

**THE ROLE OF GROUP 3 INNATE LYMPHOID CELLS AND  
TUMOUR NECROSIS FACTOR RECEPTORS IN THE  
SURVIVAL AND FUNCTION OF REGULATORY T CELLS**

**By**

**EMILY ELISABETH HALFORD**

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

The ability to therapeutically manipulate regulatory T (Treg) cell survival/function would have far reaching implications; with the potential to limit immune pathology in autoimmune disease, allergy and transplantation; and to reduce regulation of anti-tumour responses in cancer. This study has established a method to study the survival and function of antigen specific Treg cells *in vivo*, adapting an existing approach in which an endogenous naïve T cell population is expanded and tracked. Multiple immunisation of antigen, and an agonistic anti-DR3 antibody were used to ensure a sufficient number and proportion of Treg cells could be expanded. Further to this, an assay for investigating Treg cell function *in vivo* was applied to this system. This approach revealed that the tumour necrosis factor receptors OX40 and CD30 may play a role in Treg function, as well as expansion. Unexpectedly, these data also revealed that in the absence of OX40 there is a gross defect in the function of CD4 T cells. A regulatory role of group 3 Innate Lymphoid cells is emerging in the literature, and in accordance with this, this study demonstrates that Treg cell expansion is grossly impaired in mice which lack ROR $\gamma$ t, their lineage defining transcription factor.

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## **ABBREVIATIONS**

ADP = Adenosine diphosphate  
AIRE = Autoimmune regulator  
AMP = Adenosine monophosphate  
APC = Antigen presenting cell  
ATP = Adenosine triphosphate  
Bcl-xL = B cell lymphoma-extra large  
DR3 = Death receptor 3  
cAMP = cyclic AMP  
CCR = Chemokine receptor  
CD = Cluster of differentiation  
CLIP = Class II associated invariant chain peptide  
CTLA-4 = Cytotoxic T lymphocyte antigen 4  
DC = Dendritic cell  
DTR = Diphtheria toxin receptor  
FOXP3 = Forkhead/winged-helix box P3  
GATA3 = GATA binding protein 3  
GFP = Green fluorescent protein  
GITR = Glucocorticoid-Induced TNF receptor  
ICER = Inducible cAMP early repressor  
IFN = Interferon  
IL = Interleukin  
ILC = Innate lymphoid cell  
KO = Knock out  
LAG-3 = Lymphocyte activation gene 3  
LPS = Lipopolysaccharide  
LTi = Lymphoid tissue inducer

MHC II = Major histocompatibility complex class II

mTEC = Medullary thymic epithelial cell

Nrp-1 = Neuropilin-1

PAMP = Pathogen associated molecular pattern

PD-1 = Programmed death-1

PRR = Pathogen recognition receptor

RAG = Recombination activating genes

RAR = Retinoic acid receptor

ROR $\gamma$ t = RAR-related orphan receptor  $\gamma$ t

SP = Single positive

Tbet = T-box transcription factor

Tconv = Conventional T

TCR = T cell receptor

TGF = Tumour growth factor

Th = T helper

TL1A = TNF-like Ligand A1

TLR = Toll-like Receptor

TNF = Tumour Necrosis Factor

Tr1 = T regulatory 1

Treg = Regulatory T

WT = Wildtype

## **1.0 GENERAL INTRODUCTION**

## **1.1 Introduction to immune responses**

The immune system, which has evolved to protect against infection, is typically divided into innate and adaptive components. If a pathogen is able to breach the barrier surfaces of the body, the cells of the innate immune system recognise common pathogen components, known as pathogen associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Upon recognition via their pathogen recognition receptors (PRRs), phagocytes such as macrophages become activated and engulf and destroy bacteria. In addition, these cells secrete pro-inflammatory cytokines which promote inflammation and facilitate immune cell accumulation. In contrast, the cells of the adaptive immune system, B and T lymphocytes, recognise specific epitopes or peptides and there is a delay of several days before they are able to contribute to the response. Dendritic cells are considered to be the key cell in initially driving adaptive immune responses as they are able to activate T cells through presentation of cognate antigen to their T cell receptors (TCR) (Banchereau and Steinman, 1998). These antigen-presenting cells do so in the context of major histocompatibility (MHC) molecules, of which there are two classes. MHC class I molecules are expressed by all nucleated cells and present endogenous antigens, such as viral peptides to the TCR of T cells expressing the co-receptor CD8, also known as cytotoxic T cells when activated. Importantly, antigen-presenting cells can also present peptides derived from the local environment. These peptides are presented on MHC class II (MHC II) molecules, to T cells expressing the co-receptor CD4, also known as T helper (Th) cells when activated. During a bacterial infection for example, bacteria are phagocytosed and processed into peptides which are loaded onto MHC II molecules and expressed at the cell surface.

## **1.2 T cell tolerance**

To ensure protection from a wide range of pathogens, humans have evolved the capacity to generate  $10^8$  different TCR (Arstila et al., 1999) each of which can recognise up to a million different peptides (Sewell, 2012). To gain this level of diversity, T (and B cell) receptors are generated through a process called V(D)J recombination, in which coding regions are assembled from germline Variable (V), Diversity (D) and Joining (J) segments (Tonegawa, 1983, Hesslein and Schatz, 2001). This process is dependent on recombination activating genes (RAG)-1 and -2 and therefore in their absence T cells cannot develop (Mombaerts et al., 1992, Shinkai et al., 1992). This process results in a high level of diversity because multiple combinations can be assembled from the pool of V, D and J segments, and variation is introduced during segment ligation (Bassing et al., 2002). During this process, however, there is the potential that TCR are generated which can recognise self-peptides. To safeguard against the development of autoimmune disease there is therefore the need for multiple layers of T cell tolerance. These can be broadly split into central tolerance, which occurs during T cell development through a process called negative selection, and peripheral tolerance which is mediated by a number of mechanisms in the periphery.

### ***1.2.1 Central tolerance***

The precursors of T cells are generated in the bone marrow, but develop in the thymus. Lymphoid progenitors enter the thymus at the cortico-medullary junction as double negative thymocytes (CD4<sup>-</sup>CD8<sup>-</sup>), and migrate through the cortex, where they undergo VDJ recombination (Germain, 2002). The TCR  $\beta$  chain is first to be



rearranged and if successful, CD4 and CD8 are upregulated and TCR  $\alpha$ -chain rearrangement occurs. Positive selection selects for CD4<sup>+</sup>CD8<sup>+</sup> (double positive) thymocytes bearing TCR that can recognise peptide in the context of MHC II. This therefore ensures that T cells which progress have the capacity to become activated by antigen-presenting cells such as dendritic cells in the periphery (Laufer et al., 1996). Double positive thymocytes which survive positive selection downregulate either CD4 or CD8 to become single positive cells. Negative selection is mediated by medullary thymic epithelial cells (mTECs) as well as dendritic cells, which present self-peptides in the context of MHC II molecules and mediate the clonal deletion of thymocytes bearing TCR with high affinity for these peptides (Gallegos and Bevan, 2004). mTECs are able to express tissue specific antigens due to the activity of the transcription factors Autoimmune regulator (AIRE) and Fezf2 (Derbinski et al., 2001, Takaba et al., 2015). Negative selection ensures that only (conventional) T cells with low affinity for self-peptide enter the periphery, and is therefore a mechanism of maintaining tolerance to self-antigens (Starr et al., 2003). It is important however, that the process of negative selection does not delete all potentially self-reactive T cells as there is a need to strike the balance between removing these cells and ensuring that there is a wide enough repertoire to protect us from infection. There is therefore the need for mechanisms to maintain tolerance in the periphery.

### *1.2.2 Peripheral tolerance*

There are multiple layers of peripheral tolerance, of which a key checkpoint is the requirement for co-stimulation in T cell activation. T cells exit the thymus as naïve CD4 or CD8 T cells, and recirculate around the blood stream, passing through secondary lymphoid tissues such as the lymph nodes and spleen (von Andrian and

Mempel, 2003). These secondary lymphoid tissues increase the chances of a lymphocyte meeting its cognate antigen by providing specialised microenvironments with discrete areas for T and B cell activation. Lymph nodes are positioned at the junctions of lymphoid vessels, which transport antigen and activated antigen-presenting cells through the lymph to the lymph nodes from the site of infection.

CD4 T cell activation requires multiple signals and results in IL-2 production and proliferation (Smith-Garvin et al., 2009). The first signal is delivered through engagement of the TCR with its cognate antigen in the context of MHC II. The second signal is delivered by a plethora of co-stimulatory molecules, and the third by cytokines, which direct differentiation into the different T helper subsets. The three main subsets and their lineage defining transcription factors are: Th1 cells (Tbet), Th2 cells (GATA-3), and Th17 cells (ROR $\gamma$ t) (O'Shea and Paul, 2010). The existence of different T helper subsets allows tailoring of the immune response towards the type of pathogen, for example Th1 cells secrete Interferon  $\gamma$  (IFN $\gamma$ ) which promotes macrophage activation and therefore aids clearance of bacteria.

Co-stimulatory ligands and MHC II are provided by antigen-presenting cells, such as dendritic cells, but are only upregulated upon recognition of PAMPs by PRRs such as Toll-like receptors (TLRs) (Banchereau and Steinman, 1998). Co-stimulatory receptors, which are expressed by the T cell, are divided into two subsets; the immunoglobulin and tumour necrosis factor (TNF) receptor super families. The initial costimulatory signal is delivered through interaction of the immunoglobulin superfamily receptor CD28, which is constitutively expressed by naïve T cells, with its ligands CD80 and CD86, expressed by mature dendritic cells (Lenschow et al., 1996). Activation of the naïve T cell leads to upregulation of further co-stimulatory

molecules including TNF receptors such as OX40, which are principally involved in promoting T cell survival during activation (Croft, 2009). In the absence of co-stimulation TCR signaling results in anergy, a state of unresponsiveness. Therefore, the requirement for signal 2 can be considered to be a safeguard against T cell activation in the absence of infection, which is known as 'passive tolerance'. This study will concentrate on the action of Treg cells, which is known as 'dominant tolerance' and will be explored in detail.

### **1.3 Treg cells**

Treg cells are a subset of CD4 T cells which are able to suppress responses against self (Sakaguchi, 2004). These cells, which comprise 5-10% of the CD4 T cell population, have been identified both in mice (Gershon and Kondo, 1970, Sakaguchi et al., 1995) and humans (Baecher-Allan et al., 2001, Dieckmann et al., 2001, Jonuleit et al., 2001). In addition to their role in preventing autoimmunity, Treg cells have been shown to play an important role in maintaining oral and feto-maternal tolerance as well as feedback control of the immune response, particularly to reduce pathogen-associated immune pathology (Corthay, 2009). The role of Treg cells in allergy, transplantation and cancer are also of great interest in the scientific community, due to the potential for therapeutic manipulation.

The identification of discriminatory markers for Treg cells is not straightforward, as the majority of markers are shared with activated T cells in mice and humans. These include CD25 (Sakaguchi et al., 1995), Cytotoxic T lymphocyte antigen 4 (CTLA-4) (Read et al., 2000, Takahashi et al., 2000), Lymphocyte Activation Gene 3 (LAG-3) (Huang et al., 2004) and glucocorticoid-induced TNF receptor (GITR) (McHugh et al.,

2002). In mice the transcription factor forkhead/winged-helix box P3 (FOXP3) is considered to be the most reliable marker of Treg cells as it is exclusively expressed by these cells. In humans however, FOXP3 is transiently expressed in T cells upon activation (Gavin et al., 2006). It has been proposed that low expression of CD127 (IL-7R $\alpha$ ) should be also be used to aid identification of Treg cells, particularly in humans (Seddiki et al., 2006, Liu et al., 2006), therefore Treg cells are generally characterised as FOXP3<sup>+</sup> CD25<sup>hi</sup> CD127 (IL-7R $\alpha$ )<sup>lo</sup> (Vignali et al., 2008).

The crucial role of Treg cells in maintaining tolerance is demonstrated in mice which lack expression of FOXP3. These 'scurfy' mice develop a CD4 T cell dependent fatal lymphoproliferative disease (Clark et al., 1999, Brunkow et al., 2001). Similarly, if Treg cells are depleted after birth, the mouse will succumb to fatal lymphoproliferative disease within 2-3 weeks (Kim et al., 2007). This is also mirrored in the human disease IPEX (Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome), which is caused by a FOXP3 mutation and is characterised by severe autoimmunity (Bennett et al., 2001, Wildin et al., 2002). This transcription factor has been shown to act both as a repressor and as an activator (Marson et al., 2007, Zheng et al., 2007) and has been referred to as the 'master regulator' of Treg cell generation and function (Fontenot et al., 2003, Hori et al., 2003). Although there appears to be little doubt over the critical role of FOXP3 in Treg cell function, its importance in Treg cell lineage commitment has been questioned (Gavin et al., 2007, Lin et al., 2007, Hill et al., 2007). In these studies, disruption of the FOXP3 locus revealed that it amplifies and stabilises the regulatory lineage rather than being the initiating factor for commitment. Consistent with this, transcriptome analysis has

revealed that only a small proportion of the gene expression 'signature' of Treg cells can be recapitulated through FOXP3 transduction (Hill et al., 2007).

### 1.3.1 Treg cell subsets

Treg cells can either be generated in the thymus or induced in the periphery from naïve T cells (Bluestone and Abbas, 2003). As a result, Treg cells have been categorised as either thymically- (tTreg) or peripherally-derived (pTreg) (Abbas et al., 2013), as shown in **Figure 1.1**. The two subsets have often been referred to as natural or induced Treg cells, however this has not always been consistent and can be deemed misleading. The term 'natural' infers that the second subset does not occur naturally, and there is the potential that the term 'induced' may cause confusion with *in vitro* induced Treg cells. Therefore, the terms tTreg and pTreg were brought in as part of a move to simplify the nomenclature. Treg cells within human peripheral blood can also be classified according to expression of CD45RA and FOXP3 (Miyara et al., 2009). CD45RA<sup>+</sup>FOXP3<sup>lo</sup>Treg cells are considered to be 'resting', CD45RA<sup>+</sup>FOXP3<sup>hi</sup>Treg cells 'activated' and CD45RA<sup>-</sup>FOXP3<sup>lo</sup> cells 'non-suppressive'.

#### 1.3.1.1 Development of tTreg cells

Jordan et al. (2001) have demonstrated that thymic development of Treg cells requires high affinity interactions between the TCR and self-peptide. This study utilised a TCR transgenic system, demonstrating that thymocytes with low affinity for antigen were not able to become tTreg cells. TCR signalling is therefore thought to have an 'instructive' role in Treg cell development, and it has been suggested that Treg cells develop from thymocytes which bind to cognate self-antigen with affinities

below the threshold for negative selection but above the threshold for positive selection (Josefowicz et al., 2012). This reliance on antigen encounter for Treg cell development is evidenced in studies in which it is shown that without thymic antigen expression, Treg cells cannot develop (Walker et al., 2003b, Itoh et al., 1999). Further evidence for this comes from a study showing that mTEC, which express self-peptides, are crucial for Treg cell development (Cowan et al., 2013). It has also been demonstrated by Tai et al. (2005) that CD28 signaling is required alongside TCR signaling to initiate FOXP3 expression and therefore promote Treg cell development.

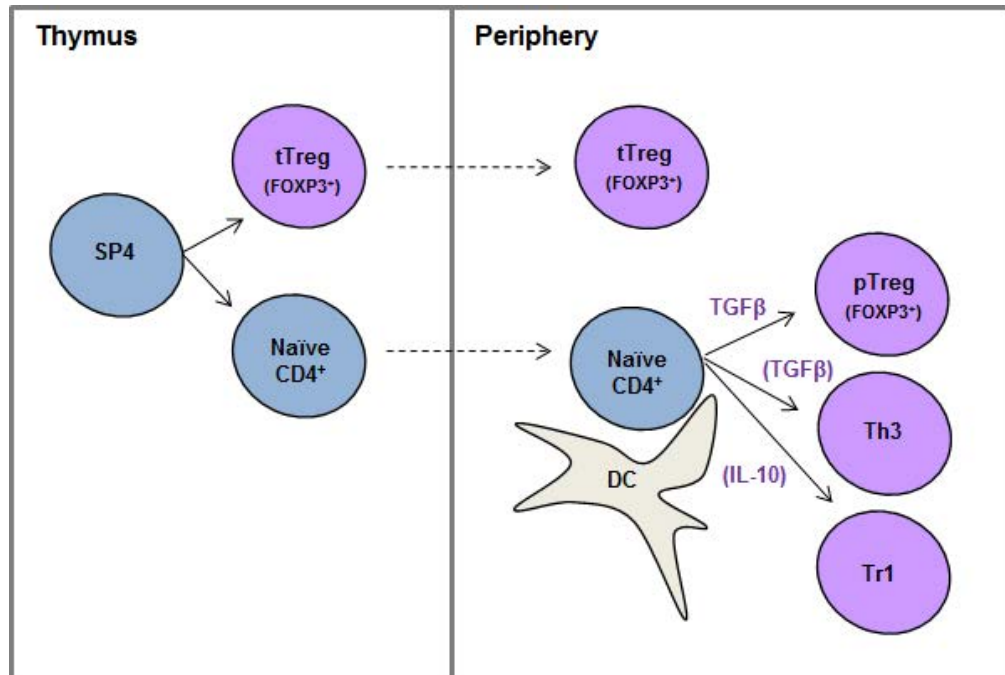
Interestingly, a sequencing study of hundreds of TCRs has shown that Treg cells do not show any more bias towards self-antigens than naïve T cells, although Treg cells do exhibit a greater diversity of TCRs (Stephens and Shevach, 2007, Pacholczyk et al., 2007). Therefore, although Treg cells can recognise self-antigen, this may not be a cardinal feature of these cells. Consistent with this, it has also been shown that immunisation of peptide can lead to the development of antigen-specific Treg cells in the thymus of mice (Zelenay et al., 2010), which demonstrates that peripheral antigen can influence tTreg development.

#### *1.3.1.2 Development of pTreg cells*

*In vitro* cell culture experiments have revealed that upon activation in the presence of TGFβ, naïve CD4 T cells can upregulate FOXP3 (Chen et al., 2003, Fantini et al., 2004). The pivotal role of this cytokine has subsequently been demonstrated through *in vivo* experiments, in which it was shown that pTreg cell induction was abrogated by administration of blocking TGFβ antibody (Mucida et al., 2005). There are also

two subsets of pTreg cells which do not express FOXP3 (Vieira et al., 2004). These are the T regulatory 1 (Tr1) and T helper 3 (Th3) subsets which have been classified as regulatory due to their high production of IL-10 and TGF $\beta$  respectively, as well as their ability to suppress chronic inflammatory responses *in vivo* (Groux et al., 1997, Chen et al., 1994). Conversion to Tr1 cells has been shown to be mediated primarily by IL-10 rather than TGF $\beta$  (Groux et al., 1997), therefore providing a positive feedback loop for Tr1 generation.

Interestingly, it has been demonstrated that naïve T cells which have recently entered the periphery from the thymus are most likely to be converted into pTreg cells (Paiva et al., 2013). pTreg cell development has been reported to be associated with low dose antigen (Apostolou and von Boehmer, 2004), and suboptimal activation of dendritic cells (Kretschmer et al., 2005). This suggests that conversion of naïve T cells to pTreg cells occurs more readily when there is weak co-stimulation. Interestingly, administration of antigen through the oral route has also been shown to be associated with pTreg generation (Mucida et al., 2005, Coombes et al., 2007). In these studies, it was shown that oral ovalbumin can induce TGF $\beta$ -dependent expansion of Treg cells in the mesenteric lymph nodes of mice. These studies used TCR transgenic (DO11.10) mice on a RAG deficient background, which are not able to produce tTreg cells (Walker et al., 2003b, Itoh et al., 1999), and therefore it was possible to demonstrate that the increase in Treg cells was due to conversion of naïve T cells rather than expansion of tTreg cells. This suggests pTreg cells have a role in oral tolerance, which interestingly has also been shown to be associated with low dose antigen (Friedman and Weiner, 1994).



**Figure 1.1: Treg cell subsets.** FOXP3<sup>+</sup> Treg cells are either generated in the thymus from CD4<sup>+</sup>CD8<sup>-</sup> thymocytes (tTregs) or in the periphery through TGFβ-dependent conversion of naïve CD4<sup>+</sup> T cells (pTregs). Two subsets of FOXP3<sup>-</sup> Treg cells also arise from naïve CD4<sup>+</sup> T cells in the periphery: T helper 3 (Th3) cells and T regulatory 1 (Tr1) cells.



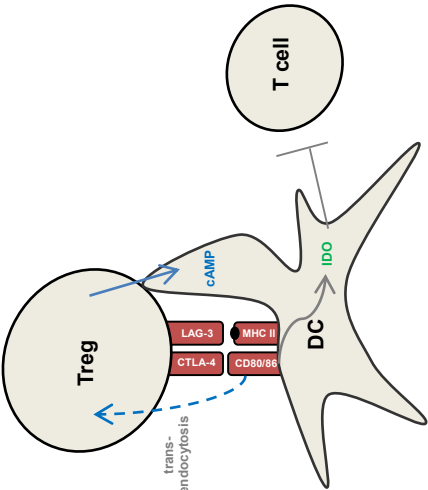
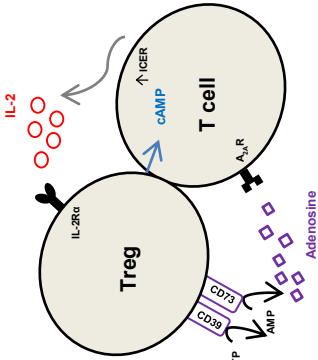
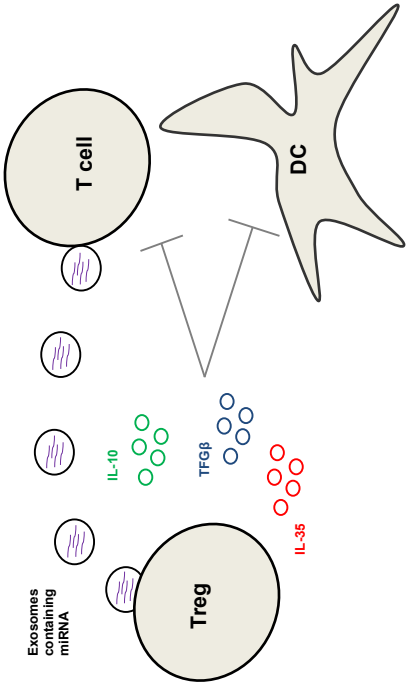
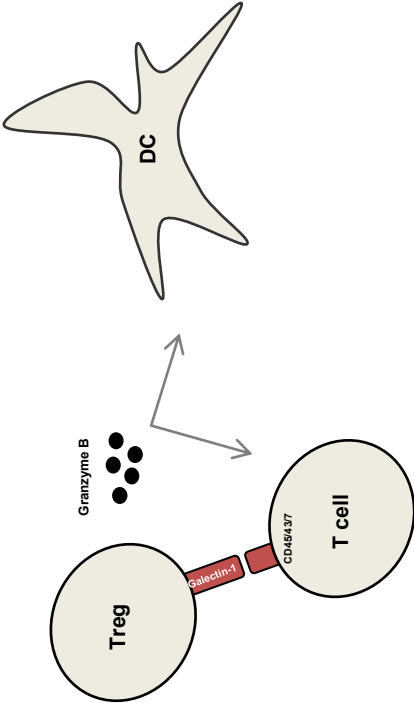
Haribhai et al. (2011b) have demonstrated that there is only 5-15% overlap between the TCR repertoire of pTreg and tTreg cells, which is perhaps unsurprising seeing as pTreg cells develop from conventional T cells. This suggests that there may functional dichotomy between these two subsets, and indeed transcriptome analysis of nTreg cells alongside pTreg cells generated by various routes has revealed that the two subsets have distinct gene expression profiles (Feuerer et al., 2010, Haribhai et al., 2009). Among the transcripts differentially expressed are those encoding chemokine receptors, effector molecules and transcription factors (Feuerer et al., 2010) indicating that these two subsets may be specialised towards different types of response and/or different locations. However, Haribhai et al. (2009) have shown that transfer of a 1:1 mix of pTreg and tTreg cells are able to better suppress the onset of T cell-induced colitis than transfer of either subset independently. Therefore, despite this functional dichotomy, these two subsets may synergise to attain maximal suppression.

#### *1.3.1.3 Discrimination between tTreg and pTreg cells*

Helios, and more recently Neuropilin-1, have been proposed as markers for discriminating tTreg cells from pTreg cells (Singh et al., 2015). Helios is a transcription factor from the Ikaros family (Hahm et al., 1998), which has been shown to be expressed by all thymic Treg cells but only 70% of Treg cells in the periphery of both mice and humans (Thornton et al., 2010). In addition, pTreg cells induced by oral ovalbumin were found to be Helios<sup>-</sup>. This has however been challenged by multiple groups (Verhagen and Wraith, 2010, Akimova et al., 2011, Gottschalk et al., 2012), including Himmel et al. (2013), who demonstrated that both Helios<sup>+</sup> and Helios<sup>-</sup> tTreg cells can be identified in human blood. Neuropilin-1 (Nrp-1) is a semaphorin III receptor which was initially characterised in relation to its role within the nervous system (Kolodkin et al., 1997). It has subsequently been shown to be expressed on the surface of Treg cells where it promotes long interactions with antigen-presenting cells (Sarris et al., 2008). It has been proposed that it is only tTreg cells which express Nrp-1 as again all thymic Treg cells were found to express Nrp-1 but only around 70% of Treg cells found in the periphery of mice. In addition, pTreg cells induced in the gut were shown to express lower levels of Nrp-1 than the overall Treg cell pool (Weiss et al., 2012, Yadav et al., 2012).

### **1.3.2 Mechanisms of suppression by Treg cells**

Treg cells can suppress many different immune cells, including natural killer cells, B cells, dendritic cells, monocytes and T cells (Schmidt et al., 2012). Their main function is often considered to be suppression of naïve T cell activation, which is thought to be by influencing both the naïve T cell, and the antigen-presenting cell (Schmidt et al., 2012), as shown in **Figure 1.2**. This ultimately leads to reduction in IL-2 production by the T cell, and therefore impaired proliferation (Thornton and Shevach, 1998, Takahashi et al., 1998). Interestingly Treg cells have also been shown to suppress effector and memory T cells (Levings et al., 2001), although they are thought to be more effective at suppressing the activation of naïve T cells (Miyara and Sakaguchi, 2007). Treg cells require stimulation through their TCR, as well as IL-2, for their activity (Thornton et al., 2004a, Thornton et al., 2004b) and thus are considered to be activated in an antigen specific manner. However, numerous studies have shown that they can suppress T cells of multiple specificities, which is termed bystander suppression (Thornton and Shevach, 2000, Takahashi et al., 1998, Thornton and Shevach, 1998). It is important to note however, that Treg cells may more efficiently suppress T cells of the same specificity (cross-regulation) (Corthay, 2009).

<p>1. Reduced T cell activation</p> 	<p>2. Metabolic disruption</p> 
<ul style="list-style-type: none"> <li>• Reduced expression of costimulatory molecules</li> <li>• Reduced antigen-presentation</li> </ul>	<ul style="list-style-type: none"> <li>• Reduced T cell proliferation</li> </ul>
<p>3. Immunomodulatory mediators</p> 	<p>4. Cytolysis/cell cycle arrest</p> 
<ul style="list-style-type: none"> <li>• Reduced DC activation</li> <li>• Reduced T cell proliferation</li> </ul>	<ul style="list-style-type: none"> <li>• Cell death/anergy</li> </ul>

**Figure 1.2: Treg cell-mediated suppression of T cells.** The suppressive mechanisms employed can be divided into four categories: reduced T cell activation, metabolic disruption, immunomodulatory mediators and cytotoxicity/cell cycle arrest. Treg: Regulatory T cell, CTLA-4: Cytotoxic T lymphocyte antigen 4, LAG-3: Lymphocyte activation gene 3, CD: Cluster of differentiation, MHC II: Major histocompatibility complex class II, DC: Dendritic cell, IDO: indoleamine 2, 3-dioxygenase, ATP: adenosine triphosphate, AMP: adenosine monophosphate, ICER: inducible cAMP early repressor, cAMP: cyclic adenosine monophosphate, A<sub>2A</sub>R: A<sub>2A</sub> receptor, IL: Interleukin, IL-2R $\alpha$ : Interleukin-2 receptor  $\alpha$ , TGF: Tumour growth factor.

### 1.3.2.1 Reduced T cell activation

Multiple mechanisms have been described for Treg cell-mediated suppression, which can be divided into four categories. The first results from interactions between Treg and dendritic cells, which have been shown to occur *in vivo* by intravital microscopy by multiple groups (Tang et al., 2006, Tadokoro et al., 2006). Interestingly Nr1, which has previously been discussed as a marker of tTreg cells, has been shown to promote long interactions between Treg cells and dendritic cells (Sarris et al., 2008), and therefore may give Treg cells an advantage over T cells.

One of the key mechanisms of Treg cell-mediated suppression is thought to be through the action of CTLA-4, particularly as selective depletion of this inhibitory receptor from Treg cells results in lymphoproliferative disease (Wing et al., 2008, Friedline et al., 2009). CTLA-4 is constitutively expressed by Treg cells (Takahashi et al., 2000, Read et al., 2000, Metzler et al., 1999) and shares its ligands CD80 and CD86 with the co-stimulatory receptor CD28. As CTLA-4 binds with higher affinity to these ligands than CD28, it has been proposed that the mechanism by which CTLA-4 interferes with T cell activation is by out-competing CD28 for binding to its ligands (Masteller et al., 2000). However, it has been shown that CTLA-4, which cycles between the surface and cytoplasm, can 'rip' CD80/86 from the surface of dendritic cells through a process called trans-endocytosis, which results in the degradation of these ligands within the Treg cell (Qureshi et al., 2011). This would suggest that rather than competing with CD28 for its ligands, CTLA-4 depletes CD80/86 from dendritic cells and as a result there is less available to bind CD28 and deliver signal 2 for T cell activation. It has also been proposed that reverse signaling occurs as a result of interactions between CTLA-4 and CD80/86, stimulating the activity of the

enzyme indoleamine 2, 3-dioxygenase (IDO) within the dendritic cell (Grohmann et al., 2002). This enzyme converts tryptophan to kynurenine, which is thought to result in inhibition of T cell proliferation due to the resulting shortage of tryptophan within the microenvironment (Mellor et al., 2002) and may cause T cell apoptosis due to the toxicity of kynurenine (Fallarino et al., 2003).

Another inhibitory receptor, LAG-3, has also been shown to interfere with T cell activation, by blocking the maturation of dendritic cells (Liang et al., 2008). As dendritic cell maturation crucially leads to the upregulation of co-stimulatory molecules and MHC II, this would prevent T cell activation. LAG-3 is upregulated on Treg cells upon activation and participates in their suppressive function (Huang et al., 2004, Workman and Vignali, 2005). LAG-3 is able to bind to MHC II with higher affinity than CD4 (Huard et al., 1995), with which it shares structural homology, and it is thought to be via this interaction that LAG-3 provides inhibitory signals to dendritic cells (Liang et al., 2008).

#### *1.3.2.2 Metabolic disruption*

Various suppressive mechanisms have been proposed which interfere with naïve T cell or dendritic cell metabolism. The high expression of CD25 on Treg cells led to the proposal that Treg cells act as an 'IL-2 sink', depriving effector T cells of this cytokine, which they need for proliferation and survival. This has been shown to occur *in vitro* (Pandiyan et al., 2007, Barthlott et al., 2005), but little evidence for this phenomenon has been ascertained from *in vivo* studies and therefore it remains controversial (Vignali, 2012). Another mechanism which results in impaired proliferation of naïve T cells is mediated by the ectoenzymes CD39 and CD73, which

are highly expressed on the surface of Treg cells (Kobie et al., 2006, Deaglio et al., 2007, Borsellino et al., 2007). These enzymes co-operate to convert extracellular adenosine tri-phosphate (ATP), which is released through cellular damage, to adenosine. Adenosine has been shown to inhibit T cell proliferation through interactions with the A<sub>2A</sub> receptor (A<sub>2A</sub>R), expressed by these cells. The mechanism by which this occurs may involve cyclic AMP (cAMP), a second messenger molecule which is increased upon binding of adenosine to A<sub>2A</sub>R (Ernst et al., 2010), but can also be transferred directly from regulatory T cells into conventional T cells through gap junctions (Bopp et al., 2007). In naïve T cells cAMP induces expression of inducible cAMP early repressor (ICER), which is a repressor at the IL-2 locus (Bopp et al., 2007). cAMP can also be transferred directly from Treg cells into dendritic cells through gap junctions (Bopp et al., 2007), which has been shown to cause downregulation of the co-stimulatory molecules CD80 and CD86 (Fassbender et al., 2010), hence suppressing naïve T cell activation.

#### *1.3.2.3 Immunomodulatory mediators*

The key immunosuppressive cytokines secreted by Treg cells are TGFβ and IL-10, which also have roles in pTreg cell induction, as previously discussed (Miyara and Sakaguchi, 2007). These cytokines act on multiple cell types, including T cells and dendritic cells, inhibiting proliferation and activation respectively. Initial *in vitro* studies showed that neither IL-10 nor TGFβ are required for Treg cell mediated suppression of conventional T cells (Thornton and Shevach, 1998, Takahashi et al., 1998). However, the absence of IL-10, which is particularly important at environmental interfaces (Rubtsov et al., 2008), has been shown to impair Treg cell-mediated suppression of colitis in multiple *in vivo* models (Kingsley et al., 2002, Annacker et



al., 2001, Asseman et al., 1999). There is also evidence from a similar colitis model that TGF $\beta$  is required for Treg-cell mediated suppression of T cells (Fahlen et al., 2005). TGF $\beta$  exists in a membrane bound, as well as a secretory form, and there is *in vitro* evidence that TGF $\beta$ -dependent suppression may be via the membrane bound form (Nakamura et al., 2001). The anti-inflammatory cytokine IL-35 can also be secreted by Treg cells and has been shown to be required for maximal suppression of T cell proliferation *in vitro* and *in vivo* (Collison et al., 2007). Interestingly, this cytokine can also induce the generation of further IL-35 producing Treg cells from naïve T cells (Collison et al., 2010). These FOXP3 negative cells have been shown to mediate suppression through IL-35 but not IL-10 or TGF $\beta$ . It is clear that there may be subsets and mechanisms of suppression which are yet to be discovered, and indeed it is emerging that Treg cells release exosomes containing microRNA which, upon delivery to effector T cells, can directly suppress proliferation and pro-inflammatory cytokine expression (Okoye et al., 2014).

#### 1.3.2.4 Cytolysis/cell cycle arrest

It has been proposed that Treg cells are able to cause lysis of activated T cells and dendritic cells via the perforin-granzyme pathway (Grossman et al., 2004), and therefore the suppressive mechanisms utilised by Treg cells include cell death. This pathway is more often associated with cytotoxic T cells and natural killer cells, which use perforin to create holes in the target cell membrane, facilitating the entry of granzymes which induce apoptosis (Dustin and Long, 2010). It has subsequently been shown that granzyme B is upregulated in Treg cells upon activation and that deficiency of this protease leads to impaired suppression (Gondek et al., 2005, Gondek et al., 2008), suggesting that this cytotoxic mediator is utilised as a

suppressive mechanism by Treg cells. These studies also revealed that this is a perforin-independent process and therefore it is not currently clear how granzyme B is able to enter the target cell, although perforin-independent mechanisms of granzyme-mediated cell death have been described (Gross et al., 2003). It has also been reported that cell cycle arrest can be induced through the interaction of Galectin-1 on Treg cells with CD45/43/7 on naïve T cells (Garin et al., 2007). This group showed that blocking of Galectin-1, which is upregulated upon Treg cell activation, impairs Treg cell suppression.

### **1.3.3 Treg cell homeostasis**

Early *in vitro* studies indicated that Treg cells are anergic and therefore do not proliferate upon TCR stimulation (Takahashi et al., 1998, Itoh et al., 1999, Kuniyasu et al., 2000). However, it has since been revealed that Treg cells are a continuously activated and highly proliferative T cell subset (Fisson et al., 2003, Walker et al., 2003a). *In vivo* cell transfer experiments performed by Fisson et al. (2003) also revealed that Treg cells are highly stable, which has since been confirmed by multiple research groups (Sakaguchi et al., 2013). It has been reported that if transferred into a lymphopenic environment, a small proportion of Treg cells lose expression of FOXP3 (Duarte et al., 2009), however this is not thought to represent conversion to conventional T cells as these cells are able to upregulate FOXP3 upon encounter with antigen (Miyao et al., 2012). It is also emerging that there may be a capacity for memory in this regulatory arm of the immune system. A previous publication has shown that foetal antigen specific Treg cells expand in the mother during pregnancy, persist after birth, and are able to rapidly accumulate in a subsequent pregnancy (Rowe et al., 2012). The key signals required for Treg cell

homeostasis are thought to be delivered via the TCR, CD28 and IL-2R $\alpha$ . There may also be a role for Nrp-1 in supporting Treg cell survival in inflammatory sites.

#### *1.3.3.1 TCR*

In addition to the requirement for TCR stimulation for Treg cell activation, it has also emerged that Treg cell homeostasis is supported by signals through the TCR (Kim et al., 2009), and hence encounter with cognate antigen. In accordance with this, Treg cells are found to proliferate and accumulate in the lymph nodes in close proximity to their target antigen (Walker et al., 2003a, Fisson et al., 2003). Sequencing studies have shown that the TCR repertoire varies based on anatomical location (Lathrop et al., 2008), again suggesting that Treg cells are enriched in the lymph node nearest their antigen, which is thought to strategically position them to maintain tolerance (Wheeler et al., 2009). Dendritic cells have been shown to present self-antigens in a draining lymph node in non-inflammatory conditions (Scheinecker et al., 2002), and therefore may be the source of antigen to support the local homeostatic control of Treg cells. Interestingly along with 'typical' antigen presenting cells such as dendritic cells, macrophages and B cells, various other cells of the immune system have been shown to be able to present antigen in various settings and may therefore be termed 'atypical' antigen presenting cells (Kambayashi and Laufer, 2014). This category includes Innate Lymphoid Cells (ILCs), which are haematopoietic cells with lymphoid morphology but which lack T cell and B cell-receptors, as well as molecules expressed by myeloid cells, such as CD11c (Spits and Cupedo, 2012). They can be identified by the absence of such markers, as well as expression of Interleukin-7 Receptor  $\alpha$  (IL-7R $\alpha$ ) (Vonarbourg and Diefenbach, 2012).

#### *1.3.3.1.1 Innate Lymphoid Cells*

ILCs have been categorised into three groups based on the expression of lineage defining transcription factors and signature cytokines, of which Group 3 ILCs (ILC3s) express the transcription factor ROR $\gamma$  and the cytokines IL-22 and IL-17 (Spits et al., 2013). ILC3s are primarily found in the gut but also reside in the lymph nodes and spleen (Walker et al., 2013). In secondary lymphoid tissue they are located at the interface between T and B cell areas, such as the interfollicular regions (Mackley et al., 2015). Multiple roles for ILCs within the innate immune system have been described, including defence against microbial infections, maintenance of epithelial barrier integrity and tissue repair. Further to this, it is becoming apparent that ILCs are able to influence the adaptive immune system (Artis and Spits, 2015). Interestingly, ILC3s have been shown to process and present antigen in the context of MHC II as a mechanism to prevent T cell responses to commensal bacteria in the gut (Hepworth et al., 2015, Hepworth et al., 2013). This therefore provides evidence that these atypical antigen presenting cells, which do not express CD80/86, have a regulatory role in the gut, and it is important to ascertain whether this extends to the periphery, where these cells are well placed to interact with recirculating lymphocytes.

#### *1.3.3.2 CD28*

Initial studies showed that despite the role of CD28 in T cell activation, a model of autoimmune diabetes was exacerbated in the absence of this receptor (Salomon et al., 2000). The number of Treg cells was markedly decreased in these CD28 deficient mice, and transfer of CD28-sufficient Treg cells was able to delay disease

onset. This indicated that CD28 has a role in Treg cell homeostasis, however this is challenging to dissect due to the role of CD28 in T cell activation and Treg cell development in the thymus (Tang et al., 2003). To overcome these complicating factors two models have been generated. The first allows inducible CD28 ablation and has revealed that in the absence of CD28, the number of Treg cells in the periphery is reduced (Gogishvili et al., 2013). This group demonstrated that this was unaffected by removal of the thymus or addition of CD28-sufficient T cells, and therefore these data suggest that CD28 is important for Treg cell homeostasis. The second model is a mouse in which CD28 selectively depleted from Treg cells, and revealed that these mice succumb to severe autoimmune disease, which is reversible by addition of CD28-sufficient Treg cells (Zhang et al., 2013). Interestingly, this group showed that the number of Treg cells was not affected by the loss of CD28. This suggesting instead that the absence of CD28 has a qualitative effect on Treg cells, and indeed expression of CTLA-4 was shown to be reduced by approximately 50%. In terms of the source of CD28 signaling, it may be that the low level of CD80/86 which is expressed on immature dendritic cells in non-inflammatory settings serves to maintain Treg cell homeostasis (Bluestone and Abbas, 2003). Indeed Bar-On et al. (2011) demonstrated that in mice that lack CD80 and CD86 expression on CD11c<sup>+</sup> dendritic cells, there is a twofold reduction in the number of Treg cells in the periphery.

