

Investigating the *in vivo*
requirements for the generation and
survival of CD4⁺ memory T cells

by

Clare Louisa Marriott

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School of Immunity and Infection
College of Medical and Dental Sciences
University of Birmingham

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Abstract

Much work has been done to elucidate the role of costimulatory molecules in CD4⁺ T cell responses. However the advent of major histocompatibility class II tetramers now allows endogenous polyclonal populations to be tracked from the naive pool through the primary response to the memory phase. I have utilised this method to dissect the role of OX40, ICOS and CD28 in the 2W1S:I-A^b CD4⁺ T cell response. Additionally I have developed a model of local immunisation in photoconvertible Kaede transgenic mice, allowing the migration of antigen-specific memory cells to be tracked from a given site.

Following infection with *Listeria monocytogenes* expressing 2W1S peptide, OX40 ligation specifically expanded T effector (Teff) cells which resulted in a memory cell pool skewed towards T effector memory (Tem) cells, while T follicular helper (TFH) cell and germinal centre formation was abrogated. ICOS was required only for the formation of TFH cells in the primary response and the subsequent generation of both T central memory and Tem cells. However signals through OX40, ICOS and CD28 were dispensable for the persistence of memory cells once formed. Upon secondary challenge again OX40 and ICOS were specifically required for the proliferation of Teff and TFH cells respectively while CD28 had a more general role in the optimal expansion of all T cell subsets. Thus a complex set of temporally separated costimulatory molecule interactions are required for optimal CD4⁺ memory T cells responses *in vivo*. My results indicate that amongst this CD4⁺ memory T cell pool, a secondary lymphoid tissue resident population reside.

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Abbreviation List

-/- deficient
AIRE autoimmune regulator
Alum ppt aluminium precipitated
APC antigen presenting cell
Bcl B cell lymphoma
Bcl-xL B cell lymphoma- extra large
C constant
CCL C-C chemokine ligand
CCR C-C chemokine receptor
CD cluster of differentiation
CDR complementarity determining region
CFA complete Freund's adjuvant
CTLA4 cytotoxic T lymphocyte antigen 4
CXCL CXC chemokine ligand
CXCR CXC chemokine receptor
D diversity
DAPI 4,6-diamidino-2-phenylindole
DC dendritic cell
DD death domain
DNA deoxyribonucleic acid
dpi days post infection/immunisation
EAE experimental allergic encephalomyelitis
ER endoplasmic reticulum
ERK extracellular signal-related kinase
FACS fluorescence activated cell sorting
FBS fetal bovine serum
Foxp3 forkhead box p3
GATA3 GATA binding protein 3
GC germinal centre
HEV high endothelial venule
ICOS inducible costimulator
ICOSL inducible costimulator ligand
IFN interferon
Ig immunoglobulin
IL interleukin
ILC innate lymphoid cell
i.p. intra-peritoneally
iTreg induced T regulatory
i.v. intravenous
J joining
KLRG1 Killer cell lectin-like receptor G 1
LCMV lymphocytic choriomeningitis virus
LFA-1 lymphocyte function-associated antigen-1
LN lymph node
LPS lipopolysaccharide
LTi lymphoid tissue inducer

MAP mitogen-activated protein
MHC major histocompatibility complex
mRNA messenger ribonucleic acid
mTEC medullary thymic epithelial cell
NFATc2 nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2
NK natural killer
NK- κ B nuclear factor kappa B
NOD non-obese diabetic
ns not significant
OVA ovalbumin
OX40L OX40 ligand
PBS phosphate buffered saline
PD-1 programmed death-1
PI3K phosphatidylinositol 3-kinase
pMHC peptide major histocompatibility complex
PNAd peripheral node addressin
PRR pattern recognition receptor
PSGL-1 P-selectin glycoprotein ligand 1
RA rheumatoid arthritis
RAG 1 recombinase activating gene 1
RAG RAG recombinase
ROR γ t retinoic acid receptor-related orphan receptor gamma t
s.c. sub-cutaneously
T-bet T-box transcription factor
Tcm T central memory
TCR T cell receptor
Teff T effector
Tem T effector memory
TFH T follicular helper
TGF β Transforming growth factor beta
Th T helper
TNF tumour necrosis factor
TNFR tumour necrosis factor receptor
TNFSF tumour necrosis factor superfamily
TRA tissue restricted antigen
TRAF TNF receptor associated factor
Treg T regulatory
Trm tissue resident memory
V variable
WT wildtype
ZAP-70 zeta chain associated protein kinase

CHAPTER 1: INTRODUCTION

1.1 The Adaptive Immune system

The immune system has evolved to combat the wide range of pathogenic organisms in the environment in which we reside. The key feature of the immune system is its ability to distinguish self from non-self and therefore eliminate potential threats without harming self-tissues. This is achieved through two arms of the immune system: innate and adaptive. The complexity of pathogenic microbes has prompted the evolution of increasing complex immune defence mechanisms against these. Central to its function is the ability to detect structural features of pathogens that mark them as threats. Broadly these can be divided into innate and adaptive recognition mechanisms. Innate mechanisms rely upon the identification of molecular patterns that are common to many microbes by receptors encoded in the germ line. Adaptive mechanisms are able to detect a virtually infinite variety of antigens, as a result of gene rearrangement (Chaplin, 2010).

Adaptive immune mechanisms evolved more recently than innate mechanisms and are present only in vertebrates. Unlike innate immunity, which is characterised by swift responses, the development of adaptive immunity takes several days. This is because the adaptive system is composed of small populations of cells, which collectively are able to recognise a vast range of pathogens, which must proliferate upon recognition of their cognate antigen (Chaplin, 2010). A key function of the adaptive immune system is its ability to generate long-lived cells, which upon reencounter of their cognate antigen are able to respond more rapidly and robustly and therefore eliminate the threat

before it has taken hold of the host (Figure 1.1). Due to the long-term persistence of this population these cells are able to respond to re-infection years after their initial formation. This feature, termed immune memory, was thought to be specific to adaptive immune responses, however more recently mechanisms of innate immune memory have been described. Natural killer (NK) cells, a component of innate immunity, were found to exhibit surprising longevity following initial priming and enhanced responses upon re-exposure to antigen (Sun et al., 2014, Boehm, 2012).

Antigen receptors expressed by T and B cells are key to the diverse range of pathogens that the adaptive immune system can recognise. These receptors undergo somatic gene rearrangement to produce randomly generated receptors that can respond to a large and diverse array of antigens. Although the innate and immune arms are described as separate entities they work in concert to effectively target pathogens. Innate cells respond rapidly to infection while adaptive cells proliferate to produce large effector populations. Many of the adaptive immune responses rely on contribution from innate cells for their activation (Chaplin, 2010). For example dendritic cells (DC) undergo maturation, upregulating MHC molecules and costimulatory ligands, to become effective antigen presenting cells (APC) in response to signals from pattern recognition receptors (PRR) (Joffre et al., 2009). The remainder of this thesis will focus on adaptive immune responses, specifically those brought about by T cells.

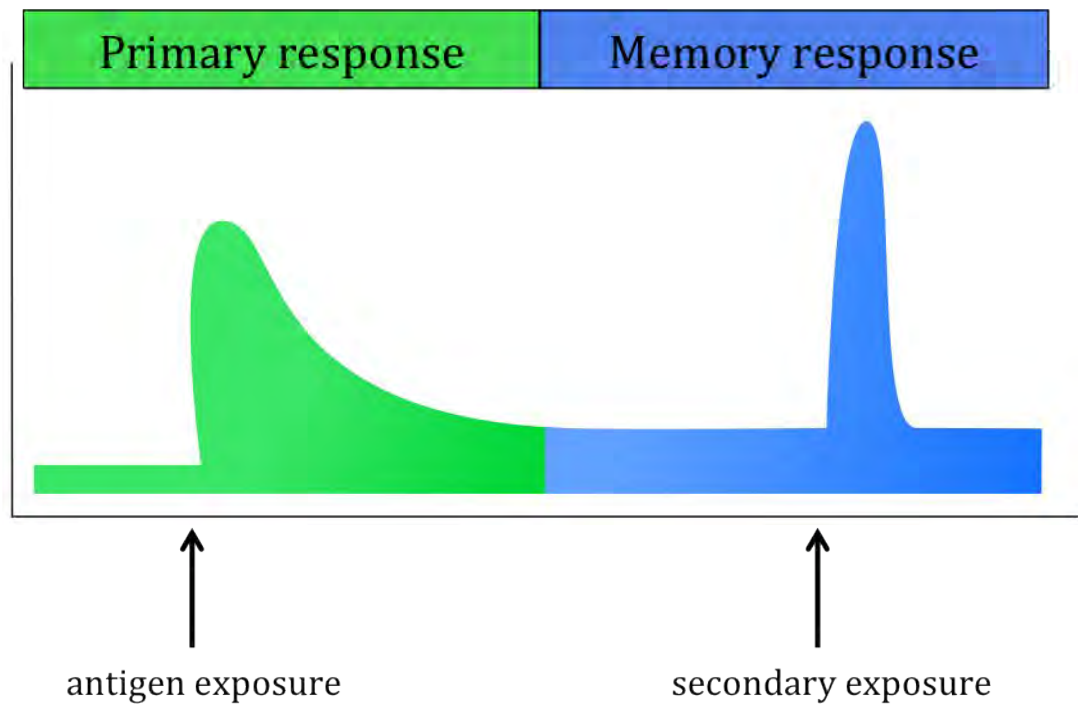


Figure 1.1: Kinetics of the immune response following antigen exposure.

Upon antigen recognition, proliferation of adaptive immune cells takes several days before the pathogen is eliminated. Upon secondary exposure memory cells respond more rapidly and robustly to eliminate the pathogen before any deleterious effects on the host.

1.2 T lymphocytes

T lymphocytes, or T cells, are a vital component of the adaptive immune system; they provide specificity for antigens, target pathogens for destruction and provide help to other cells to activate humoral and cellular arms of the immune response. A major function of T cells is the recognition and destruction of infected cells to prevent the multiplication of pathogens within them. T cells can be divided into classes with different functions; cluster of differentiation 8⁺ (CD8⁺) T cells primarily recognise and destroy infected cells, while CD4⁺ T cells coordinate and activate other cell types to regulate immune responses. Both CD8⁺ and CD4⁺ T cells recognise antigens when they are displayed on the cell surface by a family of major histocompatibility (MHC) molecules. CD8⁺ T cells recognise antigen displayed by MHC class I (MHC I) molecules and CD4⁺ T cells by MHC class II (MHC II) (Chaplin, 2010). These T cell subsets will be discussed in more detail in section 1.7. NK-T cells are a minor class of $\alpha\beta$ T cells that express the receptor NK 1.1 and are negative for both CD4 and CD8 co-receptor expression. Unlike CD4⁺ and CD8⁺ T cells they recognise glycolipid antigens presented by CD1d and secrete immunoregulatory cytokines (Godfrey et al., 2004). 60 % to 70 % of T cells in the blood and secondary lymphoid tissue are CD4⁺ while 30-40% are CD8⁺ T cells (Chaplin, 2010).

1.3 Generation of TCR diversity

T cells are defined by their expression of an antigen-specific T cell receptor (TCR), which enables them to target responses against specific pathogens through recognition of cognate antigen. The major class of T cells have a TCR that consists of an alpha and a beta subunit ($\alpha\beta$ T cells), joined by disulphide bonds. Another minor class of T cells have a TCR that consists of a gamma and a delta chain ($\gamma\delta$ T cells) which is structurally similar to the $\alpha\beta$ TCR but varies in the mechanism by which antigen is recognised (Vantourout and Hayday, 2013). Although both T cell classes develop from a common progenitor in the thymus it is unknown what drives their lineage commitment at present (Kreslavsky et al., 2010). Discussion of T cells will focus on $\alpha\beta$ T cells from this point onwards. Each α and β chain of the TCR consists of a constant (C) and a variable (V) region (Saito et al., 1984). The variable region encodes 3 complementarity determining regions (CDR) of the receptor and therefore governs antigen specificity (Figure 1.2). CDR3 is the main region responsible for determining variation in the antigen receptor. TCR genes that encode the α and β chains are located on chromosomes 14 and 7 respectively. The gene segments that encode the β chain are variable (V), diversity (D) and joining (J), which exist in a non-functional state in the germ line until they are assembled by recombination (Schatz and Ji, 2011). The α chain is encoded by V and J gene segments only. The process of V(D)J recombination takes place in the thymus to generate the wide diversity of TCR that allow T cells to recognise such a large number of foreign antigens. These gene segments are rearranged during the process of somatic

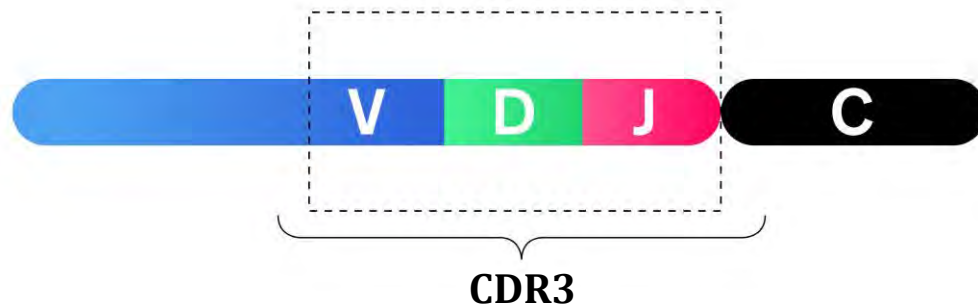


Figure 1.2: V β genes of the T cell receptor (TCR). The V β chain of the TCR contains genes encoding the variable (V), diversity (D) and joining (J) segments as well as the constant (C) region. The complementarity determining region 3 (CDR3) contains genes from each of these segments and governs the diversity of the TCR.

recombination to form functional genes. Recombination activating gene 1 (RAG1) and RAG 2 produce a protein complex termed RAG recombinase (RAG), which initiates this process. RAG introduces double stranded DNA breaks that allow the rearrangement of these genes, which are subsequently re-joined. V(D)J segments are subsequently joined to C regions to produce messenger ribonucleic acid (mRNA) from which α and β chain proteins will be transcribed. Further diversity is produced by the introduction of nucleotides, imperfect gene splicing and frame shifts (Gilfillan et al., 1993, Delves and Roitt, 2000). Although this process produces a wide variety of receptors, some of the genes produced are non-functional. Selection in the thymus eliminates these non-functional receptors and ensures they are not released into the periphery.

1.4 T cell development in the thymus

The thymus is a primary lymphoid organ that supports the development and maintenance of the diverse T cell repertoire found in the periphery (Miller and Osoba, 1967). With age the thymus undergoes atrophy and consequently the development of newly formed T cells is impaired. The role of the thymus also includes educating the T cell repertoire to become tolerant to self through the presentation of self-antigens and deletion. The development of CD4⁺ and CD8⁺ T cells from haematopoietic stem cells takes place in the thymus through the processes of positive and negative selection. T cell precursors originate from a common lymphoid haematopoietic stem cell in the bone marrow before exiting

to the thymus. Initial proliferation and rearrangement of the TCR β chain of prothymocytes takes place in the subcapsular zone (Petrie and Zuniga-Pflucker, 2007). Prothymocytes then migrate into the thymic cortex where they undergo the process of positive selection. At this stage thymocytes do not express either the CD4 or CD8 co-receptor (double negative). They then upregulate both CD4 and CD8 to become double positive before undergoing selection. Following rearrangement of the α chain to form a mature TCR, thymocytes interact with MHC:self antigen complexes, presented by specialised cortical thymic epithelial cells (cTEC), to determine their affinity for this molecule (Nitta et al., 2008). CD4 and CD8 have a role in maintaining a stable interaction between the developing thymocytes and MHC molecule; cells that interact with MHC II become CD4⁺CD8⁻ and those that interact with MHC I become CD4⁻CD8⁺. Single positive thymocytes, that now have a TCR with a sufficiently high affinity, upregulate C-C chemokine receptor type 7 (CCR7) and are able to migrate to the thymic medulla to continue their maturation (Nitta et al., 2008). Thymocytes with low affinity TCR undergo apoptosis and are cleared by macrophages, therefore they are deleted from the peripheral T cell repertoire (Surh and Sprent, 1994). In the medulla the process of negative selection identifies and eliminates TCR that have high affinity for self-antigen and therefore the potential to be autoreactive in the periphery. This is an important step in establishing central tolerance. This process is mediated by medullary thymic epithelial cells (mTEC) expressing autoimmune regulator (AIRE) and involves the presentation of a huge array of tissue-specific antigens. As well as direct presentation of tissue restricted antigens (TRA) to thymocytes, mTEC can transfer antigen to DC which is cross presented to mediate deletion of

potentially self-reactive T cells (Kyewski and Klein, 2006). Again, cells that fail this selection are deleted through apoptosis and the remaining mature T cells enter the periphery. Absence of AIRE results in self-reactive thymocytes escaping this negative selection and exiting into the periphery resulting in autoimmunity (Mathis and Benoist, 2009).

1.5 Antigen Recognition by T cells

The ability of the immune system to control and eliminate infecting pathogens relies on the recognition and destruction of infected cells. Viruses and bacteria exploit host cells for proliferation and in an attempt to evade the immune system. T cells bring about the recognition and destruction of these cells. To enable T cells to detect and target infected cells they must recognise both foreign antigen and a self-molecule. Therefore recognition of antigen by the TCR requires it to be presented on MHC I or MHC II molecules. MHC I is expressed ubiquitously while MHC II expression is mainly restricted to DC, macrophages and B cells, known as professional APC. MHC I molecules present intracellular antigens to CD8⁺ T cells. Antigens are degraded by the proteasome to produce peptides that are translocated to the endoplasmic reticulum (ER). Here the foreign peptide is inserted into the peptide binding groove of an MHC I heterodimer (Neefjes et al., 2011). Peptide-MHC I complexes are then presented at the cell surface (Vyas et al., 2008). This process targets cells infected with viruses or bacteria, or alternatively cells that have undergone transformation, for

destruction by the immune system. The process of cross presentation can be used to display extracellular antigens on MHC I molecules. This is required to eliminate viruses that don't infect cells or that suppress the normal process of antigen presentation by DC, and can also be used for tumour antigens (Heath and Carbone, 2001).

MHC II molecules primarily present exogenous antigens that have been phagocytosed and processed in the endocytic pathway to CD4⁺ T cells (Neefjes et al., 2011). Exogenous antigens are taken up by the cell into phagosomes and processed into peptides by proteolysis. As with MHC I, MHC II molecules are assembled in the ER, however they are then transported in lysosomes and fuse with the phagosome to form the phagolysosome where peptide loading takes place (Vyas et al., 2008).

1.6 TCR signalling

Upon engagement of the TCR with peptide:MHC complexes a complex signalling cascade is initiated to activate effector T cell functions. As the cytoplasmic region of the $\alpha\beta$ TCR is short, accessory molecules are required to transduce downstream signalling. This process is mediated by the CD3 complex, a series of accessory chains associated with the TCR. To transduce the signal this complex contains transmembrane chains CD3 γ , CD3 δ , CD3 ϵ and the cytoplasmic homodimer CD3 ζ . The cytoplasmic regions of the CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAM). Recognition of

peptide:MHC complexes by the TCR induces members of the Src family of kinases such as Lck and Fyn to phosphorylate tyrosines contained within these motifs (van Oers et al., 1996, Lovatt et al., 2006). This recruits ζ chain associated protein (ZAP-70) (Chan et al., 1992), which phosphorylates a number of downstream molecules such as phospholipase C-gamma 1 (PLC- γ 1). Subsequently second messengers are produced such as inositol triphosphate (IP₃) and diacylglycerol (DAG), which cause the release of intracellular calcium (Lovatt et al., 2006). This calcium flux then activates protein kinase C (PKC) (for IP₃) and Ras guanine nucleotide releasing protein (Ras-GRP) (for DAG). A mitogen associated protein (MAP) kinase phosphorylation cascade is also activated which results in the activation of transcription factors that control transcription (Smith-Garvin et al., 2009). This coordinated activation of multiple pathways leads to the transcription of genes that subsequently control lymphocyte proliferation and differentiation.

1.7 T cell subsets

1.7.1 Cytotoxic CD8⁺ T cells

CD8⁺ T cells are cytotoxic and target cells infected with pathogens and tumour cells, however they can also be immunosuppressive to prevent tissue injury through production of IL-10 (Palmer et al., 2010). CD8⁺ T cells are activated by endogenous antigens presented on MHC I molecules derived from viruses and microbes that enter the cytosol (Pamer and Cresswell, 1998). Upon activation

CD8⁺ T cells undergo rapid proliferation to produce an expanded population of effector cells that aid in pathogen clearance, most of which will die by apoptosis, while some survive to produce long-lived memory cells (Butz and Bevan, 1998). CD8⁺ T cells exhibit a range of effector functions to destroy pathogens including cytolysis and the secretion of chemokines, cytokines and microbiocides. Two pathways can mediate the cytolysis of infected cells (Berke, 1995). Firstly the release of perforins forms pores in the cell membrane through which granzymes can penetrate thereby activating the caspase cascade, which leads to apoptosis of the infected cell. Alternatively FasL, expressed on the surface of cytotoxic T cells, interacts with Fas on the target cell, which also leads to apoptosis. CD8⁺ T cells can recruit other cells, such as macrophages and neutrophils, to the infected site by production of chemokines (Harty et al., 2000).

1.7.2 Helper CD4⁺ T cells

Conventionally defined as “helper” cells CD4⁺ T cells are a heterogeneous population also containing regulatory and cytotoxic cells. CD4⁺ T cells are important in the generation of adaptive immune responses to pathogens such as intracellular bacteria; they provide help to B cells to produce antibodies and to sustain CD8⁺ T cell responses (Claman et al., 1966). T helper (Th) cell subsets can be divided into subsets based upon their function, which is thought to be determined, in part, by expression of key lineage defining transcription factors (Mosmann et al., 1986) (Figure 1.3). Each subset produces distinct cytokines and

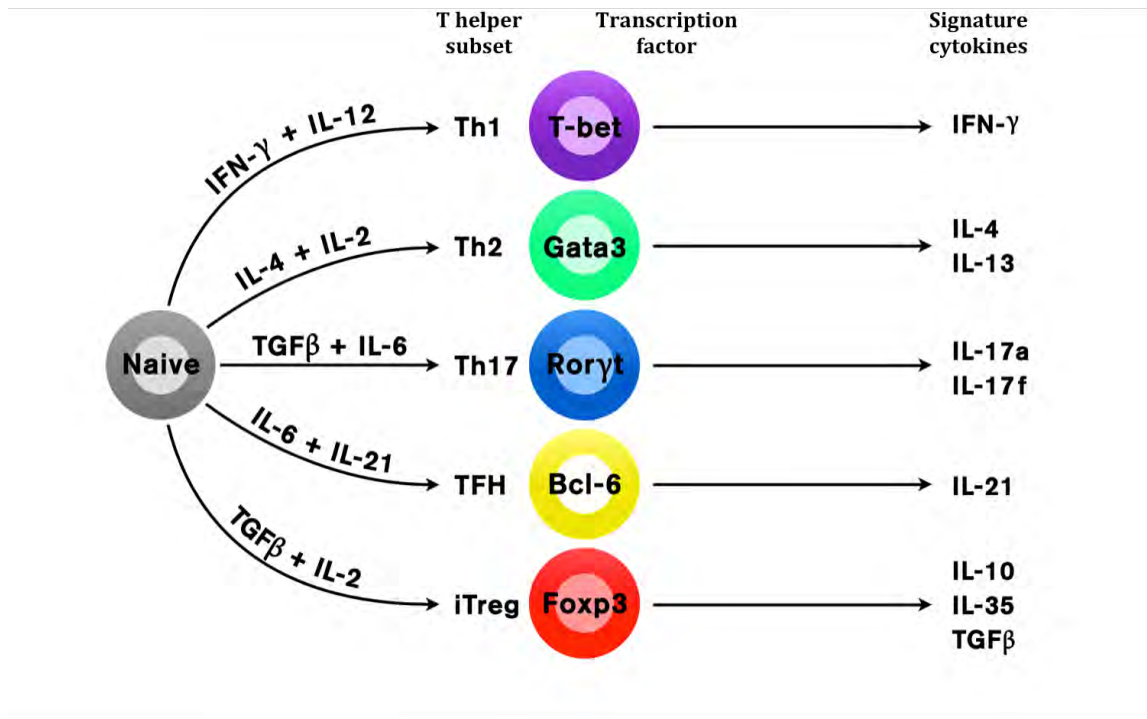


Figure 1.3: Differentiation of T helper cell subsets. Following antigen recognition CD4⁺ T cells differentiate into distinct cell lineages dependent upon a number of factors including TCR signalling and the cytokine milieu. This process of differentiation depends upon the expression of key lineage defining transcription factors and signal transducer and activation of transcription (STAT) proteins. Each cell lineage produces a distinct combination of cytokines to exert effector functions.

exerts distinctive effector functions. In response to stimulation through their TCR, T cells differentiate depending on the cytokines that are present in the environment and the level of TCR stimulation. *In vitro* experiments concluded the differentiation of Th subsets could be induced by different combinations of cytokines (Mosmann and Coffman, 1989), however more recent work has shown a dominant role for TCR signalling in regulating differentiation *in vivo* (van Panhuys et al., 2014). Using intravital imaging this study showed that high concentrations of antigen, and therefore TCR signalling, could induce a Th1 response even in the presence of Th2 polarising adjuvant. Furthermore LPS adjuvant induced the Th1 cell lineage by increasing the DC:T cell interaction time and therefore enhanced signalling through the TCR. Much focus has been put on identifying the lineage defining transcription factors sufficient to induce Th cell gene expression programmes. Differentiation of Th1, Th2, Th17, T follicular helper (TFH) and induced T regulatory cells (iTreg) was found to be dependent on: T-box transcription factor (T-bet), GATA binding protein 3 (GATA3), retinoic acid receptor-related orphan nuclear receptor (ROR γ t), B cell lymphoma-6 (Bcl-6) and forkhead box p3 (Foxp3) respectively (Zhu et al., 2010). However expression of these transcription factors is not absolute; the context in which they are expressed is vital in defining the lineage differentiation path the cell will embark upon (Oestreich and Weinmann, 2012). The existence of two other cell lineages has recently been recognised: Th9 cells which produce interleukin (IL)-9 (Veldhoen et al., 2008) and Th22 cells, which produce IL-22 and are located in the skin (Eyerich et al., 2009).

Although lineage defining transcription factors have been used to identify Th cell subsets with distinctive functions, more recently it has been recognised that co-expression of these molecules occurs (Chung et al., 2011, Hegazy et al., 2010). In these circumstances other environmental factors may influence the lineage pathway that is taken. Epigenetic modifications, micro RNAs, cytokines and costimulatory signals may all impact upon the stability of a T cell lineage (Bluestone et al., 2009). This plasticity may allow the development of more diverse T cell phenotypes. This is currently an area of active research and the precise mechanisms by which plasticity occurs will likely be elucidated in the future.

Th1 cells differentiate in the presence of IL-12 produced in large amounts by APC upon activation (Hsieh et al., 1993) and interferon (IFN)- γ produced by NK cells (Luckheeram et al., 2012). Th1 effector cells secrete IFN- γ , IL-2 and lymphotoxin to eliminate viruses, intracellular pathogens and tumours but left uncontrolled they can lead to autoimmunity. T-bet is the master regulator of Th1 differentiation, functioning to induce genes that promote commitment to this pathway but also suppressing the development of other lineages (Afkarian et al., 2002, Lazarevic et al., 2011, Djuretic et al., 2007). Th2 cells differentiate in response to IL-4 produced by NK T cells, mast cells and basophils (Wang et al., 1999). They secrete IL-4, IL-5 and IL-13 which aid in the expulsion of helminths (Le Gros et al., 1990). Th2 cells can cause allergic responses through the induction of immunoglobulin (Ig) E class switching in B cells. IgE binds to receptors on mast cells and basophils, which leads to the secretion of products

such as histamine that promote allergic inflammation (Zhu and Paul, 2008). More recently Th17 cells were also found to differentiate from naive T cells in response to transforming growth factor-beta (TGF β) and IL-6, producing IL-17A, IL-17F and IL-22 to enhance resistance to extracellular bacteria and fungi (Park et al., 2005, Harrington et al., 2005). They function through the recruitment of neutrophils and direct inflammatory responses. In combination with Th1 cells they are a key cause of autoimmunity. As well as natural T regulatory (nTreg) cells, which emerge from the thymus iTreg can differentiate in the periphery under the influence of IL-2 and TGF- β (Curotto de Lafaille and Lafaille, 2009). Tregs act to modulate the immune response through their suppressive mechanisms, production of anti-inflammatory cytokines such as TGF- β and IL-10, ensuring responses are not mounted against self-tissues.

1.7.2.1 T follicular helper cells

TFH cells have recently been shown to constitute a cell lineage that differentiates under the control of *bcl-6* (Johnston et al., 2009, Nurieva et al., 2009, Yu et al., 2009). They can be identified by high expression of the CXC chemokine receptor 5 (CXCR5), which facilitates homing to the B cell follicle of lymph nodes (Johnston et al., 2009), programmed death-1 (PD-1) and inducible costimulator (ICOS) relative to other T cell subsets (Vinuesa et al., 2005b). The signals required for TFH cell differentiation were recently elucidated by Choi et al. (Choi et al., 2011). During T cell:DC interaction, signals through ICOS induce expression of Bcl-6, which subsequently causes the upregulation of CXCR5. Bcl-6 also prevents

differentiation into the Th1 cell lineage through transcriptional repression of Blimp-1 (Tunayaplin et al., 2004). Expression of ICOS ligand (ICOSL) by B cells is required for the maintenance of TFH cells (Nurieva et al., 2008).

Co-localisation of B and T cells in the follicle is essential to their function of providing help to B cells. Migration to the follicle is controlled by expression of the chemokine receptor CXCR5 and ICOS (Xu et al., 2013, Vinuesa and Cyster, 2011). CXC chemokine ligand 13 (CXCL13), expressed by follicular stromal cells, binds CXCR5 to direct movement into the follicle (Vinuesa and Cyster, 2011). TFH cells provide help to B cells through molecules such as CD40L, IL-4, IL-21 and PD-1 (Crotty, 2011). Interaction with B cells at the T-B border initiates the formation of germinal centres (GC) by inducing the differentiation of GC B cells (Allen et al., 2007). GC are dense structures within B cell follicles consisting of TFH cells, GC B cells, follicular dendritic cells (FDC), macrophages and stromal cells. In the absence of TFH cells GC form but are aborted suggesting TFH cells are required for the maintenance of these structures (de Vinuesa et al., 2000). Therefore TFH cells are required for the generation of high affinity plasma cells and memory B cells, which are produced by the GC response. The process of affinity maturation takes place in the GC to generate B cells with higher affinity for antigen (Victora and Nussenzweig, 2012). GC B cells present antigen to TFH cells presented on MHC II; TFH cells in turn provide survival and proliferation signals to B cells. As TFH cells preferentially provide signals to B cells displaying the most antigen they select for the highest affinity B cells. Uncontrolled provision of T cell help to B cells results in the production of lower affinity

antibodies (Victora et al., 2010); this process is tightly regulated by limiting the number of TFH cells thereby promoting competition among B cells. IL-21 produced by TFH cells can induce IgG and IgA class switch recombination in human B cells (Avery et al., 2008).

There is some debate over whether TFH cells survive long-term in the absence of antigen as memory cells. A study tracking endogenous T cells responding to *Listeria monocytogenes* infection concluded that TFH cells do not form memory cells as CXCR5^{hi}PD-1^{hi} cells were absent at memory timepoints (Pepper et al., 2011). However other studies have suggested TFH cells do enter the memory cell pool but down-regulate key markers such as PD-1, ICOS and Bcl-6 compared to their effector counterparts, while maintaining low levels of CXCR5 expression (Kitano et al., 2011, Hale et al., 2013, Hale and Ahmed, 2015). TFH memory cells were identified by their CXCR5⁺Ly6c^{lo} phenotype while Th1 memory cells were CXCR5⁻Ly6c^{hi}. Analysis of gene expression in CXCR5⁺Ly6c^{lo} cells found that common genes involved in TFH cell function such as *Il6ra*, *Pdcd1* (PD-1), *Cd200* and *Sh2d1a* (SAP) were highly expressed in these cells compared to CXCR5⁻Ly6c^{hi} memory cells (Hale et al., 2013). This suggests that resting TFH memory cells have a less activated phenotype and were also found to recirculate in the blood or reside in spleen or lymph nodes (Hale et al., 2013). Discrepancies between these studies may be due to different uses of nomenclature and infection models. For example Pepper et al. designated CXCR5⁺ cells as T central memory (T_{cm}) cell precursors while TFH cells were CXCR5⁺PD-1⁺ (Pepper et al.,

2011). More recently it has been suggested that all CXCR5⁺ cells are TFH cells while those that express high levels of PD-1 are GC TFH cells (Crotty, 2011).

Unlike TFH cells responding to primary infection, antigen experienced TFH cells responding to secondary challenge are not confined to the GC, like B cells and are able to freely move between the follicle and neighbouring GC (Suan et al., 2015). Interaction with B cells is not required to generate TFH effector cells from memory TFH cells unlike in the primary response (Hale et al., 2013). CXCR5⁺ memory cells are able to provide enhanced help to B cells to facilitate rapid production of high affinity antibody following antigen encounter (MacLeod et al., 2011).

1.8 Costimulation

Following engagement of the TCR with cognate antigen a complex pathway of signalling mechanisms is initiated, as described in section 1.5. However the recognition of antigen by the TCR is not sufficient to fully activate a T cell, and this action alone results in anergy, whereby the cell becomes unresponsive to antigenic stimulation, or apoptosis (Harding et al., 1992). The two signal model of T cell activation was proposed by Bretscher and Cohn in 1970 (Bretscher and Cohn, 1970). They postulated that, as well as a specific signal, a non-specific signal was also required to distinguish self from non-self and prevent anergy of the responsive cells. This theory was extended by Lafferty and colleagues, who found that only haematopoietic cells were capable of activating T cells *in vitro*

(Lafferty et al., 1974). They proposed that APC provide the second signal to activate T cells. This second signal, termed costimulation, is dependent on environmental factors such as IL-2, which induce expression of costimulatory molecules. When a T cell receives both of these signals in concert it becomes fully activated and proliferates to carry out effector functions (Figure 1.4). Although the two signal model elegantly explains the ability of the immune system to modulate its functions, it oversimplifies a complex system of interactions. The strength of the signal from the TCR influences the activation and differentiation of the T cell and is integrated with an array of cell receptor:ligand interactions (Viola and Lanzavecchia, 1996). These include other members of the CD28 family such as ICOS, PD-1 and cytotoxic T lymphocyte antigen 4 (CTLA4) as well as other diverse receptor:ligand families. If the TCR signal is very strong or maintained, T cell activation can occur in the absence of a costimulatory signal (Bachmann et al., 1996).

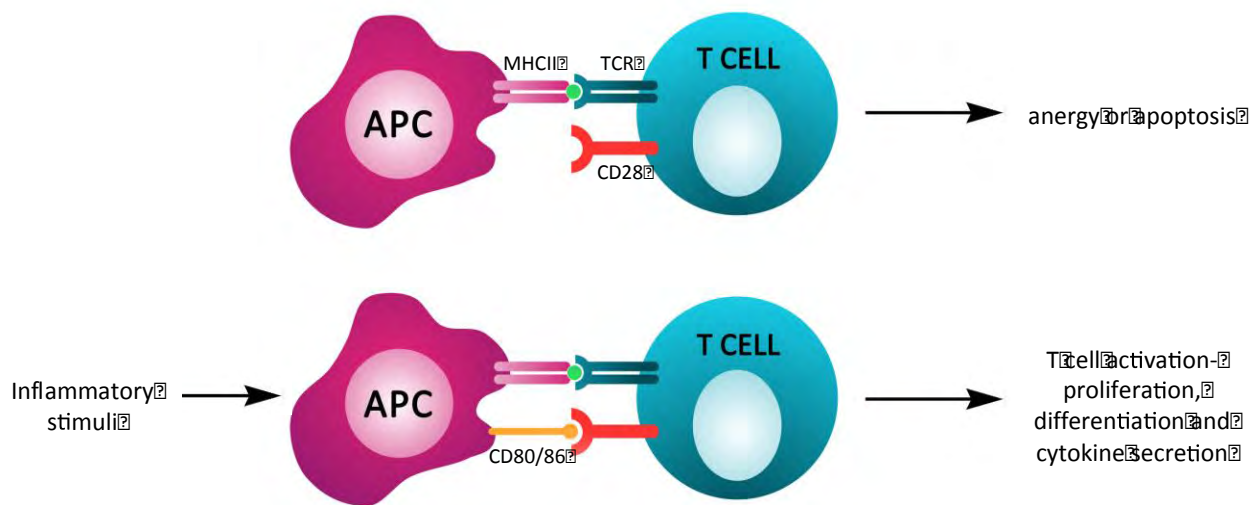


Figure 1.4: The mechanism of T cell activation. Engagement of peptide:major histocompatibility complex (pMHC) expressed on APC and T cell receptor (TCR) expressed on T cells, along with CD28 and its ligands CD80 and CD86 results in optimal T cell activation. In the absence of costimulation apoptosis or anergy of the cell is induced (adapted from Alegre et al., 2001) (Alegre et al., 2001).

1.8.1 Immunoglobulin family

1.8.1.1 CD28

CD28 was identified in the 1980s as a receptor that enhanced the proliferation of T cells (Gmunder and Lesslauer, 1984). It is now the best characterised costimulatory receptor and is known to bind two B7 family member ligands CD80 (B7.1) and CD86 (B7.2) expressed primarily by APC. CD28 is expressed constitutively on the surface of most murine T cells, naive and activated, and on 80 % of human T cells (Gross et al., 1992). Expression of CD80 and CD86 is regulated on APC such as DC, B cells and macrophages; although CD86 mRNA is found constitutively at low levels while CD80 mRNA is absent both ligands are rapidly upregulated following activation due to inflammatory stimuli (Freeman et al., 1993, Hathcock et al., 1994). It is now thought that CD80 and CD86 have overlapping functions although this has not always been the case; the higher affinity of CD86 and differences in structure suggested they might have distinct roles (Lanier et al., 1995, Borriello et al., 1997).

CD28 encodes a 44 kDa glycoprotein that forms homodimers (Aruffo and Seed, 1987). Interaction of CD28 with its ligands CD80 and CD86 is determined by a MYPPPY motif (Harper et al., 1991, Kariv et al., 1996). Following ligand engagement CD28 associates with intracellular signalling proteins such as phosphatidylinositol 3-kinase (PI3K). These initiate a cascade of interactions such as AKT (also known as protein kinase B) activation which exert downstream effects on T cell functions (Riha and Rudd, 2010). One pathway that

is activated by CD28 signalling, but not TCR signalling alone, is the nuclear factor- κ B (NF- κ B) pathway (Cheng et al., 2011). Effector functions induced by signalling through this pathway play a key role in immune responses to infection demonstrated by multiple impacts of dysregulation.

Another CD28 superfamily member, CTLA4, binds the same ligands as CD28 but with greater avidity. CTLA4 is not constitutively expressed and is upregulated on ligation of the TCR, providing an inhibitory signal to T cell responses (Linsley et al., 1992). CTLA4^{-/-} mice suffer from fatal autoimmunity with multi-organ lymphocyte infiltration due to over-activation of T cells (Tivol et al., 1995).

CD28 was found to be highly expressed on thymocytes (Gross et al., 1992), although its role in thymic development is unknown as mice deficient in CD28 show normal T cell development (Shahinian et al., 1993). The major role of CD28 is to provide a costimulatory signal to activate T cells; in effect lowering the activation threshold so T cells become responsive to lower levels of antigen (Viola and Lanzavecchia, 1996). Binding of TCR in the absence of CD28 ligation can cause apoptosis or anergy of the T cell (Harding et al., 1992). Costimulation through CD28 sustains T cells responses by promoting T cell survival. *In vitro* experiments showed that the addition of agonist anti-CD28 monoclonal antibody (mAb) to cell cultures enhanced the survival of T cells and this was due to increased expression of the anti-apoptotic Bcl-extra large (xL) protein (Boise et al., 1995). This results in the production of increased levels of cytokines (such as IL-2), proliferation and differentiation (Thompson et al., 1989, Boise et al., 1995).

In one study a modest induction of Bcl-2 was observed, another anti-apoptotic protein, however this was not as significant as Bcl-xL (Mueller et al., 1996).

Mice lacking CD28 show immune defects resulting from impaired T cell activation (Shahinian et al., 1993). Development of T and B cells in these mice is normal, however following stimulation T cells did not produce IL-2. Addition of exogenous IL-2 only partially restored proliferation indicating CD28 works through a combination of mechanisms. Furthermore basal antibody titres and isotype switching were significantly decreased due to diminished T cell help. GC formation is abrogated in CD28^{-/-} mice due to a failure of B cell proliferation in the follicle (Ferguson et al., 1996). These effects were also observed in the absence of CD80 and CD86, yet mice deficient in CD80 or CD86 only were able to isotype switch and form GC when immunised with complete Freund's adjuvant (CFA) (Borriello et al., 1997). Therefore costimulation through CD28 enhances the survival and function of helper CD4⁺ T cells which provide signals to B cells vital for the formation of GC. Following infection with lymphocytic choriomeningitis virus (LCMV) CD8⁺ T cells proliferated normally indicating that not all responses are dependent on CD28 costimulation. Repeated antigenic stimulation, such as in chronic viral infection, can bypass the requirement for CD28 costimulation (Shahinian et al., 1993, Kundig et al., 1996).

Until recently it was thought that expansion of memory T cells upon secondary challenge was independent of costimulation due to a higher affinity for antigen. This idea was based on *in vitro* studies involving stimulation of cells and the use of CD28^{-/-} mice (Croft et al., 1994, London et al., 2000). New *in vivo* studies have

contested this with evidence that both CD4⁺ and CD8⁺ memory T cells require CD28 costimulation for optimal expansion (Ndejemi et al., 2006, Fuse et al., 2011). One study found a differential effect of blocking costimulation with CTLA4Ig depending on the memory cell subset; it was found that T effector memory (Tem) cells were more inhibited than Tcm cells (Ndejemi et al., 2006).

1.8.1.2 ICOS

ICOS is a costimulatory receptor in the CD28 family, which interacts with ICOSL constitutively expressed on B cells, DC, macrophages and is also inducible on some non-haematopoietic cells (Hutloff et al., 1999, Yoshinaga et al., 1999, Swallow et al., 1999). Although ICOSL shows homology with B7 ligands CD80 and CD86, ICOS does not bind these ligands (Yoshinaga et al., 1999, Beier et al., 2000). Unlike CD28, ICOS is not expressed on naive T cells, but is upregulated upon activation (Mages et al., 2000, Hutloff et al., 1999). ICOS expression is controlled by a combination of signals from the TCR and CD28 (McAdam et al., 2000). Following activation, signalling through Src kinase Fyn and the MAP kinase extracellular signal-related kinase (ERK) control upregulation of ICOS (Tan et al., 2006). Fyn causes activation of the phosphatase calcineurin, which consequently causes dephosphorylation of nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2 (NFATc2) and translocation to the nucleus. In the nucleus binding of NFATc2 and ERK to the ICOS promoter enhance transcription of ICOS. T-bet and GATA3 additionally regulate expression in Th1 and Th2 cells respectively by binding to the ICOS promoter or a 3' untranslated

region element alongside NFATc2 (Tan et al., 2008). Expression of ICOSL on a wide range of cell types including B cells, macrophages and DC indicates the effects of ICOS costimulation may be wide ranging (Swallow et al., 1999, Yoshinaga et al., 2000). Upon engagement with its ligand ICOS signals by recruiting PI3K, which activates downstream MAP kinases (Fos et al., 2008, Feito et al., 2003). Consequently this causes the activation of AKT which is known to aid T cell survival, suggesting ICOS could be involved in this function.

ICOS was initially found to have potent costimulatory effects on T cells following activation, enhancing proliferation, cytokine production and providing B cell help (Hutloff et al., 1999). However experiments *in vivo* showed modest effects in comparison to CD28 on proliferation and production of IL-2 (McAdam et al., 2000, McAdam et al., 2001, Tafuri et al., 2001). ICOS was thought to have a role in promoting Th2 differentiation as blockade of ICOS during stimulation enhanced Th1 differentiation (McAdam et al., 2000). However this is now attributed to the role of ICOS interactions in controlling production of IL-4 and IL-13 by effector cells (Tafuri et al., 2001, McAdam et al., 2001). Evidence suggests that in contrast to CD28, ICOS regulates the production of cytokines such as IL-4 and IL-13 by effector cells, rather than the initial proliferation following antigenic stimulation. Initial reports from ICOS^{-/-} mice also indicated that ICOS was important in providing B cell help; mice showed defects in GC formation and class switching to IgA, IgE and some IgG isotypes (Dong et al., 2001, McAdam et al., 2001, Tafuri et al., 2001). These defects were also seen in common variable immunodeficient (CVID) patients that lack ICOS (Grimbacher et al., 2003). Further work using

adoptive transfers showed that this was as a result of a direct defect in T cell help due to the loss of TFH cells (Bossaller et al., 2006, Akiba et al., 2005). TFH cells are absent in ICOS^{-/-} mice due to reduced transcription of c-Maf and production of IL-21, which is made by TFH cells themselves (Bauquet et al., 2009). ICOS has also been implicated in the development of Th17 cells (Park et al., 2005), however a subsequent study found that the regulation of IL-21 by ICOS was essential only for the maintenance and function of Th17 cells (Bauquet et al., 2009). ICOS has shown to be protective in several different responses to bacteria, parasites and viruses, probably due to provision of B cell help (Mittrucker et al., 2002, Greenwald et al., 2002, Bertram et al., 2002). The interaction between CD40L on T cells and CD40 on B cells aids B cells maturation and survival. Maintained expression of CD40L on T cells, and therefore the duration of engagement with CD40, is controlled by ICOS costimulation in combination with CD28 (Kaminski et al., 2009).

Sanroque mice, in which ICOS is constitutively expressed, exhibit increased IL-21 production, TFH cell and GC formation and develop lupus like autoimmune disease (Vinuesa et al., 2005a, Linterman et al., 2009). These mice express a mutated form of Roquin, a RING-type ubiquitin ligase family member that mediates the post-transcriptional degradation of ICOS mRNA. Normally Roquin degrades ICOS mRNA through a micro-RNA dependent process however the mutated form of Roquin present in *Sanroque* mice is unable to perform this function (Yu et al., 2009). ICOS has also been implicated in the development of autoimmune disease in several animal models. ICOS mRNA and protein were

found to be upregulated on T cells following induction of a model of experimental allergic encephalomyelitis (EAE) (Rottman et al., 2001). Blockade of ICOS after the period of antigen priming resulted in a complete abrogation of the disease. Blocking anti-ICOSL mAb administered to mice with Collagen type II induced arthritis, a model of human rheumatoid arthritis (RA), ameliorated inflammation in synovial joints (Iwai et al., 2002). In a model of airway inflammation induced by *Schistosoma mansoni* eggs lymphocyte infiltration into the lungs, and thereby inflammation, was reduced by treatment with ICOS-Ig to block ICOS interactions (Tesciuba et al., 2001). As ICOS has been shown to be essential for the development of TFH cells and consequently the production of high affinity antibody by B cells the most promising targets of ICOS blockade are antibody mediated autoimmune diseases. Another study showed in two models of autoimmune disease, systemic lupus erythematosus and collagen-induced arthritis, that attenuation of the TFH cell response following anti-ICOSL mAb was responsible for amelioration of disease (Hu et al., 2009). Given the dominant role of ICOS in promoting immune responses and its involvement in autoimmune diseases it is a promising target for immunotherapy.

ICOS expression can be detected on some Tregs suggesting there may be a role for ICOS in regulating immune responses (Herman et al., 2004). ICOS^{-/-} mice show reduced numbers of Foxp3⁺ Tregs indicating ICOS may be involved in their generation or survival (Burmeister et al., 2008). ICOS was essential for establishing mucosal tolerance to EAE attributable to a role in regulating the effector function of Treg cells (Miyamoto et al., 2005).

1.8.3 Tumour necrosis factor super family (TNFSF)

The tumour necrosis factor super family (TNFSF) consists of 19 ligands and 30 receptors (Croft, 2009). The tumour necrosis factor receptor (TNFR) family are characterised by type 1 membrane proteins which contain cysteine rich repeats. These receptors can be divided into two groups depending on the presence of a 70-80 amino acid region in the cytoplasmic domain: death domain (DD) containing and non-DD containing (Screaton and Xu, 2000). DD containing receptors, such as Fas, usually function to activate the caspase cascades, which lead to apoptosis, while non-DD containing receptors enhance proliferation, differentiation and survival. I will focus on non-DD containing receptors such as CD27, CD30 and OX40, which have been described to have important roles in T cell function. TNFR family members function by binding TNF receptor-associated factors (TRAF) to initiate signalling pathways such as NF- κ B. TNF receptors also activate the PI3K and AKT pathways which contribute to T cell activation (So and Croft, 2013). TNF ligands, such as CD70 (CD27L), CD30L (CD153) and OX40L (CD134L) are type II membrane proteins that bind to TNFR members to exert effector functions. However these ligands have also been reported to receive signals thereby acting as receptors for cells expressing them, known as reverse signalling (Bowman et al., 1994, Stuber et al., 1995, Wiley et al., 1996, Lens et al., 1999). This bi-directional signalling allows both the cell expressing the receptor and the ligand to benefit from signalling induced by receptor:ligand interactions. Interactions between TNFR family members and their ligands result in activation of multiple signalling pathways that contribute to the activation or suppression

of T cell responses. TNFR can signal through the same pathways, for example OX40, CD30 and 4-1BB all signal through TRAF 2 (Arch and Thompson, 1998), indicating there may be redundancy between receptors. This has been demonstrated for some receptors, for example OX40 and CD30 (Gaspal et al., 2005), however others have shown non-overlapping functions (Dawicki et al., 2004). 4-1BB and OX40 were found to exert non-redundant effects on CD8⁺ and CD4⁺ T cells respectively. Additionally some TNFR, such as OX40, are constitutively expressed on Tregs, which complicates the analysis of their function *in vivo* (Takeda et al., 2004).

1.8.3.1 CD27

CD27 is expressed on the majority of CD4⁺ T cells, naive and memory, NK cells and on a small population of B cells (Gravestine et al., 1995, Sugita et al., 1992). Although there is constitutive expression on naive T cells, following TCR stimulation CD27 is rapidly upregulated (de Jong et al., 1991). It binds the ligand CD70, a member of the TNF superfamily that is expressed on activated T and B cells (Tesselaar et al., 1997). Expression of CD70 is strictly controlled to ensure responses only occur in the presence of antigen. Pro- and anti- inflammatory cytokines can also act to increase or decrease CD70 expression respectively (Lens et al., 1997). This interaction produces a costimulatory signal that enhances effector cell responses to suboptimal stimulation *in vitro* (Gravestine et al., 1995). Engagement of CD27 with its ligand enables binding to TRAF 2 and 5 which subsequently initiates signalling through the NF- κ B pathway and stress-

activated protein kinase (SAPK)/ c-Jun N-terminal kinase (JNK) (Akiba et al., 1998). CD27 does not enhance IL-2 production and cell cycle progression as has been shown for the immunoglobulin family member CD28 (Fraser et al., 1991, Boonen et al., 1999). Functions ascribed to CD27 are production of tumour necrosis factor (TNF) (Hintzen et al., 1995) and enhanced T cell survival (Hendriks et al., 2000). Mice deficient in CD27 exhibit defects in both CD4⁺ and CD8⁺ effector T cell expansion in response to infection with influenza virus (Hendriks et al., 2000). The most profound effects though were seen in memory cells, which showed delayed kinetics of response as well as a reduction in absolute number following influenza challenge. Constitutive expression of CD70 in B cells caused increased numbers of CD4⁺ and CD8⁺ T cells in secondary lymphoid tissue and downregulation of CD27 on these cells (Arens et al., 2001). In addition CD70 also drove the differentiation of T cells with effector memory phenotype (CD44^{hi}CD62L⁻).

1.8.3.2 CD30

CD30, alongside OX40, appears to be important for CD4⁺ T cell responses. Expression of CD30 is found on activated T cells following CD28 costimulation or in response to IL-4 (Gilfillan et al., 1998, Toennies et al., 2004). *In vitro* expression of CD30 on CD4⁺ T cells peaks 1-2 days after stimulation but for CD8⁺ T cells this is delayed until 4-5 days (Bowen et al., 1996). CD30L is expressed primarily by activated CD4⁺ T cells, some group 3 innate lymphoid cells (ILC3) and B cells (Shanebeck et al., 1995, Shimozato et al., 1999, Kim et al., 2003,

Cerutti et al., 2000). *In vitro*, CD30 has been shown to enhance T cell proliferation following sub-optimal stimulation with anti-CD3 (Gilfillan et al., 1998). Signals through CD30 can either enhance T cell survival or induce apoptosis, depending upon the situation. CD30^{-/-} mice have increased numbers of thymocytes due to impaired negative selection but the number of T and B cells in the periphery were not altered (Amakawa et al., 1996). Following stimulation proliferation of T cells was normal, probably due to compensatory mechanisms by other TNFR family members, making it difficult to elucidate the role of CD30. However CD30 has been shown to signal through TRAF 2 and 5 to activate the NF-κB pathway as other members of the TNFR family do (Aizawa et al., 1997).

1.8.3.3 OX40

OX40 was initially identified on rat CD4⁺ T cells in 1990 (Mallett et al., 1990) before being identified in the mouse (Calderhead et al., 1993). Unlike CD28, which is constitutively expressed, OX40 is upregulated on CD4⁺ T cells following antigenic stimulation indicating an important role in fine-tuning of T cell responses. Expression of OX40 *in vitro* and *in vivo* is transient, usually occurring within 24 hours of TCR stimulation and peaking at 2- 3 days before being lost 4-5 days post stimulation (Gramaglia et al., 1998, Rogers et al., 2001, Dawicki et al., 2004). As OX40 expression is closely linked to TCR stimulation the context in which antigen is presented will likely dictate the kinetics of expression; for example in chronic viral infections expression can be maintained for 20 days (Boettler et al., 2012). Expression of OX40 is dependent on CD28; although not

absent in CD28^{-/-} CD4⁺ T cells OX40 expression is delayed and sub-optimal (Rogers et al., 2001, Walker et al., 1999).

The ligand for OX40, OX40 ligand (OX40L), is highly expressed on APC such as DC and to a lower extent on B cells, T cells and some ILC (Chen et al., 1999, Kim et al., 2003). Expression of OX40L is transient and is induced by inflammatory stimuli such as signals through CD40 and Toll-like receptors (TLR). Interaction with OX40L initiates signalling through TRAFs 1, 2, 3 and 5 which activates the NF- κ B pathway and PI3K/AKT signalling (Song et al., 2004, Song et al., 2008, Kawamata et al., 1998). This results in the upregulation of anti-apoptotic proteins such as Bcl-2, Bcl-x_L and molecules that regulate proliferation, survivin and aurora B (Song et al., 2004, Rogers et al., 2001, Song et al., 2007, Song et al., 2005).

In vitro experiments have shown that, in contrast to CD28, OX40 is not required for initial CD4⁺ T cell proliferation and production of IL-2 however is required later on for survival, clonal expansion and therefore cytokine production (Rogers et al., 2001, Gramaglia et al., 1998). OX40L^{-/-} mice showed impairment in the T cell/ DC mediated contact hypersensitivity response, which was due to decreased T cell expansion and cytokine production (Chen et al., 1999). T cell homing, antibody isotype switching and GC formation were all normal in these mice. Early studies in OX40^{-/-} mice showed no differences in susceptibility to *Leishmania major*, *Nippostrongylus brasiliensis* or Theiler's murine encephalomyelitis virus due to normal T cell and antibody responses (Pippig et al., 1999). However since then multiple studies have shown a requirement for

OX40 in CD4⁺ T cell responses *in vivo* (Gramaglia et al., 2000, Gaspal et al., 2005, Kopf et al., 1999). There is known to be redundancy among the TNF family members and consistent with this OX40^{-/-}CD30^{-/-} mice show further impairment in CD4⁺ T cell responses (Gaspal et al., 2008, Gaspal et al., 2005). Deficiency of both OX40 and CD30, but not CD28 alone, is protective against fatal autoimmune disease in mice that lack Treg cells (Gaspal et al., 2011, Singh et al., 2007). This highlights the importance of these molecules in driving CD4⁺ T cell responses.

Although some studies have shown normal B cell responses in OX40^{-/-} mice, others have found a defect in the T cell dependent humoral response. *In vitro* studies showed that activated B cells receive signals through OX40L to proliferate and produce antibodies (Stuber et al., 1995). In the T dependent B cell response to 2, 4, 6 trinitro-phenyl-keyhole limpet haemocyanin, blocking OX40 decreased the anti-hapten IgG response while IgM titre and GC formation were unaffected (Stuber and Strober, 1996). However multiple *in vivo* studies have shown normal B cell and TFH cell responses in the absence of OX40:OX40L interactions (Chen et al., 1999, Pippig et al., 1999, Kopf et al., 1999). Expression of OX40 has been reported on TFH cells in some situations, depending on the mouse strain and tissue in which they are located (Akiba et al., 2005). Yet normal TFH cell and GC formation in the absence of OX40 indicates these processes are not reliant on OX40:OX40L interactions (Akiba et al., 2005). Other studies have shown a requirement for OX40 in the upregulation of CXCR5 and migration of T cells to the follicle, where they provide help to B cells (Walker et al., 1999, Fillatreau and Gray, 2003, Flynn et al., 1998, Brocker et al., 1999). These data

indicate the relative contribution of OX40 to TFH cell and B cell responses may rely upon the mouse strain and type of immunisation.

The size of the memory cell compartment is significantly reduced in OX40^{-/-} mice indicating a role for OX40 in memory cell responses (Figure 1.5) (Gramaglia et al., 2000). CD4⁺ memory T cells were absent from the lamina propria in CD30^{-/-} OX40^{-/-} mice but only reduced in either CD30^{-/-} or OX40^{-/-} mice (Withers et al., 2009). This suggests combined signals from both CD30 and OX40 are responsible for the optimal generation of the CD4⁺ memory T cell pool. It is however difficult to elucidate whether differences in memory cell populations derive from defective primary responses or if OX40 is essential for the long-term survival of memory cells. To elucidate the exact time in an immune response when OX40 is needed more sophisticated methods such as temporal application of blocking antibodies or inducible conditional knockout mice are required.

Reactivation of memory cells is thought to be independent of costimulation but recent studies have challenged this idea. Rapid upregulation of OX40 on memory cells following stimulation, within 4 hours, indicates there may be a role for OX40 in secondary responses (Gramaglia et al., 1998). One study found the recall response of TCR transgenic OTII cells was absent in OX40L^{-/-} mice following

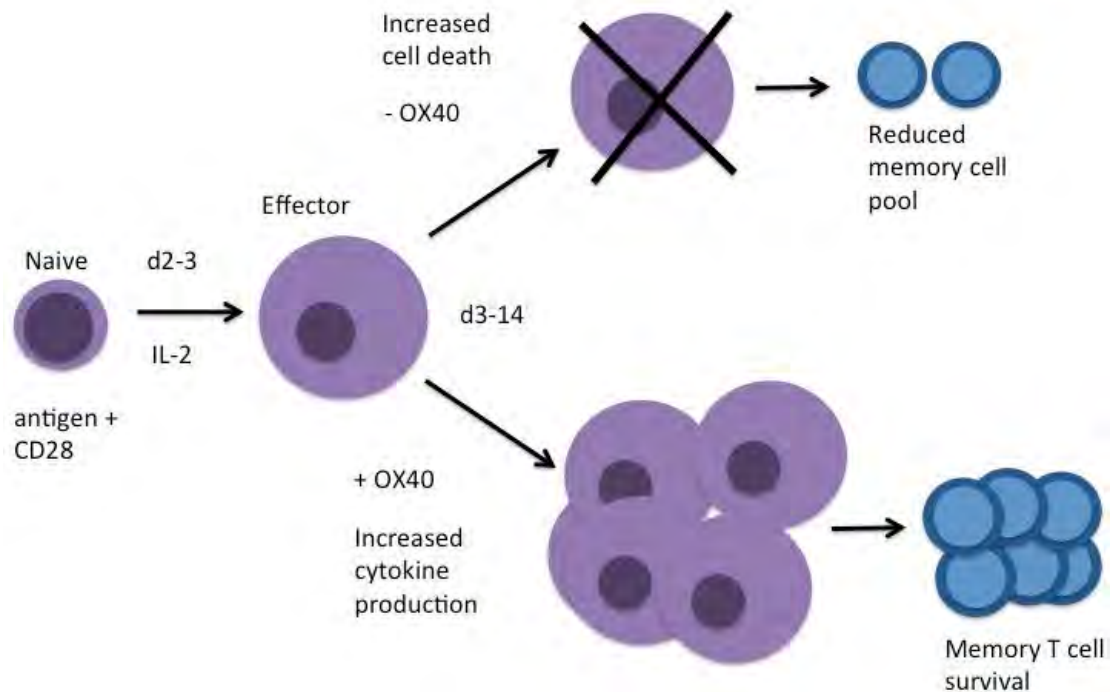


Figure 1.5: The role of OX40 in the generation of the CD4⁺ memory T cell pool. Signals received during the primary response to antigen impact upon the size and function of the memory cell pool. T cells activated in the absence of OX40 costimulation show impaired survival and cytokine production during the latter stages of the primary response. This results in a memory cell pool that is reduced in size and recall function.

challenge with ovalbumin (OVA) and lipopolysaccharide (LPS), despite there being no impairment in the primary response (Dawicki et al., 2004). In a mouse model of asthma, blocking OX40L mAb following challenge of sensitised animals resulted in reduced numbers of Th2 effector memory cells and lung inflammation (Salek-Ardakani et al., 2003).

OX40 has also been implicated in the development of autoimmune diseases. OX40 is constitutively expressed on Tregs, which suppress immune responses to self-tissues (Takeda et al., 2004). Administration of a blocking OX40L mAb decreased the severity of collagen-induced arthritis by decreasing IFN- γ production (Yoshioka et al., 2000). In a murine model of asthma OX40^{-/-} mice were unable to develop a robust Th2 response and lung inflammation was reduced (Jember et al., 2001). These studies highlight the potential of OX40 as a therapeutic target.

A human patient deficient in OX40 has recently been identified and described (Byun et al., 2013). Here functional OX40 deficiency resulted in a reduced proportion of CD4⁺ Tem cells circulating in peripheral blood however CD8⁺ T cells were less impaired. Recall response to previously vaccinated antigens or encountered pathogens were significantly impaired; IFN- γ production following stimulation for bacillus Calmette-Guerin (BCG), tetanus toxoid and cytomegalovirus (CMV) was not above the background level found in healthy control patients. This was due to deficiency in the memory cell population and not due to a requirement for OX40 upon recall. Although the number of memory B cells in peripheral blood was reduced, antibody responses to vaccine remained

normal and the proportion of CXCR5⁺ TFH cells was similar to healthy controls. Thus these data indicate OX40 functions in humans are closely aligned to the role OX40 plays in murine T cell responses.

1.9 CD4⁺ T cell memory

The ability of the adaptive immune system to form long-lived memory cells enables antigen-specific protection against secondary pathogenic challenge. CD4⁺ memory T cells are generated after infection with pathogens and provide rapid and enhanced protection upon reencounter (Seder and Ahmed, 2003). Following antigen recognition naive T cells undergo clonal expansion to form a primary effector cell population. The majority of this population die through apoptosis but a small number of cells remain and form a long-lived memory cell pool (Sprent and Surh, 2002).

Memory cells are a quiescent population, persisting in greater numbers than naive cells of the same specificity, and are long-lived with a half-life of around 40 days in mice (Pepper et al., 2010). They have the ability to migrate throughout the body, through both lymphoid and non-lymphoid tissue, to survey for pathogens. Following activation naive T cells upregulate CD44, required for survival of Th1 cells through the contraction phase, which remains highly expressed on memory cells (Baaten et al., 2010). Memory T cells can be divided into subsets based upon expression of key lymphocyte homing molecules and function (Sallusto et al., 1999). Tcm cells express CCR7 and CD62L (L-selectin),

migrate through lymphoid tissue and proliferate to produce more effector cells upon antigenic stimulation as well as secreting IL-2. Tem cells lack lymphocyte homing molecules, are thought to migrate through non-lymphoid tissue and blood and rapidly produce effector cytokines such as IFN- γ upon stimulation. More recently a population of CD8⁺ tissue resident memory (Trm) cells has been identified which are protective at the major sites of pathogen entry such as the skin and mucosal surfaces (Gebhardt et al., 2009, Masopust et al., 2010), however less is known about this population for CD4⁺ T cells.

When investigating T cell memory it is important to consider the different phases of the response. Generation, survival and reactivation of memory cells are separated temporally and therefore may show different requirements for signals.

1.9.1 Generation of CD4⁺ T cell memory

An understanding of how memory T cells are generated is key for the design of efficacious vaccines. Difficulties have arisen in deciphering the mechanism of CD4⁺ memory T cell development due to the low frequencies of responding CD4⁺ effector T cells compared to CD8⁺ T cells (Homann et al., 2001).

It is still to be determined what factors dictate whether or not a T cell enters the memory cell pool or dies by apoptosis. Initial ideas proposed that expression of IL-7R α might mark those cells that survive to form memory cells, as IL-7 is a survival factor. Arguing against this, ablation of IL-7 signalling did not affect the

normal contraction of effector cells and consequent survival of memory cells (Tripathi et al., 2007). Other candidates that may dictate this decision include TCR signal strength and asymmetric cell division (Williams et al., 2008, Chang et al., 2007). Optimal activation and clonal expansion of T cells depends on sustained TCR:pMHCII interactions (Celli et al., 2007). The strength and duration of TCR stimulation influences the resultant population of effector cells and therefore determines the functional properties of the memory cell pool (Gett et al., 2003). Although there are no definitive surface markers for identifying cells that will enter the memory cell pool, it has been shown that CD4⁺ memory T cells originate from cytokine producing cells that have undergone clonal expansion following exposure to antigen (Harrington et al., 2008, Lohning et al., 2008). Effector cells expressing IFN- γ in response to LCMV and *Listeria monocytogenes* infection were genetically marked and found to persist in the memory cell population. Another study tracking an endogenous CD4⁺ T cell population responding to *Listeria monocytogenes* found that two effector cell populations, one Tbet^{hi} and one Tbet^{lo}, both entered the memory cell pool (Pepper et al., 2010). These data suggest that some effector T cells survive the contraction phase to become memory cells.

The generation of a functional memory T cell population depends upon a number of factors. The size of the naive T cell population and the signals received in the primary response both dictate the size and function of the resulting memory cell pool. Naive T cell pools can consist of between 20 to 1500 cells in the mouse (Moon et al., 2007, Tubo et al., 2013, Jenkins and Moon, 2012, Whitmire et al.,

2006). Interestingly high precursor frequency can inhibit the formation of memory T cells. High numbers of adoptively transferred TCR transgenic T cells are not as efficient at forming memory cells as low numbers mimicking an endogenous population (Hataye et al., 2006). This is probably due to a decreased number of DC interactions through increased competition (Blair and Lefrancois, 2007). Two-photon imaging revealed that as the number of T cell precursors was increased, the interaction time between antigen-specific T cells and DC were decreased (Garcia et al., 2007). This resulted in impaired activation and clonal expansion.

A number of key factors, including costimulatory molecules, contribute to the optimal activation of CD4⁺ T cells in the primary response which impacts upon the memory cell pool. Signals through CD28 mediate primary proliferation of effector CD4⁺ T cells and production of cytokines such as interleukin 2 (IL-2) (Shahinian et al., 1993, Fraser et al., 1992). Deficiency of CD28 results in a significantly reduced CD4⁺ memory T cell population following bacterial infection (Pagan et al., 2012). Additionally a host of other receptor:ligand pairs, including members of the TNFSF, are also involved in the formation of an optimal effector population and functional memory cell pool.

Cytokines can also play a role in the development of T cell memory. The cytokines present within the environment influence the proliferation and differentiation of effector cells. Depending on the T cell subset they may also affect the survival of effector cells to enter the memory cell pool (Pepper et al., 2011).

1.9.2 Survival of CD4⁺ T cell memory

Memory T cells are a quiescent population that persist long-term due to slow turnover, unlike naive T cells which rarely divide. They show distinct requirements for survival from naive T cells, which likely reflects their raised state of activation, ready to rapidly respond to pathogens (Tough and Sprent, 1994).

The mechanisms regulating the survival of CD4⁺ memory T cells have been difficult to elucidate. Low cell frequency *in vivo* makes the study of these cells challenging. Additionally many factors involved in the optimal primary expansion of effector cells impact upon the memory cell population. Therefore the study of memory cells in genetically deficient mice does not distinguish between effects during priming and long-term survival of memory cells. TCR transgenic cells have been used to circumvent this problem and increase the frequency of antigen-specific cells to a level detectable by flow cytometry (Harrington et al., 2008). However problems have arisen with this method as clonal competition at high precursor frequency can be detrimental to memory cell generation and survival (Marzo et al., 2005, Blair and Lefrancois, 2007, Hataye et al., 2006).

Initial studies focused on the use of CD44 and CD62L to identify memory cells based on the expression of surface markers. Naive mice contain a population of memory phenotype cells, thought to be due to responses to environmental antigens. However the unknown origin of these memory phenotype cells confounded conclusions drawn from them. Recent studies have shown that

memory phenotype cells are a heterogeneous population consisting of a rapidly dividing population and a quiescent long-lived population similar to antigen-specific memory cells (Purton et al., 2007). Studies then transitioned to following antigen-specific cells that have undergone clonal expansion in response to a known antigen. TCR transgenic T cells were often used to circumvent problems associated with low cell frequency, however as described above such experiments have their own caveats.

Initial studies with memory phenotype cells indicated that persistence of low levels of antigen and therefore TCR:MHCII interactions were key to the long-term survival of memory cells (Gray and Matzinger, 1991). However the survival of CD4⁺ memory T cells in MHC II^{-/-} mice was not impaired, suggesting TCR signals may not be required (Swain et al., 1999). These experiments were performed using CD4⁺ effector T cells activated *in vitro* and transferred into T cell deficient hosts. Therefore a lack of competition from host T cells for non-antigen specific signals may mask a requirement for TCR signalling. Purton et al. showed that the survival of antigen-specific CD4⁺ memory T cells was independent of TCR signalling (Purton et al., 2007). They demonstrated that discrepancies between studies might be due to the heterogeneous nature of memory phenotype cells; this population was found to consist primarily of a subset of rapidly dividing cells that required MHC II interactions for survival. Antigen-specific cells responding to LCMV were slowly proliferating and their survival was independent of MHC II. Distinct requirements for memory phenotype and antigen-specific memory cells mean these populations cannot be

used interchangeably to elucidate the mechanisms regulating CD4⁺ memory T cell survival.

Experiments with CD8⁺ T cells were the first to show a requirement for cytokines for memory cells. Type I IFN induced the proliferation of CD8⁺ memory T cells *in vivo* (Tough et al., 1996). Other cytokines that have been shown to exhibit similar effects are IL-12, IL-15, IL-18 and IFN- γ (Zhang et al., 1998, Tough et al., 2001).

The survival and homeostatic proliferation of CD8⁺ memory T cells have now been shown to rely on both IL-15 and IL-7 (Schluns and Lefrancois, 2003).

However data indicated that CD4⁺ memory T cell survival was independent of these cytokines; memory phenotype cells are not reduced in IL-15^{-/-} mice (Kennedy et al., 2000) and persistence of memory cells in common γ chain ^{-/-} mice is normal (Lantz et al., 2000). However multiple studies have since shown a dominant role for IL-7; blockade of IL-7 using mAb decreased the number of surviving CD4⁺ memory phenotype cells (Seddon et al., 2003). Studies using antigen-specific memory cells have corroborated this finding. IL-7 was found to enhance the survival of OTII memory cells *in vitro* and caused them to upregulate the survival factor Bcl-2 (Kondrack et al., 2003). Impaired survival was also observed *in vivo* using IL-7^{-/-} mice or by mAb mediated blockade of IL-7.

Experiments by Purton et al. showed the number of TCR transgenic memory cells recovered from IL-7^{-/-} hosts was five fold less than wild-type (WT) mice (Purton et al., 2007). These findings were confirmed with endogenous polyclonal CD4⁺ memory T cells responding to LCMV. They also found that IL-15 was required for both homeostatic proliferation and survival. IL-15 and IL-7 both contributed to

driving homeostatic proliferation in non-lymphopenic hosts. The presence of normal numbers of memory phenotype cells previously observed in IL-15^{-/-} mice is thought to be due to habituation to an environment deficient in IL-15. (Purton et al., 2007).

The role of costimulation in memory T cell survival is not fully understood. One possibility is that the survival of memory cells is enhanced by continuous or periodic interactions with costimulatory molecules. Alternatively the effects exerted during the priming and contraction phases may enhance the survival of effector cells that go on to form the memory cell pool. Absence of CD28 does not alter the long-term survival of memory cells (Pagan et al., 2012). Members of the TNFSF may enhance the survival of memory cells thorough promotion of anti-apoptotic proteins such as Bcl-2. CD27⁻ memory cells were found to survive poorly following adoptive transfer compared to CD27⁺ counterparts (Pepper et al., 2010). OX40 and CD30 have also been implicated in the long-term survival of memory T cells. CD4⁺ memory T cells in the lamina propria were reduced in the absence of these molecules despite normal priming (Withers et al., 2009). This appeared to be independent of Bcl-2 or Bcl-x_L as CD30^{-/-}OX40^{-/-} cells expressed comparable levels to WT cells. However, evidence for the periodic provision of costimulatory signals is lacking, as many studies have been unable to separate the requirement for costimulation for survival through the contraction phase from long-term persistence. Given the differences in costimulatory requirements in different types of primary response, this may be reflected in different requirements for the survival of CD4⁺ memory T cells

1.10 T cell migration

The migration of lymphocytes around the body is key to immunosurveillance. Active circulation increases the chance of antigen-specific T cells, which exist at low frequency, of coming into contact with cognate antigen. The migration of T cells is controlled by a number of surface molecules such as chemokine receptors, selectins and integrins and is dependent upon activation state. Naive T cells express CCR7 and CD62L, which allow them to recirculate through lymph nodes (Forster et al., 1999, Bradley et al., 1994). Up-regulation of molecules such as CXCR3 and CCR9 allow effector cells to enter inflamed tissues at peripheral sites where they carry out effector functions to eliminate threats (Groom and Luster, 2011, Papadakis et al., 2000). Following clearance of antigen memory cells either retain expression of non-lymphoid homing molecules or re-express lymphoid homing molecules such as CD62L. Naive T cells are thought only to be able to enter lymph nodes from blood through high endothelial venules (HEV) (von Andrian and Mackay, 2000) or from another lymph node connected in a chain (Braun et al., 2011). Antigen-experienced cells however are able to enter lymph nodes through HEV or afferent lymphatics from peripheral tissues (Mackay et al., 1990). The superior migratory properties of memory cells and their increased presence in both lymphoid and non-lymphoid tissues enable enhanced responses to secondary infections.

1.10.1 Migration into lymph nodes

Expression of chemokines by stromal cells within the lymph node and chemokine receptors on T cells act to control the migration of T cells into lymph nodes. C-C chemokine ligand (CCL) 19 and CCL21 are expressed by fibroblastic reticula cells (FRCs), which form the T cell zone of the lymph node (Luther et al., 2000). DC associate closely with this structure therefore T cells expressing the receptor for these chemokines, CCR7, migrate towards DC. This migration facilitates T cell:DC interaction and thereby T cell activation (Friedman et al., 2006). Naive T cells enter lymph nodes through a process dependent on CCR7 and CD62L. T cells in the blood have to tether to the endothelium as blood flow exerts shear forces on lymphocytes. CD62L expressed by T cells binds to its ligand peripheral node addressin (PNAd) expressed by HEV (Berg et al., 1991, Streeter et al., 1988). This interaction allows T cells to roll on endothelial cells. Endothelial cells of HEV express CCL21 which is bound by CCR7 on T cells (Stein et al., 2000). This causes the activation of integrins such as lymphocyte function-associated antigen-1 (LFA-1) to enable entry through HEV. Once inside T cells remain for between 6-18 hours while they search for cognate antigen before leaving via the efferent lymphatics (Tomura et al., 2008, Grigorova et al., 2010).

Upon initiation of an immune response exit of T cells from the lymph node is inhibited and entry of cells into the lymph node is increased (Hall and Morris, 1965, Cahill et al., 1976). Entry of effector and memory T cells into the lymph node is also increased. Inflammatory signals activate stromal cells, which induce expression of chemokines and adhesion molecules on HEV (Guarda et al., 2007,

Martin-Fontecha et al., 2008). Once activated, effector cells express a range of chemokine receptors and adhesion molecules that enable their migration into inflamed tissues. For example migration to mucosal tissues requires expression of the integrin $\alpha 4\beta 7$, which binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on endothelium (Hamann et al., 1994, Gorku et al., 2009). Exit from lymph nodes is mediated by sphingosine-1-phosphate receptor 1 (S1P₁) (Cyster and Schwab, 2012). *S1pr1*^{-/-} T cells are able to enter lymph nodes through HEVS but are unable to exit highlighting that different molecules regulate these processes (Matloubian et al., 2004). Although CCR7 is required for entry into lymph nodes it is not required for exit, in fact it facilitates retention (Pham et al., 2008). However CCR7 is required for exit from non-lymphoid tissues since entry into the draining lymphatics is CCR7 dependent (Bromley et al., 2005, Debes et al., 2005).

A striking feature of T cells is that they preferentially home to the site where they were activated. For example T cells responding to antigen in the mLN, which drain the intestine, upregulate $\alpha 4\beta 7$ and CCR9 facilitating homing back to this site (Campbell and Butcher, 2002, Mora et al., 2003, Zabel et al., 1999). This ability is imprinted on T cells by the surface molecules expressed by the DC during priming.

1.10.2 Memory T cell migration

As described in section 1.8, memory cells can be sub-divided into subsets based upon their migratory capacity. Tcm cells express CCR7 and CD62L and are able to traffic through secondary lymphoid tissue (Reinhardt et al., 2001). Tem cells lack these molecules and therefore are preferentially found in non-lymphoid tissues (Masopust et al., 2001). Trm cells express unique cell surface markers that facilitate their homing to peripheral tissues. These cells permanently reside here even after clearance of the pathogen ready to respond to reinfection at these sites (Gebhardt et al., 2009, Masopust et al., 2010). Trm cell populations have been described in many tissues for CD8⁺ but are less well studied for CD4⁺ T cells. It is possible that trafficking of CD4⁺ memory cells from peripheral tissues varies from CD8⁺ memory T cells. In the steady state a large number of CD4⁺ memory T cells were found to traffic to draining lymph nodes from the skin (Tomura et al., 2010). Yet a population of CD4⁺ T cells was observed to persist in the lungs of mice for several months following infection and express higher levels of CD69 and CD11a compared to recirculating memory cells (Hogan et al., 2001, Teijaro et al., 2011). These cells were found to preferentially migrate back to the lungs when adoptively transferred into naive recipients (Teijaro et al., 2011).

