INVESTIGATING COSTIMULATORY MOLECULE PROVISION AND REGULATION FOR CD4 T CELL RESPONSES *IN VIVO*

by

Dominika Weronika Gajdasik

A thesis submitted to the University of Birmingham

for the Degree of DOCTOR OF PHILOSOPHY

Institute of Immunology and Immunotherapy

College of Medical and Dental Sciences

University of Birmingham

September 2019

ABSTRACT

Interactions between the innate and adaptive immune systems have pivotal roles in the generation of effector and memory lymphocyte responses. This thesis focuses on the costimulatory pathways that regulate CD4 T cells development, specifically OX40:OX40L as the critical mechanism downstream of CD28 signalling. Whilst it is clear that CD4 T cell responses require signals through OX40 pathway, its role in T cell function as well as the cellular sources of its ligand *in vivo* and how its expression is regulated are poorly defined.

Here we reveal our studies across various *in vivo* immune response models, including attenuated *Listeria* and *Salmonella* models and the clinically approved immunostimulant AS01 adjuvant. Collectively, these approaches all demonstrated that OX40 signals were particularly important for the generation of effector CD4 T cell populations. We have shown that in responses to *Listeria*, the number of effector CD4 T cells expressing IFNγ was highly OX40 dependent. In the context of acute bacterial infection, we discovered that OX40L is rapidly upregulated on DC within the first 24hrs post infection and this is controlled by the innate cell production of IFNγ and in particular, dependent on IL-12 signals affecting the IFNγ production by NK cells. Furthermore, using the novel OX40L conditional knockout mice we have successfully shown that CD11c+ DC were the critical cellular sources of OX40L during the primary response to *Listeria* and that OX40L expression by T cells, ILC3 and B cells was redundant. Using the AS01 model we have demonstrated that although important for optimal CD4 T cell responses, the rapid production of innate IFNγ induced by the AS01 was not required for neither the upregulation of OX40L on DC nor the B cell responses generated post immunisation, confirming multiple mechanisms for OX40L regulation in a context dependent manner. Collectively, these studies highlight the importance of OX40 pathway across various in vivo immune responses and reveal new data on regulation of OX40L expression and its cell specific provision.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr David Withers for all of his help throughout the last 4 years. He has really been a great and dedicated supervisor and I cannot thank him enough for his support and expertise which allowed me to grow as a scientist. Most importantly, I thank him for his infectious enthusiasm for immunological research which has inspired me to continue with my scientific career.

Next, I would like to thank everyone from the Withers group for their support and some of the craziest and most interesting discussions. I would like to thank Fabrina Gaspal for being the best and most patient PostDoc in the world, Remi Fiancette for great discussions about science and Brexit, and Zhi Li and Rhys Jones for making the lab work even more fun.
Special thanks to Emma Dutton for sharing one of the greatest memories of my life, the amazing conferences, sleep talking, games nights and the constant singing and dancing in the lab. Finally, to an absolutely amazing and talented person, Claire Willis: thank you for all of your hard work, supporting me at every step and for always being there for me when I needed it most. Best Maid of Honour ever!

I would also like to thank people within the IBR for all of their support and expertise. Special thanks also to Arnaud Didierlaurent and Margherita Coccia from GSK for supporting me throughout my studies and giving me the opportunity to work in a beautiful and sunny Siena as part of my industrial placement.

I would like to thank my family, especially my siblings for the endless phone calls and fun weekends and my mum for supporting me through the good and tough times and for never asking me to explain what my thesis was about.

Finally, I would like to thank my husband Tom for his continuous support and for always believing in me. Thank you for all those conversations, Netflix binge-watching sessions, concerts and amazing holiday trips. I would have never completed this without you.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

CHAPTER 5: CELLULAR SOURCES OF OX40L IN VIVO

CHAPTER 6: THE SIGNIFICANCE OF INNATE IFNγ IN AS01 INDUCED RESPONSES

CHAPTER 7: GENERAL DISCUSSION

APPENDIX AND REFERENCES

LISTS OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER4

CHAPTER 5

CHAPTER 6

CHAPTER 7

APPENDIX

LISTS OF TABLES

LIST OF ABBREVIATIONS

$^{-/-}$	deficient
AIRE	autoimmune regulator
APC	antigen presenting cell
AP-1	activator protein 1
AS01	adjuvant system 01
Bcl	B cell lymphoma
Bcl-xL	B cell lymphoma- extra large
BALT	bronchus-associated lymphoid tissue
BFA	Brefeldin A
C	constant
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDR	complementarity determining region
CFA	complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CpG-DNA	CpG oigodeoxynucleotide
CSP	circumsporozoite protein
cTEC	cortical thymic epithelial cells
CTLA4	cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand

CXCR	CXC chemokine receptor
D	diversity
DAMPS	damage-associated molecular patterns
DC	dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dLN	draining lymph node
DNA	deoxyribonucleic acid
Dpi	days post infection/immunisation
dsRNA	double stranded RNA
DT	diptheria toxin
eYFP	enhanced yellow fluorescent protein
EAE	experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EOMES	Eomesodermin
ER	endoplasmic reticulum
ERK	extracellular signal-related kinase
FBS	fetal bovine serum
FCS	fetal calf serum
Foxp3	forkhead box p3
FRC	fibroblastic reticular cell
FTY720	Fingolimod
GALT	gut-associated lymphoid tissue

GATA3	GATA binding protein 3
GC	germinal centre
GDP	guanosine diphosphate
CFU	colony forming units
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GS	Great x Smart17
HBSS	Hank's Balanced Salt Solution
HD	Human Dose
HEV	high endothelial venule
ICAM	intracellular adhesion molecule
ICOS	inducible costimulator
ICOSL	inducible costimulatory ligand
IFA	Incomplete Freund’s Adjuvant
IFN	interferon
IFNγR	interferon gamma receptor
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILF	isolated lymphoid follicles
iNKT	invariant natural killer T cell
ITAM	immunoreceptor tyrosine-based activation motif
iTreg	induced T regulatory
i.v.	intravenous

J	joining
LAT	linker for activation of T cells
LCMV	lymphocytic choriomeningitis virus
LCK	lymphocyte-specific protein tyrosine kinase
LN	lymph node
Lm-2W1S	Listeria monocytogenes-2W1S
LPS	lipopolysaccharide
LTi	lymphoid tissue inducer
MALT	mucosal associate lymphoid tissues
MARCO	macrophage receptor with collagenous structure
MAP	mitogen-activated protein
MCMV	mouse cytomegalovirus
MFI	mean fluorescence intensity
mG	membrane-targeted green fluorescent protein
MHC	major histocompatibility complex
mLN	mesenteric lymph node
MPLA	monophosphoryl lipid A
mRNA	messenger ribonucleic acid
mT	membrane-targeted tandem dimer Tomato
mTEC	medullary thymic epithelial cell
MZ	marginal zone
MZM	marginal zone macrophages
NK	natural killer

NK-κB	nuclear factor kappa B
NKT	natural killer T cell
NLR	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
NS	not significant
nTreg	natural Treg
OPSI	overwhelming post splenectomy infection
OVA	ovalbumin
OX40L	OX40 ligand
pMHC	peptide MHC
PAMPS	pathogen associated molecular patterns
PALS	periarteriolar lymphatic sheaths
PBS	phosphate buffered saline
PD-1	programmed death-1
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol 12-myristate 13-acetate
pMHC	peptide major histocompatibility complex
PRR	pattern recognition receptor
RAG1	recombinase activating gene 1
RAG	RAG recombinase
RIG-I	retinoic acid-inducible gene-I
RNA	ribonucleic acid
RORγt	retinoic acid receptor-related orphan receptor gamma t

RSS	recombination signal sequence
$S1P_1$	sphingosine-1 phosphate receptor
SB	staining buffer
SEA	staphylococcal enterotoxin A
SOS	Son of Sevenless
SR-A	scavenger receptor A
TAP	transporter associated with antigen processing
T-bet	T-box transcription factor
Tcm	T central memory
TCR	T cell receptor
Teff	T effector
Tem	T effector memory
Tfh	T follicular helper
TGFβ	Transforming growth factor beta
Th	T helper
TM	true memory
TLR	toll-like receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TNFRSF	tumour necrosis factor receptor superfamily
TRAF	TNF receptor associated factor
Treg	T regulatory
TSLP	thymic stromal lymphopoietin

V	variable
VM	virtual memory
WHO	World Health Organisation
WT	wildtype
XCR1	XC chemokine receptor 1
ZAP-70	zeta chain associated protein kinase

CHAPTER 1: INTRODUCTION

1.1 From innate to adaptive immunity

Over the course of evolution, multicellular eukaryotic organisms developed a wide range of defence mechanisms to protect themselves from highly specialised disease-causing agents with which they share the environment. Due to the complex nature of their cellular organisation and relatively weak barriers protecting them from environmental stresses, multicellular organisms dedicated high number of cells to fight pathogens.

The immune system is composed of highly compound and advanced mechanisms which evolved to target the immediate threats of infection in a quick and a broad response manner (innate immunity) followed by the more specific and thorough protective mechanism (adaptive immunity). The cells of the immune system work tightly together to recognise pathogens and activate the effector mechanisms like phagocytosis and antibody production. Although, the majority of mechanisms are common to many immune cells, there are some that will be only performed by specialised cells possessing receptors capable of binding a variety of pathogen associated molecules. Understanding mechanisms that mediate immune responses to foreign antigens will enhance our ability to develop superior approaches to combatting infectious disease. In particular, the development of new immune therapies and vaccines relies heavily upon an understanding of the interaction between the innate and adaptive immune cells.

Innate immunity is often described as the first line of defence and indeed innate cells are clustered within the sites of entry into the body. The cells involved in innate immunity include the widely studied and evolutionarily ancient hematopoietic cell types: natural killer (NK) cell, eosinophils, basophils, mast cells, macrophages, dendritic cells (DC) and neutrophils as well as cells of the non-hematopoietic origin, including stromal cells (LeibundGut-Landmann et al., 2011). In recent years, many studies highlighted the importance and function of another group

of cells, crucial in maintaining tissue barrier integrity and homeostasis. These cells are electively known as Innate Lymphoid Cells (ILCs) and include NK cells. They have been shown to be key producers of cytokines within tissues such as the intestine, lung and skin. In turn these cells are directly regulated by a wide range of locally produced cytokines in tissues (Artis and Spits, 2015). Although some of the functional aspects still require further assessment *in vivo*, there is a growing evidence supporting the involvement of ILCs in both the regulation of the innate immunity as well as modulation of the adaptive responses. Thus beyond controlling tissue homeostasis these cells are most likely important regulators of adaptive immunity (Hepworth and Sonnenberg, 2014a).

1.1.1 Innate immune recognition strategies

Cells of the innate immune system augment protection provided by barrier sites, relying on a limited number of recognition strategies. These strategies enable detection of conserved components of a pathogen which are crucial for its viability and virulence and therefore less prone to change in case of rapid pathogen evolution.

Unlike cells of the adaptive immune system, cells described as part of the innate immune system lack receptors formed by RAG-mediated combinatorial gene rearrangements. However, they do clearly respond to signals, possessing a discrimination system which allows them to differentiate between the self and non-self molecules (Akira et al., 2006; Medzhitov and Janeway, 2000). These structures of bacterial, fungal or viral origin are referred to as pathogen associated molecular patterns (PAMPs). PAMPs include peptidoglycan, lipopolysaccharide (LPS), flagellin, lipoteichoic acid as well as bacterial and viral genetic material including the dsRNA, CpG-DNA and unmethylated CpG (Janeway and Medzhitov, 2002). Innate cells can also detect immunological damage caused by an infection, including endogenous alarmins and heat-shock proteins. These are often referred to as damage-associated molecular patterns (DAMPs). Innate cells can recognise PAMPs and DAMPs

through the pattern recognition receptors (PRRs). PRRs are highly primitive and include Toll-like receptors (TLR), C-type lectin receptors, cytoplasmic RIG-I-like receptors and NOD like receptors. TLRs are well studied PRRs, able to recognise limited but very specific range of microbial products, for instance TLR4 is able to detect LPS while the TLR9 readily binds to the CpG DNA. TLRs were discovered in mid-1990s, when the findings of Drosophila research highlighted the importance of Toll protein in protecting the fruit fly against fungal infections (Lemaitre et al., 1996). TLRs act through initiation of signal transduction pathways that trigger gene expression and the subsequent upregulation of the innate immunity (Hoffmann, 2003). Understanding TLR function and identifying their potential ligands contributed greatly to the development of more efficient vaccine adjuvants. For instance, monophosphoryl lipid A (MPLA) have been used in both Adjuvant Systems 01 (AS01) and 04 (AS04) as a much less toxic ligand of the TLR4 inducing efficient innate immune responses (Casella and Mitchell, 2008; A. M. Didierlaurent et al., 2014; McKeage and Romanowski, 2011).

Although PRRs are classically considered as innate like recognition receptors, several studies have identified their expression levels on T lymphocytes or T cells and B lymphocytes or B cells. It has been shown that particularly TLR1, TLR2, TLR3, TLR6 were expressed by both CD4 and CD8 T cells (Caramalho et al., 2003; Rahman et al., 2009). B cells localised to the peritoneal and plural cavities, were found to express TLR1-9 while the marginal B cells demonstrated prevalence for expression of TLR2, TLR6 and TLR7 (Barr et al., 2007).

1.1.2 Interaction of innate and adaptive immunity

PRR signalling has two important consequences: the generation of innate responses in the form of cytokine production and induction of antigen presentation. The ability to induce adaptive responses along with several other features of the innate defences are especially well developed in DC. They represent a group of Antigen Presenting Cells (APCs) and are involved in antigen uptake and its presentation. DC were discovered by Ralph Steinman and

Zanvil Cohn who using a phase-contrast microscopy found a small group of very motile and branched cells colocalised with macrophages (Steinman and Cohn, 1973).

Murine DC can be divided into 5 groups: plasmacytoid DC, XC chemokine receptor 1 (XCR1)+ DC, CD11b+ DC, monocyte-derived DC and Langerhans cells (Crozat et al., 2010; Guilliams et al., 2010; Shortman and Liu, 2002). DC originate in bone marrow from DC precursors and circulate between blood and lymphoid and non-lymphoid tissues. Upon their maturation, which will be described in detail in later sections, DC are known to deliver three types of signals to induce activation of naive T cells: 1. Presentation of antigen to naive T cells; 2. Stimulation via the costimulatory molecules; 3. Initiation of differentiation via the cytokine secretion (Burnet, 1959; Cunningham and Lafferty, 1977; Curtsinger et al., 1999; Keppler et al., 2012; Linsley et al., 1990; Zinkernagel, 1974). The antigen presentation and provision of stimulatory signals by DCs marks the transition between the innate and adaptive immunity. The recognition of antigen by majority T cells is only possible when the antigen in presented as a part of Major Histocompatibility Complex (MHC) (Doherty and Zinkernagel, 1975). This interaction activates naive T cells via the T cell receptor (TCR) mediated signalling process which ultimately results in the activation of key transcription factors. The activation of T cells results in their subsequent differentiation, a process known as clonal expansion during which cells proliferate at the highest rate, producing great number of antigen specific subtypes of T cells, each with specific effector cytokine profiles (Goldrath and Bevan, 1999).

1.2. The adaptive immune system

The evolution of adaptive immune system occurred much later in the development of multicellular organisms and has specifically been found in all jawed fish and higher vertebrates (Schluter et al., 1999). Cells of the adaptive immunity are commonly defined as the cells expressing antigen specific receptors able to identify and act upon wide range of pathogens derived molecules presented to them by the APCs. These receptors are randomly

generated through the process of somatic gene rearrangements. T and B lymphocytes are the primary members of the adaptive immunity. They work very closely with the cells of the innate immune system to orchestrate the best type of defence to a particular threat.

Unlike the very rapid responses of the innate immune system, adaptive immunity has been characterised by slow but very specific process of organised events which usually take several days to develop (Chaplin, 2010). This is due to the relatively low number of adaptive immune cells that recognise a given peptide. Upon recognition of their cognate antigen enter the cycle of proliferation and differentiation before acquiring their effector functions.

The fundamental difference between innate and adaptive immunity lies in the formation of long-lived cells which are able to act upon secondary encounters with the specific antigen. This is often referred to as immune memory. In recent years, however, there have been reports of a NK cell derived innate memory. It has been observed that C57BL/6 mice were able to generate mouse cytomegalovirus (MCMV) specific long-lived innate memory cells. In these mice, approximately half of the NK cell population expresses Ly49H, activating receptor linked to resistance to MCMV infection. The adoptive transfer of $Ly49H^+$ NK cells into DAP12 deficient mice (defective in Ly49H function) and the their subsequent challenge with the MCMV virus, resulted in a rapid cell expansion and increased effector functions of the NK cell population (Sun et al., 2009). Thus, like $CD8^+$ T cells, the NK cells can also be subjected to viral antigen driven activation, expansion and generation of memory cells. Although compelling and potentially beneficial, the NK innate memory constitutes a small portion of the highly efficient memory responses driven by the cells of the adaptive immune system.

1.2.1 T cell receptors and their repertoire

As stated previously, the T cell receptors, or TCRs are heterodimers found on the surface of T cells where they recognise antigens presented as a part of the MHC. Both human and murine TCRs were first discovered in 1984 (Hedrick et al., 1984; Yanagi et al., 1984). Initial

descriptions of TCRs related more to the similarities with immunoglobulins but since then their structure and function have been a subject to many research.

TCRs are classed according to the type of T cells they are expressed on, the TCR$\alpha\beta$ and TCR$\gamma\delta$ (Davis and Bjorkman, 1988). The TCR$\alpha\beta$ are noticeably more abundant with majority of conventional T cells belonging to the $\alpha\beta$ lineage. The TCR α and γ chains contain variable (V), joining (J) and constant (C) regions. These regions are also present in TCR β and δ chains with an additional diversity (D) region. The variable regions are the main site of gene rearrangements which involve differential assembly of the V, D, J segments and additions or deletions of individual nucleotides before the final completion of the exon.

The constant region, situated proximally to the cell membrane, determines the effector properties and types of chains, while the distal variable region plays a predominant role in antigen recognition as it contains exceptionally variable complementarity-determining regions (CDR), CDR1, CDR2 and CDR3. The CDR3, contained among the J and D segments, is the most variable and it has the most interaction with the antigen. The other two CDRs are known for interacting with the MHC complex (Wu, 1970). As naive T cells require interaction with the MHC, mutations within the CDR3 hypervariable region are therefore particularly important and can contribute to stronger affinity for specific antigen (Xu and Davis, 2000).

Next to the V, D, J segments lies the main regulator of the gene rearrangements, the recombination signal sequence (RSS). The enzymes RAG-1 and RAG-2 act directly on the RSS and initiate gene rearrangements (Berg et al., 2002). The two enzymes are expressed by both B and T cells, with very limited evidence for expression on any other hematopoietic cells (Borghesi et al., 2004; Månsson et al., 2007). RAG-1 and RAG-2 pair together and are both required to cleave double stranded DNA of all TCR genes while the DNA repair machinery completes the recombination (McBlane et al., 1995).

The gene rearrangements occur at random, resulting in a population of highly diverse T cells with the ability to recognise extensive range of antigens. It is estimated that potential diversity

of the TCRs generated in the murine thymus could reach 10^{15} of TCR clonotypes (Casrouge et al., 2000). This range is however unrealistic due to the limited overall number of cells within one mouse. Therefore, the process of gene rearrangement contributes mainly to a differential distribution of the TCR frequencies, with some of the TCRs being more common and often shared between individuals and species (Miles et al., 2011; Venturi et al., 2008). The actual size of TCR repertoire for humans and mice is still unknown.

1.2.2 Development of T lymphocytes

The development of T cells starts in the bone marrow, where haematopoietic stem cells give rise to haematopoietic progenitors that populate the thymus and begin the process of their maturation (Germain, 2002). The thymus is a primary lymphoid organ that is crucial for maintaining a stable microenvironment and conditions required for development of T cells. It is present in all jawed vertebrates and is located in the superior mediastinum of the thorax, above the heart and behind the sternum. Functionally, the thymus is most active during the neonatal stages of life and after the puberty it begins to decrease in size, undergoing an atrophy which affects further development of thymocytes (Shanley et al., 2009).

The developing T cells undergo several important events with the initial stages of their development involving changes in the expression of cell surface markers as well as genetic mutations.

At the beginning of their development, thymocytes lack CD4 and CD8 expression and are known as ‘double- negative’ cells. Those cells undergo gene segment rearrangement within the TCR coding region which is essential to diversify its specificity, ultimately generating an enormous range of TCRs. The double negative thymocytes give rise to two distinct lineages: αβ and γδ T cells (Robey and Fowlkes, 1994). The majority of T cells express αβ TCRs. The γδ T cells comprise a minority and their numbers steadily decrease as the formation of αβ T cells progresses, making up maximum of 4% of total T cell number in the thymus (Chien et

al., 2014). The γδ T cells reside in distinct anatomical compartments, particularly the mucosa of the gut and express a distinct set of TCRs that have entirely different antigen biding properties than those of the αβ T cells. The γδ T cells are able to recognize antigens without the requirement for the MHC molecules, relying on the complementarity of the antigen and the biding site (Vantourout and Hayday, 2013).

As the result of rearrangements within the β chain, thymocytes become double positive, expressing both CD4 and CD8 cell surface markers and are tested for their affinity towards the MHC complexes, in a process known as positive selection. They enter the subcapsular region of the thymus and are presented with self-MHC molecules bound to self-peptides in a process mediated by the cortical thymic epithelial cells (cTEC) (Nitta et al., 2008). The surviving cells, which express a TCR able to bind MHCI or MHCII and peptide become single positives: either the $CD4^-CD8^+$ or $CD4^+CD8^-$. The $CD4^-CD8^+$ T cells are able to recognize antigens presented by MHCI, while the $CD4^+CD8^-$ are able to interact with the MHCII.

The rearrangements within α chain enhance the process known as the negative selection which takes place in the medulla. Thymocytes interact there with medullary thymic epithelial cells (mTECs) which present them with MHC:self-antigen complexes. The expression of self-antigens is regulated by Autoimmune regulator (AIRE) (Anderson and Su, 2011; Shi and Zhu, 2013). This allows to eliminate any T cells that might have developed strong affinity for the self-antigens and may therefore be involved in autoimmunity.

Following the selection process, thymocytes emigrate from the thymus via the sphingosine-1 phosphate receptor ($S1P_1$) (Carlson et al., 2006; Matloubian et al., 2004). Mice deficient in the $S1P_1$ lack peripheral populations of T cells caused by retention of mature T cells within the thymus (Matloubian et al., 2004).

Naive T cells recirculate through secondary lymphoid tissues searching for cognate antigens.

1.2.3 T cell migration and secondary lymphoid tissues

First reports of lymphocytes recirculation can be traced back to 1960s when Gowans and others showed that cannulation of the thoracic duct in rats induced loss of lymphocytes which was subsequently rescued by intravenous injections of the collected lymph. Moreover, lymph collected from the thoracic duct, incubated *in vitro* with tritiated adenosine for RNA and DNA labelling, and transfused back into the blood, could later be recovered from the thoracic duct (Gowans, 1957; Gowans and Knight, 1964; Mann and Higgins, 1950). This clearly indicated the recirculating nature of lymphocytes. Further *in vivo* research in sheep characterised the circulating lymphocytes (Smith et al., 1970).

High motility of lymphocytes is key to immunosurveillance and scanning for foreign antigens. Evolution of the immune system has optimised this process by forming highly organised structures, located at strategic locations allowing capturing of antigens and interaction between migrating antigen experienced DC, B cells and T cells. These finely designed structures are referred to as the secondary lymphoid tissues and they include lymph nodes (LNs), spleen and mucosal associated lymphoid tissue (MALT).

1.2.3.1 Lymph nodes

The LNs are organised lymphoid tissues that filter the lymph for signs of infection. They consist of discrete B-cell areas termed follicles (found in the cortex), T cell areas (found within the paracortex) and medullary cords, rich in plasma cells.

Afferent lymphatics draining the peripheral tissues merge into LNs, and circulating lymphocytes enter the LNs via the high endothelial venules (HEV). DC and antigen enter the subcapsular sinus and using cortical and medullary sinuses they pass through the cortex, paracortex and medullary cords (Gretz et al., 1997).

The entry of lymphocytes is highly regulated by a number of adhesion molecules, selectins, chemokine receptors and integrins which promote lymphocyte arrest and homing (Springer,

1994; Stamper and Woodruff, 1976). The migration of T and B cells into the LNs is dependent on CD62L and CCR7 (Cyster, 1999; Debes et al., 2005; Springer, 1994). The L-selectin CD62L allows for recognition of GlyCAM-1 and CD34 (vascular addressins) found on endothelium of HEVs inducing cell arrest. The chemokine signalling of CCL19 and CCL21 induces the action of integrins, like LFA-1 which binds to the ICAM-1 and -2 that are found on endothelial cells, and promotes the entry of the lymphocyte into the LN (Masopust and Schenkel, 2013). Within the LN, T cells expressing CCR7 use fibroblastic reticular cell (FRC) networks which directs them as well as DC to the paracortex via expressions of CCL19 and CCL21.

B cells migrate into B cell follicles via the network of follicular DC, a non-migratory population found within the B cell areas of secondary lymphoid tissues. These cells are known to produce high levels of CXCL13, an attractant chemokine for the CXCR5 expressing B cells.

Lymphocytes leave the LN via efferent lymphatics in a sphingosine-1-phosphate receptor 1 ($S1P_1$) dependent manner. The importance of $S1P_1$ signalling was characterised in early studies on Fingolimod (FTY720) action (Fujita et al., 1994; Yanagawa et al., 1998). FTY720 is a drug made by changing the structure of the fungal Myriocin and it modulates the egress of lymphocytes from the LN. It becomes phosphorylated by the sphingosine kinase and acts as a agonist, activating the $S1P_1$ (Brinkmann et al., 2002). Its immunomodulating characteristics have classed FTY720 as a possible therapeutic agent which has proven valuable in transplantation and multiple sclerosis treatments (Brinkmann et al., 2002, 2001; Cohen et al., 2010).

1.2.3.2 Spleen

Spleen is the largest organ of the immune system. Interposed within the blood stream, spleen plays an important role in filtering of blood and thereby monitoring for invading disease-causing pathogens as well as emergence of abnormal cells.

The importance of spleen was first recognised in animal studies involving a splenectomy. It was observed that the animals could survive the surgery and recover, however they were more prone to developing diseases later on, particularly parasitaemia (Morris and Bullock, 1919; Yadava et al., 1996). Similar observations apply to human individuals who had their spleens surgically removed. Postsurgical infection is most likely to occur within the first couple of years after surgery and usually poses a 5% lifetime risk. One of the rare but also most serious complications of the splenectomy is the overwhelming post splenectomy infection (OPSI) which is usually caused by Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis (Chong et al., 2017; Okabayashi and Hanazaki, 2008). The OPSI leads to sepsis, which unfortunately, despite aggressive treatments is associated with high mortality rates.

The spleen is organised into three main areas, the white and red pulps and the marginal zone (MZ) separating the two. Structurally, splenic white pulp is very similar to LN, as it contains T and B cell zones being involved in initiation of immune responses. On the other hand, the red pulp, rich in macrophages, is involved in blood filtering and recycling.

Blood enters the spleen via afferent splenic artery and filters through trabecular arteries entering the splenic parenchyma. Branching arterioles of the trabecular arteries enter red pulp and form central arterioles which branch further to deliver blood to the white pulp (Groom et al., 1991). Early studies on spleen function, proposed two circulatory systems, closed and open (Schmidt et al., 1993). In closed circulation, some of the arterioles branch out to the marginal zones, allowing blood to travel through the adjacent venous sinuses, completely bypassing the red pulp. Open circulation is characterised by the blood entering and filtering through a meshwork of the red pulp.

The red pulp and marginal zones, rich in macrophages form the first line of defence. The marginal zone macrophages (MZM) are particularly important for trapping antigens being equipped with various scavenger receptors including macrophage receptor with collagenous structure (MARCO), scavenger receptor A (SR-A) and SIGNR1. Studies have shown that

mice deficient in MZMs were unable to control early infection to lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* (Aichele et al., 2003; Seiler et al., 1997).

Antigen-experienced DCs and other lymphocytes pass through the marginal zones before entering the white pulp and the T cell rich periarteriolar lymphatic sheaths (PALS). Similarly to LNs, the stromal cells within the spleen, particularly the FRCs mediate the migration of cells into the white pulp by expressing CCL19 and CCL21 which interact with CCR7 receptor expressed on T cells (Förster et al., 1999). Migration of B cells into the B cell follicles is CXCL13 and CXCR5 dependent (Allen et al., 2004). Following the antigen exposure, T and B cells downregulate their homing receptors and exit the white pulp via the bloodstream.

In both circulations, blood continues to flow through the sinuses which merge into trabecular veins, which in turn form a splenic vein allowing the blood to exit into the hepatic circulation.

1.2.3.3 Mucosal associated lymphoid tissue

MALTs are located at the sites of mucosal tissues and are often referred to as the inductive sites for mucosal immunity. MALTs are involved in IgA class switching in antigen specific manner (Gormley et al., 1998). They include for instance the gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT). GALTs consist of Payer's Patches and isolated lymphoid follicles (ILFs) in which majority of the lymphocytes are contained. They are important for immune tolerance as well as defence against pathogens (Jung et al., 2010).

1.2.4 Antigen recognition and TCR signalling

The pioneering work by Doherty and Zinkernagel highlighted the importance of MHC and TCR interactions in recognising and generating pathogen specific T cell responses. Their initial experiments involved infecting the mice with meningitis virus, isolating the T killer cells and culturing them with different virus infected cells (Doherty and Zinkernagel, 1975;

Zinkernagel and Doherty, 1997). They concluded that in order for the T cells to attack the infected cells, they had to recognize not only the antigen but also the specific MHC molecule. This dual-recognition model has been used since then and inspired further research into the T cell-mediated immunity.

As mentioned previously, DCs are thought to be main players in the initiation and progress of T cell mediated responses. Their exceptional design combines the ability to intake, process and present foreign antigens with the expression of costimulatory molecules, secretion of cytokines and migration into various tissues. As shown by many *in vitro* studies, other APCs, like for instance B cells, are much less efficient in activating T cells than DC (Cassell and Schwartz, 1994; M. Croft et al., 1992).

Function of DC as immunoregulators is dependent on their state of maturation which is induced by various environmental factors. Pathogen induced inflammation recruits DC to the site of infection, where they begin the process of antigen uptake. Chemotaxis is key to their recruitment, with majority of DC expressing chemokine receptors like CCR1, CCR2, CCR3, CCR5 and CCR6 and CXCR1. These receptors are predominantly stimulated by macrophage derived proinflammatory chemokines, the CCL3 (MIP-1 alpha), CCL7 (or MCP-3), CCL5 (RANTES), CCL20 (MIP-3 alpha), and CCL23 (or MPIF-1) (Sallusto et al., 1998; Sozzani et al., 1995).

Immature DC are able to recognize the antigen and other PAMPs expressed by microorganisms by the PRR receptors found on their surface. Among those receptors, C-type or calcium dependent lectins (CLR) play a particularly important role in DC function (Soilleux et al., 2002). For instance, one of the mostly studied receptors, the DC-SIGN is capable of recognising glycosylated residues found on the surface of many pathogens (Curtis et al., 1992). The immature DC have been shown to express very weak affinity towards the T cells but great capacity for endocytosis. The interaction between antigen and CLR stimulates the DCs to take up the pathogen derived molecules and internalise them into phagosomes. Following the initial oxidative burst caused by NADPH oxidase and myeloperoxidase, NOX2

subunit of NADPH oxidase is recruited into the phagosome to alkalise the environment (Savina et al., 2006). This leads to an arrest of further degradation process, ensuring that antigens remain intact and ready for presentation. Inside the Endoplasmic Reticulum (ER), the MHCII associates with invariant chain (Ii) to form $\alpha\beta Ii_3$ complex. This complex is then transported via the trans-Golgi network into the endosomes, which assemble with the foreign peptides containing phagosomes. This results in Ii degradation and exchange of Ii subunit CLIP for foreign antigen (Bénaroch et al., 1995; Landsverk et al., 2009; Watts, 2004). The exchange of CLIP requires chaperone HLA-DM (Mosyak et al., 1998). The fully assembled MHCII peptide complex is then transported onto the surface of the DC.

Presentation of self-antigens follows the same principles however the process here is more simplistic. Following the proteolytic degradation, the peptides are translocated into the ER by the action of transporter associated with antigen processing (TAP). The assembly of peptide, MHCI, calreticulin and ERp57 is mediated by Tapasin (Williams et al., 2002). The complex is then transported via the Golgi apparatus to the plasma membrane. Upon their maturation, antigen experienced DC migrate into the secondary lymphoid organs, towards the T cell areas. They downregulate their chemokine receptors, apart from the CXCR4, becoming unresponsive to further proinflammatory signals, while upregulating CCR7 associated with cell migration and LN entry (Yanagihara et al., 1998). Cell surface proteins expressed on the surface of DC and T cells facilitate clustering between the two cells. For instance, ICAM-3 receptor, one of DC-SIGN, has a strong affinity for ICAM-3 expressed on T cells promoting DC/T cell primary interactions (Geijtenbeek et al., 2000). Upon establishing the pMHC/TCR contact, DC provide further support for T cells in the form of additional direct interaction via the CD40 signalling or by secretion of soluble cytokines which can determine T cell fate (Caux et al., 1994; Ma and Clark, 2010).

1.2.4.1 TCR signalling

TCR signalling process is very complex and it begins when a TCR engages with its cognate pMHC. TCR lacks intracellular domains but can still interact with CDδε, CD3γε and CD3ζζ subunits, which express immunoreceptor tyrosine-based activation motifs (ITAMs). Engagement with the pMHC recruits the Lck kinase to the TCR where it phosphorylates immunoreceptor ITAM motifs of two TCR components, the associated invariant CD3 and ζ chains. The colocalization of CD4 or CD8 molecules and the pMHC allows for Lck recruitment to the TCR (Bell et al., 1991; Shaw et al., 1990). Mice deficient in Lck have their T cell development impaired at early stages (Molina et al., 1992). The phosphorylated tyrosines of ITAM motifs form a binding site for Zap70 kinase. After being recruited to the plasma membrane, ZAP70 binds via SH2 domain. Lck phosphorylates ZAP70, leading to its activation and subsequent phosphorylation of two adaptor proteins: linker for activation of T cells (LAT) and cytosolic protein SH2 (Wardenburg et al., 1996). Together these proteins form a complex which directs activation of further signalling events. Phosphorylated LAT recruits PLCγ1, p38, Grb2 and Gads. The interaction between Grb2 and Son of Sevenless (SOS), guanine nucleotide exchange factors, activates the Ras-GTPs by encouraging the release of guanosine diphosphate (GDP) (Liu et al., 1999). Ras-GTP induced signalling cascade results in activation of MAPK/ERK Kinase and MAPKs kinases which act on transcription factors NF-κB and AP-1 involved in many aspects of T cell mediated responses, like migration, proliferation and effector functions.

The strength and quality of TCR signalling has a definitive impact on the differentiation of effector T cells, with the antigen dose playing important role. Previous studies on *Leishmania major* and *L. monocytogenes* infections have shown that differentiation of the CD4 effector populations was highly dependent on the dose of bacteria administered (Bretscher et al., 1992; Darrah et al., 2007; Pepper et al., 2011; Tubo et al., 2013). BALB/c mice are known to generate poor Th1 responses and are especially susceptible to *L. major* infections. However,

administration of low dose of *L. major* results in a strong Th1 response and protects the mice from bacteria (Bretscher et al., 1992). In *L. monocytogenes* infections, low doses of bacteria generally cause differentiation of T helper 1 (Th1) and T follicular helper (Tfh) cells, however, limit the formation of germinal centres (GC). The increased of the bacterial dose corresponds to a reduction in Th1 population and increase in both Tfh and GC formation (Tubo et al., 2013).

The quality of the TCR signalling is often explained through its durability which usually correlates with antigen persistence. For instance, in Tfh differentiation, the presence of increasing and persisting pMHCII enhances Tfh proliferation and induces CG formation (Baumjohann et al., 2013). While in the Th1 responses, although high levels of pMHCII increase proliferation, their persistence often results in cell death (Kallies et al., 2006).

1.2.5 T cell subsets

T cells can be divided into two large general groups according to their differential functions and properties: helper CD4 and cytotoxic CD8 T cells. Third smaller group consists of $\gamma\delta$ T cells and Natural Killer T (NKT) cell which, as previously stated, are classed as cells of the innate immune system. Invariant NKT (iNKT) cells are thought to be mediators between innate and adaptive immunity. Although, they express TCRs generated through rearrangement events, they can only recognise a limited number of glycolipid antigens (Van Kaer et al., 2011). Upon their activation, iNKT cells elicit very rapid responses and like majority of innate cells, fail to generate memory populations.

One of the first evidences for the separation of CD4 and CD8 T cells on a phenotypic and functional basis advanced from experimental data showing that depletion of Ly-2 (CD8α) and Ly-3 (CD8β) expressing lymphocytes by using antisera and compliment resulted in cell specific loss of cytotoxic function (Cantor and Boyse, 1975; Kisielow et al., 1975; Shiku et al., 1975).

1.2.5.1 Cytotoxic CD8 T cells

The function of CD8 T cells is mainly focused on eradication of infected cells by the use of their cytotoxic properties (Cantor and Boyse, 1975; Cerottini et al., 1970). Similar to one of the subclass of CD4 T cells, CD8 cell differentiation is T-box transcription factor (T-bet) dependent and as demonstrated previously IL-12 can promote its expression (Curtsinger et al., 2003; Takemoto et al., 2006). Another transcription factor, Eomesodermin (EOMES) also assists CD8 differentiation (Pearce et al., 2003).

Naive CD8 T cells recirculate through secondary lymphoid and non-lymphoid tissues, until they encounter cognate antigen (Cose et al., 2006; Weninger et al., 2001). They recognise endogenous antigens of viral or microbial origin present in the cytosol and expressed as part of MHCI (Pamer and Cresswell, 1998).

Once activated, CD8 T cells differentiate and initially were classed into two main groups, the preferential Interferon γ (IFNγ) and Tumour necrosis factor α (TNFα) producing Tc1 cells, and the IL-4, IL-5, IL-6 and IL-10 producing Tc2 cells (Mosmann et al., 1997). Tc1 CD8 T cells are involved in cell lysis, releasing secretory vesicles containing perforins and granzymes. Further studies have described a third group Tc17, characterised by production of IL-17 (Yen et al., 2009).

Therapeutically, they represent a very powerful group of cells capable of highly destructive functions which are of great benefit to tumours treatments as well as control of responses during the chronic infections. However, the same properties can be the cause of major difficulties, particularly in transplantology and autoimmunity treatments (Delfs et al., 2001; Fazou et al., 2001; Jacobsen et al., 2002).

CD8 T cell responses result in formation of long-lived and antigen-specific memory populations which express memory-like phenotype and persist in the absence of antigen (Doherty et al., 1994; Lau et al., 1994).

1.2.5.2 Helper CD4 T cells and their subsets

Helper CD4 T cells are highly important modulators of the immune responses, particularly to intracellular and extracellular bacteria, parasites and fungi. They aid in generation of effective responses by producing cytokines that can act directly or indirectly upon the infection, targeting pathogens or stimulating the proliferation of B cells. They provide help for B cells in production of antigen specific antibodies and are involved in activation of macrophages, recruitment of neutrophils, eosinophils, basophils, as well as produce vast range of cytokines and chemokines which influence the course of immune responses (Carside et al., 1998; MacLennan et al., 1997). This group of T cells also contains regulatory cells which ensure minimal tissue damage as well as the memory cells which induce a rapid response to any reoccurring infections. The basis for different 'subtypes' of CD4 T cells comes from distinct cytokine profiles.

The fundamental research conducted by Mossman and Coffman in 1986 confirmed the ability of CD4 T cell lines to differentiate into two groups of cells, that could be characterised by two different sets of cytokines they were producing, the IFNγ and IL-4 (Coffman et al., 1986). Later on, those cells were named as T helper 1 (Th1) and T helper 2 (Th2) cells. To date, we understand that CD4 T cells can take on many different functions. They have been classified into several types according to such functions, including Th1, Th2, Th9, Th17, Th22, regulatory T (Treg) cells and Tfh cells. Moreover, plasticity between the subtypes has been reported.

Differentiation of each of the CD4 T cell subsets requires several important factors, namely the transcription factors and cytokines produced in response to the infection that together shape and determine CD4 T cell fate (Figure 1.1).

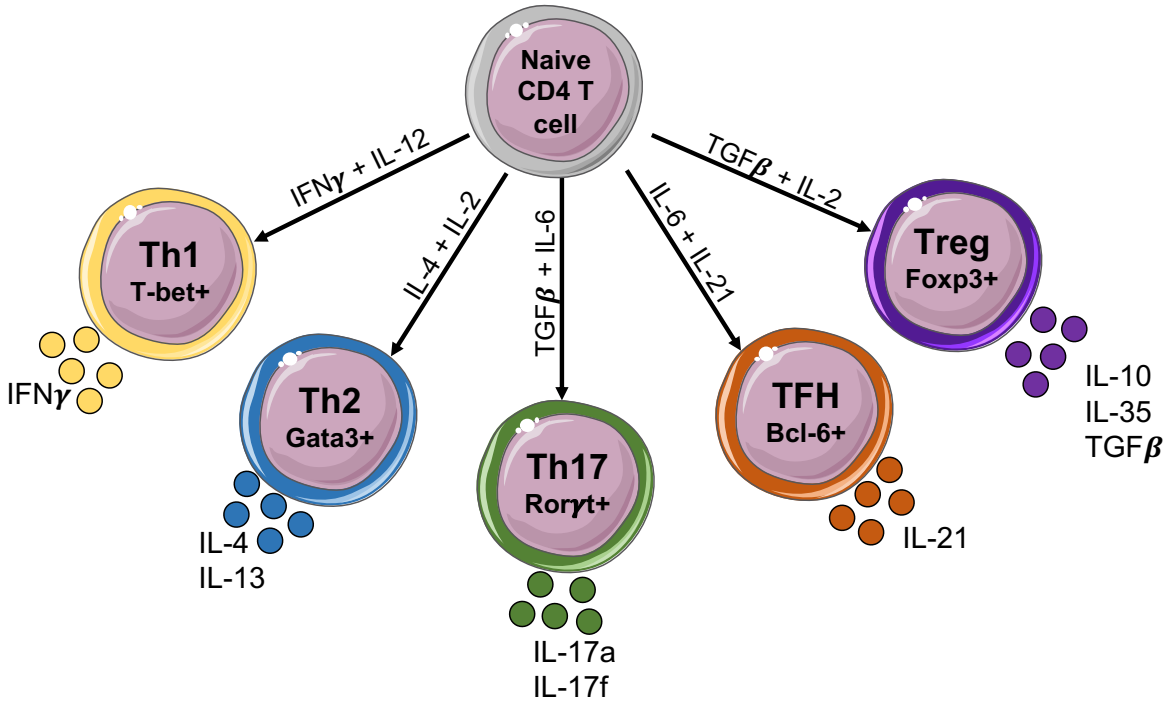


Figure 1. 1 CD4 T cell differentiation.

1.2.5.2.1 Th1 cells

Th1 cells are involved in proinflammatory responses to intracellular bacterial and viral infections. Alongside IL-2, those cells produce high levels of INFγ and TNFα which play important role in phagocyte responses by activating macrophages and stimulating cell-mediated immunity. The interaction with macrophages via CD40 and IFN-γ has a mutual effect. *In vitro* studies showed that activation of macrophages induces them to secrete high levels of IL-12 which in turn regulates Stat4 signal transducer which induces IFN-γ secretion without TCR stimulation (C S Hsieh et al., 1993; W.-R. Park et al., 2004). Increase in the IFNγ production upregulates T-bet transcription factor strengthening Th1 fate and administration of anti-IFNγ results in inhibition of the Th1 cell type differentiation yet having no effect on the proliferation rate of CD4 T cells (Sad and Mosmann, 1994). T-bet is upregulated via the IFNγ

mediated activation of Stat1. The conserved T-box domain contained within the T-bet binds specifically to the promoter as well as the enhancer of IFNγ gene, inducing its transcription and mediating IFNγ production in Th1 responses. This process is then amplified by Stat4 activation triggered by IL-12 (Thierfelder et al., 1996).

Studies have shown that, sustained expression of T-bet in differentiated Th2 cells can redirect their fate programme and polarise them towards a Th1 cell-like phenotype. This is ultimately caused by the reduction in IL-4 expression (Lametschwandtner et al., 2004; Szabo et al., 2000; Zhuang et al., 2009). Moreover, the CD4 T cell responses in T-bet deficient mice are much more biased towards a Th2 type immunity, leading to a higher expression of GATA3 (Finotto et al., 2002; Nakamura et al., 1999). These mice however are able to maintain relatively normal Th1 differentiation in the absence of IL-4 pathway (Usui et al., 2006). T-bet also has been shown to play an important role in generating effector Th1 T cells and promoting development of experimental autoimmune encephalomyelitis (EAE), as this autoimmune disease was prevented in T-bet deficient mice (Bettelli et al., 2004; Nath et al., 2006; O'Connor et al., 2013).

1.2.5.2.2 Th2 cells

Th2 cells are main effector helper cells found during responses to parasites like helminths. They are known to produce high levels of IL-4, IL-5 and IL-13 and have positive effect on the activation of eosinophils and B cell stimulation followed by strong antibody production (Coffman et al., 1986). The cytokine induced production of IgE and IgA specific to the antigen enables neutralisation of parasites during the possible re-challenge.

Th2 differentiation is highly dependent on high levels of both IL-2 and IL-4. Similarly, to the IFNγ in Th1 responses, the concept of IL-4 involvement is particularly interesting due to the formation of feedback loop in which the main product of Th2 immunity is also a crucial fate determinant. IL-4 activates Stat6, which in turn upregulates GATA3 which stabilises the Th2

differentiation using three proposed mechanisms: 1. Increased proliferation; 2. Increased cytokine production; 3. Prevention of T-bet expression (Zhu et al., 2006, 2001). In mouse studies, the absence of GATA3 led to an abolishment of Th2 differentiation and a shift in the cell fate towards the Th1 phenotype without IL-12 or IFNγ stimulation (Pai et al., 2004; Zhu et al., 2006, 2004).

Th2 responses have also been studies with regards to asthma due to the highly important role of their effector product IL-4. Abrogation of IL-4 responses with the use of monoclonal antibodies or mice deficient in IL-4 pathway resulted in reduced airway hyperactivity to an antigen (Corry et al., 1996; Coyle et al., 1995).

1.2.5.2.3 Th17 cells

Th17 population of CD4 T cells have been found to be involved in infections caused by extracellular bacteria, fungi and viruses at mucosal sites. They were first identified as one of the main factors contributing to the EAE. Early studies by Cua et al. showed that EAE development was abrogated in mice lacking IL-23, but no IL-12. By producing knockout mice for the IL-23p19 subunit, targeting only IL-23 or the IL-12p40 subunit, targeting both IL-23 and IL-12, they were able to show that both genotypes gave similar results, remaining unaffected by the EAE, while mice lacking IL-12p35 and deficient only in IL-12 showed significant progression of the disease (Cua et al., 2003).

Further studies linked the expression of IL-23 with an increase in the IL-17 producing population of CD4 T cells which in turn correlated with an increase in the severity of EAE (Langrish, 2005; Murphy et al., 2003).

Th17 cells secrete IL-17A, IL-17F, IL-21 and IL-22 which promote recruitment of neutrophils and production of antimicrobial agents and other proinflammatory cytokines like IL-1 and IL-6 (Chen et al., 2013; Harrington et al., 2005; Park et al., 2005).

Differentiation of Th17 cells is highly dependent on the expression of RORγt transcription factor which is reinforced by optimal levels of IL-6, IL-21, IL-23 and Transforming growth factor β (TGF-β) (Manel et al., 2008; Veldhoen et al., 2006). IL-6 and IL-21 activate Stat3 and its suppressor SOCS3. TGF-β plays the critical role, enhancing activation of Stat3 by inhibiting SOCS3 action (Qin et al., 2009). Stat3 in turn, regulates expression of RORγt, which orchestrates further differentiation and its absence results in the Th17 deficiency (Ivanov et al., 2006; Yang et al., 2007). Although RORγt is crucial for Th17 differentiation, another member of the ROR family, the RORα can also be involved. Deletion of both RORγt and RORα have been shown to exacerbate the impairment in the differentiation of Th17 (Yang et al., 2008).

1.2.5.2.4 Regulatory T cells

Tregs were first identified in the 1990s and are critical regulators of the immune system, orchestrating tolerance towards self-antigens and commensal microbiota. Sakaguchi et al. have shown their importance by transferring CD4 T cells depleted of Tregs into the BALB/c athymic nude mice. The results showed a dramatic impairment in the immune tolerance characterised by development of autoimmune diseases, including thyroiditis and gastritis as well as wasting disease. The autoimmunity was however prevented when Treg sufficient CD4 T cells and not CD8 T cells were transferred shortly after (Sakaguchi et al., 1995).

Tregs act on both T cells, regulating the antigen-specific responses, and at the same time remodelling the function of DCs. The two types include natural (nTreg) and induced (iTreg) Tregs which differ in their origin. The nTregs are produced during the selection process within the thymus while the iTregs are differentiated due to the proinflammatory cytokines (Chen et al., 2003). Both the TGF- β and Forkhead box p3 (Foxp3) play crucial role in the development and function of Tregs (Chen et al., 2003; Fontenot et al., 2003; Fu et al., 2004; Hori et al., 2003). Expression of Foxp3 is induced in antigen-specific manner, with Tregs having higher

affinity for self-antigens than other conventional T cells (Jordan et al., 2001; Kawahata et al., 2002). The lack of FoxP3 expression has severe effects on the functionality of immune system, causing fatal autoimmune diseases in both humans and mice (C. L. Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001). Mice that lack this crucial transcription factor do not survive for more than 5 weeks. TGFβ can upregulate Foxp3 expression, stimulating naive CD4 T cells to become Tregs. As a result, Tregs have been of main focus to transplantation studies which specified the high number of Treg could be beneficial in preventing allograft rejections (Dai et al., 2004).

Differentiation of Tregs is proportional to the level of stimuli received by the T cells from antigen-activated DCs. Very low level may not reach the threshold and prevent Treg differentiation, leading to autoimmunity. In contrast, a very strong stimulus increases their differentiation and may result in immunosuppression. The function of Tregs is highly dependent on IL-2 cytokine levels, produced by naive T cells during the antigen encounter (Malek et al., 2002). The lack of IL-2 stimuli results in Bim mediated apoptosis.

1.2.5.2.5 T follicular helper cells

Tfh cells are crucial for survival and development of B cells, being involved in guiding the class switching and maintenance of the GCs. They provide an activation signal through the CD40L molecule which is essential for sustained GC formation (Breitfeld et al., 2000; Elgueta et al., 2013; King et al., 2008). GCs are the primary sites for B cell production of high affinity antibodies (Tas et al., 2016).

There are reports of IL-6, IL-2 and ICOS driven Tfh. Signalling through the IL-6 receptor stimulates expression of Bcl-6 which in turn induces early expression of CXCR5 chemokine (Choi et al., 2011). As shown with the use of IL-6 deficient mice, absence of IL-6 has a profound impact of the Tfh differentiation (Choi et al., 2013). On the other hand, ICOS and IL-2 act as regulators of Tfh differentiation. Inhibition of ICOS by Roquin1 and Roquin2 and the

action of IL-2 during priming induces strong Tfh phenotype and GC formation (Ballesteros-Tato et al., 2012; Vogel et al., 2013).

1.2.6 Costimulatory signals

As discussed in previous sections, activation of naive T cells and their transition into effector populations is highly depended on their interaction with APCs. Although, specific recognition of antigens presented as a part of MHCII initiates signalling through the TCR receptor and activates signalling cascade, it is the costimulatory signals that stabilise the interaction and direct T cells function.

The classical two-signal model introduced in late 1970s by Lafferty and colleagues proposed that efficient T cell activation required two types of signals, including the TCR engagement as well as other stimuli (Lafferty et al., 1980). The pioneering *in vitro* studies by Schwartz group identified APCs as main providers of costimulatory signals and defined these signals as crucial for full T cell activation (Jenkins and Schwartz, 1987). They compared the stimulatory potential of live versus fixed antigen-activated APCs, assessing T cell proliferation and production of IL-2. Despite the TCR:MHCII engagement, fixed APCs were unable to drive efficient T cell responses and the interaction resulted in T cell anergy. Further reports of T cell anergy and limited function of TCR:MHCII complexes have also been shown (Brunet et al., 1987; Harding et al., 1992; June et al., 1990).

Costimulatory molecules functionally define a number of receptor:ligand interactions that typically occur between the T cells and APCs (Figure 1.2). T cells express broad repertoire of costimulatory receptors which can transduce vast number of cell specific signalling pathways upon interaction with ligand or counter-ligand.

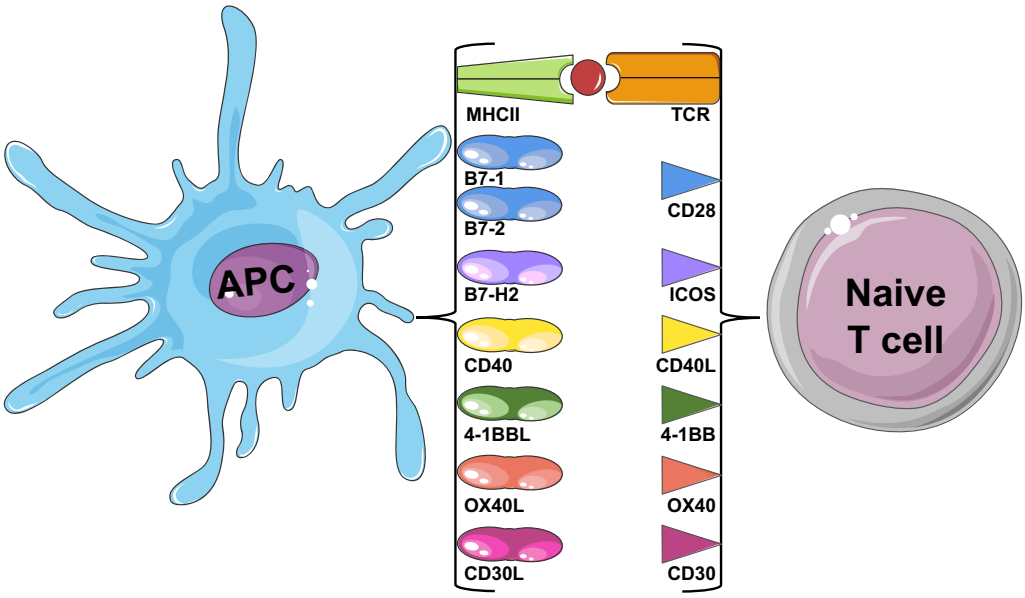


Figure 1. 2 Costimulatory interactions in T cells.

1.2.6.1 The B7-CD28 superfamily

Early studies identified the critical role of two costimulatory molecules belonging to the family of B7 ligands. Both the B7.1 (CD80) and B7.2 (CD86) proteins are expressed on the surface of APCs and have been shown to interact with the CD28 receptor that is found on majority of murine naive CD4 and CD8 T cells (Gross et al., 1992). The interaction between CD28 and B7 molecules mediates proliferation and survival of activated T cells, by enhancing the IL-2 production (Srinivasan et al., 2001). Administration of anti-CD28 prevented T cell anergy, proving the importance of this costimulation in T cell responses (Harding et al., 1992). However, it has also been shown that in prolonged acute infections and in cases where the TCR signal is very strong, T cells can become activated in the absence of crucial CD28 signalling (Kündig et al., 1996). Furthermore, the presence of functional alloreactive responses to skin grafts and their subsequent rejection supports the limited function of CD28 signalling (Ha et al., 2001; Kawai et al., 1996).

CD28 is a 44 kDa type I transmembrane glycoprotein homodimer encoded by genes found on chromosomes 1 and 2q33 in mouse and humans respectively (Aruffo and Seed, 1987;

Lafage-Pochitaloff et al., 1990). The receptor was first identified in 1980s when its enhancing effect on T cell differentiation and expansion was shown (Gmünder and Lesslauer, 1984). The importance of CD28 signalling was supported by studies in CD28-deficient mice where despite seemingly normal T and B cell development, both adaptive responses to lectins and vasicular stomatitis virus were significantly reduced (Shahinian et al., 1993). Furthermore, T cell activation was impaired due to reduced levels of IL-2. CD28 signalling has also been associated with increased expression on Bcl-2 and Bcl- extra large (Bcl-xL) protein, the two important anti-apoptotic proteins which promote cell survival (Kündig et al., 1996).

CD28 signalling is initiated by interaction between the extracellular MYPPPY motif and CD80 and CD86 (Harper et al., 1991). CD80 and CD86 expression is highly regulated by the presence of inflammatory signals, with the translation of their mRNA being enhanced following an antigen driven immune response (Paine et al., 2012; Subauste et al., 1998). The affinities of the two interactions differ with a stronger affinity for the CD86 which proved to be particularly important in transplant rejection studies (Collins et al., 2002; Weaver et al., 2008). The resulting interactions form an immunological synapse between T cell and the APC which induces recruitment of phosphoinositide 3-kinase (PI3K). This in turn enhances the signalling of AKT pathway which promotes T cell survival and differentiation (Lee et al., 2010; Wu et al., 2005). CD28 costimulation also initiates activation of Lck and Fyn protein tyrosine kinases and guanosine exchange factor VAV1 which are important for the nuclear factor NF-κB involved in enhanced expression of Bcl-xL (Khoshnan et al., 2000; Takeda et al., 2008).

Activation of naive T cells induces expression of cytotoxic lymphocyte-associated antigen-4 (CTLA4) molecule which is a homolog of the CD28. Like its family member, CTLA4 binds the CD80 and CD86 but acts as a negative regulator, whereby it inhibits proliferation of overactive T cells. It binds the two B7 molecules with much higher affinity and therefore its dysfunction has been linked to the development of autoimmune diseases (Karandikar, 1996). Deficiency in the CTLA4 molecule causes fatal autoimmunity due to the increased activity of effector T

cells (Tivol et al., 1995). Similar results were shown in tumour studies, where CTLA4 blockade with monoclonal antibodies enhanced the antitumor T cell activity (Leach et al., 1996). Additionally, in human studies, treatment with anti-CTLA4 increased survival of patients suffering from advanced melanoma (Hodi et al., 2010). Interestingly, the increased survival was associated with increase in ICOS expression on T cells (Carthon et al., 2010).

ICOS is another member of the CD28 family, and it has also been shown to have a costimulatory function in T cell activation. It interacts with the ICOS ligand (ICOSL) that is expressed on majority of DC, macrophages and B cells (Steven K. Yoshinaga et al., 1999). Unlike the CD28, expression of ICOS is induced on the surface of active T cells upon stimulation through the TCR and CD28 (Coyle et al., 2000; A Hutloff et al., 1999; McAdam et al., 2000; S K Yoshinaga et al., 1999).

ICOS signalling may be considered as a type of reassurance for T cell activation and subsequent proliferation and differentiation. It enhances low level IL-2 production by the CD4 T cell which stabilises the initial proliferation (Riley et al., 2001). It is important for regulating effector T cells responses, particularly the cytokine production including IL-4, IL-5, IL-10, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ and TNFα as well as generation of Tfh with the provision of ICOSL by B cells being critical (Bauquet et al., 2009; Andreas Hutloff et al., 1999).

The last member of the CD28 superfamily is the Programmed cell death 1 (PD1) receptor which binds homologues of the B7 molecules, namely PD-L1 (B7-H1) and PD-L2 (B7-DC). Those ligands are very specific and expressed on the lymphoid and non-lymphoid tissues as well as in some tumours. The PD1 receptor is expressed on the surface of activated T cells, B cells, DCs and NKT cells. Signalling through the PD-1 receptor inhibits CD28 induced activation of AKT pathway which results in an overall suppression of TCR signal transduction (Parry et al., 2005). PD-1 is highly important in maintaining self-tolerance and preventing

autoimmunity as C57BL/6 mice deficient in this inhibitory receptor develop an arthritis-like autoimmune condition (Nishimura et al., 1999).

The cells of many tumours express high levels of PD-L1 ligand, preventing the development of effector T cell responses and thereby impairing patient outcomes. Blocking the interaction via the PD-L1 ligand has been shown to be paramount to the immunotherapy treatment of many types of cancer, including the glioblastomas, melanomas, breast and kidney cancers (Nomi et al., 2007; Thompson et al., 2007).

1.2.6.2 Tumour Necrosis Factor Receptor Superfamily

Tumour Necrosis Factor Receptor Superfamily (TNFRSF) consists of a set of receptors and their cognate ligands which provide an essential signalling network for many aspects of immune cell functions (Gravestein and Borst, 1998). Tumour Necrosis Factor Receptors (TNFRs) are involved in modulating the antigen-specific T cell responses, including production of cytokines as well as T cell survival.

The founding member of the TNFRSF, TNF ligand, was identified four decades ago as one of the agents produced by activated monocytes and macrophages and it had detrimental effects on tumour cells *in vitro* causing them to undergo necrosis (Carswell et al., 1975). Further reports showed that it had potential in controlling the tumour growth in both *in vitro* and *in vivo* models (Haranaka et al., 1984; Matthews, 1979). TNF exists in two forms, as a transmembrane protein and as a soluble factor released by the TNFα converting enzyme induced proteolysis. All of the ligands in the TNFRSF form a homo-trimeric structure and are primarily designed for cell-cell contact, however their secreted forms can have more disperse effects (Bodmer et al., 2002). TNF binds two receptors the TNFR1 and TNFR2. Although majority of the cells express TNFR1 and only some express TNFR2, the transmembrane form of TNF binds with much higher affinity to the latter (Grell et al., 1995).

The TNFRs constitute a group of 29 type 1 transmembrane monomer receptors, which associate into trimeric structures upon the ligand binding. As the definition implies, they contain both extracellular and intracellular domains. The structure of the ectodomain, extending into the extracellular space, is highly conserved within the superfamily and is characterised by its cysteine-rich region (Locksley et al., 2001).

The TNF receptors can be divided into two groups, according to the nature of the signal transduction initiated by their intracellular domain. They can be either activating, like CD40 and induce the NF-κB and MAPK pathways, or they can act as death receptors (DRs), like Fas receptor, initiating apoptosis (Li et al., 2013). Fas receptor is one of the most well-known death receptors. It contains an intracellular death domain which becomes activated upon the interaction with FAS ligand and recruits caspase 8 (Carrington et al., 2006). Caspase 8 potentiates the apoptotic signals via the release cytochrome c and activation of caspase 9 which in turn results in apoptosis or programmed necroptosis. Hence TNFRs can provide both costimulatory and coinhibitory signals having a direct effect on the course of immune responses. However, their expression can be regulated by types of interferons, like for instance Interferon γ (IFNγ) (Aggarwal et al., 1985).

In humans, the majority of genes encoding the costimulatory TNFRs reside within one locus on Chr 1p36 (Granger and Ware, 2001). These include the OX40, CD30, HVEM, DR3, GITR, 4-1BB, TNFR2 with their corresponding ligands located within the paralogues regions among Chr 1, 6, 9 and 19.

For CD4 T cell function, OX40 and CD30 are likely the key TNFRSF members required for productive immune responses (Gaspal et al., 2011; Withers et al., 2011).

1.2.6.2.1 CD30 receptor

CD30, also known as TNFRSF8, was identified in 1980s through the analysis of Hodgkin and Stenberd-Reed cell lines. The large set of monoclonal and polyclonal antibodies used in the study enabled an in-depth analysis of the cell expression profile (Stein et al., 1982).

CD30 receptor shares the structural characteristics with other TNFRSF members and is expressed on activated B and T cells (Gilfillan et al., 1998; Stein et al., 1982). In mice, T cell expression of CD30 is enhanced in response to CD3 and CD28 signalling, and can be downregulated by IFNγ (Gilfillan et al., 1998).

CD30 ligand (CD30L/TNFSF8) is also inducible and expressed on DCs including some rare populations of splenic CD4 DCs, as well as NK cells, B and T cells, eosinophils and mast cells. *In vitro* mouse studies revealed that CD30L expression is also highly dependent on the TCR engagement and is absent on resting lymphocytes (Shimozato et al., 1999).

Costimulation via the CD30 receptor has an impact on T cell proliferation, differentiation and survival. The costimulatory nature of the receptor is reflected in the signalling pathways activated upon its engagement. The c-terminal of the cytoplasmic tail of CD30 receptor recruits TNFR-associated factors (TRAFs). Particularly, TRAF2 and TRAF5 have been shown to mediate the CD30 induced activation of NF-κB and MAPK pathways (Aizawa et al., 1997). Activation of those pathways was diminished when dominant negative mutants of TRAF2 and TRAF5 were generated (Aizawa et al., 1997; Duckett and Thompson, 1997). TRAFs associate with E2 and E3 ligase complexes which lead to RIP1 ubiquitination and subsequent activation of IKK complex. This complex, in turn, phosphorylates IκB which enables translocation of the NF-κB/Rel into the nucleus and activates transcription of target genes. Alternative pathway of CD30 signalling involves TRAF2 and TRAF5 induced activation of MAPK cascade and the activator protein 1 (AP-1), which regulate the course of cell cycle and promote cell survival.

Classification of CD30 as a costimulatory molecules resulted from early *in vitro* studies which identified it as one of the enhancers of T cell responses (Bowen et al., 1996; Del Prete et al., 1995). Other studies also highlighted the fact that CD30 is expressed late in the response, about 3-4 days post TCR engagement and therefore can be crucial in maintaining efficient T cell responses and promoting T cell survival (Nakamura et al., 1997).