#### 1.3.3.3 IL-2

As previously discussed, *in vitro* experiments have demonstrated that the presence of IL-2, as well as TCR stimulation, is necessary for Treg cell activation (Thornton et al., 2004b, Thornton et al., 2004a). Further to this, *in vivo* experiments using IL-2/IL-

2R $\beta$  deficient mice have shown that IL-2 has a central role in Treg cell homeostasis (Fontenot et al., 2005, D'Cruz and Klein, 2005, Malek et al., 2002). Activated T cells are the major source of IL-2, which is not produced by Treg cells themselves (Almeida et al., 2006). As a result, T cell activation and subsequent IL-2 production supports Treg cell activation and survival, ensuring that there is adequate regulation of the response. As contraction of the effector response occurs, there is a reduction in IL-2 availability and therefore the regulatory response. In support of this theory, Almeida et al. (2006) demonstrated that there is a direct relationship between the number of Treg cells in the periphery and the number of activated T cells.

#### *1.3.3.4 Nrp-1*

Signaling through Nrp-1 has been shown to enhance Treg cell stability at inflammatory sites in a process dependent on the ligand semaphorin-4a (Delgoffe et al., 2013). This group showed that although Nrp-1 is dispensable for Treg cell homeostasis, Treg cells lacking this receptor are unable to suppress an anti-tumour response. Semaphorin-4a expression by dendritic cells is known to enhance T cell activation (Kumanogoh et al., 2002), and this group found that the major cellular source of Nrp-1 at the tumour site was dendritic cells. Like Nrp-1, which is a semaphorin III receptor, this ligand has also been shown to be important for axonal guidance. As such it is enriched in sites such as the eye, where it is expressed by retinal epithelial cells (Rice et al., 2004).

#### **1.3.4 Control of suppression**

Interestingly it has been shown that Treg cells can upregulate transcription factors associated with different T helper responses, tailoring them towards that response

(Koch et al., 2009, Chaudhry et al., 2009, Zheng et al., 2009). For example, Treg cells have been shown to upregulate Tbet in response to agonistic anti-CD40, which stimulates a strong Th1 response (Koch et al., 2009). Experiments with Tbet-deficient Treg cells demonstrated that this transcription factor supports Treg cell homeostasis, as well as expression of the chemokine receptor CXCR3, which is postulated to direct Treg cell migration towards the site of the Th1 response (Koch et al., 2009). Interestingly 40-60% of Treg cells within the colon have been shown to be ROR $\gamma$ t<sup>+</sup>, and this population is dependent on the presence of commensal bacteria (Sefik et al., 2015). Introduction of various species of bacteria back into germ free mice has shown that there is not a strong correlation between the bacterial species which induce Th17 cells and those which induce ROR $\gamma$ t<sup>+</sup> Treg cells. Therefore, rather than tailoring colonic Treg cells towards Th17 responses, this may be a distinct mechanism, which relates to tolerance to commensal bacteria.

The strength of the immune response is known to influence the magnitude of suppression, with high doses of antigen rendering effector T cells resistant to suppression (Baecher-Allan et al., 2002, Georger et al., 2003). Interestingly, there are multiple reports that during autoimmune disease, rather than there being an impairment in regulatory T cell function, conventional T cells become resistant to suppression. For example, this has been reported in animal models of type 1 diabetes (Clough et al., 2008, D'Alise et al., 2008) as well as human studies (Lawson et al., 2008, Schneider et al., 2008). High levels of PAMPs, pro-inflammatory cytokines and co-stimulatory molecules are thought to contribute to resistance to suppression, as detailed below, and therefore this may be a mechanism to restrict suppression whilst the antigen-load is high and the effector response is developing.

As an infection is cleared, the antigen load will drop, along with the levels of these mediators, which is thought to allow the Treg cells to function to prevent immune pathology. This is termed the 'tuned suppression' model (Walker, 2009).

#### *1.3.4.1 Toll-like receptor signaling*

As explored previously, dendritic cells become activated in response to signaling through their PRRs, which include the family of Toll-like receptors. Upon activation, dendritic cells produce pro-inflammatory cytokines such as IL-6. This cytokine has been shown to partially hinder suppression by Treg cells (Pasare and Medzhitov, 2003, Iwasaki and Medzhitov, 2004) therefore, TLR stimulation may indirectly affect Treg cell activity. Interestingly, various TLRs are also expressed by conventional and regulatory T cells and signaling through TLR9, which recognises bacterial or viral DNA, has been shown to directly release effector T cells from Treg cell mediated suppression (LaRosa et al., 2007). Similarly, signaling through TLR2 and TLR8, which are constitutively expressed by Treg cells, impairs suppression (Peng et al., 2005, Suttmüller et al., 2006). These TLRs recognise bacterial lipoproteins and viral single stranded RNA respectively. In contrast, signalling through TLR5, which recognises bacterial flagellin, enhances Treg cell function (Crellin et al., 2005). Therefore, bacterial and viral PAMPS have the ability to reduce suppression via interactions with PRRs expressed by antigen presenting cells, as well as conventional and regulatory T cells, with one exception noted.

#### *1.3.4.2 Cytokines*

Multiple pro-inflammatory cytokines have been shown to contribute to resistance to suppression, particularly those expressing the common gamma chain such as IL-6



and IL-21 (Attridge and Walker, 2014). It has been suggested that these cytokines release T cells from their dependence on CD28 signals, a feature which is exploited by Treg cells (Attridge and Walker, 2014). As previously discussed, Treg cells constitutively express CTLA-4, which interferes with signalling between CD28 and its ligands CD80/86. Therefore, releasing T cells from their dependence on CD28 signals would deprive Treg cells of a key mechanism of suppression. Alternatively, these cytokines may indirectly affect suppression by interfering with Treg cell homeostasis. Attridge et al. (2012) demonstrated that IL-21 can reduce IL-2 production by T cells and that although Treg cells are critically dependent on IL-2, effector T cell proliferation can be supported by IL-21. Therefore, this cytokine can remove survival signals from Treg cells without adversely affecting effector T cell homeostasis.

#### *1.3.4.3 TNF receptors*

Interactions between TNF receptors and their ligands have also been shown to influence suppression. This family is known to contribute to a wide range of processes within immune system via regulation of cell apoptosis, survival, proliferation and differentiation (Aggarwal et al., 2012). TNF receptors are transiently expressed on T cells during activation, where they act as co-stimulatory molecules, and various TNF receptors are constitutively expressed by Treg cells (Croft, 2009). Interactions between the TNF receptor GITR and its ligand have been shown to reduce suppression via effects on conventional rather than regulatory cells (Stephens et al., 2004). It has also been reported that interactions between OX40 and its ligand directly regulates Treg cell function (Vu et al., 2007). However the role of

OX40 on Treg cells is controversial within the literature and there is also evidence that OX40 and IL-2 signal co-operatively to support Treg cells (Xiao et al., 2012).

### **1.3.5 Therapeutic avenues**

There are many situations in which boosting Treg cell activation, survival or function would be beneficial, such as autoimmune disease, allergy and transplantation. Indeed, proposed approaches for the treatment of autoimmune disease include *in vivo* or *ex vivo* Treg cell expansion, as well as enhancement of suppressive ability (Miyara et al., 2014). Conversely, because many tumours contain high numbers of Treg cells which are able to suppress the anti-tumour response (Nishikawa and Sakaguchi, 2010), inhibition of Treg cells would aid clearance by the immune system. Current advances in anti-tumour immunotherapy have included strategies to release effector T cells from regulation, for example by blocking CTLA-4 and Programmed Death-1 (PD-1), which is another negative regulator of T cell activation expressed by T cells (Curran et al., 2010). Interestingly there are reports that anti-CTLA4 treatment can cause Treg cell depletion in certain contexts (Selby et al., 2013, Simpson et al., 2013). An ideal therapeutic would boost the effector response whilst inhibiting Treg cells. It is clear that the ability to manipulate the activation, survival or function of Treg cells would have far reaching implications, either tipping the balance in favour of the effector or regulatory T cells.

### **1.4 Aims of the study**

- Establish a method to study Treg cell survival and function *in vivo*.
- Investigate the sources of cellular support, and the role of TNF receptors in Treg cell survival and function.

## **2.0 MATERIALS AND METHODS**

## 2.1 Mice

Animals were bred in accordance with Home Office Guidelines at the

. C57BL/6 mice were used as wildtype controls in all experiments using adult mice. In embryonic spleen experiments BALB/c embryos were used due to availability. Mice were age matched when possible, and all genetically modified strains were compared to wildtype mice which had been bred in-house. External wildtype mice, from Harlan or Charles River, were utilised in all other circumstances. All manipulated mice strains were on a C57BL/6 background and are described in **Table 2.1**. Mice were used at 6-12 weeks of age and were sacrificed by a rising concentration of CO<sub>2</sub>.

Strain	Phenotype	Source
FOXP3 <sup>GFP</sup>	Regulatory T cells identified by GFP expression.	(Bettelli et al., 2006)
ROR $\gamma$ t KO	Mice lack lymph nodes, Th17 cells, ROR $\gamma$ t <sup>+</sup> ILCs, and exhibit altered thymocyte development.	Ivanov et al., 2006
FOXP3 <sup>GFP</sup> x ROR $\gamma$ t KO	Mice lack lymph nodes, Th17 cells, ROR $\gamma$ t <sup>+</sup> ILCs, and exhibit altered thymocyte development. Regulatory T cells identified by GFP expression	Cross performed by Dr Fiona McConnell, unpublished
OX40/CD30 dKO	T cells lack the co-stimulatory receptors OX40 and CD30, affecting T cell activation.	Gaspal et al., 2005
OX40 KO	T cells lack the co-stimulatory receptor OX40, affecting T cell activation.	Kopf et al., 1999

*Table 2.1: Genetically modified mouse strains*

## 2.2 Solutions

Gey's solution was used to remove red blood cells from spleen cell suspensions (5 ml). The solution contained 20 ml of Solution A, 5 ml of Solution B, 5 ml of Solution C

and 70 ml of distilled H<sub>2</sub>O (**Table 2.2**). Each solution was made up to 1 L with distilled H<sub>2</sub>O and autoclaved. The composition of other solutions used is shown in **Table 2.3**.

Solution	Composition
A	35 g NH <sub>4</sub> Cl 1.85 g KCl 1.5 g Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O 0.119 g KH <sub>2</sub> PO <sub>4</sub> 5 g Glucose 25 g Gelatin 1.5 ml 1% Phenol red
B	4.2 g MgCl <sub>2</sub> .6H <sub>2</sub> O 1.4 g MgSO <sub>4</sub> .7H <sub>2</sub> O 3.4 g CaCl <sub>2</sub>
C	22.5 g NaHCO <sub>3</sub>

*Table 2.2: Composition of Gey's solution*

Solution	Composition
RPMI	Complete RPMI 1640 (Gibco)
RPMI + EDTA	Complete RPMI 1640 (Gibco) supplemented with 2.5 mM EDTA (Fluka Analytical)
Culture Medium	Complete RPMI 1640 (Gibco) supplemented with 2% foetal bovine serum (Biosera or Invitrogen), 2 mM L-glutamine (Sigma) and 1% penicillin and streptomycin (Gibco)
PBS	Dulbecco's phosphate buffered saline +MgCl <sub>2</sub> +CaCl <sub>2</sub> (Gibco)
Flow cytometry staining buffer	Dulbecco's phosphate buffered saline +MgCl <sub>2</sub> +CaCl <sub>2</sub> (Gibco) supplemented with 2% foetal bovine serum (Biosera or Invitrogen) and 2.5 mM EDTA (Fluka Analytical)
Immunofluorescence staining solution	Phosphate buffered saline (Sigma) supplemented with 1% bovine serum albumin (Sigma).

*Table 2.3: Solutions for cell/tissue preparation*

### **2.3 Tissue dissection and cell isolation**

For T cell isolation, the thymus or spleen +/- a pool of lymph nodes, were harvested from mice and placed in 3 ml RPMI + EDTA. The pool of lymph nodes included: cervical, brachial, auxiliary, inguinal and mesenteric. Fat was cleaned off the tissues using forceps under a dissection microscope. Tissues were crushed through a 70 µm cell strainer (Falcon) using RPMI + EDTA to wash. After centrifuging at 4°C for 6 minutes at 394 relative centrifugal force (rcf), red blood cell lysis was performed (spleen or spleen and lymph node suspensions only). To lyse red blood cells 5 ml Gey's solution was added to the cell pellet, the suspension was mixed and incubated on ice for 5 minutes. Cells were washed in 10 ml RPMI + EDTA to prevent further cell lysis. As with all washes, cells were centrifuged for 6 minutes at 394 rcf. Cells were re-suspended in Flow Cytometry Staining Buffer (SB) and filtered through a 70 µm cell strainer (Falcon).

For Innate Lymphoid Cell isolation, spleen and inguinal and mesenteric lymph nodes were harvested from mice, placed in 3 ml RPMI, and fat was removed. The lymph nodes were teased apart with forceps and the spleens cut up using scissors. For digestion 7.5 µl DNase I (10 mg/ml stock, Sigma) and 7.5 µl collagenase/ dispase (100 mg/ml stock, Roche Diagnostics) were added and tissues were incubated at 37°C for 20 minutes. To stop digestion, 60 µl 0.5 M EDTA (Fluka Analytical) was added. Cells were crushed through a 70 µl cell strainer (Falcon) and red blood cell lysis was performed on spleen samples as previously described. All samples were subsequently re-suspended in SB and filtered through a 70 µm cell strainer (Falcon).

## **2.4 Embryonic spleen culture**

Embryonic spleens were dissected from BALB/c embryos at day 15 of gestation. A maximum of six spleens were placed on a sterile 0.8 µm nucleopore filter (Millipore) laid on a 1 cm<sup>2</sup> piece of sterile antiwrap sponge. Each sponge-filter pair was placed in 4ml Culture medium containing 100 ng/ml IL-7 (Peprotech) in a 90 mm petri dish (Sterilin). These were placed in sterile humidified chambers and incubated for 7 days at 37°C. Embryonic spleens were removed from the filters, placed in groups of six, and teased apart with forceps. Cells were crushed through a 70 µm cell strainer (Falcon) and re-suspended in 1 ml culture medium. Cells were cultured overnight with 100 ng/ml IL-7 +/- 100 ng/ml TL1A (R&D Systems) (Kim et al., 2006) or 10 µg/ml agonistic anti-DR3 (Biolegend). The following day cells were washed twice in PBS and incubated for 15 minutes at 4°C with Livedead® fixable near-IR dead cell stain kit (Life Technologies, 1/2000). Cells were then washed again in PBS before addition of antibodies.

## **2.5 Identification of antigen-specific T cells**

Cells from secondary lymphoid tissue were incubated with MHC class II tetramers to detect specific T cell populations. These consist of four biotinylated peptide-MHC class II molecules attached to a fluorescently conjugated streptavidin core. Tetramers were obtained from the National Institute of Health Tetramer Facility or generated by the Protein Expression Facility at the University of Birmingham. To detect 2W1S-specific T cells, 2W1S: I-Ab MHC class II tetramers conjugated to PE or APC were utilised. To detect LLO<sub>190-201</sub>-specific T cells LLO: I-Ab MHC class II tetramers conjugated to APC were utilised. Tetramers loaded with MHC class II-associated

invariant chain peptide were used to exclude non-specific tetramer binding during the gating strategy. Cells were incubated with 9.3 µg/ml MHC class II tetramers at room temperature (water bath) in the dark for 1hr, and agitated every 15 minutes. Where there was a high frequency of 2W1S<sup>+</sup>-specific T cells, half of the total sample was incubated with tetramers.

Where there was a low frequency of 2W1S<sup>+</sup>- specific T cells, all isolated cells were incubated for 15 minutes at 4°C with magnetic anti-PE or anti-APC microbeads (1:10, Miltenyi Biotech), and positive selection was performed as described by Moon et al. (2009). This was achieved by passing cell suspensions through MACS separation columns held on a magnet (Miltenyi Biotech). The columns were washed 3 times with 3 ml SB and the enriched fraction was collected by removing the columns from the magnet and flushing using 5 ml SB. To identify 2W1S-specific T cells in naïve mice, the enriched fraction from two FOXP3<sup>GFP</sup> mice were pooled prior to addition of antibodies in order to increase the number of antigen-specific Treg cells.

## **2.6 Immunisation**

All injections were carried out in 200 µl sterile PBS. When multiple immunisations took place they were performed 7 days apart and mice were sacrificed 7 days after the final injection. Mice were injected intravenously with 50 or 100 µg 2W1S peptide (>70% purity, Proimmune). For anti-DR3 injections, mice were injected intravenously with 10µg agonistic anti-TNFRSF25 (DR3) monoclonal antibody (Biolegend) 2W1S peptide, or separately by intraperitoneal injection. To measure IFN $\gamma$  production an *in vivo* peptide re-stimulation was performed (Rowe et al., 2012). Mice were immunised intravenously with 50 µg 2W1S peptide and 2.5µg Lipopolysaccharide and sacrificed



4 hours later. To block cytokine release, and hence facilitate detection of IFN $\gamma$ , 10  $\mu$ g Brefeldin A (Sigma, B6542-5MG) was added during incubation at room temperature with MHC class II tetramers.

*ActA deficient Listeria monocytogenes*-expressing 2W1S peptide (a kind gift from Dr Marc Jenkins) was grown overnight at 37°C on plates containing Lennox Luria-Bertani broth with agar (Sigma Aldrich) supplemented with 20  $\mu$ g/ml chloramphenicol. A single colony was then cultured overnight in Luria-Bertani broth supplemented with 20  $\mu$ g/ml chloramphenicol at 37°C in a shaking incubator. Bacteria were diluted in Luria-Bertani broth and cultured with 20  $\mu$ g/ml Chloramphenicol at 37°C in a shaking incubator until an optical density (OD<sup>600</sup>) of over 0.1 was reached, as determined using a Spectrophotometer (Jenway). Bacteria were washed twice and re-suspended in sterile PBS such that the OD<sup>600</sup> was 0.1 and therefore equal to 10<sup>8</sup> bacteria/ml. 10<sup>7</sup> bacteria were subsequently injected intravenously into each mouse.

Administration of IL-2/anti-IL-2 complexes was used to stimulate proliferation of Innate Lymphoid Cells (Van Gool et al., 2014). Prior to injection, IL-2 (1  $\mu$ g per mouse, Peprotech) and anti-IL-2 (5  $\mu$ g per mouse, Bio Legend JES6-1A12), were incubated in 200  $\mu$ l PBS for 30 minutes at 37°C. Complexes were diluted in PBS immediately prior to intraperitoneal injection such that each mouse was given 1  $\mu$ g IL-2 complexed with 5  $\mu$ g anti-IL-2 in 200  $\mu$ l PBS. Complexes were administered daily for three days (Webster et al., 2009) and control mice were administered PBS.

## 2.7 Flow Cytometry

Cells were incubated with antibodies at 4°C for 30 minutes in 50µl Staining Buffer (SB) in a 96 U bottomed plate. After incubation with antibodies directed to cell surface antigens, cells were washed twice in 200 µl SB. As for all washes, plates were centrifuged at 4°C for 3 minutes at 394 rcf. Cells were fixed and permeabilised using a FOXP3 staining buffer kit (eBioscience). As described in the manufacturer's instructions, cells were incubated with 100 µl fixation/permeabilisation solution (1-part concentrate to 3-parts diluent) at 4°C for 30 minutes, before being washed with permeabilisation buffer (diluted to 1x with distilled H<sub>2</sub>O). Cells were incubated with antibodies directed against intracellular antigens in 50 µl of permeabilisation buffer at 4°C for 30 minutes. Cells were then washed in 200 µl permeabilisation buffer, followed by 200 µl SB. Cells were re-suspended in 250 µl SB and transferred to polystyrene fluorescence-activated cell sorting tubes (Becton). To facilitate cell quantification, 10000 Accucount blank particles (Spherotech) were added to each sample. Forward and side scatter were used to exclude dead cells and 2-3 million events were acquired per sample. Cell suspensions which had each been incubated with antibodies conjugated to one of the fluorochromes used were acquired prior to the samples and used set up the compensation parameters. Isotype controls were used to set the lower threshold for expression of a particular marker. Samples were acquired using an LSR Fortessa X-20 or LSR II, running FACS Diva software (BD Biosciences). FlowJo software (Tree Star) was then used to analyse the data. Antibodies utilised are listed in **Table 2.4** and **Table 2.5**.

Antigen	Conjugated fluorochrome	Dilution	Clone	Manufacturer
<b>Primary antibodies</b>				
B220	Pacific Blue	1/200	RA36B2	eBioscience
	FITC	1/300		eBioscience
CCR6	BV 421	1/50	29-2L17	BioLegend
CD11b	Pacific Blue	1/200	MI/70	eBioscience
CD11c	Pacific Blue	1/200	N418	eBioscience
	FITC	1/300		eBioscience
CD3	A700	1/100	500A2	eBioscience
	FITC	1/100	154-2C11	eBioscience
CD4	V500	1/300	RM4-5	BD Biosciences
	BV 605	1/800		BioLegend
	BV 785	1/300		BioLegend
CD5	FITC	1/100	53-7.3	eBioscience
CD8	FITC	1/800	53-6.7	eBioscience
	V500	1/200		BioLegend
CD25	APC	1/500	PC61	BD Biosciences
	BV 650	1/200	UC10-4B9	BioLegend
CD39	APC	1/100	Duha59	BioLegend
CD44	PerCP Cy5.5	1/300	IM7	eBioscience
	A700	1/100		eBioscience
	PE Cy7	1/800		eBioscience
CD45.1	PE	1/200	A20	eBioscience
CD73	PerCP Cy5.5	1/200	TY/11.8	BioLegend
DR3	PE	1/100	4C12	BioLegend
I-A <sup>b</sup> (MHC II)	BV 510	1/500	M5/114.15.2	BioLegend
I-A <sup>d</sup> (MHC II)	FITC	1/800	39-10-8	BioLegend
IFN $\gamma$	PE-Cy7	1/200	XMG1.2	eBioscience
IL-7R $\alpha$	PE Cy7	1/100	A7R34	eBioscience
IL-7R $\alpha$	BV 421	1/100	A7R34	BioLegend

Ki67	PE-Cy7	1/400	SolA15	eBioscience
Ly6c	BV 711	1/500	HK1.4	BioLegend
Neuropilin-1 (goat)	Unconjugated	1/200	AF566	R&D Systems
NKp46	BV 605	1/200	29A1.4	BioLegend
OX40L	PE	1/50	RMB4L	eBioscience
TCR $\beta$	APC Cy7	1/200	H57-597	eBioscience
<b>Secondary antibodies</b>				
Donkey anti-goat	AF647	1/800	A21447	Invitrogen

*Table 2.4: Antibodies used for identification of surface antigens*

<b>Antigen</b>	<b>Conjugated fluorochrome</b>	<b>Dilution</b>	<b>Clone</b>	<b>Manufacturer</b>
CD3	A700	1/100	500A2	eBioscience
CTLA-4	PE-Cy7	1/200	UC10-4B9	Bio Legend
FOXP3	FITC	1/100	FJK-16s	eBioscience
	PE	1/100		eBioscience
GATA-3	PerCP 710	1/50	TWAJ	eBioscience
Helios	PB	1/100	22F6	eBioscience
ROR $\gamma$ t	PE	1/25	AFKJS-9	eBioscience
	APC	1/50		

*Table 2.5: Antibodies used for identification of intracellular antigens*

## 2.8 Immunofluorescence and Image Analysis

Mesenteric lymph nodes were frozen in Optimal Cutting Temperature compound (Tissue-Tek) on dry ice and stored at -80°C. 6  $\mu$ m sections of frozen tissue were mounted on 4 spot slides (Hendley-Essex) prior to immunofluorescence. Sections were air dried for 1 hour before fixing for 20 minutes in acetone (J. T. Baker) at 4°C.

After being air dried for a further 30 minutes, slides were stored at -20°C. When required, slides were air dried for 30 minutes and hydrated in PBS for 5 minutes. They were blocked with 10% horse serum in Immunofluorescence Staining Solution (SS) for 15 minutes to prevent non-specific binding of antibodies. Following removal of blocking solution, primary antibodies were diluted in SS and added to sections for 20 minutes. After washing for 5 minutes in PBS, further application of antibodies, interspersed with washing was carried out. Each subsequent antibody was cross absorbed with 10% mouse serum in 100 µl SS for 30 minutes on ice before being diluted to the appropriate concentration. Antibodies and reagents used for immunofluorescence staining are listed in **Table 2.6**.

Step	Conjugate	Dilution	Clone	Manufacturer
<b>Primary</b>				
ROR $\gamma$ t (rat)	-	1/30	Poly6362	Bio Legend
CD3	Biotin	1/100	S11A2	eBioscience
MHC II	AF647	1/150	AF6-120.1	Bio Legend
Neuropilin-1 (goat)	Unconjugated	1/200	AF566	R&D Systems
<b>Secondary</b>				
Donkey anti-rat IgG	FITC	1/150	-	Strattech Scientific Ltd
Donkey anti-goat IgG	AF647	1/800	A21447	Invitrogen
<b>Quaternary</b>				
Rabbit anti-FITC	AF488	1/200	-	Invitrogen
<b>Tertiary</b>				
Donkey anti-rabbit IgG	AF488	1/200	-	Life Technologies
Streptavidin	AF555	1/500	-	Invitrogen

*Table 2.6: Antibodies/reagents used for immunofluorescence*

Sections were then washed, and incubated in DAPI (4',6-diamidino-2-phenylindole) solution for 20 seconds. Sections were then washed three times in PBS, before mounting using ProLong Gold Antifade reagent (Invitrogen). Coverslips were placed on top of each slide, sealed with clear nail varnish and air dried overnight. Analysis was performed using a LSM510 confocal microscope or LSM780 Zen microscope (Zeiss) and images were viewed using LSM Image Browser (Zeiss).

## **2.9 Statistical Analysis & Graphs**

All graphs presented within this study were created using Prism 4 software. Statistical significance was tested using the Mann–Whitney U test (non-parametric), and a two tailed p value was calculated. P values < 0.05 were considered significant. Median values were calculated and used in all analyses except where stated.

### **3.0 DEVELOPMENT OF A MODEL TO STUDY REGULATORY T CELLS *IN VIVO***

### 3.1 INTRODUCTION

Although a wealth of knowledge has been built up about Treg cells, there has been some disparity in findings between *in vitro* and *in vivo* experiments (Vignali, 2012). The most fundamental example of this is that *in vitro*, Treg cells were shown to be rendered anergic upon TCR stimulation (Takahashi et al., 1998, Itoh et al., 1999, Kuniyasu et al., 2000), whereas the majority of data from *in vivo* experiments reveals that these cells are in fact highly proliferative and continuously activated (Fisson et al., 2003, Walker et al., 2003a). In addition, there are concerns that strong conclusions have been made about the range of suppressive mechanisms deployed by Treg cells without sufficient evidence (Vignali, 2012). For example, the evidence that Treg cells act as an 'IL-2 sink' all comes from *in vitro* experiments. There have also been occasions of false negatives arising from *in vitro* work, for example little IL-10 and TGF $\beta$  can be identified *in vitro*, and blocking these cytokines has little effect (Miyara and Sakaguchi, 2007), but a clear role for these immunosuppressive cytokines has been revealed *in vivo* (Miyara and Sakaguchi, 2007). This study therefore aims to investigate Treg cell survival and function *in vivo*.

In this study antigen-specific Treg cells have been assessed to aid clarification of specific effects on survival versus function, since the precise time of expansion can be controlled. Although it is technically challenging to study Treg cell function *in vivo*, an approach which successfully achieves this in an antigen-specific manner has recently been described (Rowe et al., 2012). Here Treg cells were expanded to approximately 50% of the antigen-specific population, and the effect on a subsequent response was measured. The principles from this suppression assay will therefore be applied within this study.



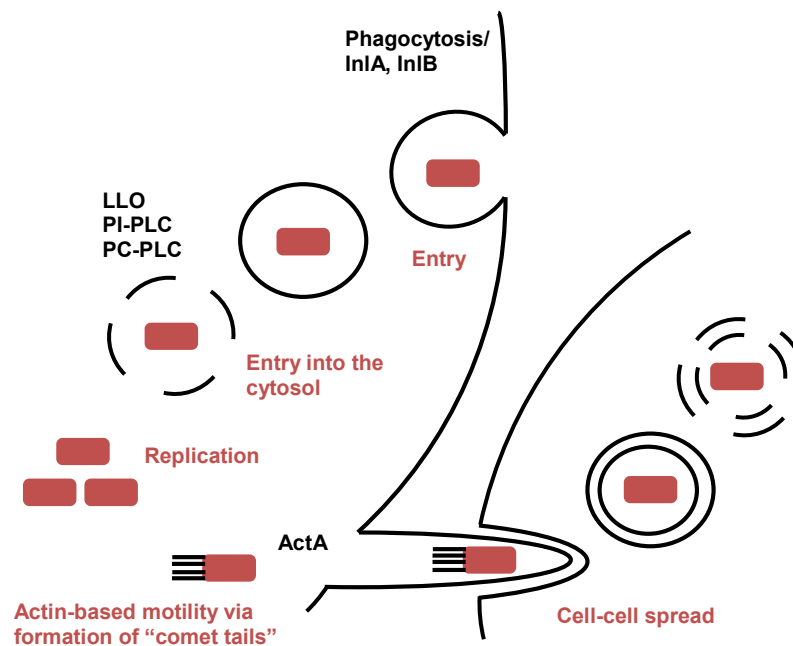
One widely used method to study T cells in an antigen-specific manner is the use of TCR-transgenic mice, which have many benefits, such as the potential to transfer and track large numbers of T cells. However it has been reported that there is an inverse relationship between the size of a clonal population and their survival (Hataye et al., 2006), therefore cell survival may be adversely affected. In addition, TCR transgenic mice on a T cell deficient background (RAG KO), which are ideal for isolating cells for transfer, lack Treg cells due to the importance of antigen in Treg cell selection in the thymus (Walker et al., 2003b, Itoh et al., 1999). Rather, I sought to develop a strategy where an endogenous polyclonal population could be tracked, using approaches pioneered by Marc Jenkins (Moon et al., 2007, Rees et al., 1999).

The Jenkins lab have demonstrated that an endogenous naïve T cell population is expanded upon encounter with 2W1S peptide, a variant of peptide 52-68 of the I-E alpha chain (Rudensky et al., 1991). The number of naïve T cells which are specific to this antigen is relatively large, at approximately 200 cells per mouse (Moon et al., 2007). In contrast, two other potential model antigens, peptide 427-441 from the FLiC protein of *Salmonella typhimurium* and peptide 323-339 from chicken ovalbumin each have a naïve T cell pool of approximately 20 cells per mouse (Moon et al., 2007). This is important as it has been found that within the primary response CD4<sup>+</sup> T cell populations expand in proportion to their naïve T cell frequency (Moon et al., 2007). It is also possible to track naïve 2W1S<sup>+</sup> Treg cells whereas this would be almost impossible for OVA and FLiC specific endogenous populations. To identify these antigen-specific T cells, fluorochrome conjugated MHC class II tetramers can be used (Moon et al., 2007). The presence of the fluorochrome enables enrichment of this T cell population to be performed using magnetic bead separation, as well as

detection by flow cytometry (Moon et al., 2009). Enrichment is particularly important when identifying a small subset of responding T cells. Therefore, in this study, this antigen-specific approach will be optimised for the study of Treg cells. Further to this an assay will be developed to study Treg cell function *in vivo*, based on work by Rowe et al. (2012).

The 2W1S peptide can also be provided through infection with ActA-deficient *Listeria monocytogenes*-expressing 2W1S (Lm-2W1S) (Ertelt et al., 2009). This approach enables the study of Treg cells within the context of an infection, which is important since the nature and strength of an immune response is known to influence the magnitude of suppression (Miyara and Sakaguchi, 2007). *Listeria monocytogenes* is a gram positive intracellular bacteria, which is able to survive within macrophages after phagocytosis, but also can invade most cell types (Portnoy et al., 2002). **Figure 3.1** provides a representation of the life cycle of this bacterium, with the role of ActA shown. To gain entry to non-phagocytic cells, the bacterial surface proteins Internalin A (InlA) and Internalin B (InlB) interact with cell surface receptors E-Cadherin and Met to trigger receptor mediated internalisation (Mengaud et al., 1996, Shen et al., 2000). Once inside the cell, the bacterium is able to escape into the cytosol through the action of the haemolysin listeriolysin-O (LLO), as well as two phospholipase C enzymes (Vázquez-Boland et al., 2001). Once in the cytoplasm the bacterium replicates and is able to spread to other cells through the action of bacterial actin assembly-inducing (ActA) protein. This protein mediates reorganisation of the actin cytoskeleton, to form “Comet tails” which allow the bacterium to move into neighbouring cells via plasma membrane protrusions (Tilney and Portnoy, 1989). As the ActA protein facilitates movement along the cytoskeleton, the absence of this

protein prevents the spread of this bacterium, facilitating its clearance by the immune system. Infection with ActA-deficient *Listeria monocytogenes*-expressing 2W1S therefore results in a sub-clinical acute infection, which is cleared within five days (Goossens and Milon, 1992). Replication of this bacterium primarily occurs in macrophages, which secrete TNF $\alpha$  and IL-12 in response to infection. This stimulates IFN $\gamma$  production by NK cells, which increases the bactericidal activity of macrophages and is thought to be the key cytokine in controlling *Listeria monocytogenes* infection (Zenewicz and Shen, 2007).

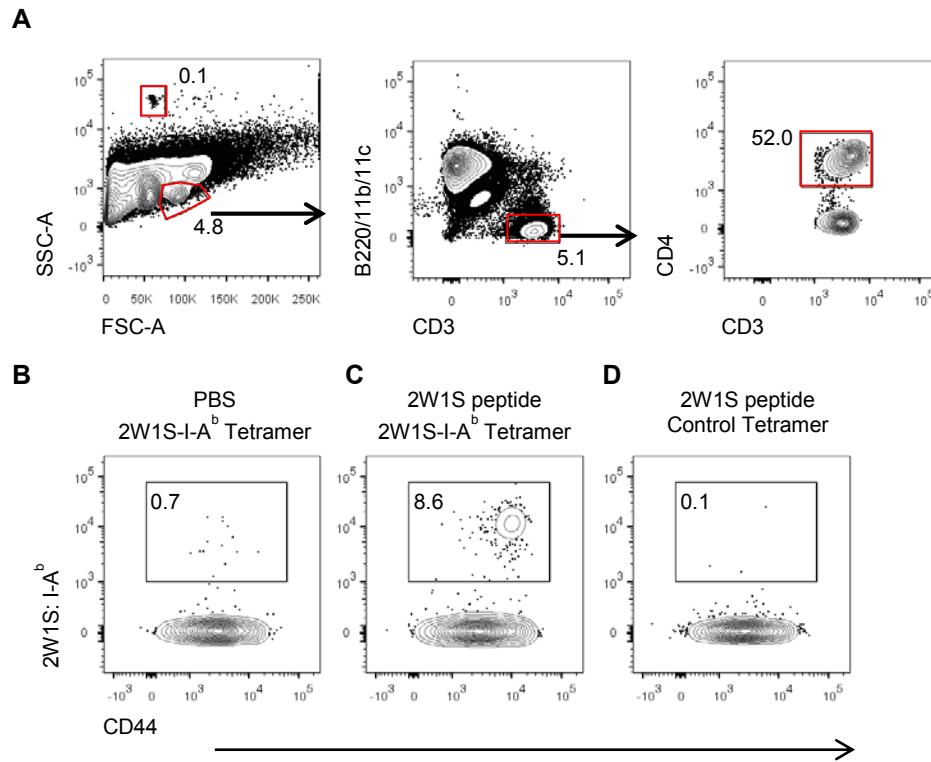


**Figure 3.1: Life-cycle of *Listeria monocytogenes*.** The bacterium (red) enters macrophages through phagocytosis, or uses Internalin A (InlA) and Internalin B (InlB) to gain entry to non-phagocytic cells. Escape from the vacuole is mediated by the haemolysin listeriolysin-O (LLO), and two phospholipase C (PLC) enzymes produced by the bacterium (PI-PLC and PC-PLC). Once in the cytosol, the bacterium replicates and travels along the actin cytoskeleton in an ActA dependent process involving the formation of “comet tails”. This allows spreading to adjacent cells within which the bacterium is once again able to escape the vacuole, which has a double membrane. Adapted from Tilney and Portnoy (1989).

## 3.2 RESULTS

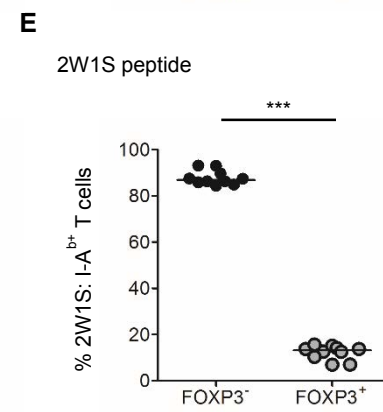
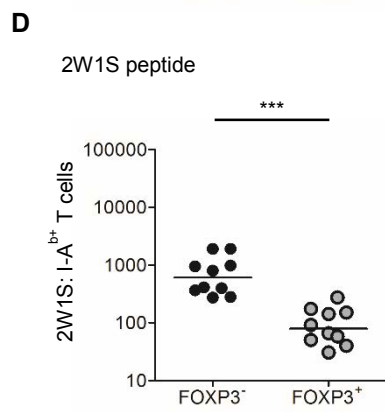
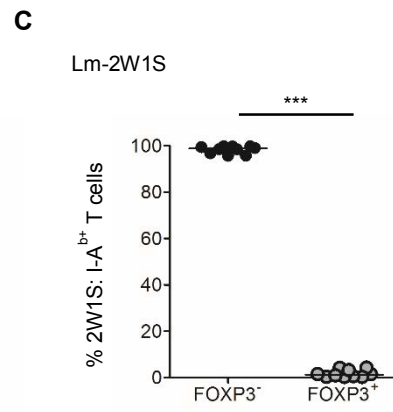
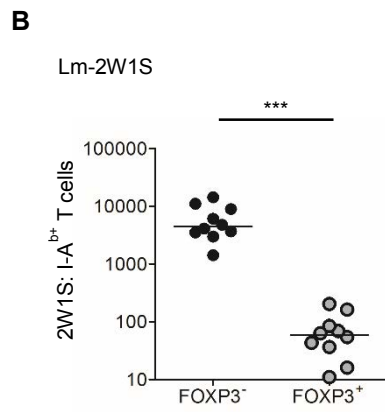
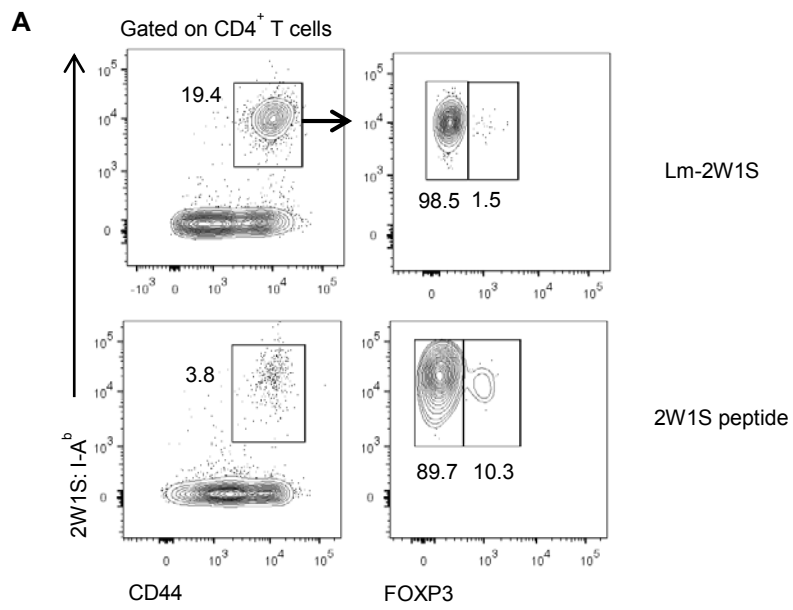
### *Expanding antigen-specific regulatory T cells*

To first identify 2W1S<sup>+</sup> T cells, wildtype (WT) mice were immunised with 100 µg 2W1S peptide or PBS as a control. In previous publications between 50-125 µg 2W1S peptide have been used to expand 2W1S<sup>+</sup> T cells (Ertelt et al., 2009, Moon et al., 2007), therefore a concentration within this range was used. Seven days after immunisation, the spleen and a pool of peripheral lymph nodes were harvested from mice and tetramer staining and enrichment was performed. The gating strategy is shown in **Figure 3.2a**, in which T cells are identified by CD3 and CD4 expression, as well as the absence of B cell and myeloid cell markers B220, CD11b and CD11c. In PBS immunised mice (naïve) there is a small population of CD44<sup>lo</sup> 2W1S<sup>+</sup> T cells, which is expanded upon immunisation with 2W1S peptide (**Figure 3.2b, c**). These 2W1S<sup>+</sup> T cells express the activation marker CD44, indicating that these cells have been activated in response to immunisation. To control for non-specific tetramer binding, cells from 2W1S peptide-immunised mice were stained with MHC class II tetramers loaded with MHC class II-associated invariant chain peptide (CLIP) (**Figure 3.2d**). During antigen processing CLIP is transiently held in the peptide groove, before being exchanged for the processed peptide (Roche and Furuta, 2015).



**Figure 3.2: Identification of 2W1S-specific T cells.** (A) Gating strategy for CD4 T cells: CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> (B) Identification of 2W1S: I-A<sup>b</sup> T cells in WT mice immunised intravenously with PBS (left) or 2W1S peptide (middle and right). After 7 days, a pool of lymph nodes and spleen were analysed. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. Either control (CLIP: I-A<sup>b</sup>) or 2W1S: I-A<sup>b</sup> tetramers were used. Plots are representative of at least 2 mice.