CD4⁺ memory T cells are found in the afferent lymph draining skin, unlike naive T cells which can only enter through HEV from the blood or from other lymph nodes (Mackay et al., 1990, Yawalkar et al., 2000). This indicates that CD4⁺ memory T cells can utilise additional mechanisms to gain entry to lymph nodes

that naive T cells cannot exploit. This is supported by studies in CCR7^{-/-} mice in which lymph nodes contain reduced numbers of naive T cells but a significant population of memory T cells (Forster et al., 1999). A recent study found that expression of CCR7 was dispensable for the entry of Tcm cells into lymph nodes (Vander Lugt et al., 2013). Using mixed bone marrow chimeras to mitigate the effects on lymphoid architecture found in CCR7^{-/-} mice, they showed that expression of CCR7 on Tcm cells did not infer any advantage in homing to lymph nodes.

1.11 Tracking antigen-specific CD4⁺ T cells *in vivo*

There are currently no specific surface markers for identifying antigen-specific CD4⁺ T cells responding to infection *in vivo*. This has made it difficult to track a polyclonal CD4⁺ T cell response from expansion through to the memory cell phase. Low frequency of naive and memory CD4⁺ T cells means they have been below the level of detection for flow cytometry. Previously *in vitro* methods have been used to isolate total CD4⁺ T cell populations and study their responses (Boise et al., 1995). Methods using peptide loaded on cultured APC can over-ride antigen processing and presentation by MHC II. These methods do not reflect the TCR stimulus that would be present in a physiological response and therefore the requirement for costimulation may be reduced or abrogated completely. Additionally *in vitro* studies cannot reflect the complex microenvironments or replicate the effects of inflammation that naturally occur *in vivo*.

A number of methods have been developed which enable antigen-specific T cells to be followed from activation through proliferation, contraction and memory phases of the response. One of these methods uses immunisation with pigeon cytochrome c peptide 81-104 in mice expressing H-2 I-Ek. Around 70% of CD4⁺ T cells that respond to this have a characteristic TCR enabling a population of antigen-specific cells to be tracked (McHeyzer-Williams and Davis, 1995). TCR transgenic T cells have been widely used to elucidate the characteristics of T cell response *in vivo* and have greatly enhanced our understanding of this area (Pape et al., 1997). However problems have arisen with the use of this method. The transfer of large numbers of TCR transgenic T cells into mice has been used to enhance detection, however this elevated frequency is also a disadvantage. Increasing the size of the precursor pool can upset the fine balance of signals and interactions that take place during an immune response. Unphysiological numbers of TCR transgenic T cells have shown poor conversion to memory cell populations due to increased clonal competition (Hataye et al., 2006). Limiting numbers of mature DC mean that T cells must compete with each other for ligation, therefore T cell:DC interaction times are shortened and T cells are sub-optimally activated (Celli et al., 2007).

The use of TCR transgenic T cells has been refined to limit these problems and reflect a physiological response. Transferring low numbers of T cells, between 10³-10⁴, mimics more closely the size of an endogenous polyclonal T cell pool as only around 10 % of transferred cells will survive (Hataye et al., 2006, Blattman et al., 2002). Adoptive transfer of low numbers of TCR transgenic SM1 CD4⁺ T

cells provides a population that can be tracked in allogeneic hosts (McSorley et al., 2002, Hataye et al., 2006).

More recently MHC tetramers have been developed to track peptide specific T cells. This method has been successful for CD8⁺ T cells but has proved more technically challenging for CD4⁺ T cells, where the TCR shows low affinity for peptide:MHC complexes. However recent refinements in tetramer design and staining techniques have increased the use of MHC II tetramers to track antigen-specific CD4⁺ T cells (Landais et al., 2009).

1.12 Aims

The role of costimulatory molecules in CD4⁺ T cells responses has been widely investigated however conflicting reports have arisen, likely due to differences in methodology. It has become clear that studies tracking endogenous polyclonal populations of antigen-specific CD4⁺ T cells are now required to clarify these roles. The study of CD4⁺ memory T cells in particular has been confounded by low cell frequency which necessitated the analysis of larger 'memory phenotype' populations. Memory T cell subsets have conventionally been defined by surface receptors that dictate their ability to home to secondary lymphoid tissue.

However the recent discovery of Trm cells suggests that memory cell populations may be more heterogeneous than originally thought. We therefore sought to clarify the role of costimulatory receptors in CD4⁺ T cell responses and

investigate the migratory capacity of CD4⁺ memory T cells by addressing the following aims:

- Clarify the role of costimulatory receptors OX40, ICOS and CD28 in the generation and long-term survival of CD4⁺ memory T cells
- Explore the therapeutic potential of agents that block or stimulate signalling through costimulatory pathways
- Investigate the location of memory cell subsets and if their migratory capabilities are intrinsically tied to their expression of key surface molecules

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

All of the animals used throughout this study were used in accordance with Home Office regulations at the [REDACTED]

[REDACTED] WT mice were purchased from external suppliers (Harlan or Charles River) or bred in house. Details of mice used are listed in Table 2.1.

Mice used were between 6-12 weeks of age and were sacrificed by cervical dislocation of the neck or a rising concentration of CO₂. All mice used were on a C57BL/6 background.

Table 2.1 Mouse strains used in study

Mouse Strain	CD45 isotype	Phenotype	Source
C57BL/6	CD45.2	WT	██████████ or externally sourced from Harlan or Charles River
B6.SJL- <i>Ptprc^a Pepc^b</i> /BoyJ	CD45.1	Congenic C57BL/6 WT	██████████
CTLA4Ig transgenic	CD45.2	Limited signalling through CD28 due to circulating CTLA4Ig fusion protein	P. J. Lane, ██████████ ██████████
<i>Cd30^{-/-} Ox40^{-/-}</i>	CD45.2	Deficient in TNF family members CD30 and OX40 resulting in impaired survival of CD4 ⁺ T cells in a primary response	Novel cross by P. J. Lane, ██████████ ██████████
B6.129S4- <i>Cd80^{tm1Shr} Cd86^{tm2Shr}</i> JJ	CD45.2	Deficient in CD80 and CD86 costimulatory ligands resulting in failure to generate optimal CD4 ⁺ T cell responses	The Jackson Laboratory
B6129P2- <i>Icosl^{tm1Mak}</i> JJ	CD45.2	Deficient in costimulatory ligand ICOSL which results in impaired T cell dependent B cell responses	The Jackson Laboratory

Kaede	CD45.2	All cells express the photoconvertible fluorescent Kaede protein that changes from green to red upon exposure to violet light.	J. Brewer, University of Glasgow
Kaede <i>Ccr7</i> ^{-/-}	CD45.2	Express the photoconvertible Kaede protein in all cells. CCR7 deficient, which impairs migration of lymphocytes in response to CCL19 and CCL21.	Novel cross at   
<i>Rag1</i> -Cre <i>Tbx21</i> fl/fl <i>Rosa26</i> -tdRFP	CD45.2	Cells expressing RAG (T and B cells) are deficient in T-bet resulting in impaired IFN- γ production and express red fluorescent protein.	S. Reiner courtesy of M. Veldhoen, Babraham Institute, Cambridge
<i>Rag2</i> ^{-/-} SM1	CD45.1 CD45.2	Deficient in B and T cells with TCR transgenic SM1 CD4 ⁺ T cells	S. McSorely, University of California

2.2 Medium and Reagents

All media were prepared in advance and stored at 4 °C except for the stock solutions for Haemolytic Gelatin Gey's Solution which were stored at room temperature.

Table 2.2 Preparation of RPMI with EDTA

Medium and Additives	Volume	Final Concentration
RPMI-1640 (1x) + L-Glutamine	500 ml	-
0.5 M Ethylenediaminetetraacetic acid (EDTA)	2.5 ml	2.5 mM

Table 2.3 Preparation of Culture Medium

Medium and Additives	Final Concentration
RPMI-1640 (1x) + L-Glutamine	-
Penicillin and Streptomycin	1 %
L-Glutamine	2 mM
Heat-inactivated foetal bovine serum (FBS)	10 %

Table 2.4 Preparation of Staining Buffer

Medium and Additives	Volume	Final Concentration
Dulbecco's Phosphate Buffered Saline +CaCl ₂ +MgCl ₂	500 ml	-
Heat-inactivated foetal bovine serum (FBS)	10 ml	2 %
0.5 M Ethylenediaminetetraacetic acid (EDTA)	2.5 ml	2.5 mM

Table 2.5 Preparation of Immunofluorescence Staining Solution

Medium and Additives	Volume	Final Concentration
Phosphate buffered saline	20 ml	-
Bovine serum albumin (BSA)	0.2 g	1 %

2.2.1 Haemolytic Gelatin Gey's Solution

Solutions were made up to 1 L in distilled water and autoclaved. Components of the solutions are listed in Table 2.6. For the working solution 10 ml of Solution A, 2.5 ml of Solution B, 2.5 ml of Solution C and 35 ml of distilled H₂O were mixed. For red blood cell lysis 5 ml of working solution was used per sample.

Table 2.6 Haemolytic Gelatin Gey's Solution components

Solution	Additive	Quantity
A	NH ₄ Cl	35 g
	KCl	1.85 g
	Na ₂ HPO ₄ .12H ₂ O	1.5 g
	KH ₂ PO ₄	0.119 g
	Glucose	5.0 g
	Gelatin	25 g
	1% Phenol red	1.5 ml
B	MgCl ₂ .6H ₂ O	4.2 g
	MgSO ₄ .7H ₂ O	1.4 g
	CaCl ₂	3.4 g
C	NaHCO ₅	22.5 g

2.3 Dissection of Mouse Tissue

Spleen, lymph nodes or lung were dissected from adult mice and placed in RPMI with EDTA. Tissues were cleaned using forceps under a dissection microscope to remove fat. Lungs were perfused with Dulbecco's Phosphate Buffered Saline prior to dissection to wash out lymphocytes in circulation. At days 2 and 3 post

immunisation lymph nodes were teased apart using forceps to enhance detection.

2.4 Cell Isolation

Spleen and lymph node tissues were crushed through a 70 μ m Falcon cell strainer and washed with RPMI with EDTA to obtain a single cell suspension and centrifuged to form a pellet. Red blood cells lysis was carried out by resuspending the pellet in 5 ml Haemolytic Gelatin Gey's solution for 5 minutes on ice. The lysis was stopped by addition of 10 ml RPMI with EDTA before centrifugation. Typical centrifugation conditions were 6 minutes, 4 °C, 394 rcf, unless stated.

Following perfusion lung tissue was chopped into small pieces using scissors and suspended in 2 ml of culture medium in a falcon tube. 10 μ l DNase (10 mg/ml-Roche Life Sciences) and 500 μ l Liberase TM Research Grade (42.4 μ g/ml-Roche Life Sciences) were added to 3.5 ml culture medium, 3 ml of this mixture was added to the lung tissue and it was then incubated for 40 minutes at 37 °C in a shaking incubator (250 rpm). Tissues were then crushed through a 70 μ m Falcon cell strainer and washed with culture medium before being centrifuged. Red blood cell lysis was carried out in 3 ml Haemolytic Gelatin Gey's solution for 5 minutes on ice. Addition of 10 ml culture medium was used to stop the lysis before centrifugation.

2.5 Cell separation by MACS cell enrichment

For identification of rare 2W1S:I-A^b or SM1-specific cell populations, samples were enriched using the Miltenyi MACS enrichment method at day 2, day 3, day 4 and day 28 onwards as described previously (Moon et al., 2007). For identification of endogenous 2W1S:I-A^b-specific cell populations, samples were stained with 10 µg/ml APC conjugated 2W1S:I-A^b MHC II tetramer, consisting of four biotinylated peptide:I-A^b MHC molecules, for 1 hour in the dark in a room temperature water bath; cells were agitated periodically. MHC II tetramers were obtained from National Institute Health (NIH) Tetramer facility or generated by the Protein Expression Facility, University of Birmingham after the kind provision of 2W1S:I-A^b producing S2 cells by Dr. Marc Jenkins. For identification of TCR transgenic SM1-specific cell populations, samples were stained with anti-CD45.1 PE or anti-CD45.2 PE, depending upon host allotype, and anti-CD16/32 both at a concentration of 1:50 for 30 minutes on ice. The samples were then incubated with 25 µl magnetic anti-PE or anti-APC beads (Miltenyi Biotec) for 15 minutes at 4 °C. Cell suspensions were washed with 10 ml staining buffer and centrifuged. To enrich, cells were filtered through a 70 µm cell strainer and passed through an LS column (Miltenyi Biotec) mounted on a magnet, previously primed with 3 ml staining buffer, and washed 3 times with 3 ml staining buffer. To elute the enriched fraction columns were removed from the magnet and 5 ml staining buffer was pushed through. Enriched and run through fractions were incubated with the same cocktail of surface antibodies to enable calculation of cell frequencies.

2.6 Cell Culture

For analysis of OX40 expression on 2W1S:I-A^b CD4⁺ memory T cells, spleen and lymph node cells were cultured overnight at 37 °C with or without IL-7. Cells were prepared as described in section 2.4 and resuspended in culture medium. 10 million cells in 1 ml of culture media were incubated overnight in a 6 well plate at 37 °C alone or in addition to 100 ng IL-7. The following day enrichment for 2W1S:I-A^b CD4⁺ memory T cells was performed as described in section 2.5 and cells were analysed for expression of OX40.

2.7 Flow Cytometry

2.7.1 Antibodies and Immunolabeling

Primary antibodies used for flow cytometry are listed in Table 2.7. Prior to use antibodies were titrated for optimal concentrations. The amount of cells stained was dependent upon the frequency of the population of interest; for low frequencies the whole of the enriched fraction or tissue was stained, for higher frequencies 10-15 % of the tissue was stained.

For the identification of TFH cells an anti-CXCR5 antibody was included alone or alongside MHC II tetramer for 1 hour in a room temperature water bath.

For *in vivo* peptide restimulation experiments 10 µg/mL brefeldin A (Sigma) was added at this stage to prevent secretion of cytokines and enhance signal detection.

Lung tissue were stained with LIVE/DEAD fixable near-IR dead cell stain kit (Life Technologies) for 20 minutes at 4 °C prior to surface staining. This was to aid in the exclusion of dead cells, which were increased due to the additional digestion step at 37 °C. Anti-CD45.2 antibody was also included to aid in the identification of lymphocytes

All cell surface staining was done in a 96 well U bottomed plate to enable pelleting of cells. Plates were centrifuged for 2 minutes, 4 °C, 394 rcf. The supernatant was flicked off and cell pellets were resuspended in 50 µl of the desired antibody cocktail. All antibody cocktails were made in staining buffer. Cells were incubated on ice for 30 minutes. Detection of CCR7 expression was performed at 37 °C for 30 minutes. A single colour control for each fluorochrome was used to set compensation parameters on the flow cytometer. Isotype controls were used where necessary to delineate negative populations; these are listed in Table 2.8. Cell pellets were washed twice with 150 µl staining buffer. Samples were resuspended in approximately 250 µl staining buffer and transferred into polystyrene fluorescence-activated cell sorting (FACS) tubes (Becton) for analysis on the flow cytometer.

2.7.2 Intracellular Immunolabeling

Following surface staining some samples were stained for intracellular transcription factors using the Foxp3/ Transcription factor staining buffer set (eBioscience) following the manufacturers instructions. Surface stains were fixed

and cells were permeabilised in 100 µl of fixation/permeabilisation solution made to a 1:3 ratio of fixative concentrate to diluent. Samples were incubated for 30 minutes on ice before being washed 3 times with 150 µl 10 % permeabilisation buffer, prepared with distilled water. Cells were then incubated with antibodies against the desired transcription factors for 30 minutes on ice with 50 µl antibody cocktail made up in 10 % permeabilisation buffer. 150 µl of 10 % permeabilisation buffer was used to wash twice before flow cytometric analysis.

Intracellular cytokine production was analysed in experiments where an *in vivo* peptide restimulation had been performed. 10 µg/ml brefeldin A was used in all staining performed at room temperature or 37 °C to prevent secretion of cytokines. Following surface staining cells were fixed and permeabilised with Cytofix/Cytoperm Plus (BD Biosciences) following manufacturer's instructions. Cells were then incubated with anti-IL-2 and anti-IFN-γ directly conjugated antibodies in 10 % permeabilisation buffer for 30 minutes on ice. Cells were washed twice with 10 % permeabilisation buffer before flow cytometric analysis.

2.7.3 Flow Cytometry Analysis

Samples were acquired using either the LSR II or LSR Fortessa X-20 using FACS diva software (BD Biosciences). Single colour controls were acquired to set compensation parameters to accommodate bleed over of fluorochromes into other channels prior to acquisition of experimental samples. Forward and side

scatter gates were set to exclude non-viable cells and debris. The number of events acquired was dependent upon the frequency of the population of interest; due to the low frequency of antigen-specific CD4⁺ T cells this was usually between 2-5 million events per sample. Analysis of data was performed using FlowJo software (Miltenyi Biotec). Addition of 10 000 Spherotech Accucount blank particles was used to calculate cell frequencies.

Table 2.7 Primary antibodies used for flow cytometry

Specificity (clone)	Conjugate	Dilution	Manufacturer
Anti-B220	efluor 450	1:200	eBioscience
(RA3-6B2)	FITC	1:400	eBioscience
Anti-CCR7	PE	1:100	eBioscience
(4B12)	Brilliant Violet 650	1:100	BD Biosciences
Anti-CD3	Alexa fluor 700	1:200	eBioscience
(17A2)			
Anti-CD4	V500	1:300	BD Biosciences
(RM4-5)	Brilliant violet 510	1:300	Biolegend
Anti-CD8	APC efluor 780	1:200	eBioscience
(53-6.7)	Brilliant Violet 711	1:200	BD Biosciences
Anti-CD11b	efluor 450	1:200	eBioscience
(M1170)	FITC	1:400	eBioscience
Anti-CD11c	efluor 450	1:200	eBioscience
(N418)	FITC	1:400	eBioscience
Anti-CD25	APC	1:100	eBioscience

(PC61)			
Anti-CD27 (LG.7F9)	PerCP-eFluor 710	1:200	eBioscience
Anti-CD44 (1M7)	APC-eFluor 780	1:200	eBioscience
	Brilliant violet 785	1:200	BD Biosciences
Anti-CD45.1 (A20)	FITC	1:200	eBioscience
	PerCP-Cy5.5	1:200	eBioscience
	PE	1:200	eBioscience
Anti-CD45.2 (104)	FITC	1:300	eBioscience
	PerCP-Cy5.5	1:200	eBioscience
	PE	1:500	eBioscience
Anti-CD62L (MCL-14)	PE	1:500	eBioscience
	PE Cy7	1:200	eBioscience
	APC	1:200	eBioscience
Anti-CD69 (HL2F3)	PE Cy7	1:100	eBioscience
Anti-CXCR5 (2G8)	Biotin	1:50	BD Biosciences
	PE Cy7	1:50	BD Biosciences
	PE	1:50	BD Biosciences
Anti-CD16/CD32 (2.4G2)	n/a	1:50	BD Biosciences
Anti-FOXP3 (FJK-16s)	FITC	1:100	eBioscience
Anti-ICOS (15F9)	PerCP-eFluor 710	1:100	eBioscience
	Alexa fluor 647	1:100	Biolegend
Anti-OX40	APC	1:10	eBioscience

(OX-86)	Brilliant violet 421	1:10	Biolegend
Anti-PD-1	PE Cy7	1:100	Biolegend
(RMPI-30)	Brilliant violet 421	1:100	Biolegend
Anti-IL-2	Alexa fluor 488	1:100	eBioscience
(JES6-5H4)			
Anti-IFN- γ	PE Cy7	1:200	eBioscience
(XMG1.2)			
Anti-T-bet	eFluor 660	1:25	eBioscience
(4B10)			

Table 2.8 Isotype controls used for primary antibodies in flow cytometry

Antibody	Isotype control	Clone	Manufacturer
Anti-OX40 APC	Rat IgG1 APC	eBRG1	eBioscience
Anti-CXCR5 PE Cy7	Rat IgG2a PE Cy7	eBR2a	eBioscience
Anti-ICOS PerCP 710	Syrian Hamster IgG PerCP 710	n/a	eBioscience
Anti-ICOS 647	Hamster IgG APC	eBio299Arm	eBioscience
Anti-CCR7 PE	Rat IgG2a κ PE	eBM2a	eBioscience
Anti-CCR7 Brilliant violet 650	Rat IgG2a κ Brilliant violet 650	RTK2758	Biolegend

2.8 Immunohistology

2.8.1 Sectioning and fixation of tissue

Tissues from experimental mice were frozen on dry ice and stored at -80 °C.

Lymph nodes were frozen in Optimal Cutting Temperature (OCT) compound due to their small size. 6 µm sections were cut and mounted onto 4 spot slides (Hendley-Essex) and dried for 1 hour at room temperature. Slides were fixed in acetone (J.T. Baker) for 20 minutes at 4 °C then air-dried for 30 minutes. Slides were stored face to face in grip seal polythene bags at -20 °C.

2.8.2 Immunolabeling of frozen tissue sections

Slides were air-dried for 30 minutes at room temperature prior to commencement of immunolabeling then hydrated in PBS for 5 minutes. Primary antibodies used for immunofluorescent staining are listed in Table 2.9 and secondary through to quaternary reagents are listed in Table 2.10. All antibodies and reagents were diluted in Immunofluorescence staining solution containing 1 % bovine serum albumin (BSA) in PBS. Slides were blocked for 10 minutes with 75 µl 10 % horse serum in staining solution. Excess blocking solution was aspirated from slides before addition of 75 µl primary antibodies for 1 hour in a humidified chamber. Slides were washed in PBS for 10 minutes between staining steps. Secondary antibodies were cross adsorbed in 10 % mouse serum before incubation for 30 minutes, then diluted further to the

required concentration before incubation. Tertiary, quaternary and quinary antibodies and reagents were prepared and incubated as for secondary antibodies. If required, slides were immersed in 50 µg/mL DAPI (4',6-diamidino-2-phenylindole) in PBS solution for 20 seconds to stain nuclei, then washed 3 times in PBS. Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen) and sealed with clear nail varnish. Slides were allowed to air dry overnight before analysis or storage at -20 °C.

2.8.3 Confocal analysis of immunofluorescent stained tissue sections

Confocal analysis was completed using an LSM 510 Meta confocal microscope (Zeiss) and analysed using LSM software (Zeiss) except for tile scans which were taken using an LSM 780 ZEN confocal microscope (Zeiss) and analysed using ZEN lite (Zeiss). Four channels were used which were each scanned separately, with no overlap in detection between each channel. Alexa 555 conjugated antibodies were excited with a 561 nm helium laser, FITC/Alexa fluor 488 conjugated antibodies were excited with a 488 nm argon laser, Alexa fluor 647 conjugated antibodies were excited with a 633 nm helium laser and DAPI were excited by a 405 nm diode laser.

For quantitation of TCR transgenic SM1 CD4⁺ T cells Zeiss LSM image browser (version 4.2.0.121) was used to draw around the T zone and B cell follicle. The number of SM1 CD4⁺ T cells, identified by CD45 isotype staining, located in these

areas was counted. To enumerate the number of SM1 CD4⁺ T cells located at the B/T interface the border between the T zone and B cell follicle was drawn. An area 20 µm either side of this line was allocated as the B/T interface and any SM1 CD4⁺ T cells lying within this were included in the count. Percentage of SM1 CD4⁺ T cells in these areas was then calculated.

Table 2.9 Primary antibodies used for immunofluorescent staining

Specificity (clone)	Conjugate	Host/Isotype	Manufacturer	Dilution
Anti-B220 (RA3-682)	Biotin	Rat IgG2a, κ	eBioscience	1:200
Anti-Bcl-6 (K112-91)	Alexa fluor 488	Mouse IgG1, κ	BD Biosciences	1:25
Anti-CD3 (eBio500A2)	Biotin	Armenian Hamster IgG	eBioscience	1:100
Purified anti-CD4 (GK 1.5)	Conjugated to Alexa fluor 647 in house	Rat IgG2b, κ	eBioscience	1:150
Anti-CD45.1 (A20)	FITC	Mouse IgG2a, κ	eBioscience	1:200
Anti-IgG2a	FITC	Goat	Southern Biotech	1:100

Table 2.10 Secondary, tertiary, quaternary and quinternary reagents used for immunofluorescent staining

Specificity (clone)	Host/Isotype	Manufacturer	Dilution
Anti-Alexa Fluor 488	Rabbit	Invitrogen	1:200
Anti-FITC Alexa fluor 488 (polyclonal)	Rabbit	Invitrogen	1:200
Anti-rabbit IgG Alexa fluor 488	Donkey	Invitrogen	1:200
Anti-rabbit FITC	Goat	Southern Biotech	1:100
Streptavidin Alexa fluor 555	-	Invitrogen	1:500

2.9 Immunisation and infection

2.9.1 Growth of *Listeria monocytogenes* for infection

Attenuated *Listeria monocytogenes* expressing peptide were used to infect mice so that a population of antigen-specific CD4⁺ T cells could be tracked from a primary response through to the memory phase. This infection route involves the uptake and processing of peptide by APC, which then display peptide fragments via their MHC II molecules. As described previously (Pepper et al., 2010), 10⁷ *actA*-deficient *Listeria monocytogenes* expressing the recombinant protein chicken OVA fused to either 2W1S₅₂₋₆₈ (Rees et al., 1999, Ertelt et al., 2009) (EAWGALANWAVDSA (Lm-2W1S)- kind gift from Professor Marc Jenkins) or FliC₄₂₇₋₄₄₁ (McSorley et al., 2000, Johanns et al., 2011) (VQNRFNSAITNLGN- (Lm-FliC) kind gift from Sing Sing Way) peptides were injected intravenously (i.v.) in the tail vein. Bacteria were grown on chloramphenicol selective Luria Bertani (LB) agar at 37 °C overnight. LB agar plates were made by dissolving

17.5 g LB broth with agar (Lennox) powder (Sigma-Aldrich) in distilled water and shaking to dissolve. The mixture was autoclaved and cooled to around 50 °C, determined by being able to comfortably touch the bottle for 10 seconds. Chloramphenicol was then added to a final concentration of 20 µg/ml. 25 ml of LB agar was poured into large petri dishes and left to set before being stored at 4 °C. A single colony was subcultured in 10 ml LB broth supplemented with 20 µg/ml chloramphenicol overnight at 37 °C in a shaking incubator. Bacteria were diluted 1/10, 1/20, 1/50 in LB broth with 20 µg/ml chloramphenicol and grown until optical density (OD)⁶⁰⁰=0.1 to ensure bacteria were used when in the log growth phase. Optical density measurements were taken on a spectrophotometer (Jenway 6405) calibrated against a blank containing LB medium only. 1 ml aliquots of bacteria were washed twice with PBS and centrifuged at 300 rcf for 10 minutes at room temperature to remove dead bacteria. Optical density measurements were taken again post washing to calculate the correct number of bacteria for injection. Bacteria were diluted in sterile PBS so each mouse received 10⁷ in 200 µl i.v. infection with Lm-FliC was carried out 24 hours post adoptive transfer of TCR transgenic SM1 CD4⁺ T cells, as described in section 2.10.

2.9.2 Peptide and protein immunisation

For *in vivo* restimulation experiments mice that had previously been infected with Lm-2W1S or Lm-FliC were given 2W1S or FliC peptide and LPS to assess cytokine production and therefore identify cells based on their function. On day

28 post infection mice were injected with 100 µg peptide and 2.5 µg LPS i.v. in the tail vein. Mice were sacrificed 4 hours later and tissues were harvested for analysis.

Aluminium precipitated (alum ppt) peptide or protein was used to elicit a strong Th2 response (Grun and Maurer, 1989). 500 µg 2W1S peptide in 25 µl dimethyl sulfoxide (DMSO) was mixed with 125 µl sterile PBS and 150 µl 10 % aluminium sulphate before addition of 7 µl 10 M NaOH and mixed. Alternatively 125 µl of 1 mg/mL OVA protein conjugated to Alexa fluor 647 (Life Technologies) was substituted for 2W1S peptide and the amount of sterile PBS was adjusted accordingly to 25 µl. This was incubated in the dark at room temperature for 30 minutes before washing 3 times with 500 µl sterile PBS. Samples were centrifuged at 400 rcf for 5 minutes at room temperature. 5 µg or 20 µg of alum ppt peptide or protein was then injected into the paw pad of the mouse in 10 µl or 20 µl sterile PBS.

2.10 Cell Transfer

The TCR transgenic SM1 CD4⁺ T population was used as an alternative way to track antigen-specific CD4⁺ T cells. This model system allowed the generation of CD4⁺ memory T cells in WT mice, which were then transferred into genetically deficient hosts to assess specific effects on memory cell survival rather than formation. Spleen and peripheral lymph nodes were harvested from TCR transgenic Rag^{-/-} SM1 mice and prepared as described in section 2.4. Briefly,

tissues were cleaned of excess fat, crushed through a 70 μ m cell strainer and red blood cells lysed with Haemolytic Gelatin Gey's solution under sterile conditions. CD45.1⁺ and CD45.2⁺ allotype was confirmed by the staining technique described in section 2.7.1. The frequency of CD45.1⁺ and CD45.2⁺ TCR transgenic SM1 CD4⁺ T cells among total cells was calculated by flow cytometry using forward and side scatter to gate on live cells. Mice were injected i.v. with 10⁴ TCR transgenic SM1 CD4⁺ T cells suspended in 200 μ l sterile PBS in the tail vein. Consistent with previous studies (Hataye et al., 2006) we have shown that approximately 90 % of transferred cells do not survive (see Chapter 4, Figure 4.3 B), so this transfer was expected to form a naive population of around 1000 SM1 CD4⁺ T cells.

For transfer of WT memory cells into genetically deficient hosts, TCR transgenic SM1 CD4⁺ T cells were prepared from spleen and lymph nodes as described above from WT mice which had received 10 000 TCR transgenic SM1 CD4⁺ T cells and been immunised with Lm-FliC 28 days previously. Typically SM1 CD4⁺ memory T cells were harvested from 4 donor mice. Cells were enriched as described in section 2.5 by MACS cell enrichment. Samples were then pooled and a small aliquot, around 2 % of the sample, was removed and stained to check for the presence and frequency of TCR transgenic SM1 CD4⁺ memory T cells, using expression of CD45.1, CD45.2 and CD44. Again the frequency of TCR transgenic SM1 CD4⁺ T cells among live cells was determined using forward and side scatter gates. SM1 CD4⁺ memory T cells were then transferred into donors by i.v. injection in the tail vein. Typically there were twice as many recipients as

donors, therefore ensuring a physiological number of cells were transferred, usually between 20,000-40,000 cells.

2.11 Antibody Injection

Temporal blockade of costimulatory pathways was achieved using mAb to circumvent the problems associated with impaired CD4⁺ T cell formation in genetically deficient mice and to model therapeutic administration of these substances. Blocking mAb against mouse OX40L (clone RM134L) and CD30L (clone RM153) were provided by Hideo Yagita (Akiba et al., 2000). Control rat IgG was purchased from Sigma-Aldrich. Mice were injected intraperitoneally (i.p.) with 0.25 mg anti-OX40L or control rat IgG. To assess memory cell survival mice were injected twice weekly for 4 weeks at 28 days post infection (dpi). To assess the effect of blocking OX40L on primary responses, mice were injected 1 and 3 dpi. To enhance signalling through OX40:OX40L mice were injected i.p. with 100 µg agonistic anti-OX40 mAb (clone OX86) 1 dpi or on the day of secondary challenge for memory experiments. Anti-OX40 mAb was also given the day following secondary challenge when stated to assess the time period in which it could be effective.

Blocking anti-ICOS and isotype control mAb were provided by MedImmune (Yusuf et al., 2014). Mice were injected i.p. with 0.25 mg anti-ICOS or isotype control mAb. Mice were injected 0 and 3 dpi for primary responses, 0 and 4 dpi

for memory responses and 1 day pre and post secondary challenge. To assess memory cell survival mice were injected twice weekly for 4 weeks from 28 dpi.

2.12 Photoconversion surgery

To investigate the migration of antigen-specific CD4⁺ T cells we used photoconvertible Kaede mice. These mice are engineered to express the green fluorescent Kaede protein cloned from the stony coral *Trachyphyllia geoffroyi* (Ando et al., 2002) in every cell under the control of a CAG promoter (Tomura et al., 2008). When exposed to violet light in the spectrum 350-400 nm a peptide cleavage occurs and the formation of a double bond causes the Kaede protein to become red fluorescent in an irreversible reaction (Mizuno et al., 2003). Once converted the Kaede red protein exhibits a long half-life *in vivo* although after multiple cell divisions it can be difficult to detect due to dilution with newly synthesised Kaede green protein (Tomura et al., 2008). To monitor the migration of antigen-specific cells from a site of local immunisation we developed a surgical method to expose the brachial lymph node to violet light and irreversibly label the cells within (Figure 2.1). The brachial lymph node was chosen due to our ability to target this lymph node with local paw pad immunisation and the ease with which it can be exposed to violet light compared with other lymph nodes. Mice undergoing surgery were 8-12 weeks of age and were weighed prior to the procedure. 2 mg/kg pre-operative Burprenorphine (Tamgesic, Animal Care UK) was administered sub-cutaneously (s.c.) 1 hour prior to surgery for analgesia.

Mice were anaesthetised within a chamber containing 4 % Isoflurane (May and Barker, Dagenham UK) carried with oxygen. Once mice were anaesthetised and breathing rate had slowed they were transferred to a face mask with 2 % Isoflurane. Throughout the procedure mice were kept on a 37 °C heat pad and breathing patterns were monitored to ensure a sufficient depth of anaesthesia. Pedal withdrawal reflex was checked prior to the first incision and at intervals throughout. Mice were shaved in the armpit region and located on their back with the left arm held above the head with micropore surgical tape to expose the area above the brachial lymph node. The area was cleaned using Hibitane disinfectant (Bioglan) and sterile gauze. An incision approximately 0.5-1 cm in length was made above the site of the brachial lymph node. Once located the brachial lymph node was exposed to 365 nm violet light (Campells) for 3 minutes. The incision was then closed with coated vicryl 13 mm round bodied sutures (Ethicon). Mice were given 500 µl saline s.c. in the back to replace fluids and placed in a warm chamber at 24 °C to recover and monitored at regular intervals by [REDACTED] staff. Mice were sacrificed 24 hours post surgery. In experiments to assess the migration of 2W1S:I-A^{b+} CD4⁺ T cells mice were immunised s.c. in the left paw pad with 5 µg alum ppt 2W1S peptide 8, 21 or 42 days previous to photoconversion surgery.



Figure 2.1: Surgery to expose the brachial lymph node to violet light. (A)

The mouse is anaesthetised and positioned so the shaved armpit area is exposed.

(B) An incision is made around 0.5-1 cm to expose the brachial lymph node. (C)

The lymph node is exposed to violet light for 3 minutes before incision is closed with suture.

2.13 Statistical Analysis

Data were analysed using GraphPad Prism (version 6.0e). Non parametric Mann-Whitney test was used to determine significance which was set at $p \leq 0.05$.

Kruskal-Wallis non-parametric one-way ANOVA was used to compare multiple groups with post hoc Dunn's test where stated. Median values were calculated and used in all analyses except where stated.

CHAPTER 3: THE ROLE OF OX40 IN PRIMARY AND
MEMORY CD4⁺ T CELL RESPONSES

3.1 Introduction

Much progress has been made in elucidating the mechanisms of CD4⁺ T cell responses through the use of TCR transgenic T cells, however new physiological methods to track endogenous polyclonal populations are now required to refine and validate these findings. Combining MHC II tetramers and magnetic bead enrichment has successfully been used to track an endogenous population of antigen-specific CD4⁺ T cells responding to infection with *Listeria monocytogenes* (Pepper et al., 2010, Pepper et al., 2011). This method allows an antigen-specific population to be tracked from infection, through priming and into the memory cell phase. Tracking an antigen-specific population in this way also means that memory cells can be defined by time and reduces the reliance of defining these populations on surface markers such as CD44 and CD62L. Despite these obvious advantages the use of MHC II tetramers has some limitations; due to their binding of the TCR they cannot be used to isolate cells for downstream applications as this may affect the activation state of the cell. Additionally, labelling cells with fluorescent MHC II tetramers can be problematic and incubation time and temperatures must be adjusted accordingly.

Although an essential function for OX40 has been shown in the formation of robust primary CD4⁺ T cell responses, the role of this receptor for memory cell populations is less clear. Clearly signals received during the primary response, including through OX40, impact upon the resultant memory cell population. Tem cells were reduced in OX40^{-/-} mice while Tcm cell number was normal, indicating a differential requirement for OX40 signals by CD4⁺ memory T cell subsets

(Soroosh et al., 2007). However whether periodic signalling through this receptor is required for the long-term persistence of CD4⁺ memory T cells once formed is unknown. Expression of OX40 has been reported on memory phenotype (CD44^{hi}CD62L⁻) CD4⁺ T cells and TCR transgenic memory cells, indicating a possible role for OX40 in memory cell responses, yet studies tracking endogenous polyclonal populations are lacking (Gaspal et al., 2005). Addition of IL-7 to cell cultures induced CD4⁺ T cells to upregulate OX40. As IL-7 is known to promote the persistence of memory cells (Kondrack et al., 2003, Purton et al., 2007), this may provide a mechanism by which CD4⁺ memory T cells receive survival signals through OX40 independent of TCR:MHC II interactions. CD4⁺ memory T cells were found to be reduced in the lamina propria of CD30^{-/-}OX40^{-/-} mice, while priming appeared normal suggesting that OX40 is important for the survival of CD4⁺ memory T cells (Withers et al., 2009). It was found that the number of antigen-specific CD4⁺ memory T cells was reduced in RORγt^{-/-} mice, which lack group 3 ILC (Withers et al., 2012). ILC3 constitutively express OX40L, therefore it was proposed they might provide periodic signals to CD4⁺ memory T cells through OX40 to enable their long-term persistence.

It has also been suggested that OX40 is important for the generation of TFH cells, which maintain GC and high affinity antibody production. OX40 expression has been shown on TFH cells, but appears to be highly variable depending upon the tissue and mouse strain (Akiba et al., 2005). OX40 was found to be essential for the upregulation of CXCR5 and therefore the movement of T cells towards the follicle, where they are located to provide survival and proliferation signals to B

cells (Walker et al., 1999, Fillatreau and Gray, 2003, Flynn et al., 1998). However several recent studies, one using antigen-specific T cells, have shown that GC formation and antibody production is normal in the absence of OX40 (Akiba et al., 2005, Kopf et al., 1999, Pippig et al., 1999, Chen et al., 1999). Furthermore a recent study showed that administration of an OX40 agonist mAb was detrimental to the formation of TFH cells and GC, due to the diversion of cells towards the Th1 lineage, in response to LCMV infection (Boettler et al., 2013).

The role of OX40 in augmenting CD4⁺ T cell responses highlighted its potential for use as a therapeutic to either promote immune responses to infection or tumour cells. Anti-OX40 mAb has undergone phase I clinical trials to test the efficacy against cancer (Curti et al., 2013). More recently combination of OX40 with other immunotherapies is being considered as the best way to target tumour cells. As expression of OX40 can be rapid and transient, depending upon the context of the antigen, the timing of administration of mAb is vital to the clinical outcome.

In this chapter the aim was to determine the specific points at which OX40 signals are required by an endogenous, polyclonal CD4⁺ T cell population. We have tracked an endogenous CD4⁺ T cell population responding to infection with Lm-2W1S. Using blocking anti-OX40L mAb to study memory cell persistence, we were able to study CD4⁺ memory T cells primed within a normal environment. Additionally as anti-OX40 mAb has shown promise as a therapeutic we dissected the effect of over-provision of OX40 signalling on both primary effector and memory CD4⁺ T cell populations.

3.2 Results

3.2.1 Utilising Infection with *Listeria monocytogenes* to track an antigen-specific CD4⁺ T cell response

Previously many studies that have investigated antigen-specific T cell responses have used TCR transgenic cells to achieve this (Rogers et al., 2001, Gaspal et al., 2005). Much progress has been made in defining T cell responses using this approach, for example the pivotal finding that antigen-specific CD4⁺ T cells are reliant upon CD28 for optimal proliferation (Lucas et al., 1995). However the limitations of using TCR transgenic T cells mean that more physiological methods are now required to fully elucidate the mechanisms of CD4⁺ T cell responses *in vivo*. TCR transgenic T cell populations are monoclonal and therefore do not have the clonal variation in responses that would be seen for an endogenous T cell pool. They are often transferred in abnormally large numbers, between 10⁵ and 10⁷ cells, thousands of times greater than a naive endogenous T cell pool, to enable ease of detection (Kearney et al., 1994). It has been shown that around 10 % of transferred cells survive within the host 24 hours after transfer and this has been termed the “park rate” (Hataye et al., 2006, Blattman et al., 2002). As the size of the effector cell population is linked to that of the precursor population (Moon et al., 2007), this results in intraclonal competition due to the presence of large numbers of identical T cells (Badovinac et al., 2007, Ford et al., 2007, Hataye et al., 2006, Foulds and Shen, 2006).

TCR transgenic T cells have been useful to advance the study of memory cells, as using surface markers such as CD44 and CD62L to distinguish them has associated caveats. Confusion over the survival requirements for memory cells has been caused by the use of memory phenotype cells, which have since been shown to have different survival requirements to antigen-specific T cells (Lenz et al., 2004, Purton et al., 2007). Memory phenotype cells can arise spontaneously, as opposed to antigen-specific memory cells, which form in response to immunisation with antigen. They consist of a heterogeneous population; some of which resemble antigen-specific cells and a small subset that undergo rapid homeostatic proliferation (Purton et al., 2007, Lenz et al., 2004). This proliferation is driven by TCR interaction, unlike antigen-specific memory cells, which are more reliant on cytokines IL-7 and IL-15 for their survival (Lenz et al., 2004, Kondrack et al., 2003, Purton et al., 2007). Although TCR transgenic T cells have been useful in the analysis of primary responses, studies have shown poor conversion to memory cells with large cell number transfers (Hataye et al., 2006), probably due to increased competition for dendritic cell interactions. Furthermore T cell differentiation has also been shown to be unphysiological in this situation resulting in a bias towards Tcm phenotype cells (Badovinac et al., 2007, Ford et al., 2007, Marzo et al., 2005).

Therefore we utilised a method optimised by the Jenkins laboratory to track a population of antigen-specific CD4⁺ T cells from the primary through to the memory response using MHC II tetramers. The immunogenic peptide 2W1S (Rees et al., 1999) was chosen as the precursor frequency of CD4⁺ T cells that

recognise this peptide is larger than for other populations that can be studied with MHC II tetramers (Moon et al., 2007). The number of cells responding to this peptide will therefore be larger making it less technically challenging. A vaccine strain of *Listeria monocytogenes* was engineered to secrete a fusion protein containing a region of OVA and the 2W1S peptide under the control of the *hly* promoter (Ertelt et al., 2009). This strain has been attenuated by a mutation in the *actA* gene (Portnoy et al., 2002), preventing cell-to-cell movement and therefore producing a mild acute infection that is rapidly cleared by the mouse. Following infection, 2W1S peptide is produced by antigen processing and presented on MHC II molecules. CD4⁺ T cells with TCR specific for 2W1S were identified by staining spleen and lymph node cells with PE or APC labelled 2W1S:I-A^b MHC II tetramers. Cells were stained with antibodies specific for CD3 to identify T cells and a dump channel of non-T cell specific markers (B220, CD11b and CD11c) were used to exclude cells that may be binding tetramer non-specifically or phagocytic cells. Staining for co-receptors CD8 and CD4 and the activation marker CD44 were also used to positively identify activated 2W1S:I-A^b CD4⁺ T cells following infection (Figure 3.1 A). Uninfected mice contained a small population of around 100 CD3⁺CD4⁺ cells that bound 2W1S:I-A^b tetramer; the majority of these were CD44^{lo}, which is consistent with a naive phenotype (Figure 3.1 B). A tetramer binding population was not detected in CD8⁺ T cells (Figure 3.1 B). This was also the case when CD4⁺ T cells were stained with class II-associated invariant peptide (CLIP) control tetramer (Figure 3.1 C). This irrelevant peptide is used as a negative control tetramer to rule out non-specific staining. Following infection with Lm-2W1S, the naive

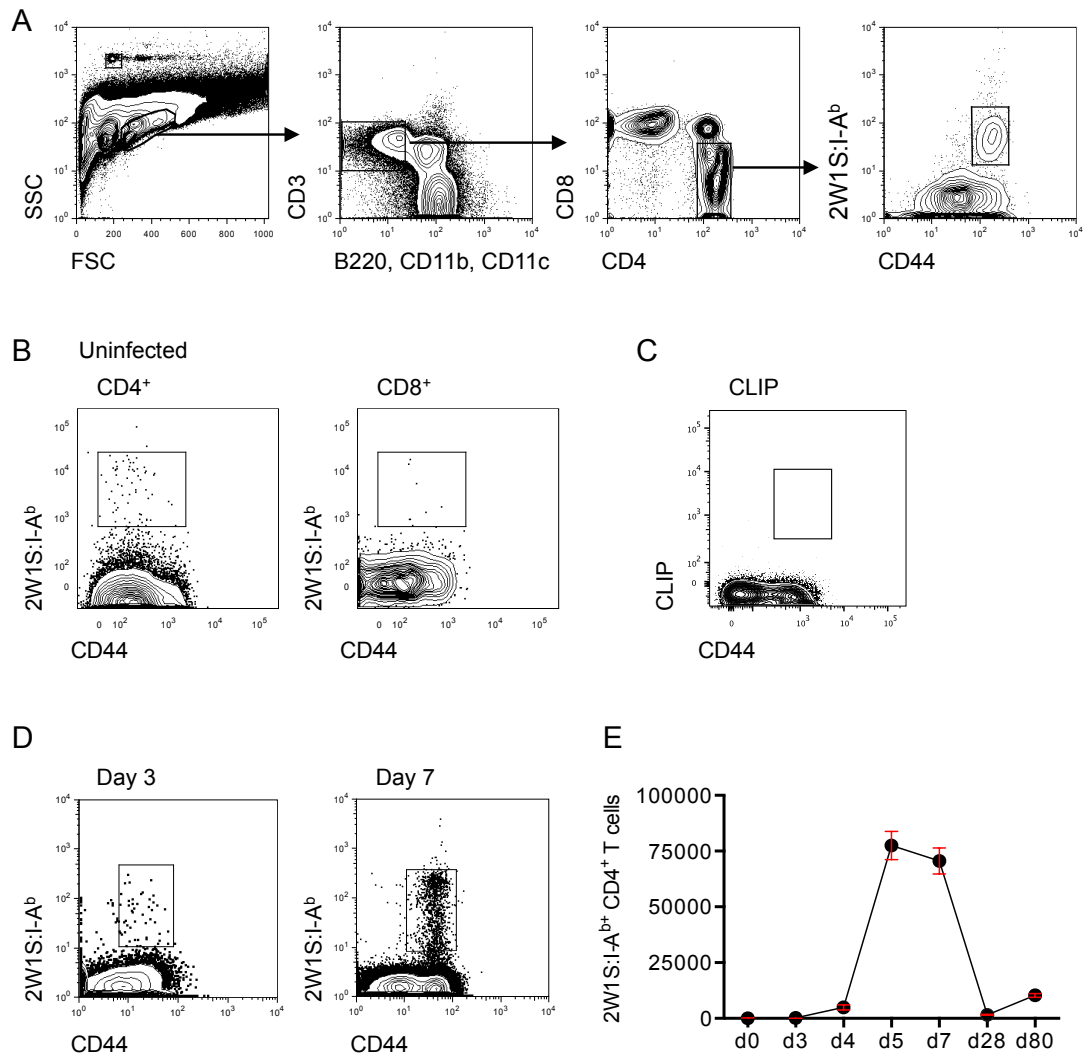


Figure 3.1: Infection with Lm-2W1S forms a population of antigen-specific CD4⁺ T cells that can be tracked using MHC class II tetramers. (A) Gating strategy for the identification of 2W1S:I-A^b+ CD4⁺ T cells; gated on lymphocytes, CD3⁺B220⁻CD11b⁻CD11c⁻, CD4⁺CD8⁻, CD44^{hi}2W1S:I-A^b+. (B) CD4⁺ (left) or CD8⁺ (right) T cells identified as in (A) from an uninfected WT mouse with gates showing 2W1S:I-A^b+ cells. (C) CD4⁺ T cells identified as in (A) from a WT mouse 7 dpi mouse stained with CLIP control tetramer. (D) CD4⁺ T cells from day 3 (left) and day 7 (right) post infection with Lm-2W1S with gating showing 2W1S:I-A^b+ CD4⁺ T cells. (E) Time course showing number of 2W1S:I-A^b+ CD4⁺ T cells following infection with Lm-2W1S (n= 4-13 mice for each data point).

(A,B and D) Plots are representative of ≥5 mice from 2 independent experiments. (C) Plot is representative of 3 mice from 2 independent experiments. (E) Graph shows pooled data from ≥2 independent experiments. Data points represent mean and error bars show SEM.

population expands rapidly through cell proliferation. We investigated the kinetics of this response by analysing spleen and lymph node cells following i.v. infection. At day 3 post infection it was necessary to pool cells from 3 mice to provide a large enough population for phenotyping; there was then an exponential proliferation of cells between day 3 and day 7 when a median of around 70 000 2W1S:I-A^b CD4⁺ T cells can be found in the spleen alone (Figure 3.1 D and E). We extended this analysis to look at the memory phase of the response; following the initial proliferation after infection there is a drastic contraction of the population until day 20, which then slowly declines over the following year (Pepper et al., 2011). A time course looking at day 0, day 3, day 4, day 5, day 7, day 28 and day 80 showed a similar pattern of expansion and contraction as has previously been reported (Figure 3.1 E), with a quiescent population of memory cells surviving at 80 dpi. Unusually there were more 2W1S:I-A^b CD4⁺ memory T cells recovered at day 80 compared to day 28 post infection (Figure 3.1 E). This is likely an artefact resulting from fixation at day 28 to stain for intracellular cytokines, a protocol which results in cell loss.

We chose to use day 28 for all analyses of memory cells to ensure the response had entered the quiescent memory phase. At this timepoint a population of 2W1S:I-A^b binding cells were identified using the magnetic enrichment method described previously (Moon et al., 2007). Following staining with fluorescent tetramers, 2W1S:I-A^b CD4⁺ T cells were enriched as described in Section 2.5. The 2W1S:I-A^b CD4⁺ T cells bound to the column were eluted and then stained with surface markers as described above. To identify memory cell subsets based

on their function we performed an *in vivo* restimulation with 2W1S peptide and LPS, which induces cytokine production (Pepper et al., 2011). Infection with Lm-2W1S produces both a population of IL-2⁺ Tcm cells and IL-2⁺IFN- γ ⁺ Tem cells at 28 dpi following restimulation with peptide (Figure 3.2 A), consistent with previous studies (Pepper et al., 2011). The Tcm cell population was around 3 fold larger than the Tem cell population (Figure 3.2 B and C).

3.2.2 Expression of OX40 on 2W1S:I-A^b CD4⁺ T cells is transient and heterogeneous

The role of OX40 in primary CD4⁺ T cells responses has been extensively studied, however much of this data has not been shown for endogenous, polyclonal T cell populations. In addition studies tracking memory cell responses in OX40^{-/-} and/or CD30^{-/-} mice have not taken into account the effect of impaired priming in these mice (Gaspal et al., 2005). OX40 is induced upon TCR activation however the pattern of expression varies depending upon the model studied; expression can be detected anywhere from 2 to 20 days post activation depending on whether it is an acute or chronic response (Boettler et al., 2013, Gramaglia et al., 1998). Knowledge of the timing of OX40 expression is essential to therapeutic interventions that target this receptor and are therefore only effective within a short window of time.

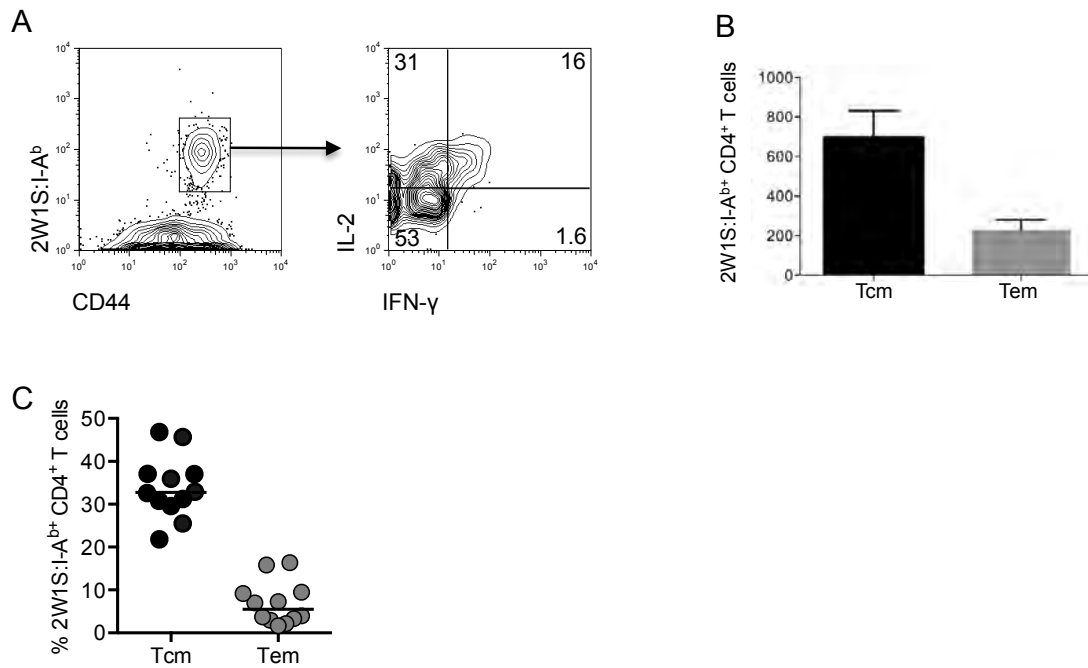


Figure 3.2: Infection with Lm-2W1S forms a population of antigen-specific CD4⁺ memory T cells. WT mice were infected with Lm-2W1S and analysed 28 dpi. Mice were restimulated *in vivo* with 2W1S peptide and LPS and analysed for cytokine secretion after 4 hours. (A) Gating to identify 2W1S:I-A^b CD4⁺ memory T cells based on functional secretion of IL-2 and IFN-γ post *in vivo* restimulation. (B) Number of 2W1S:I-A^b CD4⁺ Tcm and Tem cells 28 dpi with Lm-2W1S. (C) Percentage of 2W1S:I-A^b CD4⁺ Tcm and Tem cells 28 dpi with Lm-2W1S.

(A) Plots are representative of ≥5 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 3 independent experiments. Bars show mean and error bars show SEM. (C) Graph shows pooled data from 3 independent experiments. Bars show median.

To investigate the role of OX40 in CD4⁺ T cell responses we first determined the kinetics of expression on the endogenous 2W1S:I-A^b⁺ CD4⁺ T cell population. Total CD4⁺ Treg cells were used as a positive control as a proportion of this population constitutively express OX40 (Figure 3.3 A). At 2 dpi there was a small number of 2W1S:I-A^b⁺ CD4⁺ T cells, which meant that it was necessary to pool cells from 2 mice. Among the 2W1S:I-A^b⁺ CD4⁺ T cells present at this time, there was no detectable OX40 expression (Figure 3.3 A).

OX40 expression was only detected at 3 dpi however still less than 50 % of 2W1S:I-A^b⁺ CD4⁺ T cells expressed OX40; by 4 dpi almost all OX40 expression was absent and there was no detectable expression at 7 dpi (Figure 3.3 A and B). These data are in contrast to that published for TCR transgenic cells where OX40 expression is uniform amongst the population (Boettler et al., 2013, Boettler et al., 2012, Rogers et al., 2001). As TCR transgenic T cells are monoclonal they do not show the natural variation in response that would be present in a polyclonal endogenous population due to the presence of clones of different affinities (Ertelt et al., 2011). High levels of TCR stimulation have been shown to be important for expression of OX40 (Verdeil et al., 2006), suggesting that only 2W1S:I-A^b⁺ CD4⁺ T cells with high affinity TCR express OX40. T cells receiving different levels of TCR stimulation may differentially express costimulatory receptors and progress down different differentiation pathways. This highlights the requirement for studies assessing endogenous polyclonal populations.

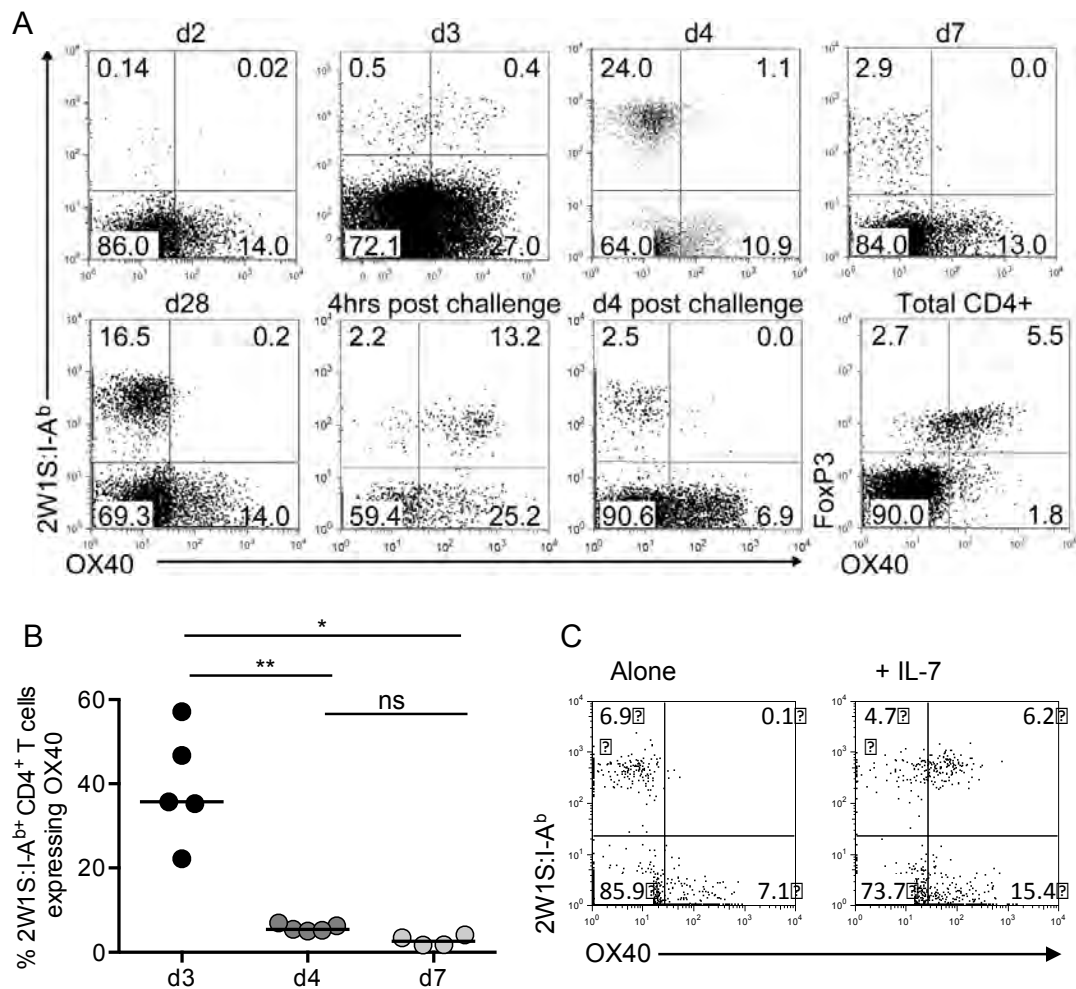


Figure 3.3: Expression of OX40 on endogenous 2W1S:I-A^b+ CD4⁺ T cells is transient and heterogeneous in response to infection with Lm-2W1S. WT mice were infected with Lm-2W1S and analysed at various timepoints as indicated. (A) Expression of OX40 on 2W1S:I-A^b+ CD4⁺ T cells at day 2, day 3, day 4, day 7 and day 28 post infection with Lm-2W1S, 4 hours post peptide restimulation and 4 days post-secondary challenge, and on Foxp3⁺ CD4⁺ Treg cells which were used as a positive control for OX40 staining. (B) Percentage of 2W1S:I-A^b+ CD4⁺ T cells expressing OX40 at day 3, day 4 and day 7 post infection. (C) WT mice were infected with Lm-2W1S and 28 dpi spleen and lymph node cells were harvested and cultured overnight alone or with IL-7. Samples were then enriched for 2W1S:I-A^b+ CD4⁺ T cells and stained for OX40. Gated on CD4⁺ T cells.

(A) Plots are representative of ≥ 5 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 2 independent experiments. Bars show median. (C) Plots are representative of 5 mice from 1 experiment. Values on plots are percentages.

2W1S:I-A^{b+} CD4⁺ memory T cells, at 28 dpi, did not express OX40 directly *ex vivo* (Figure 3.3 A), however these cells could rapidly upregulate OX40 when restimulated *in vivo* with 2W1S peptide and LPS. Four hours following peptide restimulation the vast majority of 2W1S:I-A^{b+} CD4⁺ T cells expressed OX40 indicating a rapid inducement of OX40 expression upon TCR stimulation and a less restricted expression pattern than in a primary response (Figure 3.3 A). Again OX40 expression was not maintained for a long period; 4 days post secondary challenge with Lm-2W1S all 2W1S:I-A^{b+} CD4⁺ T cells were OX40⁻ (Figure 3.3 A). These data are in line with that previously published showing OX40 is not expressed on the majority of CD4⁺ memory T cells but rather is linked to antigenic stimulation, however some studies have found low levels of OX40 on memory cells, so it is likely context dependent (Croft, 2010, Salek-Ardakani et al., 2003). Addition of IL-7 to *in vitro* cultures has been shown to upregulate OX40 expression on CD4⁺ memory T cells (Gaspal et al., 2005). To test if this was also true for 2W1S:I-A^{b+} CD4⁺ memory T cells, WT mice were infected with Lm-2W1S to form a memory cell population. Spleen and lymph node cells were harvested 28 dpi and cultured overnight with IL-7. Addition of IL-7 caused around 70 % of 2W1S:I-A^{b+} CD4⁺ memory T cells to express OX40 (Figure 3.3 C). This shows that memory cells receiving IL-7 signals *in vivo* may express OX40, however does not explain why expression cannot be detected on these cells directly *ex vivo*.

3.2.3 OX40 is primarily expressed on 2W1S:I-A^b CD4⁺ T cells with an effector phenotype following infection

Effector cells responding to Lm-2W1S can be divided into 3 populations based on their expression of CXCR5 and PD-1 (Pepper et al., 2011); CXCR5⁻PD-1⁻T-bet⁺ Teff cells, CXCR5⁺PD-1⁻Bcl-6⁺ cells that give rise to central memory cells and CXCR5⁺PD-1⁺Bcl-6⁺ TFH cells. These 3 populations could be identified in WT mice 7 dpi with Lm-2W1S by expression of the chemokine receptor CXCR5 and CD28 family member PD-1 (Figure 3.4 A). It has previously been shown that these populations present at day 7 go on to form distinct memory cell populations from Teff and T central memory precursors (Pepper et al., 2011). In contrast to previous studies using TCR transgenic T cells we found that expression of OX40 was heterogeneous among the responding 2W1S:I-A^b CD4⁺ T cell population, indicating a certain subset may specifically benefit from OX40 signals. As OX40 was only expressed early in the primary response, 3 dpi, then rapidly lost we analysed effector and TFH cell markers at this timepoint to determine if OX40 is preferentially expressed on one subset.

On day 3 post infection there are very few 2W1S:I-A^b CD4⁺ T cells present in the spleen and lymph nodes, as the population has not yet undergone exponential expansion. Therefore it was necessary to pool samples from 3 mice following magnetic enrichment to ensure there were enough cells to correctly identify expression levels. We sought to investigate if OX40 was specifically expressed by the previously identified CXCR5⁻ Teff cells or CXCR5⁺PD-1⁺ TFH cells or if there was no bias in the expression pattern. T-bet is the key lineage defining

transcription factor expressed by Th1 effector cells (Szabo et al., 2000), however at 3 dpi 2W1S:I-A^b CD4⁺ T cells uniformly express T-bet therefore it cannot be used to differentiate Teff cells from TFH cells (Figure 3.4 B). The IL-2 receptor CD25 is expressed on CXCR5⁻ Teff cells but not on CXCR5⁺ Tcm precursors or TFH cells (Pepper et al., 2011). CD25 was found to be required for the development of CXCR5⁻ Teff cells; CXCR5⁻ cells developed at 1/10 the rate of WT in the absence of CD25 but Tcm precursors and TFH cells developed normally (Pepper et al., 2011). CD25 can therefore be used to identify Teff cells at 3 dpi, the timepoint at which OX40 expression is present. CD25 is expressed on around half of 2W1S:I-A^b CD4⁺ T cells at 3 dpi identifying a population of Teff cells (Figure 3.4 B). TFH cells can be identified by a lineage defining transcription factor Bcl-6, however it is not expressed on 2W1S:I-A^b CD4⁺ T cells as early as 3 dpi therefore cannot be used to identify TFH cells when OX40 expression is present (Figure 3.4 B). CXCR5 is also used as a key marker to identify TFH cells (Ansel et al., 1999). 3 dpi with Lm-2W1S around 80% of 2W1S:I-A^b CD4⁺ T cells were CXCR5⁺ (Figure 3.4 B), meaning it is not a useful marker to distinguish Teff cells from TFH cells at this point. Therefore we could identify Teff cells based upon their expression of CD25. CD25 and OX40 are co-expressed on 2W1S:I-A^b CD4⁺ T cells (Figure 3.4 C). The majority of cells expressing OX40 at 3 dpi are CD25⁺ and conversely the majority of OX40⁺ cells are CD25⁺ (Figure 3.4 D and E). This indicated that they are committed at an early stage to the effector cell lineage, not the TFH cell lineage. This was surprising as OX40 has previously been implicated in the development and survival of TFH cells (Gaspal et al., 2005).

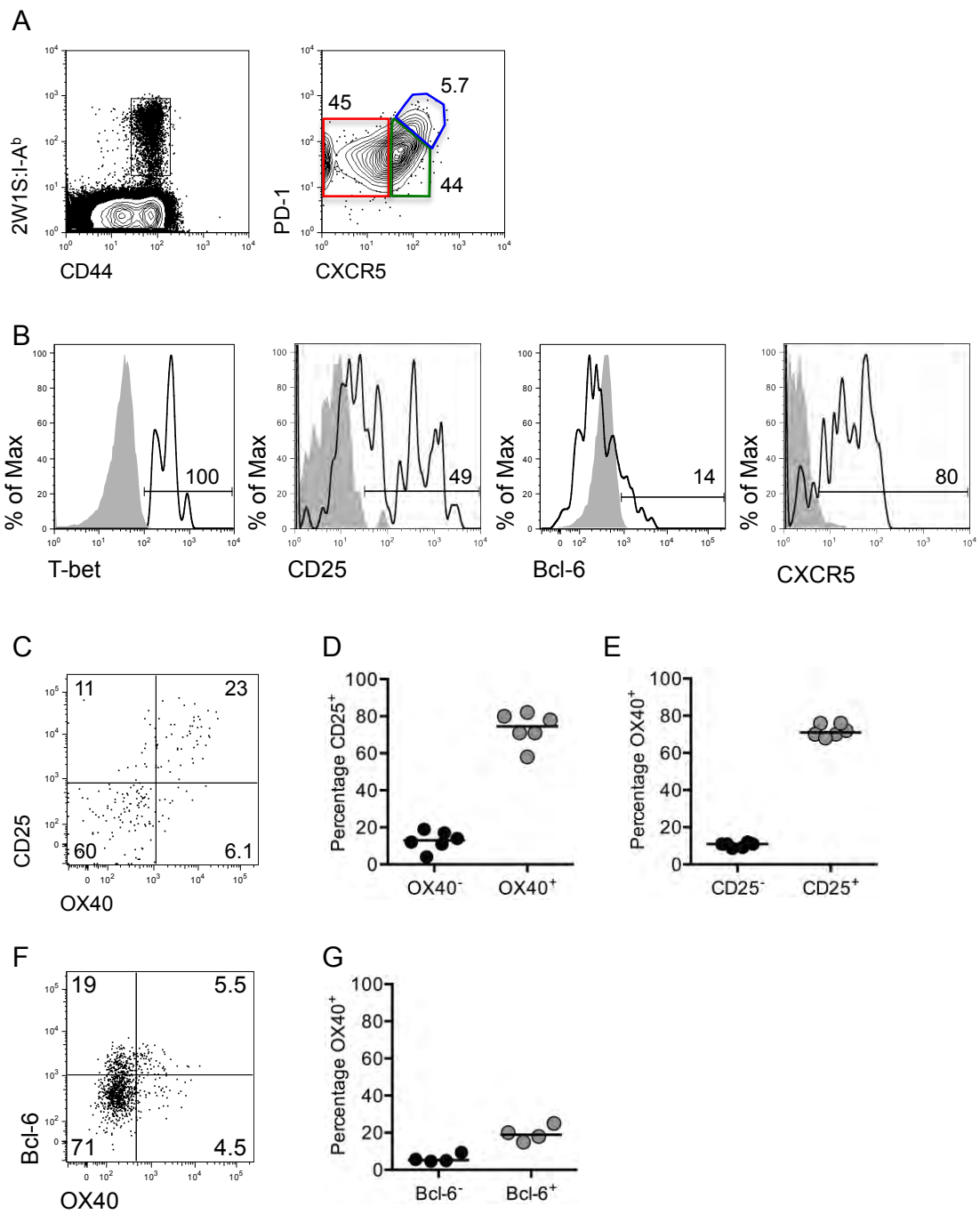


Figure 3.4: Expression of T-bet, CD25, Bcl-6 and CXCR5 on 2W1S:I-A^b CD4⁺ T cells. WT mice were infected with Lm-2W1S and analysed at the specified timepoints. On day 3 post infection samples were enriched and 3 mice were pooled due to low cell number. (A) 2W1S:I-A^b CD4⁺ T cells can be split into subsets 7 dpi based on expression of CXCR5 and PD-1; CXCR5⁻ Teff cells (red), CXCR5⁺ T central memory precursors (green) and CXCR5⁺PD-1⁺ TFH cells (blue). (B) Expression of T-bet, CD25, Bcl-6 and CXCR5 on 2W1S:I-A^b CD4⁺ T cells 3 dpi. (C) Co-expression of OX40 and CD25 on Teff cells 3 dpi. (D) Percentage of OX40⁻ and OX40⁺ cells expressing CD25 and (E) Percentage of CD25⁻ and CD25⁺ cells expressing OX40 3 dpi. (F) Expression of OX40 and Bcl-6 on 2W1S:I-A^b CD4⁺ T cells 4 dpi. (G) Percentage of Bcl-6⁻ and Bcl-6⁺ cells expressing OX40 at 4 dpi.

(A-C and F) Plots are representative of of ≥ 4 mice from ≥ 2 independent experiments. Values on plots are percentages. (B) Grey shaded areas on histograms represent relevant isotype control and values show percentage of positive cells. (D, E and G) Graphs show pooled data from 2 independent experiments. Bars show median.

Studies have shown a defect in the migration of CXCR5⁺ CD4⁺ T cells into the B cell follicle in the absence of OX40, implying a detrimental effect on TFH cells, which may complete their differentiation in the B cell follicle (Walker et al., 1999, Fillatreau and Gray, 2003, Crotty, 2011). However more recent work has shown normal TFH cell differentiation and GC development in OX40^{-/-} mice, OX40L^{-/-} mice and using blocking anti-OX40L mAb (Kopf et al., 1999, Akiba et al., 2005, Ekkens et al., 2003). Bcl-6 expression could be detected at 4 dpi however by this point the majority of 2W1S:I-A^b⁺ CD4⁺ T cells were OX40⁻. However a small percentage of Bcl-6⁺ cells were OX40⁺ indicating that OX40 is not exclusively expressed by Teff cells in the primary response (Figure 3.4 F and G).

3.2.4 Peptide restimulation rapidly induces OX40 expression on 2W1S:I-A^b⁺ CD4⁺ T cells

To investigate the kinetics of OX40 expression following TCR stimulation we used 2W1S peptide and LPS to stimulate mice that had previously been infected with Lm-2W1S. Restimulation at 7 dpi caused the majority of 2W1S:I-A^b⁺ CD4⁺ T cells to upregulate OX40 (Figure 3.5 A). This included not only CXCR5⁻ Teff cells, but also a large proportion of CXCR5⁺ Tcm precursor cells and TFH cells (Figure 3.5 B). This indicated that OX40 is more broadly expressed following restimulation and not mainly restricted to Teff cells as seen at day 3 post infection.

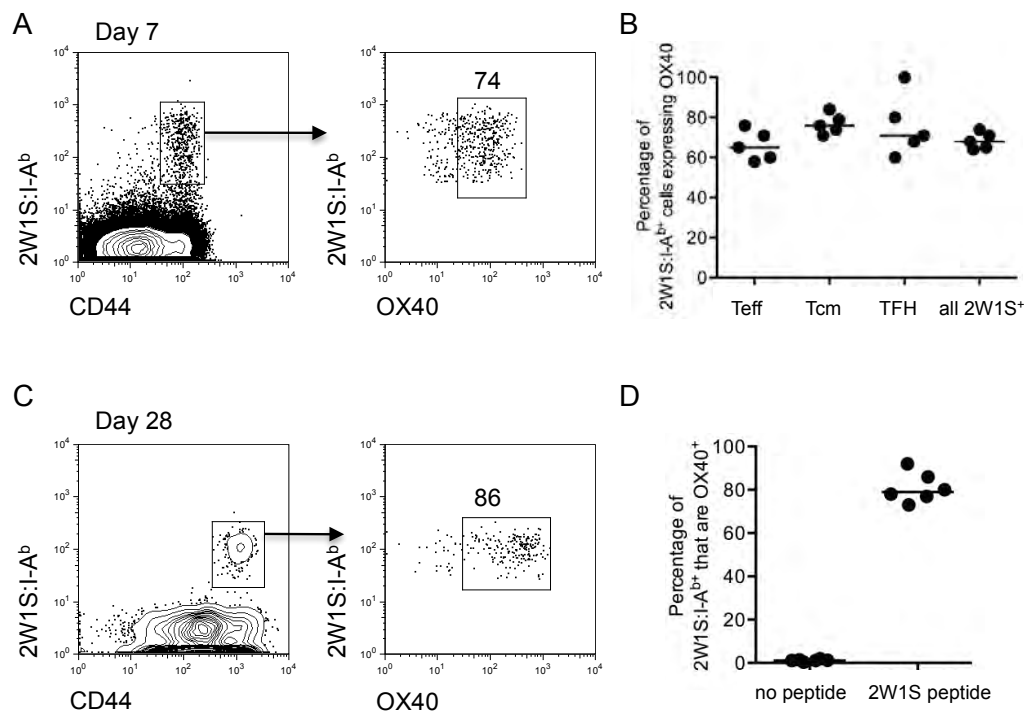


Figure 3.5: Upon re-encounter of antigen, OX40 is more broadly expressed on 2W1S:I-A^b CD4⁺ T cells. WT mice were infected with Lm-2W1S then restimulated 28 dpi *in vivo* with 2W1S peptide and LPS. Tissues were harvested 4 hours post restimulation for analysis of OX40 expression. (A) Expression of OX40 on 2W1S:I-A^b CD4⁺ T cells following restimulation with 2W1S peptide 7 dpi. (B) Percentage of T cell subsets expressing OX40 following restimulation with 2W1S peptide 7 dpi. Shown are 2W1S:I-A^b CD4⁺ T cells that are Teff cells, T central memory precursor cells and TFH cells. (C) Expression of OX40 on 2W1S:I-A^b CD4⁺ T cells following restimulation with 2W1S peptide 28 dpi. (D) Percentage of 2W1S:I-A^b CD4⁺ T cells expressing OX40 28 dpi directly *ex vivo* (no peptide) and following restimulation with 2W1S peptide.

(A and C) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B and D) Graphs show pooled data from 2 independent experiments. Bars show median.

OX40 was not detected on 2W1S:I-A^b CD4⁺ memory T cells directly *ex vivo* (Figure 3.5 A), however following restimulation around 80% of 2W1S:I-A^b CD4⁺ T cells are OX40⁺ (Figure 3.5 C and D).

3.2.5 Blocking OX40 signalling does not impair memory cell survival, however does affect expansion upon secondary challenge

Previous work in our laboratory has shown impaired survival of CD4⁺ memory T cells in mice that lack group 3 ILC. This was postulated to be due to a deficiency in signalling through OX40, as ILC3 constitutively express OX40L (Withers et al., 2012). Although the majority of CD4⁺ T cells do not express OX40, surface expression can be upregulated following incubation with IL-7 (Gaspal et al., 2005), so this was a possible mechanism by which OX40 may provide survival signals. To test this hypothesis we used blocking anti-OX40L mAb to temporally control signalling through the OX40:OX40L pathway. This approach was used to circumvent the caveats associated with using OX40^{-/-} mice where primary T cell survival is impaired and therefore defects in the precursor population impact upon the memory cell pool. As there is redundancy in the OX40 signalling pathway we combined this with blockade of CD30L (Gaspal et al., 2005). We infected mice with Lm-2W1S and rested for 28 days to form a population of endogenous 2W1S:I-A^b CD4⁺ T cells that could be tracked. Once the memory cell population had formed mice were given blocking anti-OX40L, blocking anti-

CD30L, a combination of both blocking mAb or control IgG mAb twice weekly for 4 weeks (Figure 3.6 A). Although the half-life of the 2W1S:I-A^b+ CD4⁺ memory T cell population is 40 days (Pepper et al., 2010), if OX40 signals play a key role in the survival of memory cells we would expect to see a reduction in their number after a 4 week period of antibody treatment. Blocking anti-OX40L mAb did not cause a significant reduction in the number of 2W1S:I-A^b+ CD4⁺ memory T cells recovered (Figure 3.6 B; median for control: 6794, anti-OX40L: 4509).

Combination of OX40L and CD30L blockade however caused a modest, but statistically significant reduction in the number of 2W1S:I-A^b+ CD4⁺ memory T cells (Figure 3.6 B; median for control: 6794, anti-OX40L & CD30L: 2505).

