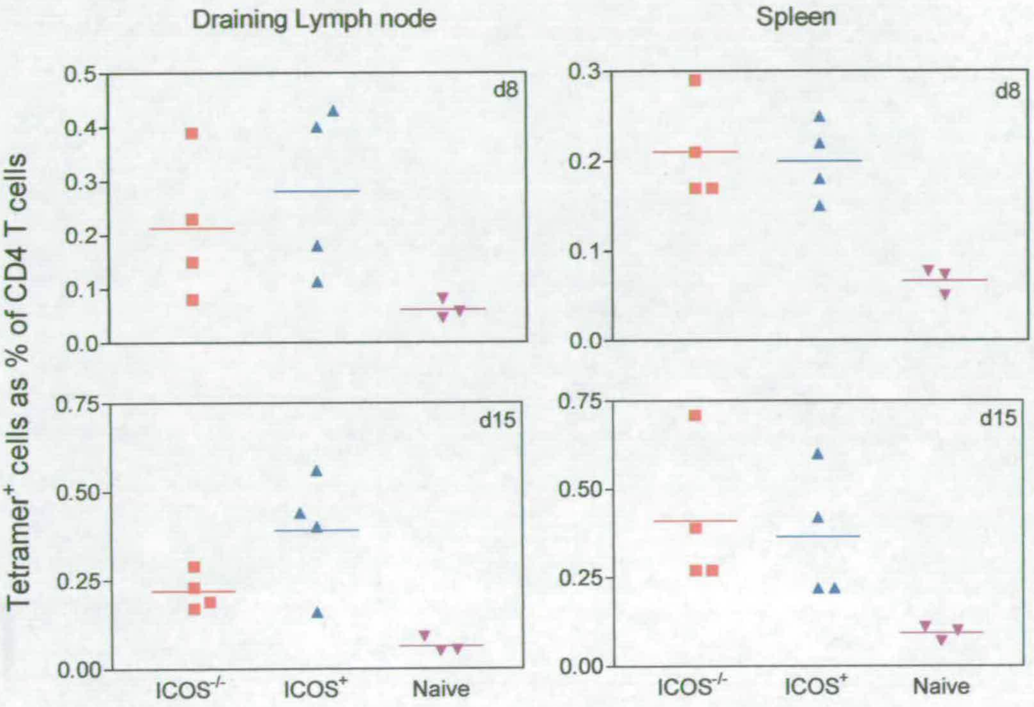


Figure 4.11 T cell priming is unaffected in the absence of ICOS. Tetramer positive cells expressed as percent of CD4$^+$ T cells in the spleens and draining lymph nodes of ICOS$^+$ and ICOS$^{-/-}$ mice 8 and 15 days after s.c. immunisation with H19env-CFA. Each point represents an individual mouse and the line shows the mean of the group. There was no significant difference between both groups at d8 or d15 by unpaired Student's t-test. Data is representative of two independent experiments.

$CD4^+$ T cells that are double positive for $V\alpha_2$ and $V\beta_5$ as enumerated by flow cytometry, indicate the extent of clonal expansion (fig 4.12).

T cell expansion following intra-peritoneal immunisation with ovalbumin-alum

Since I wanted to ascertain the role of ICOS in T cell clonal expansion, $ICOS^{-/-}$ mice expressing the OT-II transgenic TCR were generated so that peptide-specific T cell proliferation could be examined directly in adoptive transfer experiments. A cohort of either ICOS-deficient or ICOS-sufficient transgenic OT-II lymph node cells were transferred into naïve C57Bl/6 mice followed by i.p. immunisation with alum-precipitated ovalbumin 24h later. The expansion of $CD4^+$ T cells in these mice was followed over a time course between 3-8 days post-immunisation (fig 4.12).

In this adoptive transfer system, the peak of the clonal expansion is at day 4. My results show that T cell priming in the spleen is significantly reduced in the absence of ICOS-ICOSL interaction. The percent of $V\alpha_2V\beta_5$ positive $CD4^+$ T cells was significantly lower in the clonal expansion phase when transferred transgenic T cells lacked ICOS (fig 4.13C). The reduction in the number of OT-II $ICOS^{-/-}$ T cells was also significant in comparison to OT-II $ICOS^+$ at the later time point of d8, which corresponds to the contraction, activation-induced cell death (AICD) phase. The kinetics of the response was similar in both groups except that expansion of ICOS-deficient T cells did not peak at d4 (fig 4.13D). Unlike wildtype OT-II T cells, $ICOS^{-/-}$ transgenic T cells did not proliferate further after initial expansion at d3 and entered the clonal deletion phase. Since the *in vitro* proliferation of the OT-II splenocytes was unaffected in the absence of ICOS, these results highlight the importance of studying cellular interactions in the more complex environment of *in vivo* models (fig 4.13A).

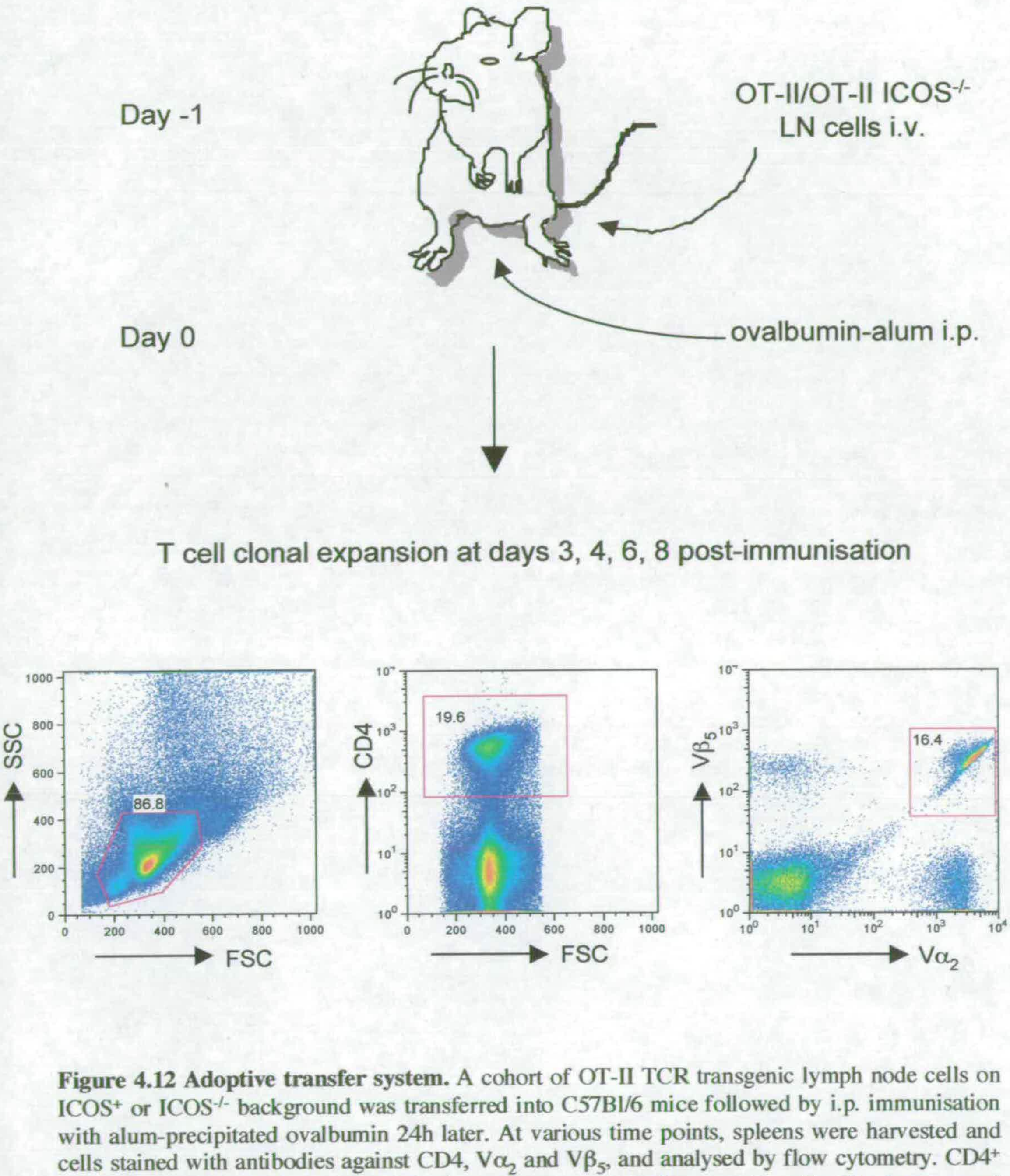


Figure 4.12 Adoptive transfer system. A cohort of OT-II TCR transgenic lymph node cells on $ICOS^+$ or $ICOS^{-/-}$ background was transferred into C57Bl/6 mice followed by i.p. immunisation with alum-precipitated ovalbumin 24h later. At various time points, spleens were harvested and cells stained with antibodies against CD4, $V\alpha_2$ and $V\beta_5$, and analysed by flow cytometry. $CD4^+$ cells expressing both $V\alpha_2$ and $V\beta_5$ indicate the expansion of transgenic cells. The lower panel shows the gating procedure to measure the expansion and the numbers show the percentage of cells in the gated box.

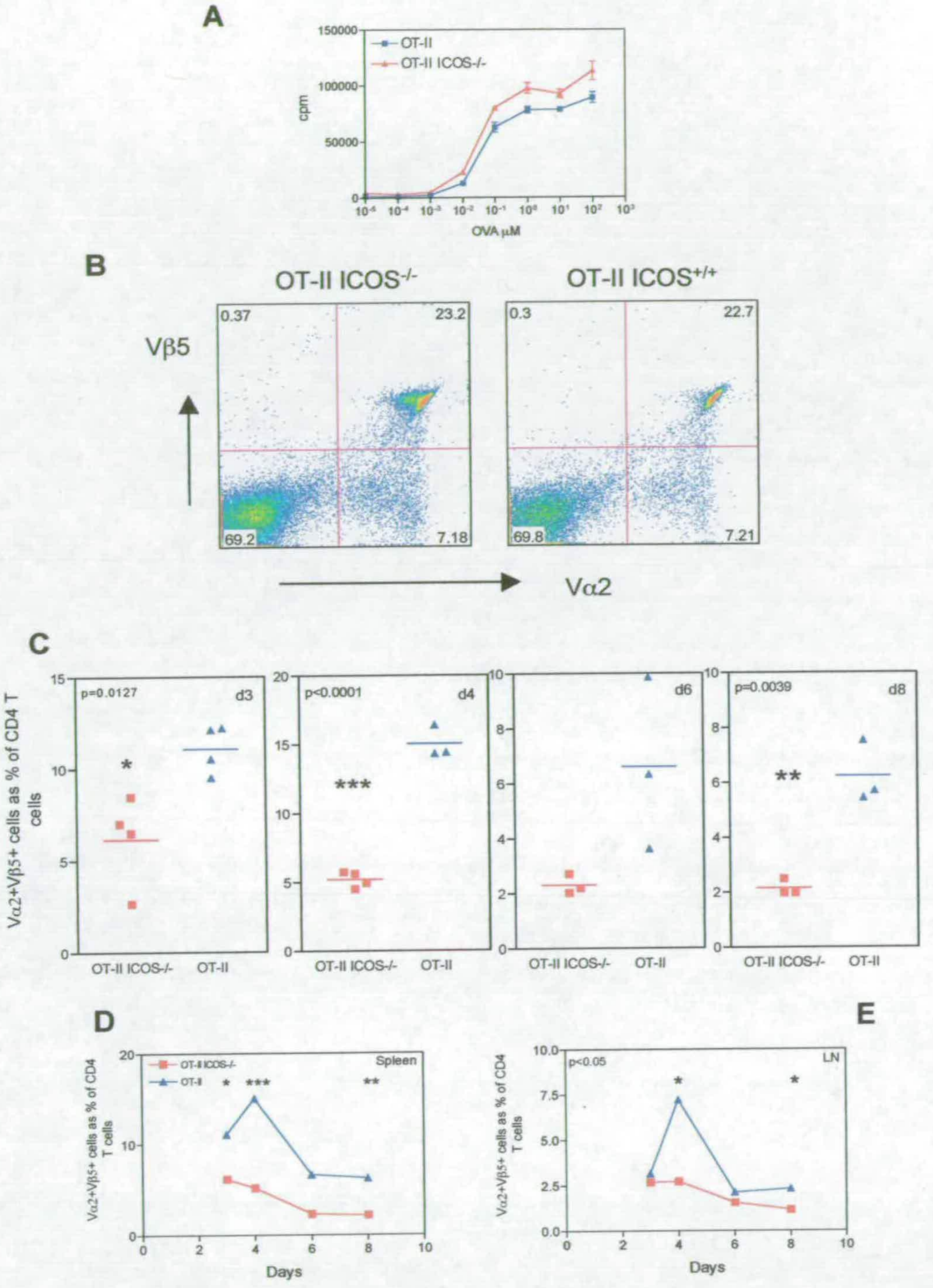


Figure 4.13 Role of ICOS in T cell clonal expansion. C57Bl/6 mice received OT-II ICOS^{+} or OT-II ICOS$^{-/-}$ lymph node cells before immunisation with ovalbumin-alum i.p., and the expansion of Vα_2Vβ_5^+ cells measured at days 3, 4, 6 and 8 in the spleen and inguinal lymph nodes. The data is representative of three independent experiments, each using 3-4 mice per group per time point. (A) *In vitro* proliferation of splenocytes from OT-II ICOS^{+} and OT-II ICOS$^{-/-}$ donor mice, measured by ^{3}H-thymidine incorporation at d3. (B) FACS plots depicting similar proportion of Vα_2Vβ_5^+ cells in the lymph node suspension used for adoptive transfer. Cells are gated on live lymphocytes. (C) Vα_2Vβ_5^+ cells as % of CD4 cells in the spleen of recipient mice at indicated time points. Both groups were statistically compared using unpaired student's t-test. The % of OT-II cells in spleens of control mice was invariably between 0.5-1%. (D) Time course of the T cell proliferative response in spleen. (E) Time course of T cell response in inguinal lymph node.

The expansion of $V\alpha_2V\beta_5$ positive $CD4^+$ T cells was observed in the inguinal lymph nodes as well, though to a much lesser extent than in spleen which is the draining lymphoid organ. The peak expansion of OT-II cells in these lymph nodes was around 7% of the $CD4^+$ T cells at d4, compared to around 15% in the spleen (fig 4.13E). The difference in the percent of $V\alpha_2V\beta_5^+$ cells in the lymph nodes between OT-II and OT-II $ICOS^{-/-}$ groups was significant at the peak of response (d4) as well as in the deletion phase (d8). Thus, ICOS has a critical and non-dispensable role in T cell activation.

CFSE

To assess quantitatively the dynamics of T cell proliferative response, OT-II and OT-II $ICOS^{-/-}$ T cells were CFSE labelled before transfer into syngeneic C57Bl/6 hosts and their division followed. Figure 4.14 shows that at d3 after immunisation, although ICOS deficient transgenic T cells underwent similar number of divisions as their ICOS sufficient counterparts, the mean percentage of $ICOS^{-/-}$ OT-II cells that had undergone more than four divisions was 41.23% compared to 54.21% $ICOS^+$ OT-II T cells. This suggests that although T cells can enter the proliferative phase in the absence of ICOS, $ICOS^{-/-}$ cells undergo less proliferation in comparison to $ICOS^+$ cells.

Activation status of T cells

The CD44 and CD62L expression on $V\alpha_2V\beta_5^+$ cells was measured to assess their activation status. The OT-II T cells that lacked ICOS had fewer cells with $CD44^{high}$ $CD62L^{low}$ phenotype compared to the wildtype T cells (fig 4.15). This difference was significant at the peak of the response on d4 suggesting that ICOS is important for the full activation of the T cells during primary immune response.

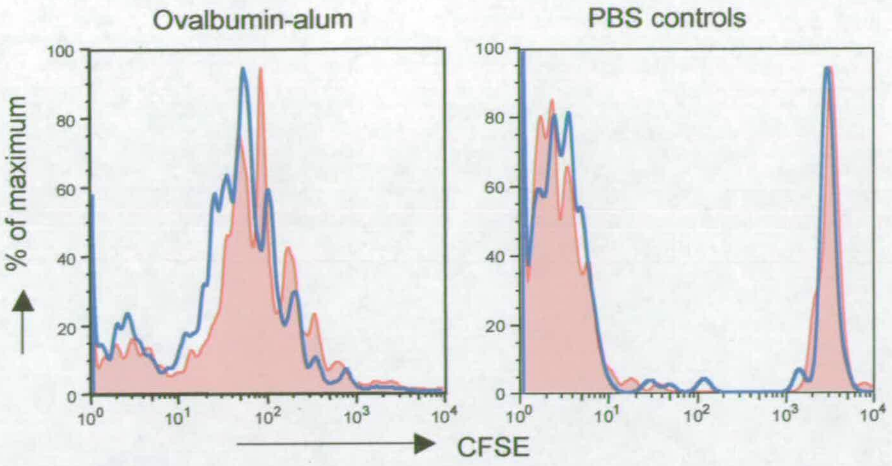


Figure 4.14 Dynamics of T cell proliferative response illustrated by the loss of CFSE. ICOS$^+$ or ICOS$^{-/-}$ OT-II T cells were labelled with CFSE before transfer into recipient C57Bl/6 mice. At d3 after i.p. immunisation with ovalbumin-alum, the division of T cells between both groups was compared by the loss of CFSE. The histograms show the CFSE staining of $V\alpha_2V\beta_5^+$ CD4$^+$ T cells in spleen. The left panel is a histogram overlay of CFSE staining one mouse from each group immunised with ovalbumin-alum, while the right panel depicts the PBS controls from each group. Blue line-OT-II; Red tinted-OT-II ICOS$^{-/-}$.

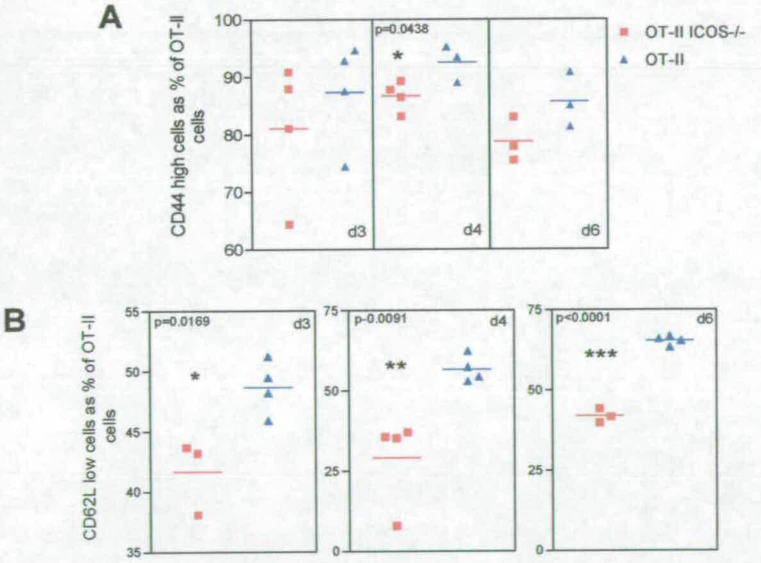


Figure 4.15 T cell activation status. At d3, d4 and d6, splenocytes of the recipient mice were stained with antibodies against CD4, $V\alpha_2$, $V\beta_5$ and CD44 or CD62L and analysed by flow cytometry. Data shown indicates the percent of $V\alpha_2V\beta_5^+$ CD4 T cells with CD44^{high} (A) and CD62L^{low} phenotype (B) from separate experiments. Each point represents an individual mouse. Unpaired Student's t-test was applied to compare both groups.