To assess generation/expansion of 2W1S<sup>+</sup> Treg cells, WT mice were immunised with 50 µg 2W1S peptide or infected with 10<sup>7</sup> Lm-2W1S. After 7 days the spleen and a pool of peripheral lymph nodes were harvested from these mice and enriched for 2W1S-specific T cells. Treg cells were identified within the 2W1S<sup>+</sup> T cell population by expression of the transcription factor FOXP3. Consistent with previous reports (Ertelt et al., 2009), Lm-2W1S induced a robust 2W1S<sup>+</sup> T cell response, with a large number of conventional 2W1S<sup>+</sup> T (Tconv) cells (median 4455) (**Figure 3.3a, b**), but a low number of Treg cells (median 59) (**Figure 3.3c**). Following administration of 2W1S peptide, fewer 2W1S<sup>+</sup> Tconv cells were expanded, but the number of Treg cells was similar (median 69 and median 79 respectively) (**Figure 3.3d**). As a result, there was a greater proportion of regulatory to conventional 2W1S<sup>+</sup> T cells than observed after Lm-2W1S administration (median 13.2% vs median 1.2%) (**Figure 3.3e**). Although Treg cell numbers are similar between the two approaches, administration of 2W1S peptide induces a greater proportion of 2W1S<sup>+</sup> Treg cells relative to 2W1S<sup>+</sup> Tconv cells than infection with Lm-2W1S.



**Figure 3.3: 2W1S<sup>+</sup> regulatory T cells can be expanded to 10% of the population by 2W1S peptide but not Lm-2W1S.** WT mice were infected intravenously with ActA deficient *Listeria monocytogenes*-expressing 2W1S peptide (Lm-2W1S), or were immunised with 2W1S peptide intravenously. After 7 days, a pool of lymph nodes and spleen were analysed. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells after either treatment. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells after infection with Lm-2W1S. (C) Percentage of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells after infection with Lm-2W1S. (D) Enumeration of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells after immunisation with 2W1S peptide. (E) Percentage of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells after immunisation with 2W1S peptide. (B-E) Bars show medians. Mann–Whitney test, \*\*\**p* < 0.001. At least 10 samples per group were pooled from two independent experiments.

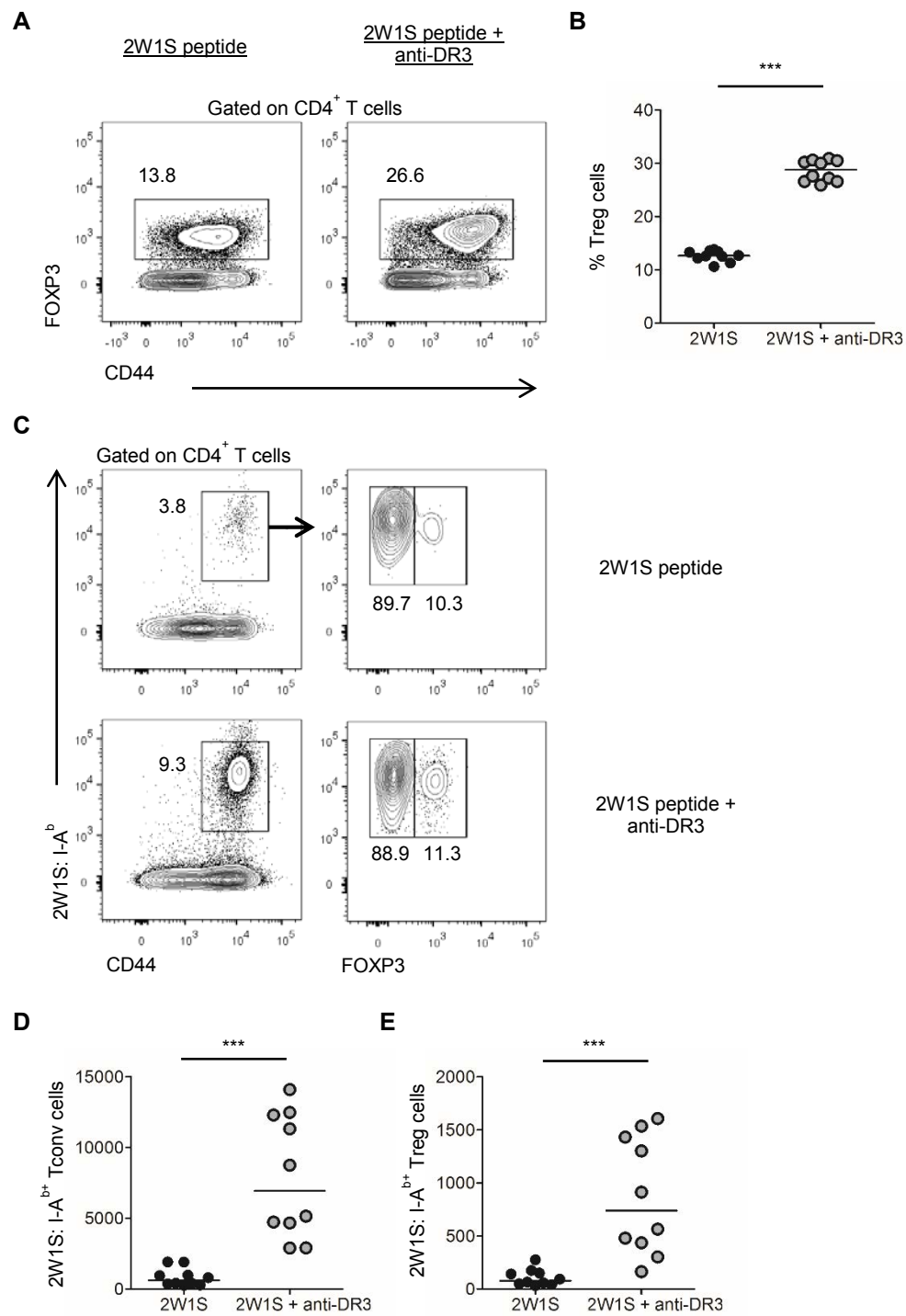


### *Investigating methods to increase 2W1S<sup>+</sup> Treg cell numbers*

Although 2W1S peptide can be used to expand Treg cells, the number detected was very low, which may limit survival experiments. To enhance the ability to detect 2W1S<sup>+</sup> Treg cells over time, tools for enhancing the total number of cells were explored. An agonistic anti-Death Receptor 3 (DR3) antibody (4C12) has previously been shown in mice to selectively increase the percentage of CD4<sup>+</sup> T cells which are FOXP3<sup>+</sup> in the blood to 30-35% (Schreiber et al., 2010). This antibody signals via the Tumour Necrosis Factor receptor DR3, which is expressed primarily on T cells, including Treg cells, and is thought to expand existing Treg cells by increasing their efficacy for IL-2 (Schreiber et al., 2010). To investigate whether agonistic anti-DR3 can be used to selectively boost Treg cells within the 2W1S<sup>+</sup> T cell population, WT mice were injected with 10 µg agonistic anti-DR3 antibody and 50 µg 2W1S peptide. 2W1S<sup>+</sup> Treg cells were then enumerated at day 7 and compared with mice administered 50 µg 2W1S peptide alone. This concentration of agonistic anti-DR3 was administered because it has been demonstrated to elicit maximal expansion of Treg cells (Schreiber et al., 2010). Again the spleen and a pool of peripheral lymph nodes were harvested from these mice and enriched for 2W1S-specific T cells.

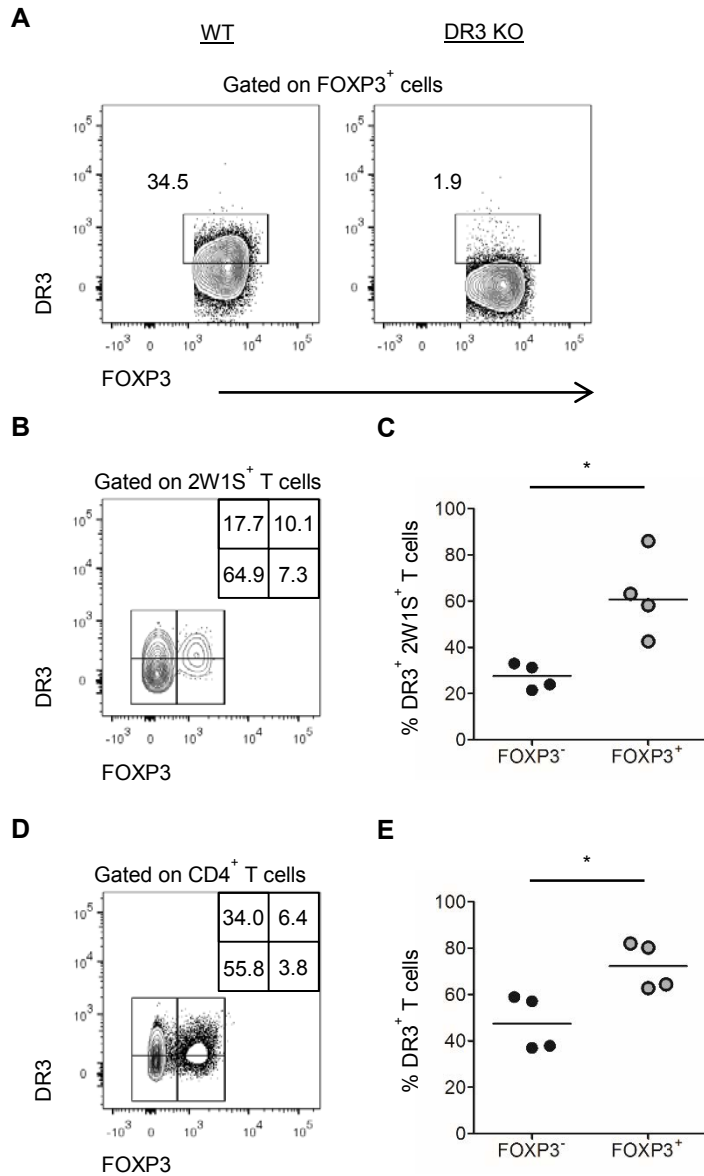
Upon agonistic anti-DR3 administration, a 2.5-fold increase in the overall Treg cell percentage was observed (median 12.8% vs median 29.8%) (**Figure 3.4a, b**). This was therefore similar to the effect observed by Schreiber et al. (2010). However, both the regulatory and conventional 2W1S<sup>+</sup> T cell populations were found to be increased, and as a result the percentage of 2W1S<sup>+</sup> Treg cells remained at approximately 10% (**Figure 3.4c**). The number of 2W1S<sup>+</sup> Tconv cell numbers increased by 11.4-fold (median 609 vs median 6946) (**Figure 3.4d**) and the number

of 2W1S<sup>+</sup> Treg cells increased by 9.3-fold (median 79 vs median 739) (**Figure 3.4e**). Therefore, administration of agonistic anti-DR3 increased both the number of regulatory and conventional 2W1S<sup>+</sup> T cells to a similar degree, rather than eliciting a selective increase in 2W1S<sup>+</sup> Treg cells.



**Figure 3.4: Agonistic anti-DR3 can boost 2W1S<sup>+</sup> regulatory T cell numbers.** WT mice were immunised intravenously with 2W1S peptide +/- agonistic anti-DR3 antibody. After 7 days, a pool of lymph nodes and spleen were analysed. (A) Identification of FOXP3<sup>+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Percentage of T cells which are FOXP3<sup>+</sup> (Treg cells). (C) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (D) Enumeration of FOXP3<sup>-</sup>CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Tconv cells) (E) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, D, E) Bars show medians. Mann–Whitney test, \*\*\**p* < 0.001. At least 10 samples per group were pooled from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

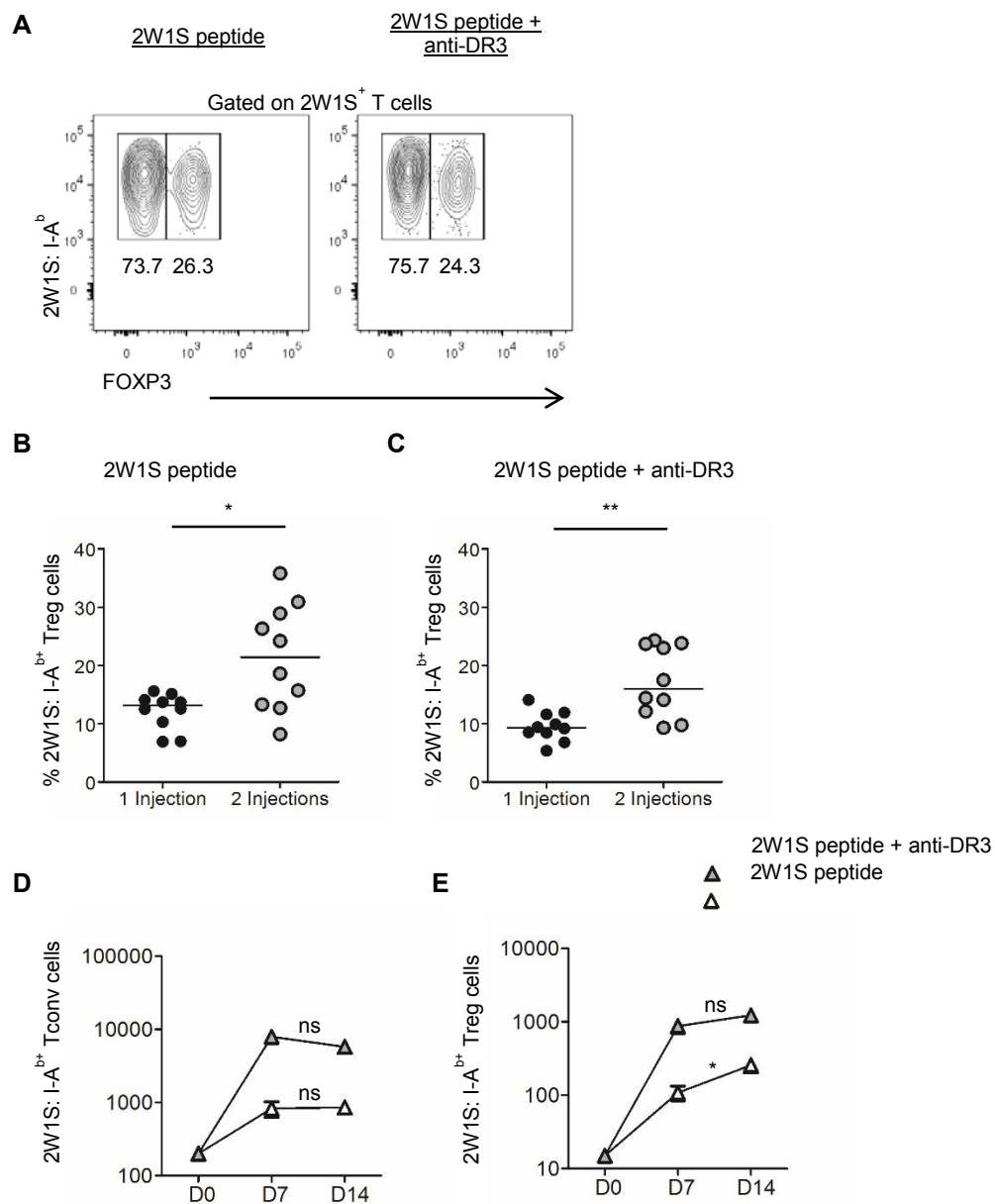
To further understand the effects of agonistic anti-DR3, the expression of DR3 on 2W1S<sup>+</sup> T cells was characterized. To expand 2W1S<sup>+</sup> T cells, WT mice were immunised with 100 µg 2W1S peptide. The spleen and a pool of peripheral lymph nodes were harvested 7 days later and enriched for 2W1S-specific T cells. The DR3 antibody was validated using cells isolated from DR3 KO mice (**Figure 3.5a**). The number of DR3<sup>+</sup> 2W1S<sup>+</sup> Treg cells found to express DR3 was 2.0-fold greater than the number of DR3<sup>+</sup> 2W1S<sup>+</sup> Tconv cells (median 63.2% vs median 31.2%) (**Figure 3.5b, c**). Similarly, significantly more regulatory than conventional T cells were shown to express DR3 in the overall T cell population (median 72.3% vs median 47.4%) (**Figure 3.5d, e**). As both conventional and regulatory 2W1S<sup>+</sup> T cells express DR3, it is understandable that agonistic anti-DR3 expands both.



**Figure 3.5: DR3 is expressed on 60% of 2W1S<sup>+</sup> regulatory T cells.** (A) Validation of anti-DR3 antibody on FOXP3<sup>+</sup> cells from the mesenteric lymph node of WT and DR3 KO mice. Plots are representative of at least three mice. (B) WT mice were immunised intravenously with 2W1S peptide and a pool of lymph nodes and spleen were analysed 7 days later. Identification of DR3 on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (C) Percentage of DR3<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (D) Identification of DR3 on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (E) Percentage of DR3<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> T cells. (C, E) Bars show medians. Mann-Whitney test, \**p* < 0.05. At least 4 mice were pooled from two independent experiments

Repeated exposure to an antigen has been shown to boost Treg cell numbers, and therefore it has been proposed that repeated administration of an autoantigen could be used to induce tolerance in autoimmune disease (Sabatos-Peyton et al., 2010). To determine whether repeated administration of 2W1S peptide and agonistic anti-DR3 can affect the proportions of regulatory and conventional 2W1S<sup>+</sup> T cells, WT mice were immunised once or twice with 50 µg 2W1S peptide +/- 10 µg agonistic anti-DR3. Seven days after their final injection, the spleen and a pool of peripheral lymph nodes were harvested and tetramer staining and enrichment was performed.

After two immunisations of 2W1S peptide +/- agonistic anti-DR3 the regulatory T cells within the 2W1S<sup>+</sup> T cell population were indeed boosted (**Figure 3.6a**). The percentage of Treg cells increased by 1.6-fold following two immunisations of 2W1S peptide (median 13.2% vs median 21.4%) (**Figure 3.6b**) and by 1.7-fold following two immunisations of 2W1S peptide and agonistic anti-DR3 (median 9.3% vs median 16.0%) (**Figure 3.6c**). In the mice administered 2W1S peptide, this increase in percentage was also associated with a significant increase in regulatory but not conventional 2W1S<sup>+</sup> T cell number (**Figure 3.6d, e**). However, in the mice given 2W1S peptide and agonistic anti-DR3 this increase in percentage did not translate to a significant increase in the number of 2W1S<sup>+</sup> Treg cells (**Figure 3.6d, e**). There was considerable variation in the percentage after two injections; however, this protocol clearly has the potential to boost the percentage of Treg cells within the 2W1S<sup>+</sup> T cell population. Therefore, to expand the highest number of 2W1S<sup>+</sup> Treg cells, and to increase their percentage, two immunisations of 2W1S peptide and agonistic anti-DR3 can be used.

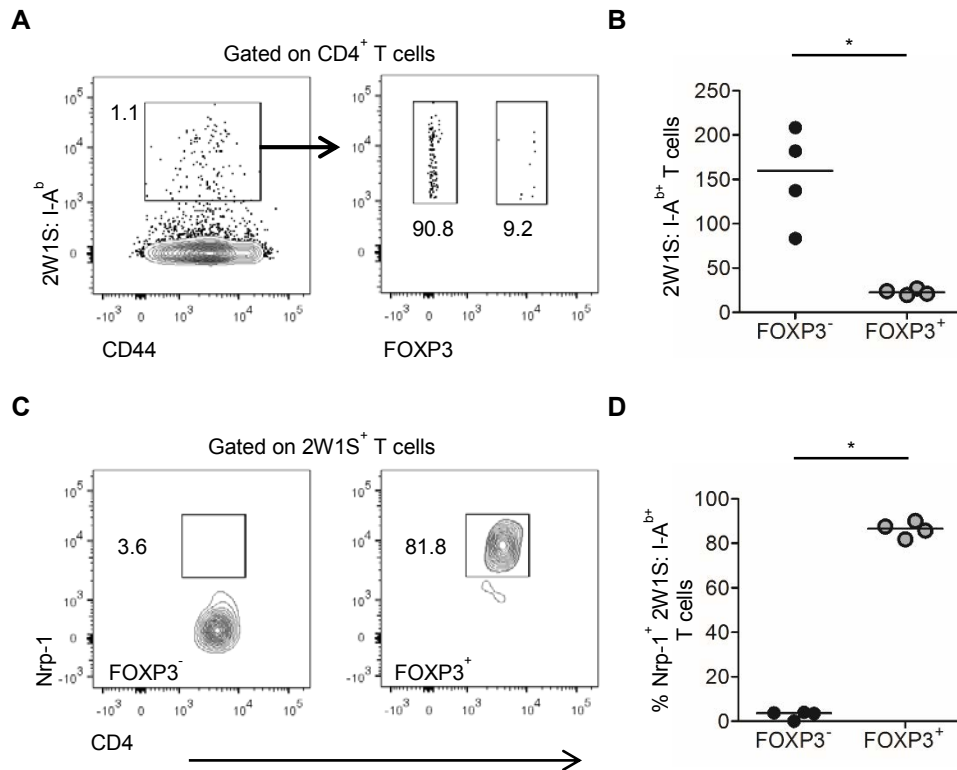




**Figure 3.6: Multiple immunisation can boost the percentage of 2W1S<sup>+</sup> regulatory T cells.** WT mice were immunised intravenously with 2W1S peptide +/- agonistic anti-DR3 antibody and this was repeated after 7 days. After another 7 days a pool of lymph nodes and spleen were analysed. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Percentage of FOXP3<sup>+</sup>CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells) after immunisation with 2W1S peptide. (C) Percentage of FOXP3<sup>+</sup>CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells) after immunisation with 2W1S peptide and agonistic anti-DR3. (D) Enumeration of FOXP3<sup>-</sup>CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (E) Enumeration of FOXP3<sup>+</sup>CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, C) Bars show medians. Mann–Whitney test, \**p* < 0.05, \*\**p* < 0.01. At least 10 samples per group were pooled from two independent experiments. (D, E) Means ± SEM are shown. Mann–Whitney test, \**p* < 0.05, ns = non-significant. At least 10 samples per group were pooled from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells. White filled triangles = 2W1S, Light grey filled triangles = 2W1S and agonistic anti-DR3.

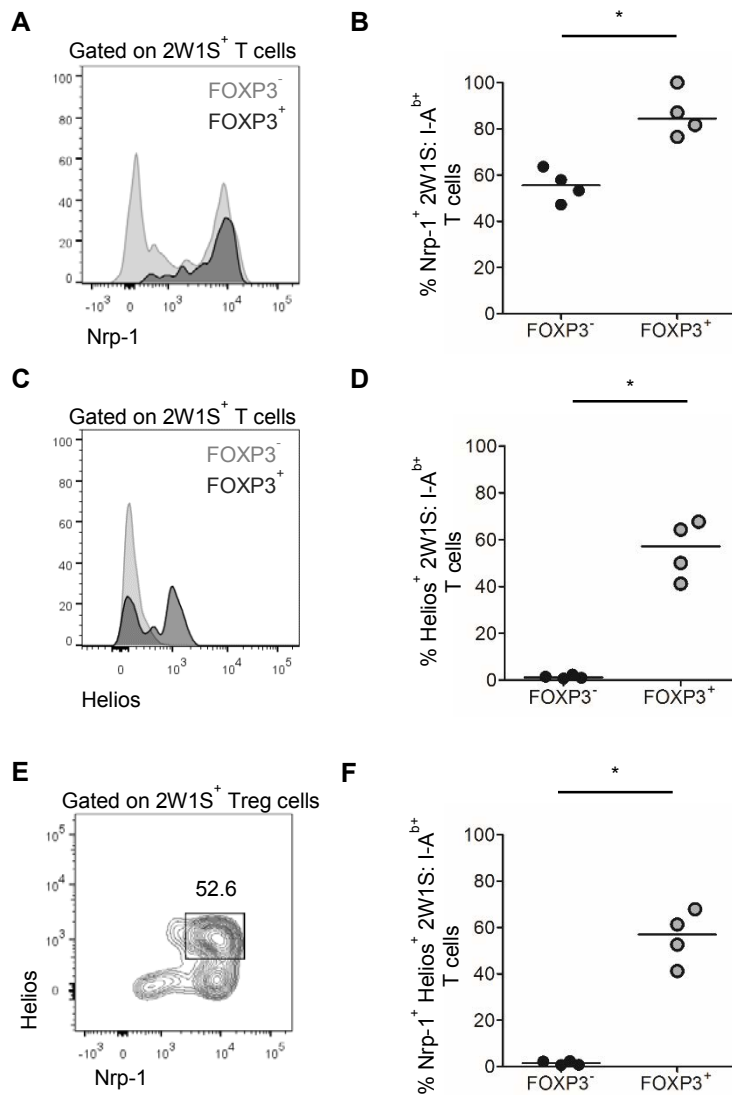
### *Determining the origin of 2W1S Treg cells*

To better understand the nature of 2W1S<sup>+</sup> Treg cells it was important to investigate whether they were thymically derived or generated in the periphery from naïve T cells. One way to investigate this was to stain for 2W1S<sup>+</sup> Treg cells in naïve mice, therefore the spleen and a pool of peripheral lymph nodes were harvested from naïve FOXP3 GFP mice and enriched for 2W1S-specific T cells. The use of reporter mice enhances the number of 2W1S<sup>+</sup> Treg cells which can be detected as cells do not need to be fixed to identify Treg cells. To further increase the number of 2W1S<sup>+</sup> Treg cells that could be detected, cells were pooled from two mice after enrichment for each sample. Similar to previous studies (Ertelt et al., 2009, Moon et al., 2011), both conventional and regulatory 2W1S<sup>+</sup> T cells could be identified (**Figure 3.7a**) (median 160 and median 23 respectively) (**Figure 3.7b**). It has been previously shown that the majority of 2W1S<sup>+</sup> Treg cells identified in naïve mice express Helios (Moon et al., 2011), a proposed marker of thymically derived Treg cells (Thornton et al., 2010). Neuropilin-1, which is another proposed marker of Treg cells (Weiss et al., 2012, Yadav et al., 2012), was found to be expressed by the majority of 2W1S<sup>+</sup> Treg cells (median 86.6%) (**Figure 3.7c, d**).



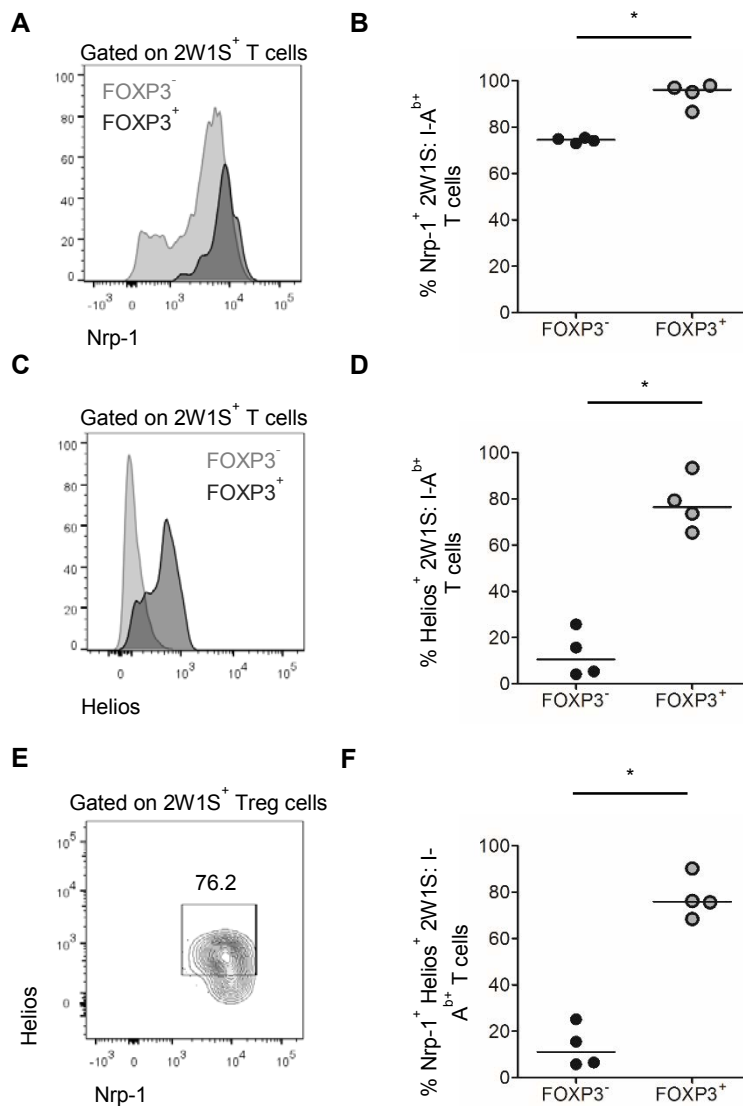
**Figure 3.7: 2W1S<sup>+</sup> regulatory T cells can be identified in naïve mice.** (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> 2W1S: I-A<sup>b</sup> T cells in naïve FOXP3<sup>GFP</sup> mice. Plots are from two mice combined and are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> 2W1S: I-A<sup>b</sup> T cells. (C) Identification of Nrp-1 on FOXP3<sup>-</sup> (left) and FOXP3<sup>+</sup> (right) 2W1S: I-A<sup>b</sup> T cells. (D) Percentage of Nrp-1<sup>+</sup> FOXP3<sup>+</sup> and FOXP3<sup>-</sup> 2W1S: I-A<sup>b</sup> T cells. (B, D) Bars show medians. Mann–Whitney test, \**p* < 0.05. At least 4 samples, each from two mice, were pooled from two independent experiments.

To further investigate whether 2W1S<sup>+</sup> Treg cells expand from thymically derived Treg cells or are induced from naïve T cells in the periphery, Helios and Nrp-1 were stained for on 2W1S<sup>+</sup> Treg cells in WT mice seven days after immunisation with 100 µg 2W1S peptide. The spleen and a pool of peripheral lymph nodes were harvested and enriched for 2W1S-specific T cells. The majority of 2W1S<sup>+</sup> Treg cells were again found to express Neuropilin-1 (median 84.4%) (**Figure 3.8a, b**); suggesting that they are expanded from Treg cells within the naïve 2W1S<sup>+</sup> T cell population. Nrp-1 is also expressed on conventional T cells after activation (Weiss et al., 2012) and indeed at this time point a proportion of 2W1S<sup>+</sup> Tconv cells expressed Nrp-1 (median 55.6%). In contrast Helios was not expressed by 2W1S<sup>+</sup> Tconv cells, but was expressed by approximately 2/3 of 2W1S<sup>+</sup> Treg cells (median 57.2%) (**Figure 3.8c, d**). Those 2W1S<sup>+</sup> Treg cells which expressed Helios also expressed Nrp-1 (**Figure 3.8e, f**).



**Figure 3.8: 2W1S<sup>+</sup> regulatory T cells expanded by 2W1S peptide express markers associated with generation in the thymus.** WT mice were immunised intravenously with 2W1S peptide. After 7 days, a pool of lymph nodes and spleen were analysed. (A) Identification of Nrp-1 on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells in WT mice. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. Filled light grey line: FOXP3<sup>-</sup> cells, filled dark grey line: FOXP3<sup>+</sup> cells. (B) Percentage of Nrp-1<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (C) Identification of Helios on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Filled light grey line = FOXP3<sup>-</sup> cells, filled dark grey line = FOXP3<sup>+</sup> cells. (D) Percentage of Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (E) Identification of Nrp-1<sup>+</sup> Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b</sup> T cells. (F) Percentage of Nrp-1<sup>+</sup> Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (B, D, F) Bars show medians. Mann–Whitney test, \**p* < 0.05. At least 4 mice were pooled from two independent experiments

The expression of Helios and Nrp-1 on 2W1S<sup>+</sup> Treg cells has also been characterized with the addition of agonistic anti-DR3. WT mice were immunised with 100 µg 2W1S peptide and 10 µg agonistic anti-DR3, the spleen and a pool of peripheral lymph nodes were harvested and enriched for 2W1S-specific T cells. Consistent with previous data, the majority of 2W1S<sup>+</sup> Treg cells expressed Neuropilin-1 (median 96.1%) (**Figure 3.9a, b**) and approximately 3/4 expressed Helios (median 76.5%) (**Figure 3.9c, d**). Again the cells which expressed Helios also expressed Nrp-1 (**Figure 3.9e, f**). There was little Helios expression on 2W1S<sup>+</sup> Tconv cells and Nrp-1 was expressed by approximately 3/4 of these cells (median 74.5%). Together, these data suggest that the 2W1S<sup>+</sup> Treg cells identified upon immunisation arise due to expansion of thymically derived Treg cells within the naïve 2W1S<sup>+</sup> T cell pool.



**Figure 3.9: 2W1S<sup>+</sup> regulatory T cells expanded by 2W1S peptide and agonistic anti-DR3 express markers associated with generation in the thymus.** WT mice were immunised intravenously with 2W1S peptide and agonistic anti-DR3. After 7 days, a pool of lymph nodes and spleen were analysed. (A) Identification of Nrp-1 on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells in WT mice. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. Filled light grey line = FOXP3<sup>-</sup> cells, filled dark grey line = FOXP3<sup>+</sup> cells. (B) Percentage of Nrp-1<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (C) Identification of Helios on FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Filled light grey line: FOXP3<sup>-</sup> cells, filled dark grey line: FOXP3<sup>+</sup> cells. (D) Percentage of Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (E) Identification of Nrp-1<sup>+</sup> Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b</sup> T cells. (F) Percentage of Nrp-1<sup>+</sup> Helios<sup>+</sup> FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (B, D, F) Bars show medians. Mann-Whitney test, \**p* < 0.05. At least 4 mice were pooled from two independent experiments

### *Development of an in vivo assay to assess 2W1S<sup>+</sup> Treg cell function*

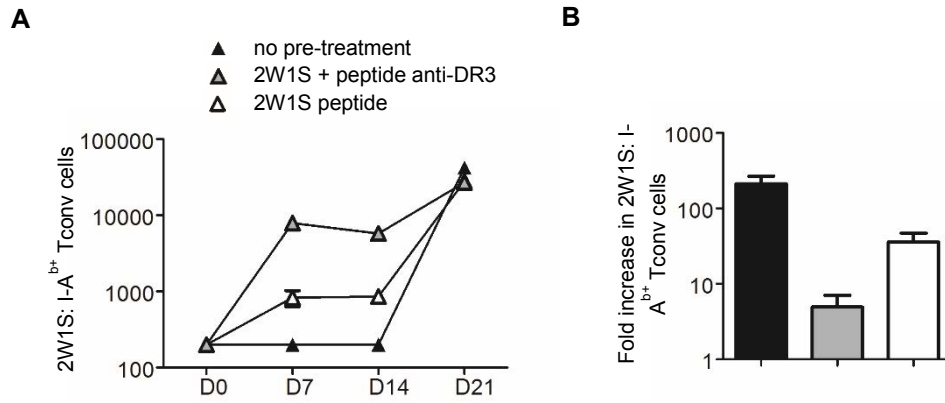
Having expanded 2W1S<sup>+</sup> Treg cells using two immunisations of 2W1S peptide and agonistic anti-DR3, it was then necessary to test the function of these cells. An *in vivo* assay was therefore developed, based on experiments in which the function of 2W1S<sup>+</sup> Treg cells was determined by measuring IFN $\gamma$ <sup>+</sup> production by 2W1S<sup>+</sup> T cells in response to Lm-2W1S (Rowe et al., 2012). In these experiments 2W1S<sup>+</sup> Treg cells expanded in response to constitutive expression of 2W1S peptide as a fetal antigen, and peaked at 50% of the 2W1S<sup>+</sup> T cell population. As the protocol for Treg expansion developed in this study results in a percentage of up to 36%, it was considered likely that a similar impaired response might be observed.

2W1S<sup>+</sup> Treg cells were therefore expanded using two immunisations of 50  $\mu$ g 2W1S peptide and 10  $\mu$ g agonistic anti-DR3, seven days apart. After another seven days mice were then infected with  $10^7$  Lm-2W1S. Any reduction in the number of 2W1S<sup>+</sup> Tconv cells, compared to mice infected with Lm-2W1S was interpreted as evidence of suppression, along with IFN $\gamma$ <sup>+</sup> production. To stimulate IFN $\gamma$ <sup>+</sup> production mice were injected with 50  $\mu$ g 2W1S peptide and 2.5  $\mu$ g LPS on the day of analysis. The 2W1S-specific response was then assessed in a pool of lymph nodes and spleen, which were harvested four hours later. It was not necessary to enrich these samples as Lm-2W1S expands a large number of 2W1S<sup>+</sup> Tconv cells.

When performing this assay, it is important to remember that this method of expanding 2W1S<sup>+</sup> Treg cells also expands 2W1S<sup>+</sup> Tconv cells. To take this into account a timeline has been plotted of the number of 2W1S<sup>+</sup> Tconv cells in mice given either of the two pre-treatments compared to mice which are infected with Lm-

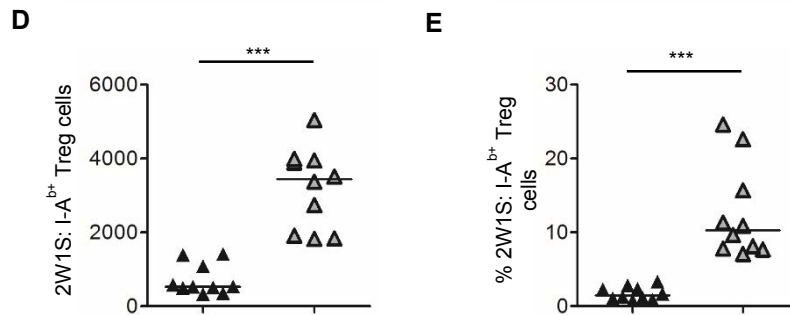
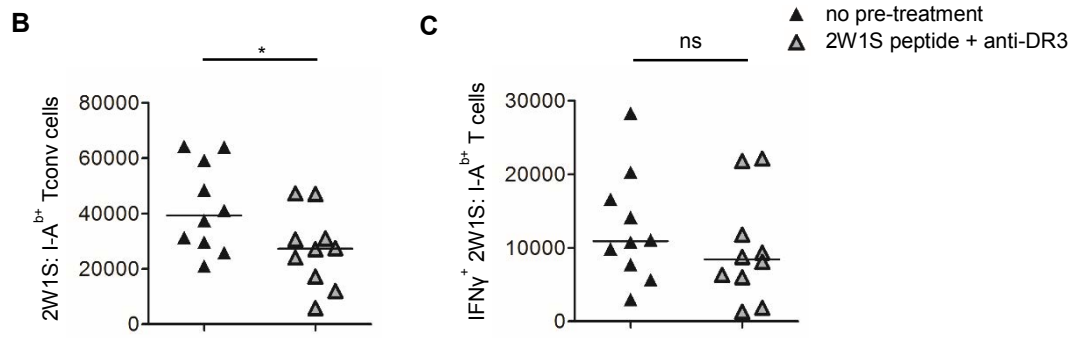
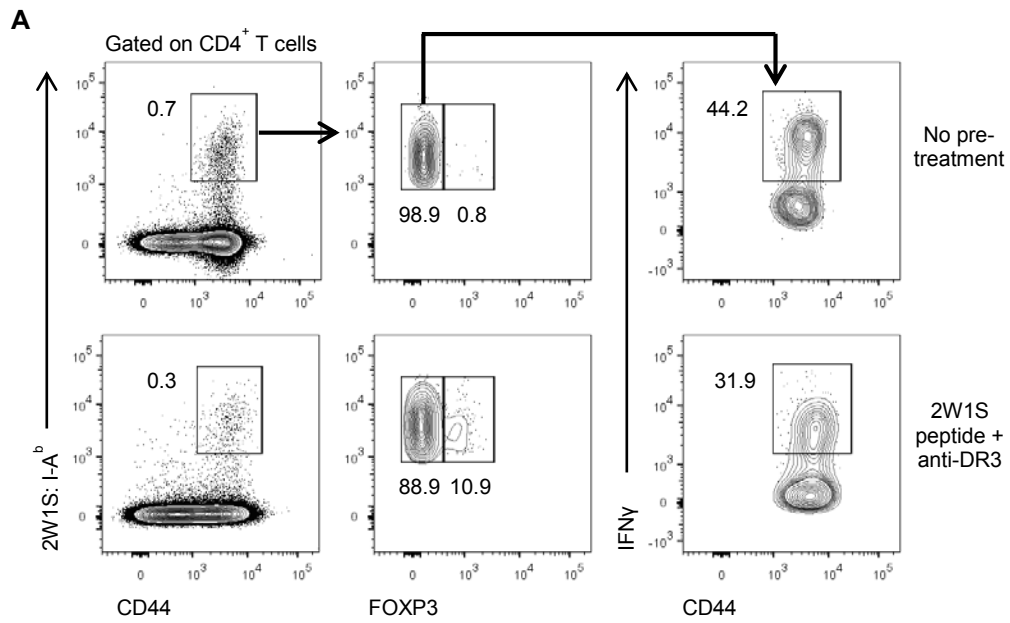


2W1S alone (**Figure 3.10a**). This has been constructed using data from earlier multiple immunisation experiments, as well as this experiment. Although there is only a slight difference between the numbers of 2W1S<sup>+</sup> Tconv cells after Lm-2W1S is administered to pre-treated mice compared to naïve mice (D21), the precursor frequency of 2W1S<sup>+</sup> Tconv cells is markedly different. There are thought to be approximately 200 2W1S<sup>+</sup> T cells in a naïve mouse (Moon et al., 2007), which would amount to a 211-fold increase in median number from D14 to D21 in the mice which had not received pre-treatment (**Figure 3.10b**). In contrast, in the mice which received two immunisations of 2W1S peptide and agonistic anti-DR3, there was a 4.9-fold increase in median number from D14 to D21. With 2W1S peptide pre-treatment alone, which expands fewer 2W1S<sup>+</sup> Treg cells as well as 2W1S<sup>+</sup> Tconv cells, there was a 35.8-fold increase in median number from D14 to D21. Therefore, although the difference in 2W1S<sup>+</sup> Tconv cells is not very large at D21, the limited nature of the expansion from D14 to D21 in pre-treated mice may be consistent with suppression, or blunting of the response.