We then investigated whether providing additional signalling through the OX40:OX40L pathway using agonistic anti-OX40 mAb could enhance memory cell survival. Agonistic OX40 mAb have the potential for use in the clinic to enhance CD4⁺ T cell responses; for example they are currently being trialled in models of cancer (Weinberg et al., 2000, Curti et al., 2013). They have also been considered for use as adjuvants in vaccination due to their efficacy in enhancing the immune response in several different viral and fungal models (Munks et al., 2004, Humphreys et al., 2003). However the number of 2W1S:I-A^b+ CD4⁺ memory T cells recovered from mice receiving anti-OX40 mAb for 4 weeks was not significantly greater than from mice that received control IgG mAb (Figure 3.6 C; median for control: 5673, anti-OX40: 5211).

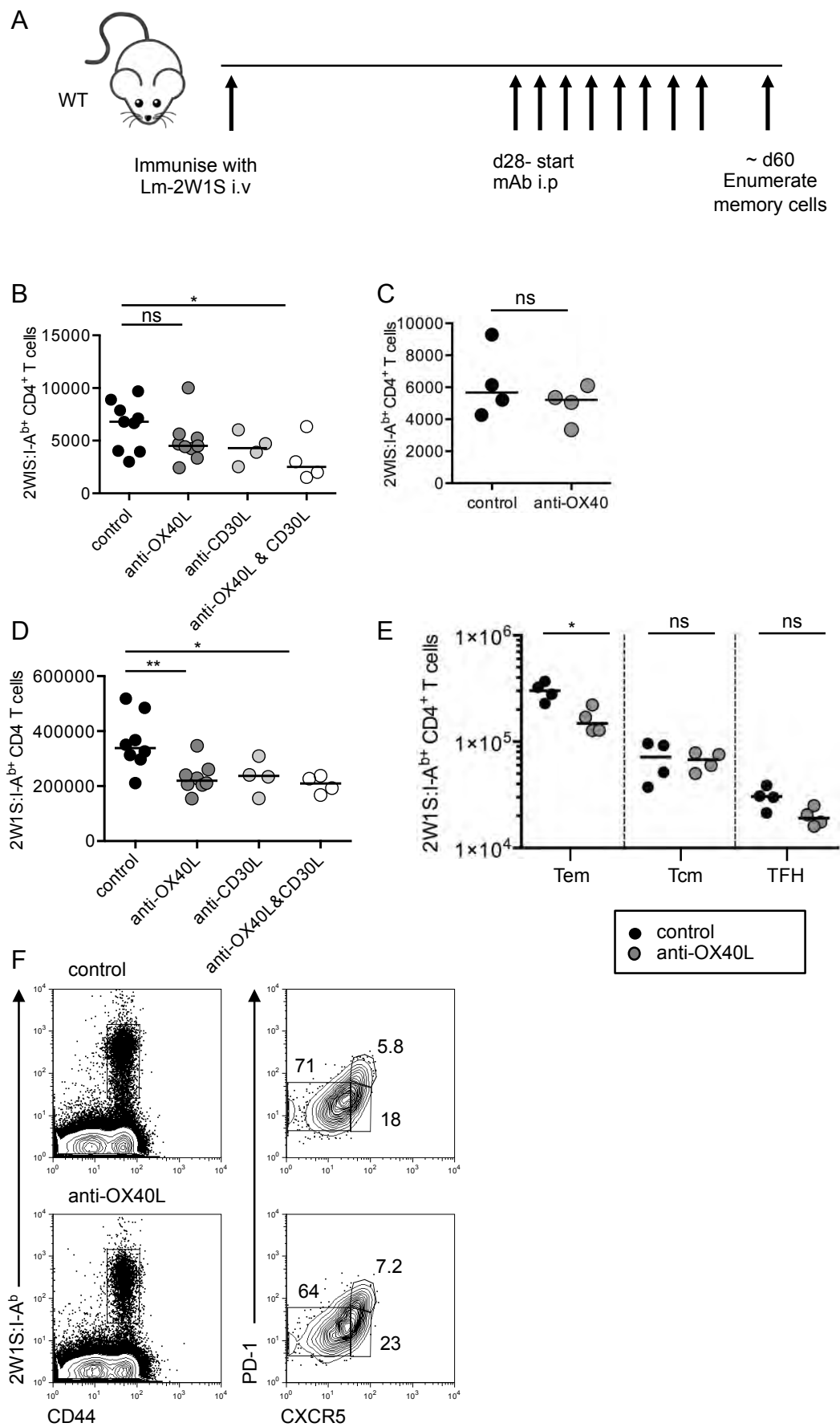


Figure 3.6: Blocking OX40L mAb impairs expansion of memory cells upon challenge but not memory cell survival. WT mice were infected with Lm-2W1S and at 28 dpi blocking anti-OX40L, blocking anti-CD30L, combined blocking anti-OX40L and anti-CD30L or control IgG mAb were administered twice weekly for 4 weeks. 2W1S:I-A^b CD4⁺ memory T cells were then enumerated from spleen and lymph nodes. (A) Schematic showing the immunisation protocol. (B) Number of 2W1S:I-A^b CD4⁺ memory T cells recovered following mAb treatment.

WT mice were infected with Lm-2W1S and at 28 dpi agonistic anti-OX40 or control IgG mAb were administered twice weekly for 4 weeks. 2W1S:I-A^b CD4⁺ memory T cells were then enumerated from spleen and lymph nodes. (C) Number of 2W1S:I-A^b CD4⁺ memory T cells recovered following mAb treatment.

WT mice were infected with Lm-2W1S and at 28 dpi challenged again with Lm-2W1S. Blocking anti-OX40L, blocking anti-CD30L, combined blocking anti-OX40L and anti-CD30L or control IgG mAb were administered 1 day pre and post secondary challenge and spleen and lymph nodes were analysed 4 days post secondary challenge. (D) Number of 2W1S:I-A^b CD4⁺ memory T cells recovered following secondary challenge. (E) Number of 2W1S:I-A^b CD4⁺ Tem, Tcm and TFH from mice receiving blocking anti-OX40L or control IgG mAb following challenge. (F) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells following secondary challenge.

(B and D) Graphs show pooled data from 2 independent experiments except for blocking anti-CD30L and blocking anti-OX40L & CD30L which are from 1 experiment. Bars show median. (C and E) Data are from 1 experiment. Bars show median. (F) Plots are representative of 4 mice from 1 experiment. Mann-Whitney test: *p<0.05, **p<0.01, ns= non-significant.

These data suggest that in the response to Lm-2W1S the survival of CD4⁺ memory T cells was independent of OX40, however a modest effect on survival was seen when CD30L was also blocked. 2W1S:I-A^b⁺ CD4⁺ memory T cells did not constitutively express the OX40 costimulatory receptor suggesting signals through OX40 are an unlikely survival mechanism. However 2W1S:I-A^b⁺ CD4⁺ memory T cells were able to rapidly upregulate OX40 following restimulation with 2W1S peptide. This led to the question of whether manipulating OX40 signals at this timepoint could affect the re-expansion of memory cells. The rapid expansion of memory cells following antigenic stimulation is one of their defining characteristics. Previous work has shown an important role for OX40 in the memory recall response in an asthma model (Salek-Ardakani et al., 2003). We examined whether there is a requirement for OX40 costimulatory signals upon secondary challenge. Mice were challenged with Lm-2W1S at day 28 post infection and spleen and lymph node tissues were analysed 4 days post secondary challenge. Blocking anti-OX40L, blocking anti-CD30L or control IgG mAbs were administered 1 day pre and post secondary challenge due to the rapid and transient expression of OX40 on 2W1S:I-A^b⁺ CD4⁺ T cells. In contrast to their survival, OX40 was required for the optimal expansion of 2W1S:I-A^b⁺ CD4⁺ T cells following secondary challenge, however combined blockade of CD30L did not cause an additive effect (Figure 3.6 D; median for control: 338 051, anti-OX40L: 219533, anti-OX40L & CD30L: 209 632). Furthermore when 2W1S:I-A^b⁺ CD4⁺ T cells were broken down into subsets, Tem, Tcm and TFH cells, this effect was specifically seen on the Tem cell subset (Figure 3.6 E and F).

3.2.6 Ligation of OX40 upon secondary challenge

enhances Tem cell expansion

Since blocking OX40 signalling caused a reduction in the expansion of memory cells post secondary challenge we investigated whether ligation of OX40 could enhance expansion. As before WT mice were infected with Lm-2W1S and challenged again 28 dpi. Agonistic anti-OX40 or control mAb were administered on the day of secondary challenge. Enumeration of 2W1S:I-A^b⁺ CD4⁺ memory T cells from spleen and lymph nodes showed a significant increase in number (approximately 6.4 fold) from mice treated with anti-OX40 mAb (Figure 3.7 B). Interestingly the phenotype of the expanded antigen-specific pool was biased in favour of CXCR5⁻ Tem cells (Figure 3.7 A) with a significant increase in both their proportion and absolute number (Figure 3.7 C). This was accompanied by a decrease in the proportion of TFH cells however the absolute number was not significantly affected, indicating that agonistic anti-OX40 mAb have specific effects on CXCR5⁻ cells (Figure 3.7 D).

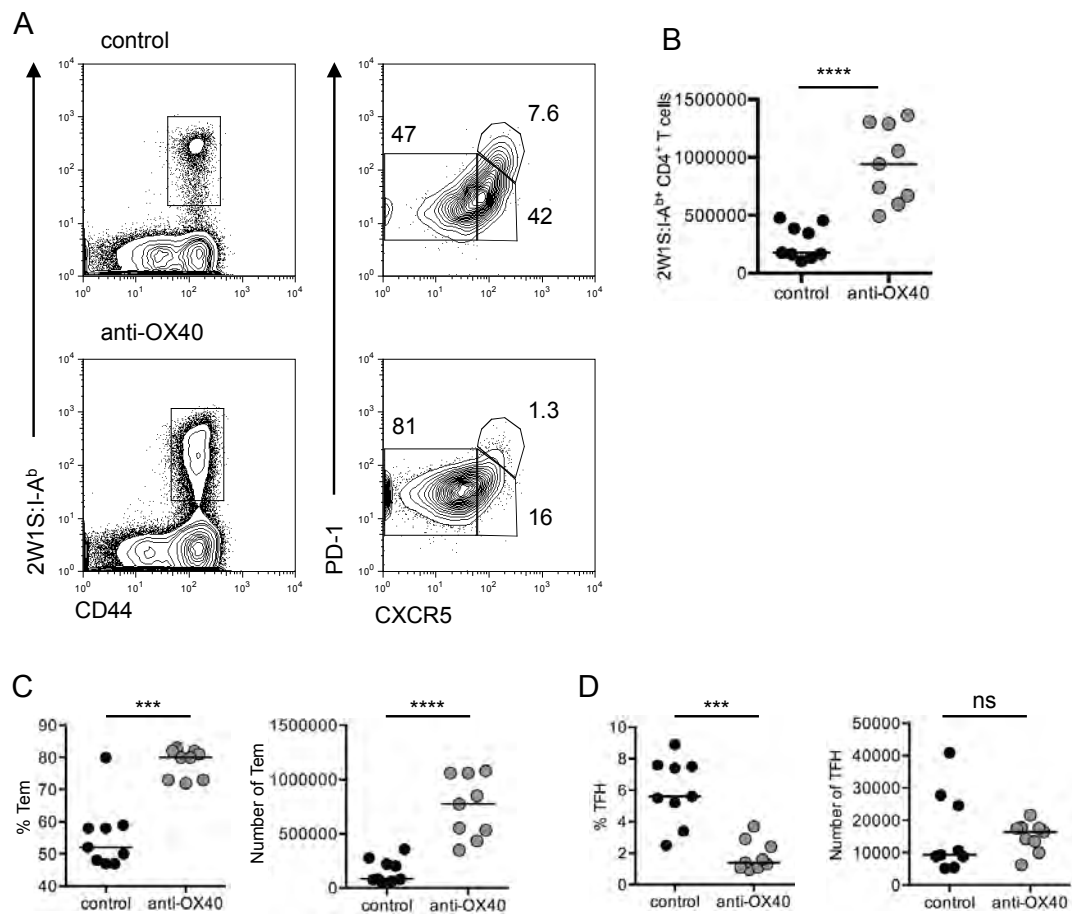


Figure 3.7: Ligation of OX40 upon challenge enhances Tem cell expansion. WT mice were infected with Lm-2W1S and at 28 dpi challenged with Lm-2W1S. Agonistic anti-OX40 or control IgG mAb were administered on the day of secondary challenge. 2W1S:I-A^b CD4⁺ T cells were enumerated from spleen and lymph nodes 3 days later. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ memory T cells. (C) Percentage (left) and number (right) of Tem cells. (D) Percentage (left) and number (right) of TFH cells.

(A) Plots are representative of >5 mice from 2 independent experiments. (B-D) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ***p<0.001, ****p<0.0001 ns= non-significant.

The expression of OX40 in this response is rapid and transient therefore we investigated whether the timing of administration of the mAb had a bearing on the response; an important point to consider when implementing their use therapeutically. WT mice were infected with Lm-2W1S and challenged on day 28 as before, however administration of anti-OX40 or control mAb was delayed until the following day. Despite a modest increase in number in mice receiving anti-OX40 mAb this difference was not significant indicating mAb must be given at the point of secondary challenge for the greatest effect, given the rapid loss of OX40 expression (Figure 3.8 A and B).

Ligation of OX40 upon secondary challenge biased the resulting population towards Tem cells and reduced the proportion of TFH cells. As TFH cells are essential for the maintenance of GC we cut spleen sections from mice post secondary challenge to identify what affect this would have. Sections were stained for Bcl-6 (the transcription factor expressed by GC B cells and TFH cells), CD3 and CD4 and counter-stained with the nuclear marker DAPI. Tile scans were taken of whole spleen sections to accurately assess the number and size of GC. GC were identified as dense clusters of Bcl-6⁺ cells within the B cell follicle which was identified by absence of CD3⁺ and CD4⁺ stained cells. GC were identified in both mice receiving control and anti-OX40 mAb however in the latter there were considerably fewer present and they were reduced in size (Figure 3.9 A). This difference was quantified by drawing around the GC in each spleen section and calculating their area; the total GC area was then divided by area of the spleen section to account for any differences in spleen size.

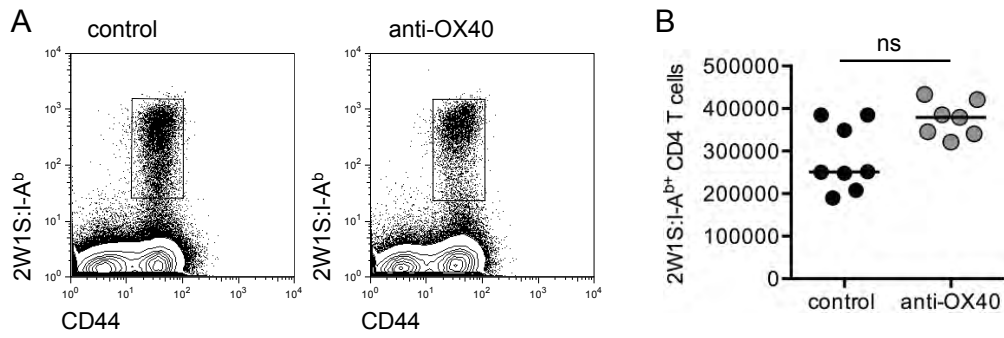


Figure 3.8: Timing of anti-OX40 mAb administration impacts response. WT mice were infected with Lm-2W1S and at 28 dpi challenged with Lm-2W1S. Agonistic anti-OX40 or control IgG mAb were administered 1 day post secondary challenge. 2W1S-specific memory cells were enumerated from spleen and lymph nodes. (A) Flow cytometry plots showing the population of CD44^{hi} 2W1S:I-A^b⁺ CD4⁺ T cells following secondary challenge from control and anti-OX40 treated mice. (B) Number of 2W1S:I-A^b⁺ CD4⁺ memory T cells recovered secondary challenge from control and anti-OX40 treated mice.

(A) Plots are representative of 8 mice from 2 independent experiments. (B) Graphs shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ns= non-significant.

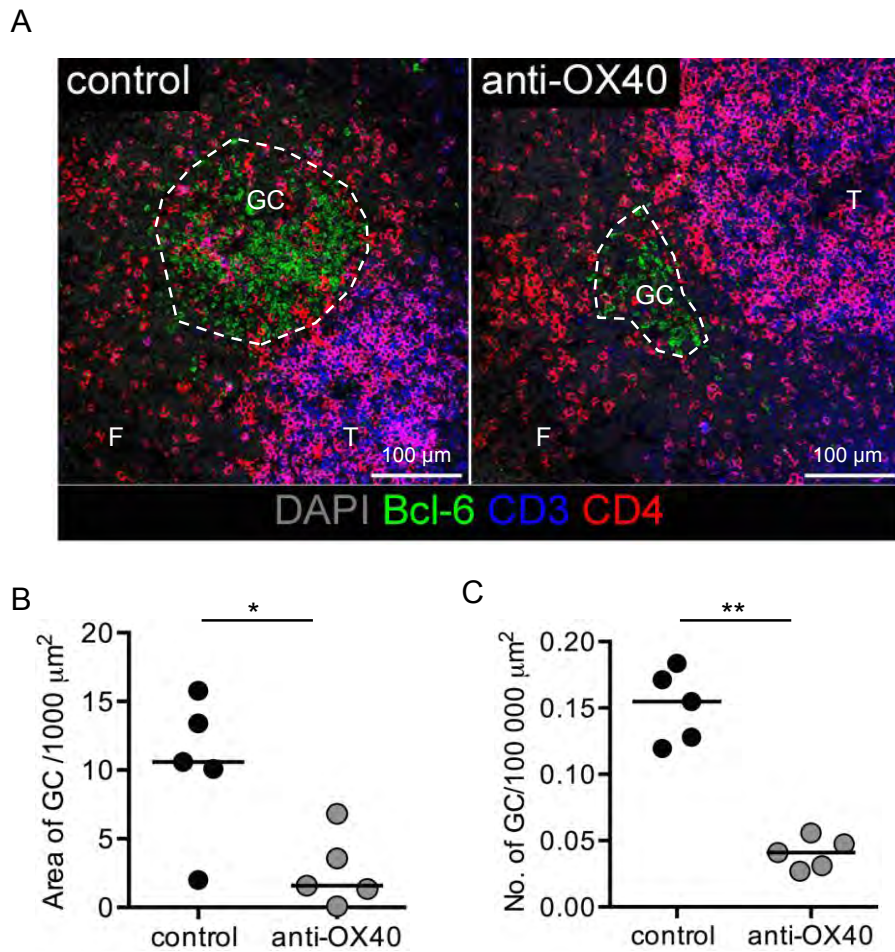


Figure 3.9: Ligation of OX40 upon challenge decreases the area and total number of GC. Mice were infected with Lm-2W1S and at 28 dpi challenged again with Lm-2W1S. Agonistic anti-OX40 or control IgG mAb were administered on the day of secondary challenge. (A) Spleen sections were cut and stained for expression of Bcl-6 (green), CD3 (blue) and CD4 (red) and counterstained with DAPI to identify GC by dense clusters of Bcl-6⁺ cells. “GC” denotes germinal centre outlined by white dashed line; “T” denotes T cell zone and “F” denotes B cell follicle. (B) GC area from control and anti-OX40 treated mice was calculated by drawing around Bcl-6⁺ areas on Zen lite and dividing by total splenic area to adjust for differences in spleen size. (C) Total number of GC per section were counted and divided by total splenic area to adjust for differences in spleen size.

(A) Confocal images are representative of 5 mice from 1 experiment. Magnification 25x. Scale bar represents 100 μ m. (B and C) Graphs show data from 1 independent experiment. Bars show median. Mann-Whitney test: * p <0.05, ** p <0.01.

The total area (Figure 3.9 B) and number (Figure 3.9 C) of GC were both significantly reduced in mice that received anti-OX40 mAb compared to control mice. This effect was seen despite there being a reduction only in the proportion of TFH cells but not in the absolute number (Figure 3.7 D). This may be due to alterations in the fine balance of T cell subsets and spreading of CD4⁺ T cells into the B cell follicle impacting upon the response.

3.2.7 Manipulation of OX40 signalling shapes the primary response to Lm-2W1S

Agonistic anti-OX40 mAb have previously been shown to increase the numbers of TCR transgenic T cells following primary immunisation (Gramaglia et al., 1998, Williams et al., 2007). Studies using OX40^{-/-} mice have shown a defect in the primary expansion of CD4⁺ T cells due to poor survival late in the primary response (Gramaglia et al., 2000, Murata et al., 2000). Some studies have inferred a role for OX40 in the generation of TFH cells however this is from the study of total, not antigen-specific, CXCR5⁺ T helper cells and therefore does not separate effects on T cell survival from TFH cell differentiation (Walker et al., 1999, Fillatreau and Gray, 2003). Since provision of agonistic anti-OX40 mAb at challenge specifically benefitted the CXCR5⁺ Teff cell population, we manipulated OX40 signalling in the primary response to determine which subsets are reliant on this pathway. WT mice were infected with Lm-2W1S and received either agonistic anti-OX40, blocking anti-OX40L or control mAb and were analysed 7

dpi to determine the effect on proliferation of antigen-specific CD4⁺ T cells in a primary response. Data using the Lm-2W1S model was consistent with previous studies; provision of agonistic anti-OX40 mAb 1 dpi resulted in a 5.5-fold increase in the number of 2W1S:I-A^b⁺ CD4⁺ T cells at 7 dpi (Figure 3.10 B; median for control 53 972, anti-OX40 316 688). Ligation of OX40 using anti-OX40 mAb specifically expanded the CXCR5⁺ Teff cell subset as seen in response to secondary challenge (Figure 3.10 A). This increase was observed in both the proportion (Figure 3.10 D) and absolute number (Figure 3.10 E) of Teff cells among the 2W1S:I-A^b⁺ population. Interestingly the TFH cell subset was completely abrogated (Figure 3.10 A), despite reports that OX40 may be important for the generation of TFH cells (Gaspal et al., 2005). Both the proportion and absolute number of TFH cells were significantly decreased following anti-OX40 treatment (Figure 3.10 F and G). Blocking anti-OX40L mAb recapitulated data shown for OX40^{-/-} mice; the number of 2W1S:I-A^b⁺ CD4⁺ T cells was 2-fold lower than mice receiving control mAb (Figure 3.10 C). Blockade of OX40L caused a significant decrease in the number and proportion of Teff cells, consistent with a role for OX40 in survival of CD4⁺ T cells, but there was no significant effect on TFH cells (Figure 3.10 D-G).

T-bet is the key transcription factor that directs lineage commitment to the Th1 response. As we observed a bias towards expansion of Teff cells with ligation of OX40 we analysed intracellular expression of T-bet in mice that received anti-OX40, anti-OX40L or control mAb.

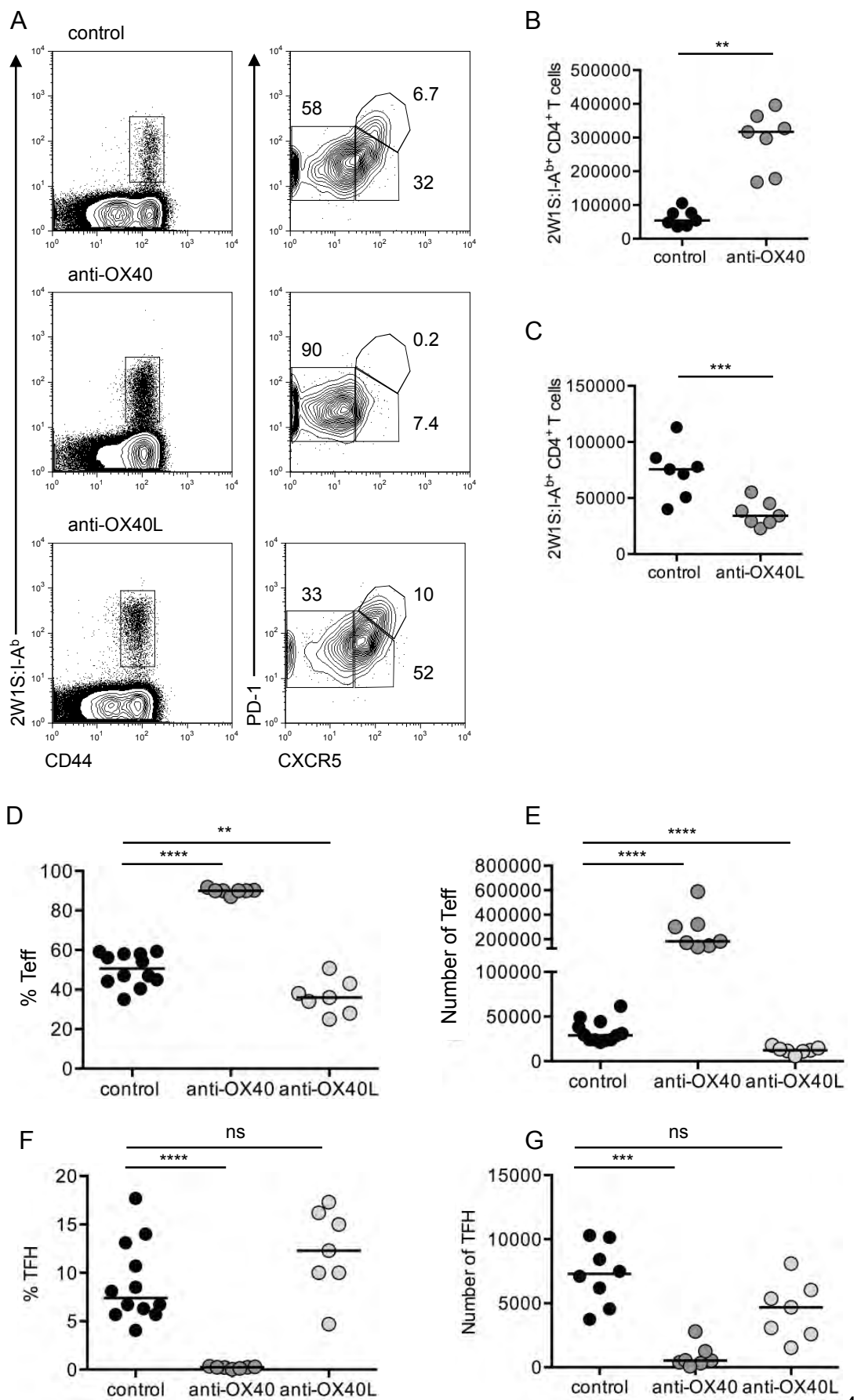


Figure 3.10: Manipulation of signalling through OX40 shapes the 2W1S:I-A^b CD4⁺ T cell response to Lm-2W1S. WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi, or blocking anti-OX40L or control IgG mAb 1 and 3 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells 7 dpi from mice receiving control IgG, agonistic anti-OX40 or blocking anti-OX40L mAb. (B) Number of 2W1S:I-A^b CD4⁺ T cells 7 dpi from mice receiving control IgG or agonistic anti-OX40 mAb. (C) Number of 2W1S:I-A^b CD4⁺ T cells 7 dpi from mice receiving control IgG or blocking anti-OX40L mAb. (D) Percentage of Teff cells among 2W1S:I-A^b CD4⁺ T cells 7 dpi from mice receiving control IgG, agonistic anti-OX40 or blocking anti-OX40L mAb. (E) Number of Teff 2W1S:I-A^b CD4⁺ T cells cells 7 dpi from mice receiving control IgG, agonistic anti-OX40 or blocking anti-OX40L mAb. (F) Percentage of TFH cells among 2W1S:I-A^b CD4⁺ T cells 7 dpi from mice receiving control IgG, agonistic anti-OX40 or blocking anti-OX40L mAb. (G) Number of TFH cells 7 dpi from mice receiving control IgG, agonistic anti-OX40 or blocking anti-OX40L mAb.

(A) Plots are representative of ≥ 7 mice from ≥ 2 independent experiments. (B-G) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns= non-significant.

Within the 2W1S:I-A^b+ CD4⁺ T cell population expression of T-bet was primarily by CXCR5⁺ Teff cells (Figure 3.11 A). Ligation of OX40 resulted in the vast majority (99%) of 2W1S:I-A^b+ CD4⁺ T cells expressing T-bet at 7 dpi, confirming that the CXCR5⁺ Teff population are specifically expanded. Conversely blocking OX40 caused a reduction in the percentage of cells expressing T-bet (Figure 3.11 B).

3.2.8 Intrinsic expression of T-bet is not required for the enhanced expansion of Teff cells in response to OX40 ligation

As we observed an increase in the number of 2W1S:I-A^b+ CD4⁺ T cells expressing T-bet when mice were treated with agonistic anti-OX40 mAb we investigated whether endogenous expression of T-bet was required for expansion of Teff cells responding to OX40. To this end mice with a selective deletion of the gene encoding T-bet in cells that express RAG1 (B and T cells) were utilised. WT and Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP were immunised with Lm-2W1S, given control or anti-OX40 mAb at 1 dpi and analysed at 7 dpi. As seen in WT mice provision of agonistic anti-OX40 mAb in Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice resulted in significantly more 2W1S:I-A^b+ CD4⁺ T cells 7 dpi (Figure 3.12 B), due to the specific expansion of Teff cells (Figure 3.12 A and D). Again this was accompanied by the complete loss of TFH cells (Figure 3.12 A and C).

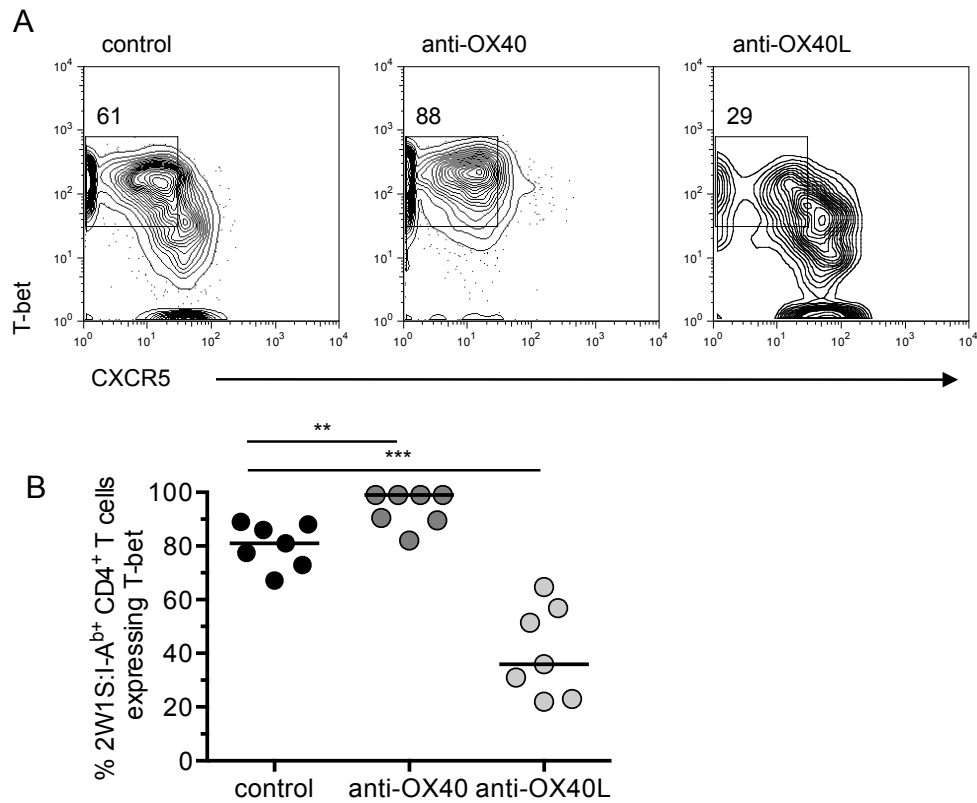


Figure 3.11: Ligation of OX40 increases the proportion of T-bet⁺ 2W1S:I-A^{b+} CD4⁺ T cells. WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi, or blocking anti-OX40L mAb or control IgG 1 and 3 dpi. (A) Flow cytometry plots showing expression of CXCR5 and T-bet on 2W1S:I-A^{b+} CD4⁺ T cells. Values show percentage of Tbet⁺CXCR5⁻ cells. (B) Percentage of 2W1S:I-A^{b+} CD4⁺ T cells expressing T-bet.

(A) Plots are representative of ≥ 6 mice from 2 independent experiments. (B) Graph shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ** $p < 0.01$, *** $p < 0.001$.

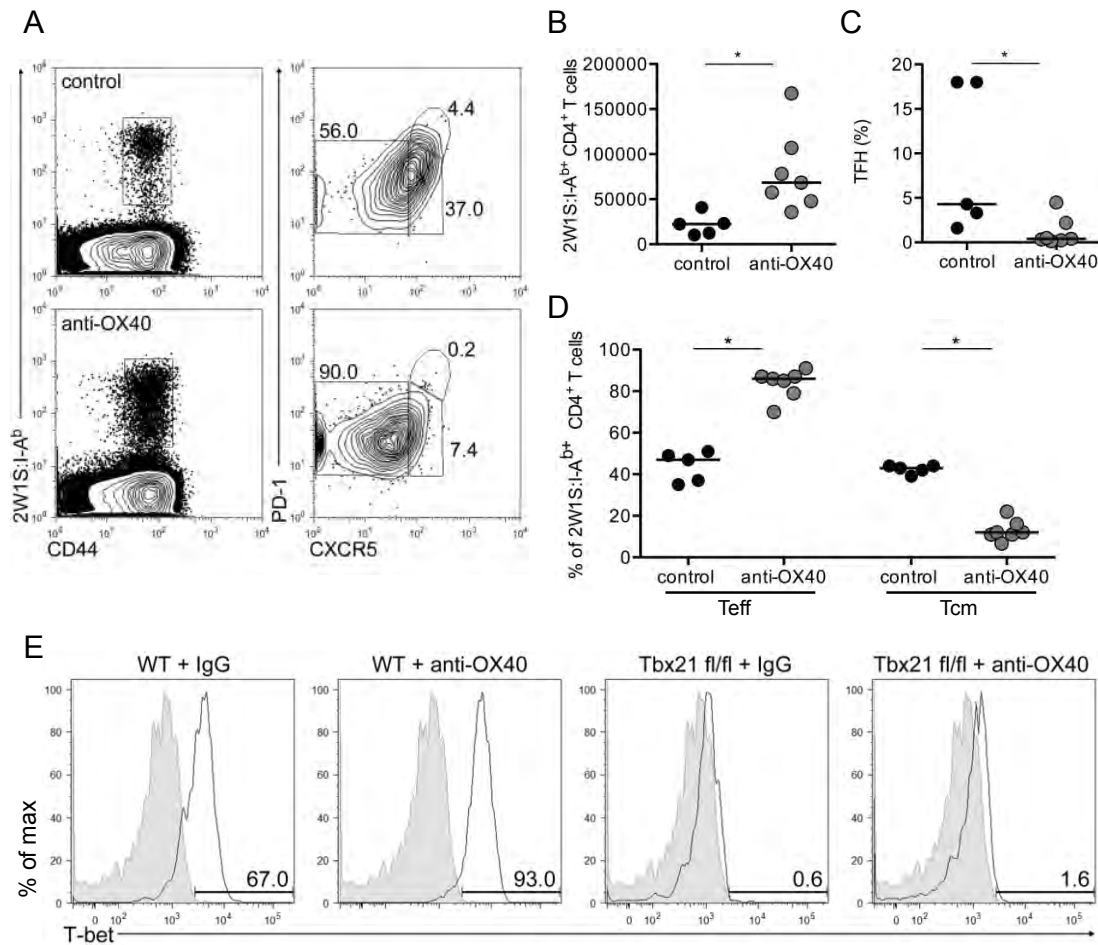


Figure 3.12: Intrinsic expression of T-bet is not required for the enhanced Teff cell response to agonistic anti-OX40 mAb. WT and Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP (Tbx21 fl/fl) mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells from Tbx21 fl/fl mice receiving agonistic anti-OX40 or control IgG mAb. (B) Number of 2W1S:I-A^b CD4⁺ T cells from Tbx21 fl/fl mice receiving agonistic anti-OX40 mAb or control IgG. (C) Percentage of TFH cells among 2W1S:I-A^b CD4⁺ T cells from Tbx21 fl/fl mice receiving agonistic anti-OX40 or control IgG mAb. (D) Percentage of 2W1S:I-A^b CD4⁺ Teff and T central memory precursor cells from mice receiving agonistic anti-OX40 or control IgG mAb. (E) Histograms of expression of T-bet on WT and Tbx21 fl/fl mice receiving agonistic anti-OX40 or control IgG mAb. Values on plots are percentages of Tbet⁺ cells.

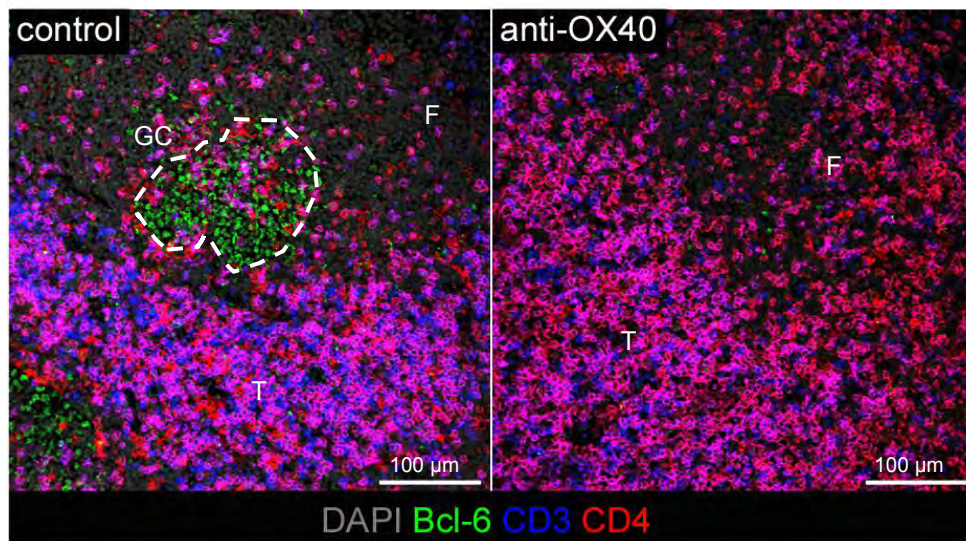
(A and E) Plots are representative of ≥ 5 mice from 2 independent experiments. (B-D) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: *p $\leq$ 0.05

Staining for expression of T-bet confirmed its absence in Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice and that ligation of OX40 did not induce T-bet expression (Figure 3.12 E), demonstrating that the effects of agonistic anti-OX40 mAb on T cell phenotype do not require T-bet expression. This finding is consistent with experiments with non-antigen specific CD4⁺ T cells where T-bet was not required for proliferation and acquisition of effector function in response to OX40 costimulation (Williams et al., 2007).

3.2.9 Agonistic anti-OX40 mAb impairs GC formation

TFH cells are essential for the maintenance of GC; although they can form spontaneously in the absence of T cell help they are quickly lost (de Vinuesa et al., 2000). To assess the effect of a complete loss of TFH cells following treatment with agonistic anti-OX40 mAb spleen sections were taken at 7 dpi and stained for Bcl-6. GC B cells can be identified by nuclear expression of Bcl-6 and TFH cells by co-expression of Bcl-6, CD3 and CD4. There was a complete absence of GC structures in mice that received agonistic anti-OX40 mAb and CD4⁺ T cells had spread from the T cell zone into the B cell follicle (Figure 3.13 A). Class switching, the process of rearrangement to change one antibody isotype to another, occurs in the GC (Liu et al., 1996). Infection with Lm-2W1S generates a Th1 response, conventionally marked by switching to the IgG2a antibody isotype (Toellner et al., 1998). Spleen sections were analysed for expression of IgG2a to determine if antibody switching had taken place.

A



B

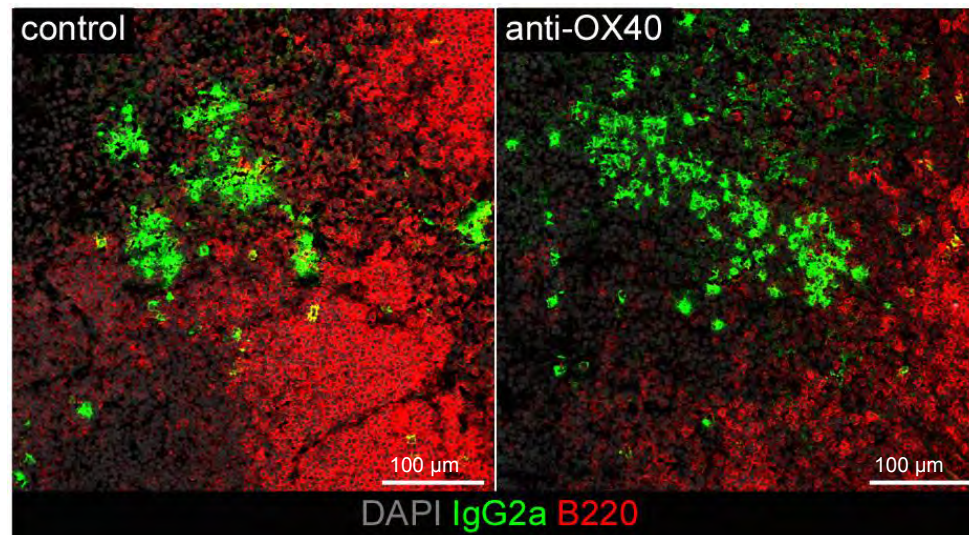


Figure 3.13: GC are absent in mice treated with agonistic anti-OX40 mAb. WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi. (A) Spleen sections were cut and stained for expression of Bcl-6 (green), CD3 (blue) and CD4 (red) and counterstained with DAPI to identify GC by dense clusters of Bcl-6⁺ cells. Scale bar represents 100 μ m. “GC” denotes germinal centre outlined by white dashed line; “T” denotes T cell zone and “F” denotes B cell follicle. (B) Spleen sections were cut and stained for expression of IgG2a (green) and B220 (red) and counterstained with DAPI.

Confocal images are representative of 5 mice from 1 experiment (A) or 2 mice (B). Magnification 25x, scale bar represents 100 μ m.

IgG2a was found to be present in both control and anti-OX40 treated mice indicating that some antibody switching had occurred in the absence of the GC (Figure 3.13 B). However, this is consistent with previous data indicating that GC are not required for initial antibody switching but are necessary for generation of high affinity antibody (MacLennan et al., 2003).

3.2.10 OX40 is not required for the generation of 2W1S:I-A^b+ CD4⁺ TFH cells

OX40 has previously been implicated in the generation of TFH cells however these studies looked at total CXCR5⁺ T helper cells, not antigen-specific cells, which makes it difficult to dissect impacts on survival from TFH cell differentiation (Fillatreau and Gray, 2003, Walker et al., 1999). Previously it has been shown that GC cannot be maintained in CD30^{-/-}OX40^{-/-} mice (Gaspal et al., 2005). This was attributed to a deficient T cell population in the B cell follicle and therefore a lack of cognate help for B cells. On 2W1S:I-A^b+ CD4⁺ T cells OX40 is expressed at 3 dpi, a time when TFH cells could not yet be identified due to a lack of Bcl-6 and PD-1 expression. To further investigate whether OX40 signals were required for the formation of TFH cells in the response to Lm-2W1S, mice deficient in both OX40 and CD30 were used, since there may be redundancy in these signalling pathways (Gaspal et al., 2005). WT and CD30^{-/-}/OX40^{-/-} mice were immunised with Lm-2W1S and the number and phenotype of 2W1S:I-A^b+ CD4⁺T cells were analysed at 7 dpi. There was an overall decrease in the number

of 2W1S:I-A^b CD4⁺ T cells (Figure 3.14 B) resulting from a significantly reduced number and proportion of T_{eff} cells (Figure 3.14 C and D). This finding is consistent with decreased survival of effector cells, however the generation of T_{FH} cells was not impaired (Figure 3.14 A, E and F) indicating that the generation of these cells did not require OX40 or CD30 signals.

3.2.11 Agonistic anti-OX40 mAb does not deplete OX40⁺ cells

The agonistic anti-OX40 mAb has been used extensively in previous studies and clinical trials to augment CD4⁺ T cell responses (Weinberg et al., 2000, Curti et al., 2013). However, some mAb can target cells for deletion through antibody dependent cell mediated cytotoxicity. To test if the effects we saw with mAb treatment were due to depletion of OX40⁺ cells WT mice were infected with Lm-2W1S and given either anti-OX40 mAb or control 1 dpi. We then analysed the total number of Foxp3⁺ CD4⁺ Treg cells and 2W1S:I-A^b CD4⁺ Treg cells in the spleen, as it is known that the majority of these cells constitutively express OX40 (Takeda et al., 2004). Treg cells were identified by intracellular expression of the lineage defining transcription factor Foxp3 and analysed for expression of OX40 (Figure 3.15 A). Over 70 % of both total and 2W1S:I-A^b CD4⁺ Treg cells expressed OX40 (Figure 3.15 B). The 2W1S:I-A^b CD4⁺ T cell pool consists of around 10 % Treg cells, although it has previously been shown that infection with Lm-2W1S poorly expands this Foxp3⁺ population (Ertelt et al., 2009).

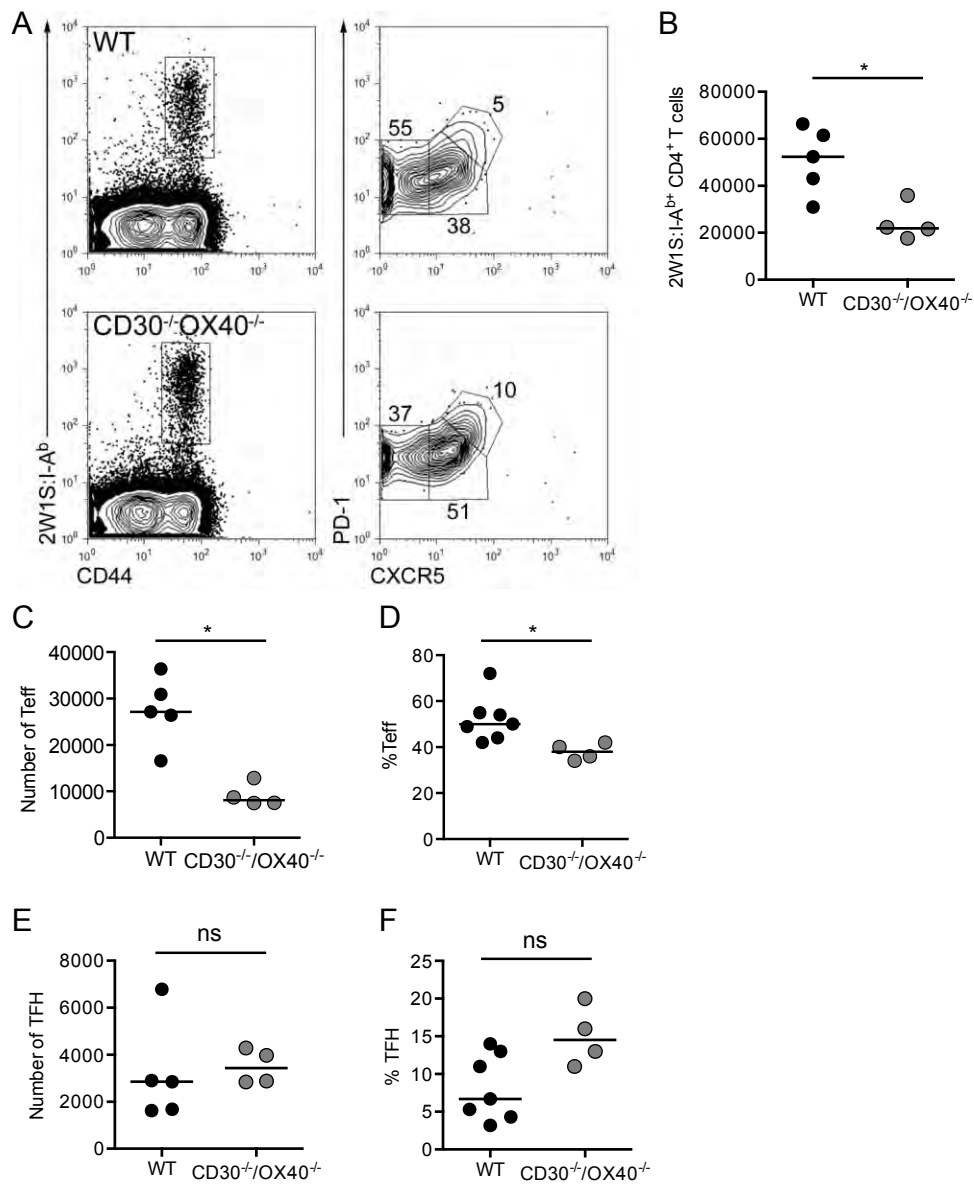


Figure 3.14: OX40 signals are not required for the generation of 2W1S:I-A^b CD4⁺ TFH cells. WT and CD30^{-/-}/OX40^{-/-} mice were infected with Lm-2W1S and taken 7 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ T cells. (C) Number of 2W1S:I-A^b CD4⁺ Teff cells. (D) Percentage of Teff cells among 2W1S:I-A^b CD4⁺ T cells. (E) Number of 2W1S:I-A^b CD4⁺ TFH cells. (F) Percentage of TFH cells among 2W1S:I-A^b CD4⁺ T cells.

(A) Plots are representative of 5 mice from 2 independent experiments. (B-F) Graphs show pooled data from 2 independent experiments, representative of 3 independent experiments. Bars show median. Mann-Whitney test: *p≤0.05, ns= non-significant.

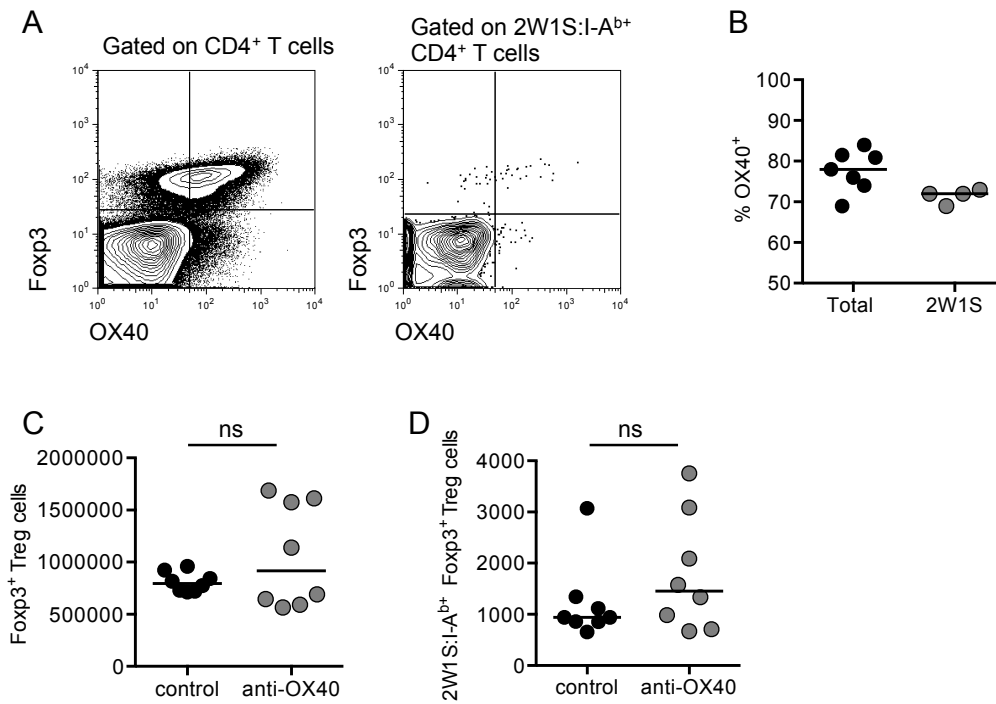


Figure 3.15: Agonistic anti-OX40 mAb do not deplete OX40⁺ cells. WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi. Spleen cells were fixed and stained for intracellular Foxp3 to identify Treg cells. (A) Flow cytometry plots of expression of Foxp3 and OX40 on $CD4^+$ T cells (left) and $2W1S:I-A^b$ $CD4^+$ T cells (right). (B) Percentage of total and $2W1S:I-A^b$ $CD4^+$ Treg expressing OX40. (C) Number of Foxp3⁺ $CD4^+$ Treg cells from mice receiving agonistic anti-OX40 or control IgG mAb. (D) Number of Foxp3⁺ $2W1S:I-A^b$ $CD4^+$ Treg cells from mice receiving agonistic anti-OX40 or control IgG mAb.

(A) Plots are representative of 7 mice from 2 independent experiments. (B-D) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ns= non-significant.

A very small population of these cells expressing OX40 are present following infection with Lm-2W1S (Figure 3.15 A). The number of both total CD4⁺ Treg cells and 2W1S:I-A^b⁺ Treg cells were not significantly different between control and treated mice (Figure 3.15 C and D). This indicates that anti-OX40 mAb is not acting upon OX40⁺ cells to cause their depletion.

3.2.12 The primary response to anti-OX40 mAb depends upon timing of antibody administration

Expression of OX40 on 2W1S:I-A^b⁺ CD4⁺ T cells was restricted to a brief window of time. However as a proportion of Treg cells constitutively express OX40 it remained possible that anti-OX40 mAb indirectly affected the 2W1S response by affecting 2W1S:I-A^b⁺ Treg cells. Therefore we administered anti-OX40 mAb 4 dpi, after 2W1S:I-A^b⁺ CD4⁺ T cells have lost expression of OX40, to assess whether the effects we previously observed are lost, thereby supporting a direct role for anti-OX40 mAb. Mice receiving anti-OX40 mAb 4 dpi showed a similar phenotype of CXCR5 and PD-1 expression to control mice (Figure 3.16 A). The number of 2W1S:I-A^b⁺ CD4⁺ T cells recovered was significantly increased however this difference was less than 2-fold, so was not as dramatic as when mAb was administered 1 dpi (Figure 3.16 B; median for control 46 097, anti-OX40 59 423). Both the number and proportion of Teff and TFH cells were not significantly different in anti-OX40 treated mice compared to controls indicating the mAb is not effective when administered 4 dpi (Figure 3.16 C-F).

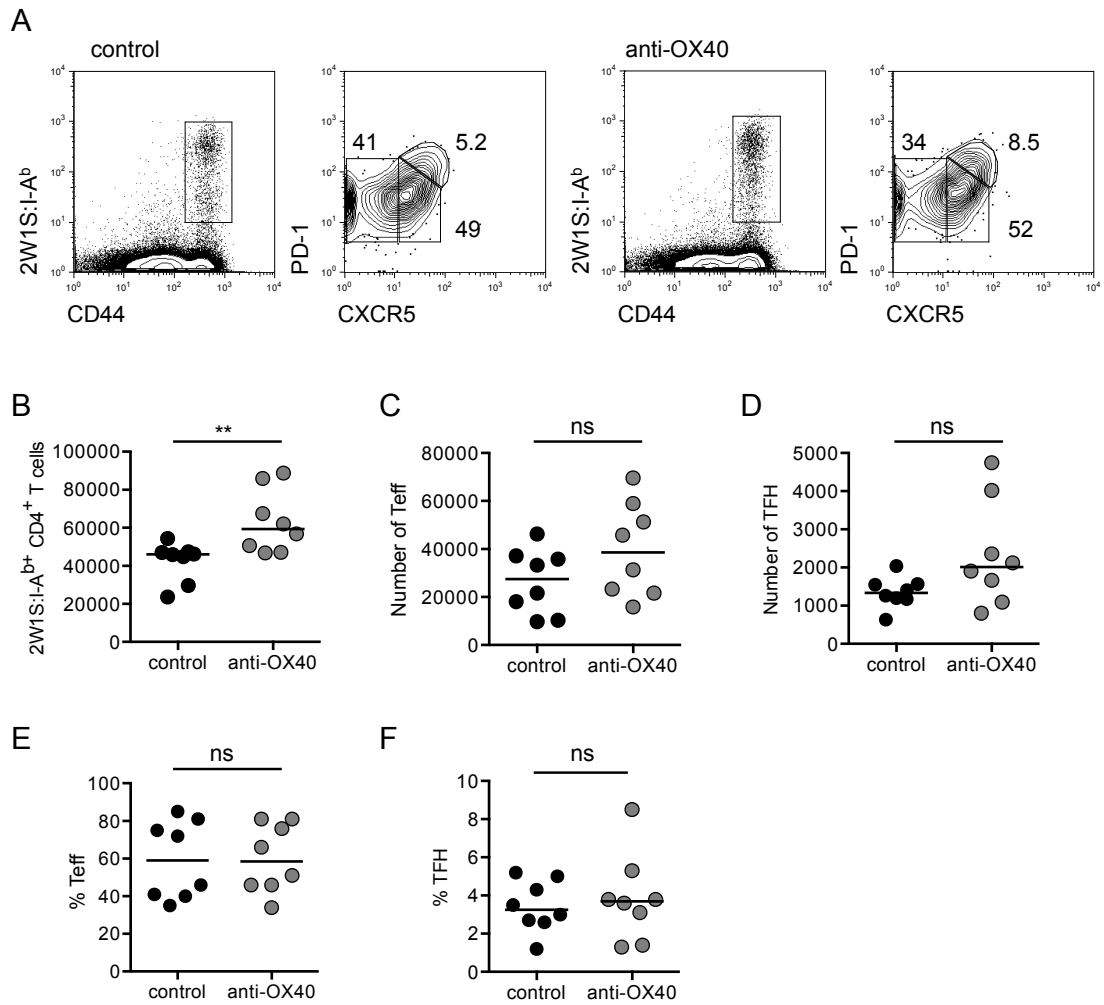


Figure 3.16: OX40 ligation only affects Teff cell expansion and phenotype when administered during the early stages of the primary response. WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 4 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ T cells. (C) Number of 2W1S:I-A^b CD4⁺ Teff cells. (D) Number of 2W1S:I-A^b CD4⁺ TFH cells. (E) Percentage of Teff cells among 2W1S:I-A^b CD4⁺ T cells. (F) Percentage of TFH cells among 2W1S:I-A^b CD4⁺ T cells.

(A) Plots are representative of 8 mice from 2 independent experiments. (B-F) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ns= non-significant

3.2.13 Ligation of OX40 post infection biases the memory cell pool towards Tem cells

As ligation of OX40 post infection had specific effects on the effector cell population we wanted to investigate if there would be a similar impact upon the resulting memory cell population. In the primary response, anti-OX40 mAb caused the vast expansion of Teff cells and the abrogation of TFH cells (Figure 3.10). Previous studies have found that increased numbers of effector cells following priming resulted in an enlarged memory cell population (Evans et al., 2001, Gramaglia et al., 2000) and this attribute may be useful for exploiting in vaccine development. To investigate this idea WT mice were immunised with Lm-2W1S as before and treated with control or agonistic anti-OX40 mAb at 1 dpi. Mice were left for 28 days to form 2W1S:I-A^{b+} CD4⁺ memory T cells and were then restimulated *in vivo* with 2W1S peptide and LPS before being sacrificed 4 hours later. This method has been shown to rapidly and synchronously stimulate 2W1S:I-A^{b+} CD4⁺ T cells to produce cytokines (Pepper et al., 2011). 2W1S:I-A^{b+} CD4⁺ memory T cells were then enriched through columns and analysed for secretion of cytokines. Tem and Tcm cell populations can then be functionally defined by their production of IFN- γ and IL-2 or IL-2 only respectively, as infection with Lm-2W1S elicits a Th1 biased response (Pepper et al., 2011). Both of these populations could be identified by cytokine secretion following restimulation with 2W1S peptide (Figure 3.17 A). The size of the 2W1S:I-A^{b+} CD4⁺ Tcm cell population was around 4 fold larger than the Tem cell population (Figure 3.17 B; median for Tcm 563, Tem 125).

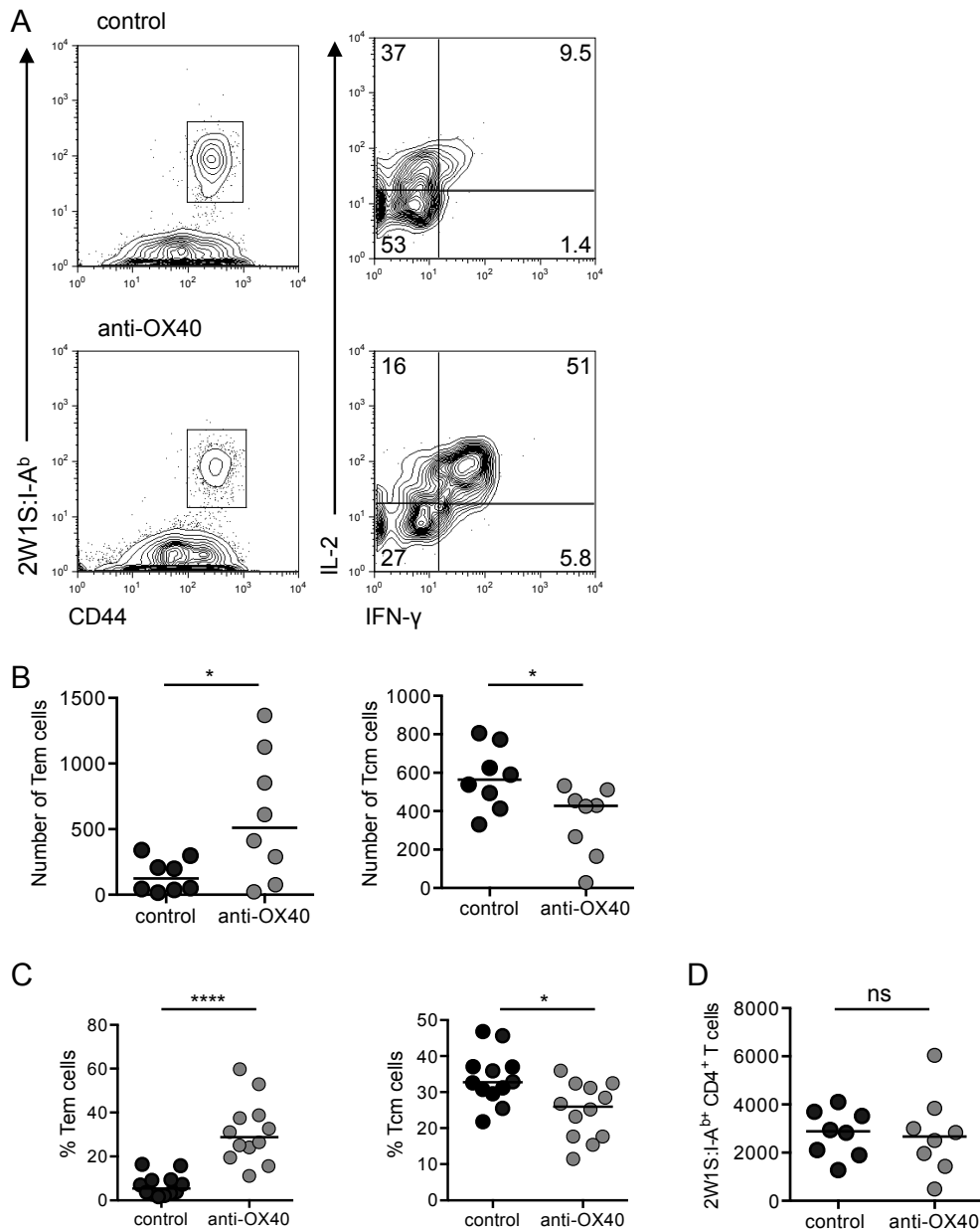


Figure 3.17: Ligation of OX40 post infection alters the phenotype, but does not increase the size of the memory cell pool. WT mice were infected with Lm-2W1S and restimulated 28 dpi *in vivo* with 2W1S peptide and LPS. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi. Spleen and lymph nodes were harvested 4 hours post restimulation for analysis. (A) Flow cytometry plots showing expression of IL-2 and IFN- γ on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ T cells that are Tem (IL-2⁺ IFN- γ ⁻ left) and Tcm (IL-2⁺ IFN- γ ⁺ right) cells. (C) Percentage of 2W1S:I-A^b CD4⁺ T cells that are Tem (IL-2⁺ IFN- γ ⁻ left) and Tcm (IL-2⁺ IFN- γ ⁺ right) cells. (D) Total number of 2W1S:I-A^b CD4⁺ T cells.

(A) Plots are representative of 12 mice from 3 independent experiments. (B and D) Graphs show pooled data from 2 independent experiments. Bars show median. (C) Graph shows pooled data from 3 independent experiments. Bars show median. Mann-Whitney test: * $p \leq 0.05$, **** $p \leq 0.0001$, ns = non-significant.

Treatment with agonistic anti-OX40 mAb resulted in a significant increase in the number of Tem cells and a corresponding decrease in Tcm cells (Figure 3.17 B). This difference was more pronounced when looking at the proportion of cells that were Tem or Tcm (Figure 3.17 C). Although the memory cell population had been skewed towards cells with an effector memory phenotype there was not an overall increase in the total number of memory cells, in contrast to previous findings (Figure 3.17 D) (Weinberg et al., 1998, Maxwell et al., 2000, Gramaglia et al., 2000). The 2W1S:I-A^b CD4⁺ T cell population must therefore have undergone substantial contraction given the large expansion of cells in the primary response.

3.2.14 Expression of OX40 depends upon the route of immunisation

Lm-2W1S is an attenuated strain of intracellular bacteria and is therefore cleared from the mouse by innate and adaptive immune mechanisms in a short period of time. Infection with bacteria elicits a Th1 response characterised by CD4⁺ T helper cells which produce IFN- γ and IL-2 which consequently activate macrophages that initiate cell mediated and phagocyte dependent responses. However Th2 responses to gastrointestinal nematodes are characterised by cells producing IL-4, IL-5 and IL-13 and robust antibody production (Mosmann et al., 1986). The expression of OX40 in response to Lm-2W1S is rapid and transient therefore we investigated if the response to ligation of OX40 would be the same

when the route of infection was varied. Aluminium precipitation of antigen is a common method used to elicit a robust Th2 biased response (Grun and Maurer, 1989, Brewer et al., 1999), yet the intricacies of antigen delivery in this system are not fully understood. It has been suggested that aluminium precipitation provides a depot from which antigen is released in a controlled manner (Glenny et al., 1931), however this mode of action has been questioned by more recent work (Hutchison et al., 2012). Studies do show that aluminium precipitation elicits a prolonged, robust response compared to peptide alone (Glenny et al., 1931, Ghimire et al., 2012). We used this method as a comparison to the acute bacterial infection elicited by Lm-2W1S. WT mice were immunised i.p. with 100 µg 2W1S peptide precipitated in aluminium sulphate and taken 7 dpi. In contrast to infection with Lm-2W1S, expression of OX40 was not detected on 2W1S:I-A^b CD4⁺ T cells until day 4 and was maintained at day 9 post immunisation (Figure 3.18 A). Unlike following infection with Lm-2W1S, OX40 was more broadly expressed on T cells subsets with Teff, Tcm precursors and TFH cells all showing some expression at 9 dpi (Figure 3.18 B).

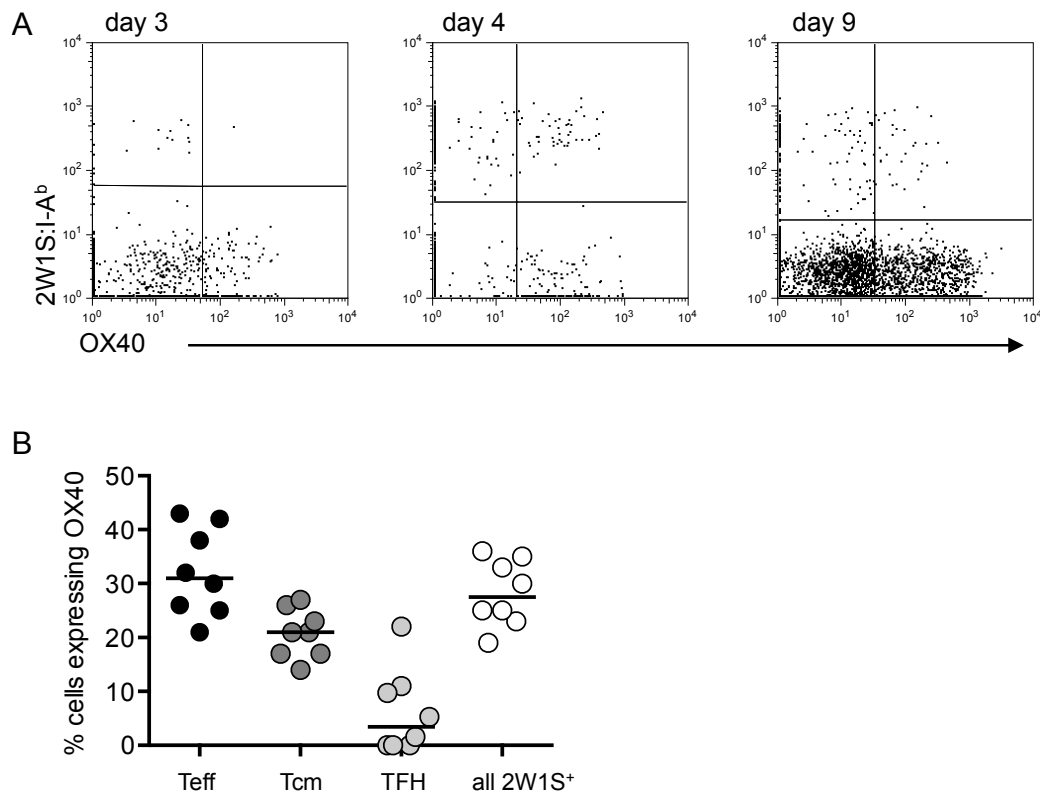


Figure 3.18: OX40 expression is dependent on the route of immunisation. WT mice were immunised with 100 µg alum ppt 2W1S peptide i.p. and analysed at 3, 4 and 9 dpi. (A) Flow cytometry plots showing expression of OX40 on 2W1S:I-A^b CD4⁺ T cells. (B) Percentage of Teff, Tcm, TFH and total 2W1S:I-A^b CD4⁺ T cells expressing OX40 at 9 dpi.

(A) Plots are representative of 3 (day 3) or 4 (day 4) mice from 1 experiment or 7 mice (day 9) from 2 independent experiments. (B) Graph shows pooled data from 2 independent experiments. Bars show median.

To investigate the effect of ligating OX40 in this system WT mice were immunised with alum ppt 2W1S and given anti-OX40 or control IgG mAb 1 dpi and analysed at 9 dpi as the kinetics of this response are slower. Despite this, and different timing of OX40 expression ligation of OX40 still caused a significant increase in the number of 2W1S:I-Ab⁺ CD4⁺ T cells (Figure 3.19 B). The same effect was seen as in the Lm-2W1S response whereby Teff cells were increased in proportion and number (Figure 3.19 A and C). However although TFH cells were significantly reduced in proportion their number was not altered (Figure 3.19 D). This could be due to the broader expression of OX40 affecting the expansion of other cell types apart from Teff cells.

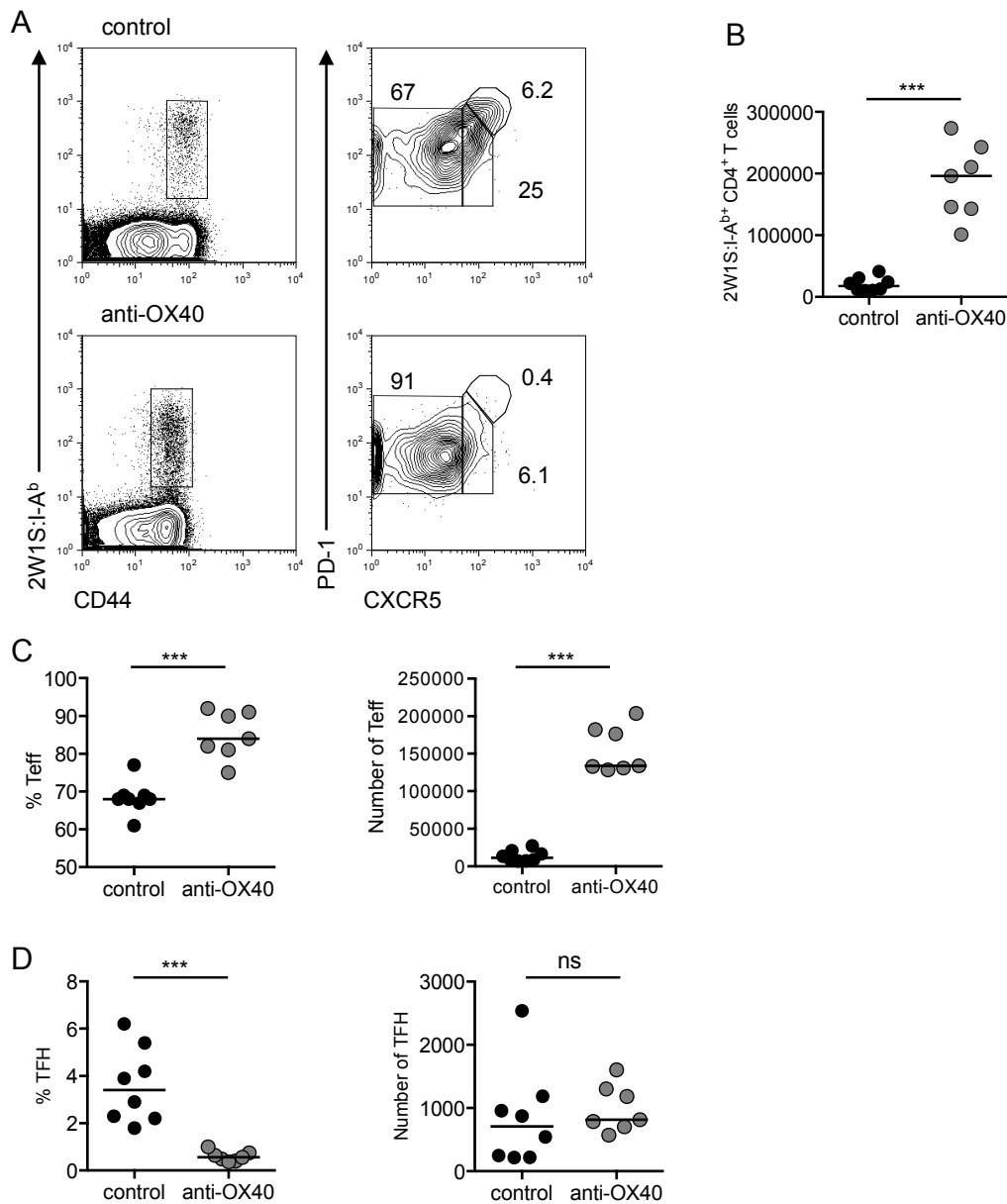


Figure 3.19: Anti-OX40 still causes Teff cell expansion with alum ppt peptide immunisation. WT mice were immunised with 100 µg alum ppt 2W1S peptide i.p and taken 9 dpi. Mice received either agonistic anti-OX40 or control IgG mAb 1 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ T cells. (C) Percentage (left) and number (right) of Teff cells among 2W1S:I-A^b CD4⁺ T cells. (D) Percentage (left) and number (right) of TFH cells among 2W1S:I-A^b CD4⁺ T cells.

(A) Plots are representative of 7 mice from 2 independent experiments. (B-D) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ***p≤0.001, ns= non-significant.

3.3 Discussion

Using a model to track an endogenous population of antigen-specific CD4⁺ T cells I have shown that signals through OX40 specifically benefit the Teff cell population. Following infection with Lm-2W1S OX40 expression is transient and expressed primarily on cells with the Teff phenotype. Ligation of OX40 at this point specifically expands the Teff cell population and biases the memory cell pool towards Tem cells, while TFH cell and GC formation is abrogated. Upon secondary challenge OX40 is more widely expressed, however ligation of OX40 again specifically expanded Tem cells. Once formed, OX40 was dispensable for the long-term persistence of the memory cell population. Despite reports that OX40 may be required for the optimal formation of TFH cells (Gaspal et al., 2005, Walker et al., 1999, Fillatreau and Gray, 2003), CD30^{-/-}/OX40^{-/-} mice did not show any impairment in this population.

Although the use of TCR transgenic T cells has greatly enhanced our understanding of CD4⁺ T cell responses and enabled the study of antigen-specific memory cells, there is a now need for more refined physiological methods. Problems associated with the injection of large numbers of TCR transgenic T cells have recently come to light, particularly with regard to the study of memory T cell populations. Clonal competition, poor conversion to memory cells and unphysiological differentiation have been observed meaning there is now a requirement to study endogenous CD4⁺ T cell populations. Previously this has been challenging due to low precursor frequency, however the refinement of MHC II tetramers has now made this approach feasible. Infection with Lm-2W1S

can be used as a model to track an endogenous, polyclonal antigen-specific population of CD4⁺ T cells through from the primary to the memory response. This method was highly reproducible; a small naive population could be detected that rapidly expanded following i.v. infection, then contracted to form a stable memory cell pool. Restimulation with 2W1S peptide and LPS enabled the identification of Tcm and Tem cell subsets defined functionally by the production of cytokines, as has been shown previously (Pepper et al., 2011).

Expression of OX40 on 2W1S:I-A^b CD4⁺ T cells was not unlike that previously reported from *in vitro* experiments and for TCR transgenic T cells, being very tightly linked to antigen exposure (Gramaglia et al., 1998, Rogers et al., 2001, Dawicki et al., 2004). OX40 was detected early at 3 dpi but was rapidly lost and was absent by day 7, around the peak of this response. The brief expression of OX40 on 2W1S:I-A^b CD4⁺ T cells was likely due to the acute nature of the infection, with attenuated bacteria being rapidly cleared. Chronic infections such as lymphocytic choriomeningitis virus (LCMV) can maintain OX40 expression for up to 20 days (Boettler et al., 2012). Injection with alum ppt 2W1S peptide resulted in the expression of OX40 on 2W1S:I-A^b CD4⁺ T cells at 9 dpi. Use of aluminium precipitation as an adjuvant has been shown to elicit prolonged immune responses (Kool et al., 2008) and therefore the kinetics of OX40 expression may reflect a chronic infection rather than attenuated Lm-2W1S. This demonstrates that the expression of OX40 is very context dependent and should be considered when trying to target this receptor therapeutically.

At the only timepoint when OX40 was detected in the primary response, around 40% of 2W1S:I-A^b CD4⁺ T cells expressed the costimulatory receptor. This observation was notably distinct to previous studies with TCR transgenic T cells where OX40 expression was uniform among the population (Boettler et al., 2013, Boettler et al., 2012, Rogers et al., 2001). This is perhaps due to the polyclonal nature of the endogenous 2W1S:I-A^b population compared to TCR transgenic T cells, which are monoclonal. T cells with higher affinity TCR may receive additional signals due to an increased interaction time with APC that may affect the expression of costimulatory receptors downstream. High levels of TCR stimulation were shown to be important for expression of OX40 (Verdeil et al., 2006), therefore cells with low affinity TCR that do not receive sufficient signalling will not express OX40. However TCR transgenic T cell populations are monoclonal and therefore will receive identical signalling through the TCR resulting in uniform downstream responses such as expression of OX40. Such observations reinforce the need to study endogenous, polyclonal T cell populations.