In vitro restimulation

OT-II ICOS$^{-/-}$ and OT-II cells recovered from the spleens of C57Bl/6 recipients at different time points after immunisation were restimulated *in vitro* with varying concentrations of pOVA. OT-II cells proliferated around 3-fold higher than OT-II ICOS$^{-/-}$ cells at the maximal antigen concentration when the number of transgenic cells was different in both populations (fig 4.16A). However, when the number of transgenic cells were equalised between ICOS$^{-/-}$ and wildtype groups in a separate experiment, there was no difference in their proliferation *in vitro* (fig 4.16B).

Role of ICOS in T cell differentiation: cytokine profile

T cell clonal expansion after priming is only one component of the immune response. To establish whether the reduced T cell proliferation in the absence of ICOS was accompanied by any defect in the cytokine response, cell-based cytokine ELISA was done for IL-2, IL-4, IL-10 and IFNγ.

At all concentrations of the pOVA, the OT-II ICOS$^{-/-}$ cells produced less IL-10 and IL-4 as compared to OT-II cells. The difference in the levels of IL-2 and IFNγ was even more pronounced with OT-II cells secreting more of these cytokines except at lowest concentration of pOVA (0.01μM) (fig 4.17). However, since the proportion of OVA-specific T cells was not equal in OT-II ICOS^{+} and OT-II ICOS$^{-/-}$ cultures to start with, it is difficult to definitely state anything about the role of ICOS in effector T cell differentiation from these results. Hence, the data was transformed and the cytokine secreted by same number of OVA-specific $V\alpha_2V\beta_5^+$ T cells in both groups compared.

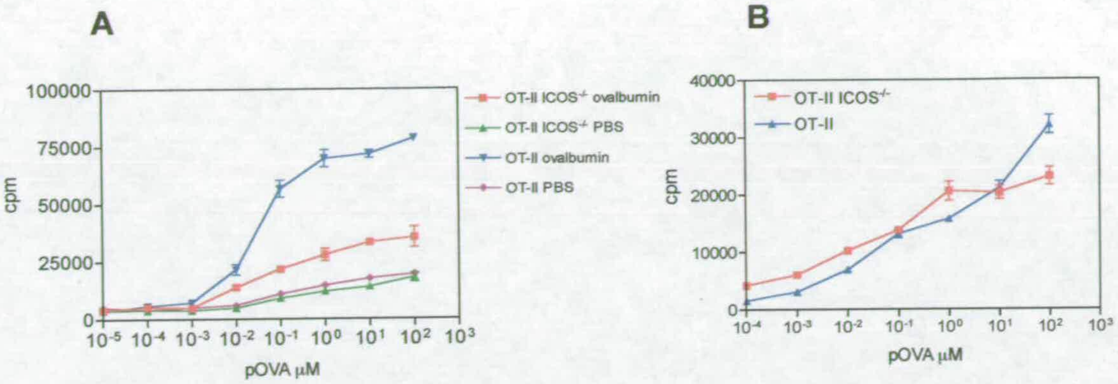


Figure 4.16 ***In vitro*** **restimulation of OT-II cells.** The splenocytes from the recipient mice of ICOS$^+$ or ICOS$^{-/-}$ OT-II cells were restimulated at d3 *in vitro* in the presence of titration of pOVA. Proliferation was measured by ^{3}H-thymidine incorporation during the last 16h of a 3d culture. Data is shown as the mean of triplicate wells ± SEM. The proportion of OT-II cells was different (A) or equalised between groups (B) in separate experiments. Proliferation was less than 2500cpm in the absence of antigen.

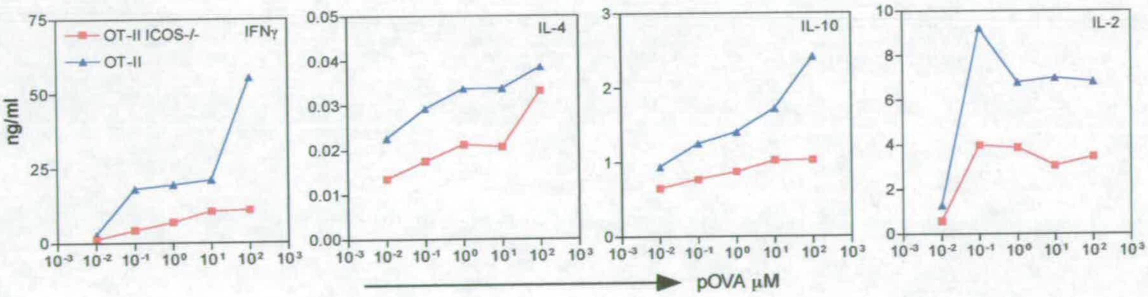


Figure 4.17 Cytokine secretion by OT-II cells in the presence or absence of ICOS. Following adoptive transfer of ICOS$^+$ or ICOS$^{-/-}$ OT-II cells and i.p. immunisation with ovalbumin, the splenocytes from the recipient C57Bl/6 mice were assessed for cytokine secretion by cell-based ELISA at d4 (section 2.10.3). Splenocytes, 8x10^5/well were cultured *in vitro* for 3 days in 96-well plate and the cytokine secreted during the last 20h of culture measured by ELISA. Amount of cytokine secreted was measured by interpolation of absorbance from the standard curve.

At 100μM pOVA, the calculated amount of cytokines produced by 10^3 $V\alpha_2V\beta_5^+$ is depicted in the following table:

Table 4.1 Cytokine production by OT-II ICOS$^{-/-}$ and OT-II ICOS$^+$ cells.

Cytokine per 10^3 $V\alpha_2V\beta_5^+$ cells	OT-II ICOS$^{-/-}$	OT-II ICOS$^+$
IL-2 (pg/ml)	111.0	76.0
IL-4 (pg/ml)	1.06	0.432
IL-10 (pg/ml)	33.0	26.7
IFNγ (pg/ml)	352.0	614.0

However, it is important to note that in order to normalise the data, a linear relation between the number of $V\alpha_2V\beta_5^+$ cells per well and the cytokine secreted, was assumed, which may not actually be the case. In a separate experiment, cultures for cell-based cytokine ELISA were set up with equal number of $V\alpha_2V\beta_5^+$ cells from both groups, in which case the OT-II ICOS$^{-/-}$ group had around 3 times more cells than the OT-II ICOS$^+$ group. ICOS-deficient OT-II cells secreted similar or slightly higher amounts of IL-2, IL-4 and IL-10 in comparison to wildtype OT-II cells in this experiment. The amount of IFNγ secreted by OT-II ICOS$^{-/-}$ cells, however, was higher than OT-II cells at all titrations of the antigen. This was probably due to additional number of T cells (added to equalise the number of $V\alpha_2V\beta_5^+$ cells between both groups) in ICOS-deficient cultures responding to the pOVA.

Thus, from these experiments, it can be summarised that ICOS-deficient T cells are defective in the production of IFNγ, but not other cytokines.

T cell expansion following subcutaneous immunisation with ovalbumin-CFA

In contrast to above adoptive transfer experiment, I had not found any defect in CD4 T cell priming in the absence of ICOS using tetramers. The reason for different outcomes might be that the two approaches used different immunisation protocols; antigen emulsified in CFA given subcutaneously in tetramer studies, and alum-precipitated antigen given intraperitoneally in the adoptive transfer system. CFA is a strong Th1 inducing adjuvant while alum is a Th2 inducing adjuvant. To investigate if this might be the reason for difference in results, an adoptive transfer experiment was undertaken with immunisation regime used in tetramer studies. Either OT-II or OT-II ICOS$^{-/-}$ lymph node cells were transferred into C57Bl/6 mice followed 24h later by s.c. immunisation with ovalbumin emulsified in CFA in both the thighs. The expansion of the OVA-specific transgenic CD4 T cells in the inguinal lymph nodes was examined at different days after immunisation by staining with antibodies against $V\alpha_2$ and $V\beta_5$.

The expansion of OT-II cells was equivalent in the both groups in the priming and expansion phase (d3 and d4), however, significant difference in the number of $V\alpha_2V\beta_5^+$ cells was noticed during the clonal deletion phase. Significantly lower number of OT-II cells were found in the draining inguinal lymph nodes in the absence of ICOS (fig 4.18A & B). Similar trend was observed in the spleen at all the day points (fig 4.18C).

CFSE

To determine whether the difference in the $V\alpha_2V\beta_5^+$ cell numbers in both groups was due to less T cells entering division or T cells undergoing fewer divisions, OT-II and OT-II ICOS$^{-/-}$ T cells were CFSE labelled before transferring into C57Bl/6 hosts and their division followed after immunisation. The overlay of CFSE histograms at d4 shows that ICOS-deficient OT-II cells in the lymph nodes had undergone fewer divisions in

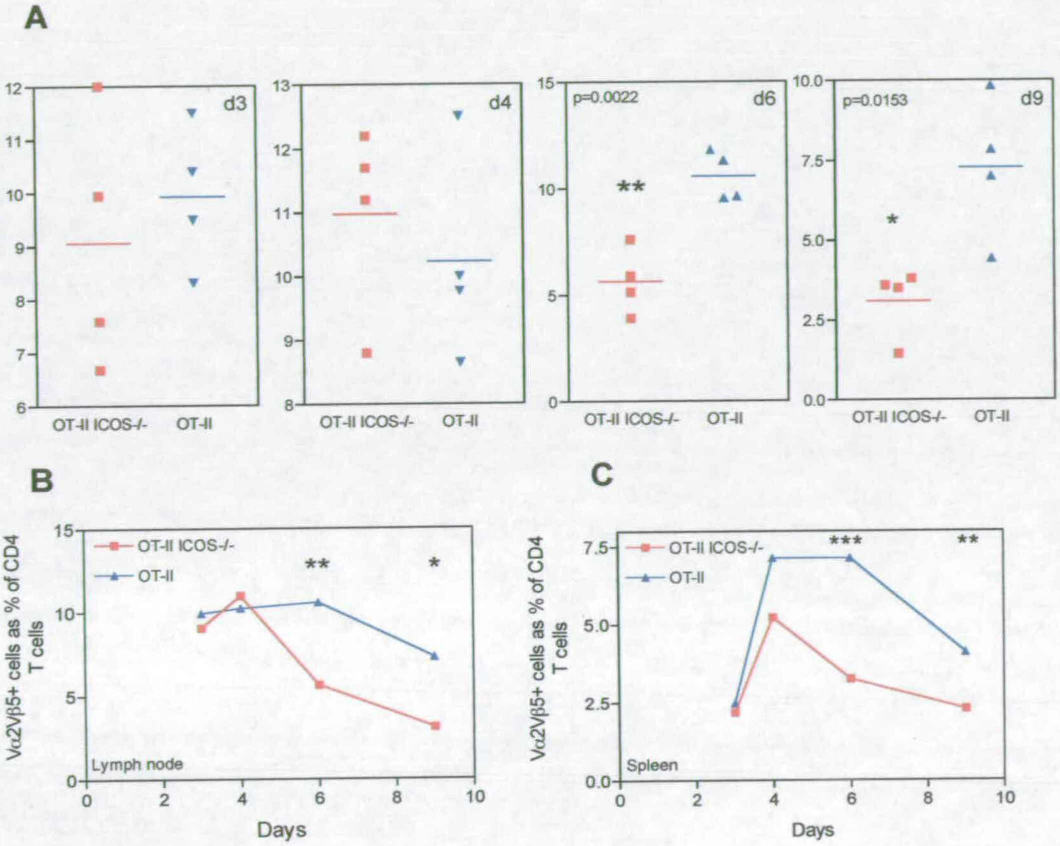


Figure 4.18 Role of ICOS in T cell clonal expansion. C57Bl/6 mice received OT-II ICOS$^+$ or OT-II ICOS$^{-/-}$ lymph node cells before immunisation with ovalbumin-CFA s.c., and the expansion of $V\alpha_2V\beta_5^+$ cells measured at days 3, 4, 6 and 9 in the draining and spleen. This experiment is done once using 4 mice per group per time point. (A) $V\alpha_2V\beta_5^+$ cells as % of CD4 cells in the inguinal lymph nodes of recipient mice at indicated time points. Both groups were statistically compared using unpaired Student's t-test. The % of OT-II cells in LN of control mice was invariably between 0.5-1% of CD4 T cells. (B) Time course of T cell proliferative response in inguinal lymph nodes. (C) Time course of the T cell response in spleen.

comparison to ICOS-sufficient OT-II T cells (fig 4.19). Thus, in the absence of ICOS, T cells are slower to divide.

Role of ICOS in T cell apoptosis

Once a T cell response is generated, clonal expansion is followed by clonal deletion phase where in majority of the activated T cells undergo apoptosis. It is difficult to detect the apoptotic cells as the phagocytic and scavenger cells of the body are very efficient at phagocytosing them quickly. One of the earliest signs of apoptosis is the translocation of membrane phospholipid phosphatidylserine (PS) from inner membrane to outer plasma membrane. Annexin-V binds to the PS and therefore is a sensitive tool to detect early apoptotic cells. The OT-II cells in the inguinal lymph nodes were examined for Annexin-V binding at various days after immunisation. At d4 post-immunisation, the ICOS-deficient OT-II cells had slightly higher proportion of apoptotic cells as compared to ICOS$^+$ OT-II group, but this difference was not significant (fig 4.20). At d6 and d9 after immunisation, however, there was not much difference in the number of apoptotic cells in both the groups. Thus, ICOS does not seem to play a role in regulating the apoptosis of T cells.

From the results described so far, it can be concluded that the difference in the number of $V\alpha_2V\beta_5^+$ cells between ICOS-sufficient and -deficient groups is due to fewer ICOS$^{-/-}$ T cells undergoing proliferation, since proportion of $V\alpha_2V\beta_5^+$ cells undergoing apoptosis is similar in both groups.

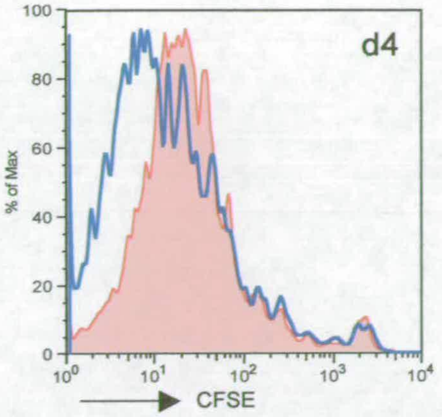


Figure 4.19 T cell proliferative response illustrated by the loss of CFSE. ICOS$^+$ or ICOS$^{-/-}$ OT-II T cells were labelled with CFSE before transfer into recipient C57Bl/6 mice. At d4 after s.c. immunisation with ovalbumin-CFA, the division of T cells in the LN between both groups was compared by the loss of CFSE. The histogram shows the CFSE staining of cells gated on $V\alpha_2V\beta_5^+$ $CD4^+$ T cells. The histogram overlay depicts CFSE staining from one mouse per group immunised. Blue line-OT-II; Red tinted-OT-II ICOS$^{-/-}$.

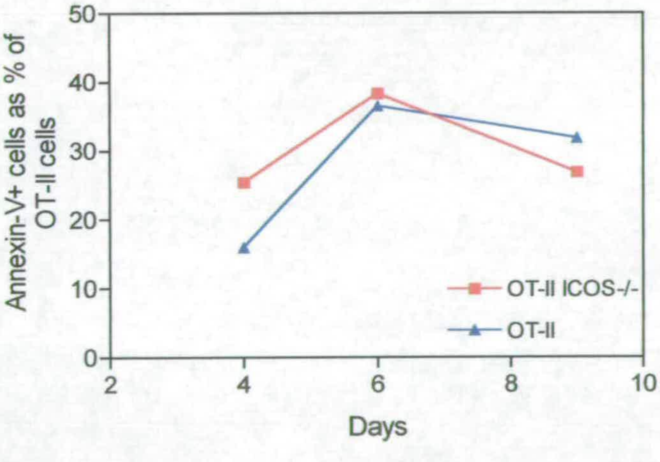


Figure 4.20 Role of ICOS in T cell apoptosis. The inguinal LNs of the recipient mice in a group were pooled and OT-II cells undergoing apoptosis assessed at days 4, 6 and 9 post-immunisation. The cells were stained with CD4, $V\alpha_2$, $V\beta_5$ and Annexin-V and analysed by flow cytometry. Data represented show Annexin-V$^+$ cells as the percentage of $V\alpha_2V\beta_5^+$ $CD4^+$ T cells. This experiment was done once.

4.2.6. Role of ICOS in $CD4^+$ effector T cell generation

Antigen presentation in combination with costimulation drives T cells to enter cell cycle and to produce IL-2 [5]. Proliferating CD4 T cells initially express a spectrum of cytokine genes, but within a short period, a population of cells, each producing a broadly similar array of cytokine, emerges as a dominant type. These T cells have a stable, inheritable phenotype characterised by the production of signature effector cytokines that define the immune response. ICOS has been implicated in Th2 responses, though it also plays critical role in Th1 responses as shown recently in *in vivo* infectious Th1 models [114-116].

I aimed to elucidate the role of ICOS in T cell differentiation *in vivo* by using polarising antigens: heat killed *Propionebacterium acnes* (Pa, Th1) and *Schistosoma mansoni* eggs (Th2). Schistosome eggs release proteins into the tissues surrounding their site of deposition during infection [265]. Soluble Egg Antigen (SEA) contains these released antigens, and is widely used to assess CD4 T responses during infection [266]. Similarly, infection with Pa involves exposure of the host to the whole bacteria. These polar opposite CD4 T responses mimic the dominant responses seen during infection with these bacterial and helminth pathogens.

As outlined in figure 4.21, $ICOS^{-/-}$ and $ICOS^+$ mice were immunised s.c. in the footpad with either Pa (50µg/footpad) or *S. mansoni* eggs (1500eggs/footpad) or PBS. The popliteal lymph nodes were harvested a week after the immunisation. $7.5x10^5$ cells/well from the lymph nodes were restimulated *in vitro* in the presence of Pa (5µg/ml) or SEA (25µg/ml) or medium for 3 days before supernatants were harvested. The amount of different cytokines such as IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ, secreted by the cells in the culture supernatant was measured by cytokine specific ELISA.

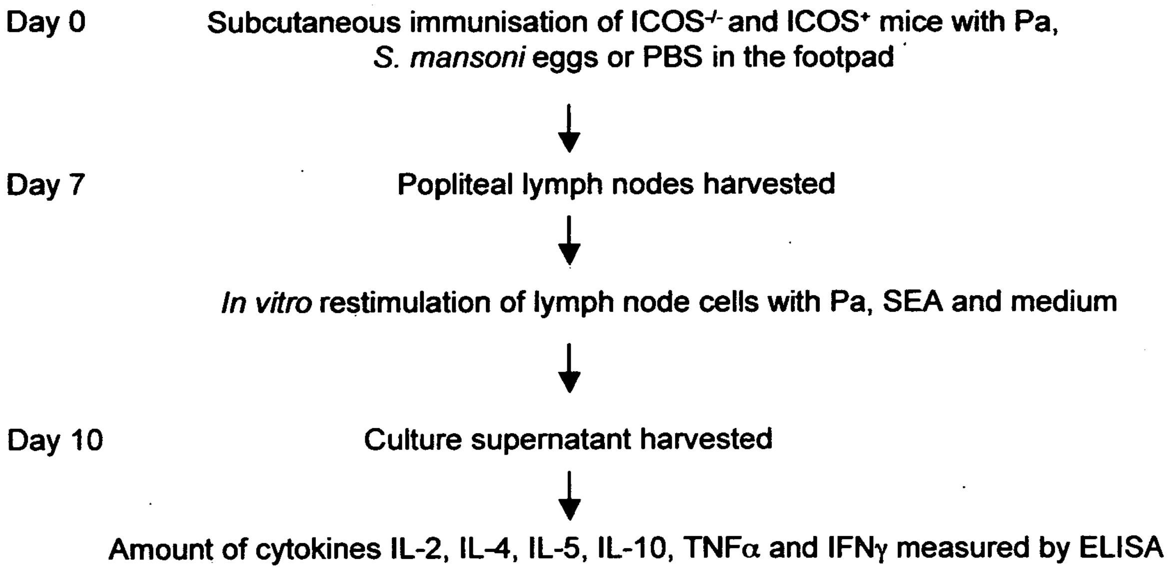


Figure 4.21 Flow diagram of the experimental outline. Role of ICOS in effector T cell differentiation was assessed using polarising antigens, Pa - Th1 and SEA - Th2.