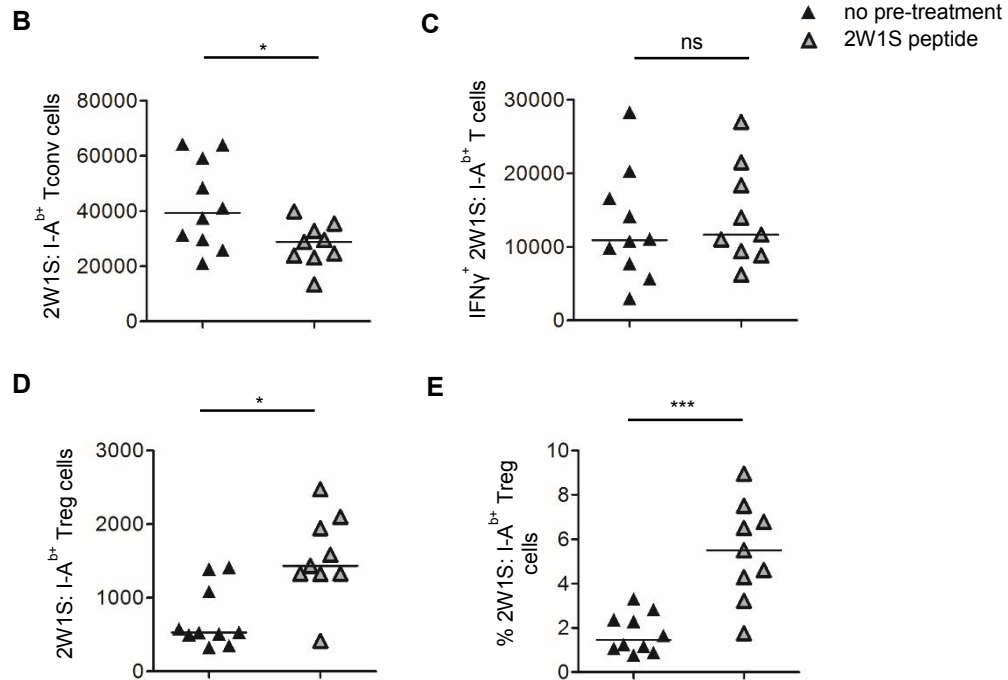
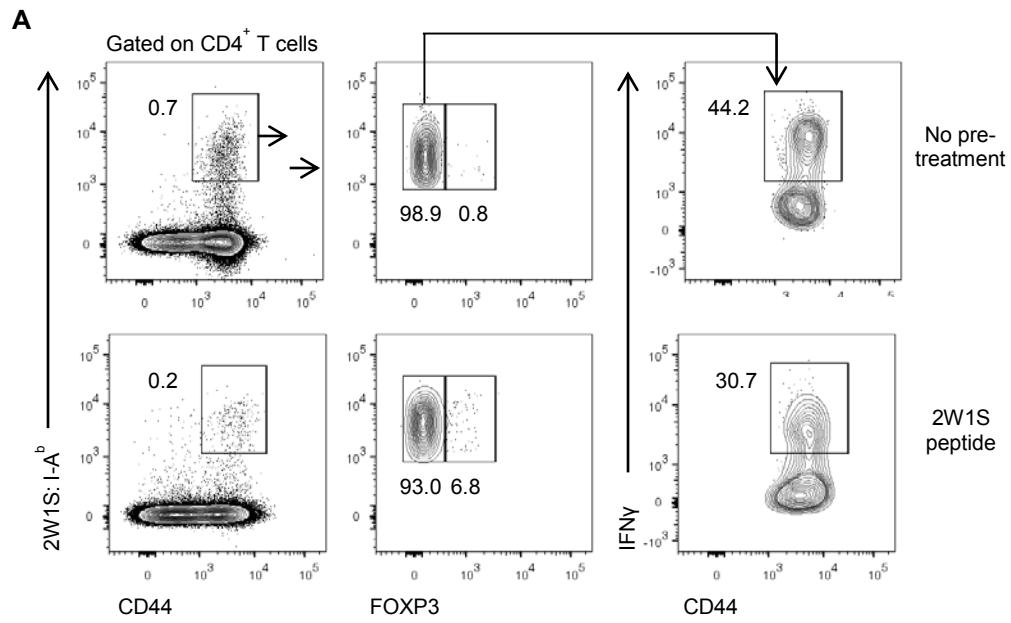
**Figure 3.10: 2W1S<sup>+</sup> regulatory T cell expansion blunts the subsequent response to Lm-2W1S.** WT mice were infected intravenously with Lm-2W1S. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. A group of each cohort were immunised intravenously twice with 2W1S peptide +/- agonistic anti-DR3 prior to infection with Lm-2W1S, 7 days apart. (A) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). Plots are pre-gated on CD3<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> cells. Black filled triangles = no pre-treatment, grey filled triangles = 2W1S and agonistic anti-DR3, white filled triangles = 2W1S peptide. (B) Fold increase in FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells) after administration with Lm-2W1S (from D14-D21). Black filled bar = no pre-treatment, grey filled bar = 2W1S and agonistic anti-DR3, white filled bar = 2W1S. (A-B) Means  $\pm$  SEM are shown. At least 10 samples per group were pooled from two independent experiments. Tconv = conventional T cells.

There was a statistically significant difference in 2W1S<sup>+</sup> Tconv cell number between the mice administered Lm-2W1S only and those given pre-treatment, with 1.4-fold less in the pre-treated mice (median 39275 vs median 27244) (**Figure 3.11a, b**). This is consistent with a tolerised state, supporting Treg cell-mediated suppression. IFN $\gamma$  production was identified in both groups but there was not a significant difference between the two (**Figure 3.11c**). In accordance with a role for these cells, the number of 2W1S<sup>+</sup> Treg cells identified within the pre-treated mice was 6.5-fold higher than the mice given Lm-2W1S only (median 3444 vs median 528) (**Figure 3.11d**). Further to this, the percentage of 2W1S<sup>+</sup> Treg cells within the pre-treated mice was much higher than in the mice given Lm-2W1S only (median 13.3% vs median 1.5%) (**Figure 3.10e**). Therefore, blunting of a subsequent response following Treg cell expansion can be observed in this assay, consistent with Treg cell-mediated suppression. It is however, important to note that this experimental approach does not directly demonstrate Treg cell involvement.



**Figure 3.11: Expansion of 2W1S<sup>+</sup> regulatory T cells using 2W1S peptide and agonistic anti-DR3 blunts the subsequent response to Lm-2W1S.** WT mice were infected intravenously with Lm-2W1S. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. A group of each cohort were immunised intravenously twice with 2W1S peptide and agonistic anti-DR3 prior to infection with Lm-2W1S, 7 days apart. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells, as well as IFN $\gamma$  expression by CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells. (D) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Treg cells). (E) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B-E) Black filled triangles = no pre-treatment, grey filled triangles = 2W1S peptide and agonistic anti-DR3. Mann–Whitney test, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ns = non-significant. At least 10 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

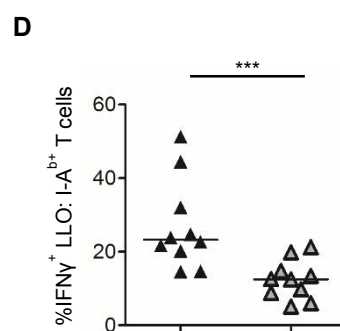
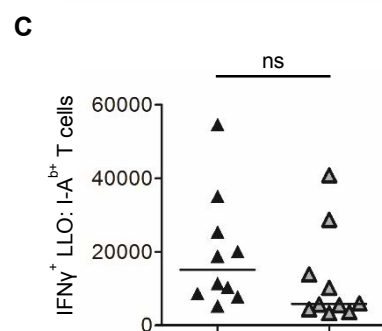
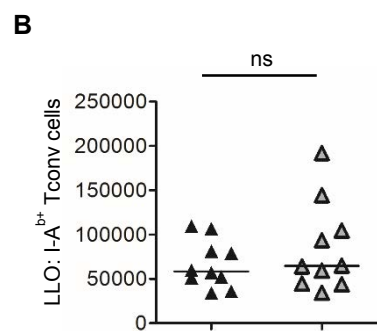
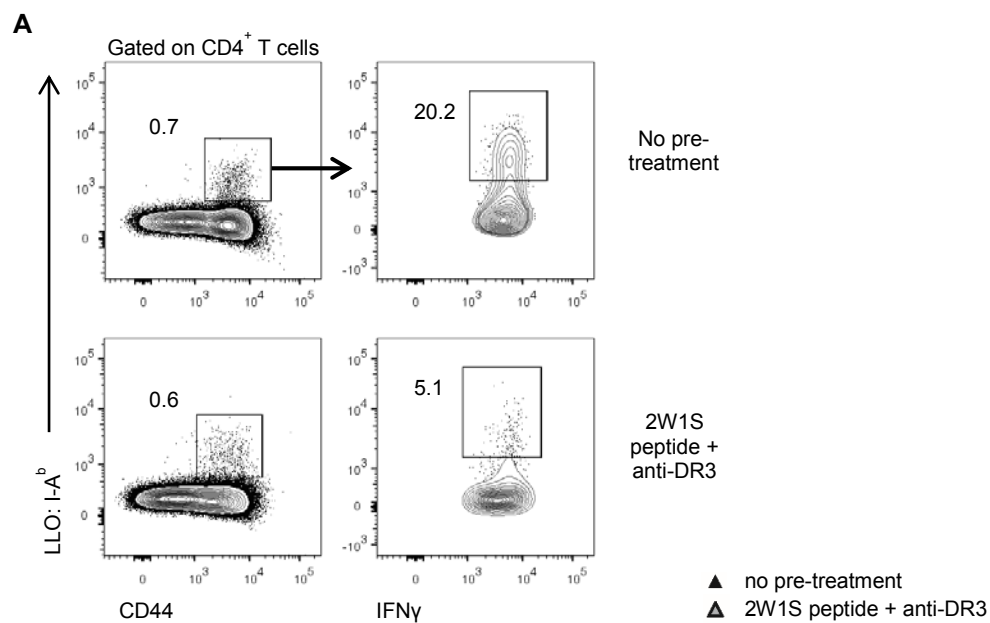
This assay has also been performed using two immunisations of 2W1S peptide to expand Treg cells to ensure that the difference observed in 2W1S<sup>+</sup> Tconv cell number is not solely due to an effect mediated by the agonistic anti-DR3 antibody. Therefore, 2W1S<sup>+</sup> Treg cells were expanded using two immunisations of 50 µg 2W1S peptide, seven days apart. After another seven days mice were then infected with 10<sup>7</sup> Lm-2W1S. To stimulate IFNγ<sup>+</sup> production mice were injected with 50 µg 2W1S peptide and 2.5 µg LPS on the day of analysis. The 2W1S-specific response was then assessed in a pool of lymph nodes and spleen, which were harvested four hours later. Again, there was a statistically significant 1.4-fold reduction in the number of 2W1S<sup>+</sup> Tconv cells in the pre-treated mice (median 39275 vs median 28743) (**Figure 3.12a, b**), consistent with a tolerised state. There was not a significant difference in the median number of IFNγ<sup>+</sup> 2W1S<sup>+</sup> T cells (**Figure 3.12c**). Again the number of 2W1S<sup>+</sup> Treg cells identified within the pre-treated mice was 2.7-fold higher than the mice given Lm-2W1S only (median 1433 vs median 528) (**Figure 3.12d**). Further to this, the percentage of 2W1S<sup>+</sup> Treg cells within the pre-treated mice was higher than in the mice given Lm-2W1S only (median 5.5% vs median 1.5%) (**Figure 3.12e**). Therefore, as this impairment in the 2W1S<sup>+</sup> T cell response was again observed after two immunisations of 2W1S peptide, these data show that the effect observed in this assay are not due to the addition of agonistic anti-DR3.



**Figure 3.12: Expansion of 2W1S<sup>+</sup> regulatory T cells using 2W1S peptide blunts the subsequent response to Lm-2W1S.** WT mice were infected intravenously with Lm-2W1S. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. A group of each cohort were immunised intravenously twice with 2W1S peptide prior to infection with Lm-2W1S, 7 days apart. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells, as well as IFN $\gamma$  expression by CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (D) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (E) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B-E) Black filled triangles = no pre-treatment, grey filled triangles = 2W1S peptide. Bars show medians. Mann–Whitney test, \* $p < 0.05$ , \*\*\* $p < 0.001$ , ns = non-significant. At least 10 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

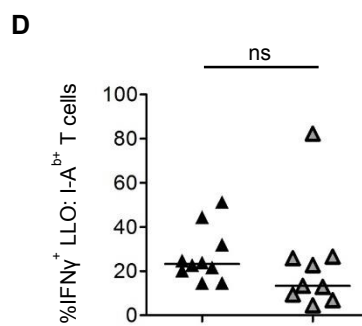
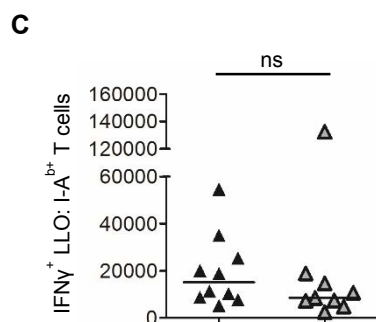
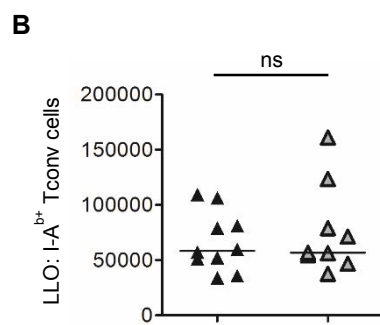
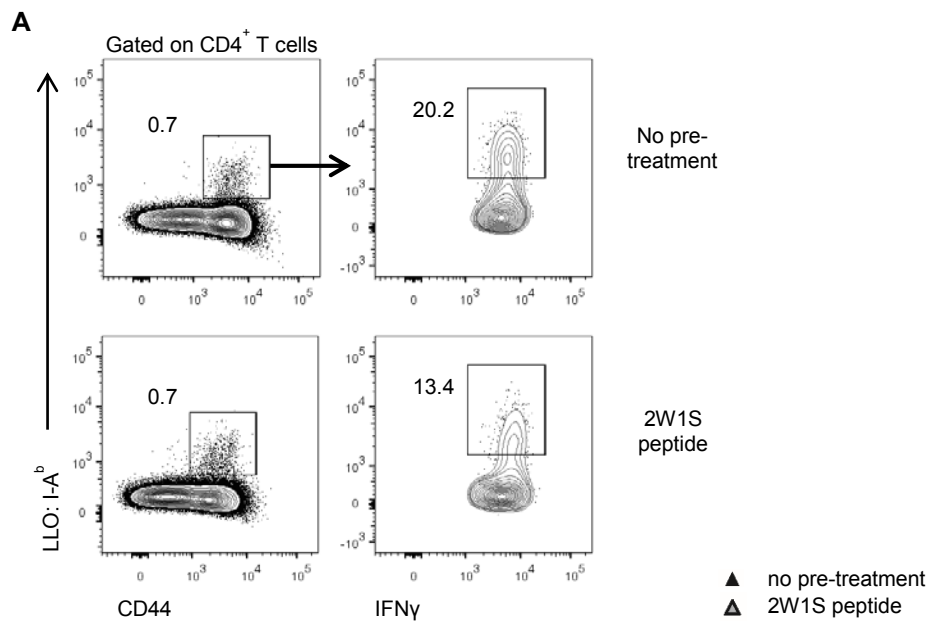


There are reports that although Treg cells are activated in an antigen-specific manner they are able to suppress T cells of a different specificity (Schmidt et al., 2012). To assess whether 2W1S<sup>+</sup> Treg cells can suppress another T cell population, MHC tetramers loaded with peptide 190-201 of LLO were used to detect T cells responding to Listeriolysin (LLO), which is a major virulence factor expressed by *Listeria monocytogenes* (Hamon et al., 2012). To assess whether the expansion of 2W1S<sup>+</sup> Treg cells can affect the subsequent expansion of these LLO<sup>+</sup> Tconv cells, 2W1S<sup>+</sup> Treg cells were first expanded using two immunisations of 50 µg 2W1S peptide and 10 µg agonistic anti-DR3, seven days apart. After another seven days mice were then infected with 10<sup>7</sup> Lm-2W1S, and seven days later the LLO-specific response was assessed in a pool of lymph nodes and spleen. There was not, however, a statistically significant difference in the number of LLO<sup>+</sup> Tconv cells between pre-treated mice and those given Lm-2W1S only (**Figure 3.13a, b**). Similarly, there was not a statistically significant difference in the number of IFNγ<sup>+</sup> LLO<sup>+</sup> T cells, although the median number was reduced by 2.6-fold in the mice given pre-treatment (**Figure 3.13c**). However, the percentage of IFNγ<sup>+</sup> LLO<sup>+</sup> T cells was reduced by 1.9 fold (median 23 vs median 13), an effect which was statistically significant (**Figure 3.13d**). Therefore, there is some evidence of bystander suppression of LLO<sup>+</sup> Tconv cells by 2W1S<sup>+</sup> Treg cells after administration of 2W1S peptide and agonistic anti-DR3.



**Figure 3.13: Expansion of 2W1S<sup>+</sup> regulatory T cells using 2W1S peptide and agonistic anti-DR3 does not affect the LLO<sup>+</sup> specific response to Lm-2W1S.** WT mice were infected intravenously with Lm-2W1S. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. A group of each cohort were immunised intravenously twice with 2W1S peptide and agonistic anti-DR3 prior to infection with Lm-2W1S, 7 days apart. (A) Identification of CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells, as well as IFN $\gamma$  expression by CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (D) Percentage of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (B-D) Black filled triangles = no pre-treatment, grey filled triangles = 2W1S peptide and agonistic anti-DR3. Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , ns = non-significant. At least 10 mice were pooled per group from two independent experiments. Tconv = conventional T cells.

This experiment was also carried out with two immunisations of 2W1S peptide, and no agonistic anti-DR3. Therefore, 2W1S<sup>+</sup> Treg cells were expanded using two immunisations of 50 µg 2W1S peptide, seven days apart. After another seven days mice were then infected with 10<sup>7</sup> Lm-2W1S, and seven days later the LLO-specific response was assessed in a pool of lymph nodes and spleen. Again there was not a statistically significant difference in the number of LLO<sup>+</sup> Tconv cells between pre-treated mice and those given Lm-2W1S only (**Figure 3.14a, b**). Similarly, there was not a statistically significant difference in the number of IFNγ<sup>+</sup> LLO<sup>+</sup> T cells, although the median number was reduced by 1.8-fold in the mice given pre-treatment (**Figure 3.14c**). The percentage of IFNγ<sup>+</sup> LLO<sup>+</sup> T cells was reduced by 1.7 fold (median 23 vs median 13), although this was not statistically significant (**Figure 3.14d**). Therefore, there is little evidence of bystander suppression of LLO<sup>+</sup> Tconv cells by 2W1S<sup>+</sup> Treg cells after 2W1S peptide administration.



**Figure 3.14 Expansion of 2W1S<sup>+</sup> regulatory T cells using 2W1S peptide does not affect the LLO<sup>+</sup> specific response to Lm-2W1S.** WT mice were infected intravenously with Lm-2W1S. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. A group of each cohort were immunised intravenously twice with 2W1S peptide prior to infection with Lm-2W1S, 7 days apart. (A) Identification of CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells, as well as IFN $\gamma$  expression by CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (D) Percentage of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (B-D) Black filled triangles = no pre-treatment, grey filled triangles = 2W1S peptide. Bars show medians. Mann–Whitney test, ns = non-significant. At least 10 mice were pooled per group from two independent experiments. Tconv = conventional T cells.

*Determining the effect of Lm-2W1S infection on subsequent 2W1S<sup>+</sup> Treg cell expansion*

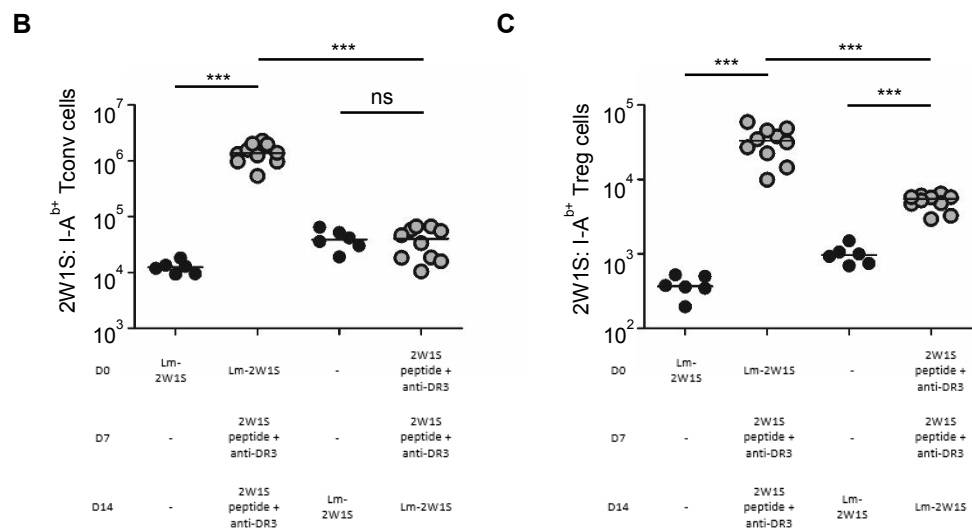
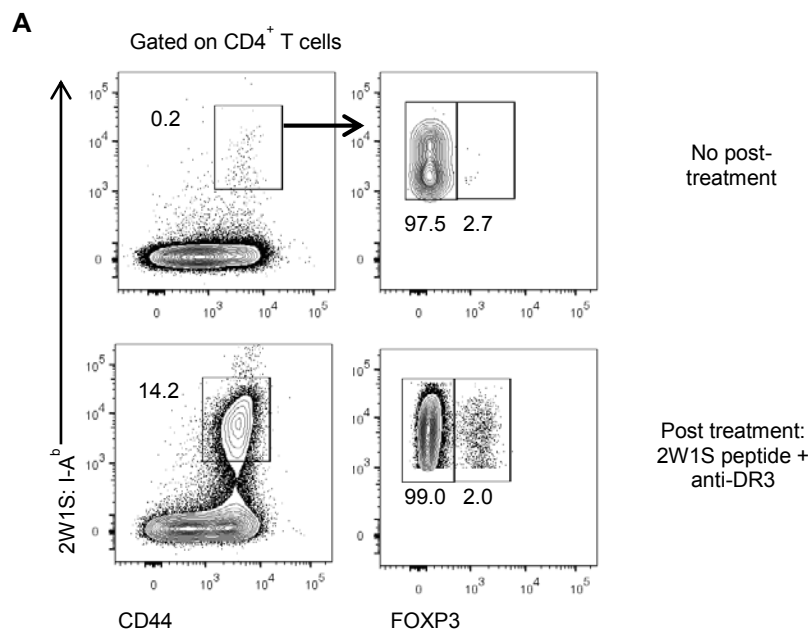
Treatment with 2W1S peptide +/- agonistic anti-DR3, but not Lm-2W1S, has been shown to successfully expand Treg cells from the naïve 2W1S<sup>+</sup> T cell population. To determine whether 2W1S<sup>+</sup> Treg cells can be expanded after Lm-2W1S treatment, 10<sup>7</sup> Lm-2W1S was administered to WT mice prior to two injections of 50 µg 2W1S peptide and 10 µg agonistic anti-DR3, 7 days apart. Within the same experiment, this has been compared to the original protocol of 2W1S<sup>+</sup> Treg cell expansion prior to Lm-2W1S infection. Therefore, 2W1S<sup>+</sup> Treg cells were first expanded using two immunisations of 50 µg 2W1S peptide and 10 µg agonistic anti-DR3, seven days apart. After another seven days mice were then infected with 10<sup>7</sup> Lm-2W1S, and any reduction in the number of 2W1S<sup>+</sup> Tconv cells, compared to mice which are infected with Lm-2W1S without pre-treatment, was interpreted as evidence of suppression. Lm-2W1S was again administered at the same time to control mice as to the pre-treated mice (D14), and also to another group of control mice at the same time as the mice receiving Lm-2W1S prior to 2W1S<sup>+</sup> Treg cell expansion. On the day of analysis, the 2W1S-specific response was assessed in a pool of lymph nodes and spleen.

When Lm-2W1S was administered prior to the two injections of 2W1S peptide and agonistic anti-DR3, there was a very large expansion of 2W1S<sup>+</sup> Tconv cells, despite the presence of a large number of 2W1S<sup>+</sup> Treg cells (**Figure 3.15a**). This striking difference in mice administered 2W1S peptide and agonistic anti-DR3, compared to those administered Lm-2W1S only, translated to a 109.8-fold increase in the median number of 2W1S<sup>+</sup> Tconv cells (**Figure 3.15b**). This also constituted a 33.8-fold increase when compared to mice given 2W1S peptide and agonistic anti-DR3 twice

prior to Lm-2W1S infection (median 1362494 vs median 40279), despite both sets of mice receiving the same treatments in a different order. In contrast to the previous experiment, there was not a statistically significant difference in the number of 2W1S<sup>+</sup> Treg cells in mice given pre-treatment, compared to those given Lm-2W1S only.

Despite such a striking expansion of 2W1S<sup>+</sup> Tconv cells, a large number of 2W1S<sup>+</sup> Treg cells could be identified in mice given 2W1S peptide and agonistic anti-DR3 after Lm-2W1S infection (**Figure 3.15c**). This number was 90-fold higher than mice given Lm-2W1S at the same time (median 33148 vs median 368), and 6-fold higher when than mice infected with Lm-2W1S after pre-treatment (median 5489). It is important to note however that the percentage of 2W1S<sup>+</sup> Treg cells in these mice was very low (median 2.3%). Therefore, these data demonstrate that if Lm-2W1S is administered prior to multiple injections of 2W1S peptide and agonistic anti-DR3, a very large expansion of 2W1S<sup>+</sup> Tconv cells occurs despite the presence of 2W1S<sup>+</sup> Treg cells.





**Figure 3.15: 2W1S<sup>+</sup> regulatory T cells can be expanded subsequent to Lm-2W1S administration, but are unable to prevent a large 2W1S<sup>+</sup> conventional T cell response.** WT mice were infected intravenously with Lm-2W1S, either 7 days before or after two intravenous immunisations of 2W1S peptide and agonistic anti-DR3, 7 days apart. Control mice were infected intravenously Lm-2W1S at either time-point but had no pre or post-treatment. After 7 days mice were immunised intravenously with 2W1S peptide and LPS and a pool of lymph nodes and spleen were analysed 4 hours later. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, C) Black filled triangles = no pre/post-treatment, grey filled triangles = 2W1S peptide and agonistic anti-DR3. Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , ns = non-significant. At least 10 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

### 3.3 DISCUSSION

This study aimed to establish a method for generating and tracking antigen specific Treg cells, and assessing their function *in vivo*. This method was based on work pioneered by Marc Jenkins, in which an endogenous population of naïve T cells are expanded by exposure to their cognate peptide, 2W1S, and identified *ex vivo* using MHC class II tetramers (Moon et al., 2007). This approach has previously been successfully used to study CD4 memory T cells (Marriott et al., 2015, Marriott et al., 2014), as well as foetal antigen-specific Treg cells (Rowe et al., 2012).

In this study, administration of 2W1S peptide was shown to induce greater expansion/generation of 2W1S<sup>+</sup> Treg cells relative to 2W1S<sup>+</sup> Tconv cells than infection with Lm-2W1S. This is consistent with previous reports that Treg cell expansion/generation is associated with low dose antigen (Friedman and Weiner, 1994). Notably, this also limits the expansion of Tconv cells due to the low level of expression of co-stimulatory molecules, and therefore has a favourable effect on the proportion of Treg cells, as observed in this study. In contrast, *Listeria monocytogenes* infection produces a strong type 1 immune response, with a low percentage of Treg cells. Therefore, immunisation with 2W1S peptide was used in this study to expand Treg cells, rather than Lm-2W1S administration.

Although Treg cells comprised approximately 13% of the 2W1S<sup>+</sup> T cell population following 2W1S peptide administration, the number was very low. Therefore, it was necessary to investigate strategies to expand Treg cells. Ideally such a strategy would increase the percentage of Treg cells as well as the number, to ensure that the function of Treg cells might be assessed. For example, *in vitro* suppression assays

often start at a 2:1 ratio of conventional to regulatory T cells (Collison and Vignali, 2011). Administration of an agonistic anti-DR3 antibody has previously been shown to selectively expand Treg cells to 30-35% of the T cell population, and as a result to prevent the onset of an animal model of asthma (Schreiber et al., 2010), and rejection of a cardiac allograft (Wolf et al., 2012). DR3 is a tumor necrosis factor receptor, which is well characterized as a costimulatory molecule for T cell activation (Croft, 2009). This agonistic anti-DR3 antibody is thought to cause Treg cell expansion by increasing their sensitivity to IL-2 (Schreiber et al., 2010). Consistent with these findings, when given along with 2W1S peptide, an increase in the overall Treg cell population to 30-35% was observed. However, this antibody increased both the regulatory and conventional 2W1S<sup>+</sup> T cell populations and therefore did not have a selective effect on Treg cells within this T cell population.

Despite the selective effect of agonistic anti-DR3 on the overall Treg cell population, regulatory and conventional T cells have been reported to express similar levels of DR3 (Taraban et al., 2011a, Meylan et al., 2011). Further to this, constitutive expression of its ligand, TL1A, on dendritic cells has been shown to expand both regulatory and conventional T cells, with the net effect being more inflammatory than regulatory (Taraban et al., 2011b). To investigate this further, DR3 expression was studied on these cells. The overall conventional and regulatory T cell populations both exhibited DR3 expression, with approximately 50% of conventional T cells and 70% of regulatory T cells found to be DR3<sup>+</sup>. Interestingly, it has been proposed that it is not relative expression of DR3 that causes the global increase in the Treg cell proportion, but relative availability of cognate antigen (Schreiber and Podack, 2013). Therefore, because at steady-state the global Treg cell population regularly

encounters self-antigen, this is the population which expands upon administration of the antibody. This theory would fit with the observation within this study that agonistic anti-DR3 increased the regulatory and conventional 2W1S<sup>+</sup> populations both by the same degree when administered with 2W1S peptide as both subsets would have encountered cognate antigen. Within the 2W1S<sup>+</sup> population, approximately 30% of 2W1S<sup>+</sup> Tconv cells were found to express compared to 60% of 2W1S<sup>+</sup> Treg cells seven days after immunisation with 2W1S peptide. That DR3 expression can be observed on both 2W1S<sup>+</sup> conventional and regulatory T cells is consistent with them both being expanded by this agonistic anti-DR3 antibody, particularly as DR3 is upregulated on T cells upon activation (Croft, 2009).

Therefore, a strategy to boost the number of 2W1S<sup>+</sup> Treg cells was established and further experiments were performed to identify a strategy which would also increase the proportion of these cells. Repeated exposure to antigen has been shown to increase the percentage of Treg cells either due to anergy induction within the conventional T cell population (Eroukhmanoff et al., 2009) or as a result of Treg cell proliferation (Rosenblum et al., 2011). In this study, the percentage of 2W1S<sup>+</sup> Treg cells was indeed increased following two injections of 2W1S peptide, reaching approximately 20%. This reflected a selective increase in the number of 2W1S<sup>+</sup> Treg cells, and was also observed when 2W1S peptide and agonistic anti-DR3 was administered twice. This observation has important implications in terms of autoantigen-specific immunotherapy for autoimmune disease, where the challenge is to boost the number of Treg cells specific for an autoantigen, without exacerbating the effector response to that autoantigen. These experiments revealed that the greatest number and percentage of Treg cells could be expanded using two

immunisations of 2W1S peptide and agonistic anti-DR3 and therefore this protocol should be used in subsequent experiments.

To investigate whether Treg cells are thymically (tTreg cells) or peripherally derived (pTreg cells), expression of the Ikaros family transcription factor Helios (Thornton et al., 2010) and the Semaphorin III receptor Neuropilin-1 (Weiss et al., 2012, Yadav et al., 2012) can be used. Further to this, identification of Treg cells which are specific to a particular antigen in naïve mice can also provide an indication that the Treg cells are thymically derived. It has previously been shown that approximately 7.5% of the naïve 2W1S<sup>+</sup> T cell population is FOXP3<sup>+</sup> and that these Treg cells express Helios (Moon et al., 2011). In this study, 2W1S<sup>+</sup> Treg cells were also identified in naïve mice and the majority were shown to express Neuropilin-1 (Nrp-1). In addition, after immunisation with 2W1S peptide +/- agonistic anti-DR3 the majority of 2W1S<sup>+</sup> Treg cells expressed Nrp-1 (85-95%) and 55-75% expressed Helios. Together this suggests that 2W1S<sup>+</sup> Treg cells which we are able to identify upon immunisation arise due to expansion of thymically derived Treg cells within the naïve 2W1S<sup>+</sup> T cell pool.

As previously described, there are concerns about the reliability of *in vitro* assays, such as suppression assays. Therefore in this study, an assay was developed to assess the function of antigen-specific Treg cells *in vivo*, based on work published by Rowe et al. (2012). To first expand 2W1S<sup>+</sup> Treg cells, the previously established protocol was used: two immunisations of 2W1S peptide and agonistic anti-DR3. Following Treg cell expansion, mice were infected with Lm-2W1S and any resulting impairment in the response, compared to mice given Lm-2W1S only, was considered to reflect suppression. Indeed, when the number of 2W1S<sup>+</sup> Tconv cells was

compared between the two groups, there was an impaired response in the mice given pre-treatment. Perhaps more strikingly, despite immunisation with 2W1S peptide and agonistic anti-DR3 expanding 2W1S<sup>+</sup> Tconv cells, and therefore having a much larger precursor frequency than naïve mice, both groups of mice infected with Lm-2W1S had similar numbers of 2W1S<sup>+</sup> Tconv cells. This is consistent with a tolerised state, and therefore with the action of 2W1S<sup>+</sup> Treg cells. Further to this, the response was also blunted when the pre-treatment consisted of two immunisations of 2W1S peptide alone, which demonstrates that it is not solely mediated by agonistic anti-DR3. Therefore, these data indicate that the response to Lm-2W1S is impaired following Treg cell expansion, which is consistent with suppression. It is however possible that T cell anergy may be instead occurring, a phenomenon which may occur upon antigen presentation in the absence of adequate co-stimulation (Harding et al., 1992). Further experiments therefore needed to confirm that the impairment in the response is a direct result of Treg cell activity, rather than effects on the Tconv cell population. Such experiments could be performed using FOXP3<sup>DTR</sup> mice, in which Treg cells can be specifically depleted by addition of diphtheria toxin (Kim et al., 2007). It is also important to note that variability has been observed in the augmentation of the response in WT mice across all experiments performed. This may however, be due to variability in Treg cell percentage, with an impairment observed only if the Treg percentage is above a certain threshold. Together these data establish a method to study the survival and function of thymically-derived Treg cells in an antigen-specific manner.

Treg cells are also known to have the capacity to suppress T cells of multiple specificities, (Thornton and Shevach, 2000, Takahashi et al., 1998, Thornton and

Shevach, 1998), although they are activated in an antigen-specific manner and are thought to most efficiently suppress T cells of the same specificity (Corthay, 2009). In this study, MHC tetramers loaded with peptide 190-201 of LLO were used to detect T cells responding to Listeriolysin (LLO), which is expressed by Lm-2W1S (Hamon et al., 2012). It was therefore possible to investigate whether 2W1S<sup>+</sup> Treg cells augment Lm-2W1S mediated expansion of LLO<sup>+</sup> Tconv cells. Upon 2W1S peptide and agonistic anti-DR3 administration, the number of LLO<sup>+</sup> Tconv cells was similar in both groups and the number of IFN<sup>+</sup>LLO<sup>+</sup> Tconv cells was modestly reduced, although this was not statistically significant. However, the percentage of IFN<sup>+</sup>LLO<sup>+</sup> Tconv cells was significantly reduced, which is be consistent with suppression of the response. Upon 2W1S peptide administration, the number of LLO<sup>+</sup> Tconv cells was similar in both groups and there were modest reductions in the number and percentage of IFN<sup>+</sup>LLO<sup>+</sup> Tconv cells, although this was not statistically significant. Therefore, some evidence of bystander suppression was observed within these experiments, although the number of LLO<sup>+</sup> Tconv cells was not affected

One potential therapeutic avenue to reduce tissue damage in autoimmune disease is to expand Treg cells either *in vivo*, or *ex vivo* prior to transfer (Miyara et al., 2014). However, as clinical symptoms occur after the immune response is already underway; it is not always possible to expand Treg cells beforehand. Therefore, in this study, 2W1S<sup>+</sup> Treg cells have been expanded following Lm-2W1S infection, again using 2W1S peptide and agonistic anti-DR3. Using this protocol, 2W1S<sup>+</sup> Treg cell expansion was observed, but these cells comprised only a very small percentage of the 2W1S<sup>+</sup> T cells, as there was a very large expansion of 2W1S<sup>+</sup> Tconv cells. The fact that the 2W1S<sup>+</sup> Tconv cells expand to such a degree in the presence of 2W1S<sup>+</sup>



Treg cells suggests that after a strong Th1 response, suppression is more difficult to achieve and therefore this approach to the treatment of autoimmune disease may be technically challenging. This may reflect differences in the ratio of Treg cells to effector cells or enhanced resistance of effector cells to Treg cell-mediated suppression. There are indeed reports that high doses of antigen can render effector T cells resistant to suppression (Baecher-Allan et al., 2002, Georger et al., 2003), with contributions from PAMPS, pro-inflammatory cytokines and co-stimulatory molecules (Walker, 2009). However, it is important to note that in these experiments, Treg cells were successfully expanded after clearance of Lm-2W1S, and therefore it is at unclear whether these mechanisms are involved in the effect observed.

Therefore, in addition to establishing a method to study Treg cells in an antigen-specific manner these data explore the concept of 'bystander suppression' and demonstrate that suppression of a Th1 response is more readily achieved if Treg cells are induced before challenge than after.

## **4.0 THE ROLE OF TNF RECEPTORS OX40 AND CD30 IN REGULATORY T CELL SURVIVAL AND FUNCTION**

## 4.1 INTRODUCTION

TNF receptors such as OX40, which is constitutively expressed on Treg cells (Croft, 2009), have been proposed as attractive targets for cancer immunotherapy as blocking of these receptors may have dual anti-tumor effects, expanding effector T cells as well as turning off Treg cell function (Kitamura et al., 2009). OX40 is expressed on naïve T cells 12-24 hours after stimulation through the T cell receptor and CD28 (Gramaglia et al., 1998, Walker et al., 1999, Gilfillan et al., 1998), where it promotes survival (Rogers et al., 2001, Song et al., 2005). Further to this, OX40 has been shown to be important for the generation of CD4 T cell memory (Gramaglia et al., 2000, Withers et al., 2009). Therefore, in the absence of OX40, CD4 T cell expansion is impaired, which may lead to the formation of less memory T cells. Interestingly, these effects are enhanced if the TNF receptor CD30 is also absent, which suggests that there may be some redundancy in the signalling pathways between these two receptors (Gaspal et al., 2005, Gaspal et al., 2008). This suggests that considering CD30 as well as OX40 when developing new therapeutics may be beneficial, however little is currently known about the importance of CD30. In addition, although the hope is that agonistic anti-OX40 would suppress Treg cell function; there is controversy in the literature about the role of OX40 on these cells.

It has been reported that 7-8 week old OX40 KO mice have lower numbers of Treg cells (Takeda et al., 2004). Further to this, OX40 expression by Treg cells, as well as Tconv cells, has been shown to be associated with survival upon treatment with anti-lymphocyte serum (Kroemer et al., 2007). These data indicate that OX40 may play a role in the survival and/or generation of Treg cells. However, it is now well established that the number of Treg cells in adult OX40 KO mice is largely similar to

WT (Vu et al., 2007, Tang et al., 2003, Valzasina et al., 2005, Takeda et al., 2004, Kroemer et al., 2007).

In terms of function, Treg cells from OX40 KO mice have been shown to be less effective *in vitro* at suppressing effector T cell proliferation and cytokine production, whereas Treg cells from mice overexpressing OX40L have been shown to be more effective (Takeda et al., 2004). Further to this, OX40 KO Treg cells are unable to prevent T cell-induced colitis (Griseri et al., 2010, Piconese et al., 2010). Together this suggests that OX40 is necessary for Treg cell function. In contrast another publication has shown that OX40 is dispensable for suppression (Vu et al., 2007). Additionally, stimulation of OX40 *in vitro* using an agonistic OX40 antibody (OX86) or mice overexpressing OX40L, has been shown to abrogate the suppressive ability of Treg cells (Kitamura et al., 2009, Vu et al., 2007, Xiao et al., 2012, Valzasina et al., 2005, Piconese et al., 2008, Takeda et al., 2004, Kroemer et al., 2007). This has been reported to be coupled with FOXP3 downregulation (Vu et al., 2007, Kitamura et al., 2009) and it has been shown that suppression can be restored if Treg cells are depleted of OX40 (Vu et al., 2007). Treg mediated suppression has also been shown to be impaired upon OX40 stimulation in murine models of skin allograft rejection (Vu et al., 2007), graft versus host disease (Valzasina et al., 2005) and colitis (Takeda et al., 2004), as well as anti-tumor responses (Houot and Levy, 2009, Kitamura et al., 2009, Piconese et al., 2008). Notably, OX40 signaling also inhibits conversion of naïve T cells into regulatory T cells *in vitro* and *in vivo* (Vu et al., 2007, So and Croft, 2007, Ito et al., 2006, Duan et al., 2008).