Heterogeneous expression of OX40 suggested cells might receive different signals, dependent upon expression of costimulatory receptors, and differentiate into distinct T cells subsets. Common markers of Th1 and TFH cells such as T-bet, CXCR5 and Bcl-6 were not useful in identifying these populations at the early timepoint when OX40 was present as they were either expressed on the majority of cells or not present at this time. However, co-expression of OX40 with CD25, a marker of Th1 effector cells (Pepper et al., 2011), indicated that OX40 may be

important specifically for Teff cells and not Tcm precursors or TFH cells.

Consistent with this finding, ligation of OX40 post Lm-2W1S infection using agonistic anti-OX40 mAb specifically expanded CXCR5⁺ Teff cells but abrogated TFH cell generation. This resulted in a complete lack of GC structures in the spleen of treated mice. A recent study on LCMV found a similar effect; injection of anti-OX40 abrogated development of LCMV-specific TFH cells. This was attributed to expression of the transcriptional repressor Blimp-1 in the majority of effector cells, driven by enhanced IL-2 production, which subsequently antagonises Bcl-6 and therefore prevented TFH cell development (Boettler et al., 2013). This resulted in a significant decrease in antibody titre due to impaired GC responses. This effect was observed in the response to both acute and chronic infections. However in the response to Lm-2W1S, cells expressing OX40 were already committed to the Teff phenotype at an early stage, evidenced by co-expression with CD25 3 dpi, indicating these cells benefited from antibody ligation of OX40 at this time. It is possible that OX40 promotes the proliferation and survival of the 2W1S:I-A^b⁺ CD4⁺ Teff cell population rather than impairing TFH cell formation and their loss is due to out-competition in this niche.

Alternatively extensive proliferation of Teff cells may lead to high levels of IFN- γ , as seen in CD70 transgenic mice, which may deplete TFH cells (Arens et al., 2001). Constitutive expression of CD27 on B cells led to an increase in peripheral effector T cells and progressive reduction in B cell number due to IFN- γ . Another study also found that when anti-OX40 and blocking anti-PD-L1 mAb were administered simultaneously during *P.yoelii* Malaria infection, excessive production of IFN- γ by Teff cells resulted in a reduction in TFH cells that led to

increased parasite burden (Zander et al., 2015). Therefore although IFN- γ is required to control parasitaemia, in excess it acts to disrupt TFH cell differentiation or survival leading to impaired GC responses. Agonistic anti-OX40 mAb have already been trialled to enhance immune responses to tumour models in phase 1 clinical trials and are being considered to enhance vaccine responses (Croft, 2010, Weinberg et al., 2011). These data suggest caution should be employed when considering the use of anti-OX40 mAb in the clinic, especially when a robust humoral response is desired.

We observed that ligation of OX40 post infection caused the majority of 2W1S:I-Ab⁺ CD4⁺ T cells to express T-bet, the key transcription factor regulating Th1 cell differentiation. Mice where T-bet was selectively removed from cells expressing RAG were used to investigate if the selective expansion of effector cells and loss of TFH cells was dependent on expression of this transcription factor.

Administration of agonistic anti-OX40 mAb in these mice had the same effect as in WT mice indicating this mechanism is not dependent on intrinsic expression of T-bet, consistent with a previous study showing T-bet is not required for effector cell cytokine production in response to OX40 (Williams et al., 2007). In this study signalling through the IL-2 receptor was required for T cells to produce IFN- γ in response to antigen and OX40 costimulation, however expression of T-bet and IL-12 receptor were dispensable.

Studies have previously implicated OX40 in the generation and maintenance of TFH cells and GC. In CD30^{-/-}/OX40^{-/-} mice secondary antibody responses were impaired due to a lack of T helper cells in the follicle and GC were not maintained

at 14 dpi (Gaspal et al., 2005). Compromised OX40 function has also been shown to impair development of CXCR5⁺ CD4⁺ T cells and their migration into the B cell follicle (Walker et al., 1999, Fillatreau and Gray, 2003). Here we found that generation of TFH cells 7 days post infection with Lm-2W1S in CD30^{-/-}/ OX40^{-/-} mice was normal as has been seen in another antigen-specific study (Akiba et al., 2005). Furthermore, blockade of OX40 signalling using mAb resulted in a decreased Teff population but did not affect the generation of TFH cells. This does not rule out a role for OX40 in the maintenance of TFH cells however in this model TFH cells lack OX40 expression at 7 dpi, therefore do not interact with OX40L at the time when they are located in the GC. There was however a 2 fold reduction in the number of Teff cells, consistent with previous reports showing a role for OX40 in survival of CD4⁺ T cells (Rogers et al., 2001). Ligation of OX40 did not enhance the number of TFH cells, but conversely abrogated their development. Previous studies have shown that OX40 is important for control of LCMV and generation of high affinity antibody (Boettler et al., 2012, Gaspal et al., 2005). This requirement may depend on the context in which the antigen is delivered, as LCMV can be chronic this may induce prolonged expression of OX40 on a wider range of T cell subsets. Other studies have shown normal GC centre development, antibody production and control of infection with *L. major*, *N. brasiliensis* and murine encephalomyelitis virus in the absence of OX40 signalling (Kopf et al., 1999, Chen et al., 1999, Pippig et al., 1999). Therefore it is likely that the role of OX40 in TFH cell generation and survival is highly dependent upon the context in which antigen is delivered, which dictates expression of OX40 and OX40L.

Expression of OX40 was absent on 2W1S:I-A^b+ CD4⁺ T memory cells directly *ex vivo* as has been reported for other memory cell populations (Croft, 2010). Addition of IL-7 in culture has been used to induce expression of OX40 on memory cells (Gaspal et al., 2005) and was successful in upregulating OX40 expression on 2W1S:I-A^b+ CD4⁺ memory T cells. IL-7 has been implicated in the long term survival of memory T cells once formed indicating that memory cells receive signals from IL-7 in a physiological setting (Kondrack et al., 2003, Purton et al., 2007). This raises the possibility that signalling through IL-7 upregulates OX40 expression on CD4⁺ memory T cells therefore enhancing survival through an antigen independent mechanism. However this is hard to reconcile with the fact that OX40 expression is not detected on 2W1S:I-A^b+ CD4⁺ T cells directly *ex vivo*. ROR γ t^{-/-} mice, which lack ILC3, were shown to have a defect in survival of CD4⁺ memory T cells (Withers et al., 2012). This was attributed to the lack of provision of OX40L by lymphoid tissue inducer (LTi) cells. Blockade of OX40L once memory cells had formed did not inhibit their survival however a modest reduction was seen when this was combined with CD30L blockade. CD30 is technically challenging to stain on CD4⁺ T cells and the lack of OX40 expression on 2W1S:I-A^b+ CD4⁺ memory T cells make a direct interaction between memory cells and LTi an unlikely mechanism regulating their survival. Furthermore provision of OX40 signals through anti-OX40 mAb did not enhance the survival of 2W1S:I-A^b+ CD4⁺ memory T cells. OX40 expression can be rapidly induced within 4 hours of peptide restimulation indicating it may play an important role in secondary effector T cell responses. This also demonstrates that the absence

of OX40 on 2W1S:I-A^b+ CD4⁺ memory T cells is not due to the method of preparation *ex vivo*.

Although OX40 expression on 2W1S:I-A^b+ CD4⁺ T cells was restricted to a brief period, they rapidly re-express the receptor upon TCR engagement. Four hours post restimulation with 2W1S peptide, at either 7 or 28 dpi, the majority of 2W1S:I-A^b+ CD4⁺ T cells were OX40⁺. At this time OX40 was more broadly expressed across T cell subsets indicating a less restricted pattern of expression in secondary compared to primary responses. Again expression of OX40 was rapidly lost; by day 4 post secondary challenge there was no detectable expression. We temporally manipulated provision of OX40 signals at the point of challenge using mAb; due to the short window of OX40 expression timing of antibody administration was important. When agonistic anti-OX40 mAb were given on the day of secondary challenge, they specifically expanded 2W1S:I-A^b+ CD4⁺ Tem cells. However the number of 2W1S:I-A^b+ CD4⁺ T cells was only modestly increased when administration of mAb was delayed until the day after secondary challenge, indicating OX40 is required early after antigen exposure. The specific expansion of Tem cells was associated with a reduction in the percentage of TFH cells, however the total number remained the same in control and treated mice. Confocal analysis of spleen sections revealed a significant reduction in the number and area of GC in the mice receiving anti-OX40 mAb. As the total number of TFH cells was not altered this suggests there may be increased competition due to a specific expansion of Tem cells in anti-OX40 treated mice, inhibiting GC formation. Conversely blocking OX40 interactions

after secondary challenge using mAb resulted in a specific decrease in Tem but there was no significant difference in TFH cell number. Addition of blocking CD30L mAb did not further impair the expansion of Tem. These data are consistent with previous studies showing OX40 is required for accumulation of CD4⁺ memory T cells and induction of lung inflammation following secondary antigen exposure (Salek-Ardakani et al., 2003).

Administration of agonistic anti-OX40 mAb immediately following infection with Lm-2W1S resulted in a memory cell population that was biased towards Tem cells. This is consistent with OX40 being important for the survival of Teff cells, some of which survive to form the memory cell pool. Surprisingly, despite the vast increase in cell number observed at day 7 following anti-OX40 mAb treatment, the number of 2W1S:I-Ab⁺ CD4⁺ memory T cells recovered 28 dpi was not significantly altered. This is in contrast to other studies that have shown administration of anti-OX40 mAb enhances the accumulation of CD4⁺ memory T cells (Gramaglia et al., 2000, Maxwell et al., 2000). These studies used adoptive transfers of TCR transgenic T cells, which may show different survival requirements to the endogenous 2W1S:I-Ab⁺ CD4⁺ memory cell population. Additionally one of these studies used immunisation with CFA, which is highly immunogenic and likely elicits a different response to infection with attenuated Lm-2W1S. The large population contraction we observed may be due to poor conversion to memory cells when there are abnormally high numbers of T cells, as seen when non-physiological numbers of TCR transgenic cells are used

(Hataye et al., 2006). This is thought to be due to increased competition among T cell clones for DC interactions (Blair and Lefrancois, 2007).

Overall these data show that OX40 expression by an endogenous, polyclonal antigen-specific population of CD4⁺ T cells is very tightly regulated temporally and not uniformly expressed amongst responding cells in a primary response. Upon re-encounter of antigen OX40 is rapidly and more broadly expressed, indicating a role in secondary responses. Restriction of OX40 signalling through administration of blocking mAb or in genetically deficient mice specifically impaired the Teff or Tem cell population. Overprovision of OX40 signalling specifically expanded Teff cells in the primary response or Tem cells following secondary challenge, while being detrimental to the TFH cell population, however the overall size of the memory cell pool was not altered. It will be important to consider these implications when applying the use of OX40 agonists in the clinic or for vaccine design. While it may be desirable to enhance CD4⁺ T cells responses in this way, if high affinity antibody responses are required other options may need to be considered.

CHAPTER 4: THE ROLE OF COSTIMULATORY
RECEPTORS ICOS AND CD28 IN CD4⁺ T CELL
RESPONSES

4.1 Introduction

4.1.1 Therapeutic Interventions

Targeting costimulatory pathways for therapeutic benefit has been considered since the discovery of the two signal model for T cell activation. Targeting this second signal allowed T cell responses to be manipulated without knowing the exact nature of the antigen, which is often the case in transplantation or autoimmune disease. Controlling the effector T cell response is essential in preventing graft rejection and controlling autoimmune diseases such as systemic lupus erythematosus, RA and type 1 diabetes. As T cell help is vital for optimal B cell responses and production of high affinity antibody, this method can also target B cell mediated disease. A number of different costimulatory molecules have been considered as therapeutic targets, such as CD40, but the interaction of CD28 with its B7 ligands has become the focus of attention. Clinical treatment with a human form of CTLA4Ig known as Abatacept has been trialled in autoimmune diseases such as RA and psoriasis with some degree of success (Ruderman and Pope, 2005, Abrams et al., 1999). Abatacept was approved for the treatment of RA in 2005 following trials showing amelioration of RA symptoms when combined with the existing treatment methotrexate (Kremer et al., 2003). However trials of abatacept in type 1 diabetes (Orban et al., 2014, Orban et al., 2011), allergen-induced airway inflammation (Parulekar et al., 2013) and ulcerative colitis (Sandborn et al., 2012) have not been as successful. This suggests that T cell mediated autoimmune diseases show varying responses to abatacept and highlights the need for additional therapeutic pathways. A

modified form of CTLA4Ig has been developed, Belatacept, with decreased dissociation rates from CD80 and CD86. In non-human primates this mutated form has been shown to be significantly more effective than abatacept in preventing graft rejection (Larsen et al., 2005).

ICOS has also been exploited as a therapeutic target to control activated T cells as, unlike CD28, ICOS is not expressed on naive T cells but is rapidly upregulated following antigen recognition (Hutloff et al., 1999, Mages et al., 2000). This is advantageous as memory cells cause pathogenesis in T cell mediated autoimmune diseases. Blocking ICOS signalling has proven effective in limiting ongoing CD4⁺ T cell responses in some murine models of disease. Blockade of ICOS signals, using ICOS-Ig, following development of EAE symptoms ameliorated disease; this was thought to be due to decreased IL-10 and IFN- γ production by T cells that mediate pathology (Sporici et al., 2001). However timing of administration was found to be vital to the clinical outcome; blocking anti-ICOS mAb given during the priming phase increased disease severity due to increased IFN- γ production (Rottman et al., 2001). ICOS blockade prevented the development of autoimmune diabetes in non-obese diabetic (NOD) mice and prolonged allograft survival but was unable to reverse diabetes after onset (Ansari et al., 2008).

4.1.2 The role of ICOS in CD4⁺ memory T cell responses

Although ICOS has a well-documented role in the formation of TFH cells in the primary response, the requirement of CD4⁺ memory T cells for costimulation through this receptor is less clear. Initial reports identifying ICOS expression on previously activated CD44^{hi} T cells indicated that this receptor might be important in memory cell responses (Yoshinaga et al., 1999). ICOSL fusion protein administered during secondary challenge resulted in an augmented hypersensitivity response, superior to when administered during the primary response, supporting the idea that ICOS costimulates memory T cells. In agreement with this, another study also found that contact hypersensitivity was enhanced following challenge when ICOSL-Ig was injected and was more effective than CD86-Ig (Guo et al., 2001). More recently Mahajan and colleagues found that the reactivation of an endogenous, polyclonal CD4⁺ memory T cell population was impaired in ICOS^{-/-} mice (Mahajan et al., 2007). This was despite normal priming, generation of memory and survival at 10 weeks post infection.

There have been conflicting reports for the requirement of ICOS signalling by Tcm and Tem cells. Several studies have indicated that ICOS is required for the formation of the Tem cell population. Expression of ICOS was found to be highest on Tem phenotype cells, defined by expression of CD44 and absence of CD62L, while Tcm phenotype cells showed only intermediate ICOS expression (Burmeister et al., 2008). Although there was no overall difference in the total number of CD4⁺ memory T cells in ICOS^{-/-} mice, CD44^{hi}CD62L^{lo} Tem phenotype cells were significantly reduced. In addition the initial priming of TCR transgenic

OTII cells was not affected by the absence of ICOS signalling but the expansion of effector cells 5 dpi was reduced by half compared to WT mice (Burmeister et al., 2008). They concluded that this impaired survival is the cause of the observed reduction in Tem cells. Another study tracking antigen-specific cells found very similar results. The total number of influenza-specific memory cells was not reduced in ICOS^{-/-} mice however there was a significant defect in Tem cells attributable to enhanced rate of decline following resolution of infection (Moore et al., 2011). However ICOS signalling has also been implicated in the development and survival of Tcm cells. An endogenous CD4⁺ T cell population responding to infection with *Listeria monocytogenes* was analysed in mixed bone marrow chimeras reconstituted with an equal mixture of WT and ICOS^{-/-} cells (Pepper et al., 2011). The generation of Tcm cell precursors was significantly impaired in the absence of ICOS and this population declined more quickly than in WT mice. Two human patients with ICOS deficiency were described to have decreased CD4⁺ memory T cell populations in peripheral blood; this resulted from a decrease in both Tcm and Tem cells (Takahashi et al., 2009). Production of Th1, Th2 and Th17 cytokines were all impaired upon *in vitro* stimulation.

We sought to clarify the requirement for ICOS and CD28 signals in the generation, survival and reactivation of memory cells. We have tracked an endogenous, polyclonal CD4⁺ memory T cell population and used TCR transgenic SM1 CD4⁺ T cells to analyse the survival of CD4⁺ memory T cells generated in WT mice. A combination of genetically deficient mice and blocking mAb were used to

temporally assess the impact of loss of costimulatory signals on CD4⁺ T cell responses.

4.2 Results

4.2.1 CTLA4Ig mice as a model of limited costimulation through CD28

Therapeutic targeting of T cell responses has shown some clinical success, where key costimulatory molecules are blocked to limit T cell activation and proliferation. As CD28 is a potent costimulatory molecule involved in the proliferation and differentiation of T cells it is an attractive therapeutic target. Injection of CTLA4Ig has been used to abrogate signalling through the CD28 pathway (Linsley and Nadler, 2009). Here we aimed to establish a mouse model in which constitutive expression of CTLA4Ig could be used to investigate the impact of limited costimulation on CD4⁺ T cells responses. CTLA4Ig mice produce a fusion protein consisting of the extracellular domain of CTLA4 and the Fc region of human IgG1, which competitively binds to the ligands of the CD28 receptor, CD80 and CD86 (Lane et al., 1994). CTLA4 binds to these ligands with around 20-100 fold greater affinity than CD28 and therefore dramatically reduces the number of available ligands, restricting signalling through the CD28 pathway (Linsley et al., 1994). The CTLA4Ig fusion protein is present at 10-30 µg/ml in the serum therefore is maintained at a therapeutically relevant level; i.v. abatacept therapy is administered at 3-10 mg/kg periodically (Lane et al., 1994, Keating, 2013). This mouse model is analogous to abatacept therapy, which has been trialled for use in autoimmune diseases such as RA and psoriasis with some degree of success (Ruderman and Pope, 2005, Abrams et al., 1999).

Signalling through the CD28 pathway is well characterised and is required for the optimal expansion of CD4⁺ T cells following TCR stimulation (Shahinian et al., 1993, Viola and Lanzavecchia, 1996). We sought to test the efficacy of CTLA4Ig in this mouse model using infection with Lm-2W1S to track the expansion of an endogenous polyclonal CD4⁺ T cell population. CD80^{-/-}CD86^{-/-} mice were included as a control as these mice lack both of the ligands to which CD28 binds, thus establishing the baseline for no signalling through CD28. In these mice signalling through CD28 is abrogated and therefore T cell proliferation is severely stunted (Chang et al., 1999). WT, CTLA4Ig and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S and the number of 2W1S:I-A^b⁺ CD4⁺ T cells were enumerated from the spleen 7 dpi. As expected CD80^{-/-}CD86^{-/-} mice showed a significant impairment in the expansion of 2W1S:I-A^b⁺ CD4⁺ T cells, which were almost 20 fold reduced compared to WT mice (Figure 4.1 A; median for WT: 48 659, CD80^{-/-}CD86^{-/-}: 2328). There was also a significant reduction in the number of 2W1S:I-A^b⁺ CD4⁺ T cells in CTLA4Ig mice (Figure 4.1 A; median for CTLA4Ig: 16495). The 2.5-fold reduction in number of 2W1S:I-A^b⁺ CD4⁺ T cells observed indicates that constitutive production of CTLA4Ig fusion protein affects the optimal expansion of CD4⁺ T cells, however blockade of CD28 appears to be incomplete.

We also tracked the TCR transgenic SM1 CD4⁺ T cell response to Lm-FliC. An advantage of this method is that CD4⁺ T cells are adoptively transferred into hosts, since endogenous CD4⁺ T cells in CTLA4Ig mice may be subject to developmental defects in the thymus due to the absence of CD28 signalling.

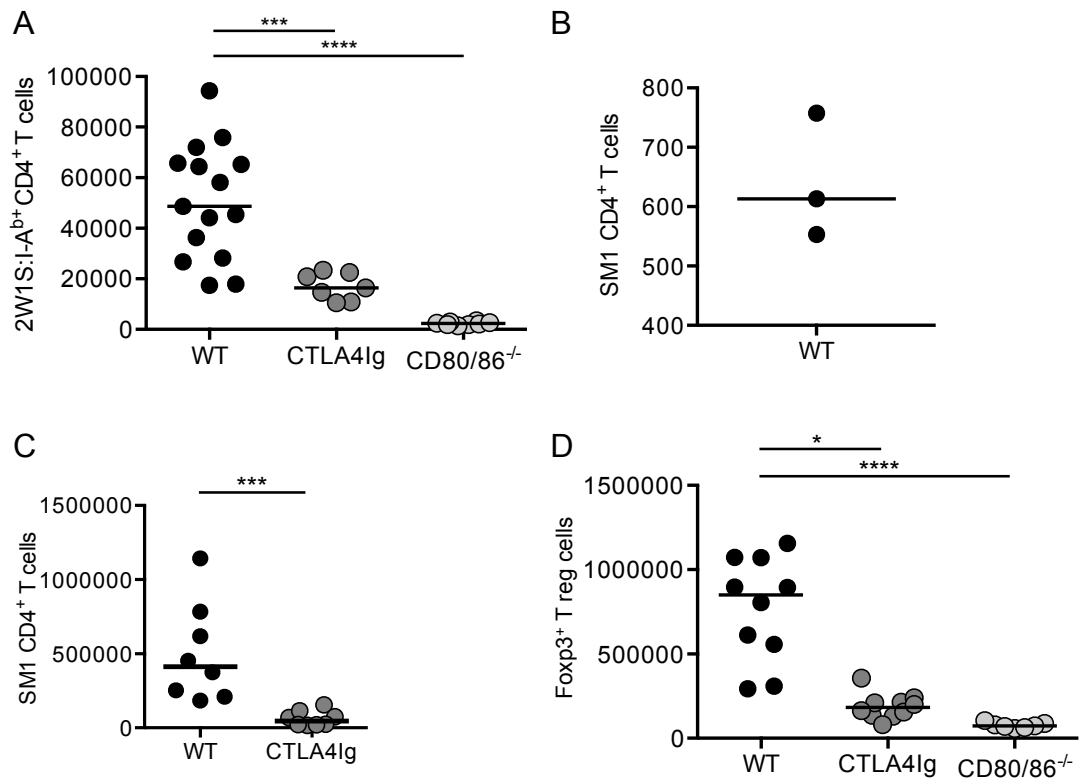


Figure 4.1: CTLA4Ig mice can be used as a model of limited CD28 costimulation. (A) WT, CTLA4Ig and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S and spleen was analysed for the number of 2W1S:I-A^b CD4⁺ T cells 7 dpi. (B) WT mice received 10 000 SM1 CD4⁺ T cells i.v. and the following day spleen and lymph nodes were analysed. Number of SM1 CD4⁺ T cells surviving is shown, defined as the “park rate”. (C) WT and CTLA4Ig mice were injected with 10 000 SM1 CD4⁺ T cells and infected with Lm-FluC the following day. Spleen was analysed for the number of SM1 CD4⁺ T cells 7 dpi. (D) Number of Foxp3⁺ CD4⁺ Treg cells from the spleen of WT, CTLA4Ig and CD80^{-/-}CD86^{-/-} mice.

(A and D) Graph shows pooled data from ≥ 2 independent experiments. (B) Graph shows data from 1 independent experiment. (C) Graph shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ***p<0.001, ****p<0.0001.

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Some studies have found that CD28 is highly expressed on thymocytes (Gross et al., 1992), however others have not shown any impairment in the peripheral T cell repertoire in CD28^{-/-} mice (Shahinian et al., 1993). The use of TCR transgenic T cells circumvents problems that may be associated with abnormal thymic development. Following i.v. transfer of TCR transgenic cells, the number of cells surviving after 24 hours has been described as the “park rate” (Hataye et al., 2006). This has been shown to equal 10 % of the original population that was adoptively transferred (Hataye et al., 2006, Blattman et al., 2002). To test this using the SM1 TCR transgenic model WT mice were injected with 10 000 SM1 CD4⁺ TCR transgenic T cells and the spleen and lymph nodes were analysed 24 hours later to enumerate surviving cells. A median of around 600 SM1 CD4⁺ T cells were remaining following transfer, equalling 6 %, similar to previous reports (Figure 4.1 B). Injection with a low number of SM1 CD4⁺ T cells is designed to mimic the size of an endogenous T cell pool, which circumvents caveats associated with the transfer of large numbers of TCR transgenic T cells. Assuming a park rate of 10 %, this results in a naive starting population of around 1000 cells, which only modestly exceeds endogenous T cell pools, which have been measured at between 20-1500 cells per mouse (Moon et al., 2007, Obar et al., 2008, Kotturi et al., 2008). WT and CTLA4Ig mice were injected with 10 000 SM1 CD4⁺ T cells and infected with Lm-FliC the following day. The number of SM1 CD4⁺ T cells in spleen was then enumerated. The expansion of SM1 CD4⁺ T cells was significantly impaired in CTLA4Ig mice, by almost 9 fold (Figure 4.1 C; median for WT: 413 679, CTLA4Ig: 46 966). These data

demonstrate that constitutive expression of CTLA4Ig can significantly impair the primary expansion of antigen-specific CD4⁺ T cells in multiple model systems.

Development of Treg cells in the thymus and their survival in the periphery are both thought to be CD28 dependent (Tai et al., 2005, Salomon et al., 2000, Tang et al., 2003). To evaluate how effective constitutive expression of CTLA4Ig fusion protein is at blocking signalling through CD28 we analysed the Treg population in CTLA4Ig mice. The total number of CD4⁺ Treg cells in the spleen was calculated from WT, CTLA4Ig and CD80^{-/-}CD86^{-/-} mice. Again CD80^{-/-}CD86^{-/-} mice were used as a control, in which Treg cell development would be severely impaired due to lack of CD28 signalling. As expected there was a significant reduction in the number of Treg cells in CD80^{-/-}CD86^{-/-} when compared to WT mice (Figure 4.1 D; median for WT: 848 998, CD80^{-/-}CD86^{-/-}: 74 078). This was also the case in CTLA4Ig mice, demonstrating that circulating CTLA4Ig fusion protein impairs Treg cell development or survival by blocking signals through CD28 (Figure 4.1 D; median for CTLA4Ig 182 043).

To further assess the degree of impairment in the T cell response in CTLA4Ig mice, effector T cell populations were assessed in these animals. The generation of TFH cells has been shown to require signals through CD28; in the absence of this pathway TFH cell formation is impaired or abrogated (Salek-Ardakani et al., 2011, Akiba et al., 2005). For this reason the formation of 2W1S:I-A^b and SM1 TFH was evaluated in CTLA4Ig mice. TFH cells were identified by expression of the highest levels of CXCR5 and PD-1 (as shown in Figure 3.4). Both the number and the percentage of 2W1S:I-A^b TFH cells were significantly reduced in

CTLA4Ig mice compared to WT mice (Figure 4.2 A and B). TFH cells were also reduced in CTLA4Ig mice following adoptive transfer of SM1 CD4⁺ T cells and infection with Lm-FliC; the number and percentage of SM1 TFH cells were both significantly reduced (Figure 4.2 C and D).

TFH cells have been shown to be essential for the maintenance of GC, providing surviving signals to B cells (de Vinuesa et al., 2000). Although spontaneous GC formation can occur in the absence of T cell help these are aborted early after induction (de Vinuesa et al., 2000). To investigate the effect of a reduced TFH cell population, spleen sections from WT and CTLA4Ig mice infected with Lm-2W1S 7 days previously were cut and stained for confocal microscopy analysis. As described for Figure 3.9 in section 3.2.6, spleen sections were stained for Bcl-6 to identify GC and CD3 and CD4 to identify T cells. In WT mice large clusters of Bcl-6⁺ cells were identified indicating GC are present at this timepoint (Figure 4.3 A). Consistent with the significant decrease in TFH cells observed in Figure 4.2, the size of GC in CTLA4Ig mice appeared reduced (Figure 4.3 A). TFH cells can be identified by co-staining of CD3 and CD4 and nuclear staining of Bcl-6. A number of TFH cells were present in and around the GC in WT mice (Figure 4.3 B- identified by yellow circles). Some TFH cells could be identified in the follicle of CTLA4Ig mice however their number appeared to be reduced (Figure 4.3 B).

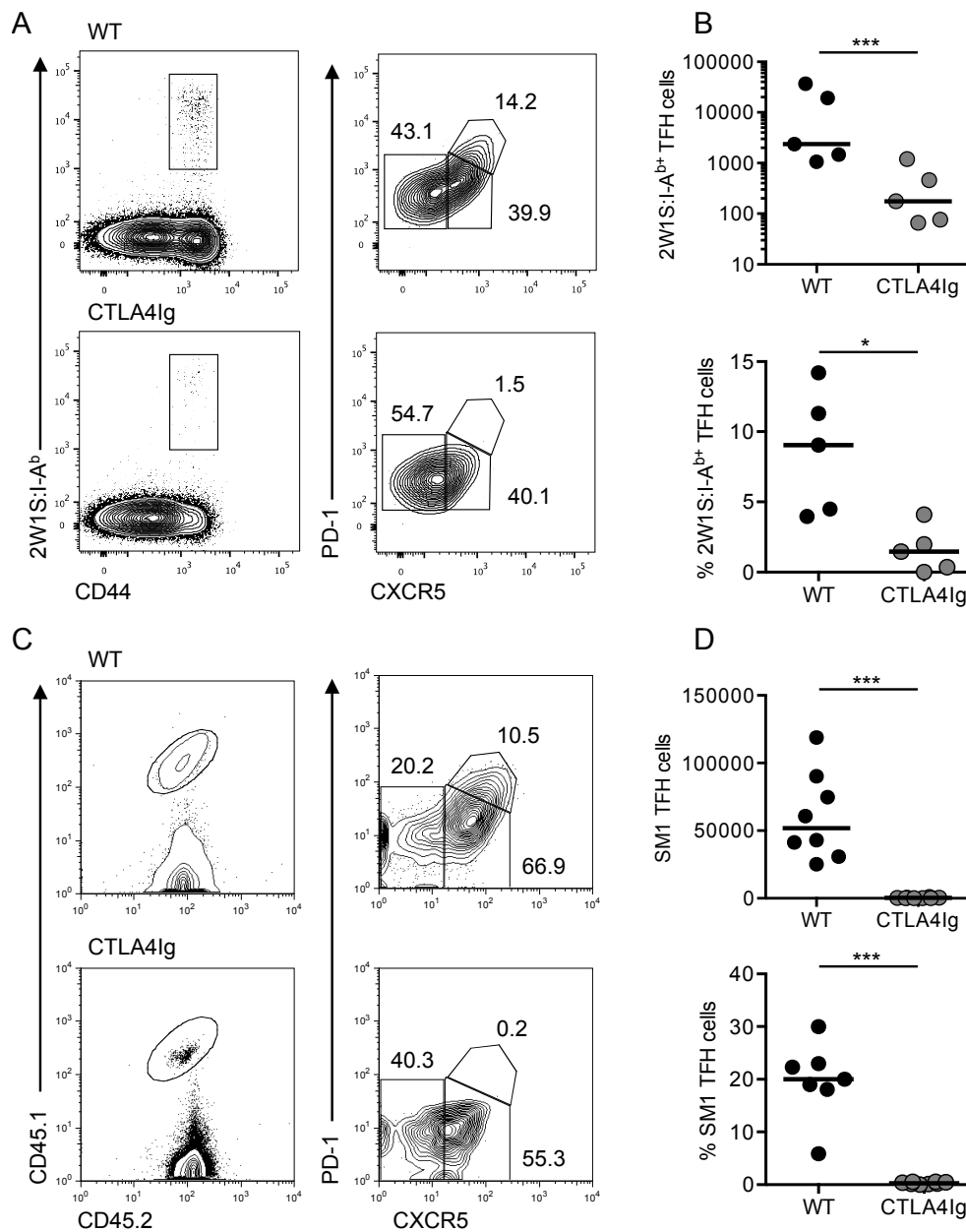


Figure 4.2: TFH cell formation is severely impaired in CTLA4lg mice. WT and CTLA4lg mice were infected with Lm-2W1S and analysed 7 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number (top) and percentage (bottom) of 2W1S:I-A^b TFH cells.

WT and CTLA4lg mice were injected i.v. with 10 000 SM1 CD4⁺ T cells and infected with Lm-FluC the following day. Mice were analysed 7 dpi. (C) Flow cytometry plots showing expression of CXCR5 and PD-1 on SM1 CD4⁺ T cells (D) Number (top) and percentage (bottom) of SM1 TFH cells.

(A and C) Plots are representative of ≥ 5 mice from 2 independent experiments. Values on plots are percentages. (B and D) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: *** $p < 0.001$.

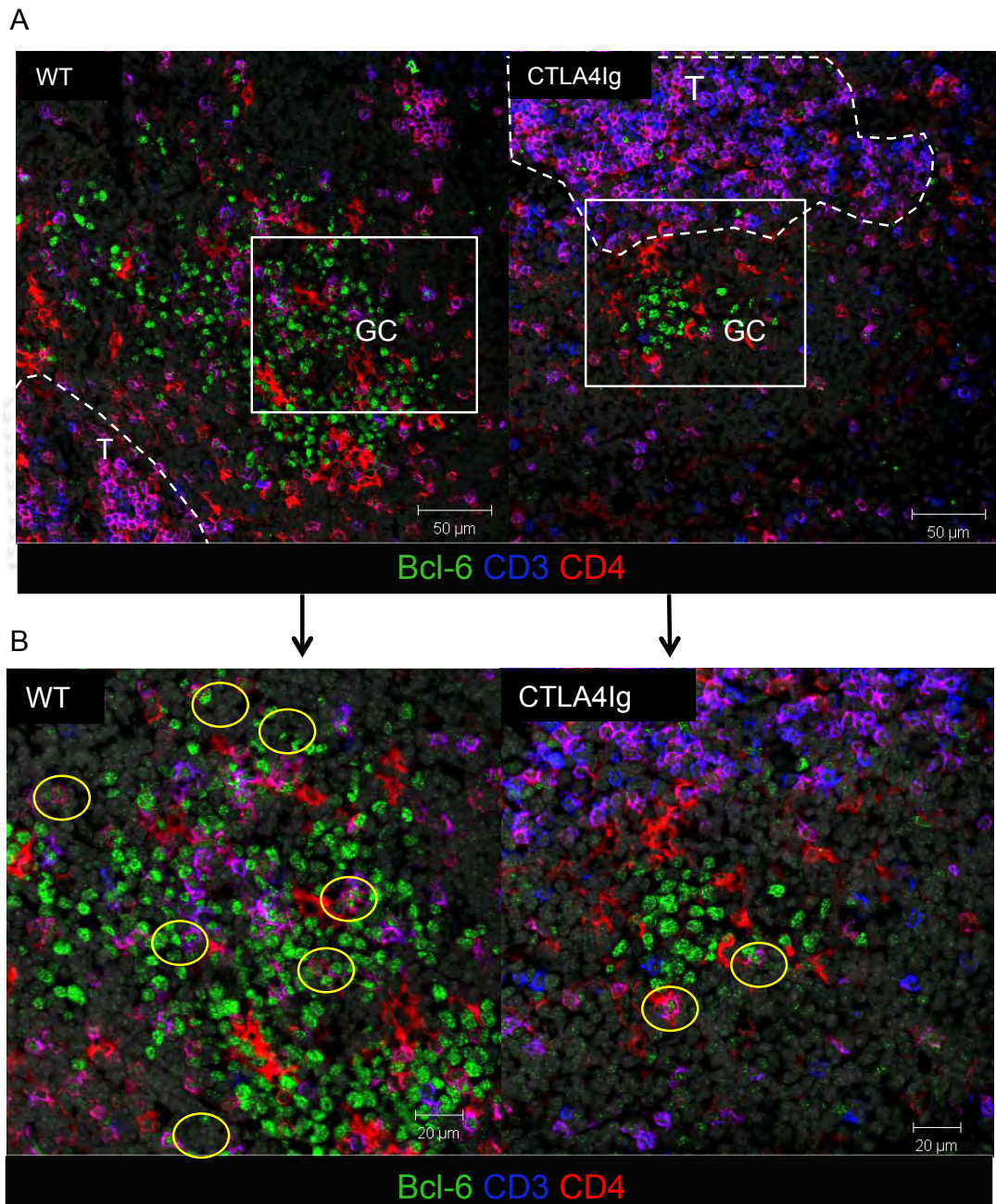
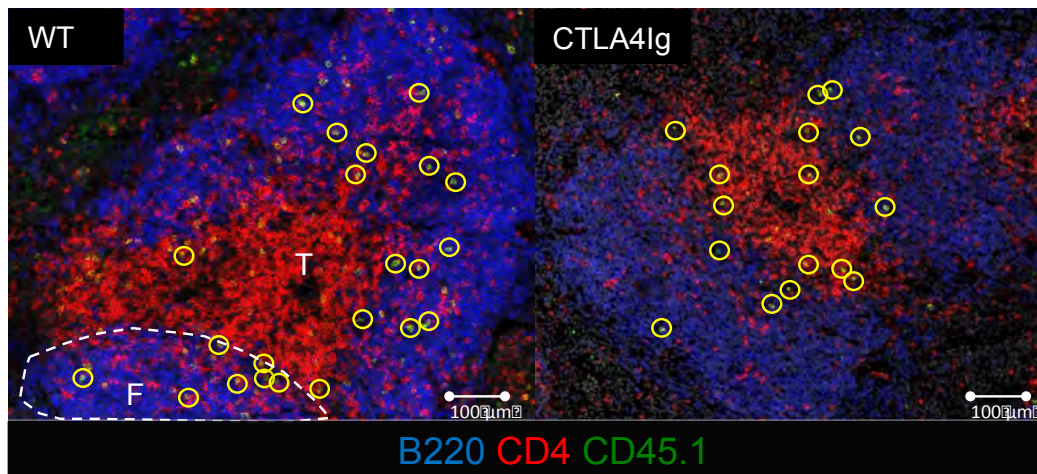


Figure 4.3: GC are reduced in size in CTLA4Ig mice. WT and CTLA4Ig mice were infected with Lm-2W1S and analysed 7 dpi. (A) Spleen sections were cut and stained for expression of Bcl-6 (green), CD3 (blue) and CD4 (red) and counterstained with DAPI to identify GC. “GC” indicates germinal centre. “T” denotes T cell zone outlined by white dashed line. Magnification 25x, scale bar represents 50 μ m. White boxes indicate the area shown magnified in (B). (B) Spleen sections were cut and stained as in (A). Yellow circles indicate examples of TFH cells identified by nuclear Bcl-6 staining surrounded by CD3 and CD4 co-staining. Magnification 40x, scale bar represents 20 μ m.

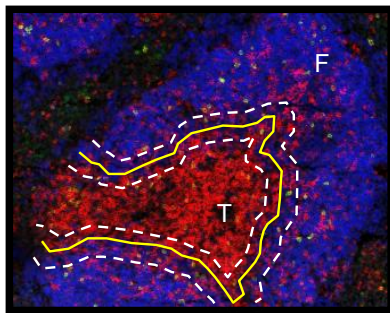
4.2.2 Migration of CD4⁺ T cells into the B cell follicle is impaired in CTLA4Ig mice

To investigate the location of TFH cells further we used CD45.1⁺CD45.2⁺ TCR transgenic SM1 CD4⁺ T cells which could be identified in sections by allotype marking. Current limitations in tetramer staining methods mean it is not possible to identify 2W1S:I-A^b⁺ CD4⁺ T cells in tissue sections. WT (C57BL/6, CD45.2⁺) and CTLA4Ig (C57BL/6, CD45.2⁺) mice received an adoptive transfer of 10 000 SM1 CD4⁺ T cells and were infected with Lm-FliC the following day. Sections of spleen were analysed 7 dpi for the location of responding SM1 CD4⁺ T cells within the white pulp areas. SM1 CD4⁺ T cells were identified by expression of CD45.1. B220 and CD4 were used to identify the B cell follicle and T cell zone respectively. For quantitation of SM1 CD4⁺ T cells in the B cell follicle, the area occupied by B220⁺ cells was marked and the number of CD45.1⁺ SM1 CD4⁺ T cells within were counted (Figure 4.4 A). This was repeated for the T cell zone and the percentage of total SM1 cells that were located in the follicle was calculated. In WT mice a high proportion of responding SM1 CD4⁺ T cells (around 80%) were located within the B cell follicle, however in CTLA4Ig mice this was significantly reduced to around 40% (Figure 4.4 C). It was observed that a large number of SM1 CD4⁺ T cells in CTLA4Ig mice were located around the border of the T cell zone and the B cell follicle, an area known as the B/T interface. To quantify this, a zone 20 µm either side of this border was designated as the B/T interface and the number of SM1 CD4⁺ T cells within it was counted.

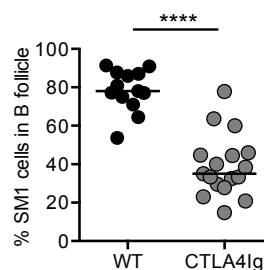
A



B



C



D

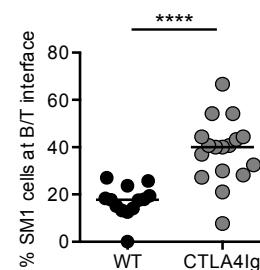


Figure 4.4: Migration of SM1 CD4⁺ T cells to the follicle is impaired in CTLA4Ig mice. WT and CTLA4Ig mice were injected with 10 000 SM1 cells and infected with Lm-FliC the following day. Mice were analysed 7 dpi. (A) Spleen sections were cut and stained for expression of B220 (blue), CD4 (red) and CD45.1 (green) and counterstained with DAPI to identify white pulp areas. SM1 CD4⁺ cells were identified by co-staining for CD4 and CD45.1 in green. Yellow circles indicate examples of SM1 cells located in or close to the B cell follicle. “F” denotes B cell follicle outlined by white dashed line. “T” denotes T cell zone. Magnification x10, scale bar represents 100 μm. (B) Diagram showing how the B/T interface was designated. The solid yellow line marks the B/T border and the white dashed lines denotes the edge of the B/T interface 20 μm either side. (C) Percentage of SM1 CD4⁺ T cells located in B follicle of splenic white pulp area. (D) Percentage of SM1 CD4⁺ T cells located at the B/T interface designated as shown in (B).

(A) Confocal images are representative of 3 mice from 1 independent experiment. (C and D) Graphs show pooled data from 3 mice from 1 independent experiment. Each data point represents a white pulp area. Bars show median. Mann Whitney test: ****p<0.0001.

A diagram describing how the B/T interface was designated is shown in Figure 4.4 B. In CTLA4Ig mice significantly more SM1 CD4⁺ T cells were located in this area than in WT mice (Figure 4.4 D) indicating a defect in the migration of these cells past this point. This is consistent with a lack of CXCR5 expression (shown in Figure 4.2 C), which is essential for the migration of CD4⁺ T cells into the B cell follicle (Breitfeld et al., 2000).

4.2.3 ICOS expression is induced on antigen-specific CD4⁺ T cells following TCR stimulation

ICOS is a member of the CD28 family of costimulatory receptors however, unlike CD28 it is not constitutively expressed on CD4⁺ T cells. Although the role for ICOS in the formation of TFH cells has been clearly identified (Akiba et al., 2005, Choi et al., 2011), it's function for other CD4⁺ T cell subsets, including memory cells, is unclear. Conflicting reports have proposed a role for ICOS in the formation of Tem cells but not Tcm cells (Moore et al., 2011, Burmeister et al., 2008) and vice versa (Pepper et al., 2011). To dissect the role of ICOS:ICOSL signalling in the generation of CD4⁺ memory T cells we utilised ICOSL^{-/-} mice in which all cells lack the ligand required to initiate the signalling cascade downstream of ICOS in CD4⁺ T cells. ICOS only interacts with ICOSL, which is expressed on APC, therefore these mice show impaired GC and humoral responses associated with a deficiency in T cell help (Yoshinaga et al., 1999, Hutloff et al., 1999). The naive 2W1S:I-A^{b+} CD4⁺ T cell population was analysed in these mice and found to be

similar to WT mice; a small population of tetramer binding CD4⁺ T cells could be identified that were CD44^{lo} (Figure 4.5 A). The median size of this population in ICOSL^{-/-} mice was around 100 cells, which is not significantly different from WT mice (Figure 4.5 D). Expression of ICOS is induced on activated CD4⁺ T cells following TCR stimulation and is dependent on signals through the costimulatory receptor CD28 (Yoshinaga et al., 1999, Tafuri et al., 2001, Hutloff et al., 1999, McAdam et al., 2001, Gonzalo et al., 2001, McAdam et al., 2000). Our data are consistent with this finding; expression of ICOS was detected on CD44^{hi} CD4⁺ T cells in both WT and ICOSL^{-/-} mice (Figure 4.5 B). 2W1S:I-A^b CD4⁺ T cells that had not been activated with antigen lacked expression of ICOS (Figure 4.5 C). An isotype control for anti-ICOS staining was found to have a high level of background staining and therefore under-represented the level of ICOS expression. As expression of ICOS is dependent on CD28 signalling (McAdam et al., 2000), CD80^{-/-} CD86^{-/-} mice were used as a negative control to set the gating for ICOS⁺ cells (Figure 4.5 E).

To dissect the requirements for CD28 and ICOS signals during a CD4⁺ T cell response we used a combination of antigen-specific models. To analyse an endogenous polyclonal population, 2W1S:I-A^b CD4⁺ T cells were tracked from primary proliferation through to the memory stage of the response using MHC II tetramers. This was combined with the use of TCR transgenic SM1 cells as this method allowed us to generate memory cells in WT mice which could be transferred into genetically deficient hosts to monitor their persistence and re-expansion upon challenge.

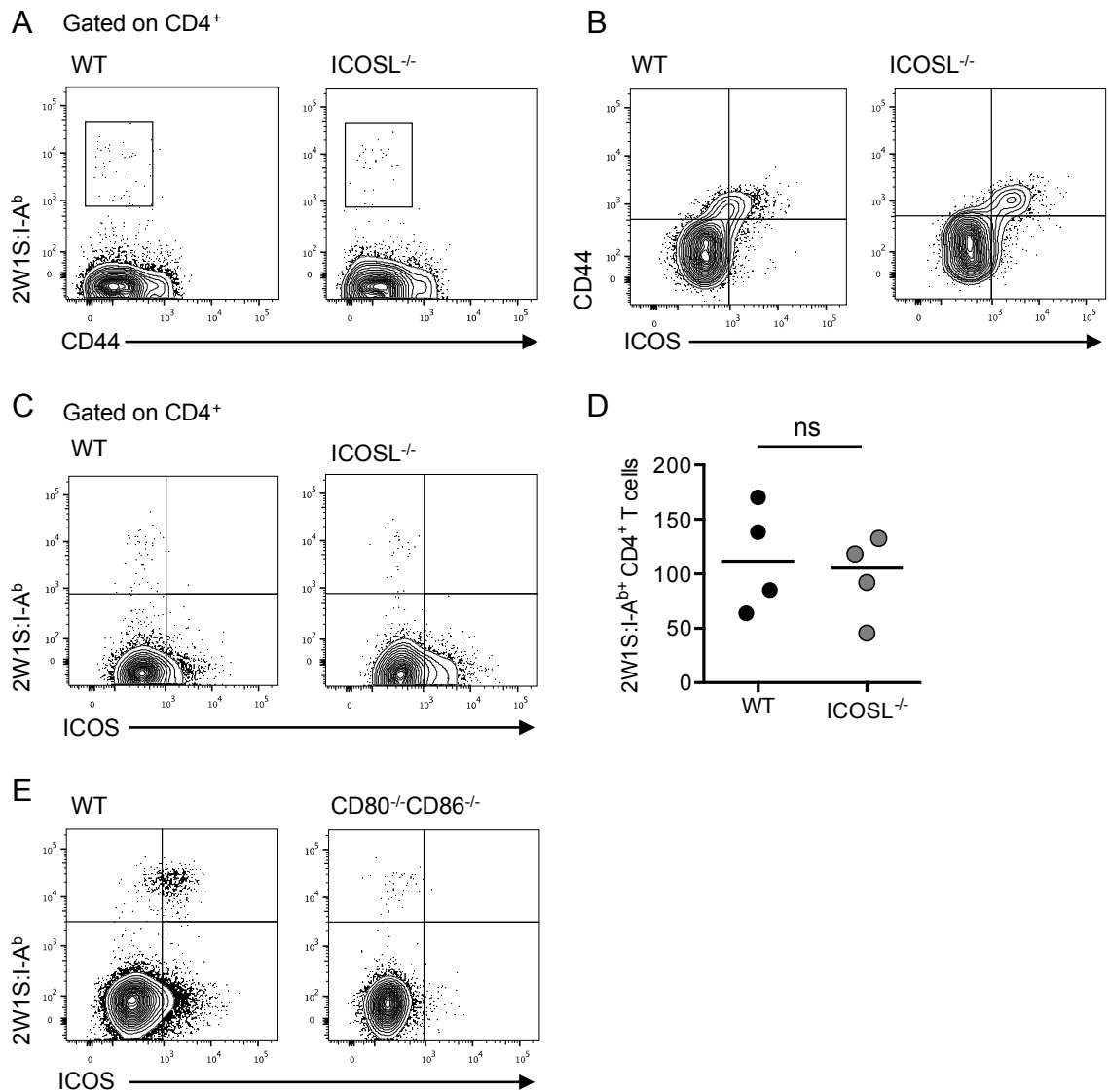


Figure 4.5: Naive 2W1S:I-A^b+ CD4⁺ T cells lack ICOS expression. Naive WT and ICOSL^{-/-} mice were analysed for expression of CD44 and ICOS. (A) Expression of CD44 on 2W1S:I-A^b+ CD4⁺ T cells. Plots are gated on CD4⁺ T cells and show 2 mice pooled. (B) Expression of CD44 and ICOS by CD4⁺ T cells. (C) Expression of ICOS on 2W1S:I-A^b+ CD4⁺ T cells. Plots are gated on CD4⁺ T cells and show 2 mice pooled. (D) Number of 2W1S:I-A^b+ CD4⁺ T cells.

(E) WT and CD80^{-/-}CD86^{-/-} mice were analysed for ICOS expression on 2W1S:I-A^b+ CD4⁺ T cells 28 dpi with Lm-2W1S. Gating for the ICOS⁻ population was set using 2W1S:I-A^b+ CD4⁺ T cells from CD80^{-/-}CD86^{-/-} mice which do not express ICOS.

(A-C and E) Plots are representative of ≥4 mice from 2 independent experiments. (D) Graph shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ns= p>0.05.

This approach allows a WT memory cell population to be studied, without the caveats associated with the sub-optimal generation of memory cells in costimulation deficient mice. Due to previous reports showing poor conversion to memory when large numbers of TCR transgenic cells are transferred (Hataye et al., 2006), we injected WT mice with 10 000 SM1 CD4⁺ T cells i.v. and infected mice with Lm-FliC to form a population of SM1 CD4⁺ memory T cells. SM1 CD4⁺ memory T cells were harvested from donor mice as described in section 2.9 and transferred into ICOSL^{-/-}, CD80^{-/-}CD86^{-/-}, CTLA4Ig or WT mice for analysis.

As we saw that OX40 expression was transient and closely linked to antigen exposure in the 2W1S model system we analysed ICOS expression on both 2W1S:I-A^{b+} CD4⁺ T cells and SM1 CD4⁺ T cells at different stages of the response. ICOS expression was examined on primary effector cells (7 dpi), memory cells (28 dpi), memory cells 4 hours post peptide restimulation and on recently activated memory cells (3 days post secondary challenge). Naive 2W1S:I-A^{b+} CD4⁺ T cells do not express ICOS (shown in Figure 4.5) however at all subsequent timepoints analysed after activation with Lm-2W1S, ICOS was expressed by all or the majority of responding cells (Figure 4.6 A). Unlike OX40, ICOS was expressed on resting memory cells, albeit at a lower level than primary effector cells or recently activated memory cells. ICOS expression on TCR transgenic SM1 CD4⁺ T cells responding to their antigen Lm-FliC was comparable to polyclonal 2W1S:I-A^{b+} CD4⁺ T cells (Figure 4.6 B). Again the level of expression on resting memory cells was lower than SM1 cells at other stages of the response.

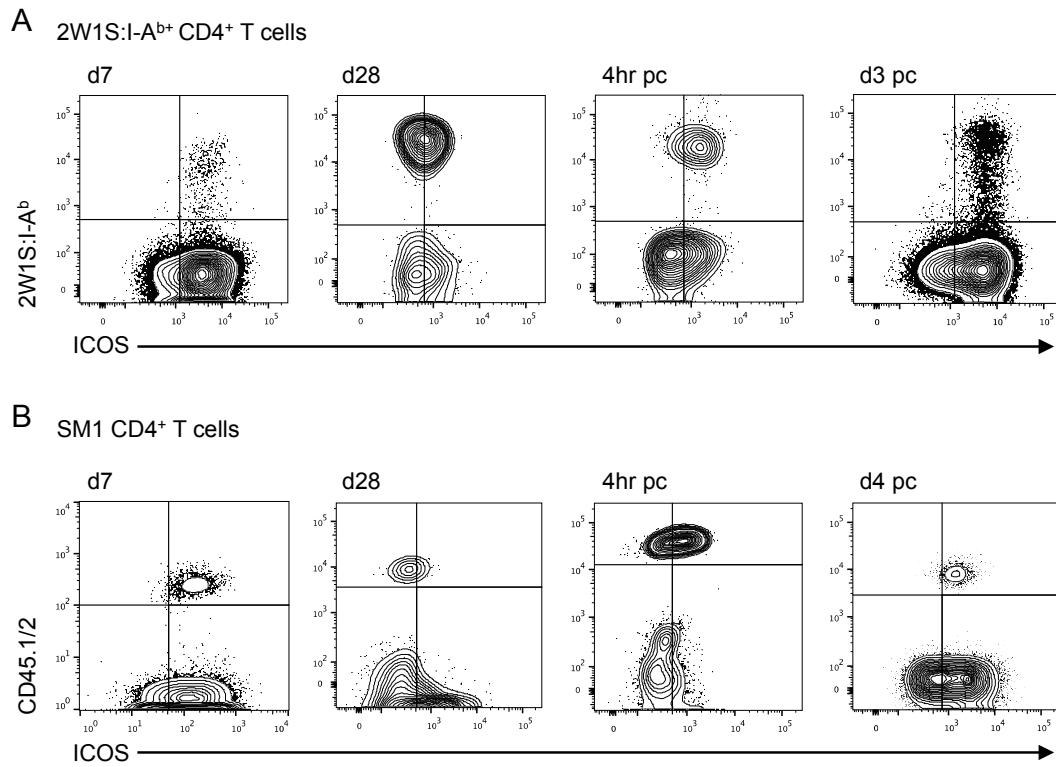


Figure 4.6: Timecourse of ICOS expression by 2W1S:I-A^b CD4⁺ T cells and TCR transgenic SM1 CD4⁺ T cells. (A) WT mice were infected with Lm-2W1S and analysed at the indicated timepoint. ICOS expression on 2W1S:I-A^b CD4⁺ T cells at 7 dpi, on memory cells at 28 dpi, 4 hours post restimulation with 2W1S peptide and 3 days post secondary challenge with Lm-2W1S 28 dpi. (B) WT mice received an adoptive transfer of 10 000 TCR transgenic SM1 CD4⁺ T cells and were infected the following day with Lm-FliC. ICOS expression was analysed at 7 dpi, on memory cells at 28 dpi, 4 hours post restimulation with FliC peptide and 4 days post secondary challenge with Lm-FliC 28 dpi.

(A) Plots are representative of ≥ 8 mice from 2 independent experiments. (B) Plots are representative of ≥ 6 mice from 2 independent experiments except for 7 dpi and 4 hours post challenge which represent 2 mice from 1 independent experiment.

4.2.4 ICOS is required for the formation of 2W1S:I-A^b+

TFH cells

One of the key functions of the costimulatory receptor ICOS is in the differentiation of TFH cells (Choi et al., 2011, Akiba et al., 2005) and this has also been demonstrated in human patients (Bossaller et al., 2006). Expression of ICOSL on B cells is essential for the generation and maintenance of TFH cells (Nurieva et al., 2008, Choi et al., 2011). To test if ICOS is required for the generation of 2W1S:I-A^b CD4⁺ T cells, WT and ICOSL^{-/-} mice were infected with Lm-2W1S. The CXCR5⁺PD-1⁺ TFH cell population was essentially absent in ICOSL^{-/-} mice and the Tcm precursor population was also significantly reduced (Figure 4.7 A and B). There was however no significant effect seen on Teff cells (Figure 4.7 B). These data are consistent with previous studies analysing the 2W1S:I-A^b CD4⁺ T cell response (Pepper et al., 2010, Pepper et al., 2011). Therapeutic targeting of the ICOS:ICOSL pathway has been successfully trialled to prolong cardiac allograft survival (Harada et al., 2003, Schenk et al., 2009). Combination of multiple pathways including ICOS and CD40L has been used to promote allograft tolerance and has prevented autoimmune diabetes in NOD mice (Ford et al., 2014, Orban et al., 2011). We sought to test the efficacy of mAb blocking ICOS:ICOSL interactions by targeting the ICOS receptor. Blocking anti-ICOS mAb were provided by MedImmune and administered at 0 and 3 dpi. The half life of these mAb is 7-10 days (Gianluca Carlesso- unpublished observations) therefore these reagents could reasonably be expected to block ICOS:ICOSL signalling throughout the primary CD4⁺ T cell response.

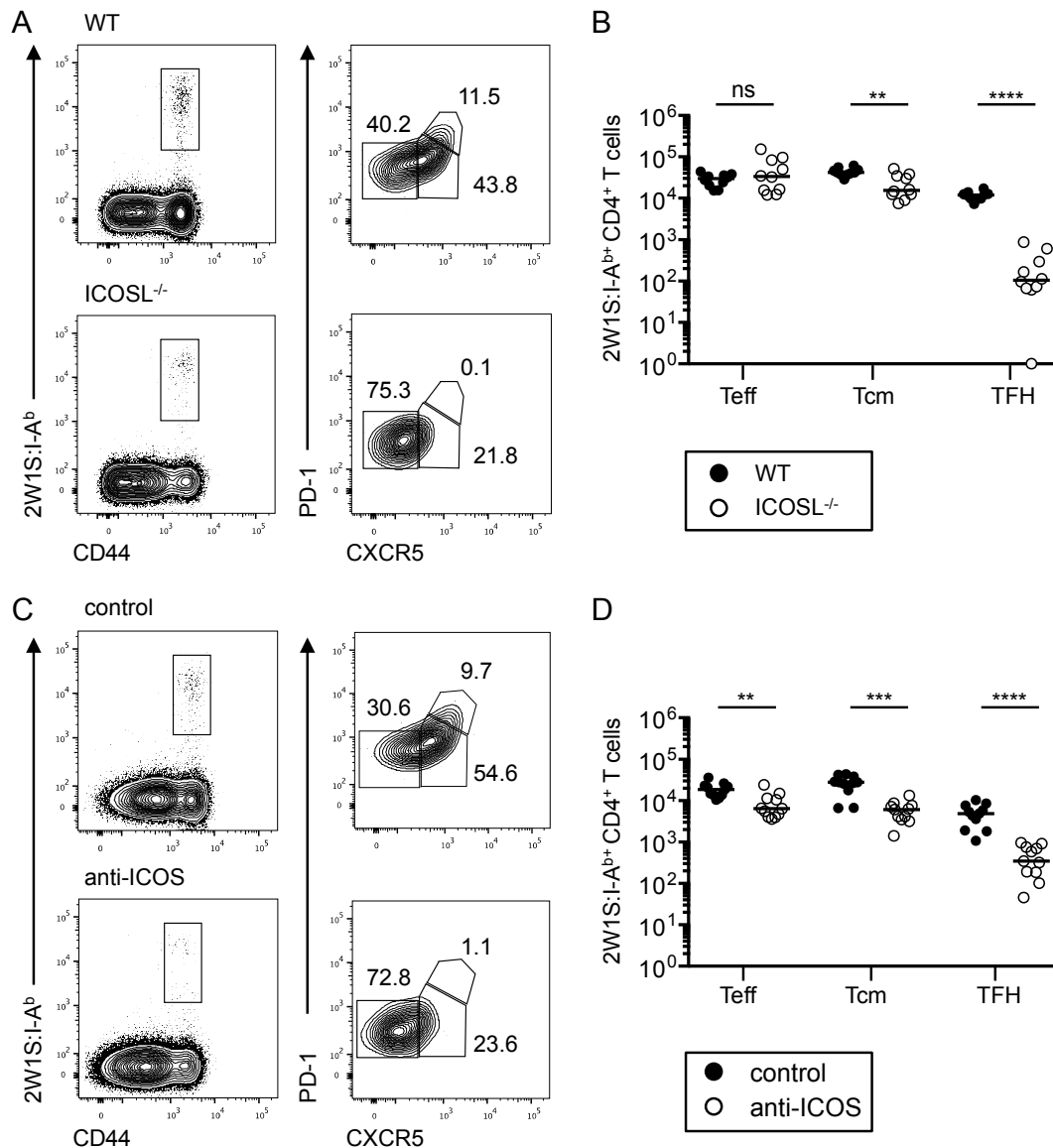


Figure 4.7: 2W1S:I-A^b CD4⁺ TFH cells require ICOS signals for their formation. WT and ICOSL^{-/-} mice were infected with Lm-2W1S and taken 7 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells 7 dpi. (B) Number of 2W1S:I-A^b CD4⁺ Teff, T central memory precursor (Tcm) and TFH cells.

WT mice were infected with Lm-2W1S and taken 7 dpi. Mice received either blocking anti-ICOS or control mAb 0 and 3 dpi. (C) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Teff, T central memory precursor (Tcm) and TFH cells.

(A and C) Plots are representative of 12 mice from 3 independent experiments. Values on plots are percentages. (B and D) Graphs show pooled data from 2 independent experiments. Values of 0 were assigned as 1. Bars show median. Mann-Whitney test: **p<0.01, ***p<0.001, ****p<0.0001, ns= p>0.05.

Blocking anti-ICOS mAb recapitulated the effects seen in ICOSL^{-/-} mice; generation of TFH cells and Tcm precursors was significantly impaired, although the reduction in Tcm precursors was less dramatic than in TFH cells (Figure 4.7 C and D). However unlike in ICOSL^{-/-} mice, blocking anti-ICOS mAb caused a significant reduction in Teff cells. The blocking anti-ICOS mAb was made by linking the variable region sequences from Rat IgG2a anti-mouse ICOS with sequences of human IgG1-TM (Yusuf et al., 2014). The human IgG1 contains 3 mutations in the Fc region that have been shown to significantly inhibit binding to Fcγ receptors and therefore prevent mAb dependent cytotoxicity (Herbst et al., 2010, Oganessian et al., 2008). For this reason the blocking anti-ICOS mAb is not expected to deplete ICOS⁺ cells and is unlikely to account for the reduced population of Teff cells following mAb treatment.

4.2.5 CD28 is required for the optimal expansion of all 2W1S:I-A^b CD4⁺ T cell subsets in the primary response

We sought to compare the effects of blocking ICOS signalling to another costimulatory receptor, CD28. CD28 is well characterised as the 2nd signal required for T cell activation. Interaction of CD28 with two B7 family member ligands, CD80 and CD86, provides an important signal to sustain T cell responses following TCR stimulation (Lenschow et al., 1996). CD80^{-/-}CD86^{-/-} mice were used to assess the primary 2W1S:I-A^b CD4⁺ T cell response in the absence of CD28 signalling. GC formation and immunoglobulin class switching are impaired

in these mice due to an absence of T cell help (Borriello et al., 1997). There was a significant reduction in all 2W1S:I-A^b CD4⁺ T cell subsets 7 dpi infection with Lm-2W1S and TFH cell formation was almost completely abrogated in the absence of CD28 signalling (Figure 4.8 A and B). Demonstrated in Figures 4.1 and 4.2, CTLA4Ig mice can be used as a model of limited CD28 costimulation, such as experienced by patients receiving Abatacept therapy. Optimal expansion of all T cell subsets was also impaired in these mice and TFH cells were again the most severely affected (Figure 4.8 C and D).

4.2.6 Generation of both Tcm and Tem populations requires signalling through ICOS as well as CD28

Signals received in the primary response are key to the generation of a functional memory cell pool. Sub-optimal expansion at this early stage reduces both the size of the memory cell pool and it's ability to rapidly expand upon secondary challenge and exhibit effector cell functions (Hataye et al., 2006, Blair and Lefrancois, 2007). Currently the literature surrounding the role of ICOS in the generation of CD4⁺ memory T cells is conflicted. Recently human ICOS-deficient patients have been identified and characterised. They were found to be deficient in both Tcm and Tem cell populations indicating that ICOS is involved in the generation or survival of CD4⁺ T cell memory (Takahashi et al., 2009).

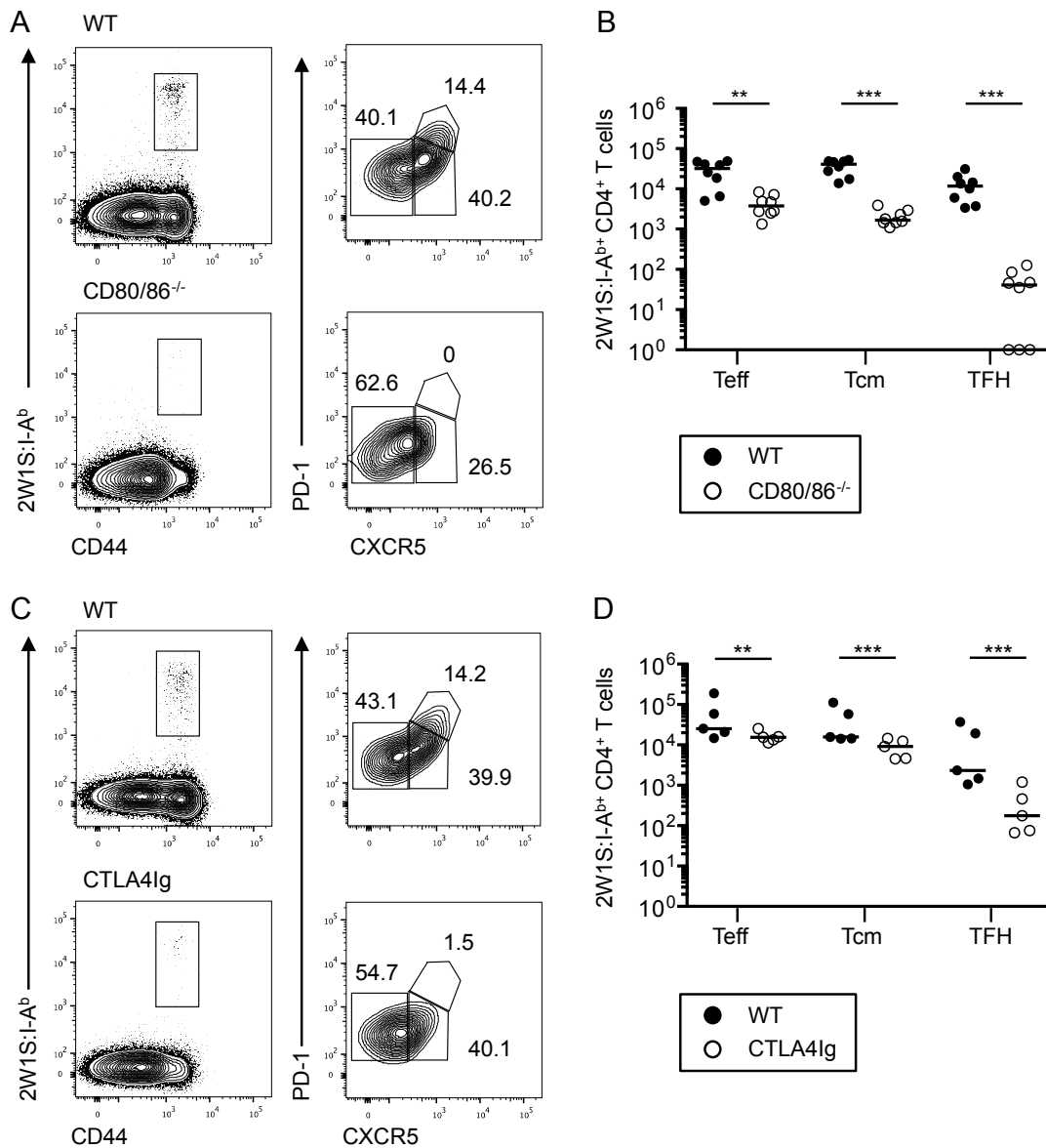


Figure 4.8: CD28 signals are required for the optimal expansion of all 2W1S:I-A^b + CD4⁺ T cell subsets. WT and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S and taken 7 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Teff, T central memory precursor (Tcm) and TFH cells.

WT and CTLA4Ig mice were infected with Lm-2W1S and taken 7 dpi. (C) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Teff, T central memory precursor (Tcm) and TFH cells.

(A) Plots are representative of 12 mice from 3 independent experiments. Values on plots are percentages. (C) Plots are representative of 5 mice from 2 independent experiments. Values on plots are percentages. (B and D) Graphs show pooled data from 2 independent experiments. Values of 0 were assigned as 1. Bars show median. Mann-Whitney test: **p<0.01, ***p<0.001. [2]

Studies of ICOS^{-/-} and ICOSL^{-/-} mice however have noted a specific reduction in Tem cells, but identification of memory cells in these studies has relied upon the use of CD44 and CD62L as well as adoptive transfer of non-physiological numbers of TCR transgenic T cells (Moore et al., 2011, Burmeister et al., 2008). Another study utilising bone marrow chimeras reconstituted with an equal mix of WT and ICOS^{-/-} bone marrow came to a different conclusion. Using infection with Lm-2W1S they found that only Tcm cells were affected by the absence of ICOS (Pepper et al., 2011). Therefore we sought to clarify if CD4⁺ memory T cells require ICOS signals and whether there is a differential requirement for Tcm versus Tem cells.

We tracked the 2W1S:I-A^b⁺ CD4⁺ T cell population at the memory cell timepoint in ICOSL^{-/-} mice to identify which, if any, memory cell subsets were affected. As infection with Lm-2W1S induces a Th1 response, Tem cells were functionally identified by the rapid production of both IL-2 and IFN- γ upon restimulation with 2W1S peptide. Tcm cells were identified by the production of IL-2 only; they do not produce IFN- γ until after proliferation into effector cell types (Pepper et al., 2011). Strikingly, we found that the number of both Tcm and Tem cells were both significantly reduced in ICOSL^{-/-} mice yet neither population was completely abrogated (Figure 4.9 A and B). At 28 dpi there was approximately a 10-fold decrease in the total number of 2W1S:I-A^b⁺ CD4⁺ T cells compared to only a 3.5-fold difference at day 7 (Figure 4.9 C and D). This indicated that either ICOS:ICOSL signalling was required after day 7 or that ICOS signalling in the primary response is required to form an optimal CD4⁺ memory T cell pool.

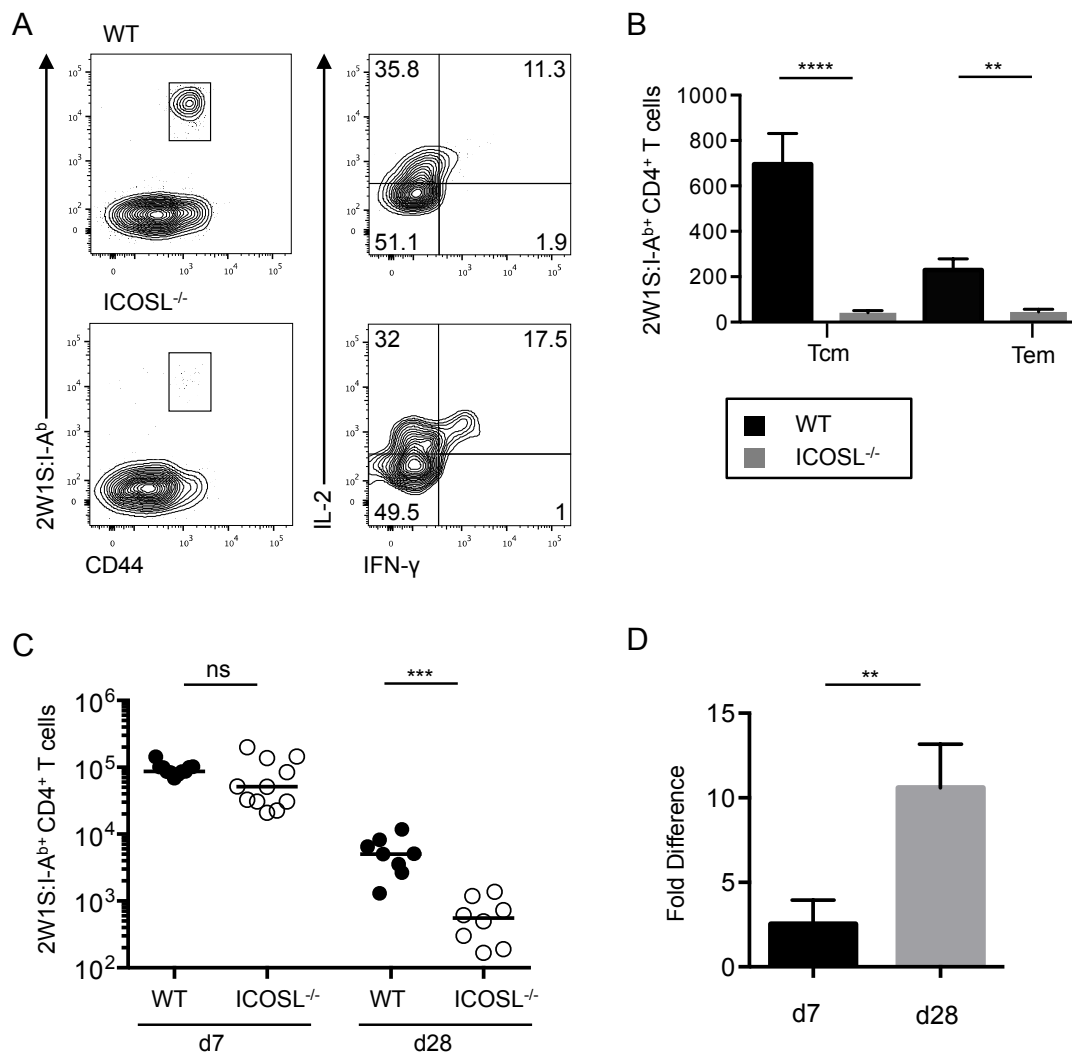


Figure 4.9: Generation of both Tem and Tcm cells is impaired in the absence of signals through ICOS. WT and ICOSL^{-/-} mice were infected with Lm-2W1S and 28 dpi restimulated *in vivo* with 2W1S peptide and LPS. Spleen and lymph nodes were harvested 4 hours post restimulation for analysis. (A) Flow cytometry plots showing expression of IL-2 and IFN- γ on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Tcm (IL-2⁺ IFN- γ ⁻) and Tem (IL-2⁺ IFN- γ ⁺) cells. (C) Number of 2W1S:I-A^b CD4⁺ T cells at 7 and 28 dpi in WT and ICOSL^{-/-} mice. (D) Fold difference in the median number of 2W1S:I-A^b CD4⁺ T cells at 7 and 28 dpi between WT and ICOSL^{-/-} mice.

(A) Plots are representative of 12 mice from 3 independent experiments. Values on plots are percentages. (B and C) Graphs show pooled data from ≥ 2 independent experiments. Bars show median. Mann-Whitney test: **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001, ns= p>0.05. (D) Graph shows pooled data from 3 independent experiments. Unpaired T-Test: **p $\leq$ 0.01.

The extent to which 2W1S:I-A^b CD4⁺ memory T cell formation was impaired in the absence of ICOS was surprising as previously ICOS was thought to be involved in the generation of either Tem or Tcm cells only. Additionally, only a minimal impact was observed on non-TFH cells in the primary response. CD28 however is a powerful costimulatory molecule involved in the activation of T cells and in the absence of CD28 T cell proliferation is significantly reduced. As CD80^{-/-}CD86^{-/-} mice show significant impairment in the expansion of CD4⁺ T cells in a primary response this will likely impact upon the memory cell pool. To compare memory cell generation in the absence of ICOS signals with a lack of CD28 signalling, WT and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S and the number of 2W1S:I-A^b CD4⁺ memory T cells analysed 28 dpi. The number of 2W1S:I-A^b CD4⁺ memory T cells recovered from CD80^{-/-}CD86^{-/-} was significantly diminished compared to WT mice (Figure 4.10 A and B). As well as a dramatic decrease in the number of 2W1S:I-A^b CD4⁺ memory T cells, they were also non-functional in that they were unable to produce cytokines IL-2 and IFN-γ (Figure 4.10 A and C).

4.2.7 Blocking anti-ICOS mAb inhibits CD4⁺ memory T cell formation

The ICOS:ICOSL pathway is a potential therapeutic target; in the primary response blocking ICOS impairs TFH cell development and therefore limits the humoral response.