Early *in vivo* studies focused on assessing the effects of CD30 deletion on the development of T cells. Mice deficient in CD30 were first shown to have an impaired thymic negative selection determined by an increase in the total number of thymocytes and thereby suggesting that CD30 was involved in promoting cell death (Amakawa et al., 1996). This however proved controversial and was later disproved by DeYoung et al. who used both class I and class II- restricted TCR transgenic mouse strains that were crossed with $CD30^{-/-}$ mice to outline the role of CD30 in negative selection (DeYoung et al., 2000). They showed that the lack of CD30 signals had no effect on the negative selection and that it had a minimal effect on thymic apoptosis. In their study, DeYoung et al. discussed the possible caveats of the previously published data and narrowed them down to the problems with housing and breeding conditions of the mice used in the study.

Further *in vivo* studies demonstrated the important role of CD30 signals in particularly the CD4, CD8 T cell and B cell responses. Some of the key findings of bacterial infection models, highlighted the role of CD30/CD30L signalling in controlling pathogens and the course of infection, in particular generation of long-lived memory T cell populations. Across different types of infections, the lack of CD30/CD30L signalling was associated with an increase in bacterial burden, reduction in the number of memory effector CD4 T cells capable of secreting IFNγ as well as with a significant impairment in the CD8 T cell memory (Nishimura et al., 2005; Podack et al., 2002; Tang et al., 2008; Umeda et al., 2011). Furthermore, CD30 signals have been shown to be important in $\gamma\delta$ T cell mediated immunity, particularly their ability to produce IL-17A (Guo et al., 2013).

In regards to the humoral responses, the absence of CD30 signals has been linked to a reduction in the tiers of high affinity antibodies post immunisation with NP-chicken gammaglobulin (NP-CγG) (Gaspal et al., 2005). Moreover, at 14 days post NP-CγG treatment, the $CD30^{-/-}$ mice as well as $CD30^{-/-}OX40^{-/-}$ mice failed to sustain germinal centres and lost plasma cells.

In clinical studies, soluble CD30 has been used as one of the markers for monitoring the progress of immune diseases, like for instance leishmaniasis or HIV viremia, however it has also been used as a readout for the efficacy of hepatitis c therapy (Ajdary et al., 2006; Biswas et al., 2006; Yang et al., 2006). Due to the increased CD30 expression on many lymphomas, the signalling pathways have been targeted in various therapeutic treatments (Ansell et al., 2007; Bhatt et al., 2016).

1.2.6.2.2 OX40 receptor

OX40 receptor and its costimulatory potential were first identified in 1987, using monoclonal antibodies against rat T cell blasts (Paterson et al., 1987). The study showed that blocking OX40 *in vitro* reduced T cell proliferation. Further cloning experiments identified it as a member of TNFRSF (Calderhead et al., 1993). OX40, also known as TNFRSF4, is a 50kD transmembrane protein which shares the cysteine-rich domain with other TNFRSF members. Ligand for OX40, the OX40L or TNFSF4, is also a transmembrane protein which was first identified as gp34 molecule present on T cells carrying the human T cell leukemia virus (Miura et al., 1991). So far, there is no clear indication suggesting that OX40L could be an alternative ligand for other receptors.

Following antigen presentation and subsequent CD28 signalling, OX40 is induced on activated T cells with a peak of expression at 2-3 days post stimulation (Marriott et al., 2014; Stüber and Strober, 1996). CD40 activated APCs and APC and T cell-derived IL-2 production stabilise its expression (Rogers et al., 2001). The extent of OX40 expression on activated T

cells is highly dependent on the strength of TCR stimulation, however other factors like IL-2 and IL-4 can also affect it (Gramaglia et al., 1998; Rogers et al., 2001; Toennies et al., 2004). Following repeated signalling through the TCR , the memory T cells upregulate OX40 more rapidly, often within 1-4 hours of reactivation (Croft, 2010). Constitutive expression of OX40 has only been reported on nTregs. Several groups showed that OX40 ligation reduces suppressive function of nTregs specifically via downregulation of FoxP3 transcription factor (Kitamura et al., 2009; Takeda et al., 2004; Valzasina et al., 2005; Vu et al., 2007).

Like CD30 receptor, OX40 is activated upon engagement with its ligand and acts via the TRAF2 and TRAF5 to initiate NF-κB, PI3K and AKT signalling pathways and thereby exacerbating its pro-survival properties (So et al., 2011) (Figure 1.3).

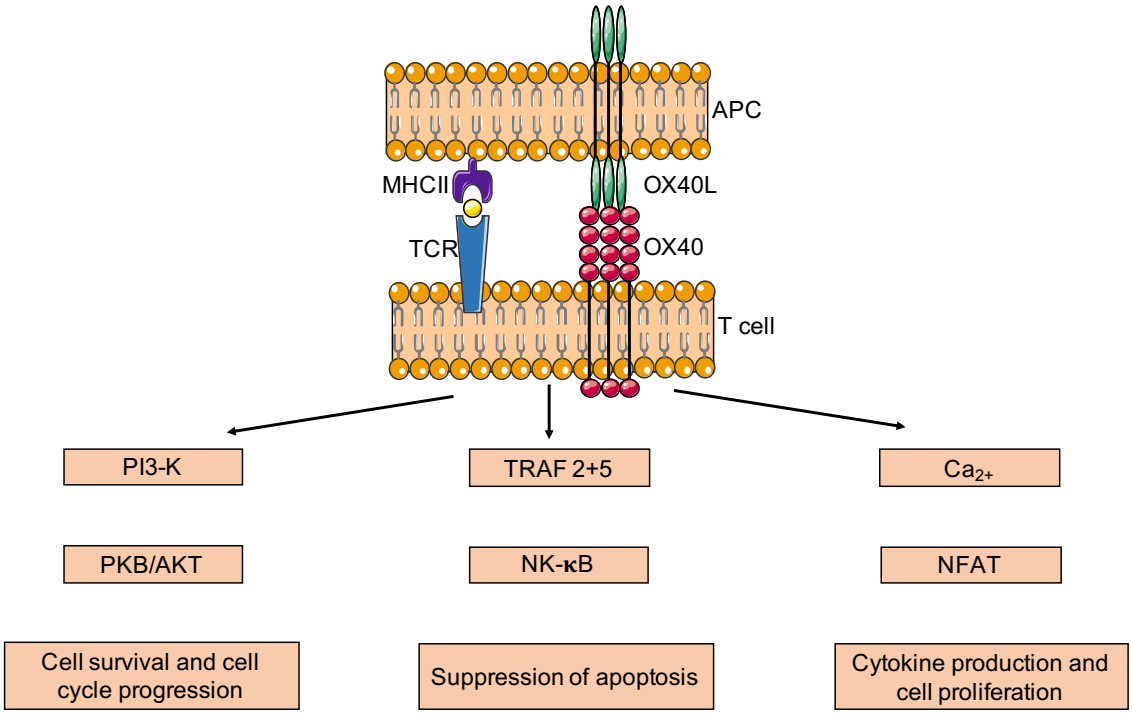


Figure 1. 3 OX40-OX40L signalling.

Primary sources of OX40L include activated APCs, like DCs and B cells, where its expression is induced by maturation factors like CD40, and inflammatory factors like thymic stromal

lymphopoietin (TSLP) and signals through the BCR. OX40L, however, has also been found on other cell types including macrophages, endothelial, mast and NK cells (Imura et al., 1996; Karulf et al., 2010; Kashiwakura et al., 2004; Zingoni et al., 2004). Additionally, $CD4^{+}CD3^{-}CD11c^{-}$ accessory cells known as lymphoid tissue inducer (LTi) cells, belonging to the group 3 of ILCs were shown to provide both OX40L and CD30L, particularly at adult stages of mouse development (Kim et al. 2005). The expression of those two ligands is absent at the neonatal stages and reaches normal levels within the first weeks of life. The diversity of cell types providing OX40L suggests the pathway is involved in many aspects of immune responses and therefore poses as a potential target for various immunotherapies.

Studies have shown that the close evolutionary relationship and sharing of signalling pathways between the CD30 and OX40 receptors supports redundancy in case of deficiency of one of the signals (Chung et al., 2002; M.-Y. Kim et al., 2005; Locksley et al., 2001). Despite this synergistic function of OX40 and CD30 receptors, previous data has shown the effects of the OX40 signalling to be more dominant in challenge CD8 T cell responses to OVA (Bekiaris et al., 2009a). In FoxP3-dependent autoimmunity, the lack of OX40 more than CD30 signals decreased the function of effector T cells and thereby delayed the onset of the disease (Gaspal et al., 2011).

OX40 signals are important for expansion and survival of both CD4 and CD8 T cells, by maintaining the survival signals and facilitating transition through effector phases (Gramaglia et al., 1998; I. Gramaglia et al., 2000; Murata et al., 2000a; Weinberg et al., 1999). The lack of early OX40 costimulation and the subsequent reduction in T cell proliferation translates into an impaired generation and reactivation of memory T cells (Salek-Ardakani and Croft, 2006; Takeda et al., 2004; Valzasina et al., 2005; Vu et al., 2007) (Figure 1.4).

Administration of agonistic OX40 antibody can overturn the defect and augment T cell responses. Similar results were obtained in mice constitutively expressing OX40L on DCs, which showed elevated numbers of CD4 T cells present within the B cell areas in GCs (Brocker et al., 1999).

Reports of OX40 mediated control of cytokines production describe its contribution to the T cell derived IL-2 (Gramaglia et al., 1998). In this *in vitro* study, I-E^K fibroblast lines were transfected with murine OX40L cDNA which enabled generation of APCs expressing either OX40L, CD28 or OX40L and CD28, and then were used to activate CD4 T cells. The results at 40 and 60 hours post stimulation showed the combined effect of OX40L and CD28 signalling which promoted significantly higher levels of IL-2 production that either of the molecules alone.

Furthermore, OX40 signals have also been shown to enhance expression of CD25, also known as IL-2R αchain (Lathrop et al., 2004). In study of peripheral self-tolerance, transgenic CD4 T cells transferred into mice expressing pMHC ligand under the control of MHCII promoter expanded and became unresponsive to the antigen *in vitro* (Lathrop et al., 2004). Interestingly, 5 days post administration of agonist anti-OX40, T cells were defined by an increase in cell granularity and high expression of CD25. Mice died within 12 days of treatment due to the profound accumulation of effector T cells and the resulting cytokine storm. Administration of anti-OX40 augmented T cell responsiveness to IL-2 which in turn resulted in enhanced production of IFNγ through stimulation with IL-12 and IL-18, which proved more effective than treatment with phorbol 12-myristate 13-acetate (PMA) and Ionomycin.

Due to its enhancing properties, OX40 has been used as an adjuvant to increase immune protection against various infections. For instance, treatment with OX40L.Ig fusion protein mediated clearance of *Cryptococcus neoformans* (Humphreys et al., 2003). Moreover, OX40 is also highly important in a poxvirus vaccine, where along with 4-1BB enhanced priming *in vivo* of both CD4 and CD8 T cells (Munks et al., 2004). Cancer therapy studies have focused on OX40 signalling, because of reports of OX40 expression on T cells infiltrating tumours and the protective properties of agonist OX40 against melanomas and breast and colon

carcinomas (Weinberg et al., 2000). Anti-OX40 enhanced tumour specific priming as well as generation of memory T cells. The enhancing effect was however dose dependent.

The Th2 infection models outlined the role of OX40 signalling in mediating the T cell specific in asthmatic patients and increasing airway sensitivity (Di et al., 2015; Hong et al., 2015; Siddiqui et al., 2010).

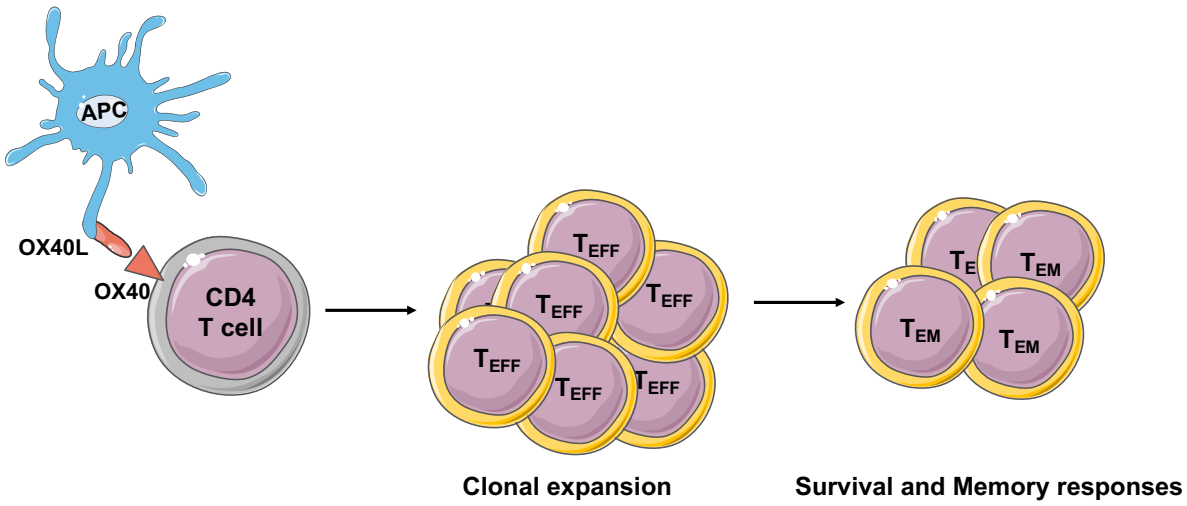


Figure 1. 4 The role of OX40 signals in CD4 T cells responses.

1.2.7 CD4 T cell memory

Typical antigen-specific T cell responses are characterised by distinct phases during which they become activated (priming), proliferate (expansion), undergo apoptosis (contraction) with the remaining cells being maintained as a quiescent population (memory). T cell responses usually peak at day 7-15 post antigen encounter and after that initial phase, effector cells enter a long contraction phase during which the majority of them die through apoptosis (Krammer et al., 2007). This phase is characterized by an increased action of Fas signalling which is responsible for inducing apoptosis in the T lymphocytes (Nagata, 1997). Although, Fas receptor is found on both effector and naive T cells, only the active cells are

subjected to its negative effects due to the absence of the protective FLIP molecule (Tschopp et al., 1998). The effector cells capable of escaping the process survive and become memory cells. The T memory cells are residual and quiescent cells and can survive for many years after the infection has been cleared. In mice, the half-life of memory T cells fluctuate around 40 days (Pepper et al., 2010). In this study the use of MHCII tetramers enabled tracking of antigen-specific CD4 T cells post infection with *L. monocytogenes* and revealed that transcription factor $Tbet^{high}$ and $Tbet^{low}$ effector T cells were both found to survive and enter the memory pool.

Memory T cells are often divided into two groups: the effector memory (Tem) and central memory (Tcm) cells. This classification is based on the expression of CCR7 and CD62L which characterise their migratory capabilities. Tcm express both CCR7 and CD62L which enables them to move via the lymphoid tissue (Pepper and Jenkins, 2011). They secrete more IL-2 which results in increased proliferative potential. In comparison Tem are thought to move via the nonlymphoid tissue or blood and are secreting effector cytokines. Tem upregulate other adhesion molecules which allow the entry to the nonlymphoid tissues, for instance CCR4, E-selectin and $\alpha 4\beta 1$ which allows entry to the skin (Gehad et al., 2012).

Memory and secondary responses have been described for all subsets of CD4 T cells. Generation of memory T cells in Th1 type of responses is well understood, unlike the generation of Th2 and Th17 memory which still proves problematic, primarily due to the lack of good antigen specific models. Moreover, the plasticity between the populations makes the research even more challenging. There have also been reports of Treg memory showing similar phases of activation, contraction and persistence in viral infections (Sanchez et al., 2012).

The factors responsible for promoting the CD4 memory T cell generation are still being established, due to the low abundance of those cells compared to the CD8 T cells. There are however two strong candidates that could enhance their formation and survival. Despite initial

controversies arising from the use of TCR transgenic mice to assess the influence of TCR:pMHC interaction on generation of memory T cells, it has been shown that this association could play a significant role (Gray and Matzinger, 1991; Purton et al., 2007; Swain et al., 1999). The determining factor is the heterogeneity of the memory cells resulting from the asymmetric division which may affect their dependence on the TCR:pMHC interaction. This is visible in the turnover rate for the two identified populations the MHCII depended memory phenotype cells and MHC independent antigen-specific cells (Purton et al., 2007). Although the optimal TCR:pMHCII interaction is important, studies have shown that it is durability of the interaction of some TCR clones that gives them a competitive advantage and enables survival (Celli et al., 2008; Kim et al., 2013).

Other factors like the initial size of the pool of naive T cells can regulate the expansion and size of the CD4 memory T cell population (Moon et al., 2007). Previous reports have shown that the number of naive CD4 T cell precursors specific for a single epitope in both mice and humans ranges between 1-100 cells per 10^6 naive CD4 T cell (Kwok et al., 2012; Whitmire et al., 2006).

Costimulatory signals and cytokines present during priming and expansion can also contribute to the establishment of memory population. The early CD28 and CD40 signals are particularly important as they both promote T cell expansion and minimise the non-specific T cell activation (Grewal et al., 1995; Jenkins et al., 2001; Pagan et al., 2012). As discussed before CD28 upregulates IL-2 production which in turn enhances the proliferation of effector T cells. Particularly, in Th1 and Th2 responses the strength of the IL-2 correlates with the CD4 T cell proliferation which in turn promotes survival of higher number of effector T cells and their entry into the memory phase (Pepper et al., 2011). Furthermore, TNFRSF plays a crucial role in maintaining effector T cells and promoting generation of memory T cells (Withers et al., 2009).

Maintenance of CD4 T cell memory is dependent on a combination of TCR signalling, IL-7 and IL-15 cytokines. Studies have shown that lack of homeostatic signals via the TCR affects

memory cells, specifically their responsiveness to IL-7 signals (Bushar et al., 2010). Similar to the CD8 memory T cells, under normal conditions, maintenance of CD4 memory T cells is IL-15 dependent (Purton et al., 2007). IL-7 and IL-15 are mainly produced by stromal tissues in secondary lymphoid organs, bone marrow, epithelial cells and antigen presenting cells (Fehniger and Caligiuri, 2001; Mazzucchelli et al., 2009).

Developed memory T cells move throughout the body and upon encounter of a cognate antigen they drive a more rapid and specific secondary response, which results in a greater number of antigen-specific cells as well as greater responsiveness to the antigen.

1.3 Adjuvants and their role in enhancing immune responses

Development of successful vaccination systems requires many years of research, studying different aspects of immune responses. It is crucial to understand the mechanisms behind the development of highly efficient immunity and generation of memory T cell and B cells which will guard an individual against reoccurring infections. Interaction between innate and adaptive immunity including cell activation and recruitment, cytokine secretion and upregulation of costimulatory molecules are all key parameters which collectively influence the course of the immune response and the establishment of immune memory. Previous studies have shown that priming of CD4 T cells can be affected by many factors including the cytokine milieu surrounding the cells as well as the components and types of vaccines (Jelley-Gibbs et al., 2008; Lanzavecchia and Sallusto, 2005).

Vaccination involves administration of an immunologic agent which leads to the development of long-term immunity. It was first described in 1796 when Edward Jenner conducted his pioneering experiment. He inoculated 8-year-old boy, James Phipps with the matter from a cowpox lesion found on a dairymaid's arm. He observed that James developed signs of mild infection which begun resolving after 10 days. Approximately 2 months later James received another inoculum this time from a freshly isolated smallpox lesion. To Jenner's astonishment,

James did not develop smallpox as a result of treatment, which led to a conclusion that exposure to the cowpox virus provided protection against the smallpox virus. Since Jenner's discovery, further advances in biological and immunological research led to establishment of worldwide immunisation programs which aimed to control the spread of infectious diseases. In 1980, following global immunisation campaign led by the World Health Organisation (WHO), smallpox was declared eradicated.

Although we have been able to controls the vast majority of infections, we are still faced with several challenged like rapid evolution of disease-causing organisms, cancer, the complications of past diseases as well as public, social and financial challenges. All of those make the development of new vaccines that much more difficult.

One of the most important stages in the history of vaccination was the transition between the live-attenuated and the more advanced inactivated vaccines as well as discovery of recombinant DNA technology which enabled formation of subunit vaccines. Although still in use, some of the early vaccines have been associated with insufficient bacterial inactivation which may be dangerous for recipients (Zepp, 2010). With the discovery of immunostimulatory effects of bacterial molecules and other antigen stability enhancing agents, inactivated vaccines became the main focus. In these vaccines microorganisms and viral particles are subjected to treatments with heat, chemicals or radiation and are therefore more safe for use (Pasquale et al., 2015). However, the absence of strong stimulation from living organisms makes those vaccines relatively inefficient and therefore they are heavily depended on either large doses which are not desired or more commonly are supported by adjuvants.

Adjuvants were first discovered in 1920s, when observations of coinjection of diphtheria toxoid with unknown substances showed significantly higher rates of antibody production (Glenny and Südmersen, 1921). Shortly after this, it was found that both aluminium salts and emulsions could intensify immune responses.

Administration of antigen/adjuvant complexes has been shown to increase the expansion of CD4 T cells and production of proinflammatory cytokines (Curtsinger et al., 1999; Pape et al., 1997). In studies on the responses to pigeon cytochrome c protein in B10.BR mice, the different adjuvants were shown as major determinants in antigen-driven CD4 T cell diversity (Baumgartner and Malherbe, 2010; Malherbe et al., 2008).

1.3.1 Types of adjuvants and their mechanisms of action

The action of majority adjuvants is based on two immune response inducing factors: stimulation of immune system and antigen delivery fraction. The immunostimulatory molecules consists of TLR, CLR and NLR ligands as well as saponins, providing co-stimulation. The delivery systems comprise mainly the aluminium salts, which are most commonly used, and emulsions and lipid vessels (Reed et al., 2013).

1.3.2 Aluminium salts

Aluminium salts are far the mostly studied adjuvants. They were the first adjuvants that received approval to be used in human vaccines including vaccines against Hepatitis A (HAV), Hepatitis B (HBV), diphtheria/tetanus/pertussis (DTP) and human papillomavirus (HPV) (Apostólico et al., 2016).

The mode of action of aluminium salts was first described in 1926, when precipitation of antigen with aluminium potassium sulphate proved effective in inducing antibody responses (Glenny et al., 1926). They have been shown to promote efficient antigen uptake and to stimulate components of the complement system (Gupta, 1998; Gupta et al., 1995). Aluminium salts were shown to work as terminals and bind antigens via electrostatic forces, allowing for their slow release rates and prolonging its effect on the immune system. This theory was widely accepted for many years, however in recent decade further research suggested that other mechanism might be involved in the action of aluminium salts. For

instance, removal of slow release-depot properties did not alter the immune response (Hutchison et al., 2011). Moreover, additional reports also demonstrated that aluminium salts led to a release of uric acid which acts as DAMP activating innate immune system (Kool et al., 2008). In addition, they can also be sensed by NOD-like receptors (NLRs) and activate NLRP3/NALP3 inflammasome complex (Eisenbarth et al., 2008). The key issues associated with aluminium adjuvants include weak responses, preferentially inducing Th2 responses, limited range of injection sites and possible allergic reactions (Grun and Maurer, 1989). Therefore, aluminium salts cannot be used in every vaccine formulation.

1.3.3 Emulsions

Emulsions are the most commonly used adjuvants. They have several classes depending on the dispersion phase of oil or water components (water-in-oil, oil-in-water, water-oil-water). They often act as antigen depot, slowing down its release yet their mechanisms are still not fully understood. The three representative adjuvants of this group are Freund's Adjuvants, MF59 and AS03. Complete Freund's adjuvant (CFA) is a water-in-oil emulsion, containing heat-killed mycobacteria. CFA induces immunogenicity and promotes primarily Th1 responses (Yip et al., 1999). It has been used particularly in experimental induction of autoimmune diseases, like arthritis which could be induced through immunisation with type II heterologous collagen in CFA (Inglis et al., 2007). One major limitation of the use of CFA are the inflammatory side effects which often result in painful ulceration around the injection site (Billiau and Matthys, 2001).

The Incomplete Freund's adjuvant (IFA) is also a water-in-oil emulsion, however it does not contain mycobacteria. Similarly to CFA, the efficiency of IFA adjuvants is overshadowed by the possible side effects which for some individuals can be severe including sterile abscesses (Miller et al., 2005). Nevertheless, IFA has been shown to induce local immunity leading to a

skewed Th2 responses (Yip et al., 1999). Alongside CFA it also enhances the antigen uptake by APCs through the increase in phagocytic activity (Nicol et al., 1966).

The frequent side effect of both CFA and IFA has made them unsafe for human use and are progressively considered hurtful for the animals undergoing research, with some reports suggesting the use of analgesics to minimise the side effects (Kolstad et al., 2012).

Another adjuvant, MF59 is a water-in-oil emulsion, containing squalene oil, and surfactants Tween 80 and Span85 which together induce robust cellular and humoral responses (Stephenson et al., 2005). It has been licensed for more than 19 years and has been used in influenza vaccine (Fluad™, Seqirus) which showed significant effects for adults and individuals over the age of 65 and adults suffering from chronic illness (O'Hagan, 2007). It has also been used to induce the immunogenicity of the trivalent inactivated influenza vaccine in 14-20 months children where it increased antibody titers and enhanced antigen-specific CD4 T cell responses (Nakaya et al., 2016). In PBMC cultures, MF59 induced secretion of chemokines, like CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CXCL8 (IL-8) which act as chemoattractant for monocytes and granulocytes (Seubert et al., 2008). Similarly, it was also shown to promote monocyte differentiation characterised by upregulation of MHCII, CD86 and increased expression of CCR7.

Finally, AS03 a GSK licensed oil-in-water adjuvant emulsion, containing α-tocopherol (vitamin E), squalene, and polysorbate 80, was first used in Prepandrix vaccine against avian influenza H5N1. Later it was also included in two other influenza vaccines, the Pandemrix and Arepanrix. The AS03 adjuvant has been shown to induce high antibody titers, even with reduced antigen dose. It has been shown to enhance the function of innate cells, particularly antigen presentation and increase the humoral and antigen-specific CD4 T cell responses (Garçon et al., 2012; Roman et al., 2011).

As described above both MF59 and AS03 are considered safe for human use and have been incorporated into various vaccines due to the absence of major adverse side effects.

1.3.4 Challenges of modern vaccines: Adjuvant system 01 (AS01) and its potential in promoting effective innate and adaptive immune responses

The continuous evolution of disease-causing pathogens poses the biggest challenge of modern vaccines. One of such organisms is the parasite from genus *Plasmodium* responsible for 212 million clinical cases of Malaria worldwide in 2015 leading to 429 000 deaths (WHO, 2017). Recently, great focus has been placed on the development of vaccine targeting the deadliest *P. falciparum* infection. GlaxoSmithKline Biologicals (GSK), the PATH Malaria Vaccine Initiative (MVI), Bill & Melinda Gates Foundation and a network of African research centres came together to produce first efficient malaria vaccine, the RTS,S/AS01 (RTS, S).

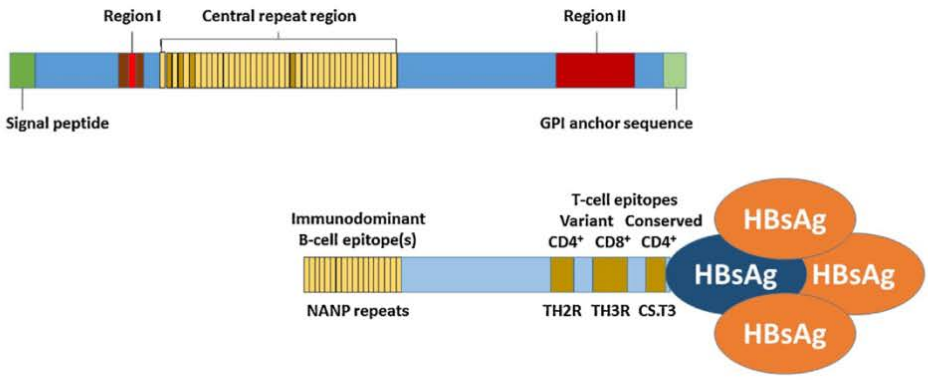


Figure 1. 5 Graphical depiction of circumsporozoite (CSP) and RTS,S structures (Kaslow and Biernaux, 2015).

RTS,S vaccine is composed of 189 amino acids from circumsporozoite protein (CSP). This protein is found on the surface of the sporozoites or on infected hepatocyte (Offeddu et al., 2012) (Figure 1.5). The Hepatitis B virus surface antigen (HBsAg) monomers are fused to CSP and serve as protein carriers (Bairwa et al., 2012). As RTS,S alone has been shown to have poor immunogenicity, the AS01 adjuvant has been added to improve the resulting immune protection (Stewart et al., 2007).

The design of AS01 adjuvant gives this vaccine a great potential at targeting Malaria, however it has also been selected for development of candidate Zoster vaccine where it induced immunity in people over 50 years of age (Chlibek et al., 2013). The adjuvant itself began development more than 20 years ago and overtime has gained some excellent immune enhancing qualities.

AS01 belongs to a group of liposomes-based adjuvants. This group of adjuvants combines two functions of being able to deliver the vaccine as well as induce immune responses. AS01 is composed of two immuno-stimulants: MPLA and QS-21 (licensed by GSK from Antigenics Inc., a wholly-owned subsidiary of Agenus Inc., a Delaware, USA corporation).

MPLA has been used as an immune stimulant for many years. It is a derivative of lipopolysaccharide (LPS) from *Salmonella minnesota* and therefore it acts on the innate immunity via the TLR4. It is however approximately 100-fold less toxic than LPS and therefore used as a preferred stimulant (Thompson et al., 2005).

QS-21 is a saponin extract from soap bark tree *Quillaja saponaria Molina*. QS-21 is a water-soluble triterpene glycoside. So far, the there is no clear understanding of the mechanisms and signalling pathways used by QS-21 to induce immune response, however it has been shown to trigger CD8 T cell and other antibody responses (Kashala et al., 2002; Newman et al., 1997). It also activates the ASC-NLRP3 inflammasome pathway inducing release of IL-18. The cholesterol added to the liposome core removes its haemolytic activity (Didierlaurent et al., 2017).

Previously, Diedierlaurent et al (2014) showed the impact AS01 had particularly on the innate immunity. They identified the response to be especially rapid, with the drainage from the muscle occurring within the first 24 hours post injections, concluding that the administration of antigen and adjuvant at the location and same time was pivotal to efficient establishment of antigen specific immunity (Arnaud M Didierlaurent et al., 2014). Flow cytometry analyses revealed elevated numbers of $Ly6C^{high}$ monocytes and neutrophils within the muscle just 3 hrs post injections, whereas in the draining lymph nodes (dLN) the level of DCs increased 16

hrs post injections (A. M. Didierlaurent et al., 2014). This suggests that AS01 promotes recruitment of innate cells into the site of injections and the dLNs. Specifically, the recruitment and activation of DCs and subsequent upregulation of costimulatory molecules is important to induce T cell priming and begin the process of establishing T and B cells memory populations.

Recently, the group has focused on assessing the earliest stages of vaccination using the HBs antigen and AS01 (Coccia et al., 2017). They successfully identified cell populations involved in producing early production of IFNγ which is well known to be highly important in creating Th1 polarising milieu, allowing establishment of antigen-specific CD4 T cells responses. They assessed the IFNγ production in draining iliac LNs within the first 6 hrs of immunisation. The analyses revealed the main source of IFNγ to include dLN resident NK cells and CD8 T cells which have been identified to respond in antigen-independent fashion. These initial findings have emphasised the potential and efficacy of the AS01 adjuvant in inducing immune responses against antigens.

1.4 Aims

Several hypotheses were stated prior the investigation:

1. The CD4 T cells and their effector functions generated in response to *Listeria monocytogenes-2W1S*, *Salmonella typhimurium*-2W1S and AS01 adjuvant are dependent on OX40 signals.
2. Cellular provision of OX40L by ILC3, T cells, DCs and B cells is crucial for the generation of effector CD4 T cell responses to *Listeria monocytogenes-2W1S*.
3. Early cytokines produced in responses to *Listeria monocytogenes-2W1S* are involved in the regulation of OX40L expression on DC post infection.
4. AS01 adjuvant induces early production of proinflammatory cytokines by innate immune cells and thereby promotes adaptive CD4 T cell and B cell responses.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

Mice were bred at the University of Birmingham Biomedical Services Unit (BMSU) except for IL-12p35$^{-/-}$ mice which were generated at the University of Manchester and shipped to Birmingham. All mice were used in accordance with Home Office regulations. Efforts were made to ensure that mice were age and sex- matched for the individual experiments. The age of the mice ranged from 6 to 12 weeks at the start of the procedure. All genetically modified strains were compared to WT controls or adequate littermate controls bred in-house. The mice were sacrificed using a Schedule 1 technique, cervical dislocation of the neck. All mice used in this research were on a C57BL/6 background and their details can be found in Table 2.1.

Table 2. 1 Original mice strains used in this investigation

Mouse strain	Formal names	CD45 Allotype	Back ground	Phenotype	Source
BoyJ	B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ https://www.jax.org/strain/002014	CD45.1	C57BL/6	WT	BMSU
C57BL/6	C57BL/6J https://www.jax.org/strain/000664	CD45.2	C57BL/6	WT	BMSU
CD11c^{Cre}	B6N.Cg-Tg(Itgax-cre)1-1Reiz/J https://www.jax.org/strain/008068	CD45.2	C57BL/6	Control strain expressing dendritic cell	Provided by Remi Fiancette

				specific Cre recombinase	
CD30$^{-/-}$ OX40$^{-/-}$	CD30$^{-/-}$ Tnfrsf8^{tm1Mak} http://www.informatics.jax.org/allele/key/2438 OX40$^{-/-}$ B6.129S4-*Tnfrsf4*^{tm1Nik}/J https://www.jax.org/strain/012838	CD45.2	C57BL/6	Deficient in expression of CD30 and OX40 receptor (Gaspal et al., 2005)	Provided by Peter Lane, UoB
CD80$^{-/-}$ CD86$^{-/-}$	B6.129S4-*Cd80*^{tm1Shr} *Cd86*^{tm2Shr}/J https://www.jax.org/strain/003610	CD45.2	C57BL/6	Deficient in CD80 and CD86 costimulatory molecules. Impaired T cell responses.	The Jackson Laboratory
E8III^{Cre}	Tg(Cd8a*cre)B8Asin http://www.informatics.jax.org/allele/key/617541	CD45.2	C57BL/6	Control strain expressing Cre recombinase under the CD8a promoter (McCaughtry et al., 2012)	The Jackson Laboratory

Great	Ifng$^{tm3(EYFP)Lky}$ http://www.informatics.jax.org/allele/key/817862	CD45.2	C57BL/6	IFNγ reporter mice (Price et al., 2012; Reinhardt et al., 2009)	Kind gift from Richard Locksley
H2-Ab1$^{f/f}$	B6.129X1-*H2-Ab1*^{tm1Koni}/J https://www.jax.org/strain/013181	CD45.2	C57BL/6	Strain has floxed exon 1 of the *H2-Ab1* locus, used to study MHCII restricted antigen presentation (Hashimoto et al., 2002)	Provided by Daniela Finke
IFNγ$^{-/-}$	B6.129S7-*Ifng*^{tm1Ts}/J https://www.jax.org/strain/002287	CD45.2	C57BL/6	Deficient in IFNγ.	Provided by Adam Cunnigham, UoB
IFNγ$^{-/-}$R	C57BL/6N-*Ifngr1*$^{tm1.1Rds}$/J https://www.jax.org/strain/025394	CD45.2	C57BL/6	Deficient in IFNγ receptor (Lee et al., 2013)	The Jackson Laboratory
IL-12p35$^{-/-}$	C.129S1(B6)-*Il12a*^{tm1Jm}/J	CD45.2	C57BL/6	Deficient in p35 subunit of IL-12 cytokine	Provided by Andrew Macdonald,

	https://www.jax.org/strain/002691				University of Manchester
JCD1	B6.129S6-Del(3Cd1d2-Cd1d1)1Sbp/J https://www.jax.org/strain/008881	CD45.2	C57BL/6	Deficient in CD1d, glycoprotein found on NKT cells.	Provided by Nick Jones, UoB
$Mb1^{Cre}$	B6.C(Cg)-*Cd79a*$^{tm1(cre)Reth}$/EhobJ https://www.jax.org/strain/020505	CD45.2	C57BL/6	Control strain expressing Cre recombinase under the CD79 promoter	Provided by Michael Reth
mTmG	*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J https://www.jax.org/strain/007576	CD45.2	C57BL/6	Double-fluorescent reporters used for phenotypic analysis of Cre-expression (Muzumdar et al., 2007)	The Jackson Laboratory
$OX40^{-/-}$	B6.129S4-*Tnfrsf4*^{tm1Nik}/J https://www.jax.org/strain/012838	CD45.2	C57BL/6	Deficient in expression of OX40 receptor.	Provided by Peter Lane, UoB

OX40L$^{f/f}$	TNFSF4$^{fl/fl}$	CD45.2	C57BL/6	Strain possess floxed *Tnfsf4* locus (Cortini et al., 2017)	Provided by Marina Botto
PGK^{Cre}	B6.C-Tg(Pgk1-cre)1Lni/CrsJ https://www.jax.org/strain/020811	CD45.2	C57BL/6	Ubiquitous Cre recombinase strain (Lallemand et al., 1998)	BMSU
Rag$^{-/-}$	B6(Cg)-*Rag2*$^{tm1.1Cgn}$/J https://www.jax.org/strain/008449	CD45.2	C57BL/6	Deficient in T and B cells.	The Jackson Laboratory
RORc^{cre}	B6.FVB-Tg (Rorc-cre)1Litt/J https://www.jax.org/strain/022791	CD45.2	C57BL/6	Control strain expressing Cre recombinase in RORγt cells (Eberl and Littman, 2004).	Provided by Dan Littman, NYU
Smart 17A	Il17a$^{tm1.1Lky}$ http://www.informatics.jax.org/allele/MGI:5439016	CD45.2	C57BL/6	IL-17A reporter mice (Price et al., 2012)	Kind gift from Richard Locksley

Table 2. 2 Mice strains generated for this investigation

Mouse strain	CD45 Allotype	Background	Phenotype
C57BL/6 x BoyJ	CD45.1 CD45.2	C57BL/6	WT
$CD11c^{Cre}$ x $H2\text{-}Ab1^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking the expression of MHCII on dendritic cells
$CD11c^{Cre}$ x $OX40L^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking the expression of OX40 ligand on dendritic cells
$CD11c^{Cre}$ x $IFN\gamma R^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking IFNγR on dendritic cells
$CD11c^{Cre}$ x mTmG	CD45.2	C57BL/6	Reporter mice allowing to fatemap the expression of Cre in dendritic cells
$E8III^{Cre}$ x $OX40L^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking the expression of OX40 ligand on T cells
$E8III^{Cre}$ x mTmG	CD45.2	C57BL/6	Reporter mice allowing to fatemap the expression of Cre in T cells.
Great x Smart17A	CD45.2	C57BL/6	Dual reporter mice for IFNγ and IL-17A
Great x Smart17A x $CD30^{-/-}OX40^{-/-}$	CD45.2	C57BL/6	Dual reporter mice for IFNγ and IL-17A deficient in CD30 and OX40 receptors.

Great x Smart17A x $CD30^{-/-}$ $OX40^{+/-}$	CD45.2	C57BL/6	Dual reporter mice for IFNγ and IL-17A deficient in CD30 receptor and expressing one copy of OX40 receptor.
Great x Smart17A x $CD30^{+/-}$ $OX40^{-/-}$	CD45.2	C57BL/6	Dual reporter mice for IFNγ and IL-17A deficient in OX40 receptor and expressing one copy of CD30 receptor.
$Mb1^{Cre}$ x $OX40L^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking the expression of OX40 ligand on B cells.
PGK^{Cre} x $OX40L^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice with ubiquitous deletion of OX40 ligand.
$RORc^{Cre}$ x $OX40L^{f/f}$	CD45.2	C57BL/6	Conditional knockout mice lacking the expression of OX40 ligand on RORγt cells.
$RORc^{Cre}$ x mTmG	CD45.2	C57BL/6	Reporter mice allowing to fatemap the expression of Cre in RORγt cells.

2.2 Medium and Reagents

2.2.1 Medium

A list of reagents used in the research is provided below in Table 2.3

Table 2. 3 List of reagents used in this investigation

Medium	Reagents and concentrations
Culture Medium	RPMI-1640 with L- Glutamine (ThermoFisher), 1% Penicillin and Streptomycin (Sigma-Aldrich), 1% L-Glutamine (Sigma-Aldrich), 10% Heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich).

Staining buffer (SB)	Dulbecco's Phosphate Buffered Saline (DPBS) +$CaCl_2$ +$MgCl_2$ (Sigma-Aldrich), 2% Heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich), 2.5 M Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich)
Staining solution for immunofluorescence microscopy	Phosphate buffered saline (PBS) (Sigma-Aldrich), 1% Bovine serum albumin (Sigma-Aldrich)
Haemolytic Gey's Solution	Solution A: 35 g NH_4Cl, 1.85 g KCl, 1.5 g $Na_2HPO_4.12H_2O$, 0.119 g KH_2PO_4, 5.0 g Glucose, 25 g Gelatin, 1.5 mL 1% Phenol red Solution B: 4.2 g $MgCl_2.6H_2O$, 1.4 g $MgSO_4.7H_2O$, 3.4 g $CaCl_2$ Solution C: 22.5 g $NaHCO_5$, distilled H2O
Hank's Balanced Salt Solution (HBSS) with EDTA	500 mL Calcium and Magnesium free HBSS (Sigma-Aldrich), 2.5 M EDTA (Sigma-Aldrich)
HBSS with FCS	500 mL Calcium and Magnesium free HBSS (Sigma-Aldrich), 2% FCS
Culture media with Collagenase VIII (from C.histolyticum)	15 mL culture media, 0.015 g Collagenase from C. histolyticum (Sigma-Aldrich)

2.2.2 Gey's solution

Gey's solution was used in preparation of spleen samples as a red blood cell lysis buffer. It was made from solution described in Table 2.3. Stock solutions A, B and C were prepared, as described above, to a final volume of 1 L and autoclaved. Gey's solution was made by

combining 10 mL Solution A, 2.5 mL Solution B, 2.5 mL Solution C and 35 mL of distilled water. The buffer was kept at 4-8 °C.

2.3 Cell isolation from murine tissues

2.3.1 Preparation of cell suspension from mouse lymph nodes

The dissected lymph nodes were placed in small petri dishes (Nunclon, ThermoScientific) containing 3 mL RPMI medium. The lymph nodes were cleaned under the dissecting microscope to remove the fat depositions around the tissues using forceps.

Lymph nodes were teased using forceps and incubated in 3 mL RPMI with 250 µg/mL Collagenase/ Dispase (Roche), 25 µg/mL DNase I (Sigma-Aldrich) for 20 min at 37 °C. The reaction was stopped with 60 µL of 10 mM EDTA (Sigma-Aldrich). The tissues were crushed through a 70 µm Falcon cell strainer (Falcon, Corning) and washed thoroughly with RPMI. Cell suspensions were centrifuged for 6 min at 4 °C, 1500 rpm, unless stated otherwise. Pellets obtained from lymph node samples were then resuspended in appropriate volume of Staining Buffer (SB).

2.3.2 Preparation of cell suspension from mouse spleen

Spleen were dissected and placed in RPMI medium. When required, the spleens were teased using forceps and digested as described in section above with the digest lasting 30 min.

The spleens were crushed through a 70 µm Falcon cell strainer and washed thoroughly with RPMI. The single cell suspensions were centrifuged and obtained pellets were resuspended in 5 mL Gey's solution red lysis buffer. Pellets were incubated for 5 min on ice. After addition of 10 mL RPMI, the samples were centrifuged and pellets were resuspended in appropriate volume of SB. Splenocytes were re-filtered prior to use.

Cells prepared for flow cytometry took place in non-sterile conditions, whereas the preparation of cells for overnight culture involved the use of safety cabinets and sterile reagents.

2.3.3 Preparation of cell suspension from mouse small intestine

The small intestine was dissected (end of the stomach to the cecum) and the fat surrounding it was removed using forceps. Peyer's Patches were excised and the small intestine was cut length-wise and cleaned in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) 2 % FCS. Next the intestine was cut into small fragments and placed in 50 mL falcon tube containing 20 mL HBSS 2 % FCS. The sample was filtered through a nylon mesh and incubated in 20 mL HBSS EDTA for 20 min at 37 °C shaking incubator. The sample was filtered and washed with 20 mL HBSS before incubating it for another 20 min in 20 mL HBSS EDTA in the same condition as above. Both HBSS EDTA and HBSS were prewarmed in 37 °C water bath prior the experiment.

After second incubation, small intestine was washed 3 times with 20 mL HBSS. It was then placed in 15 mL of prewarmed culture media containing 1 mg/mL Collagenase VIII (Sigma-Aldrich) and incubated for 15 min at 37 °C shaking incubator. After the initial 10 min incubation, the samples were shaken manually until most tissue fragments were digested, however at no point exceeding the 5 min.

The digested tissue was placed on ice and then filtered through a 100 µm cell strainer (Greiner Bio-One) and then a 70 µm Falcon cell strainer each time washing it twice with 5 mL SB. Samples were centrifuged and pellets resuspended in appropriate volume of SB and placed on ice.

2.3.4 Preparation of cell suspension from mouse colon

The colons were dissected and the fat surrounding them was removed using forceps. The tissues were cut lengthwise and cleaned in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) 2 % FCS. In general, the preparation followed similar protocol to the one used for small intestine, with some fundamental differences in enzyme mix used to digest the tissue. The enzymes used in colon and cecum preparation included: 0.85 mg/mL Collagenase V (sigma C9263-1G), 1.25 mg/mL Collagenase D (Roche 11088882001), 1 mg/mL Dispase (Gibco 17105-041), 30 μg/mL DNAse (Roche 101104159001). The enzyme mix was made in culture media and 10 mL was used per sample. Samples were digested between 30-40 min at 37 °C.

Similarly, to the small intestine preparation, the single cell suspensions were passed through a 100 μm cell strainer (Greiner Bio-One) and then a 70 μm Falcon cell strainer each time washing it twice with 5 mL SB. Samples were centrifuged and pellets resuspended in appropriate volume of SB and placed on ice.

2.4 Cell Culture

Cell suspensions were prepared in sterile conditions using the method described in section 2.3.1 and 2.3.2. The cells were cultured overnight (24 hrs) at 37 °C, 5 % $C0_2$ in 24 well plate in 1 mL culture media. Those conditions were also true for the 48 and 72 hrs cultures. Both culture types involved culturing 2 x 10^6 splenocytes/1 mL, unless stated otherwise.

Assessment of OX40L expression on Antigen Presenting Cells, like dendritic cells and B cells required overnight culture with CD40 stimulation. For this purpose, purified anti-CD40 antibody was added to at 1 μg/mL.

To assess the expression of OX40L on T cells, 2 x 10^6 splenocytes were cultured at 37 °C with 5 % $C0_2$ for 72 hrs in the presence of 10 μg/mL Abatacept and 0.5 μg/mL Functional Grade anti-CD3e.

In experiments assessing expression and regulation of OX40L on splenic DC, the $2x10^6$ splenocytes from uninfected or *actA*-deficient *Listeria monocytogenes*-2W1S (Lm-2W1S) infected mice, were cultured overnight (18-24 hrs) at 37 °C, 5 % $C0_2$ in 24 well plate in 1 mL culture media, either alone or with 1 µg anti-CD40 Abs or 100 ng recombinant IFNγ (Peprotech).

Unspecific stimulations with PMA and Inonomycin required culturing splenocytes at 37 °C in the presence of PMA (50 ng/mL) and Iononomycin (1.5 µM). After the initial hour, BFA (BD Biosciences) was added to a final concentration of 10 ug/mL and cells were incubated for further 3 hrs.

In AS01 experiments, assessment of the early IFNγ production involved incubation of cell suspensions for 3 hrs in culture media in the presence of Brefeldin A (BFA) (10 µg/mL, BD Biosciences) and Monensin (10 µg/mL, BD Biosciences) at 37 °C 5 % $C0_2$.

Similarly, splenocytes or colon cells from mice infected with Lm-2W1S or *Salmonella typhimurium* respectively, were cultured for 3 hrs in culture media in the presence of BFA (10 µg/mL, BD Biosciences) to assess IFNγ production.

2.5 Flow Cytometry

List of antibodies used in flow cytometric analyses can be found in Table 2.4.

The fraction of cells stained for specific markers was depended on the experiment and the population that needed to be identified. For the AS01 experiments whole pools of iliac and inguinal lymph nodes were stained, while in the Lm-2W1S model the staining was performed on 1/3 of pooled LNs (mesenteric, 2 inguinal, 2 brachial, 2 auxiliary) and 1/10 or 1/12 of spleen. Typically, 1/2 of mesenteric LN was stained, unless stated otherwise. When MACS cell separation was performed, whole enriched fraction and 1/10 of the run-through fraction were stained.

Any samples subjected to cell culture and also gut samples were stained with APC eFluor 780 viability dye (1:1000, eBiosciences) in PBS for 20 min on ice prior to antibody staining. For assessing OX40L on DC, prior to surface staining the cells were treated with 2.4G2 antibody for 10 min to block FC receptor.

Surface staining was performed in 96-well plates (ThermoScientific) in 50 μl or 100 μl SB for 30 min on ice. Plates were centrifuged for 2-3 min to pellet the cells. Staining for CXCR5 was performed for 1 hr at room temperature (RT), usually alongside the 2W1S:IA^b MHCII Tetramer staining. Single stain controls were set up using WT splenocytes stained in 50 μl with individual fluorochromes used in the antibody cocktails. Staining for lineage markers involved the use of either individual antibodies or collection of antibodies conjugated to the same fluorochrome. Following the staining, 100 μl of SB was added and the cells were centrifuged. Pellets were washed twice with 200 μL SB.

Staining with biotinylated antibodies required isotype control staining to assist correct gating. For this type of staining, both antibody cocktails were prepared in SB containing 5 % of rat and 5 % mouse sera to block any non-specific avidin binding. The cells were incubated for 30 min on ice. Detection of biotinylated antibodies was achieved using Streptavidin conjugated to specific fluorochrome, prepared in SB with 5 % of rat and 5 % mouse sera and incubating the samples for 30 min on ice. Following the staining, 100 μl of SB was added and the cells were centrifuged. Pellets were washed twice with 200 μL SB.

Analysis of transcription factors and intracellular cytokines required the use of intracellular labelling. Following the surface staining, cells were fixed and permeabilised using either Foxp3 (eBioscience) or BD Cytofix (BD Bioscience) kits which were used according to the manufacturer's instructions. Foxp3 (eBioscience) fixation and permeabilisation buffers were used for majority of transcription factor staining, with exception to experiments were reporter mice were used or overnight staining carried out. For those experiments as well as for majority of intracellular cytokine staining BD Cytofix (BD Bioscience) fixation and permeabilisation

buffers were used. The cells were fixed on ice for 30 min or overnight in 100 µL of fixation/permeabilisation solution prior to addition of 100 µL permeabilisation buffer and centrifugation. The cells were washed 3 times with 200 µL permeabilisation buffer. The cells were then stained with intracellular antibodies in 100 µL permeabilisation buffer for 40-60 min or overnight at RT. 100 µL of permeabilisation buffer was added before centrifuging the cells and washing twice with 200 µl of permeabilisation buffer. Pellets were resuspended in 200-400 µL SB for analysis.

The samples were transferred into fluorescence-activated cell sorting (FACS) tubes (Becton) and Spherotech Accucount blank particles (Spherotech) were added to each sample allowed calculation of total cell numbers factoring in the proportion of the samples analysed.

Samples were analysed using LSR Fortessa X-20 with a FACS diva software (BD Biosciences). Compensations were set up manually using the single stain controls. The acquired events were first analysed using the forward and side scatter. The gate was placed appropriately to exclude dead cells and debris. Collected data was analysed using the FlowJo software (FlowJo LLC)

Table 2. 4 List of antibodies used in this investigation

Specificity	Clone	Conjugate	Dilution	Manufacturer
B220	RA3-6B2	eFluor 450	1:200	eBioscience
		FITC	1:300	eBioscience
		PE-Cyanine7	1:200	eBioscience
		APC eFluor 780	1:200	eBioscience
CD3	17A2	FITC	1:100	eBioscience
		Alexa Fluor 700	1:200	eBioscience
		Brilliant violet 650	1:200	Biolengend
	145-2C11	PE-Cyanine7	1:100	eBioscience
		PE	1:200	eBioscience
CD4	RM4-5	Brilliant violet 510	1:300	Biolegend
		APC	1:200	eBioscience
		Brilliant violet 786	1:200	Biolegend
CD8	53-6.7	Brilliant Violet 711	1:200	eBioscience
		APC	1:100	eBioscience
CD11b	M1/70	eFluor 450	1:200	eBioscience
		FITC	1:300	eBioscience
CD1d	PBS-57	APC	1:100	NIH
CD11c	N418	eFluor 450	1:200	eBioscience
		FITC	1:300	eBioscience
		PE-Cyanine7	1:200	eBioscience
CD24	M1/69	Brilliant violet 605	1:600	Biolegend
CD25	PC61	Brilliant Violet 650	1:200	Biolegend
CD38	90	PerCP-eFluor 710	1:1000	eBioscience

CD44	IM7	Brilliant violet 785	1:200	eBioscience
CD45	30-F11	Brilliant violet 786	1:200	BioLegend
CD45.2	104	Brilliant violet 786 PE	1:200 1:200	BioLegend eBioscence
CD49b	DX5	FITC	1:200	eBioscence
CD86	GL-1	FITC Brilliant Violet 650	1:100 1:700	BioLegend BioLegend
CCR6	29-2L17	Brilliant violet 605	1:100	eBioscience
CXCR5	2G8	PE-Cyanine7	1:50	BD Biosciences
EOMES	Dan11mag	PE	1:50	eBioscience
GL7	GL-7	eFluor 450	1:100	eBioscience
hNGFR	ME20.4	PE	1:50	BioLegend
IL-7Rα	A7R34	Brilliant violet 421 Eflour 660	1:100 1:200	BioLegend eBioscience
IFN-γ	XMG1.2	PE-Cyanine7 Brilliant violet 510 Alexa Fluor 700	1:200 1:200 1:100	eBioscience BioLegend BioLegend
IFN-γR	2E2	Biotin	1:100	eBioscience
IL-2	JES6-5H4	Alexa Fluor 488	1:100	eBioscience
Ly6c	HK1.4	Brilliant Violet 711	1:800	BioLegend
NK1.1	PK136	Brilliant Violet 650 FITC	1:100 1:100	BD Bioscences eBioscience
NKp46	29A1.4	Brilliant Violet 605	1:100	BioLegend
MHCII	M5/114.15.2	Brilliant Violet 510 Alexa Fluor 700	1:600 1:100	BioLegend eBioscience
OX40	OX86	APC	1:20	eBioscience

		Brilliant violet 421	1:20	BioLegend
OX40L	RM134L	PE	1:50	Biolegend
RORγt	AFKJS-9	PE APC	1:50 1:50	eBioscience eBioscience
SIRPα	P84	PerCP-eFluor 710	1:200	eBioscience
Straptavidin		PerCP-eFluor 710 PE-Cyanine7	1:500 1:500	eBioscience eBioscience
TCRβ	H57-597	Alexa Fluor 700	1:100	Biolegend
T-bet	4B10	PE-Cyanine7 eFluor 660 PE	1:50 1:50 1:50	eBioscience eBioscience Biolegend
XCR1	ZET	Alexa Fluor 647	1:100	Biolegend

2.5.1 MHCII Tetramer staining

MHC II tetramers were obtained from National Institute Health (NIH) Tetramer facility. The staining was performed for 1 hr at RT. Control MHC II tetramer was used regularly to stain a fraction of cells to assess the specificity of 2W1S:I-A^b tetramer binding.

Additionally, experiments carried out to identify different memory T cell populations and T follicular helper cells required staining with CXCR5 antibody along with the 2W1S:I-A^b tetramer.

When performing the *in vivo* peptide stimulation it was important to preserve the detection of intracellular cytokines, therefore 1 µL of Brefieldin A (10 µg/mL) was added to each sample stained with 2W1S:I-A^b tetramer.

The CD1d tetramer was added to the cocktail of extracellular antibodies and stained on ice for 30 min.

2.5.2 MACS cell separation

MACS cell separation was performed to identify and analyse the rare 2W1S:I-A^b populations at day 2-4 post immunization with Lm-2W1S. After 1 hr of staining with 10 µg/mL APC conjugated 2W1S:I-A^b MHC II tetramer in the dark in a RT water bath, the samples were incubated with 25 µL anti-APC beads (Miltenyi Biotec) for 15 min on ice. Cells were then washed and centrifuged and filtered through a 30 µm pre-separation filters (Miltenyi Biotec). The LS columns (Miltenyi Biotec) were assembled onto a magnet and washed with 3 mL staining buffer. The cells were passed through the columns and washed with 9 mL staining buffer (in 3 consecutive repeats). The magnetically labelled cells were flushed through the column with 5 mL SB by firmly applying the plunger. Both the enriched and 'run-through' fractions were stained to assess the efficiency of the enrichment.

2.6 Immunisations and infections

2.6.1 Growth of *Listeria monocytogenes* for infection

Mice were infected with *actA*-deficient strain of *Listeria monocytogenes*-2W1S. This strain was engineered to express recombinant protein OVA fused to $2W1S_{52\text{-}68}$ (EAWGALANWAVDSA). This enabled detection of antigen-specific $CD4^+$ T cell responses.

2.6.1.1 Fresh stock preparation

Bacterial cultures were plated and grown on agar with 20 µg/mL of selection antibiotic, chloramphenicol at 37 °C overnight. Agar plates were prepared by autoclaving agar powder (Sigma-Aldrich) in distilled water and adding the antibiotic once the medium has cooled to around 50 °C. The 20 mL of LB agar was poured into large petri dishes and were stored at 4 °C. Single colonies of Lm-2W1S were subcultured in 10 mL LB broth containing 20 µg/mL chloramphenicol overnight at 37 °C in a shaking incubator. Next day the subcultures were diluted in LB broth with 20 µg/mL chloramphenicol to give 1/10, 1/20, 1/50 fractions of the

original culture. Those were incubated for approximately 3 hrs to reach the optical density $(OD)^{600}$ of 0.1 which was measured using a spectrophotometer (Jenway 6405). The spectrophotometer was calibrated using a LB medium. The culture that showed required OD^{600} was divided into 1 mL aliquots and centrifuged at 10000 rpm for 10 min. Pellets were washed with sterile PBS and centrifuged again to remove dead bacteria. The resulting pellets were resuspended in sterile PBS and further checks were performed to ensure the OD^{600} remained within the limit of 0.1.

2.6.1.2 Glycerol stock preparation

Bacteria were pre-cultured in 10 mL of LB broth containing 20 µg/mL chloramphenicol from a single colony and grown overnight at 37 °C at 200 to 250 rpm. Next day, 1 mL of Lm-2W1S was seeded into 200 mL of LB broth containing 20 µg/mL chloramphenicol and grown at 37 °C at 200 to 250 rpm until OD600 of 0.6-0.7 was reached. The liquid bacterial cultures were then centrifuged for 20 min at 4 °C, 4000 rpm. The supernatant was discarded and pellets resuspended in LB broth with 15 % glycerol. Glycerol stocks of Lm-2W1S were aliquoted into 1 mL cryovials and stored at -80 degrees. Three randomly chosen samples were used to assess the viability of bacterial stocks by plating serial dilution of bacteria on agar containing 20 µg/mL chloramphenicol. Prior administration, the 1 mL Lm-2W1S was centrifuged and washed with sterile PBS before resuspending in adequate volume of sterile PBS to obtain 10^7 bacteria per 200 µL.

The bacteria were diluted in sterile PBS to 10^7 in 200 µL. Mice were infected with 10^7 Lm-2W1S through intravenous injection in the tail vein.

2.7.4 *In vivo* restimulation

The mice that had been infected with Lm-2W1S received 100 µg 2W1S peptide (20 mg/1 mL) and 2.5 µg LPS i.v. in the tail vein. The mice were then sacrificed 4 hrs later and their spleens and pool of mesenteric, brachial, auxiliary and inguinal LNs were harvested for analysis.

2.6.2 Growth of *Salmonella typhimurium* for infection

Glycerol stocks were prepared using the same method as described in previous section. Streptomycin was used as a selection antibiotic. The viability of the stocks was tested by randomly choosing 3 samples and plating the bacteria on MacConkey agar (Sigma-Aldrich) containing 100 µg/mL of streptomycin (Sigma-Aldrich).

The mice were given 20 mg of streptomycin in 100 µL sterile PBS by oral gavage 24 hrs prior to the administration of *Salmonella*.

The glycerol stock of bacteria were centrifuged and washed with sterile PBS before resuspending in adequate volume of sterile PBS to obtain 10^9 bacteria per 100 µL and administered by oral gavage.

To determine the prevalence of *Salmonella* infection, stool sampling was carried out at various timepoints. To collect the stools, individual mice were placed in an isolated cage to allow time for stools production. The stools were weighed and resuspended in appropriate volume of PBS (e.g. 0.0321 g stool was resuspended in 321 µL of PBS) and left for 40 min. Once homogenised, the stools were pulsed in a centrifuge for 3 sec. The collected supernatants were used in serial dilutions, later plated on MacConkey agar (Sigma-Aldrich) containing 100 µg/mL of streptomycin and grown overnight at 37 °C.

2.6.5 AS01 adjuvant immunizations

To test the effectiveness of AS01 adjuvant, the injections were performed using the 2W1S peptide conjugated to OVA protein. The antigens and adjuvant were administered through

intramuscular injections. The injections were performed in sterile conditions with mice under anaesthetic. The site of the injection, the gastrocnemius muscle, was shaved to allow better visualization of the site. Approximately 3 mm of the needle was inserted into the muscle. Mice were left to recover while being monitored for any signs of possible damage to the sciatic nerve.

Initial experiments involved administration of 30 µL Evan Blue dye at 10 mg/ml of PBS in gastrocnemius muscle of one leg.

Later experiments progressed to test the effect of AS01 on enhancing the immune response. The subsequent refinements involved injecting the OVA-2W1S protein with or without the AS01 as well as reduction of the injection volume to 10 µL administered into gastrocnemius muscle of both hind legs. The injections contained either 20 µg OVA-2W1S (10 mg/1 mL) and 1/50 Human Dose (HD) AS01 or 20 µg OVA-2W1S and PBS. A pool of iliac and draining inguinal lymph nodes were analysed to assess the changes in immune response. Cell suspensions were prepared according to the protocol in section 2.3.1.

2.7 Statistics

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

CHAPTER 3: THE ROLE OF OX40 SIGNALS IN TH1 CD4 T CELL RESPONSES

3.1 Introduction

Effective CD4 T cell responses, both primary and secondary, rely on activation and most importantly subsequent expansion of antigen-specific cells. In the absence of expansion, the insufficient numbers of activated antigen-specific cells can lead to poor immune responses or tolerance which often result in cells death (M. Croft et al., 1992).

APCs play a crucial role in eliciting CD4 T cell responses, by providing essential signals that promote both activation and expansion. The discovery of CD28 signalling in the late 1980s marked an emergence of a new two-signal model of T cell activation. Although oversimplified, this theory described the importance of both the formation of TCR and Ag-MHCII complexes as well as the signalling via various costimulatory pathways (Jenkins et al., 1988; Mueller et al., 1989).

The costimulatory interactions have different levels of significance, with some of them like the CD28 signalling being absolutely essential for T cell activation as represented by the constitutive expression of CD28 receptor on the surface of all naive T cells. Other costimulatory pathways, however, can be inducible, being expressed or regulated after the initial TCR and CD28 signalling. These include for instance, ICOS, a member of the CD28 and CTLA-4 receptor family, which is similarly efficient in enhancing T cell responses like CD28, increasing proliferation and secretion of cytokines, except for IL-2 (A Hutloff et al., 1999). Others include members of the TNFRSF, like OX40 and CD30, which are involved in potentiating and maintaining the survival of Ag-specific CD4 T cells.

Although, it is known that co-signalling downstream of the TCR is important in all stages of T cell response, like the priming, effector and memory phases as well as for both CD4 and CD8 subtypes of T cells, yet still its influence on T cell fate decision and remains unclear. Similarly,

the exact timing and contributions of individual signalling pathways impacting T cell function still require further understanding.

Since its discovery in 1980s, much progress has been made in understanding the role of OX40 signalling pathway in various immunological models. The preferential expression of OX40 on activated CD4 T cells has highlighted its potential in promoting and augmenting immune responses as well as in generation of long-term memory. It has provided a particularly attractive target for cancer immunology, with many studies emphasising the benefits of using OX40 agonists in targeting tumours (Gough et al., 2012; Redmond et al., 2014). Phase I clinical trials of patients with advanced form of cancer showed OX40 to be a potent costimulatory pathway, with a regression of metastatic lesions after just one treatment with monoclonal OX40 antibody (Curti et al., 2013).

Many *in vitro* and *in vivo* studies showed OX40 to be crucial in the secondary mechanisms of T cells activation, maintaining proliferation and enhancing their survival by activating Bcl-xL and Bcl-2 molecules (Rogers et al., 2001; Soroosh et al., 2014, 2007).

Despite early questions regarding polarisation signals and OX40, there is growing evidence suggesting that all effector T cell populations are indeed OX40 dependent, regardless of the differences in the polarisation signals.