Despite these findings, OX40 stimulation, either due to OX86 or OX40L overexpression, has been shown to stimulate Treg cell proliferation *in vivo* (Hippen et

al., 2008, Ruby et al., 2009, Hirschhorn-Cymerman et al., 2009, Xiao et al., 2012, Takeda et al., 2004). This can result in 'exhaustion' and therefore downregulation of FOXP3 and CD25, as well as impaired suppressive ability (Xiao et al., 2012). The provision of exogenous IL-2 can however prevent exhaustion, and if IL-2/anti-IL-2 complexes are given with OX86, long term allograft survival can be observed (Xiao et al., 2012). This suggests that the previously described negative effects of OX40 stimulation on Treg function *in vitro* and during rejection, colitis and anti-tumor responses may be due to the limited availability of IL-2 to Treg cells.

IL-2 is known to be vital for Treg cell survival (Malek and Castro, 2010) and interestingly Treg cells are thought to display altered IL-2 signaling to effector T cells, with co-operation with other receptors such as the T cell receptor required for full signaling (Bensinger et al., 2004). OX40 signaling on Treg cells has been shown to result in Akt activation, which is necessary for full signaling, and is not achieved through IL-2 in Treg cells (Xiao et al., 2012). This suggests that signals through OX40 and IL-2R may co-operate towards responsiveness to IL-2, and indeed, OX40 KO Treg cells have been shown to be hypo responsive to IL-2 (Piconese et al., 2010, Xiao et al., 2012). This may explain why OX40 KO Treg cells have been shown to be less effective in T cell induced colitis (Griseri et al., 2010, Piconese et al., 2010), as the absence of OX40 puts them at a disadvantage against effector T cells, with which they are competing with for IL-2.

Therefore the role of these TNF receptors in Treg cell survival and function is unclear and it is often difficult to uncouple these two characteristics (Vignali, 2012). An antigen-specific T cell approach will aid clarification of specific effects on survival versus function, since the precise time of expansion can be controlled. It is also

important to test function *in vivo* where possible, due to the previously described limitations of *in vitro* suppression assays. This is technically challenging but recent studies have provided clear evidence that such approaches are possible (Rowe et al., 2012). As previous data is consistent with synergy between OX40 and CD30 (Gaspal et al., 2005, Gaspal et al., 2008, Withers et al., 2009, Gaspal et al., 2011), experiments have been performed using OX40/CD30 double Knock Out (dKO) mice to best reveal key Treg cell requirements.

## 4.2 RESULTS

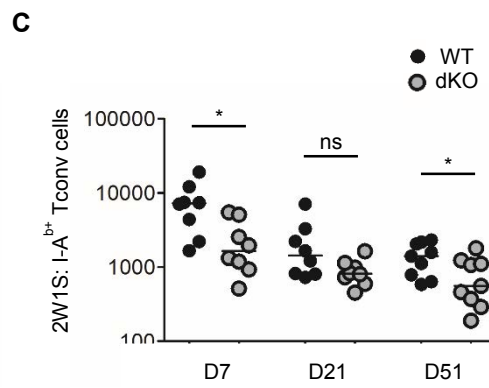
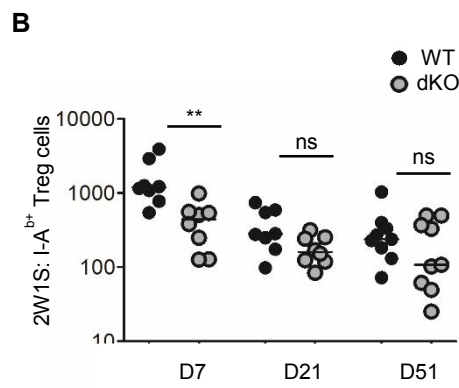
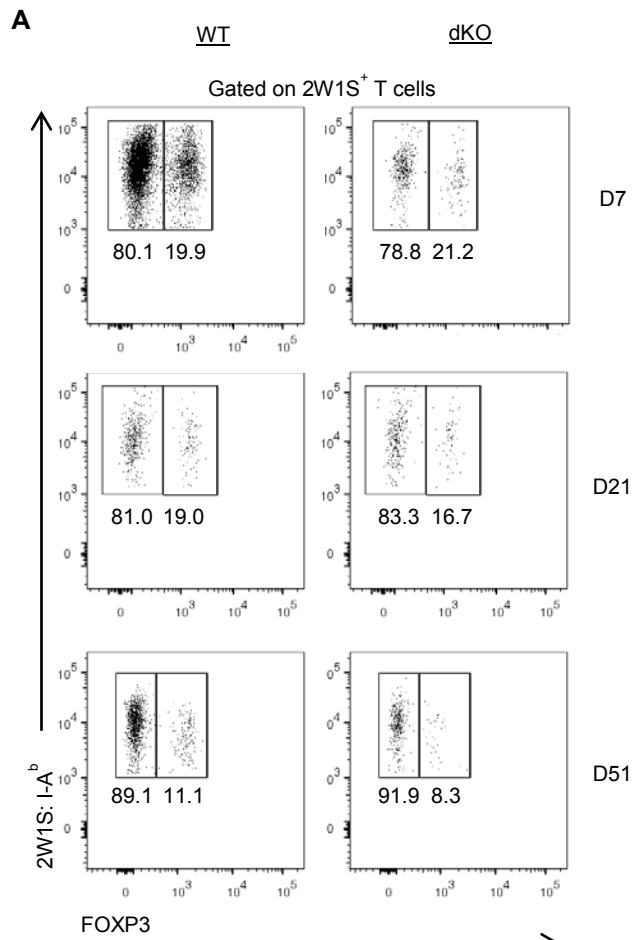
### *Treg cell expansion and survival in OX40/CD30 dKO mice*

To investigate whether OX40 and CD30 are involved in the expansion and persistence of 2W1S<sup>+</sup> Treg cells, OX40/CD30 dKO and WT mice were immunised twice with 50 µg 2W1S peptide and 10 µg agonistic anti-DR3, seven days apart. This protocol for 2W1S<sup>+</sup> Treg cell expansion was used as it expands the greatest number, aiding detection at longer time points, anticipating low numbers of 2W1S<sup>+</sup> Treg cells. On either day 7, 21 or 51 after the second injection of 2W1S peptide and agonistic anti-DR3, the spleen and a pool of peripheral lymph nodes were harvested from these mice and enrichment of 2W1S<sup>+</sup> T cells was performed.

Treg cell numbers were analysed along with the number of conventional T cells as a comparison, due to the well characterized role of TNF receptors in promoting T cell survival during a primary response (Croft, 2009). At all timepoints studied, both regulatory and conventional 2W1S<sup>+</sup> T cells could be detected (**Figure 4.1a**). At day 7 after the final immunisation, there were significantly fewer 2W1S<sup>+</sup> Treg cells in the OX40/CD30 dKO mice than in WT mice, which amounted to a 2.7-fold reduction in number (**Figure 4.1b**) (median 1188 vs median 444). Similarly, a 4.4-fold reduction in the number of 2W1S<sup>+</sup> Tconv cells was observed between WT and OX40/CD30 dKO mice (median 7242 vs median 1649) (**Figure 4.1c**), indicating that the optimal expansion of both regulatory and conventional T cells is dependent on OX40 and CD30. These data do suggest, however, that Treg cells are marginally less dependent upon these signals at this stage.

After 51 days, the significant reduction in the number of 2W1S<sup>+</sup> Tconv cells (2.5 fold; median 1399 vs median 557) but not 2W1S<sup>+</sup> regulatory T cells remained. Therefore, long term survival of these antigen specific Treg cells appears to be comparable in the absence of OX40 and CD30 despite reduced numbers at day 7. A time point between day 7 and 51 was also studied to determine whether signaling through OX40 and CD30 may have a role within this time period. At day 21 after the final immunisation, there was again no significant difference in the numbers of 2W1S<sup>+</sup> Treg cells in OX40/CD30 dKO mice compared to WT. The number of 2W1S<sup>+</sup> Tconv cells was also unchanged between dKO and WT mice. Overall these data indicate that following expansion of 2W1S<sup>+</sup> T cells, the survival of conventional but not regulatory T cells is impaired.



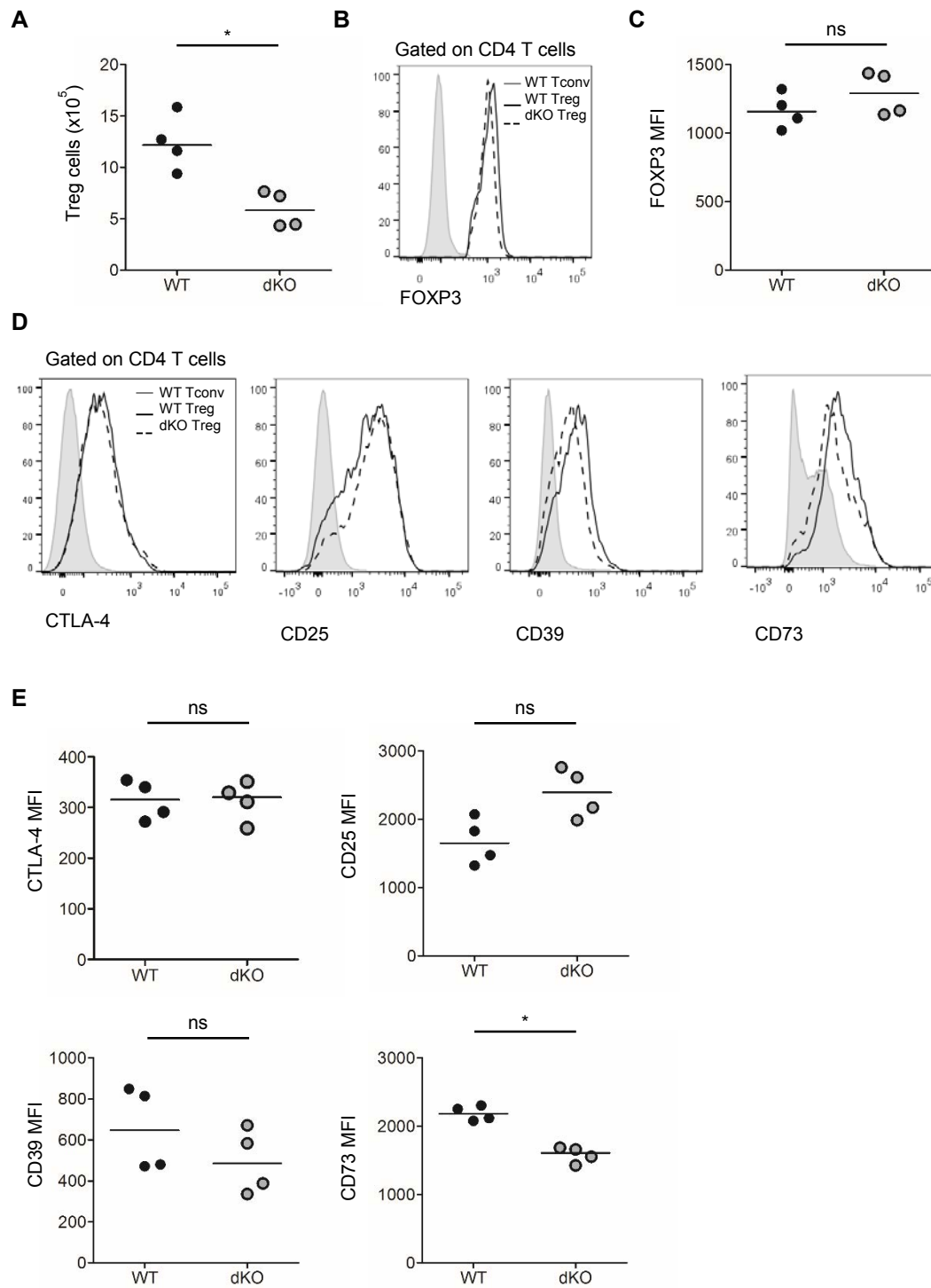


**Figure 4.1: Expansion of regulatory and conventional antigen-specific T cells is impaired in mice which lack OX40 and CD30.** WT and OX40/CD30 dKO mice were immunised twice with 2W1S peptide and agonistic anti-DR3 antibody intravenously, 7 days apart. 7, 21 or 52 days after the second immunisation, a pool of lymph nodes and spleen were analysed. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, C) Bars show medians. Mann–Whitney test, \* $p < 0.05$ , \*\* $p < 0.01$ , ns = non-significant. At least 8 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

### *Further investigation of the role of OX40 and CD30 in Treg cell function*

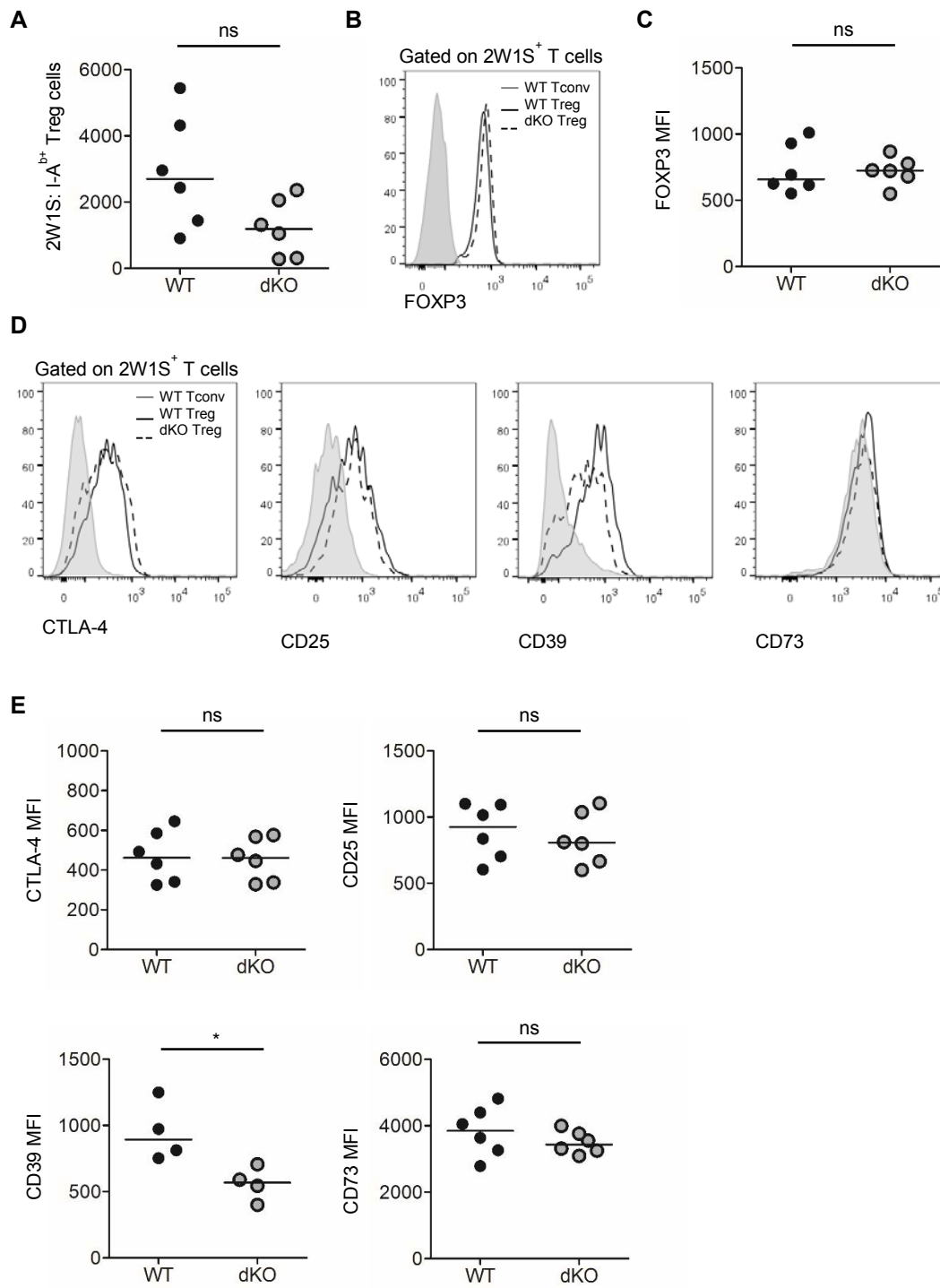
Given the modest effects of the absence of OX40 and CD30 on Treg cell survival, possible defects in Treg function were investigated. Treg cells from the spleen and lymph nodes of OX40/CD30 dKO mice were therefore analysed for expression of FOXP3, which is critical for Treg cell function (Fontenot et al., 2003, Hori et al., 2003). In addition, the expression of CTLA-4, CD25, CD39 and CD73, which are key molecules involved in suppression (Schmidt et al., 2012), was also analysed. Total Treg cells were first studied, followed by the 2W1S<sup>+</sup> Treg cell population.

Treg cell enumeration revealed that the median number of Treg cells in mice lacking OX40 and CD30 was 2.1 fold lower than WT mice (**Figure 4.2a**). In this total Treg cell population, there was not a significant difference in the expression of FOXP3, suggesting that Treg cell function is intact (**Figure 4.2b, c**). In addition, there were not any significant differences in expression of CTLA-4, CD25 or CD39 between OX40/CD30 dKO and WT mice (**Figure 4.2d, e**). There was however a modest but significant reduction in the expression of CD73, which amounted to a 1.4-fold reduction in median fluorescence intensity (2186 vs 1608). Again conventional T cells have been used for comparison within this experiment. As expected within the global Tconv cell population, there was little expression of CTLA-4, CD25 and CD39, which are upregulated upon activation. CD73 was, however, expressed by a proportion of total Tconv cells.



**Figure 4.2: Expression of key molecules involved in regulatory cell function is not dependent on OX40 and CD30.** (A) Enumeration of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells (Treg cells). (B) Identification of FOXP3 expression within Treg cells from WT and OX40/CD30 dKO mice. Expression within WT conventional T cells is also shown for comparison. (C) Median Florescence Intensity (MFI) of FOXP3 within Treg cells from WT and OX40/CD30 dKO mice. (D) Identification of CTLA-4, CD25, CD39 or CD73 expression on Treg cells from WT and OX40/CD30 dKO mice. Expression on WT conventional T cells is also shown for comparison. (E) Median Florescence Intensity (MFI) of CTLA-4, CD25, CD39 and CD73 on Treg cells from WT and OX40/CD30 dKO mice. (B, D) Plots are gated on FOXP3<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. Filled grey line = WT Tconv cells, Solid black line = WT Treg cells, Dotted black line = dKO Treg cells. (C, E) Bars show medians. Mann–Whitney test, \**p* < 0.05, ns = non-significant. At least 4 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

To characterize expression of these markers on 2W1S<sup>+</sup> Treg cells, WT and OX40/CD30 dKO mice were immunized twice with 50 µg 2W1S peptide and 10 µg agonistic anti-DR3 7 days apart. The spleen and a pool of peripheral lymph nodes were harvested seven days later from these mice and enrichment of 2W1S-specific T cells was performed. In accordance with the data from figure 4.2, the median number of 2W1S<sup>+</sup> Treg cells was 2.3 fold lower in mice lacking OX40 and CD30 compared to WT, although this did not reach statistical significance (**Figure 4.3a**). There were not any significant differences in the expression of FOXP3 (**Figure 4.3b, c**), CTLA-4, CD25 or CD73 (**Figure 4.3d, e**). However, there was a modest but significant reduction in the expression of CD39, which amounted to a 1.6-fold reduction in median fluorescence intensity (893 vs 568). These data therefore indicate that Treg cells from OX40/CD30 dKO mice are not substantially impaired in their expression of key molecules involved in Treg cell function. Expression of these markers on 2W1S<sup>+</sup> Tconv cells has again been displayed on the histograms for comparison. In contrast to the total Tconv cells (Figure 4.2d, e) CD25 was expressed by a proportion of these cells as it is upregulated on T cells upon activation (Malek and Castro, 2010). In addition, CD73 was found to be expressed by all 2W1S<sup>+</sup> Tconv cells. This is perhaps surprising as CD73 has previously been described as a marker of Treg cells (Deaglio et al., 2007). However, it has also been reported to be expressed on CD25- primed uncommitted precursor cells (Kobie et al., 2006, Bono et al.).



**Figure 4.3: Expression of key molecules involved in (2W1S<sup>+</sup>) regulatory cell function is not dependent on OX40 and CD30.** WT and OX40/CD30 dKO mice were immunised twice with 2W1S peptide and agonistic anti-DR3 antibody intravenously. (A) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B) Identification of FOXP3 expression within 2W1S<sup>+</sup> Treg cells from WT and OX40/CD30 dKO mice. Expression within WT 2W1S<sup>+</sup> conventional T cells is also shown for comparison. (C) Median Florescence Intensity (MFI) of FOXP3 within 2W1S<sup>+</sup> Treg cells from WT and OX40/CD30 dKO mice. (D) Identification of CTLA-4, CD25, CD39 or CD73 expression on 2W1S<sup>+</sup> Treg cells from WT and OX40/CD30 dKO mice. Expression on WT 2W1S<sup>+</sup> conventional T cells is also shown for comparison. (E) Median Florescence Intensity (MFI) of CTLA-4, CD25, CD39 and CD73 on 2W1S<sup>+</sup> Treg cells from WT and OX40/CD30 dKO mice. (B, D) Plots are pre-gated on Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells and then FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). Filled grey line = WT Tconv cells, Solid black line = WT Treg cells, Dotted black line = dKO Treg cells. (C, E) Bars show medians. Mann–Whitney test, \**p* < 0.05, ns = non-significant. At least 4 mice were pooled per group from three independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

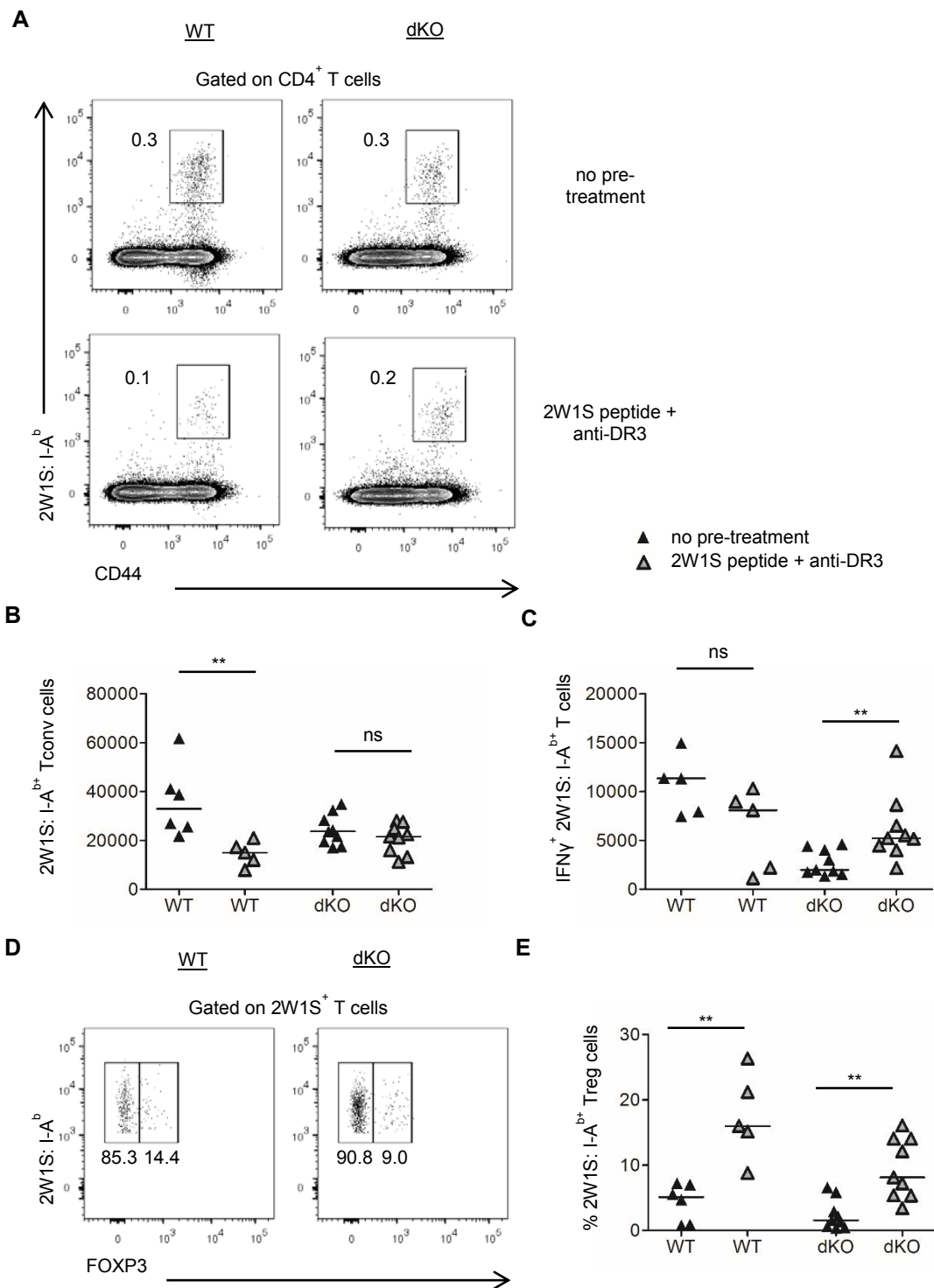


This study has shown that 2W1S<sup>+</sup> Treg cells do not exhibit gross defects in persistence or expression of some key suppressive molecules in OX40/CD30 dKO mice. To further assess function, experiments were performed to test whether expanded 2W1S<sup>+</sup> Treg cells are able to suppress a subsequent 2W1S<sup>+</sup> T cell response. This *in vivo* assay was based on experiments in which the function of 2W1S<sup>+</sup> Treg cells was determined by measuring IFN $\gamma$ <sup>+</sup> production by 2W1S<sup>+</sup> T cells in response to Lm-2W1S (Rowe et al., 2012). In these experiments 2W1S<sup>+</sup> Treg cells expanded in response to constitutive expression of 2W1S peptide as a fetal antigen, and peaked at 50% of the 2W1S<sup>+</sup> T cell population. As the protocol for Treg expansion developed in this study results in a percentage of up to 36%, it was likely that a similar impaired response would be observed. 2W1S<sup>+</sup> Treg cells were therefore expanded in WT and OX40/CD30 dKO mice using two immunisations of 50  $\mu$ g 2W1S peptide and 10  $\mu$ g agonistic anti-DR3 seven days apart. To determine whether the presence of 2W1S<sup>+</sup> Treg cells augments the subsequent response to Lm-2W1S, seven days after the final injection of 2W1S peptide and agonistic anti-DR3, mice were infected with 10<sup>7</sup> Lm-2W1S. On the day of analysis, seven days later, mice were injected with 50  $\mu$ g 2W1S peptide and 2.5  $\mu$ g LPS to stimulate cytokine production. The 2W1S-specific response was then assessed in a pool of lymph nodes and spleen, which were harvested four hours later.

In the WT controls, fewer 2W1S<sup>+</sup> Tconv cells were detected in mice which had been previously immunised with 2W1S peptide and agonistic anti-DR3 compared to than those infected with Lm-2W1S without pre-treatment (**Figure 4.4a**), and this 2.2-fold reduction in numbers was statistically significant (median 32942 vs median 15014) (**Figure 4.4b**). In contrast, there was not a significant difference in 2W1S<sup>+</sup> Tconv cell

number in the OX40/CD30 dKO mice between those pre-treated and those which were not. This augmentation of the response in WT but not OX40/CD30 dKO mice is consistent with impairment in the function of OX40/CD30 dKO Treg cells. In WT mice, there was a trend towards reduced IFN $\gamma$  production by 2W1S<sup>+</sup> Tconv cells between those pre-treated and those which were not, although this was not statistically significant (**Figure 4.4c**). Strikingly, these data revealed that IFN $\gamma$  production was very low in OX40/CD30 dKO mice, which could be significantly increased upon pre-treatment.

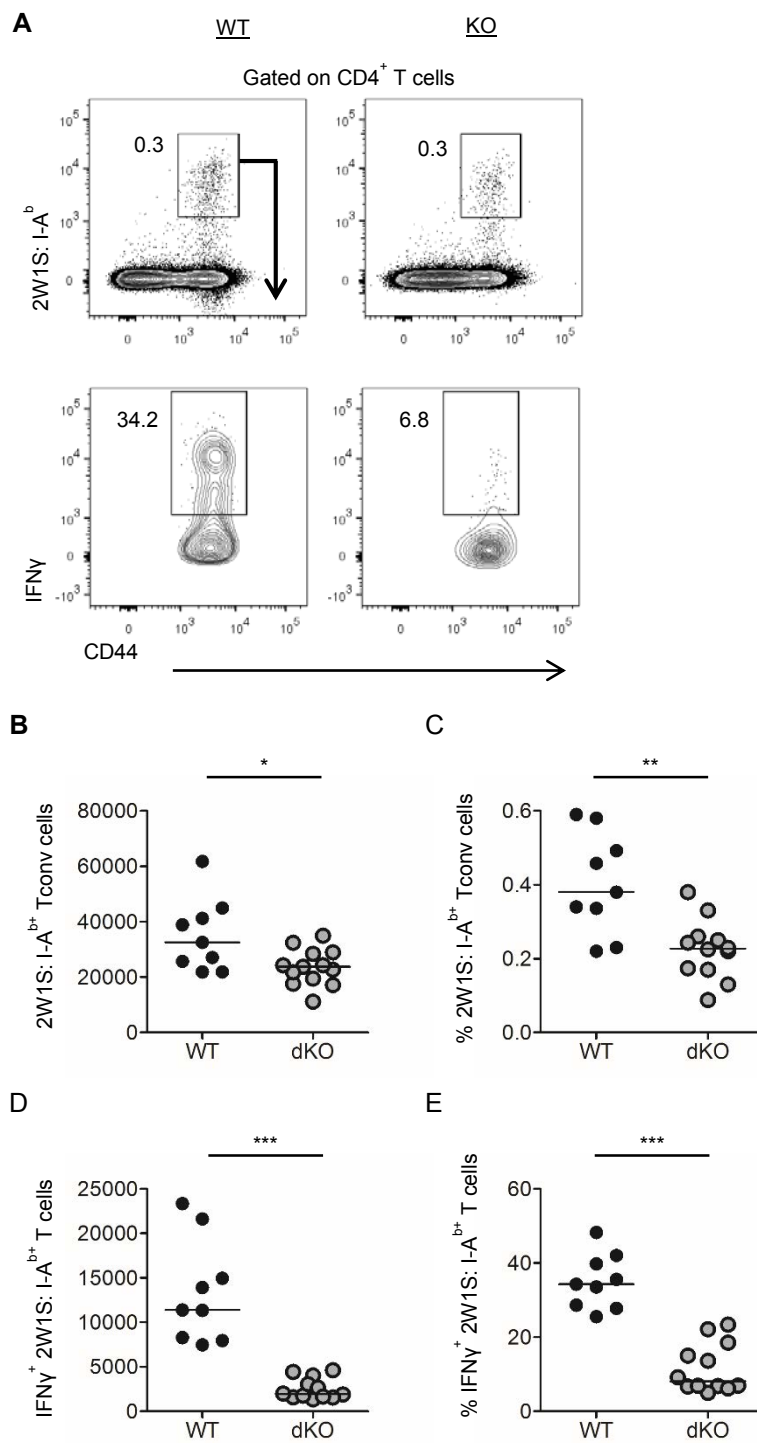
It was important to confirm that Treg cells had been successfully expanded in WT and OX40/CD30 dKO mice given the pre-treatment (two immunisations of 2W1S peptide and agonistic anti-DR3). A population of 2W1S<sup>+</sup> Treg cells could indeed be identified in WT and dKO mice (**Figure 4.4d**), with a median percentage of 16 in WT mice and 8.1 in OX40/CD30 dKO mice (**Figure 4.4e**). Therefore, these data show augmentations of the 2W1S<sup>+</sup> Tconv cell response in WT mice, which is not detected in OX40/CD30 dKO mice. This is consistent with the function of OX40/CD30 dKO Treg cells being impaired. It is however, important to note that this experimental approach does not directly demonstrate Treg cell involvement. Furthermore, variability has been observed in the augmentation of the response in WT mice across all experiments performed. This may however, be due to variability in Treg cell percentage.



**Figure 4.4: The absence of OX40 and CD30 impairs regulatory T cell function.** WT and OX40/CD30 dKO mice were infected with Lm-2W1S intravenously. After 7 days a four hour *in vivo* stimulation with 2W1S peptide was performed and a pool of lymph nodes and spleen were analysed. A group of each cohort were immunised twice with 2W1S peptide and agonistic anti-DR3 antibody intravenously prior to infection with Lm-2W1S, 7 days apart. (A) Identification of CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Enumeration of IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (D) Identification of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells) in the group of WT and OX40/CD30 dKO mice immunised twice with 2W1S peptide and agonistic anti-DR3. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (E) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, C, E) Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns = non-significant. Between 5-10 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

### *Investigating the role of OX40 and CD30 in effector cytokine production*

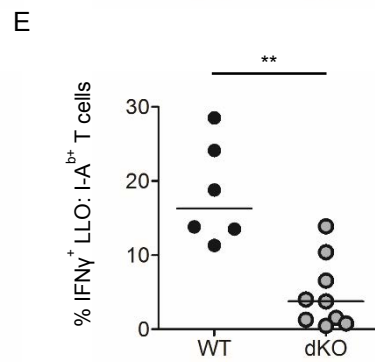
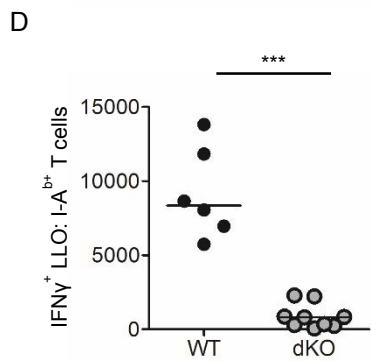
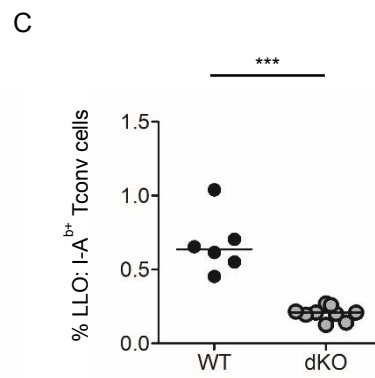
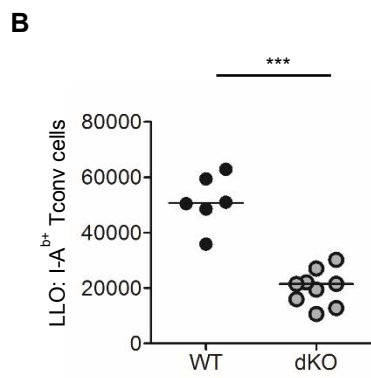
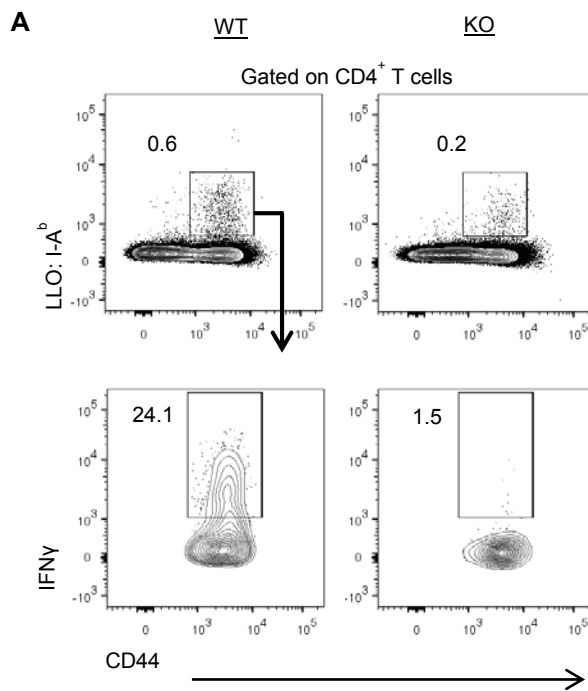
A striking observation from this *in vivo* assay is the impaired production of IFN $\gamma$  by 2W1S<sup>+</sup> Tconv cells from OX40/CD30 dKO mice. As earlier data showed that Tconv cell expansion and survival was impaired in OX40/CD30 dKO mice but only by 4.4 and 2.5 fold respectively, it may be that the key role of OX40 and CD30 on Tconv cells is in their function. To study this in more detail, WT and OX40/CD30 dKO mice were infected with 10<sup>7</sup> Lm-2W1S and were injected with 50  $\mu$ g 2W1S peptide and 2.5  $\mu$ g LPS seven days later to stimulate cytokine production. The 2W1S-specific response was then assessed in a pool of lymph nodes and spleen, which were harvested four hours later. Significantly fewer 2W1S<sup>+</sup> Tconv cells were identified in the OX40/CD30 dKO than WT mice (**Figure 4.5a**). This 1.4-fold difference in median number (**Figure 4.5b**) and 1.7-fold difference in median percentage (**Figure 4.5c**) demonstrates that although reduced, 2W1S<sup>+</sup> Tconv cell expansion does occur in these mice in response to Lm-2W1S. Their ability to produce the effector cytokines upon *in vivo* stimulation with 2W1S peptide and LPS however was grossly impaired, as is demonstrated by the 5.9-fold reduction in the number of IFN $\gamma$ <sup>+</sup> 2W1S<sup>+</sup> Tconv cells (median 11382 vs median 1936) (**Figure 4.5d**) and 4.3-fold reduction in the percentage of 2W1S<sup>+</sup> Tconv cells which express this effector cytokine (median 34.3% vs median 8.1%) (**Figure 4.5e**). This suggests that signals through OX40 and CD30 are fundamental to the function of CD4 T cells, rather than their expansion.



**Figure 4.5: OX40 and CD30 are required for effector cytokine production by 2W1S<sup>+</sup> T cells.** WT and OX40/CD30 dKO mice were infected intravenously with Lm-2W1S. After 7 days a four hour *in vivo* stimulation with 2W1S peptide was performed and a pool of lymph nodes and spleen were analysed. (A) Identification of CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells, followed by IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Percentage of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (D) Enumeration of IFN $\gamma$ <sup>+</sup> FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (E) Percentage of IFN $\gamma$ <sup>+</sup> FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. (B-E) Bars show medians. Mann–Whitney test, \*\*\* $p$  < 0.001, ns = non-significant. At least 9 mice were pooled per group from three independent experiments. Tconv = conventional T cells.

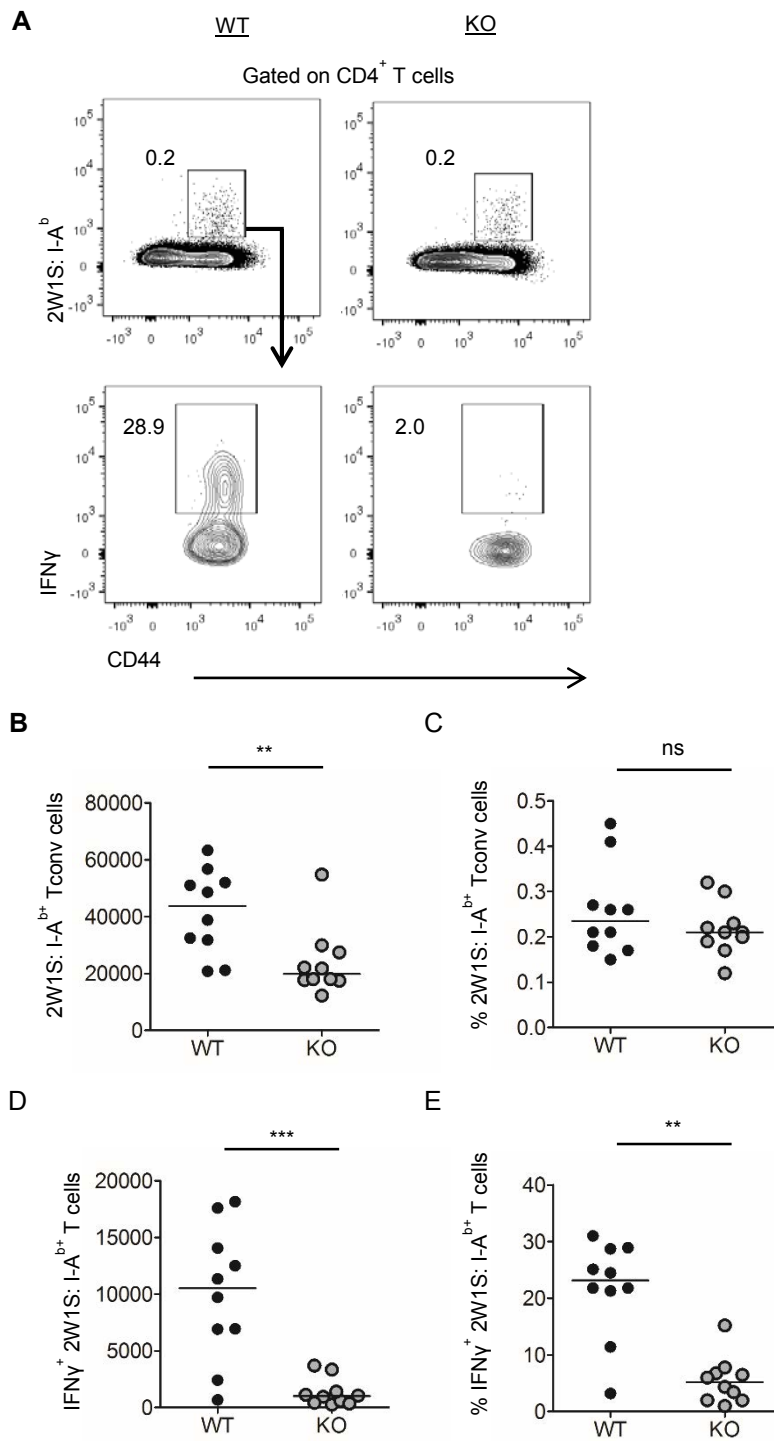
To provide further evidence that Tconv cells from OX40/CD30 dKO mice were fundamentally impaired in effector cytokine production; another endogenous T cell population was studied after infection with Lm-2W1S. MHC class II tetramers loaded with peptide 190-201 of LLO were used to detect T cells responding to Listeriolysin (LLO), which is a major virulence factor expressed by *Listeria monocytogenes* (Hamon et al., 2012). Therefore, WT and OX40/CD30 dKO mice were infected with  $10^7$  Lm-2W1S and the LLO-specific response was assessed in a pool of lymph nodes and spleen, which were harvested seven days later. Again, significantly fewer LLO<sup>+</sup> Tconv cells were identified in the OX40/CD30 dKO than WT mice (**Figure 4.6a**). This 2.4-fold difference in median number (**Figure 4.6b**) and 3-fold difference in median percentage (**Figure 4.6c**) demonstrates that although reduced, LLO<sup>+</sup> Tconv cell expansion does occur in these mice in response to Lm-2W1S. However, the striking difference was again the 10.4-fold reduction in the number of IFN $\gamma$ <sup>+</sup> LLO<sup>+</sup> Tconv cells (median 8343 vs median 804) (**Figure 4.6d**) and 4.4-fold reduction in the percentage of LLO<sup>+</sup> Tconv cells which express this effector cytokine (median 16.3% vs median 3.8%) (**Figure 4.6e**). Combined, these data demonstrate that in the absence of OX40 and CD30, effector T cell cytokine production is abrogated.