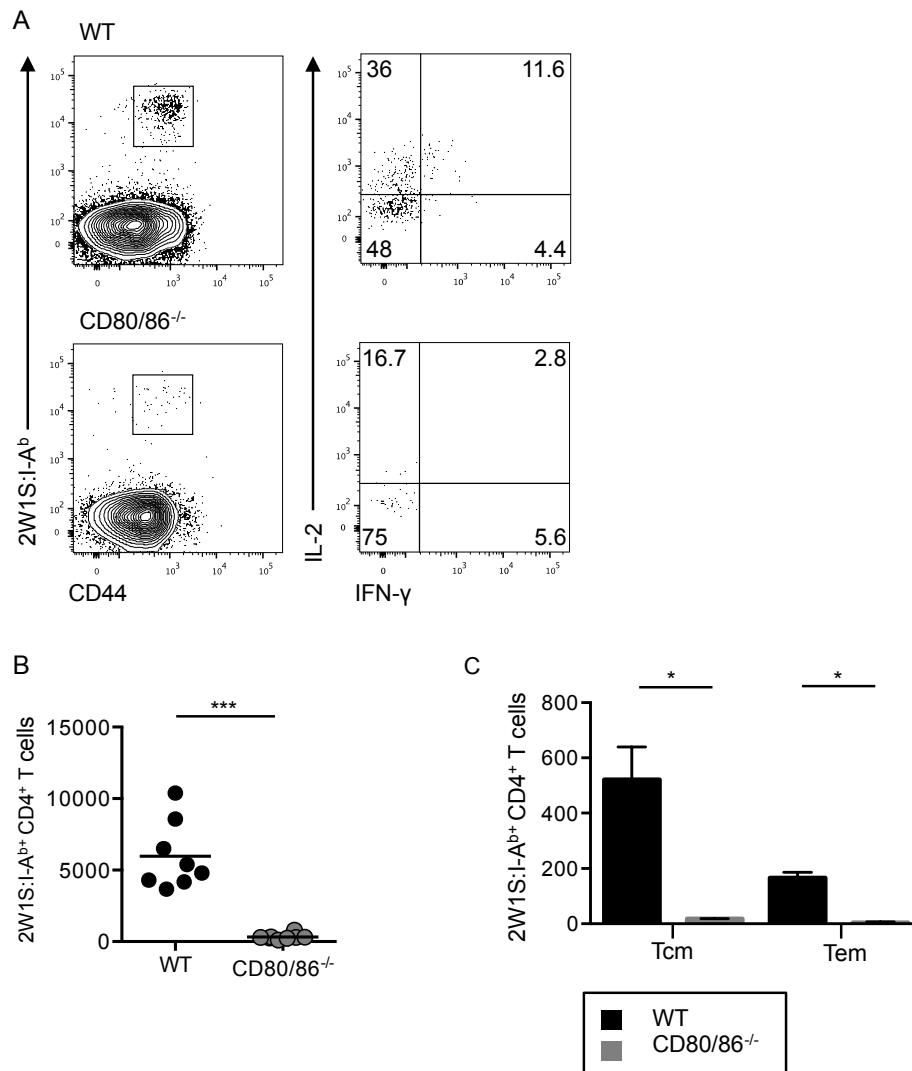


Figure 4.10: Formation of functional 2W1S:I-A^b+ CD4⁺ memory T cells requires CD28 costimulation. WT and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S and restimulated *in vivo* with 2W1S peptide and LPS 28 dpi. Spleen and lymph nodes were harvested 4 hours post restimulation for analysis. (A) Flow cytometry plots showing expression of IL-2 and IFN- γ on 2W1S:I-A^b+ CD4⁺ T cells. (B) Number of 2W1S:I-A^b+ CD4⁺ memory T cells. (C) Number of 2W1S:I-A^b+ CD4⁺ Tcm (IL-2⁺ IFN- γ ⁺) and Tem (IL-2⁺ IFN- γ ⁺) cells.

(A) Plots are representative of 8 mice from 2 independent experiments except for cytokine staining which is representative of 4 mice from 1 independent experiment. Values on plots are percentages. (B) Graph shows pooled data from 2 independent experiments. Bars show median. (C) Graph shows data from 1 independent experiment. Bars show median. Mann-Whitney test: * $p \leq 0.05$, *** $p \leq 0.001$.

Blocking this costimulatory pathway may alleviate autoimmune diseases that are antibody mediated. Generation of the 2W1S:I-A^b CD4⁺ memory T cell population was severely impaired in ICOSL^{-/-} mice and it is clear that memory T cells may play an important role in autoimmune diseases such as systemic lupus erythematosus and type 1 diabetes (Devarajan and Chen, 2013). Since blocking anti-ICOS mAb were successfully used to mimic the effect of genetic absence of ICOSL^{-/-} on the primary CD4⁺ T cell response, the effect of these reagents on the memory cell response was investigated following infection with Lm-2W1S. Blocking anti-ICOS mAb were administered on day 0 and 4 following infection and mice were again analysed for 2W1S:I-A^b memory T cells 28 dpi. As seen in ICOSL^{-/-} mice, although both functional populations of memory cells were present (Figure 4.11 A), there was a significant decrease in the overall number of 2W1S:I-A^b CD4⁺ memory T cells (Figure 4.11 B). Again, this resulted from a significant decrease in both T_{cm} and T_{em} cell populations (Figure 4.11 C).

4.2.8 The persistence of antigen-specific CD4⁺ memory T cells does not require ICOS or CD28 signalling

2W1S:I-A^b CD4⁺ memory T cells clearly require signals through both ICOS and CD28 for their generation. It is less clear whether CD4⁺ memory T cells continue to require costimulatory signals once this population has formed. Cytokines such as IL-7 and IL-15 are known to be required for CD4⁺ memory T cell persistence (Purton et al., 2007, Kondrack et al., 2003).

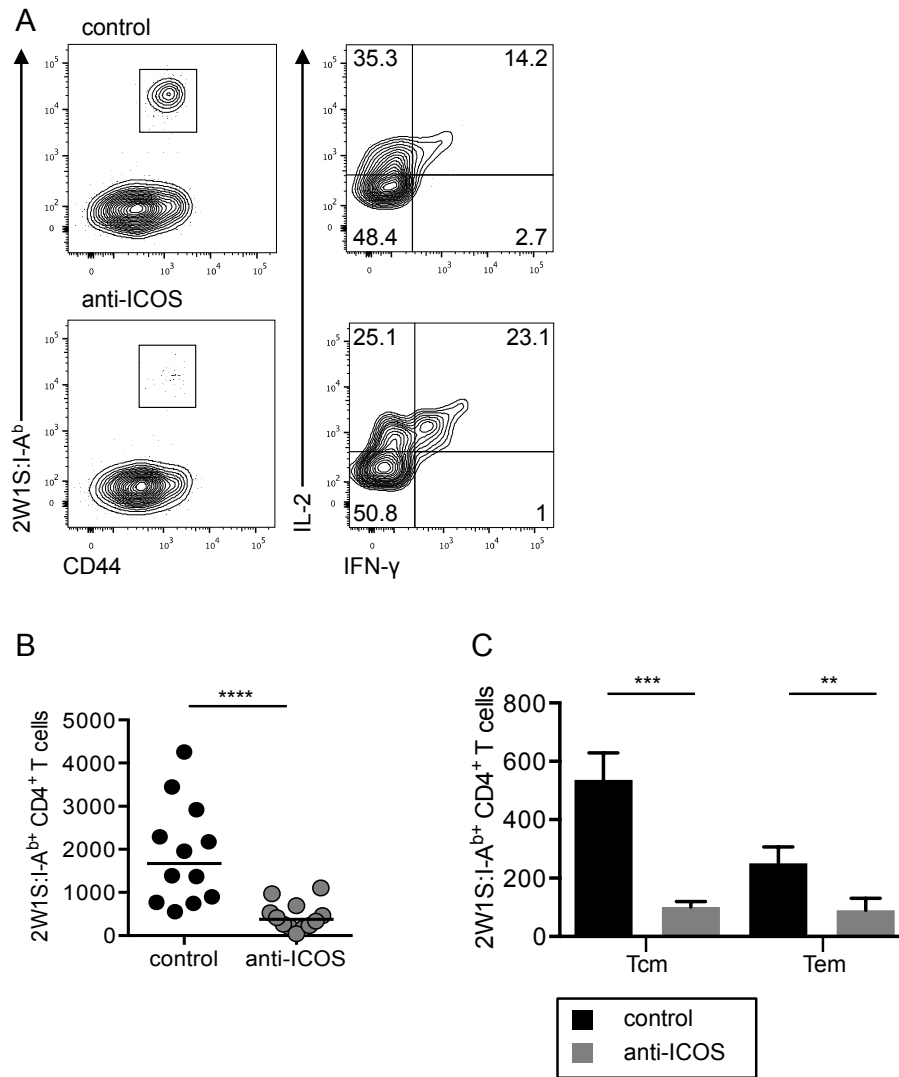


Figure 4.11: Blocking anti-ICOS mAb prevents formation of 2W1S:I-A^b CD4⁺ memory T cells. WT mice were infected with Lm-2W1S and restimulated *in vivo* with 2W1S peptide and LPS 28 dpi. Mice received either blocking anti-ICOS or control mAb 0 and 4 dpi. Spleen and lymph nodes were harvested 4 hours post restimulation for analysis. (A) Flow cytometry plots showing expression of IL-2 and IFN- γ on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ memory T cells. (C) Number of 2W1S:I-A^b CD4⁺ Tcm (IL-2⁺ IFN- γ ⁻) and Tem (IL-2⁺ IFN- γ ⁺) cells.

(A) Plots are representative of 12 mice from 3 independent experiments. Values on plots are percentages. (B and C) Graphs show pooled data from 3 independent experiments. Bars show median. Mann-Whitney test: **p<0.01, ***p<0.001, ****p<0.0001.

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Whether CD4⁺ memory cells require periodic signals through costimulatory receptors for their continued survival is less well understood. OX40 has been suggested as a candidate for enhancing the survival of memory cells (Withers et al., 2009) however we were unable to see a significant effect on 2W1S:I-A^b⁺ CD4⁺ memory T cell survival in the absence of OX40 costimulation.

To investigate the contribution of CD28 signals in CD4⁺ memory T cell persistence, the 2W1S:I-A^b⁺ CD4⁺ T cell population in CTLA4Ig mice was assessed following infection with Lm-2W1S. The number of 2W1S:I-A^b⁺ CD4⁺ T cells was tracked in WT and CTLA4Ig mice from the primary response through to the memory phase. 7 dpi there was around a 2.5 fold difference in the number of 2W1S:I-A^b⁺ CD4⁺ T cells (Figure 4.12 A; median for WT: 44 221, CTLA4Ig: 16495). This difference was increased to approximately 4-fold at day 28 but was then maintained until day 80 post infection indicating that partial blockade of the CD28 pathway with constitutive production of CTLA4Ig does not seem to impair the survival of an endogenous memory cell population (Figure 4.12 A; d28 median for WT: 12 415, CTLA4Ig: 3235; d80 median for WT: 9583, CTLA4Ig: 2151).

Blocking anti-ICOS mAb was effective in limiting the size of primary and memory CD4⁺ T cell populations when administered following antigenic stimulation. To determine if CD4⁺ memory T cells require continued interactions for survival, blocking anti-ICOS mAb was used to temporally block ICOS signalling in WT mice once memory T cells had been formed.

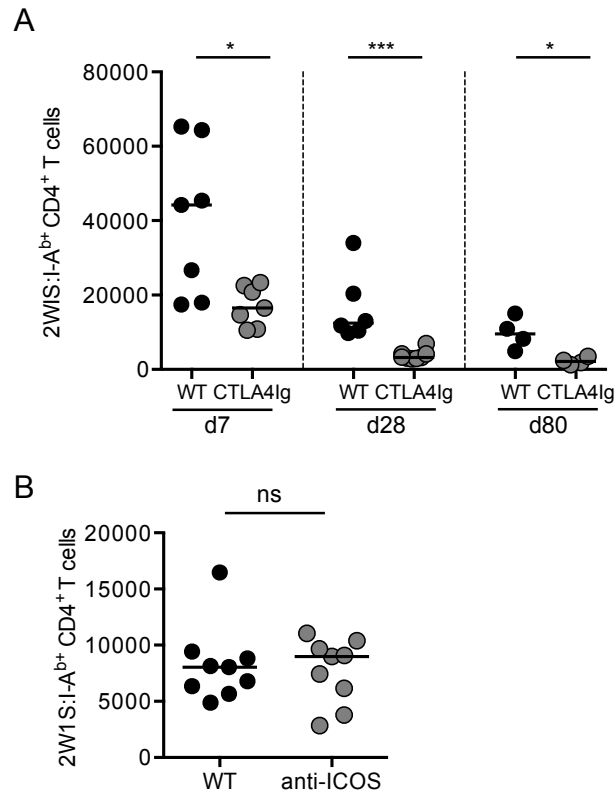


Figure 4.12: Combined blockade of ICOS and CD28 signals does not affect the persistence of 2W1S:I-A^b CD4⁺ memory T cells once formed. (A) WT and CTLA4Ig mice were infected with Lm-2W1S and 2W1S:I-A^b CD4⁺ T cells were enumerated 7, 28 and 80 dpi. (B) WT mice were infected with Lm-2W1S and at 28 dpi blocking anti-ICOS or control IgG mAb were administered twice weekly for 4 weeks. 2W1S:I-A^b CD4⁺ memory T cells were then enumerated from spleen and lymph nodes.

(A and B) Graph shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: * $p \leq 0.05$, *** $p \leq 0.001$, ns= $p > 0.05$.

This approach would circumvent the problems associated with analysing memory T cell populations in mice where priming is impaired. For example experiments using CD30^{-/-}OX40^{-/-} mice concluded a problem with memory cell survival in the absence of these TNFR, however it is difficult to differentiate this from the effect of the absence of these receptors on the survival of effector CD4⁺ T cells following priming (Gaspal et al., 2005). CD4⁺ T cells responding to Lm-2W1S undergo a period of intense proliferation peaking 7 dpi. The population then undergoes a massive contraction due to apoptosis ending around 20 dpi, before forming a stable population of memory cells that decrease gradually in number over the following year (Pepper et al., 2011). To model a lack of ICOS signals only after memory cell formation, WT mice were infected with Lm-2W1S and after 28 days blocking anti-ICOS or control mAb were administered twice weekly for 4 weeks. The number of 2W1S:I-A^b CD4⁺ memory T cells recovered from mice receiving blocking anti-ICOS or control mAb was not significantly different, indicating ICOS signals are not required for their survival in this time period (Figure 4.12 B).

Since blocking ICOS mAb blockade may not be complete and CTLA4Ig is a model only of limited costimulation we sought to confirm these results using another model system that enables the survival of WT CD4⁺ memory T cells to be assessed. The transfer of tetramer specific CD4⁺ T cell populations is technically challenging due to their low frequency. Moreover, the magnetic enrichment method used to purify 2W1S:I-A^b CD4⁺ T cells binds via the TCR, adding additional caveats associated with TCR stimulation. For these reasons allotype

marked TCR transgenic SM1 CD4⁺ memory T cells were used to evaluate WT memory cell survival in genetically deficient hosts. Outlined in the schematic in Figure 4.13 A, 10 000 allotype marked SM1 CD4⁺ T cells were transferred into WT mice that were then immunised with Lm-FliC the following day to expand the SM1-specific population. Mice were left until day 28 post infection to form an SM1 CD4⁺ memory T cell population that was then harvested from spleen and lymph nodes and subsequently enriched using allotype markers. Approximately 20 000- 40 000 SM1 CD4⁺ memory T cells were transferred into WT and genetically deficient hosts for comparison. SM1 CD4⁺ memory T cells were identified by the gating strategy shown in Figure 4.13 B. SM1 CD4⁺ memory T cells are CD3^{lo} so a large gate is drawn to take account of this; other cell lineages are excluded using the markers B220, CD11b and CD11c. They are then identified by expression of CD4, CD45.1 and CD45.2. All SM1 CD4⁺ memory T cells were checked for expression of CD44 and found to be CD44^{hi} (Figure 4.13 C). Following transfer of SM1 CD4⁺ memory T cells into WT, ICOSL^{-/-}, CTLA4Ig and CD80^{-/-}CD86^{-/-} mice there were no significant differences in the number of memory cells recovered (Figure 4.13 D-F). Data shown in Figure 4.13 D and E are representative of 2 independent experiments, as due to the variable nature of memory cell transfers it was unfeasible to pool data. Within experimental repeats, no difference was observed in the number of recovered SM1 CD4⁺ memory T cells in costimulation deficient mice compared to WT controls. These data are consistent with our previous findings using endogenous 2W1S:I-A^{b+} CD4⁺ T cells; blockade of ICOS or CD28 signals did not impair the persistence of established memory CD4⁺ T cells once formed.

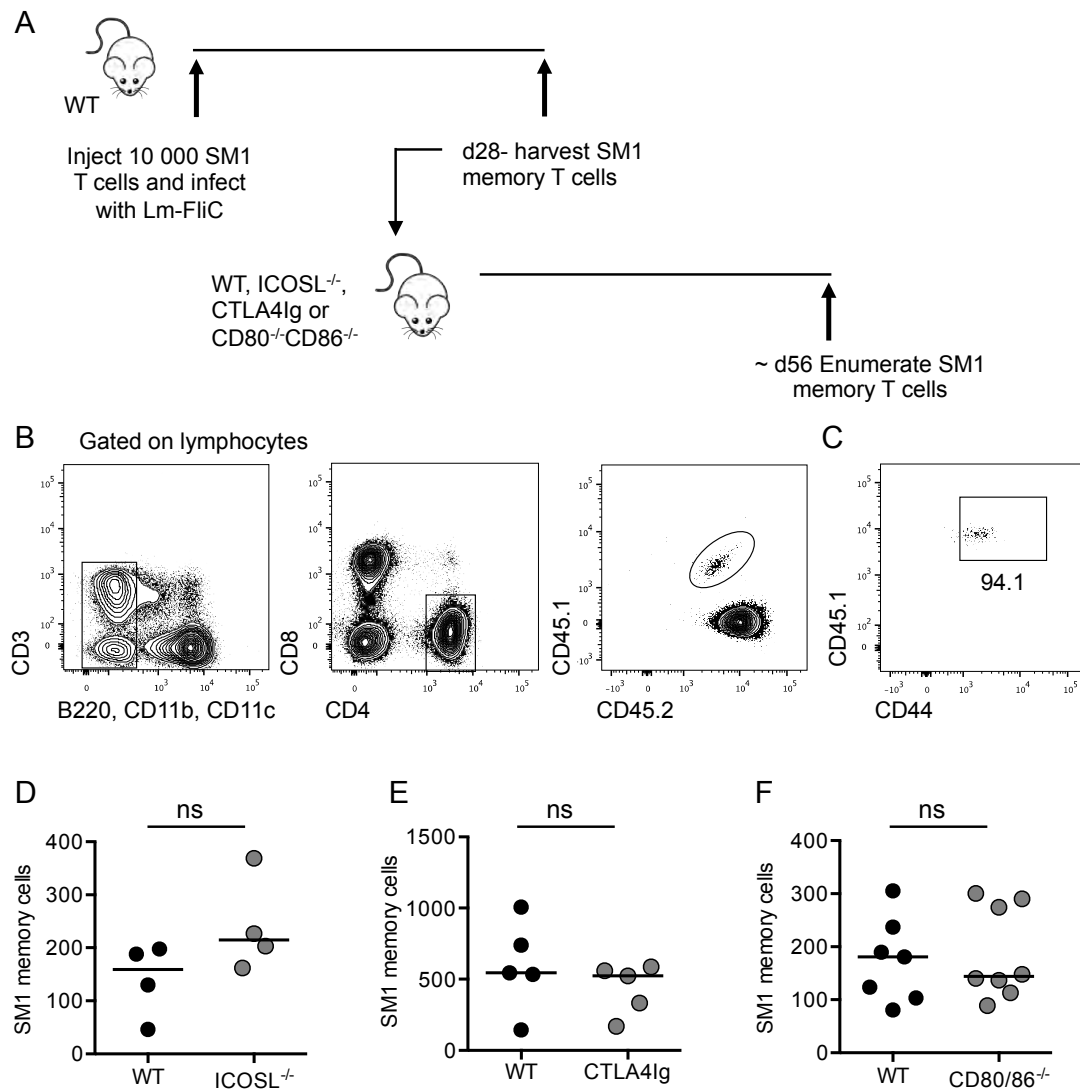


Figure 4.13: Persistence of CD4⁺ memory T cells generated in WT mice is not dependent on ICOS or CD28 signals. WT mice were injected with 10 000 SM1 CD4⁺ T cells and infected the following day with Lm-FliC. Mice were rested for 28 days enabling formation of an SM1 CD4⁺ memory T cell population. Spleen and lymph nodes were harvested and enriched for SM1 CD4⁺ memory T cells, which were injected back into new hosts (approximately 25 000 SM1 memory cells per mouse). Mice were analysed 28 days post transfer. (A) Schematic showing the protocol for transfer of SM1 CD4⁺ memory T cells. (B) Flow cytometry plots showing the gating strategy for identification of SM1 CD4⁺ memory T cells; gated on lymphocytes, B220⁻CD11b⁻CD11c⁻, CD4⁺CD8⁻, CD45.1⁺CD45.2⁺. (C) Representative plot showing expression of CD44 on SM1 CD4⁺ memory T cells. (D) Number of SM1 CD4⁺ memory T cells recovered from WT and ICOSL^{-/-}. (E) Number of SM1 CD4⁺ memory T cells recovered from WT and CTLA4lg mice. (F) Number of SM1 CD4⁺ memory T cells recovered from WT and CD80^{-/-}CD86^{-/-} mice.

(B and C) Plots are representative of >12 mice from >3 independent experiments. Values on plots are percentages. (D-E) Graphs show representative data from 2 independent experiments. (F) Graph shows pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: ns = p > 0.05.

4.2.9 Following secondary challenge ICOS is required for the generation of TFH cells

Analysis of the requirements for the generation and survival of 2W1S:I-A^b CD4⁺ memory T cells following Lm-2W1S infection indicated that ICOS signals were required for the formation of this population, but not for subsequent survival following the contraction phase. Expansion of CD4⁺ memory T cells upon secondary challenge is thought to be less reliant on costimulatory signals due to the enhanced state of activation in which they reside (London et al., 2000, Croft et al., 1994). To investigate the specific requirement of antigen-specific CD4⁺ memory T cells for ICOS signals upon re-encounter of antigen, blocking mAb was used to temporally control ICOS signalling. WT mice were infected with Lm-2W1S and challenged again with Lm-2W1S 28 dpi. Blocking anti-ICOS or control mAb were administered the day before and after secondary challenge to ensure blockade at the point of memory cell expansion. There was no difference in the expansion of T_{cm} cells, however a small, but significant, difference was observed in the expansion of T_{em} cells (Figure 4.14 A and B). Interestingly 2W1S:I-A^b TFH cell expansion was severely impaired by ICOS blockade, as was seen in the primary response (Figure 4.14 A and B).

To additionally assess CD4⁺ memory T cell expansion in the genetic absence of ICOSL, SM1 CD4⁺ memory T cells were generated in WT mice and transferred into WT and ICOSL^{-/-} hosts. Mice were then challenged with Lm-FliC 28 days post transfer and SM1 CD4⁺ T cells were enumerated 4 days post secondary challenge.

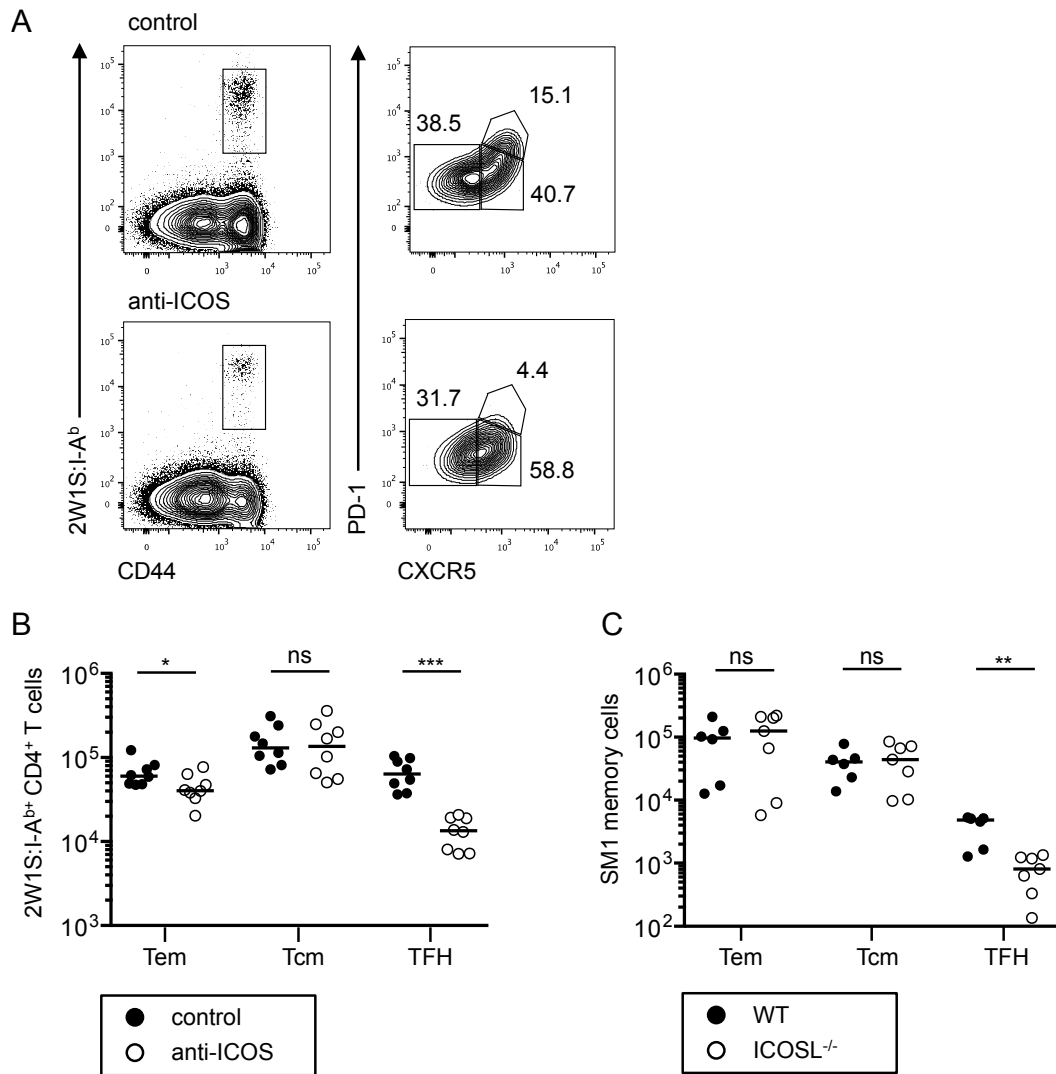


Figure 4.14: ICOS is required for the generation of TFH cells following secondary challenge. WT mice were infected with Lm-2W1S and at 28 dpi challenged with Lm-2W1S. Mice received blocking anti-ICOS or control mAb the day before and after challenge. 2W1S:I-A^b CD4⁺ T cells were enumerated from spleen and lymph nodes 3 days later. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Tem, Tcm and TFH cells.

(C) WT and ICOSL^{-/-} received approximately 25 000 WT SM1 CD4⁺ memory T cells. Mice were then challenged with Lm-FliC 28 days post transfer. SM1 CD4⁺ memory T cells were enumerated from spleen and lymph nodes 4 days later. Shown is the number of SM1 CD4⁺ Tem, Tcm and TFH cells.

(A) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages. (B-C) Graphs show pooled data from 2 independent experiments. Bars show median. Mann-Whitney test: *p≤0.05, **p≤0.01, ***p≤0.001, ns= p>0.05.

As observed with blockade of ICOS, the generation of TFH cells from SM1 CD4⁺ memory cells was dependent upon ICOSL, however the expansion of the other T cell populations was not affected (Figure 4.14 C). Combined, these two approaches indicate that TFH cells remain reliant on ICOS for expansion following secondary challenge despite the convention that the costimulatory requirements of memory cells are reduced.

To further investigate the costimulatory requirement of CD4⁺ memory T cells upon secondary challenge, the requirement for CD28 signals at this stage was also assessed. Previously it has been thought that the expansion of CD4⁺ memory T cells is independent of CD28 costimulation due to a reduced requirement for TCR stimulation for activation (Salomon and Bluestone, 2001). These ideas were mostly based on *in vitro* experiments showing that memory cells could proliferate in the absence of B7 ligands (London et al., 2000, Croft et al., 1994). However more recently published data has challenged this paradigm showing that influenza virus- and OVA-specific memory cell proliferation was inhibited by CTLA4Ig treatment (Ndejmbi et al., 2006). SM1 CD4⁺ memory T cells generated in WT mice were transferred into WT and CD80^{-/-}CD86^{-/-} hosts then challenged 28 days post transfer with Lm-FliC. The number of responding cells was enumerated from the spleen and lymph nodes 4 days post secondary challenge. Unlike in ICOSL^{-/-} hosts, the expansion of all subsets of SM1 CD4⁺ memory T cells were reliant on signals through CD28, not just TFH cells (Figure 4.15 A and B).

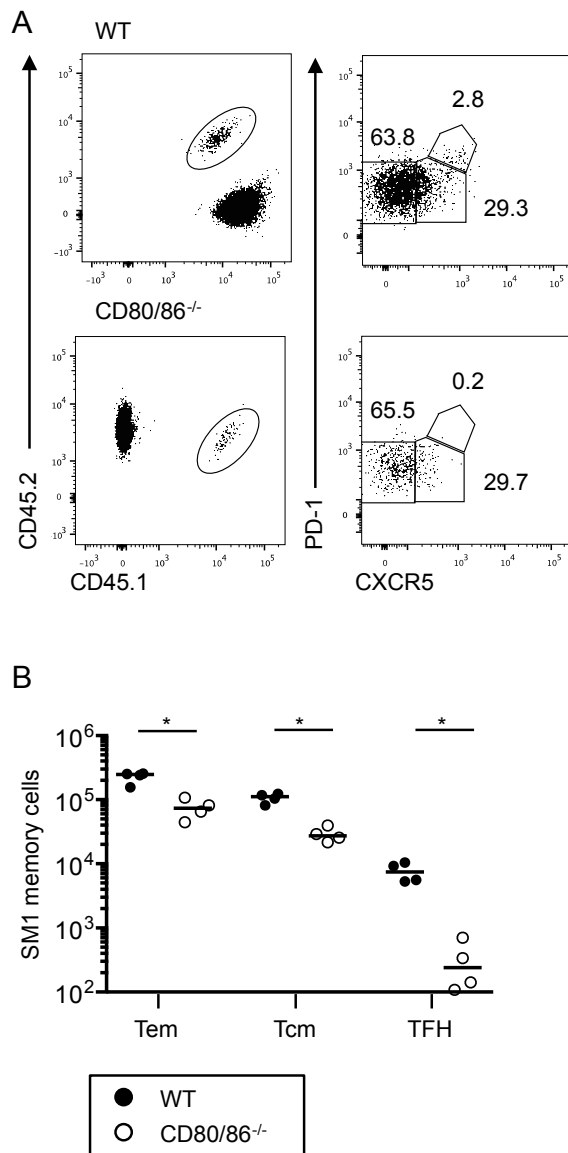


Figure 4.15: CD28 is required for the optimal generation of all T cell subsets following secondary challenge. WT and CD80^{-/-}CD86^{-/-} mice received approximately 25 000 SM1 CD4⁺ memory T cells. Mice were challenged with Lm-FliC 28 days post transfer. SM1 CD4⁺ T cells were enumerated from spleen and lymph nodes 4 days later. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on SM1 CD4⁺ T cells. (B) Number of SM1 CD4⁺ Tem, Tcm and TFH cells.

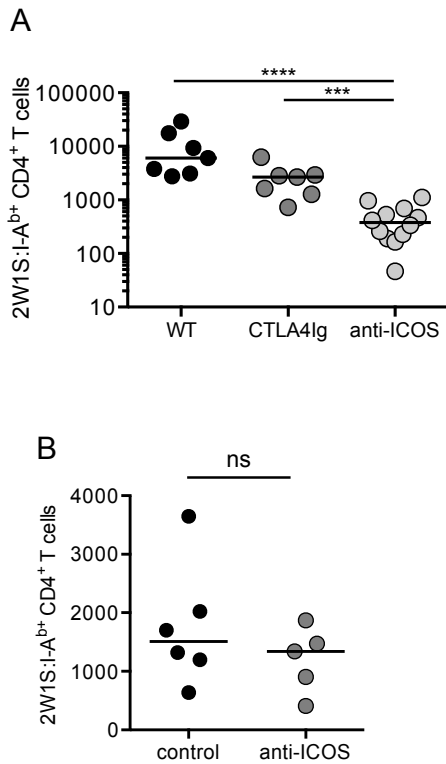
(A) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows representative data from 2 independent experiments. Bars show median. Mann-Whitney test: *p≤0.05.

These data are consistent with recent studies indicating that memory cells require costimulatory signals for optimal expansion upon re-encounter of antigen (Ndejembu et al., 2006).

4.2.10 Combination of blocking anti-ICOS mAb and CTLA4Ig enhances blockade of CD4⁺ T cell response

Antibody mediated blockade of ICOS in the primary response comprehensively blocked TFH cell formation, however was not as effective at limiting proliferation of Teff cell and Tcm cell precursor populations. Despite this, the generation of memory cells was significantly impaired. Constitutive expression of CTLA4Ig inhibited the proliferation of all T cell subsets in the primary response but was not as effective as would be expected considering the potent effect of CD28 on T cell proliferation; 2W1S:I-A^b CD4⁺ T cells were reduced by only 2.5 fold.

Surprisingly blocking anti-ICOS mAb caused a greater reduction in the memory cell population at day 28 than constitutive provision of CTLA4Ig (Figure 4.16 A; median for WT: 6076, CTLA4Ig: 2671, anti-ICOS: 377). There was a 10-fold reduction in the number of 2W1S:I-A^b CD4⁺ memory T cells following blocking anti-ICOS mAb compared to approximately 4-fold reduction in CTLA4Ig mice. A therapeutic approach combining these treatments may be the optimal way to effectively dampen CD4⁺ T cell responses. Furthermore, possible redundancy in the signalling cascades elicited by B7 family members may be circumvented by the combination of two blocking agents.



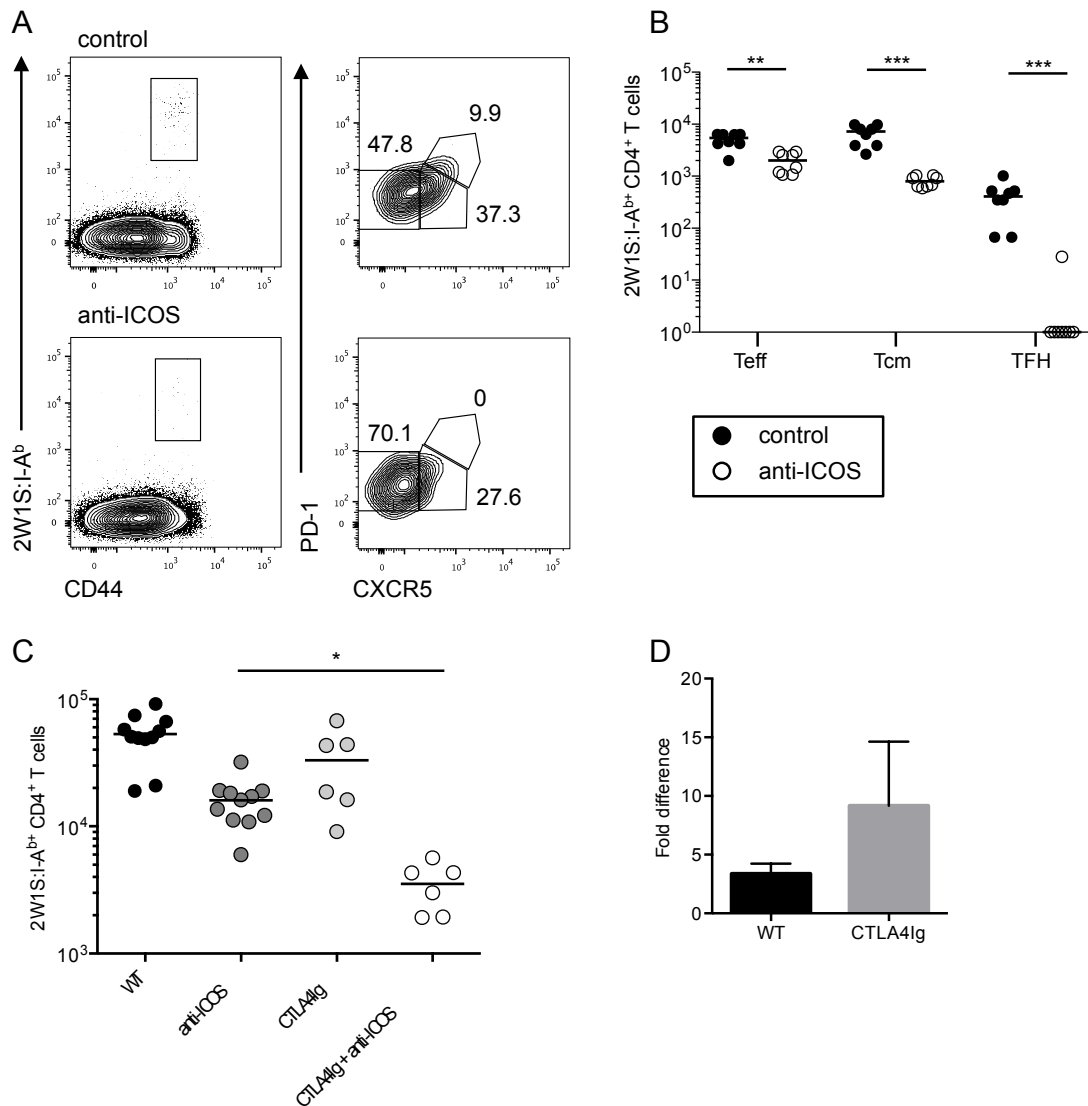
4.16: Blockade of ICOS and CD28 signalling does not impair 2W1S:I-A^{b+} CD4⁺ memory cell T survival. (A) WT, CTLA4Ig and anti-ICOS mAb treated mice were infected with Lm-2W1S and analysed for enumeration of 2W1S:I-A^{b+} CD4⁺ memory T cells 28 dpi. Blocking anti-ICOS mAb were administered at day 0 and 4 post infection. (B) CTLA4Ig mice were infected with Lm-2W1S and at 28 dpi blocking anti-ICOS or control mAb were administered twice weekly for four weeks. 2W1S:I-A^{b+} CD4⁺ memory T cells were then enumerated from spleen and lymph nodes.

(A-B) Graph shows data pooled from 2 independent experiments. Bars show median. Mann Whitney test: *** $p < 0.001$, **** $p < 0.0001$, ns= $p > 0.05$.

Previously, a combination of blocking anti-ICOS mAb and CTLA4Ig has been used to successfully prevent cardiac allograft rejection in transplant models (Kosuge et al., 2003). Blocking ICOS:ICOSL interactions also suppressed ongoing T cell responses in autoimmune EAE (Sporici et al., 2001). Combining blocking anti-ICOS mAb and CTLA4Ig targets both cellular and humoral responses, through their impact on T cell responses. Since the survival of 2W1S:I-A^b CD4⁺ memory T cells was not impaired in CTLA4Ig mice or with blocking anti-ICOS mAb alone we combined these approaches to investigate whether this reflected redundancy in the requirement for these signals. CTLA4Ig mice infected with Lm-2W1S received blocking anti-ICOS mAb for 4 weeks from 28 dpi, however the number of 2W1S:I-A^b CD4⁺ memory T cells recovered was not significantly different from control mice (Figure 4.16 B). This demonstrates that the persistence of 2W1S:I-A^b CD4⁺ memory T cells in the absence of either CD28 or ICOS is not due to compensatory signalling from ICOS or CD28 respectively.

In the primary response to Lm-2W1S only a modest reduction in 2W1S:I-A^b CD4⁺ T cell number was observed in both CTLA4Ig mice and with the use of blocking anti-ICOS mAb. To investigate a dual therapeutic approach CTLA4Ig mice were infected with Lm-2W1S and received blocking anti-ICOS or control mAb. 7 dpi there was a significant decrease in all 2W1S:I-A^b CD4⁺ T cell subsets and TFH cells were absent (Figure 4.17 A and B). In CTLA4Ig mice treated with blocking anti-ICOS mAb there was a significant decrease in the number of 2W1S:I-A^b CD4⁺ T cells compared to WT mice treated with anti-ICOS mAb alone (Figure 4.17 C). There was a 9-fold decrease for CTLA4Ig anti-ICOS mAb treated

mice compared to only a 3.5-fold decrease for WT anti-ICOS mAb treated mice (Figure 4.17 D). This indicates that combined CTLA4Ig and anti-ICOS mAb treatment is superior to either reagent alone for therapeutic blockade of the primary CD4⁺ T cell response.



4.17: Therapeutic combination of blocking anti-ICOS mAb and CTLA4Ig enhances blockade of primary CD4⁺ T cell response. CTLA4Ig mice were infected with Lm-2W1S and analysed 7 dpi. Mice received either blocking anti-ICOS or control mAb 0 and 3 dpi. (A) Flow cytometry plots showing expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells. (B) Number of 2W1S:I-A^b CD4⁺ Teff, T central memory precursor (Tcm) and TFH cells.

WT and CTLA4Ig mice were infected with Lm-2W1S and analysed 7 dpi. Mice received blocking anti-ICOS or control mAb 0 and 3 dpi. (C) Number of 2W1S:I-A^b CD4⁺ T cells enumerated from the spleen. (D) Fold difference in median number of 2W1S:I-A^b CD4⁺ T cells between mice treated with blocking anti-ICOS and control mAb for both WT and CTLA4Ig mice.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 3 independent experiments. Bars show median. Mann-Whitney test: **p < 0.01, ***p < 0.001. (C) Graph shows pooled data from 2 independent experiments. Bars show median. Kruskal Wallis test with post host Dunn's test: *p < 0.05. (D) Graph shows data from ≥ 2 independent experiments. Bars show median.

4.3 Discussion

Although there is a clear role for ICOS in the formation and maintenance of TFH cell populations, the importance of this receptor for other T cell subsets is less well defined, hindered by conflicting data. In this chapter I have dissected the requirement for ICOS during the generation and subsequent responses of antigen-specific CD4⁺ memory T cells and contrasted this with the role of CD28 signals. These data show that, unlike CD28 which is required for the optimal expansion of all T cell subsets, ICOS is required only for the development of TFH cells in the primary response as well as following secondary challenge. Both T_{cm} and T_{em} cells are dependent on ICOS for their generation, but not for their subsequent persistence once formed. The combination of blocking anti-ICOS mAb and CTLA4Ig was more effective at blocking antigen-specific CD4⁺ T cell expansion than either reagent alone, indicating this may provide a useful therapeutic option.

Originally thought to control the differentiation of Th2 cells, ICOS is now thought to have a more general role in controlling cytokine production by effector cells (McAdam et al., 2000, Tafuri et al., 2001, McAdam et al., 2001). However, the exact time in the response at which ICOS is required for an endogenous antigen-specific CD4⁺ T cell population responding to infection has not been dissected. In addition there are conflicting reports for the requirement of ICOS signalling for memory cell populations. Conventionally, memory cells are thought to be less reliant on costimulation for reactivation than naive T cells, however in Chapter 1 I have demonstrated that a lack of signalling through OX40 specifically impacts

upon the Tem cell population. Therefore in this chapter we sought to determine when and which memory cell subsets require signalling through ICOS and compare this to CD28, the molecule on which much of the early work suggesting costimulation is dispensable for memory cells was performed.

As observed previously, ICOS was not present on naive T cells but following activation expression of ICOS was detected at all timepoints analysed on both 2W1S:I-A^{b+} and SM1 CD4⁺ T cells, with a slightly lower level observed on memory cells (Hutloff et al., 1999, Mages et al., 2000). This expression of ICOS after TCR engagement indicates a prominent role for this costimulatory receptor in effector T cell responses. Unlike OX40, ICOS was expressed on 2W1S:I-A^{b+} CD4⁺ memory T cells at low levels suggesting they may receive either continuous or periodic signals through this receptor that contribute to their survival. To precisely dissect the requirements for ICOS, a combination of ICOSL^{-/-} mice and blocking anti-ICOS mAb were used, alongside the models established in the previous chapter, to investigate the role of this costimulatory receptor in CD4⁺ T cell responses. Formation of TFH cells was significantly impaired in the absence of ICOS for both 2W1S:I-A^{b+} and SM1 CD4⁺ T cells, consistent with previous data (Akiba et al., 2005, Nurieva et al., 2008, Choi et al., 2011). Teff cell formation was not affected in ICOSL^{-/-} mice suggesting that ICOS functions specifically to promote the generation or survival of TFH cells. As a comparison CD80^{-/-}CD86^{-/-} mice were used to show the effect of a lack of signalling through CD28. As expected from previous studies all CD4⁺ T cell subsets are reliant on CD28 for optimal primary expansion (Akiba et al., 2005, Shahinian et al., 1993).

The data surrounding the requirement of ICOS for CD4⁺ memory T cells is unclear since different studies have made conflicting conclusions on whether Tcm or Tem require ICOS signals for their development or survival. One study found a significant reduction of Tem cells in the steady state in ICOS^{-/-} mice, an effect which was amplified with age (Burmeister et al., 2008). However, this conclusion was drawn from the analysis of memory phenotype cells, which have been shown to be an independent population from antigen-specific memory cells with different requirements for survival signals (Purton et al., 2007, Lenz et al., 2004). These findings were corroborated by an antigen-specific study showing Tem cells depend on ICOS for survival (Moore et al., 2011). Adoptive transfer of TCR transgenic cells and influenza-specific tetramers were used to show ICOS was required specifically for the long-term survival of Tem cells and not their development. In contrast, a study by Pepper et al. revealed dependence on ICOS signals for generation of Tcm precursors and maintenance of this population once formed (Pepper et al., 2011).

My data demonstrate a clear role for ICOS signals in the generation of both 2W1S:I-A^b⁺ CD4⁺ Tcm and Tem cell populations. Further experiments are required to conclusively dissect whether this requirement is during the initial formation of these populations, or in the maintenance during the contraction phase of the response. Notably, amongst the small 2W1S:I-A^b⁺ CD4⁺ memory T cell population formed in the absence of ICOS signals, the percentage of functional Tcm and Tem cells was not altered in comparison to WT mice. This is consistent with data described for human patients with ICOS deficiency;

peripheral blood T cells were found to contain a reduced percentage of both Tcm and Tem cells (Takahashi et al., 2009). The 2W1S:I-A^b CD4⁺ memory T cell pool was significantly more impaired than at 7 dpi; a 10 fold reduction in the number of memory cells in ICOSL^{-/-} mice was observed compared to a 3.5 fold reduction at day 7. This finding suggests that ICOS signals may support the generation of CD4⁺ memory T cells by enhancing the initial development of antigen-specific cells that will survive long-term. Alternatively, ICOS signals may be required after initial proliferation to directly support the survival of cells that will enter the memory cell pool. Both of these effects could be achieved through the upregulation of anti-apoptotic molecules, such as Bcl-2, that permit survival through the contraction phase. Further careful manipulation of the timing of blocking anti-ICOS mAb administration could be used to dissect this.

Previous data have suggested there may be a role for costimulatory signals in the long-term persistence of memory CD4⁺ T cells. RORγt^{-/-} mice infected with Lm-2W1S showed normed CD4⁺ T cell expansion in the primary response, but had a significantly reduced memory cell pool indicating a role for RORγt⁺ ILC in memory cell survival (Withers et al., 2012). Constitutive expression of OX40L by ILC prompted us to test this observation in Chapter 3, however expression of OX40 was tightly linked to antigen-exposure. ICOSL is expressed by ILC3, (D. Withers unpublished observations), which are absent in RORγ^{-/-} mice, making ICOS:ICOSL interactions a possible mechanism enhancing memory CD4⁺ T cell survival.

We observed that ICOS, and also CD28, signals were dispensable for the persistence of both polyclonal 2W1S:I-A^b CD4⁺ memory T cells once formed and TCR transgenic SM1 memory cells formed in WT mice and transferred into new hosts. Furthermore a combined approach using blocking anti-ICOS mAb in CTLA4Ig mice did not affect memory cell survival, indicating that the lack of effect is not due to redundancy in these pathways. These data suggest that it is likely that signals from costimulatory molecules received during priming impact upon the survival of CD4⁺ T cells through the latter stages of the primary response, to form a functional memory cell pool. Clear evidence that periodic signals through costimulatory receptors regulate the survival of the memory cell population once formed is lacking and at present cytokines such as IL-7 and IL-15 appear to play the dominant role (Kondrack et al., 2003, Purton et al., 2007).

Upon secondary challenge we observed that antigen-specific CD4⁺ memory T cells show a differential requirement for signals through CD28 and ICOS.

Although many studies have previously thought CD28 to be unnecessary for reactivation of memory cells (Croft et al., 1994, London et al., 2000), here we show for WT TCR transgenic SM1 memory cells proliferation is significantly impaired in the absence of B7 ligands CD80 and CD86. These data are consistent with a study showing blocking CD28 signalling through injection of CTLA4Ig inhibited the expansion of CD4⁺ T cells following secondary challenge (Ndejembu et al., 2006). However in the absence of ICOS signals only SM1 TFH cells were significantly reduced with T_{cm} and T_{em} cells both expanding normally. This was also the case for polyclonal 2W1S:I-A^b CD4⁺ memory T cells, which showed a

significant impairment in TFH cell expansion when blocking anti-ICOS mAb were administered at secondary challenge. Secondary expansion of tetramer-specific CD4⁺ T cells has previously been shown to be impaired in ICOS^{-/-} mice (Mahajan et al., 2007). In this study mice were immunised s.c. with peptide in CFA, a robust adjuvant, which may account for the differences seen in our study. It is not yet known if the initial priming of CD4⁺ T cells impacts upon the costimulatory requirements of the resulting memory cell population upon secondary challenge.

We explored the use of mice constitutively expressing CTLA4Ig fusion protein as a model of limited costimulation analogous to treatment of human patients with Abatacept. Abatacept has had some success in the treatment of RA and has been promising in clinical trials to treat psoriasis (Ruderman and Pope, 2005, Kremer et al., 2003, Abrams et al., 1999). However other disease models have not been ameliorated by CTLA4Ig treatment, for example type 1 diabetes and ulcerative colitis (Orban et al., 2014, Orban et al., 2011, Sandborn et al., 2012). We sought to clarify the efficacy of CTLA4Ig in a mouse model by analysing the endogenous 2W1S:I-A^b CD4⁺ T cell population and comparing this to adoptively transferred SM1 CD4⁺ T cells. CTLA4Ig mice exhibited a reduced Treg cell compartment and impaired primary CD4⁺ T cells responses to infection with *L. monocytogenes*, both CD28 dependent processes. These mice also had smaller GC than WT mice following immunisation with Lm-2W1S and migration of SM1 CD4⁺ T cells to the follicle was impaired. CD80^{-/-}CD80^{-/-} mice analysed as a control in which CD28 signalling is absent were significantly more impaired, indicating that CTLA4Ig fusion protein causes a partial blockade. These data show that constitutive

expression of CTLA4Ig is effective in limiting CD28 costimulation and therefore provides a mouse model of Abatacept treatment.

As we observed that blocking anti-ICOS mAb were highly efficient at recapitulating the phenotype of ICOSL^{-/-} mice, we explored their potential for use as a therapeutic to limit CD4⁺ T cell responses. Following infection with Lm-2W1S, TFH cell formation was abrogated but non-TFH cells were largely unaffected. As constitutive expression of CTLA4Ig resulted in only partial blockade, we investigated whether combination of CTLA4Ig and anti-ICOS mAb could enhance immune blockade. Previously, combination of CTLA4Ig and anti-ICOS mAb have been used to successfully prevent cardiac allograft rejection in transplant models indicating this may be a possible way to enhance immune blockade (Kosuge et al., 2003). Following treatment of CTLA4Ig mice with anti-ICOS mAb a further reduction in the primary effector responses was seen compared to the use of either therapeutic alone.

In summary, unlike OX40 ICOS expression is maintained on 2W1S:I-A^b⁺ CD4⁺ T cells throughout the course of the response. This prompted us to investigate when ICOS signals are required by CD4⁺ T cells for either generation or survival and if there is a preferential requirement among T cells subsets. I have shown a clear role for ICOS signals in the generation of both Tcm and Tem cells alongside CD28. Although required for their initial formation, ICOS and CD28 were dispensable for the persistence of memory cells. The re-expansion of memory cells upon secondary challenge was dependent on CD28 but not ICOS, except for TFH cells, which remained ICOS dependent. Furthermore, therapeutic targeting

using anti-ICOS mAb was superior to CTLA4Ig in limiting memory cell generation. Dual targeting of CD28 and ICOS was more effective at blocking the primary response than using CTLA4Ig alone, highlighting the need for combinatorial approaches in the treatment of disease.

CHAPTER 5: INVESTIGATING THE MIGRATION OF

CD4⁺ MEMORY T CELLS

5.1 Introduction

Memory T cells have distinct migratory patterns from their naive counterparts.

Naive T cells are thought to recirculate throughout the lymph system, ready to encounter cognate antigen. Within 24 hours 70% of naive lymphocytes within a lymph node will have exited and migrated to other sites (Tomura et al., 2008).

Tcm cells also recirculate through lymphoid tissue and blood due to their expression of CCR7 and CD62L while Tem cells are thought to recirculate between the blood and non-lymphoid tissues providing a rapid response to reinfection. A third subset of memory cells, Trm, has recently been described which do not recirculate but are maintained within tissues (Masopust et al., 2010, Gebhardt et al., 2009). This population does not exhibit the recirculating properties characteristic of conventional memory cell subsets, but reside in non-lymphoid sites to mediate rapid responses to pathogenic challenge. A population of CD4⁺ memory T cells found in the skin appear to recirculate; exit from this tissue is mediated by low level expression of CCR7 therefore these cells are distinct from recirculating CCR7⁻ Tem cells (Bromley et al., 2013). This population has been termed recirculating memory T cells (Trcm) and also express CD62L and E-selectin ligand to enable recirculation between lymph nodes and peripheral tissue.

The migration of effector and memory CD4⁺ T cells is thought to be more diverse than their naive counterparts. This is due to a range of surface molecules that mediate their entry into peripheral tissues. CD44, LFA-1, $\alpha 4\beta 1$ -integrin and P-selectin glycoprotein ligand (PSGL-1) are more highly expressed on antigen-

experienced T cells than naive T cells (Ostermann et al., 2002, Hirata et al., 2000, Bonder et al., 2006, Shimizu et al., 1990). Other adhesion molecules and chemokine receptors are expressed on specific subsets of memory cells, which direct their migration to distinct sites. For example expression of cutaneous lymphocyte-associated antigen (a modified form of PSGL-1) and CCR4 enable T cells to home to the skin (Fuhlbrigge et al., 1997, Campbell et al., 1999).

Although Tcm cells have been defined by expression of CCR7 and CD62L, memory cells have been identified in lymph nodes that express a low or undetectable level of these molecules (Brinkman et al., 2008, Unsoeld and Pircher, 2005, Marzo et al., 2005). Therefore the composition of memory cell populations in the lymph node may be more complex than just Tcm cell alone. The entry of effector and memory cells into lymph nodes may be less dependent on CCR7 than for naive T cells due to the expression of additional surface molecules. E-selectin and $\alpha 4\beta 1$ integrin were found to enable CD8⁺ effector and memory T cells to enter lymph nodes even though they displayed low expression of CD62L (Brinkman et al., 2013). The convention that memory cells can be divided into Tcm and Tem based on expression of CD62L and CCR7, molecules that determine their migration patterns, has been questioned by a study analysing CD8⁺ memory T cells responding to infection with LCMV (Unsoeld and Pircher, 2005). Not only were expression of CD62L and CCR7 mixed, they also found significant number of Tcm cells present in non-lymphoid tissues. Many human memory cells expressing adhesion molecules required for entry into non-lymphoid tissues, such as $\alpha 4\beta 7$, were found to also express CCR7 (Campbell et

al., 2001). In addition, another study found that entry of CD4⁺ memory T cells into the lymph nodes from the blood did not require expression of CCR7 (Vander Lugt et al., 2013). Using mixed bone marrow chimeras to circumvent anomalies seen in CCR7^{-/-} mice, they showed that expression of CCR7 on antigen-experienced CD4⁺ T cells did not increase their representation in lymph nodes compared to CCR7⁻ cells. Antigen experienced cells were defined by expression of CD44 so this population will likely contain a mixture of effector cells and true memory cells that persist in the absence of antigen.

In Chapters 3 and 4 we noted that both Tcm and Tem cells defined functionally by cytokine production were present in secondary lymphoid tissue. This is despite the conventional definition of these subsets by differential expression of lymphocyte homing molecules, indicating they are located in distinct areas. This prompted us to investigate the location of Tem cells in secondary lymphoid tissue, specifically lymph nodes, and if these cells were recirculating through these areas or residing here. To this end we analysed the migratory behaviour of CD4⁺ T cells to determine the movement of naive, Tcm and Tem cells, using Kaede transgenic mice. These mice express the photoconvertible Kaede protein in every cell, which upon exposure to violet light irreversibly switches to red fluorescence. Unlike previous studies analysing memory T cell migration this method allows us to track the migration of a labelled population of antigen-specific that have originated from a known site. Utilising the model to track antigen-specific CD4⁺ T cells established in Chapters 3 and 4, our aim was to

better understand the composition of CD4⁺ memory T cell populations within secondary lymphoid tissue.

5.2 Results

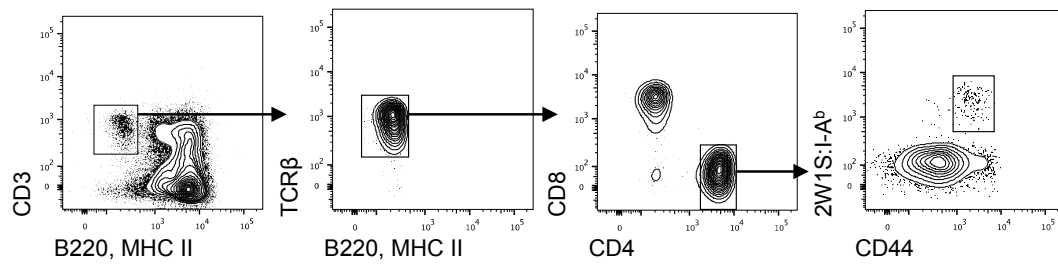
5.2.1 Exploring the location of 2W1S:I-A^b CD4⁺ memory

T cells

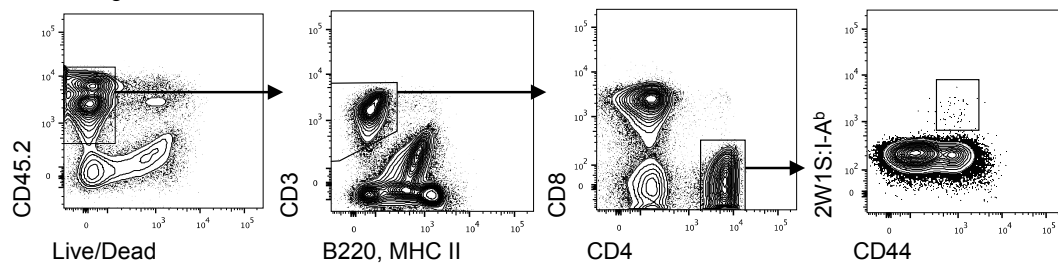
Following i.v. infection with Lm-2W1S two distinct memory cell populations have been identified that can be subdivided into Tcm and Tem cells based on their production of cytokines (Pepper et al., 2011). Conventionally Tcm cells have been identified as CCR7⁺CD62L⁺ cells, therefore express the molecules required for trafficking into lymph nodes and white pulp areas in the spleen (Sallusto et al., 1999, Forster et al., 1999). Tem cells however are CCR7⁻CD62L⁻, therefore should lack this ability, and are usually considered to traffic through peripheral, non-lymphoid tissues (Sallusto et al., 1999). Homing of lymphocytes to lymphoid tissue is an essential function required for immunosurveillance and is regulated by the expression of surface molecules such as integrins and L-selectin (CD62L) (Weninger et al., 2001, Stein et al., 2000). Entry of lymphocytes into lymph nodes takes place through HEV and this process is mediated by CD62L, which allows lymphocytes to roll on HEV (Marchesi and Gowans, 1964). Lymphocytes expressing CD62L bind PNA^d expressed by endothelial cells, which halts their circulation in the blood stream. In previous experiments we identified the presence of IL-2⁺ IFN- γ ⁺ 2W1S:I-A^b CD4⁺ Tem cells in lymph nodes and spleen. This was surprising, as Tem cells do not express CD62L and CCR7, markers required to enter these tissues. The presence of Tem cells in lymph nodes raises two possibilities. Firstly they are generated in this location following interaction with antigen, proliferation and differentiation and have

remained resident since. Secondly they have migrated into lymphoid tissue by an alternate mechanism independent of CCR7 and CD62L. This observation prompted us to further investigate the migratory properties of CD4⁺ memory T cells. To establish where 2W1S:I-A^b⁺ CD4⁺ memory T cells are located following infection with Lm-2W1S, tissues were analysed from WT mice 28 dpi. Previous studies found that the majority of 2W1S:I-A^b⁺ CD4⁺ memory cells were located in secondary lymphoid tissue but a small population were identified in the bone marrow (Pepper et al., 2010). Therefore in addition to lymph nodes and spleen, the lungs were chosen to analyse as a representative non-lymphoid tissue. As memory cells have been shown to express a number of integrins that enable homing to specific tissues including CD11b (Sallusto et al., 1999, Baron et al., 1993), we excluded anti-CD11b and anti-CD11c from the stain to ensure we were not selectively gating out cells. Anti-B220 was retained to exclude B cells and anti-MHC II was included to label and gate out APC (Figure 5.1 A). After gating on CD3⁺B220⁻MHCII⁻ cells they were checked for expression of TCR β to ensure only T cells were analysed. 2W1S:I-A^b⁺ cells were then identified as CD4⁺CD8⁻CD44^{hi} (Figure 5.1 A). This gating strategy was used for the analysis of spleen and lymph node tissues and blood. To analyse lung tissue addition of a Live/Dead marker and anti-CD45.2 antibody were used to gate on viable lymphocytes before gating on CD3⁺B220⁻MHCII⁻, CD4⁺CD8⁻CD44^{hi} 2W1S:I-A^b⁺ T cells (Figure 5.1 B). This was necessary due to a high proportion of non-lymphoid cells present in the lungs and increased cell death in the preparation of lung tissue. To identify Tem and Tcm cells based on their cytokine production mice were restimulated *in vivo* with 2W1S peptide and LPS as described in section 2.9.2.

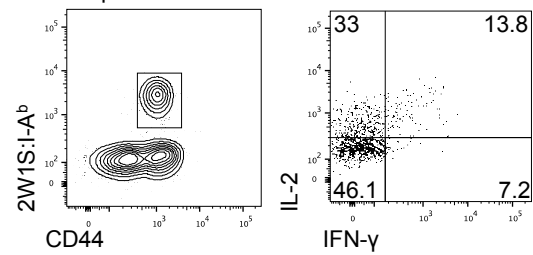
A Gated on lymphocytes
Spleen, LN and Blood



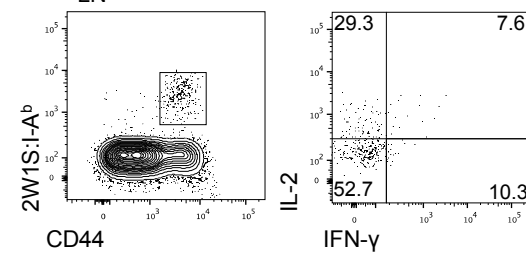
B Gated on lymphocytes
Lungs



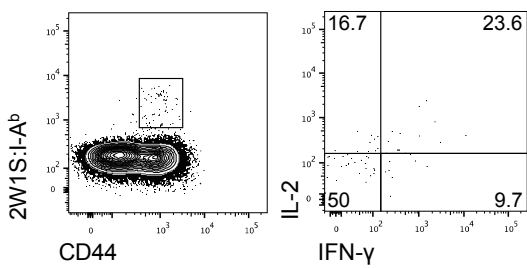
C Spleen



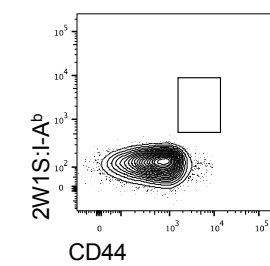
D LN



E Lungs



F Blood



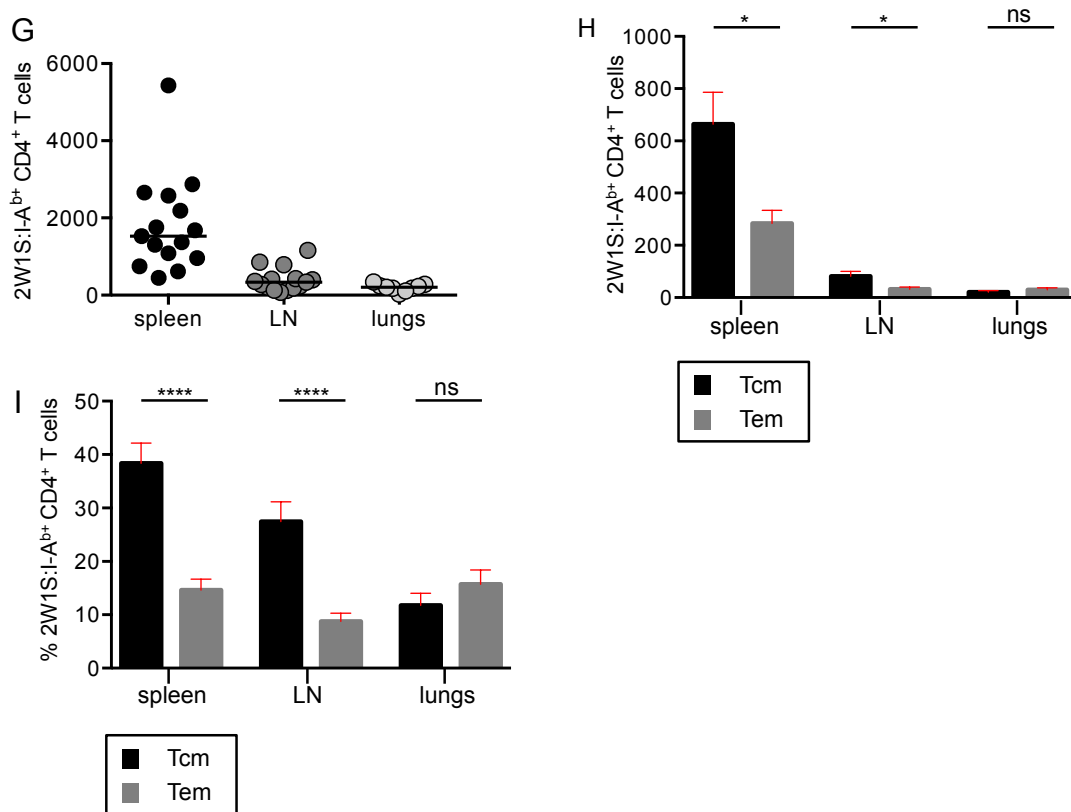


Figure 5.1: 2W1S:I-A^b CD4⁺ memory T cell populations are present in the spleen, lymph nodes and lungs but not in the blood. (A) Gating strategy for analysis of 2W1S:I-A^b CD4⁺ memory T cells in spleen, lymph nodes and blood. Gated on CD3⁺ B220⁻ MHCII⁻; TCRβ⁺; CD4⁺CD8⁻; 2W1S:I-A^b CD44^{hi} cells. (B) Gating strategy for analysis of 2W1S:I-A^b CD4⁺ memory T cells in the lungs. Gated on viable CD45.2⁺ cells using Live/Dead marker then CD3⁺ B220⁻ MHCII⁻; CD4⁺CD8⁻; 2W1S:I-A^b CD44^{hi} cells.

WT mice were infected i.v. with Lm-2W1S and analysed 28 dpi for location of 2W1S:I-A^b CD4⁺ memory T cells. Mice were restimulated *in vivo* with 100 µg 2W1S peptide and LPS and analysed after 4 hours to identify Tcm and Tem cells based on cytokine production. (C) IL-2 and IFN-γ production by 2W1S:I-A^b CD4⁺ memory T cells in the spleen, (D) lymph nodes, and (E) lungs. Values on plots are percentages. (F) Absence of 2W1S:I-A^b CD4⁺ memory T cells in blood. (G) Number of 2W1S:I-A^b CD4⁺ memory T cells in the spleen, lymph nodes and lungs. (H) Number and (I) Percentage of 2W1S:I-A^b CD4⁺ Tcm and Tem cells in the spleen, lymph nodes and lungs.

(A-F) Plots are representative of ≥9 mice from ≥2 independent experiments except E which is representative of 6 mice. Values on plots are percentages. (G) Graph shows pooled data from ≥2 independent experiments. Bars show median. (H and I) Graphs show pooled data from ≥2 independent experiments. Bars show mean and error bars show SEM. Mann Whitney Test: *p≤0.05, ****p≤0.0001, ns= non-significant.

As mice were infected with *Listeria monocytogenes*, which elicits a Th1 response, Tcm cells were identified as IL-2⁺IFN- γ ⁻ and Tem cells were identified as IL-2⁺IFN- γ ⁺. A population of CD44^{hi} 2W1S:I-A^b⁺ CD4⁺ memory T cells could be identified in spleen, lymph node and lung tissue, however none were present in the blood samples analysed (Figure 5.1 C-G). A population of Tcm and Tem cells were present in the spleen and lymph nodes however were less clear in the lungs (Figure 5.1 C-F and H). In the spleen and lymph nodes there was a significantly higher proportion of Tcm cells (Figure 5.1 I). Lung tissue showed a bias towards Tem cells however this was not a significant increase (Figure 5.1 I). Although there appeared to be a bias toward IFN- γ ⁺ cells in the lungs the overall number of 2W1S:I-A^b⁺ CD4⁺ T cells was very low making analysis of memory cell populations somewhat unreliable. Furthermore the method to stimulate cytokine produce in spleen and lymph node cells through i.v. injection may not be appropriate for use when trying to stimulate cells in the lung. Intranasal instillation with LPS has been shown to be more reliable in targeting the lungs than by the i.v. route (Szarka et al., 1997).

Analysis of CCR7 expression on 2W1S:I-A^b⁺ CD4⁺ T cells from spleen, peripheral lymph nodes and lungs was performed, as this chemokine receptor is required for entry into lymphoid tissue (Forster et al., 1999). Tcm cells have been defined by their expression of CCR7 and Tem cells by its absence (Sallusto et al., 1999). Expression of CCR7 was more widespread in lymphoid tissues. In the spleen and lymph nodes around 70% of 2W1S:I-A^b⁺ CD4⁺ T cells are CCR7⁺ whereas in the lungs only 25-45% are CCR7⁺ (Figure 5.2 A and B).

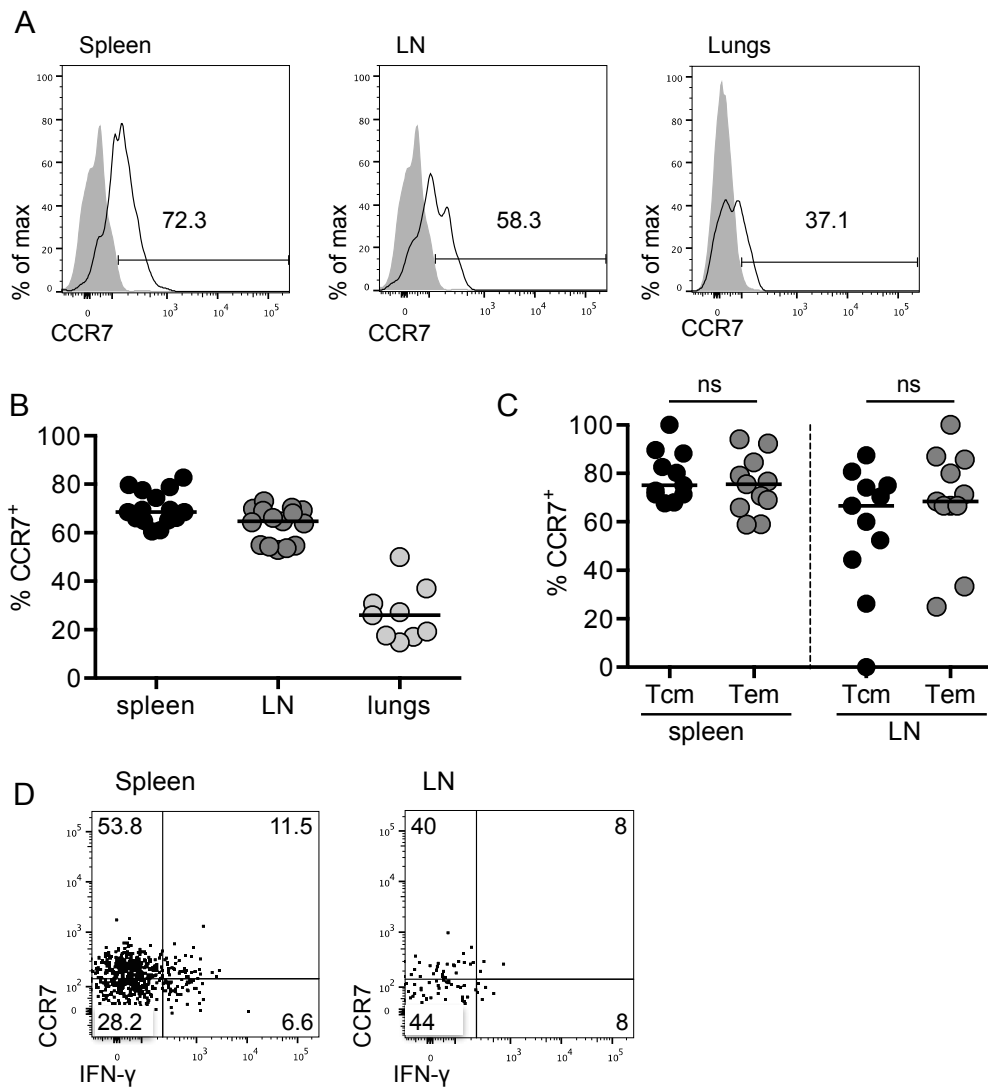


Figure 5.2: CCR7 expression does not correlate with Tcm cell phenotype. WT mice were infected i.v. with Lm-2W1S and analysed 28 dpi. Mice were restimulated *in vivo* with 100 µg 2W1S peptide and LPS and analysed after 4 hours to identify Tcm and Tem cells based on cytokine production. (A) Expression of CCR7 on 2W1S:I-A^b⁺ CD4⁺ memory T cells from the spleen, lymph nodes and lungs. (B) Percentage of 2W1S:I-A^b⁺ CD4⁺ memory T cells expressing CCR7 in the spleen, lymph nodes and lungs. (C) Percentage of 2W1S:I-A^b⁺ CD4⁺ Tcm and Tem cells expressing CCR7 from spleen and lymph nodes. (D) Expression of IFN-γ and CCR7 on 2W1S:I-A^b⁺ CD4⁺ memory T cells from the spleen and lymph nodes.

(A and D) Plots are representative of 15 mice from 3 independent experiments except for lungs which are representative of 9 mice from 2 independent experiments. Grey shaded areas on histograms represent relevant isotype control and values show percentage of CCR7⁺ cells. (B-C) Graphs show pooled data from ≥ 2 independent experiments. Bars show median. Mann Whitney Test: ns= non-significant.

This finding is consistent with previous data showing Tcm cells recirculate through lymph nodes, not through non-lymphoid sites (Reinhardt et al., 2001, Masopust et al., 2001). However, within the 2W1S:I-A^b CD4⁺ T cell population no significant difference in CCR7 expression was observed between IL-2⁺IFN- γ ⁻ Tcm cells and IL-2⁺IFN- γ ⁺ Tem cells, with IFN γ ⁺CCR7⁺ cells evident (Figure 5.2 C and D). Thus, expression of CCR7 on CD4⁺ memory T cells following Lm-2W1S infection does not seem to correlate with a Tcm phenotype.

5.2.2 Establishing a model to investigate the location and migration of 2W1S:I-A^b CD4⁺ memory T cells

To further investigate the location and migration of CD4⁺ memory T cells, a model in which antigen-specific cells could be tracked from their point of origin through different lymphoid and non-lymphoid tissues was required. To this end local immunisation in the paw pad was used, which initiates an immune response in the draining brachial and axillary lymph nodes. This method would allow us to track CD4⁺ T cells responding to cognate antigen in the brachial lymph node and their movement to other lymph nodes and the spleen. Previous observations in our laboratory have shown that infection with attenuated Lm-2W1S in the foot pad (an approach that can be used to model a local route of immunisation) did not elicit a large 2W1S:I-A^b CD4⁺ T cell response, perhaps due to a combination of the attenuated nature of the antigen and a non-physiological infection route (Emma Mackley- unpublished observations).

Therefore, immunisation with 2W1S peptide precipitated with aluminium was used given the robust response induced by this adjuvant (Glenny et al., 1931, Brewer et al., 1996).

WT mice were immunised in the right paw pad only, with 20 µg 2W1S peptide alum ppt or PBS as a control. 8 dpi draining brachial lymph node, contralateral lymph nodes (consisting of a pool of brachial, axillary and inguinal lymph nodes), mesenteric lymph node and spleen were harvested for analysis. This timepoint was chosen as aluminium precipitation of peptide has been shown to produce a prolonged response (Kool et al., 2008). This study found that after immunisation with alum ppt OVA, antigen-specific T cells were present in the draining lymph node at 7 dpi, however these cells were virtually absent at this timepoint after immunisation with OVA alone. We found that OX40 expression was maintained at day 9 following immunisation with alum ppt 2W1S peptide, indicating the possible continued presence of antigen (Chapter 3, Figure 3.15). Contralateral lymph nodes were analysed to assess trafficking from the site of immunisation via the bloodstream. This would exclude possible drainage solely through the lymphatics to lymph nodes that are connected in chains (Tomura et al., 2008, Braun et al., 2011).

Following immunisation in the paw pad with 20 µg 2W1S peptide, the 2W1S:I-A^b CD4⁺ T cell population was analysed for expression of CXCR5 and PD-1 to monitor the migration of different T cell subsets. A large population of 2W1S:I-A^b CD4⁺ T cells could be identified in the draining brachial lymph node and within this T_H1, T_H2 central memory precursors and T_H17 cells were present

(Figure 5.3 A and B; median for bLN 29 799). There was also a considerable population of 2W1S:I-A^b CD4⁺ T cells in the spleen and a smaller number in the contralateral and mesenteric lymph nodes (Figure 5.3 A and B; median for cLN 502, mLN 526, spleen 18 177). TFH cells could be identified in all tissues, with the majority found in the draining brachial lymph node and the spleen (Figure 5.3 A, C and D). TFH cells are thought to differentiate in the presence of antigen and reside in the follicle without migrating to other tissues (Suan et al., 2015). The presence of a small number of TFH cells in the contralateral and mesenteric lymph nodes and a large population in the spleen suggested that antigen had disseminated beyond the draining lymph node. Therefore this method cannot be used as a model of local immunisation to study the migration of 2W1S:I-A^b CD4⁺ T cells, as their origin would be unknown.

To refine this approach, the quantity of 2W1S peptide was reduced to 5 µg to restrict dissemination from the site of immunisation. Also, the injection site was moved further towards the palm of the paw to ensure the antigen would be contained within this area and not enter the circulation through blood vessels in the wrist. Following immunisation with 5 µg alum ppt 2W1S peptide a population of 2W1S:I-A^b CD4⁺ T cells could be identified in the draining brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen (Figure 5.4 A and B; median for bLN 8334, cLN 537, mLN 652, spleen 4370). The number of 2W1S:I-A^b CD4⁺ T cells in the draining brachial lymph node was reduced compared to mice that were immunised with 20 µg 2W1S peptide (median 8334 compared to 29 799).