Upon *in vitro* restimulation with Pa, T cells from ICOS$^{-/-}$ mice immunised with Pa secreted less IFNγ and TNFα as compared to ICOS^{+} mice (fig 4.22). Reduced levels of IL-2 in the ICOS$^{-/-}$ culture supernatants were noted as compared to ICOS^{+} cultures. I did not find any defect in Th2 differentiation in terms of IL-4 and IL-5. The amount of IL-4 in the supernatants of lymph node cells from the mice that were immunised with Schistosome eggs and further cultured with SEA was similar in both groups. Though surprisingly, the levels of IL-5 in the ICOS$^{-/-}$ supernatants were higher compared to the ICOS^{+} supernatants. Although ICOS has been reported to be a superinducer for IL-10 [104], this experimental protocol did not yield similar observations. No difference in the levels of IL-10 in both culture supernatants was observed (fig 4.22). This experiment, although done only once, suggests that costimulation via ICOS is required for the induction of Th1 responses.

4.3. Discussion

ICOS is a CD28 homologue implicated in regulating T cell differentiation. Costimulatory signals are critical for regulating T cell activation, and thus an understanding of these signals may enable the design of rational therapies for immune mediated diseases. In this chapter, I have characterised the ICOS$^{-/-}$ mice and using them, I have shown that interaction between ICOS and ICOSL is critical for activation and clonal expansion of both T and B lymphocytes. Moreover, this interaction is crucial for the provision of T cell help for B cells to produce class switched antibodies and form GC.

4.3.1. T cell activation

The role of ICOS in T cell activation has been a contentious issue due to differing reports published since its identification five years ago. Initial studies reported that ICOSL-Ig fusion protein along with anti-CD3 could costimulate T cells through ICOS,

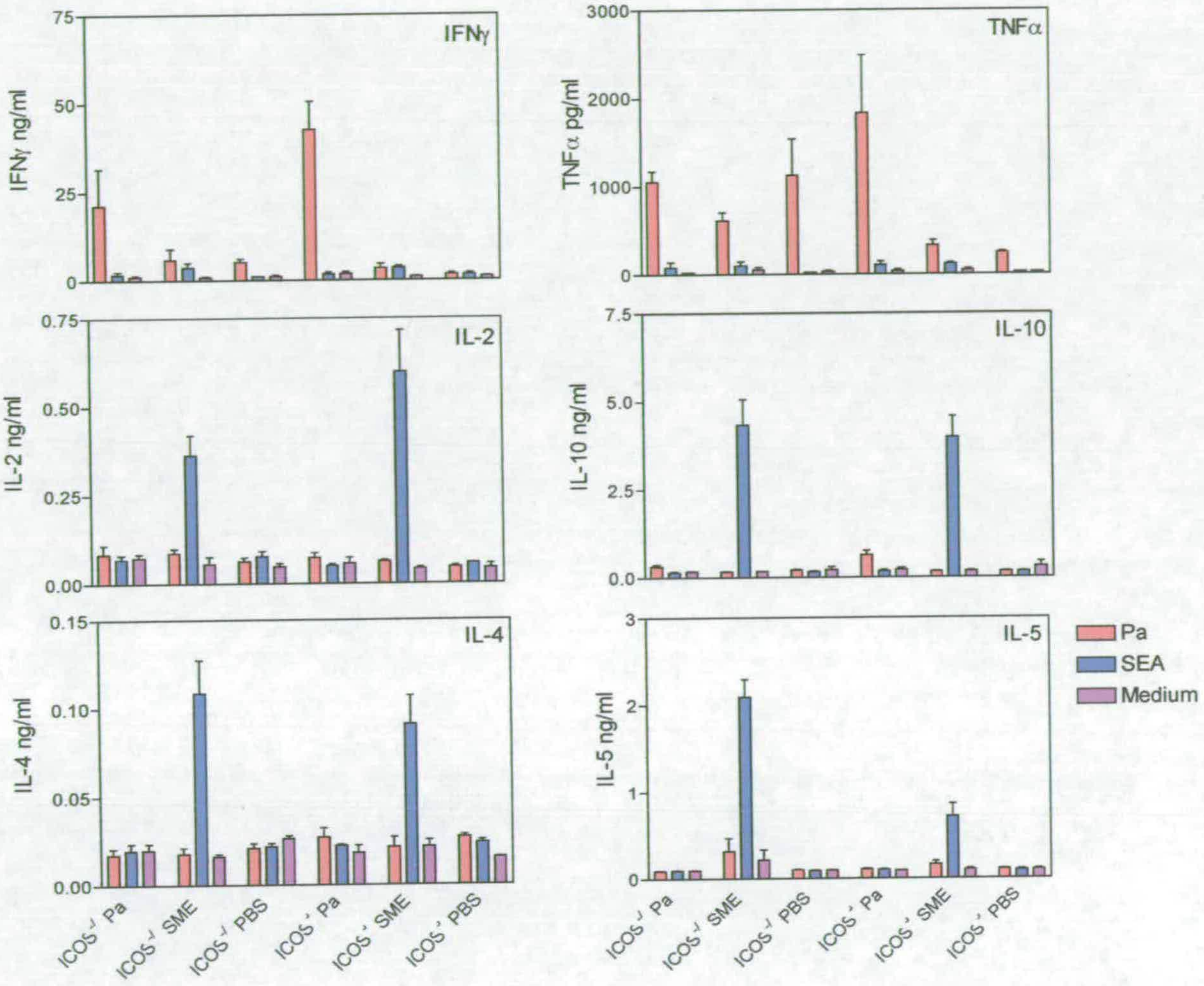


Figure 4.22 Role of ICOS in effector T cell differentiation. As per the experiment outlined in fig. 4.21, ICOS^{+} and ICOS$^{-/-}$ mice were immunised s.c. in the footpad with Th1 inducing Pa (50µg/footpad), Th2 inducing *S. mansoni* eggs (1500 eggs/footpad) or PBS. Popliteal LNs were harvested a week later and 7.5x10^{5} cells/well restimulated *in vitro* in the presence of Pa (5µg/ml) or SEA (25µg/ml) or medium for 3 days before the supernatants were harvested. The immunisations are labelled on x-axis and colour of the bars depict the culture conditions. The amount of cytokines in the supernatants was measured by cytokine-specific ELISA (section 2.10.4). This experiment was done once with 3 mice per group. The error bars represent the SEM.

even in CD28$^{-/-}$ mice, showing that it can regulate T cell function independent of CD28 [89,100]. However, Gonzalo *et al.* observed that ICOS-Ig fusion protein markedly reduced the expansion of CD4 and CD8 cells *in vivo* as well as *in vitro* [105] while McAdam *et al.* did not find any difference in T cell proliferation in the presence of blocking ICOS-Ig fusion protein [100]. Later reports using ICOS knockout and ICOSL knockout mice did not make the issue any clearer. McAdam *et al.* noticed equal proliferation of T cells from either ICOS$^{-/-}$ or wildtype mice in the presence of wildtype APCs [93], while profoundly defective proliferation was reported when ICOS$^{-/-}$ T cells were stimulated by anti-CD3 alone [95] or in presence of B7RP-1-Fc [94]. Nurieva *et al.* observed reduced T cell proliferation when activated by B7h$^{-/-}$ APCs [267].

This issue has been mostly addressed either by *in vitro* studies or *in vivo* experiments involving blocking of ICOS-ICOSL interactions by antibodies or fusion proteins. I studied the role of ICOS in T cell activation by an adoptive transfer system, whereby we could track the expansion of the transgenic T cells directly *ex vivo*. My finding that clonal expansion of OT-II cells lacking ICOS was significantly deficient compared to ICOS^{+} OT-II cells came as a surprise, since ICOS$^{-/-}$ splenocytes restimulated *in vitro* proliferated similarly to ICOS^{+} cells (fig 4.7). One possible explanation may be that a minor defect in the ICOS$^{-/-}$ T cell priming from normal (non-transgenic) mice gets accentuated in transgenic mice where most of the T cells are antigen-specific. But given that even OT-II and OT-II ICOS$^{-/-}$ splenocytes proliferated similarly *in vitro* (figs 4.13A and 4.16B), this does not seem to be the reason. It also indicates the unlikelihood of an intrinsic qualitative defect in naïve CD4 T cells in ICOS$^{-/-}$ mice.

Possible explanations for reduced expansion of transgenic T cells when ICOS was lacking are: decreased migration of transgenic T cells to spleen; increased death of transgenic T cells; or decreased division of transgenic T cells. We addressed the division issue by injecting CFSE labelled CD4^{+} T cells into recipient mice. Although both types of cells underwent a similar number of divisions, fewer ICOS$^{-/-}$ transgenic T cells compared to ICOS^{+}, divided at d3. Similar observations have been reported by Smith *et al.* in a double adoptive transfer system [97]. They transferred HEL-specific B cells and DO11.10 T cells into BALB/c mice followed by immunisation with HEL-OVA and treatment with anti-ICOS mAb. Although treatment with anti-ICOS mAb did not prevent transgenic T cells from dividing, T cell expansion was nearly half than that found in the non-treated group at d3 post-immunisation. Although ICOS may not be involved in the initial interactions between T cell and APC, it seems important in the generation of primary T cell responses. Since OT-II cells were unaffected in the absence of ICOS *in vitro*, these results highlight the importance of studying responses in the more complex environment of *in vivo* models.

I did not find much difference in the Annexin-V binding of ICOS^{+} and ICOS$^{-/-}$ CD4^{+} T cells during the clonal expansion and deletion phases. Mak *et al.* observed similar staining for Annexin-V and 7-AAD by CD4^{+}CD25^{+} T cells from wildtype and ICOSL$^{-/-}$ mice [106]. Therefore, the reason for the reduced number of $V\alpha_2V\beta_5^{+}$ cells in the recipients of ICOS-deficient OT-II cells might be that ICOS$^{-/-}$ T cells underwent less proliferation and not because of more cells dying.

Fewer CD4 T cells with activated phenotype in terms of CD44^{high}, CD62L^{low}, CD69^{+} and CD43^{+} expression were observed in ICOS$^{-/-}$ mice in comparison to the wildtype mice. Conflicting reports are available regarding the effect of ICOS on upregulation of activation markers on T cells. Tafuri *et al.* observed reduced levels of CD40L, CD25 and CD69 on ICOS$^{-/-}$ CD4 T cells 24h after *in vitro* stimulation with anti-CD3/B7RP1-Fc [94]. On the other hand, Hutloff *et al.* observed upregulation of CD69, CD25 and CD71 on human T cells to be normal when stimulated *in vitro* in the presence of anti-ICOS mAb [104]. Probably, this is due to technical differences, ICOS$^{-/-}$ mice versus blocking anti-ICOS mAb, and mouse versus human study. In my study, similar number of cells expressed CD40L and OX40 in both groups. Normal upregulation of CD40L has been observed in ICOS$^{-/-}$ mice *in vivo* [93,96] as well as on human T cells treated with anti-ICOS mAb *in vitro* [104]. Recently, Suh *et al.* have reported similar levels of CD40L induction on T cells from wildtype, ICOS$^{-/-}$, CD28$^{-/-}$ and ICOS/CD28 double knockout mice [103]. This is in line with existing literature that CD40L induction is largely independent of costimulatory signals [96,268,269].

Using MHC-tetramer binding assays, there was not significant difference in the number of antigen-specific cells in ICOS$^{-/-}$ and wildtype mice at d8 and d15 after immunisation. However, while studying the role of ICOS in the reactivation of CD4 memory T cells, Megan MacLeod in the lab, observed significantly reduced number of tetramer positive cells in the draining lymph nodes of ICOS$^{-/-}$ mice compared to C57Bl/6 mice at 10 weeks after immunisation. These mice were initially immunised with BMDCs pulsed with H19env peptide and boosted with peptide-CFA subcutaneously after 10 weeks. The tetramer positive cells were measured 5 days after the boost. Thus, using this method, ICOS seems to be required for generation or reactivation of the memory CD4 T cells.

Although the adoptive transfer system has been invaluable in understanding T cell activation *in vivo*, it is far from physiological as it results in an artificially higher proportion of antigen-specific T cells. Moreover, different TCR-transgenic T cells can have different characteristics such as affinity for MHC and costimulatory requirements as discussed in chapter 3. The OT-II transgenic T cells have low affinity for the MHC class-II [252,253], which might increase their requirement for costimulation in comparison to the polyclonal population in non-transgenic mice. On the other hand, tetramer staining allows detection of antigen-specific T cells in more physiological settings, but due to exceedingly small number of antigen-specific cells, they have to undergo clonal expansion before they can be detected by tetramers. Hence, soon after the initiation of the immune response, the number of antigen-specific cells is too low to analyse reliably. Thus, transgenic cells provide more useful information at early times of T cell activation. Both techniques demonstrated that T cell activation could occur in the absence of ICOS-ICOSL interactions, however, the defects in the transgenic system were greater than those in the polyclonal system and this illustrates the importance of examining more than one system of T cell activation before assigning definitive roles to particular molecular interactions.

4.3.2. Effector T cell differentiation

Earlier work using anti-ICOS antibodies and fusion proteins suggested that ICOS signalling induces the secretion of IFNγ but not IL-2 [89,99,102,104,105]. Later, studies with ICOS$^{-/-}$ mice have reported both normal [94], and even increased production of IFNγ in the *in vitro* culture supernatants of ICOS$^{-/-}$ T cells, as compared to ICOS$^{+/+}$ T cells [93]. It was reasoned that ICOS enhances Th2 response, so its absence might increase generation of Th1 cells. As both these groups have used similar immunisation protocols, the reason for different findings is unclear. Dong *et al.* observed that *in vitro* differentiated ICOS$^{-/-}$ CD4^{+} T cells could produce IFNγ and IL-10 but failed to secrete IL-4 and IL-2 [95]. Also, ICOS has also been implicated for the production of IL-4, IL-5, GM-CSF and TNFα [102,104,105]. ICOS costimulation has been reported to induce several fold higher secretion of IL-10 as compared to modest stimulatory capacity of CD28, hence called a superinducer for IL-10 [104,111]. Hence, the role of ICOS in cytokine induction remains slightly confused.

I tried to clarify whether ICOS is instrumental in both Th1 and Th2 effector cell generation by immunising ICOS$^{-/-}$ and wildtype mice with polarising antigens. Heat killed Pa induces strong Th1 response while *Schistosoma mansoni* eggs elicit Th2 response. Using this protocol, I did not observe reduction in any of the Th2 cytokines, but levels of IFNγ, TNFα and IL-2 were reduced in the ICOS$^{-/-}$ culture supernatants. This suggests that ICOS is important for the Th1 differentiation. As this experiment has only been done once, these results await further validation. Also, considering that schistosome eggs are very strong Th2 inducing antigen, it can possibly overcome the need for ICOS costimulation. Since from other experiments, it appears that ICOS is more important in the secondary responses, the ideal way to do this experiment would be to measure the cytokines in the *in vitro* restimulated culture supernatants after boosting the immunised mice with the antigen. Also, Pa-specific and SEA-specific antibody response in the sera of ICOS-deficient or -sufficient mice would be informative regarding the helper T cells generated. Another way to address the role of ICOS-ligation in effector T cell generation would be to inject ICOSL$^{-/-}$ or wildtype DCs pulsed with Pa or *S. mansoni* eggs into wildtype mice. This protocol would take care of any differences in the APCs, if any, in mice lacking ICOS.

My results correlate with Dong *et al.* in terms of IL-2, IL-5 and IL-10. They immunised ICOS$^{-/-}$ and wildtype mice s.c. with KLH in alum or CFA. *In vitro* restimulation of the draining lymph node cells with peptide revealed reduced IL-2, IL-5 and IL-4 with no difference in IL-10 response. They also observed similar amounts of IL-5 and IL-10 and reduced IL-4 and IL-13 by *in vitro* restimulated lung lymph node cells from OVA-sensitised ICOS$^{-/-}$ mice followed by airway boost with antigen [95].

Recently Lohning *et al.* reported a tight link between ICOS and IL-10, as cells expressing high levels of ICOS were the main secretors of IL-10 [111]. They observed a relation between low expression of ICOS and early cytokine secretion and medium-high ICOS expression corresponded to late cytokine production. The authors argued that ICOS is gradually upregulated in the course of progressing T cell differentiation. So, first T cells would utilise ICOS to release Th2 cytokines, and later a population of T cells would differentiate to produce IL-10 and would utilise high ICOS expression for an effective downregulation of the immune response. Though the observation that T cells from ICOS$^{-/-}$ mice are fully capable of secreting IL-10 upon CD3 stimulation *in vitro* seems to indicate that ICOS is not directly involved in the differentiation of T cells to IL-10 secreting cells, but rather controls the release of this cytokine [95].

Using CD80-CD86 double knockout mice, Schweiter and Sharpe have shown that CD28 is required by naïve T cells for IL-2 production and clonal expansion but recently activated T cells do not need these signals for secretion of IL-4 or IFNγ [149]. Furthermore, ICOS has been shown to function as an important costimulatory molecule for effector cytokine production by recently activated T cells in CD28-B7 independent response [99]. Low levels of ICOSL are expressed constitutively on peripheral tissues, for example, endothelial cells and its expression increases upon inflammation [270]. Under these conditions, ICOS-ICOSL is the main pathway for cytokine release by T cells upon antigen presentation by endothelial cells, since the inflamed endothelial cells remain negative for CD80/CD86 [111,270].

In the adoptive transfer experiments, I studied the role of ICOS on effector T cell differentiation and cytokine secretion by cell based ELISAs. Difficulty in analysing these results arose from the unequal number of OT-II cells in the starting populations in both $ICOS^+$ and $ICOS^{-/-}$ groups. When equal number of OT-II cells were cultured in both groups, the $ICOS^{-/-}$ cultures had 3 times more cells as compared to $ICOS^+$ cultures, which might have accounted for the increased secretion of IFNγ. An ideal way would be to measure the amount of cytokine secreted by same number of OT-II and OT-II $ICOS^{-/-}$ T cells. This can be done by sorting these cells from spleens of the adoptive transfer recipients and the amount of cytokine measured by quantitative real time PCR.

4.3.3. T cell-B cell collaboration

ICOS-ICOSL costimulation is a key interaction in humoral responses to T-dependent antigens. Our findings that $ICOS^{-/-}$ mice have reduced levels of DNP-specific class switched antibodies in their sera as compared to $ICOS^+$ mice confirm the earlier reports.

$ICOS^{-/-}$ mice have been shown to mount normal levels of IgM and markedly reduced IgG_1 and IgG_{2a} antibodies [93,94,103]. Furthermore, class switching is highly dependent on ICOS-mediated T cell help when CD28 is not present. A recent study using CD28/ICOS double knockout mice has shown that in the absence of CD28, ICOS plays a critical role in promoting switching to IgG_1. Basal IgG_1 levels in $CD28^{-/-}$ mice was 10% of the wildtype mice, while in double knockout mice, it was 1.2%. Also, GCs are totally absent in CD28/ICOS double knockout mice [103].