**Figure 4.6: OX40 and CD30 are required for effector cytokine production by LLO<sup>+</sup> T cells.** WT and OX40/CD30 dKO mice were infected intravenously with Lm-2W1S. After 7 days a four hour *in vivo* stimulation with 2W1S peptide was performed and a pool of lymph nodes and spleen were analysed. (A) Identification of CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells, followed by IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells (Tconv cells). (C) Percentage of FOXP3<sup>-</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells (Tconv cells). (D) Enumeration of IFN $\gamma$ <sup>+</sup> FOXP3<sup>-</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (E) Percentage of IFN $\gamma$ <sup>+</sup> FOXP3<sup>-</sup> CD44<sup>hi</sup> LLO: I-A<sup>b+</sup> T cells. (B-E) Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , ns = non-significant. At least 9 mice were pooled per group from three independent experiments. Tconv = conventional T cells.

The roles of OX40 and CD30 have been investigated in this study as there are numerous reports that these two receptors display synergy (Gaspal et al., 2005, Gaspal et al., 2008, Withers et al., 2009, Gaspal et al., 2011). To investigate whether OX40 deficiency alone results in grossly impaired effector cytokine production, WT and OX40 KO mice were infected with  $10^7$  Lm-2W1S and seven days later mice were injected with 50  $\mu$ g 2W1S peptide and 2.5  $\mu$ g LPS to stimulate cytokine production. The 2W1S-specific response was then assessed in a pool of lymph nodes and spleen, which were harvested four hours later. Significantly fewer 2W1S<sup>+</sup> Tconv cells were identified in the OX40 KO than WT mice (**Figure 4.7a**), which amounted to a 2.2-fold reduction in median number (**Figure 4.7b**). There was not a significant difference in the percentage of 2W1S<sup>+</sup> Tconv cells between OX40 KO and WT mice, however (**Figure 4.7c**). Their ability to produce the effector cytokines upon *in vivo* stimulation with 2W1S peptide and LPS however was again abrogated, with a 10.5-fold reduction in the number of IFN $\gamma$ <sup>+</sup> 2W1S<sup>+</sup> Tconv cells (median 10538 vs median 1004) (**Figure 4.7d**) and 4.5-fold reduction in the percentage of 2W1S<sup>+</sup> Tconv cells which express this effector cytokine (median 23.2% vs median 5.2%) (**Figure 4.7e**). Thus the impaired effector cytokine production observed in OX40/CD30 dKO mice was attributable to OX40 expression, which is a key determinant of CD4 T cell function in a primary response.



**Figure 4.7: OX40 expression is key to the ability of T cells to produce effector cytokines.** WT and OX40 KO mice were infected intravenously with Lm-2W1S. After 7 days a four hour *in vivo* stimulation with 2W1S peptide was performed and a pool of lymph nodes and spleen were analysed. (A) Identification of CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells, followed by IFN $\gamma$ <sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Percentage of FOXP3<sup>-</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (D) Identification of FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Treg cells). (E) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup>2W1S: I-A<sup>b+</sup> T cells (Treg cells. (B, C, E) Bars show medians. Mann–Whitney test, \*\*\**p* < 0.001, ns = non-significant. At least 9 mice were pooled per group from three independent experiments. Tconv = conventional T cells.

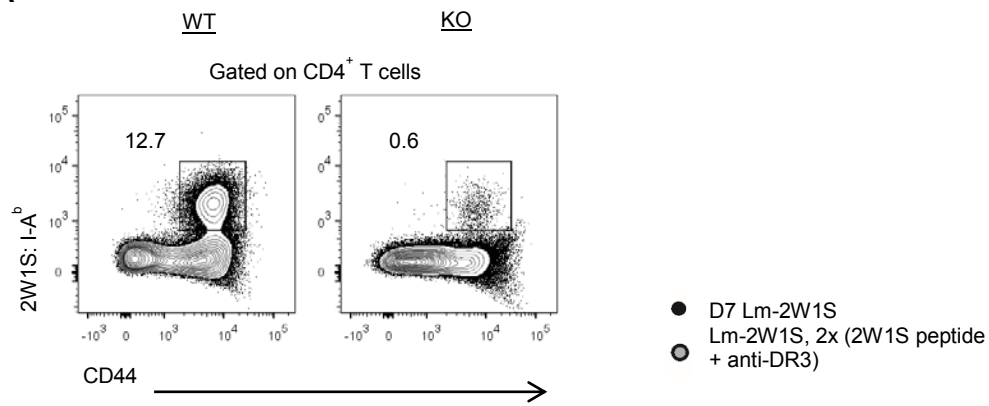
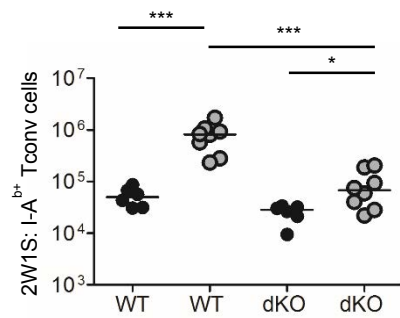
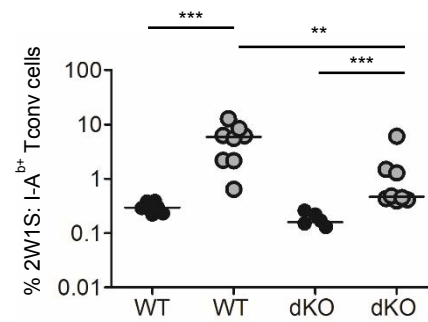
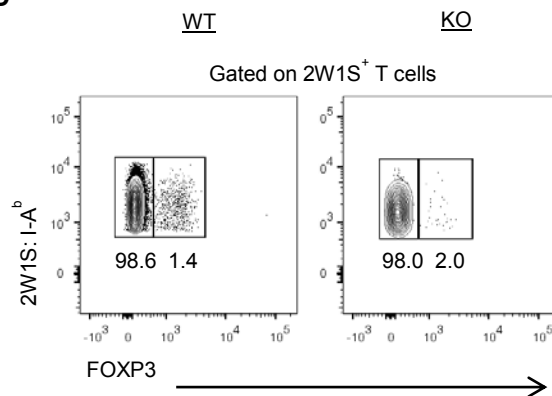
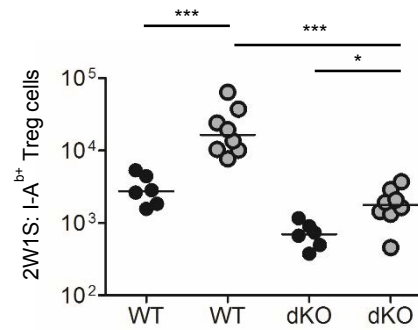
### *Repeated expansion of 2W1S<sup>+</sup> T conventional cells in OX40/CD30 dKO mice*

This study has shown that 2W1S<sup>+</sup> T cells display grossly impaired effector cytokine production, building on previously published data which indicates that OX40 and CD30 are required for functional autoreactive CD4 T cell responses (Gaspal et al., 2011). In this study it was shown that Treg cells deficient in OX40 and CD30 fail to succumb to autoimmunity which results from the absence of Treg cells. To further investigate the importance of these TNF receptors in Tconv cell function, chronic antigen exposure, as occurs in autoimmunity, was modelled through repeated expansion of 2W1S<sup>+</sup> Tconv cells. WT and OX40/CD30 dKO mice were therefore infected with 10<sup>7</sup> Lm-2W1S and two injections of 50 µg 2W1S and 10 µg agonistic anti-DR3 were then administered seven days apart, starting seven days after the Lm-2W1S. The 2W1S-specific response was assessed in a pool of lymph nodes and spleen, which were harvested seven days later. These two groups of mice were also compared to a group of WT and a group of OX40/CD30 dKO mice which were infected with 10<sup>7</sup> Lm-2W1S and analysed seven days later to allow acute antigen exposure to be compared to chronic antigen exposure.

In the WT mice, there was a very large expansion of 2W1S Tconv cells after the three injections (**Figure 4.8a**). The median number of 2W1S<sup>+</sup> Tconv cells was 16.5 fold higher than the WT mice that had been infected with Lm-2W1S seven days previously (**Figure 4.8b**). Additionally, the median percentage of CD4 T cells which were 2W1S-specific was 19.8 fold higher in the WT mice which encountered antigen repeatedly. In contrast in the OX40/CD30 dKO mice administered the three injections; there was only a 2.4-fold increase in the median number of 2W1S<sup>+</sup> Tconv cells compared to mice infected with Lm-2W1S seven days previously. Similarly, the

median percentage of CD4 T cells which were 2W1S-specific was increased by only 2.9 fold (**Figure 4.8c**). Therefore, after repeated exposure to antigen, the number of 2W1S Tconv cells was 12.2 fold higher in the WT mice than OX40/CD30 dKO mice (median 820714 vs median 67160), and the percentage was 12.6 fold higher (median 5.9% vs median 0.5%) (**Figure 4.8b, c**). These data indicate that in the absence of OX40 and CD30, there is a striking impairment in the ability of the antigen-specific T cell pool to expand in response to repeated exposure to antigen.

In this protocol, two immunisations of 2W1S peptide and agonistic anti-DR3 have been administered after Lm-2W1S infection. Accordingly, 2W1S<sup>+</sup> Treg cells have also been expanded within this experiment, although at a low percentage of the 2W1S-specific T cell population (**Figure 4.8d**). The number of 2W1S<sup>+</sup> Treg cells followed the same trend as the 2W1S<sup>+</sup> Tconv cell population, with greater expansion observed in the WT mice than the OX40/CD30 dKO mice. A 6-fold increase in median 2W1S<sup>+</sup> Treg cell number was observed in the WT mice, and a 2.5-fold increase in OX40/CD30 dKO mice between those administered the three injections and those infected with Lm-2W1S seven days previously (**Figure 4.8e**). Therefore, these data demonstrate that OX40 and CD30 are required for T cells to be able to expand in response to repeated antigen exposure.

**A****B****C****D****E**



**Figure 4.8: OX40 and CD30 are required for the repeated expansion of T cells.** WT and OX40/CD30 dKO mice were infected intravenously with Lm-2W1S, and either analysed 7 days later, or subsequently given two immunisations of 2W1S peptide and agonistic anti-DR3 intravenously, 7 days apart. 7 days after the final injection, a 4 hour *in vivo* stimulation with 2W1S peptide was performed on all mice and a pool of lymph nodes and spleen were analysed. (A) Identification of CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells in the group of WT and OX40/CD30 dKO mice immunised twice with 2W1S peptide and agonistic anti-DR3 after Lm-2W1S administration. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (C) Percentage of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (D) Identification of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells) in the group of WT and OX40/CD30 dKO mice immunised twice with 2W1S peptide and agonistic anti-DR3 after Lm-2W1S administration. Plots are pre-gated on CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (E) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B, C, E) Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , \* $p < 0.05$ , ns = non-significant. Between 6-8 samples per group were pooled from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

### 4.3 DISCUSSION

The role of OX40 signaling in Treg cell biology is controversial within the literature, with reports that OX40 negatively regulates Treg cell function, and conversely that OX40 and IL-2 signal co-operatively to support Treg cells (Xiao et al., 2012). OX40 is known to have an important role in CD4 T cell expansion (Rogers et al., 2001, Pippig et al., 1999, Chen et al., 1999, Kopf et al., 1999) and therefore the potential that OX40 signaling may turn off Treg cell function has led to proposals that agonistic OX40 antibodies would have dual anti-tumor effects, by expanding effector T cells and turning off Treg cell function (Kitamura et al., 2009). However, with controversy surrounding the role of OX40, it is important to fully understand the role of this TNF receptor in Treg biology. An antigen-specific system has been used within this study, to allow effects on survival and function to be dissected. As OX40 and CD30 have been reported to exhibit synergy (Gaspal et al., 2005, Gaspal et al., 2008, Withers et al., 2009, Gaspal et al., 2011) the role of both of these TNF receptors has been addressed within this study.

OX40 KO mice have been reported to have similar numbers of Treg cells as WT mice (Vu et al., 2007, Tang et al., 2003, Valzasina et al., 2005, Takeda et al., 2004, Kroemer et al., 2007), although one study shows that the number is less in 7-8 week old OX40 KO mice (Takeda et al., 2004). Treg cell generation in the thymus is therefore thought to be independent of OX40. In this study, however, total and 2W1S<sup>+</sup> Treg cell numbers in OX40/CD30 dKO mice were found to be reduced by approximately 2 fold. It has also been reported that OX40 promotes Treg cell survival (Kroemer et al., 2007). Within this study, persistence of antigen-specific Treg cells was monitored following expansion. After seven weeks, there was a significant

reduction in the median number of conventional but not regulatory 2W1S<sup>+</sup> T cells, which indicates that survival of Treg cells is not highly dependent on OX40 and CD30. It is important to note that it is possible that there is also synergy between these TNF receptors and other members of this superfamily, and therefore other TNF receptors may be compensating for the loss of OX40 and CD30.

OX40 stimulation has been shown to result in Treg cell proliferation *in vivo*, similar to the effect on Tconv cells (Hippen et al., 2008, Ruby et al., 2009, Hirschhorn-Cymerman et al., 2009, Xiao et al., 2012, Takeda et al., 2004). In addition, there is evidence that the absence of OX40 renders Treg cells less responsive to IL-2 (Piconese et al., 2010, Xiao et al., 2012), which is required for Treg cell activation and survival (Malek and Castro, 2010). The initial expansion of 2W1S<sup>+</sup> Treg cells in response to 2W1S peptide and agonistic anti-DR3 was shown in this study to be significantly reduced in OX40/CD30 dKO mice, although less so than the number of 2W1S<sup>+</sup> Tconv cells. As evidence within this study suggests that 2W1S<sup>+</sup> Treg cells are thymically derived, the difference observed in 2W1S<sup>+</sup> Treg cell numbers cannot be accounted for by this reduction in 2W1S<sup>+</sup> Tconv cells. This therefore suggests that there is a role for OX40 and CD30 in expansion of Treg cells; however, it is important to note that Treg cells were less affected than Tconv cells.

The role of OX40 in Treg cell function is also controversial. There are conflicting reports from *in vitro* suppression assays using OX40 KO Treg cells, with one study showing suppression is impaired in the absence of OX40 (Takeda et al., 2004), and another showing that OX40 is dispensable (Vu et al., 2007). Consistent with the latter, OX40 KO Treg cells are unable to prevent the onset of T cell-induced colitis (Griseri et al., 2010, Piconese et al., 2010). This suggests that OX40 is required for

Treg function, although paradoxically, stimulation of OX40 *in vitro* using an agonistic OX40 antibody (OX86) or mice overexpressing OX40L, has been shown to abrogate the suppressive ability of Treg cells (Kitamura et al., 2009, Vu et al., 2007, Xiao et al., 2012, Valzasina et al., 2005, Piconese et al., 2008, Takeda et al., 2004, Kroemer et al., 2007). Additionally, Treg mediated suppression is impaired upon OX40 stimulation in murine models of skin allograft rejection (Vu et al., 2007), graft versus host disease (Valzasina et al., 2005), colitis (Takeda et al., 2004) as well as anti-tumor responses (Houot and Levy, 2009, Kitamura et al., 2009, Piconese et al., 2008). Interestingly, it appears that much of the dichotomy within the literature may be due to the availability of IL-2. The majority of studies which suggest that OX40 is a negative regulator of Treg function include experiments in which OX40 is stimulated by OX86. This leads to Treg proliferation, and hence an increased requirement for IL-2, which can lead to exhaustion if IL-2 levels are limiting, and a resulting functional impairment. IL-2 may become limiting *in vitro* or if the antibody is given *in vivo* during an immune response, when Treg cells must compete with activated T cells, which also express OX40, for IL-2.

This study revealed no differences in FOXP3 expression by total and 2W1S<sup>+</sup> Treg cells from OX40/CD30 dKO mice, which is a crucial transcription factor for Treg cell function (Fontenot et al., 2003, Hori et al., 2003). Further to this, the expression of key molecules involved in suppression was largely similar between total and 2W1S<sup>+</sup> Treg cells from OX40/CD30 dKO and WT mice. This included CTLA-4, which reduces antigen presentation through interactions with the co-stimulatory molecules CD80 and CD86 on antigen presenting cells (Qureshi et al., 2011), and CD25, which proposed to deplete effector T cells of IL-2 (Pandiyani et al., 2007, Barthlott et al.,

2005). The ectoenzymes CD39 and CD73 are also thought to disrupt the metabolism of effector T cells, in this case through production of adenosine, which inhibits their proliferation (Kobie et al., 2006, Deaglio et al., 2007, Borsellino et al., 2007). The expression of CD73 was found to be significantly lower on total Treg cells but not 2W1S<sup>+</sup> Treg cells from OX40/CD30 dKO mice. Conversely, expression of CD39 was found to be significantly lower on 2W1S<sup>+</sup> Treg cells but not total Treg cells from OX40/CD30 dKO mice. The absence of OX40 and CD30 therefore may affect the expression of ectoenzymes which Treg cells utilize to disrupt effector T cells, however no evidence was found of other mechanisms being affected. This supports the findings of Vu et al. (2007), who postulate that OX40 is dispensable for suppression. However, it is important to note that Treg cells employ a range of mechanisms (Schmidt et al., 2012), within which there may be redundancy. In addition, Treg cells also suppress a variety of cell types (Schmidt et al., 2012) and it has been reported that OX40 expression on Treg cells mediates suppression of mast cell degranulation (Gri et al., 2008).

There has been concern within the field that too strong conclusions have been drawn about Treg cell function from *in vitro* assays, which do not provide sufficient evidence (Vignali, 2012). In this study, an *in vivo* assay was therefore used to investigate Treg cell function. This *in vivo* assay demonstrated that after expansion of 2W1S<sup>+</sup> Treg cells, the subsequent response to Lm-2W1S is augmented in WT but not OX40/CD30 dKO mice. This suggests that the function of OX40/CD30 dKO Treg cells is impaired. Further experiments in mice which can be conditionally depleted of Treg cells are needed to confirm that the impairment in the response to Lm-2W1S after Treg cell expansion is indeed dependent on the presence of Treg cells. The

most effective model for studying this would be to use 'FOXP3<sup>DTR</sup>' mice, which are engineered so that the Diphtheria Toxin Receptor (DTR) is expressed by Treg cells, and therefore these cells can be specifically ablated by administration of Diphtheria Toxin (Kim et al., 2007).

As previously discussed, OX40 and CD30 have been shown to promote survival during T cell activation and the generation of CD4 T cell memory. Further to this, the absence of OX40 and CD30 has been shown to protect mice from lethal autoimmunity which results from the absence of Treg cells (Gaspal et al., 2011), implying a role in CD4 T cell function. T cells from these OX40/CD30 dKO mice were shown to produce significantly less IFN $\gamma$  upon stimulation. This effector cytokine is the key mediator produced by T helper 1 (Th1) cells, particularly through its effects on macrophages where signaling through the IFN $\gamma$  receptor leads to increased antigen presentation and bactericidal activity (Schroder et al., 2004). Interestingly, a human patient deficient in OX40 has been shown to display impaired IFN $\gamma$  production upon exposure to previously encountered antigens (Byun et al., 2013). This therefore suggests that the generation of a functional CD4 T cell response is dependent on OX40 and CD30.

Data within this study show that seven days after infection with *Listeria monocytogenes*-expressing 2W1S, which induces a strong Th1 response (Spellberg and Edwards, 2001), the ability of 2W1S<sup>+</sup> Tconv cells from OX40/CD30 dKO mice to produce IFN $\gamma$  is abrogated. This was also confirmed by studying another endogenous T cell population which recognise peptide 190-201 of Listeriolysin O (LLO), a major virulence factor of *Listeria monocytogenes* (Hamon et al., 2012). Together these data suggest that these TNF receptors have a crucial role in T cell

function, which can be separated from any effects on survival. There was also a significant reduction in the number of T cells within both populations in OX40/CD30 dKO mice, consistent with the established role these TNF receptors play in promoting the survival of T cells during activation (Croft, 2009). Despite this, the effect was much greater on the production of IFN $\gamma$ , and therefore these data suggest that CD4 T cell function, rather than primary expansion is particularly dependent on OX40 and CD30. There is thought to be partial redundancy in the signaling pathways used by OX40 and CD30, as CD4 T cell responses are significantly more impaired in mice in which both TNF receptors are absent (Gaspal et al., 2008, Gaspal et al., 2005). Despite this, the effect on IFN $\gamma$  production by 2W1S<sup>+</sup> Tconv cells observed in OX40/CD30 dKO mice could be recapitulated in OX40 KO mice. This therefore suggests that OX40 plays a key role in CD4 T cell function.

This study has demonstrated that CD4 T cell function, rather than primary expansion is highly dependent on OX40 and CD30. To further investigate the role of these TNF receptors, repeated expansion was studied in OX40/CD30 dKO mice, and compared to primary expansion. During acute infection, OX40 is expressed for a short window of time (Marriott et al., 2014), but during chronic infection its expression is more prolonged (Boettler et al., 2012). Strikingly, expansion of 2W1S<sup>+</sup> Tconv cells within OX40/CD30 dKO mice after infection with Lm-2W1S, followed by two immunisations with 2W1S peptide and agonistic anti-DR3 was grossly reduced compared to WT. Interestingly, the number of 2W1S<sup>+</sup> Tconv cells in OX40/CD30 dKO mice was only approximately 3 fold higher after repeated antigen exposure compared to primary exposure. This suggests that CD4 T cells are impaired in their capacity to expand in

response to repeated antigen exposure, and this is affected much more than primary expansion.

Therefore, data within this study suggest that the expansion and function of Treg cells is partially dependent on OX40 and CD30. Further to this, the data confirms that there is a role for OX40 and CD30 in CD4 T cell expansion. However, these data show that CD4 T cells from OX40/CD30 dKO mice are fundamentally impaired in both their capacity to produce effector cytokines and to expand in response to repeated antigen exposure, as may occur during persistent infection or autoimmune disease.



## **5.0 THE ROLE OF GROUP 3 INNATE LYMPHOID CELLS IN REGULATORY T CELL SURVIVAL**

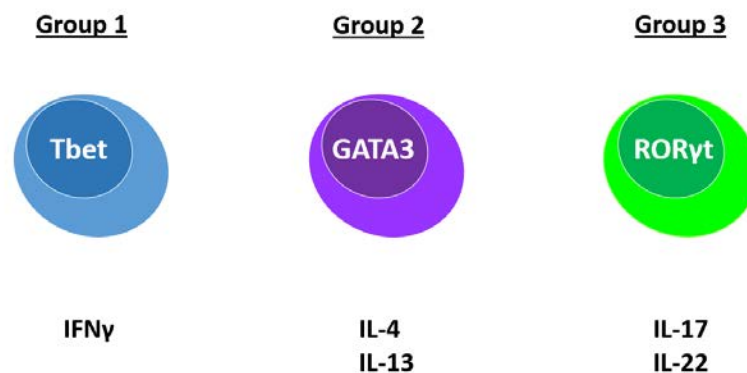
## 5.1 INTRODUCTION

Regulatory T cells, like conventional T cells recirculate around the blood and lymph, although, interestingly, it has also been shown that Treg cells can be enriched in the lymph node nearest their antigen (Lathrop et al., 2008). Treg cells are highly stable (Sakaguchi et al., 2013) and are considered to be a continuously activated and highly proliferative T cell subset (Fisson et al., 2003, Walker et al., 2003a). The cytokine IL-2 is a critical survival factor for Treg cells (Malek and Castro, 2010), which express high levels of the high affinity subunit of the IL-2 receptor (CD25). Further to this, signals through the co-stimulatory receptor CD28 and the T cell Receptor (TCR) have been shown to contribute to Treg cell homeostasis (Attridge and Walker, 2014). Immature dendritic cells have been proposed as a potential source of TCR and CD28 stimulation, through presentation of self-antigen (Scheinecker et al., 2002) and low levels of CD80/CD86 expression (Bluestone and Abbas, 2003). However, further characterisation of the signals required, and the source of such signals is needed to fully understand Treg cell homeostasis.

Interestingly, preliminary data has shown that Treg cells can be found in close association with group 3 Innate Lymphoid Cells (ILCs) in the mesenteric lymph node (Dr Fiona McConnell, unpublished data), which suggests that these cells interact. ILCs are haematopoietic cells with lymphoid morphology but which lack T cell and B cell-receptors, as well as molecules expressed by myeloid cells, such as CD11c (Spits and Cupedo, 2012). They can be identified by the absence of such markers, as well as expression of Interleukin-7 Receptor  $\alpha$  (IL-7R $\alpha$ ) (Vonarbourg and Diefenbach, 2012). Multiple roles for ILCs within the innate immune system have been described, including defence against microbial infections, maintenance of

epithelial barrier integrity and tissue repair. Further to this, it is becoming apparent that ILCs are able to influence the adaptive immune system (Artis and Spits, 2015).

ILCs have been categorised into three groups based on the expression of lineage defining transcription factors and signature cytokines (Spits et al., 2013), as is shown in **Figure 5.1**. It is interesting to note that these lineage defining transcription factors and signature cytokines mirror those of T cell subsets. Th1 cells depend on Tbet and express IFN $\gamma$ ; Th2 cells depend on GATA3 and express IL-4 and IL-13; Th17 cells depend on ROR $\gamma$ t and express IL-17 and/or IL-22 in response to IL-23 (Spits et al., 2013).

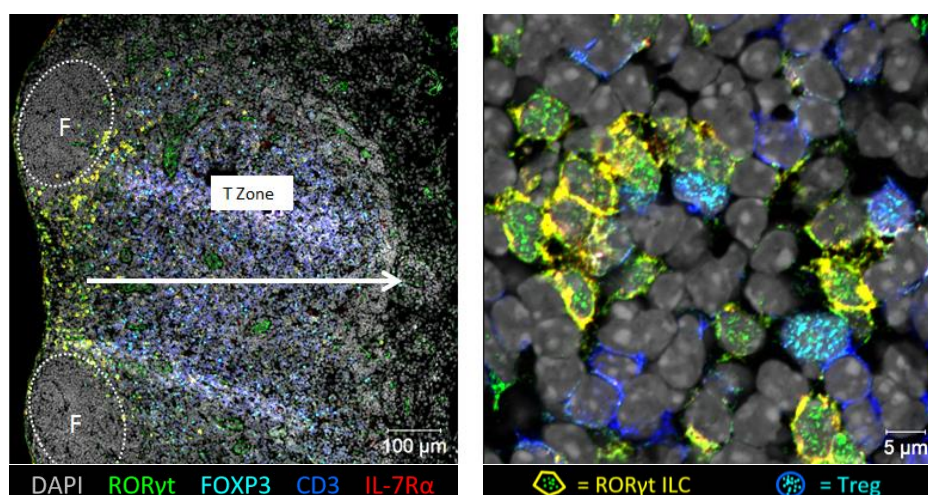


**Figure 5.1: Innate lymphoid cell subsets.** ILCs can be categorised into three groups based on expression of lineage defining transcription factor and signature cytokine expression.

This study will concentrate on group 3 ILCs, which include the Lymphoid Tissue Inducer (LTi) cells, which play an essential role in lymph node organogenesis (Mebius, 2003). Group 3 ILCs in the adult are often referred to as ILC3s and can be further subdivided on the basis of Chemokine Receptor-6 (CCR6) expression. CCR6<sup>+</sup> ILC3s are considered to be LTi-like whereas CCR6<sup>-</sup> ILC3s arise postnatally and have

the capacity to exhibit plasticity through downregulation of ROR $\gamma$ t and upregulation of Tbet as well as the natural cytotoxicity receptor NKp46 (Klose et al., 2013).

ILC3s are primarily found in the gut but also reside in the lymph nodes and spleen (Walker et al., 2013). In secondary lymphoid tissue they are located at the interface between T and B cell areas, such as the interfollicular regions (Mackley et al., 2015). As shown in **Figure 5.2**, it was in this location that ILC3s, identified as CD3<sup>+</sup>IL-7R $\alpha$ <sup>+</sup>ROR $\gamma$ t<sup>+</sup> cells, were found in close association with FOXP3<sup>+</sup> Treg cells. It has been proposed that in this location, ILC3s provide survival signals to memory T cells as persistence of CD4 memory T cells has been shown to be hindered in mice which lack ILC3s (Withers et al., 2012). Therefore, it may be that ILC3s in the interfollicular areas of the lymph nodes and spleen also provide survival signals to Treg cells as they recirculate.



**Figure 5.2: Association between ILC3s and Treg cells.** Location of ROR $\gamma$ t<sup>+</sup> ILCs (ILC3s) (identified as CD3<sup>+</sup>ROR $\gamma$ t<sup>+</sup>IL-7R $\alpha$ <sup>+</sup>) within the mesenteric lymph node and proximity to regulatory T cells (identified as CD3<sup>+</sup> Foxp3<sup>+</sup>). High magnification insert shows a section of an interfollicular region (unpublished, Fiona McConnell). F: Follicle.

Interestingly, ILC3s have been shown to express MHC II and to have the ability to process and present antigen, but do so in a manner which does not lead to T cell proliferation (Hepworth et al., 2013). Further to this, mice which lack MHC II on ILC3s exhibit increased CD4 T responses to commensal bacteria, which leads to gut inflammation, suggesting that ILC3s limit CD4 T cell responses to commensal bacteria in a MHC II-dependent manner (Hepworth et al., 2013). Indeed, it has subsequently been shown that presentation of commensal bacteria-derived antigens by ILC3s can cause T cell death in a process similar to negative selection in the thymus (Hepworth et al., 2015). The ability of ILC3s to present antigen as a mechanism of preserving tolerance opens up the possibility that presentation of self-antigen by ILC3s could be a mechanism by which Treg cells are maintained, particularly due to the key role of TCR stimulation in Treg cell homeostasis. In this study, the role of ILC3s in Treg cell expansion and survival has been investigated using an antigen-specific approach in mice which lack ILC3s (ROR $\gamma$ t KO mice). Further to this, the expression pattern of potential interaction partners on ILC3s has been characterised.

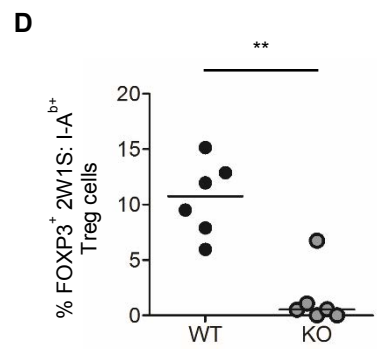
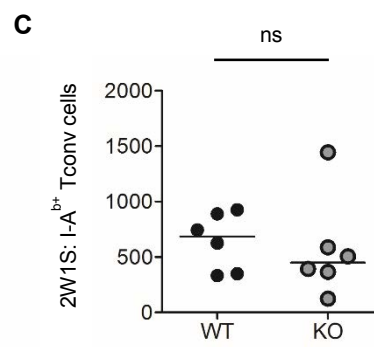
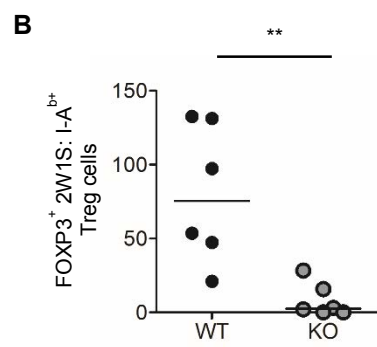
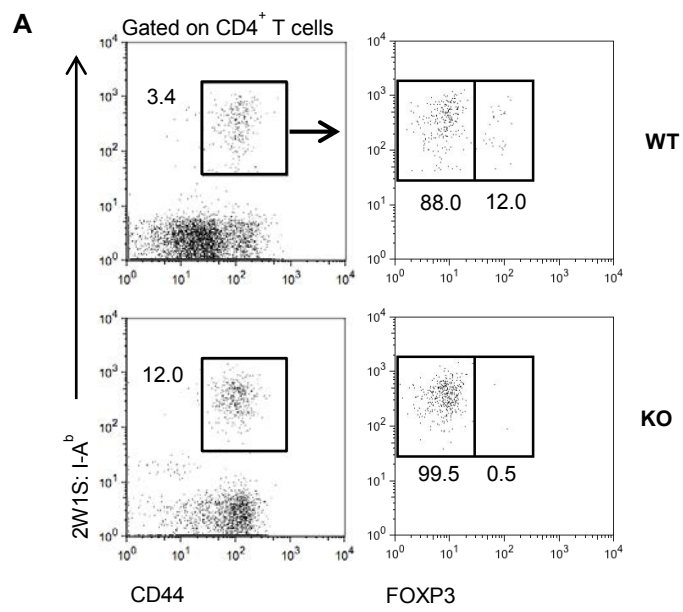
## 5.2 RESULTS

### *Antigen-specific Treg cell expansion in ROR $\gamma$ t KO mice*

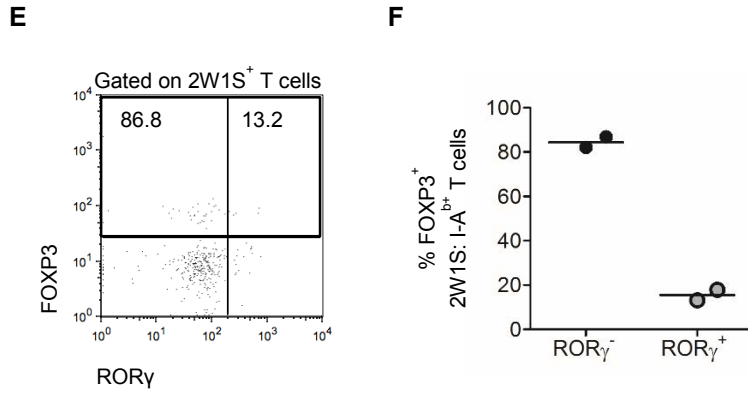
To determine whether ILC3s may be involved in 2W1S<sup>+</sup> Treg cell expansion, ROR $\gamma$ t KO and WT mice were immunised with 100  $\mu$ g 2W1S peptide and analysed seven days later. ROR $\gamma$ t KO mice lack lymph nodes (Sun et al., 2000, Kurebayashi et al., 2000) and it has been suggested that the loss of the lymph node niche may result in redistribution of T cells to the spleen (Zhang et al., 2003). Therefore, the spleen was compared to the spleen and a pool of lymph nodes from WT mice. Following harvesting of tissue, enrichment of 2W1S<sup>+</sup> T cells was performed. Strikingly, very few 2W1S<sup>+</sup> Treg cells could be identified in the ROR $\gamma$ t KO mice upon immunisation with 2W1S peptide (**Figure 5.3a**). The percentage of Treg cells within the 2W1S<sup>+</sup> T cell population was 20.1-fold lower in the ROR $\gamma$ t KO mice than WT (median 0.5% vs median 10.7%) (**Figure 5.3b**). In accordance with this, the number of 2W1S<sup>+</sup> Treg cells was reduced by 29.8-fold (median 3 vs median 76) (**Figure 5.3c**), but there was not a significant difference in the number of 2W1S<sup>+</sup> Tconv cells (**Figure 5.3d**). This therefore argues against simply an impaired 2W1S<sup>+</sup> T cell population.

It has been reported that a proportion of Treg cells express ROR $\gamma$ t (Lochner et al., 2008), without negative impacts on their stability or function (Tartar et al., 2010, Yang et al., 2015). Indeed, it is now emerging that Treg cells can upregulate transcription factors associated with different T helper responses, tailoring them towards that response (Koch et al., 2009, Chaudhry et al., 2009, Zheng et al., 2009). Although ROR $\gamma$ t<sup>+</sup> Treg cells were shown to be in the minority, 2W1S<sup>+</sup> Treg cells from WT mice in this experiment were analysed for ROR $\gamma$ t expression. However only a small

percentage of 2W1S<sup>+</sup> Treg cells (median 16%) were found to express ROR $\gamma$ t (**Figure 5.3e, f**), and therefore its absence cannot explain the low number of 2W1S<sup>+</sup> Treg cells identified in ROR $\gamma$ t KO mice.