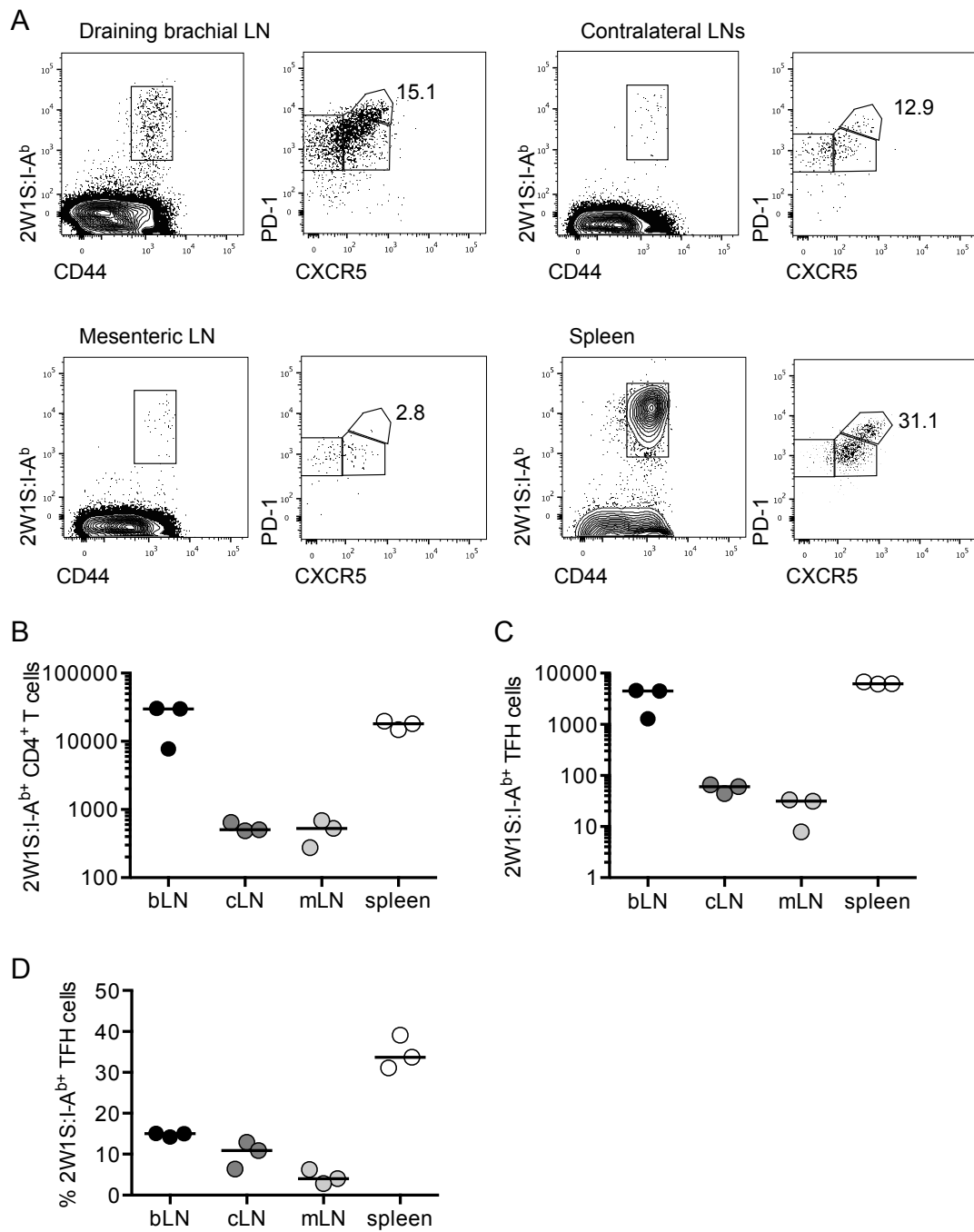


Figure 5.3: A model to investigate the location and migration of 2W1S:I-A^b CD4⁺ memory T cells. WT mice were immunised with 20 µg alum ppt 2W1S peptide in the right paw pad. Draining brachial lymph node, contralateral lymph nodes (brachial, axillary and inguinal), mesenteric lymph node and spleen were analysed 8 dpi. (A) Expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells from each tissue respectively. (B) Number of 2W1S:I-A^b CD4⁺ T cells from each tissue. (C) Number and ;(D) Percentage of 2W1S:I-A^b CD4⁺ TFH cells from each tissue.

(A) Plots are representative of 3 mice from 1 independent experiment. Values on plots are percentages. (B-D) Graphs show data from 1 independent experiment. Bars show median.

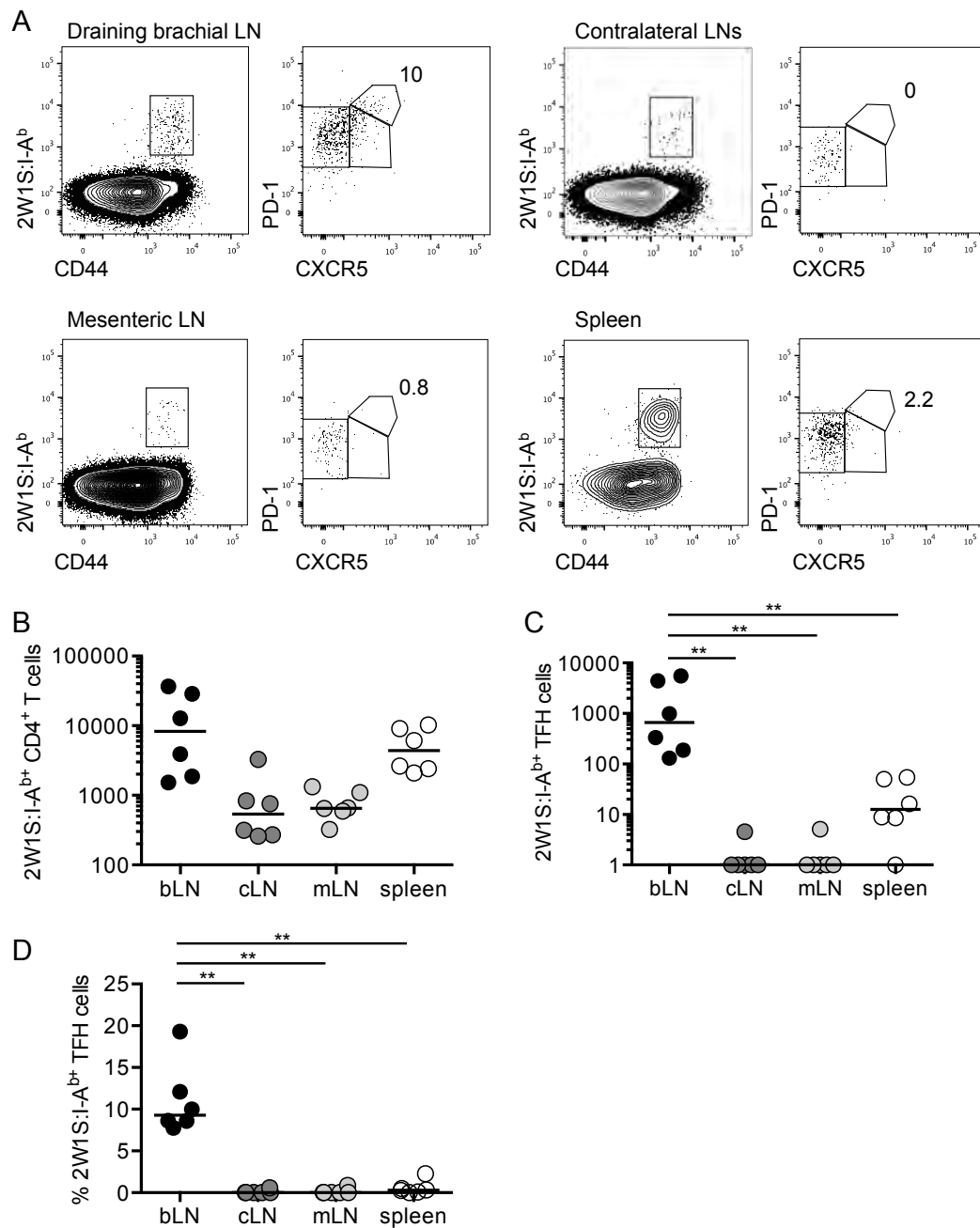


Figure 5.4: Optimising a model to investigate the location and migration of 2W1S:I-A^b CD4⁺ memory T cells. WT mice were immunised with 5 µg alum ppt 2W1S peptide in the right paw pad. Draining brachial lymph node, contralateral lymph nodes (brachial, axillary and inguinal), mesenteric lymph node and spleen were analysed 8 dpi. (A) Expression of CXCR5 and PD-1 on 2W1S:I-A^b CD4⁺ T cells from each tissue respectively. (B) Number of 2W1S:I-A^b CD4⁺ T cells from each tissue. (C) Number of 2W1S:I-A^b CD4⁺ TFH cells from each tissue.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B-C) Graphs show pooled data from 2 independent experiments. Values of 0 were assigned 1. Bars show median. Mann Whitney test: **p<0.01.

2

The number of 2W1S:I-A^b CD4⁺ T cells was also reduced in the spleen, however the number of cells found in the contralateral and mesenteric lymph nodes were comparable. Despite this, TFH cells were only found in significant numbers in the draining brachial lymph node and were almost absent in non-draining tissues (Figure 5.4 A, C and D). These findings indicated that antigen had not disseminated beyond the draining lymph nodes, as was the case when mice were immunised with 20 µg 2W1S peptide and that this method could be used as a model of local immunisation to monitor the migration of antigen-specific CD4⁺ T cells.

5.2.3 The presence of 2W1S:I-A^b CD4⁺ T cells in distal tissues from the site of immunisation is not due to dissemination of antigen

To further assess whether antigen had disseminated beyond the draining brachial lymph node, fluorescently labelled antigen was injected to enable direct analysis of its spread. Injection with chicken OVA labelled with Alexa Fluor 647 (AF647) was used to fluorescently label cells that bind or phagocytose antigen. DC take up antigen by phagocytosis and present peptides on MHC molecules to activate naive T cells. DC can express several integrins and chemokine receptors on their surface, which determine their migration into secondary lymphoid tissue (Guermonprez et al., 2002). OVA-AF647 was aluminium precipitated as performed for the 2W1S peptide in an attempt to maintain the nature of the

immunisation. After injection of alum ppt OVA-AF647 into the right paw pad we analysed the draining brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen for the presence of AF647⁺ DC. The timepoints we chose to analyse were 6 and 24 hours post injection of OVA, as the movement of DC from the site of immunisation to lymphoid tissues has been shown to occur within this period (Kool et al., 2008, Ciabattini et al., 2011). The gating strategy used to identify AF647⁺ DC is defined in Figure 5.5 A. As DC are larger than T cells the forward scatter gate was extended. Putatively DC were then positively identified based on expression of CD11c and MHCII. CD3⁺ and B220⁺ cells were gated out to remove contaminating T and B cells. In the draining brachial lymph node 6 hours post injection a population of OVA⁺ DC could be identified which constituted approximately 40% of total DC (Figure 5.5 A). A control mouse receiving PBS in the right paw pad was used to set the gating and eliminate the possibility of background staining (Figure 5.5 B). 6 hours post injection of OVA in the right paw pad, none of the distal tissues analysed contained a population of OVA⁺ DC (Figure 5.5 B). This was also the case 24 hours post injection, yet a population could still be identified in the draining brachial lymph node (Figure 5.5 C). These data indicate that antigen does not reach other lymphoid tissues within the initial 24 hours post immunisation, an observation consistent with the restriction of TFH cells to the draining lymph node at 8 dpi (Figure 5.4 A and C).

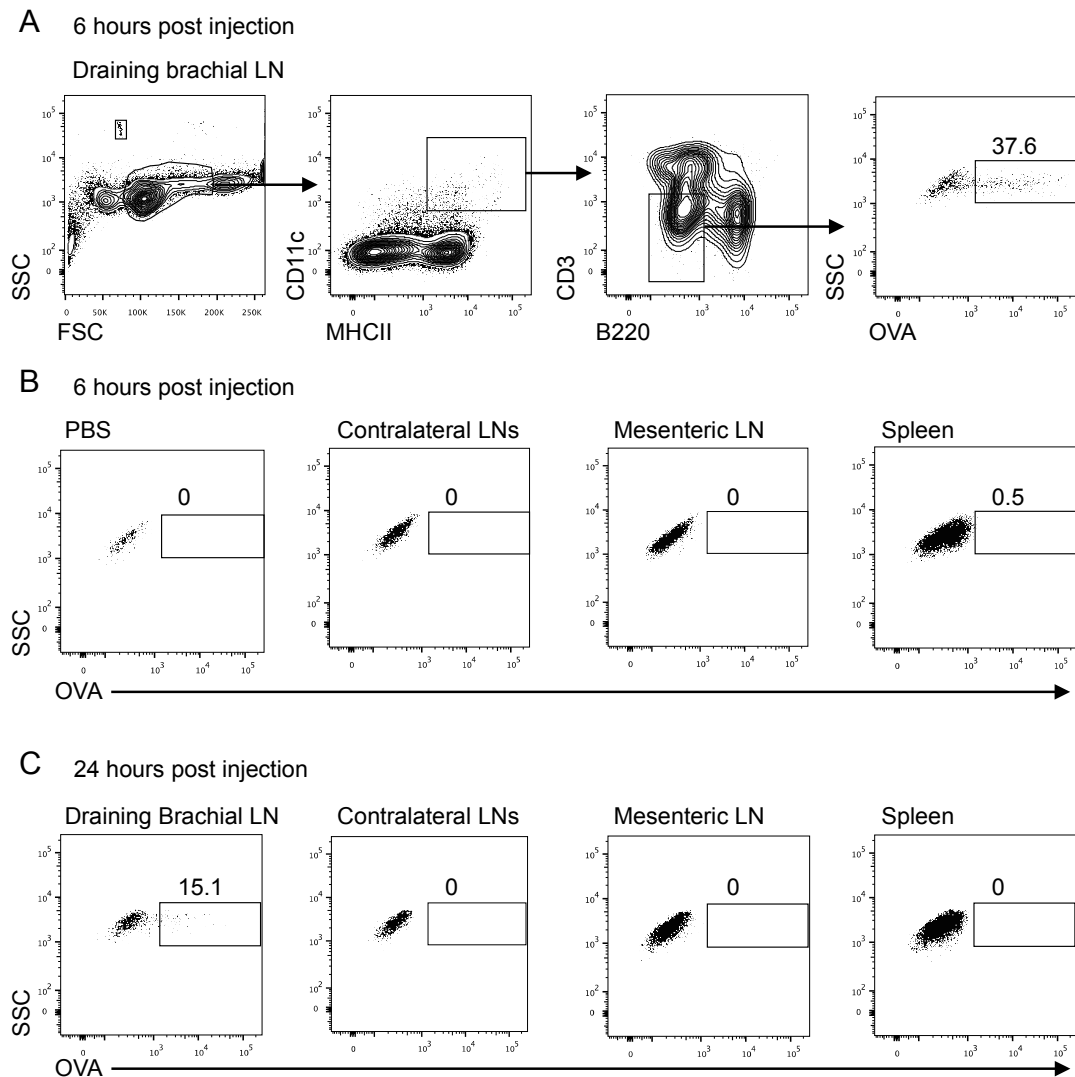


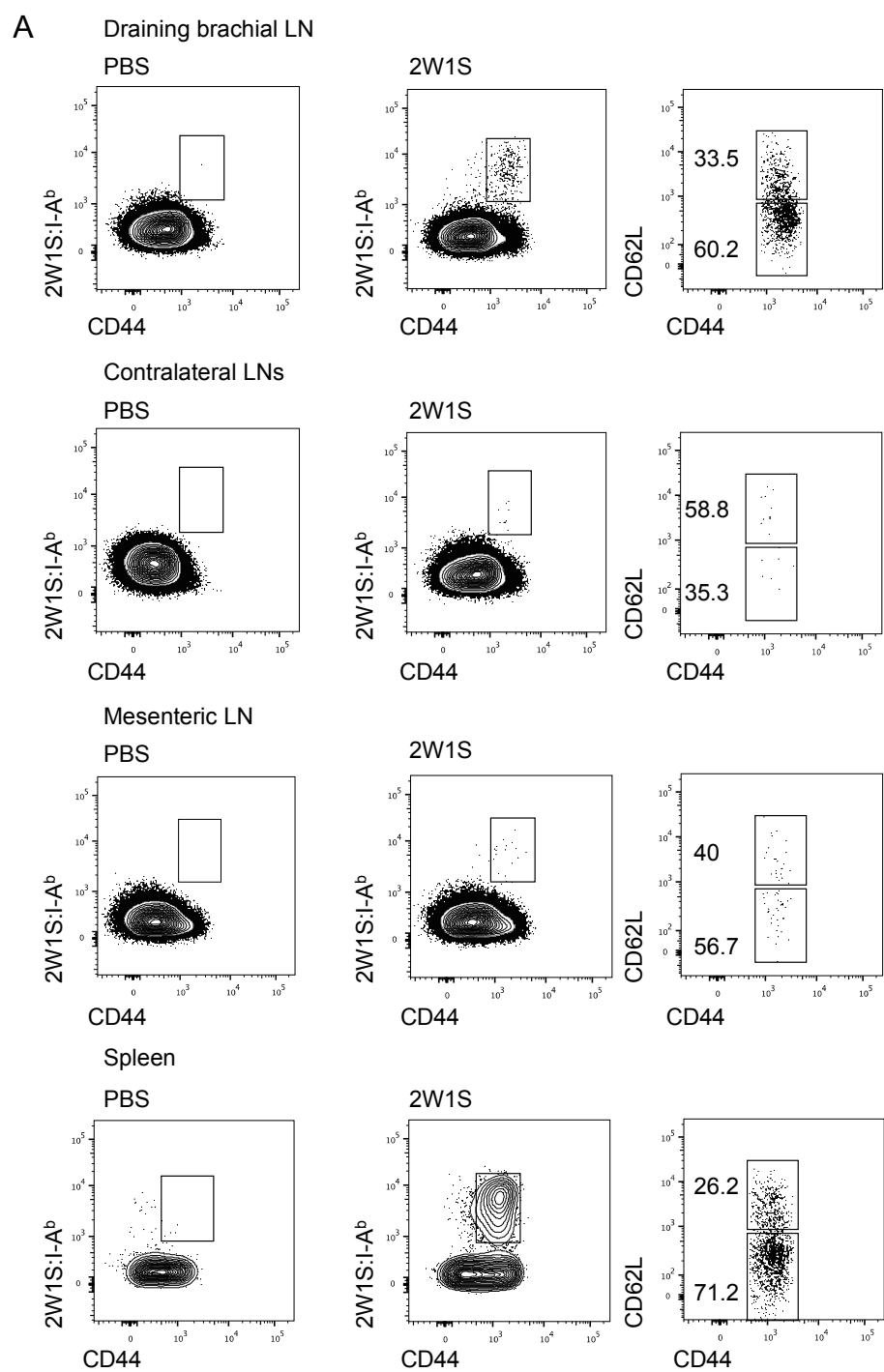
Figure 5.5: Antigen does not disseminate beyond the draining lymph node. WT mice were injected with 5 μ g alum ppt OVA Alexa Fluor 647 in the right paw pad. Draining brachial lymph node, contralateral lymph node, mesenteric lymph node and spleen were then analysed 6 and 24 hours later as indicated. (A) Gating strategy to identify OVA⁺ DC in the draining brachial lymph node 6 hours post injection. Gated on extended lymphocyte gate, MHCII⁺CD11c⁺, B220⁻CD3⁻, OVA⁺. (B) OVA⁺ DC gated as shown in (A) from the draining brachial lymph node PBS control, contralateral lymph nodes, mesenteric lymph node and spleen 6 hours post injection. (C) OVA⁺ DC gated as shown in (A) from the draining brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen 24 hours post injection.

(A-C) Plots are representative of 3 mice from 1 independent experiment. Values on plots are percentages.

5.2.4 Analysing the migration of 2W1S:I-A^b CD4⁺

memory T cells

Having assessed the 2W1S:I-A^b CD4⁺ T cell response at 8 dpi during the primary phase we sought to investigate the migration of memory cell populations. Whilst memory cells may be present 8 dpi, identification of these cells is challenging due to a lack of specific surface markers. Therefore, memory cells were defined by time since immunisation and we analysed the migration of this population at later timepoints when definition of this population is more reliable. To directly study antigen-specific CD4⁺ memory T cell migration is technically challenging due to low cell frequency, which is exacerbated in a local immunisation model where only one lymphoid site is responding. Mice were immunised with 5 µg 2W1S peptide in the right paw pad and analysed 21 dpi. This timepoint was chosen as following systemic immunisation with Lm-2W1S effector cells have contracted to form a memory cell population by this stage (Pepper et al., 2010). As we would expect a very low cell frequency at this time, gating for 2W1S:I-A^b CD4⁺ T cells was set using a control mouse that received PBS in the right paw pad and therefore will not have a 2W1S:I-A^b CD4⁺ memory T cell population (Figure 5.6 A). Draining brachial, contralateral, mesenteric lymph node and spleen were analysed for the presence of a 2W1S:I-A^b CD4⁺ memory T cell population. The low frequency of cells at this timepoint made conclusions of phenotype based on cytokine secretion unreliable, so this approach was not used. Therefore, surface markers CD44 and CD62L were used to putatively define T_{cm} and T_{em} cells.



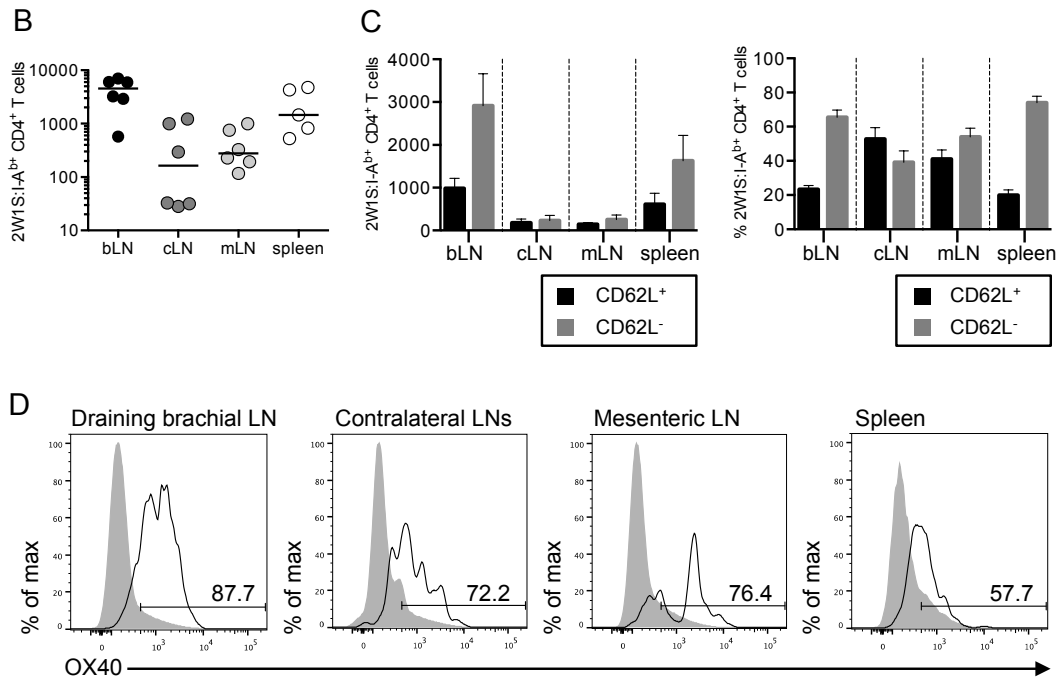


Figure 5.6: Investigating the migration of 2W1S:I-A^b+ CD4⁺ memory T cells. WT mice were immunised with 5 µg alum ppt 2W1S peptide in the right paw pad. Draining brachial lymph node, contralateral lymph nodes (brachial, axillary and inguinal), mesenteric lymph node and spleen were analysed 21 dpi. (A) Expression of CD44 and CD62L on 2W1S:I-A^b+ CD4⁺ T cells from each tissue respectively. PBS control shows how gating was set for 2W1S:I-A^b+ cells. (B) Number of 2W1S:I-A^b+ CD4⁺ T cells from each tissue. (C) Number (left) and percentage (right) of 2W1S:I-A^b+ CD4⁺ CD62L⁺ and CD62L⁻ cells from each tissue. (D) Expression of OX40 on 2W1S:I-A^b+ CD4⁺ T cells from each tissue.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 2 independent experiments. Bars show median. (C) Graphs show pooled data from 2 independent experiments. Bars show mean and error bars show SEM. (D) Plots are representative of 3 mice from 1 experiments. Values on plots are percentages. Grey shaded areas on histograms represent CD4⁺ T cells, the majority of which are OX40⁺.

Both populations of memory cells express the activation marker CD44; Tcm cells are defined by expression of CD62L, required for entry into lymph nodes, while Tem cells lack this molecule (Sallusto et al., 1999). Therefore Tcm phenotype cells were identified as CD62L⁺ and Tem phenotype cells were CD62L⁻ cells. A population of 2W1S:I-A^b⁺ CD4⁺ memory T cells could be detected in all tissues, however this was very small in the contralateral and mesenteric lymph nodes (Figure 5.6 A and B; median for bLN 4567, cLN 164.4, mLN 277, spleen 1460). In the draining lymph node and spleen there was a significantly higher proportion of CD62L⁻ Tem cells (Figure 5.6 A and C). This and the high cell number suggested that the response may be ongoing and that the 2W1S:I-A^b⁺ CD4⁺ T cell population might contain both effector and memory cells. To further investigate this, 2W1S:I-A^b⁺ CD4⁺ T cells were stained for expression of OX40. OX40 expression is thought to be tightly linked to antigen exposure and in the acute response to attenuated Lm-2W1S OX40 expression is transient and lost by 4 dpi (Chapter 3, Figure 3.3). Since in chronic responses expression of OX40 can be maintained until 20 dpi (Boettler et al., 2013), it was possible that alum ppt 2W1S peptide may persist for several weeks at the site of immunisation, driving a sustained primary response (Glenny et al., 1931, Ghimire et al., 2012). Total CD4⁺ T cells were used as a negative control for OX40 staining as the isotype control under-represents the background level. OX40 expression was detected on the majority of 2W1S:I-A^b⁺ CD4⁺ T cells from all tissues (Figure 5.6 D). This indicated that the kinetics of the response to alum ppt 2W1S peptide are different to that of attenuated Lm-2W1S and analysis at a later timepoint would likely enrich for only memory 2W1S:I-A^b⁺ CD4⁺ T cells.

A caveat of defining T cell populations by expression of CD62L and CCR7 is that they are intrinsically linked to their ability to enter lymphoid tissue (Forster et al., 1999, Marchesi and Gowans, 1964). Analysis of Tem cell migration would be enhanced by identification of other surface markers expressed specifically by these cells. To this end we investigated other possible phenotypic markers that could be used to identify Tcm and Tem cells. Killer cell lectin-like receptor G 1 (KLRG1) and CD27 have both been used in the study of CD8⁺ memory T cells. KLRG1 expression is associated with short-term survival and is therefore present at lower levels on CD8⁺ T cells that will enter the memory cell pool (Sarkar et al., 2008). CD27⁻ CD8⁺ memory T cells showed an enhanced ability to produce effector cytokines, characteristic of Tem cells (De Jong et al., 1992). In addition CD27⁻ cells were found to be enriched in CCR7⁻ and CD62L⁻ human memory T cell populations suggesting lack of this receptor is correlated with an inability to home to secondary lymphoid tissue (Campbell et al., 2001). Therefore we analysed expression of these markers on 2W1S:I-A^b⁺ CD4⁺ T cells to determine if they could also be applicable to CD4⁺ memory T cells. Following immunisation with alum ppt 2W1S peptide in the paw pad the majority of CD62L⁺ and CD62L⁻ cells were KLRG1⁻ indicating this would not be a useful phenotypic marker (Figure 5.7 A). The majority of both CD62L⁺ and CD62L⁻ cells were CD27⁺; there was a small population of CD27⁻ cells within the CD62L⁻ cell population which was not seen within CD62L⁺ cells (Figure 5.7 A). This was consistent in all tissues analysed where a proportion of CD62L⁻ cells, but not CD62L⁺ cells, were CD27⁻ (Figure 5.7 B). These data indicate that any CD27⁻ 2W1S:I-A^b⁺ CD4⁺ T cells identified were likely Tem cells.

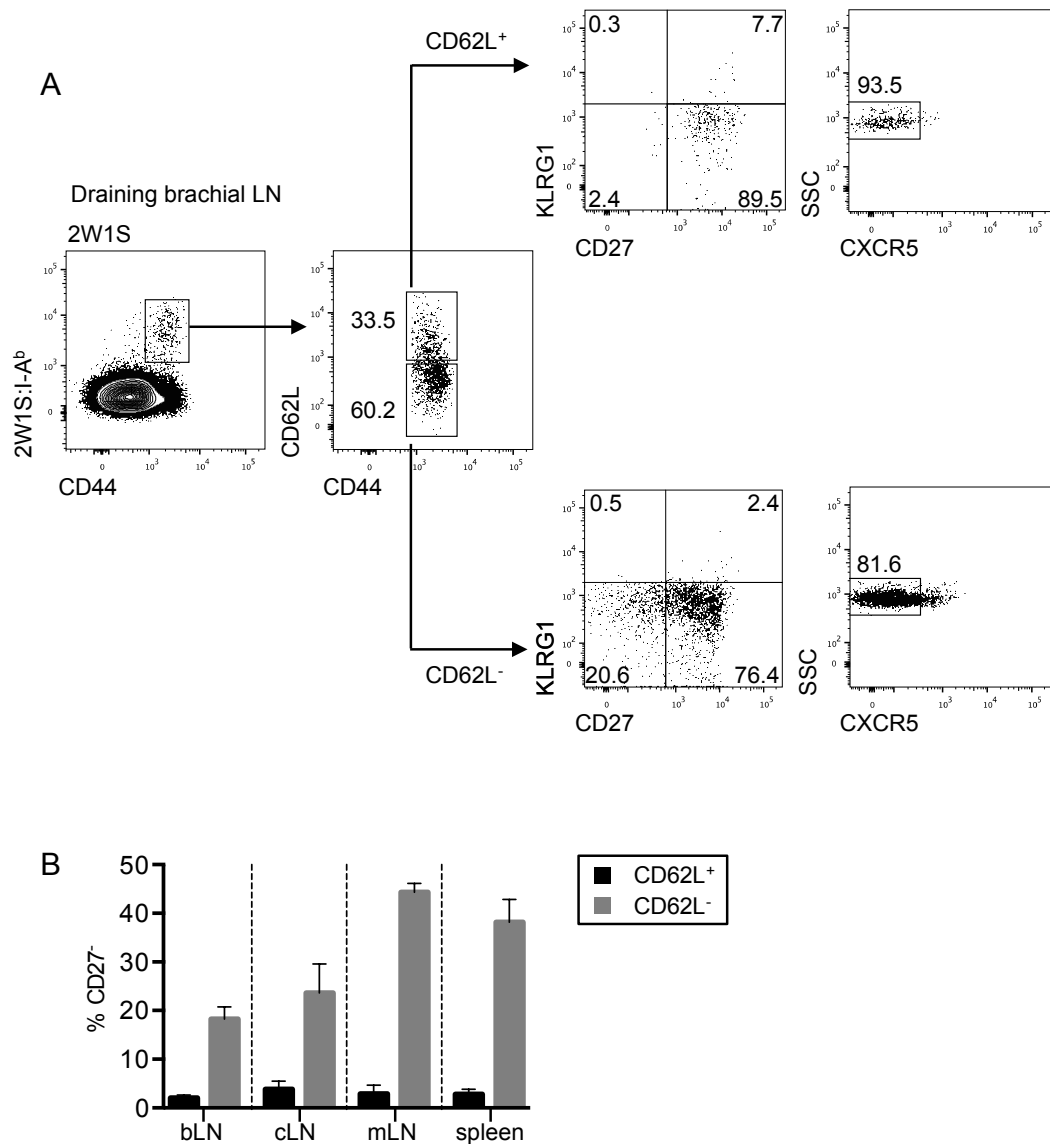
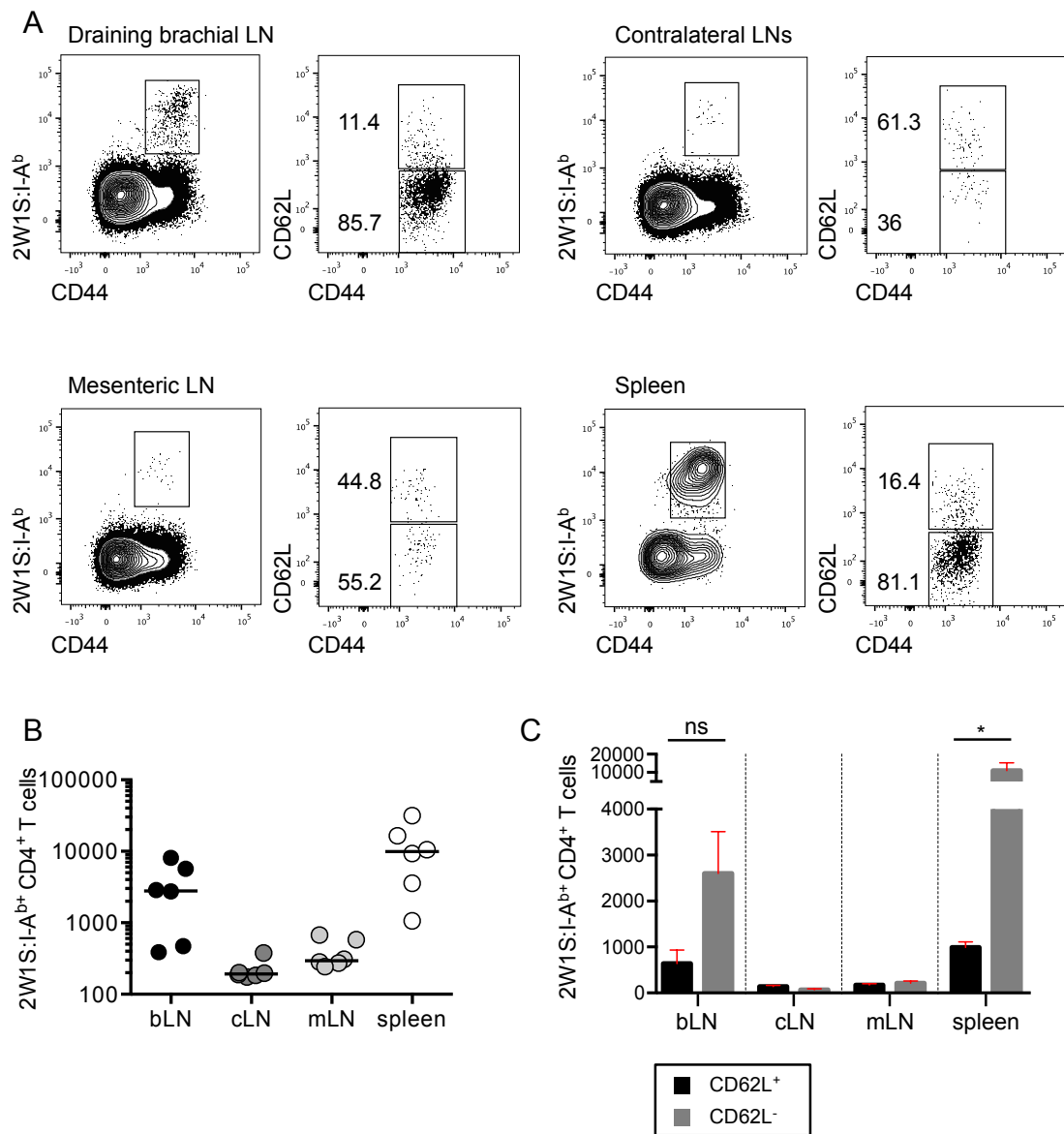


Figure 5.7: Investigating alternative phenotypic markers for Tem cells. WT mice were immunised with 5 μ g alum ppt 2W1S peptide in the right paw pad. Draining brachial lymph node, contralateral lymph nodes (brachial, axillary and inguinal), mesenteric lymph node and spleen were analysed 21 dpi. (A) Expression of CD27, KLRG1 and CXCR5 on 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells from the draining lymph node. (C) Percentage of 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells that are CD27⁻ in each tissue.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 2 independent experiments. Bars show mean and error bars show SEM.

We and others have used CXCR5 to subdivide 2W1S:I-A^b CD4⁺ T cells into Teff cells and Tcm precursor cells following infection with Lm-2W1S (Marriott et al., 2014, Marriott et al., 2015, Pepper et al., 2011). For this reason we looked for expression of CXCR5 following immunisation with alum ppt 2W1S peptide. However the majority of 2W1S:I-A^b CD4⁺ T cells did not express CXCR5 indicating this cannot be used as a marker to differentiate Tcm and Tem populations (Figure 5.7 A).

Since OX40 expression on 2W1S:I-A^b CD4⁺ T cells at 21 dpi indicated that the response was still in the effector phase we waited a further 3 weeks before analysing the memory cell population; mice were immunised with alum ppt 2W1S peptide and analysed at 42 dpi. A population of around 3000 2W1S:I-A^b CD4⁺ T cells could be recovered from the draining brachial lymph node at this time, however, in the contralateral and mesenteric lymph nodes the population was very small (Figure 5.8 A and B; median for bLN 2806, cLN 192, mLN 296). There was also a significant population recovered from the spleen (Figure 5.8 A and B; median for spleen 9909). Similar to what was observed at 21 dpi the number and proportion of CD62L⁻ cells were higher than CD62L⁺ cells in the draining brachial lymph node and the spleen (Figure 5.8 A, C and D). However, the proportion of 2W1S:I-A^b CD4⁺ T cells expressing OX40 was decreased compared to 21 dpi in the draining, contralateral and mesenteric lymph nodes and the spleen indicating the response has entered the memory phase (Figure 5.8 E).



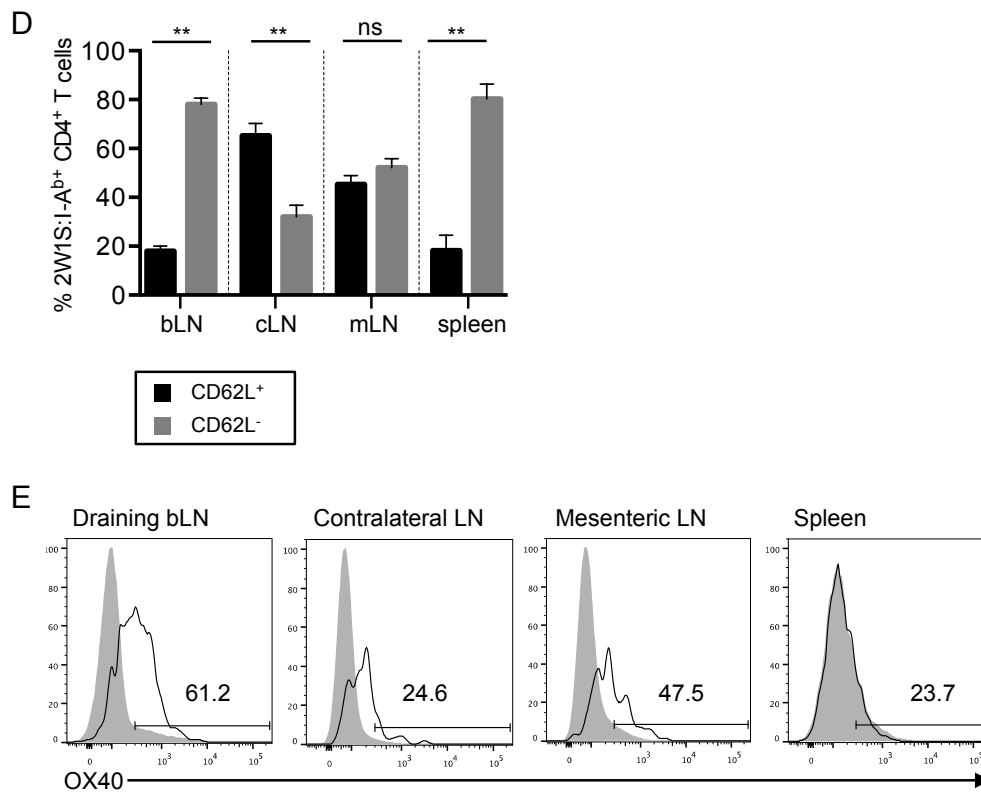


Figure 5.8: 2W1S:I-A^b CD4⁺ Tcm and Tem phenotype cells can be identified in draining and contralateral tissues following local immunisation. WT mice were immunised in the right paw pad with 5 µg 2W1S alum ppt peptide. Draining brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen were analysed 42 dpi. (A) Expression of CD44 and CD62L on 2W1S:I-A^b CD4⁺ T cells from each tissue respectively. (B) Number of 2W1S:I-A^b CD4⁺ T cells from each tissue. (C) Number of 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells from each tissue. (D) Percentage of 2W1S:I-A^b CD4⁺ T cells that are CD62L⁺ and CD62L⁻ from each tissue. (E) Expression of OX40 on 2W1S:I-A^b CD4⁺ T cells from each tissue.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 2 independent experiments. Bars show median. (C-D) Graphs show pooled data from 2 independent experiments. Bars show mean and error bars show SEM. (E) Plots are representative of 3 mice from 1 experiment. Values on plots are percentages. Grey shaded areas on histograms represent CD4⁺ T cells. Mann Whitney Test: *p<0.05, **p<0.01, ns= non-significant.

In the contralateral lymph nodes the proportion of CD62L⁺ cells was significantly higher than CD62L⁻ cells, consistent with their migratory roles, however this was not the case in the mesenteric lymph node where the ratio was around 50:50 (Figure 5.8 A and D). However previous studies have shown that entry into the mesenteric lymph node is mediated by surface molecule such as $\alpha 4\beta 7$, in addition to CD62L (Hamann et al., 1994, Gorfu et al., 2009).

We further analysed the phenotype of 2W1S:I-A^b CD4⁺ memory T cells 42 dpi to determine if other surface markers correlated with CD62L expression.

Expression of CCR7 was not restricted to CD62L⁺ cells, again indicating that on memory cells CCR7 may be expressed by both Tcm and Tem cell populations (Figure 5.9 A and B). This is in contrast to initial studies that defined Tcm cells based on expression of CD62L and CCR7 and Tem cells on their absence (Sallusto et al., 1999), however another study has indicated that expression of CCR7 may be more complex (Unsoeld and Pircher, 2005). Expression of CCR7 may not be exclusive to CD62L⁺ cells or alternatively changes in expression of CCR7 may occur depending upon location. As seen at 21 dpi, in all lymph nodes, the majority of both CD62L⁺ and CD62L⁻ cells were CD27⁺ but a small number of CD62L⁻ cells were CD27⁻ (Figure 5.9 A and C). Between 21-43% of CD62L⁻ cells were CD27⁻ depending upon the tissue, with the highest number found in the spleen (Figure 5.9 C). CD27⁻ cells were essentially absent from the CD62L⁺ population, but clearly detected amongst CD62L⁻ cells, independent of CCR7 expression (Figure 5.9 A and C).

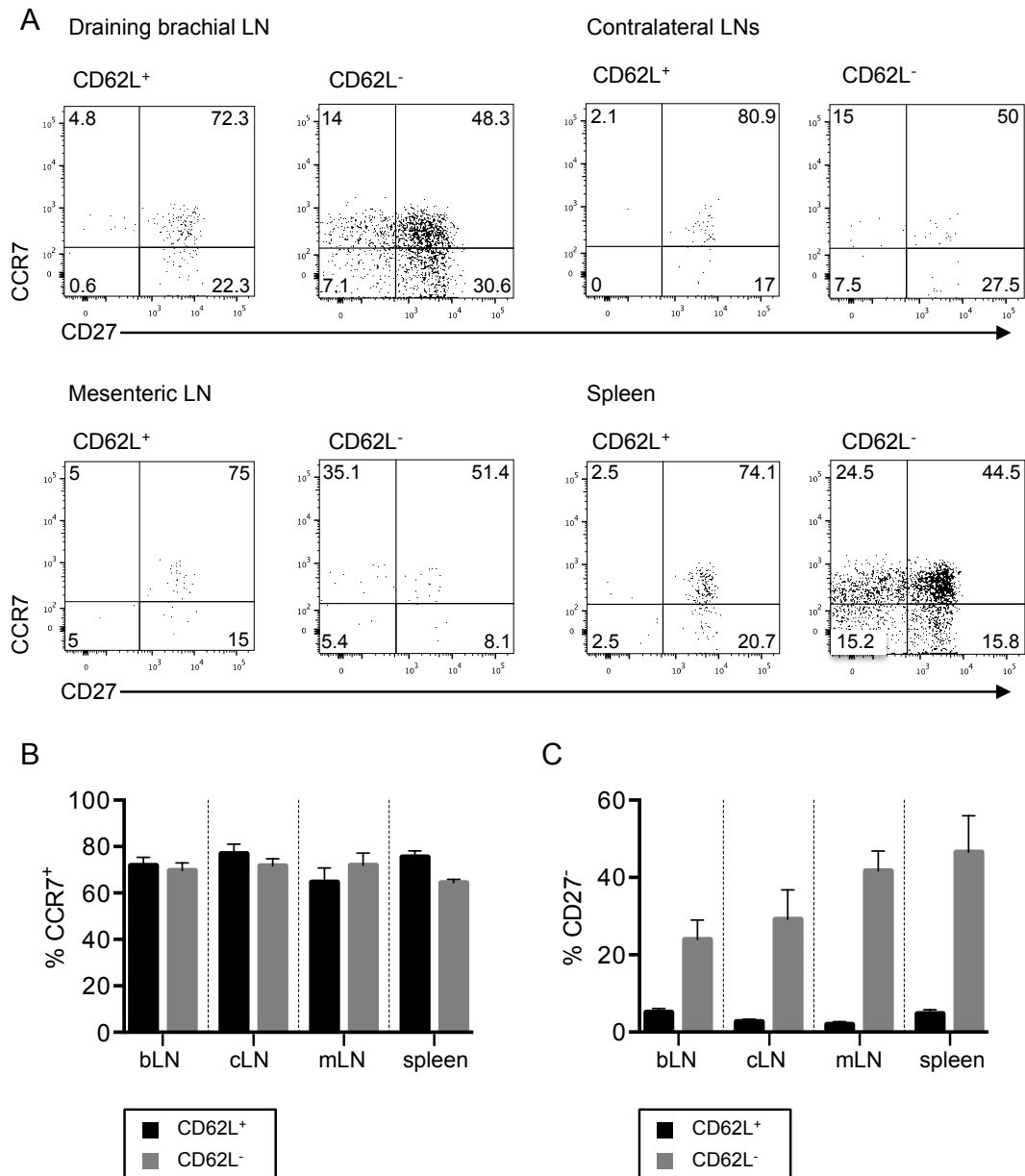


Figure 5.9: Phenotype of 2W1S:I-A^b CD4⁺ Tcm and Tem cells following local immunisation. WT mice were immunised in the right paw pad with 5 µg 2W1S alum ppt peptide. Draining brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen were analysed 42 dpi. (A) Expression of CD27 and CCR7 on 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells from each tissue respectively. (B) Percentage of 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells expressing CCR7 from each tissue. (C) Percentage of 2W1S:I-A^b CD4⁺ CD62L⁺ and CD62L⁻ cells that are CD27⁻ from each tissue.

(A) Plots are representative of 6 mice from 2 independent experiments. Values on plots are percentages. (B-C) Graphs show pooled data from 2 independent experiments. Bars show mean and error bars show SEM.

5.2.5 Utilising photoconvertible Kaede protein to track cell migration

Having established a model to track an antigen-specific response to local immunisation we further refined this approach to investigate the migration of cells from the site of immunisation within a distinct period of time. This key additional information would better establish trafficking of cells from a given site. We used Kaede transgenic mice, which are genetically modified to express the fluorescent Kaede protein in every cell (Ando et al., 2002). This Kaede protein fluoresces green at 518 nm under normal conditions, however following exposure to violet light (wavelength 350-400 nm) formation of a double bond within the structure causes a change in fluorescence to red (wavelength 582 nm) (Mizuno et al., 2003, Ando et al., 2002). This irreversible reaction labels cells exposed to violet light and the red Kaede protein has a long half-life *in vivo* enabling their migration to be tracked (Tomura et al., 2008).

To enable us to specifically label only the cells in the draining brachial lymph node we developed a surgical technique to expose this site to violet light. Under anaesthesia a small incision was made in the armpit area of the mouse and the brachial lymph node was located. This site was then exposed to violet light for 3 minutes, following testing *in vitro* and on sacrificed mice to determine the optimal time for photoconversion. Mice were then sacrificed immediately post photoconversion to analyse the efficacy of this method. Mice undergoing photoconversion (switched) were compared to control mice (unswitched) that had not been photoconverted. Following 3 minutes of exposure to violet light

almost 100 % of lymphocytes were converted to Kaede red (Figure 5.10 A and B). Consistent with other reports (Ando et al., 2002, Tomura et al., 2008), we have found that even with prolonged exposure to violet light, not all of the Kaede green protein is converted to red and therefore “switched” cells are defined as double positive for Kaede green and red protein.

As local immunisation in the paw pad not only targets the draining brachial lymph node but also the axillary lymph node we assessed the level of photoconversion in this tissue. Surgical exposure of the brachial lymph node resulted in variable labelling of the axillary lymph node due to scatter of light (Figure 5.10 C and D). For this reason the axillary lymph node was excluded and only the brachial lymph node was analysed. We then investigated the level of photoconversion in different cell subsets. B cells, CD4⁺ and CD8⁺ T cells were all equally efficient at photoconverting with over 99 % of cells switched in the brachial lymph node (Figure 5.10 E-G).

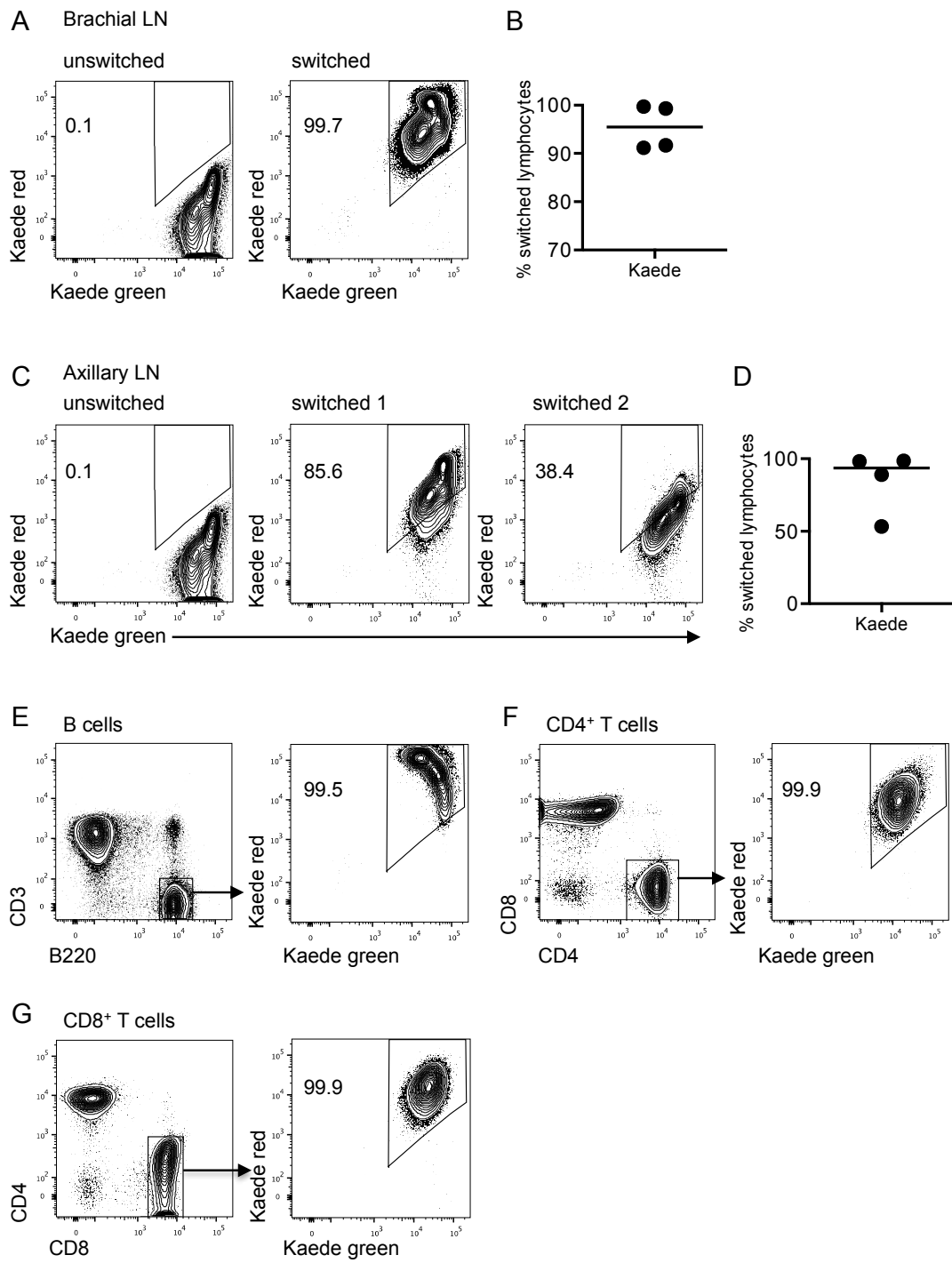


Figure 5.10: Photoconversion of Kaede protein. Kaede mice underwent surgery to expose the brachial lymph node which was then exposed to violet light for 3 minutes. Mice were sacrificed and analysed immediately post surgery to assess switching in brachial and axillary lymph nodes. (A) Gated on total lymphocytes to show the level of expression of Kaede red protein in the brachial lymph node of control mice (unswitched- left) and following exposure to violet light (switched-right). (B) Percentage of switched lymphocytes in the brachial lymph node following violet light exposure. (C) Gated on total lymphocytes to show the level of expression of Kaede red protein in the axillary lymph node of control mice (unswitched- left) and in 2 mice following exposure to violet light (switched-right). (D) Percentage of switched lymphocytes in the axillary lymph node following violet light exposure. (E) Plot showing level of Kaede red expression in B cells following violet light exposure. (F) Plot showing level of Kaede red expression in CD4⁺ T cells following violet light exposure. (G) Plot showing level of Kaede red expression in CD8⁺ T cells following violet light exposure.

(A, C, E-G) Plots are representative of 4 mice from 1 independent experiment. Values on plots are percentages. (B and D) Graphs show data from 1 experiment.

5.2.6 Tracking the migration of cells in 24 hours

following photoconversion

An initial step in tracking the migration of antigen-specific CD4⁺ T cells was to first analyse total lymphocytes to validate the method on a large population of cells. The left brachial lymph node was exposed to violet light using the surgical technique described in section 5.2.5 and the wound was sutured and the mice left to recover. Mice were sacrificed 24 hours after photoconversion and the brachial lymph node was analysed. Approximately 15 % of lymphocytes in the brachial lymph node were positive for Kaede red (switched) indicating that around 85 % of lymphocytes had migrated into or out of the lymph node in the previous 24 hours (Figure 5.11 A and B). These data were highly reproducible between mice; consistently between 10 and 20 % of cells within the brachial lymph node were switched (Figure 5.11 B). This was also the case for B cells or CD4⁺ T cells which show similar kinetics of migration; around 85 % of cells in the brachial lymph node were unswitched indicating a large migration has taken place (Figure 5.11 C-F). CD8⁺ T cells however showed much faster kinetics of migration as less than 1 % of cells in the lymph node were switched after 24 hours (Figure 5.11 G and H). This is in contrast to a previous study monitoring the migration of lymphocytes from the inguinal lymph node, where the kinetics of migration of CD8⁺ T cells were similar to CD4⁺ T cells (Tomura et al., 2008).

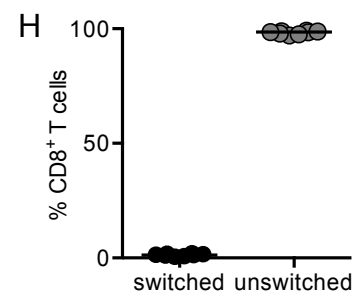
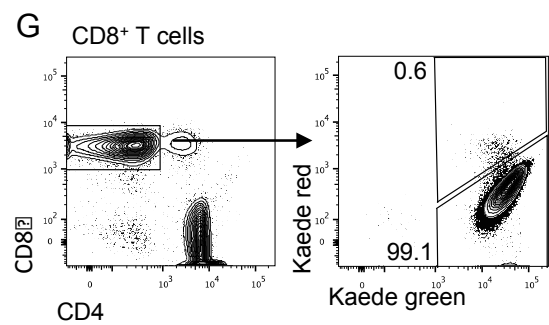
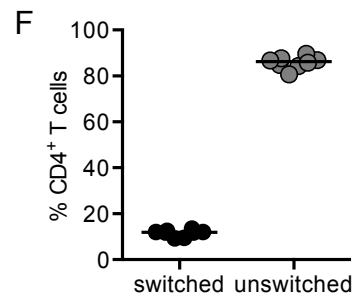
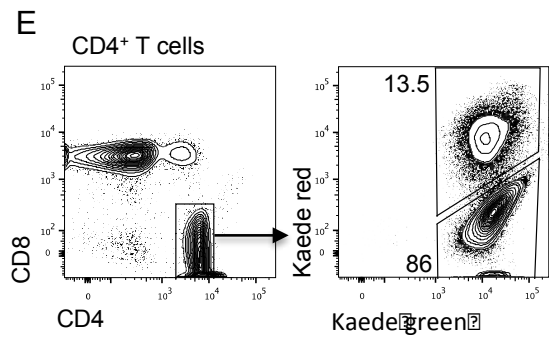
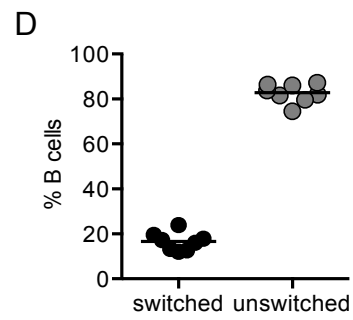
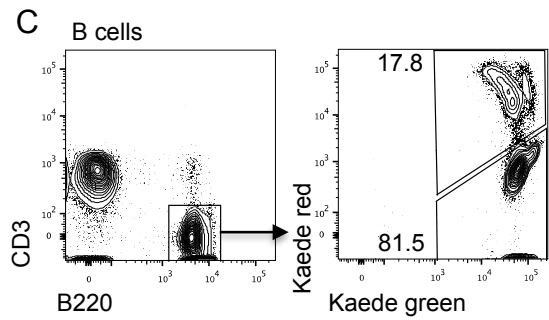
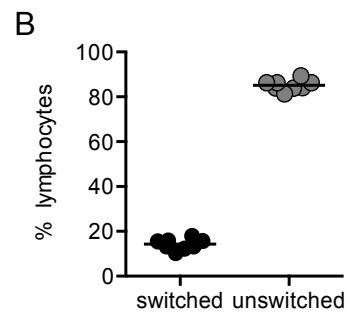
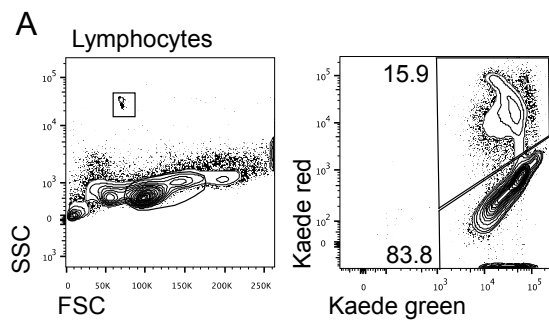


Figure 5.11: Migration of cells to and from the brachial lymph node in 24 hours.

The left brachial lymph node was exposed to violet light for 3 minutes then analysed 24 hours later for migration of lymphocytes. (A) Expression of Kaede green and Kaede red protein in lymphocytes from the brachial lymph node. (B) Percentage of switched (resident) and unswitched (migrating) lymphocytes from the brachial lymph node. (C) Expression of Kaede green and Kaede red protein in B220⁺ B cells from the exposed brachial lymph node. (D) Percentage of switched (resident) and unswitched (migrating) B cells from the brachial lymph node. (E) Expression of Kaede green and Kaede red protein in CD4⁺ T cells from the brachial lymph node. (F) Percentage of switched (resident) and unswitched (migrating) CD4⁺ T cells from the brachial lymph node. (G) Expression of Kaede green and Kaede red protein in CD8⁺ T cells from the brachial lymph node. (H) Percentage of switched (resident) and unswitched (migrating) CD8⁺ T cells from the brachial lymph node.

(A, C, E, G) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages. (B, D, F, H) Graphs show pooled data from 2 independent experiments.

Using this method we can track the migration of cells that were in the brachial lymph node at the time of photoconversion to other tissues in the body. The presence of any switched cells in the spleen and lymph nodes indicates that their point of origin was the brachial lymph node 24 hours previously. In both the contralateral lymph nodes and the spleen a small proportion of lymphocytes, around 2-3 %, were switched, validating the use of this method to investigate cell migration (Figure 5.12 A). Around 2.5 % of B cells in the contralateral lymph nodes and spleen were switched (Figure 5.12 B), however this was less than 0.5 % for CD8⁺ T cells (Figure 5.12 C). This could not be accounted for by cells remaining in the brachial lymph node as CD8⁺ T cells showed faster kinetics of migration. It is possible that CD8⁺ T cells preferentially migrate to a tissue that we have not analysed.

Approximately 2 % of CD4⁺ T cells in the contralateral lymph nodes and spleen were switched indicating similar migratory properties to B cells (Figure 5.13 A and B). Again the number of switched cells was highly reproducible between mice, indicating that this will be a reliable method when analysing smaller, antigen-specific populations. Naive T cells are CD44^{lo}CD62L⁺ while Tcm and Tem phenotype cells were defined as CD44^{hi} cells that were CD62L⁺ or CD62L⁻ respectively. Based on their lack of CD62L and CCR7 expression it is assumed that Tem cells are unable or less efficient at entering lymph nodes through HEV. We investigated the migratory capacity of T cell subsets by analysing their proportions within the switched and unswitched populations of CD4⁺ T cells 24 hours post photoconversion of the brachial lymph node.

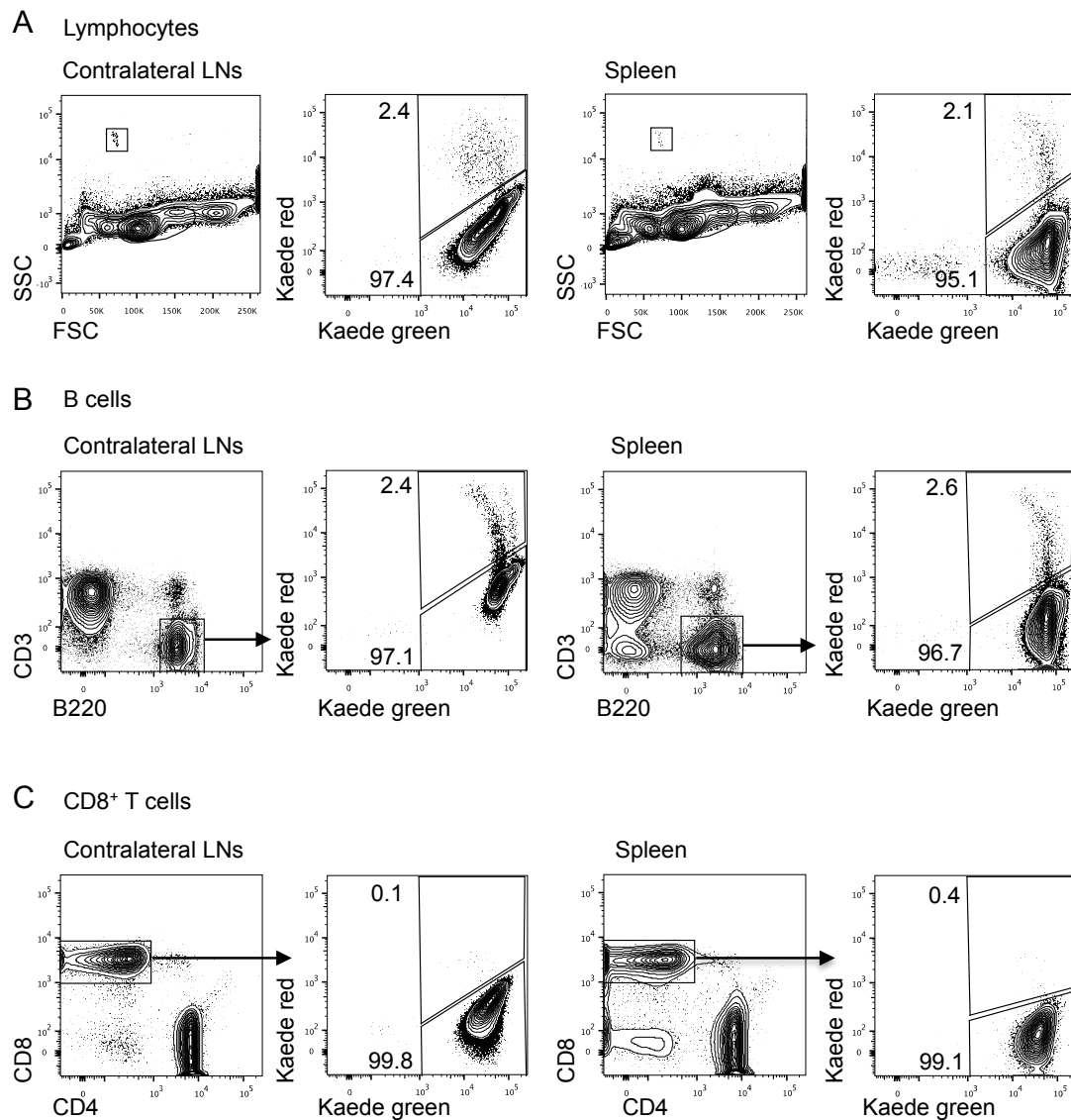


Figure 5.12: Migration of cells from the brachial lymph node to contralateral lymph nodes and spleen in 24 hours. The left brachial lymph node of Kaede mice was exposed to violet light for 3 minutes. Mice were analysed 24 hours later for migration of lymphocytes to the contralateral lymph nodes and spleen. (A) Expression of Kaede green and Kaede red protein in lymphocytes each tissue. (B) Expression of Kaede green and Kaede red protein in B cells from each tissue. (C) Expression of Kaede green and Kaede red protein in CD8⁺ T cells from each tissue.

(A-C) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages.

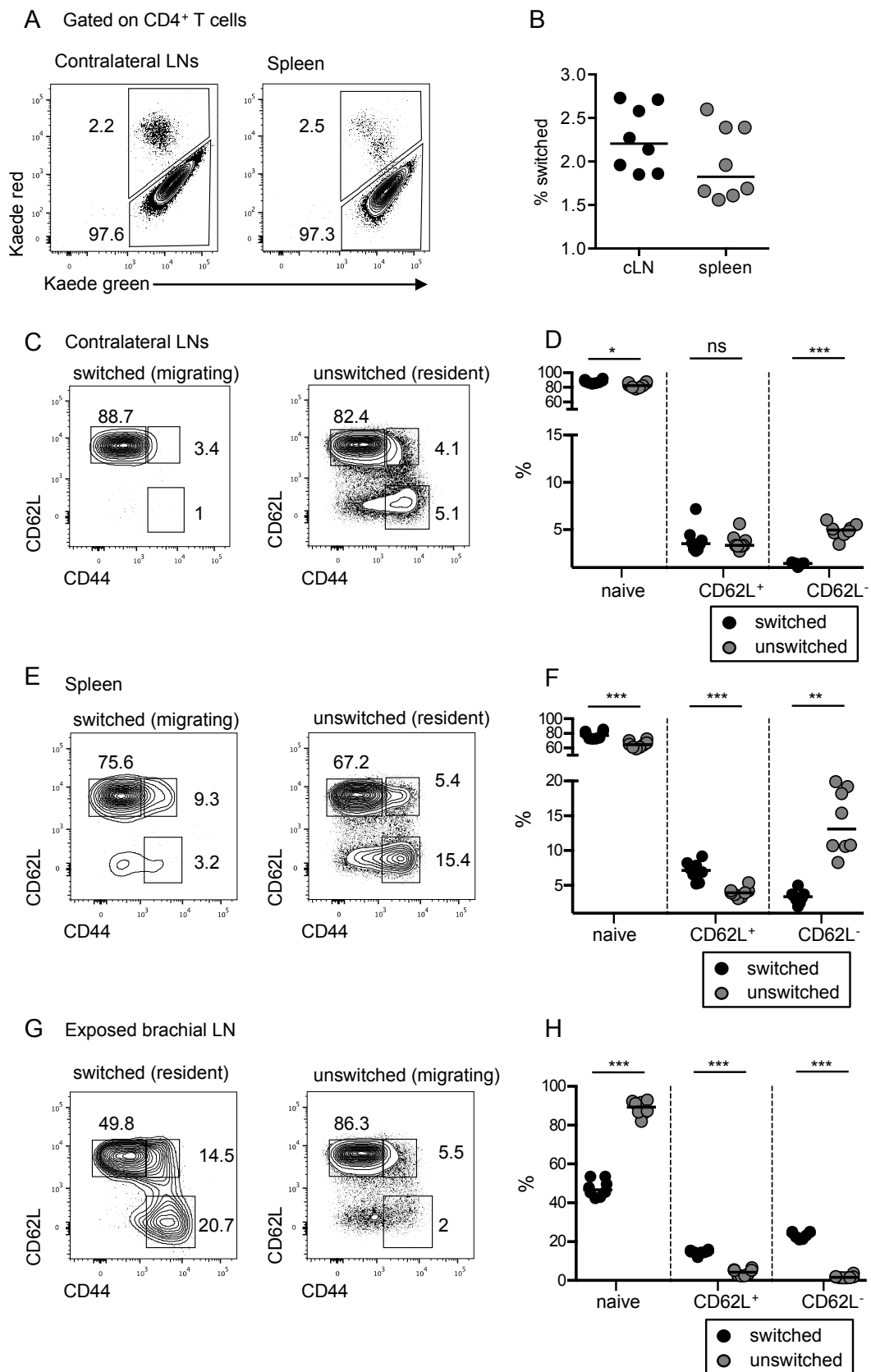


Figure 5.13: Migration of CD4⁺ T cell subsets 24 hours following photoconversion of the brachial lymph node. The left brachial lymph node of Kaede mice was exposed to violet light for 3 minutes. Mice were analysed 24 hours later for migration of CD4⁺ T cells. (A) Expression of Kaede red and Kaede green in CD4⁺ T cells from the contralateral lymph nodes and spleen. (B) Percentage of switched CD4⁺ T cells in the contralateral lymph nodes and spleen. (C) Expression of CD44 and CD62L on CD4⁺ T cells from the contralateral lymph nodes that are switched (left) and unswitched (right). (D) Percentage of switched and unswitched cells that are either naive T cells, CD62L⁺ or CD62L⁻ cells in the contralateral lymph node. (E) Expression of CD44 and CD62L on CD4⁺ T cells from the spleen that are switched (left) and unswitched (right). (F) Percentage of switched and unswitched cells that are either naive T cells, CD62L⁺ or CD62L⁻ cells in the spleen. (G) Expression of CD44 and CD62L on CD4⁺ T cells from the exposed brachial lymph node that are switched (left) and unswitched (right). (H) Percentage of switched and unswitched cells that are either naive T cells, CD62L⁺ or CD62L⁻ cells in the exposed brachial lymph node.

(A, C, E, G) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages. (B, D, F, H) Graphs showed pooled data from 2 independent experiments. Bars show median. Mann Whitney Test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns= non-significant.

In the contralateral lymph nodes naive T cells constitute the majority of CD4⁺ T cells for both switched and unswitched populations (Figure 5.13 C and D). There was a small proportion of CD62L⁺ cells in both switched and unswitched populations indicating that cells with Tcm phenotype have migrated from the exposed brachial lymph node into the contralateral lymph node (Figure 5.13 C and D). CD62L⁻ cells however are significantly reduced in the switched population compared to the unswitched (Figure 5.13 D), consistent with a significant impairment in their ability to migrate into the contralateral lymph nodes. It is possible that the small population of Tem cells in the lymph node, identified by lack of CD62L, are actually Tcm cells that have down-regulated this molecule following lymph node entry. Alternatively, it is possible that Tem cells express other surface molecules that enable inefficient entry into lymph nodes from the blood. In the spleen there were similar proportions of T cell subsets to the contralateral lymph nodes; naive T cells were most abundant and there was a small population of CD62L⁺ cells in both the switched and unswitched populations (Figure 5.13 E and F).

In the exposed brachial lymph node the switched population are cells that are resident and have not migrated in the previous 24 hours. Within this population the proportion of naive T cells was much lower than in the migrating unswitched cells (Figure 5.13 G and H; median for switched 47 %, unswitched 89 %). This is consistent with naive CD4⁺ T cells being highly migratory, patrolling through secondary lymphoid tissue in search of cognate antigen. In the brachial lymph node CD62L⁻ cells were significantly enriched in the switched population

suggesting these cells are resident within this lymph node, however, there is also an increase in CD62L⁺ cells which may indicate that memory cells in general show slower kinetics of emigration than naive cells (Figure 5.13 G and H).

5.2.7 Tracking the migration of cells in CCR7^{-/-} mice

To extend our analysis of cell trafficking we crossed Kaede mice with CCR7^{-/-} mice to create a model in which we could track the migration of cells that lack the chemokine receptor CCR7. CCR7 is important for several immune functions including the trafficking of leukocytes, establishing cognate interactions between cells and the organisation of lymphocytes to form lymphoid structures such as spleen and lymph nodes. The movement of naive CD4⁺ T cells (Campbell et al., 1998a, Campbell et al., 1998b, Gunn et al., 1999) and Tcm cells (Sallusto et al., 1999) from blood into lymph nodes is dependent on CCR7, although this has been disputed for antigen-experienced T cells (Vander Lugt et al., 2013). CCR7^{-/-} mice display impaired immune phenotypes including T cell responses due to the absence of distinct microenvironments within secondary lymphoid tissue (Forster et al., 1999). These secondary lymphoid structures are small and do not have distinct T and B cell zones which are required for the correct positioning of lymphocytes during an immune response. Therefore although the phenotypic defects observed in CCR7^{-/-} mice have been attributed to impairment in cell trafficking they may be due to development within abnormal secondary lymphoid structures.

Cohorts of CCR7^{-/-} Kaede mice were analysed and compared to WT mice that were previously photoconverted and analysed after 24 hours. Photoconversion of the brachial lymph node of CCR7^{-/-} Kaede mice and analysis 24 hours later revealed a significantly increased proportion of switched CD4⁺ T cells residing here compared to WT Kaede mice (Figure 5.14 A and B). This demonstrates that there is an impairment in the movement of CD4⁺ T cells into or out of lymph nodes in these mice. Naive T cells were significantly reduced in the unswitched CD4⁺ T cell population that is migrating into the brachial lymph node of CCR7^{-/-} Kaede mice (Figure 5.14 D). The proportion of CD62L⁺ and CD62L⁻ memory phenotype cells however was increased compared to WT Kaede mice, indicating that naive T cells are more reliant on CCR7 for entry into the lymph node (Figure 5.14 C and D). The total number of naive, CD62L⁺ and CD62L⁻ T cells migrating into this tissue was decreased in CCR7^{-/-} mice showing that all these population utilise CCR7 to some extent to enter lymph nodes (Figure 5.14 E). The switched (resident) CD4⁺ T cell population was altered in constitution compared to WT Kaede mice; the proportion of CD62L⁻ cells was significantly increased with corresponding decreases in naive T cells and CD62L⁺ T cells (Figure 5.14 C and F). When analysing total number of cells it is important to consider that the naive CD4⁺ T cell population is significantly reduced in the lymph nodes of CCR7^{-/-} mice (Forster et al., 1999). This is due to their inability to enter through HEV, therefore the number of CD4⁺ T cells that were exposed to violet light and initial number of switched cells would be decreased in CCR7^{-/-} Kaede mice. For this reason it was not informative to analyse the number of switched (resident) cells present in the brachial lymph node.

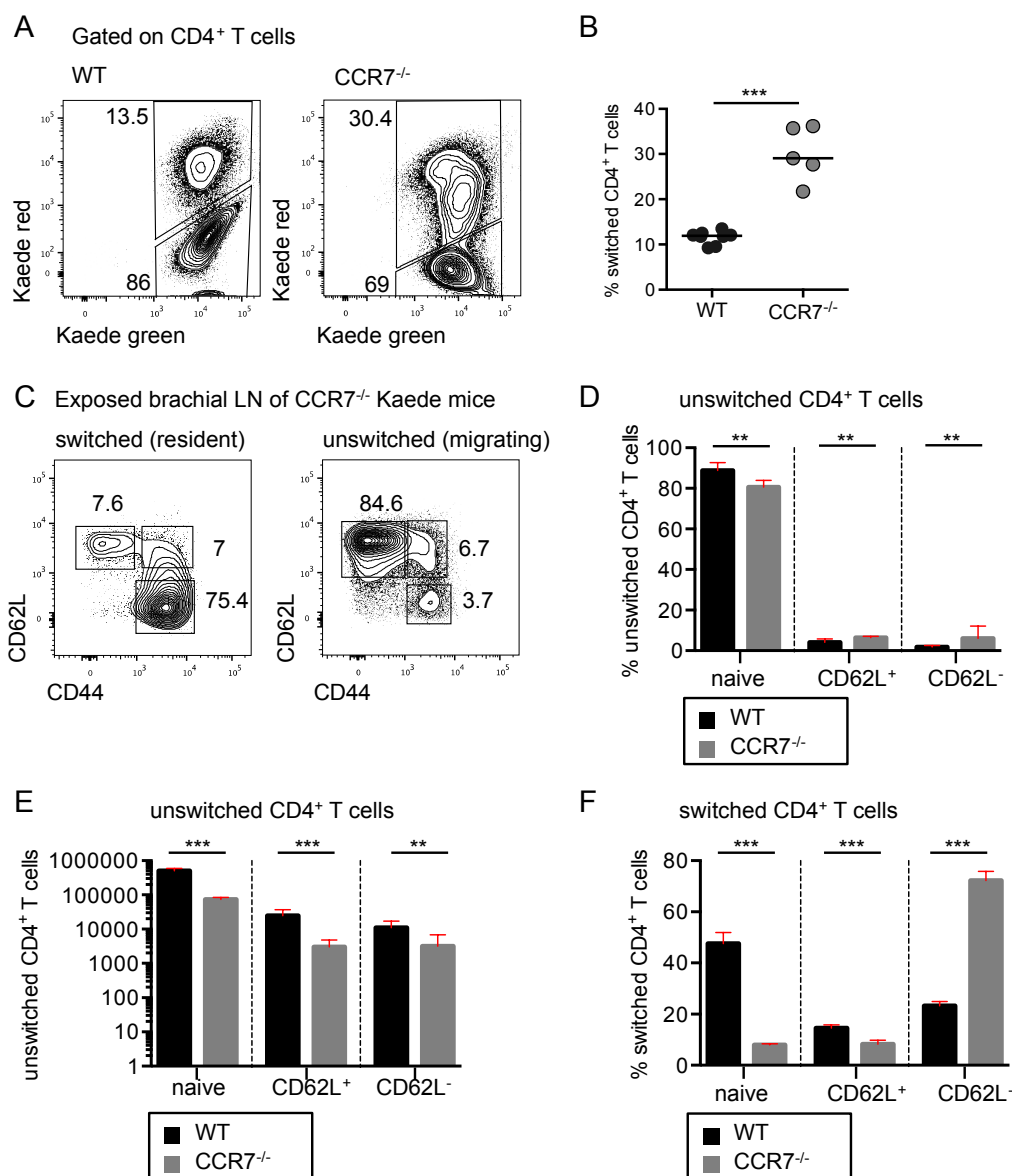


Figure 5.14: Investigating the migratory capacity of cells within the brachial lymph node 24 hours post photoconversion in WT and CCR7^{-/-} Kaede mice. Cohorts of WT and CCR7^{-/-} Kaede mice were analysed separately. The left brachial lymph node was exposed to violet light for 3 minutes then analysed 24 hours later. (A) Expression of Kaede green and Kaede red protein in CD4⁺ T cells. (B) Percentage of switched CD4⁺ T cells. (C) Expression of CD44 and CD62L on CD4⁺ T cells that are switched (left) and unswitched (right). (D) Percentage of unswitched CD4⁺ T cells that are naive, CD62L⁺ or CD62L⁻ cells. (E) Number of unswitched CD4⁺ T cells that are naive, CD62L⁺ or CD62L⁻ cells. (F) Percentage of switched CD4⁺ T cells that are naive, CD62L⁺ or CD62L⁻ cells.