Initial studies suggested that OX40 was particularly important for regulating the balance between Th1 and Th2 immunity, particularly during T cell priming where it was shown to promote Th2 differentiation. For instance, activated naive T cells engaging in OX40/OX40L signalling were shown to produce low but stable levels of IL-4 cytokine, promoting development of Th2 effector cells (Ohshima et al., 1998). Moreover, disease models revealed that OX40 deficient mice appeared to be resistant to allergic inflammation in mouse model of asthma, while in WT mice blockage of OX40 signals prevented progression of *Leishmania major* induced infection which was linked to reduced Th2 responses (Akiba et al., 2000; Jember et al., 2002). Additionally, overexpression of OX40L acted as an adjuvant, augmenting Th2 immune responses to OVA antigen (Ishii et al., 2003). Further studies

described provision of OX40L by DC *in vivo* to be potent costimulatory signal promoting Th2 responses, however with no effect on their optimal polarisation (Jenkins et al., 2007). On the other hand, studies also showed that in presence of IL-12, OX40L lost the ability to mediate Th2 immunity and instead increased Th1 inflammatory responses by inducing release of TNFα, while immunotherapy with OX40L-Fc (chimeric fusion protein) increased levels of Th1 cytokines (Ito et al., 2005a; Zubairi et al., 2004). However, further studies are still required to fully understand the role of OX40/OX40L signalling in Th1 responses.

In regard to Th17 responses, the published data is contradictory, since some reports propose that OX40 signals are supportive for Th17 cells *in vivo*, however, other studies conclude that OX40 signals inhibit Th17 formation through histone modifications that result in closure of Il17 locus (Xiao et al., 2016, 2012; Xin et al., 2014; Zhang et al., 2010).

With all of the conflicting data, one particular study investigated the role of several costimulatory molecules, including OX40, in T cell differentiation, also assessing the role of the initial antigen dose as well as affinity of the peptide presented as a part of MHCII during CD4 T cell priming. They concluded that rather than one individual receptor intrinsically controlling differentiation of CD4 T cells, it was the combination of many costimulatory pathways and the peptide affinities that led to acquisition of specific T cell fate (Rogers and Croft, 2014). Therefore, perhaps OX40 alone functions as a regulator of T activation rather than one of the cell fate determinants.

Many of the existing studies investigating the role of OX40 signals in various immune responses have utilised TCR transgenic T cells often at completely unphysiological precursor frequencies, having a profound effect on the nature of the response. Assessments of the T cell function in the presence and absence of OX40 have also been limited, with the analysis of cytokine production often relying heavily upon *ex vivo* restimulation. This can distort our understanding of where the defects really are and might explain why OX40 signals have been linked to so many aspects of the T cell responses.

In this chapter, we aimed to further define the role of OX40 signals, particularly focusing on their requirement in generating functional CD4 T cell responses in a polyclonal endogenous response and investigate which T cell populations are impacted the most by the lack of OX40 signalling. Using previously widely utilised 2W1S peptide, either incorporated into bacterial genome or conjugated to OVA protein, we were able to track antigen specific CD4 T cell responses to *Listeria monocytogenes*, *Salmonella* serovar *typhimurium* as well as adjuvanted vaccines. Those three very different models enabled us assessing the effects of OX40 deletion on 2W1S-specific effector CD4 T cells in systemic and acute intestinal Th1 responses as well as responses induced by non-live immunostimulants.

3.2 Results

3.2.1 IFNγ producing effector CD4 T cells generated in response to Listeria monocytogenes-2W1S are OX40 dependent

Early studies have established the essential role of the OX40 pathway in the formation of robust primary CD4 T cell responses (Gaspal et al., 2005; Rogers et al., 2001). One such study has shown that primary responses to antigen pigeon cytochrome *c,* in particular the effector cell proliferation and functions were highly dependent on OX40 signals (I Gramaglia et al., 2000). However, many of the previous research investigating the antigen-specific CD4 T cells responses used TCR transgenic models and did not explored the endogenous T cell populations. As monoclonal populations, having no variations in their clonal expansion, TCR transgenic T cells are limiting as they distort the real representation of the endogenous *in vivo* responses. Moreover, the experimental design of the TCR transgenic models often involves a transfer of a considerably large numbers of TCR transgenic T cells, creating not only a competitive environment but also further altering the effector responses due to the large precursor populations (Ford et al., 2007; Hataye et al., 2006; Kearney et al., 1994).

As previously established in our laboratory, we have used a more refined method of tracking the antigen specific CD4 T cell responses. This method was optimised by the Jenkins laboratory, where they used MHCII Tetramers to an immunogenic peptide 2W1S which has been previously shown to be recognised by a large endogenous population of naïve CD4 T cells

The immunogenic potential of the 2W1S peptide was utilised by creating an attenuated strain of *Listeria monocytogenes* with a mutation in the actA gene preventing bacterial motility, that was engineered to secrete an OVA-2W1S conjugate protein under the control of *hly* promoter (Ertelt et al., 2009; Portnoy et al., 2002). Infection with an attenuated *Listeria monocytogenes*-2W1S (Lm-2W1S) strain has been extensively studied and has been characterised by a robust 2W1S specific CD4 T cell response expressing Th1 phenotype (Pepper et al., 2011, 2010).

The initial studies in our laboratory have shown the significance of OX40 signals in establishing the antigen specific CD4 T cells responses to Lm-2W1S. In particular, previously published data on Lm-2W1S induced memory CD4 T cell responses have determined an important role for OX40 pathway, specifically in generating 2W1S effector memory T cell populations (Marriott et al., 2014). Interestingly, in the absence of OX40 signals, generation of T follicular helper cells occurred without any complications. These results provided useful insight into the costimulatory requirements of 2W1S specific CD4 T cells in producing efficient and long-term responses to *Listeria* infections.

We therefore aimed to build on this existing data by investigating the primary CD4 T cell responses to Lm-2W1S in the absence or presence of OX40 signals using IFNγ reporter mice to better asses the functional requirements of CD4 T cells. Furthermore, we also focused on expanding the investigation to include other infection models to determine if OX40 signals could have the same impact across different Th1 responses and tissues.

Although CD30 and OX40 clearly control the outcome of CD4 T cell responses, understanding their effect within an endogenous response with survival versus function carefully dissected has not been done, but is crucial to really understanding exactly what these signals do.

We began our investigations into the requirements for OX40 signals in establishing efficient CD4 T cell responses to Lm-2W1S focusing on the specific role of these signals in the function of CD4 T cells defined as their ability to make IFNγ. For these analyses we generated mice deficient in both CD30 and OX40 ($CD30^{-/-}$ x $OX40^{-/-}$) and crossed with Great x Smart17A dual IFNγ and IL-17A reporter (GS) mice (Price et al., 2012). The two costimulatory pathways for CD30 and OX40 have previously been shown to have possible redundancy in their expression, showing a synergistic effect on the survival of Th2 cells in responses to alum-precipitated NP-chicken gammaglobulin, thus we decided initially to use the CD30 and OX40 double knockout mice (Gaspal et al., 2005).

The use of GS mice allowed for accurate and unmanipulated assessment of the impact of CD30 and OX40 ablation on the IFNγ expression following the infection as well as cell differentiation and survival. The GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice alongside WT controls were infected with Lm-2W1S and the responses assessed in the spleen at the peak of the response, at 7 days post infection (dpi). WT mice were used as negative controls for the eYFP expression. The analysis of 2W1S specific CD4 T cells, identified as lin^- ($B220^-$ $CD11b^-$ $CD11c^-$), $CD3^+$, $CD4^+$, $CD44^{hi}$, revealed a robust population of $CD44^{hi}$ 2W1S-specifc CD4 T cells characterised with the use of 2W1S tetramer (Figure 3.1 A).

To distinguish between the effector and Tfh populations of 2W1S-specific CD4 T cells, we assessed the expression of eYFP and CXCR5 which were previously identified to be dominant in the two populations respectively (Pepper et al., 2011). The gates were set using infected WT controls which were then applied to identify population of interests in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.1 B). Our analyses showed that absence of CD30 and OX40 resulted in modest reduction (approximate two-fold) in total number of 2W1S-specific CD4 T cells (Figure 3.1 C). However, the most prominent contribution of OX40 signals was observed when assessing the eYFP expression, where the numbers and percentages of effector T cells, identified as $eYFP^+CXCR5^-$, were highly reduced in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.1 B, D, E). The reduction in numbers was approximately 8.7-fold comparing to the GS mice. Interestingly, the numbers of Tfh $CXCR5^+$ cells were not affected by the lack of CD30 and OX40 signals (Figure 3.1 F), consistent with the previously published data on the OX40 independent Tfh responses (Marriott et al., 2014).

Due to the eYFP reporter expression being relatively low, we next sought to assess Th1 effector responses using another method, namely assessment of the T-bet expression which was previously identified as transcription factor required for efficient Th1 responses and IFNγ production (Lugo-Villarino et al., 2003).

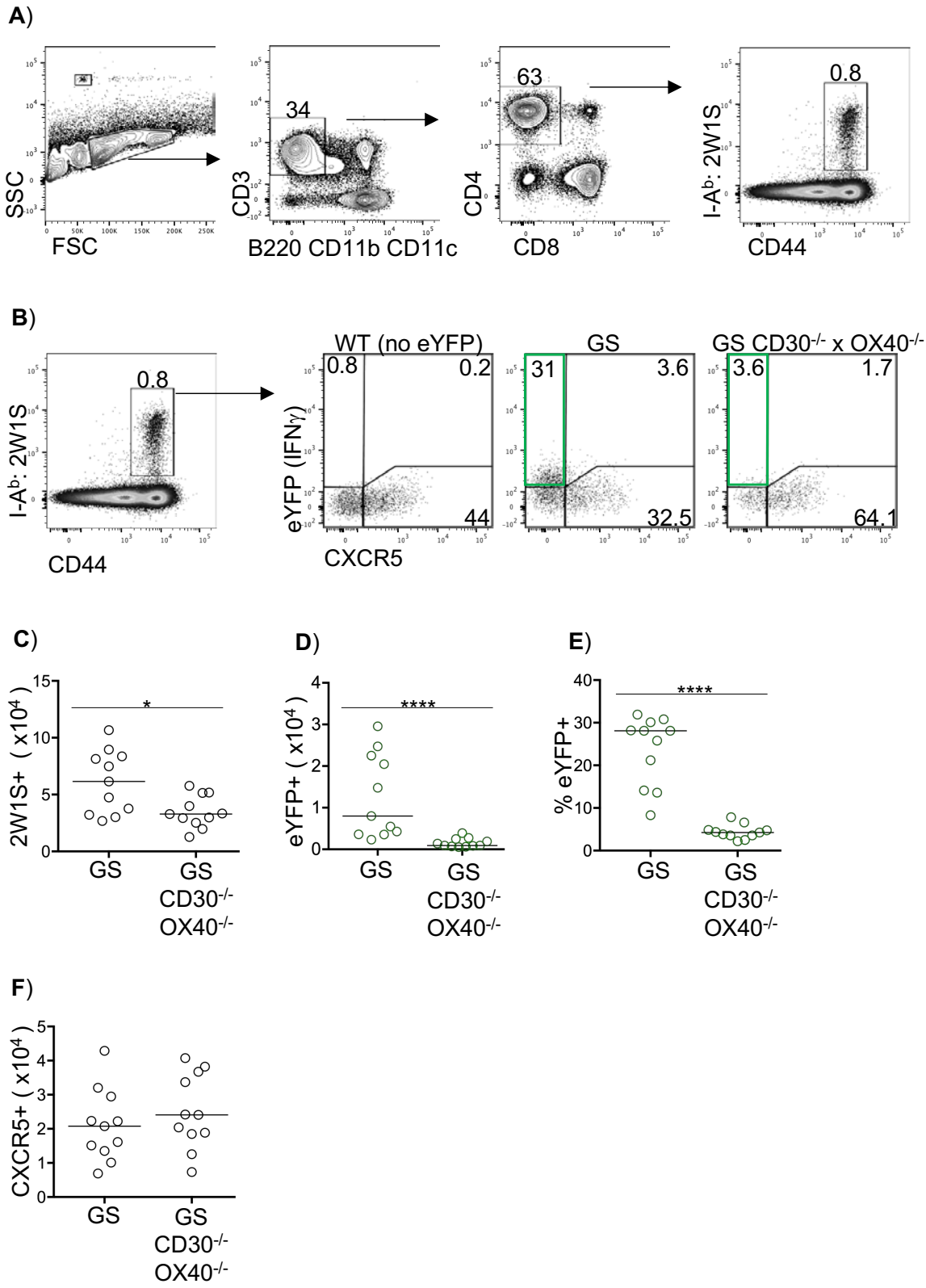

A)
SSC
FSC
34
CD3
B220 CD11b CD11c
63
CD4
CD8
0.8
I-Ab: 2W1S
CD44
B)
0.8
I-Ab: 2W1S
CD44
WT (no eYFP)
GS
GS CD30-/- x OX40-/-
0.8
0.2
44
31
3.6
32.5
3.6
1.7
64.1
eYFP (IFNγ)
CXCR5
C)
2W1S+ (x10^4)
15
10
5
0
*
GS
GS CD30-/- OX40-/-
D)
eYFP+ (x10^4)
4
3
2
1
0
****
GS
GS CD30-/- OX40-/-
E)
% eYFP+
40
30
20
10
0
****
GS
GS CD30-/- OX40-/-
F)
CXCR5+ (x10^4)
5
4
3
2
1
0
GS
GS CD30-/- OX40-/-

Figure 3. 1 Mice deficient in CD30 and OX40 show a dramatic decrease in reporting of IFNγ production

To determine the requirement for CD30 and OX40 on CD4 T cell effector function without *ex vivo* manipulation, dual IFNγ (eYFP) and IL-17A reporter mice (Great x Smart17A, GS) sufficient or deficient in CD30 and OX40 (GS $CD30^{-/-}$ x $OX40^{-/-}$) were infected i.v. with Lm-2W1S and the splenic response analysed at 7 days post infection. (**A, B**) Gating strategy showing identification of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) and their expression of CXCR5 and eYFP in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice. Gated on WT cells (lacking any eYFP) used to establish eYFP gating. (**C**) Enumeration of 2W1S-specific CD4 T cells. (**D**) Enumeration of $eYFP^{+}$ 2W1S-specific CD4 T cells. (**E**) Percentage of 2W1S-specific CD4 T cells expressing eYFP. (**F**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from three independent experiments, n=11. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

These analyses ensured that all effector cells were identified regardless of their functional abilities. The 2W1S specific CD4 T cells were identified as previously [lin^-($B220^-CD11b^-$ $CD11c^-$), $CD3^+$, $CD4^+$, $CD44^{hi}$] and their expression of T-bet assessed against the expression of CXCR5 in both GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.2 A, B). Numbers of effector T cells characterised as $Tbet^+CXCR5^-$ and their percentages were significantly reduced (approximately 2.1-fold) in the absence of CD30 and OX40 signals (Figure 3.2 C, D). Similarly, to previous data the numbers of Tfh cells, $T\text{-}bet^-CXCR5^+$, where unaffected in none of the mouse strains assessed, again supporting the nonessential role of OX40 signals in Tfh formation (Figure 3.2 E).

We aimed to determine whether the absence of IFNγ-producing Th1 cells at 7 dpi, identified in Lm-2W1S as peak of the response, was due to the reduced survival of the effector T cells. We therefore performed identical assessment of the 2W1S-specific CD4 T cell responses at 4 dpi in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice, looking at both the ability to make IFNγ and expression of T-bet.

Consistently with the analyses conducted at 7 dpi, also in this case the number of 2W1S-specific CD4 T cell response was significantly impaired in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.3 B). Furthermore, the numbers and percentages of $eYFP^+$ 2W1S-specific CD4 T cells were heavily reduced in the absence of CD30 and OX40 signals, as oppose to no difference in the number of $CXCR5^+$ 2W1S specific T cells (Figure 3.3 A, C, D, E). This data was further supported by the considerable reduction of T-bet expression on 2W1S-specific effector CD4 T cells but not the CXCR5 expression on Tfh population in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.3 F, G, H, I). Altogether, these results have highlighted the crucial role of CD30 and OX40 signals in primary responses to Lm-2W1S, being particularly important for the function and to a lesser extend the number of Th1 effector CD4 T cells. Majority of research has concluded the role of OX40 to be specifically important for the survival of T cells and memory responses (Byun et al., 2013; Gaspal et al., 2005; I. Gramaglia et al., 2000).

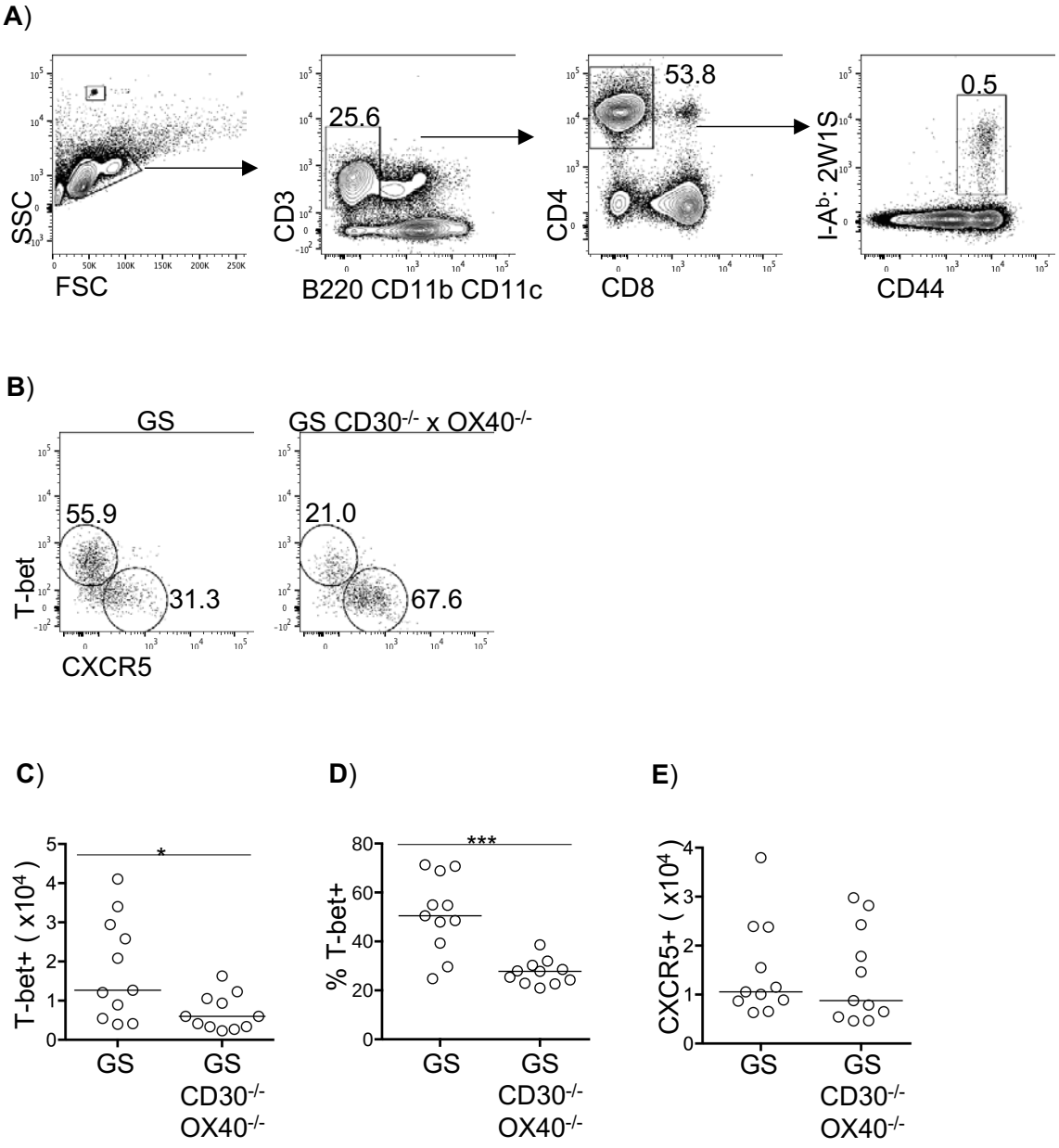

A)
SSC
FSC
CD3
B220 CD11b CD11c
25.6
CD4
CD8
53.8
I-Ab: 2W1S
CD44
0.5
B)
GS
GS CD30-/- x OX40-/-
T-bet
CXCR5
55.9
31.3
21.0
67.6
C)
T-bet+ (x10^4)
*
GS
GS CD30-/- OX40-/-
D)
% T-bet+
***
GS
GS CD30-/- OX40-/-
E)
CXCR5+ (x10^4)
GS
GS CD30-/- OX40-/-

Figure 3. 2 Generation of Th1 effector T cells, but not CXCR5^{+} populations is highly dependent on CD30 and OX40 signals

To test the role of CD30 and OX40 signals in the generation of Th1 effector CD4 T cells, the GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ mice were infected i.v. with Lm-2W1S and the responses assessed at 7 days post infection. (**A, B**) Gating strategy showing identification of 2W1S-specific CD44^{hi} CD4 T cells (CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}) and their expression of CXCR5 and T-bet in GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ mice. (**C**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**D**) Percentage of 2W1S-specific CD4 T cells expressing T-bet. (**E**) Enumeration of CXCR5^{+} 2W1S-specific CD4 T cells. Data are pooled from three independent experiments, n=11. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

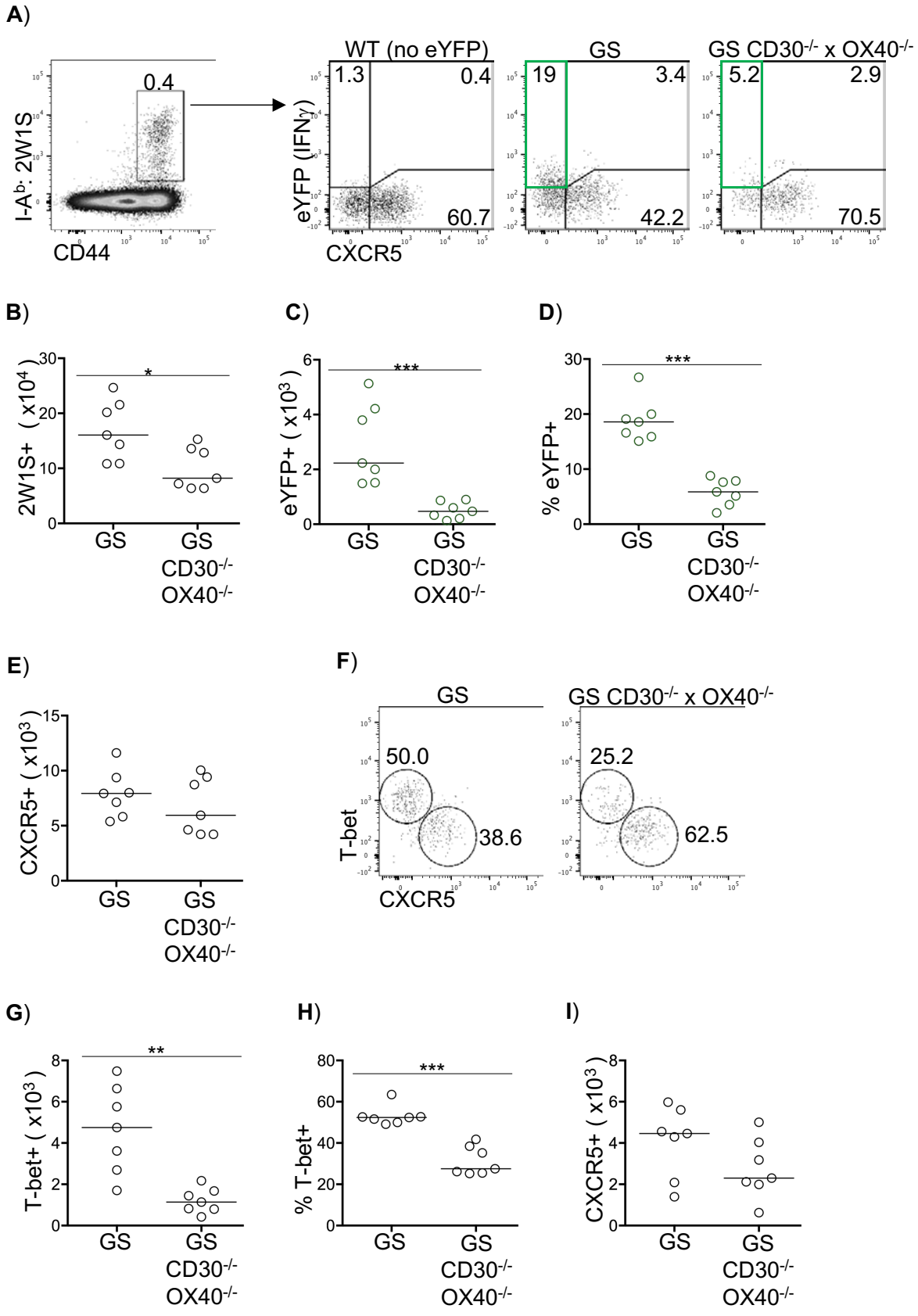

A)
0.4
I-Ab: 2W1S
CD44
WT (no eYFP)
GS
GS CD30-/- x OX40-/-
1.3
0.4
60.7
19
3.4
42.2
5.2
2.9
70.5
eYFP (IFNγ)
CXCR5
B)
2W1S+ (x10^4)
*
C)
eYFP+ (x10^3)
***
D)
% eYFP+
***
GS
GS CD30-/- OX40-/-
E)
CXCR5+ (x10^3)
F)
GS
GS CD30-/- x OX40-/-
50.0
38.6
25.2
62.5
T-bet
CXCR5
G)
T-bet+ (x10^3)
**
H)
% T-bet+
***
I)
CXCR5+ (x10^3)

Figure 3. 3 Requirements for OX40 signals in generation of effector T cells and their function are evident early in the response

To determine the requirement for CD30 and OX40 on CD4 T cell effector function without *ex vivo* manipulation, GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ were infected i.v. with Lm-2W1S and the splenic response analysed at 4 days post infection. (**A**) Gating strategy showing identification of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) and their expression of CXCR5 and eYFP in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice. Gated on WT cells (lacking any eYFP) used to establish eYFP gating. (**B**) Enumeration of 2W1S-specific CD4 T cells. (**C**) Enumeration of $eYFP^{+}$ 2W1S-specific CD4 T cells. (**D**) Percentage of 2W1S-specific CD4 T cells expressing eYFP. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. (**F**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**G**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**H**) Percentage of T-bet^{+} 2W1S-specific CD4 T cells. (**I**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from two independent experiments, n=7. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

Here we successfully showed that responses were diminished from the very early stages and were maintained throughout, thereby linking the requirements of OX40 to the function of T cells rather than only to the expansion and survival.

3.2.2 OX40 signals play the dominant role in generating functional effector CD4 T cells

Having established the importance of OX40 and CD30 signalling pathways for effector T cell responses, we sought to determine if OX40 was indeed the dominant signal as previously indicated (Bekiaris et al., 2009b; Gaspal et al., 2011).

For these analyses we firstly focused on comparing the effector T cells in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice by the means of the T-bet and CXCR5 expression which were used as determinants of the effector and Tfh populations respectively (Figure 3.4 A). We observed significant reduction in the 2W1S-specific CD4 T cell response in spleens of infected $CD30^{-/-}$ x $OX40^{-/-}$ mice as compared to WT and $OX40^{-/-}$ mice (Figure 3.4 B). These results were consistent with the previously described 2W1S-specific CD4 T cells responses in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice reporter mice.

Although, $OX40^{-/-}$ mice showed minimal but not statistically significant defect in the total 2W1S response, the analyses of their 2W1S-specific CD4 effector T cells evidently indicated the requirement for OX40 signals represented by approximately 3-fold reduction in the number of T-bet expressing 2W1S-specific T cells (Figure 3.4 C). Similar defect in the effector T cell responses was also observed in the $CD30^{-/-}$ x $OX40^{-/-}$ mice, approximately 4.5 reduction when compared to the WT control mice (Figure 3.4 C), demonstrating the effector T cell responses to be in larger part dependent on OX40 rather than the CD30 signals. Significant defect in the total numbers of T-bet expressing cells were further supported by similar statistically verified reduction in percentage of 2W1S-specific CD4 T cells expressing T-bet at 7dpi (Figure 3.4 D). On this occasion we found the numbers of Tfh, T-$bet^{-}CXCR5^{+}$ population to be significantly decreased in both $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.4 E).

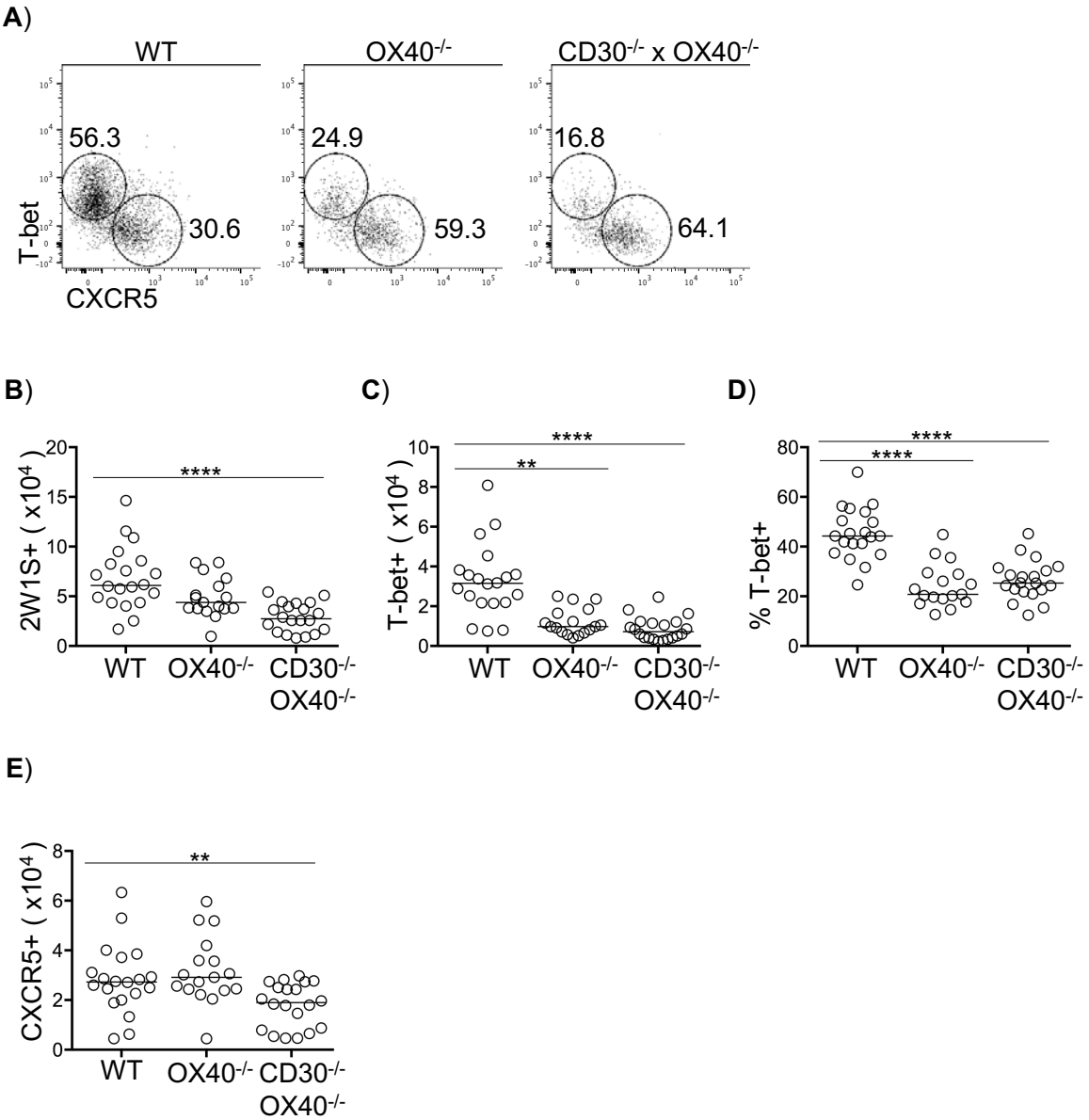

A)
WT
OX40-/-
CD30-/- x OX40-/-
56.3
30.6
24.9
59.3
16.8
64.1
T-bet
CXCR5
B)
2W1S+ (x10^4)
****
WT
OX40-/-
CD30-/- OX40-/-
C)
T-bet+ (x10^4)
****
**
WT
OX40-/-
CD30-/- OX40-/-
D)
% T-bet+
****
****
WT
OX40-/-
CD30-/- OX40-/-
E)
CXCR5+ (x10^4)
**
WT
OX40-/-
CD30-/- OX40-/-

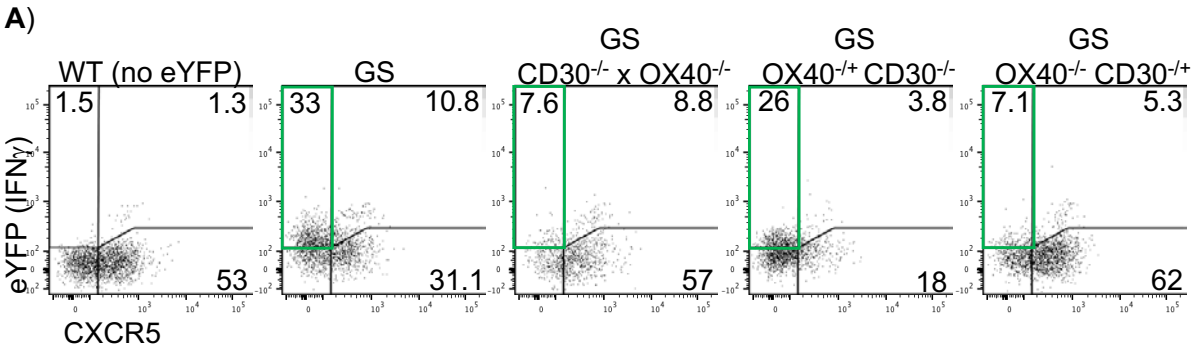
Figure 3. 4 Generation of effector T cells is dependent primarily on OX40:OX40L interactions in response to Lm-2W1S

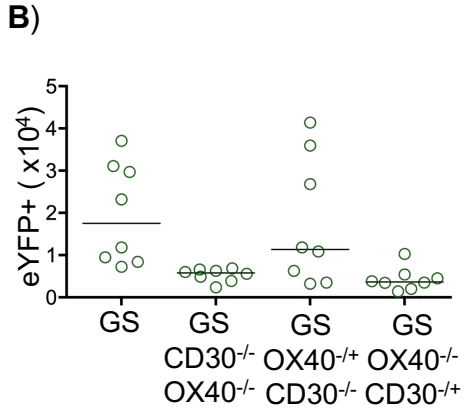
The specific contribution of OX40:OX40L interactions in the generation of Th1 effector CD4 T cells was determined in the response at 7 days post infection with Lm-2W1S. The 2W1S effector T cell response was assessed in WT, $OX40^{-/-}$ versus $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**B**) Enumeration of 2W1S-specific CD4 T cells. (**C**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**D**) Percentage of T-bet^{+} 2W1S-specific CD4 T cells. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from four independent experiments, n=20 WT, n=17 OX40KO, n=20 dKO. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

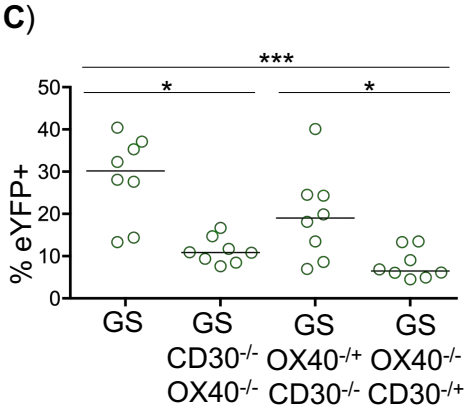
To further investigate the dominant role of OX40 in establishing effector T cell, particularly in their functional capabilities, we generated GS reporter mice that were deficient in either CD30 or OX40. The obtained GS $OX40^{-/-}$ $CD30^{-/+}$ (sufficient in CD30 but lacking OX40 signals) and GS $OX40^{-/+}$ $CD30^{-/-}$ (sufficient in OX40 but lacking CD30 signals) were infected with Lm-2W1S and the responses assessed in spleens at 7 dpi. Similarly, to the previous data, we found that approximately 30% of 2W1S-specific CD4 T cells in GS mice expressed eYFP (proportional to IFNγ) at 7 dpi (Figure 3.5 A). Comparisons in the single knockout mice revealed once again a OX40 dependent formation of effector T cell population shown by the significant reduction in the numbers and percentages of $eYFP^{+}$ cells in both GS $CD30^{-/-}$ x $OX40^{-/-}$ and GS $OX40^{-/-}$ $CD30^{-/+}$ mice as opposed to the GS control and GS $OX40^{-/+}$ $CD30^{-/-}$ mice (Figure 3.5 A, B, C). No significant differences were found in the expression of CXCR5, defining the Tfh population to be OX40 independent (Figure 3.5 D).

Moreover, analyses of T-bet expression in infected GS mice further indicated the OX40 specific, rather than CD30, generation of effector T cells post infection with Lm-2W1S (Figure 3.6 A). In comparison to the GS control mice, the enumeration and percentages of T-bet expressing cells were evidently reduced in the absence of OX40 signals in both GS $CD30^{-/-}$ x $OX40^{-/-}$ and GS $OX40^{-/-}$ $CD30^{-/+}$, while utterly unaffected in GS $OX40^{-/+}$ $CD30^{-/-}$ mice (Figure 3.6 A, B, C). Typically, the Tfh responses were not influenced by the differences the provision of OX40 and CD30 signals.

Together these data demonstrate the dominant nature of OX40 signals and their crucial requirement for generating primary Th1 response in Lm-2W1S induced infection, with the signals via the CD30 pathway being redundant.


A)
WT (no eYFP)
GS
GS
CD30-/- x OX40-/-
GS
OX40-/+ CD30-/-
GS
OX40-/- CD30-/+
1.5
1.3
53
33
10.8
31.1
7.6
8.8
57
26
3.8
18
7.1
5.3
62
eYFP (IFNγ)
CXCR5


B)
eYFP+ (x10^4)
GS
GS CD30-/- OX40-/-
GS OX40-/+ CD30-/-
GS OX40-/- CD30-/+


C)
***
*
*
% eYFP+
GS
GS CD30-/- OX40-/-
GS OX40-/+ CD30-/-
GS OX40-/- CD30-/+

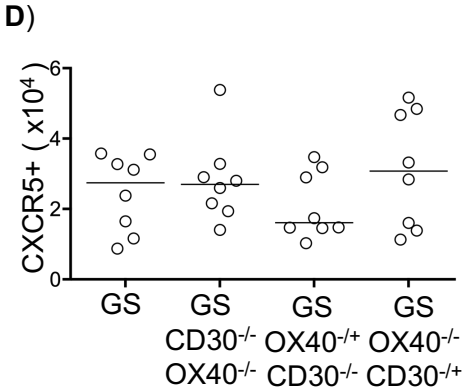

D)
CXCR5+ (x10^4)
GS
GS CD30-/- OX40-/-
GS OX40-/+ CD30-/-
GS OX40-/- CD30-/+

Figure 3. 5 OX40 rather than CD30 is critical for the CD4 effector T cell response to infection with Lm-2W1S

To dissect the individual requirement for CD30 versus OX40 in the production of IFNγ by effector CD4 T cells, GS $CD30^{-/-}$ x $OX40^{-/-}$ mice were crossed with $CD30^{-/-}$ or $OX40^{-/-}$ mice to generate GS mice with only a single functional copy of OX40 (GS $CD30^{-/-}$ x $OX40^{+/-}$) or CD30 (GS $CD30^{+/-}$ x $OX40^{-/-}$). The 2W1S-specific CD4 T cell response was assessed at 7 days post infection with Lm-2W1S. (**A**) Expression of CXCR5 and eYFP; on 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in WT (lacking any eYFP), GS, GS $CD30^{-/-}$ x $OX40^{-/-}$, GS $CD30^{-/-}$ x $OX40^{+/-}$ and GS $CD30^{+/-}$ x $OX40^{-/-}$ mice. (**B**) Enumeration of 2W1S-specific CD4 T cells expressing eYFP **(C)** Percentage of 2W1S-specific CD4 T cells expressing eYFP. (**D**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from three independent experiments, n=8. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

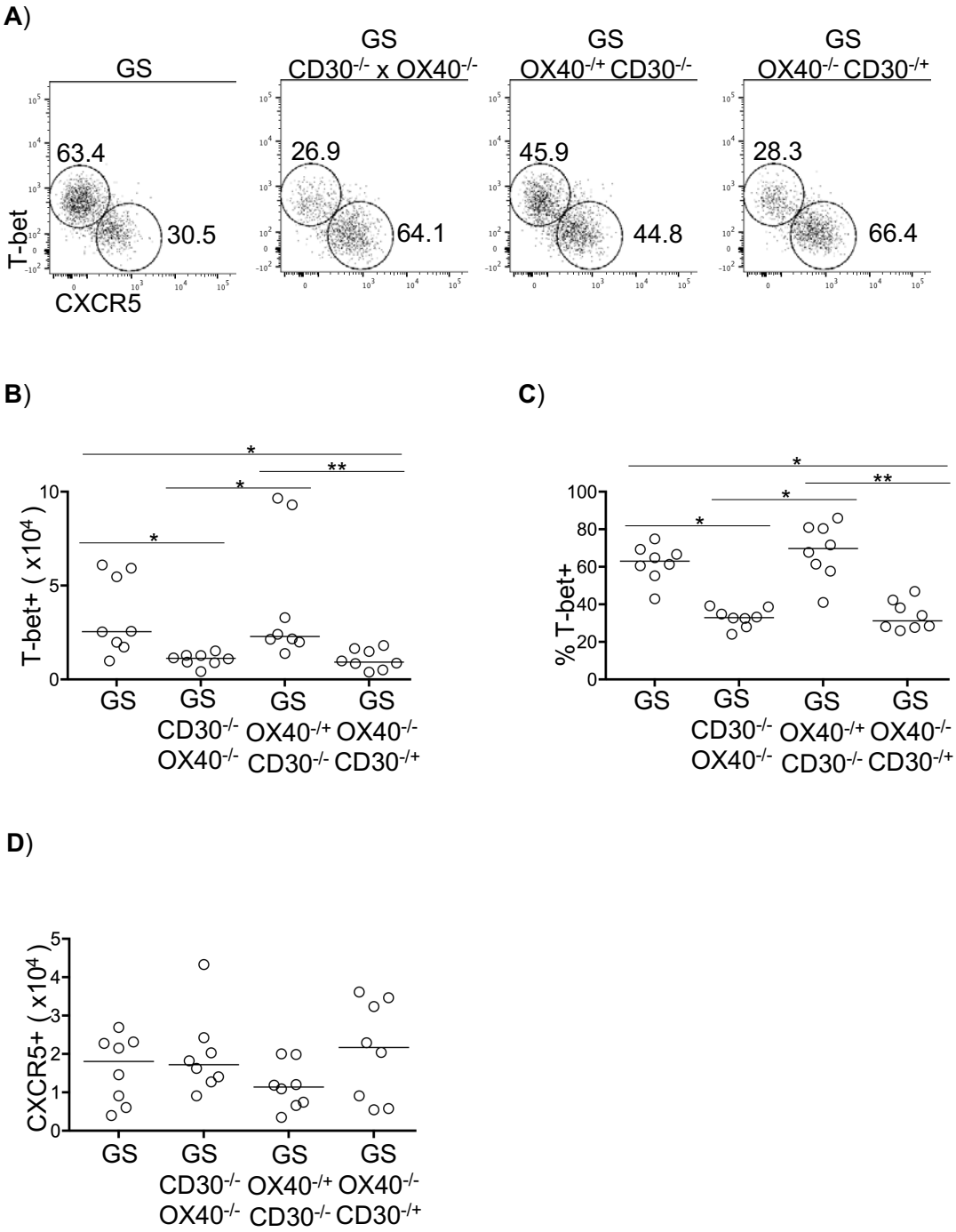

A)
GS
GS CD30-/- x OX40-/-
GS OX40-/+ CD30-/-
GS OX40-/- CD30-/+
63.4
30.5
26.9
64.1
45.9
44.8
28.3
66.4
T-bet
CXCR5
B)
T-bet+ (x10^4)
C)
% T-bet+
D)
CXCR5+ (x10^4)
GS
GS CD30-/- OX40-/-
GS OX40-/+ CD30-/-
GS OX40-/- CD30-/+

Figure 3. 6 Dominant role of OX40 in generation of CD4 effector T cells

To dissect the individual requirement for CD30 versus OX40 in the generation of Th1 effector CD4 T cells, GS, GS $CD30^{-/-}$ x $OX40^{-/-}$, GS $CD30^{-/-}$ x $OX40^{+/-}$ and GS $CD30^{+/-}$ x $OX40^{-/-}$ mice were infected i.v. with Lm-2W1S and responses were assessed at day 7 post infection. (**A**) Expression of T-bet versus CXCR5 on 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$); (**B**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**C**) Percentage of T-bet^{+}2W1S-specific CD4 T. (**D**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from three independent experiments, n=8. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

3.2.3 The requirement for OX40 signals persists throughout the response

Previous reports concluded the role of OX40 to be specifically important for the survival of T cells and memory responses (Byun et al., 2013; Gaspal et al., 2005; I. Gramaglia et al., 2000). Having established that T-bet expression could be used to track effector T cells responses to Lm-2W1S and most importantly was reflecting the OX40 requirement of 2W1S-specific CD4 T cells to generate efficient immune responses, we aimed to investigate further time points in the infection to determine whether the defective primary responses could persists later on, having a particular impact on the memory responses upon challenge.

We firstly assessed responses at 14 dpi in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice infected with Lm-2W1S and found no significant difference in the 2W1S-specific CD4 T cell response in all infected strains (Figure 3.7 A, B). However, despite the initial observations, analyses of T-bet versus CXCR5 expression identified a persisting defect in the effector T cell population in mice that lack both OX40 and CD30 signals. Both the total numbers and percentages of effector T cells were highly reduced in both $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice, with minimal changes observed in Tfh $CXCR5^{+}$ populations (Figure 3.7 C, D, E). This once more has indicated a dominant role of OX40 signals, as the defect in effector T cells in $CD30^{-/-}$ x $OX40^{-/-}$ replicated the results obtained in straight $OX40^{-/-}$ mice.

We next wanted to determine whether requirements for OX40 signals were also crucial in generating effector T cells responses upon secondary exposure to the antigen. For this we infected WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice at day 0 and then administered another dose of the Lm-2W1S at 42 dpi. The mice were then left for 4 days to enable for secondary expansion of 2W1S-specific CD4 T cells and were immunised again with 2W1S peptide adjuvanted with LPS to induce IFNγ production, a method previously described (Marriott et al., 2014). The responses were assessed 4 hrs later. The enumeration of 2W1S-specific CD4 T cells showed no significant differences between the mice comparable to the results observed at 14 dpi (Figure 3.8 B). Subsequent analyses of the effector functions revealed

clear dependency on the availability of OX40 signals at the time of secondary challenge. First of all, we observed a significant reduction in the number of T-bet expressing effector T cells in $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.8 A, C). The number of Tfh cells was again unaffected by the lack of OX40 and CD30 signals (Figure 3.8 D).

The most notable results, defining the crucial role of OX40 signals in memory responses upon challenge emerged from the assessment of functional capabilities of the 2W1S-specific CD4 T cells, namely their ability to produce IFNγ. We compared the IFNγ versus IL-2 production following the 2W1S peptide *in vivo* restimulation in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.8 E). The results showed a significant impairment in the production of IFNγ in the absence of both OX40 and CD30 signals as represented by the reduction in total numbers as well as percentages of IFNγ expressing 2W1S-specific CD4 T cells (Figure 3.8 F, G).

Concluding, our analyses successfully demonstrated an OX40 dependent generation of 2W1S specific CD4 effector T cell responses to Lm-2W1S which were diminished from the very early stages and maintained throughout the response. In particular, we were able to link the requirements for OX40 to the function of CD4 T cells and not just their survival as previously reported.

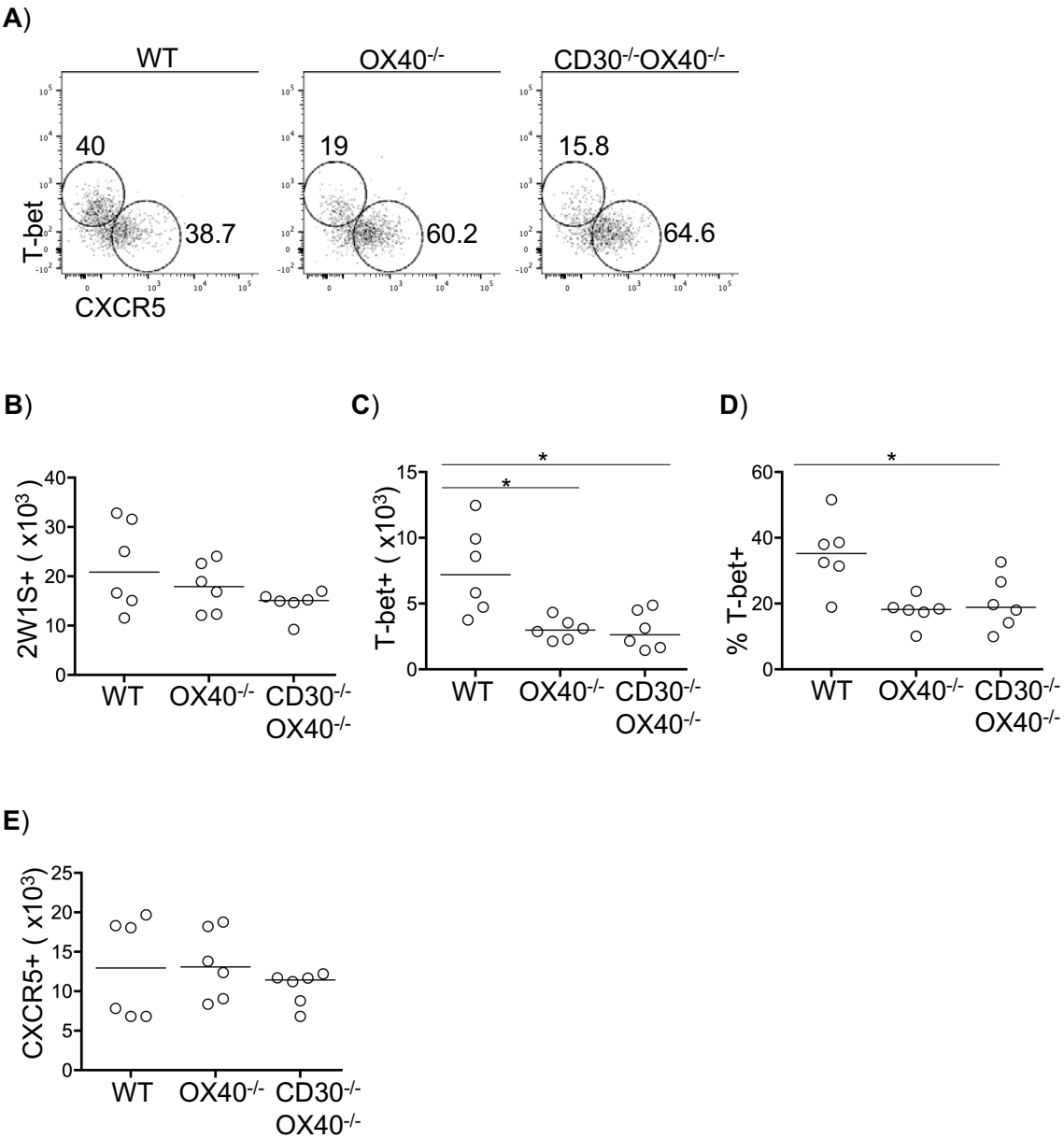

A)
WT
OX40-/-
CD30-/-OX40-/-
40
38.7
19
60.2
15.8
64.6
T-bet
CXCR5
B)
2W1S+ (x10³)
C)
T-bet+ (x10³)
D)
% T-bet+
WT
OX40-/-
CD30-/-
OX40-/-
E)
CXCR5+ (x10³)

Figure 3. 7 The defect in the generation of Th1 effector T cells is present later in the response

The effect of OX40:OX40L interactions in the generation and survival of antigen-specific Th1 effector CD4 T cells was determined at 14 days post infection with Lm-2W1S. The 2W1S effector T cell response was assessed in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**B**) Enumeration of 2W1S-specific CD4 T cells. (**C**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**D**) Percentage of T-bet^{+} 2W1S-specific CD4 T cells. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data are pooled from two independent experiments, n=6. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

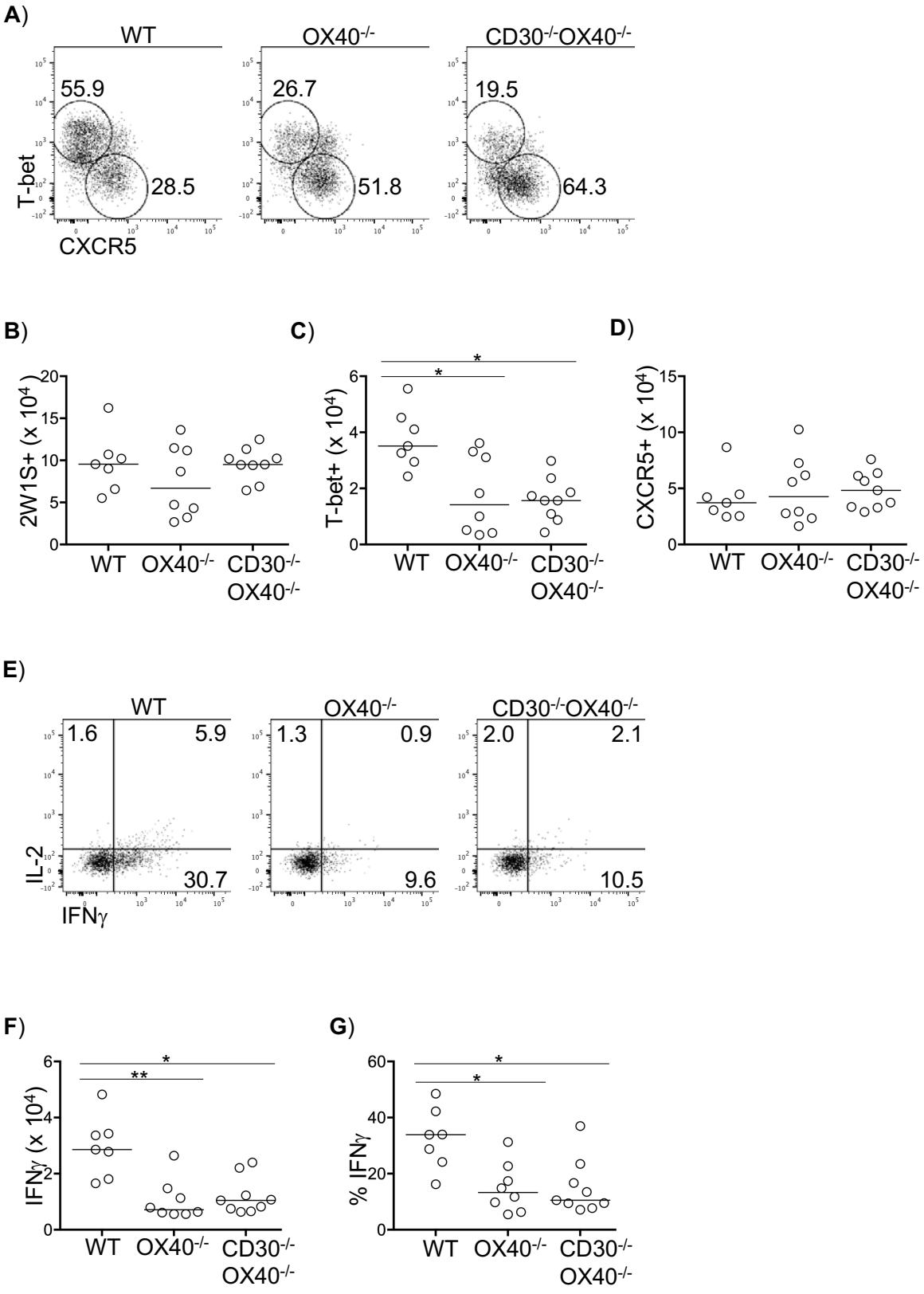

A)
WT
OX40-/-
CD30-/-OX40-/-
55.9
28.5
26.7
51.8
19.5
64.3
T-bet
CXCR5
B)
2W1S+ (x 10^4)
C)
T-bet+ (x 10^4)
D)
CXCR5+ (x 10^4)
WT
OX40-/-
CD30-/-
OX40-/-
E)
1.6
5.9
30.7
1.3
0.9
9.6
2.0
2.1
10.5
IL-2
IFNγ
F)
IFNγ (x 10^4)
G)
% IFNγ

Figure 3. 8 The defect in the Th1 effector CD4 T cell numbers and function persist throughout the Lm-2W1S response

The contribution of OX40:OX40L interactions to the survival of antigen-specific Th1 effector CD4 T cells at challenge responses to Lm-2W1S. The mice were infected i.v. with Lm-2W1S, followed by administration of a second dose of Lm-2W1S at day 42. At day 46 the mice received 2W1S peptide adjuvanted with LPS via i.v. route and were sacrificed 4 hrs later. The 2W1S effector T cell response was assessed in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**B**) Enumeration of 2W1S-specific CD4 T cells. (**C**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**D**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. (**E**) Expression of IL-2 versus IFNγ^{+} by 2W1S-specific $CD44^{hi}$ CD4 T cells in WT, $OX40^{-/-}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**F**) Enumeration of IFNγ^{+} 2W1S-specific CD4 T cells. (**G**) Percentage of IFNγ^{+} 2W1S-specific CD4 T cells. Data are pooled from two independent experiments, WT n=7, $OX40^{-/-}$ n=8, $CD30^{-/-}$ x $OX40^{-/-}$ n=9. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

3.2.4 Vaccine adjuvant AS01 requires OX40 pathway to promote efficient CD4 T cells responses to OVA-2W1S

Costimulation plays a pivotal role in T cell biology, providing essential secondary signals which trigger activation and subsequent proliferation of antigen-specific T cells. What cells provide these signals and when is crucial for the development of new therapeutic approaches to better manipulate these responses. Vaccination is our most successful prophylactic approach for combatting infectious diseases, however vaccines containing recombinant antigen require the use of adjuvant to improve their immunogenicity. How adjuvants affect innate populations and which mechanisms and costimulatory pathways are required for generating effective responses remain unclear.

Through an extensive collaboration with a British pharmaceutical company GlaxoSmithKline Biologicals (GSK), we were able to test one of the current adjuvant systems used in both Malaria and Zoster candidate vaccines and other vaccines in development (Didierlaurent et al., 2017). The Adjuvant System 01 (AS01) has been developed for over 20 years and has already proved to have some excellent immune enhancing qualities. It has been included in the Malaria vaccine RTS,S/AS01 which is used to induce long term protection against parasite *Plasmodium falciparum*. The design of the AS01 adjuvant improves the immune protection of this vaccine, however it has also been licensed for the use in Shingrix, vaccine used to prevent shingles caused by the varicella-zoster virus as a complication of past chickenpox infection, where it induced immunity in people over 50 years of age (Didierlaurent et al., 2017; Lal et al., 2015). Zoster vaccine has now been licensed and has been widely used in USA with now more than 10 million doses administered (personal communication, Arnaud Didierlaurent, Area Medical Director for Shingrix, GSK)

AS01 belongs to a group of liposomes-based adjuvants. Those consist of lipid bilayer surrounding the vaccine mixture which also ensures the stability of the antigen (Coccia et al., 2017). In AS01, however the liposome core carries the two immunostimulants: MPL and QS-

21 (licensed by GSK from Antigenics Inc., a wholly owned subsidiary of Agenus Inc., a Delaware, USA corporation).

Here we aimed to establish a model of intramuscular immunisation in mice to test the effects of AS01 adjuvant on antigen-specific CD4 T cell responses to OVA-2W1S and most importantly investigate the potential role of OX40 signals in the mechanisms used by the AS01 to elicit immune responses.

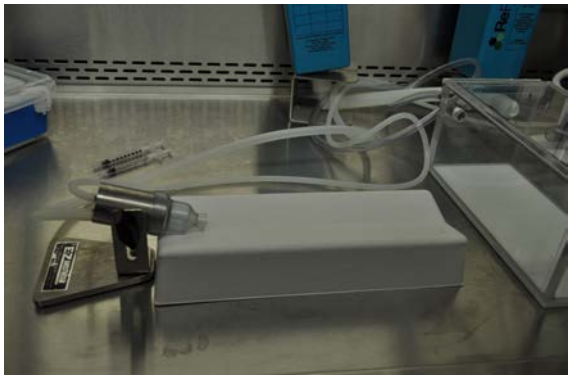
Given the predominance of intramuscular delivery for vaccines in humans, initial experiments aimed to validate an intramuscular injection model. The mice were first anaesthetised with Isoflurane according to the Home Office regulations (Figure 3.9 A). Following the initial exposure to anaesthesia, the mice were transferred onto a platform where they continued to receive anaesthetic via a mouthpiece and had their hind legs shaved to expose the gastrocnemius muscle (Figure 3.9 B, C). The OVA-2W1S/AS01 was administered in a volume of 10 μL using a syringe with specially cut lid allowing only 3 mm of the needle to be exposed. This ensured the uniformity of the immunisations.

To assess drainage from the injection site, injections of Evans blue dye into the gastrocnemius muscle of the hind leg were performed. The results showed a clear drainage into the iliac lymph node (LN), while the drainage to inguinal LN was absent (Figure 3.9 D).

After initial pilot experiments, the model was refined by injecting into the gastrocnemius muscle of both hind legs, enabling collection of both iliac and inguinal LNs and obtaining sufficient cell numbers. For the purpose of analysis, the iliac and inguinal LNs of each mouse were pooled. Rather than pure 2W1S peptide, 2W1S peptide conjugated to OVA was used to better model peptide frequencies amongst protein antigens. Finally, OVA-2W1S was given alone (in PBS) or with ASO1 to directly assess the contribution of the adjuvant. The mice were sacrificed at day 5 of the response, when a T cell response should have already been established.

A)

B)



C)

D)

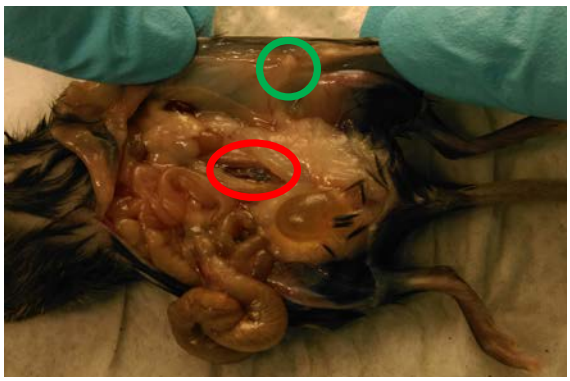


Figure 3. 9 AS01 model and its potential in tracking local responses

Intramuscular injection in C57Bl/6 mice. (**A-C**) Set up of intramuscular injections under local anaesthetic. Mice were placed in an anaesthetic chamber filed with Isoflurane. Once under anaesthetic, mice were transferred onto a platform where they continued receiving anaesthetic via breather. Both hind legs were shaved to remove the hair, enabling visualisation of the injection site. Following the assessment of the pedal reflex, the mice were immunised in both hind legs in the gastrocnemius muscle. The orange lid was cut and placed back onto the syringe to expose only 3mm of the needle, ensuring the position of the needle. (**D**) Lymphatic drainage into the iliac LN following the intramuscular injection of Evans blue dye into the gastrocnemius muscle.

We firstly carried out an assessment of the 2W1S-specific CD4 T cell responses in both iliac and inguinal draining LNs in which AS01 induced expansion of the 2W1S population as compared to the OVA-2W1S/PBS control mice (Figure 3.10 A). This was demonstrated further by statistically significant increase in the number of 2W1S-specific CD4 T cells within the draining LNs of mice immunised with OVA-2W1S/AS01 (Figure 3.10 B). Overall, immunisation with AS01 induced increase in the total number of CD4 T cells within the draining LNs with additional significant changes in their proportion in the iliac and minimal increase in the inguinal LNs (Figure 3.10 C, D).

Interestingly we have consistently observed greater response in the inguinal rather than the iliac LNs at day 5 post immunisation with OVA-2W1S/AS01 which didn't not correlate with our initial observations obtained in experiments with Evans blue dye. The initial action taken to understand this phenomenon involved testing the quality of the intramuscular injections as one of the suspected reasons included a possible subcutaneous administration of the antigen. This hypothesis was however rejected after the results for both subcutaneous and intramuscular injections did not show any significant differences (data not shown). The response in inguinal LN may result entirely from the lymphatic drainage pathway following the intramuscular injections. Despite the very limited literature on murine lymphatic drainage, there are data suggesting that both rear paw pad injections as well as injections to the base of the tail involve drainage to both inguinal and iliac LNs. In both of those cases however, there is a strong indication that the drainage might diverge and may not be necessarily sequential (Harrell et al., 2008).

Although TCR:pMHC complexes are crucial stimulants of T cell function, other signals are required for full activation of T cells. To fully understand the mechanisms behind adjuvant function it is important to dissect different stages on the immune response, including the requirement for costimulatory molecules. We therefore carried out additional analyses to investigate whether AS01 induced CD4 T cell responses 'obeyed' the requirements for crucial CD80 and CD86 costimulation.