I observed slightly reduced IgM response at d7 in $ICOS^{-/-}$ mice. Nurieva *et al.* observed significantly reduced IgM responses in $B7h^{-/-}$ mice [267] as well as in $ICOS^{-/-}$ mice [126]. They suggested that ICOS-ICOSL interactions might have a role in the initial B cell response before Ig class switching occurs. DNP-specific IgM response at d0 may be due to cross-reactivity between DNP and a ubiquitous environmental antigen. Interestingly, the defect in Ig isotype switch could be overcome when CFA was used in the immunisation regime, suggesting that given enough inflammation and CD80, CD86 upregulation, CD28-mediated costimulation provides enough T cell help for B cells to undergo class switching [93]. This is consistent with previous observations demonstrating a role for CD28 in providing B cell help [61].

I found B cell clonal expansion to be markedly reduced in $ICOS^{-/-}$ mice immunised with PE as compared to $ICOS^{+}$ mice. This finding validates a previous study by Smith *et al.* using ICOS-Ig fusion protein in a double adoptive transfer system [97]. The authors showed that antigen-specific B cell clonal expansion was reduced approximately 3-fold in mice treated with ICOS-Ig fusion protein in comparison to untreated mice. Moreover, these mice had lower HEL-specific serum IgM levels at d10 post-immunisation. Since B cell expansion is dependent on T cell help, these findings further validate the crucial role of ICOS-ICOSL interactions T-B cell collaboration.

Indeed, following immunisation with T-dependent antigen, the main site of B cell clonal expansion is the GC in the B cell follicles. Since the size and number of the GC is reduced in ICOS$^{-/-}$ mice, it implies that ICOS, although not essential for GC formation, is critical for B cell expansion in GC [93,94,96]. No GCs were formed in ICOS$^{-/-}$ mice in secondary response [96]. Interestingly, signalling via CD28 is required for the development of the GC response, but the established GCs can be maintained without CD28 signalling [271,272]. The organisation of B and T lymphocytes is normal in spleen of ICOS$^{-/-}$ mice, and surprisingly, these mice even have normal number of CD21^{+} FDCs [93,96]. Possibly, in the absence of ICOS-ICOSL interaction, the B cells are poorly stimulated in the T cell areas and fail to migrate into FDC network to form GC.

As the immune response progresses T cells migrate into the follicles to provide help to B cells to class switch and form GC. Using carrier-hapten based *in vitro* help assay, I observed that ICOS$^{-/-}$ T cells were unable to help wildtype B cells to class switch to either IgG$_1$ or IgG$_{2a}$. The importance of ICOS in T-B cell interaction is evident from a study on transgenic mice expressing soluble and secreted B7RP-1-Fc. Such mice had lymphoid hyperplasia in T as well as B cell areas, high circulating IgG and plasmacytosis [89].

As the capacity of T cells to help B cells for activation and antibody production is generally correlated to their ability to migrate into B cell follicles, the number of T cells in the follicles would quantitatively reflect the T cell help for B cells. A pilot study done in the lab suggested reduced number of T cells in the splenic B cell follicles of the ICOS$^{-/-}$ mice 6-9 days after immunisation (A. Cervera, unpublished data). I intended to study the T cell migration in ICOS$^{-/-}$ mice over a period of time, but this was not possible due to shortage of time. Contrary to this, Smith *et al.* found that disruption of ICOS-ICOSL interaction does not affect the migration of T cells in B cell follicles, although the T cell help to B cells was markedly reduced [97]. They blocked the ICOS-ICOSL interaction by injecting anti-ICOS mAb in mice transferred with DO11.10 T cells and MD4 B cells and found reduced IgM serum responses in these mice.

ICOS expressing T cells interact with ICOSL during two different phases of a response to a T cell dependent antigen: first, during the T cell priming and differentiation phase, in which professional or semi-professional APCs promote T cell acquisition of helper function; and second, during the delivery of T cell help to B cells. Mak *et al.* dissected the role of this interaction in both situations using adoptive transfer system of T and B cells from either ICOSL$^{-/-}$ or wildtype mice into irradiated recipients. Abrogation of ICOS-ICOSL engagement during either APC-T interaction or secondary T-B interaction resulted in substantial defects in the antibody production in the recipient mice [106].

Long-lived plasma cells are responsible for the continuous maintenance of serum antibody levels [192]. Our ELISPOT data to enumerate ASCs confirms that ICOS is a critical costimulatory molecule for the B cells to become class switched antibody producing cells, especially in the secondary response. Presumably, this is partly due to the reduced B cell expansion following the primary immunisation. Given that ICOS is predominantly expressed on GC T cells located in the apical part of the light zone where the T cells are known to induce terminal differentiation of the B cells into plasma cells or memory cells, these results are not surprising [104]. Hutloff *et al.* while studying human SLE patients, found clusters of B cells and plasma cells in close contact with ICOS^{+} T cells in the kidneys. They suggested ICOS to be one of the forces driving the formation of memory B cells and plasma cells in SLE [273]. Since ICOS has been implicated in maintenance of immune responses, it will be interesting to enumerate the ASCs in ICOS$^{-/-}$ and wildtype mice over a period of time in the absence of boosting.

Reduced B cell recall responses in terms of clonal expansion and number of ASC along with reduced T cell primary clonal expansion and T cell helper function in ICOS$^{-/-}$ mice suggest a crucial role of this interaction in the generation and/or reactivation of functional antigen-specific memory cells.

4.4. Conclusion

The experiments carried out in this chapter show that in the absence of ICOS-ICOSL interactions, $CD4^+$ T cells were unable to provide help for B cells to undergo clonal expansion as well as class switching to IgG_1 and IgG_{2a}. Thus, $ICOS^{-/-}$ mice have markedly reduced levels of class switched immunoglobulins in their serum and significantly less antibody secreting cells in spleen and bone marrow. Using adoptive transfer studies with ICOS-deficient transgenic T cells, I have shown that ICOS is important for the comprehensive activation, clonal expansion and differentiation of $CD4^+$ T cells.

5. Role of B cells in the development of T cell helper function

Background

Initial activation of naïve CD4 T cells depends upon prolonged signalling through TCR engagement of peptide/MHC complexes as well as ligation of costimulatory molecules on APCs. Following peptide/MHC-TCR and CD28-CD80/CD86 interactions [274], additional receptors that promote costimulation are induced on T cells, including CD40L (CD154) (371), OX40 (CD134) [10] and ICOS [94], which engage with counter-receptors on APC to promote optimal T cell stimulation. Productive interaction can be achieved by sustained as well as serial encounters of T cells with APC [275]. The duration of stimulation can determine the commitment of CD4 cells to division, which typically correlates with the development of the effector functions [151]. Although cell division *per se* does not appear requisite for epigenetic remodelling of cytokine gene transcription sites [276], several *in vitro* studies reveal a strong correlation between CD4 cell expansion and the frequency of cells that produce such cytokine [151,277]. Other studies have shown that secretion of cytokines IFNγ and IL-2 and expression of markers of T cell differentiation, such as CD44, CD45RB and CD62L, all depend primarily on cell division rather than receipt of costimulatory signals [278].

Once division commences, effector cells can expand and differentiate independently of antigen [200,279]. Optimal induction of costimulation and cytokine production are two major mechanisms that contribute to growth, differentiation and survival of CD4 T cells. So, APCs are integral not only for initiating a CD4 response but also for determining the magnitude of expansion and development of effector cells with Th1 and Th2 phenotypes.

Although initial priming of the naïve T cells occurs on DCs and various DC subsets can promote development of Th1 and Th2 CD4 T cells [280], recruitment of DCs diminishes after the initial phase of a response [275]. DCs can affect T cell differentiation via antigen presentation and cytokine production for only a limited duration before further maturation and exit from lymphoid organ [281].

The role of B cells in T cell priming and differentiation has been strongly debated for a number of years. Literature is available supporting the critical role of B cells as APCs in deciding the fate of CD4 T cell response [82,200,203,205,218,282], though there are reports showing normal CD4 T cell expansion and differentiation in the absence of B cells as well [205,222,283,284]. B cells have been found to be necessary as APC for protein antigens [202] but not for peptide antigens [285]. Peptide antigens do not require antigen processing and can bind directly to surface MHC-II molecules and therefore are much more efficient antigens, which makes them less dependent on antigen-specific B cells as APC [286,287].

Early work done *in vitro* has shown that B cells can present peptide-MHC-II complexes and stimulate naïve T cells [288]. The stimulatory capacity is greatly enhanced if B cells possess a BCR that crosslinks an antigen. For example, TNP-specific B cells have been shown to present TNP-conjugated antigen at 1000-10,000-fold lower concentration than non-TNP coupled proteins [193]. B cells have been implicated in CD8 T cell responses as well. Christensen *et al.* have shown that $CD8^+$ T cell response to LCMV initially is qualitatively similar in wildtype and B cell-deficient mice but CD8 T cells loose functional capacity in the absence of B cells [198]. They also observed markedly impaired priming of virus-specific $CD4^+$ T cells in B cell-deficient mice. Furthermore, in $relB^{-/-}$ mice, that are deficient in myeloid and functional lymphoid DCs, CD4 T cells can be primed in response to soluble protein antigen, suggesting that B cells play a role in T cell priming in the absence of DC functions [206].

B cells require T cell help to clonally expand, class switch and undergo GC reaction in response to a protein antigen. Although it has long been realised that delivery of help by T cells require cognate recognition of antigen presented by B cells, the mechanisms by which this is achieved are unclear. However, it seems unlikely that T cells primed by DCs should immediately express all the functions of a competent helper cell, as this would allow help to be given to all the B cells, regardless of the antigen specificity. Instead, there may be further signals which are required in order for T cells to mature as helper cells, or which trigger delivery of help. This could be delivered during secondary interactions between recently primed T cells and other APCs such as B cells.

Earlier work in the lab, in an *in vivo* model, has shown that T cells primed in CD40 deficient mice are unable to help normal B cells to class switch [289]. Taking this as the baseline, I studied further the molecular basis of this observation in a less laborious *in vitro* model. The aim of this study was to investigate the role of B cells in the differentiation of helper T cells for antibody class switching.

Results

5.1. CD40-deficient T cells do not help B cells to produce class switched antibodies

Initial experiments were done to repeat the previous experiments [289] using *in vitro* system. The experimental model involved hapten-carrier system (fig 5.1). B cells were purified from the spleens of C57Bl/6 mice primed with alum-precipitated DNP-KLH along with killed *Bordetella pertussis* as adjuvant. CD4$^+$ T cells were purified by positive selection from spleens of CD40-deficient mice or wildtype C57Bl/6 mice immunised with ovalbumin-alum and killed *B. pertussis*. A minimum of three mice constituted a group and T cells were purified from the pooled splenocyte population. Purified B and T cells were cultured *in vitro* in the presence of soluble DNP-ovalbumin.

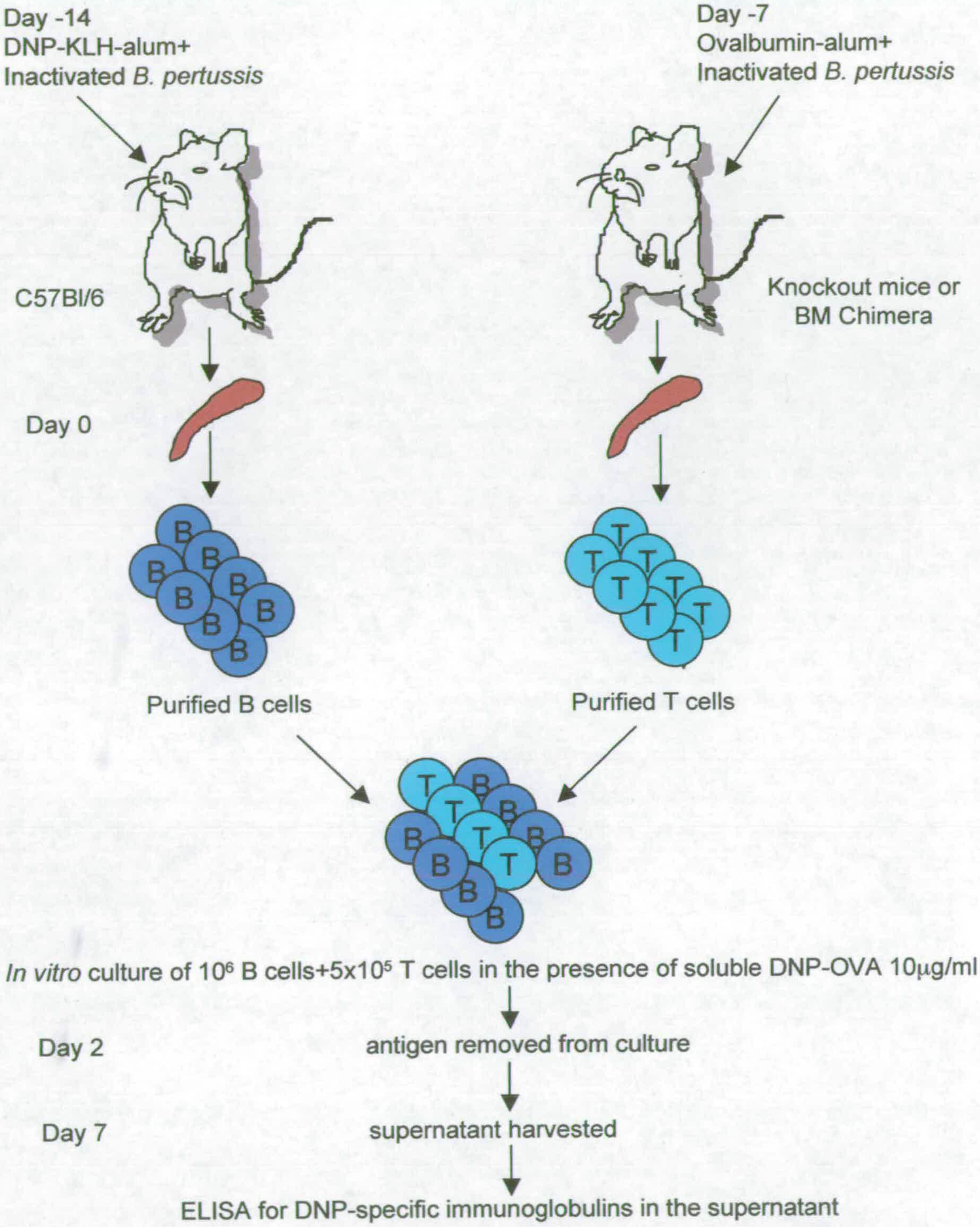


Figure 5.1 Experimental protocol of help assay. B cells were purified from C57Bl/6 mice immunised with alum-precipitated DNP-KLH. Mice, either knockout or chimeric with defective B cell compartment, immunised with alum-precipitated ovalbumin, were the source of T cells. Purified B and T cells were cultured *in vitro* in the presence of DNP-ovalbumin. The medium containing antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later. DNP-specific immunoglobulins in the supernatant were measured by ELISA. DNP-specific immunoglobulins were not detectable when B cells were cultured in the absence of T cells.

After 2 days, the medium containing the antigen was removed and replaced with fresh medium. The culture supernatant was harvested after 5 days and DNP-specific immunoglobulin isotypes measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted.

In this experimental system, naïve B cells did not make measurable quantities of DNP-specific IgG, so in all the experiments DNP-primed B cells were cultured with ovalbumin-primed T cells. The DNP-specific IgG synthesis observed in help assays using normal control T cells was dependent upon cognate T-B interactions, and only occurred when T cells, B cells and specific antigen were all present.

Figure 5.2 shows that there is no significant difference in the amount of DNP-specific IgM produced in CD40$^{-/-}$ and C57Bl/6 cultures. However, the amount of IgG was significantly reduced in the presence of CD40$^{-/-}$ T cells in comparison to the wildtype T cells. One very obvious question arising from this finding was whether the reduced help to B cells was due to smaller clonal expansion of the CD4^{+} T cells in CD40$^{-/-}$ mice as compared to C57Bl/6 mice, and hence less antigen-specific T cells interacting with B cells. To address this question, lymph node cells from OVA-specific TCR transgenic OT-II mice crossed onto CD40$^{-/-}$ background, were adoptively transferred into C57Bl/6 and CD40$^{-/-}$ mice. These mice were immunised 24h later with alum-precipitated ovalbumin intraperitoneally. The expansion of transgenic T cells was measured 7 days after the immunisation by staining the splenocytes with antibodies against CD4, Vα_2 and Vβ_5, and analysed by flow cytometry. Figure 5.3 depicts that the transgenic T cell expansion was 2-fold higher in C57Bl/6 mice as compared to the CD40$^{-/-}$ hosts (C57Bl/6, 7.73%; CD40$^{-/-}$, 3.26%). As shown in chapter 3, the initial T cell priming at day 3 is equivalent in the presence or absence of CD40-CD40L interactions, though it is not sustained in mice lacking either CD40 or CD40L [73].

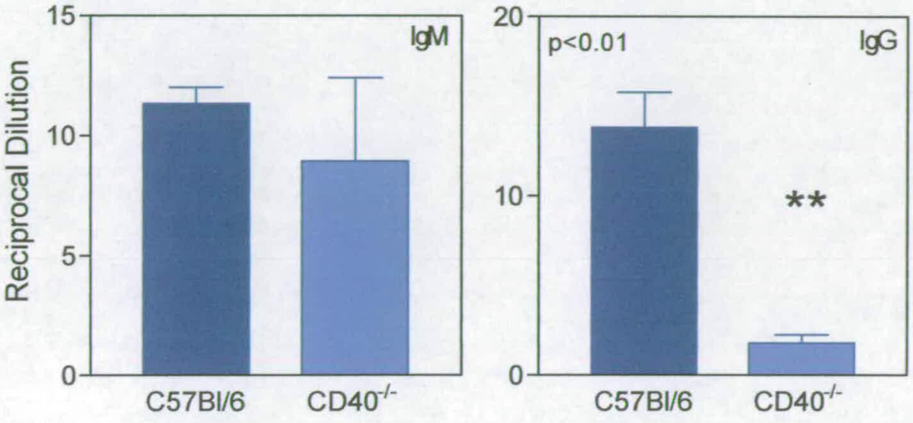


Figure 5.2 CD40-deficient T cells do not help B cells to class switch. Purified CD4 T cells from C57Bl/6 and CD40$^{-/-}$ mice immunised with ovalbumin were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The medium containing antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later and DNP-specific immunoglobulins in the supernatant measured by ELISA. Both groups consisted of 3 mice each and cells purified from pooled splenocytes. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents four independent experiments. Both groups were compared statistically by unpaired Student's t-test.

To compensate for the reduced number of antigen specific T cells in CD40$^{-/-}$ mice, the protocol for help assay was modified slightly. CD4^{+} T cells were purified from the spleens of ovalbumin-immunised C57Bl/6 and CD40$^{-/-}$ recipient mice and the proportion of double positive cells ($V\alpha_2^{+}V\beta_5^{+}$) assessed by FACS. The number of T cells from CD40$^{-/-}$ and C57Bl/6 mice cultured with the DNP-KLH-specific B cells in the help assay were equalised on the basis of proportion of $V\alpha_2V\beta_5$ positive cells. Similar amount of DNP-specific IgM was observed in both cultures, while IgG and IgG_1 were below the detection limits in CD40$^{-/-}$ culture supernatants (fig 5.3). Thus, T cells from CD40$^{-/-}$ mice do not provide help to the normal B cells to class switch to IgG *in vitro*.

5.2. T cells differentiated in the absence of OX40L are unable to help B cells for antibody production

Given the findings of Linton *et al.* [82] that reconstitution with wildtype B cells but not OX40L$^{-/-}$ B cells could restore CD4 T cell responses in μMT mice, I next studied the role of OX40L for T cell helper function development.

When OX40L$^{-/-}$ ovalbumin-primed T cells were cultured with DNP-KLH-specific C57Bl/6 B cells in the presence of DNP-ovalbumin, the amount of DNP-specific antibodies in the supernatants was significantly lower as compared to C57Bl/6 cultures. Significantly lower IgM and undetectable levels of IgG, IgG_1 and IgG_{2a} in culture supernatants from OX40L$^{-/-}$ T cells highlight the critical role of this costimulatory molecule in the development of T cell helper function (fig 5.4).