(A and C) Plots are representative of 5 mice from 2 independent experiments. Values on plots are percentages. (B) Graph shows pooled data from 4 independent experiments. Bars show median. (D-F) Graphs show pooled data from 4 independent experiments. Bars show mean and error bars show SEM. Mann Whitney Test: **p ≤ 0.01, ***p ≤ 0.001.

Both the proportion and the number of switched CD4⁺ T cells that had migrated into the contralateral lymph nodes and spleen were significantly reduced in CCR7^{-/-} Kaede mice, consistent with a defect in the migration of these cells into lymphoid tissue (Figure 5.15 A and B). The fold change in the proportion of switched CD4⁺ T cells appeared to be greater in the contralateral lymph nodes than in the spleen (Figure 5.15 C). This is consistent with the requirement for CCR7 to enter lymph nodes and white pulp areas in the spleen but not for entry into the red pulp (Forster et al., 1999). The switched CD4⁺ T cell population in the contralateral lymph nodes of CCR7^{-/-} Kaede mice contained a smaller proportion of naive T cells than in WT mice (Figure 5.15 D and E). There was also a significant decrease in the number of naive T cells entering the contralateral lymph nodes in CCR7^{-/-} mice (Figure 5.15 F; mean for naive T cells: WT 19511, CCR7^{-/-} 899). However as a number of naive T cells were able to enter the lymph node this shows that expression of CCR7 isn't an absolute requirement for entry. There was a small, but statistically significant, increase in the proportion of switched CD62L⁺ Tcm phenotype cells and CD62L⁻ Tem phenotype cells, again indicating a higher reliance on CCR7 for naive T cells (Figure 5.15 E). Consistent with this, the fold change in number of cells entering the contralateral lymph nodes between WT and CCR7^{-/-} Kaede mice was higher for naive T cells than for CD62L⁺ or CD62L⁻ CD44^{hi} cells (Figure 5.15 G).

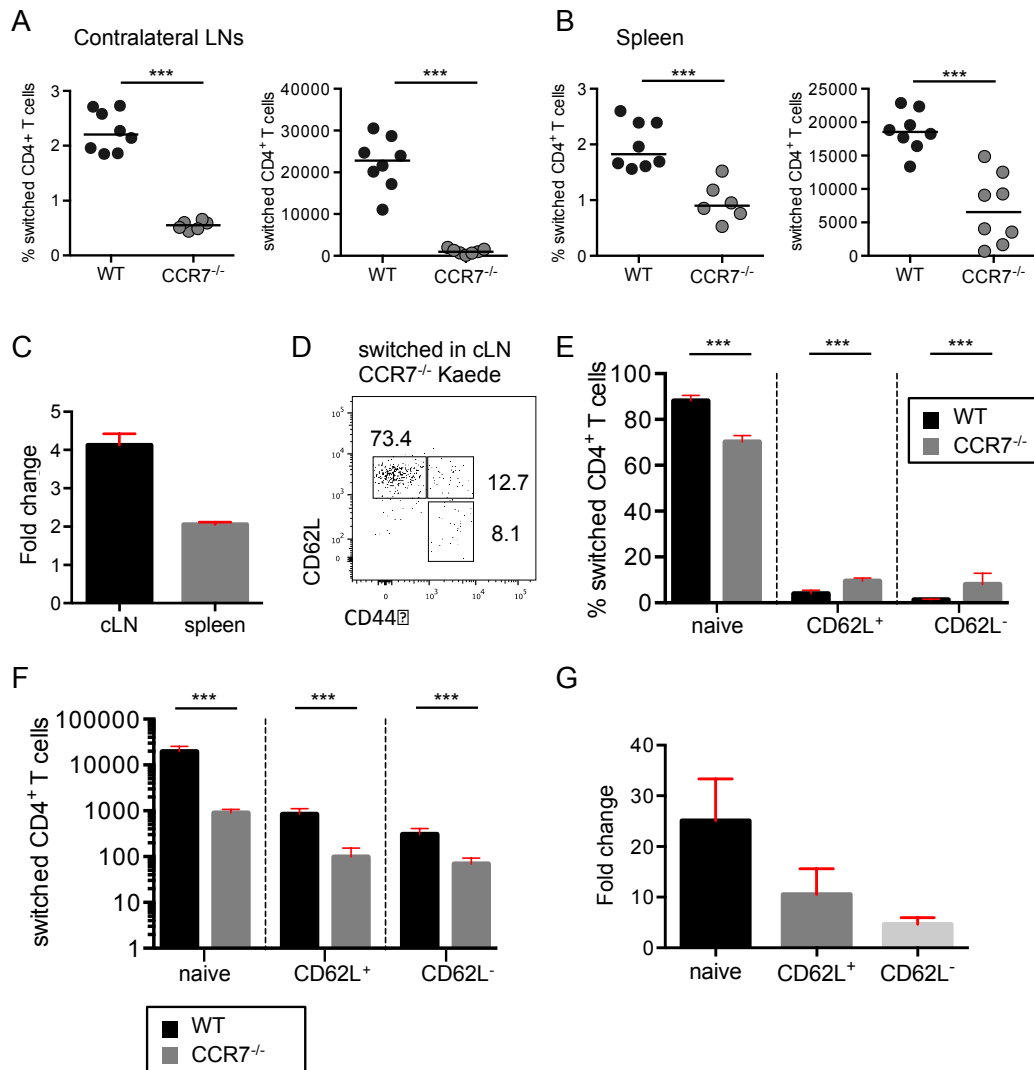


Figure 5.15: Investigating the migratory capacity of switched cells within the contralateral lymph nodes and spleen 24 hours post photoconversion in WT and CCR7^{-/-} Kaede mice. Cohorts of WT and CCR7^{-/-} Kaede mice were analysed separately. The left brachial lymph node was exposed to violet light for 3 minutes. Contralateral lymph nodes and spleen were then analysed 24 hours later. (A) Percentage (left) and number (right) of switched CD4⁺ T cells in the contralateral lymph nodes and ;(B) Spleen. (C) Fold change between WT and CCR7^{-/-} Kaede mice in the percentage of switched CD4⁺ T cells in the contralateral lymph nodes and spleen. (D) Expression of CD44 and CD62L on CD4⁺ T cells that are switched in the contralateral lymph nodes from CCR7^{-/-} Kaede mice. (E) Percentage and ;(F) Number of switched CD4⁺ T cells that are naive, CD62L⁺ or CD62L⁻ cells in the contralateral lymph nodes. (G) Fold change between WT and CCR7^{-/-} Kaede mice in the number of switched naive, CD62L⁺ and CD62L⁻ CD4⁺ T cells in the contralateral lymph nodes.

(D) Plot is representative of 7 mice from 2 independent experiments. Values on plots are percentages. (A and B) Graphs show pooled data from 4 independent experiments. Bars show median. (C-F) Graphs show pooled data from 4 independent experiments. Bars show mean and error bars show SEM. Mann Whitney Test: ***p ≤ 0.001.

5.2.8 Tracking the migration of 2W1S:I-A^b CD4⁺ T cells

24 hours following photoconversion

To assess migration of CD4⁺ memory T cell populations, we combined the use of our local immunisation model and Kaede transgenic mice to track the migration of antigen-specific CD4⁺ T cells. Previously it has been difficult to elucidate the migration patterns of antigen-specific T cells due to their low frequency.

Experiments have been carried out using TCR transgenic cell transfers, however injection with abnormally high numbers of cells may not represent the normal migratory behaviour of endogenous T cell populations. Additionally lymphoid tissues contain populations of T cells that are resident, as well as recirculating T cells that have altered expression of adhesion molecules upon entry to these sites (Luettig et al., 2001) . Therefore cell suspensions prepared from lymphoid tissues will contain cells that are not ready to leave and may not display normal migration (Westermann et al., 2003). To establish a model to investigate CD4⁺ T cell migration in Kaede mice, we immunised the left paw pad with 5 µg 2W1S peptide alum ppt. Surgery was performed to expose the draining lymph node to violet light and therefore switch all 2W1S:I-A^b CD4⁺ T cells contained within in it 8 dpi. We chose this timepoint as this is the peak of the effector response and therefore a large 2W1S:I-A^b CD4⁺ T cell population will be present to aid in analysis of migrating cells.

Twenty four hours following photoconversion 80 % of 2W1S:I-A^b CD4⁺ T cells in the exposed brachial lymph node were switched (Figure 5.16 A and B). This is a significant inhibition of migration compared to total CD4⁺ T cells in the naive

state where only 15 % remained switched after 24 hours (Figure 5.16 B). During an immune response lymph node shutdown describes the process whereby lymphocyte egress is inhibited to concentrate cells in the draining lymph node (Hall and Morris, 1965, Cahill et al., 1976). The mechanisms of this process are unknown but it is thought to increase the likelihood of cognate interactions occurring. Lymph node shutdown has been reported to last for 24 hours following antigen exposure, after which egress of lymphocytes is increased (Mackay et al., 1992). Another possibility is that there is antigen still present in the lymph node as alum ppt peptide can induce a prolonged immune response, demonstrated by maintained expression of OX40 following immunisation with 2W1S alum ppt peptide (Chapter 3, Figure 3.18). To determine if the migration of non-antigen specific T cells was also affected we determined the percentage of total CD4⁺ T cells that were switched in the brachial lymph node 8 dpi. 20 % of CD4⁺ T cells were switched which was significantly increased from 15 % seen in the naive state (Figure 5.16 A and B). This implies that the egress of multiple cell populations from the draining lymph node is inhibited due to the ongoing immune response. The percentage of 2W1S:I-A^b⁺ CD4⁺ T cells that were switched was higher still, indicating fundamental changes in antigen-specific cells that are responsible for their residency within the draining lymph node. It is also possible that the number of recirculating of 2W1S:I-A^b⁺ CD4⁺ T cells at this timepoint is very low due to their residence in the draining lymph node where cognate antigen is present. This would mean there are few incoming unswitched 2W1S:I-A^b⁺ CD4⁺ T cells to decrease the proportion of switched cells, as is the case for total CD4⁺ T cells.

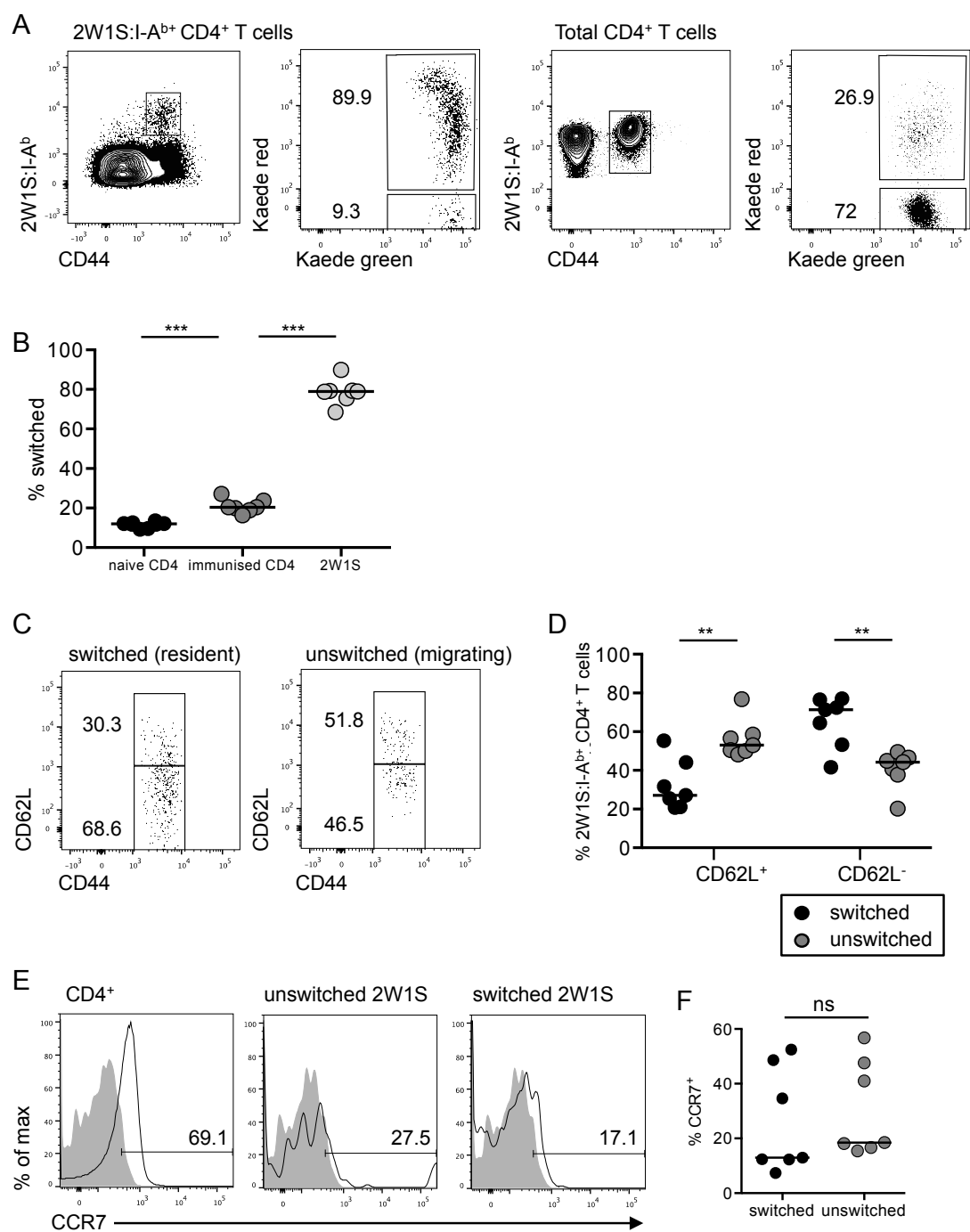


Figure 5.16: Tracking the migration of 2W1S:I-A^b CD4⁺ T cells from the brachial lymph node. Kaede mice were immunised in the left paw pad with 5 µg 2W1S alum ppt peptide. 8 dpi mice underwent surgery to photoconvert the left brachial lymph node which was then analysed 24 hours later. (A) Expression of Kaede green and Kaede red protein in 2W1S:I-A^b CD4⁺ T cells (left) and total CD4⁺ T cells (right). (B) Percentage of switched CD4⁺ T cells from naive mice, CD4⁺ T cells 8 dpi and 2W1S:I-A^b CD4⁺ T cells 8 dpi. (C) Expression of CD44 and CD62L on 2W1S:I-A^b CD4⁺ T cells that are switched (left) and unswitched (right). (D) Percentage of 2W1S:I-A^b CD4⁺ T cells that are switched and unswitched that are either CD62L⁺ or CD62L⁻. (E) Expression of CCR7 on total CD4⁺ T cells, unswitched and switched 2W1S:I-A^b CD4⁺ T cells. (F) Percentage of switched and unswitched 2W1S:I-A^b CD4⁺ T cells that are CCR7⁺.

(A, C, E) Plots are representative of 7 mice from 2 independent experiments. Values on plots are percentages. Fluorescence minus one control is represented by solid grey peak. (B, D, F) Graphs show pooled data from 2 independent experiments. Bars show median. Mann Whitney Test: **p ≤ 0.01, ***p ≤ 0.001, ns=non-significant.

We examined the expression of CD44 and CD62L on switched and unswitched 2W1S:I-A^b CD4⁺ T cells in the brachial lymph node to see if these surface markers determine their migratory capabilities (Figure 5.16 C). Eight dpi 2W1S:I-A^b CD4⁺ T cells have not yet undergone the contraction phase therefore the CD62L⁻ population will consist of effector cells and those that will survive as Tem phenotype cells. Unswitched 2W1S:I-A^b CD4⁺ T cells that have migrated into the brachial lymph node in the previous 24 hours are enriched in CD62L⁺ cells and have fewer CD62L⁻ cells compared to switched cells, which are resident in the lymph node (Figure 5.16 C and D). Although expression of CCR7 could be detected on 70 % of total CD4⁺ T cells, the majority of which are naive cells that highly express CCR7, the level of expression on activated 2W1S:I-A^b CD4⁺ T cells was very low (Figure 5.16 E). Due to the presence of two endogenous fluorescent markers in Kaede transgenic mice that cause bleed over into other channels, the fluorochromes that can be used to label surface markers are more limited. Combined with this CCR7 is notably difficult to detect upon the cell surface hence staining is performed at 37 °C to enhance detection. The percentage of switched and unswitched 2W1S:I-A^b CD4⁺ T cells that were CCR7⁺ was calculated and was highly variable between two experimental repeats (Figure 5.16 F). Therefore within Kaede transgenic mice detection of CCR7 expression by 2W1S:I-A^b CD4⁺ T cells was beyond the technical limits of the experiment.

A population of 2W1S:I-A^b CD4⁺ T cells could be detected in the contralateral lymph nodes, mesenteric lymph node and spleen (Figure 5.17 A).

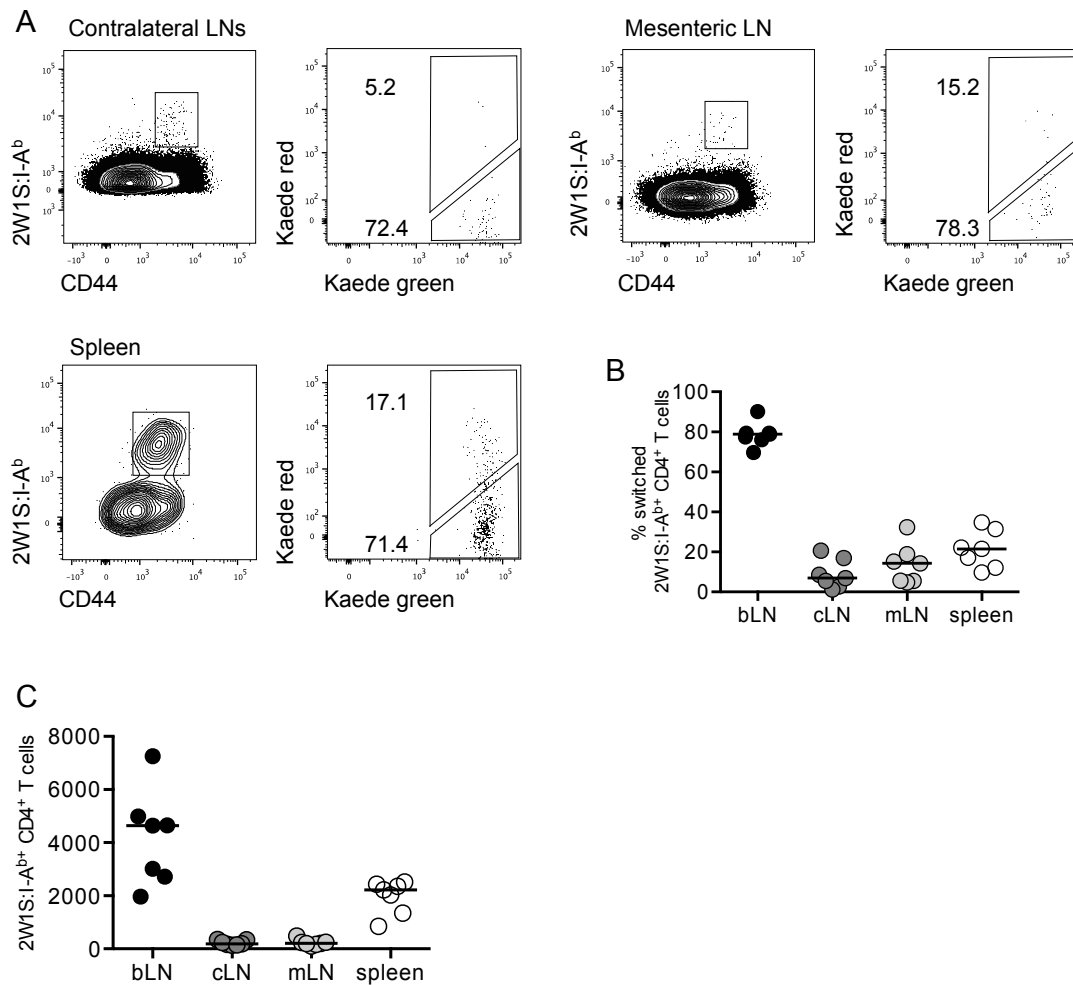


Figure 5.17: Tracking the migration of 2W1S:I-A^b CD4⁺ T cells throughout the body. Kaede mice were immunised in the left paw pad with 5 µg 2W1S alum ppt peptide. 8 dpi mice underwent surgery to photoconvert the left brachial lymph node and were analysed 24 hours later. (A) Expression of Kaede green and Kaede red protein in 2W1S:I-A^b CD4⁺ T cells from the contralateral lymph nodes, mesenteric lymph node and spleen. (B) Percentage of switched 2W1S:I-A^b CD4⁺ T cells from the exposed brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen. (C) Number of 2W1S:I-A^b CD4⁺ T cells from the exposed brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen.

(A) Plots are representative of 7 mice from 2 independent experiments. Values on plots are percentages. (B-C) Graphs show pooled data from 2 independent experiments. Bars show median.

The level of Kaede green expression was variable between experiments so gating was set on a larger population (CD4⁺ T cells) where positive and negative populations could be more easily delineated. A small proportion of 2W1S:I-A^{b+} CD4⁺ T cells were switched in all contralateral tissues (Figure 5.17 A). Migration of 2W1S:I-A^{b+} CD4⁺ T cells into these tissues appeared to be higher than for total CD4⁺ T cells in the naive state (contralateral LNs median: 7% compared to 2% and spleen median: 22 % compared to 2%- see Figure 5.13 A). For the contralateral lymph nodes this may be confounded by the very low cell number, resulting in an inflated percentage of switched cells (Figure 5.17 C; contralateral LNs median: 183). In the spleen however a significant population of 2W1S:I-A^{b+} CD4⁺ T cells was identified (Figure 5.17 C: spleen median 2219). Approximately 22 % of these cells had migrated into the spleen in the previous 24 hours, a significantly higher migration than for total CD4⁺ T cells in the naive state.

The majority of switched cells in the contralateral lymph nodes and mesenteric lymph nodes were CD62L⁺ (Figure 5.18 A and B). Although this observation is consistent with their ability to migrate into lymphoid tissue, the number of 2W1S:I-A^{b+} CD4⁺ T cells present was very low. In the spleen there was a more even split between CD62L⁺ and CD62L⁻ cells with a slight bias towards the latter (Figure 5.18 A and B).

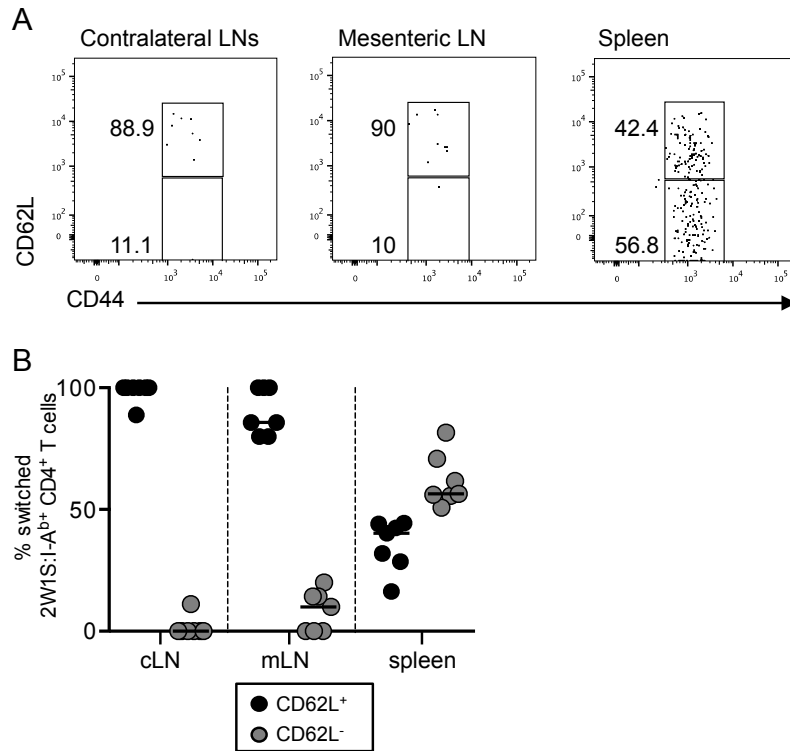


Figure 5.18: Phenotyping migrating 2W1S:I-A^b CD4⁺ T cells. Kaede mice were immunised in the left paw pad with 5 μ g 2W1S alum ppt peptide. 8 dpi mice underwent surgery to photoconvert the left brachial lymph node and contralateral lymph nodes, mesenteric lymph nodes and spleen were analysed 24 hours later. (A) Expression of CD44 and CD62L on switched 2W1S:I-A^b CD4⁺ T cells from each tissue respectively. (B) Percentage of switched 2W1S:I-A^b CD4⁺ T cells that are CD62L⁺ or CD62L⁻ from each tissue.

(A) Plots are representative of 7 mice from 2 independent experiments. Values on plots are percentages. (B) Graphs show pooled data from 2 independent experiments. Bars show median.

To extend our analysis to CD4⁺ memory T cells we immunised Kaede mice with 2W1S alum ppt peptide in the left paw pad and rested for 42 days. This timepoint was chosen as we have previously shown in WT mice the response to alum ppt 2W1S peptide takes longer to enter the memory phase than with Lm-2W1S infection (Figures 5.5 and 5.6). At 42 dpi mice underwent surgery to photoconvert the draining brachial lymph node and were analysed 24 hours later. In the exposed brachial lymph node approximately 70 % of 2W1S:I-A^{b+} CD4⁺ memory T cells were switched indicating that there had been little migration of these antigen-specific CD4⁺ T cells when compared to migration of total CD4⁺ T cells in a naive mouse where the percentage of switched cells remaining after 24 hours was only 15 % (Figure 5.19 A; naive CD4⁺ T cell migration shown in Figure 5.11 E and F). The reduced proportion of 2W1S:I-A^{b+} CD4⁺ T cells expressing OX40 at this timepoint indicated that the response may have entered the memory phase, however this lack of migration indicated that cell movement might still be restricted. To determine if this lack of migration was due to prolonged effects of lymph node shutdown, we analysed the migration of total CD4⁺ T cells in Kaede mice that had been immunised 42 days previously. The median percentage of switched CD4⁺ T cells in the brachial lymph node was 7 % demonstrating that migration of naive cells has been restored to normal levels (Figure 5.19 A and B). This suggests that the lack of migration in 2W1S:I-A^{b+} CD4⁺ T cells is due to fundamental differences between naive and memory cells.

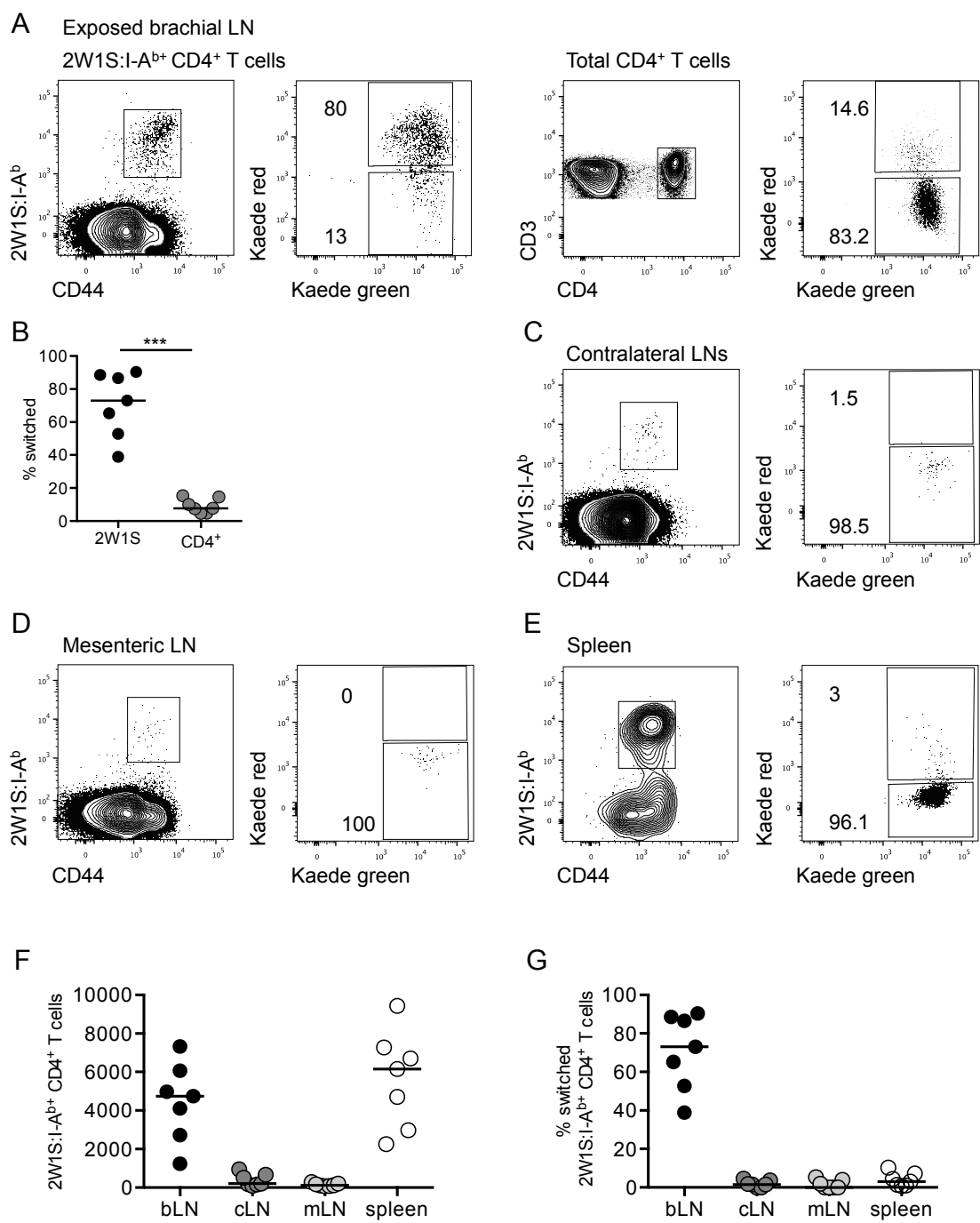


Figure 5.19: Tracking the migration of 2W1S:I-A^b CD4⁺ memory T cells. Kaede mice were immunised in the left paw pad with 5 µg 2W1S alum ppt peptide. 42 dpi mice underwent surgery to photoconvert the left brachial lymph node and were analysed 24 hours later. (A) Expression of Kaede green and Kaede red protein in 2W1S:I-A^b CD4⁺ T cells (left) and total CD4⁺ T cells (right) from the exposed brachial lymph node. (B) Percentage of switched 2W1S:I-A^b CD4⁺ T cells and total CD4⁺ T cells from the exposed brachial lymph node. (C) Expression of Kaede green and Kaede red protein in 2W1S:I-A^b CD4⁺ T cells from the contralateral lymph nodes; (D) mesenteric lymph node and (E) spleen. (F) Number of 2W1S:I-A^b CD4⁺ T cells from the exposed brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen. (G) Percentage of switched 2W1S:I-A^b CD4⁺ T cells from the exposed brachial lymph node, contralateral lymph nodes, mesenteric lymph node and spleen.

(A, C-E) Plots are representative of 7 mice from 2 independent experiments. Values on plots are percentages. (B, F and G) Graphs show pooled data from 2 independent experiments. Bars show median. Mann Whitney Test: *** $p \leq 0.001$

A population of 2W1S:I-A^b⁺ CD4⁺ memory T cells could be clearly identified in contralateral lymph nodes, mesenteric lymph node and spleen but as expected was small in contralateral and mesenteric lymph nodes (Figure 5.19 C-F). Nearly all 2W1S:I-A^b⁺ CD4⁺ memory T cells identified in these tissues were unswitched meaning they had not migrated from the draining brachial lymph node in the previous 24 hours (Figure 5.19 C-E and G). However their presence at these sites indicated that 2W1S:I-A^b⁺ CD4⁺ memory T cells are migrating but perhaps 24 hours is not a sufficient amount of time for them to move from the draining brachial lymph node. Alternatively, as the most switched cells could be found in the spleen, it may be that there is not a large enough population circulating through the lymph nodes to detect. Fundamentally, these data indicate that a CD4⁺ memory T cell population in the draining lymph node is maintained which displays very limited migration within 24 hours.

Again we investigated the phenotypic makeup of the switched and unswitched populations in the exposed brachial lymph node using expression of CD62L. Unswitched 2W1S:I-A^b⁺ CD4⁺ memory T cells that had migrated into the lymph node in the previous 24 hours contained a higher proportion of CD62L⁺ cells, 40 % compared to 14 %, than switched cells that were resident (Figure 5.20 A and B). Switched 2W1S:I-A^b⁺ CD4⁺ memory T cells that are resident in the draining lymph node were enriched for CD62L⁻ cells (Figure 5.20 A and B; median for switched Tem 85 %, unswitched Tem 56 %). Approximately 56% of unswitched 2W1S:I-A^b⁺ CD4⁺ T cells in the brachial lymph node were CD62L⁻ and therefore of

Tem phenotype indicating that CD62L⁻ cells do recirculate through lymph nodes (Figure 5.20 B).

Nearly all 2W1S:I-A^b⁺ CD4⁺ memory T cells in the contralateral lymph node were unswitched meaning that these cells had not migrated from the brachial lymph node in the previous 24 hours, but must still be a recirculating population to have reached this distal site. We analysed this population for expression of CD62L and CD27. Although there was a significantly higher proportion of CD62L⁺ cells, around 35 % of this population was CD62L⁻ (Figure 5.20 C and D). In addition a proportion of this population were CD27⁻ and therefore defined as Tem cells (Figure 5.20 E and F). These data indicate that although the majority of recirculating 2W1S:I-A^b⁺ CD4⁺ memory T cells are of the conventional CD62L⁺ Tcm phenotype, a small proportion are Tem cells, which are not thought to recirculate through lymph nodes.

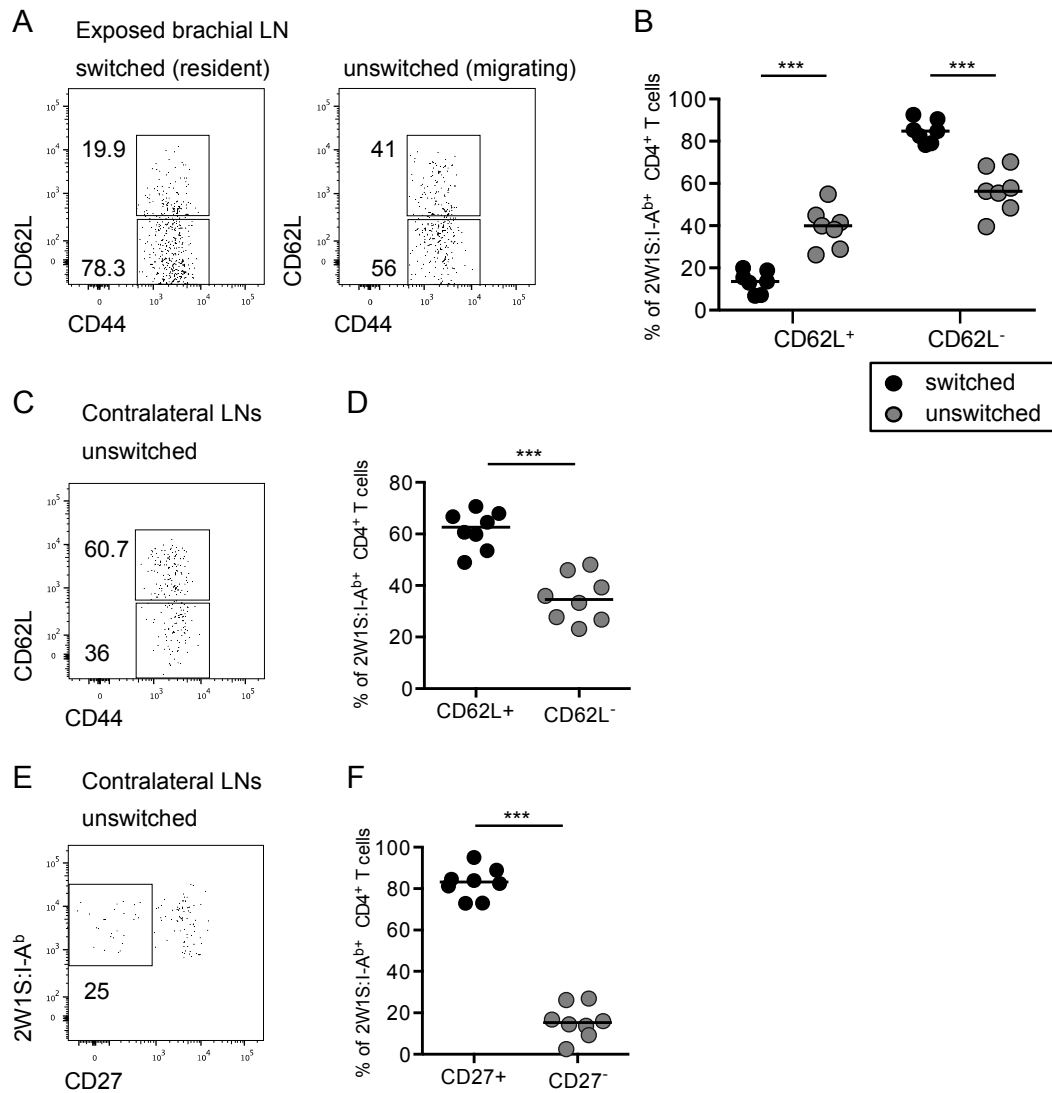


Figure 5.20: Phenotyping migrating 2W1S:I-A^b CD4⁺ memory T cells. Kaede mice were immunised in the left paw pad with 5 µg 2W1S alum ppt peptide. 42 dpi mice underwent surgery to photoconvert the left brachial lymph node and were analysed 24 hours later. (A) Expression of CD44 and CD62L on switched and unswitched 2W1S:I-A^b CD4⁺ memory T cells from the exposed brachial lymph node. (B) Percentage of 2W1S:I-A^b CD4⁺ memory T cells that are switched and unswitched that are either CD62L⁺ or CD62L⁻ phenotype from the exposed brachial lymph node. (C) Expression of CD44 and CD62L on unswitched 2W1S:I-A^b CD4⁺ memory T cells from the contralateral lymph nodes. (D) Percentage of 2W1S:I-A^b CD4⁺ memory T cells that are CD62L⁺ and CD62L⁻ from the contralateral lymph nodes. (E) Expression of CD27 on unswitched 2W1S:I-A^b CD4⁺ memory T cells from the contralateral lymph nodes. (F) Percentage of 2W1S:I-A^b CD4⁺ memory T cells that are CD27⁺ and CD27⁻ from the contralateral lymph nodes.

(A, C and E) Plots are representative of 8 mice from 2 independent experiments. Values on plots are percentages. (B, D and F) Graphs show pooled data from 2 independent experiments. Bars show median. Mann Whitney Test: ***p ≤ 0.001

5.3 Discussion

Throughout my analysis of CD4⁺ memory T cell populations, a surprising observation was the location of cells possessing a Tem phenotype within secondary lymphoid tissue. These data suggested that some Tem cells might persist within secondary lymphoid tissue rather than traffic through non-lymphoid tissue as is conventionally thought. To further investigate this, in this chapter I have established a novel model to track the migration of an antigen-specific CD4⁺ T cell population in photoconvertible Kaede transgenic mice. This model clearly demonstrates that, following immunisation with alum ppt peptide, a population of CD4⁺ memory T cells displaying a non-circulatory phenotype within the draining lymph node is generated and persists at this site for an extended period of time.

Both Tcm and Tem cells, identified by cytokine secretion, could be found in secondary lymphoid tissue following systemic infection with Lm-2W1S. To better understand the distribution of CD4⁺ memory T cells following infection with Lm-2W1S, I looked in the lymph nodes, spleen, lungs and blood samples. Within the spleen and lymph nodes the 2W1S:I-A^b⁺ CD4⁺ memory T cell population was enriched in Tcm cells as would be expected for lymphoid sites. In contrast to this memory cells in the lung were biased towards IFN- γ producing Tem cells. Although expression of CCR7 was more prevalent in lymphoid tissues it was not restricted to Tcm cells as would be expected based on their definition (Sallusto et al., 1999). This raises the possibility that CCR7 is not only expressed by Tcm cells, at least in the context of some responses. A recent paper assessing

the contribution of CCR7 in competitive mixed bone marrow chimeras found that expression of CCR7 conferred no advantage for CD4⁺ memory T cells entering lymph nodes from the blood (Vander Lugt et al., 2013). This, combined with my data, is suggestive that CCR7 may not be the most useful marker for functionally defining Tcm cells.

To further investigate the migratory capacity of CD4⁺ memory T cells, I have established a local immunisation model in which an antigen-specific population of CD4⁺ T cells was expanded in the draining lymph node and their subsequent migration into contralateral tissue then tracked. This model assessed the movement of CD4⁺ T cells from the blood through HEV into lymph nodes and is not influenced by intra-nodal migration of cells through the lymph system. The amount of antigen administered was central to the success of this model; low quantities of peptide restricted the immune response to the draining lymph node and antigen was not disseminated beyond this within 24 hours. Crucially this meant that the differentiation of Teff, Tcm precursors and TFH cells took place only in the draining lymph node and the migratory component of these populations could be followed in contralateral tissues. Evidence for this was generated through the use of OVA AF647, which indicated that antigen did not disseminate beyond the draining lymph node 24 hours after immunisation. Although it cannot be ruled out that small quantities of antigen may be dispersed after this time, the absence of TFH formation in contralateral lymph nodes, mesenteric lymph node and spleen indicates that little antigen spreads beyond the draining lymph node.

We then extended this model to investigate the migration of antigen-specific CD4⁺ memory T cells. As there are caveats associated with the study of memory phenotype cells we followed the endogenous 2W1S:I-A^b CD4⁺ T cell population and defined memory cells based on time since immunisation. Due to the prolonged response induced by alum ppt peptide memory cells were studied 42 dpi, a later timepoint than in the systemic response to Lm-2W1S. Conventional definition of Tcm and Tem cells by CD62L and CCR7 has recently been questioned by several studies (Unsoeld and Pircher, 2005, Vander Lugt et al., 2013, Brinkman et al., 2013, Debes et al., 2004). CD8⁺ memory T cells responding to LCMV were frequently found to be CD62L⁻CCR7⁺ in both lymphoid and non-lymphoid tissue, a phenotype not classically associated with Tcm or Tem cells (Unsoeld and Pircher, 2005). Following i.v. infection with Lm-2W1S CCR7 expression was more widespread in lymphoid tissue however a proportion of CCR7⁺ cells were present in the lungs. This indicates that expression of lymph node homing surface molecules cannot solely be used to predict the localisation of CD4⁺ memory T cells. IFN-γ⁺ Tem cells that expressed CCR7 at a similar level to IFN-γ⁻ Tcm cells could be identified, as has been described for IFN-γ⁺ cells in the lung draining mediastinal lymph nodes following influenza infection (Debes et al., 2004). At 42 dpi with alum ppt 2W1S peptide, CCR7⁺CD62L⁻ cells could also be identified in the lymph nodes and spleen demonstrating that CCR7 expression is not restricted to CD62L⁺ cells. Although it is possible that these represent Tcm cells that have down-regulated CD62L, co-expression of IFN-γ and CCR7 suggest CCR7 expression is more widespread than previously thought.

For the reasons outlined above, reliance on CD62L and CCR7 for the definition of CD4⁺ memory T cell populations may be unreliable. Furthermore these molecules mediate the entry of CD4⁺ T cells into lymph nodes, therefore investigation of migration may be confounded by definition of memory T cells by these markers. For this reason we investigated alternative markers that could be used to define Tem cells. KLRG1 has been used to discriminate CD8⁺ memory T cells with the ability to rapidly produce effector cytokines (Sarkar et al., 2008). However KLRG1 was not expressed by 2W1S:I-A^{b+} CD4⁺ T cells and therefore is not useful for discrimination of memory cell populations. CD27⁻ cells are contained within CCR7⁻ and CD62L⁻ circulating human memory T cell populations suggesting lack of this TNF receptor correlates with Tem phenotype (Campbell et al., 2001). Consistent with this we found that a population of CD27⁻ cells was present in the CD62L⁻, but not CD62L⁺, 2W1S:I-A^{b+} CD4⁺ T cell population, indicating that CD27⁻ cells are Tem cells.

To study the migration of 2W1S:I-A^{b+} CD4⁺ memory T cells we used photoconvertible Kaede transgenic mice. Irreversible photoconversion of cells upon exposure to violet light labels these cells and their migration can be tracked throughout the body. We have successfully developed a surgical model to expose the brachial lymph node to violet light and combined this with local immunisation to track the migration of photoconverted 2W1S:I-A^{b+} CD4⁺ T cells. This method is highly advantageous compared to previous studies of memory cell migration as it enables antigen-specific cells to be tracked from a known

origin in a specified period of time. This model allowed us to better establish the kinetics of cell trafficking to and from lymphoid sites.

Initial analysis of CD4⁺ T cells in a resting state revealed that these cells are highly migratory; in a 24 hour period approximately 85 % of the cells present at the time of photoconversion had exited the lymph node. Analysis of 8 similarly treated mice showed little variation indicating that this is a robust model for tracking cell migration. A small, but consistent, population of switched cells could then be identified in the contralateral lymph nodes and spleen demonstrating that these cells have migrated from the brachial lymph node and validating this model as a method to track CD4⁺ T cell migration from a given site. Among the migrating switched cells found in the contralateral lymph node the CD62L⁻ population are under-represented, consistent with the role of this molecule in lymph node entry. Within the switched population resident in the exposed brachial lymph node CD44^{hi} cells are enriched, indicating that the antigen-experienced population is less likely to or less efficient at recirculating than naive T cells.

To track the migration of an antigen-specific CD4⁺ T cell population mice were immunised with alum ppt 2W1S peptide and underwent surgery to expose the draining lymph node to violet light 8 dpi or 42 dpi to study memory T cells. Previously it has been difficult to study the migration of antigen-specific CD4⁺ memory T cell populations due to their low frequency. The development of photoconvertible proteins has provided new ways to investigate cell trafficking (Tomura et al., 2008). Targeted exposure to violet light allows cells within a

given site to be irreversibly labelled at a specific point in time. Photoconversion of the draining lymph node 8 dpi and analysis after 24 hours revealed that 80 % of 2W1S:I-A^b CD4⁺ T cells were switched, showing that the kinetics of migration of responding antigen-specific cells in an inflamed lymph node are much slower than CD4⁺ T cells in the steady state. This could be due to a number of reasons; 8 dpi with alum ppt 2W1S peptide there may still be antigen present within the draining lymph node which is inhibiting the egress of T cells from this site. In support of this the egress of naive CD4⁺ T cells was also decreased compared to the steady state indicating that this effect is not restricted to responding antigen-specific cells. Alternatively, or in addition, the number of circulating 2W1S:I-A^b CD4⁺ T cells will likely be low as the majority are contained within the draining lymph node, actively involved in a response. Therefore there are few migrating cells to replace the switched cells within the draining lymph node.

The migration of 2W1S:I-A^b CD4⁺ T cells out of the draining lymph node remained low for memory cells; at 42 dpi 70 % of cells within this site were switched. OX40 expression at this timepoint was significantly reduced compared to 21 dpi, however was still present on a number of 2W1S:I-A^b CD4⁺ T cells. This may suggest that there is still antigen present, but the migration of total CD4⁺ T cells was restored to the level observed in the steady state, indicating they were not under the effect of lymph node shutdown. It is possible that expression of OX40 in the response to alum ppt 2W1S peptide shows different kinetics to infection with Lm-2W1S, independent of antigen availability. Unexpectedly there appears to be a resident population of 2W1S:I-A^b CD4⁺ memory T cells present

in the lymph node 42 dpi. Conventional recirculating Tcm and Tem populations may have been expected to leave the lymph node by this point. This resident population was composed of significantly more CD62L⁻ cells, a characteristic displayed by tissue resident CD8⁺ T cells in the small intestine (Schenkel and Masopust, 2014). Trm cells have been described for both CD4⁺ (Teijaro et al., 2011) and CD8⁺ (Schenkel and Masopust, 2014) T cell populations, poised to respond to infection at non-lymphoid sites. Also using photoconversion to label cells, a recent study has described a resident population of CD4⁺ T cells in the mesenteric lymph node and Peyer's patches (Ugur et al., 2014). These cells were also CD62L⁻ and accumulated in response to chronic antigen administered orally (Ugur et al., 2014). This population included TFH cells as well as other T cell subsets and expressed CD69, a commonly used marker of CD8⁺ Trm cells (Schenkel and Masopust, 2014). This suggests that lymph node resident populations may form in response to persistent antigen, however resident CD8⁺ T cells have also been described in the absence of antigen using parabiosis experiments. A small population in the spleen and lymph nodes, between 1-5 % of CD8⁺ memory cells, remained within these tissues and were phenotypically similar to resident cells in the small intestine (Schenkel et al., 2014). The function of a lymph node resident population is unknown and presumably would have to decrease in size over time to make way for subsequent responses. CD8⁺ Trm cells were found to be positioned at sites of pathogen entry in the spleen and lymph nodes (Schenkel et al., 2014), perhaps located here to most effectively activate immune responses for pathogen control.

Although the proportion of switched cells in the draining lymph node 8 dpi is high suggesting slow egress of cells, 2W1S:I-A^b⁺ CD4⁺ T cells appear to migrate into contralateral lymph nodes and the spleen at a higher rate than naive T cells in the steady state. This is likely because these sites originally contain very few 2W1S:I-A^b⁺ CD4⁺ T cells and they are being seeded with circulating cells from the draining lymph node. The majority of these cells in contralateral lymph nodes are CD62L⁺ as would be expected for cells able to enter the lymph node from the bloodstream. At 42 dpi switched 2W1S:I-A^b⁺ CD4⁺ memory T cells could not be detected in the contralateral lymph nodes. This could be because 24 hours is not sufficiently long enough for memory cells to migrate from the brachial lymph node, however the presence of switched 2W1S:I-A^b⁺ CD4⁺ memory T cells in the spleen argues against this notion. It is more likely that the number of 2W1S:I-A^b⁺ CD4⁺ memory T cells migrating into contralateral lymph nodes is below our limit of detection, as a small population could be identified in the spleen, which is a larger tissue. There were however many unswitched 2W1S:I-A^b⁺ CD4⁺ memory T cells present in the contralateral lymph nodes that represent a recirculating population, although they were not in the brachial lymph node at the time of photoconversion. As expected this population was enriched in CD62L⁺ cells, however contained a significant proportion, around 35 %, of CD62L⁻ cells. Furthermore a small proportion of these cells were CD27⁻ and therefore displayed the phenotype of T_{em} cells, which are not thought to recirculate through lymph nodes. Unswitched cells migrating into the brachial lymph node contained an increased proportion of CD62L⁺ cells compared to cells resident in the lymph node at both primary and memory timepoints. Yet approximately half

of this population was CD62L⁻, again indicating that recirculating effector and memory 2W1S:I-A^{b+} CD4⁺ memory T cells may enter lymph nodes from the bloodstream using a CD62L independent mechanism as well as through afferent lymph.

CCR7^{-/-} Kaede transgenic mice were used to study the migration of T cells that lack this chemokine receptor, thought to be required for multiple immune functions including entry into lymph nodes (Forster et al., 1999). Consistent with a defect in migration, the exposed brachial lymph node of CCR7^{-/-} Kaede mice contained an increased proportion of switched cells. As the number and proportion of naive T cells migrating into lymph nodes was reduced this was due to impaired entry, not exit. The proportion of CD44^{hi} cells was increased in lymph nodes implying that memory cells are less reliant on CCR7 than naive T cells. This is consistent with a study showing that CCR7 confers no advantage for entry of Tcm cells into lymph nodes (Vander Lugt et al., 2013). The migration of CD4⁺ T cells into contralateral lymph nodes was more impaired than their migration into spleen. Entry into the spleen is not impaired in the absence of CCR7 but T cell localisation is severely perturbed (Forster et al., 1999).

In summary I have developed a robust method to track the migration of both CD4⁺ T cells and 2W1S:I-A^{b+} T cells using photoconvertible Kaede transgenic mice. Migration of naive T cells into lymph nodes is CCR7 dependent and is significantly impaired in mice that lack this chemokine receptor. However antigen-experienced cells are less reliant on CCR7 for entry into lymph nodes and likely use other CCR7-independent mechanisms. Expression of CCR7 was not

restricted to Tcm and was also found on Tem cells, both functionally defined by cytokine secretion. Following local immunisation migration of 2W1S:I-A^{b+} CD4⁺ T cells, the majority of which were CD62L⁻, out of the draining lymph node was low at both 8 and 42 dpi. This suggests that a population of cells with Tem phenotype persist in the draining lymph node and do not recirculate as classical Tcm and Tem cells do.

CHAPTER 6: GENERAL DISCUSSION

6.1 Discussion

Vaccines have been highly successful in reducing morbidity and mortality since they were first developed by Edward Jenner in 1796. Major diseases that used to cause significant mortality such as smallpox, measles and diphtheria are now eradicated or reported rarely in vaccinated populations (Hilleman, 2000).

Underpinning the success of vaccines is the induction of long-term immunological memory. Although the driving force behind the development of many vaccines was the induction of a high-titre antibody response, the role that the cell-mediated arm plays in protective immunity is now better understood. Conventionally, vaccines have been effective as the pathogens they target have been antigenically stable therefore have induced robust antibody responses, as for smallpox and polio. The majority of currently licensed vaccines induce antibody responses, which are reliant on T cell help (Esser et al., 2003). These vaccines are successful at providing long-term protection against viruses such as influenza and smallpox. However, vaccine development for chronic infections or those able to evade immune recognition through switching of surface antigens has been more problematic, as in the case of *Mycobacterium tuberculosis* (TB), human immunodeficiency virus (HIV) and hepatitis C virus (HCV). The protective responses to these pathogens are cell mediated and consist of both CD4⁺ and CD8⁺ T cells.

Thus, memory T cells play a key role in protective immunity and are the cornerstone of successful vaccines. CD4⁺ memory T cells are a fundamental aspect of recall immunity as they activate and recruit other components of the

immune response to clear pathogens before they are able to spread systemically. Elucidating the mechanisms involved in the generation and subsequent survival of memory cells is vital to the understanding and production of more efficient vaccination strategies. To date this has been challenging due to the lack of sensitive methods to track antigen-specific memory T cells *in vivo*, impeding our understanding of the precise mechanisms controlling their generation and survival.

The division of CD4⁺ memory T cells from primary effector cells has been problematic due to a deficit of distinctive surface receptors. Upregulation of CD44 following antigen recognition is often used to distinguish antigen-experienced T cells from naive T cells, however this marker is unable to differentiate between effector cells in an active response from memory cells persisting in the absence of antigen. Other activation markers such as CD69 and CD25 have also been employed to identify recently activated cells, however expression of these molecules can be transient and therefore their absence does not necessarily identify memory cells (Sallusto et al., 1999, Dutton et al., 1998). Furthermore memory phenotype cells defined by these criteria have been shown to possess different survival and homeostatic properties from antigen-specific T cells (Purton et al., 2007, Lenz et al., 2004). For this reason the study of antigen-specific memory T cells that have proliferated in response to a known antigen and undergone contraction to form a stable memory cell population is necessary. TCR transgenic cells have been employed to enhance detection however studies have now found they may not be representative of endogenous, polyclonal

memory cell populations (Marzo et al., 2005, Foulds and Shen, 2006). For this reason tracking endogenous populations using MHC II tetramers has become the “gold standard” for analysis of CD4⁺ memory T cells. Amongst these endogenous populations, CD4⁺ T cells responsive to 2W1S peptide have been demonstrated as a robust model due to reliable detection methods and the development of an infectious agent expressing the 2W1S peptide (Pepper et al., 2010, Pepper et al., 2011). Infection with Lm-2W1S induces expansion of CD4⁺ T cells responding to the 2W1S peptide, a larger than average endogenous T cell pool that facilitates the study of this population. Combination of magnetic enrichment and MHC II tetramers enable this population to be tracked in naive mice, following infection through the primary response and after contraction of the effector population into the memory cell phase. Detailed analysis of the kinetics of this response by the Jenkins laboratory has facilitated the definition of memory T cells by time since infection. This stable population consists of cells undergoing slow homeostatic proliferation, persisting in the absence of antigen (Pepper et al., 2010, Pepper et al., 2011). The establishment of this model now enables the role of multiple molecules and signalling pathways to be investigated for their involvement in CD4⁺ memory T cell generation and survival.

Memory cells have been broadly divided into subsets based upon expression of key lymph node homing molecules CCR7 and CD62L (Sallusto et al., 1999). Tcm cells were defined by their expression of CCR7 and CD62L, circulating through lymphoid tissue. Upon activation they produce IL-2 and proliferate to produce numerous effector cells to respond to reinfection. Tem cells lack these lymph

node homing receptors, instead circulating through blood and non-lymphoid tissue. Upon activation they rapidly produce effector cytokines such as IFN- γ to eliminate threats. However since their description it has been shown that these definitions are not absolute; memory T cells lacking CCR7 and CD62L have been observed within lymphoid tissue (Unsoeld and Pircher, 2005). Additionally, the recent identification of Trm cells has shown that memory cell populations may be more complex than this simple definition based upon expression of homing molecules. It is becoming increasingly clear that Trm cells, which are not present in human peripheral blood samples, are an important part of adaptive immunity and protection against infection. The requirements for survival and maintenance of these populations are yet to be defined, but may be different to those of circulating Tcm and Tem cells. Development of new vaccines may hinge upon a greater understanding of how protective immunity is initiated at specific sites, whether this is due to migration of memory cells or tissue resident populations. The putative resident CD4⁺ memory T cell population that we have identified within a peripheral skin draining lymph node following local immunisation may represent a Trm cell population found within secondary lymphoid organs. This population is similar to a recently described resident population located in Peyer's patches and mesenteric lymph node (Ugur et al., 2014). These cells did not recirculate and were found to accumulate in the Peyer's patches following chronic antigen exposure. Although the function of such a population is currently unclear, it may be poised at sites of pathogen entry to efficiently recruit other immune cells to this site and rapidly clear pathogens before they can spread systemically. The goal of vaccine design will be to induce high numbers of both

Tem and Trm cells, to provide immediate effector responses, and Tcm cells to provide a pool of new effector cells through proliferation. A greater understanding of the factors that govern the generation and survival of each of these populations is essential to improved vaccine design in the future. Definition of these populations based on chemokine receptors, cytokine secretion and adhesion molecules will help to determine what type of response a vaccine is eliciting and therefore be tailored to provide the most efficacious combination.

The generation of a functional memory cell pool is defined by the integration of numerous signals received during T cell priming. CD4⁺ T cells undergo 3 distinct phases after encounter of cognate antigen: extensive proliferation and differentiation, contraction due to cell death and the memory phase, which is maintained by slow homeostatic proliferation. Signals received during priming dictate the differentiation pathway and are determined by the context in which antigen is recognised, which will shape the response due to activation of different PRR, and the duration of antigen contact (Iezzi et al., 1998). CD4⁺ T cells then integrate signals from a number of different costimulatory receptors to determine the differentiation of T cell subsets. Costimulatory molecules can act to enhance signalling through the TCR or may provide additional signals that affect the proliferation or differentiation of T cells. There may be temporal separation of these signals; for example CD28 is constitutively expressed on naive T cells therefore acts immediately upon antigen recognition to influence TCR signalling (Tuosto and Acuto, 1998). Other receptors such as OX40 and ICOS

are upregulated following T cell activation therefore may exert their influence in the days following initial antigen recognition (Gramaglia et al., 1998, Hutloff et al., 1999, Mages et al., 2000). When analysing the costimulatory requirements of CD4⁺ T cell populations it will be important to consider the nature of the response. Monoclonal TCR transgenic populations will likely show different requirements to endogenous polyclonal populations, due to the presence of T cells with a range of affinities. Therefore a better understanding of how the resulting memory cell pool is affected by signals received in the primary response may require the study of endogenous populations, as they most closely resemble those in a human patient.

Previous studies have shown a clear role for ICOS in the generation of TFH cells but its importance for other T cell subsets was less clear (Akiba et al., 2005, Bossaller et al., 2006). My studies have shown that ICOS signals received during the primary response to Lm-2W1S enable the optimal formation of the memory cell pool. Importantly, ICOS signals were required for the generation of both Tcm and Tem cells subsets. Human patients with ICOS deficiency have been reported to show a similar defect in both Tcm and Tem cell populations indicating our murine studies show close parallels to human immunity (Takahashi et al., 2009). Additionally, signals received through OX40 appeared to specifically benefit the Teff cell population and were actually detrimental to TFH cells when given in excess. Agonistic anti-OX40 mAb caused the vast expansion of Teff cells in the primary response and skewed the memory cell pool towards Tem cells. Understanding the role these different costimulatory molecules play in

controlling T cell differentiation and memory cell formation will enable the manipulation of vaccine responses to achieve the desired T cell response.

The factors mediating the long-term survival of memory T cells have been a contentious issue; initial ideas suggested that continuous provision of TCR signals through peptide:MHC sustained memory cells (Gray and Matzinger, 1991). However studies in MHC II^{-/-} mice have shown normal survival of CD4⁺ memory T cells (Swain et al., 1999). Since CD8⁺ memory T cells are more abundant and therefore easier to study much work has revolved around signals regulating the survival of this population. It was determined that the cytokines IL-7 and IL-15 were essential for the persistence of this population (Schluns and Lefrancois, 2003), so these were proposed as likely candidates regulating the survival of CD4⁺ T cells. However contradictory studies found that the survival of CD4⁺ memory T cells was not impaired in common γ chain^{-/-} mice (Lantz et al., 2000) and also that memory cell survival was impaired in IL-7^{-/-} mice (Kondrack et al., 2003, Purton et al., 2007). This confusion was perhaps partly explained by studies showing that memory phenotype cells, classically used in the study of memory cell survival, showed different signalling requirements to antigen-specific memory cells (Purton et al., 2007, Lenz et al., 2004). IL-7 and IL-15 were both found to play a role in both CD4⁺ and CD8⁺ memory T cell survival in non-lymphopenic mice. Following on from this it was suggested that periodic signals from costimulatory molecules might enhance memory cell survival. Clearly the generation and survival of memory cells through the contraction phase of the response is dependent on signals from costimulatory molecules such as OX40,

but whether they are involved in long-term persistence once they are formed is unknown. Impaired survival of CD4⁺ memory T cells in CD30^{-/-}/OX40^{-/-} mice and RORγt^{-/-} mice suggested a possible mechanism where interactions between ILC and CD4⁺ memory T cells supported survival. ILC3 are strategically located in the lymph nodes to interact with cells trafficking through this tissue (Mackley et al., 2015). Constitutive expression of OX40L (Kim et al., 2003) and ICOSL (Maazi et al., 2015) on some ILC prompted us to investigate the role of these receptors, OX40 and ICOS, in CD4⁺ memory T cell survival. The kinetics of expression of OX40 and ICOS were distinctive; OX40 expression was transient and tightly linked to antigen availability, whereas ICOS expression was more sustained once initial TCR activation had taken place. This suggested a temporal separation of the roles of OX40 and ICOS in CD4⁺ T cell responses. Although not detected directly *ex vivo*, expression of OX40 could be upregulated on 2W1S:I-A^b CD4⁺ memory T cells following culture with IL-7. ICOS expression was maintained at low levels on memory cells, from both endogenous and TCR transgenic populations. Despite this, the survival of antigen-specific CD4⁺ memory T cells was not impaired by the specific absence of signalling through OX40, ICOS or CD28. To date there is no clear evidence that, once formed, CD4⁺ memory T cells require costimulatory signals for their long-term persistence.

The response of T cells upon secondary challenge is an important consideration for boosting of protective immunity through multiple dose vaccination. Prime-boost vaccination can be used to generate a large population of memory T cells. Initial priming with antigen is done in one vector, distinct from the 2nd vector

used for boosting. This approach is more effective at generating antigen-specific memory T cells than repeated doses of the same vaccine as antigen presentation and inflammatory signals are not impaired by existing immunity (Woodland, 2004). Although conventionally considered dispensable for expansion of memory cells, costimulation was an important dictator of the secondary response to Lm-2W1S. CD28 was required for the optimal expansion of all subsets following challenge, while OX40 and ICOS again appeared to exert effects on specific subsets. ICOS was not required for expansion except for TFH cells, similar to what was observed in the primary response. Again OX40 signalling specifically benefited the effector cell population by enhancing expansion of Tem cells. Understanding what costimulatory signals are required upon recall will aid in the development of optimal vaccination strategies for control of diseases in which traditional humoral vaccines have been unsuccessful.

6.2 Concluding remarks

Through the use of a model to track an antigen-specific CD4⁺ T cell population we have elucidated the differing requirements for costimulatory molecules and the migratory behaviour of T cell subsets. The formation of an optimal memory T cell pool was dependent upon signals through CD28, ICOS and OX40. However these costimulatory signals were not required for the long-term persistence of memory cells once formed. Upon secondary challenge a differential requirement is seen; CD28 is important for the expansion of all T cell subsets whereas OX40 and ICOS were important for Tem cell and TFH cell expansion respectively. We have identified a putative lymphoid tissue resident memory cell population that does not appear to recirculate from the draining lymph node.

6.3 Future Work

To fully elucidate the mechanisms regulating the long-term persistence of CD4⁺ memory T cells once formed more refined methods are required. Ideally this would include the use of inducible conditional knockout mice to selectively remove costimulatory receptors, OX40 and ICOS, from CD4⁺ memory T cells once the primary response has resolved. For example CD4 cre/ERT2 mice, which have a tamoxifen inducible Cre recombinase under the control of the *Cd4* promoter, crossed with either OX40 fl/fl or ICOS fl/fl mice. This would directly assess whether CD4⁺ memory T cells require continuous or periodic signals through

costimulatory receptors for their persistence. This strategy can be employed to dissect the role of costimulatory receptors in multiple CD4⁺ T cell responses. Understanding the precise requirements of different responses will facilitate therapeutic manipulation for clinical benefit.

Fundamentally, it is still unclear whether signals through the TCR are required for the persistence of CD4⁺ memory T cells; experiments in MHC^{-/-} mice demonstrating TCR signals are dispensable used adoptive transfer of TCR transgenic T cells into T cell deficient mice. Therefore more sophisticated methods such as inducible conditional knockout mice could again be applied to induce deletion of MHC II expression after the formation of CD4⁺ memory T cells in WT mice.

In this thesis I have identified a putative resident CD4⁺ memory T cell population in a peripheral skin draining lymph node. The function of this population is currently unknown. Thus experiments to test whether this population persists for the lifetime of the mouse or occupies a niche that will be vacated upon subsequent responses should be performed. For example, mice could be immunised as presented in Chapter 5 with 2W1S peptide in the paw pad and following formation of the memory cell population immunised with a distinct antigen, listeriolysin (LLO) peptide for which MHC II tetramers are available. Analysis of the draining lymph node 42 days after this second immunisation would then identify if the original 2W1S:I-A^b CD4⁺ T cell population remains or if this has been replaced by a LLO:I-A^b CD4⁺ T cell population. Additionally I would like to assess if these resident cells display Tem cell like functions upon

immunisation. To do this mice will be re-immunised at a distal site with cognate antigen and the migration of 2W1S:I-A^b CD4⁺ T cells away from the resident site will be assessed. These experiments would provide key further data on the function of this putative lymph node resident CD4⁺ memory T cell population.

Papers arising from this thesis:

Marriott, C. L., Mackley, E. C., Ferreira, C., Veldhoen, M., Yagita, H. and Withers, D. R. (2014), OX40 controls effector CD4⁺ T-cell expansion, not follicular T helper cell generation in acute *Listeria* infection. Eur. J. Immunol., 44: 2437–2447.

doi: 10.1002/eji.201344211

Marriott, C. L., Carlesso, G., Herbst, R. and Withers, D. R. (2015), ICOS is required for the generation of both central and effector CD4⁺ memory T-cell populations following acute bacterial infection. Eur. J. Immunol., 45: 1706–1715.

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Appendix

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Immunomodulation 2437

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OX40 controls effector CD4⁺ T-cell expansion, not follicular T helper cell generation in acute *Listeria* infection

Clare L. Marriott¹, Emma C. Mackley¹, Cristina Ferreira²,
Marc Veldhoen², Hideo Yagita³ and David R. Withers¹

¹ MRC Centre for Immune Regulation, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

² Babraham Institute, Babraham Research Campus, Cambridge, United Kingdom

³ Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

To investigate the importance of OX40 signals for physiological CD4⁺ T-cell responses, an endogenous antigen-specific population of CD4⁺ T cells that recognise the 2W1S peptide was assessed and temporal control of OX40 signals was achieved using blocking or agonistic antibodies (Abs) in vivo. Following infection with *Listeria monocytogenes* expressing 2W1S peptide, OX40 was briefly expressed by the responding 2W1S-specific CD4⁺ T cells, but only on a subset that co-expressed effector cell markers. This population was specifically expanded by Ab-ligation of OX40 during priming, which also caused skewing of the memory response towards effector memory cells. Strikingly, this greatly enhanced effector response was accompanied by the loss of T follicular helper (TFH) cells and germinal centres. Mice deficient in OX40 and CD30 showed normal generation of TFH cells but impaired numbers of 2W1S-specific effector cells. OX40 was not expressed by 2W1S-specific memory cells, although it was rapidly up-regulated upon challenge whereupon Ab-ligation of OX40 specifically affected the effector subset. In summary, these data indicate that for CD4⁺ T cells, OX40 signals are important for generation of effector T cells rather than TFH cells in this response to acute bacterial infection.

Keywords: CD4⁺ T cells • Costimulation • Germinal centre • Memory • OX40



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Introduction

Secondary lymphoid tissue supports the adaptive immune responses that provide effector cells to clear danger whilst also generating memory cells that establish protection for the future. Whilst peptide:MHC complexes and B7 family member costimulation are critical for productive T-cell responses, members of the TNF receptor superfamily are also involved. Amongst the TNF

receptor superfamily, OX40 is a key determinant for the success of T-cell responses [1, 2]. Expression of OX40 is absent on naive T cells, but rapidly expressed upon activation although reportedly only maintained for a further 3–4 days [1–3]. Signalling through OX40 was found to inhibit apoptotic pathways through expression of survivin [4] and Bcl-2 family members, tipping the balance in favour of enhanced survival and thus aiding clonal expansion [5]. Consistent with this, OX40^{-/-} mice show impaired CD4⁺ T-cell responses, with reduced numbers of antigen-specific CD4⁺ T cells late in the primary response and the memory cell compartment is greatly reduced [6, 7]. Mice deficient in both

Correspondence: Dr. David R. Withers
e-mail: d.withers@bham.ac.uk

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OX40 and CD30 show even more impaired CD4⁺ T-cell responses and loss of memory cells, indicating redundancy due to shared signalling pathways [8, 9]. Provision of agonistic anti-OX40 antibodies (Abs) during a primary response resulted in greatly expanded numbers of antigen-specific CD4⁺ T cells and a larger memory cell compartment [6].

Expression of OX40 has been reported on memory phenotype (CD44^{hi}CD62L⁺) CD4⁺ T cells within the spleen as well as on memory TCR transgenic cells [8], suggesting that OX40 signalling may also be important for memory cell survival. Provision of OX40L by ROR- γ^+ innate lymphoid cells (ILCs), which constitutively express this molecule, has been proposed to explain the loss of memory CD4⁺ T cells in mice lacking these cells [10]. T follicular helper (TFH) cells have also been described as OX40⁺ [11], at least in some situations [12] and OX40 expression is thought to be linked to their generation [11]. Treg cells constitutively express OX40 although the functional consequences of this *in vivo* are not completely clear. In studies with OX40^{-/-} Treg cells, it is difficult to dissect impaired survival with impaired suppressive function *in vivo* [13, 14].

Whilst a wealth of experiments on the importance of OX40 for T-cell responses have been performed, antigen-specific responses have used monoclonal TCR transgenic T cells [5, 8], transferred in numbers thousands of times greater than a natural antigen-specific naive T-cell pool, a methodology that can alter the survival kinetics [15]. In this study, we have analysed the response of an endogenous polyclonal population, with the aim to test *in vivo* when exactly OX40 was expressed, by what T-cell subsets and what the *in vivo* consequences of ligating or blocking this receptor were. We show that ligation of OX40, which is expressed principally by effector CD4⁺ T cells during priming, dramatically skewed the CD4⁺ T-cell response towards the formation of effector cells. Crucially, this occurred at the expense of TFH cells, resulting in a loss of germinal centres (GCs). Combined, these data clarify the role of OX40 signals for a physiological cohort of antigen-specific CD4⁺ T cells during an acute bacterial infection.

Results

Antigen-specific memory CD4⁺ T-cell survival is not impaired by blocking OX40L Abs

Signals through OX40 are clearly important for the survival of CD4⁺ T cells after priming [5], however, whether they are important for the continued survival of formed memory cells is controversial [2]. We sought to investigate the importance of OX40 signals for the survival of an endogenous antigen-specific memory cell pool, using the well-characterised response to an attenuated *Listeria monocytogenes* strain expressing the 2W1S peptide (Lm-2W) [16]. In this response, the memory phase occurs from 3–4 weeks post-infection, after rapid clearance of the bacteria. Therefore, WT mice were immunised with Lm-2W and after 4 weeks given twice weekly injections of anti-OX40L (or control) Abs for a further 28 days. At this point, numbers of CD44^{hi} 2W1S:I-A^{b+}

CD4⁺ T cells were enumerated (Fig. 1A). Whilst there was a modest reduction in the number of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells recovered from the control and treated mice, this difference was not significant (Fig. 1B; WT vs. OX40L: $p = 0.2973$; median for control: 6794, anti-OX40L: 4509).

Heterogeneous expression of OX40 by 2W1S:I-A^{b+} CD4⁺ T cells

Given that the survival of 2W1S-specific memory T cells was not significantly impaired by anti-OX40L Abs, expression of OX40 by 2W1S-specific CD4⁺ T cells during the response to Lm-2W infection was assessed, with total CD4⁺ Treg cells used as a positive control for OX40 detection (Fig. 1C). Although only a small number of 2W1S:I-A^{b+}CD4⁺ T cells were detectable 2 days post-infection (dpi) with Lm-2W, these lacked expression of OX40 (Fig. 1C). By 3 dpi, OX40 expression was detected on the 2W1S:I-A^{b+} CD4⁺ T cells, however <50% of the cells were OX40⁺ (Fig. 1C and D) and this represented the peak of detectable OX40 expression since by 4 dpi approximately 5% of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells expressed this receptor. These data were notably different to that described for TCR transgenic T cells, where OX40 was expressed by all the antigen-specific cells [5, 17, 18]. Following Lm-2W infection, three subsets of 2W1S-specific CD4⁺ T cells have been elegantly described [19]: CXCR5⁺PD-1⁺T-bet⁺ effector T cells (where PD-1 is programmed death-1), CXCR5⁺PD-1⁺Bcl-6⁺ cells that give rise to central memory cells and CXCR5⁺PD-1⁺Bcl-6⁺ TFH cells. Expression of CD25 can be used at 3 dpi to identify the CXCR5⁺PD-1⁺T-bet⁺ effector T-cell subset [20]. Strikingly, the majority (>70%) of CD25⁺ 2W1S-specific T cells at 3 dpi expressed OX40 and accounted for the majority (>70%) of the OX40-expressing CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells (Fig. 1E–G). By 7 dpi, no OX40 expression was detected on CD44^{hi}2W1S:I-A^{b+} CD4⁺ T cells (Fig. 1C and D), including the TFH population. Since OX40 signals have been implicated in TFH formation and survival [8], we investigated whether OX40⁺ cells co-expressed markers of TFH cells. Expression of Bcl-6 was detected from 4 dpi and although only a fraction of the CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells expressed OX40 at this time, a minority of the cells co-expressed Bcl-6 (Supporting Information Fig. 1). Therefore, whilst OX40 is expressed mostly by 2W1S-specific CD4⁺ T cells with an effector phenotype, a subset of Bcl-6-expressing 2W1S-specific CD4⁺ T cells do also express OX40.