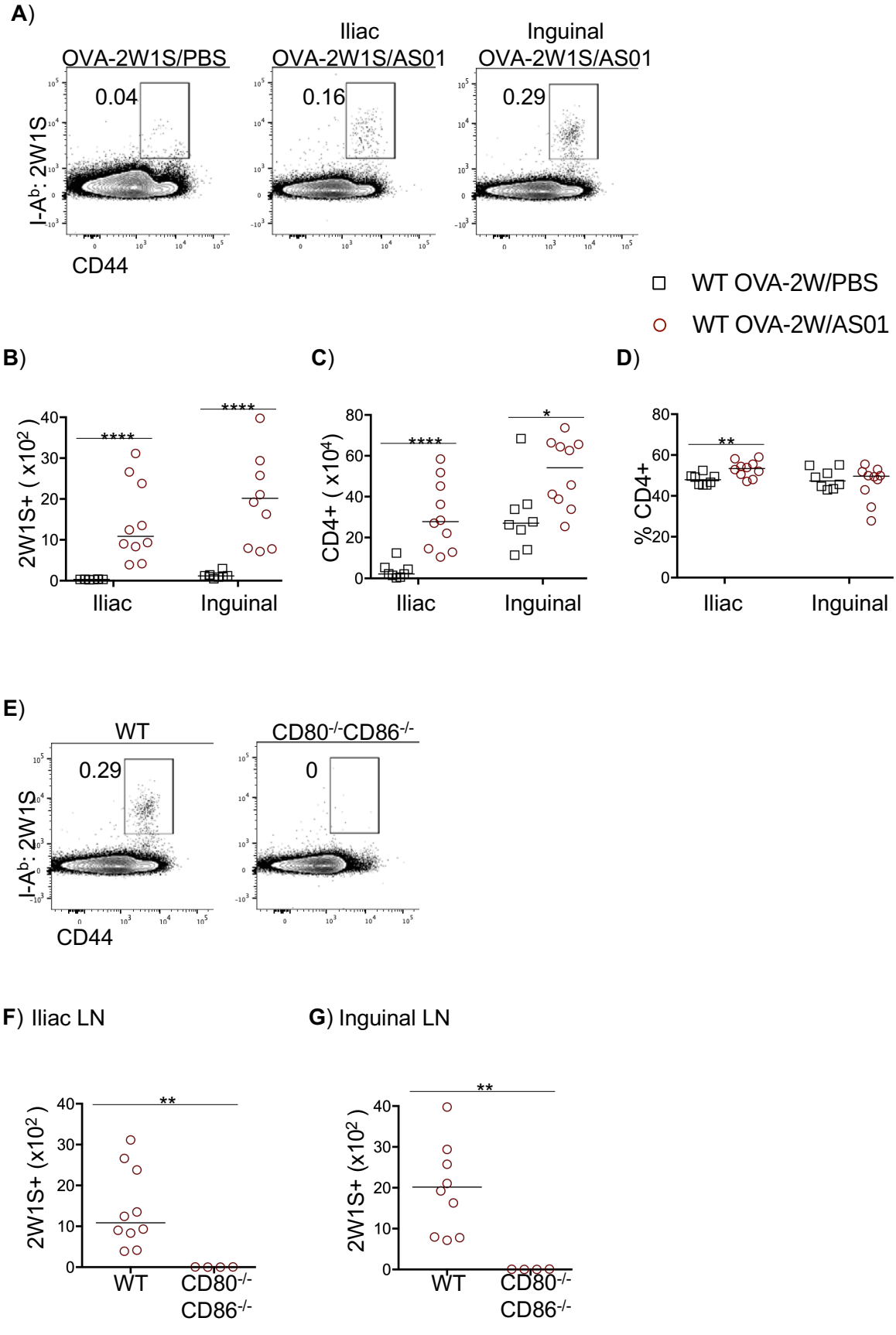

A)
OVA-2W1S/PBS
Iliac
OVA-2W1S/AS01
Inguinal
OVA-2W1S/AS01
0.04
0.16
0.29
I-Ab: 2W1S
CD44
WT OVA-2W/PBS
WT OVA-2W/AS01
B)
C)
D)
2W1S+ (x10²)
CD4+ (x10⁴)
% CD4+
****
*
**
Iliac
Inguinal
E)
WT
CD80-/-CD86-/-
0.29
0
F) Iliac LN
G) Inguinal LN
2W1S+ (x10²)
WT
CD80-/-
CD86-/-

Figure 3. 10 AS01 drives a robust CD4 T cell response to OVA-2W1S in draining LNs in a CD80 and CD86 dependent manner

Intramuscular injection in WT control and $CD80^{-/-}86^{-/-}$ mice. Mice were immunised with 20μg OVA-2W/PBS or 20μg OVA-2W/AS01 (1/50 HD) and sacrificed at D5 post immunisation. (**A**) Gating strategy showing identification of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in control mice (OVA-2W1S/PBS) and immunised mice (OVA-2W1S/AS01). (**B**) Enumeration of 2W1S-specific CD4 T cells in iliac and inguinal dLNs. **(C)** Enumeration of total CD4 T cells in iliac and inguinal dLNs. (**D**) Percentage of total CD4 T cells in iliac and inguinal dLNs. Data pooled from three independent experiments OVA-2W1S/PBS n=8, OVA-2W1S/AS01 n=10. (**E**) Gating strategy showing identification of 2W1S-specific $CD44^{hi}$ CD4 T cells in inguinal dLNs of immunised WT and $CD80^{-/-}86^{-/-}$ mice. Representative flow cytometry plots for iliac dLNs can be found in Appendix Figure 8.1 (**F**) Enumeration of 2W1S-specific CD4 T cells in iliac dLNs. (**G**) Enumeration of 2W1S-specific CD4 T cells in inguinal dLNs. Data pooled from two independent experiments WT n=10; $CD80^{-/-}86^{-/-}$ n=4. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

This requirement is well established for infection/T cell response in general but not yet for AS01, although previous reports have shown upregulation of those costimulatory molecules on APCs post immunisation with AS01 (Coccia et al., 2017). These investigations involved immunisation of WT and $CD80^{-/-}86^{-/-}$ knockout mice with OVA-2W1S/AS01 and assessment of the resulting 2W1S-specific CD4 T cells response. As anticipated, lack of those two crucial costimulatory molecules had significant effect on stimulation of CD4 T cells with the total ablation of the 2W1S responses in both iliac and inguinal LNs of $CD80^{-/-}86^{-/-}$ knockout mice as represented by severely reduced total numbers of 2W1S-specific CD4 T cells (Figure 3.10 E, F).

We then focused on another costimulatory pathway, namely the OX40L-OX40 signalling, given the key role played in many other CD4 T cell responses (M.-Y. Kim et al., 2005; Withers et al., 2012). The efficacy of vaccines is often measured by the induction of efficient effector and memory responses, hence we investigated the potential role of OX40 signalling, as previous studies have linked OX40 signals to the robust memory responses (Gaspal et al., 2005; Withers et al., 2011).

For these analyses we immunised WT and $OX40^{-/-}$ mice with OVA-2W1S/AS01 and assessed the antigen-specific CD4 T cell responses at day 7 in both iliac and inguinal draining LNs. First of all, we observed a significant reduction in the magnitude of the 2W1S-specific CD4 T cell response in iliac and inguinal LNs of $OX40^{-/-}$ mice which correlated strongly with our previously discussed Lm-2W1S data (Figure 3.11 A, B, C). The differences between responses in iliac and inguinal LNs were minimal. Secondly, again identically to the Lm-2W1S model, we performed analyses of the T-bet versus CXCR5 expression to determine whether lack of OX40 signals had any effect on the generation of effector CD4 T cells. Similarly, to the Lm-2W1S model, also these analyses demonstrated the promotion of effector CD4 T cell responses by AS01 to be OX40 dependent, as represented by highly significant decrease in the number and proportion of T-bet expressing 2W1S-specific CD4 T cells in both iliac and inguinal LNs (Figure 3.11 D, E).

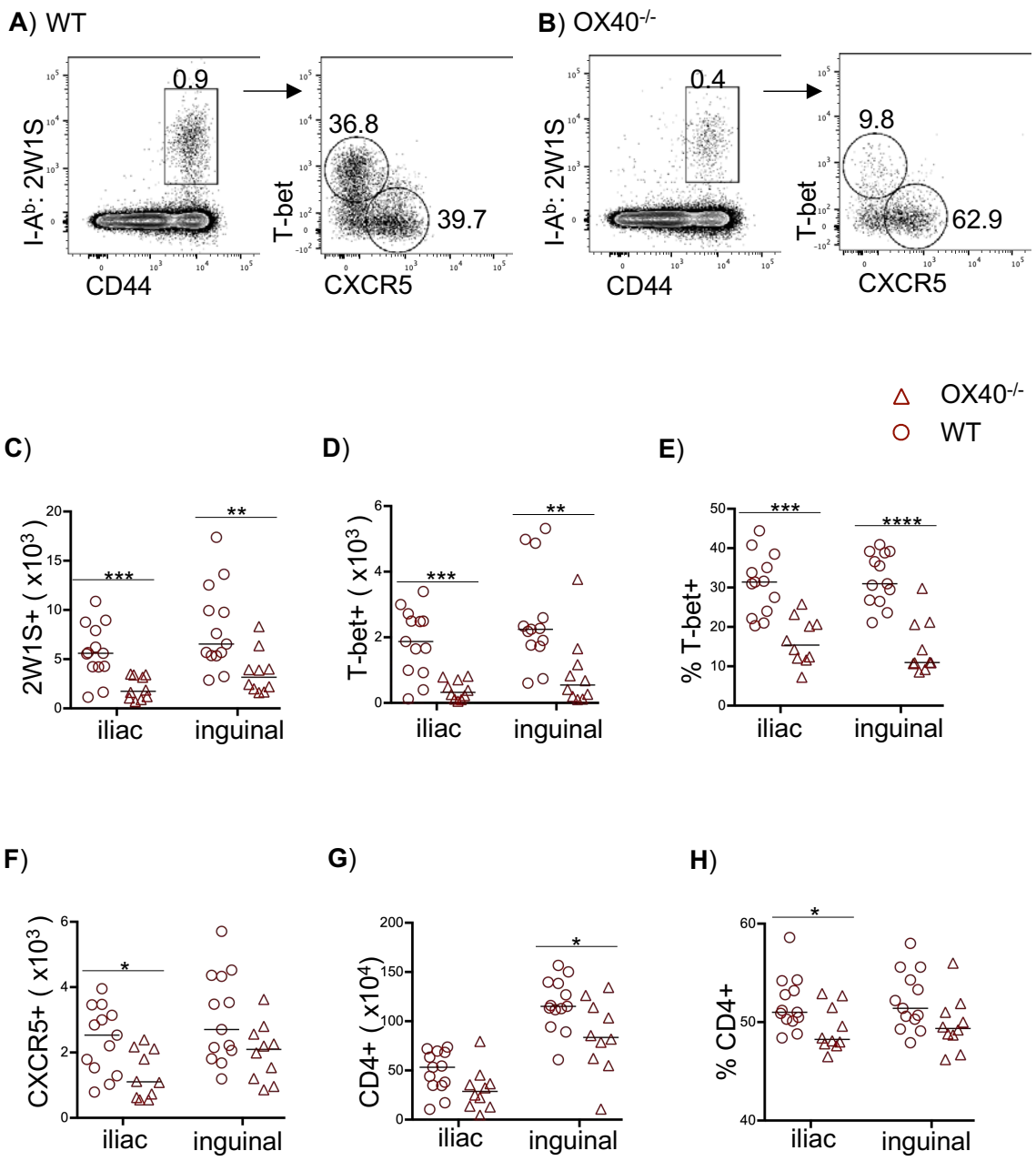

A) WT
B) OX40-/-
0.9
36.8
39.7
0.4
9.8
62.9
I-Ab: 2W1S
CD44
T-bet
CXCR5
OX40-/-
WT
C)
D)
E)
2W1S+ (x10³)
T-bet+ (x10³)
% T-bet+
***
**
****
iliac
inguinal
F)
G)
H)
CXCR5+ (x10³)
CD4+ (x10⁴)
% CD4+
*

Figure 3. 11 CD4 T cell responses to OVA-2W1S in draining LNs at D7 post immunisation are OX40 dependent

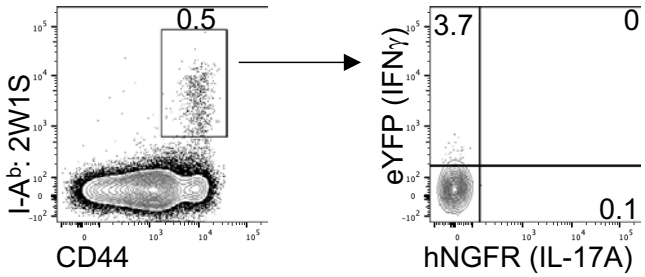
Intramuscular injection in C57Bl/6 and $OX40^{-/-}$ mice. Mice were immunised with 20μg OVA-2W/AS01 (1/50 HD) and sacrificed at D7 post immunisation. (**A, B**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^+CD4^+B220^-CD11b^-CD11c^-$) and expression of T-bet versus CXCR5 in inguinal dLNs of WT and $OX40^{-/-}$ mice. Representative flow cytometry plots for iliac dLNs can be found in Appendix Figure 8.2. (**C**) Enumeration of 2W1S-specific CD4 T cells in iliac and inguinal dLNs. (**D**) Enumeration of T-bet^+ 2W1S-specific CD4 T cells. (**E**) Percentages of T-bet^+ 2W1S-specific CD4 T cells. (**F**) Enumeration of $CXCR5^+$ 2W1S-specific CD4 T cells. (**G**) Enumeration of total CD4 T cells in iliac and inguinal dLNs. (**D**) Percentage of total CD4 T cells in iliac and inguinal dLNs. Data pooled from 4 independent experiments, WT n=13, $OX40^{-/-}$ n=10. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

We observed a partial defect in the numbers of Tfh cells, defined by expression of CXCR5, in iliac LNs which was not apparent in inguinal LNs (Figure 3.11 F). Finally, we assessed the numbers and percentages of the total CD4 T cell population present in both draining LNs which were reduced in $OX40^{-/-}$ mice, suggesting a fundamental problem with the expansion of the CD4 T cells in the absence of OX40 signals (Figure 3.11 G, H).

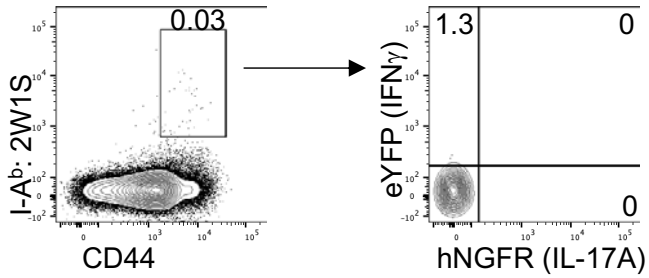
In addition to assessing T-bet expression, we also aimed to extend our analysis of AS01 driven effector T cell responses to include their function, namely production of cytokines. As AS01 is known for generating rapid IFNγ responses, we decided to investigate the effect of OX40 deletion on the functional CD4 T cells responses, namely the production of IFNγ at day 7 post immunisation with OVA-2W1S/AS01. To have a more in situ view, our initial experiments focused on testing the AS01 model in Great x Smart17A (GS) dual IFNγ reporter mice to determine whether they were suitable for future analyses of AS01 induced 2W1S-specific CD4 T cell responses. Using control WT mice, expressing no eYFP, immunised with OVA-2W1S/AS01, we set the gates and assessed IFNγ production in both iliac and inguinal draining LNs of GS mice immunised with either OVA-2W1S/PBS or OVA-2W1S/AS01 (Figure 3.12 A). Unfortunately, despite sustainable 2W1S responses, we were unable to report for IFNγ in GS mice immunised with OVA-2W1S/AS01, with the analysis showing a minimal increase in eYFP expression comparing to the PBS control mice (Figure 3.12 B, C, D).

We therefore left the idea of using GS mice and moved towards an already existing approach for assessing T cell function *ex vivo* involving potent unspecific stimulation with phorbol 12-myristate 13-acetate (PMA) and Ionomycin. PMA is an analog of diacylglycerol and together with Ionomycin they activate key signalling pathways and stimulate release of Ca^{2+} from the ER.

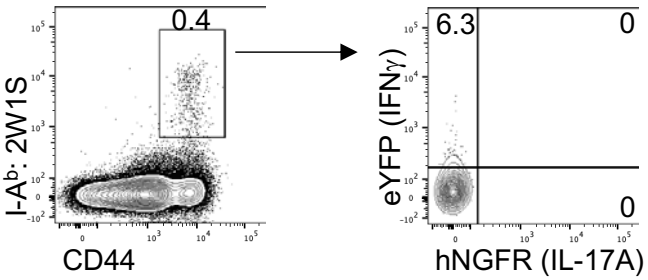
A)


0.5
I-Ab: 2W1S
CD44
3.7
0
0.1
eYFP (IFNγ)
hNGFR (IL-17A)

B)


0.03
I-Ab: 2W1S
CD44
1.3
0
0
eYFP (IFNγ)
hNGFR (IL-17A)

C)


0.4
I-Ab: 2W1S
CD44
6.3
0
0
eYFP (IFNγ)
hNGFR (IL-17A)

D)

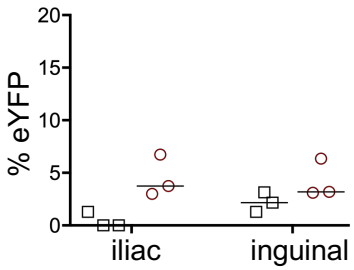

20
15
10
5
0
% eYFP
iliac
inguinal

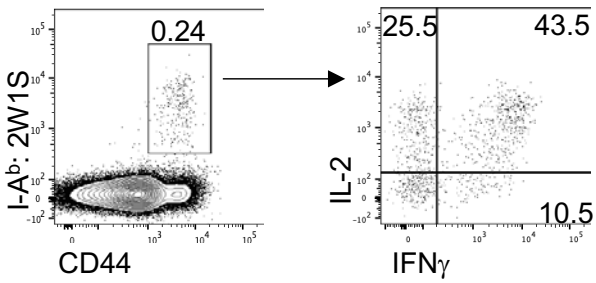
Figure 3. 12 AS01 driven IFNγ response is undetectable at D7 post immunisation with OVA-2W1S/AS01

To determine the CD4 T cell derived IFNγ (eYFP) production with no further manipulation, the GS mice were immunised with 20μg OVA-2W1S/AS01 (1/50 HD) or OVA-2W1S/PBS and the responses assessed 7 days later in draining iliac and inguinal LNs. (**A**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) and their expression of eYFP and IL-17A in inguinal LNs of WT control mice (no eYFP). (**B**) 2W1S-specific $CD44^{hi}$ CD4 T cell response and the expression of eYFP and IL-17A in inguinal LNs of GS mice immunised with OVA-2W1S/PBS. (**C**) 2W1S-specific $CD44^{hi}$ CD4 T cell response and the expression of eYFP and IL-17A in inguinal LNs of GS mice immunised with OVA-2W1S/AS01. (**D**) Percentage of $eYFP^{+}$ 2W1S-specific CD4 T cells in iliac and inguinal dLNs. Data represents one experiment, n=3. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

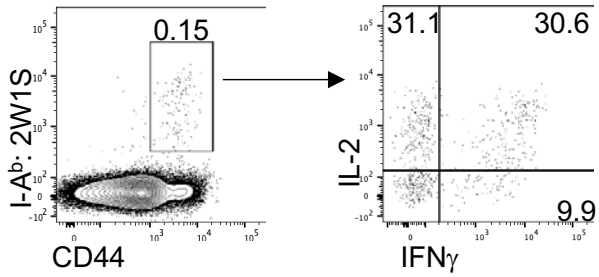
In this approach we immunised WT and OX40$^{-/-}$ mice with OVA-2W1S/AS01 and sacrificed them 7 days later. The cell suspensions of the collected iliac and inguinal LNs were then *ex vivo* cultured in the presence of PMA and Ionomycin. Similarly, to previously discussed data, we observed a reduced magnitude of the 2W1S-specific CD4 T cell response in OX40$^{-/-}$ mice as oppose to the WT mice (Figure 3.13 A, B, C). This reduction reached statistical significance in the analysis of the 2W1S response in inguinal LN (Figure 3.13 C). However, most importantly, total numbers of IFNγ producing 2W1S-specific CD4 T cells were also reduced in both draining LNs in OX40$^{-/-}$ mice following immunisation with OVA-2W1S/AS01 (Figure 3.13 D). The enumeration of IL-2 only producing cells was not included in this analysis but can be found in the appendices. Past studies have shown that simultaneous production of IFNγ and IL-2 could be used to define effector T cells generated upon secondary responses, while IL-2 is predominantly being produced by the central memory cells also often referred to as Tfh (Pepper et al., 2011).

Collectively, these analyses provided strong evidence for OX40 dependency in AS01 induced CD4 T cell responses including the generation and function of effector T cells.

A)


0.24
I-Ab: 2W1S
CD44
25.5
43.5
10.5
IL-2
IFNγ

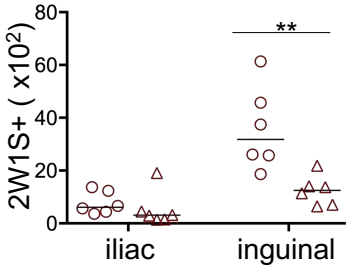
B)


0.15
I-Ab: 2W1S
CD44
31.1
30.6
9.9
IL-2
IFNγ

△ OX40$^{-/-}$

○ WT

C)


2W1S+ (x10^2)
80
60
40
20
0
**
iliac
inguinal

D)

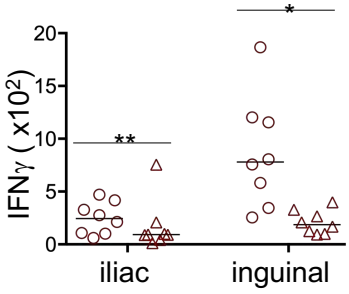

IFNγ (x10^2)
20
15
10
5
0
**
*
iliac
inguinal

Figure 3. 13 OX40 signalling is required for optimal functional CD4 T cell responses elicited by AS01

To investigate the mechanisms through which AS01 induces robust CD4 T cell responses, WT and OX40$^{-/-}$ mice were immunised intramuscularly with 20μg OVA-2W/AS01 (1/50 HD) and the draining LNs assessed 7 days later. The expression of IFNγ was assessed following 4hr *ex vivo* restimulation with PMA and Ionomycin (**A**) Identification of 2W1S-specific CD44^{hi} CD4 T cells and their expression of IL-2 and IFNγ in inguinal dLNs of WT mice. Representative flow cytometry plots for iliac dLNs can be found in Appendix Figure 8.3. (**B**) Identification of 2W1S-specific CD44^{hi} CD4 T cells and their expression of IL-2 and IFNγ in inguinal dLNs of OX40$^{-/-}$ mice. (**C**) Enumeration of 2W1S-specific CD4 T cells in iliac and inguinal dLNs. (**D**) Enumeration of IFNγ^{+} 2W1S-specific CD4 T cells. Data pooled from three independent experiments, WT n=6, OX40$^{-/-}$ n=6. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

3.2.5 Antigen-specific responses to the intestinal pathogen *Salmonella typhimurium* are impaired in the absence of OX40 signals

The gastrointestinal tract is a unique series of organs, primarily involved in digestion and uptake of nutrients. However, it also has the difficult task of maintaining immune homeostasis in an antigen-rich and bacteria dominated environment. The difficulty lies in establishing tolerance to commensal bacteria, self-antigens and food antigens, yet still recognising harmful pathogens and defending against their actions. To achieve these specialised functions, GI tract has evolved the largest and most advanced network of immune cell interactions.

The intestinal immune system is highly compartmentalised and forms several levels of regulation, starting with GALT, like Peyer's patches, scattered throughout the intestinal wall and the associated mesenteric lymph nodes (mLNs). Moreover, multiple lymphocyte populations residing in the small intestine lamina propria, including IgA-secreting plasma cells and T cells, also mediate defence and homeostatic mechanisms.

The major population of intestinal T cells is represented by CD4 subset which can be found in all intestinal compartments, in either naive or effector states (Shale et al., 2013). Majority of naive intestinal T cells usually reside within the inductive sites like mLNs and Peyer's patches, where they can easily interact with DC and undergo tissue-specific priming and activation. The costimulatory requirements of intestinal T cells in both steady state and during intestinal infections, however, have yet to be properly understood. Moreover, the limited number of experimental models tracking antigen specific CD4 T cells responses in intestinal infections has posed another challenge in trying to understand their function.

We aimed to use one of the few models available *Salmonella enterica* serovar *typhimurium*, an intestinal pathogen, to track antigen specific CD4 T cell responses and investigate the role of OX40 signals in generating Th1 effector T cells. With the overall goal established, we firstly asked a simple question whether intestinal CD4 T cell responses to commensal bacteria (steady state) required OX40 and CD30 signals for cytokine production.

Previous work in our laboratory provided evidence for the crucial requirement of OX40 and CD30 signals in intestinal CD4 T cell immunity, showing a significant reduction in the CD4 T cell compartment in small intestine lamina propria as well as impaired survival of the memory CD4 T cells generated in response to OVA and R848 (Withers et al., 2009).

To determine the role played by OX40 and CD30 signals in generating Th1 and Th17 responses to commensal bacteria we assessed CD4 T cells responses in small intestine lamina propria of GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ mice prior to and after weaning. As expected, the analysis prior to weaning in both strains showed no significant Th1 or Th17 responses, represented by minimal percentages of both IFNγ and IL-17A respectively (Figure 3.14 A, B, C).

In contrast, the analysis of CD4 T cell responses post weaning (5 weeks) showed a clear expression of these cytokines was reported in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 3.14 D). This was further reflected in the significant reduction in percentages of both IFNγ and IL-17A found in GS $CD30^{-/-}$ x $OX40^{-/-}$ mice compared to GS mice (Figure 3.14 E, F).

The above results highlighted the importance of OX40 signals in generation of CD4 T cell responses to commensal bacteria and to build on those initial observations we decided to investigate whether OX40 and CD30 signals were similarly required for generation of effector CD4 T cell response using *Salmonella*.

Salmonella enterica serovar *typhimurium* (STM) is a gram-negative bacterium predominantly found in intestinal lumen. Non-typhoid serovars of *Salmonella enterica* are one of the most common causes of foodborne diseases around the globe (Balasubramanian et al., 2018; Graham et al., 2000; Hedberg, 2011). Bacterium can be ingested with food or water, causing gastroenteritis. It infects macrophages and prevents their acidification (Alpuche Aranda et al., 2006).

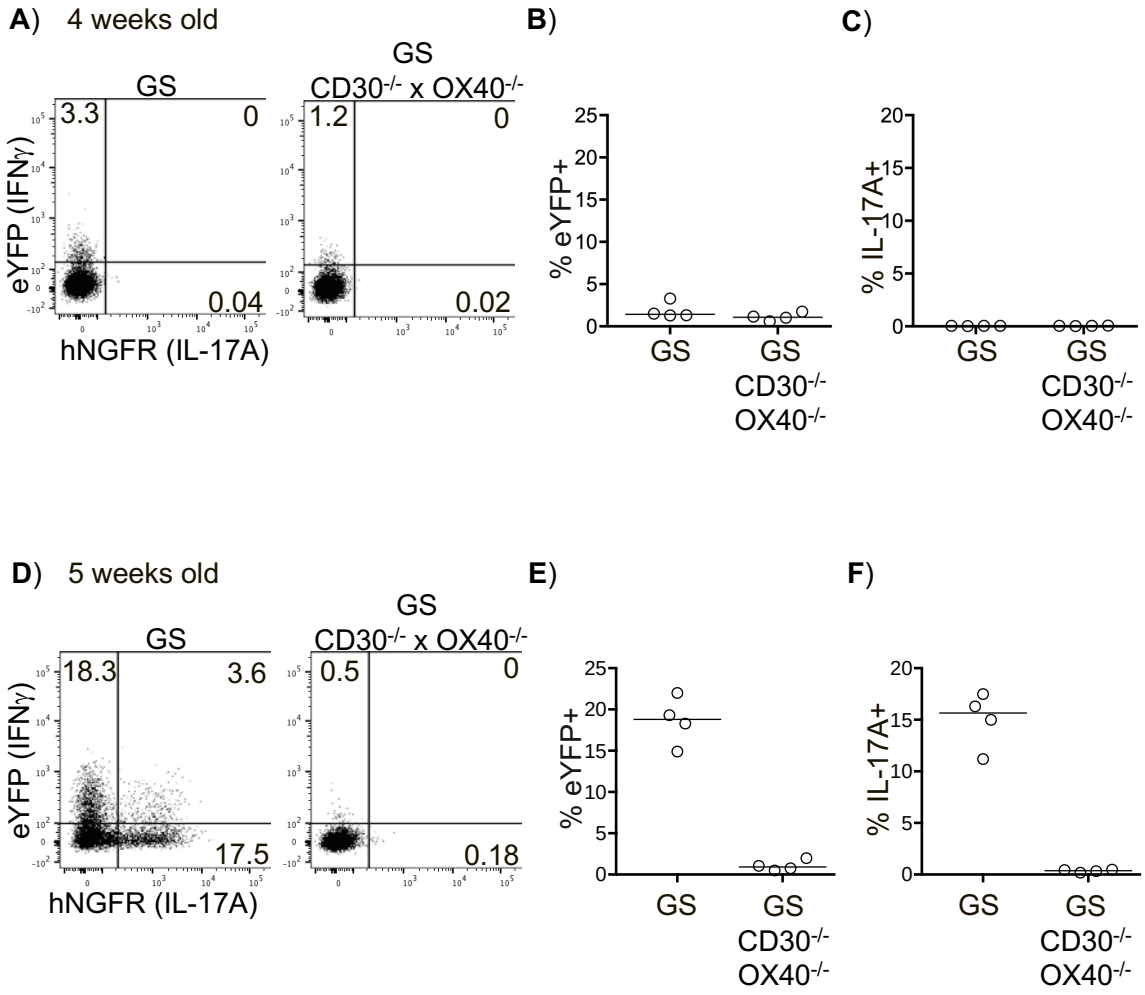

A) 4 weeks old
GS
GS CD30-/- x OX40-/-
3.3
0
0.04
1.2
0
0.02
eYFP (IFNγ)
hNGFR (IL-17A)
B)
% eYFP+
GS
GS CD30-/- OX40-/-
C)
% IL-17A+
GS
GS CD30-/- OX40-/-
D) 5 weeks old
GS
GS CD30-/- x OX40-/-
18.3
3.6
17.5
0.5
0
0.18
eYFP (IFNγ)
hNGFR (IL-17A)
E)
% eYFP+
GS
GS CD30-/- OX40-/-
F)
% IL-17A+
GS
GS CD30-/- OX40-/-

Figure 3. 14 OX40 signalling is required for intestinal CD4 T cell response to commensal microbiota

To investigate the role of OX40 signals in maintaining efficient responses commensal microbiota, the small intestine lamina propria of 4 and 5 weeks of age GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice was analysed for the production of IFNγ and IL-17A at steady state. (**A**) Expression of IFNγ (eYFP) and IL-17A by intestinal CD4 T cells at steady state in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice at 4 weeks of age, prior weaning. (**B**) Percentage of eYFP expressing intestinal CD4 T cells. (**C**) Percentage of IL-17A expressing intestinal CD4 T cells. (**D**) Expression of eYFP and IL-17A by intestinal CD4 T cells at steady state in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice at 5 weeks of age, post weaning. (**E**) Percentage of eYFP expressing intestinal CD4 T cells. (**F**) Percentage of IL-17A expressing intestinal CD4 T cells. Data from two independent experiments, GS n=4, GS $CD30^{-/-}$ x $OX40^{-/-}$ n=4. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Over the years, several *in vivo* animal models have been established to study the host-pathogen interaction. Initial studies showed murine STM to be especially pathogenic only in susceptible mice, where it caused a typhoid-like fever symptoms followed by the spread of bacteria to other organs (Santos et al., 2001). In specific- pathogen free mice, however, bacteria met with colonisation resistance, a process mediated by host and commensal bacteria to defend against pathogens (Van Der Waaij et al., 1971). As a result, current studies use antibiotic pretreatment method to prevent this phenomenon and allow assessment of intestinal responses to this potent pathogen (Barthel et al., 2003).

As previously established, defensive mechanisms against infections with intracellular pathogens rely heavily on Th1-like CD4 T cell responses including production of IFNγ and TNFα (Reiner and Locksley, 2003; Schaible et al., 2008). This is particularly apparent in studies using mice deficient in either MHCII (required for priming of CD4 T cells) or in TCR$\alpha\beta$ chains, with mice becoming susceptible to the *Salmonella* infection (Hess et al., 1996). In contrast, mice lacking MHCI (required for priming CD8 T cells) or lacking TCRδ chains, were resistant to the infection.

Many initial studies provided evidence of *in vivo* CD4 T cells activation and expansion following infection with Salmonella, however these were obtained by performing TCR transgenic adoptive transfer (Chen and Jenkins, 1999). We decided to use an attenuated strain of STM, expressing 2W1S peptide (STM-2W1S) to aid tracking of antigen-specific CD4 T cell responses following the infection. Additionally, we focused on assessing the 2W1S-secific responses in mLN as well as colon to obtain broader understanding of the effect of STM infection on inductive and effector sites. Finally, despite previous reports on the importance of OX40 signals in generating CD4 Th1 responses to STM, we aimed to investigate *in vivo,* the requirements of 2W1S-specific CD4 T cells for generation of effector functions (Gaspal et al., 2008).

We utilised GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ dual reporter mice which enabled assessment of both IFNγ and IL-17A expression without further *ex vivo* manipulation. The responses were assessed at 7dpi. We identified 2W1S-specific CD4 T cells, as CD45.2^{+}, CD3^{+}, CD4^{+}, CD44^{high}, using same strategy as the one applied in Lm-2W1S model (Figure 3.15 A, B). The same strategy was used for both mLN and colon and showed the magnitude of the 2W1S response to be impaired in GS CD30$^{-/-}$ x OX40$^{-/-}$ mice with a significant reduction in the total numbers of 2W1S-specific CD4 T cells (Figure 3.15 C, D, E, F). Overall, the 2W1S response was more robust in colon than mLN. There was no difference in the bacterial burden present in stool samples collected from GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ mice.

Using WT control mouse, expressing neither eYFP nor hNGFR, we assessed the production of IFNγ and IL-17A respectively, in mLNs of GS versus GS CD30$^{-/-}$ x OX40$^{-/-}$ mice (Figure 3.16 A). We observed a definite upregulation in expression of IFNγ by the 2W1S-specific CD4 T cells in GS mice following infection with STM, compared to the hardly detectable production of IL-17A, consistent with Th1 driven program for pathogen clearance (Figure 3.16 A, B, C). The numbers and percentages of IFNγ producing effector T cells were significantly reduced in GS CD30$^{-/-}$ x OX40$^{-/-}$ mice most probably partially reflecting the lower magnitude of the 2W1S response (Figure 3.16 A, B, C). The Th17 responses were insignificant in both mouse strains, despite the apparent increase in the percentage of IL-17A producing cells in GS CD30$^{-/-}$ x OX40$^{-/-}$ mice (Figure 3.16 D, E). The defects in production of IFNγ found in mLN of GS CD30$^{-/-}$ x OX40$^{-/-}$ mice were further exacerbated in colon showing highly significant reduction in total numbers of IFNγ (Figure 3.16 F, G). The differences in proportion of cells expressing IFNγ were however minimal (Figure 3.16 H). Assessment of colonic IL-17A responses revealed statistically significant increase in the percentage of IL-17 expressing 2W1S-specific CD4 T cells in GS CD30$^{-/-}$ x OX40$^{-/-}$ mice, with no indicative changes found in the total numbers (Figure 3.16 I, J).

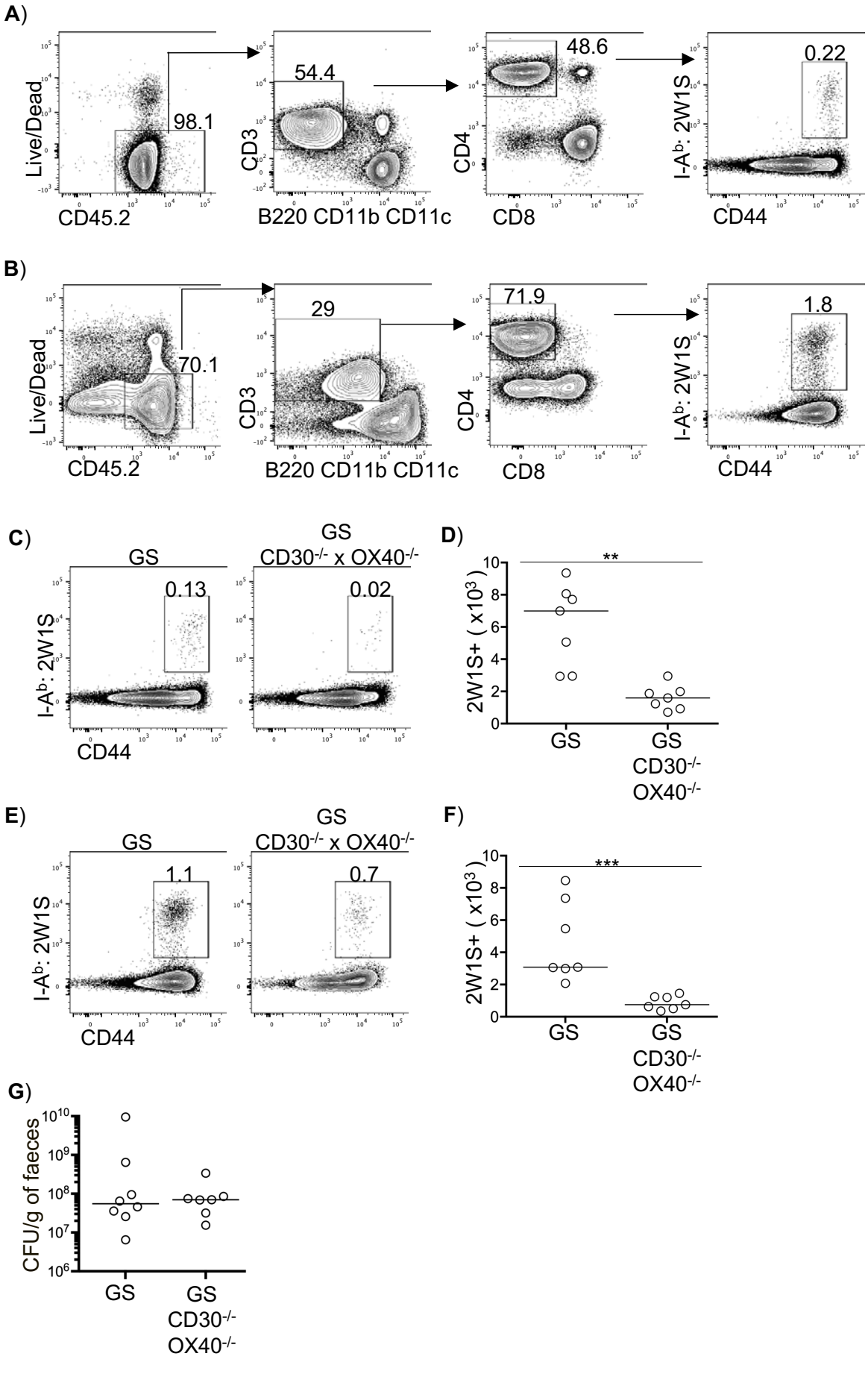

A)
Live/Dead
98.1
CD45.2
54.4
CD3
B220 CD11b CD11c
48.6
CD4
CD8
0.22
I-Ab: 2W1S
CD44
B)
Live/Dead
70.1
CD45.2
29
CD3
B220 CD11b CD11c
71.9
CD4
CD8
1.8
I-Ab: 2W1S
CD44
C)
GS
GS CD30-/- x OX40-/-
0.13
0.02
I-Ab: 2W1S
CD44
D)
2W1S+ (x10^3)
**
GS
GS CD30-/- OX40-/-
E)
GS
GS CD30-/- x OX40-/-
1.1
0.7
I-Ab: 2W1S
CD44
F)
2W1S+ (x10^3)
***
GS
GS CD30-/- OX40-/-
G)
CFU/g of faeces
GS
GS CD30-/- OX40-/-

Figure 3. 15 Salmonella typhimurium-2W1S induces strong 2W1S specific responses in both colon and draining mesenteric LNs which are CD30 and OX40 dependent

To investigate the role of CD30 OX40 signals in other Th1 infection models, GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ reporter mice were infected with intestinal pathogen STM-2W1S via oral gavage 24 hrs post administration of streptomycin and the responses in colon and mLN assessed 7 days later. (**A, B**) Gating strategy showing identification of 2W1S-specific CD44^{hi} CD4 T cells (live, CD45^{+}, B220^{-}CD11b^{-}CD11c^{-}, CD3^{+}, CD4^{+}, CD44^{+}) in mLN and colon, respectively. (**C**) Flow cytometry plots showing 2W1S-specific CD44^{hi} CD4 T cell response in mLN in GS and GS CD30$^{-/-}$ x OX40$^{-/-}$mice at D7 post infection. (**D**) Enumeration of 2W1S-specific CD4 T cells in mLN. (**E**) Flow cytometry plots showing 2W1S-specific CD44^{hi} CD4 T cell response in colon in GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ mice at D7 post infection. (**F**) Enumeration of 2W1S-specific CD4 T cells in colon. (**G**) Bacterial burden present in feaces of GS and GS CD30$^{-/-}$ x OX40$^{-/-}$ mice. Data pooled from two independent experiments, n=7. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

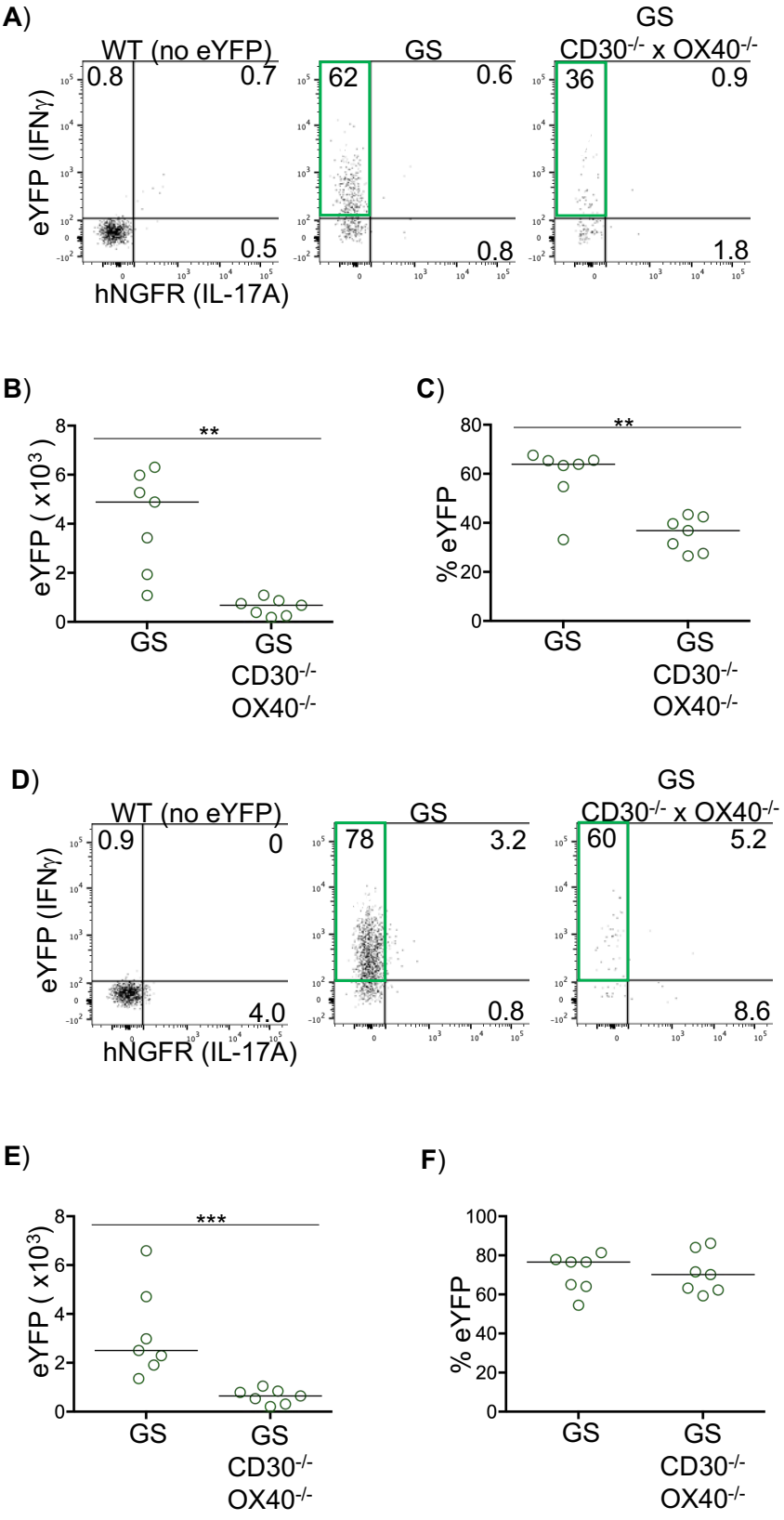

A)
WT (no eYFP)
GS
GS CD30-/- x OX40-/-
0.8
0.7
0.5
62
0.6
0.8
36
0.9
1.8
eYFP (IFNγ)
hNGFR (IL-17A)
B)
eYFP (x10³)
**
GS
GS CD30-/- OX40-/-
C)
% eYFP
**
GS
GS CD30-/- OX40-/-
D)
WT (no eYFP)
GS
GS CD30-/- x OX40-/-
0.9
0
4.0
78
3.2
0.8
60
5.2
8.6
eYFP (IFNγ)
hNGFR (IL-17A)
E)
eYFP (x10³)
***
GS
GS CD30-/- OX40-/-
F)
% eYFP
GS
GS CD30-/- OX40-/-

Figure 3. 16 CD30 and OX40 are required for the generation of effector T cells in responses to Salmonella typhimurium-2W1S but not for their function

To investigate the role of OX40 signals in the function of effector CD4 T cells generated post infection with STM-2W1S, responses in GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice were assessed 7 days post infection. (**A**) Expression of eYFP and IL-17A on 2W1S-specific $CD44^{hi}$ CD4 T cells (live, $CD45^{+}$, $B220^{-}CD11b^{-}CD11c^{-}$, $CD3^{+}$, $CD4^{+}$, $CD44^{+}$) in mLN of GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice. WT mouse was used as a negative control for the eYFP expression. (**B**) Enumeration of eYFP expressing CD4 T cells in mLN. (**C**) Percentage of eYFP expressing CD4 T cells in mLN. (**D**) Expression of eYFP and IL-17A on 2W1S-specific $CD44^{hi}$ CD4 T cells (live, $CD45^{+}$, $B220^{-}CD11b^{-}CD11c^{-}$, $CD3^{+}$, $CD4^{+}$, $CD44^{+}$) in colon of GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice. WT mouse was used as a negative control for the eYFP expression. (**E**) Enumeration of eYFP expressing CD4 T cells in colon. (**F**) Percentage of eYFP expressing CD4 T cells in mLN. Enumeration of IL-17 expressing CD4 T cells can be found in the Appendix Figure 8.4. Data pooled from two independent experiments, n=7. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Collectively, this data showed a clear requirement for OX40 and CD30 signals in generating antigen-specific effector CD4 T cells responses to acute intestinal infections. Comparing to the Lm-2W1S and AS01 models, which lack anatomical dissection of the generation and effector sites, STM-2W1S model enabled investigation of the two sites. We showed that effector cells CD4 T cells were predominantly found within the colon where their proved to be highly dependent on OX40 and CD30 pathways, however these results might have reflected the expansion and survival of antigen-specific T cells.

3.3 Discussion

Upon interaction with antigen-presenting cells, naïve CD4 T cells receive instructions which direct their further development into distinct and functionally diverse populations. Early work on CD4 T cell clonal cell lines has demonstrated a clear division between the IL-2 and IFNγ producing CD4 T cells, known as Th1 cells and the IL-4 producing cells, belonging to the Th2 population (Mosmann et al., 1986). Since then our understanding of different CD4 T cell subsets has improved greatly, in particular further characterisation of Th populations like Th17 and Tfh cells. Despite those advances, the process of cell fate acquisition remains complex, with many factors influencing the course of CD4 T cell development either directly or indirectly. Furthermore, the costimulatory requirements of particular subsets of CD4 T cells to acquire their effector functions are still unclear. Costimulatory molecules, broadly defined as co-signalling receptors can transduce both positive and negative signals into T cells, ultimately altering their TCR activity.

In this investigation we have investigated the role of OX40 is establishing CD4 T cell function across three different Th1 responses and have shown that it was particularly important for generation of effector T cell population. The three Th1 response models chosen for this investigation utilised the significance of 2W1S immunogenicity in C57BL/6 mice, with its sequence incorporated either into bacterial genome of *L. monocytogenes* and *Salmonella typhimurium* or used as an adjuvanted vaccine.

3.3.1 The role of OX40 signalling in *in vivo* CD4 T cell responses to Listeria monocytogenes

L. monocytogenes is an important model pathogen used to study innate and adaptive immune responses. When given a sub-lethal dose of this gram-positive bacterium, mice can generate efficient immune responses, resulting in bacterial clearance and resistance to future infections. This has been known since 1960s, when cellular immunity, in particular the role of peritoneal macrophages, was shown to be greatly important in inducing resistance in mice

susceptible to infection with *L. monocytogenes* (Mackaness, 1962). Moreover, T cell responses were undoubtedly required in generating effector and memory responses following infection with *L. monocytogenes* (Bhardwaj et al., 1998; Lara-Tejero and Pamer, 2004).

With the advances in molecular biology and subsequent generation of bacterial strains carrying specific peptides, our understanding of T cell responses, against Listeria infections has vastly improved. Initial studies aimed to dissect the paramount antigen-specific CD4 T cell responses using TCR-transgenic models (C S Hsieh et al., 1993; C. S. Hsieh et al., 1993). However, taking into consideration all potential limitation of this approach, for instance T cell precursor frequencies and T cell competition for APCs, the evaluation and interpretation of resulting data could have ignored important aspects of the immune response (Smith et al., 2000). Furthermore, the use of OVA as an antigen for transgenic T cells might have limited biological relevance and would never fully reflect T cell responses against a pathogen (Zinkernagel and Hengartner, 2001).

Utilising already established model of Lm-2W1S we were able to track an endogenous, polyclonal antigen-specific population of CD4 T cells throughout the course of infection. Alongside the use of a highly reproducible infection method, we also took advantage of the IFNγ reporter mice, GS and GS x $CD30^{-/-}OX40^{-/-}$, which allowed for accurate and unmanipulated assessment of IFNγ production following the infection and revealed crucial importance of OX40 and CD30 signals in establishing effector T cell function.

Following infection with Lm-2W1S, the pool of expanded antigen-specific CD4 T cells in GS or WT mice at 7 dpi acquired previously described phenotype, with a clearly distinct effector and Tfh (also known as central memory) populations (Pepper et al., 2011; Tubo et al., 2013; Yang et al., 2015). The absence of OX40 and CD30 ligation had a profound effect specifically on the effector T cell population, characterised by expression of IFNγ, involved in the clearance of intracellular pathogen like *L. monocytogenes*, and T-bet, the key transcription factor regulating Th1 cell differentiation.

We observed an approximately 8-fold reduction in 2W1S-specific CD4 T cells were expressing eYFP in GS x CD30$^{-/-}$OX40$^{-/-}$ mice which reflected production of IFNγ. With similar results observed at 4 dpi, the data indicate that OX40 and CD30 signals were pivotal to the generation of effector CD4 T cell function. At both timepoints, the level of IFNγ production in GS mice translated into eYFP expression was detectable, however its level was relatively low. The double reporter mice used in this investigation have been produced by crossing Great (IFNγ reporter) mice with Smart17A (IL-17A reporter) mice. The reporter gene construct used to make Great mice consisted of an IRES-eYFP reporter cassette which was inserted between the translational stop codon and 3' UTR/polyA tail of the *Ifng* gene. Previous studies have shown this construct to be highly functional and specific, showing no spontaneous off-target expression of IFNγ, unlike other IFNγ reporter models (Reinhardt et al., 2015). For instance, Yeti mice show elevated levels of IFNγ due to the stabilising BGH polyA tail contained within the reporter gene construct which enhances eYFP expression (Reinhardt et al., 2015; Stetson et al., 2002). Upon infection with *Listeria* or *Leishmania,* heterozygous and homozygous Yeti mice were resistant to the infection or failed to survive respectively, while Great mice and WT mice were both equally susceptible to the infection. However, most importantly, results of steady state analyses showed that Yeti mice generated significantly more IFNγ than Great mice, with most of the IFNγ being expressed by naïve T cells. In Great mice the main source of IFNγ consisted of innate cells, specifically NK cells which was representative of IFNγ regulation similar to the WT mice (Reinhardt et al., 2015). Therefore, the relatively low level of eYFP expression we observed was most likely reflecting the physiology of the immune response to Lm-2W1S which is heavily attenuated and usually cleared by 7 dpi.

Fundamentally, the results obtained in IFNγ analyses were more significant than the differences found in the expression of T-bet, however both could be used as an evidence of a defect in effector T cell population. Previous studies indicated that in T-bet$^{-/-}$ mice the

function of some effector CD4 T cells was unaffected and that in response to agonistic anti-OX40, IL-2 signals were inducing production of IFNγ rather than either T-bet or IL-12 (Williams et al., 2007). Despite this data the production of IFNγ in mice lacking T-bet was still substantially diminished. Our data showed a significant reduction in the expression of T-bet in GS x $CD30^{-/-}OX40^{-/-}$ mice as compared to GS and WT controls, but although effective, those analyses could have camouflaged the substantial loss in the effector CD4 T cell function. The use of IFNγ reporter mice have therefore developed further understanding of the costimulatory requirements of the effector T cell function following infection with Lm-2W1S. Further improvements would involve reporting for IFNγ combined with simultaneous assessment of T-bet expression, which was not possible in this investigation due to the unstable nature of eYFP under the action of the two fixative solutions used. Additionally, a brighter reporter construct would also be beneficial. Nevertheless, the use of T-bet as an effector T cell marker in Lm-2W1S model remained highly effective, allowing assessment of responses in all mouse strains described in later chapters.

OX40 and CD30 receptors have previously been shown to share signalling pathways, being involved in upregulating antiapoptotic proteins, Bcl-2 and Bcl-x_L (Rogers et al., 2001). Both pathways were shown to act in synergy at the memory T cell responses. These data suggest that OX40 and CD30 signals act together to control the development of memory T cells and their absence has a significant impact on secondary antibody production (Gaspal et al., 2005). Further studies also identified the crucial role of both signals in CD8 T cell activation and maintenance of the effector functions (Bekiaris et al., 2009a). However, in case of FoxP3 deficient mice, which suffer from lethal autoimmune disease due to the absence of Tregs, OX40 signals were shown to play dominant role (Gaspal et al., 2011). In this investigation we have shown that generation of effector CD4 T cells responses to Lm-2W1S as well as their function were predominantly OX40 dependent, as shown by the comparisons of the effector 2W1S-specific CD4 T cell responses in WT, $OX40^{-/-}$, $CD30^{-/-}OX40^{-/-}$ mice. The minimal

differences in the T-bet expression between $OX40^{-/-}$ and $CD30^{-/-}OX40^{-/-}$ mice supported the dominant requirement of the OX40 signals in the effector CD4 T cell responses, while the use of novel GS x $CD30^{-/+}OX40^{-/-}$ or GS x $CD30^{-/-}OX40^{-/+}$ mice advanced our understanding of the costimulatory requirements for the functional properties of CD4 T cells responding to Lm-2W1S.

The crucial role of OX40 signals has been associated particularly with enhanced memory CD4 T cell responses, as shown by increased numbers of memory T cells after the treatment with agonistic anti-OX40 antibody (Gramaglia et al., 2000; Weinberg et al., 2000). Additionally, studies in $OX40^{-/-}$ mice have demonstrated the significant impact of OX40 deletion on the resulting pool of memory T cell, which was significantly reduced with the function of the effector memory T cell (Tem) population being predominantly affected (Soroosh et al., 2007). Our observations supported the requirement of OX40 for generating efficient memory responses to Lm-2W1S. The defect in effector T cell responses originating at the primary stages persisted throughout the course of immune responses, showing reduced numbers of effector T cells present at 14 dpi. Furthermore, we observed a clear expansion of Tem cells, expressing T-bet, following secondary challenge with Lm-2W1S in WT mice which was consistent with published data on secondary antigen exposures (Salek-Ardakani et al., 2003). Furthermore, mice lacking either OX40 or OX40 and CD30 signals failed to generate functional effector CD4 T cell responses shown by the reduced IFNγ production. Together, this data indicates that the cells that made it past the initial OX40-dependent stages persist but are fundamentally impaired in cytokine production.

As previously stated, antigen-specific CD4 T cells generated in response to Lm-2W1S can be phenotyped as effector and Tfh populations. The Tfh cells, specialized in providing help to B cells, were distinguished from the effector T cells on the basis of the chemokine receptor CXCR5 expression. This chemokine enables the cells to migrate into the B cell follicle and reside in the GCs (Breitfeld et al., 2000).

Production of IFNγ can positively regulate differentiation of Tfh cells as shown by overactivity of the germinal centres in sanroque lupus model (Lee et al., 2012). Mice homozygous for the 'san' allele of Roquin gene, suffer from autoimmune responses caused by the accumulation of Tfh cells and lupus. Past studies have shown that development of lupus in *Roquin*$^{san/san}$ mice could be prevented by abrogation of Tfh responses (Linterman et al., 2009). Other types of interferons, for instance IFNα and IFNβ, are involved in inducing Tfh formation, however the resulting population fails to complete the entire developmental programme (Nakayamada et al., 2014).

Past studies highlighted the importance of OX40 signals in the optimal generation of the Tfh cells (Gaspal et al., 2005; Walker et al., 1999). Decreased numbers of Tfh cells present in CD30$^{-/-}$OX40$^{-/-}$ mice were responsible for defective secondary antibody responses (Gaspal et al., 2005). This however, seemed to be highly infection-specific with conflicting reports suggesting that increased signalling via the OX40 receptor was important for reduction of Tfh function and thereby controlling malaria clearance and LCMV infections but on the other hand in *L. monocytogenes* infections OX40 signalling was dispensable, with its increased expression or its absence having no effect on Tfh formation (Boettler et al., 2013; Marriott et al., 2014; Zander et al., 2015). Consistent with this previously published data, our observations showed the Tfh population to be formed independently of OX40 signals. The numbers of Tfh cells were not significantly different in both OX40$^{-/-}$ and CD30$^{-/-}$OX40$^{-/-}$ mice throughout the Lm-2W1S response.

3.3.2 AS01 induced CD4 T cells responses and its dependency on OX40 signalling

The significance of OX40 in vaccine immunology has not yet been fully understood. Induction of strong effector T cell responses is crucial to generating effective and long-lasting immunity. As shown in this investigation and other studies, antigen-specific T cells require OX40 signals to differentiate into functional population. Past studies explored the role of agonistic OX40 as

a potent stimulant, inducing increased activity of the CD4 T cells. For instance, administration of agonistic OX40 alongside synthetic long peptide vaccines during booster vaccination resulted in significantly higher numbers of polyfunctional T cells and improved efficacy against mouse CMV infection (Panagioti et al., 2017). Furthermore, OX40L was identified as one of the costimulatory molecules which in combination with Fc portion of human immunoglobin served as a treatment for murine gliomas significantly increasing the cure rates (Murphy et al., 2012). Further studies have also shown that vaccination with irradiated granulocyte macrophage colony stimulating factor (GM-CSF)-expressing GL261 cells (GVAX) alongside the agonistic OX40, increased the antitumor Th1 responses and subsequently prolonged the survival of glioma-bearing mice (Jahan et al., 2018). Altogether, those results supported the adjuvant-like function of OX40 signals in enhancing immune responses. We have however asked a different question of whether OX40 signals were indeed required for the optimal efficacy of adjuvanted vaccines. This project was developed in collaboration with GSK who kindly provided their licensed Adjuvant System 01 (AS01). The combination of the two immunostimulatory components of AS01 adjuvant, MPLA and QS-21, enabled investigation into the role of OX40 in local Th1 responses to OVA-2W1S as oppose to the systemic responses induced by Lm-2W1S. As previously described, the use of highly immunogenic 2W1S antigen allowed for identification of the polyfunctional antigen-specific CD4 T cell responses using MHCII tetramers.

To accurately model the antigen exposure route, the AS01 model involved intramuscular injections into the gastrocnemius muscle of both hindlimbs. The WHO recommends administration of adjuvanted vaccines via the intramuscular route in order to reduce or eliminate possible adverse effects like induration and inflammation which was supported by clinical trials (Diez-Domingo et al., 2015; WHO, 2019). Past studies have shown that muscle contain very low number of immune cells, particularly DC, yet delivery of vaccines into a muscles can optimise their immunogenicity due to the increased vascularity (Arnaud M Didierlaurent et al., 2014; Korthuis, 2011). The subcutaneous regions contain few immune

cells but are very closely positioned to the dermis, which is rich in various types of immune cells like DC and macrophages (Dupasquier et al., 2004). However administration of certain vaccines subcutaneously have been shown to have increased side effects as well as for instance injection of hepatitis B vaccine subcutaneously resulted in lower antibody responses and vaccine failure as compared to the intramuscular route (Diez-Domingo et al., 2015; Shaw et al., 1989).

In AS01 model, injections into both hindlimbs allowed for reduction in the volume of the vaccine administered and optimisation of the animal use.

Following intramuscular injections, AS01 was highly efficient at inducing strong 2W1S-specific CD4 T cells responses as well as increasing total numbers of the CD4 T cells in both iliac and inguinal draining LNs at 5 dpi. The presence of increased 2W1S responses in both draining lymph nodes validated the previously suggested cell trafficking between the muscle injection site and those two local LNs. Past studies indicated the possible simultaneous drainage from the injection site to both iliac and inguinal LNs, however the injections sites used in that study were either the base of the tail or a footpad which might have induced different draining patterns (Harrell et al., 2008). A very recent study using near-infrared fluorescence imaging demonstrated three lymphatic systems present in the hindlimb of mice, namely the deep medial, superficial medial and superficial lateral systems (Nakajima et al., 2018). Most importantly, this study also detected a lymphatic system connecting inguinal and iliac LN.

It is not clear whether this connection differs between species as previous studies on AS01 induced responses in sheep focused entirely on the cannulation of the iliac LN post intramuscular immunisation (Neeland et al., 2016). Nevertheless, consistent with our data, AS01 immunisation in sheep induced significant increase in the number of HBsAg-specific CD4 T cells present at 3-7 dpi in the efferent lymphatics. This also coincided with higher titres of the HBsAg-specific Ig detected (Neeland et al., 2016).

In humans, vaccination with AS01 induced higher geometric mean frequencies of CD4 T cells present in peripheral blood following immunisation with either the gE (varicella zoster virus glycoprotein) or HBs (recombinant hepatitis B virus surface protein) antigens used in Zoster and RTS,S/AS01 Malaria vaccines respectively (Chlibek et al., 2013; Leroux-Roels et al., 2016). In case of the RTS,S/AS01 vaccine, the CD4 T cell stimulating effects were observed at 14 dpi, while in the Zoster vaccine the increase was recorded at 3 months post infection. The antibody titres were induced similarly in both vaccines at approximately 30 dpi. Additionally comparisons between the $AS01_B$ and $AS01_E$ ($AS01_E$ contains reduced amount of MPLA and QS-21) showed no significant differences for both vaccines (Chlibek et al., 2013; Leroux-Roels et al., 2016).

With the use of $CD80^{-/-}86^{-/-}$ mice we confirmed that AS01 induced CD4 T cell responses were CD28 signalling dependent. We found no 2W1S-specific CD4 T cells in the absence of the two ligands. The importance of this particular costimulatory pathway in adjuvant function has previously been described and it is simply a result of the critical role CD28 signalling plays in antigen-driven T cell activation and subsequent proliferation. Similar results were observed in mice lacking CD28 receptor, where administration of LPS did not induce formation of the antigen-specific T cells (Khoruts et al., 1998). Additionally, immunisation of OVA in Incomplete Freund's Adjuvant (IFA) in $CD28^{-/-}$ mice showed highly reduced expansion of the antigen-specific CD4 T cells as oppose to and $CD40L^{-/-}$ and WT mice (Howland et al., 2000). Ultimately our investigation provided further support to the already existing data, with the additional advantage of being conducted *in vivo* and with the use of clinically relevant adjuvant whereas two described studies relied on the use of transgenic T cells.

The OX40 pathway has been of interest to adjuvant related research due to its enhancing effect on CD4 T cell function and memory cell formation. In our investigation, we have presented strong evidence supporting the OX40 dependent generation of the effector CD4 T cell population in AS01 induced Th1 responses. We found that following the

immunisation with OVA-2W1S/AS01, 2W1S-specific CD4 T cell responses were significantly impaired in $OX40^{-/-}$ mice as oppose to the WT controls. Most importantly, however, it was the total number and percentages of effector T cells (identified on the basis of T-bet expression) which were predominantly reduced in the absence of OX40 signals. This was consistent with our previous data showing a defect in the effector population generated in response to Lm-2W1S.

Studies on Th2 responses demonstrated the significant role of OX40 signals in ameliorating adjuvant induced T cell responses. In particular, the TCR transgenic T cell responses to OVA in the presence of Complete Freund's Adjuvant (CFA) or Alum were shown to be highly OX40L dependent when comparing the $OX40L^{-/-}$ mice to the WT controls (Ishii et al., 2003). On the other hand, overexpression of OX40L signals resulted in augmented CD4 T cells responses in absence and presence of the two adjuvants, once again demonstrating the T cell stimulating nature of OX40 signals.