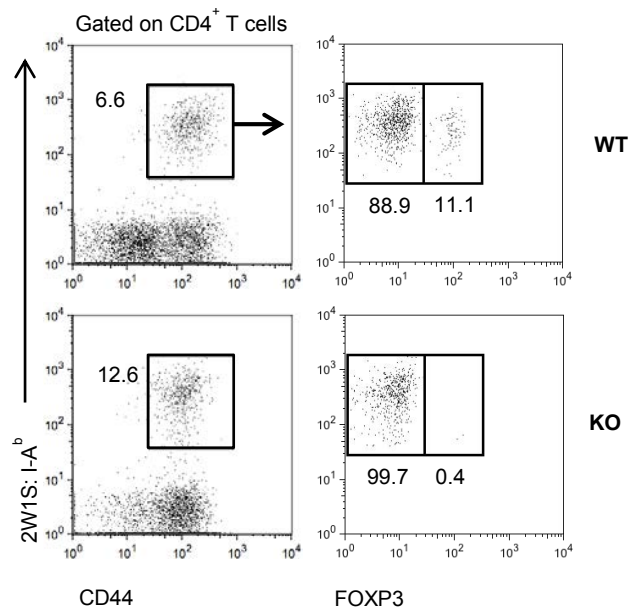
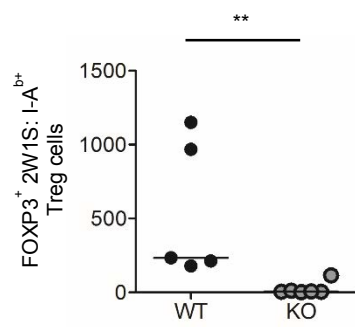
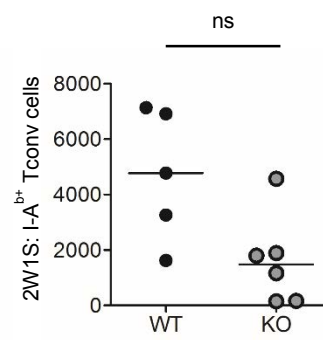
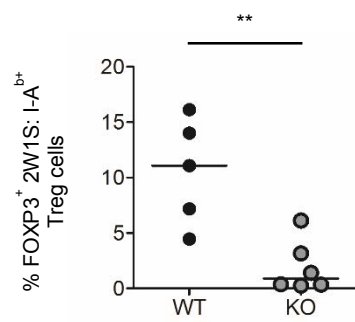






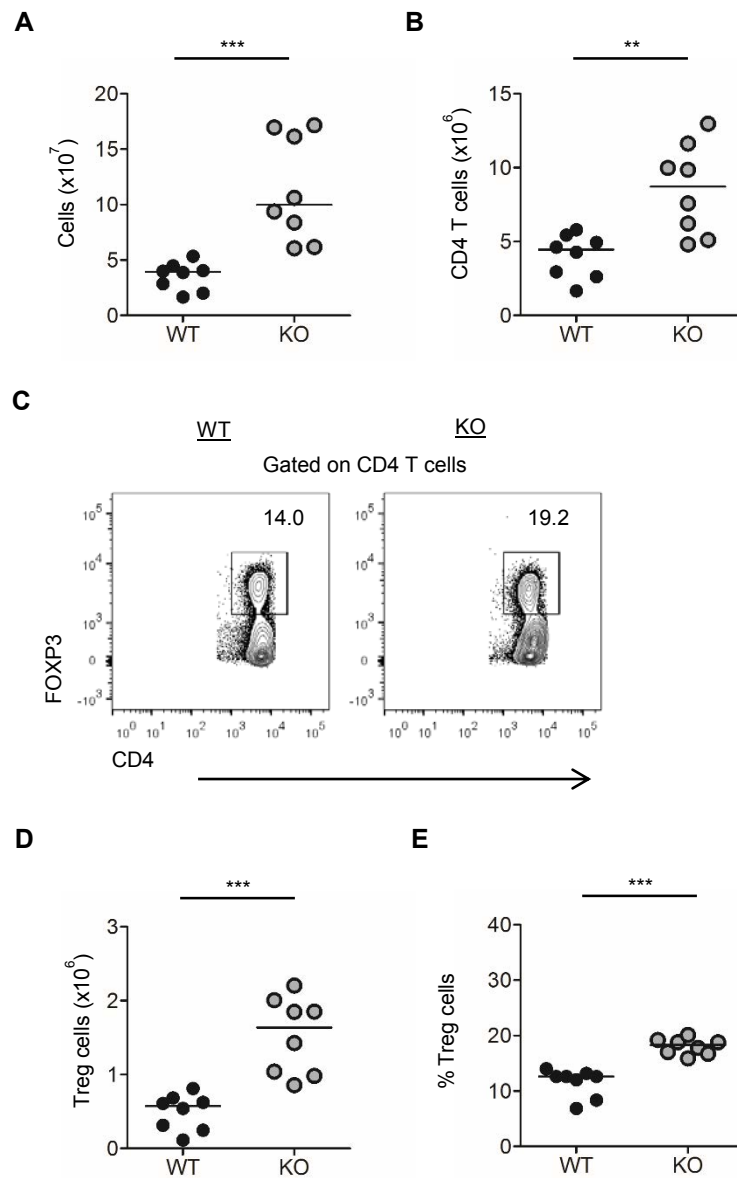
**Figure 5.3: Expansion of regulatory T cells by 2W1S peptide is attenuated in ROR $\gamma$ t KO mice.** WT and ROR $\gamma$ t KO mice were immunised intravenously with 2W1S peptide and analysed 7 days later. A pool of spleen and lymph nodes from WT mice were compared to spleen from ROR $\gamma$ t KO mice. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (C) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (D) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (E) Identification of FOXP3 and ROR $\gamma$ t expression by CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (wildtype mice). Plots are pre-gated on CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (F) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells) which express ROR $\gamma$ t (wildtype mice). Bars show medians, at least 2 mice analysed per group. (B, C, D, F) Bars show medians. Mann–Whitney test  $^{**}p < 0.01$ , ns = non-significant. At least 6 mice were pooled per group from three independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

Agonistic anti-DR3 was then used to further expand the 2W1S-specific population in ROR $\gamma$ t KO mice given the low numbers of 2W1S<sup>+</sup> Treg cells. Therefore, ROR $\gamma$ t KO and WT mice were immunised with 100  $\mu$ g 2W1S peptide and 10  $\mu$ g agonistic anti-DR3. Seven days later the spleen (ROR $\gamma$ t KO) or spleen and a pool of lymph nodes (WT) were harvested and enrichment of 2W1S<sup>+</sup> T cells was performed. As observed previously agonistic anti-DR3 was able to increase the number of regulatory and conventional 2W1S<sup>+</sup> T cells in WT mice. However, the number of 2W1S<sup>+</sup> Treg cells was again very low, consistent with the original experiment (**Figure 4.4a**). The percentage of Treg cells within the 2W1S<sup>+</sup> T cell population was 12.4-fold lower in the ROR $\gamma$ t KO mice than WT (median 0.9% vs median 11.1%) (**Figure 5.4b**), and the number of 2W1S<sup>+</sup> Treg cells was reduced by 41.1-fold (median 6 vs median 234) (**Figure 5.4c**). Again, there was not a statistically significant difference in the number of 2W1S<sup>+</sup> Tconv cells between ROR $\gamma$ t KO mice and WT (**Figure 5.4d**). It is interesting to note, however, that the median number of 2W1S<sup>+</sup> Tconv cells in ROR $\gamma$ t KO mice after 2W1S peptide and agonistic anti-DR3 administration was 3.2-fold lower than WT. Although not statistically significant, this may suggest that the effects of agonistic anti-DR3 are dependent on ROR $\gamma$ t. Together, these data indicate that following immunisation there are far fewer 2W1S<sup>+</sup> Treg cells in the spleen of ROR $\gamma$ t KO mice, compared with WT. These data indicate that in the absence of ILC3s Treg cell expansion is impaired. However, it is important to note that due to the role of ROR $\gamma$ t in the thymus (Sun et al., 2000) there may be fundamental effects on Treg cells generation in ROR $\gamma$ t KO mice.

**A****B****C****D**

**Figure 5.4: Expansion of regulatory T cells by 2W1S peptide and agonistic anti-DR3 is attenuated in RORyt KO mice.** WT and RORyt KO mice were immunised intravenously with 2W1S peptide and agonistic anti-DR3 antibody and analysed 7 days later. A pool of spleen and lymph nodes from WT mice were compared to spleen from RORyt KO mice. (A) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells. Plots are pre-gated on CD3<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> cells. (B) Enumeration of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (C) Enumeration of FOXP3<sup>-</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Tconv cells). (D) Percentage of FOXP3<sup>+</sup> CD44<sup>hi</sup> 2W1S: I-A<sup>b+</sup> T cells (Treg cells). (B-D) Bars show medians. Mann–Whitney test \*\* $p < 0.01$ , ns = non-significant. At least 6 mice were pooled per group from three independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

The specific defect in 2W1S<sup>+</sup> Treg cells may reflect an effect on all Treg cells in ROR $\gamma$ t KO mice. Therefore, to determine whether the overall Treg cell population is affected by the loss of ROR $\gamma$ t, spleens were harvested from ROR $\gamma$ t KO and WT mice and phenotyping took place. The median cellularity of the spleen of ROR $\gamma$ t KO mice was 2.5-fold higher than WT (**Figure 5.5a**), consistent with previous studies (Kurebayashi et al., 2000). The median number of CD4 T cells was also increased by 2-fold (**Figure 5.5b**) and the median number of Treg cells by 2.9-fold (**Figure 5.5c**). In accordance with this, the percentage of Treg cells within the CD4 T cell population was significantly increased (1.5-fold; median 12.5% vs median 17.6%) (**Figure 5.5d**). This suggests that not all Treg cells are disproportionately affected by the loss of ROR $\gamma$ t.



**Figure 5.5: Overall regulatory T cell numbers are not reduced in RORyt KO mice.** Cells were isolated from the spleen of naïve RORyt KO and WT mice.  $4 \times 10^6$  cells were antibody stained. (A) Cellularity of the spleen. (B) Enumeration of FOXP3<sup>-</sup> CD4<sup>+</sup> T cells (Tconv). (C) Identification of FOXP3<sup>+</sup> T cells (Treg cells). Plots are pre-gated on CD3<sup>+</sup> CD4<sup>+</sup> B220<sup>-</sup> CD11b<sup>-</sup> CD11c<sup>-</sup> cells. (D) Enumeration of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells (Treg cells). (E) Percentage of FOXP3<sup>+</sup> CD4<sup>+</sup> T cells (Treg cells). (A-B, D-E) Bars show medians. Mann–Whitney test, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . At least 8 mice were pooled from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

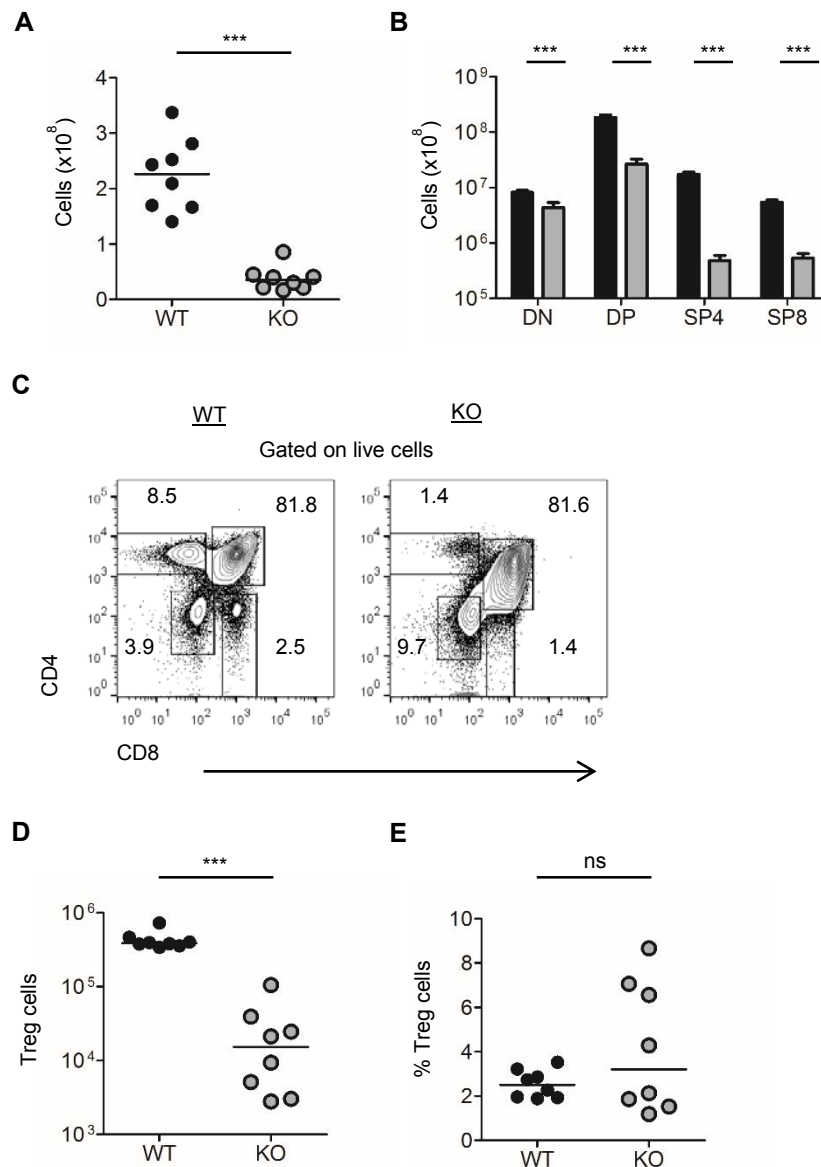
### *Treg cell development in ROR $\gamma$ t KO mice*

As ROR $\gamma$ t is involved in T cell development in the thymus (Sun et al., 2000) it is important to investigate whether the defects seen in the 2W1S<sup>+</sup> Treg cell population reflect fundamental alterations to Treg cell generation in the thymus. Thymocytes can be separated into four populations on the expression of CD4 and CD8 (Borgulya et al., 1991). Thymocytes enter the thymus as Double Negative (DN) cells, mature into Double Positive (DP) cells and undergo positive selection. If successful, DP cells then become Single Positive (SP) 4 or 8 cells and undergo negative selection in the medulla. ROR $\gamma$ t is expressed by DP thymocytes, within which it facilitates survival via production of Bcl-xL (Sun et al., 2000). This is thought to give these cells an opportunity to interact with MHC II/peptide and if appropriate, go through positive selection. As a result, V $\alpha$  to J $\alpha$  recombination has been shown to be altered in ROR $\gamma$ t KO mice (Guo et al., 2002).

Initially, phenotyping of the thymus of ROR $\gamma$ t KO mice was performed to investigate whether Treg cell development was impaired in the absence of ROR $\gamma$ t. The median cellularity of the thymus from these mice was 6.4-fold lower than that of the WT mice (**Figure 5.6a**), which is consistent with previous studies (Sun et al., 2000). This reduction was reflected in all thymocyte populations, particularly the SP4 population. The median number of SP4 cells was reduced by 42.2-fold and the median number of cells in the SP8 population was reduced by 11.3-fold (**Figure 5.6b**). The dramatic reduction in the SP4 population in ROR $\gamma$ t KO mice is also displayed in **Figure 5.6c**, which demonstrates the gating strategy for the four thymocyte populations. Within the SP4 population, the median number of Treg cells was reduced by 25.4-fold in ROR $\gamma$ t KO mice (**Figure 5.6d**), and therefore Treg cells do not appear to be affected more

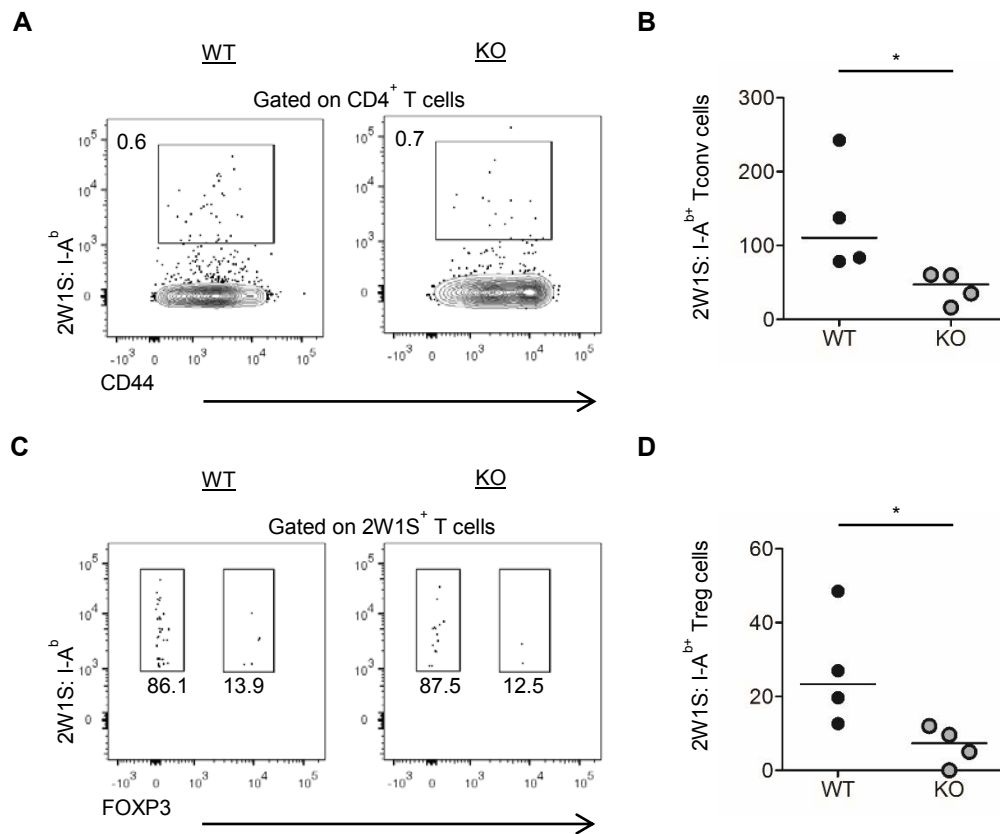
than the rest of the SP4 population. Consistent with this, the percentage of Treg cells within the SP4 population was not significantly different in RORyt KO mice compared to WT (median 2.5% vs median 2.9%) (**Figure 5.6e**).





**Figure 5.6: Regulatory T cell development is not disproportionately affected by the absence of ROR $\gamma$ t.** Cells were isolated from the thymus of naïve ROR $\gamma$ t KO and WT mice.  $4 \times 10^6$  cells were antibody stained. (A) T cell profile in ROR $\gamma$ t KO thymus. Plots are gated on live cells. (B) Cellularity of the thymus. (C) Enumeration of Double Negative (DN), Double Positive (DP), Single Positive CD4 (SP4) and Single Positive CD8 (SP8) cells. (D) Number of FOXP3<sup>+</sup> cells (Treg cells). (E) Percentage of FOXP3<sup>+</sup> cells (Treg cells) within the SP4 population. (B, E) Bars show medians. Mann–Whitney test, \*\*\* $p < 0.001$ , ns = non-significant. (C) Means  $\pm$  SEM are shown. At least 8 mice were pooled per group from two independent experiments. Tconv = conventional T cells, Treg = regulatory T cells.

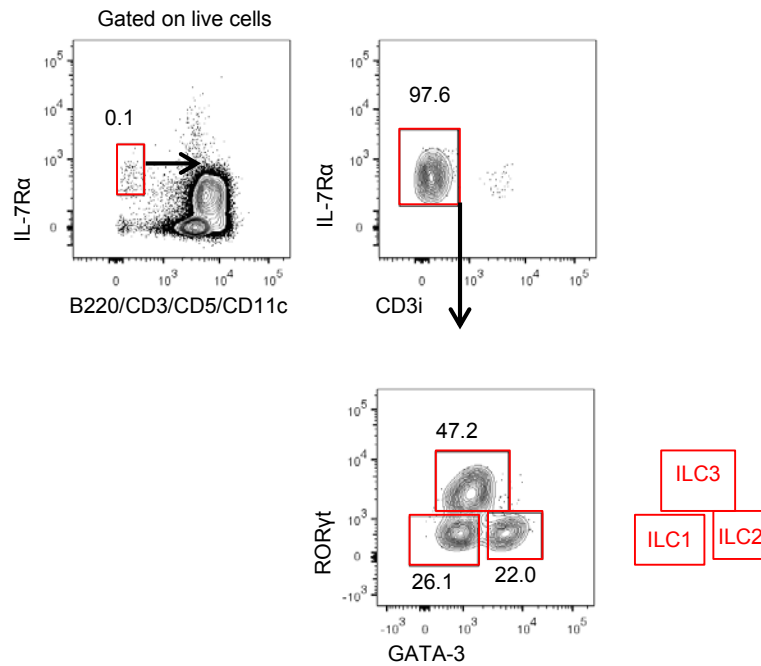
Although total Treg cells were not disproportionately impaired in ROR $\gamma$ t KO mice, such studies may not identify changes in Treg cell selection in the thymus. Therefore, numbers of 2W1S<sup>+</sup> Treg cells may still be reduced. To investigate this, the 2W1S<sup>+</sup> T cell population was assessed in naïve WT and ROR $\gamma$ t KO mice. Given concerns over low numbers, FOXP3<sup>GFP</sup> reporter mice were used to optimise the yield of 2W1S<sup>+</sup> T cells, which are rare. The spleens were harvested from naïve ROR $\gamma$ t KO FOXP3<sup>GFP</sup> mice and the spleen and lymph nodes were harvested from naïve FOXP3<sup>GFP</sup> mice, which served as the WT controls. Tetramer staining and enrichment was then performed. For each sample, the cells were pooled from two mice after enrichment to increase the number of 2W1S<sup>+</sup> T cells. Conventional 2W1S<sup>+</sup> T cells could be identified in ROR $\gamma$ t KO mice (**Figure 5.7a**), although the median number was 2.3-fold lower than WT (**Figure 5.7b**). Similarly, regulatory 2W1S<sup>+</sup> T cells were identified in ROR $\gamma$ t KO mice (**Figure 5.7c**) and the median number was 3.2-fold lower than WT (**Figure 5.7d**). Although there was a modest reduction in the median number of 2W1S<sup>+</sup> Treg cells, this reflects a reduction in the overall 2W1S-specific T cell population, and fundamentally 2W1S<sup>+</sup> Treg cells could be identified. This suggests that 2W1S<sup>+</sup> Treg cells can develop in mice which are deficient in ROR $\gamma$ t.



**Figure 5.7:  $2W1S^+$  Treg cells can develop in mice which are deficient in ROR $\gamma$ t.** (A) Identification of  $2W1S: I-A^b$  T cells in naïve FOXP3 GFP mice and ROR $\gamma$ t KO x FOXP3<sup>GFP</sup> mice. Plots are from two mice combined and are pre-gated on CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (B) Identification of FOXP3<sup>-</sup> and FOXP3<sup>+</sup>  $2W1S: I-A^b$  T cells in naïve FOXP3<sup>GFP</sup> mice and ROR $\gamma$ t KO x FOXP3<sup>GFP</sup> mice. Plots are from two mice combined and are pre-gated on  $2W1S: I-A^b$  CD3<sup>+</sup>CD4<sup>+</sup>B220<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>-</sup> cells. (C) Enumeration of FOXP3<sup>-</sup>  $2W1S: I-A^b$  T cells. (D) Enumeration of FOXP3<sup>+</sup>  $2W1S: I-A^b$  T cells. (C, D) Bars show medians. Mann–Whitney test, \* $p < 0.05$ . At least 4 samples, each from two mice, were pooled from two independent experiments.

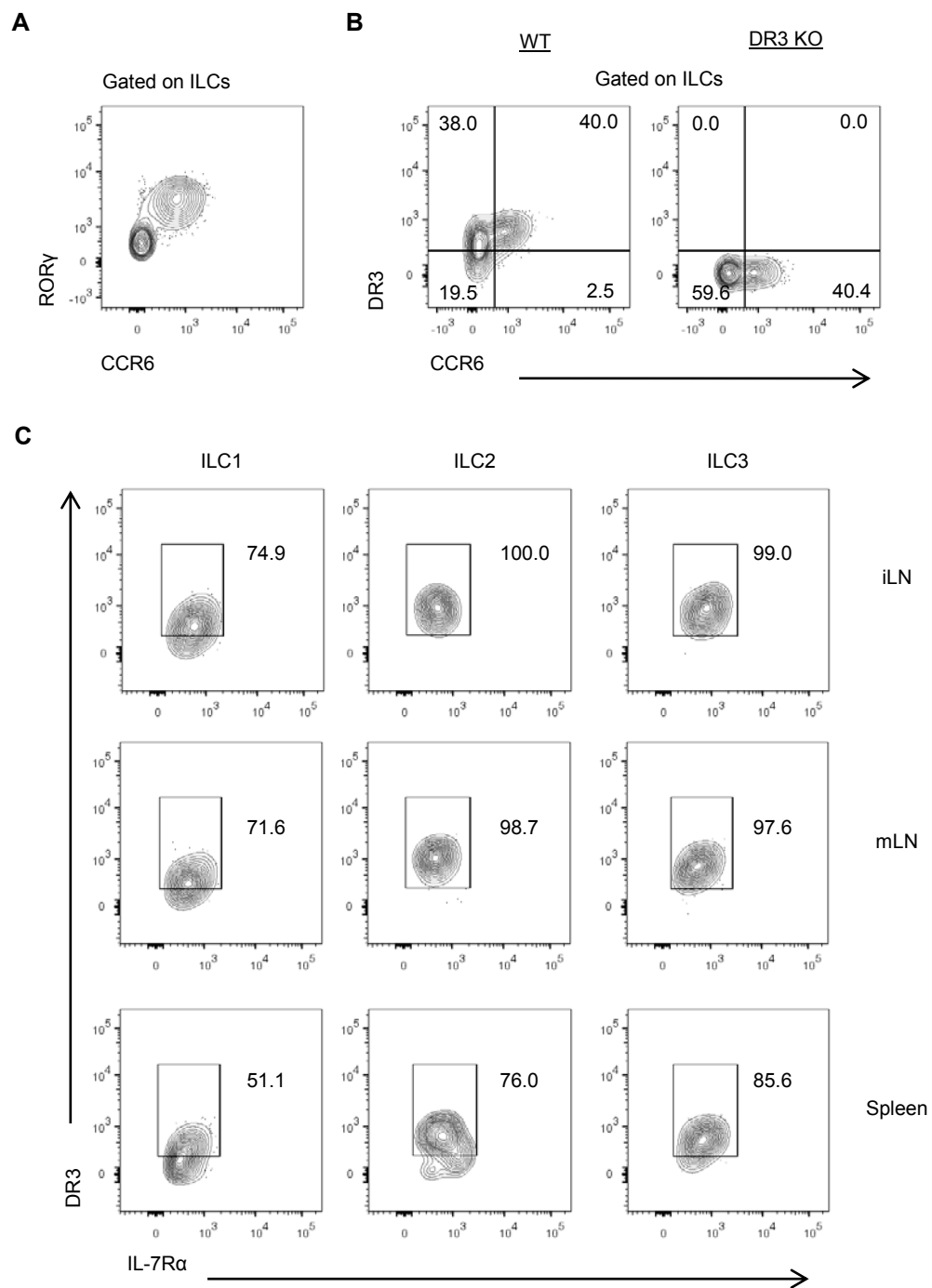
### *DR3 expression on Innate Lymphoid cells*

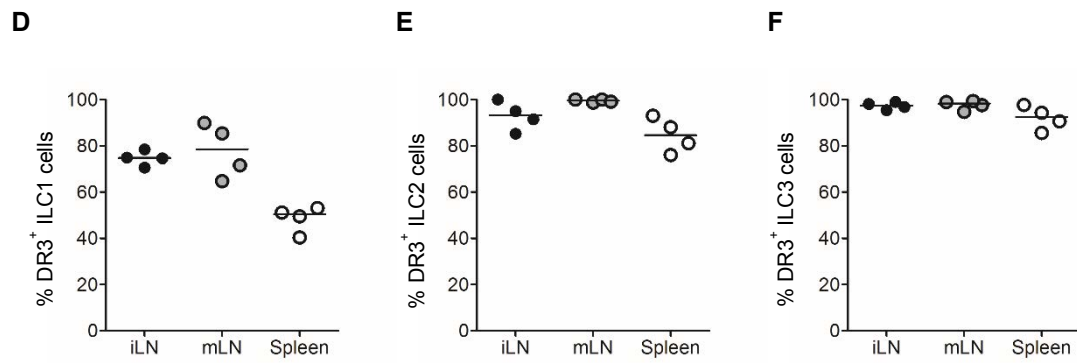
Previous data from Figure 5.4 indicated that the effect of agonistic anti-DR3 on Treg cell numbers is reduced in ROR $\gamma$ t KO mice. Interestingly along with regulatory and conventional T cells, embryonic LT $\alpha$ i, which are part of the group 3 ILC subset, have been shown to express DR3 (Kim et al., 2003). Further to this, DR3 expression has been shown on adult cells which resemble these embryonic cells (Kim et al., 2003), now classified as ILC3s. ILC populations are known to differ between different lymph nodes (Mackley et al., 2015), therefore DR3 was stained for in inguinal lymph nodes, mesenteric lymph nodes and spleen. These tissues were harvested from naïve mice, teased under a microscope and digested using collagenase dispase and DNase prior to staining to increase the yield of cells. ILCs were identified by flow cytometry as IL-7R $\alpha$ <sup>+</sup> cells lacking lineage markers associated with T cells, B cells and myeloid cells. To ensure that T cells were not included in this population, cells which contained intracellular CD3 were gated out. The three ILC populations were then separated into ROR $\gamma$ t<sup>+</sup> (ILC3s), GATA-3<sup>+</sup> (ILC2s) and the double negative population (ILC1s) (**Figure 5.8**). This method of staining enables clear gating of ILC3s and enables comparison with the other ILC subsets (Mackley et al., 2015).



**Figure 5.8: Gating strategy for ILC Subsets.** Cells were isolated from the spleen, mesenteric lymph node and inguinal lymph nodes of C57BL/6 mice. Cells were surface stained, fixed, permeabilised and analysed by flow cytometry. Gating strategy for ILCs and for ILC subsets: ILC1 ( $\text{ROR}\gamma\text{t}^- \text{GATA3}^-$ ), ILC2 ( $\text{ROR}\gamma\text{t}^- \text{GATA3}^+$ ), and ILC3s ( $\text{ROR}\gamma\text{t}^+ \text{GATA3}^-$ ).

As previously described, adult ILC3s can be divided into two subsets based on CCR6 expression (Klose et al., 2014). The CCR6<sup>+</sup> (LTi-like) ILC3s are of principal interest within this study, and make up the majority of ILC3 population in the mesenteric lymph node (Mackley et al., 2015). Thus CCR6 can be used as a surrogate marker to identify ILC3s in place of ROR $\gamma$ t, as shown in **Figure 5.9a**. This surrogate marker was used when validating the anti-DR3 antibody using DR3 KO mice, as the anti-DR3 antibody was conjugated to the same fluorochrome as the anti-ROR $\gamma$ t antibody (**Figure 5.9b**). As shown in **Figure 5.9c**, DR3 could be identified on all three subsets of ILCs. In all three tissues, the majority of ILC3s were found to express DR3 (medians 94-98%) (**Figure 5.9d**). Similarly, the majority of ILC2s expressed DR3 in the lymph nodes (medians 95-99%) and the spleen (median 85%) (**Figure 5.9e**). A high percentage of ILC1s in the lymph nodes expressed DR3 (medians 75-79%), and approximately half of IL1s in the spleen (median 50%) (**Figure 5.9f**). Therefore, the majority of ILC3s express DR3 in peripheral and mesenteric lymph nodes, as well as the spleen, and is therefore likely to have a key role on these cells.





**Figure 5.9: DR3 is highly expressed by ILC3s.** Cells were isolated from the spleen, mesenteric lymph node and inguinal lymph nodes of C57BL/6 mice. Cells were surface stained, fixed, permeabilised and analysed by flow cytometry. (A) (B). (C) Identification of DR3 on ILC subsets from spleen, mesenteric lymph nodes and inguinal lymph nodes. (D) Percentage of each ILC subset which expresses DR3 in each lymphoid organ analysed. Bars show medians. At least four mice were pooled from two independent experiments.

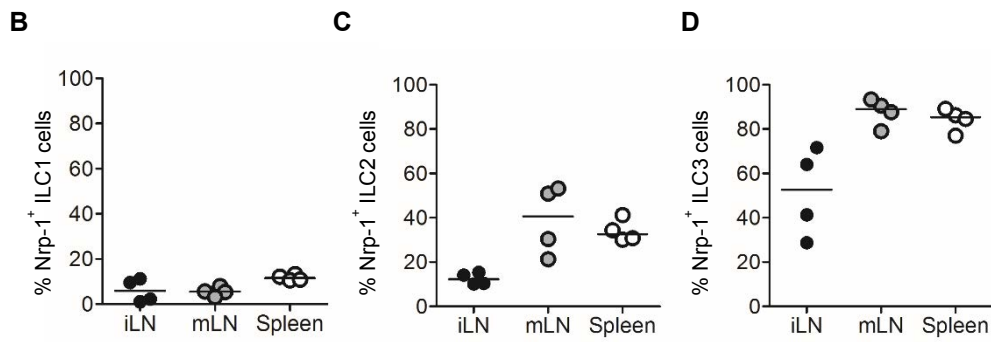
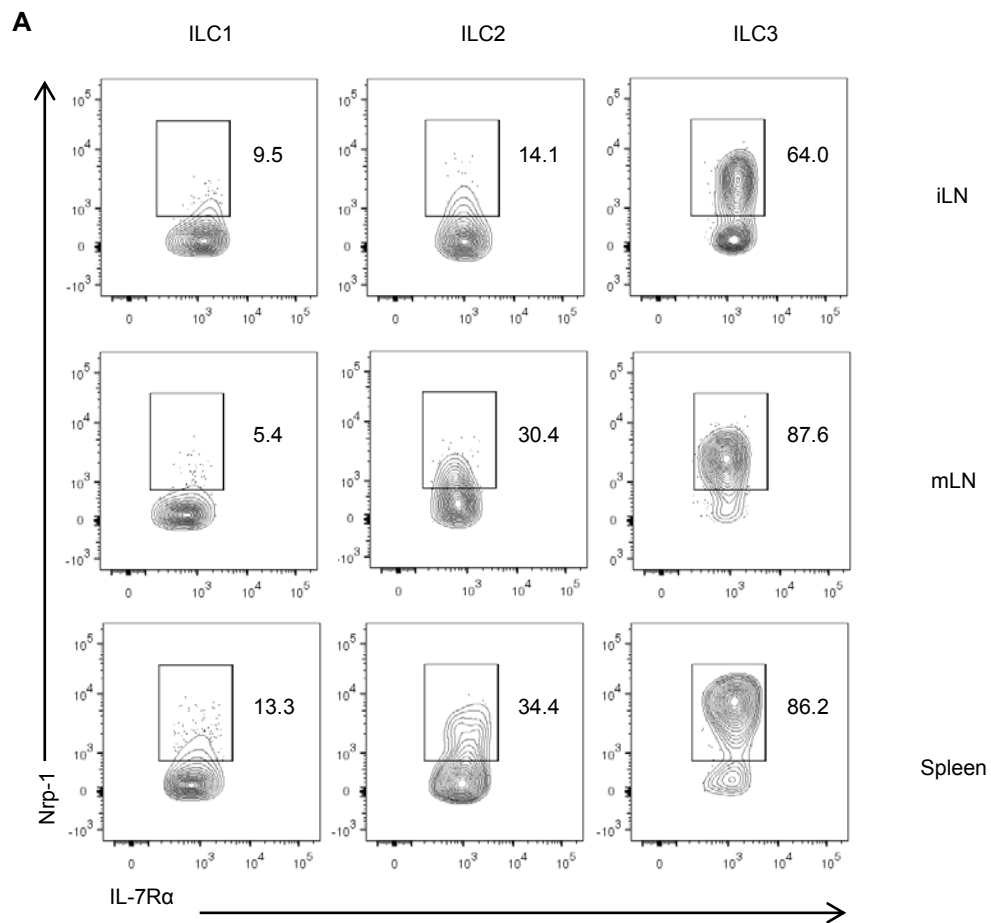


### *Determining potential interaction partners between Treg cells and Innate Lymphoid Cells*

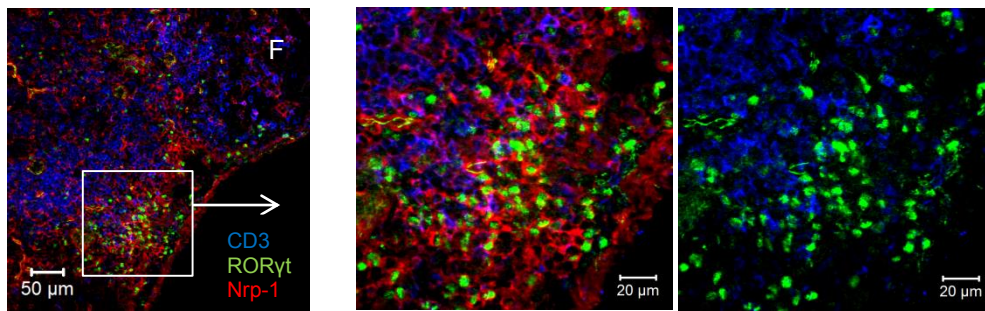
Impaired Treg responses have been observed in ROR $\gamma$ t KO mice within this study, which suggests that Treg cells may interact with ILC3s. This is consistent with preliminary data which shows that ILC3s can be found in close association with Treg cells in the interfollicular areas of the mesenteric lymph node (Figure 5.1). In this study, the expression of potential interaction partners between ILC3s and Treg cells has been explored to further investigate this. These include Neuropilin-1 (Nrp-1), which has been shown to enhance Treg cell stability (Delgoffe et al., 2013) and can signal through homotypic interactions (Sarris et al., 2008); and MHC II as Treg cell homeostasis is known to be supported by encounter with cognate antigen (Attridge and Walker, 2014). Further to this, there is thought to be a switch in the function of group 3 ILC3s from lymph node organogenesis in the embryo, to modulation of the adaptive immune system, as well as roles in the innate immune system, in the adult (Kim et al., 2006). This study therefore also investigated whether MHC II is expressed on group 3 ILCs in the embryo (LTi), and hence this functional switch.

To identify Nrp-1 expression, the inguinal lymph nodes, mesenteric lymph nodes and spleen were harvested from naïve mice, teased under a microscope and digested using collagenase dispase and DNase. The three ILC subsets were gated upon using the previously strategy. ILC3s were found to be the predominant Nrp-1 expressing subset of ILCs (**Figure 5.10a**). There was little Nrp-1 on ILC1s in all three tissues (**Figure 5.10b**). A proportion of ILC2s in the spleen and mesenteric lymph nodes expressed Nrp-1 (medians 33-41%), but there was little expression in the inguinal lymph nodes (**Figure 5.10c**). In the mesenteric lymph nodes and spleen the

majority of ILC3s were found to express Nrp-1 (medians 85-89%), and approximately half of the ILC3s in inguinal lymph nodes (median 53%) (**Figure 5.10d**). To confirm these data, confocal microscopy was used. ILC3s were identified in the interfollicular areas of mesenteric lymph node sections as IL-7R $\alpha$ <sup>+</sup> ROR $\gamma$ t<sup>+</sup> CD3<sup>-</sup> cells. **Figure 5.10e** shows a magnification of the interfollicular area (marked by a white box). Nrp-1 positive ILC3s can be identified in this area, and have been circled in white. The panel on the right allows confirmation that these cells are not CD3<sup>+</sup>. This demonstrates that the majority of ILC3s in the mesenteric lymph nodes express Nrp-1, and shows that the interfollicular area is rich in Nrp-1.

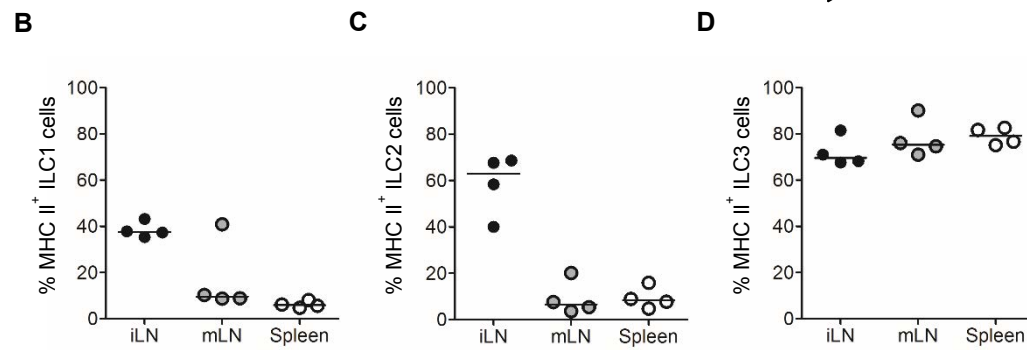
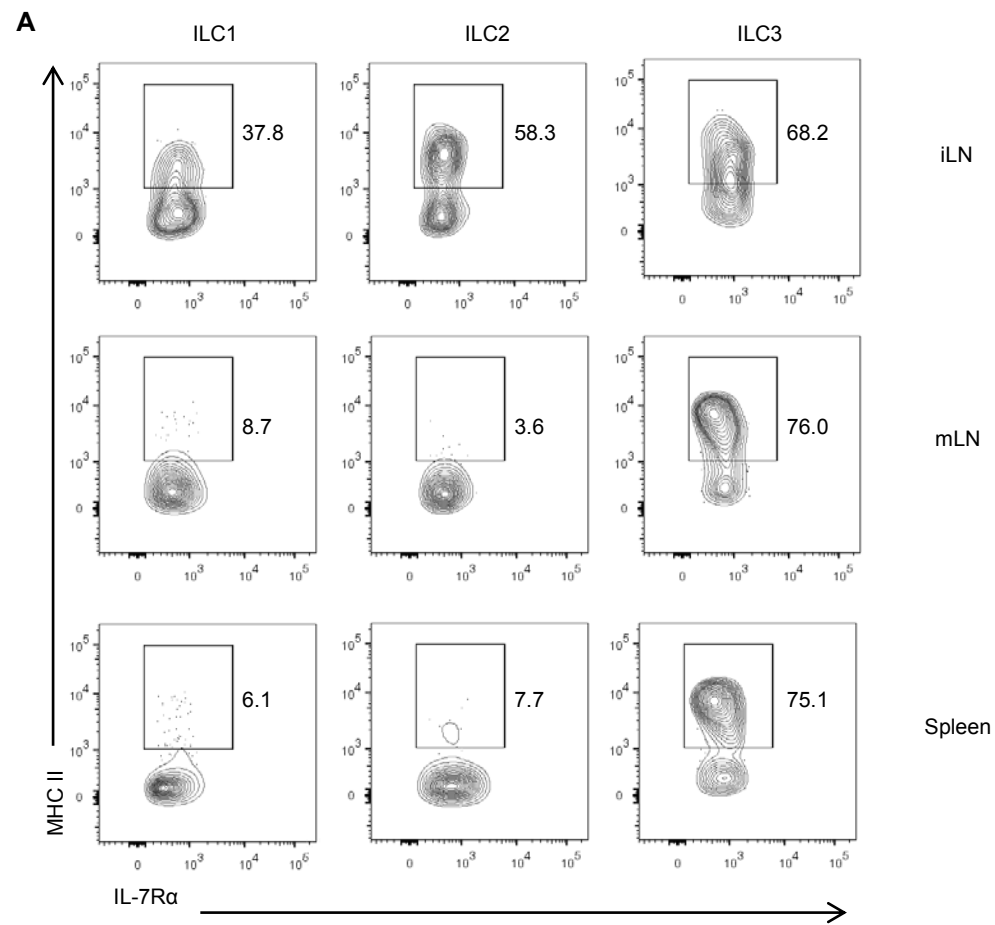


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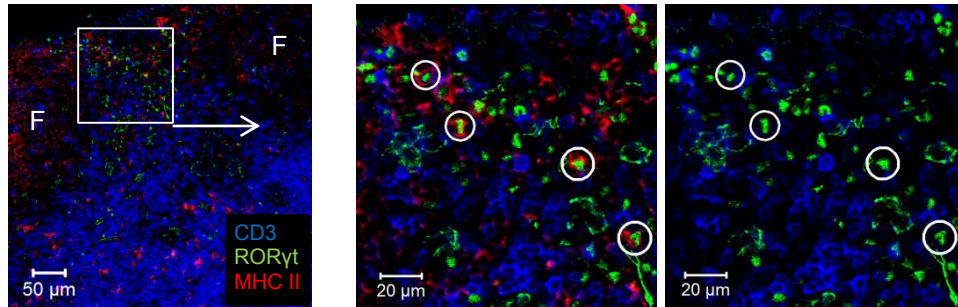


**Figure 5.10: Neuropilin-1 is highly expressed by ILC3s.** (A-B) Cells were isolated from the spleen, mesenteric lymph node and inguinal lymph nodes of C57BL/6 mice. Cells were surface stained, fixed, permeabilised and analysed by flow cytometry. (A) Identification of Neuropilin-1 (Nrp-1) on ILC subsets from spleen, mesenteric lymph nodes and inguinal lymph nodes. Gating of ILC subsets was performed as shown previously. (B) Percentage of each ILC subset which expresses Nrp-1 in each lymphoid organ analysed. Bars show medians. At least four mice were pooled from two independent experiments. (C) Mesenteric lymph node sections from naïve wildtype mice were cut and stained for Nrp-1 on ILC3s. Interfollicular area (white box) of image on left is magnified with and without Nrp-1 on right. F indicates follicle. ILC3s are identified as IL-7Rα<sup>+</sup> RORγt<sup>+</sup> CD3<sup>-</sup> cells. Images are representative of four mice.