It has been reported recently that inbred mouse strains with the Igh1-b allele do not have the gene for IgG_{2a} but instead express the isotype IgG_{2c} and due to inadequate cross-reactivity, commercial anti-IgG_{2a} sera are unsuitable to detect this subclass in C57Bl/6

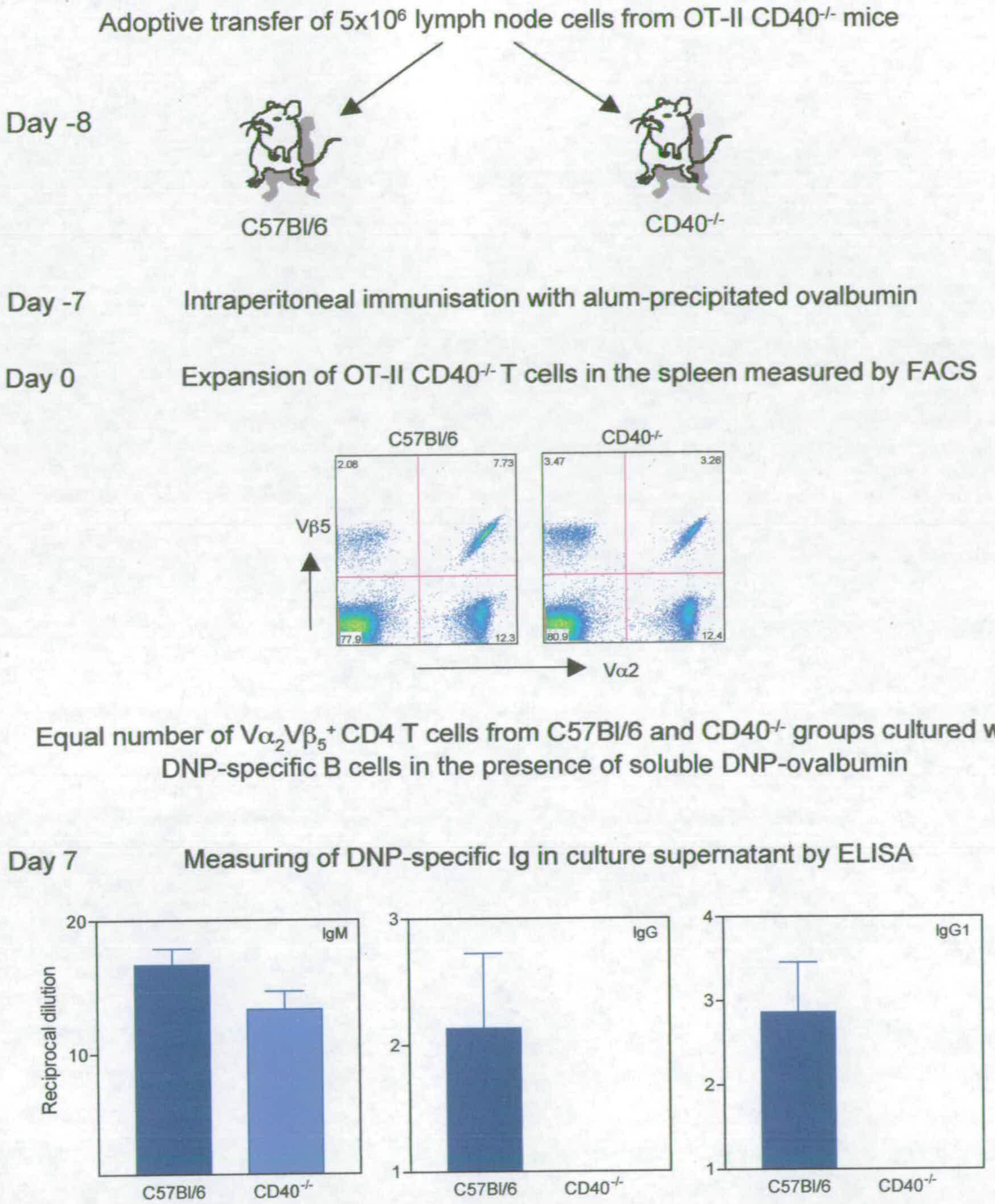


Figure 5.3 CD40-deficient T cells do not help B cells to class switch. $5x10^6$ OT-II $CD40^{-/-}$ lymph node cells were adoptively transferred to C57Bl/6 and $CD40^{-/-}$ recipient mice followed by i.p. immunisation with ovalbumin-alum 24h later. The expansion of OT-II cells in both groups was assessed by flow cytometry and equal number of $V\alpha_2V\beta_5^+$ CD4 T cells from both groups were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The antigen was removed 48h later and culture supernatant harvested 5 days later. DNP-specific immunoglobulins in the supernatant were measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. Both groups consisted of 3 mice each. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments. Since the amount of IgG and IgG_1 were below the detection limits in $CD40^{-/-}$ cultures, their dilution required to achieve half-maximal OD was assumed to be 1.

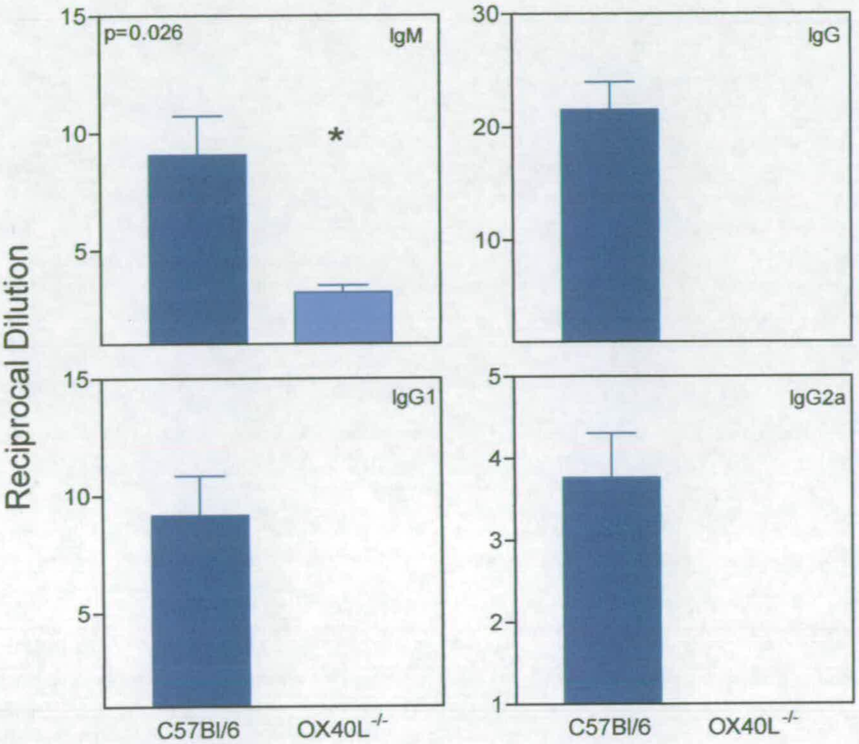


Figure 5.4 T cells differentiated in the absence of OX40L are unable to help B cells for antibody production. Purified CD4 T cells from C57Bl/6 and OX40L$^{-/-}$ mice immunised with ovalbumin were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The medium containing antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later and DNP-specific immunoglobulins in the supernatant measured by ELISA. Both groups consisted of 3 mice each. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments. Both groups were compared statistically by unpaired Student's t-test. Since the amount of class switched Ig were below the detection limits in OX40L$^{-/-}$ cultures, their dilution required to achieve half-maximal OD was assumed to be 1.

mice [290]. In our study however, we did not observe any difference in absorbance values of culture supernatants when measured for either IgG_{2a} or IgG_{2c} (not shown).

5.3. B cells are critical for T cells to acquire helper function

This study aimed at the role of B cells in helper T cell differentiation. Since there have been conflicting reports as to whether T cells primed in B-cell deficient mice can provide help to B cells [202,205,242], I first addressed this issue in *in vitro* system.

Ovalbumin-primed T cells from B cell-deficient μMT and C57Bl/6 mice were cultured with DNP-KLH-primed B cells in the presence of soluble DNP-ovalbumin. 7 days later, the supernatant was harvested and the amount of DNP-specific IgM, IgG, IgG_1 and IgG_{2a} measured by ELISA. Figure 5.5 shows that C57Bl/6 T cells provided help for DNP-specific antibody production, while μMT T cells induced little IgG secretion. When assessed for IgG_1 and IgG_{2a} levels in the supernatants, the levels of both isotypes were below the detection limits in μMT culture supernatants. In all the three experiments done, the levels of IgM in μMT cultures were significantly and consistently lower in comparison to wildtype cultures.

The priming of $CD4^+$ T cell in B cell-deficient mice has been a contentious issue. Available literature from experiments done with different types of B cell deficient mice (μMT-$H2^b$, μMT-$H2^d$, JHD) and using B cell-depleting antibodies, reach conflicting conclusions [199,200,204,205,218,291].

Experiments were done as per the modified protocol of help assay to compensate for any defect in T cell clonal expansion. Thus, equal numbers of antigen-specific T cells from both groups were placed in wells. $3.5x10^6$ purified $CD4^+$ T cells from OT-II $Ly5.1^{+/-}$ transgenic mice were adoptively transferred through the tail vein into 4 each of μMT and

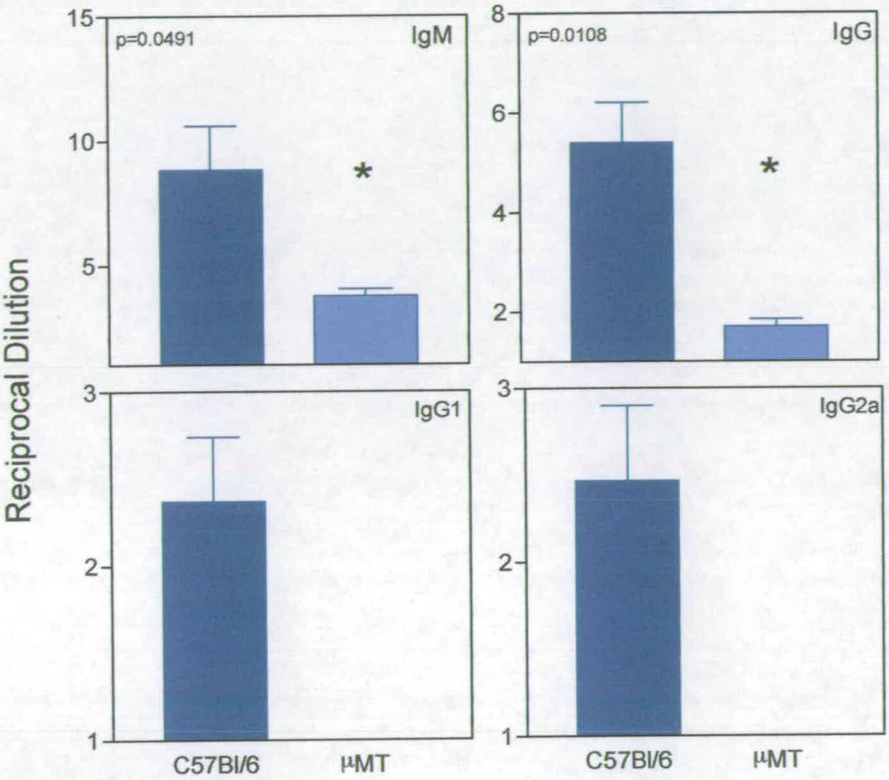


Figure 5.5 B cells are critical for T cells to acquire helper function. Purified CD4 T cells from C57Bl/6 and μMT mice immunised with ovalbumin were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The medium containing antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later and DNP-specific immunoglobulins in the supernatant measured by ELISA. Both groups consisted of 3 mice each. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents three independent experiments. Both groups were compared statistically by unpaired Student's t-test.

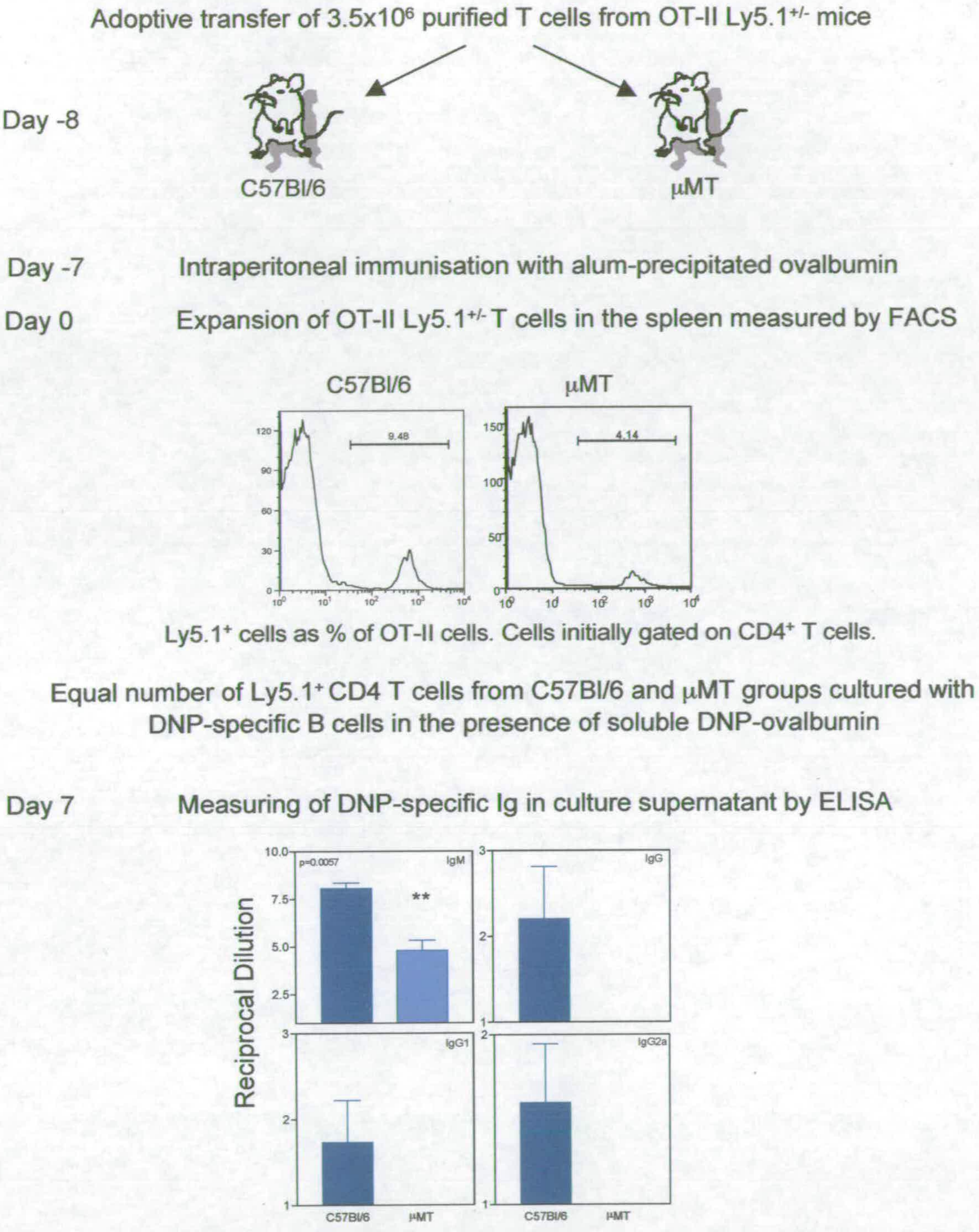


Figure 5.6 T cells differentiated in B cell-deficient mice are unable to help normal B cells for antibody production. 3.5x10^6 OT-II Ly5.1$^{+/-}$ lymph node cells were adoptively transferred to C57Bl/6 and μMT recipient mice followed by i.p. immunisation with ovalbumin-alum 24h later. Expansion of OT-II cells in both groups was measured by FACS and equal number of Ly5.1$^+$ T cells were cultured with DNP-KLH primed B cells in the presence of DNP-ovalbumin. The culture supernatant was harvested at d7 and DNP-specific immunoglobulins measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. Both groups consisted of 3-4 mice each. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments. Since the amount of class switched Ig were below the detection limits in μMT cultures, their dilution required to achieve half-maximal OD was assumed to be 1. Both groups were compared statistically by unpaired Student's t-test.

C57Bl/6 mice, followed by i.p. immunisation with alum-precipitated ovalbumin 24h later. Seven days later, the expansion of OT-II-Ly5.1$^+$ CD4$^+$ T cells in the spleens of the recipients was measured by staining with antibodies against CD4 and Ly5.1, and analysed on FACS. The number of Ly5.1$^+$ OT-II T cells in μMT mice was half the number in wildtype mice (fig 5.6). For example, in one out of the two experiments done, transgenic cells were 4.14% of the CD4$^+$ T cells in μMT mice while in wildtype mice, it was 9.48%. These transgenic T cells were purified using biotinylated anti-Ly5.1 mAb, followed by streptavidin microbeads, and magnetic separation on QuadroMACS (Miltenyi).

DNP-KLH-primed B cells were then cultured with purified Ly5.1$^+$ cells from C57Bl/6 and μMT mice in the presence of DNP-ovalbumin. The B:T ratio in these experiments was 4:1 and not 2:1 as in earlier experiments due to lower yield of Ly5.1$^+$ cells. Figure 5.6 shows that, despite compensating for the reduced priming of T cells in μMT mice, T cells differentiated in μMT mice did not provide help to the B cells for antibody production. Levels of DNP-specific IgM were significantly lower in μMT culture supernatants; IgG and IgG$_1$ were below the detection limits (fig 5.6).

Since the full helper activity does not develop over the 7 days in the presence of normal B cells in the *in vitro* culture, it seems that presence of B cells is crucial during the initial T cell priming event. Thus, B cells are important for T cell acquisition of helper function.