An important mechanism of adjuvant action involves induction of antibody responses. Specifically, emulsion-based adjuvants, like MF59 oil-in-water adjuvant has been shown to promote potent Tfh responses and thereby enhancing GC B cell responses in 3 week old mice (Gavillet et al., 2015). Moreover in human trials, the inducing effects of MF59 immunisation were shown by the increased frequencies of H1-specific Tfh (identified as $CD4^{+}IL\text{-}21^{+}ICOS^{+}CXCR5^{+}$) present in peripheral blood at day 7 post administration of the A/California/7/2009 avian flu vaccine (Spensieri et al., 2016). Other adjuvants like Aluminium salts were shown to have limited Tfh polarisation properties as shown by the minimal expansion of the $CD4^{+}CXCR5^{+}$ population in murine draining LNs following immunisation with Aluminium hydroxide (Riteau et al., 2016). Our data indicated that in AS01 system, antigen-specific CD4 T cells formed previously described population of effector and Tfh ($CXCR5^{+}$) cells. Unlike Lm-2W1S data, the total numbers of Tfh in $OX40^{-/-}$ mice were reduced. This however this could have been a result of the overall reduction in the magnitude of the response in the draining LNs.

Consistent with Lm-2W1S data, lack of OX40 signals has a significant effect on the functional properties of the effector CD4 T cells generated in responses to AS01 immunisation. Those analyses required the use of *ex vivo* stimulation with PMA/Ionomycin, as initial attempt with immunisation in GS mice showed hardly any IFNγ production. This result was however unsurprising, considering previous studies showing a very rapid production of IFNγ by CD4 and CD8 T cells during the first 6-8 hrs post immunisation with AS01 which decreased shortly after (Coccia et al., 2017; Neeland et al., 2016). The levels of IFNγ present in efferent lymphatics increased slightly again between day 5 and 7 post AS01 immunisation in sheep, however did not reach nearly as significant levels as detected at early timepoints (Neeland et al., 2016). Following *ex vivo* stimulation, we observed a significant reduction in the IFNγ expression by the 2W1S-specific CD4 T cells in $OX40^{-/-}$ mice immunised with OVA-2W1S/AS01 as compared to immunised WT control mice, suggesting that OX40 pathway was required for AS01 efficacy in inducing effector CD4 T cell responses. Similar results were obtained in previous studies using TCR transgenic T cells, where $OX40L^{-/-}$ mice had their IFNγ responses significantly reduced following immunisation with both CFA and Alum (Ishii et al., 2003). Overall, future studies would benefit greatly from further investigation into the OX40 dependent generation of AS01 induced responses.

3.3.3 The requirement for OX40 signals in responses to intestinal pathogen *Salmonella enterica* serovar *typhimurium*

Finally, this investigation also examined the role of OX40 pathway in tissue specific responses following acute infection with *Salmonella enterica* serovar *typhimurium.* This particular pathogen, like in case of *Listeria monocytogenes,* is a facultative anaerobic intracellular bacterium which induces highly specific Th1 responses. Upon ingestion, it invades host gut mucosa and becomes captured by phagocytic cells. One of the most important differences

between the two bacteria, is the structure of their cell walls, with *Salmonella* being classed as a Gram-negative bacterium.

The possible dependency on OX40 signals in *Salmonella* induced infections was indicated by previously published data showing reduced Th1 CD4 T cell survival in the absence of OX40 or CD30 signals as well as significant impairment in the CD4 T cell responses to attenuated *Salmonella enterica* serovar *typhimurium* in mice lacking both costimulatory pathways (Gaspal et al., 2008). Although, highly suggestive, that particular study used an unphysiological route of infection, namely intraperitoneal injections. The resulting systemic infection was not representative of the usual transmission mode, where the bacteria are ingested with contaminated food and water. In humans, nontyphoidal *Salmonella* causes bloodstream infections only in 6% cases, affecting mostly immunocompromised patients (Parry et al., 2013; Vugia et al., 2004). *Salmonella enterica* serovar *typhimurium* is considered less invasive, yet hospitalisation rates following the infection are still significant (Jones et al., 2008). As a result of intraperitoneal infections, dissemination of the bacteria might have been skewed and aberrant, in particular with recent reports showing significantly higher loads of bacterial colony forming units (CFU) present in mLN, ileum and cecum than spleen post oral gavage (Bravo-Blas et al., 2018; Nilsson et al., 2019). Furthermore, the lack of better identification system for the antigen specific CD4 T cells than the expression of CD62L, prevented delineation of functionally distinct CD4 T cell subpopulations (Gaspal et al., 2008). We therefore used a novel *Salmonella enterica* serovar *typhimurium*-2W1S (STM-2W1S), which was administered by oral gavage with a pre-treatment of streptomycin. Pre-treatments with antibiotics are not necessary but act to reduce variability between the mice as well as resulting colitis resembles human Salmonellosis (Griffin and McSorley, 2011; Nilsson et al., 2019).

Prior to those experiments, we observed an essential role of OX40 and CD30 signals in generating effective CD4 T cell responses to commensal bacteria which appeared absent post weaning. Although further experiments are required to fully examine this phenomenon,

it did highlight the requirement of those key costimulatory pathways in gut mucosal responses.

Consistent with previously published data, antigen-specific CD4 T cell responses following infection with STM-2W1S were significantly reduced in mesenteric LN and colon of GS x $CD30^{-/-}OX40^{-/-}$ mice as opposed to WT controls. Interestingly, again like in the previous study, we also found that mice lacking both CD30 and OX40 signals had comparable numbers of CFU present in their faeces at 7 dpi. This might have been however a result of the bacteria entering the stationary phase where their growth plateaus for instance due to spatial requirements.

The effector CD4 T cell responses following *Salmonella* infections were firstly studied using TCR transgenic adoptive transfer systems, where following the infection with *Salmonella*-OVA, OVA-specific CD4 T cells expanded and begun secreting IFNγ by day 5 of the response (Chen and Jenkins, 1999). With the use of reporter mice, we were able to accurately assess the function of effector CD4 T cells, which once again proved to be OX40 and CD30 dependent. The requirement for those signalling pathways was particularly evident in CD4 T cells responses generated in the mesenteric LNs. Interestingly, the eYFP expression in GS mice observed in those experiments comparing to the Lm-2W1S model was significantly higher. This was most likely caused by the acute state of the STM-2W1S infection which is more invasive and not cleared, eventually being fatal for the mice.

3.3.4 Summary

Overall, data presented in this chapter, clarified the role of OX40 in supporting functional effector CD4 T cells. Across different Th1 infection and immunisation models, the requirements for OX40 signals were crucial for the efficient expansion of 2W1S-specific CD4 T population. However, most importantly, the impairment in the functional properties of effector T cells in the absence of OX40, highlighted the vital role of this costimulatory pathway

in the generation of effective immune responses and indicated that further research into their function would be beneficial in designing better immunotherapies. The following chapters will aim to provide better mechanistic understanding of how is OX40L expression regulated and who provides it *in vivo* which would further aid understanding of how to better manipulate this pathway.

CHAPTER 4: REGULATION OF OX40L EXPRESSION ON DC POST INFECTION WITH LM-2W1S

4.1 Introduction

Disease causing pathogens entering the body are treated as threat and instantly challenged by the cells of the innate immune system, a collection of specialised cells often with sentry roles (Janeway and Medzhitov, 2002). These cells act promptly to recognise infectious agents, initiate measures to limit the spread and expansion of the invader and initiate adaptive immune responses that can clear the infection should the innate response prove insufficient. DC are critical cells in this regard, located in tissues throughout the body but particularly at barrier sites, which upon their activation transform into highly potent APCs. DC can become activated by both pathogen derived molecules as well as by the signals their receive from other innate immune cells, and can be involved in mediating both primary and memory responses (Hart, 1997; Zammit et al., 2005). It is widely accepted, that activated DC are critical initiators of T cell responses. Their exceptional potential has been shown by both *in vitro* and particularly the *in vivo* studies, in which conditional or constitutive ablation of DC function resulted in abrogation of T cell responses (Jung et al., 2002; Steinman and Witmer, 1978). Antigen experienced DC provide several signals to naïve T cells, including presenting foreign antigens via the MHCII complexes, and most importantly costimulatory and co-inhibitory molecules which are also required for T cells activation. Interaction of T cells with DC via the pMHC in the absence of costimulatory signals leads to a peripheral tolerance (Hawiger et al., 2001). The repertoire of costimulatory molecules is versatile, with many of them inducible like OX40 or ICOS, becoming expressed later on during T cell priming (M. Croft et al., 1992; A Hutloff et al., 1999). Their diverse expression patterns can represent specific functions of individual costimulatory pathways at different phases of T cell responses. Furthermore, DC maturation, cytokine production and expression of costimulatory signals can be influenced by changes in their local microenvironment and depending on the pathogen,

some of the pathways may be modulated to aid the course of infection (Foti et al., 2006; Sallusto and Lanzavecchia, 2002). Additionally, for some of the costimulatory molecules, like OX40L, there is not enough evidence describing who expresses them *in vivo* and how it is regulated. Hence, the complexity of DC function in the process of T cells activation, has highlighted the importance of assessing roles of separate costimulatory pathways but also determining mechanisms involved in their regulation, specifically mechanisms behind their upregulation.

Throughout the last 30 years of research, our understanding of the costimulation paradigm has improved greatly. Given the importance of CD28 signalling in T cell activation, many studies focused on costimulatory molecules belonging to the B7/CD28 family and TNFRSF which identified wide range of possible ligand-receptor interactions and revealed the requirement and necessity of certain pathways in generating efficient T cells responses, also highlighting their potential use in T cell mediated immunotherapies. For instance, it has been shown that in many cancer patients, costimulatory molecule CD80 was expressed in very low levels on circulating monocytes, suggesting a possible impairment in antigen presentation and T cell priming (Bella et al., 2003; Chaux et al., 1996). Low level of CD80 expression are usually associated with immune tolerance where ICOSL expression dominates and resulting stabilisation of IL-10 receptor and production of IL-10 cytokine induces differentiation of Tregs (Herman et al., 2004; Rottman et al., 2001). Previous work also unveiled a mutual function of certain costimulatory pathways like for instance CD40L which can induce activation of both T cells and DC and result in subsequent further enhancement of T cell responses (Cella et al., 1996). The interaction between CD40 and CD40L promoted much greater production of IL-12 than after stimulation with TNFα and LPS which then in turn led to enhanced DC activation and expression of other costimulatory molecules. Ligation through CD40 receptor enhances expression of CD80, CD86, OX40L, although other signals including proinflammatory cytokines can also be involved (Ohshima et al., 1997). For instance, IFN family of cytokines

has been shown to be particularly important in fighting pathogens by promoting antiviral and antimicrobial immune responses (Samuel, 2001). This family includes two types of interferons, namely type I IFNα/β and type II IFNγ which bind their cognate receptors and like other cytokines receptors, signal via Jak/Stat pathway (Darnell et al., 1994; Stark et al., 1998). Both types of interferons have been specifically shown to induce expression of CD80 on primary monocytes cultured *ex vivo*, as shown by the increase in mRNA and protein levels (Bauvois et al., 2009). Furthermore, transcriptomic and proteomic analyses of JNK-specific inhibitor SP600125 (reversible ATP-competitive inhibitor which inhibits phosphorylation of c-Jun and thereby expression of IL-2, IFNγ, TNFα and COX-2) in human monocytic cell line revealed that expression of CD80 was also regulated by interferon regulatory factor 7 (B. L. Bennett et al., 2001; Lim et al., 2005). Additionally, another study on human monocytic cell line, linked IFNγ to an enhanced expression of another member of the B7 family, the CD86 molecule (Curiel et al., 1999). Innate immune cells produce proinflammatory cytokines in response to danger signals they receive from environment, particularly the invading pathogens. As mentioned before, bacteria and viruses can act to modulate the costimulation and impair T cell responses. Previous reports on infection with *Mycobacterium leprae* showed a downregulation in CD40 signalling, while in chronic mycobacterial infections CD40, CD80 and CD86 expression was significantly lower in all infected cells, while PD-L1 and PD-L2 expression increased (Murray et al., 2007; Sakai et al., 2010; Schreiber et al., 2010). In viral infections, caused by for example HIV, expression of CD80, CD86, 4-1BBL was downregulated (Chaudhry et al., 2005; Keersmaecker et al., 2011; Venkatachari et al., 2007). In CD4 T cell responses, the crucial OX40:OX40L interaction, in particular OX40L expression *in vivo* is poorly understood and there is little understanding of how such expression is regulated. In this chapter, we sought to understand the kinetics of OX40L expression on DC following infection with *Listeria monocytogenes* and determine how was its expression regulated.

4.2 Results

4.2.1 OX40L is upregulated on DC at 24hrs post infection with Lm-2W1S

Having established the crucial role of OX40 signals in generating functional CD4 T cell responses to pathogens and vaccine adjuvants, we sought to determine how this key effector costimulatory pathway was regulated *in vivo*.

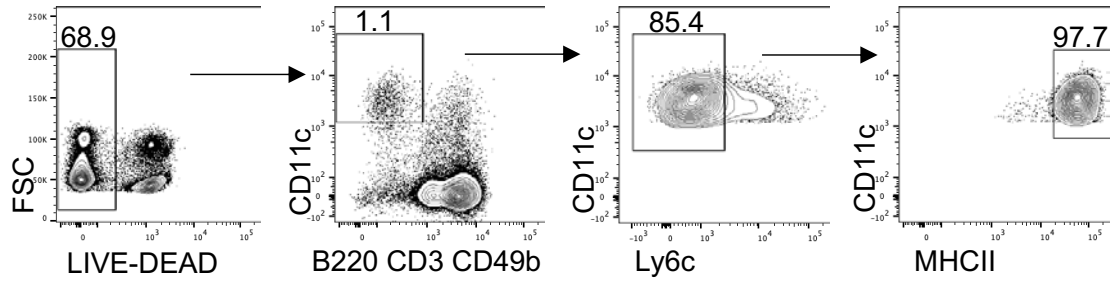
The expression of OX40 and OX40L has been found primarily on activated T cells and APC, respectively. However, their inducible and transient nature has made it particularly difficult to understand the mechanisms behind their interaction and in particular the signals involved in the upregulation of OX40L.

Previous reports in $Rag^{-/-}$ mice, identified DC as one of the main cellular sources of OX40L signals alongside LTi-like cells (Kim et al., 2003). Furthermore, studies have also shown that OX40L expression on DC could be elevated upon stimulation (Godfrey, 2004; Murata et al., 2000a; Ohshima et al., 1997; Stüber et al., 1995). We therefore hypothesised that DC expression of OX40L was important for the CD4 T cell response, given the crucial role these play in T cell priming. There is a lack of any data describing OX40L expression in the response to *L. monocytogenes*, therefore we firstly decided to simply track the kinetics of OX40L expression on DC post infection with Lm-2W1S in order to provide foundation for better understanding of OX40:OX40L pathway in this infection.

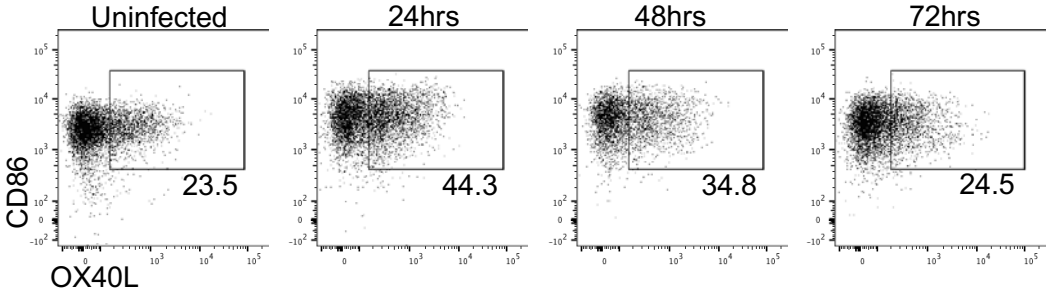
Therefore, WT mice were infected with Lm-2W1S and sacrificed at selected time points (24, 48 and 72 hrs) post infection. The splenocytes from infected and control WT mice were cultured overnight and stained for OX40L. The culturing process was required to obtain optimal OX40L expression on DC, which is usually absent on freshly isolated splenic DC (M.-Y. Kim et al., 2005). The assessment of OX40L expression on DC was controlled using untreated WT mice as well as the PGK^{Cre} x $OX40L^{f/f}$ mice (ubiquitous OX40L deletion) which were used as negative controls, facilitating setting accurate gates.

Initial phenotyping of splenic DC included cell surface proteins associated with all conventional DC, specifically CD11c, MHCII and CD86 (Figure 4.1 A).

A)


68.9
FSC
LIVE-DEAD
1.1
CD11c
B220 CD3 CD49b
85.4
CD11c
Ly6c
97.7
CD11c
MHCII

B)


Uninfected
24hrs
48hrs
72hrs
CD86
OX40L
23.5
44.3
34.8
24.5

C)

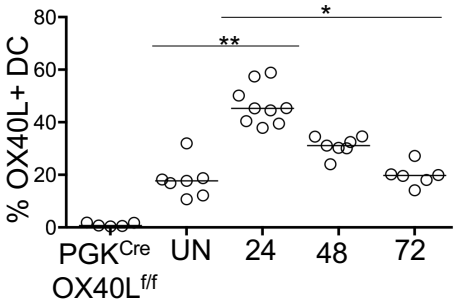

% OX40L+ DC
80
60
40
20
0
*
**
PGKCre
OX40Lf/f
UN
24
48
72

Figure 4. 1 OX40L is upregulated on DC in response to Lm-2W1S

To investigate regulation of DC expression of OX40L, splenocytes were isolated from mice at different times post infection with Lm-2W1S. (**A**) Gating strategy used to identify splenic DC ($B220^{-}CD3^{-}CD49b^{-}$, $CD11c^{+}$, $Ly6C^{low}$, $MHCII^{+}$). (**B**) Expression of OX40L by DC from uninfected mice and mice infected with Lm-2W1S 24, 48 and 72 hrs previously. (**C**) Proportion of DC expressing OX40L at different times post infection with Lm-2W1S, versus uninfected (UN) and PGK^{cre} x $OX40L^{f/f}$ controls. Data pooled from 2 independent experiments, n=5 PGK^{cre} x $OX40L^{f/f}$, n=7 uninfected, n=9 24 hrs post Lm-2W1S, n=7 48 hrs post Lm-2W1S, n=6 72 hrs post Lm-2W1S infection. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

This particular gating strategy for splenic DC in untreated WT mice showed that approximately 20% of splenic DCs expressed OX40L at steady state comparing to the PGK^{Cre} x $OX40L^{f/f}$ control mice (Figure 4.1 B, C). However, infection with Lm-2W1S resulted in significant upregulation of OX40L on DC which peaked at 24 hrs post infection, reaching a median of 45.3 %. This then gradually decreased with the percentage of DC expressing OX40L returning back to the baseline by 72hrs (Figure 4.1 B, C). Thus, DC appeared to rapidly upregulate OX40L expression after Lm-2W1S infection, however this expression was short lived.

To determine the phenotype of the OX40L expressing DC, splenocytes from Lm-2W1S infected WT mice were further analysed. In particular, the expression of cell surface proteins characteristic for the two conventional DC subsets, cDC1 and cDC2 was assessed. The 'conventional' group of DC includes all DC except for the plasmacytoid DC (pDC) and can be found in most lymphoid and nonlymphoid tissues. In the spleen they reside within marginal and T cell zones, screening for tissue and blood for foreign antigens. The two subsets have been associated with differential role in eliciting immune responses. The overall view presents cDC1 subset as important for efficient cross-present antigens to $CD8^+$ T cells, while the cDC2s are involved in the stimulation of $CD4^+$ T cells. However, there is some evidence suggesting that either DC subset can stimulate both types of T cells (den Haan and Bevan, 2002; Dudziak et al., 2007).

The identification of DC populations was first carried out in untreated WT mice and followed the previously described gating strategy used to recognize $OX40L^+$ DC, classing them as $B220^-CD3^-CD49B^-$, $CD11c^+$, $Ly6C^{low}$, $MHCII^+$, $CD86^+$ (Figure 4.2 A). Next, the $OX40L^+$ DC were further phenotyped on the basis of CD24 and SIRPα expression, typical for cDC1 and cDC2, respectively. Additional analyses validated their anticipated phenotypes, with the $CD24^+$ DC expressing CD8 and XCR1, while the SIRPα^+ DC classed as $CD4^+$ and $CD11b^+$ (Figure 4.2 B).

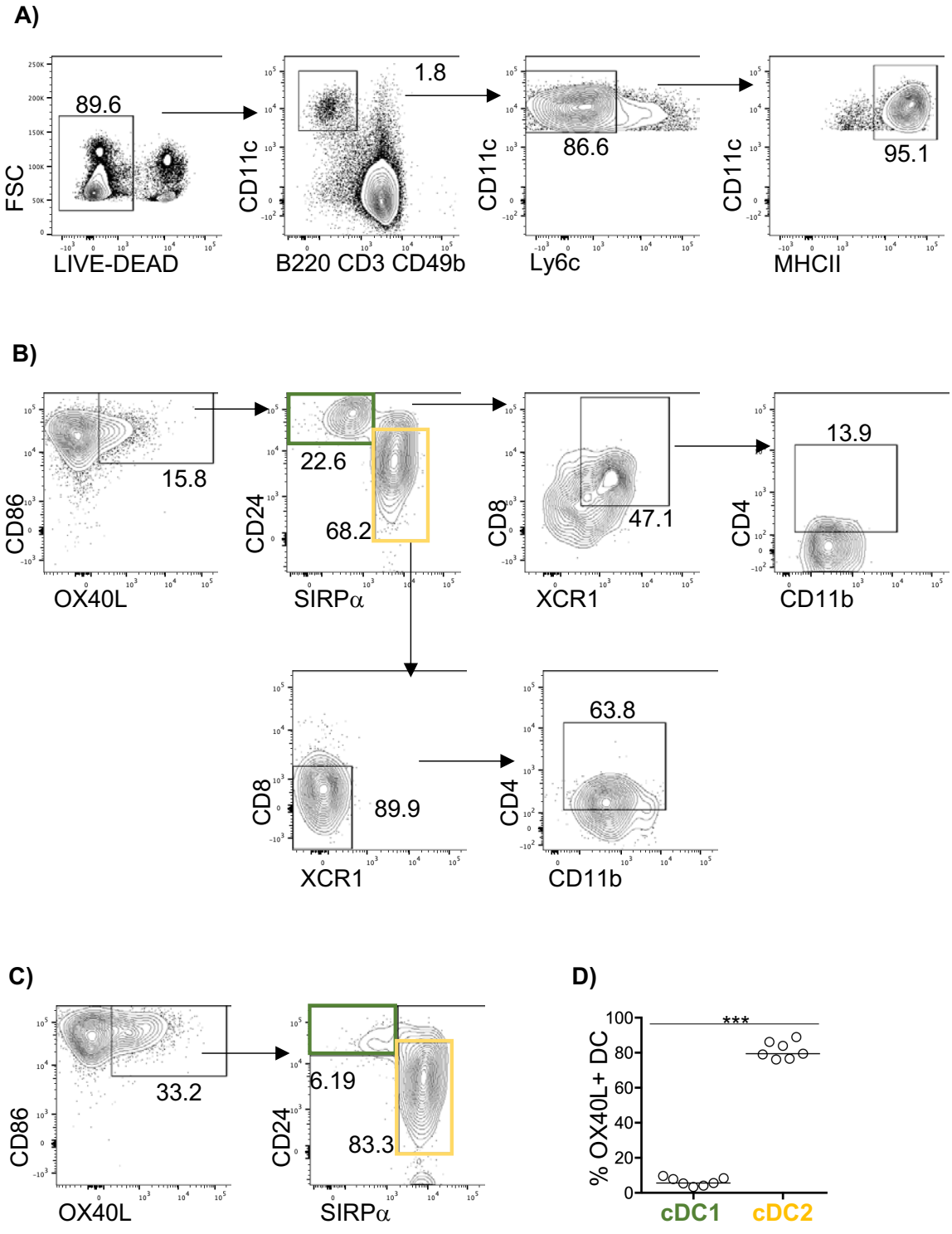

A)
89.6
FSC
LIVE-DEAD
1.8
CD11c
B220 CD3 CD49b
86.6
CD11c
Ly6c
95.1
CD11c
MHCII
B)
15.8
CD86
OX40L
22.6
68.2
CD24
SIRPα
47.1
CD8
XCR1
13.9
CD4
CD11b
89.9
CD8
XCR1
63.8
CD4
CD11b
C)
33.2
CD86
OX40L
6.19
83.3
CD24
SIRPα
D)
% OX40L+ DC
100
80
60
40
20
0
***
cDC1
cDC2

Figure 4. 2 Population of DC expressing OX40L at 24 hrs post Lm-2W1S can be phenotyped as type 2 conventional DC (cDC2)

To determine the phenotype of DC expressing OX40L, WT mice were infected with Lm-2W1S. The mice were sacrificed 24 hrs later and their splenocytes were cultured overnight. (**A**) Gating strategy used to identify splenic DC ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, MHCII). (**B**) Phenotyping of splenic DC expressing OX40L from uninfected mice, showing cDC1 (Green-$CD24^{+}SIRP\alpha^{-}CD8^{+}XCR1^{+}CD4^{-}$) and cDC2 (Yellow-$CD24^{-}$ $SIRP\alpha^{+}$ $CD8^{-}XCR1^{-}CD4^{+}$) DC. (**C**) Phenotypes of DC expressing OX40L at 24 hrs post infection with Lm-2W1S. (**D**) Proportion of cDC1 versus cDC2 expressing OX40L in infected WT mice. Data pooled from two independent experiments, n=7. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

The cDC2 population was undoubtedly the dominant population of splenic DC expressing OX40L. This has remained true for the analyses conducted in WT mice infected with Lm-2W1S 24 hrs prior the spleen harvest (Figure 4.2 C). The phenotypic analyses evidently identified the cDC2 population to account for the majority of the OX40L expressing splenic DC (Figure 4.2 D).

The analyses of OX40L kinetics post infection with Lm-2W1S were extended to additionally include the dynamics of OX40 expression on antigen-specific 2W1S:I-A^{b+} CD4 T cells measured within the same spleen. This assessment was particularly important, enabling broader understanding of the relative expression of the receptor to its cognate ligand.

The expression of OX40 was assessed at day 2, 3 and 4 post infection with Lm-2W1S as previously published data showed OX40 expression to be detectable between 2 to 20 days depending whether the activation was induced by acute or chronic infection (Boettler et al., 2013; Gramaglia et al., 1998). Additionally, previous work from our lab showed the OX40 expression post Lm-2W1S infection to peak at day 3 (Marriott et al., 2014). MACS cell separation system was used to enhance the MHCII Tetramers facilitated detection of 2W1S:I-A^{b+} CD4 T cells.

At day 2 post infection in WT mice there was a very small but evident population of 2W1S:I-A^{b+} CD4 T cells, as compared to uninfected controls (Figure 4.3 A, E). Among those cells approximately 40% had detectable expression of OX40 which was validated using $OX40^{-/-}$ mice as negative controls. Importantly, the expression was unaffected in Lm-2W1S infected PGK^{Cre} x $OX40L^{f/f}$ mice, which expressed comparable levels to the infected WT mice. Interestingly, majority of the $OX40^{+}$ cells also co-expressed CD25 (Figure 4.3 A-C). This receptor has been previously recognised as an important developmental requirement for the TH1 effector T cells and as a marker to aid their identification (Pepper et al., 2011).

Similar OX40 expression levels were detected at day 3 post infection in both WT and PGK^{Cre} x $OX40L^{f/f}$ mice maintained by approximately 45% of 2W1S:I-A^{b+} CD4 T cells (Figure 4.3 B, E).

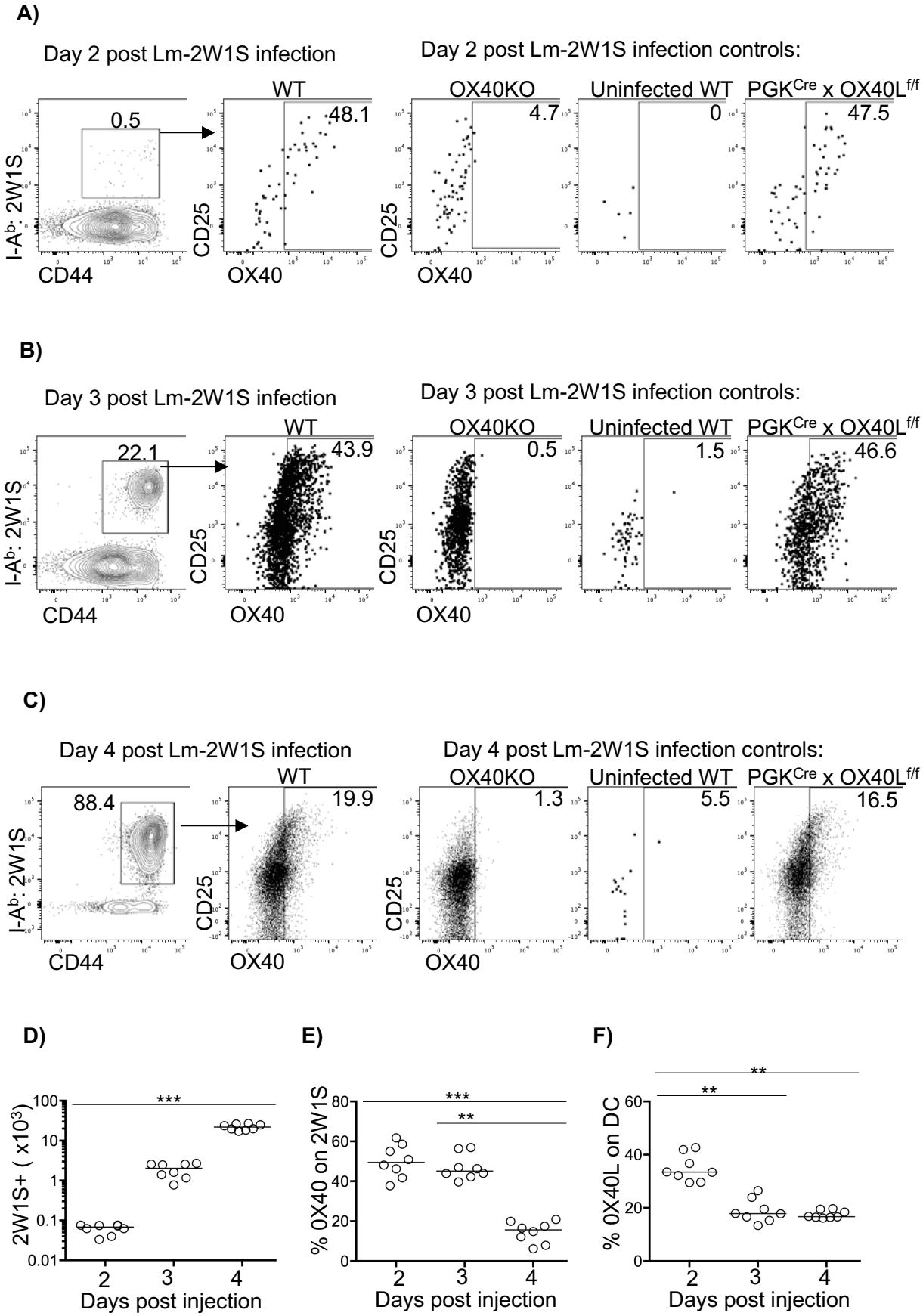

A)
Day 2 post Lm-2W1S infection
Day 2 post Lm-2W1S infection controls:
WT
OX40KO
Uninfected WT
PGKCre x OX40Lf/f
0.5
48.1
4.7
0
47.5
I-Ab: 2W1S
CD44
CD25
OX40
B)
Day 3 post Lm-2W1S infection
Day 3 post Lm-2W1S infection controls:
22.1
43.9
0.5
1.5
46.6
C)
Day 4 post Lm-2W1S infection
Day 4 post Lm-2W1S infection controls:
88.4
19.9
1.3
5.5
16.5
D)
2W1S+ (x10³)
***
Days post injection
E)
% 0X40 on 2W1S
***
**
Days post injection
F)
% 0X40L on DC
**
**
Days post injection

Figure 4. 3 Kinetics of OX40 and OX40L expression in response to Lm-2W1S

To understand the relationship between DC OX40L expression and CD4 T cell OX40 expression, mice were infected with Lm-2W1S and T cells and DC from the same spleen analysed by flow cytometry. (**A**) Expression of OX40 on 2W1S- specific CD4 T cells ($B220^{-}$ $CD11b^{-}CD11c^{-}CD3^{+}CD4^{+}$) at day 2 post infection with Lm-2W1S in WT and in uninfected and $OX40^{-/-}$ and PGK^{Cre} x $Ox40L^{f/f}$ control mice. (**B**) Expression of OX40 on 2W1S- specific CD4 T cells at day 3 post infection with Lm-2W1S in WT and in uninfected and $OX40^{-/-}$ and PGKCre x $Ox40L^{f/f}$ control mice. (**C**) Expression of OX40 on 2W1S- specific CD4 T cells at day 4 post infection with Lm-2W1S in WT and in uninfected and $OX40^{-/-}$ and PGK^{Cre} x $Ox40L^{f/f}$ control mice. (**D**) Enumeration of 2W1S-specific CD4 T cells at day 2, 3 and 4 post infection with Lm-2W1S. (**E**) Percentage of 2W1S- specific CD4 T cells expressing OX40 at 2, 3 and 4 days post infection with Lm-2W1S. (**F**) Percentage of DC expressing OX40L at 2, 3 and 4 days post infection with Lm-2W1S. Data pooled from two idependent experiments, n=8. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

First signs of a decline in OX40 expression on 2W1S:I-A^{b+} CD4 T cells were observed at day 4 post infection with *Lm*-2W1S where its percentages decreased to 20% (Figure 4.3 C, E). The expression of OX40 on 2W1S:I-A^{b+} CD4 T cells was totally absent by day 7 post infection (data not shown, (Marriott et al., 2014)). Throughout this analysis the total numbers of 2W1S:I-A^{b+} CD4 T cells in infected WT mice were increasing steadily, following the course of infection (Figure 4.3 D).

Overall, the kinetics of OX40 expression on 2W1S:I-A^{b+} CD4 T cells post infection correlate strongly with the expression of OX40L on DC which begins a decline from day 2, reaching the baseline levels and being maintained by day 3 and 4 post infection (Figure 4.3 E, F).

These analyses have shown that both OX40 receptor and its ligand are expressed early in the response to Lm-2W1S, and in parallel with previously published data, we showed that OX40 is expressed by a proportion of antigen-specific CD4 T cells being most likely linked to antigenic stimulation.

4.2.2 Early production of IFNγ promotes upregulation of OX40L on DC

Following the observation that OX40L was rapidly upregulated on DC after infection with Lm-2W1S, we sought to understand the signals that controlled this process. Past studies have shown that expression of OX40L on DC can be readily upregulated by *in vitro* stimulation through a CD40 receptor (Murata et al., 2000a; Ohshima et al., 1997; Stüber et al., 1995). CD40 belongs to the TNFRSF family of costimulatory molecules and is expressed on B cells and DC, while its ligand (CD40L) is induced rapidly on activated T cells. The transient expression of the CD40L ranges anywhere between 4 to 72 hrs following an *in vitro* αCD3 mediated T cell activation (Lee et al., 2002). In fact, the expression of CD40 has been shown to be upregulated on DC among other maturation markers, like CD86 and CD80, at 24 hrs post infection with *L. monocytogenes* (Mitchell et al., 2011). However, there is no evidence of rapid expression of CD40L on *L. monocytogenes* activated T cells within the first 24 hrs of

Lm-2W1S infection. Other cells like NK cells and macrophages could be providing CD40L at this time, however our observations have indicated that *L. monocytogenes* drives a significantly higher OX40L upregulation on DC as opposed to the one seen with *in vitro* CD40 stimulation. This has led us to a hypothesis that other signals from innate immune cells could also be involved, aiding in the upregulation of OX40L on DC.

Production of IFNγ in *L. monocytogenes* induced infections and the following chain of cellular responses to this particular cytokine have been shown to be paramount for the effective clearance of the infection, as demonstrated in both mice treated with mAb to IFNγ or IFNγ$^{-/-}$ mice (Buchmeier and Schreiber, 1985; Dalton et al., 1993). We therefore aimed to investigate the role of IFNγ in the upregulation of OX40L on splenic DC at 24 hrs post Lm-2W1S infection. Firstly, we examined the IFNγ production at 24 hrs post Lm-2W1S infection, particularly focusing on the NK cell contribution as previous studies reported a rapid IFNγ release by activated NK cells in response to adjuvanted vaccines (Coccia et al., 2017). We therefore sought to provide further data on NK derived IFNγ production *in vivo* using the GS reporter mice.

The GS reporter mice were infected with Lm-2W1S and responses assessed in spleens 24 hrs later. The analyses revealed that comparing to the untreated GS controls, in GS mice infected with Lm-2W1S, NK cells were a source of substantial IFNγ expression, which accounted for approximately 50-60% of all identified splenic NK cells (Figure 4.4 A, B). Next, we assessed the contribution of NK cells to the total IFNγ expression present in GS mice at 24 hrs post Lm-2W1S infection. Gating on total eYFP expression (proportionate to the total IFNγ) showed that approximately 70% was represented by NK cells making them the dominant source of IFNγ (Figure 4.4 C, D). Other cells accounted for remaining 30% of total IFNγ (Figure 4.4 D).

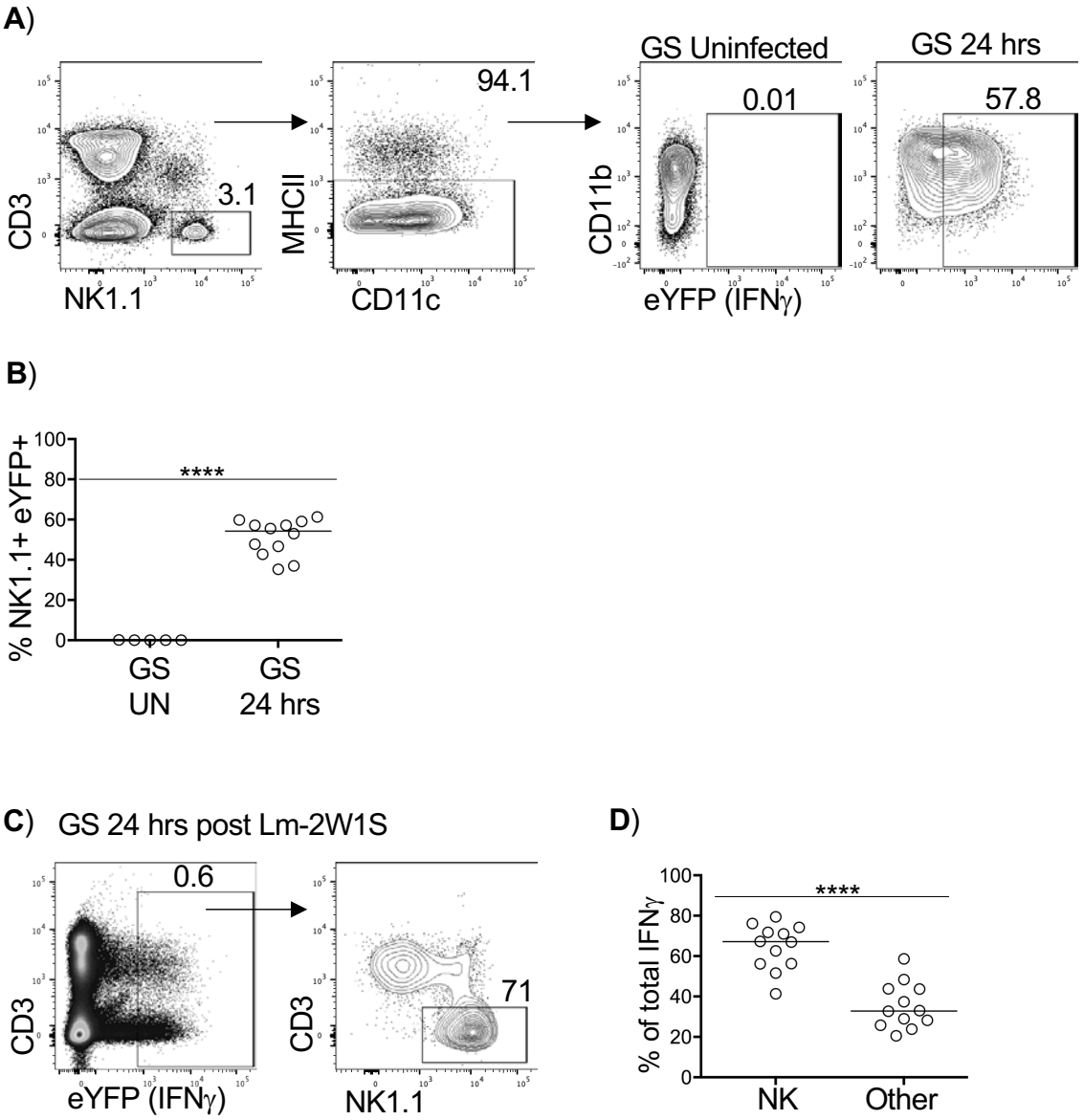

A)
CD3
NK1.1
3.1
MHCII
CD11c
94.1
GS Uninfected
GS 24 hrs
CD11b
eYFP (IFNγ)
0.01
57.8
B)
% NK1.1+ eYFP+
****
GS UN
GS 24 hrs
C) GS 24 hrs post Lm-2W1S
CD3
eYFP (IFNγ)
0.6
CD3
NK1.1
71
D)
% of total IFNγ
****
NK
Other

Figure 4. 4. NK cells are main producers of IFNγ 24 hrs post infection with Lm-2W1S

To investigate the early innate cytokine response to Lm-2W1S, GS reporter mice were used to assess IFNγ production at 24 hrs post infection. (**A**) Representative flow cytometry plots showing gating strategy and expression of IFNγ by NK cells ($CD3^{-}NK1.1^{+}MHCII^{-}$) (**B**) Percentage of NK cells expressing IFNγ (eYFP). (**C**) Representative flow cytometry plots showing expression of eYFP by different immune cells. (**D)** Percentage of total IFNγ attributable to NK cells or other immune cell populations. Data pooled from two independent experiments, n = 5 uninfected and n=12 Lm-2W1S infected mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

Having established that IFNγ was rapidly expressed in response to infection with Lm-2W1S and its high expression correlated with OX40L upregulation, we then sought to determine whether IFNγ was required for upregulation of OX40L on DC. This was especially important as past studies described a close relationship between NK and DC (Gerosa et al., 2002; Goldszmid et al., 2012).

To test the requirement for IFNγ in OX40L upregulation, WT and IFNγ$^{-/-}$ mice were infected with Lm-2W1S and DC expression of OX40L assessed 24 hrs later. The exact gating strategy was used to identify splenic DC in WT and IFNγ$^{-/-}$ mice (Figure 4.5 A). The analyses revealed an abrogation of OX40L upregulation on DC in the absence of IFNγ, as represented by a significant reduction (approximately 50% or 1.4-fold) in the percentage of DC expressing OX40L at 24 hrs post infection (Figure 4.5 B, C). This led us to a conclusion that early IFNγ plays an important role in the upregulation of OX40L on splenic DC.

Production of IFNγ is highly dependent on two important inducing cytokines, the interleukin 12 (IL-12) and interleukin 18 (IL-18). Despite previous efforts in targeting their synergism, the relationship between the two cytokines remains unclear. Past studies have shown that IL-12 upregulates the expression of IL-18 which is in turn required for IL-12 dependent activation of Th1 cells (Okamura et al., 1998). While IL-12 acts on NK cells and T cells, IL-18 is mainly a potent NK cell activation inducer (Micallef et al., 1997). Overall, the synergy between the two is required for the optimal production of IFNγ due to their differential role in transcriptional regulation (Barbulescu et al., 1998). Preceding reports have described the importance of IL-12 in responses to *L. monocytogenes*, showing that unlike live organisms, heat killed *L. monocytogenes* was only able to induce IFNγ when administered with recombinant IL-12 (Miller et al., 1995; Trinchieri, 1997). Additionally, neutralisation of IL-12 signals *in vivo* had a significant impact on the survivability of mice treated with sublethal dose of *L. monocytogenes* and the mice could be rescued by administration of recombinant IFNγ (Tripp et al., 1994).

Therefore, we decided to focus on the role of IL-12 in inducing IFNγ in responses to Lm-2W1S as well as its subsequent importance in upregulation of OX40L on DC at 24 hrs post infection. To investigate the requirement for IL-12, we infected IL-12p35$^{-/-}$ mice with Lm-2W1S and measured both the NK cell derived production of IFNγ as well as OX40L expression on splenic DC 24 hrs later.

As IL-12p35$^{-/-}$ mice did not express reporter properties, the assessment of IFNγ production by NK cells required the splenocytes to be cultured for 3 hrs in the presence of a protein transport inhibitor, BFA. NK cells were identified on the basis of their NK1.1 and EOMES expression (Figure 4.6 A). As described previously, the infection with Lm-2W1S induced IFNγ production by the NK cells, and expectedly the responses were absent in untreated WT controls (Figure 4.5 B). The impact of IL-12 deletion was evident as approximately 50% of the splenic NK cells failed to upregulate production of IFNγ (Figure 4.6 B, C). More importantly, the residual IFNγ expression by IL-12p35$^{-/-}$ NK cells was substantially lower than in WT NK cells, as evidence by a 3-fold reduction in the Mean Fluorescence Intensity (MFI) of IFNγ (Figure 4.6 D).

These data indicate that IL-12 plays an important role in NK expression of IFNγ in response to infection with Lm-2W1S. To ask whether DC isolated from these mice showed impaired OX40L expression, which would be expected given the reduced IFNγ production, DC isolated from spleens of infected WT controls and IL-12p35$^{-/-}$ mice were compared. A significant decrease in the percentage of splenic DC upregulating and expressing OX40L at 24 hrs post Lm-2W1S infection was observed in IL-12p35$^{-/-}$ mice (Figure 4.6 E, F).

Thus these analyses provided strong support for the role of IL-12 mediated production of IFNγ by the NK cells in the upregulation of OX40L on DC post infection with Lm-2W1S.

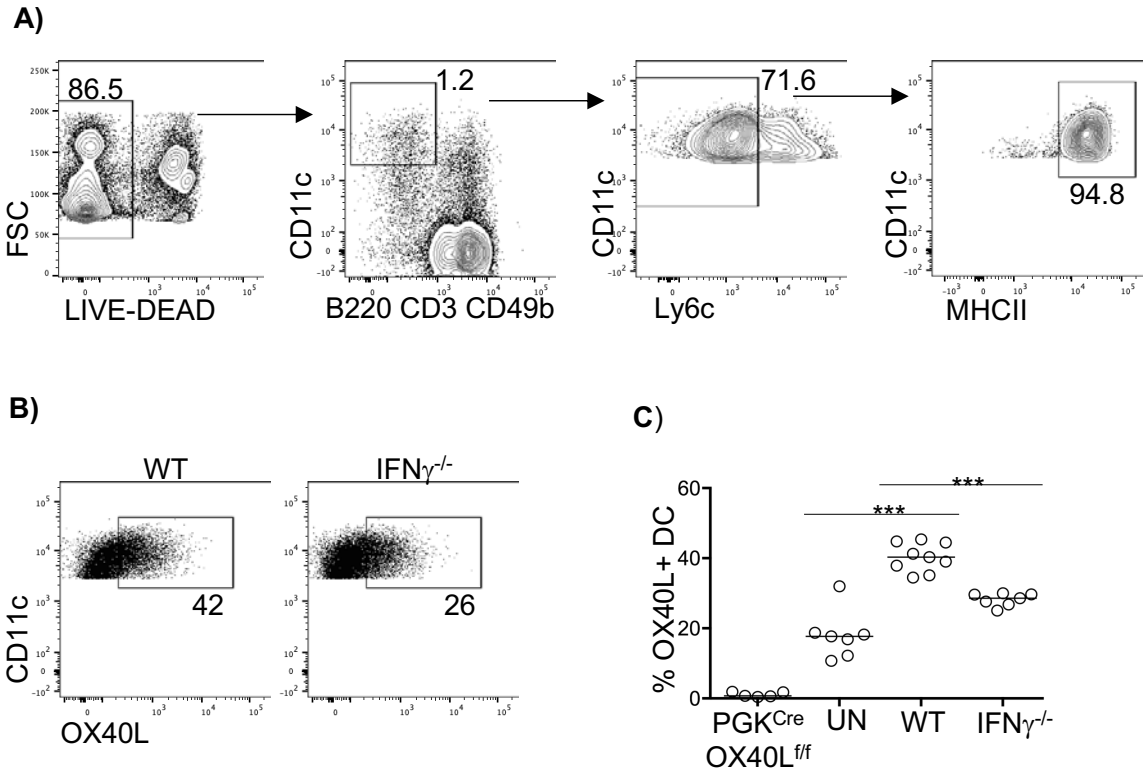

A)
86.5
FSC
LIVE-DEAD
1.2
CD11c
B220 CD3 CD49b
71.6
CD11c
Ly6c
CD11c
94.8
MHCII
B)
WT
IFNγ-/-
42
26
CD11c
OX40L
C)
% OX40L+ DC
60
40
20
0
***
***
PGKCre
OX40Lf/f
UN
WT
IFNγ-/-

Figure 4. 5 Early production of IFNγ is required for optimal expression of OX40L by DC 24 hrs post infection with Lm-2W1S

To test whether DC expression of OX40L was influenced by IFNγ, splenocytes from WT and IFNγ$^{-/-}$ mice were assessed for OX40L expression at 24 hrs post infection with Lm-2W1S, alongside PGK^{cre} x OX40L$^{f/f}$ and uninfected (UN) controls. (**A**) Gating strategy used to identify splenic DC (B220^{-}CD3^{-}CD49b^{-}, CD11c+, Ly6C^{low}, MHCII^{+}). (**B**) Representative flow cytometry plots showing expression of OX40L on DC in WT and IFNγ$^{-/-}$ mice. (**C**) Percentage of DC expressing OX40L in PGK^{cre} x OX40L$^{f/f}$, UN, WT and IFNγ$^{-/-}$ mice. Data pooled from two independent experiments, n=5 PGK^{cre} x OX40L$^{f/f}$, n=7 uninfected, n=9 WT, n=7 IFNγ$^{-/-}$. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance as tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

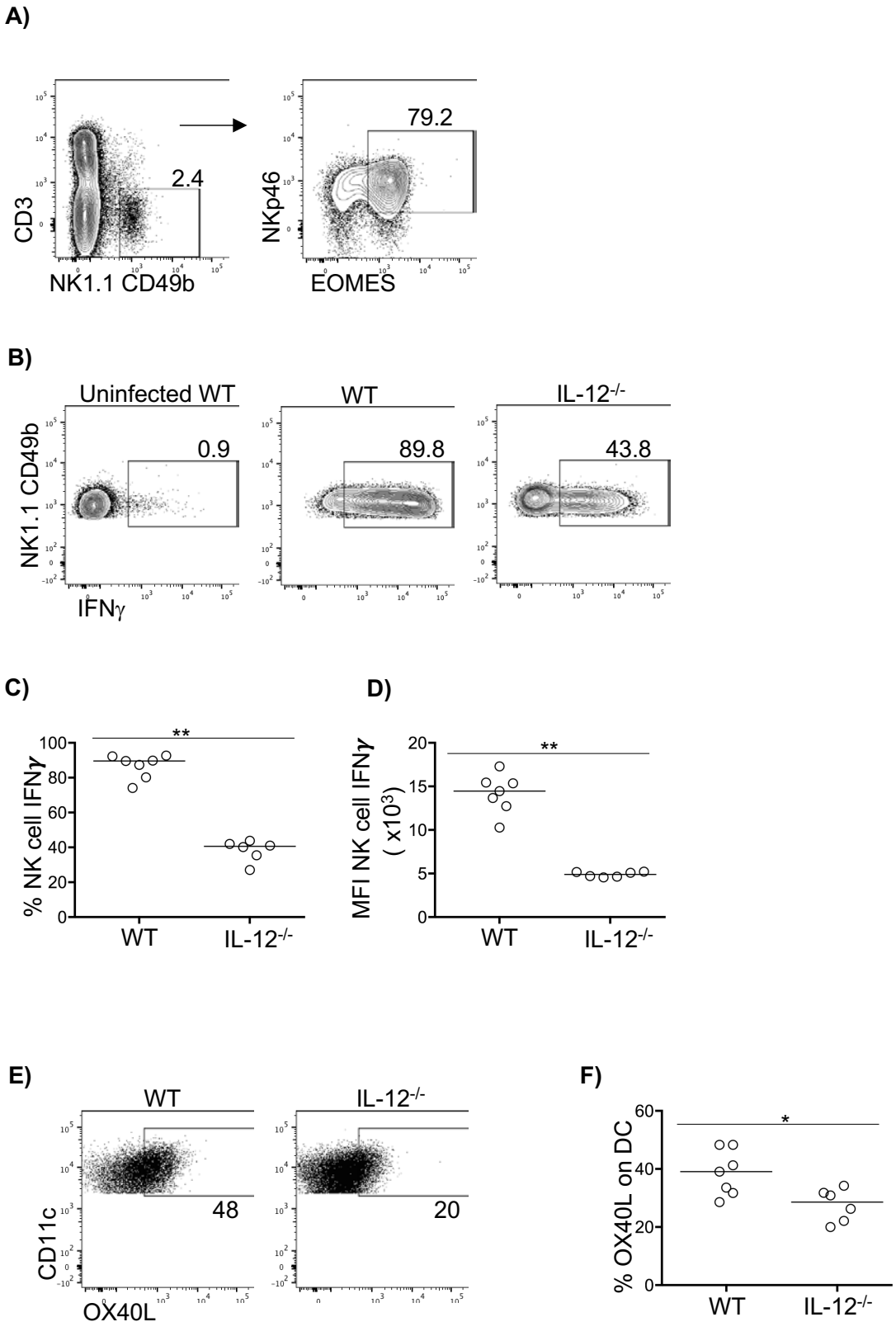

A)
CD3
NK1.1 CD49b
2.4
NKp46
EOMES
79.2
B)
Uninfected WT
WT
IL-12-/-
NK1.1 CD49b
IFNγ
0.9
89.8
43.8
C)
% NK cell IFNγ
WT
IL-12-/-
**
D)
MFI NK cell IFNγ
(x10³)
WT
IL-12-/-
**
E)
WT
IL-12-/-
CD11c
OX40L
48
20
F)
% OX40L on DC
WT
IL-12-/-
*

Figure 4. 6 OX40L is upregulated on DC in response to IL-12-mediated production of IFNγ by NK cells

To determine whether production of IFNγ by NK cells is promoted by IL-12, the WT and IL-12p35$^{-/-}$ mice were infected with Lm-2W1S and responses were assessed 24 hrs later. (**A**) Gating strategy used to identify NK cells. (**B**) Representative flow cytometry plots showing production of IFNγ by NK cells from uninfected WT and infected WT and IL-12p35$^{-/-}$ mice 24 hrs post Lm-2W1S. (**C,D**) Proportion of NK cells producing IFNγ and their MFI at 24 hrs post infection with Lm-2W1S. (**E**) Representative flow cytometry plots showing OX40L expression on DC (B220^{-}CD3^{-}CD49b^{-}, CD11c+,. Ly6C^{low}, MHCII^{+}) in WT and IL-12p35$^{-/-}$ mice 24 hrs post infection with Lm-2W1S. (**F**) Percentage of DC expressing OX40L in WT and IL-12p35$^{-/-}$ mice 24 hrs post infection with Lm-2W1S. Data from 1 experiment, n=7 WT, n=6 IL-12p35$^{-/-}$. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

4.2.3 Production of IFNγ by iNKT cells is not necessary for the upregulation of OX40L expression on DC

NK cells can be activated by a variety of signals, including stimulation they receive from NKT cells. NKT cells represent a unique subset of T cells, which express rearranged TCR and also share properties with NK cells, like the expression of NK1.1 cell surface protein. Their TCR repertoire is extremely limited and specifically designed to bind glycolipids, lipids and some proteins presented by CD1d molecules (Park and Bendelac, 2000; Porcelli and Modlin, 2002). NKT cells are thought to be able to drive NK cell responses and the sequential activation of NK cells by NKT cells has been shown in studies using the glycolipid α-galactosylceramide, α-GalCer which *in vivo* resulted in NK cell activation measured by induction of CD69 expression and IFNγ production (Carnaud et al., 1999). This effect was however absent in $Rag^{-/-}$ and $CD1d^{-/-}$ mice, indicating the role of NKT cells in NK cell activation.

Considering these findings, we hypothesised that NKT cells were involved in early activation of NK cells, their expression of IFNγ and the upregulated OX40L on splenic DC.

To investigate whether this crosstalk occurred, we first infected GS mice to compare the contribution of NKT cells and NK cells to the production of IFNγ at 6 and 24 hrs post Lm-2W1S infection. NK cells were identified as previously, while the NKT cells were described as $CD3^{+}NK1.1^{+}CD1d^{+}$ (Figure 4.7 A). Staining for the CD1d was validated using a control tetramer. The assessments at 6 hrs post Lm-2W1S failed to detect IFNγ expression by NK cells (Figure 4.7 B, F). In contrast, the NKT cells showed small but evident upregulation of IFNγ production, which accounted for 15 % (Figure 4.7 C, F). Interestingly, at 24 hrs post Lm-2W1S infection, among both NK cell and NKT cell populations, approximately 50 % of the cells were reporting IFNγ (Figure 4.7 D, E, G). Finally, we assessed the NK and NKT contribution to the total IFNγ production at 6 hrs and 24 hrs post Lm-2W1S. Indeed, NKT cells were involved in IFNγ production at 6 hrs as compared to the NK cells (Figure 4.7 H).

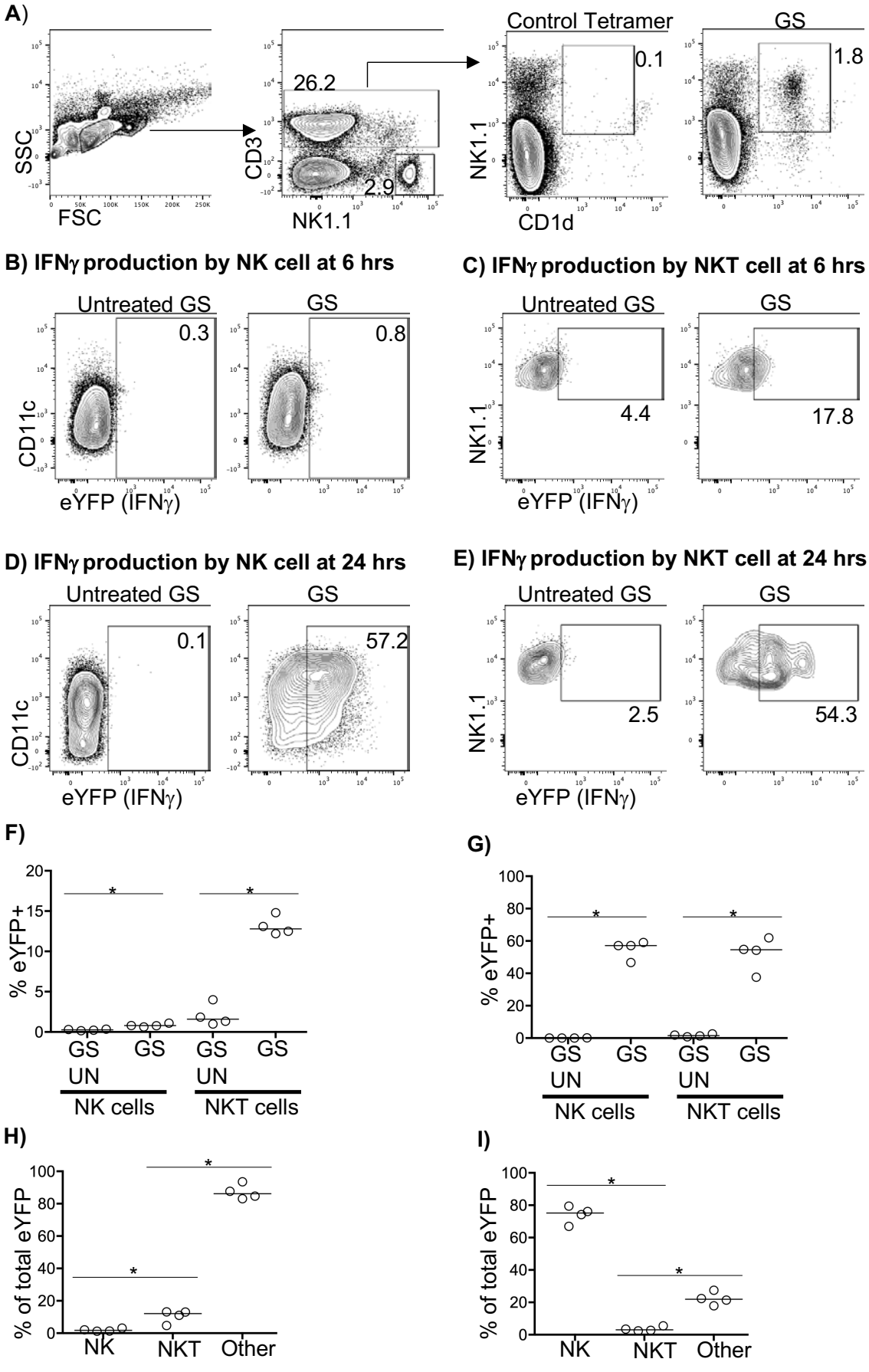

A)
SSC
FSC
CD3
NK1.1
26.2
2.9
Control Tetramer
GS
0.1
1.8
NK1.1
CD1d
B) IFNγ production by NK cell at 6 hrs
Untreated GS
GS
0.3
0.8
CD11c
eYFP (IFNγ)
C) IFNγ production by NKT cell at 6 hrs
Untreated GS
GS
4.4
17.8
NK1.1
eYFP (IFNγ)
D) IFNγ production by NK cell at 24 hrs
Untreated GS
GS
0.1
57.2
CD11c
eYFP (IFNγ)
E) IFNγ production by NKT cell at 24 hrs
Untreated GS
GS
2.5
54.3
NK1.1
eYFP (IFNγ)
F)
% eYFP+
GS
GS
GS
GS
UN
UN
NK cells
NKT cells
G)
% eYFP+
GS
GS
GS
GS
UN
UN
NK cells
NKT cells
H)
% of total eYFP
NK
NKT
Other
I)
% of total eYFP
NK
NKT
Other

Figure 4. 7 NKT cells are among IFNγ producers at 6 and 24 hrs post infection with Lm-2W1S

To investigate the possible contribution of the NKT cells to the production of IFNγ by NK cells, the GS reporter mice were infected with Lm-2W1S and the responses assessed at 6 and 24 hrs post infection. (**A**) Representative flow cytometry plots showing gating strategy to identify splenic NK and NKT cells. (**B, C**) Representative flow cytometry plots showing expression of IFNγ by NK cells and by NKT cell at 6 hrs post infection. (**D, E**) Representative flow cytometry plots showing expression of IFNγ by NK cells and by NKT T cells at 24 hrs post infection. (**F**) Percentage of NKT vs NK cells expressing eYFP at 6 hrs. (**G**) Percentage of NKT vs NK cells expressing eYFP at 24 hrs. (**H**) Percentage of total eYFP attributable to NK cells, NKT cells and other immune cell populations at 6 hrs. (**I**) Percentage of total IFNγ attributable to NK, NKT cells and other immune cell populations at 24 hrs. Data represents one experiment, n =4 uninfected and n=4 Lm-2W1S infected mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

However, other cells like for instance γδ T cells contributed to the 80% of the total IFNγ production (personal communication, Ameenah Zeglam, fellow PhD student). Furthermore, assessments of the total IFNγ production at 24 hrs showed that despite 50% of the NKT cells were producing IFNγ, comparing to the dominant NK cell population, the contribution from NKT cells was minimal (Figure 4.7 I).

Having established that NK cells were the most numerous IFNγ producing population, we asked whether the early NKT cell response was required to fuel NK cell activation.

To answer this, we infected WT, CD1d$^{-/-}$ and IFNγ$^{-/-}$ mice with Lm-2W1S and assessed NK cell IFNγ expression 24 hrs later. Similarly, to the previous experiments, the splenocytes were first cultured with BFA for 3 hrs to enhance and preserve the IFNγ expression due to the lack of reporter mice. The identified NK cells from IFNγ$^{-/-}$ mice were used as negative controls for the IFNγ expression (Figure 4.8 A, B). Strikingly, the NK cells from WT and CD1d$^{-/-}$ mice showed comparable IFNγ expression, fluctuating at around 90% for both strains (Figure 4.8 B, C). Thus, these data strongly indicate that NKT cells are not absolutely required for the early IFNγ response produced by NK cells after infection with Lm-2W1S.

We further asked whether OX40L expression on DC was impaired in the absence of NKT cells. Consistent with normal expression of IFNγ, no impairment in the expression of OX40L on DC was detected in the absence of NKT cells (Figure 4.8 D). Both WT and CD1d$^{-/-}$ mice showed comparable percentage of DC upregulating OX40L at 24 hrs post Lm-2W1S (Figure 4.8 E). Combined, these data demonstrate that crosstalk between NK and NKT cells and DC was not required of normal expression of IFNγ by NK cells nor OX40L upregulation by DC.