To identify MHC II expression, the inguinal lymph nodes, mesenteric lymph nodes and spleen were harvested from naïve mice, teased under a microscope and digested using collagenase dispase and DNase. The three ILC subsets were gated upon using the strategy previously shown. In the mesenteric lymph nodes and spleen, ILC3s were the only ILC population which expressed MHC II (**Figure 5.11a**). In the inguinal lymph nodes, however, MHC II expression was observed by all three groups of ILCs. In these peripheral lymph nodes, the median percentage of ILC1s expressing MHC II was 38% (**Figure 5.11b**) and the median percentage of MHC II<sup>+</sup> ILC2s was 63% (**Figure 5.11c**). In all three tissues, MHC II expression was observed on a high proportion of ILC3s (medians 67-79%) (**Figure 5.11d**). To confirm these data, confocal microscopy was used. ILC3s were again identified in the interfollicular areas of mesenteric lymph node sections as IL-7Rα<sup>+</sup> RORγt<sup>+</sup> CD3<sup>-</sup> cells. **Figure 5.11e** shows a magnification of the interfollicular area (marked by a white box). MHC II positive ILC3s can be identified in this area, and have been circled in white. The panel on the right allows confirmation that these cells are not CD3<sup>+</sup>. Therefore, the majority of ILC3s express MHC II in the mesenteric lymph nodes. Together these data show that MHC II expression is observed by ILC3s in peripheral and mesenteric lymph nodes, as well as spleen.



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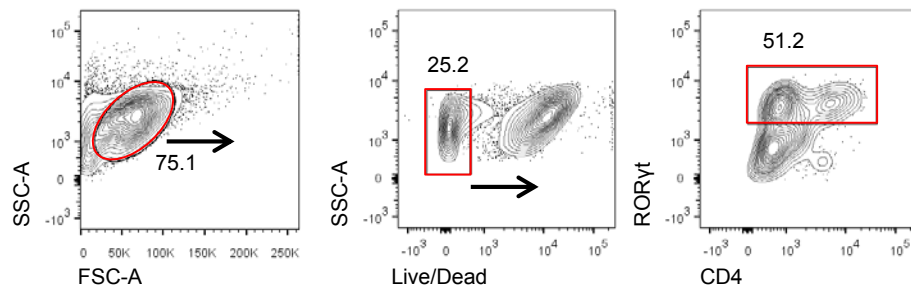
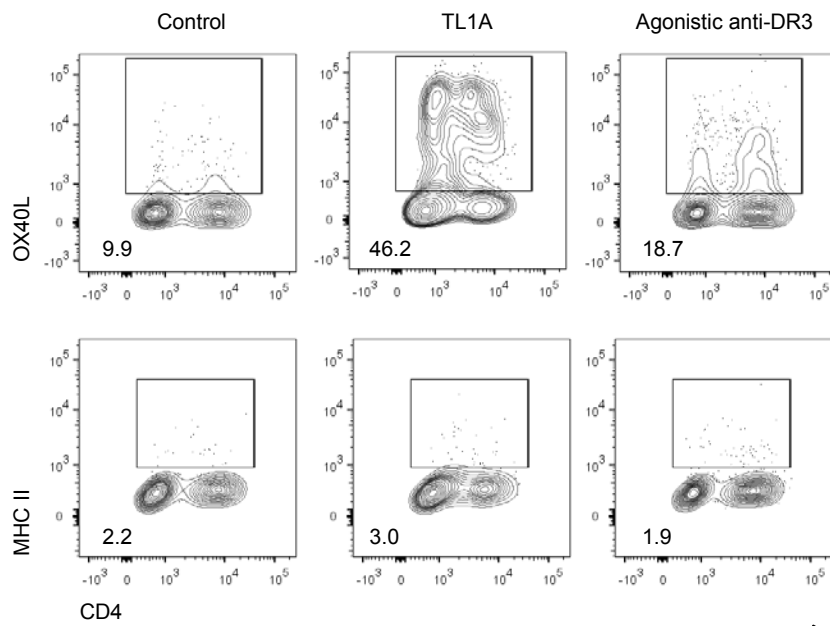
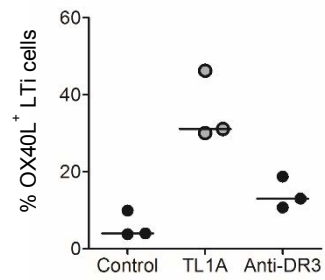
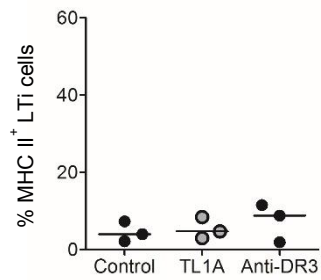
**Figure 5.11: MHC II is highly expressed by ILC3s.** (A-B) Cells were isolated from the spleen, mesenteric lymph node and inguinal lymph nodes of C57BL/6 mice. (A) Identification of MHC II on ILC subsets from spleen, mesenteric lymph nodes and inguinal lymph nodes. Gating of ILC subsets was performed as shown previously. (B) Percentage of each ILC subset which expresses MHC II in each lymphoid organ analysed. Bars show medians. At least four mice were pooled from two independent experiments. (C) Mesenteric lymph node sections from naïve wildtype mice were cut and stained for MHC II on ILC3s. Interfollicular area (white box) of image on left is magnified with and without MHC II on right. F indicates follicle. ILC3s are identified as IL-7R $\alpha$ <sup>+</sup> ROR $\gamma$ t<sup>+</sup> CD3<sup>-</sup> cells. White circles indicate MHC II<sup>+</sup> ILC3s. Images are representative of three mice.

The ligand for DR3, TL1A, has been shown to induce OX40L expression on LT<sub>i</sub> cells upon overnight culture (Kim et al., 2006). OX40L is expressed by adult ILC3s but not embryonic LT<sub>i</sub> (Kim et al., 2005) and therefore this is thought to be part of this developmental switch from embryonic to adult cells (Kim et al., 2006). MHC II has also been shown to be expressed by adult ILC3s (Hepworth et al., 2013) and may follow the same TL1A-induced developmental switch. To investigate this, spleens were removed from E15 embryos and cultured for seven days with IL-7 to increase the number of LT<sub>i</sub> cells. Spleens then were pooled into groups of six, teased under a microscope and crushed to create cell suspensions. Cells were cultured overnight with IL-7 +/- 100ng/ml TL1A in accordance with the original experiments showing that TL1A can upregulate OX40L (Kim et al., 2006). A cell suspension was cultured with 10 µg/ml agonistic anti-DR3 instead of TL1A to determine whether the antibody can recapitulate the effects of this soluble mediator.

As shown in **Figure 5.12a**, LT<sub>i</sub> cells were identified simply as ROR $\gamma$ <sup>+</sup> cells. This is possible because LT<sub>i</sub> cells are the major ROR $\gamma$ -expressing population at E15 (Eberl et al., 2004). There was little OX40L or MHC II expression on LT<sub>i</sub> which were cultured with IL-7, which confirms previous observations that embryonic LT<sub>i</sub> do not express these molecules. Upon TL1A administration OX40L was upregulated on LT<sub>i</sub> (median 31%), but the levels of MHC II remained unchanged (**Figure 5.12b**). This effect of TL1A on OX40L expression was therefore consistent with previously published data (Kim et al., 2006) and amounted to a 31.1-fold increase in the median percentage of LT<sub>i</sub> which expressed OX40L (**Figure 5.12c**). Upon treatment with agonistic anti-DR3 a small proportion of LT<sub>i</sub> were found to express OX40L (median 13%) (**Figure 5.12d**), therefore agonistic anti-DR3 is able to upregulate OX40L but to a much

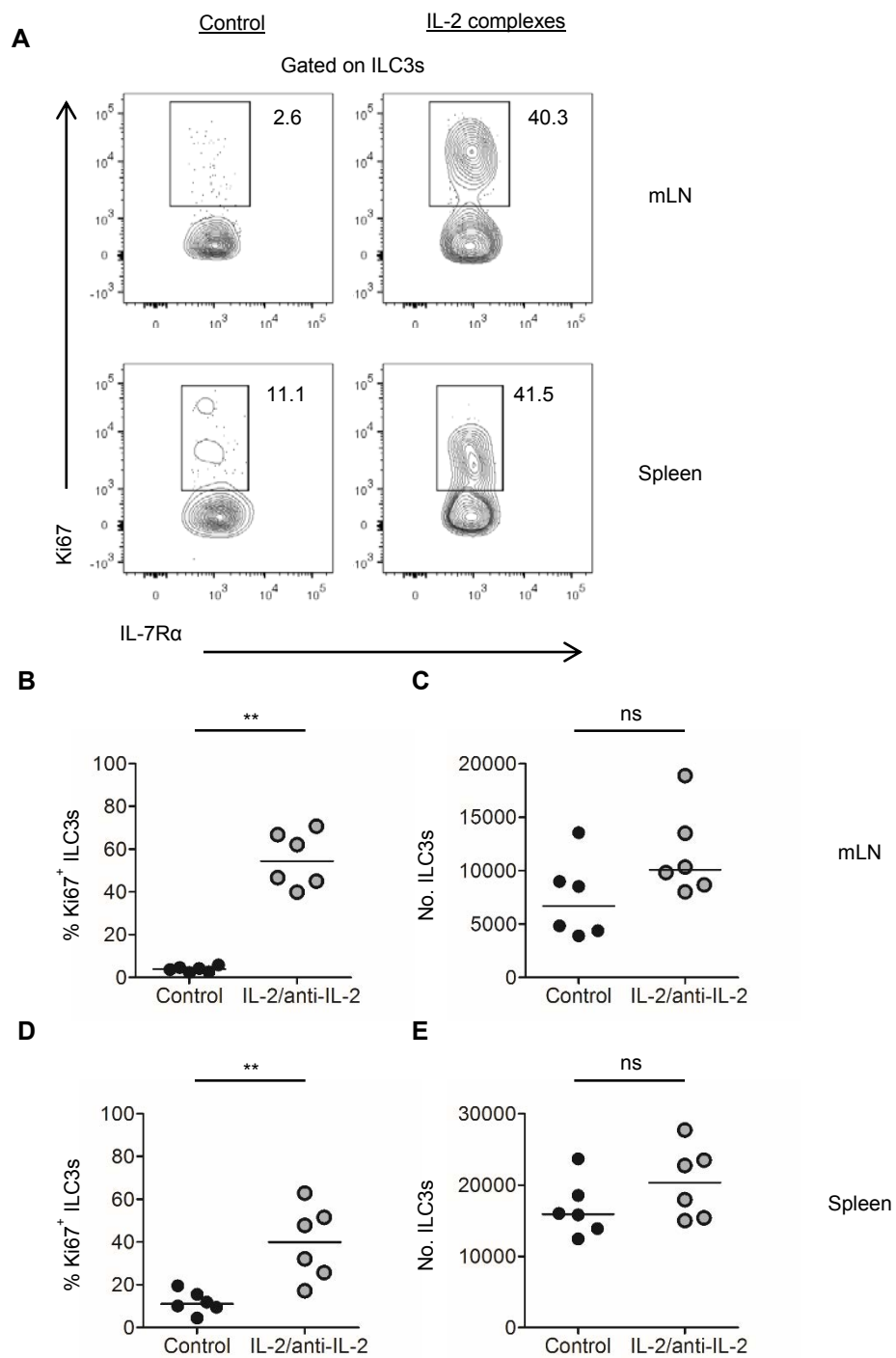


lesser extent than TL1A. MHC II expression was again unchanged. Therefore, MHC expression does not appear to be regulated by DR3 signaling.

**A****B****C****D**

**Figure 5.12: Expression of MHC II is not regulated by DR3 signaling.** E15 BALB/c embryonic spleens were placed on a sterile nucleopore filter laid on a piece of sterile antiwrap sponge (6 spleens per filter). These were placed in 4ml sterile culture medium containing IL-7 and cultured for 6-7 days at 37°C in a sterile humidified chamber. Cells were then isolated and cultured overnight with IL-7 +/- TL1A or agonistic anti-DR3. On the next day cells were stained for dead cells prior to antibody staining. (A) Gating strategy for LTi. (B) Identification of OX40L and MHC II on LTi. (C) Percentage of LTi which express OX40L. (D) Percentage of LTi which express MHC II. (A-B) Plots are representative of three independent experiments. Six spleens were pooled for each condition. Bars show medians. (C-D) Each point represents 6 pooled E15 spleens.

These data therefore show that Nrp-1 and MHC II are expressed by ILC3s in the periphery. Future study into the relationship between Treg cells and ILC3s may be facilitated by the ability to boost ILC3 numbers. The administration of IL-2/anti-IL-2 complexes has been shown to cause ILC2 proliferation (Van Gool et al., 2014). Interestingly, this is also a technique which has been used to boost Treg cells (Webster et al., 2009). To investigate whether IL-2/anti-IL-2 complexes can also induce ILC3 proliferation, WT mice were administered complexes composed of 1 µg IL-2 and 5 µg anti-IL-2, or PBS as a control, by intraperitoneal injection for three days. The mesenteric lymph node and spleen were then harvested the day after the final injection. ILC3s were identified as previously shown, and expression of Ki67 was used to identify proliferating ILC3s. As shown in **Figure 5.13a**, ILC3s from PBS injected mice displayed little Ki67 expression, but upon administration of IL-2/anti-IL-2 complexes, Ki67 expression was induced on these cells. In the mesenteric lymph nodes, the percentage of Ki67<sup>+</sup> ILC3s was found to be 13.7-fold higher in the IL-2/anti-IL-2 complex-injected mice than the PBS-injected mice (median 54.4% vs median 3.9%) (**Figure 5.13b**). At this early time point a 1.6-fold increase in ILC3 number could also be observed (median 6503 vs median 10062) (**Figure 5.13c**). In the spleen, the percentage of Ki67<sup>+</sup> ILC3s was 3.9-fold higher in the IL-2/anti-IL-2 complex-injected mice than the PBS-injected mice (median 44.7% vs median 11.6%) (**Figure 5.13d**). As observed in the mesenteric lymph node, a 1.3-fold increase in ILC3 number was observed in the spleen (median 15849 vs median 20611) (**Figure 5.13e**). Therefore, administration of IL-2/anti-IL-2 complexes can be used successfully to induce ILC3 proliferation, although later time points will need to be studied to determine whether ILC3 numbers increase as a result.



**Figure 5.13: ILC3s proliferate in response to IL-2/anti-IL2 complexes.** IL-2/anti-IL-2 complexes were administered to WT mice by intraperitoneal injection for three days and the mesenteric lymph node and spleen were analysed on the fourth day. (A) Identification of Ki-67 expression on ILC3s from the mesenteric lymph nodes and spleen. Gating of ILC3s was performed as shown previously. (B) Percentage of ILC3s which express Ki67 in the mesenteric lymph nodes. (C) Enumeration of ILC3s in the mesenteric lymph nodes. (D) Percentage of ILC3s which express Ki67. (E) Enumeration of ILC3s in the spleen. (B-D) Bars show medians. Mann–Whitney test  $**p < 0.01$ , ns = non-significant. At least six mice were pooled from two independent experiments.

### 5.3 DISCUSSION

Therapeutic manipulation of Treg cell survival, as well as function, would have far reaching implications in a range of conditions from cancer to autoimmune disease. However, the cells and signals which are required for Treg cell homeostasis are yet to be fully elucidated. It has been proposed that low level CD80 and CD86 expression on immature dendritic cells, as well as presentation of self-antigen, may promote Treg survival, through CD28 and TCR signals. Interestingly ILC3s have also been shown to have the capacity to process and present antigen (von Burg et al., 2014, Hepworth et al., 2013). Therefore, within this study, the role of this ILC subset in Treg cell biology was investigated in ROR $\gamma$ t KO mice, which lack ILC3s. An antigen-specific approach was again used so that the timing and nature of antigen exposure was known.

Using an antigen-specific approach, Treg cells can be expanded and tracked over an extended period of time to understand the signals necessary for their survival. However, within this study, very few 2W1S<sup>+</sup> Treg cells could be identified in ROR $\gamma$ t KO mice seven days after immunisation with 2W1S peptide. This impairment was more restricted to the regulatory population, with few differences observed in the number of Tconv cells between WT and ROR $\gamma$ t KO mice. This impairment in 2W1S<sup>+</sup> Treg cell expansion was also observed seven days after immunisation with 2W1S peptide and agonistic anti-DR3, which has been shown previously in this study to enhance the number of regulatory and conventional T cells within the 2W1S<sup>+</sup> T cell response. These data therefore suggest that the expansion of regulatory, but not conventional 2W1S<sup>+</sup> T cells is ROR $\gamma$ t-dependant. The signals required for Treg cell activation are similar to those required for their survival, and indeed for conventional

T cell activation. Treg cells are activated in an antigen specific manner, requiring stimulation through their TCR (Thornton et al., 2004a, Thornton et al., 2004b). It is possible that ILC3s, through expression of MHC II, could contribute to Treg cells activation, although they lack expression of CD80/86 ligands, and therefore the capacity for CD28 signaling (Hepworth et al., 2013), which has been shown to be important for Treg cell proliferation (Bour-Jordan et al., 2011).

To determine whether this impairment in Treg cell expansion is reflected in the overall Treg population, the spleen of naïve ROR $\gamma$ t KO mice was studied. However, the number and percentage of Treg cells was increased ROR $\gamma$ t KO mouse, which is not consistent with impaired Treg cell expansion. It is notable that ROR $\gamma$ t KO mice do not have lymph nodes (Kurebayashi et al., 2000, Sun et al., 2000) and the spleen of ROR $\gamma$ t KO mice is more than twice the size of WT, with significantly higher numbers of conventional and regulatory CD4 T cells. Due to the previously described role of ILC3s in the gut, a key reason for the enlargement of the spleen in ROR $\gamma$ t KO mice is thought to be due to inflammation in response to commensal bacteria (Hepworth et al., 2013). The presence of compounding factors such as this demonstrates the importance of using an antigen-specific approach. It is important to note also that ROR $\gamma$ t has an important role in thymocyte development, and therefore there is a possibility that the absence of 2W1S<sup>+</sup> Treg cells in ROR $\gamma$ t KO mice could be due to altered Treg cell selection. In the thymus, ROR $\gamma$ t facilitates the survival of double positive thymocytes via production of Bcl-xL (Sun et al., 2000). This is thought to give these cells an opportunity to interact with MHC II/peptide and if appropriate, go through positive selection. V $\alpha$  to J $\alpha$  recombination has been shown to be altered in ROR $\gamma$ t KO mice (Guo et al., 2002), which is likely therefore to lead to an altered



TCR $\alpha$  repertoire. It was therefore important to investigate whether Treg cell development is adversely affected in the ROR $\gamma$ t KO thymus. Consistent with previously published data, the single positive 4 (SP4) and single positive 8 (SP8) populations were dramatically reduced in the ROR $\gamma$ t KO mouse. However, Treg cells did not appear to be more affected than the rest of the SP4 population. Crucially, 2W1S<sup>+</sup> Treg cells were identified in the periphery of naïve ROR $\gamma$ t deficient mice, suggesting that these mice 2W1S<sup>+</sup> Treg cells can develop in the ROR $\gamma$ t KO thymus. Therefore, the specific impairment in Treg cell expansion in ROR $\gamma$ t KO is not due to the absence of 2W1S<sup>+</sup> Treg cell generation in these mice. A model in which ILC3s are specifically depleted, is necessary to confirm that it is the absence of ILC3s which leads to the defect observed.

Interestingly, the expansion of 2W1S<sup>+</sup> Tconv cells upon administration of 2W1S peptide and agonistic anti-DR3, but not 2W1S peptide alone, was reduced in ROR $\gamma$ t KO mice. As the majority of ILC3s in the spleen, inguinal and mesenteric lymph nodes were found to express DR3 in this study; there may be a role for ILC3s in the action of this agonistic anti-DR3 antibody. Interestingly the majority of ILC2s also expressed DR3, and this TNF receptor has been shown to be important for ILC2 expansion, survival and function (Yu et al., 2014). The high level of DR3 expression on ILC3s suggests that DR3 has an important role on these cells, and further investigation is needed to determine if there are any parallels with the role on ILC2s.

As previously introduced, group 3 ILCs include LTi, which are required for lymph node organogenesis. DR3 is also expressed by LTi (Kim et al., 2006) and interestingly, its ligand TL1A has been shown to induce OX40L expression on LTi cells as part of a developmental switch (Kim et al., 2006). This 'switch' is thought to

reflect the change in function of group 3 ILCs from lymph node organogenesis to their roles within the immune system, and corresponds with bacterial colonisation which occurs upon birth. It is possible that MHC II, which is expressed by ILC3s in the adult (Hepworth et al., 2013) is subject to this same developmental switch mediated by TL1A-DR3 interactions. Data within this study showed that like OX40L, MHC II is not expressed by embryonic LT $\alpha$ i. However, as MHC II was not upregulated upon culture with TL1A, it appears that MHC II is regulated by another mechanism.

This impairment of Treg cell expansion along with observations from preliminary data, suggest that Treg cells and ILC3s do indeed interact. Various candidates have emerged as potential interaction partners between ILC3s and Treg cells. This study has previously explored the role of OX40 and CD30 on Treg cell expansion, survival and function, as OX40L and CD30L are expressed by ILC3s (Lane et al., 2005). Another molecule expressed by Treg cells which has been proposed to support their survival is neuropilin-1 (Nrp-1) (Delgoffe et al., 2013), which is also a proposed marker of thymically derived Treg cells (Weiss et al., 2012, Yadav et al., 2012). These effects on Treg cell stability are reported to be through interactions with Semaphorin-4a (Delgoffe et al., 2013), but Nrp-1 has also been shown to signal through homotypic interactions to promote long interactions and therefore synapse formations between Treg cells and immature dendritic cells (Sarris et al., 2008). Interestingly Nrp-1 is expressed by the majority of ILC3s in the periphery, which leaves open the possibility that homotypic interactions between Nrp-1 on Treg cells and ILC3s may support their stability. Confocal microscopy has revealed that the interfollicular areas of the mesenteric lymph nodes are rich in Nrp-1 expression. As

this is where ILC3s reside, this gives further evidence that homotypic Nrp-1-Nrp-1 interactions may occur.

MHC II was shown to be expressed on the majority of ILC3s in the periphery. As Treg cell homeostasis is known to be supported by encounter with cognate antigen (Kim et al., 2009, Walker et al., 2003a, Fisson et al., 2003), this may be the role of ILC3-derived MHC II in the periphery. Notably, a similar role has already been proposed for dendritic cells in non-inflammatory conditions (Scheinecker et al., 2002). Interestingly, ILC3s in the gut have been shown to maintain tolerance to commensal through antigen presentation resulting in T cell anergy and death (Hepworth et al., 2015, Hepworth et al., 2013). Thus a regulatory role for MHC II-expressing ILC3s, through interactions with T cells, has been proposed, which may also translate to the periphery.

These data show that in the absence of ILC3s, Treg cell expansion is impaired. Studies using mice in which ILC3s are specifically depleted are needed to investigate further and confirm that this defect is due to the absence of ILC3s. However, along with the observation that ILC3s and Treg cells can be found in close association interfollicular areas of lymph nodes, these data suggest that ILC3s and Treg cells interact. The use of IL-2/anti-IL-2 complexes to expand ILC3 numbers will also aid future understanding of the relationship between ILC3s and Treg cells; an approach which was further developed within this study. Nrp-1 has emerged as a strong candidate for mediating the interactions between ILC3s and Treg cells, as well as presentation of peptide to Treg cells by MHC II on ILC3s. As DR3 is so highly expressed on ILC3s, and has been shown to be important for ILC2 biology, this TNF receptor is likely to also have an important role on ILC3s.

## **6.0 OVERALL DISCUSSION**

Treg cells are a subset of CD4 T cells which have an important role in maintaining tolerance to self-antigens, and provide feedback control to the immune system to limit pathogen-associated immune pathology (Corthay, 2009). Treg cells can suppress many different immune cells, but their main function is considered to be suppression of naïve T cell activation (Schmidt et al., 2012). Since their discovery (Gershon and Kondo, 1970, Sakaguchi et al., 1995) there has been great interest in the therapeutic potential of Treg cell manipulation. There are many situations in which boosting Treg cell activation, survival or function would be beneficial, such as autoimmune disease, allergy and transplantation. Conversely, inhibition of Treg cells would boost anti-tumour responses and therefore aid tumour clearance by the immune system. Current advances in anti-tumour immunotherapy have included strategies to release effector T cells from regulation, for example by blocking CTLA-4 and PD-1 (Curran et al., 2010). However, it is hoped that agonistic antibodies directed against TNF receptors would have dual anti-tumour effects by turning off Treg cell function as well as boosting effector T cell activation through co-stimulation (Kitamura et al., 2009). To better study Treg cell survival and function, and hence the cells and signals required, an *in vivo* antigen-specific approach was developed to allow specific effects on survival versus function to be dissected out.

Therefore, in this study the following aims were addressed:

- Establish a method to study Treg cell survival and function *in vivo*.
- Investigate the sources of cellular support, and the role of TNF receptors in Treg cell survival and function.

## 6.1 Development of an antigen-specific model to study regulatory T cells

In this study, an antigen-specific approach pioneered by Dr Marc Jenkins was refined specifically to study Treg cells. Using this *in vivo* approach, an endogenous naïve CD4 T cell population is expanded upon encounter with 2W1S peptide, a variant of peptide 52-68 of the I-E alpha chain, and identified using MHC II tetramers. Studying endogenous murine polyclonal populations of CD4 T cells avoids potential artefacts arising from monoclonal TCR transgenic cells, such as reported detrimental effects on survival of having a large clonal population (Hataye et al., 2006). To specifically study Treg cells within this CD4 T cell population, approaches used to expand overall Treg cells were applied to the 2W1S model.

After immunisation with 2W1S peptide, Treg cells were found to make up approximately 13% of the 2W1S<sup>+</sup> T cell population. However, by immunising twice with 2W1S peptide, seven days apart, this percentage could be boosted, through a selective increase in the number of 2W1S<sup>+</sup> Treg cells. This observation may have important implications in terms of autoantigen-specific immunotherapy for autoimmune disease, where the challenge is to boost Treg cells specific to an autoantigen, without exacerbating the effector response. As well as exploring methods to increase the proportion of antigen-specific Treg cells it was also important to ensure a high number of these cells could be expanded, for example to boost efforts to assess long term survival, where low numbers might hinder assay sensitivity. In this study it was found that an agonistic antibody which targets the TNF receptor DR3 can increase the number of Treg cells within this population by approximately ten fold, although it also increases the number of Tconv cells and therefore this is not a selective effect. Interestingly, this antibody selectively

increases the percentage of total Treg cells, and such disparity is thought to be a result of relative availability of cognate antigen (Schreiber and Podack, 2013). Therefore, after 2W1S peptide administration, expansion of regulatory and conventional T cells which recognise this peptide occurs, as well as total Treg cells which are encountering self-antigen. The protocol for expanding Treg cells was therefore set as two immunisations of 2W1S peptide and agonistic anti-DR3, hence incorporating both an increase in proportion, and number.

To study Treg cell function *in vivo*, an assay was established based on work by Rowe et al. (2012), in which the function of 2W1S<sup>+</sup> Treg cells was assessed through measurement of IFN $\gamma$  production by 2W1S<sup>+</sup> Tconv cells upon infection with *Listeria monocytogenes*-expressing 2W1S peptide (Lm-2W1S). In these experiments 2W1S<sup>+</sup> Treg cells were expanded in response to constitutive expression of 2W1S peptide as a fetal antigen, and peaked at 50% of the 2W1S<sup>+</sup> T cell population. In my study, 2W1S<sup>+</sup> Treg cells were expanded using two immunisations of 2W1S peptide and agonistic anti-DR3, which resulted in blunting of a subsequent T cell response to Lm-2W1S. Although IFN $\gamma$  production was not affected, blunting of the response is consistent with Treg cell mediated suppression.

Interestingly, this study showed that if Lm-2W1S is administered prior to the protocol for expanding Treg cells, a very large expansion of 2W1S<sup>+</sup> Tconv cells occurs despite the presence of 2W1S<sup>+</sup> Treg cells. This suggests that suppression is harder to achieve after a strong Th1 response. One proposed avenue to prevent destruction of tissue in autoimmune disease is Treg cell expansion (Miyara et al., 2014), however this may be technically challenging if these findings are confirmed, as clinical symptoms often appear after the immune response is already underway. This

observation may reflect differences in the ratio of Treg cells to effector cells, as after Lm-2W1S administration the percentage of Treg cells is very low. Alternatively, it may reflect enhanced resistance of effector cells to Treg cell-mediated suppression. Indeed, it has been reported that high doses of antigen can render T cells resistant to suppression (Baecher-Allan et al., 2002, Georger et al., 2003), and a role for PAMPS, pro-inflammatory cytokines and TNF receptors has also been reported (Walker, 2009).

## **6.2 The role of OX40 and CD30 in regulatory and effector T cell biology**

The role of OX40 signaling in Treg cell biology is controversial within the literature, with reports that OX40 negatively regulates Treg cell function, and conversely that OX40 and IL-2 signal co-operatively to support Treg cells (Xiao et al., 2012). Having established a model to assess Treg cells, I sought to study the role of TNF receptors OX40 and CD30 in in this context. Interestingly, primary expansion of Treg cells was impaired in OX40/CD30 dKO mice, although less so than Tconv cell expansion. However, a decline in Treg cell numbers was not clearly observed at 21 or 51 days after immunisation suggesting longer term survival of Treg cells in the periphery is not highly dependent on OX40 and CD30. Data within this study and others strongly indicates that the majority of 2W1S<sup>+</sup> Treg cells are thymically derived (tTreg cells). Therefore, the observed impairment in Treg cell expansion is unlikely to be explained by effects on the Tconv cell pool. There are two proposed markers of tTreg cells, Neuropilin-1 and Helios, which along with the presence of a Treg cell population in naïve mice, give a strong indication of the origin of a Treg cell population. However, the field would benefit from the characterisation of a more robust marker for tTreg cells, as neither marker is expressed only by tTreg cells and not pTreg cells (Singh et



al., 2015). This would aid understanding of the differing roles of these the two subsets which are thought to have non-overlapping functions but to synergise to attain maximal suppression (Haribhai et al., 2011a). Collectively these data showed that following peptide stimulation the persistence of Treg cells was not heavily dependent upon signals through CD30 and OX40. Whilst the cells persisted, whether these cells remained functional became the key question to address.

The previously described *in vivo* assay developed to assess Treg cell function was used to investigate the role of OX40 and CD30. Blunting of the response was again observed in the wildtype control mice, but interestingly, this was not observed in OX40/CD30 dKO mice, consistent with an impairment of the function of Treg cells in the absence of these signals. These data therefore support the body of evidence that OX40 signaling supports Treg cells. Further experiments in mice which can be conditionally depleted of Treg cells are needed to confirm that the impairment in the response to Lm-2W1S after Treg cell expansion is indeed dependent on the presence of Treg cells. The most effective model for studying this would be to use 'FOXP3<sup>DTR</sup>' mice, which are engineered so that the Diphtheria Toxin Receptor (DTR) is expressed by Treg cells, and therefore these cells can be specifically ablated by administration of Diphtheria Toxin (Kim et al., 2007).

This study has also furthered our understanding of the role of OX40 and CD30 in CD4 T cell function. Strikingly, CD4 T cells were found to be highly dependent on OX40 and CD30 as the ability of antigen-specific Tconv cells to produce IFN $\gamma$  in response to Lm-2W1S was grossly impaired in their absence. Therefore, this study has demonstrated that CD4 T cell function, rather than primary expansion is highly dependent on OX40 and CD30. These TNF receptors are upregulated rapidly on T

cells upon activation, but in acute infection OX40 is only transiently expressed, and indeed the majority of OX40 expression is restricted to a brief window after Lm-2W1S administration (Marriott et al., 2014). Thus the requirement for OX40 signalling in the function of these cells indicates that critical interactions with OX40L<sup>+</sup> cells must occur within this time. If we can pinpoint the cellular source of OX40L, we can better manipulate the process therapeutically.

Importantly, the requirement for OX40 and CD30 signals in CD4 T cell function was not restricted to the 2W1S response, and could be observed in another endogenous T cell population which responds to Listeriolysin-O, a major virulence factor of *Listeria monocytogenes* (Hamon et al., 2012). Thus, these data indicate that there is a clear requirement for OX40 in the production of effector cytokines in Th1 responses such as the response mounted to Lm-2W1S. Further experiments in other experimental settings could test whether it is true of other T helper responses, for example using Group A Streptococcus expressing 2W1S peptide (GAS-2W1S) would enable the study of Th17 responses (Dileepan et al., 2011).

The gross impairment in effector cytokine production by 2W1S<sup>+</sup> T cells was also observed in OX40 KO mice, which suggests that this effect is largely attributable to OX40, although future experiments with CD30 KO mice are required. CD30 is not as well characterised in the literature and it is technically challenging to identify CD30 ex-vivo as it appears to be expressed at low levels. However, it has previously been shown that CD4 memory T cell generation in OX40 KO mice is affected by OX40, and that this is more pronounced if both receptors are absent, which suggest that they exhibit synergy (Gaspal et al., 2005, Gaspal et al., 2008, Withers et al., 2009,

Gaspal et al., 2011). To further investigate the role of OX40 in CD4 T cell function, other pro-inflammatory cytokines could be assessed such as TNF $\alpha$ .

Interestingly, this study also revealed that OX40 and CD30 are required for CD4 T cells to be able to expand in response to repeated antigen exposure, as occurs in autoimmunity, as well as many other conditions where therapeutic manipulation of the immune response would be beneficial. To further study the contribution of OX40 and CD30, this experiment should be performed in OX40 KO mice, as well as CD30 KO mice. Together these data show fundamental differences in CD4 T cell behavior in the absence of OX40 and CD30, with reduced rapid pro-inflammatory cytokine production, as well as impaired expansion upon repeated antigen exposure. This therefore indicates that the capacity to mount functional memory CD4 T cell responses is impaired.

### **6.3 Cellular support for Treg cell survival**

Previous studies on the cells that support Treg cells has centered mainly on immature dendritic cells, which may provide survival signals through the TCR and CD28 on Treg cells (Attridge and Walker, 2014). This study has instead explored the role of group 3 ILCs, using the previously described antigen-specific approach in mice that lack ROR $\gamma$ t, the lineage defining transcription factor of this group of ILCs. Whilst technically challenging to specifically pinpoint ILC3 due to a lack of specific mouse models, the absence of ILC3s correlated with a striking impairment in Treg cell expansion. This suggests that these cells may interact, supporting preliminary data which showed Treg cells in close proximity to ILC3s in the interfollicular areas of mesenteric lymph nodes. ROR $\gamma$ t mice have many caveats, particularly the absence

of lymph nodes, and altered thymic development (Sun et al., 2000, Kurebayashi et al., 2000). However, as 2W1S<sup>+</sup> Treg cells can be identified in naïve ROR $\gamma$ t KO mice, it is possible to conclude that this effect is not simply due to the absence of 2W1S<sup>+</sup> Treg cell generation in the ROR $\gamma$ t KO mice. Studies using mice in which ILC3s are specifically depleted are therefore needed to investigate this further and confirm that this defect is due to the absence of ILC3s. Interestingly, DR3 was shown to be highly expressed on ILC3s in this study, and is therefore likely to have a role in ILC3 biology, particularly as DR3 expression has been shown to contribute to ILC2 expansion, survival and function (Yu et al., 2014).

Various candidates have emerged as potential interaction partners between ILC3s and Treg cells. OX40L and CD30L have been reported to be expressed by ILC3s (Lane et al., 2005), but Treg cells do not appear to be highly dependent on OX40 and CD30 for their survival. This study shows that Nrp-1, which has been proposed to support Treg cell survival (Delgoffe et al., 2013), is highly expressed by ILC3s in the periphery. As there are reports that Nrp-1 can form homotypic interactions (Sarris et al., 2008), this may be a mechanism by which ILC3s support Treg cell survival, particularly as the interfollicular area was shown to be rich in Nrp-1 expression. To study this further a mouse could be generated which lacks Nrp-1 specifically on ILC3s using the cre-lox system, and experiments could be performed to determine whether antigen-specific Treg cell survival is affected.

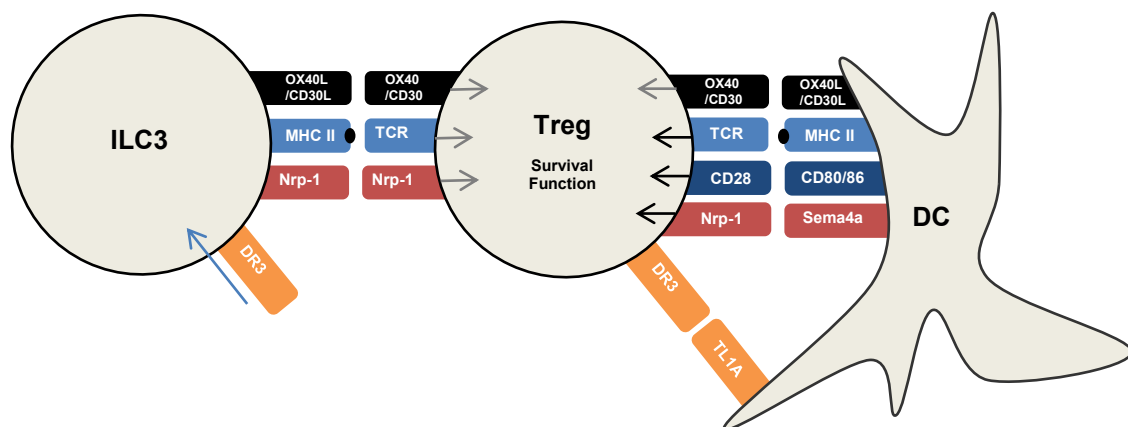
In this study it was also shown that MHC II is highly expressed by ILC3s in the periphery. As TCR signals have been shown to contribute to Treg cell survival (Kim et al., 2009, Walker et al., 2003a, Fisson et al., 2003) MHC II may be a potential interaction partner between ILC3s and Treg cells. Interestingly, ILC3s in the gut have

been shown to express high levels of MHC II and to be able to process and present antigen (Hepworth et al., 2013). Further studies with these mice, which lack MHC II on ILC3s, would aid understanding of whether MHC II on ILC3s provides survival signals to Treg cells. Whilst investigating the role of ILC3s in Treg cell survival, it is important to note that different lymph nodes contain different proportions of ILC3s (Mackley et al., 2015). The use of IL-2/anti-IL-2 complexes to expand ILC3 numbers may also aid future understanding of the relationship between ILC3s and Treg cells; an approach which was further developed within this study.

#### **6.4 Overall Summary**

This study has established a method to study the survival and function of Treg cells *in vivo*, and therefore to investigate the signals and cells which are involved. The role of ILC3s was explored, as well as the co-stimulatory molecules OX40 and CD30. **Figure 6.1** shows some of the key signals required by Treg cells, which may be provided by dendritic cells, and demonstrates potential roles for ILC3s and OX40/CD30 within this context. These experiments provided evidence for a role for OX40 and CD30 in Treg cell expansion, as well as function. In addition, effector cytokine production by Th1 cells was shown to be heavily dependent upon signals through these TNF receptors, as well as their ability to expand upon repeated antigen exposure. This therefore indicates that OX40 and CD30 are required to mount functional CD4 memory T cell responses. A regulatory role of ILC3s is emerging in the literature, and in accordance with this, this study demonstrates that Treg cell expansion is grossly impaired in mice which lack ROR $\gamma$ t, the lineage defining transcription factor for ILC3s, although this needs to be repeated in mice which specifically lack ILC3s. This study provides further evidence that MHC II is a potential

interaction partner between Treg cells and ILC3s, and suggests a new candidate: Nrp-1. The TNF receptor and co-stimulatory molecule DR3 was also shown to be highly expressed by ILC3s, and therefore may have a role in modulating their activity (**Figure 6.1**). A technique for inducing ILC proliferation was successfully applied to ILC3s, which will aid future study of the relationship between Treg cells and ILC3s.



**Figure 6.1: Dendritic Cell and Group 3 Innate Lymphoid Cell-derived signals which may contribute to regulatory T cell survival and function.** Previous studies have demonstrated a clear role for TCR and CD28 in the survival of Treg cells, and signals between Nrp-1 and Sema4a have also been implicated (black arrows). This study has provided evidence for a role for OX40 and CD30 in Treg cell expansion, as well as function, and considers the role of ILC3s which are able to provide signals through these receptors, as well as the TCR (grey arrows). In addition, this study demonstrated that Nrp-1 is highly expressed by ILC3s, as well as the TNF receptor DR3. The former has the potential to form homotypic interactions which could provide signals through Nrp-1 expressed by Treg cells, and the latter may contribute to ILC3 function (blue arrow).

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