5.4. Bone marrow chimeras

Having confirmed that B cells are important for T cell acquisition of the helper function, I next wanted to dissect out its molecular basis. I first investigated whether cognate interaction between B and T cells is necessary for the helper T cell differentiation. This

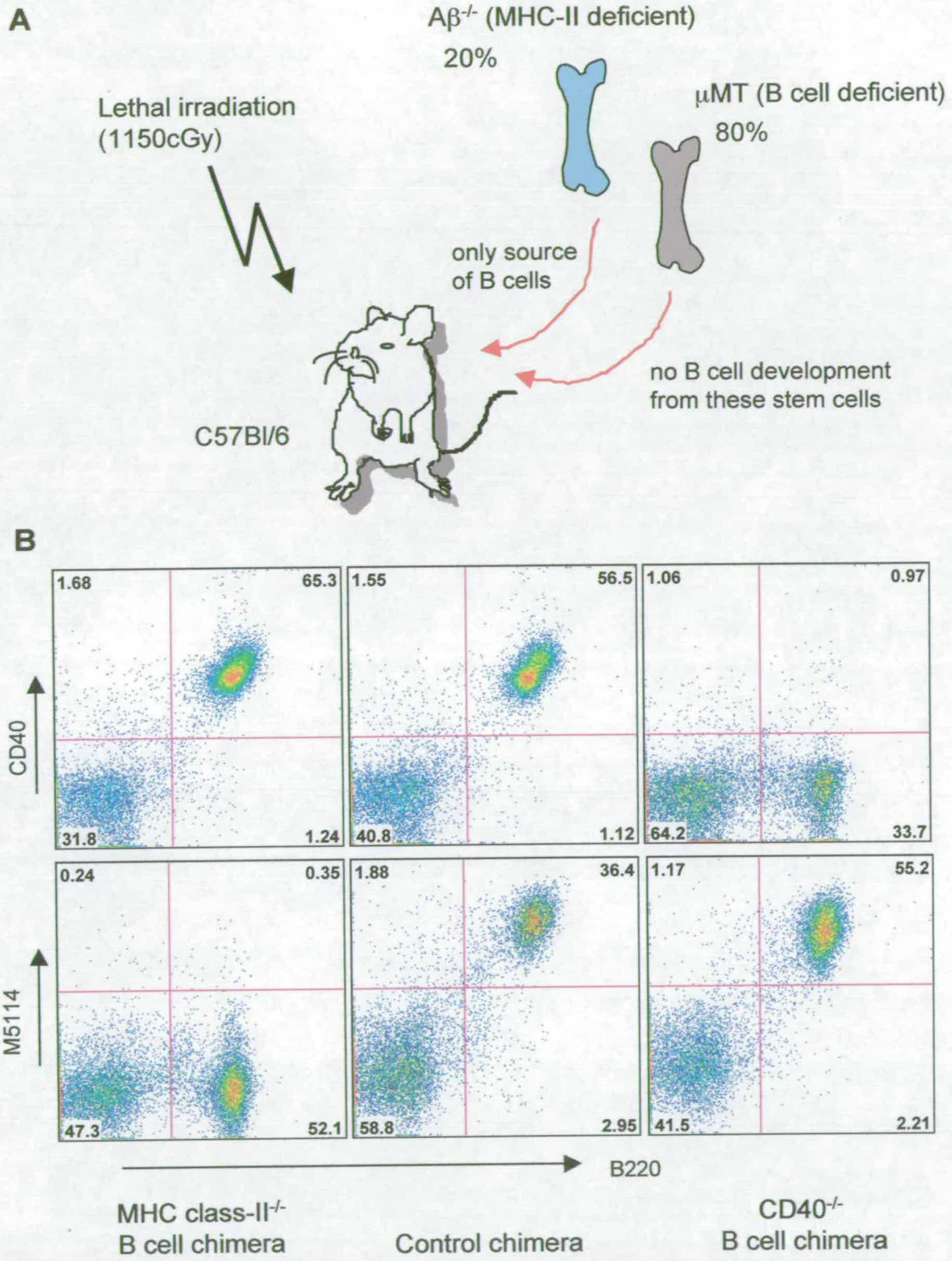


Figure 5.7 Bone marrow chimeras: Generation and reconstitution. (A) Lethally irradiated (1150cGy) C57Bl/6 mice were reconstituted with 3.5×10^6 T-depleted bone marrow cells the following day. MHC-II$^{-/-}$ chimeras received 80% μMT and 20% Aβ$^{-/-}$ bone-marrow, control chimeras 80% μMT + 20% C57Bl/6 and the CD40$^{-/-}$ chimeras got 80% μMT + 20% CD40$^{-/-}$ bone marrow. The mice were left 8 weeks to allow reconstitution of the immune system. (B) The reconstitution of the mice was confirmed by flow cytometry before use. Splenocytes from few mice sacrificed per group were stained with antibodies against B220, CD40 and MHC-II (M5114) and analysed on FACS.

was studied by making bone marrow chimeras (fig 5.7A) with the result that MHC class-II was lacking on B cells but expressed on DCs and other APCs.

Irradiated (1150cGy) C57Bl/6 mice were reconstituted with 80% bone marrow from μMT (gene for μ heavy chain deleted causing a block in B cell differentiation [196] and 20% $A\beta^{-/-}$ (MHC class-II knockout) [172]. Thus, 80% of the hematopoietic cells (except B cells) in the chimeric mice will be wildtype in gene expression whereas the B cells can only be derived from $A\beta^{-/-}$ precursors (fig 5.7A). Over an 8-week period, the 20% knockout bone marrow completely repopulated the peripheral lymphoid system with B cells while contributing only 20% to other lineages. In this way, the function of other APCs including DCs is minimally impaired whereas antigen presentation via MHC class-II by B cells was abolished. As controls, bone marrow chimeras with normal B cell compartment were made by combining 80% μMT and 20% C57Bl/6 bone marrow. Proper reconstitution of the bone marrow chimeras was confirmed before they were immunised (fig 5.7B).

5.5. Differentiation of helper T cells is defective in the absence of cognate interaction with B cells

Ovalbumin-specific $CD4^+$ T cells from MHC class-II deficient B cell chimeras and control chimeras were cultured with DNP-specific C57Bl/6 B cells in the presence of DNP-ovalbumin. ELISA for DNP-specific antibodies in the supernatant harvested a week later revealed that amount of IgG was significantly lower in the cultures with T cells from MHC class-II deficient B cell chimeras as compared to control chimeras. Level of IgG_1 was below the detection limits in $MHC\text{-}II^{-/-}$ chimera cultures (fig 5.8). These results were consistent over four experiments. In two out of four experiments, levels of IgG_{2a} were low even in control chimera group. Thus, in the absence of antigen presentation by B cells, the T cells do not acquire the ability to help B cells for class switching.

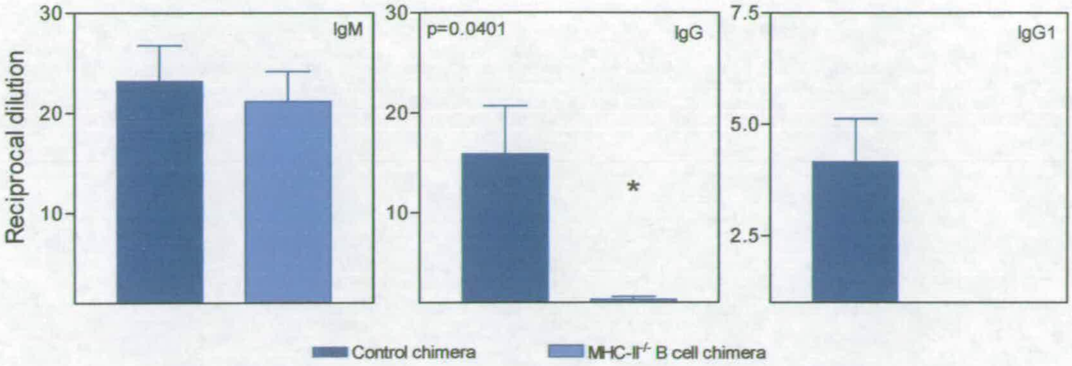


Figure 5.8 Cognate interaction between B and T cells is critical for T cells to acquire helper function. Ovalbumin-specific T cells from MHC-II$^{-/-}$ or control chimeras were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The culture supernatant was harvested at d7 and DNP-specific immunoglobulins in the supernatant measured by ELISA. Both groups consisted of 3 mice each. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents four independent experiments. Both groups were compared statistically by unpaired Student's t-test. Wherever the amount of Ig was below the detection limits, the dilution required to achieve half-maximal OD was assumed to be 1.

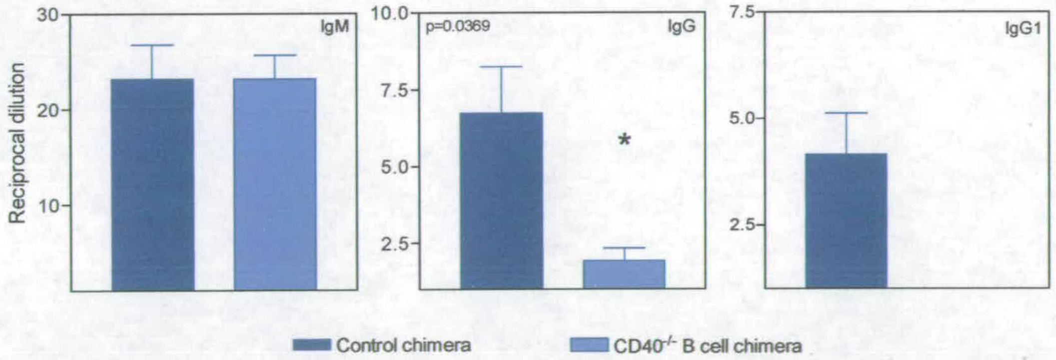


Figure 5.9 Costimulation by B cells is important for T cell acquisition of helper function. Ovalbumin-specific T cells from CD40$^{-/-}$ or control chimeras were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The culture supernatant was harvested at d7 and DNP-specific immunoglobulins in the supernatant measured by ELISA. Both groups consisted of 3 mice each. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents four independent experiments. Both groups were compared statistically by unpaired Student's t-test. Wherever the amount of Ig was below the detection limits, the dilution required to achieve half-maximal OD was assumed to be 1.

5.6. Costimulation by B cells is important for T cell acquisition of helper function

Bone marrow chimeras were generated in which only B cells lacked CD40, all other APCs being CD40 positive (fig 5.7B). The bone marrow inoculate in these chimeras constituted of 80% μMT and 20% CD40$^{-/-}$ [71].

When assessed for providing help to B cells, the T cells from these chimeras were found deficient. Figure 5.9 depicts that IgG as well as IgG_1 were markedly reduced in cultures which had T cells primed in the absence of CD40 on B cells. These results were consistent over four experiments, two of which had significant difference in IgG levels between CD40$^{-/-}$ B cell chimeras and control chimeras. Thus, CD40-CD40L interaction between B and T cell is important for T cells to acquire the ability to help B cells for class switching.

5.7. The defect in T cell help in chimeras is not due to reduced clonal expansion of T cells

One of the possible reasons for the deficient T cell help in MHC class-II$^{-/-}$ and CD40$^{-/-}$ B cell chimeras could be reduced clonal expansion of T cells and hence fewer antigen-specific T cells in the cultures as compared to wildtype cultures. To compensate for this defect, the number of antigen-specific T cells cultured with the DNP-KLH-specific B cells were equalised among various chimeras. OT-II CD40$^{-/-}$ T cells, $3x10^6$ per mouse, were transferred i.v. to MHC class-II$^{-/-}$ B cell chimeras, CD40$^{-/-}$ B cell chimeras and control chimeras. Mice were immunised i.p. with ovalbumin-alum 24h later. CD4$^+$ T cells were purified from the spleens of these mice 7 days later and equal number of $V\alpha_2V\beta_5$ double positive cells from all three groups cultured with DNP-specific C57Bl/6 B cells. Despite equalising the number of antigen-specific T cells in the cultures, the T cells from either MHC class-II$^{-/-}$ B cell chimeras or CD40$^{-/-}$ B cell chimeras fail to

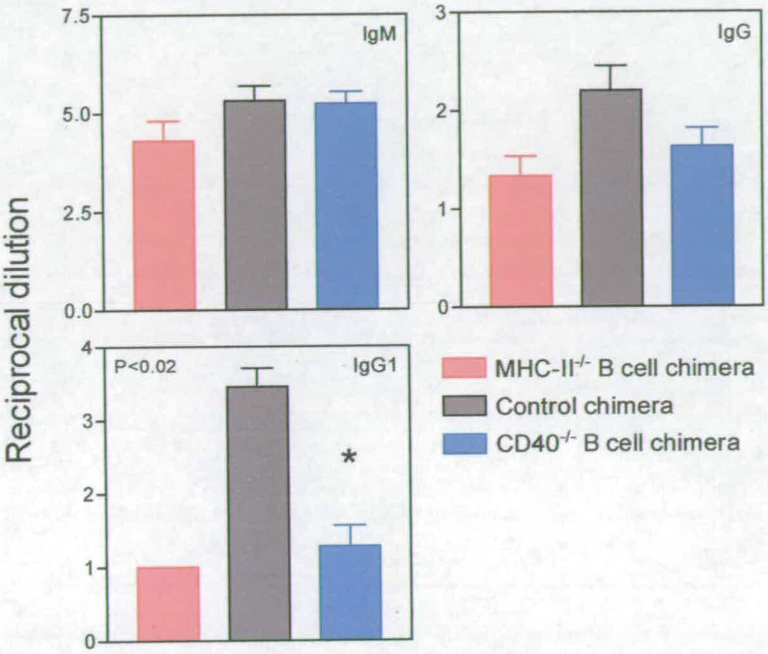


Figure 5.10 Defect in the help provided by T cells from either MHC-II or CD40$^{-/-}$ B cell chimeras is not due to reduced number of antigen-specific T cells in these mice. 3x10^6 OT-II CD40$^{-/-}$ lymph node cells were adoptively transferred to MHC-II$^{-/-}$, CD40$^{-/-}$ and control chimeras followed by i.p. immunisation with ovalbumin-alum 24h later. The expansion of OT-II cells in both groups was assessed by flow cytometry and equal number of Vα_2Vβ_5^+ CD4 T cells were cultured with DNP-KLH primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The medium containing antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later. DNP-specific immunoglobulins in the supernatant were measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. Both groups consisted of 3 mice each. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments. Wherever the amount of Ig was below the detection limits, the dilution required to achieve half-maximal OD was assumed to be 1. Statistical comparison between groups was done by unpaired Student's t-test.

efficiently help the B cells to produce DNP-specific IgG and IgG_1 compared to control chimeras (fig 5.10).

Hence, it can be inferred from above studies that in the absence of T cell-interaction with B cells, in terms of antigen-presentation and CD40 costimulation, the T cells fail to acquire the ability to help B cells for class switching *in vitro*.

5.8. Class switching in bone marrow chimeric mice

Cognate interaction between B cells and T cells is required to form GCs and undergo isotype class switching in response to protein antigens. Also, CD40-CD40L interaction between B and T cells is critical for the production of class switched antibodies. The B cells in MHC-$II^{-/-}$ B cell chimeras do not present the antigen to T cells, while in $CD40^{-/-}$ B cell chimeras, the B cells cannot provide costimulation to T cells via CD40. In both the situations, mice should not make IgG responses to protein antigens. To confirm this, mice were immunised with alum-precipitated DNP-ovalbumin and their sera examined for DNP-specific antibodies a week later by ELISA. The IgM response was comparable between all mice while only control chimeras could mount DNP-specific IgG response (fig 5.11A).

5.9. Expression of CD40L and ICOS on T cells in chimeras

An important signal required by B cells to isotype switch is CD40 cross-linking, which coincidently also provides a costimulatory signal to T cells via CD40L and enhances IL-4 synthesis [292]. I compared the CD40L upregulation on CD4 T cells from MHC-$II^{-/-}$ B cell chimeras, $CD40^{-/-}$ B cell chimeras and control chimeras immunised a week before with ovalbumin-alum. The surface staining of splenocytes for CD40L was done either directly *ex vivo* or 20min after *in vitro* stimulation with PMA and ionomycin at 37°C, a process known to translocate preformed CD40L to the cell surface [165]. Intracellular

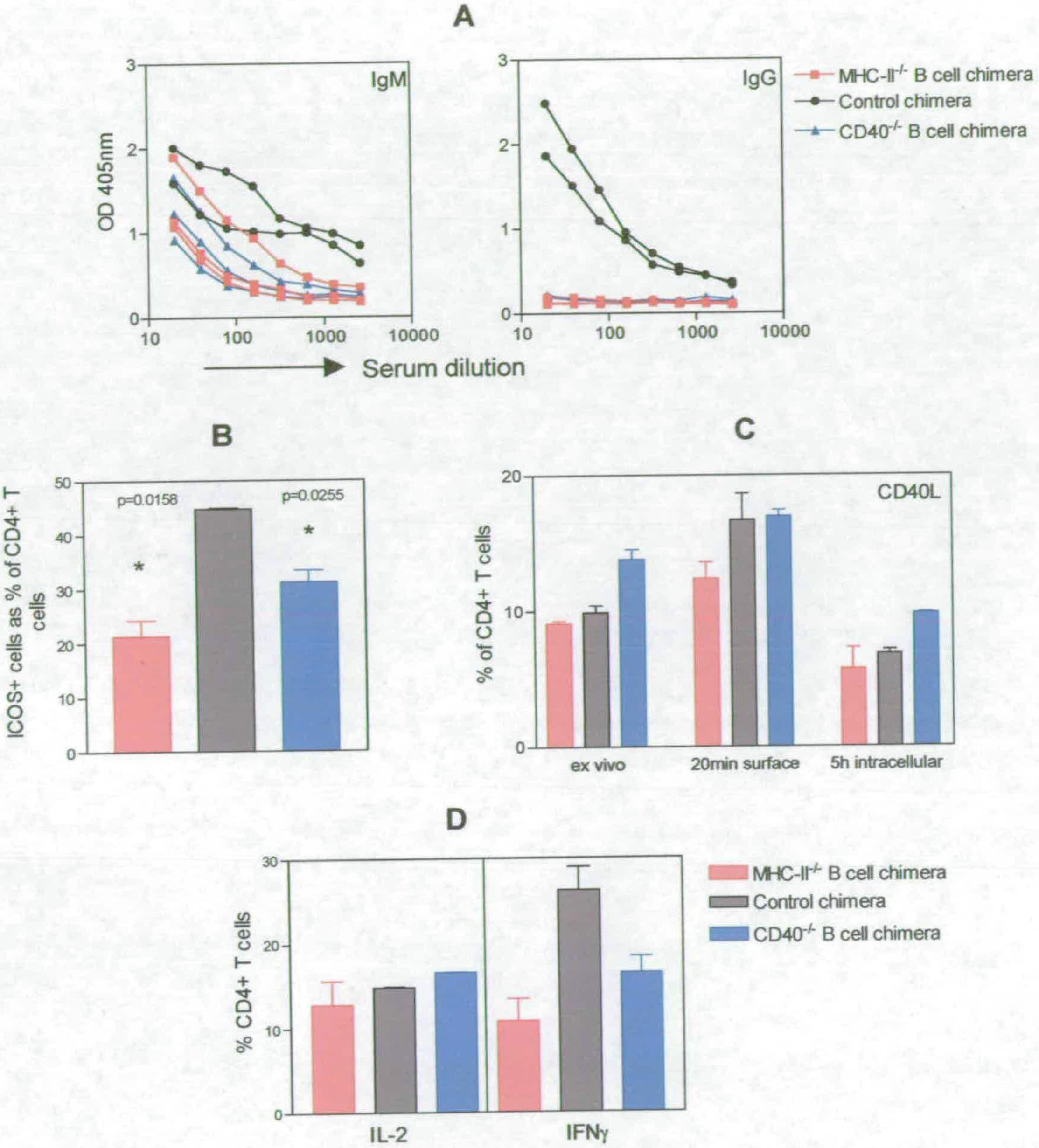


Figure 5.11 Characterisation of the bone marrow chimeras. The chimeras were immunised i.p. with alum-precipitated ovalbumin, 150μg/mouse, and their responses assessed at d7. (A) B cells in MHC-II and CD40$^{-/-}$ B cell chimeras do not produce class switched antibodies. Mice were bled before imminisation and at d7 after immunisation and serum separated. DNP-specific IgM and IgG levels were measured by ELISA. The graphs show the OD at 405nm of d7 serum samples. (B) The splenocytes were stained with antibodies against CD4 and ICOS and analysed by flow cytometry for double positive cells. The graph shows ICOS$^+$ cells as % of CD4 T cells. (C) CD40L surface expression on CD4 T cells was assessed either directly *ex vivo* or 20min after *in vitro* PMA+ionomycin activation by staining with antibodies against CD4 and CD40L and analysing on FACS. Intracellular staining for CD40L was done after 5h incubation with PMA+ionomycin and GolgiStop. (D) Cells were stimulated with PMA+ionomycin+GolgiStop and their cytokine expression measured by intracellular cytokine staining and flow cytometry (section 2.7.2). The graph shows the IL-2 and IFNγ positive cells as % of CD4 T cells. All the groups had 2-3 mice each. Data shown in A, B and D is representative of two independent experiments while C shows data from one experiment. Unpaired Student's t-test was used to compare the groups statistically.

staining for CD40L was done after 5h of incubation with PMA, ionomycin and GolgiStop to measure the proportion of T cells capable of upregulating CD40L.

No significant difference was noticed in the proportion of T cells that upregulated CD40L among different chimeras at all times apart from slightly reduced expression by T cells from MHC-II$^{-/-}$ chimera 20min after PMA and ionomycin stimulation (fig 5.11C). This study indicates that B cell antigen presentation and costimulation via CD40 does not affect the upregulation of CD40L on T cells.