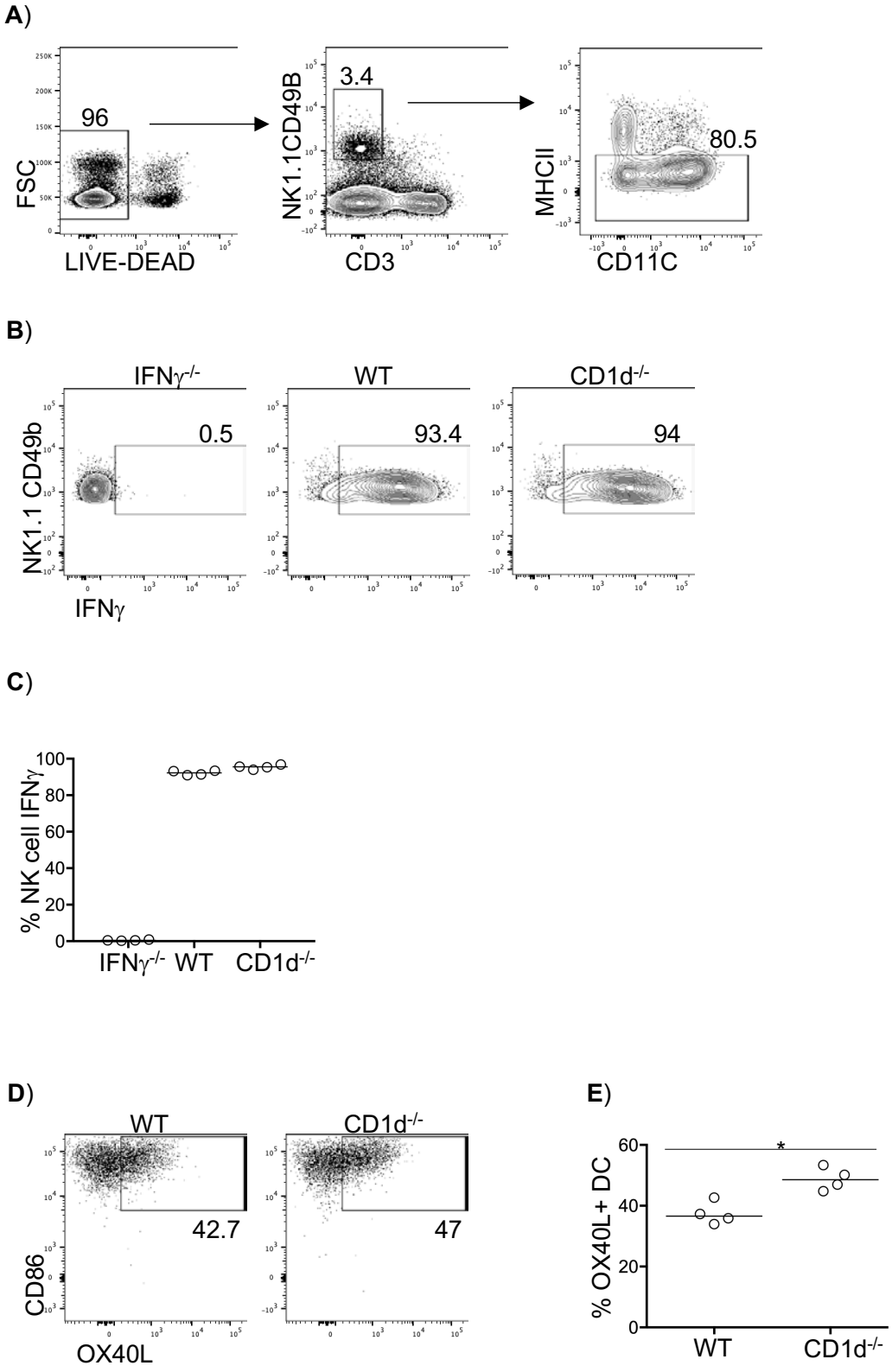

A)
96
FSC
LIVE-DEAD
3.4
NK1.1CD49B
CD3
80.5
MHCII
CD11C
B)
IFNγ-/-
WT
CD1d-/-
0.5
93.4
94
NK1.1 CD49b
IFNγ
C)
% NK cell IFNγ
100
80
60
40
20
0
IFNγ-/-
WT
CD1d-/-
D)
WT
CD1d-/-
42.7
47
CD86
OX40L
E)
% OX40L+ DC
60
40
20
0
*
WT
CD1d-/-

Figure 4. 8 NKT cells do not contribute to the NK derived IFNγ production at 24 hrs post infection with Lm-2W1S and their absence has no effect on OX40L upregulation on DC

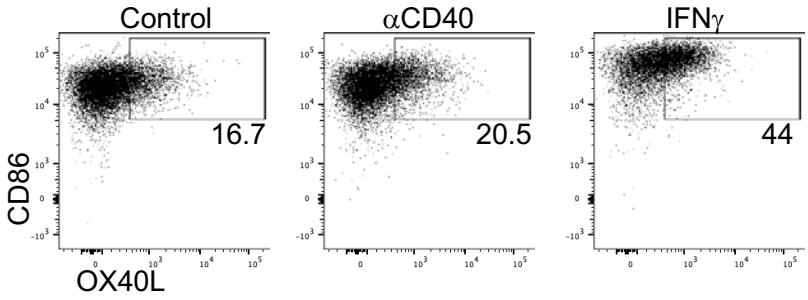
To determine whether NKT cells were required for IFNγ$^{-/-}$ production by NK cells, the CD1d$^{-/-}$ and WT mice were infected with Lm-2W1S and responses at 24 hrs post infection were assessed. The splenocytes were cultured for 3 hrs with BFA or overnight to assess IFNγ and OX40L expression, respectively. (**A**) Gating strategy used to identify NK cells. (**B**) Representative flow cytometry plots showing expression of IFNγ by splenic NK cells from IFNγ$^{-/-}$, WT and CD1d$^{-/-}$ mice at 24 hrs post infection with Lm-2W1S. (**C**) Percentage of NK cells expressing IFNγ. (**D**) Expression of OX40L by DC (B220^{-}CD3^{-}CD49b^{-}, CD11c+, Ly6C^{low}, MHCII^{+}) from WT and CD1d$^{-/-}$ mice at 24 hrs post infection with Lm-2W1S. (**E**) Percentage of DC expressing OX40L in WT and CD1d$^{-/-}$ mice 24 hrs after infection with Lm-2W1S. Data from 1 experiment, n=4. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

4.2.4. IFNγ signals directly via the IFNγR on DC to upregulate expression of OX40L

IFNγ, as an important immunomodulatory cytokine, provokes a wide range of biological effects on various cell types. Its role in modulating DC maturation and function has already been widely studied. For instance, *in vitro* and *in vivo* stimulation with recombinant IFNγ has been shown to lead to an upregulation of CD86, CD80, CD40 and MHCII and provided assistance in TLR stimulation (Sheng et al., 2013a). Furthermore, an autocrine mechanisms for DC function has been observed, with the overexpression of IFNγ leading to upregulation of CD40, CD54, CD80, CD86 and CCR7 as well as having enhancing effect on IL-1β and IL-12 production, while the lack of IFNγR led to an impairment of DC function (Pan et al., 2004). Since responsiveness to IFNγ is crucial for the clearance of *L. monocytogenes* infections and as shown in previous sections IFNγ does play an important role in upregulation of OX40L on splenic DC, we were interested to explore the possible direct role of IFNγ on the expression of OX40L by splenic DC via the interaction with IFNγR.

Firstly, we aimed to recapitulate our previous findings in *in vitro* settings and assess whether recombinant IFNγ could directly upregulate OX40L on DC. To do so, we cultured WT splenocytes *in vitro* with recombinant IFNγ alongside anti-CD40 Abs as a positive control. As anticipated, stimulation with IFNγ did have a profound effect on upregulation of OX40L on DC, significantly increasing the percentage of $OX40L^+$ DC by approximately 50% as compared to the untreated controls (Figure 4.9 A, B). When compared to the splenocytes stimulated with αCD40, it was evident that IFNγ enhanced further the upregulation of OX40L on DC, however the statistical tests used showed no significance. These analyses indicate that recombinant IFNγ is sufficient for the upregulation of OX40L on DC *in vivo*.

A)


Control
αCD40
IFNγ
16.7
20.5
44
CD86
OX40L

B)

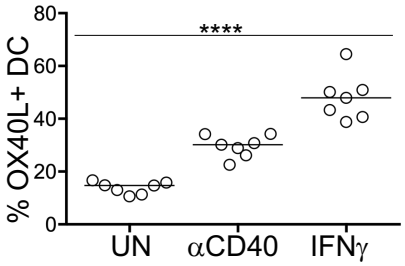

****
% OX40L+ DC
80
60
40
20
0
UN
αCD40
IFNγ

Figure 4. 9 Recombinant IFNγ directly enhances OX40L expression on DC

To determine the mechanisms of how IFNγ contributed to the upregulation of OX40L on DC, *in vitro* experiments to test a direct interaction were performed. (**A**) Representative flow cytometry plots showing expression of OX40L by DC ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, $MHCII^{+}$) isolated from WT mice and cultured with either αCD40 or recombinant IFNγ. (**B**) Proportion of DC expressing OX40L after culture with recombinant IFNγ. Data from 2 independent experiments, n= 7 uninfected control (UN) and n = 7 αCD40 and n=7 IFNγ. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

To test whether IFNγ signalled directly to DC *in vivo*, CD11c^{cre} x IFNγR$^{f/f}$ mice were generated to enable the conditional deletion of IFNγR on DC. To confirm the robust and specific deletion of the IFNγR on splenic DC, cells isolated from WT versus CD11c^{cre} x IFNγR$^{f/f}$ mice were compared. The DC were identified as CD11c^{+}, MHCII^{+} and the analysis of their expression of IFNγR in WT mice showed that approximately 80% of all splenic DC were positive for IFNγR at steady state (Figure 4.10 A, B, C). FMO controls for the IFNγR were used to set accurate gates when analysing the data. In contrast to WT mice, the expression of IFNγR in CD11c^{cre} x IFNγR$^{f/f}$ mice was severely reduced, indicating the successful incorporation of the gene construct and the predicted phenotype (Figure 4.10 B, C). We were however cautious about the NK cell expression of IFNγR due to the significant level of the CD11c expression found on these cells. Flow cytometric analysis showed that around 90% of NK cells in WT mice, described as CD3^{-}NK1.1^{+}, expressed IFNγR at steady state as compared to the FMO controls (Figure 4.10 D, E, F). The analysis of the IFNγR expression on NK cells in CD11c^{cre} x IFNγR$^{f/f}$ mice showed a significant and approximately 20% reduction in the percentage of IFNγR^{+} NK cells (Figure 4.10 D, E, F). Thus, whilst there was a modest reduction in the total proportion of NK cells expressing the IFNγR in CD11c^{cre} x IFNγR$^{f/f}$ mice, this model did allow us to assess the requirement for DC expression of IFNγR which was very robustly deleted.

To test the direct effect of IFNγR stimulation on the upregulation of OX40L on DC, we infected the CD11c^{cre} x IFNγR$^{f/f}$ alongside the CD11c^{cre} and IFNγ$^{-/-}$ controls with *Lm*-2W1S. Our observations revealed that, compared with control mice, splenic DC isolated from CD11c^{cre} x IFNγR$^{f/f}$ mice expressed significantly less OX40L on DC which was comparable to the defect we have previously reported in the IFNγ$^{-/-}$ mice and recapitulated in this instance as well (Figure 4.11 A, B).

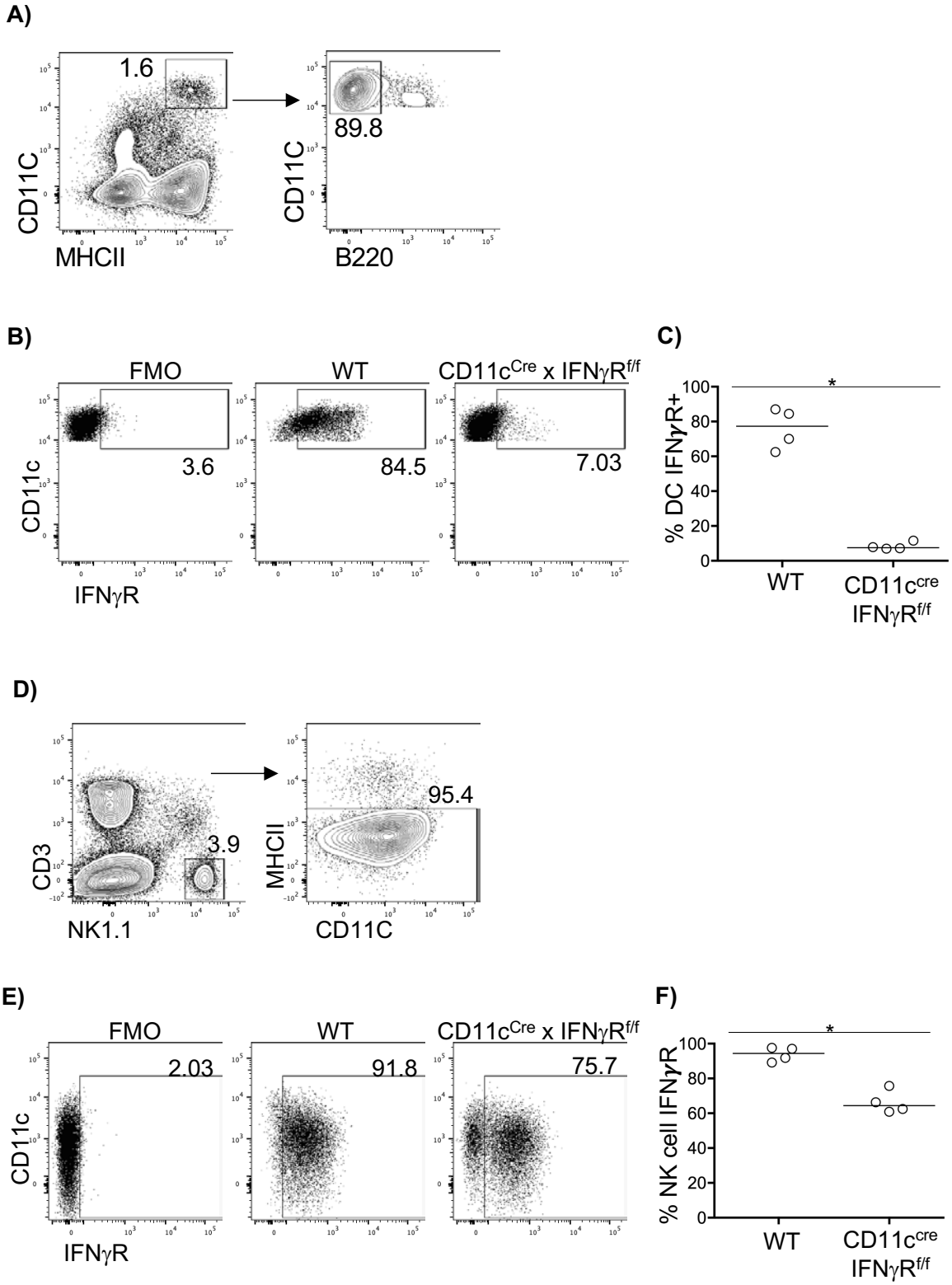
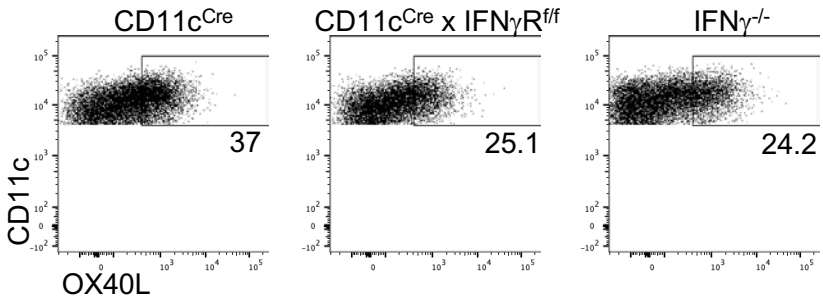

A)
1.6
CD11C
MHCII
89.8
CD11C
B220
B)
FMO
WT
CD11c^Cre x IFNγR^f/f
3.6
84.5
7.03
CD11c
IFNγR
C)
% DC IFNγR+
100
80
60
40
20
0
*
WT
CD11c^cre IFNγR^f/f
D)
3.9
CD3
NK1.1
95.4
MHCII
CD11C
E)
FMO
WT
CD11c^Cre x IFNγR^f/f
2.03
91.8
75.7
CD11c
IFNγR
F)
% NK cell IFNγR
100
80
60
40
20
0
*
WT
CD11c^cre IFNγR^f/f

Figure 4. 10 Analysis of IFNγR expression in conditional IFNγR deficient mice

To assess the DC specific deletion of IFNγR, splenocytes from $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice were stained for its expression on DC versus NK cells. (**A**) Gating strategy used to identify splenic DC. (**B**) Expression of IFNγR on splenic DC ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, $MHCII^{+}$) in WT and $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice. (**C**) Proportion of DC expressing IFNγR in WT versus $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice. (**D**) Gating strategy used to identify splenic NK cells. (**E**) Expression of IFNγR on splenic NK cells in WT and $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice. (**F**) Proportion of NK cells expressing IFNγR in WT versus $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice. Data representative of two independent experiments, n=4. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

A)


CD11c^Cre
CD11c^Cre x IFNγR^f/f
IFNγ^-/-
37
25.1
24.2
CD11c
OX40L

B)

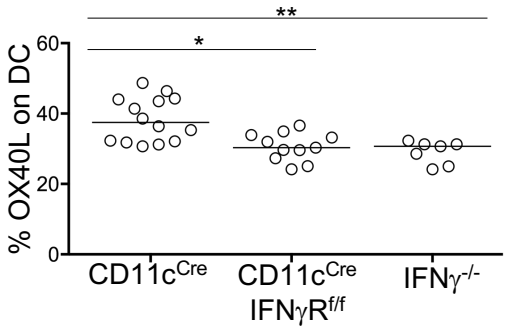

**
*
% OX40L on DC
60
40
20
0
CD11c^Cre
CD11c^Cre IFNγR^f/f
IFNγ^-/-

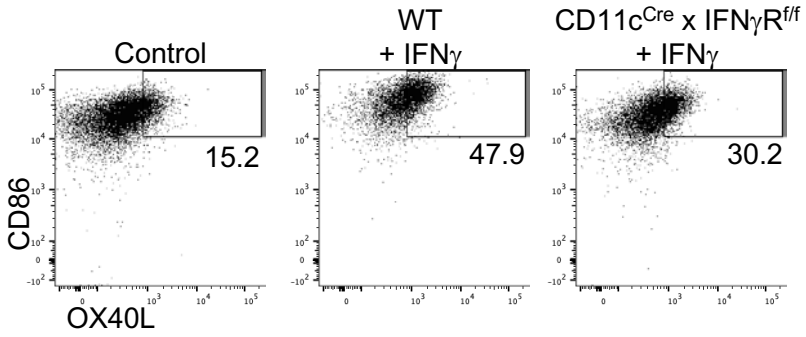
Figure 4. 11 Early IFNγ directly enhances OX40L expression on DC via IFNγR

In vivo experiments were performed to test a direct interaction between IFNγ and the upregulation of OX40L on DC. (**A**) Representative flow cytometry plots showing expression of OX40L on DC ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, $MHCII^{+}$) from $CD11c^{Cre}$, $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ and $IFN\gamma^{-/-}$ mice 24 hrs after infection with Lm-2W1S. (**B**) Proportion of DC expressing OX40L at 24 hrs post infection with Lm-2W1S. Data pooled from three independent experiments, n = 14 $CD11c^{Cre}$, n = 11 $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$, n=7 $IFN\gamma^{-/-}$. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

Finally, we stimulated splenocytes isolated from CD11c^{cre} x IFNγR$^{f/f}$ mice *in vitro* with recombinant IFNγ. Like above, the result clearly indicated an IFNγ dependent OX40L upregulation on DC via the IFNγR. In comparison to the WT mice undergoing the same treatment, the CD11c^{cre} x IFNγR$^{f/f}$ mice failed to upregulate OX40L on DC, represented by a significant reduction in the percentage of OX40L^{+} DC post overnight culture (Figure 4.12.A, B).

In summary, here we have provided a detailed *in vitro* and *in vivo* analysis of the OX40L upregulation on DC post infection with Lm-2W1S as well as the mechanisms that are crucial for its optimal expression. We concluded that early IFNγ, predominantly produced by the NK cells in an IL-12 dependent manner, is critical for optimal OX40L on DC and it acts directly on DC via the expression of IFNγR.

A)


Control
WT
+ IFNγ
CD11c^Cre x IFNγR^f/f
+ IFNγ
15.2
47.9
30.2
CD86
OX40L

B)

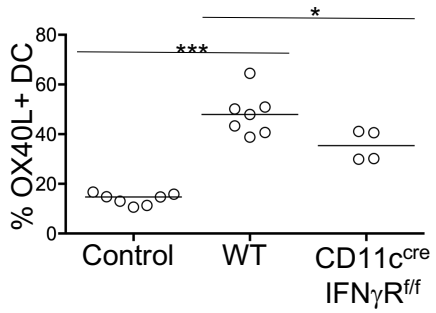

*
***
% OX40L+ DC
80
60
40
20
0
Control
WT
CD11c^cre
IFNγR^f/f

Figure 4. 12 DC can upregulate OX40L directly via IFNγR stimulation in response to recombinant IFNγ

In vitro experiments using recombinant IFNγ were performed in $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ to further support evidence for the direct interaction between IFNγ and upregulation of OX40L on DC. **(A**) Representative flow cytometry plots showing expression of OX40L by DC isolated from WT versus $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice and cultured with recombinant IFNγ. (**B**) Proportion of DC expressing OX40L after culture with recombinant IFNγ. Data from two independent experiments , n= 7 Control, n=7 WT and n = 4 $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

4.3 Discussion

The immune responses to bacterial infections involve a close interaction between the innate and adaptive immune systems. The adaptive immune system and its precision and immunological memory rely heavily on elements of the innate immune system for the efficient rapid sensing and recognition of pathogens, attempted control and for the initiation and direction of immune responses. One of the crucial properties of the innate immune cells is their ability to provide costimulatory signals which are particularly important for T cell activation. Despite the considerable amount of knowledge of how major costimulatory molecules influence the course of immune responses, it is crucial to investigate who expresses them and how they are regulated which would facilitate their careful manipulation therapeutically.

In the previous chapter we have provided detailed analyses of the CD4 T cells responses in various infection models, demonstrating the crucial role of OX40 signalling in promoting effector T cell responses to a range of Th1 responses across multiple tissues and routes of antigen exposure. In this chapter we presented data which aimed to explore the mechanisms behind OX40L upregulation at early stages of Lm-2W1S responses and identify possible stimulatory signals which enhance this crucial signalling pathway. We found that OX40L was upregulated on DC at 24 hrs post infection with Lm-2W1S in IFNγ and IL-12 dependent manner. The innate IFNγ signalled directly via the IFNγR to the DC to induce OX40L expression. We also demonstrated that within that time period, the dominant producers of innate IFNγ were NK cells.

4.3.1 DC expression of OX40L post infection with Lm-2W1S

We have focused our analyses on DC as they are one of the main providers of OX40L signals as well as being one of the myeloid cells responding at early stages of *L. monocytogenes* infection. The innate responses to *L. monocytogenes* administered via intravenous route start

within the spleen where monocyte mediated phagocytosis takes place. The intravital snapshots of infected spleens have detected bacteria within the first 2 minutes of injection (Waite et al., 2011).

In the marginal zones of the spleen, resident macrophages, DC and neutrophils facilitate the uptake of bacteria and subsequently begin the immune response. Neutrophils kill the bacteria by inducing production of reactive nitrogen and oxygen species, while resident red pulp macrophages as well as Kupffer cells in the liver can engulf the bacteria within the first minutes of the infection and are known to secrete TNFα and IL-12 which act synergistically to activate NK cells and induce their production of IFNγ (Babior et al., 1973; Tripp et al., 1994). TNFα and IFNγ are also important for the optimal activation of macrophages, as shown by impaired responses to *L. monocytogenes* post treatment with TNFα monoclonal antibodies and increase in MHCII expression post stimulation with IFNγ (Denis, 1991; Dunn and North, 1991; Higginbotham et al., 1992; van Furth et al., 1994).

Once entered via the marginal zones, *L. monocytogenes* taken up by the myeloid cells which then migrate into the white pulps of the spleen, particularly the PALS, where it has been found in close association with splenic DC. The crucial role of DC in controlling *L. monocytogenes* infections has been demonstrated in CD11c-DTR mice, which upon diptheria toxin (DT) treatment lacked $CD8^+$ DC and could not control the spread and proliferation of bacteria (Neuenhahn et al., 2006). Similarly experiments in $Batf3^{-/-}$ knockout mice emphasised the importance of $CD8^+$ DC in harbouring the bacteria and allowing its entry into the spleen, however have also been associated with increased resistance to the *L. monocytogenes* infection (Edelson et al., 2011).

Being located within the T cell zones, Lm-activated DC are at the ideal position to present antigens and support the T cells activation via costimulatory pathways. Our investigation has shown that OX40L expression on DC was upregulated at 24 hrs post infection with Lm-2W1S, which gradually decrease reaching baseline levels at 72 hrs. Interestingly we were unable to

obtain OX40L expression on freshly isolated DC and therefore systematically throughout all analyses performed under this investigation OX40L expression was always analysed following an overnight culture. These observations correlate with previously published data showing a significant increase in OX40L and CD40 expression on lung DC, following isolation with additional adherence steps and overnight culturing (Wilkes et al., 2014). Past studies identified several reasons for this phenomenon, particularly the negative action of proteolytic enzymes, like collagense D or dispase which have been shown to affect expression of several surface proteins (Merad et al., 2013). Expression of CD40L, CD11c and CD8 is readily reduced by the action of dispase, while collagenase D can reduce CD27 expression (Autengruber et al., 2012). However, there is no evidence that any of those enzymes has any effect on OX40L expression. Similar results were previously observed by the Lane lab who showed that preparation of splenic DC in the absence of any enzyme did not rescue OX40L expression and that detection of OX40L on ILC3 is only possible after overnight culture (M. Kim et al., 2005; Kim et al., 2003). Therefore, the most probable reason for the culturing requirement was the enhancing effect of overnight culture on DC maturation. A potential technical solution to these issues would be a OX40L reporter mouse, which would enable tracking OX40L expression on any cell type with no further manipulation.

The assessment of the type of DC expressing OX40L post infection with Lm-2W1S, revealed that this population predominantly consisted of cDC2 cells, showing a reduction in the proportion of cDC1 cells. The culture settings or digestion protocols had a minimal effect on the cDC1 population as shown by the significantly higher percentage in uninfected mice which were treated with the same enzymes and also cultured overnight. This contradicts the previously published data on CD8 DC involvement in the control of *L. monocytogenes* infections, yet other past reports have also observed a reduction in CD8 DC with some suggesting that they were prone to *L. monocytogenes* induced apoptosis (Edelson et al., 2011; Mitchell et al., 2011). Similar reduction in cDC1 type DC post Lm-2W1S infection was

observed by one of our collaborators (personal discussion with Dr Audrey Gerard – Senior Research Fellow at Kennedy Institute for Rheumatology, Oxford University).

To fully understand the kinetics of OX40L upregulation on DC we have also conducted simultaneous analyses of OX40 expression on 2W1S specific T cells. Consistent with previous data from our lab, we have shown that approximately 40% of 2W1S specific T cells express OX40 at D2 and D3 post infection with Lm-2W1S which was significantly reduced by D4. The expression of OX40 is transient and heavily tied to TCR and CD28 signalling hence in this case most probably reflected the acute nature of the Lm-2W1S infection which is usually cleared by D7 (Flynn et al., 1998). The previous study demonstrated that this clearance coincided with the absolute loss of OX40 expression on 2W1S-specific T cells (Marriott et al., 2014). In more chronic infections, OX40 can be maintained for prolonged periods of time (Boettler et al., 2012). Our data also revealed that cells with the highest expression of OX40 were also positive for the CD25 expression, characteristic for Th1 effector T cells, which could explain some of our data from Chapter 3 where we showed a specific effector T cell defect in mice lacking OX40 receptor. Similar findings on expression of CD25 by a portion of 2W1S-specific CD4 T cells and it co-expression with OX40 were previously published (Marriott et al., 2014; Pepper et al., 2011).

4.3.2 Regulation of OX40L expression on DC

Regulation of OX40L expression *in vivo* is not well understood, reflecting challenges in studying its expression without *in vitro* manipulation. Many reports have demonstrated the importance of CD40 signalling in upregulation of OX40L on DC in various experimental models (Dannull et al., 2005; Jenkins et al., 2007; Ohshima et al., 1997). Further reports also identified TNFα as a potent stimulant of DC maturation which also correlated with OX40L expression (Zaini et al., 2007a). Similarly a recent study showed that DC from GM-CSF treated mice induced expansion of Tregs *in vivo* in a OX40L dependent manner (Marinelarena

et al., 2018). Our investigation, however, has revealed that other signals could also upregulate OX40L. We were able to demonstrate that optimal DC OX40L expression at 24 hrs post infection with Lm-2W1S was dependent on the early IFNγ signals, demonstrating the crucial role of IFNγ in controlling primary responses to *L. monocytogenes* infection. Using IFNγ$^{-/-}$ mice, we found that *ex vivo* OX40L expression on DC was impaired in the absence of IFNγ signals. We have conducted further analyses using a reductionist approach of simply culturing splenocytes with recombinant IFNγ which resulted in greater expression of OX40L on DC than the one induced by CD40 alone. This argues that as well as being IFNγ dependent, IFNγ might directly impact DC. Consistently, other past study also demonstrated a stimulatory effect of IFNγ on DC maturation which induced expression of CD86, CD80, MHCII and CD40 aiding TLR stimulation (Sheng et al., 2013).

4.3.3 Who provides IFNγ *in vivo* in responses to Lm-2W1S

To better understand the role of IFNγ in DC OX40L expression we investigated the possible sources of this cytokine *in vivo* post infection with Lm-2W1S. Previous studies have shown that main sources of IFNγ included mainly NK cells, but other cells like CD8 T cells and γδT cells, iNKT cells and DCs also contributed (Bancroft, 1993; Berg et al., 2005; Hiromatsu et al., 1992; Kang et al., 2008; S. H. Lee et al., 2013). Our studies also identify a number of possible sources of IFNγ, although numerically NK cells are dominant IFNγ producers at 24 hrs post infection with Lm-2W1S. We additionally performed detailed analysis of the iNKT cell contribution to the total IFNγ production, which in comparison to the NK cells, was insignificant. Although iNKT cells were dominant IFNγ producers at 6 hrs post infection, their total contribution was still minimal. Those experiments benefited greatly from the use of GS mice which provided unmanipulated way of reporting for IFNγ, however later experiments with

assessing IFNγ at the protein level in WT post *ex vivo* culture with BFA also showed consistent results.

The effector function of NK cells can be mediated by many signals, although some studies highlighted the crucial role of iNKT cells and DCs in NK cells activation. *In vivo* stimulation with α-GalCer have been previously shown to induced iNKT cells to promote activation of NK cells, as $CD1d^{-/-}$ failed to generate responses (Carnaud et al., 1999). We demonstrated that in responses to Lm-2W1S, the iNKT cells were redundant in providing stimulatory signals to NK cells, as $CD1d^{-/-}$ mice responded as well as the WT mice did, including showing no impairment in neither the NK cell IFNγ expression nor upregulation of OX40L expression on DC. One past study investigating *L. monocytogenes* responses drew the same conclusions, demonstrating that iNKT cells were not essential for optimal responses (Ranson et al., 2005). The importance of DC in the activation of NK cell effector functions was shown by co-migration of the two cell types into the PALS following infection with *L. monocytogenes* (Carnaud et al., 1999). At 24 hrs post infection with *L. monocytogenes*, myeloid cells and NK cells were found clustered together within the T cell zones of the white pulp with the formation of these clusters being crucial for the NK derived production of IFNγ (Kang et al., 2008). Similarly, depletion of DC also resulted in the loss of IFNγ expression by NK cells. Other studies showed that *in vitro* co cultures of bone marrow derived DCs infected with *Lm* and naïve splenic NK cells induced IFNγ production by 12 hrs (Humann and Lenz, 2010). This would suggest that direct DC and NK cell contact is pivotal to the innate responses against *L. monocytogenes*, however other signals may also be involved. First of all, other cytokines, like IL-12 and IL-18 have been shown to induce activation of NK cells (Bellora et al., 2012; Metzger et al., 1997). Previous studies demonstrated that neutralisation of IL-12 expression with the use of monoclonal antibodies led to reduction in the NK cell mediated IFNγ production (Humann and Lenz, 2010). Furthermore, in mice lacking IL-12 subunit p40, the IFNγ production by NK and myeloid cells clusters was highly impaired post infection with *L.*

monocytogenes (Kang et al., 2008). We have shown similar results in IL-12p35 knockout mice in which the p35 subunit is unique to IL-12, unlike the p40 subunit which is shared with IL-23 and can affect both IFNγ and IL-17 (Oppmann et al., 2000). Our results revealed that in the absence of IL-12 signals, IFNγ expression by NK cells was significantly reduced post infection with Lm-2W1S. Not surprisingly, the IL-12 signals were also required for the optimal expression of OX40L on DC which related back to the importance of innate IFNγ in regulation of OX40L expression on DC. Further investigations should explore the possible sources of IL-12 signals to determine the specific role of DC in NK cell activation.

The results on both IFNγ and IL-12 dependent OX40L expression on DC led to a hypothesis that innate IFNγ responses to Lm-2W1S could also signal back to DC and induce their expression of OX40L. Expression of IFNγR on DC has previously been associated with an autocrine signalling whereby DC can induce their own maturation, as mice deficient in IFNγR had reduced DC expression of CD86, CD54 and IL-12 (Pan et al., 2004). Furthermore, some early studies have suggested that possible alternative ligands for IFNγR could mask the severity of responses in $IFN\gamma^{-/-}$ mice (Cantin et al., 1999). We therefore utilised the $CD11c^{Cre}$ x $IFN\gamma R^{f/f}$ mice to specifically prevent IFNγ signalling to DC. Our analyses showed that phenotypically the mice indeed lacked the expression of IFNγR on DC and that although CD11c expression has been shown in NK cells, we saw a minor defect in their effector function post infection with Lm-2W1S. Moreover, we also showed that OX40L expression on DC at 24 hrs post Lm-2W1S was reduced in these mice and in the $IFN\gamma^{-/-}$ controls, as compared to the optimal expression found in WT mice. Similarly, further *in vitro* analyses with recombinant IFNγ demonstrated requirement of IFNγ signalling via the IFNγR for the upregulation of OX40L on DC. One other study investigated the effect of DC specific loss of IFNγR expression on the responses to *L. monocytogenes* and showed that these mice were most susceptible to the infection (S. H. Lee et al., 2013). They found that this was related to the decreased production of IL-12 by the CD8 DC and furthermore they also showed that IL-

4 production in these mice was increased and its abrogation enhanced *L. monocytogenes* specific immune responses. This result is particularly interesting as we have also observed that *in vitro* stimulation with IL-4 significantly downregulated of OX40L expression on splenic DC (personal observations, Gajdasik and Gaspal, UoB).

4.3.4 Summary

This Chapter provided detailed analysis of OX40L upregulation on splenic DC in both *in vivo* and *in vitro* settings, highlighting the importance of innate IFNγ and IL-12 in the costimulatory function of DC. This investigation is however not fully complete. The results obtained in IFNγ$^{-/-}$ mice showed a partial reduction in OX40L expression and therefore the role of other signals in regulating OX40L expression on DC post infection with Lm-2W1S should be explored. For instance, blocking of IL-18 and TNFα signals *in vivo* as well as stimulation of splenocytes with those signals *in vitro* could provide valuable information. Moreover the previously described NK independent sources of IFNγ in *L. monocytogenes* infections, like CD8 T cells should also be considered, as they were able to maintain innate responses (Andersson et al., 1998). Similarly, CD8 T cell and DC interactions were shown to be more detailed analyses of the IFNγ induced OX40L expression would be beneficial in order to understand its relationship with the CD40 signalling.

Overall, this data set a strong basis for the further investigations into the requirement of OX40L provision by DC in CD4 T cell responses to Lm-2W1S which will be explored in the next Chapter.

CHAPTER 5: CELLULAR SOURCES OF OX40L IN RESPONSES TO LM-2W1S

5.1 Introduction

A simple question of "what a specific gene does" has led to major advances in the field of molecular biology. Ultimately, the best way to determine specific gene's function is to see what effect its ablation has on the whole organism. However, often selected genes can be differentially expressed or their transcripts post-translationally modified, resulting in cell specific expression. Additionally, often proteins encoded by those genes, like for instance receptor ligands, might be expressed in several cell types, making it extremely difficult to target specific cell type expression with a simple gene knockout. Since 1990s, our understanding of cellular and molecular interactions *in vivo* has improved tremendously. Rajewsky and colleagues introduced a new versatile genetic tool for producing genetically modified mice in which any gene could be deleted in a cell- or tissue-specific manner (Gu et al., 1994, 1993). The use of Cre- loxP system allowed immunologists to study the impact of specific gene deletions in various disease models but also assess the function of particular genes at steady state. This powerful technology involves the use of bacteriophage P1 derived Cre recombinase. This 38 kDa protein catalyses a site-specific recombination between two 34 bp long loxP sites. The loxP sequences are made of two 13 bp inverted repeats and an 8 bp nonpalindromic region. Depending on their orientation, opposite or the same, loxP sites mediate inversion or excision of the specific DNA sequence found between the sites by Cre recombinase, respectively (Orban et al., 2006; Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). The Cre recombinase was shown to be highly compatible with eukaryotes, as represented by this prokaryotic system being expressed under the cadmium induced metallothionein I gene promoter in mammalian cells (Sauer and Henderson, 1988). One of the first reports of a mouse lines generated using Cre- loxP system, described a controlled suppression of class switch recombination (Gu et al., 1993).

Throughout the last 20 years, the Cre-recombinase system has been widely used by many immunologists to study conditional gene knockouts, conditional gene expression, fate mapping or the effects of germline. Additionally, it allows for genetic ablation within a specific cell type which can also be induced at any time if Cre recombinase is for example fused with another ligand-binding domain, like estrogen (Feil et al., 1996).

There is absolutely no doubt about the significance of costimulation in the activation and maintenance of T cell responses, yet some costimulatory pathways, like OX40-OX40L pathway still remain poorly understood in terms of the critical cellular provider, if there is one, *in vivo*.

Since the discovery of OX40L and its binding properties, many studies focused on understanding the role of OX40L signalling in cell-cell interactions. This has proven somehow difficult, particularly because of its inducible nature but most importantly due to the identification of various cellular sources capable of providing OX40L signals. However, recently a floxed *Tnfsf4* ($OX40L^{f/f}$) mouse has been described which was used to generate several mouse lines using the Cre- loxP systems, showing an essential role of B cell specific provision of OX40L for the generation of T follicular cell population and promoting systemic lupus erythematosus development (Cortini et al., 2017).

In this chapter, we sought to answer the question of whether there was a critical cellular source of OX40L in the Th1 infection models used in Chapter 3, and if so what was the nature of that source. To do this we aimed to utilise a combination of the newly generated $OX40L^{f/f}$ mouse and several different Cre expressing mouse lines, each targeting a different accessory cell which could potentially be providing crucial OX40L signals.

5.2 Results

5.2.1 Novel conditional knockout mice enable assessment of OX40L provision *in vivo*

In previous chapters, we focused on establishing the importance of OX40 signals specifically in antigen specific Th1 CD4 T cells responses utilizing various infection and immunisation models. Moreover, using the Lm-2W1S model, we also showed that early innate factors, namely the rapid production of IFNγ can aid in the upregulation of OX40L expression by DC in an IL-12 dependent manner. Recently, role of OX40L provision by ILC2 was reported to be essential in Th2 and Treg responses in the lung (Halim et al., 2018). However, specific cellular provision of OX40L in Th1 responses has not yet been properly dissected. We therefore aimed to investigate further the relevance of OX40L signals provided by various accessory cells, including DC, to determine whether their provision was absolutely required for the generation of efficient effector CD4 T cell responses following infection with Lm-2W1S.

Professional APCs, like DC, B cells and macrophages, have been considered as likely sources of OX40L within the secondary lymphoid tissues, but this has not been fully determined *in vivo*. These APCs have been reported to express OX40L within hours of their activation (Murata et al., 2000a; Stüber and Strober, 1996). Many signals, including LPS/ Endotoxin, CD40 signalling controlled by the CD28/B7 axis, IL-18 and TSLP can lead to an induction of OX40L on DC and B cells (Ito et al., 2005b; Maxwell et al., 2006; Rogers et al., 2001). As previously described in Chapter 4, our data has now revealed that recombinant IFNγ can also upregulate OX40L on DC following overnight *in vitro* stimulation.

However, OX40L expression has also been found on other lymphoid and nonlymphoid cells like ILC3, NK cells, Langerhans, and mast cells and even non-hematopoietic populations such as endothelial cells, creating a wide range of interactions which potentially could play an important and different role in the generation and maintenance of T cell function.

Considering all sources of OX40L, we decided to focus our investigation on four cell types, namely DC, ILC3, T cells, and B cells and determine whether their provision of OX40L during primary responses to Lm-2W1S was absolutely required for promoting effector T cell function. To dissect this, we have generated novel conditional knockout mice, by crossing previously described $OX40L^{f/f}$ mice with cell type-specific Cre strains (Cortini et al., 2017) to target OX40L expression on DC ($CD11c^{Cre}$), ILC and T cells ($Rorc^{Cre}$) and only T cells ($E8III^{Cre}$). B cell OX40L conditional knockout mice ($Mb1^{Cre}$) were generated at later timepoints in the invetigation.

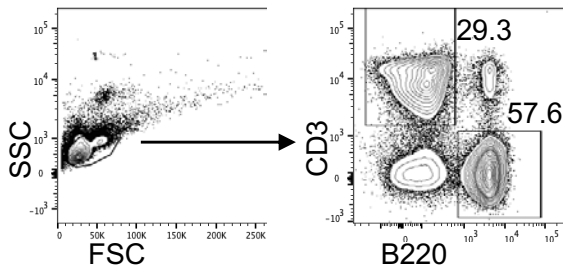
To better understand the specificity of cre expression in these mice, we additionally cross these strains with mTmG mice, a double-fluorescent Cre reporters, expressing membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision (Muzumdar et al., 2007). Recent reports showed Cre systems to be particularly inclined to germline recombination or transient expression (Song and Palmiter, 2018).

In the analysis, we identified splenic T cells as $CD3^+$, B cells as $B220^+$, DC as $CD11c^+$, $MHCII^+$ and ILC3 as $CD3^-$, $B220^-$, IL-$7R\alpha^+$, $ROR\gamma t^+$ (Figure 5.1A, B, C). Expression of Cre was assessed in all those cell types with the comparison to the mTmG control splenocytes, which did not express any mG protein due to the lack of Cre (Figure 5.2 A).

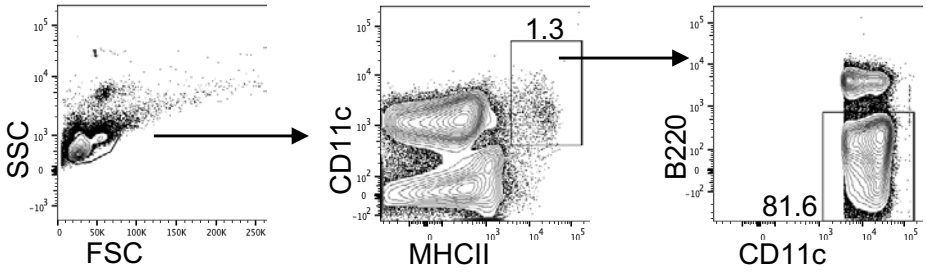
Analysis of $CD11c^{Cre}$ x mTmG mice has shown a DC specific expression of the Cre construct, reaching approximately 90% of total splenic DC (Figure 5.2 A, B). B cells and ILC3 were minimally affected, showing significantly lower levels of mG protein present. However, the expression was more prominent in T cells showing approximately 15-20% cells expressing mG protein (Figure 5.2 A, B). Analysis of $E8III^{Cre}$ x mTmG mice showed that levels of mG protein were significantly higher in T cells of the $E8III^{Cre}$ x mTmG mice, comparing to the scarce levels present in B cells, DC and ILC3 (Figure 5.2 A, C). The Cre expression in $Rorc^{Cre}$

x mTmG was absolutely restricted to T cells and ILC3, reaching approximately 70-90% (Figure 5.2 A, D).

A)


SSC
FSC
CD3
B220
29.3
57.6

B)


SSC
FSC
CD11c
MHCII
1.3
B220
CD11c
81.6

C)

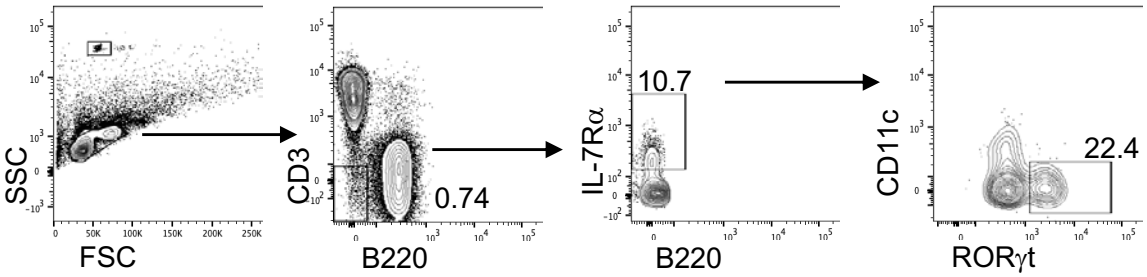
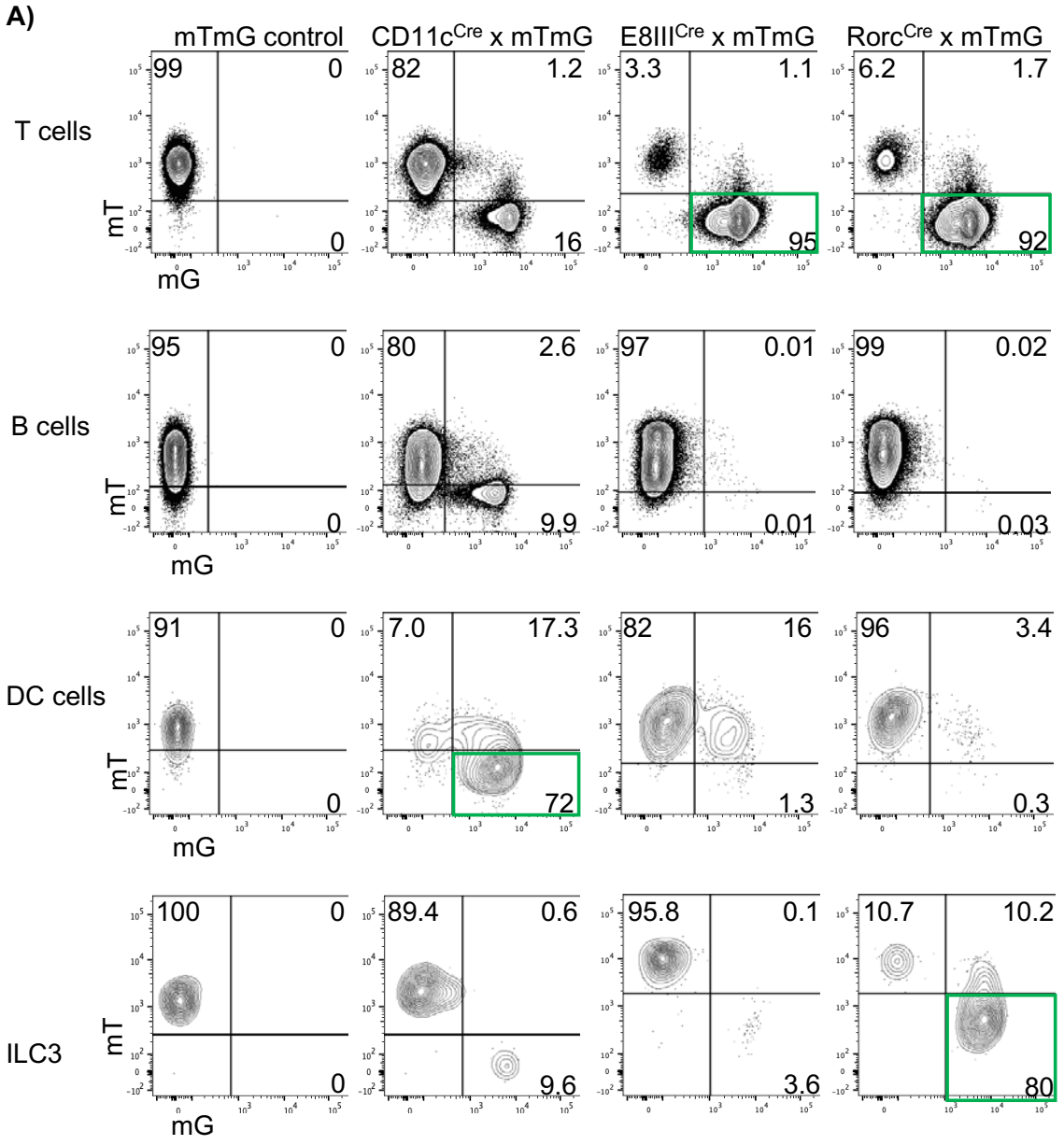

SSC
FSC
CD3
B220
0.74
IL-7Rα
B220
10.7
CD11c
RORγt
22.4

Figure 5. 1 Gating strategy used to identify different cell populations prior the analysis of the Cre induced mG (membrane tagged GFP) expression.

(**A**) Gating strategy used to identify T and B cells. (**B**) Gating strategy used to identify DC (**C**) Gating strategy used to identify innate lymphoid cells (ILC). Values on flow cytometry plots represent percentages.


A)
mTmG control
CD11c^Cre x mTmG
E8III^Cre x mTmG
Rorc^Cre x mTmG
T cells
B cells
DC cells
ILC3
mT
mG
99
0
0
82
1.2
16
3.3
1.1
95
6.2
1.7
92
95
0
0
80
2.6
9.9
97
0.01
0.01
99
0.02
0.03
91
0
0
7.0
17.3
72
82
16
1.3
96
3.4
0.3
100
0
0
89.4
0.6
9.6
95.8
0.1
3.6
10.7
10.2
80

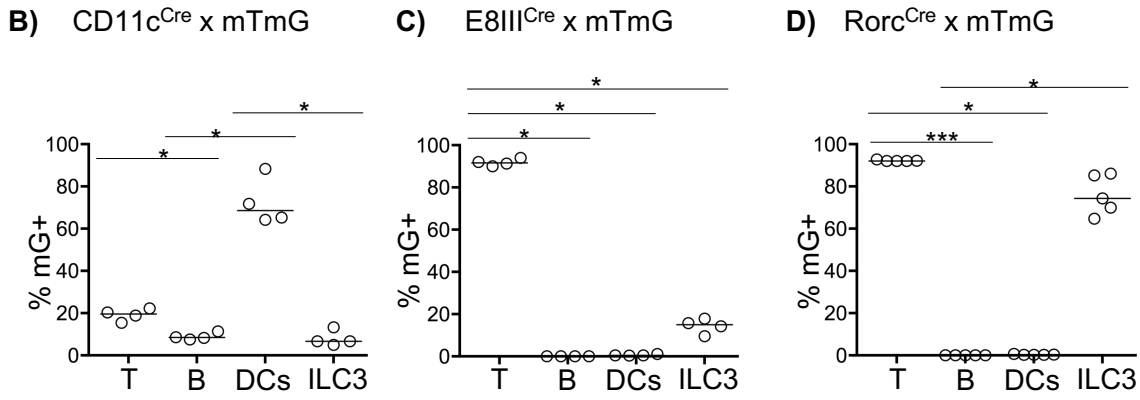

B) CD11c^Cre x mTmG
C) E8III^Cre x mTmG
D) Rorc^Cre x mTmG
% mG+
T
B
DCs
ILC3
*
***

Figure 5. 2 Analysis of Cre expression in conditional OX40L deficient mice using mTmG fate-mapping mice.

The fidelity of Cre expression in the Cre-expressing mouse strains used to selectively delete OX40L was assessed through crossing with mTmG 'fate-mapping' mice, where expression of mG (membrane tagged GFP) is induced by Cre expression. (**A**) Expression of mT vs mG in mTmG control mice, $CD11c^{Cre}$ x mTmG, $E8111^{cre}$ x mTmG, $Rorc^{cre}$ x mTmG mice. (**B**) The percentage of mG+ T cells, B cells, DCs and ILCs in the spleen of $CD11c^{cre}$ x mTmG mice. (**C**) The percentage of mG+ T cells, B cells, DCs and ILCs in the spleen of $E8111^{cre}$ x mTmG mice. (**D**) The percentage of mG+ T cells, B cells, DCs and ILCs in the spleen of $Rorc^{cre}$ x mTmG mice. Data are pooled from 2 independent experiments, n=4 $CD11c^{cre}$ x mTmG, $E8111^{cre}$ x mTmG and n=5 for $Rorc^{cre}$ x mTmG mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

Alongside the analysis of Cre expression, we also sought to confirm the specific deletion of OX40L on the target cell populations. Here we used two separate protocols for inducing OX40L expression on DC, B cells and ILC3 which involved overnight stimulation with αCD40, and on T cells involving 72 hrs culture with αCD3 and CTLA4-Ig (Abatacept). The individual cell subsets were identified similarly to the previous data, with an exception to T cells which required TCRα staining due to the downregulation of CD3 in culture, and ILC3 which were identified on the basis of the CCR6 and CD4 expression (Figure 5.3 A, B, C, D).

The OX40L expression on different cell types was assessed in comparison to expression levels found in both control WT mice as well as the pan-OX40L deficient PGK^{Cre} x $OX40L^{f/f}$ mice, which expressed Cre recombinase ubiquitously (Figure 5.4 A). Collectively, these comparisons confirmed the validity of the models we had generated, with appropriate cell-type specific deletion of OX40L. Results in WT mice showed an upregulated expression of OX40L on DC, B cells, T cells and ILC3 which in contrast was completely abrogated in PGK^{Cre} x $OX40L^{f/f}$ mice (Figure 5.4 A). As anticipated, OX40L expression in $Rorc^{Cre}$ x $OX40L^{f/f}$ mice was deleted on T cells and ILCs, with no defect found on DC and B cells. While in $E8III^{Cre}$ x $OX40L^{f/f}$ mice only T cells were affected, again with DC, B cells and ILC3 expressing normal levels of OX40L (Figure 5.4 A, B, C, D, E). Finally, assessment of OX40L expression in $CD11c^{Cre}$ x $OX40L^{f/f}$ mice showed DC specific deletion, with minimal effect on other populations. OX40L was not significantly deleted in T cells in the $CD11c^{Cre}$ x $OX40L^{f/f}$ mice (Figure 5.4 A, D).

The analyses provided valuable information about the precision of Cre expression in individual cell types, which was further supported by the protein level analysis showing the deletion of OX40L to be efficient in all conditional knockout mice. Combined these data demonstrated that we had developed appropriate tools to determine the cellular source of OX40L *in vivo*.

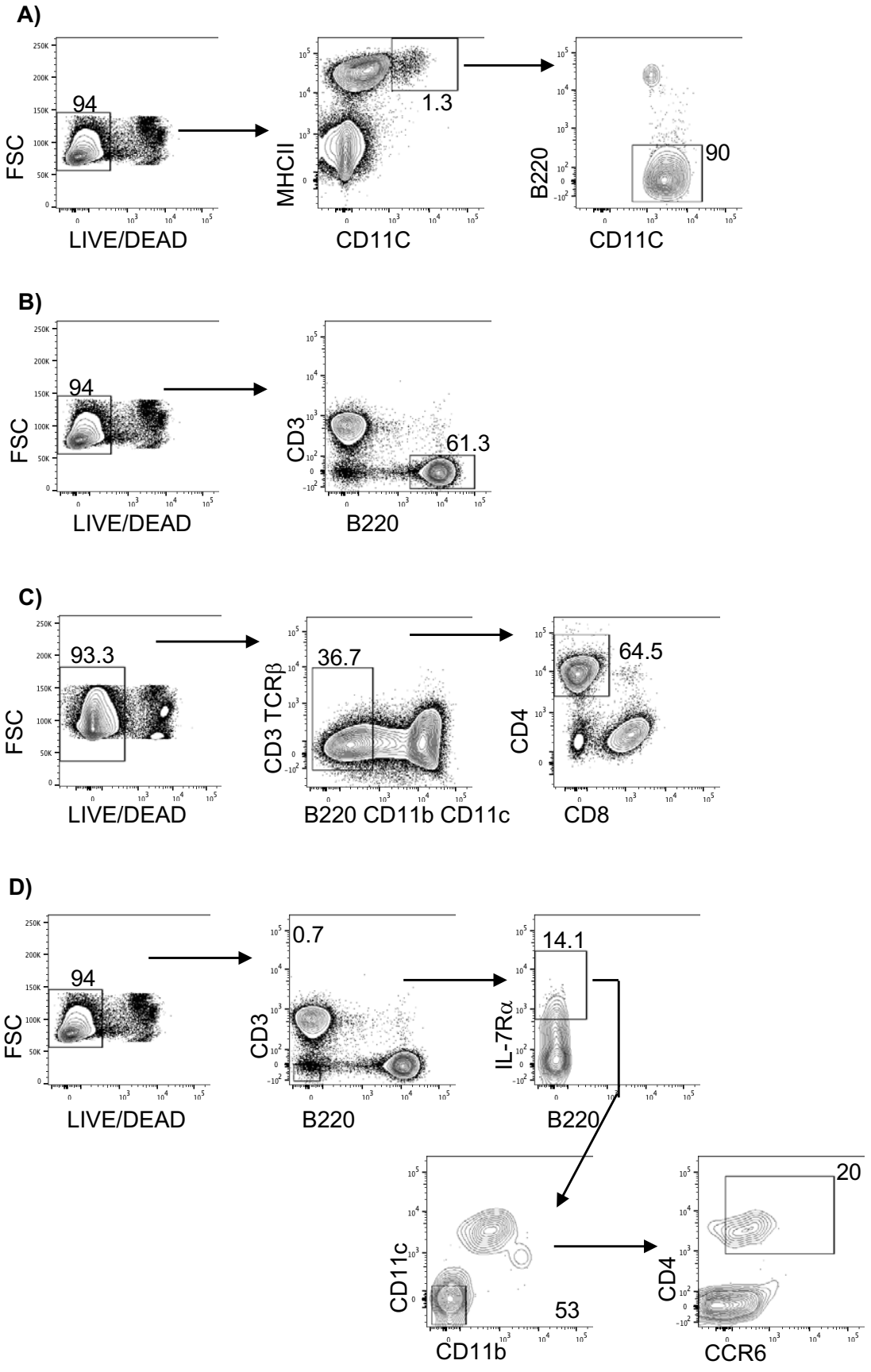

A)
94
FSC
LIVE/DEAD
1.3
MHCII
CD11C
90
B220
CD11C
B)
94
FSC
LIVE/DEAD
61.3
CD3
B220
C)
93.3
FSC
LIVE/DEAD
36.7
CD3 TCRβ
B220 CD11b CD11c
64.5
CD4
CD8
D)
94
FSC
LIVE/DEAD
0.7
CD3
B220
14.1
IL-7Rα
B220
53
CD11c
CD11b
20
CD4
CCR6

Figure 5. 3 Gating strategy used to identify different cell populations expressing OX40L.

(**A**) Gating strategy used to identify DC. (**B**) Gating strategy used to identify B cells. (**C**) Gating strategy used to identify T cells. (**D**) Gating strategy used to identify innate lymphoid cells (ILC). Values on flow cytometry plots represent percentages.

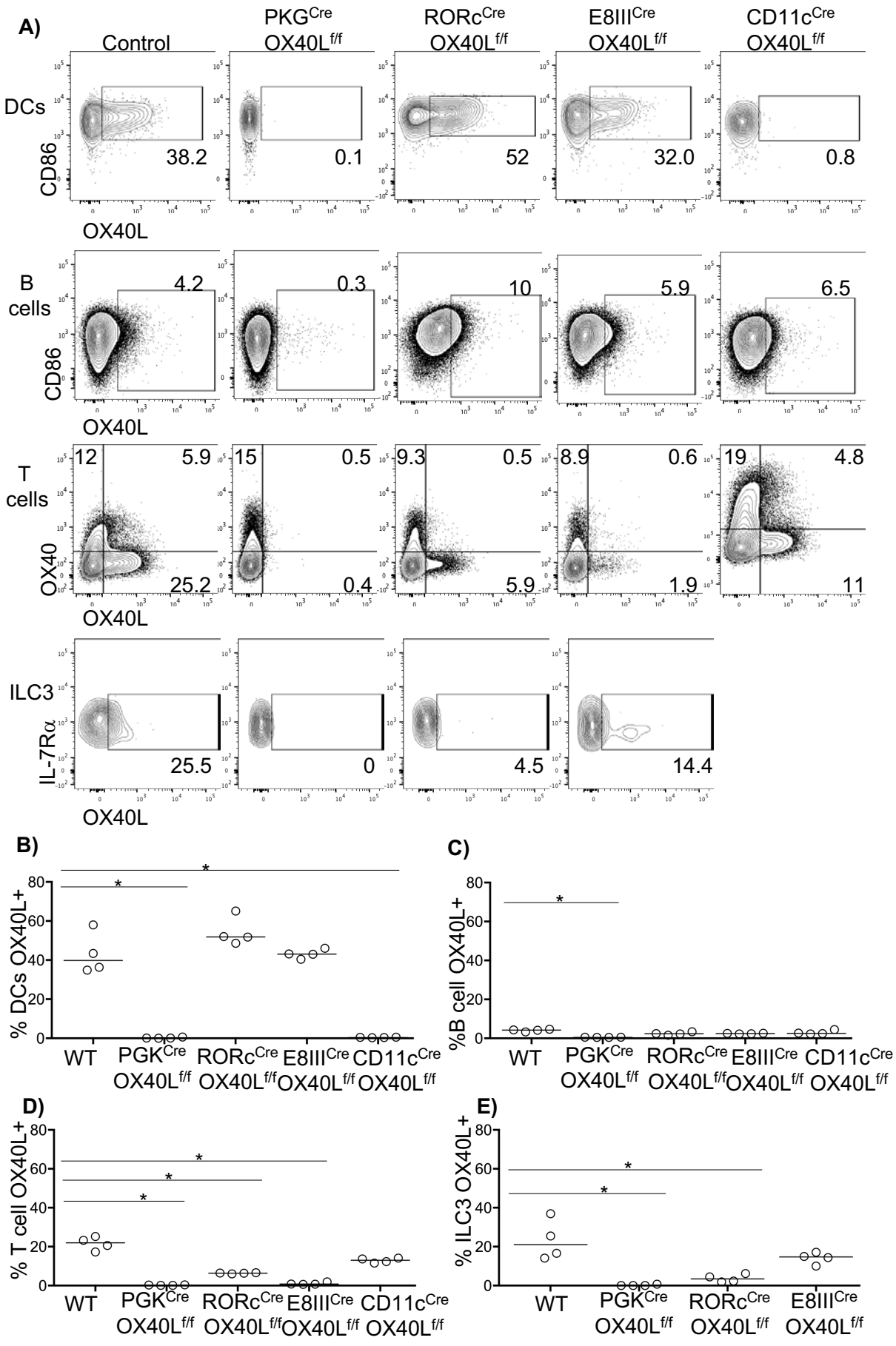

A)
Control
PKGCre OX40Lf/f
RORcCre OX40Lf/f
E8IIICre OX40Lf/f
CD11cCre OX40Lf/f
DCs
B cells
T cells
ILC3
CD86
OX40
IL-7Rα
OX40L
38.2
0.1
52
32.0
0.8
4.2
0.3
10
5.9
6.5
12
5.9
25.2
15
0.5
0.4
9.3
0.5
5.9
8.9
0.6
1.9
19
4.8
11
25.5
0
4.5
14.4
B)
% DCs OX40L+
C)
%B cell OX40L+
D)
% T cell OX40L+
E)
% ILC3 OX40L+
WT
PGKCre OX40Lf/f
RORcCre OX40Lf/f
E8IIICre OX40Lf/f
CD11cCre OX40Lf/f

Figure 5. 4 Analysis of OX40L expression in conditional OX40L deficient mice.

To confirm appropriate deletion of OX40L in novel OX40L conditional mouse strains, splenocytes were cultured overnight with αCD40 and OX40L expression of key cell types assessed. (**A**) Expression of OX40L by DCs (CD11c+, MCHII+), B cells (B220+), T cells (CD3+TCRβ+CD4+) and ILCs [lin- (CD3, B220, CD11c, CD11b), IL- 7Rα+, CD4+,CCR6+] in control versus PGK^{cre} x $OX40L^{f/f}$, $Rorc^{cre}$ x $OX40L^{f/f}$, $E8111^{cre}$ x $OX40L^{f/f}$, and $CD11c^{cre}$ x $OX40L^{f/f}$ mice. (**B**) Expression of OX40L by DC in WT mice versus conditional OX40L deficient mice. (**C**) Expression of OX40L by B cells in WT mice versus conditional OX40L deficient mice. (**D**) Expression of OX40L by T cells in WT mice versus conditional OX40L deficient mice. (**E**) Expression of OX40L by ILC3 in WT mice versus conditional OX40L deficient mice. Data pooled from two independent experiments, n=4. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

5.2.2 Provision of OX40L by LTI-like cells is redundant for generation of effector CD4 T cell responses

Following the initial assessment of our Cre models, we focused our attention on investigating the role of different cellular providers of OX40L in generating effector T cell responses to Lm-2W1S.

Although there are no clear reports of other molecules binding to OX40 beyond OX40L, we began with testing the effects of total OX40L deficiency on antigen-specific CD4 T cell responses, in particular whether they could phenocopy the effects of OX40-deficiency. We therefore infected PGK^{Cre} x $OX40L^{f/f}$ mice, alongside PGK^{Cre} only controls and $OX40^{-/-}$ positive controls with Lm-2W1S and assessed responses within spleens at 7 dpi. As previously, we identified 2W1S-specific CD4 T cells as lin^- ($B220^+CD11b^+CD11c^+$), $CD3^+$, $CD4^+$, $CD44^{high}$ and used T-bet versus CXCR5 expression to identify both effector and Tfh populations (Figure 5.5 A, B).