To further investigate whether OX40 signals were required for the formation of TFH cells in the response to Lm-2W, mice deficient in both OX40 and CD30 were used, since there may be redundancy in these signalling pathways [8]. WT and CD30^{-/-}OX40^{-/-} mice were immunised with Lm-2W and then analysed at 7 dpi. An overall decrease in the number of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells (Supporting Information Fig. 2; $p = 0.0317$; median for WT: 52 331, CD30^{-/-}OX40^{-/-}: 21 975) resulted from less CXCR5⁺ effector cells ($p = 0.0159$; median for WT: 27 164, CD30^{-/-}OX40^{-/-}: 8103) consistent with decreased survival, however generation of TFH was not impaired ($p = 0.5556$; median

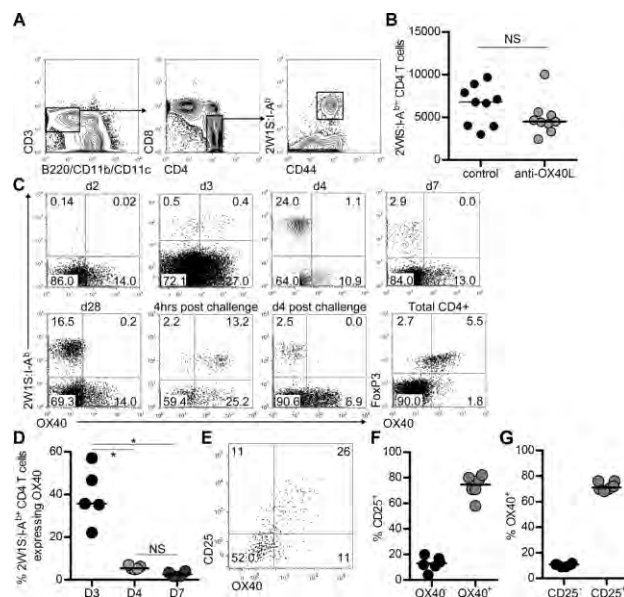


Figure 1. Blockade of OX40:OX40L interactions does not affect memory CD4⁺ T-cell survival. WT mice were immunised with Lm-2W and after 4 weeks given blocking anti-OX40L or control Abs twice weekly for 4 weeks. (A) Detection of CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells. Plots are gated on CD3⁺ B220[−]CD11b[−]CD11c[−] followed by CD4⁺CD8[−] CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells. (B) Enumeration of CD44^{hi}2W1S:I-A^b+ CD4⁺ memory T cells in mice receiving either anti-OX40L or control IgG Abs. (C) Expression of OX40 on 2W1S:I-A^b+ CD4⁺ T cells at d2, d3, d4, d7 and d28 post-immunisation with Lm-2W, 4 hours and 4 days post-secondary challenge, and on Foxp3⁺CD4⁺ Treg cells. (D) Percentage of CD44^{hi} 2W1S:I-A^b+ CD4⁺ T cells expressing OX40 at d3, d4 and d7 post-immunisation. (E) Expression of CD25 and OX40 on 2W1S:I-A^b+ CD4⁺ T cells at 3 dpi. (F) Percentage of OX40[−] and OX40⁺CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells that express CD25. (G) Percentage of CD25[−] and CD25⁺CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells that express OX40. (A, C) Plots are representative of ≥ 6 mice pooled from two independent experiments. (B, D, F, G) Data are pooled from two independent experiments, each data point represents one mouse. Bars show medians. Mann-Whitney test, * $p < 0.05$, NS = non-significant.

for WT: 2850, CD30^{−/−}OX40^{−/−}: 3430), indicating that the generation of these cells did not require OX40 signals.

Although memory phenotype CD4⁺ T cells have been shown to express OX40, amongst the memory CD44^{hi}2W1S:I-A^b+ CD4⁺ T-cell population no OX40 expression was detected. The unimpaired survival of these cells when OX40L signals were blocked is likely explained by a lack of OX40 expression by the antigen-specific CD4⁺ T cells at this time. These cells could rapidly up-regulate OX40 expression upon challenge in vivo with 2W1S peptide (Fig. 1C) as previously described [2]. Here, the vast majority of 2W1S:I-A^b+ CD4⁺ T cells expressed OX40, indicating that expression at this stage was not restricted to the effector subset. Similarly, if Lm-2W-infected mice were challenged at 7 dpi with 2W1S peptide, OX40 was rapidly expressed by almost all 2W-specific CD4⁺ T cells (Supporting Information Fig. 3). These data show that OX40 expression by an endogenous polyclonal antigen-specific population of CD4⁺ T cells is very tightly regulated temporally and not uniformly expressed amongst responding cells in a primary response. Upon re-encounter of antigen, OX40 is more broadly expressed.

Agonistic anti-OX40 Abs at challenge drive effector T-cell expansion

Although the survival of 2W1S-specific cells in the memory phase of the response was OX40 independent, the rapid expression of

OX40 within hours of seeing cognate antigen again prompted us to test the effect of OX40 ligation at this time. Provision of agonistic anti-OX40 Abs [6] on the day of challenge resulted in significantly more (approximately 6.4-fold) CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells 4 days later (Fig. 2A; $p = 0.0079$; median for control: 164 070, anti-OX40: 1 055 000). The majority of these 2W1S:I-A^b+ CD4⁺ T cells were CXCR5[−]PD-1[−] effector CD4⁺ T cells [19] and the percentage of CXCR5⁺PD-1⁺ TFH-cell population significantly reduced (Fig. 2B–D). However, total numbers of TFH cells were comparable, indicating that OX40 ligation had specifically expanded effector 2W1S-specific cells (Fig. 2E; $p = 0.222$; median for control: 8712, anti-OX40: 6673). If provision of anti-OX40 Abs was delayed until the day after challenge, no significant effect on the number of 2W1S:I-A^b+ CD4⁺ T cells was detected, indicating that as with a primary response, expression of OX40 was rapidly lost by the 2W1S:I-A^b+ CD4⁺ T cells (Fig. 2F; $p = 0.0541$; median for control: 250 936, anti-OX40: 379 040). To investigate whether the reduced percentage of TFH cells impacted the ability to sustain GCs, sections of spleen at day 4 post-challenge were stained for Bcl-6, the key transcription factor expressed by GC B cells and TFH cells. Despite no difference in absolute number of TFH cells, spleens from anti-OX40-treated mice were considerably larger than that of control mice resulting in less TFH cells per unit area. In mice given anti-OX40 Abs, fewer GCs per unit area of spleen were detected, suggesting that changes to the balance of the T-cell response had impacted the ability to sustain these structures (Fig. 2G–I).

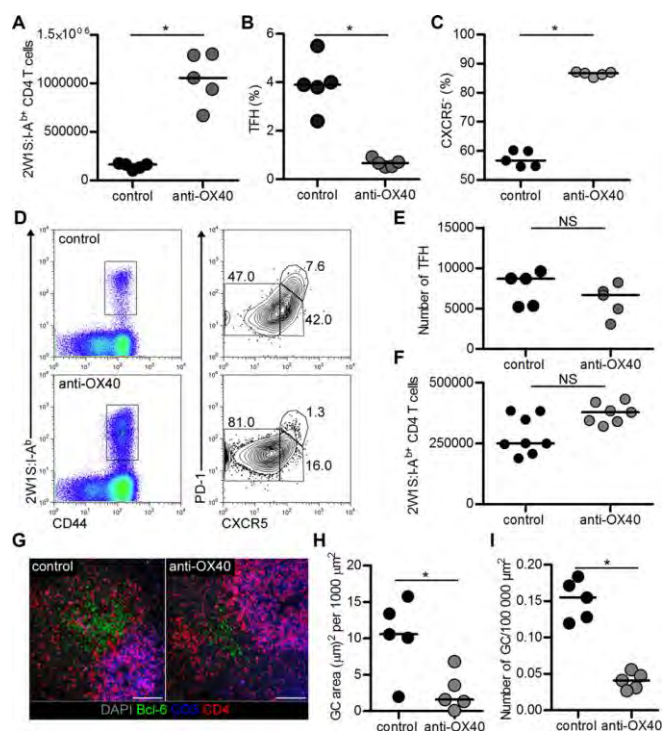


Figure 2. Ligation of OX40 upon challenge enhances memory cell expansion and skews phenotype towards T effector memory. Secondary lymphoid tissues were analysed from mice receiving agonistic anti-OX40 or control IgG Abs after challenge. (A) Enumeration of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells given Abs on the day of challenge. (B) Percentage of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells that are CXCR5⁺PD-1⁺ TFH cells or (C) CXCR5⁺PD-1⁺ effector cells. (D) Expression pattern of CXCR5 and PD-1 by CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells. (E) Total number of CD44^{hi}2W1S:I-A^{b+}CD4⁺ CXCR5⁺PD-1⁺ TFH cells. (F) Enumeration of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells from mice given Abs the day after challenge. (G) Immunofluorescence staining of spleen sections DAPI (grey) and expression of Bcl-6 (green), CD3 (blue) and CD4 (red) shown. Magnification 25 $\times$, scale bar represents 100 μ m. Confocal images are representative of five mice. (H) Area of Bcl-6⁺ germinal centre (GC) per 1000 μ m² spleen. (I) Absolute number of GCs per 100 000 μ m² spleen. (A–C, E, H, I) Data are pooled from two independent experiments, each data point represents one mouse. Bars show medians. (D) Plots are representative of ≥ 6 mice pooled from two independent experiments. Mann–Whitney test, * p < 0.05, NS = non-significant.

Ligation of OX40 drives effector T-cell formation at the expense of GCs in a primary response

OX40 expression by 2W1S-specific cells was tightly restricted to a brief window of time associated with antigen exposure and proliferation. Since OX40 ligation at challenge caused the expansion of only the effector memory subset, we sought to investigate the effects of OX40 ligation during a primary response. Previously, agonistic anti-OX40 Abs have dramatically enhanced the numbers of TCR transgenic T cells [6, 21]. Mice were infected with Lm-2W, then given agonistic anti-OX40 Abs at 1 dpi and analysed at 7 dpi. Provision of anti-OX40 Abs resulted in approximately 5.5-fold more CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells (Fig. 3A; p = 0.0006; median for controls: 57 710, anti-OX40: 316 688). In contrast, provision of the blocking anti-OX40L Abs at 1 and 3 dpi approximately halved the number of CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells at 7 dpi (Fig. 3B; p = 0.0041; median for control: 75 684, anti-OX40L: 34 167). As detected at challenge, with anti-OX40, the CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells were heavily skewed towards CXCR5⁺ effector T cells. Furthermore, anti-OX40, but not anti-OX40L, Abs dramatically reduced the percentage of the 2W1S-

specific cells with a TFH-cell phenotype (Fig. 3C–E; p = 0.0006; median for control: 6.6%, anti-OX40: 0.24%). Analysis of total numbers of TFH cells confirmed these cells were almost absent in anti-OX40-treated mice (Fig. 3F). Therefore, unlike at challenge where TFH cell numbers appeared undiminished, anti-OX40 Abs during priming result in the expansion of effector cells and the loss of the TFH-cell population.

Amongst CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells after exposure to Lm-2W, expression of T-bet is restricted to the CXCR5⁺ effector T cells [19] (Fig. 3G). To further demonstrate that OX40 ligation caused the accumulation of the effector subset, we checked expression of T-bet amongst CD44^{hi}2W1S:I-A^{b+}CD4⁺ T cells. Ligation of OX40 resulted in the vast majority of 2W-specific CD4⁺ T cells expressing T-bet at 7 dpi (Fig. 3G and H; control vs. anti-OX40: p = 0.0067; median for controls: 81%, anti-OX40: 99%; control vs. anti-OX40L: p = 0.0167; median for anti-OX40L: 56.9%). Finally, given the loss of the TFH-cell population, sections of spleen taken at 7 dpi were stained for expression of Bcl-6 to assess effects on GCs (Fig. 3I). Whilst GCs were evident in control mice, in mice given agonistic anti-OX40 no clusters of Bcl-6⁺ cells were detected. The detection of IgG2a⁺ plasma cells indicated that a

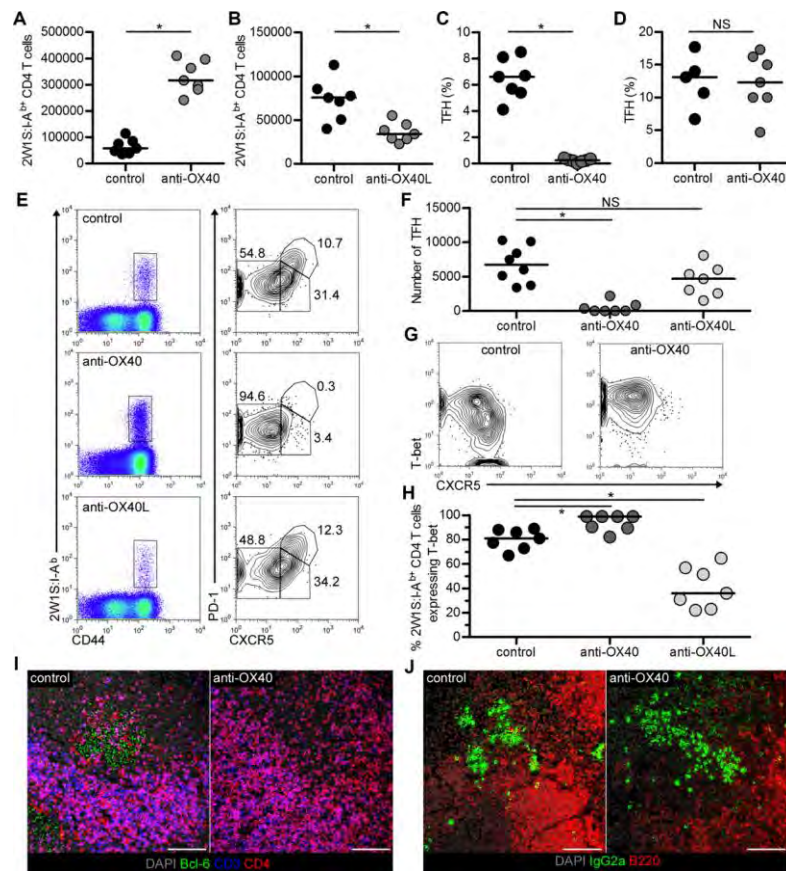


Figure 3. Therapeutic manipulation of OX40:OX40L interactions impact on the size and phenotype of the primary 2W1S:I-A^b CD4⁺ T-cell response. Analysis of CD44^{hi}2W1S:I-A^b CD4⁺ T cells at 7 dpi from mice given anti-OX40, anti-OX40L or control IgG Abs. (A) Enumeration of CD44^{hi}2W1S:I-A^b CD4⁺ T cells in spleen at 7 dpi in mice given anti-OX40 Abs; or (B) anti-OX40L Abs. (C) Percentage of CD44^{hi}2W1S:I-A^b CD4⁺ cells that are CXCR5⁺PD-1⁺ TFH cells following treatment with anti-OX40 or (D) anti-OX40L Abs. (E) Representative CXCR5 and PD-1 expression on CD44^{hi}2W1S:I-A^b CD4⁺ T cells in mice given control IgG, anti-OX40 or anti-OX40L Abs. (F) Total numbers of CD44^{hi}2W1S:I-A^b CD4⁺ CXCR5⁺PD-1⁺ TFH cells from mice receiving control IgG, anti-OX40 or anti-OX40L Abs. (G) Representative CXCR5 and T-bet expression on CD44^{hi}2W1S:I-A^b CD4⁺ T cells in mice given control IgG or anti-OX40. (H) Percentage of CD44^{hi}2W1S:I-A^b CD4⁺ T cells expressing T-bet in mice receiving control IgG, anti-OX40 Abs or anti-OX40L. (I) Immunofluorescence staining of spleen sections from 7 dpi, DAPI (grey) and expression of Bcl-6 (green), CD3 (blue) and CD4 (red) shown, and (J) DAPI (grey) and expression of IgG2a (green) and B220 (red) shown. Magnification 25 $\times$, scale bar represents 100 μ m. Confocal images are representative of five mice (I) or two mice (J). (A–D, F and H) Data are pooled from two independent experiments, each data point represents one mouse. Bars show medians. (E, G) Plots are representative of ≥ 6 mice pooled from two independent experiments. Mann–Whitney test, * $p < 0.05$, NS = non-significant.

B-cell response with switching to Th1 Ab isotypes had been initiated (Fig. 3J) consistent with previous data showing GCs are not required for initial Ab switching [22]. These data show strong parallels to the recent study of anti-OX40 Abs in the response

to lymphocytic choriomeningitis virus (LCMV). A key difference is that in this study, TCR transgenic T cells that homogeneously express OX40 during priming were used. Ligand of OX40 caused up-regulation of Blimp-1 repressing TFH-cell formation. However,

in the response to Lm-2W described here, expression of OX40 is restricted to cells with an effector phenotype, which then specifically benefit from Ab-ligation of the receptor. Whilst there is no previous evidence for the OX-86 clone depleting OX40⁺ cells, we assessed the numbers of Treg cells, which constitutively express OX40, in control and anti-OX40-treated mice. No significant differences were detected in the number of FoxP3⁺2W1S:I-A^b⁺ CD4⁺ T cells or total CD4⁺FoxP3⁺ T cells (Supporting Information Fig. 4), arguing against *in vivo* depletion of OX40⁺ cells by the anti-OX40 Abs.

These data indicated that Ab-mediated ligation of OX40 during priming caused the specific expansion of OX40⁺2W1S:I-A^b⁺ CD4⁺ T cells, which gave rise to the expanded CXCR5⁺ effector T-cell pool. Consistent with this, administration of anti-OX40 Abs at 4 dpi with Lm-2W, a time point when the CD44^{hi}2W1S:I-A^b⁺ CD4⁺ T cells had lost expression of OX40, resulted in only a very modest increase in CD44^{hi}2W1S:I-A^b⁺ CD4⁺ T cells (Supporting Information Fig. 5; $p = 0.0047$; median for control: 46 097, agonistic anti-OX40: 59 423) and the percentage of TFH cells was not affected.

Ligation of OX40 results in an enhanced effector memory T-cell pool

Given the increased effector T-cell expansion, anti-OX40 Abs likely enhanced the effector rather than central memory pool. Previous studies with OX40-deficient OTII T cells indicated that OX40 signals were important for effector rather than central memory generation [23]. Recent data from a human OX40-deficient patient reported a reduced proportion of effector memory CD4⁺ T cells [24]. To assess the memory population after OX40 ligation during priming, mice were immunised as before, treated with control or agonistic anti-OX40 Abs at 1 dpi and at 28 dpi given 2W1S peptide *in vivo* and analysed 4 hours later. *In vivo* peptide stimulation was used to functionally define effector memory cells as those able to make both IL-2 and IFN- γ , and central memory cells as those making only IL-2 [20]. In mice treated with anti-OX40 Abs, there was a significant increase in the percentage of CD44^{hi}2W1S:I-A^b⁺ CD4⁺ T cells defined functionally as effector memory cells (Fig. 4A and C). The enhanced frequency of effector memory cells was accompanied by a significant decrease in the central memory population (Fig. 4B; $p = 0.013$), therefore ligation of OX40 during priming had significantly skewed the resulting memory population. Surprisingly, the total numbers of CD44^{hi}2W1S:I-A^b⁺ CD4⁺ T cells between control and anti-OX40-treated mice, in the secondary lymphoid tissue at least, were now comparable, indicating a dramatic contraction of the primary response and normal homeostatic regulation of the memory pool size (Fig. 4D).

Intrinsic expression of T-bet is not required for effector T-cell expansion

Ligation of OX40 has been found to induce T-bet mRNA [21]. Expression of T-bet is thought to block Th2 differentiation, rather

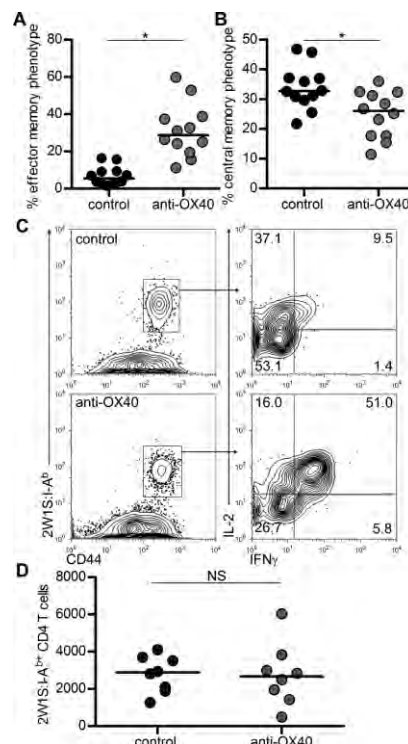


Figure 4. Ligation of OX40 alters the phenotype but not the number of 2W1S:I-A^b memory CD4⁺ T cells. Analysis of the CD44^{hi}2W1S:I-A^b memory CD4⁺ T cells. The percentages of the memory CD4⁺ T-cell population with (A) effector memory (IL-2⁺IFN- γ ⁺) or (B) central memory (IL-2⁺IFN- γ ⁻) phenotype are shown. (C) Flow cytometry staining of cytokines IL-2 and IFN- γ following *in vivo* stimulation with 2W1S peptide at 28 dpi. (D) Total number of CD44^{hi}2W1S:I-A^b CD4⁺ memory T cells recovered after *in vivo* peptide stimulation. (A, B, D) Data have been pooled from ≥ 2 independent experiments, each data point represents one mouse. Bars show medians. (C) Plots are representative of ≥ 6 mice pooled from two independent experiments. Mann-Whitney test, * $p < 0.05$, NS = non-significant.

than promote Th1 formation [25]. To investigate whether the phenotypic changes observed with OX40 ligation were dependent upon endogenous T-bet expression, WT and Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP were immunised with Lm-2W, given control or anti-OX40 Abs at 1 dpi and analysed at 7 dpi. In Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice given control IgG, the response was comparable to WT mice (Fig. 5A). Provision of anti-OX40 Abs resulted in significantly increased expansion of the CD44^{hi}2W1S:I-A^b⁺ CD4⁺ T cells (Fig. 5B; $p = 0.005$; median for control: 22 405, anti-OX40: 68 405) with again the loss of TFH cells (Fig. 5C; $p = 0.0344$) and the skewing towards CXCR5⁺ cells (Fig. 5D; CXCR5⁺, $p = 0.005$;

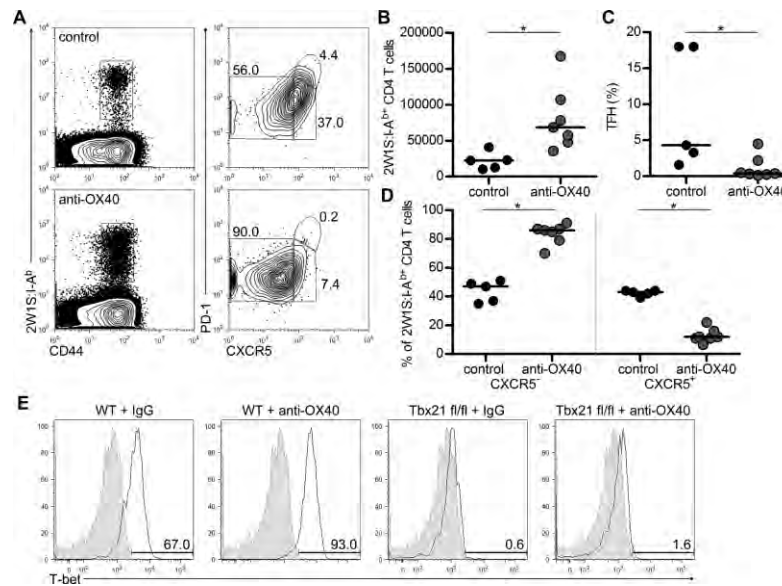


Figure 5. Intrinsic expression of T-bet is not required for the effects of agonistic anti-OX40 Abs. WT and Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP were immunised with Lm-2W and given control IgG or anti-OX40 Abs at 1 dpi and then analysed at 7 dpi. (A) Staining of CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells for CXCR5 and PD-1 in Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice. (B) Total numbers of CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells in Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice. Percentage of (C) TFH cells and (D) CXCR5⁺ and CXCR5⁻ CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells from Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP mice. (E) Expression of T-bet by CD44^{hi}2W1S:I-A^b+ CD4⁺ T cells. (A, E) Plots are representative of ≥ 5 mice pooled from two independent experiments. (B–D) Data have been pooled from two independent experiments, each data point represents one mouse. Bars show medians. Mann–Whitney test, * $p < 0.05$, NS = non-significant.

CXCR5⁺, $p = 0.005$). Staining for expression of T-bet confirmed its absence in the Tbx21 fl/fl mice, demonstrating that the effects of anti-OX40 Abs on T-cell phenotype do not require T-bet expression, consistent with experiments with non-antigen-specific CD4⁺ T cells [21].

Altered OX40 expression in a Th2 response, but still effector cell expansion upon ligation

To assess the response when 2W1S peptide was encountered within a different context, mice were immunised with 2W1S peptide precipitated with alum. This resulted in different kinetics of OX40 expression, with expression of OX40 by 2W1S-specific cells initiated at 4 dpi, and maintained until at least 9 dpi (Fig. 6A). Furthermore, at 9 dpi, expression was not exclusively confined to CXCR5⁺ cells, although very little was detected on the TFH-cell population (Fig. 6B and C). Ligation of OX40 had the same effect as in the Lm-2W infection, with the dramatic expansion of the 2W1S-specific CD4⁺ T cells (Fig. 6B and D; $p = 0.003$; median for control: 17 721, anti-OX40: 210 626). Again, this expansion was

specific to the CXCR5⁺ cells, which expanded significantly, whilst although the percentage of TFH cells was much reduced, absolute numbers were not significantly affected (Fig. 6E–H) and the number of GC structures appeared comparable (data not shown). Therefore, whilst the manner in which the antigen is encountered changed the pattern of OX40 expression by responding cells, enhanced OX40 signalling again expanded specifically the effector population.

Discussion

In this report, we show that for an endogenous polyclonal CD4⁺ T-cell population responding to *Listeria* infection, OX40 expression is induced during priming largely on responding antigen-specific T cells with an effector phenotype. Modulation of OX40 through blocking OX40L, ligation of OX40 or mice deficient in OX40, substantially affected the effector subset of 2W1S-specific CD4⁺ T cells. Although expression of OX40 has been previously linked to the persistence of GCs and TFH cells [8], within this infection model, the formation of TFH cells did not require OX40 signals and ligation of OX40 did not enhance their number.

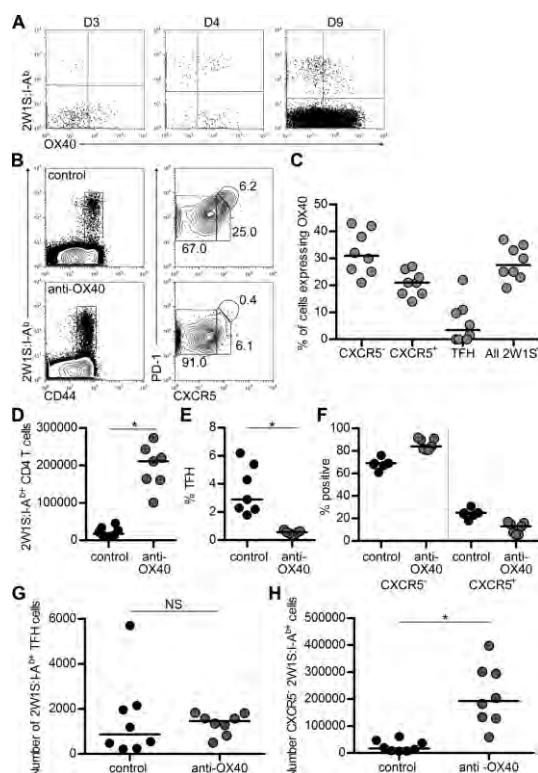


Figure 6. OX40 expression is prolonged with alum adjuvant, but ligation still causes effector cell expansion. Mice were immunised with alum-precipitated 2W1S peptide and given anti-OX40 Abs or control IgG at 1 dpi. (A) Representative plots of OX40 expression on CD44^{hi}2W1S:I-A^b*CD4⁺ T cells at d3, d4 and d9 post-immunisation. Plots are representative of ≥ 3 mice. (B) Staining of CD44^{hi}2W1S:I-A^b*CD4⁺ T cells for CXCR5 and PD-1 by flow cytometry at 9 dpi. (C) Percentage of CXCR5⁺, CXCR5⁺ and TFH cells amongst CD44^{hi}2W1S:I-A^b*CD4⁺ T cells, or all of CD44^{hi}2W1S:I-A^b*CD4⁺ T cells expressing OX40 at 9 dpi. (D) Enumeration of CD44^{hi}2W1S:I-A^b*CD4⁺ T cells at 9 dpi. (E) Percentage of TFH cells amongst CD44^{hi}2W1S:I-A^b*CD4⁺ T cells. (F) Percentage of CD44^{hi}2W1S:I-A^b*CD4⁺ T cells that are CXCR5⁺ or CXCR5⁺. Total number of CD44^{hi}2W1S:I-A^b*CD4⁺ T cells that are TFH cells (G) or CXCR5⁺ (H). (A and B) Plots are representative of ≥ 5 mice pooled from two independent experiments unless stated. (C–H) Data have been pooled from two independent experiments, each data point represents one mouse. Bars show medians. Mann–Whitney test, * $p < 0.05$, NS = non-significant.

Initially, we sought to analyse expression of OX40 by endogenous polyclonal CD4⁺ T cells, utilising MHC-II tetramers to build upon previous investigations reliant upon TCR transgenic T cells. In these studies, OX40 expression was induced within 24–48 hours and maintained for several days [1, 5, 6]. Chronic LCMV infection will maintain antigen-specific CD4⁺ T-cell expression of OX40 for weeks in contrast to acute infection [17]. Therefore, the kinetics of OX40 expression reflects the nature of the antigen and the manner in which it is encountered. The brief window of OX40 expression in the primary response to Lm-2W likely reflects the acute nature of this attenuated infection and its rapid clearance. Clearly, the other 2W1S-specific subsets can be induced to express OX40 upon re-encounter of the peptide. Immunisation with alum-precipitated 2W1S-peptide resulted in a longer window of OX40 expression by the same endogenous antigen-specific population. In all these situations, Ab-ligation of OX40 specifically enhanced the effector subset of 2W1S-specific cells. It was recently shown in LCMV infection that Ab-ligation of OX40 greatly enhanced the effector subset and caused the loss of TFH cells, an observation

linked to enhanced Blimp-1 expression, thought to block Bcl-6 and TFH formation [18]. In this interesting study, the authors propose that ligation of OX40 drives enhanced IL-2 expression, driving Blimp-1 expression and STAT5 activation and inhibiting TFH-cell formation. In the primary response to Lm-2W, the majority of the cells that express OX40 already appear committed to an effector phenotype, indicating that in this situation, OX40 ligation promotes the proliferation and survival of this effector population rather than impaired differentiation of the TFH cells. This expansion is also not dependent upon endogenous T-cell expression of T-bet. Impaired TFH-cell differentiation may well occur when OX40 is ligated upon re-exposure to antigen, where 2W1S-specific CD4⁺ T cells rapidly up-regulate OX40 regardless of their effector or TFH-cell phenotype. Notably, total numbers of TFH cells are not altered when OX40 is ligated at challenge, whilst in a primary response the effector cell expansion is accompanied by the complete loss of the TFH-cell population. These data suggest that during the primary response, the enhanced effector cell expansion is accompanied by the out-competition of the TFH cells, perhaps

due to a loss of niches within the tissue. Alternatively, the high levels of IFN- γ produced following Ab-ligation of OX40 may cause the loss of TFH cells and GCs, analogous to CD70 transgenic mice [26].

Previous studies have concluded that signals through OX40 are important for persistence of TFH cells and thus the formation of T-dependent humoral responses and GCs [8]. Boettler et al. have also shown that control of chronic LCMV through high-affinity Ab is dependent upon OX40 [17]. Therefore, it might have been anticipated that provision of anti-OX40 Abs would enhance the generation of TFH cells, however this clearly does not occur in our hands or others [18]. The injection of large quantities of anti-OX40 Abs may deregulate the normal *in vivo* control of OX40 signals and thus normal functions of OX40 signals may be obscured. However, in the response to Lm-2W at least, we show that signals through OX40 are not required to generate a TFH-cell population. Furthermore, this population did not express OX40 in WT mice at a time when established GC structures were evident. Should TFH cells require OX40 signals for persistence, presumably these cells should express OX40, which may occur in situations of chronic infection and abundant antigen. This model would also require OX40L expression by a GC-resident cell type, which has not been clearly described. In the Lm-2W model, TFH cells are clearly reliant upon B-cell-derived ICOS ligand (ICOSL) for their generation [19]. Therefore, amongst CD4⁺ T-cell subsets, distinct requirements for costimulatory molecules exist, which are also likely different for naive and memory cells.

Whilst there are considerable data supporting a role in the expansion and survival of CD4⁺ T cells during a primary response, direct evidence for memory CD4⁺ T-cell survival is lacking, particularly under physiological conditions. These studies were also prompted by efforts to understand the mechanisms by which ROR- γ ⁺ ILCs might support memory CD4⁺ T-cell survival [10]. Infection of ROR- γ ^{-/-} mice with Lm-2W results in a normal primary response, but a significantly reduced memory cell pool, consistent with the theory that ROR- γ ⁺ ILCs supported memory cells through provision of OX40L. To test this, we blocked OX40L signals *in vivo* and found that the survival of 2W1S-specific memory CD4⁺ T cells was not significantly affected. It remains possible that blockade of OX40L signals was incomplete or was not maintained for sufficient time to see significant changes in memory cell numbers. As we were unable to detect OX40 expression on these memory CD4⁺ T cells directly *ex vivo*, this would seem an unlikely mechanism through which survival signals are mediated.

Since agonistic anti-OX40 Abs are being used clinically, these data clarify that the cost of enhanced effector T-cell responses may be the loss of high-affinity Ab generated through the GC. Agonist Abs to OX40 have been used in a variety of tumour models with varying success and also in phase I clinical trials [2, 27]. Ligation of OX40 clearly dramatically enhances the number of effector CD4⁺ T cells, which may enhance tumour clearance. Stimulation through OX40 is also being considered as a means of enhancing vaccine responses [2]. Our data suggest that, depending on the context of the response, this approach may be less successful if high-affinity Ab responses are desired.

Furthermore, despite the dramatic enlargement of the effector T-cell pool, numbers of antigen-specific T cells at the memory phase of the response were comparable between treated and control mice, perhaps due to poor conversion to memory cells as observed when non-physiological numbers of TCR transgenic T cells are analysed [15]. In summary, our data clarify the importance of OX40 signals for physiological responses and provide data on the likely effects of clinical manipulation of OX40 signals in patients.

Materials and methods

Mice

Mice used were C57BL/6 (obtained from Harlan) and Rag1-Cre Tbx21 fl/fl Rosa26-tdRFP (Tbx21 fl/fl mice were kind gift from Dr. Steve Reiner to Dr. Marc Veldhoen). CD30^{-/-}OX40^{-/-} mice [8] were compared to C57BL/6 mice bred in-house. Animals were used in accordance with Home Office guidelines at the University of Birmingham and Babraham Institute, Biomedical Services Units.

Immunisation

Mice were immunised intravenously (i.v.) with 10⁷ ActA-deficient Lm-2W (kind gift from Dr. Marc Jenkins) as described [16]. Bacteria were grown at 37°C in a shaking incubator to concentration OD₆₀₀ = 0.1 in Luria-Bertani broth supplemented with 20 µg/mL chloramphenicol. To generate a Th2 response, mice were immunised intra-peritoneally (i.p.) with 100 µg aluminium hydroxide precipitated 2W1S peptide. For *in vivo* peptide challenge, mice were injected i.v. with 100 µg 2W1S peptide with 2.5 µg LPS, and secondary lymphoid tissues were harvested 4 hours later for flow cytometric analysis.

Ab injection

Blocking mAbs against mouse OX40L (clone RM134L) were prepared as described previously [28]. Control rat IgG was purchased from Sigma-Aldrich. Mice were injected i.p. with 0.25 mg anti-OX40L or control rat IgG. For memory cell experiments, mice were injected twice weekly for 4 weeks at 28 dpi. To assess effect of blocking OX40 on primary responses, mice were injected 1 and 3 dpi. Mice were injected i.p. with 100 µg anti-OX40 (clone OX86) on the day of challenge for memory experiments or as stated.

Flow cytometry

For tetramer staining, cells from secondary lymphoid tissue were pooled and stained for 1 hour at room temperature with 10 nm PE-conjugated 2W1S:I-A^b. For *in vivo* peptide experiments, 10 µg/mL brefeldin A was added at this stage. All cell surface

staining was done at 4°C for 30 min, with the exception of CXCR5 that was stained for 1 hour at room temperature. Enrichment for 2W1S:I-A^b-specific T cells was performed at d2, d3, d4 and d28, as described previously, using anti-PE MicroBeads (Miltenyi Biotech) and MACS enrichment [29]. Enriched and run-through fractions were stained with the same cocktail of surface Abs to enable calculation of cell frequencies. Samples were run using an LSR11 (BD Biosciences) and analysed using FlowJo software (Tree Star). For intracellular cytoplasmic staining, cells were fixed and permeabilised with Cytofix/Cytoperm Plus (BD Biosciences) according to manufacturer's instructions. Intracellular cytokines were stained with IL-2 488 and IFN- γ PECy7 (BD Bioscience).

Immunofluorescence and image analysis

Tissues sections from experimental mice were cut and stained as described previously [10]. Sections were counterstained with DAPI (Invitrogen) and mounted using ProLong Gold (Invitrogen). Slides were analysed on an LSM 510 Meta confocal (Zeiss). For calculations of number and size of GCs, tile scans of whole spleen sections were made, and all Bcl-6⁺ cell clusters were identified and the area was calculated using LSM (Zeiss).

Statistics

Data were analysed using GraphPad Prism (version 5.01). Non-parametric Mann-Whitney test was used to determine significance that was set at $p \leq 0.05$. Median values were calculated and used in all analyses.



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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: dpi: days post-infection · ILC: innate lymphoid cell · LCMV: lymphocytic choriomeningitis virus · Lm-2W: *Listeria monocytogenes* expressing 2W1S peptide · PD-1: programmed death-1 · TFH: T follicular helper

Full correspondence: Dr. David R. Withers, MRC Centre for Immune Regulation, College of Medical and Dental Sciences, University of Birmingham, Birmingham, B15 2TT, UK
Fax: +44(0)121-414-3599
e-mail: d.withers@bham.ac.uk

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ICOS is required for the generation of both central and effector CD4⁺ memory T-cell populations following acute bacterial infection

Clare L. Marriott¹, Gianluca Carlesso², Ronald Herbst²
and David R. Withers¹

¹ Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, UK

² Respiratory, Inflammation and Autoimmunity, Research Department, MedImmune LLC, Gaithersburg, MD, USA

Interactions between ICOS and ICOS ligand (ICOSL) are essential for the development of T follicular helper (Tfh) cells and thus the formation and maintenance of GC reactions. Given the conflicting reports on the requirement of other CD4⁺ T-cell populations for ICOS signals, we have employed a range of *in vivo* approaches to dissect requirements for ICOS signals in mice during an endogenous CD4⁺ T-cell response and contrasted this with CD28 signals. Genetic absence of ICOSL only modestly reduced the total number of antigen-specific CD4⁺ T cells at the peak of the primary response, but resulted in a severely diminished number of both T central memory and T effector memory cells. Treatment with blocking anti-ICOS mAb during the primary response recapitulated these effects and caused a more substantial reduction than blocking CD28 signals with CTLA4Ig. During the memory phase of the response further signals through ICOS or CD28 were not required for survival. However, upon secondary challenge only Tfh cell expansion remained heavily ICOS-dependent, while CD28 signals were required for optimal expansion of all subsets. These data demonstrate the importance of ICOS signals specifically for memory CD4⁺ T-cell formation, while highlighting the potential of therapeutically targeting this pathway.

Keywords: ICOS · CD28 · CD4⁺ Memory · T-cell responses · Tfh



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Introduction

Memory is a fundamental aspect of the adaptive immune response that enables rapid responses against pathogens upon reencounter. Memory T cells are the product of successful T-cell responses, existing within long-lived populations that last many months in mice [1] and years in humans [2]. T cells of any given specificity

are rare [3], requiring extensive proliferation and differentiation to form a pool of effector CD4⁺ T cells. The proliferation and differentiation of naive CD4⁺ T cells requires both TCR/MHCII interactions and costimulatory signals initially through CD28 [4, 5], although subsequently many other receptor/ligand pairs can contribute [6]. This results in effector T-cell subsets with distinct functions and developmental requirements [7]. Evidence that memory cells develop from effector cells, rather than as a separate population derives from observations that cytokine producing and nonproducing effector cells generate memory cells equally well [1, 8, 9]. The memory cell pool can be divided into two subsets

Correspondence: Dr. David R. Withers
e-mail: d.withers@bham.ac.uk

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based upon chemokine receptor expression and cytokine secretion; T central memory (Tcm) cells express CCR7, required for trafficking through secondary lymphoid tissue and need to proliferate to produce cytokines other than IL-2. T effector memory (Tem) cells lack CCR7 expression and thus are thought to be unable to recirculate through secondary lymphoid tissue; rather these cells can rapidly produce effector cytokines such as IFN- γ as well as IL-2 upon antigen recognition [10, 11].

The signals that control the development and subsequent survival of the memory cell pool require further understanding. The cytokines IL-7 and IL-15 are known to be important for the homeostasis of CD4⁺ memory T cells, while TCR/MHCII interactions are not thought to be required [12]. Whether cell surface molecules provide additional survival signals is unclear. Such experiments are technically challenging since signals required for the formation of the memory cell population must be distinguished from signals required for persistence. Impaired CD4⁺ memory T-cell survival in mice where group 3 innate lymphoid cells (ILC3s) were absent suggests that costimulatory molecules expressed by these cells such as OX40 ligand may support memory cells [13], however, this has not been determined in vivo. Previous work indicated that OX40 expression was tightly linked with antigen exposure [14] rather than consistently expressed by memory CD4⁺ T cells, suggesting other surface molecules may play a role in their survival once formed.

CD28 family members regulate T-cell responses through costimulatory and inhibitory signals [15, 16]. Previously, it has been shown that CD28 and ICOS are essential for productive primary CD4⁺ T-cell responses, however they each have distinct roles [17, 18]. CD28 is constitutively expressed on CD4⁺ T cells and is required to enhance T-cell proliferation and IL-2 production [19, 20]. CD80^{-/-}CD86^{-/-} mice, which lack both ligands of CD28, show impairments in isotype class switching and are unable to form GC [21]. ICOS is upregulated on CD4⁺ T cells following antigenic stimulation [17, 18, 22, 23] and engagement with ICOS ligand (ICOSL) provides a key signal required for the generation of T follicular helper cells (Tfh) and consequently the formation and maintenance of GC [24, 25]. Thus deficiency in ICOS also results in a reduction in class switched antibody and B-cell memory [22, 23, 26]. Studies in CD28-deficient mice demonstrated the importance of ICOS signals to CD4⁺, but not CD8⁺ T-cell responses to viral and parasite infections [27]. Recently the T cells of patients with ICOS deficiency were characterized in detail and found to be deficient in both Tcm and Tem cell populations, indicating that ICOS signals are fundamental to the generation and/or the survival of these cells [28]. However, studies in mice have indicated ICOS to be important specifically for the generation or survival of Tem cells [29–31], although this relied heavily on identifying Tem cells based only on CD44 and CD62L expression and the use of unphysiological numbers of TCR transgenic T cells. A recent study tracking endogenous antigen-specific CD4⁺ T cells revealed a requirement for ICOS signals only in the Tcm population [11].

Therefore we sought to dissect the requirements of a polyclonal CD4⁺ T-cell population for CD28 and ICOS signals during the different stages of a T-cell response. Using MHCII tetramers

an endogenous CD4⁺ T-cell population recognizing 2W1S peptide was tracked, since such cells will most closely reflect the size and polyclonality of the naive T cell pool in human patients. Our aim was to determine at what points in a CD4⁺ T-cell response CD28 and ICOS signals were required, using a combination of blocking mAb and genetically deficient mice to enable WT memory cells formed in vivo to be assessed. Genetic deficiency in ICOSL or blockade of ICOS in the primary response resulted in a grossly impaired memory cell pool with both Tcm and Tem cells reduced. The subsequent survival of these cells did not require further signals through ICOS and only the Tfh cell population was dependent on ICOS signals upon secondary challenge. While memory CD4⁺ T cells did not require further signals through CD28 for survival, all subsets of T cells were at least partially dependent on CD28 signals for expansion upon secondary challenge. Collectively our data indicate an essential role for ICOS in the formation of all memory cells and highlight the potential of therapeutic targeting of this pathway to block CD4⁺ T-cell responses.

Results

ICOS, as well as CD28, is required for optimal formation of antigen-specific CD4⁺ T cells

To dissect the precise requirements for CD28 and ICOS signals during a CD4⁺ T-cell response, we used the 2W1S model pioneered by the Jenkins laboratory to enable an endogenous, polyclonal population to be assessed. Mice were infected with an attenuated *Listeria monocytogenes* expressing 2W1S peptide (Lm-2W1S) as previously defined [1], and the 2W1S-specific CD4⁺ T-cell population was tracked from primary proliferation through to resting memory cells using MHCII tetramers. In this model, expression of OX40 is closely linked to antigen exposure and tightly regulated [14]. Naive CD44^{lo} 2W1S-specific CD4⁺ T cells lacked ICOS expression (Supporting Information Fig. 1), however, at all subsequent time points after activation (day 7, memory cells, 4 h post-secondary challenge and day 3 post secondary challenge) ICOS was expressed by all, or the majority of responding cells (Fig. 1A). Expression of ICOS was limited to CD44^{hi} CD4⁺ T cells and virtually absent in mice lacking the ligands of CD28 (Supporting Information Fig. 1). Expression of ICOS on responding TCR transgenic CD4⁺ T cells was also assessed, using low numbers of SM1 T cells to model the population size of endogenous naive T-cell pools [32]. ICOS expression was comparable with the endogenous CD4⁺ T-cell population except on memory SM1 cells, which expressed lower levels of ICOS than the memory 2W1S-specific population (Fig. 1A). Following Lm-2W1S infection, three subsets of CD4⁺ T cells can be defined: CXCR5⁺PD-1⁻T-bet⁺ effector cells (Teff) that give rise to Tem cells, CXCR5⁺PD-1⁻Bcl-6⁺ central memory precursors that give rise to Tcm cells and CXCR5⁺PD-1⁺Bcl-6⁺ Tfh cells [11]. The Tcm precursor cell subset of 2W1S-specific CD4⁺ T cells was significantly reduced in ICOSL^{-/-} mice 7 dpi (days postinfection) with Lm-2W1S and Tfh cells were almost absent (Fig. 1B and F), consistent with previous studies

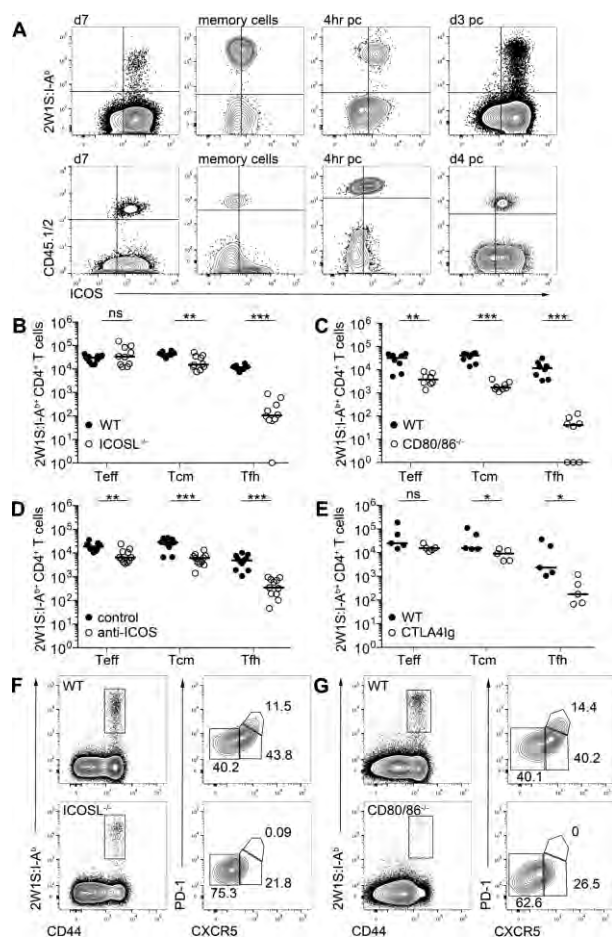


Figure 1. ICOS, as well as CD28, is required for optimal formation of antigen-specific T cells in a primary response. WT mice were infected with Lm-2W1S and 2W1S-specific T cells were detected by flow cytometry. (A) (top) Expression of ICOS on 2W1S:I-A^b CD4⁺ T cells and (bottom) TCR transgenic SM1 T cells at 7 dpi, memory cells, 4 h and 3 (2W1S:I-A^b) or 4 (SM1) days postsecondary challenge. Plots are gated on CD3⁺ B220⁺ CD11b⁺ CD11c⁺ followed by CD4⁺ CD44^{hi} T cells. (B–E) Enumeration of CD44^{hi} 2W1S:I-A^b CD4⁺ CXCR5⁺ (Teff), CXCR5⁺ (Tcm precursors), and CXCR5⁺ PD-1⁺ (Tfh) cells 7 dpi in (B) ICOSL^{-/-} mice; (C) CD80^{-/-} CD86^{-/-} mice; (D) C57BL/6 mice treated with anti-ICOS or isotype control mAb 0 and 3 dpi; and (E) CTLA4Ig mice. (F and G) Expression pattern of CXCR5 and PD-1 by CD44^{hi} 2W1S:I-A^b CD4⁺ T cells in (F) ICOSL^{-/-} mice and (G) CD80^{-/-} CD86^{-/-} mice. (A, F, and G) Plots are representative of ≥ 6 mice from two independent experiments in all experiments except (A) ICOS expression on SM1 cells 7 dpi and 4 h post secondary challenge, which represent two mice. Values on plots are percentages. (B–E) Graphs show pooled data from two independent experiments, each data point represents one mouse. Bars show medians. Values of 0 were assigned as 1. Mann–Whitney test: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

of this response [1, 11]. CD80^{-/-}CD86^{-/-} mice were also analyzed 7 dpi as a comparison and all T-cell subsets were significantly decreased in the absence of signaling through CD28 (Fig. 1C and G). We also sought to assess the effect of reagents blocking these interactions to explore therapeutic targeting of these pathways within the context of this response. Blocking anti-ICOS mAb were administered at 0 and 3 dpi before mice were analyzed 7 dpi with Lm-2W1S. Notably, this mAb recapitulated the effects seen in the ICOSL^{-/-} mice, with Tfh cell formation most significantly affected; although reductions in both the Tcm precursors and Teff cell subsets were also observed (Fig. 1D). To assess blockade of CD28 signals we investigated the 2W1S-specific response in mice

that express a CTLA4Ig fusion protein, blocking CD28 binding to CD80 and CD86, analogous to abatacept therapy. Serum levels of 10–30 $\mu\text{g/mL}$ CTLA4Ig are maintained in the CTLA4Ig mice [33]. Interestingly, while Tfh cell formation was substantially impaired, consistent with an inability to form GC within these mice [33], Teff and Tcm precursor populations were only modestly reduced compared with the CD80^{-/-}CD86^{-/-} mice, consistent with an incomplete block of available CD28 ligands (Fig. 1E). Supporting the partial blockade of CD28 signals in these mice, numbers of Treg cells are substantially reduced in CTLA4Ig mice, compared with WT controls, although not to the numbers detected in CD80^{-/-}CD86^{-/-} mice (Supporting Information Fig. 2).

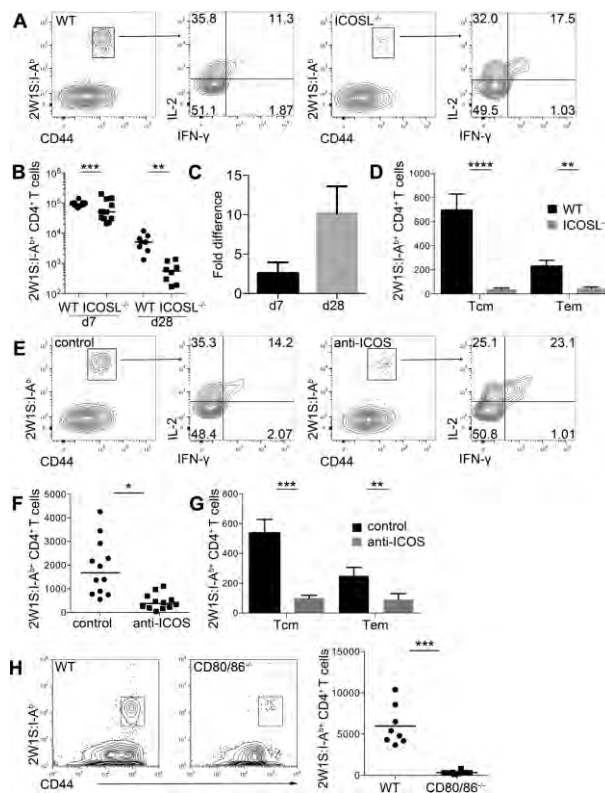


Figure 2. ICOS is required to form both Tcm and Tem cell pools. (A–D) WT and ICOSL^{-/-} mice were infected with Lm-2W1S then restimulated in vivo 28 dpi with 2W1S peptide. (A) CD44^{hi} 2W1S:I-A^{b+} memory CD4⁺ T cells were stained for cytokines IL-2 and IFN- γ and analyzed by flow cytometry. (B) Total number of CD44^{hi} 2W1S:I-A^{b+} CD4⁺ T cells recovered at 7 and 28 dpi from WT and ICOSL^{-/-} mice; bars show median. (C) Fold difference between WT and ICOSL^{-/-} mice in number of CD44^{hi} 2W1S:I-A^{b+} CD4⁺ T cells at 7 and 28 dpi. Bars show mean + SEM of fold difference between experiments. (D) CD44^{hi} 2W1S:I-A^{b+} CD4⁺ memory T cells with Tcm (IL-2⁺IFN- γ ⁺) or Tem (IL-2⁺IFN- γ ⁺) phenotype were enumerated by flow cytometry from WT and ICOSL^{-/-} mice. Bars show mean + SEM from 12 mice. (E–G) WT mice were infected with Lm-2W1S then restimulated in vivo 28 dpi with 2W1S peptide after administration of anti-ICOS or isotype control mAb at 0 and 4 dpi. (E) CD44^{hi} 2W1S:I-A^{b+} memory CD4⁺ T cells were stained for IL-2 and IFN- γ and analyzed by flow cytometry. (F) Total number of CD44^{hi} 2W1S:I-A^{b+} CD4⁺ memory T cells recovered at 28 dpi; bars show medians. (G) Tcm and Tem phenotype cells were enumerated by flow cytometry. Bars show mean + SEM from 12 mice. (H) WT and CD80^{-/-}CD86^{-/-} mice were infected with Lm-2W1S then restimulated in vivo 28 dpi with 2W1S peptide. (Left) Representative flow cytometry plots and (right) total number of CD44^{hi} 2W1S:I-A^{b+} CD4⁺ memory T cells recovered at 28 dpi. (A, E, and H) Plots are representative of ≥ 6 mice from ≥ 2 independent experiments, values on plots are percentages. (B–D and F–H) Graphs show pooled data from ≥ 2 independent experiments. Each data point represents one mouse. Mann–Whitney test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

ICOS is required for the formation of both Tcm and Tem cells

Formation of a memory CD4⁺ T-cell pool is dependent upon a robust primary response [32, 34]. Studies of ICOS^{-/-} and ICOSL^{-/-} mice have concluded a specific loss of Tem cells [29, 30], but following Lm-2W1S infection of chimeras reconstituted with an equal mix of ICOS-deficient and WT bone marrow cells, a reduced population of Tcm precursors was detected [11]. Therefore we sought to assess the 2W1S-specific memory cell pool in WT or ICOSL^{-/-} mice 28 dpi with Lm-2W1S. To functionally identify Tcm and Tem cells, mice were restimulated in vivo with 2W1S peptide prior to analysis. Strikingly, the number of 2W1S-specific memory cells was substantially reduced in ICOSL^{-/-} mice (Fig. 2A and B), with an approximate tenfold reduction in total number at this time point, compared with an approximate 3.5-fold difference at day 7 (Fig. 2C). Notably, both the Tcm and Tem populations were heavily depleted compared with controls (Fig. 2D) in con-

trast to previous reports that observed effects only on the Tem cells [11, 29, 30]. This impaired memory cell formation was also seen when anti-ICOS mAb were administered to WT mice at 0 and 4 dpi (Fig. 2E–G). As expected, in CD80^{-/-}CD86^{-/-} mice, the memory cell pool was barely detected (Fig. 2H). These data indicate that signals through ICOS are essential in the formation of a robust memory cell compartment despite modest effects on total numbers of cells at the peak of the primary response.

CD28 and ICOS signals are not required for antigen-specific memory T-cell survival

Whether memory CD4⁺ T cells require signals through ICOS and CD28 for continued survival during the memory phase of the response has not been robustly tested. To circumvent problems associated with analyzing memory cell populations in mice where priming may be impaired, WT mice were infected with Lm-2W1S

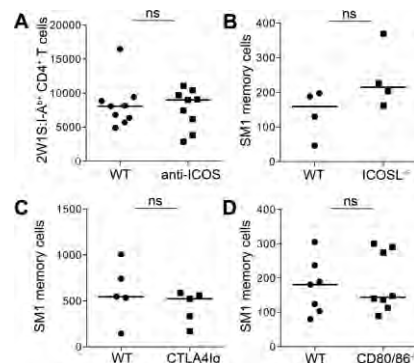


Figure 3. ICOS and CD28 costimulation are not required for antigen-specific memory cell survival. (A) WT mice were infected with Lm-2W1S, treated with blocking anti-ICOS or isotype control mAb twice weekly for 4 weeks and 28 dpi CD44^{hi} 2W1S:I-A^b CD4⁺ memory T cells were enumerated by flow cytometry. (B–D) SM1 memory cells generated in WT mice were transferred into different hosts: (B) ICOSL^{-/-} mice; (C) CTLA4Ig mice; and (D) CD80^{-/-}CD86^{-/-} mice and the number of surviving memory cells enumerated 4 weeks posttransfer by flow cytometry. (A and D) Graphs show pooled data from two independent experiments. (B, C) Graphs show representative data of two independent experiments. Each data point represents one mouse; bars show medians. Mann–Whitney test: ns $p > 0.05$.

and left for 4 weeks to form a 2W1S-specific memory population. Blocking anti-ICOS or isotype control mAb were given at 28 dpi twice weekly for 4 weeks, then the memory cell population was assessed. There was no significant difference in the number of 2W1S-specific memory cells recovered (Fig. 3A) indicating that signals through ICOS were not required to maintain the memory cell pool at this stage of the response. Since ICOS mAb blockade may be incomplete, we sought a second method by which WT memory cell survival could be assessed. Given the challenge of transferring tetramer specific CD4⁺ T-cell populations, an allo-type marked TCR transgenic SM1 memory population was generated in vivo by transferring 10^4 naive SM1 cells into WT hosts that were then infected with attenuated *Listeria monocytogenes* expressing Flic (Lm-Flic), thus keeping the agent with which the mice were infected consistent. Through transferring low numbers of TCR transgenic T cells, a physiological CD4⁺ T-cell response is recapitulated [32]. After 4 weeks, the SM1 cells were enriched and transferred into new hosts. Analysis of the memory SM1 cells confirmed their expression of ICOS at this time (Fig. 1A). Approximately 2.5×10^4 SM1 memory cells were transferred and mice were analyzed 4 weeks posttransfer. Survival of WT SM1 memory cells was not impaired in ICOSL^{-/-}, CD80^{-/-}CD86^{-/-}, or CTLA4Ig mice indicating that signals through B7 costimulatory molecules are not required for the continued survival of antigen-specific memory cells once formed (Fig. 3B–D). Therefore while formation of a robust memory population is highly dependent upon both CD28 and ICOS, the persistence of these cells does not require these signals.

ICOS is required for expansion of antigen-specific Tfh cells post secondary challenge

Our data indicated that signals through ICOS were critical for the generation of both Tcm and Tem populations, rather than the subsequent survival of these cells during the memory phase of the response. To assess the requirement for ICOS signals upon secondary challenge mice were infected with Lm-2W1S and then again at 28 dpi and analyzed 3 days later. Blocking anti-ICOS or isotype control mAb were administered day 1 pre- and postsecondary challenge. Mice receiving blocking anti-ICOS mAb showed substantially impaired Tfh cell formation, modestly impaired Tem populations and no significant effect on Tcm cells (Fig. 4A and B). To further assess the contribution of costimulatory molecules only at secondary challenge, SM1 memory cells generated in WT mice were transferred into ICOSL^{-/-} or WT hosts that were then challenged 28 days later with Lm-Flic. While expansion of Tcm and Tem cells was unaffected, again the number of number of Tfh cells was substantially reduced indicating that this cell type remains heavily ICOS dependent (Fig. 4C). In contrast, transfer of SM1 memory cells into CD80^{-/-}CD86^{-/-} hosts subsequently challenged with Lm-Flic revealed a significant reduction in all of the T-cell subsets analyzed, demonstrating that these populations remain partially dependent on CD28 signals for expansion (Fig. 4D).

Combination of CTLA4Ig and anti-ICOS mAb enhances blockade of antigen-specific CD4⁺ T-cell response

Antibody-mediated blockade of ICOS signals during the primary response comprehensively blocked Tfh cell formation and significantly impacted on the resulting memory population, although the total number of antigen-specific CD4⁺ T cells at day 7 was only modestly reduced. Blocking anti-ICOS mAb caused a greater reduction in the memory cell population at day 28 (approximate tenfold reduction versus WT controls, Fig. 2B) than observed in CTLA4Ig mice, where the fold difference was approximately 4 (Fig. 5A). Given the modest reduction in Tef and Tcm precursors observed in the CTLA4Ig mice at day 7, we sought to assess the effects of combined blockade of ICOS and CD28 signals as a dual therapeutic approach. Therefore CTLA4Ig mice were treated with blocking anti-ICOS or isotype control mAb at 0 and 3 dpi with Lm-2W1S. At 7 dpi there was a reduction in all 2W1S-specific T-cell subsets in CTLA4Ig mice that received blocking anti-ICOS treatment compared to control treated CTLA4Ig mice and Tfh cells were absent (Fig. 5B and C). Interestingly, there was an approximate 8.5-fold reduction in 2W1S-specific CD4⁺ T cells in CTLA4Ig mice treated with anti-ICOS mAb compared to a 3.5-fold reduction in WT mice indicating that combined CTLA4Ig and anti-ICOS treatment enhances blockade of the T-cell response (Fig. 5D and E).

Finally, given that blockade of ICOS or CD28 signals alone did not affect memory cell survival, we sought to test whether

this reflected redundancy in the requirement for these signals at this time. Therefore, CTLA4Ig mice were infected with Lm-2W1S and blocking anti ICOS or isotype control mAb were administered for 4 weeks starting 28 dpi. Enumeration of 2W1S-specific CD4⁺ memory T cells revealed no significant difference, indicating that rather than redundancy in the memory cell requirements for CD28 and ICOS signals, neither are necessary for survival at this stage of the response (Fig. 5F).

Discussion

Studies on the importance of ICOS signals for CD4⁺ T-cell responses have focused on the clear requirement for this molecule in the formation of Tfh cells and T-dependent Ab responses [24, 25, 31]. Here, we have dissected the different stages of a CD4⁺ T-cell response and assessed the exact time at which ICOS signals are required for the different subsets of an endogenous CD4⁺ T-cell population. While CD28 signals were critical for the early expansion of CD4⁺ T cells, the subsequent formation of a robust memory cell pool was highly dependent on signals through both CD28 and ICOS and mAb blocking ICOS during priming effectively reduced memory cell numbers. Furthermore, we show that in this systemic response, both the Tcm and Tem populations require ICOS for their generation, but not their subsequent survival nor initial expansion upon secondary challenge.

Members of the TNF receptor super family such as OX40 have been intensely studied as key signals in the survival of CD4⁺ T cells [35] and signals through these molecules are important in the survival and function of Treg cells [36, 37]. While requirements for costimulatory molecules likely depend upon the nature of the infection and the type or quantity of antigen, a striking feature of this study was the very brief expression of OX40 only at times of antigen exposure [14]. In contrast, responding 2W1S-specific CD4⁺ T cells retain expression of ICOS throughout the phases of the response, an observation that prompted us to dissect when signals through this molecule might be important. Several previous studies have indicated a requirement for ICOS only for CD4⁺ Tem cells and these have been reported to express the highest levels of ICOS compared to Tcm and naive T cells [29]. Although these studies have relied upon a surface phenotype, which does not distinguish responding cells from memory phenotype cells known to have different survival requirements [12], studies with antigen-specific CD4⁺ T cells also indicated that only Tem cells depend on ICOS for survival [30]. The data presented here clearly show that in the response to Lm-2W1S infection, generation of all memory cells was heavily dependent upon ICOS signals, which is consistent with observations from human patients with ICOS

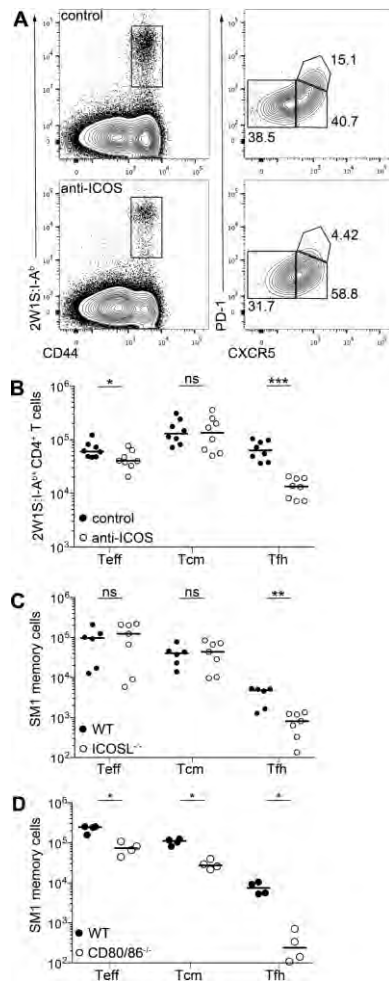


Figure 4. ICOS is required for expansion of antigen-specific Tfh cells post secondary challenge. (A and B) WT mice were infected with Lm-2W1S then challenged again at 28 dpi and treated with anti-ICOS or isotype control mAb 1 day pre- and post secondary challenge with Lm-2W1S. (A) Three days post secondary challenge CD44^{hi} 2W1S:I-A⁺ CD4⁺ T cells were stained for CXCR5 and PD-1 expression and analyzed by flow cytometry. (B) CD44^{hi} 2W1S:I-A⁺ CD4⁺ CXCR5⁻ (Teff), CXCR5⁺ (Tcm precursors), and CXCR5⁺ PD-1⁺ (Tfh) cells were enumerated 3 days post secondary challenge. (C and D) Mice were injected with SM1 memory cells and challenged 28 days posttransfer. Enumeration of SM1 memory cells that are CXCR5⁻ (Teff), CXCR5⁺ (Tcm precursors), and CXCR5⁺ PD-1⁺ (Tfh) cells from (C) ICOSL^{-/-} and (D) CD80^{-/-} mice day 4 post secondary challenge. (A) Plots are representative of ≥6 mice from two independent experiments. Values on plots are percentages. (B, C) Graphs show pooled data or (D) representative data from two independent experiments. Each data point represents one mouse; bars show medians. Mann-Whitney test: ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

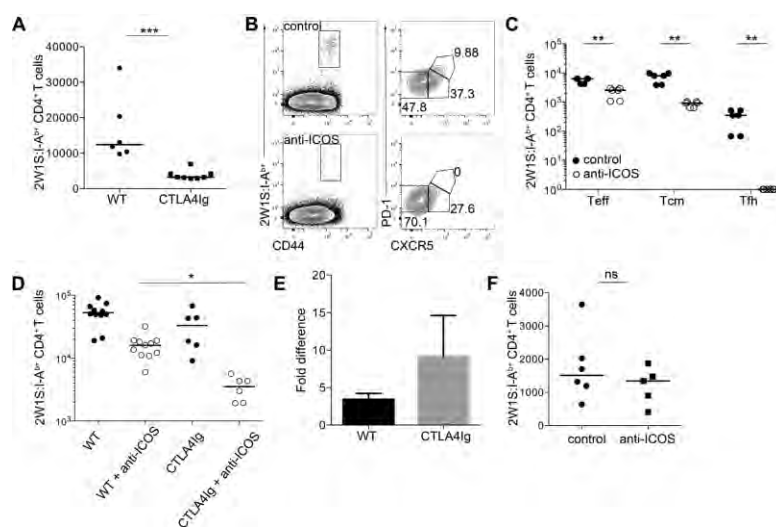


Figure 5. Therapeutic combination of blocking anti-ICOS mAb and CTLA4Ig enhances blockade of CD4⁺ T-cell response. (A) WT and CTLA4Ig mice were infected with Lm-2W1S. CD44^{hi} 2W1S:I-A^b CD4⁺ memory T cells were enumerated 28 dpi by flow cytometry. (B and C) CTLA4Ig mice were infected with Lm-2W1S and treated with anti-ICOS or isotype control mAb 0 and 3 dpi. (B) CD44^{hi} 2W1S:I-A^b CD4⁺ T cells were stained for CXCR5 and PD-1 expression 7 dpi and analyzed by flow cytometry. Plots are representative of ≥ 6 mice from three independent experiments. Values on plots are percentages. (C) CD44^{hi} 2W1S:I-A^b CD4⁺ CXCR5⁺ (Teff), CXCR5⁺ (Tcm precursors), and CXCR5⁺PD-1⁺ (Tfh) cells were enumerated 7 dpi from control and anti-ICOS treated mice. (D) CD44^{hi} 2W1S:I-A^b CD4⁺ T cells were enumerated 7 dpi from WT and CTLA4Ig mice treated with anti-ICOS or isotype control mAb 0 and 3 dpi. Kruskal–Wallis one-way ANOVA with post hoc Dunn's test: * $p \leq 0.05$. (E) Fold difference between mice treated with anti-ICOS or isotype control mAb in number of CD44^{hi} 2W1S:I-A^b CD4⁺ T cells in WT and CTLA4Ig mice. Bars show mean $\pm$ SEM of fold difference between experiments. (F) CD44^{hi} 2W1S:I-A^b CD4⁺ memory T cells were enumerated from CTLA4Ig mice 28 dpi with Lm-2W1S, treated with blocking anti-ICOS or isotype control mAb twice weekly for 4 weeks. (A, C–F) Graphs show pooled data from ≥ 2 independent experiments. Values of 0 were assigned as 1. Each data point represents one mouse; bars show medians. Mann–Whitney test: ns $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

deficiency [28]. Interestingly, studies tracking HA-specific CD4⁺ memory cells using MHCII tetramers following i.n. infection with influenza revealed no significant differences in the total memory cell population in ICOS^{-/-} mice, with a reduced Tcm population balanced by an expanded number of Tcm cells [30]. Furthermore, Mahajan et al. concluded that following subcutaneous immunization with H19-Env peptide emulsified with CFA, ICOS signals were not required for memory cell survival, but were important upon restimulation [26]. When compared with the data presented here, these studies indicate that the importance of ICOS signals in a CD4⁺ T-cell response may be dictated by the route of antigen encounter, for example i.v. or i.n. infection with Lm-2W1S results in very different memory cell responses [1]. Furthermore, responses generated within mucosal secondary lymphoid tissue may involve distinct costimulatory requirements, which should be considered when immunizing at such sites or attempting to block autoimmune T cells in such tissues. Different adjuvants or types of infection will also likely shape the costimulatory profile of the response, through the stimulation of distinct PRR. Our experiments have focused on the response to Lm-2W1S and pre-

vious studies have identified an important role for ICOS signals in the response to *L. monocytogenes* and other intracellular bacteria [38, 39]. Finally, it is not clear whether the costimulatory requirements of memory cells upon secondary challenge depend upon the nature of the initial response, which may explain the differences between data presented here and in other studies [26].

Temporary blockade of ICOS during the primary response had substantial effects on the resulting memory population, not dissimilar to that observed in ICOSL^{-/-} mice and more impressive than in CTLA4Ig mice, demonstrating the therapeutic potential of targeting this pathway to limit CD4⁺ T-cell responses. Since both genetic deficiency in ICOSL or Ab-mediated blockade of ICOS only modestly reduced the total number of antigen-specific CD4⁺ T cells at the peak of the primary response, this would indicate that either signals through ICOS are required beyond 7 dpi or the absence of these signals during the primary response caused the survival of the CD4⁺ T cells to be grossly impaired. Although the blocking anti-ICOS mAb has a half-life of approximately 7–10 days in vivo (G. Carlesso, unpublished observation), systematic blockade of ICOS signals at different time points in the

response is really required to dissect this further. While CD28 signals are clearly crucial for the initiation of CD4⁺ T-cell responses and lie upstream of ICOS expression [17], therapeutic targeting of CD28 signals using abatacept has yielded mixed results clinically in blocking CD4⁺ T-cell responses. Abatacept treatment has shown promise in autoimmune conditions such as rheumatoid arthritis and type 1 diabetes, but not multiple sclerosis or ulcerative colitis [40]. In transplantation a modified version of abatacept has been used, named belatacept, which more effectively inhibited renal transplant rejection [40–42]. Blockade of ICOS signals has also been trialed clinically and been shown to prolong cardiac allograft survival [43, 44]. To further enhance costimulatory blockade combined targeting of multiple pathways has been proposed [40, 45] and inhibition of both the ICOS and CD40 ligand pathways resulted in allograft tolerance and prevented autoimmune diabetes in NOD mice [46]. Here, we show that whilst CTLA4Ig mice produce 10–30 µg/mL serum levels of CTLA4Ig protein [33] and GC development is blocked, the effect on other T-cell subsets was surprisingly modest compared to CD80^{-/-}CD86^{-/-} mice. Our data show that while signals through CD28 are clearly critical in the generation of CD4⁺ T-cell responses, CTLA4Ig at the levels maintained in these mice does not seem to provide a robust blockade of CD80 and CD86. Strikingly, provision of only two doses of anti-ICOS mAb was sufficient to substantially reduce the resulting memory T-cell population, while modestly affecting the primary response, recapitulating data from ICOSL^{-/-} mice. The combined use of CTLA4Ig and anti-ICOS mAb in our model was far superior in blocking primary CD4⁺ T-cell responses, suggesting this dual targeting may have significantly enhanced clinical benefit in treating autoimmunity and graft rejection.

Whilst the cytokines IL-7 and IL-15 have been demonstrated to affect memory CD4⁺ T-cell survival, a role for costimulatory molecules remains unclear. To test a possible contribution from CD28 or ICOS signals we generated antigen-specific memory CD4⁺ T cells in vivo within WT hosts and subsequently transferred these into ICOSL^{-/-} or CD80^{-/-}CD86^{-/-} mice. The lack of these ligands had no significant effect on the number of memory CD4⁺ T cells subsequently recovered indicating that neither CD28 nor ICOS signals are required solely for memory cell survival within the timescale of these studies. Interestingly, this memory cell transfer model indicated that CD4⁺ memory T cells remain dependent on CD28 signals for optimal expansion upon secondary challenge, consistent with studies of memory cell recall using CTLA4Ig fusion proteins [47]. ICOS signals remain important for the generation of Tfh cells upon challenge but had no clear effect on the expansion of Tem and Tcm cells at this time point. The modest reduction in Tem cells after challenge observed with 2W1S-specific CD4⁺ T cells in ICOSL^{-/-} mice was not observed with SM1 cells, potentially reflecting differences between endogenous and TCR transgenic T cell responses.

In summary, beyond the formation of Tfh cells, signals through ICOS appear critical for the generation of a functional pool of Tem and Tcm cells following acute bacterial infection. The effectiveness of anti-ICOS blocking mAb in this response suggests that therapeutic targeting of this pathway may substantially improve

clinical attempts to control CD4⁺ T cells, perhaps in combination with blockade of CD28 signals.

Materials and methods

Mice

Mice used were BoyJ, C57BL/6 (obtained from Harlan or in house), ICOSL^{-/-}, CD80/86^{-/-}, CTLA4Ig [33], Rag^{-/-} SM1 (CD45.1⁺CD45.2⁺). Mice were only compared to those that had been bred in the same facility. Animals were bred in accordance with Home Office guidelines at the University of Birmingham, Biomedical Services Unit.

Cell transfer

A total of 10⁴ SM1 cells harvested from spleen and peripheral lymph nodes of TCR transgenic Rag^{-/-} SM1 mice were injected i.v. into C57BL/6 or BoyJ mice before i.v. infection with Lm-Flic the following day [48]. For memory cell transfers, secondary lymphoid tissues were harvested 28 dpi and cells were enriched using MACS columns and transferred i.v. into hosts [32]. Cells were transferred into twice as many recipients as donors, typically approximately 2.5 × 10⁴ memory SM1 cell were transferred.

Infection

Mice were infected i.v. with 10⁷ ActA-deficient Lm-2W1S (kind gift from Dr. Marc Jenkins) or Lm-Flic (kind gift from Dr. Sing Sing Way) as described [1, 14]. Mice were challenged at 28 dpi or post SM1 memory cell transfer and mice were sacrificed on day 3 or 4 post secondary challenge, respectively. For in vivo restimulation of memory cells mice were injected i.v. with 100 µg 2W1S peptide and 2.5 µg LPS 28 dpi and analyzed after 4 h.

Antibody injection

Blocking anti-ICOS and isotype control mAb were provided by MedImmune [49, 50]. Mice were injected i.p. with 0.25 mg anti-ICOS or isotype control mAb. Mice were injected 0 and 3 dpi for primary responses, 0 and 4 dpi for memory responses and 1 day pre- and post secondary challenge. To assess memory cell survival mice were injected twice weekly for 4 weeks from 28 dpi.

Flow cytometry

CD44^{hi} 2W1S:I-A^b CD4⁺ T cells were enumerated from spleen at 7 dpi and a pool of spleen and LN for all memory responses. Staining with PE conjugated 2W1S:I-A^b was carried out as described previously [14]. For in vivo restimulation experiments 10 µg brefeldin A was added. Cell surface staining was done at 4°C

for 30 min, except for CXCR5 that was stained for 1 h at room temperature. Enrichment for 2W1S-specific memory T cells was performed, as described previously, using anti PE MicroBeads (Miltenyi Biotec) and MACS enrichment [51]. Samples were run using a Fortessa X20 (BD Biosciences) and analyzed using FlowJo software (Tree Star). For intracellular cytoplasmic staining, cells were fixed and permeabilized with Cytofix/Cytoperm Plus (BD). Intracellular cytokines were stained with IL-2 488 and IFN- γ PECy7 (BD Biosciences). Ten microliters of Spherotech Accucount blank particles was used to calculate cell frequency.

Statistics

Data were analyzed using GraphPad Prism (version 6.0e). Non-parametric Mann–Whitney test was used to determine significance which was set at $p \leq 0.05$. Kruskal–Wallis one-way ANOVA was used to compare multiple groups with post hoc Dunn's test where stated. Median values were calculated and used in all analyses except where stated.



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Conflict of interest: R. Herbst and G. Carlesso are full-time employees of MedImmune LLC.

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Abbreviations: dpi: days post infection · ICOSL: ICOS ligand · Lm-2W1S: *Listeria monocytogenes* expressing 2W1S peptide · Tcm: T central memory · Tem: T effector memory · Tfh: T follicular helper

Full correspondence: Dr. David Withers, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK
Fax: +440-121-414-3599
e-mail: d.withers@bham.ac.uk

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