ICOS has been implicated in the T-B interactions and the mice lacking ICOS have few small GCs and no class switched antibodies [94]. When the chimeras were compared for the expression of ICOS on CD4 T cells, both MHC-II$^{-/-}$ B cell chimeras as well as CD40$^{-/-}$ B cell chimeras were found to have significantly fewer CD4 T cells expressing ICOS as compared to the control chimeras (fig 5.11B).

5.10. T cell effector function: cytokine profile in chimeras

The CD4 T cells from spleens of the chimeras immunised with ovalbumin-alum a week before were examined for the cytokines by intracellular cytokine staining. No apparent difference was noted in the number of CD4 T cells secreting IL-2. IFNγ secreting CD4 T cells were fewer in MHC-II$^{-/-}$ chimeras and CD40$^{-/-}$ chimeras in comparison to control chimeras (fig 5.11D). Although by intracellular cytokine staining, number of T cells that were positive for IL-4 was always found to be low making it difficult to draw a conclusion, there was an indication towards lower number of IL-4^{+} T cells in MHC-II$^{-/-}$ chimeras (not shown). Attempts were made to measure the cytokines such as IL-4 in *in vitro* cultures of splenocytes from various chimeras by cell based ELISAs, but those experiments did not yield conclusive data due to the low amount of IL-4 produced. Perhaps, more sensitive assay such as ELISPOT to measure cytokines can give conclusive data.

5.11. Can wildtype B cells revert the defect in differentiation of helper T cells in chimeras?

Apart from antigen presentation and costimulation, B cells can affect T cell responses by cytokines or some other B cell-derived factor. Transfer of naïve B cells [199] or antigen-pulsed activated B cells [82] have been reported to restore the T cell responses in B cell-deficient mice and OX40L$^{-/-}$ mice, respectively. From this study so far, it could be concluded that B cells affect the T cell differentiation via MHC class-II and CD40. We attempted to compensate for this defect in chimeras by transferring C57Bl/6 B cells. Between 10-20 million naïve B cells were injected into MHC-II$^{-/-}$ B cell chimeras and control chimeras on the day of immunisation, and help assay carried out as before. ELISA for DNP-specific antibodies in the supernatants indicated that wildtype B cells could compensate for the defect in T helper cell differentiation in chimeras (fig 5.12), but these results await confirmation.

In one experiment, ovalbumin-primed B cells from C57Bl/6 mice were injected in CD40$^{-/-}$ B cell chimeras and control chimeras 24h before immunisation. The level of DNP-specific IgG was highest in the group with transferred B cells, but due to inconsistency of the results especially in control chimeras, it was difficult to ascertain whether or not these B cells were instrumental in the T cell differentiation (fig 5.13). Given more time, I would like to try transferring naïve and primed wildtype B cells in the chimeras on two consecutive occasions.

5.12. Role of chemokine receptor CXCR5 in the development of T cell helper function

The chemokine receptor CXCR5 is critical for the migration of lymphocytes to the B cell follicles in secondary lymphoid tissues. As the immune response progresses, T cells

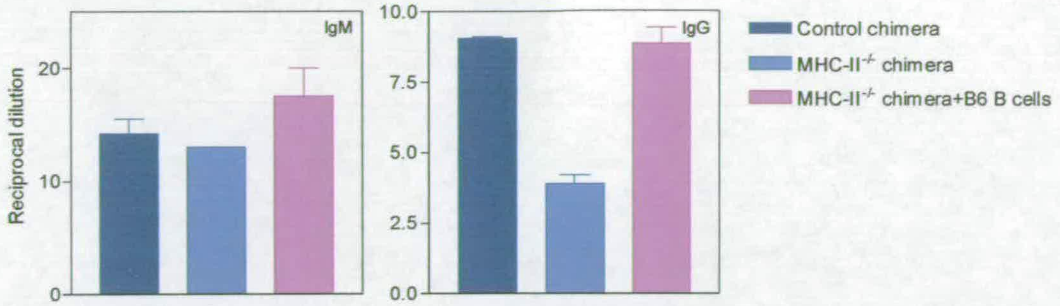


Figure 5.12 C57Bl/6 B cells may revert the T cell differentiation defect in MHC-II$^{-/-}$ B cell chimeras. 10-20x10^6 C57Bl/6 B cells were injected into MHC-II$^{-/-}$ chimeras on the same day when they were immunised with ovalbumin. A week later, T cells were purified and cultured with DNP-KLH specific-B cells in the presence of DNP-ovalbumin. The antigen was removed after 2 days and the culture supernatant harvested 5 days later. DNP-specific Ig were measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. Each group comprised of 3 mice. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments.

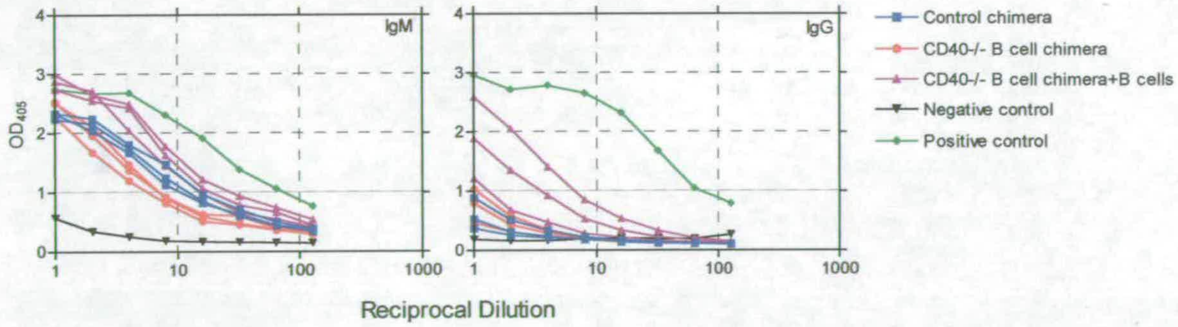


Figure 5.13 Primed C57Bl/6 B cells may revert the defect in T cell differentiation in CD40$^{-/-}$ B cell chimeras. B cells were purified from C57Bl/6 mice primed with ovalbumin 2 weeks earlier and transferred to CD40$^{-/-}$ chimeras a day before immunisation with alum-precipiated ovalbumin. A week later, T cells were purified from spleens of the recipient mice and cultured with DNP-KLH specific-B cells in the presence of DNP-ovalbumin. The antigen was removed after 2 days and the culture supernatant harvested 5 days later. DNP-specific Ig were measured by ELISA. Each group comprised of 3 mice. The graphs show the raw data from ELISA done on triplicate wells from one experiment.

migrate into the follicles to provide help to B cells to class switch and form GC. From the results discussed so far, the interaction between T and B cells in the follicles is possibly bi-directional. I intended to investigate whether the signals that induce T cells with the specialisation to help B cells are delivered prior to (by DC) or after (B cells) T cell migration to the B cell follicles. This study can be undertaken by making bone marrow chimeric mice in which T cells lack CXCR5, and thus are not able to migrate to B cell follicles. Since TCRβδ$^{-/-}$ mice do not have T cells, a combination of bone marrow inoculum from TCRβδ$^{-/-}$ mice (~80%) and CXCR5$^{-/-}$ mice (~20%) would give rise to chimeric mice that do not express CXCR5 on the T cells. Due to difficulties in acquiring TCRβδ$^{-/-}$ mice however, this study could not be done. As a prelude, I tested the B cell helping ability of CXCR5$^{-/-}$ T cells. Ovalbumin-primed CXCR5$^{-/-}$ T cells were cultured with DNP-KLH-specific wildtype B cells in the presence of DNP-ovalbumin. As shown in figure 5.14, CXCR5$^{-/-}$ T cells were as efficient as wildtype T cells to help B cells to class switch. Interestingly, the levels of IgM in CXCR5$^{-/-}$ cultures were significantly higher than C57Bl/6 cultures. I had expected a defect in help by CXCR5$^{-/-}$ T cells, considering that the lymphoid architecture in CXCR5$^{-/-}$ mice is aberrant without proper segregation of B and T cells. However, it is possible that CXCR5 is required only for migration of lymphocytes since class switching and affinity maturation do occur in CXCR5$^{-/-}$ mice in spite of disturbed organisation of the secondary lymphoid organs [166,167].

5.13. Discussion

It is a rare event for a naïve T cell to interact with a naïve B cell, and it is likely that T cells first divide to increase their number before interacting with B cells. The initial priming of the naïve T cells occurs on DCs. Besides higher expression of MHC-II, adhesion and costimulatory molecules, DCs process and present limited immunodominant peptides. While as a result of antigen internalisation and processing by

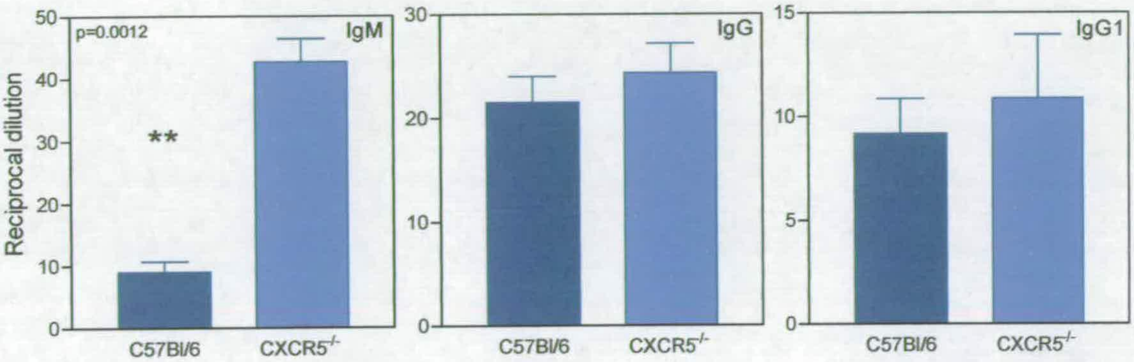


Figure 5.14 CXCR5$^{-/-}$ T cells are efficient helpers for B cell antibody production. Purified CD4 T cells from C57Bl/6 and CXCR5$^{-/-}$ mice primed with ovalbumin were cultured with DNP-KLH-primed C57Bl/6 B cells in the presence of DNP-ovalbumin. The medium containing the antigen was removed 48h later and replaced with fresh medium. The culture supernatant was harvested 5 days later and DNP-specific immunoglobulins in the supernatant measured by ELISA. The reciprocal dilution required for half-maximal OD was measured and plotted. Data shown is the mean of triplicate wells ± SEM and represents two independent experiments. Both groups were compared statistically by unpaired Student's t-test.

B cells, the complexity of antigenic epitopes made available to the T cell pool by a particular B cell increases significantly. This happens despite the frequency of a B lymphocyte of any one specificity being only in the order of $2.5\text{-}4\text{x}10^{-4}$ for murine lymphoid organs [293-295]. B cells can also increase chances of T cell survival by providing costimulatory molecules to T cells e.g. OX40L, which interacts with OX40 on T cells and suppresses apoptosis by promoting the expression of Bcl-xL and Bcl-2 [55]. B cells are also known to act as decoys by expressing Fas and regulating their own destruction instead of T cells [136].

B cells have been shown to be important for the differentiation of helper T cells on the basis of various studies done using B cell-deficient mice of various strains. Also, costimulatory molecules like CD40 and OX40L have also been proved to be critical for the T cell acquisition of helper function [209,289]. However, using more classical cellular assays, it has been difficult to determine the precise nature of the observed defects in either B cell deficient mice or mice lacking these costimulatory molecules. In particular it is unclear to what extent the defects are quantitative, qualitative or both. Therefore, to better characterise the defect in the T cell differentiation in mice with defective B cell compartment, we took advantage of a unique technology involving bone marrow chimeras. By transferring a combination of bone marrow precursor cells from μMT and $A\beta^{-/-}$ or $CD40^{-/-}$ into lethally irradiated mice, I could create chimeric mice that lacked the molecule of interest on B cells only. These mice provided me with opportunity to study the role of MHC-II and CD40 on B cells in differentiation of T helper cells.

Because I used purified populations of $CD4^{+}$ T cells and B cells, and because T and B cells were primed with different antigens, it is unlikely that DNP-specific IgG was produced by contaminating B cells in the T cell (ovalbumin-primed) population or that contaminating T cells in the B cell population induced immunoglobulin synthesis.

I found μMT T cells to lack the capability to help B cells for antibody production. This was evident by significantly lower levels of IgG, IgG_1 and IgG_{2a} in μMT culture supernatants. This study corroborates earlier findings [202,204]. Macaulay *et al.* attributed the lack of help by JHD T cells only partly to a failure to upregulate IL-4 synthesis, since addition of IL-4 to the JHD cultures did not increase IgG production to the level in wildtype cultures without exogenous IL-4. I observed low IgM levels in μMT culture supernatants. This finding differs from the earlier reports [204], though, not unexpected given that T cell help is important, if not critical, for IgM responses.

On the contrary, Rivera and colleagues reported that systemically primed T cells from μMT.BALB/c or μMT.B6 B cell-deficient mice were equally capable of providing B cell help as T cells primed in normal control mice [199]. This contrasting result may be because of differences in experimental protocols. This report had both T as well as B cells primed with SRBC before adoptive transfer into lethally irradiated recipients. The possibility of contaminating SRBC-specific T cells in the B cell population providing the help required for class switching cannot be excluded.

Given that same number of OVA-specific OT-II T cells from C57Bl/6 and $CD40^{-/-}$ mice in the culture could not compensate for the reduced help by $CD40^{-/-}$ T cells, it is likely that the defect in helper function is qualitative. Gray and co-workers observed similar findings in an *in vivo* model where KLH-primed T cells from $CD40^{-/-}$ and wildtype littermates were injected into lightly irradiated $CD40^{-/-}$ mice along with CD40 expressing B cells [289]. Only those mice that received T cells primed in $CD40^{+}$ environment produced class switched antibodies of the donor origin. The outcome was the same despite giving 15-fold excess of antigen-specific $CD40^{-/-}$ T cells compared to wildtype T cells.

Oxenius *et al.* however showed that neither CD40 expression nor the presence of B cells was necessary for the development of T cell helper function [242]. T cells from either μMT or CD40$^{-/-}$ mice infected with LCMV could facilitate isotype switching when transferred into normal naïve mice. These conflicting results may reflect a difference in the predominant cytokine and isotypes present during LCMV infection (Th1) versus priming with alum-precipitated antigen (Th2). It is also possible that viral infection activates APCs in such a way as to give different signals to responding T cells than adjuvant activation does.

Having shown that MHC-II and CD40 on B cells are required for T cells to differentiate into efficient helpers for B cells, it is important to consider why these molecules could be important. It is possible that some T cells that undergo only a short interaction with DC have not been primed enough to undergo cell division and differentiation, and the second interaction with a B cell is necessary for differentiation. It has been shown that T cells require several hours of stimulation before they can undergo proliferation. Furthermore, MHC-signalling in B cells may enhance their proliferation, upregulate costimulatory molecules and cytokine production, making them efficient APC [296-298]. Another possibility is that B cells provide cytokines or some other factor during a stable interaction with T cells as a result of peptide/MHC-TCR and CD40-CD40L interactions.

The defects I have observed in the interaction of primed T cells (from μMT mice and chimeras) with B cells *in vitro* indicate an absence of a signal normally provided by B cells during *in vivo* priming. Given the findings of van Essen *et al.* [289] that T cells primed in CD40$^{-/-}$ mice are unable to provide B cell help and that CD40L signalling can stimulate T cell IL-4 synthesis [292], CD40-CD40L interaction seems like a critical signal in the development of helper T cells. Although other APCs express CD40, B cells express this molecule at higher levels [207,299], which may be necessary for these responses. Signalling via CD40 on B cells has a number of effects, including class switching, proliferation [300] and upregulation of costimulatory molecules like CD80 and CD86 [301,302]. Since B cell activation and proliferation seem to depend critically on presence of cognate T-help [183,303], it may be possible that in MHC-II$^{-/-}$ and CD40$^{-/-}$ B cell chimeras, there are fewer activated B cells as APC and reduced costimulatory molecules, which affects the T cell differentiation.

It has also been suggested that limited expansion and differentiation of T cells in μMT mice is probably due to APC deficiency since either B cell or splenic DC transfers in μMT mice could reconstitute the expansion and Th2 cytokine response [203]. This could be one of the reasons for defective helper T cell differentiation in the MHC-II$^{-/-}$ B cell chimeras where B cells are not able to present antigen to T cells.

Interestingly, uptake of antigen *in vivo* via Ig receptor upregulates B7-2 and MHC-II expression on B cells within 4h and allows successful priming of naïve T cells [285]. However, by 24h of antigen introduction, B cells downregulate B7-2 and loose their antigen-presenting capacity over time unless they interact with an antigen-specific T cell. Between 24-48h, B cells expressing antigen complexes in the absence of costimulatory molecules are most likely to induce T cell anergy.

5.13.1. OX40-OX40L interactions in T cell differentiation

T cells differentiated in the absence OX40-OX40L interaction fail to acquire the ability to help B cells to class switch. A possible function of OX40-OX40L interaction in regulating the T cell-dependent B cell responses was proposed based on the finding that injection of rabbit anti-mouse OX40 antiserum inhibits antigen-specific B cell responses in mice [85]. Treated mice developed GCs in response to TNP-KLH but were impaired in the production of TNP-specific IgG. However, Pippig *et al.* did not find OX40 to be essential for B cell differentiation and antibody secretion in their studies done with

$OX40^{-/-}$ mice. $OX40^{-/-}$ and wildtype mice exhibited equivalent levels of serum antibody in response to various antigens [304]. Probably the use of parasitic infections like *Leishmania major* and *Nippostrongylus brasiliensis*, involving powerful antigenic stimulation, might have masked the effects due to lack of OX40. Costimulation of CD4 T cells through OX40-OX40L interaction has also been implicated in the upregulation of chemokine receptor CXCR5 *in vitro* [209]. Since CXCR5 is required for the migration of B and T cells into the follicles, OX40-OX40L interaction could be important for recruiting antigen-specific T cells into the GCs and thereby influencing antibody responses. Additional support for the involvement of OX40L on T cell responses comes from the studies done by Linton and co-workers. They showed restoration of CD4 T cell responses in μMT mice reconstituted with wildtype B cells but not $OX40L^{-/-}$ B cells [82]. Jacquot *et al.*, in a human study showed that CD27-CD70 interactions induced plasma cell differentiation once CD40-dependent clonal expansion of the B cells had taken place [214]. Morimoto *et al.*, in a similar study suggested that this is followed by OX40-OX40L mediated enhanced antibody production on per cell basis in the presence of IL-4 and IL-10 [134].

To answer whether OX40L on B cells is important for T cells to acquire the ability to help B cells, I have generated $OX40L^{-/-}$ B cell chimeras. It was not feasible to do the experiments with these mice yet due to time lost in re-derivation of $OX40L^{-/-}$ mice and the reconstitution of the bone marrow chimeras.

5.13.2. Lymphoid architecture in T cell responses

Although studies with B cell deficient mice have increased our understanding of the involvement of B cells in regulation of T cell responses, the fact that these mice have disrupted architecture of the major lymphoid organs (e.g. spleen and lymph nodes), cannot be ignored. Making chimeras was an elegant way to look at the role of B cells in particular in context of antigen presentation or costimulation, without disruption of the splenic architecture. Unlike μMT mice, these BM chimeras possess both FDCs and marginal zone macrophages. Interestingly, it is the environment of B cell zone that is more critical than the B cells themselves as impaired CD4 cell responses to LCMV have been reported in TNFR$^{1+2-/-}$ mice that have normal B cells but resemble B cell-deficient mice in that they lack splenic B cell follicles, follicular dendritic networks and germinal centres [198]. Normal lymphoid architecture hence seems critical for the optimal T cell priming.