We found the total numbers of 2W1S-specific CD4 T cells to be significantly reduced in PGK^{Cre} x $OX40L^{f/f}$ mice which was comparable with the response observed in mice deficient in OX40 signals (Figure 5.5 C). Moreover, mice lacking both PGK^{Cre} x $OX40L^{f/f}$ and $OX40^{-/-}$ mice showed an impairment in the generation of effector CD4 T cells responses, with the numbers and percentage of T-bet^+ cells significantly reduced (Figure 5.5 D, E). Although showing a modest defect in PGK^{Cre} x $OX40L^{f/f}$ and $OX40^{-/-}$ mice, the enumeration of $CXCR5^+$ cells proved statistically nonsignificant when compared to PGK^{Cre} only control mice (Figure 5.5 F).

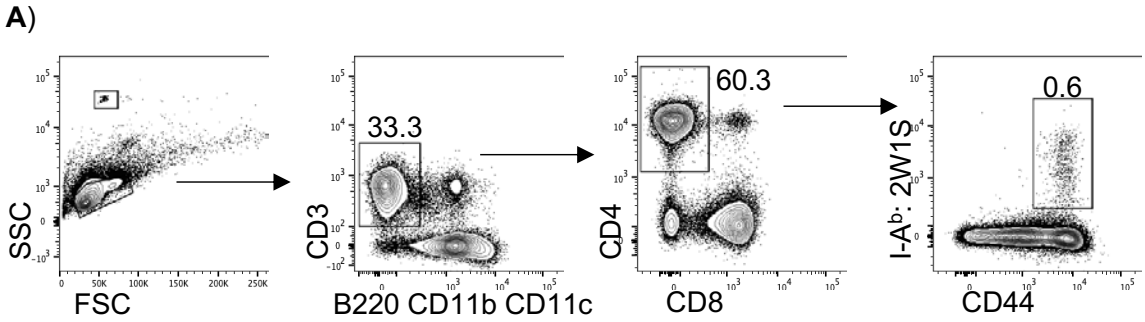
Having confirmed that the response in pan-OX40L deficient mice was comparable to mice lacking OX40, we sought to test the importance of OX40L provision by ILC3 on the effector T cells responses.

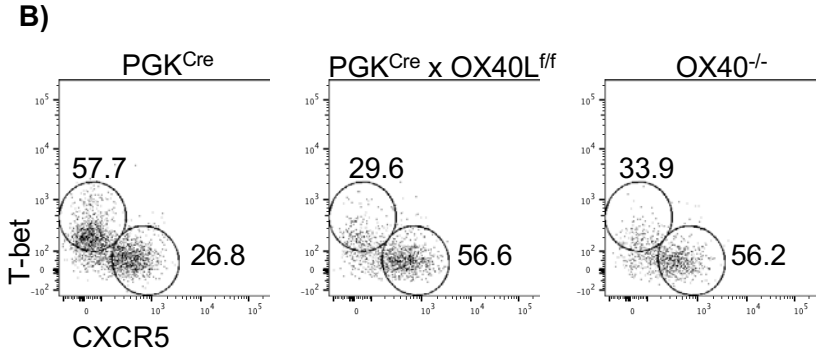
Over the last decade, there has been an increasing number of reports suggesting that ILC3s could be involved in presenting antigens and providing costimulatory signals (Hepworth and Sonnenberg, 2014b; Kim et al., 2003). Moreover, their position within the lymphoid tissue

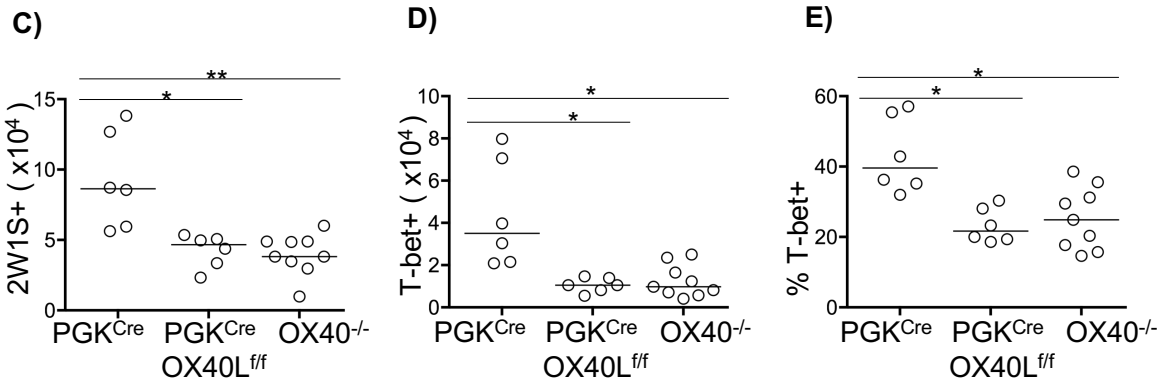
located in the interfolicular spaces between T and B cells zones, and their help in splenic CD4 T cells responses, supports the hypothesis of OX40L provision by the ILC3 to be required for functional Th1 responses (Mackley et al., 2015; von Burg et al., 2014).

To test this, we infected $Rorc^{cre}$ versus $Rorc^{cre}$ x $OX40L^{f/f}$ mice with Lm-2W1S and assessed the responses at 7 dpi, alongside $OX40^{-/-}$ controls. The *Rorc* gene encodes two isoforms Rorγ and Rorγt, with the latter being expressed in distinct immune cell population including ILC3, Th17, $\gamma\delta$T cells (Eberl et al., 2004; Eberl and Littman, 2003; He et al., 1998). Our analyses revealed no significant defect in the magnitude of the 2W1S specific CD4 T cell response between the $Rorc^{cre}$ versus $Rorc^{cre}$ x $OX40L^{f/f}$ mice (Figure 5.6 A). Although the reduction in the total number of 2W1S-specific CD4 T cells in $OX40^{-/-}$ mice was non-significant, this was at odds with all the previous experimental data and likely reflected the surprising spread of data in the Rorc controls (Figure 5.6 B).

Furthermore, total number and percentages of T-bet^{+} cells were unaffected in $Rorc^{cre}$ x $OX40L^{f/f}$ mice as compared to the $Rorc^{Cre}$ only controls, indicating that ILC3 provision of OX40L was not required for the generation of effector Th1 responses in the spleen (Figure 5.6 C, D). These data were significantly different to $OX40^{-/-}$ controls. Consistent with our previous data, we also demonstrated the expression of T-bet by 2W1S-specific CD4 T cells in $OX40^{-/-}$ to be significantly impaired, while no differences were observed in the total numbers of CXCR5^{+} Tfh cells between all mouse strains (Figure 5.6 C, D, E).


A)
SSC
FSC
33.3
CD3
B220 CD11b CD11c
60.3
CD4
CD8
0.6
I-Ab: 2W1S
CD44


B)
PGKCre
PGKCre x OX40Lf/f
OX40-/-
57.7
26.8
29.6
56.6
33.9
56.2
T-bet
CXCR5


C)
2W1S+ (x10⁴)
D)
T-bet+ (x10⁴)
E)
% T-bet+
PGKCre
PGKCre OX40Lf/f
OX40-/-

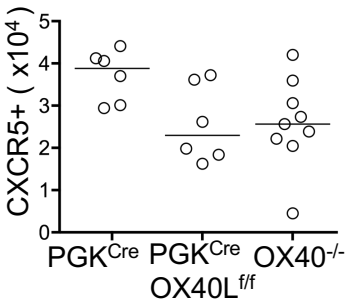

F)
CXCR5+ (x10⁴)
PGKCre
PGKCre OX40Lf/f
OX40-/-

Figure 5. 5 Th1 responses in mice lacking OX40L mimic the defect observed in OX40$^{-/-}$ mice.

To confirm that mice deficient in OX40L phenocopied mice deficient in OX40, expression of T-bet versus CXCR5 by 2W1S-specific CD44^{hi} CD4 T cells in PGK^{cre}, PGK^{cre} x OX40L$^{f/f}$ versus OX40$^{-/-}$ mice was assessed. (**A**) Gating strategy used to identify 2W1S-specific CD44^{hi} CD4 T cells (CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}). (**B**) Expression of T-bet versus CXCR5 by 2W1S-specific CD44^{hi} CD4 T cells in PGK^{cre}, PGK^{cre} x OX40L$^{f/f}$ and OX40$^{-/-}$ mice. (**C**) Enumeration of 2W1S-specific CD4 T cells. (**D**) Enumeration of T-bet^{+} 2W1S-specific CD4 T cells. (**E**) Percentage of T-bet^{+} 2W1S-specific CD4 T cells. (**F**) Enumeration of CXCR5^{+} 2W1S-specific CD4 T cells. Data are pooled from two independent experiments, n=6 PGK^{Cre}, n=6 PGK^{Cre} x OX40L$^{f/f}$, n=9 OX40$^{-/-}$. Values on flow cytometry plots represent percentages, bars on scatter plots represent the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

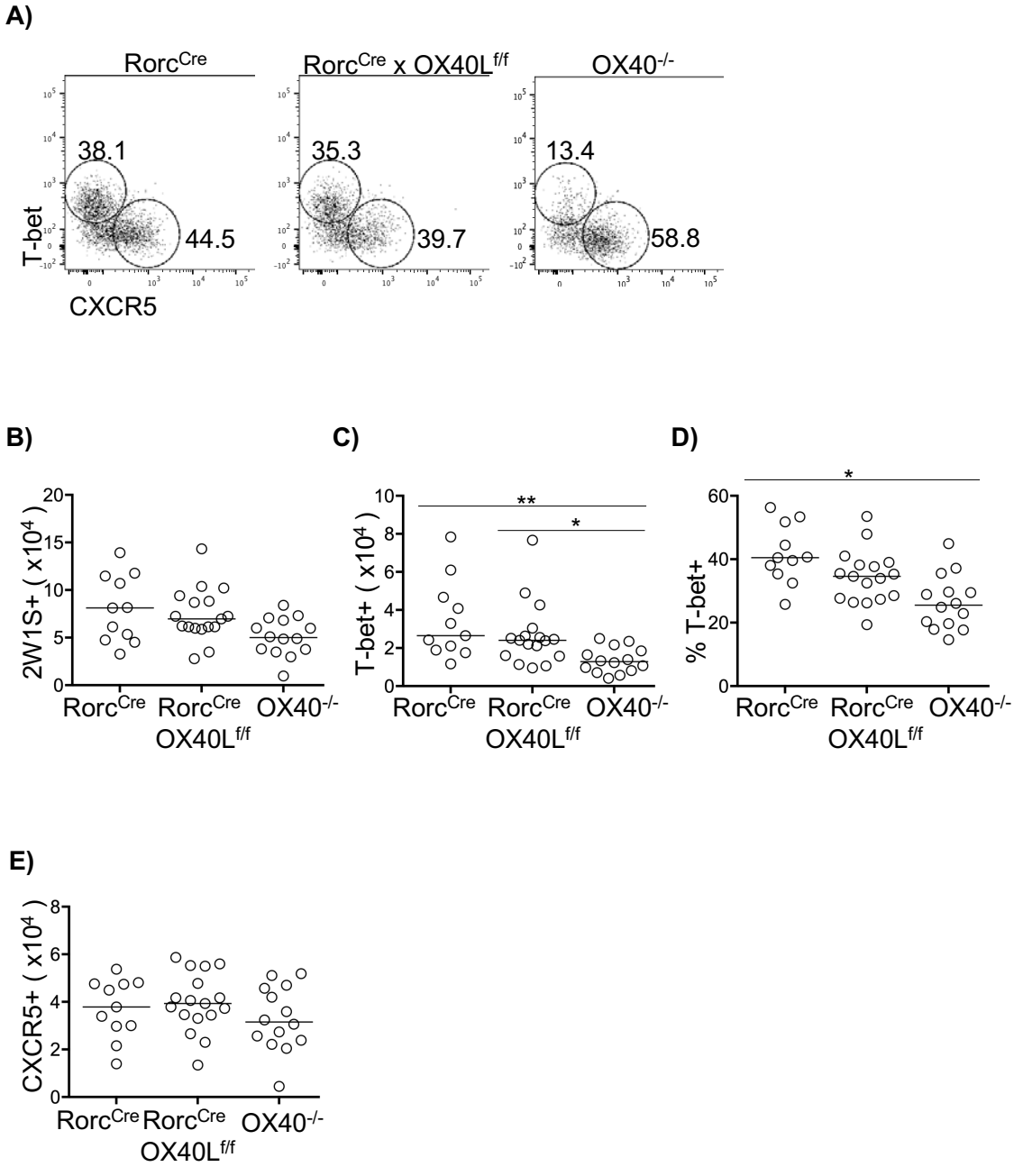

A)
Rorc^Cre
Rorc^Cre x OX40L^f/f
OX40^-/-
38.1
44.5
35.3
39.7
13.4
58.8
T-bet
CXCR5
B)
2W1S+ (x10^4)
C)
T-bet+ (x10^4)
D)
% T-bet+
Rorc^Cre
Rorc^Cre OX40L^f/f
OX40^-/-
E)
CXCR5+ (x10^4)

Figure 5. 6 Expression of OX40L by ILC3 is not required for effector Th1 responses to Lm-2W1S.

To investigate the critical cellular interaction for generation of Th1 effector T cells, conditional OX40L deficient mice, targeting ILC3 ($Rorc^{cre}$) were generated. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in $Rorc^{cre}$, $Rorc^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice. (**B**) Enumeration of $CD44^{hi}$ 2W1S-specific CD4 T cells in $Rorc^{cre}$, $Rorc^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice. (**C**) Enumeration of T-bet^{+} 2W1S-specific $CD44^{hi}$ CD4 T cells. (**D**) Percentage of T-bet^{+} 2W1S-specific CD4 T cells. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific CD4 T cells. Data pooled from three independent experiments, n=11 $Rorc^{cre}$, n =17 $Rorc^{cre}$ x $OX40L^{f/f}$ and n=14 $OX40^{-/-}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represent the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

5.2.3 DC are the critical providers of OX40L in a primary CD4 T cell response

Having demonstrated that ILC3 and T cell specific expression of OX40L were not absolutely required in the effector T cell responses to Lm-2W1S, we asked whether DC were the critical provider of OX40L in this response. Dendritic cells play a crucial role in inducing immune responses by processing and presenting antigens, providing costimulation to T cells and other inflammatory signals as well as being involved in inducing tolerance (Banchereau and Steinman, 1998; M Croft et al., 1992; Ronchese and Hausmann, 1993). They therefore interact with T cells from very early on in the response, and given past reports in $Rag^{-/-}$ mice and our previous data showing a defect in the Th1 effector response at 4 dpi with Lm-2W1S, we hypothesised that OX40L provision by DC could be a critical step in generation of effector T cell responses (Kim et al., 2006). The significance of OX40L provision by DCs has been previously shown as an important factor in generating tumour-specific immunity in mouse tumour models (Zaini et al., 2007b).

To test the importance of DC interaction in CD4 T cell responses to Lm-2W1S, we infected $CD11c^{Cre}$ x $H2Ab\text{-}1^{f/f}$ mice, in which MHCII expression on DC was specifically targeted. As anticipated, in comparison to the typical responses in $CD11c^{Cre}$ mice, the 2W1S-specific response in $CD11c^{Cre}$ x $H2Ab\text{-}1^{f/f}$ mice was completely abrogated, showing significant impairment in the total numbers of $2W1S^{+}$ cells (Figure 5.7 A, B, C). Thus, in the response to Lm-2W1S, CD4 T cells must interact with DC to induce antigen-specific CD4 T cell responses. Next asked a question whether DC were the critical source of the OX40L signals required for formation of Th1 effector T cell responses. To investigate this particular role, $CD11c^{cre}$ x $OX40L^{f/f}$ mice were infected with Lm-2W1S, alongside $CD11c^{cre}$ and $CD30^{-/-}$ x $OX40^{-/-}$ controls and the responses assessed at 7 dpi.

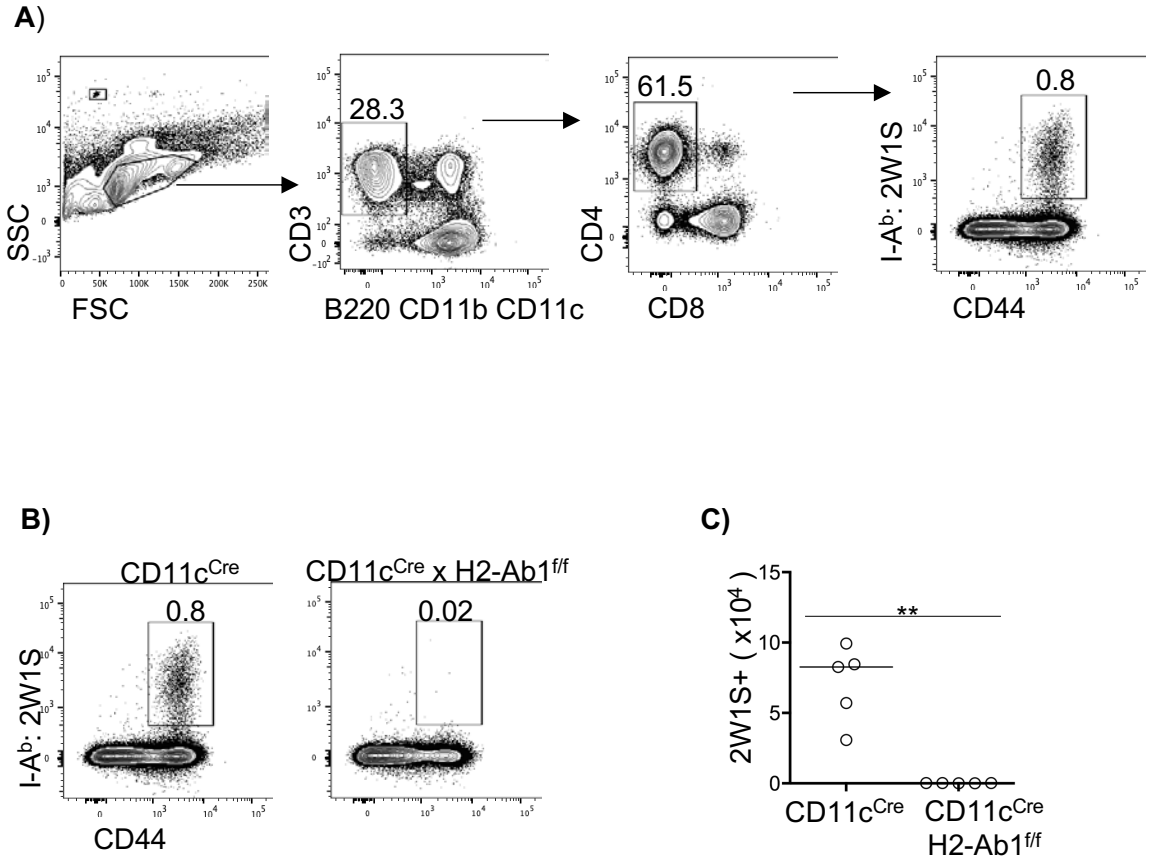

A)
SSC
FSC
28.3
CD3
B220 CD11b CD11c
61.5
CD4
CD8
0.8
I-Ab: 2W1S
CD44
B)
CD11cCre
0.8
I-Ab: 2W1S
CD44
CD11cCre x H2-Ab1f/f
0.02
C)
2W1S+ (x10^4)
15
10
5
0
**
CD11cCre
CD11cCre
H2-Ab1f/f

Figure 5. 7 Dendritic cells are pivotal to generation antigen-specific CD4 T cell responses to Lm-2W1S.

(**A**) Gating strategy used to identify 2W1S-specific $CD44^{hi}$ CD4 T cells. (**B**) Representative flow cytometry plots showing $CD44^{hi}$ 2W1S-specific CD4 T cells in the spleen of $CD11c^{cre}$ and $CD11c^{cre}$ x $H2\text{-}Ab1^{f/f}$ mice at 7 days post infection with Lm-2W1S. (**C**) Enumeration of $CD44^{hi}$ 2W1S-specific CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$). Data pooled from two independent experiments, n=5 $CD11c^{cre}$, n =5 $CD11c^{cre}$ x $H2\text{-}Ab1^{f/f}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Results clearly indicated that DC provision of OX40L was undoubtedly required for generation of efficient 2W1S-specific CD4 T cell responses, as represented by a significant reduction in the total number of $2W1S^{+}$ cells in $CD11c^{cre}$ x $OX40L^{f/f}$ mice (Figure 5.8 A, B). The total numbers of 2W1S-specific CD4 T cells in $CD30^{-/-}$ x $OX40^{-/-}$ control mice were similarly affected.

Most interestingly, assessment of the effector T cell responses, using expression of T-bet versus CXCR5, revealed substantial loss in the $T\text{-}bet^{+}$ population in $CD11c^{cre}$ x $OX40L^{f/f}$ mice which strikingly was comparable to the defect observed in $CD30^{-/-}$ x $OX40^{-/-}$ control mice (Figure 5.8 C, D). Similarly, to the previous findings, the total numbers of $CXCR5^{+}$ cells were not affected by the lack of OX40L on DCs mice (Figure 5.8 E).

These data support a model in which DC provision of OX40L is essential for the Th1 effector response to Lm-2W1S and furthermore indicate that loss of OX40L on DC, phenocopies the impact on CD4 T cells responding to Lm-2W1S in the complete absence of CD30 and OX40 at this stage of the response.

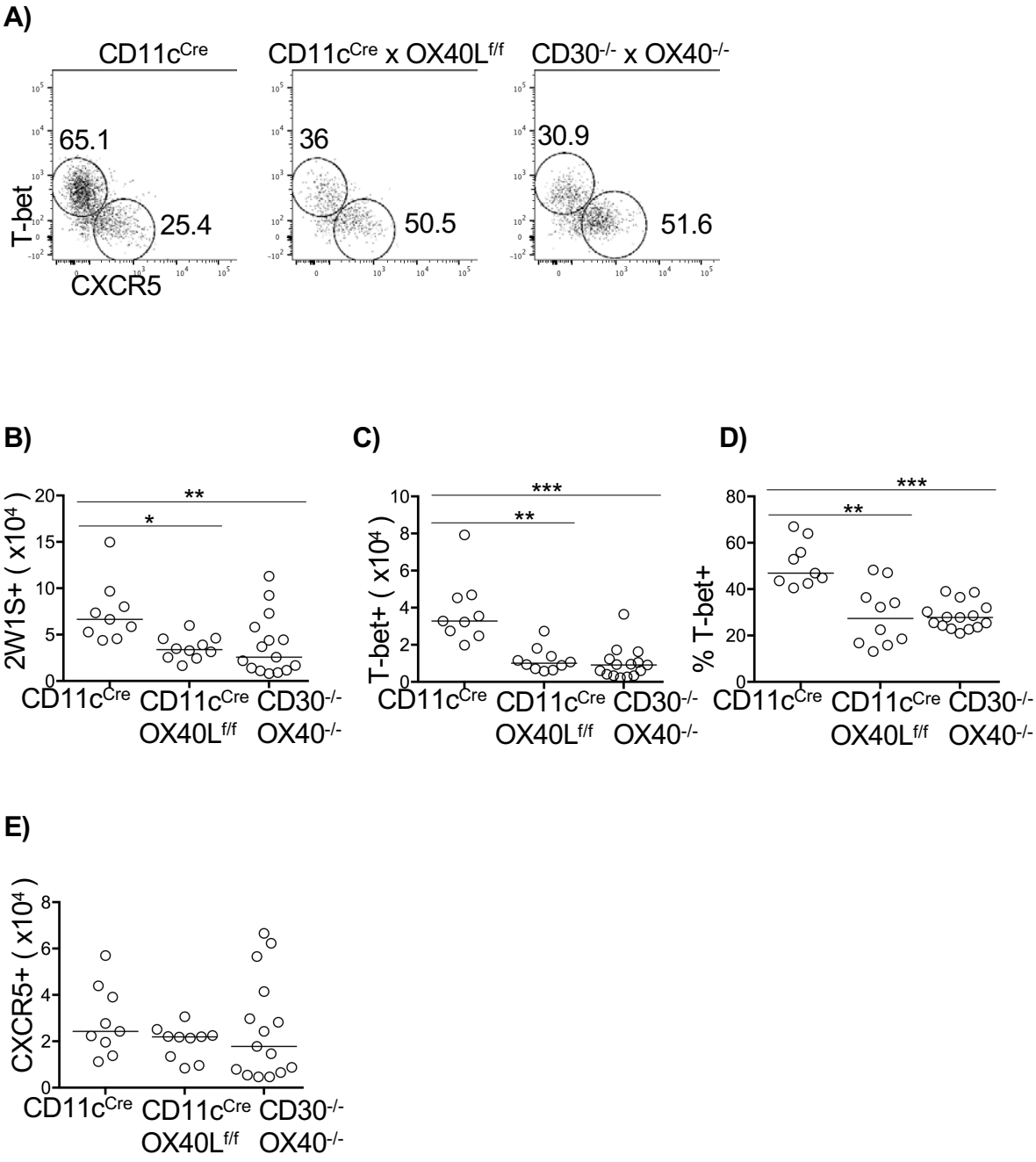

A)
CD11c^Cre
CD11c^Cre x OX40L^f/f
CD30^-/- x OX40^-/-
65.1
25.4
36
50.5
30.9
51.6
T-bet
CXCR5
B)
2W1S+ (x10^4)
C)
T-bet+ (x10^4)
D)
% T-bet+
CD11c^Cre
CD11c^Cre OX40L^f/f
CD30^-/- OX40^-/-
E)
CXCR5+ (x10^4)

Figure 5. 8 Expression of OX40L by DC is required for effector Th1 responses

To investigate the critical cellular interaction for generation of Th1 effector T cells, conditional OX40L deficient mice, DC ($CD11c^{cre}$) mice were generated. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in $CD11c^{cre}$, $CD11c^{cre}$ x $OX40L^{f/f}$ and $CD30^{-/-}$ x $OX40^{-/-}$ mice. (**B**) Enumeration of 2W1S-specific $CD44^{hi}$ CD4 T cells. (**C**) Enumeration of T-bet^{+} 2W1S-specific $CD44^{hi}$ CD4 T cells. (**D**) Percentage of T-bet^{+}2W1S-specific CD4 T cells. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific $CD44^{hi}$ CD4 T cells. Data pooled from three independent experiments, n=9 $CD11c^{cre}$, n= 10 $CD11c^{cre}$ x $OX40L^{f/f}$ and n=15 $CD30^{-/-}$ x $OX40^{-/-}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

5.2.4 Expression of OX40L by neither T cells nor B cells is required for generation of Th1 effector T cells in response to Lm-2W1S

Finally, having established the important of DC in providing OX40L, we aimed to further investigate whether that other populations could not serve as additional sources of OX40L signals, being involved after the initial DC interactions. For instance T cells themselves are able to provide the OX40L as a feedback loop, further amplifying their responsiveness (Soroosh et al., 2007). Whilst the $Rorc^{cre}$ x $OX40L^{f/f}$ mice indicated this was unlikely to be critical in the response to Lm-2W1S, we wanted to further assess this in another mouse model. To target OX40L expression on T cells we utilised a T cell-specific conditional OX40L deficient mice, which were again infected with Lm-2W1S, alongside $CD30^{-/-}$ x $OX40^{-/-}$ control mice, and the responses in their spleens assessed at 7 dpi. The Lm-2W1S induced primary CD4 T cell responses in $E8III^{Cre}$ x $OX40L^{f/f}$ mice were unaffected compared to the responses in $E8III^{Cre}$ controls, yet still observed a significant reduction in the magnitude of the response in $CD30^{-/-}$ x $OX40^{-/-}$ control mice (Figure 5.9 A, B). In regard to effector CD4 T cell responses, we found no significant defect in the T-bet^{+} expression in $E8III^{Cre}$ x $OX40L^{f/f}$ mice as compared to $E8III^{Cre}$ controls (Figure 5.9 C, D). There was, however, a significant reduction in total numbers and percentages of T-bet^{+} cells in $CD30^{-/-}$ x $OX40^{-/-}$ mice (Figure 5.9 C, D). The total numbers of Tfh cells again showed no differences between the mouse strains tested (Figure 5.9 F).

Having excluded a role for T cell expressed OX40L in the response, finally, we asked whether B cell expression of OX40L was required for optimal effector Th1 responses to Lm-2W1S. To do this, a B cell-specific OX40L conditional knockout mouse was generated through making $Mb1^{Cre}$ x $OX40L^{f/f}$ mice. In $Mb1^{Cre}$ mice, Cre recombinase is expressed under a CD79a promoter which is a B cell antigen receptor, therefore enabling expression of Cre throughout B cell development (Hobeika et al., 2006). This strain of mice was not phenotyped using mTmG fatemapping method due to a lack of mTmG expressing mice at the time, however we did check for the deletion of OX40L on B cells at the protein level. Similarly, to the previously

shown data, we cultured splenocytes of $Mb1^{Cre}$ x $OX40L^{f/f}$ mice and their $Mb1^{Cre}$ only controls overnight in the presence of αCD40 and assessed the expression of OX40L on DC, B cells, T cells and ILC3. We found the OX40L deletion to be specific to B cells, having no adverse effect on any other populations (Figure 5.10 A, B).

To assess the requirement for B cell OX40L in generating normal primary Th1 effector responses, we infected $Mb1^{Cre}$ x $OX40L^{f/f}$ mice and their $Mb1^{Cre}$ only controls and $OX40^{-/-}$ mice with Lm-2W1S. Analyses at 7 dpi revealed no significant differences in the magnitude of the 2W1S-specific CD4 T cells responses between $Mb1^{Cre}$ only controls and $Mb1^{Cre}$ x $OX40L^{f/f}$ mice (Figure 5.10 C, D). Furthermore, we observed no defect in the effector T cells responses, characterised by normal expression of T-bet (Figure 5.10 E, F). Analogous to our previous reports we found the 2W1S-specific CD4 T cell response and generation of the T-bet^{+} cells to be significantly reduced in $OX40^{-/-}$ mice, with no effect on total numbers of THF cells which were comparable between all three strains (Figure 5.10 E, F, G).

In summary, this chapter contains a detailed analysis of differential sources of OX40L *in vivo* using novel Cre models to specifically target its expression on various cell types. Our observations provided evidence for the essential role of DC OX40L in promoting primary effector CD4 T cell responses to Lm-2W1S, with OX40L provision by T cell, ILC3 and B cells redundant.

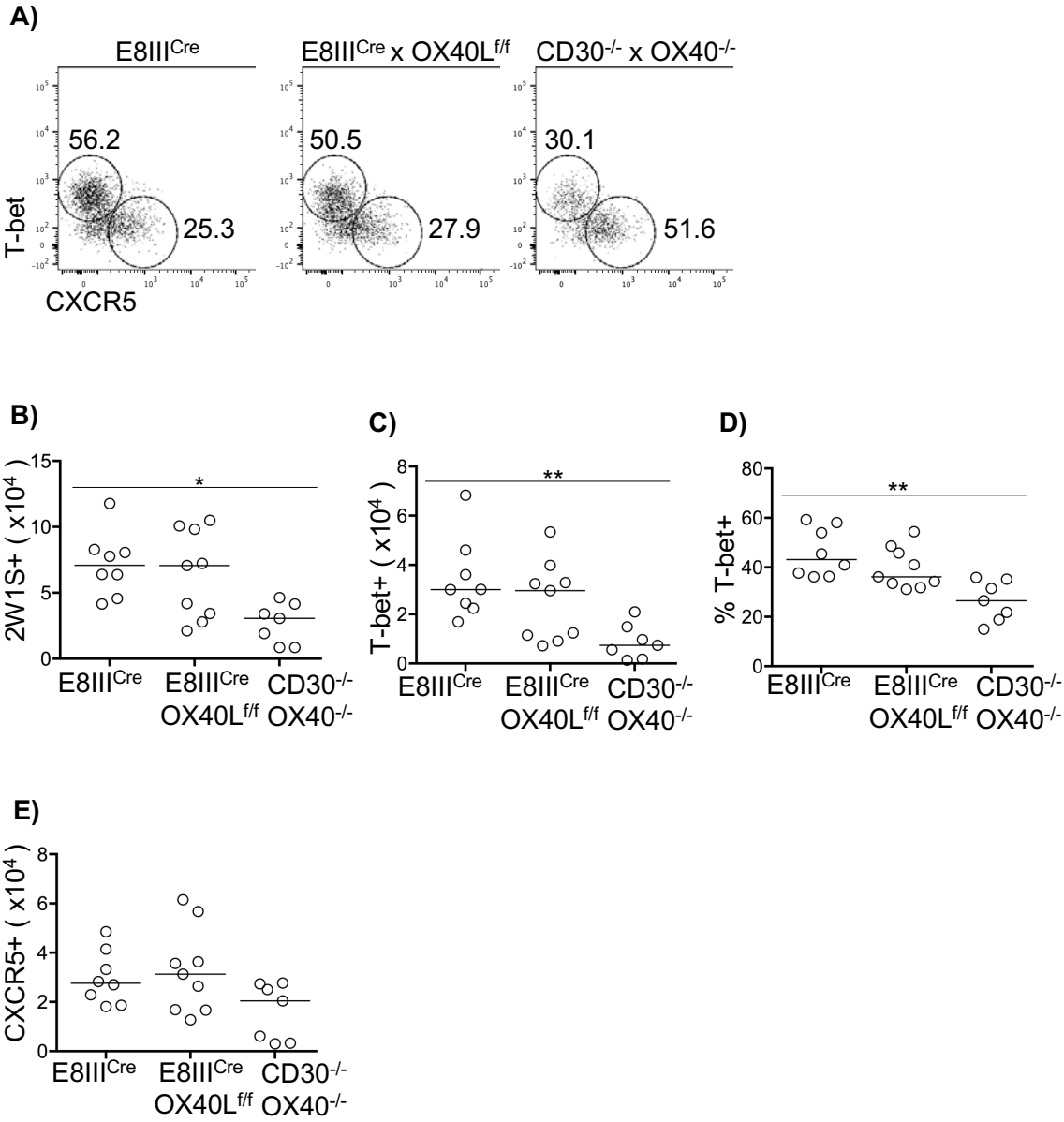

A)
E8III^Cre
E8III^Cre x OX40L^f/f
CD30^-/- x OX40^-/-
56.2
25.3
50.5
27.9
30.1
51.6
T-bet
CXCR5
B)
2W1S+ (x10^4)
*
E8III^Cre
E8III^Cre OX40L^f/f
CD30^-/- OX40^-/-
C)
T-bet+ (x10^4)
**
D)
% T-bet+
**
E)
CXCR5+ (x10^4)

Figure 5. 9 Expression of OX40L by T cells is not required for generation of Th1 effector T cells in response to Lm-2W1S

To investigate whether lymphocytes were key cellular sources of OX40L *in vivo*, the response to Lm-2W1S in T cell ($E8111^{Cre}$) conditional OX40L deficient mice was assessed. (**A**) Expression of T-bet versus CXCR5 by 2W1S-specific CD44hi CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in $E8111^{Cre}$ and $E8111^{Cre}$ x OX40Lf/f mice. (**B**) Enumeration of $CD44^{hi}$ 2W1S-specific CD4 T cells. (**C**) Enumeration of T-bet+ 2W1S- specific $CD44^{hi}$ CD4 T cells. (**D**) Percentage of $T\text{-}bet^{+}$2W1S-specific CD4 T cells. (**E**) Enumeration of $CXCR5^{+}$ 2W1S-specific $CD44^{hi}$ CD4 T cells. Data pooled from two independent experiments, n=8 $E8111^{Cre}$, n=9 $E8111^{Cre}$ x $OX40L^{f/f}$ and n=7 $CD30^{-/-}$ x $OX40^{-/-}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

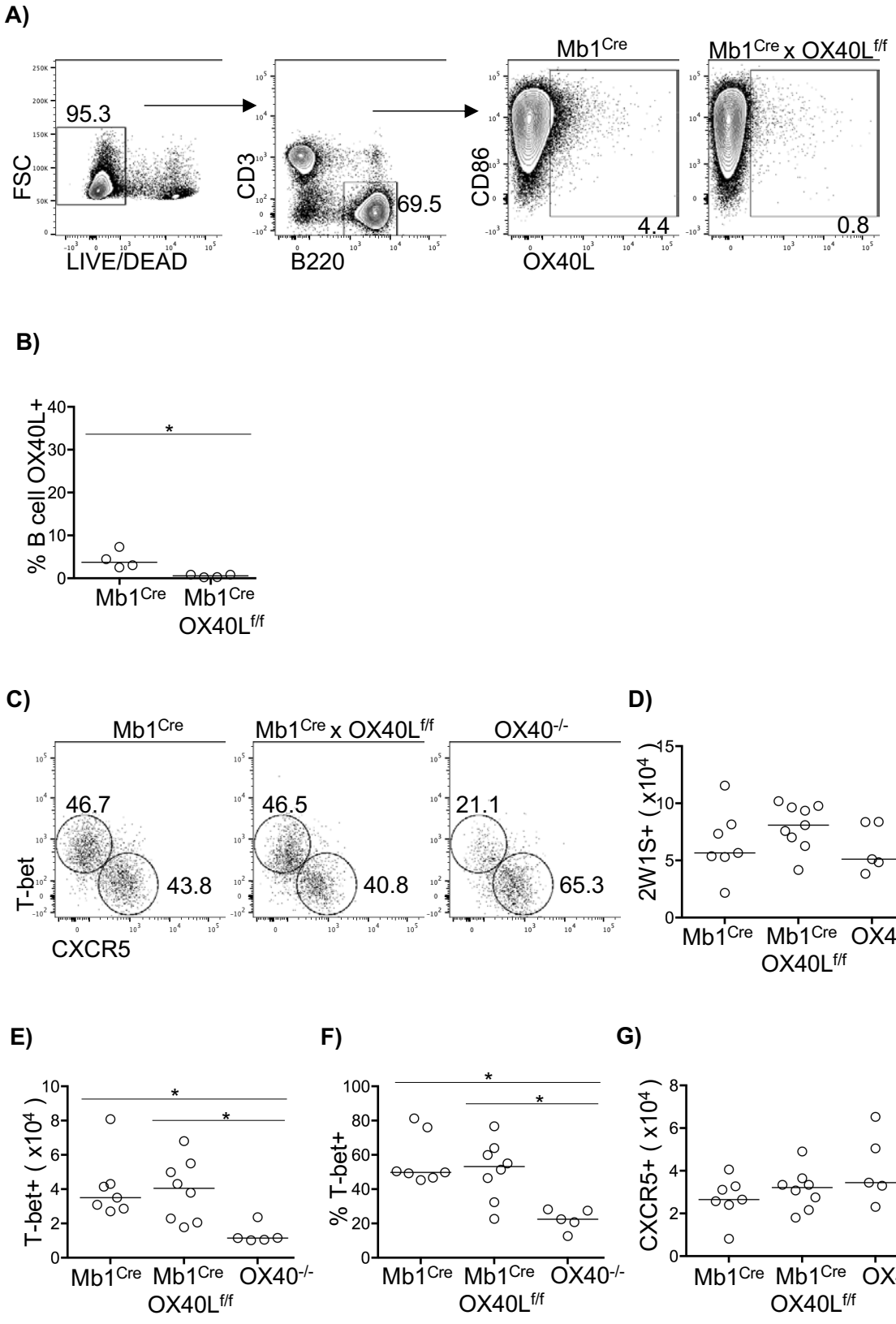

A)
95.3
FSC
LIVE/DEAD
CD3
69.5
B220
Mb1Cre
CD86
4.4
OX40L
Mb1Cre x OX40Lf/f
0.8
B)
% B cell OX40L+
Mb1Cre
Mb1Cre OX40Lf/f
C)
Mb1Cre
Mb1Cre x OX40Lf/f
OX40-/-
46.7
43.8
46.5
40.8
21.1
65.3
T-bet
CXCR5
D)
2W1S+ (x10^4)
Mb1Cre
Mb1Cre OX40Lf/f
OX40-/-
E)
T-bet+ (x10^4)
F)
% T-bet+
G)
CXCR5+ (x10^4)

Figure 5. 10 B cell provision of OX40L is not required for generation of Th1 effector T cells

To assess the OX40L expression on B cells, splenocytes were cultured overnight with αCD40. (**A**) Gating strategy used to identify B cells. (**B**) Expression of OX40L on B cells ($B220^+$) in $Mb1^{Cre}$ and $Mb1^{Cre}$ x $OX40L^{f/f}$ mice. Data pooled from two independent experiments, n=4 $Mb1^{Cre}$, n =4 $Mb1^{Cre}$ x $OX40L^{f/f}$. To investigate whether B cells were key cellular sources of OX40L *in vivo*, the response to Lm-2W1S in B cell ($Mb1^{cre}$) conditional OX40L deficient mice was assessed. (**C**) Expression of T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^+CD4^+B220^-CD11b^-CD11c^-$) in $Mb1^{Cre}$ and $Mb1^{Cre}$ x $OX40L^{f/f}$ mice. (**D**) Enumeration of $CD44^{hi}$ 2W1S-specific CD4 T cells. (**E**) Enumeration of T-bet+ 2W1S-specific $CD44^{hi}$ CD4 T cells. (**F**) Percentage of T-bet^+2W1S-specific CD4 T cells. (**G**) Enumeration of $CXCR5^+$ 2W1S-specific $CD44^{hi}$ CD4 T cells. Data pooled from two independent experiments, n=7 $Mb1^{Cre}$, n =9 $Mb1^{Cre}$ x $OX40L^{f/f}$ and n=5 $OX40^{-/-}$ mice. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

5.3 Discussion

The mammalian immune system is highly dynamic, consisting of specialised cells organised in functional networks, interacting together to promote effective host defence. The broad spectrum of interactions supports all aspects of immune responses, stimulatory, regulatory and inhibitory. The *in vivo* cellular interactions, particularly via membrane proteins can determine the course of immune responses against invading pathogens.

As described in previous chapters, the OX40 signalling pathway has been widely studied, particularly in the context of T cell activation and prolonged survival. Despite the years of research, the expression and function of its ligand *in vivo* remains poorly understood. In this chapter we conducted careful analysis of our novel OX40L conditional knockout mice, dissecting the cellular providers of OX40L and elucidating its *in vivo* provision in response to Lm-2W1S infection. We found that DC provision of OX40L in primary responses to Lm-2W1S was required for generating effector CD4 T cell population, leaving other known cellular sources of OX40L like T cell, B cells and ILC3 redundant.

5.3.1 Cre loxP technologies

All mice used in this investigation were generated using Cre-loxP technology, which has been the most widely used method for generating conditional knockout mice. In combination with the mTmG reporter mice, we were able to assess the efficiency of Cre expression in the target cells, which overall was satisfactory, ranging between 70-100%. The off targets in both $CD11c^{Cre}$ and $E8III^{Cre}$ accounted for approximately 20%. It is not uncommon for the Cre-loxP systems to produce off targets, whereby Cre is expressed in unwanted tissues or cells. In fact, it is actually one of the main criticisms of this powerful technology. The reduced specificity is caused by a transient expression of Cre in germline or during early development (Song and Palmiter, 2018). Due to increased occurrence of unintended and confound Cre-mediated results, monitoring of Cre expression has been of greatest importance to many

immunological studies. A previous report on Cre characterisation method, revealed that majority of mouse strains in the Jax Cre Repository showed some degree of unexpected Cre recombinase activity, including impaired positioning in off target tissues (Heffner et al., 2012). This study developed a specific pipeline to histologically assess the efficiency of Cre expression using reporter mice to test Cre expression in various organs and tissues at both prenatal and postnatal stages. One of the reporter strains used was the exact mTmG reporter we have used in this investigation. Although not favourable in the described report, this strain was characterised by ubiquitous expression, strong robustness and good mouse breading properties. Additionally this reporter strain was used to verify the expression patterns observed in the preferred R26R-lacZ strain (Heffner et al., 2012). Other reporter strains tested, like R26R-GFP or R26R-YFP, showed deficiencies in certain areas, making them less suitable. According to JAX, $CD11c^{Cre}$ has been found to be expressed in lymphocytes in low amounts, based on the original study in which the $CD11c^{Cre}$ transgene was generated (Caton et al., 2007). Using R26-$EYFP^{+}$ reporter mice, they showed that less than 10% of T cells and B cells expressed eYFP, compared to the 95% expression pattern in various types of DC, including their progenitors. Moreover, the expression seen in the T cells originated in early bone marrow progenitors and was not found in their activated states. On the other hand, there are several studies suggesting the existence of $CD11c^{+}CD8^{+}$ T cells. Therefore, our analyses on $CD11c^{Cre}$ expression could have also reflected the existence of such cells. The establishment of the $CD11c^{+}CD8^{+}$ T cell function have been challenging with conflicting reports implying that they played either regulatory or effector roles (Y. H. Kim et al., 2005; Lin et al., 2003; Vinay et al., 2009). They have been shown to become stimulated by the 4-1BB and antigen, producing high amounts of IFNγ as well as have been shown to stimulate production of indoleamine 2,3-dioxygenase which induces immunosuppressive action of DC (Choi et al., 2006; Soliman et al., 2010).

Assessment of the E8III^{Cre} showing 20% expression in ILC3 population were most likely a result of limited staining panel used to identify ILC3 and further analyses could increase its specificity. There is no Cre expression data available to verify it, however this Cre was chosen with the aim of specifically targeting T cells and not ILC3 given the lack of evidence showing a requirement for CD8 during their development (Melo-Gonzalez and Hepworth, 2017). Despite reports of an intermediate ILC population characterised by expression of CD8 in human fetal intestine and population of CD8^{+} ILC1 population found in human peripheral blood, murine splenic ILC have been shown not to express the CD8 receptor (Li et al., 2018; Magri et al., 2014; Roan et al., 2016).

Overall, the phenotypic analyses conducted in this investigation demonstrated high efficiency of the Cre systems used and highlighted importance of systematic assessment of Cre expression to maximise and validate the use of this powerful and versatile technology.

5.3.2 Assessing OX40L expression in conditional knockout mice

Further analyses of OX40L expression on various cell types in all tested conditional knockout mice also served as a secondary checkpoint for the Cre activity and importantly confirmed deletion of expression in the target populations. Those analyses revealed that at the protein level all conditional Cre systems were fairly specific, abrogating OX40L expression on target cells. Despite the previously concerning phenotypic data, the E8III^{Cre} x OX40L$^{f/f}$ and CD11c^{Cre} x OX40L$^{f/f}$ mice showed normal expression of OX40L on ILC3, which is not very high and minimally reduced expression of OX40L on T cells, respectively.

Overall, stimulation with anti-CD40 allowed for optimal assessment of OX40L expression on APCs, as previously described (Ohshima et al., 1997). In our analyses, DC showed the highest expression of OX40L post *in vitro* stimulation with anti-CD40, while B cells the lowest. This result was consistent with previously published data where a treatment with anti-CD40 alone had minor influence on the OX40L expression on B cells, yet in combination with anti-

IgM, led to its significant increase (Akiba et al., 1999). However, for the simple assessment of the OX40L deletion in different conditional knockout mice, anti-CD40 treatment was sufficient, showing activation of B cells by higher expression of CD86 and revealing significant differences between the WT controls and the PGK^{Cre} x $OX40L^{f/f}$ mice and later on the $Mb1^{Cre}$ x $OX40L^{f/f}$ mice.

Inducing signalling via the CD3 co-receptor and preventing signalling through the CD28 receptor enabled assessment of T cell OX40L expression. To inhibit CD28 signalling, we used CTLA4Ig (Abatacept) which is an immunosuppressive agent and a CD28 receptor analog, showing strong affinities towards murine CD80 and CD86 ligands (Holt et al., 2017). This method of OX40L induction on T cells was optimised by a fellow PhD student in the Lane group, Dr Gwilym Webb, who observed that lymphocytes obtained from $CTLA4^{-/-}$ mice and stimulated with anti-CD3, showed significantly lower expression of OX40L on CD4 T cells as compared to the littermate WT controls (Webb, 2017). On the other hand, the OX40 expression was increased in these mice, suggesting that blocking CD28 signalling and subsequent T cell activation could lead to an upregulation of OX40L on CD4 T cells. Similar observations were previously described, where *in vitro* stimulation with anti-CD3 and anti-CD28 led to an increase in OX40 and reduction in OX40L expression on splenic T cells (Akiba et al., 1999). This study also suggested that upregulation of OX40 on T cells could occur in CD28 independent manner as anti-CD3 stimulation in $CD28^{-/-}$ mice showed no significant differences in OX40 expression when compared to WT controls. However, Gwilym's experiments were more sophisticated using $CTLA4^{-/-}CD80^{-/-}CD86^{-/-}$ mice in which he showed that stimulation with anti-CD3 and anti-CD28 led to upregulation of OX40 while stimulation with anti-CD3 and CTLA4Ig led to upregulation of OX40L on CD4 T cells (Webb, 2017). In our investigation, this method proved highly effective at phenotyping T cell expression of OX40L in different conditional knockout mice, revealing cell specific knockouts in $Rorc^{Cre}$ x $OX40L^{f/f}$ and $E8III^{Cre}$ x $OX40L^{f/f}$ mice.

5.3.3 Importance of OX40L signals in responses to Lm-2W1S infection

The function of OX40L, this widely expressed 34 Kd glycosylated type II transmembrane protein, has been previously described in several studies, indicating its crucial role for instance in the pathogenesis of EAE and graft-versus-host disease (Nohara et al., 2001; Stüber et al., 1998; Weinberg et al., 1999). Moreover, constitutive expression of OX40L in OX40L-Tg mice has been linked to a development of spontaneous inflammatory diseases, like interstitial pneumonia presented with elevated numbers of neutrophils and macrophages (Murata et al., 2002). Responses to viral infections, like influenza have also been shown to exacerbate following OX40L signalling, with OX40L deficiency resulting in reduced susceptibility to the viral infection (Hirano et al., 2016). Furthermore, in tumour immunology OX40L has been associated with enhanced IFNγ production and improved survival rates post vaccination with OX86 agonistic antibody (Shibahara et al., 2015).

Since the generation of mice deficient in OX40L, the understanding of OX40L function has vastly improved. Past studies demonstrated that abrogation of OX40L signals had a significant effect particularly on the function of APCs as T cell stimulants, yet it played no role in their phenotype. Both DC and B cells isolated from OX40L$^{-/-}$ mice showed no defect in their activation properties, characterised by expression of MHCII, CD40, CD80 and CD86, in the presence of anti-CD40 or at steady state (Murata et al., 2000a). However, their costimulatory properties as APCs were severely impaired, as shown by reduced antigen-specific CD4 T cell recall responses following immunisation with Keyhole Limpet Hemocyanin. Furthermore, *in vitro* cultures with CD4 T cells and APCs isolated and purified from either OX40L$^{-/-}$ or WT mice and assessed in different combinations, showed reduced T cell proliferation and priming when either CD4 T cells or APC came from the OX40L$^{-/-}$ mice. Additionally, the effector functions of CD4 T cells were also defective as shown by reduced IFNγ production (Murata et al., 2000a). Consistently, our data provided further evidence on the crucial role of OX40L in effector CD4 T cell responses to Lm-2W1S at peak of the response (7 dpi). The OX40L$^{-/-}$

mice used in this investigation were the PGK^{Cre} x $OX40L^{f/f}$, expressing Cre ubiquitously, as shown by efficient deletion of OX40L on all APCs, T cells and ILCs. We found that overall 2W1S-specifc CD4 T cell responses and particularly their expression of T-bet^{+} (previously described as characteristic for effector population in Chapter 3) were highly impaired and mirrored the defect in generation of effector CD4 T cells found in $OX40^{-/-}$ mice. Hence together this data reproduced the well-established concept of the importance of OX40:OX40L pathway in efficient antigen specific CD4 T cell responses.

Additionally, past studies have identified the possible role of OX40L in generating primary and secondary antibody responses to a T cell-dependent antigen which could suggest that Tfh function was OX40L dependent (Kurche et al., 2010). In our investigation, the effect on Tfh population was not significant although further repeats could have strengthened the data. However, our group has previously demonstrated that blocking OX40L *in vivo* had no effect on numbers of Tfh cells (Marriott et al., 2014). Thus, it would seem that the requirement of different T cell subsets for OX40 signalling is likely dependent on the response and may reflect factors such as chronicity of antigen exposure which in turn helps control the timing of OX40 expression.

5.3.4 Cellular providers of OX40L *in vivo*: ILC3 and T cells

As previously mentioned, our investigations into the requirement of OX40L provision by different accessory cells focused on utilising the novel OX40L conditional knockout mice, establishing the requirement for OX40L provision by each cell type in the primary effector CD4 T cell responses to Lm-2W1S. First reports of OX40L expression on ILC3 showed a group of $CD4^{+}CD3^{-}$ accessory cells capable of interacting with T and B cells and lacking responsiveness to Flt3 ligand (Kim et al., 2003). In $Rag^{-/-}$ mice, alongside DC, these cells had the highest expression of OX40L (Kim et al., 2006). Following these initial observations, many reports have suggested that ILC3 could indeed be important in providing help to T cells via

various stimulatory signals. They have been shown to be important for maintaining effective responses to microbiota (Hepworth et al., 2013). Additionally, deletion of MCHII on the surface of ILC3 had a profound effect on antigen-specific CD4 T cells and T cell dependent B cell responses, showing their direct role in antigen presentation (von Burg et al., 2014). In terms of provision of OX40L, current studies have shown that TL1A-induced expression of OX40L on ILC3 during colitis played beneficial role in barrier protection and T cell activation, while others showed it to be crucial in maintaining intestinal Treg homeostasis (Castellanos et al., 2018; Deng et al., 2019). Finally, previous reports from our lab also indicated that lack of ILC3 and their expression of OX40L could be linked to the reduced maintenance of memory CD4 T cells (Withers et al., 2012). Despite previous reports on the importance of ILC3 in providing costimulatory signals to T cells, we found that, alongside T cells, their provision of OX40L played no role in establishing effector T cells responses. Data generated from the $Rorc^{Cre}$ x $OX40L^{f/f}$ on ILC3 contribution might be explained by the very limited number of ILC3 in spleen in lymphoreplete mice (Baerenwaldt et al., 2016). Similarly, earlier and more recent studies relied on $Rag^{-/-}$ mice where OX40L expression is much higher on ILC3 for reasons that are not completely clear (Castellanos et al., 2018; Kim et al., 2003). Perhaps, other models like for instance infections with *Citrobacter rodentium* or *Salmonella* serovar *typhimurium*, could be better suited for this type of analysis as ILC3 are predominantly associated with intestinal homeostasis and could play a clearer role in GALTs.

Past studies have shown that T cells had the ability to acquire some of the APC associated molecules, like CD40 and CD86, which were crucial for generation of memory CD8 T cells and activation of naïve T cells, respectively (Bourgeois et al., 2002; Hakamada-Taguchi et al., 1998). OX40L expression on T cells has been associated with promoting and augmenting T cell responsiveness. In experiments with TCR transgenic CD4 T cells, following a transfer into WT or sublethally irradiated mice, OX40L-deficient T cells had reduced survival upon antigen stimulation (Soroosh et al., 2014). Our data, however, suggested that in Lm-2W1S induced antigen-specific CD4 T cells responses, absence of OX40L on T cells had no effect

on neither the magnitude of the response nor generation of effector population. Past studies on T cell OX40L function have used OT-II TCR transgenic mice which are often associated with non-physiological conditions. Our *in vivo* analyses indicated that in both $Rorc^{Cre}$ x $OX40L^{f/f}$ and $E8III^{Cre}$ x $OX40L^{f/f}$ mice, provision of OX40L by T cells and ILC3 was redundant in the primary response to Lm-2W1S.

5.3.5 Cellular providers of OX40L *in vivo*: DC

It is well established that OX40L expression is especially linked to APCs function as they represent primary group of accessory cells interacting with and providing costimulatory signals to naïve T cells (Ohshima et al., 1997; Stüber et al., 1995). DC in particular, as most efficient APCs, have the biggest potential to be crucial providers of OX40L signals. In fact, in *L. monocytogenes* induced infections, DC have been shown to be utterly required for entry of bacteria into the spleen and the consequent generation of immune responses (Neuenhahn et al., 2006). We demonstrated their crucial role in generating CD4 T cell responses using mice deficient in MHCII expression on DC, which did not respond to the Lm-2W1S infection in antigen-specific manner. Similar results were shown previously, where OVA-specific CD4 T cell transfer into mice lacking MHCII on DC resulted in absence of antigen-specific CD4 T cell but not OT-I CD8 T cell responses following immunisation with OVA (Loschko et al., 2016). Total knockout of MHCII in mice has been associated with impaired or even absent CD4 T cell development due to the lack of $MHCII^{+}$ cTECs), mTECs and DC which mediate positive and negative selection in the thymus (Chen et al., 2017; Grusby et al., 1991).

As previously described, studies involving the use of $OX40L^{-/-}$ mice have been the main source of data supporting the critical role of OX40L expression on DC. They were based almost entirely on *in vitro* polarisation or adoptive transfer experiments, providing no evidence for the *in vivo* requirement of DC OX40L. With the documented wide expression patterns of OX40L found on many cell types, it has been the priority to investigate DC specific deletion

of OX40L and their subsequent role in effective CD4 T cell priming and effector responses. One past study explored this in Th2 type of responses, where bone marrow derived DC from WT and $OX40L^{-/-}$ mice where cultured with or without superantigen staphylococcal enterotoxin A (Jenkins et al., 2007). The antigen-experienced OX40L-deficient DC were transferred into WT mice and 7 days later, the harvested splenocytes were re-challenged *in vitro* with the SEA. The results of ELISA analyses, showed impaired Th2 responses, characterised by reduced production of IL-4, IL-5, IL-10 and IL-13 cytokines. Furthermore, this phenotype was rescued by intraperitoneal injections of the agonistic OX86 monoclonal antibody (Jenkins et al., 2007).

Due to the availability of the $CD11c^{Cre}$ x $OX40L^{f/f}$ mice, our study provided even more specific analysis of the OX40L provision by DC in *in vivo* Th1 responses. Mice lacking OX40L on DC had their 2W1S-specific CD4 T cell responses significantly reduced. Our most striking observation however was the defect in the effector CD4 T cell population which was consistent with the similar reduction in mice lacking both CD30 and OX40 receptors. Once again, the Tfh population appeared to be unaffected by the lack of OX40L on DC, strengthening our data in PGK^{Cre} x $OX40L^{f/f}$ mice.

5.3.6 Cellular providers of OX40L *in vivo*: B cells

The T cell-B cell contact via OX40:OX40L pathway has been particularly implicated in the development of T cell dependent humoral responses (Stüber et al., 1995; Stüber and Strober, 1996). Those initial studies however, did not investigate in detail which of the cells was the actual OX40L provider, as both T and B cell can express OX40 and OX40L (Akiba et al., 1999). Later reports demonstrated that adoptive transfer of OX40L deficient B cells along with WT T cells into $Rag2^{-/-}$ mice, led to fewer memory B cells and lower alloantibody numbers, but CD4 T cell numbers were maintained (Kato et al., 2004). Moreover, another study has shown that WT B cells transferred into $OX40L^{-/-}$ mice were capable of restoring defective Th2

responses, while OX40L-deficient B cells transferred into immunoglobulin μ chain knockout mice (lack mature B cells) did not rescue the phenotype (Linton et al., 2003). Together, these studies highlighted the role of B cell OX40L, however, were limited to possible caveats resulting from their experimental designs. Most recent study on B cells provision of OX40L, has used the conditional knockout $CD19^{Cre}$ x *Tnfsf4*$^{fl/fl}$ mice to specifically delete OX40L on B cells and demonstrated that it was absolutely required for optimal Tfh development and antibody affinity maturation (Cortini et al., 2017). We have received the *Tnfsf4*$^{fl/fl}$ mouse from this group and used them in generation of all of our OX40L conditional knockout mice. Interestingly, in responses to Lm-2W1S we found no defect neither in the 2W1S-specific CD4 T cell responses nor in the generation of effector population at the primary stages of the infection. Similarly, the Tfh population was unaffected by the lack of OX40L on B cells. Although conflicting, the two sets of data reflect two very different immune responses and experimental designs, therefore should be analysed depending on their context. Additionally, we have used different Cre recombinase, $Mb1^{Cre}$, to target B cells, which has been shown to present stronger B cell phenotype than the $CD19^{Cre}$ (Hobeika et al., 2006).

5.3.7 Summary

Altogether, data presented in this Chapter explored the possible cellular providers of OX40L signals in primary responses to Lm-2W1S. Among many cells, DC were identified as absolutely required for the optimal Th1 CD4 T cell responses, once again emphasising the importance of DC: T cell interactions and the crucial role of OX40:OX40L pathway in promoting T cell function. Future studies should focus on providing detailed analysis of the effector T cell responses in $CD11c^{Cre}$ x $OX40L^{f/f}$ mice, particularly the cytokine profile as well as investigate the role of DC OX40L in memory T cell responses. Next chapter will assess the role of early IFNγ signals in AS01 driven responses and determine whether DC OX40L provision is required for generating effector CD4 T cells.

CHAPTER 6: THE SIGNIFICANCE OF INNATE IFNγ IN THE EFFICACY OF AS01 INDUCED RESPONSES

6.1 Introduction

The discovery of vaccination, almost 200 years ago, has transformed our lives, allowing us to prevent multiple infectious diseases establishing themselves within large swathes of the human population. We have observed a steady decrease in the morbidity and mortality rates, with many of the diseases closely controlled and some already or nearly eradicated like Smallpox and Polio.

Initially, our understanding of how vaccine work was extremely limited, this however changed with 50 years of immunological research, leading to development of more advanced vaccine systems. One of such improvements involved the use of inactivated vaccines, which were combinations of attenuated live strains of pathogens and immunostimulants, called adjuvants. Over the last century, many adjuvants have been tested for use in human vaccines, however not many have actually been licensed. This has partially been caused by limited research methods and non-transferable results of animal research, with the mechanisms of action of many adjuvants lacking basic understanding. In recent years, however, further advances have been made and has led to generation of new adjuvant systems which have been optimized to be safe for human use. Typically, modern adjuvants act as delivery systems, usually consisting of emulsion or liposome core, carrying fragments of pathogens and other immunostimulatory agents. Over the years the focus of research has also shifted towards targeting other diseases like Malaria, flu and HIV which require generation of potent antigen specific Th1 immune responses. Previous generations of adjuvants, like aluminium salts, were known to induce poor responses, primarily and controversially of a Th2 phenotype (McKee et al., 2009; Petrovsky and Aguilar, 2004). Thus, many modern adjuvants, often referred to as PRR ligands, now include immunostimulants based on TLR agonists, generating efficient and robust Th1 responses.

One such modern adjuvant is the GSK licensed adjuvant AS01, described briefly in Chapter 3. This system incorporates the use of liposome-based structure and TLR agonist to induce APC activation and maturation, resulting in elevated immune responses (Didierlaurent et al., 2017). The TLR agonist used in AS01, as previously outlined, is MPLA. It is a TLR-4 agonist and is a LPS derivative, also used in another GSK licensed adjuvant, the AS04 which was incorporated into a hepatitis B vaccine, licensed in Europe since 2005 (Tong et al., 2005). MPLA is a detoxified version of lipid A from LPS of *Salmonella minnesota* obtained through controlled hydrolysis, which retains the immunostimulating activities of its parent molecule but with much lower toxicity rate (Ribi et al., 1979; Takayama et al., 1981; Vuopio-Varkila et al., 1988). In addition to MPLA, AS01 adjuvant also contains QS-21, a fraction of a saponin extract from soap bark tree *Quillaja saponaria*. First isolated in 1974, QS-21 has gained a lot of interest due to its inducing effects on the function of CD8 T cell as well as antibody production, in particular IgG1 and IgG2a (Dalsgaard, 1974; Katayama et al., 1999; Kensil et al., 1991; Kensil and Kammer, 2005). The combination of those two components has made AS01 into a particularly potent immune system inducer, which is currently being used in Zoster and Malaria RTS,S vaccines. The phase III trial for RTS,S took place between 2009 and 2014 in several sub-Saharan African countries. The results of those trials proved promising particularly in children who received 4 doses, reducing malaria cases by 39% (RTS,S Clinical Trials Partnership, 2015). AS01 is also included in a number of other candidate vaccines against HIV and Mycobacterium tuberculosis (Dendouga et al., 2012; Leroux-Roels et al., 2013; Van Braeckel et al., 2011). All of these reports described increase in the number of antigen specific CD4 T cells as well as increased production of antibodies. No increase in antigen specific CD8 T cells have been reported across those studies.

Although highly effective, the mechanisms behind AS01 functions are still being investigated. Recent reports on the RTS,S vaccine suggested a reduced efficacy of the vaccine over time with possible rebound in malaria cases, however more studies are necessary to fully understand AS01 function (Olotu et al., 2016). On the other hand, AS01 has also been shown

to overcome age-related decline in immunity as in the case of the Zoster vaccine, however the mechanisms behind this function still remain unclear (Chlibek et al., 2016).

Previous studies showed AS01 to play an important role in activating the innate immunity which in turn is critical for its adjuvant function (A. M. Didierlaurent et al., 2014). Investigations with gE (antigen used in Zoster vaccine)/AS01 vaccine with fluorescently labelled QS-21 and antigen showed the response to be notably rapid, with the drainage from the site of injection site, occurring within the first 24 hours post injections. Those analyses also concluded that the administration of antigen and adjuvant at the same location and same time was pivotal to efficient establishment of antigen specific immunity. The study also showed that immunisation with gE/AS01 increased the total numbers of innate cells, like $Ly6C^{high}$ monocytes and neutrophils present within the dLNs, with majority of those found within the muscle just 3 hrs post injections, whereas in dLN the level of $MHCII^{high}$ DC increased 16 hrs post injections (A. M. Didierlaurent et al., 2014). The resulting DC population included both tissue-resident and monocyte-derived cells. This suggests that AS01 promotes recruitment of innate cells into the site of injections and the dLNs. Furthermore, upregulation of costimulatory B7 molecules on DC and increase in CXCL9 and CXCL10 cytokines induced efficient T cell priming and enhanced Th1 programme.