5.13.3. Regulation of T cell help by cytokines

B cells can regulate cytokine secretion by CD4 T cells. Linton and colleagues observed that OT-II cells produced similar quantities of IFNγ, IL-2 and IL-10 irrespective of whether they were primed in normal or μMT mice. However, T cells from μMT mice produced markedly lower levels of IL-4, IL-5 and IL-13, which could be restored by provision of activated, antigen pulsed OX40L^{+} B cells [82]. Reconstitution with B cells did not affect the frequency of IFNγ producing effector cells in μMT mice suggesting that IFNγ response, which can develop before that of IL-4 [151,305] can be optimally primed without a contribution from B cells. Link between B cell antigen presentation and induction of IL-4 secretion by CD4 T cells has been suggested earlier [207,208].

Literature is available on reduced IFNγ production by T cells in B cell deficient μMT mice [203] and *in vitro* in JHD cultures [204]. In contrast, Moulin *et al.* have reported that CD4 effectors primed in μMT mice are skewed towards Th1 response with secretion of high levels of IFNγ that appeared to correlate with DC synthesis of IL-12 [306]. It is possible that without B cells to either downregulate IL-12 synthesis or DC maturation via IL-10, or assume a role as APC that do not produce IL-12, only Th1 cells would arise [307-309]. Moreover, after stimulation of naïve CD4 cells, the capacity to produce IFNγ develops early, whereas IL-4 secretion develops more slowly, and can be associated with progression through several cycles of division [151]. Thus, reduced CD4 expansion in the absence of B cells may result in aborted CD4 cell differentiation, and as a consequence, a Th1 dominated response [200].

5.13.4. Importance of B cells

Significantly less CD4 T cells in the MHC-II$^{-/-}$ chimera as well as CD40$^{-/-}$ chimera expressed ICOS as compared to control chimeras. The fact that ICOS is critical for B cell help by T cells, this finding strongly indicates the suboptimal differentiation of helper T cells in mice with defective B cell compartment. Hence, it suggests that B cells are an important regulator of T cell responses.

I did not observe any major defect in CD40L upregulation on T cells in chimeras. CD40L has been reported to be upregulated to the same extent in JHD and wildtype T cells when stimulated with anti-CD3, though it is not known if the same would be true with B cells as APC [204]. In support, CD40L induction is largely independent of costimulatory signals [96,268,269].

The absence of IgG antibodies in MHC class-II and CD40-deficient B cell chimeras can likely affect the T cell responses in these chimeras. One possible way this can happen is by reduced IgG mediated opsonization and therefore reduced activation of macrophages through FcγRI (CD64). Macrophages when activated are potent APCs and can contribute to enhanced T cell responses [75]. On the other hand, antibodies can neutralise the antigen and thereby reduce lymphocyte priming.

T_{FH} subset of T cells, characterised by expression of CXCR5, is considered to be involved in B cell help [33,164,165]. I could not characterise this subset of T cells in the chimeras in this study in want of CXCR5-specific antibody that could be used to visualise these cells on FACS. Alternatively, induction of CXCR5 mRNA in CD4 T cells can be tried [209].

Although the involvement of marginal zone (MZ) B cells in T-independent immune responses is well established, a recent report by Attanavanich and Kearney suggested that MZ B cells can present protein antigens to CD4 T cells inducing their proliferation both *in vivo* and *in vitro* [310]. Moreover, the authors observed that MZ B cells upregulate costimulatory molecules such as CD80 and CD86, and regulate effector T cell generation by stimulating CD4 T cells to produce high levels of Th1 cytokines upon *in vitro* stimulation. Since Follicular (FO) B cells have always been considered more important for the T-B interactions, it would be interesting to investigate whether MHC-II and CD40 on FO or MZ B cells are important for the T cell differentiation into helper cells for B cells. Bone marrow chimeric mice specifically lacking these molecules on MZ B cells can be created using Dedicator of cytokinesis 2 (Dock2) deficient mice or Protein kinase 2 (Pyk2) deficient mice that lack MZ B cells [17,311,312].

Jordan *et al.* have recently shown that intraperitoneal injection of alum resulted in more than 50% of the splenic B cells being primed in the context of intracellular Ca^{2+} levels [313]. IL-4 secreted by a subset of $Gr1^{+}$ cells was ascertained for this priming. How the use of alum as adjuvant in my study affect the B cells in chimeras, would be interesting to investigate.

5.14. Conclusions

In summary, B cells are crucial for the T cells to acquire helper function. The data presented in this chapter suggest that T cells that have been activated *in vivo* in the absence of B cells are impaired as they are unable to help B cells to class switch *in vitro*. Furthermore, T cells primed in the absence of CD40 and OX40L are unable to help normal B cells to class switch. By creating bone marrow chimeric mice, I have shown that in the absence of B cells that express MHC class-II or CD40, antigen induced differentiation of effector T cells that help B cells to class switch, is impaired.

6. Discussion

6.1. Role of CD40 and ICOS in CD4 T cell responses

Costimulation is more complex than it was originally thought. The immune system has lots of back up mechanisms in terms of the redundant and overlapping functions of various costimulatory molecules. In addition, costimulatory signals may not be completely distinct from TCR signals. Many studies now show that CD28, ICOS and members of TNFR family can engage signalling intermediates that are common to the TCR and to each other [314]. So, the effects of co-signalling can be quantitative, leading to the accumulation of common signalling intermediates, or qualitative, involving distinct signals that do not overlap between various costimulatory molecules, and are not engaged by the TCR.

As there are many costimulatory receptors, a reasonable hypothesis is that several costimulatory signals are required for the development of effective T cell immunity in any given situation. Acknowledging that some redundancy exists, the requirement for many or all of these molecular interactions probably reflects two things: first, that several signals are required simultaneously at any one time in the life of the responding T cell; and second, that different signals are required at different times and/or stages of the T cell response.

I have addressed the requirement of costimulation via CD40 and ICOS in T cell responses. Chapter 3 describes attempts to investigate the importance of CD40 on DCs for T cell proliferation in an *in vitro* model. I found that activation and survival of DO11.10 T cells *in vitro* was not curtailed in the absence of CD40 on DCs, since the proliferation of T cells in terms of thymidine incorporation as well as loss of CFSE was similar in the presence or absence of CD40 on DCs. However, when OT-II transgenic T cells were tested for their ability to proliferate and survive in the absence of CD40-CD40L interactions, contrasting results were observed. OT-II T cells proliferated less and underwent apoptosis when cultured with CD40$^{-/-}$ DCs in comparison to wildtype DCs. We hypothesised that owing to the low affinity of the OT-II cells for the MHC-peptide compared to DO11.10 T cells, their requirement for costimulation is greater. Using APLs, I tried to study if dependence of DO11.10 T cells on CD40 costimulation increases in the presence of peptide for which their TCR has lower affinity. However, I did not observe any difference in the proliferation of DO11.10 T cells whether or not the DCs expressed CD40. Although, this study did not shed any light on why DO11.10 T cells are independent of CD40 costimulation, it does suggest that the requirement for CD40 is much more stringent for low affinity TCR-peptide-MHC interactions of OT-II T cells than for high affinity DO11.10 T cells. Studies done in the lab using MHC class-II tetramers have shown that CD4 T cells do not get primed in CD40$^{-/-}$ mice. However, due to small number of antigen-specific T cells, they could not be detected until 7 days after immunisation in this system.

Although the *in vitro* requirements for T cell activation have been studied extensively, the initiation of the immune response *in vivo* takes place within a complex microenvironment, in which the interplay between DC and T cells is restricted by anatomical constraints. The findings of *in vitro* experiments may give an indication of the requirements for T cell activation *in vivo*, but their validation in *in vivo* settings is essential. The *in vivo* experiments involving the adoptive transfer of OT-II T cells into wildtype and CD40$^{-/-}$ mice revealed that OT-II T cells need CD40-CD40L interactions to sustain proliferation *in vivo*, thus confirmed my *in vitro* findings. Experiments are underway to confirm whether the DO11.10 T cells have similar requirements *in vivo*.

In the CD28-CD80/CD86 and CD40-CD40L costimulatory pathways for the initial activation of the lymphocytes, the signalling molecule (CD28 and CD40) is constitutively expressed and the ligand is inducible. In contrast, in the ICOS/ICOSL pathway, the putative signalling molecule ICOS is induced after activation. Therefore stimulation of T cells via ICOS will depend primarily on induction of ICOS, which can then interact with constitutively expressed ligand on the APCs. It is important to determine the role of ICOS in T cell responses and the factors that regulate ICOS induction. I addressed the former by studying the T cell responses in ICOS$^{-/-}$ mice.

I took advantage of two techniques to study whether ICOS is required for T cell activation: adoptive transfer and MHC-II tetramers. The use of TCR transgenic T cells for adoptive transfer has several advantages - first, the majority of the T cells are naïve; second, T cells are of known antigenic specificity, so they can be stimulated with physiologic signals i.e. peptide and MHC on APC. The disadvantage is that this system is far from physiological. In this study, the number of ICOS-deficient OT-II cells was significantly lower compared to wildtype OT-II cells, during both clonal expansion as well as clonal deletion phases. However, when strong adjuvant CFA was used, both types of cells expanded to similar levels, but again were significantly reduced in the deletion phase when ICOS was lacking. Using CFSE labelled cells we have shown that cells can enter proliferation in the absence of ICOS-ICOSL interactions but they undergo less division. These findings are in line with the fact that ICOS is induced after activation and hence crucial for sustained T cell expansion once they are activated.

With MHC-tetramer binding studies following immunisation with H19env peptide emulsified in CFA, I did not find much defect in T cell proliferation in ICOS$^{-/-}$ mice. Although more physiological, this technique is inherently different from the adoptive transfer system as it can detect the antigen-specific T cells only after they have expanded. The comparison of both the techniques using alum was not possible, since tetramer staining, following immunisation with H19env-alum was tried by other members of the lab without much success. Thus, it is difficult to rule out the possible role played by the different adjuvants in both models.

Costimulation can augment cell proliferation, survival and production of cytokines. Following antigen recognition, IL-2 is required for T cell clonal expansion. Once the T cell is activated by antigen and costimulation, it stimulates transcription of IL-2 gene followed by the synthesis and secretion of IL-2. It is possible that in the absence of one costimulatory molecule, T cells can still proliferate as the required amount of IL-2 is achieved by other costimulatory molecules. Generation of ICOS$^{-/-}$ IL-2$^{+/-}$ mice by crossing ICOS$^{-/-}$ mice to IL-2$^{-/-}$ mice may be an approach to determine if ICOS plays a critical role in the initial T cell proliferation, since these mice will make less IL-2 to begin with.

Activated B cells do not attract T cells to lymphoid follicles, rather it is the DCs that control the T cell migration into the follicles [9]. Considering that DCs are the APC for the majority of the T cells, it is possible that the interactions that occur in the follicle may optimise clonal expansion of T cells. There is evidence that the follicle is a major site of CD4 T cell proliferation [315,316]. Although this study has not addressed the migration of T cells to the follicles in ICOS$^{-/-}$ mice due to time constraints, this can give further insight into the role of ICOS in T cell clonal expansion. However, Smith *et al.* blocked these interactions by anti-ICOS mAb and found that T cells were able to migrate to the follicles after immunisation although the T cell clonal expansion was reduced by 50 percent [97].

That ICOS is critical in the contact-mediated T cell help to B cells was suggested by our *in vitro* help assay in which ICOS$^{-/-}$ T cells failed to help primed wildtype B cells to produce class switched antibodies. The ligation of ICOSL on B cells is critical for them to undergo clonal expansion and Ig isotype switching, since the ICOS$^{-/-}$ mice had reduced number of antigen-specific (PE^{+}) B cells and reduced serum Ig and ASC in comparison to wildtype mice. Furthermore, the induction of ICOS on T cells in MHC-II as well as CD40-deficient B cells chimeras was significantly lower in comparison to wildtype chimeras. Collectively these findings implicate the interaction between ICOS and ICOSL in the development of T cell helper function. In our quest to dissect the molecular basis of B cell dependence of differentiation of T cells, we intended to make ICOSL$^{-/-}$ B cell chimeras. Whether T cells from such chimeric mice are able to help the B cells, would be interesting to investigate. Once ICOSL$^{-/-}$ mice are available, these studies would be undertaken.

6.2. B cells can regulate T cell differentiation

There is growing interest in the fundamental roles that B cells may play in regulating immune responses. Interactions between T and B cells involve a complex network of signals mediated by communication through multiple receptors expressed by both populations of cells. One aspect of this complexity that is not well understood is the directionality, and possible bi-directionality, of signalling through specific "receptor-ligand" interactions. While the importance of these interactions for the B cell and GC response is clear, the importance of the signals that T cells gain is less apparent.

B cells require T cell help to clonally expand, class switch and undergo GC reaction in response to a protein antigen. Although it has long been realised that delivery of help by T cells require cognate recognition of antigen presented by B cells, the mechanisms by which this is achieved are unclear. Initial activation of naïve T cells occurs on DCs. However, it seems unlikely that T cells primed by DCs should immediately express all the functions of a competent helper cell, as this would allow help to be given to all the B cells, regardless of the antigen specificity. Instead, there may be further signals which are required in order for T cells to mature as helper cells, or which trigger delivery of help. This could be delivered during secondary interactions between recently primed T cells and B cells. It is also possible that some T cells that undergo only a short interaction with DC have not been primed enough to undergo cell division and differentiation, and the second interaction with a B cell enables this to occur.

van Essen *et al.* studied the helper T cell development in the absence of CD40 costimulation [289]. They found that although T cells primed in the absence of CD40 undergo normal clonal expansion, they are unable to provide help for B cells to either produce class-switched antibodies or generate GCs. The CD40-CD40L interaction cannot take place during the initial priming event on DCs, as resting T cells do not express CD40L. However, CD4 T cells rapidly induce expression of CD40L after activation, allowing subsequently encountered APCs to costimulate them via this molecule. Signalling via CD40L enables differentiation of helper T cells, and perhaps the type of APC on whose surface it is seen directs this differentiation. Hence T cells engaging CD40 on a DC or macrophage causes them to secrete IL-12, favouring differentiation to Th1 phenotype [67]. Conversely, T cells meeting CD40-expressing B cells are pushed towards the Th2 pathway. This is exemplified by the findings of Stockinger *et al.* [208]. They demonstrated that B cell presentation could override an intrinsic propensity for Th1 development and results in generation of C5 specific antibodies in self-antigen C5-specific TCR transgenic mice. The T cells from these mice otherwise induce biased Th1 response and do not generate antibody response to C5.

T cell differentiation is dependent on the number of divisions they undergo, and this also correlates with whether a T cell migrates into B cell follicle, these two functions may be related. As suggested by Garside and colleagues, T cells require passage through a B cell follicle and the cellular interactions they undergo there are necessary for maximal clonal expansion, terminal differentiation and to acquire effector and memory function [317]. The blockade of any of the molecules or interactions essential for either migration of T cells into B cell follicles, or for their interaction with B cells in the follicles, would limit the number of divisions they undergo and alter their differentiation. My experiments with MHC-II$^{-/-}$ and CD40$^{-/-}$ B cell chimeras identify two molecular interactions between B and T cells that seem to be critical for the development of helper function. From

Fillatreau and Gray's findings, T cells migrate normally to the follicles in both these chimeras [9]. This also confirms that the migration of T cells to the follicles is not solely for the purpose of helping B cells, but related to normal T cell differentiation pathways. In support, the markers that distinguish various memory T cell subsets, e.g. the chemokine receptors CCR7 and CXCR5, are important for the positioning of T cells in or out of the lymphoid follicles, suggest a link between passage through the follicle and differentiation into these subsets [33,164,165,318,319].

Given my findings, it appears that incomplete interaction of T cells with B cells in follicles due to the absence of MHC-II and CD40 on B cells, affects their differentiation into T cells that can help B cells. In MHC-II$^{-/-}$ chimeras, since the B cells are not even able to present the antigen to the T cells, both cell types are affected. In these chimeras, B cells do not make class switched antibodies and T cells do not evolve as helpers for the B cells. Thus, T cells presented with antigen by B cells have an advantage over their DC-presented counterparts in terms of acquisition of helper function. These findings provide an insight into earlier reports that T cells from B cell deficient mice do not acquire the ability to help B cells to class switch [202,204]. I found that T cells differentiated in the absence of OX40-OX40L interactions are also not able to help B cells to class switch. Costimulation by OX40 upregulates CXCR5 on T cells thereby facilitating their migration into B cell follicles where they can then provide the necessary help to B cells [209]. To answer whether B cell expression of OX40L plays any role in T cell acquisition of helper function, studies are currently underway with OX40L$^{-/-}$ B cell chimeras.

The importance of migration through follicles for T cell differentiation has been controversial, particularly for Th1, as Randolph *et al.* suggested that this subset neither upregulates CXCR5 nor migrates into follicles [160]. When CXCR5 was retrovirally transduced into Th1 cells, they remained primarily in the central PALS, suggesting that action of CCR7 is dominant over CXCR5. In contrast, Smith *et al.* observed similar number of *in vivo* generated Th1 and Th2 T cells in the B cell follicles [163]. Moreover, they found both subsets were equally able to support B cell clonal expansion and antibody production. This finding is supported by other reports [97,320,321]. Indeed, high affinity IgG_{2a} antibodies are generated, itself an indication of Th1 cells migration to follicles, since B cell clonal expansion in extrafollicular foci is not associated with somatic hypermutation [39]. Alternatively, some studies have suggested that B cell help is a function of an entirely separate Th cell subset, T_{FH}, which may not be Th1 or Th2 [165,322,323], or may become polarised only after passage through the follicle [324]. This remains a controversial though crucial area for understanding of T cell differentiation.

What signals do B cells provide to T cells that no other APC can replicate? As the immune response progresses, a switch in APC usage from DC to B cells is favoured, this is due to various reasons: first, the recruitment of DCs is diminished after the initial phase of response unless the source of antigen is chronic; second, DCs have relatively shorter life span; and third, due to clonal expansion of antigen-specific B cells, the number of B cells that can act as APC increases as the response matures. Thus, the B cells have a quantitative advantage over DCs and this property appears to be exclusive to B cells.

In addition to antigen presentation, B cells can affect T cell response by cytokines and costimulatory molecules. DCs control T cell differentiation by secretion of IL-12. B cells have been shown to be able to secrete cytokines such as IL-10, IL-6, TGFβ, TNFα and IFNγ [11] and there are some reports that B cells can make IL-12 [325]. Skok *et al.* reported that DC-derived IL-12 was required for the induction of Th2 response by a negative feedback loop involving IL-10 secreted by B cells [11]. B cell-derived IL-6 can direct the differentiation of Th2 cells by inducing IL-4 transcription [11,212]. Moreover, Macaulay *et al.* reported impaired IL-4 production by T cells in B cell deficient mice to be at least partly responsible for the failure to develop effective T cell help for B cells

[204]. These findings indicate that B cells can influence T cell differentiation either directly (IL-6) or by modulating DC function (IL-10).

There do not seem to be any B cell-specific costimulatory molecules. Initially ICOSL was reported to be expressed by B cells and not DCs, however, there are many reports now suggesting its expression on DCs [92,101]. Despite this, the differences in the timing of expression of costimulatory molecules may possibly be one of the reasons for the unique ability of B cells to affect T cell differentiation. Considering the many possible differences between DCs and B cells in terms of cytokines, chemokines, adhesion molecules, costimuli and signalling molecules, a cDNA microarray analysis would yield a global picture of qualitative, quantitative and kinetic differences in expression of these molecules in DC and B cells.

In summary, the experiments reported in this thesis shed light on some of the mechanisms involved in T cell activation and differentiation as helper T cells. At the same time, a number of new questions have arisen. Their resolution promises to be an exciting area of future research.

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