Recently, further research has focused on assessing the earliest stages of vaccination using the HBs antigen (used in RTS,S vaccine) and AS01 and successfully identified cell populations involved in producing early production of IFNγ which is well known to be highly important in creating Th1 polarising milieu, thereby allowing establishment of antigen-specific CD4 T cells responses (Coccia et al., 2017). The assessment of early IFNγ production in draining iliac LNs within the first 6hrs of immunisation revealed the main source of IFNγ to include dLN resident NK cells and CD8 T cells which have been identified to respond in antigen-independent fashion. Additionally, the data clearly indicated that those early responses rely on both IL-12 and IL-18 signals.

In this chapter we focused on exploring the mechanisms behind AS01 efficacy further, in particular its effect on innate cells and their subsequent role in promoting efficient adaptive responses. Building on existing data, we aimed to provide further insight into early IFNγ production, induced by the AS01, and its role in promoting efficient T cell and B cell responses.

6.2 Results

6.2.1 AS01 induces rapid IFNγ production in draining LNs 6hrs post immunisation

Data presented in chapter 3, clearly demonstrated the efficiency of GSK licensed adjuvant AS01 in promoting antigen specific CD4 T cell responses. Moreover, we also showed that the adjuvant properties of AS01 are OX40 dependent, which prompted the question of mechanistically how does AS01 stimulate OX40L expression and which cells provide this signal to support effector CD4 T cell responses in vivo. We therefore made it paramount for us to understand the early actions of AS01, in particular early production of cytokines like IFNγ which could promote upregulation of costimulatory molecules and thereby enhance immune responses.

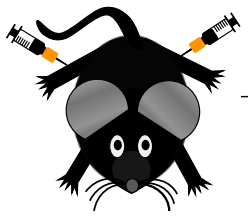
Most common adjuvants are characterised as having the ability to evoke innate signals which in turn activate antigen presenting cells, upregulating various molecules, including many costimulatory pathways, ensuring the efficacy and efficiency of antigen specific CD4 T cell responses. In particular, inflammatory cytokines, like IL-6 and IL-18 produced at the injection site form a link with enhanced antigen presentation (Li et al., 2004; S.-J. Park et al., 2004) . Notably, IFNγ can generate a wide range of cellular events which include cell activation, upregulation of molecules, induction or stabilization of effector functions and especially promoting the Th1 polarizing milieu, enabling activation and expansion of antigen specific T cell populations. Due to the previous evidence showing importance of early IFNγ in systemic responses to Lm-2W1S infections, we sought to understand what where the mechanisms behind AS01 function in local Th1 responses.

Recent reports have shown that AS01 induced very rapid production of IFNγ responses in WT mice (Coccia et al., 2017). As established earlier, all studies involving AS01 adjuvants were conducted in accordance with GSK guidelines. The injections were performed intramuscularly into the gastrocnemius muscle of both hind legs, with 10μL injections containing 20μg OVA-2W1S and 1/50 of human dose of AS01 or PBS.

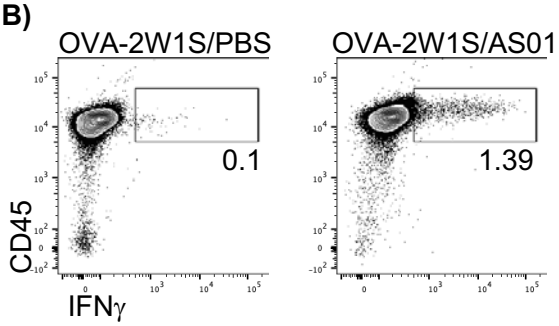
The WT mice were immunised with either 20μg OVA-2W/PBS or 20μg OVA-2W/AS01 and were culled 6hrs later (Figure 6.1 A). To identify IFNγ producing cells in iliac and inguinal draining LNs we followed the GSK optimised protocol, where the isolated cells from draining LNs were cultured for 3 hrs in the presence of BFA (Golgi transport inhibitor) and Monensin (Golgi stop). Flow cytometry analyses revealed that AS01 indeed induced rapid production of IFNγ 6 hrs post immunisation, evident by a clear population of $CD45^{+}IFN\gamma^{+}$ cells in OVA-2W/AS01 immunised mice and its minimal presence in control (OVA-2W/PBS) mice (Figure 6.1 B, C). The results were true for both iliac and inguinal LNs and were further verified by the enumeration and proportion of $CD45^{+}$ cells expressing IFNγ which were found to be significantly higher in both iliac and inguinal draining LNs of OVA-2W1S/AS01 immunised mice as compared to their PBS controls (Figure 6.1 D, E).

We next focused on assessing the phenotype of the $CD45^{+}IFN\gamma^{+}$ cells, particularly the engagement of NK cells and their innate $IFN\gamma^{+}$ responses, which were previously reported (Coccia et al., 2017). Gating on $CD45^{+}IFN\gamma^{+}$ cells, we assessed the expression of NK1.1 and CD3 amongst $IFN\gamma^{+}$ cells, enabling identification of NK cells (NK1.1+, red), putative NKT cells (NK1.1+CD3+, green), T cells (CD3+NK1.1-, yellow) and other $NK1.1^{-}CD3^{-}$ cells (purple) (Figure 6.2 A). Immunisations with AS01 enhanced the total numbers of all identified population as compared to the PBS controls, however this was due to the overall increase in the cellularity of iliac and inguinal LNs post immunisation with OVA-2W1S/AS01 (Figure 6.2 B, D). Further assessment of the proportional contribution of the individual populations showed most substantial changes, as compared to PBS controls, to be amongst the NK and T cell populations. (Figure 6.2 C, E). The increase in putative NKT cell population was more modest but still significant. These results were therefore consistent with previous data showing NK cells and T cells were the main source of IFNγ after immunisation with AS01 adjuvant (Coccia et al., 2017).

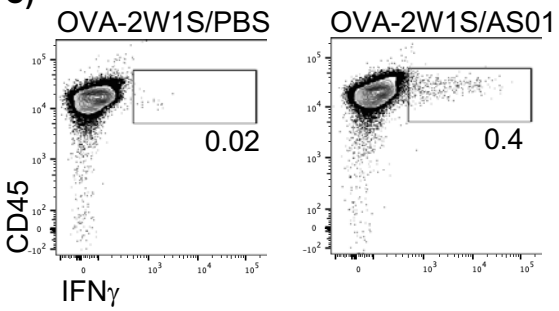
A)


Intramuscular injections
6hr
Draining LNs cultured with BFA and Monensin
3hr
Analysis
20µg OVA-2W1S + 1/50HD AS01 or PBS

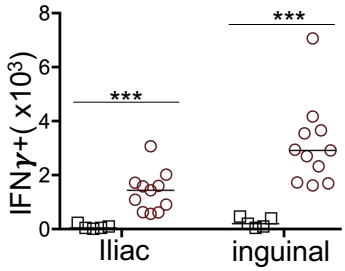
B)


OVA-2W1S/PBS
OVA-2W1S/AS01
0.1
1.39
CD45
IFNγ

C)


OVA-2W1S/PBS
OVA-2W1S/AS01
0.02
0.4
CD45
IFNγ

D)


IFNγ+(x10³)
***
***
Iliac
inguinal

E)

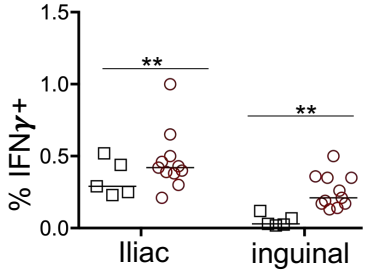

% IFNγ+
**
**
Iliac
inguinal

Figure 6. 1 AS01 induces rapid production of IFNγ

To investigate the early effects of AS01 on the immune response in the draining LNs, WT mice were injected intramuscularly with 20 µg OVA-2W/PBS or 20 µg OVA-2W/AS01, sacrificed 6hrs post immunisation and the IFNγ response assessed in both the iliac and inguinal LNs following a 3 hrs culture with BFA and Monensin. (**A**) Experimental design. (**B**) Representative flow cytometry plots showing IFNγ production at 6 hrs post immunization with OVA-2W/PBS and OVA-2W/AS01 in iliac dLNs. (**C**) Representative flow cytometry plots showing IFNγ production at 6 hrs post immunization with OVA-2W/PBS and OVA-2W/AS01 in inguinal dLNs. (**D**) Total number of $CD45^{+}IFN\boldsymbol{\gamma}^{+}$ cells in iliac and inguinal dLNs. (**E**) Percentages of $CD45^{+}IFN\boldsymbol{\gamma}^{+}$ cells in iliac and inguinal dLNs. Data pooled from 4 independent experiments, n=5 OVA-2W/PBS, n=11 OVA-2W/AS01. Values on flow cytometry plots represent percentages, bars on scatter plots represent the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

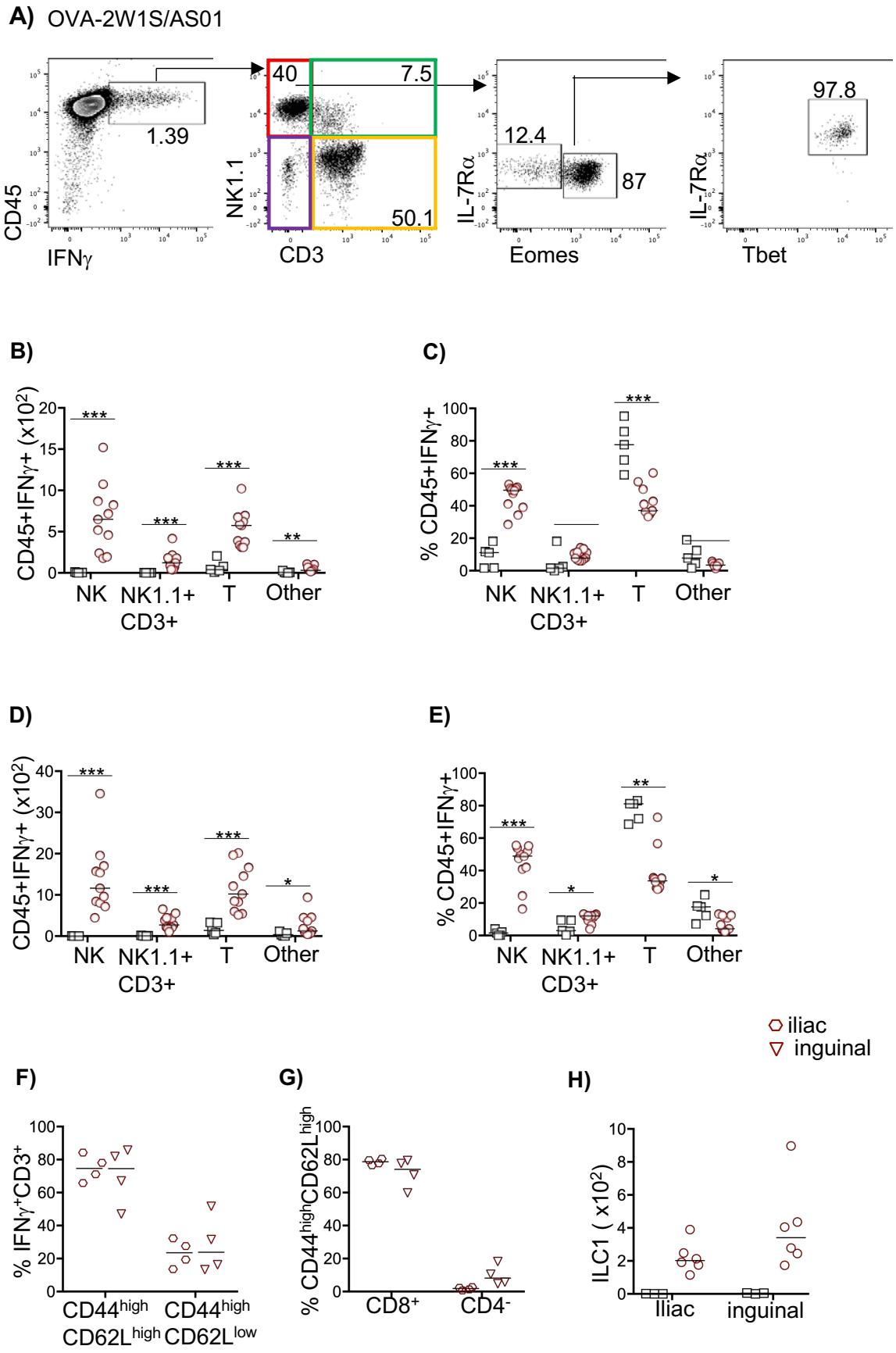

A) OVA-2W1S/AS01
CD45
IFNγ
1.39
NK1.1
CD3
40
7.5
50.1
IL-7Rα
Eomes
12.4
87
IL-7Rα
Tbet
97.8
B)
CD45+IFNγ+ (x10²)
NK
NK1.1+ CD3+
T
Other
C)
% CD45+IFNγ+
NK
NK1.1+ CD3+
T
Other
D)
CD45+IFNγ+ (x10²)
NK
NK1.1+ CD3+
T
Other
E)
% CD45+IFNγ+
NK
NK1.1+ CD3+
T
Other
iliac
inguinal
F)
% IFNγ+CD3+
CD44high CD62Lhigh
CD44high CD62Llow
G)
% CD44highCD62Lhigh
CD8+
CD4-
H)
ILC1 (x10²)
Iliac
inguinal

Figure 6. 2 AS01 immunisation have an effect of different cell subsets

To further understand the early effects of AS01 on the immune response in the draining LNs, population of the IFNγ^+ cells were phenotyped. (**A**) Flow cytometry plots showing gating strategy used to identify different CD45$^+$IFNγ^+ cell subsets (NK cells (NK) - RED, putative iNKT cells (NK1.1+ CD3+) - GREEN, T cells (T) - YELLOW, other cells (Other) - PURPLE) in iliac dLNs. These plots also show identification of ILC population within the NK cell subset. Representative flow cytometry plots for inguinal dLNs can be found in Appendix Figure 8.5. (**B, C**) Total numbers and percentages of different IFNγ producing cells subsets identified at the 6hrs post immunisation in iliac LNs. (**D, E**) Total numbers and percentages of different IFNγ producing cells subsets identified at the 6hrs post immunisation in inguinal LNs. **(F)** Percentages of CD62L$^+$ and CD62L$^-$ T cells present at 6 hrs post immunisation with OVA-2W1S/AS01. Gating strategy and representative flow cytometry plots can be found in Appendix Figure 8.6 (**G**) Percentages of CD8$^+$ versus CD4$^+$ cells found within the CD62L$^+$ T cell population at 6 hrs post immunisation with OVA-2W1S/AS01. **(H**) Total number of ILC population induced at 6 hrs post immunisation with OVA-2W1S/AS01. Data in **B-E** are pooled from four independent experiments, OVA-2W/PBS: n=5 and OVA-2W/AS01: n=11; data in **F-G** are pooled from two independent experiments n=5; data in **H** are pooled from three independent experiments n=3 OVA-2W/PBS, n=6 OVA-2W/AS01; Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

We also observed T cell phenotypes induced by AS01 identified the IFNγ^+ T cells to be expressing conventional memory markers, including CD62L and being primarily CD8$^+$ (Figure 6.2 F, G). This suggests that early T cells responses resulted from reactivation of memory T cells, likely in TCR independent manner.

Within the group of NK cells, we also assessed whether group 1 of innate lymphoid cells (ILC1) could be contributing to the production of IFNγ. Despite the lack of conventional antigen receptors, ILC represent group of lymphocytes capable of producing wide range of effector functions which correspond closely to the function of individual helper T cell subsets (Spits and Cupedo, 2012). They are primarily tissue resident and highly abundant in lymph nodes. In recent years, ILC1 have been described to include classical NK cells as well as non-NK ILC1, with different transcriptional factor requirements. To differentiate between the classical NK cells and ILC1, we used expression of EOMES, a transcription factor which has been previously identified to be primarily expressed on NK cells (Figure 6.2 A) (Gordon et al., 2012). Data showed that majority of the IFNγ^+ NK1.1 cells, approximately 85%, expressed EOMES and therefore were undoubtedly characterised as classical NK cells. Interestingly, approximately 12% of cells were negative for EOMES, yet still expressed T-bet and thus were most likely to be genuine ILC1 (Figure 6.2 H).

We next sought to determine whether putative NKT cell population included genuine NKT cells. As in the case of Lm-2W1S model, we hypothesised that NKT cells might play an important role in IFNγ production as well as stimulation of NK cells. In particular, identification of the NK1.1$^+$CD3$^+$ cells among the IFNγ^+ population alone gave an indication for the possible involvement of NKT cells in AS01 induced responses. We were therefore interested to investigate if ablation of NKT cells would have an effect on the identified phenotype and potential of the IFNγ producing cells. To do so, we immunised WT and CD1d$^{-/-}$ mice with OVA-2W1S/AS01. The mice were sacrificed 6 hrs later and the AS01 induced responses analysed in both iliac and inguinal draining LNs following the *ex vivo* culture (Figure 6.3 A). We firstly

confirmed that $CD1d^{-/-}$ mice indeed lacked NKT cells by staining for the CD1d tetramer which showed a significant reduction percentage of NKT cells present (Figure 6.3 B). Analysis of the AS01 induced innate IFNγ responses in draining LNs unveiled no difference in the population of $NK1.1^{+}CD3^{+}$ cells in WT and $CD1d^{-/-}$ mice following immunisation with OVA-2W1S/AS01 which was further represented by the absence of variation in their total numbers (Figure 6.3 D). Moreover, abrogation of NKT cell responses had no significant effect on the overall IFNγ production at 6 hrs post immunisation with OVA-2W1S/AS01(Figure 6.3 E). These results led us to a conclusion that the $NK1.1^{+}CD3^{+}$ population should not be identified as putative NKT cells and that they most likely form a subset of T cells which acquire NK cell phenotype upon stimulation, in particular the expression of NK1.1. Our observations showed that proportion of the $NK1.1^{+}CD3^{+}$ cells were also positive for the CD8 expression (Figure 6.3 C), validating previously published data on the potential of CD8 T cells in expression of NK cell associated molecules (Assarsson et al., 2000).

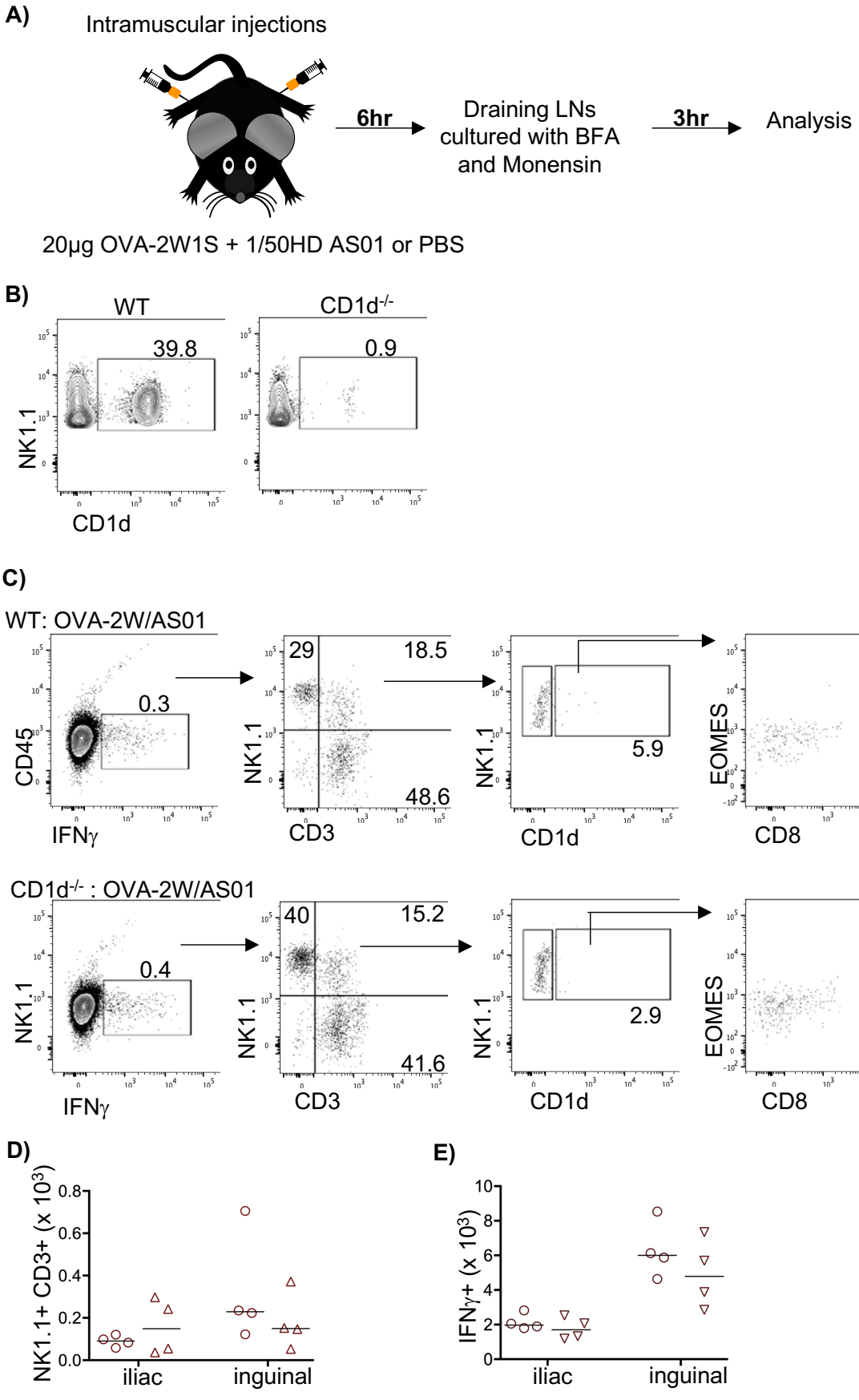

A)
Intramuscular injections
6hr
Draining LNs cultured with BFA and Monensin
3hr
Analysis
20µg OVA-2W1S + 1/50HD AS01 or PBS
B)
WT
CD1d-/-
39.8
0.9
NK1.1
CD1d
C)
WT: OVA-2W/AS01
0.3
29
18.5
48.6
5.9
CD45
IFNγ
NK1.1
CD3
CD1d
EOMES
CD8
CD1d-/- : OVA-2W/AS01
0.4
40
15.2
41.6
2.9
D)
NK1.1+ CD3+ (x 10³)
0.0
0.2
0.4
0.6
0.8
iliac
inguinal
E)
IFNγ+ (x 10³)
0
2
4
6
8
10
iliac
inguinal

Figure 6. 3 The NK1.1^{+}CD3^{+} population of IFNγ^{+} cells cannot be identified as putative iNKT cells

To investigate the phenotype of the NK1.1+CD3+ population of AS01 induced CD45^{+}IFN$\boldsymbol{\gamma}^{+}$ cells, WT and CD1d$^{-/-}$ mice were immunised intramuscularly with 20 µg OVA-2W/AS01, sacrificed 6hrs post immunisation and the IFNγ response assessed in both the iliac and inguinal LNs following 3hrs culture in the presence of BFA and Monensin. (**A**) Experimental design. (**B**) Representative flow cytometry plots showing CD1d$^{-/-}$ deletion in spleen of the mice tested and compared to WT mice. **(C, D**) Representative flow cytometry plots showing IFNγ production in iliac dLN of WT and CD1d$^{-/-}$ mice 6 hrs post immunization with OVA-2W/AS01. Representative flow cytometry plots for inguinal dLNs can be found in Appendix Figure 8.7 (**D**) Enumeration of NK1.1^{+}CD3^{+} subset of IFN$\boldsymbol{\gamma}^{+}$ cells in iliac and inguinal dLNs. (**E**) Total numbers of CD45^{+}IFN$\boldsymbol{\gamma}^{+}$ cells in iliac and inguinal dLNs. Data pooled from two independent experiments, n=4 WT, n=4 CD1d$^{-/-}$. Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

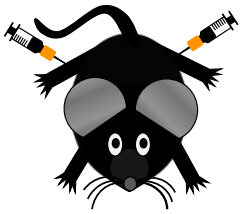
6.2.2 AS01 promotes IFNγ production by innate cells and antigen-specific CD4 T cells in response to OVA-2W

Having established that AS01 induced early IFNγ from different innate and memory cells, we next aimed to move on to developing the same approach in GS reporter mice to enable more dynamic assessment of IFNγ expression which could be further explored also in prime boost approach. We have previously established the effectiveness of GS mice in reporting of IFNγ (eYFP) and IL-17A (hNGFR). To confirm that our experimental approach would work with the GS mice, WT and GS mice were injected with OVA-2W1S/AS01 and their draining LNs analysed at 6hr post immunisation (Figure 6.4 A). WT mice immunised with OVA-2W/AS01 served as negative controls for identifying eYFP expression (Figure 6.4 B). The gate set in the control mouse was then applied to the samples obtained from GS mice. The analyses showed a clear population of $eYFP^+$ (proportional to IFNγ expression) cells when compared to the background signal detected in the WT controls (Figure 6.4 C), identified in both iliac and inguinal draining LNs (Figure 6.4 D).

The established surface staining panel allowed identification of the four IFNγ producing populations. These again were identified as NK cells (red), $NK1.1^+CD3^+$ (green), T cells (yellow) and other $NK1.1^-CD3^-$ cells (purple) (Figure 6.4 C). The results obtained from these analyses were comparable to those observed in WT mice with *ex vivo* culture, showing NK cells and T cells among the dominant population of IFNγ cells (Figure 6.4 E, F).

Having established more robust analysis that enabled direct *ex vivo* assessment with no further manipulation, we next sought to address two further questions, regarding the effect of a second booster injection of AS01 and whether responding CD4 T cells in the draining LN contributed to the IFNγ production.

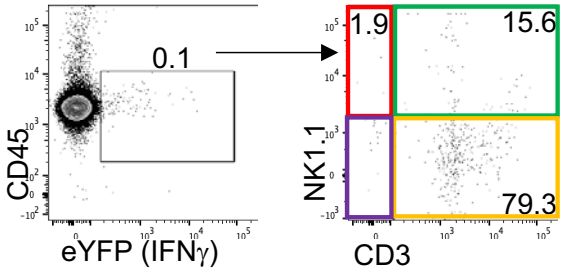
A) Intramuscular injections



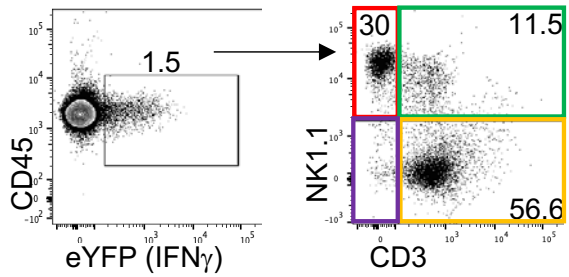
6hr → Analysis

20µg OVA-2W1S + 1/50HD AS01 or PBS

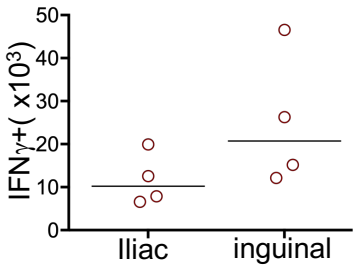
B) WT: OVA-2W1S/AS01



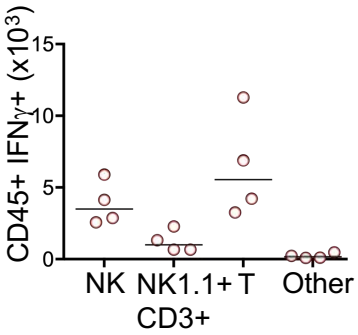
C) GS: OVA-2W1S/AS01



D)



E)



F)

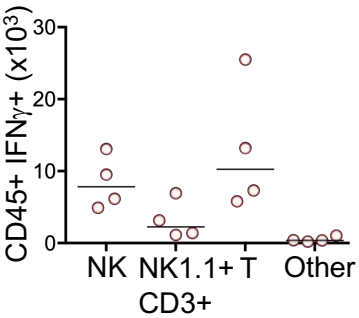


Figure 6. 4 Assessment of IFNγ production induced by AS01 using IFNγ reporter mice

To directly assess IFNγ production without further manipulation, GS mice dual reporter mice were injected intramuscularly with 20 µg OVA-2W/AS01, sacrificed 6hrs post immunisation and response was assessed in both iliac and inguinal dLNs. (**A**) Experimental design. (**B**) Representative flow cytometry plots showing eYFP expression in WT mice following the intramuscular injections and the gating strategy used to identify different cell subsets in iliac LNs. Representative flow cytometry plots for inguinal dLNs can be found in Appendix Figure 8.8 (**C**) Representative flow cytometry plots showing eYFP expression GS mice following the intramuscular injections and the gating strategy used to identify different cell subsets in iliac LNs. (**D**) Total numbers of $CD45^+$eYFP cells in iliac and inguinal dLNs. (**E**) Total numbers of different $CD45^+$eYFP cell subsets identified in iliac dLNs. (**F**) Total numbers of different $CD45^+$eYFP cell subsets identified in inguinal dLNs. Data pooled from 2 independent experiments, n=4. Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

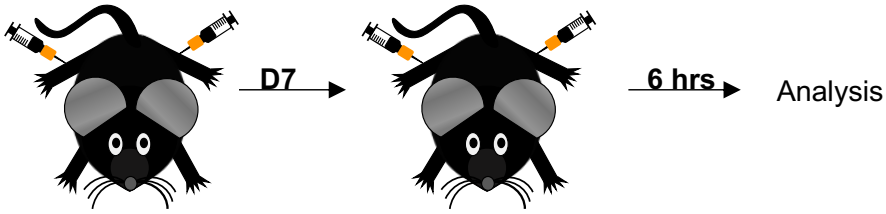
To try to investigate this further, we established 4 treatment groups (Figure 6.5 A). The GS mice were injected at D0 with either OVA-2W/PBS or OVA-2W/AS01 or had no procedure carried out and then received a second dose of either OVA-2W/PBS or OVA-2W/AS01 at D7, anticipating that the early innate IFNγ would have finished but new T cells from the initial immunisation might have formed effector population. A group of mice that received only OVA-2W/PBS at D7 (group 1) served as a negative control for the effects of AS01 and helped with gating on both eYFP^{+} cells but also 2W1S-specific CD4 T cells. To assess the effect of boost on the innate responses, the analyses were conducted 6 hrs post second immunisation at D7 in both draining LNs.

Analysis of eYFP expression in the different mice revealed several interesting observations. First of all, the use of GS control mice (group 1) proved paramount to the accurate identification of the eYFP expressing cells. It indicated a presence of eYFP^{low} expressing cells which most probably reflected the impact of the injection procedure or more homeostatic levels in the LN (Figure 6.6 A). This population also appeared comparable between groups, with the eYFP^{high} population particularly apparent in mice that had received AS01 within last 6hrs prior analysis (group 2 and 4) (Figure 6.6 A). This was further confirmed by the significant increase in the total number of eYFP^{+} cells in both iliac and inguinal draining LNs in mice belonging to treatment group 2 (Figure 6.6 B, C), revealing a clear enhancement of the innate response elicited by a second booster injection with AS01 present.

To further assess whether boosting with AS01 altered the populations that produced high levels of IFNγ, we phenotyped the eYFP^{high} populations using previous gating strategy assessing expression of NK1.1 and CD3 in both iliac and inguinal draining LNs (Figure 6.7 A, E). We then compared the individual cell subsets identified in draining LNs between the different treatment groups. Amongst the eYFP^{+} population, total numbers of NK cells, NKT cells and T cells were significantly increased in mice that received OVA-2W/AS01 at D0 and D7 (group 2) as compared to other treatment groups (Figure 6.7 B, C, D, F, G, H).

A)

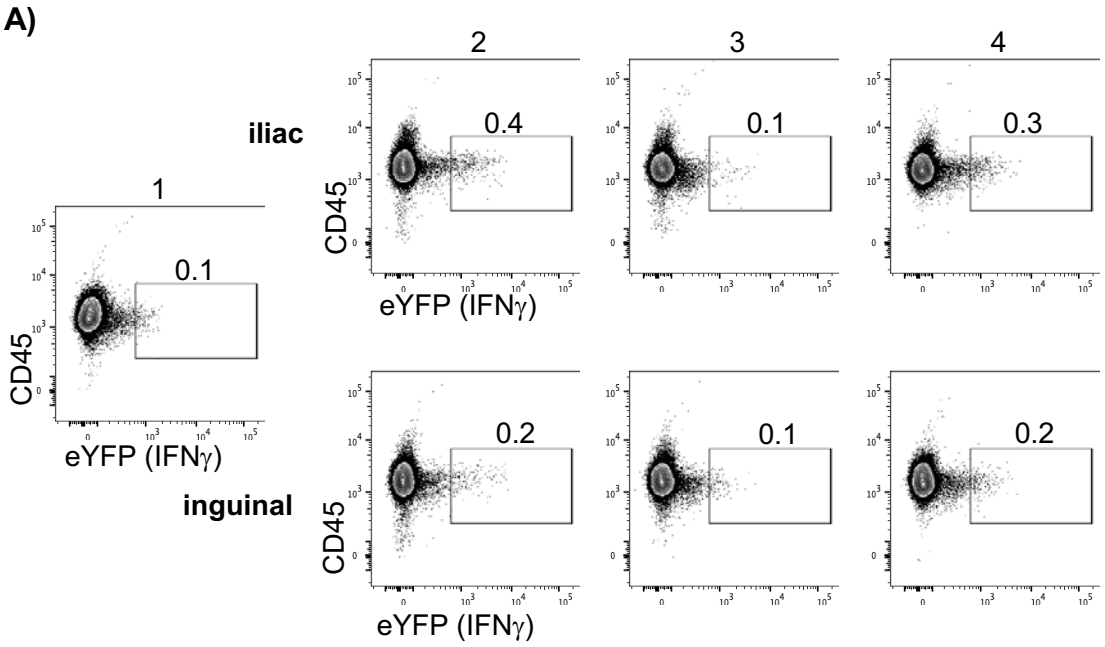
Intramuscular injections



1	**No injection**	**2W1S/PBS**
2	**2W1S/AS01**	**2W1S/AS01**
3	**2W1S/AS01**	**2W1S/PBS**
4	**2W1S/PBS**	**2W1S/AS01**

Figure 6. 5 Model for tracking AS01 induced recall responses in draining LNs

To investigate the effects of AS01 on the efficacy of NK cell and T cell responses, the GS were immunised intramuscularly at D0 and D7 with either 20 µg OVA-2W/PBS or 20 µg OVA-2W/AS01 according to 4 independent experimental conditions: 1= D7: 2W/PBS; 2= D0: 2W/AS01➔D7: 2W/AS01; 3= D0: 2W/AS01➔ D7: 2W/PBS; 4= D0: 2W/PBS➔D7: 2W/AS01. (**A**) Experimental design.


A)
iliac
inguinal
1
2
3
4
0.1
0.4
0.1
0.3
0.2
0.1
0.2
CD45
eYFP (IFNγ)

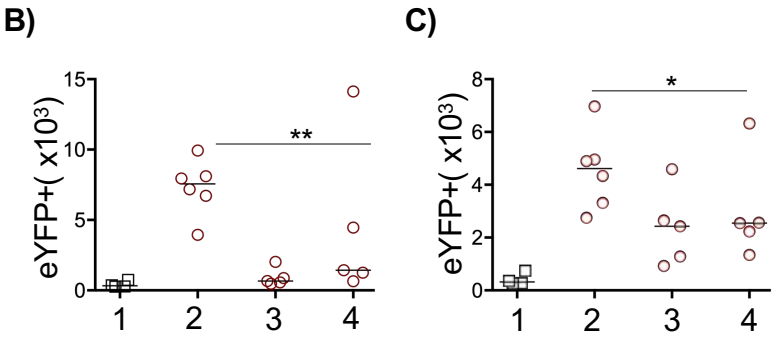

B)
C)
eYFP+(x10³)
**
*
1
2
3
4

Figure 6. 6 Boost with AS01 at D7 increases significantly NK derived IFNγ production

The production of IFNγ was assessed using GS mice which were prior immunised either with 20 µg OVA-2W/PBS or 20 µg OVA-2W/AS01 according to 4 independent experimental conditions: 1= D7: 2W/PBS; 2= D0: 2W/AS01➔D7: 2W/AS01; 3= D0: 2W/AS01➔ D7: 2W/PBS; 4= D0: 2W/PBS➔D7: 2W/AS01. The mice were sacrificed 6hrs post immunisation at D7 and the response was assessed in both iliac and inguinal dLNs. (**A**) Representative flow cytometry plots showing eYFP expression in iliac and inguinal dLNs following the intramuscular injections. (**B**) Total numbers of $CD45^{+}$eYFP cells in iliac dLNs. (**C**) Total numbers of $CD45^{+}$eYFP cells in inguinal dLNs. Data pooled from 2 independent experiments, n=4 Condition 1 (iliac and inguinal LNs pooled); n=6 Condition 2, n=5 Condition 3, n=5 Condition 4. Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

Responses post boost were more prominent in iliac draining LNs than in inguinal LNs, however further repeats could reinforce sample size and provide sufficient statistical power to carry out further analyses. Here we showed that boost with AS01 enhanced innate IFNγ production which supported better adjuvant effect.

Our investigations revealed that approximately half the IFNγ producing cells in the draining LN were T cells. Whilst these may be a mixture of previously activated or memory cells responding directly to the adjuvant or indirectly in response to other signals, we sought to test whether responding antigen experienced CD4 T cell within the draining LN were responsive to AS01. To do this, we assessed the endogenous CD4 T cell response to the OVA-2W1S, using MHCII tetramers. Consistent with previous experiments, administration of AS01 upon initial encounter of antigen resulted in a far more robust CD4 T cell response than the one detected in OVA-2W/PBS controls (Figure 6.8 A, B, C). Whilst boosting with OVA-2W/AS01 did not result in a greater number of 2W1S-specific CD4 T cells, as might be expected given the 6hr time point assessed, interestingly, a significant increase in the IFNγ production was detected versus mice that only received OVA-2W/PBS at boost (Figure 6.8 D, E). Thus, boosting with AS01 not only resulted in enhanced innate IFNγ production but also by antigen-specific CD4 T cells. At this stage, we do not know the impact of boost upon the resulting memory response, however, combined, these initial data indicate that a second booster injection with OVA-2W1S/AS01 induces an enhanced response compared with initial priming with AS01 followed by OVA-2W1S/PBS.

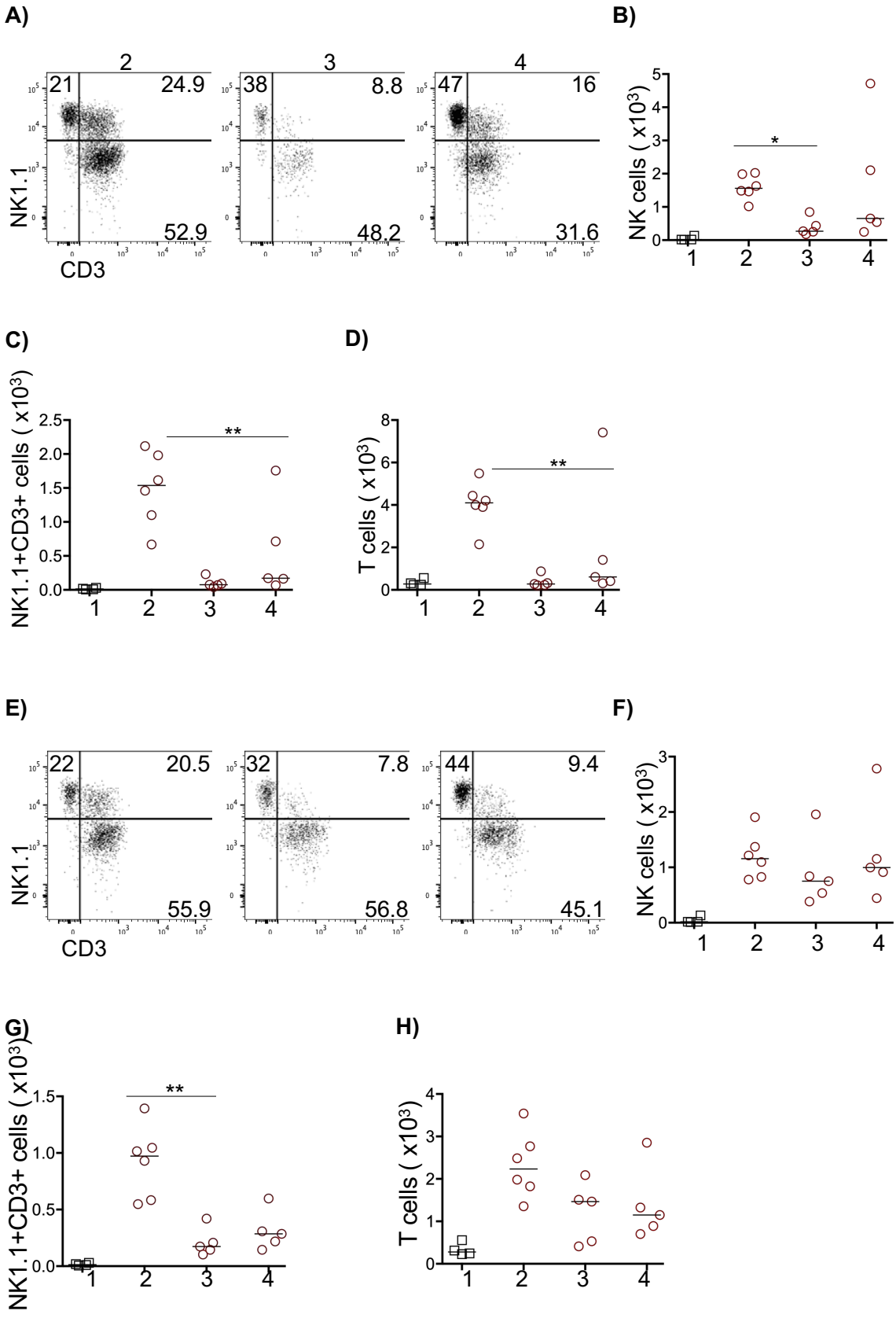
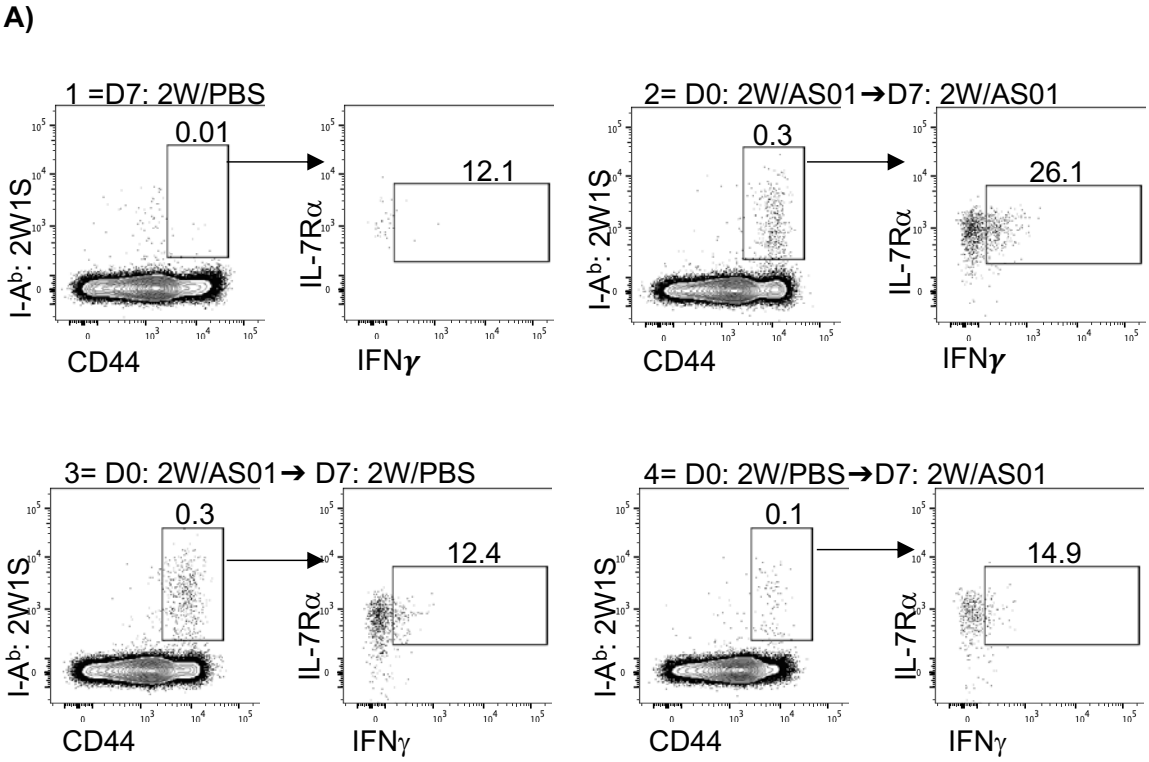

A)
2
3
4
21
24.9
52.9
38
8.8
48.2
47
16
31.6
NK1.1
CD3
B)
NK cells (x10³)
*
C)
NK1.1+CD3+ cells (x10³)
**
D)
T cells (x10³)
**
E)
22
20.5
55.9
32
7.8
56.8
44
9.4
45.1
NK1.1
CD3
F)
NK cells (x10³)
G)
NK1.1+CD3+ cells (x10³)
**
H)
T cells (x10³)

Figure 6. 7 Analysis of IFNγ-producing populations induced by AS01

Phenotyping of the IFNγ^+ cells following immunisation with 20 µg OVA-2W/PBS or 20 µg OVA-2W/AS01 according to 4 independent experimental conditions: 1= D7: 2W/PBS; 2= D0: 2W/AS01➔D7: 2W/AS01; 3= D0: 2W/AS01➔ D7: 2W/PBS; 4= D0: 2W/PBS➔D7: 2W/AS01. The mice were sacrificed 6hrs post immunization at D7 and the response was assessed in both iliac and inguinal dLNs. (**A**) Representative flow cytometry plots showing different CD45$^+$eYFP cell subsets in iliac dLNs following the intramuscular injections. (**B**) Total numbers of NK cells identified in iliac dLNs. (**C**) Total numbers of NK1.1+CD3+ cells identified in iliac dLNs. (**D**) Total numbers of T cells identified in iliac dLNs. (**E**) Representative flow cytometry plots showing different CD45$^+$eYFP cell subsets in inguinal dLNs following the intramuscular injections. (**F**) Total numbers of NK cells identified in inguinal dLNs. (**G**) Total numbers of NK1.1+CD3+ cells identified in inguinal dLNs. (**H**) Total numbers of T cells identified in inguinal dLNs. Data pooled from 2 independent experiments, n=4 Condition 1 (iliac and inguinal LNs pooled), n=6 Condition 2, n=5 Condition 3, n=5 Condition 4. Data assessed by Mann-Whitney, two tailed, non-parametric test, *p<0.05, ** p<0.005, ***p<0.0005, bars show median.


A)
1 =D7: 2W/PBS
0.01
12.1
I-Ab: 2W1S
CD44
IL-7Rα
IFNγ
2= D0: 2W/AS01➔D7: 2W/AS01
0.3
26.1
3= D0: 2W/AS01➔ D7: 2W/PBS
0.3
12.4
4= D0: 2W/PBS➔D7: 2W/AS01
0.1
14.9

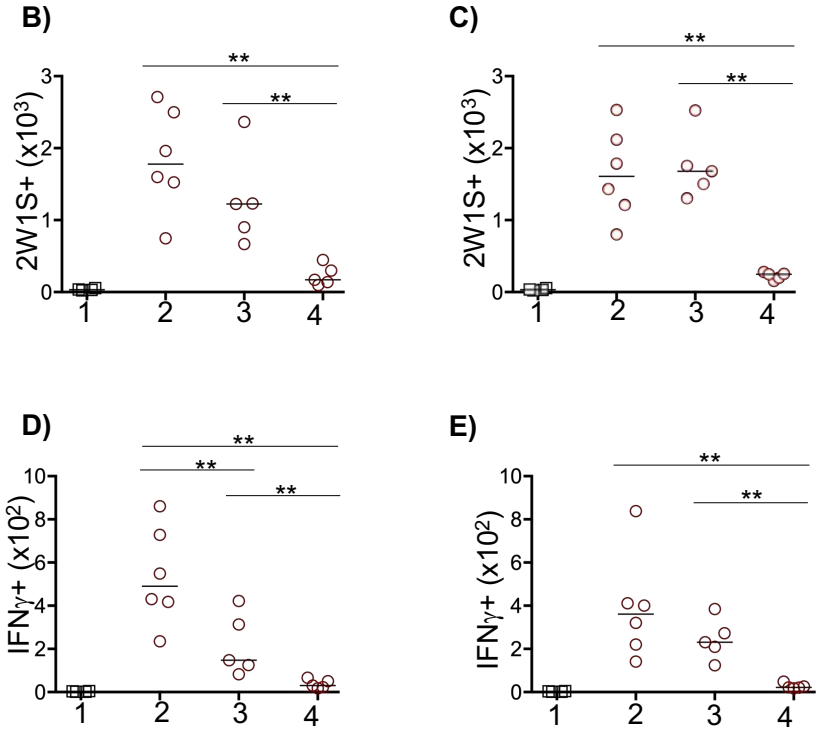

B)
2W1S+ (x10³)
C)
D)
IFNγ+ (x10²)
E)
**

Figure 6. 8 AS01 promotes IFNγ production by responding CD4 T cells in the draining LN

To test whether AS01 induced IFNγ production by responding $CD4^+$ T cells in the draining LN GS mice were injected intramuscularly at D0 and D7 with either 20 µg OVA-2W/PBS or 20 µg OVA-2W/AS01 according to 4 independent experimental conditions: 1= D7: 2W/PBS; 2= D0: 2W/AS01➔D7: 2W/AS01; 3= D0: 2W/AS01➔ D7: 2W/PBS; 4= D0: 2W/PBS➔D7: 2W/AS01. The mice were sacrificed 6hrs post immunization at D7 and the 2W1S-specific response assessed in both iliac and inguinal dLNs using MHCII tetramers ($I\text{-}A^b$:2W1S). (**A**) Representative flow cytometry plots identifying 2W1S-specific CD4 T cells ($CD3^+CD4^+B220^-CD11b^-CD11c^-$) and showing eYFP (IFNγ) expression in iliac dLNs for the tested experimental conditions. Representative flow cytometry plots for inguinal dLNs can be found in Appendix Figure 8.9 (**B**) Total numbers of 2W1S-specific CD4 T cells in iliac dLNs. (**C**) Total numbers of 2W1S-specific CD4 T cells in inguinal dLNs. **(D)** Total numbers of $eYFP^+$ 2W1S-specific CD4 T cells in iliac dLNs. **(E)** Total numbers of $eYFP^+$ 2W1S-specific CD4 T cells in inguinal dLNs. Data pooled from 2 independent experiments, n=4 Condition 1 (iliac and inguinal LNs pooled); Condition 2: n=6; Condition 3: n=5; Condition 4: n=5. Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

6.2.3 AS01-dependent germinal centres formation requires interaction between T and B cells but not IFNγ

So far, our data showed that AS01 induced rapid IFNγ from both innate and adaptive immune cell populations. We next began studies assessing how AS01 elicits B cell responses to build on our understanding of the mechanisms through which this adjuvant supports establishment of robust adaptive immunity and whether they were IFNγ dependent.

The role of IFNγ in creating the environment for effector T cells and regulating both T cell-independent and T cell-dependent B cell differentiation has been already well established (Abed et al., 1994; Berg et al., 2005; Huang et al., 1993). Induction of long-term immune responses is paramount to the efficacy of vaccines, especially, generation of memory B cells and antigen responses. However, antigen-specific B cell responses are still not well understood. Previous studies identified MF59 oil-in-water emulsion adjuvant as an effective inducer of HlaH35L, a Staphylococcus aureus Ag, specific B cell responses (Salvatore et al., 2015). To assess antigen-specific B cell responses following AS01 immunisation, mice were immunised intramuscularly with 10 μg PE/AS01, a method previously shown to allow detection of endogenous antigen-specific B cell populations (Pape et al., 2011). In these initial experiments, we sought to ask whether the B cell response induced by AS01, assessed simply by the formation of PE-specific GCs was a) dependent on T cells and b) dependent upon IFNγ, given the prominent role this plays in supporting the CD4 T cell response. We planned to do this *in vivo* using mice lacking αβ T cells or IFNγ.

The assessment involved immunisation of WT, TCRα$^{-/-}$ and IFNγ$^{-/-}$ mice with either 10 μg PE/PBS or 10 μg PE/AS01 and the responses in a pool of iliac and inguinal draining LNs assessed at D10 of the response (Figure 6.9 A).

In WT mice immunised with AS01/PE, a distinct and expanded population of PE specific B cells was identified, with this population nearly absent in PE/PBS control mice consistent with this population expanding in response to immunisation (Figure 6.9 B, C, D). This population

was significantly decreased in TCR$\alpha^{-/-}$ mice indicating that in absence of $\alpha\beta$ T cells, the enhanced B cell response was blocked (Figure 6.9 D, E, F). We observed no difference in B cells response in IFN$\gamma^{-/-}$ mice which argued that IFNγ was not required for generation of robust B cells responses post immunisation with AS01 adjuvant (Figure 6.9 D, E, F).

GC B cells were defined as $CD38^{low}GL7^{+}$, as previously described (Oliver et al., 1997; Taylor et al., 2012). Consistent with requirement for T cell help, a discrete GC B cell population, identified as $GL7^{+}CD38^{-}$ was evident in AS01 immunised WT mice, but not PE/PBS controls nor TCR$\alpha^{-/-}$ mice (Figure 6.10 A, B, C). In IFN$\gamma^{-/-}$ mice, this population was not diminished, indicating this was independent of IFNγ mediated effects of AS01 (Figure 6.10 A, B, C).

Data described above, defined the function of AS01 adjuvant as an effective immunostimulatory agent and its role in mediating the CD4 T cell and B cell responses in draining LNs.

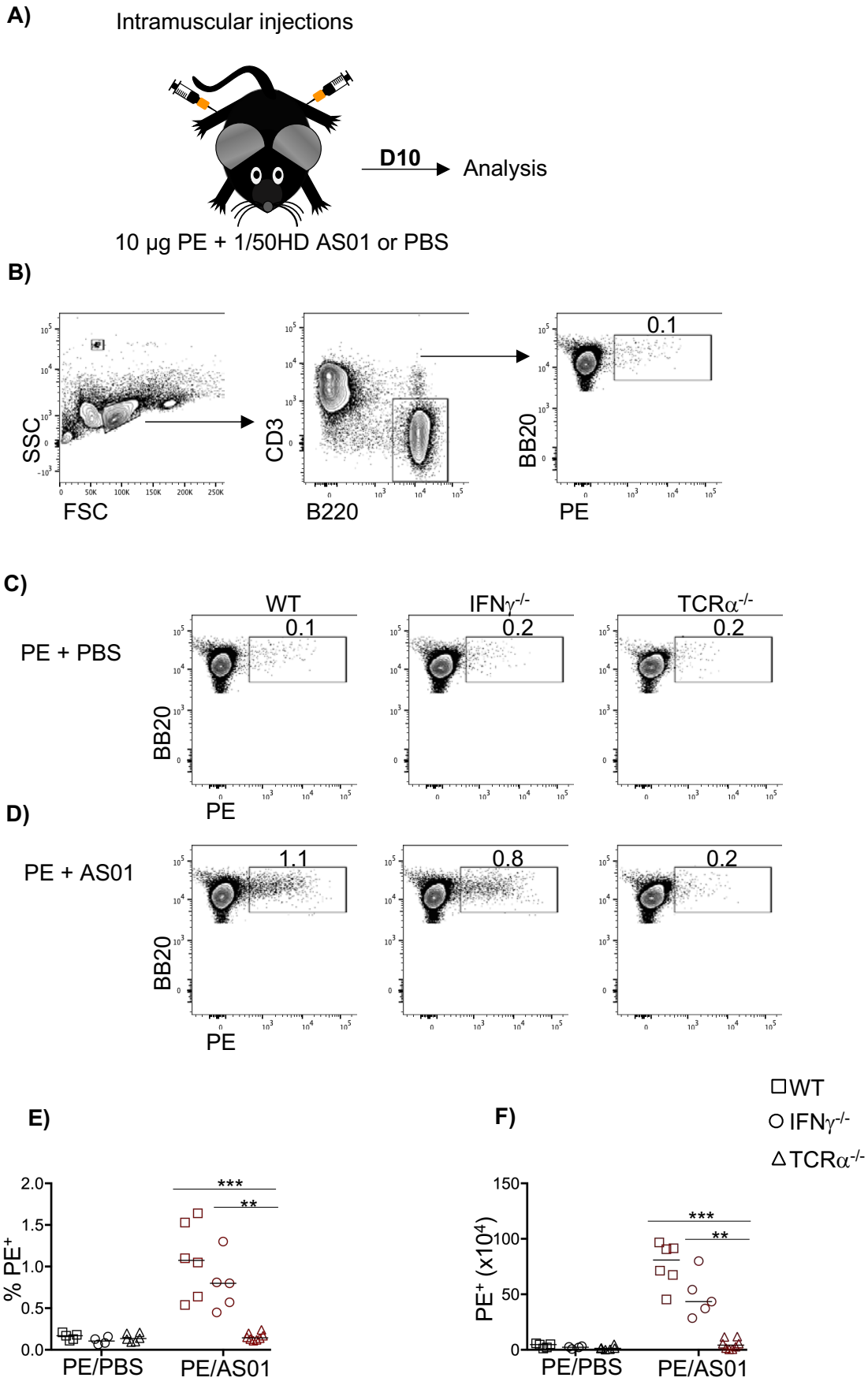

A)
Intramuscular injections
D10
Analysis
10 µg PE + 1/50HD AS01 or PBS
B)
SSC
FSC
CD3
B220
BB20
PE
0.1
C)
PE + PBS
WT
IFNγ-/-
TCRα-/-
0.1
0.2
0.2
BB20
PE
D)
PE + AS01
1.1
0.8
0.2
BB20
PE
E)
% PE+
PE/PBS
PE/AS01
***
**
F)
PE+ (x10^4)
PE/PBS
PE/AS01
***
**
WT
IFNγ-/-
TCRα-/-

Figure 6. 9 AS01-induced B cell responses to PE are T cell dependent

To investigate the mechanisms through which AS01 induces robust B cell responses, WT, IFN$\gamma^{-/-}$ and TCR$\alpha^{-/-}$ mice were injected intramuscularly with either PE/PBS or PE/AS01 and the draining LNs assessed 10 days later. (**A**) Experimental design. (**B**) Gating strategy used to identify PE specific B cells. (**C**) Representative flow cytometry plots showing B cell responses in dLNs (iliac and inguinal combined) of PE/PBS control WT, IFN$\gamma^{-/-}$ and TCR$\alpha^{-/-}$ mice. (**D**) Representative flow cytometry plots showing B cell responses in dLNs (iliac and inguinal combined) of PE/AS01 immunised WT, IFN$\gamma^{-/-}$ and TCR$\alpha^{-/-}$ mice. (**E**) Percentages of PE^+ B cells in dLNs. (**F**) Total numbers of PE^+ B cells in dLNs. Data pooled from two experiments, PBS control: n=5 C57Bl/6; n=4 IFN$\gamma^{-/-}$ and n=6 TCR$\alpha^{-/-}$; AS01 immunised: n=6 C57Bl/6; n=5 IFN$\gamma^{-/-}$ and n=8 TCR$\alpha^{-/-}$.Values on flow cytometry plots represent percentage, bars on scatter plots represent the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

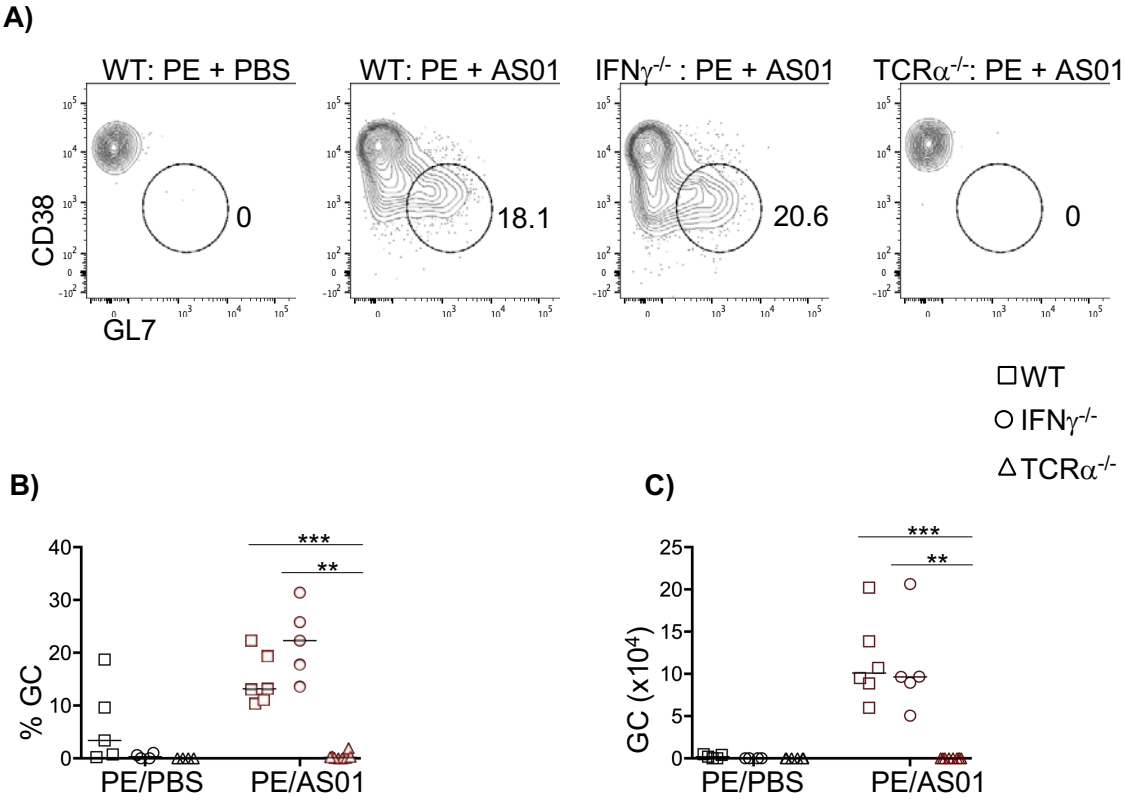

A)
WT: PE + PBS
WT: PE + AS01
IFNγ-/- : PE + AS01
TCRα-/-: PE + AS01
CD38
GL7
0
18.1
20.6
0
WT
IFNγ-/-
TCRα-/-
B)
% GC
PE/PBS
PE/AS01
***
**
C)
GC (x10⁴)
PE/PBS
PE/AS01
***
**

Figure 6. 10 AS01-induced germinal centre formation is T cell dependent

To investigate the AS01 induced germinal centre formation WT, IFN$\gamma^{-/-}$ and TCR$\alpha^{-/-}$ mice were injected intramuscularly with either PE/PBS or PE/AS01 and the draining LNs assessed 10 days later. (**A**) Representative flow cytometry plots showing germinal centre B cells identified as GL7^{+} CD38^{low} at D10 post immunisation in dLNs (iliac and inguinal combined) of PE/PBS control WT and PE/AS01 immunised WT, IFN$\gamma^{-/-}$ and TCR$\alpha^{-/-}$ mice. (**B**) Percentages of GC^{+} B cells in dLNs. (**C**) Total numbers of GC^{+} B cells in dLNs. Data pooled from two experiments, PBS control: n=5 C57Bl/6; n=4 IFN$\gamma^{-/-}$ and n=6 TCR$\alpha^{-/-}$; AS01 immunised: n=6 C57Bl/6; n=5 IFN$\gamma^{-/-}$ and n=8 TCR$\alpha^{-/-}$. Values on flow cytometry plots represent percentage, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001

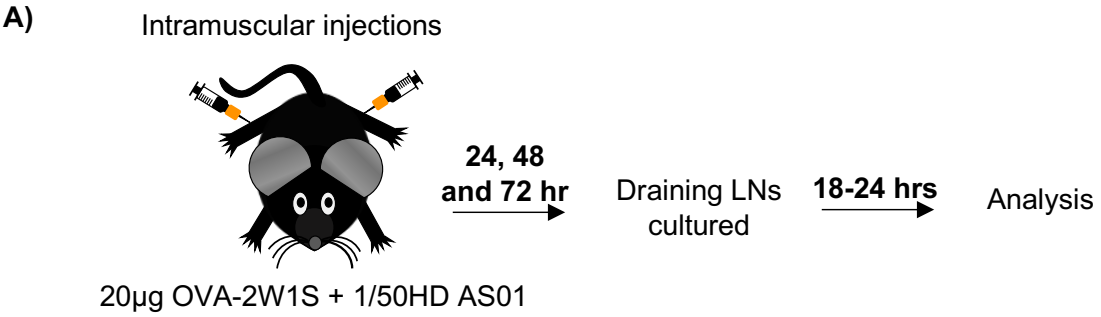
6.2.4. Provision of OX40L by DC is moderately important for generation of effector CD4 T cells responses following immunisation with AS01 and OVA-2W1S

Following our previous findings, we then aimed to investigate the effect of AS01 on the DC function in particular whether it had the potential to upregulate OX40L on DC and moreover whether this interaction could feed into the antigen- specific CD4 T cell responses.

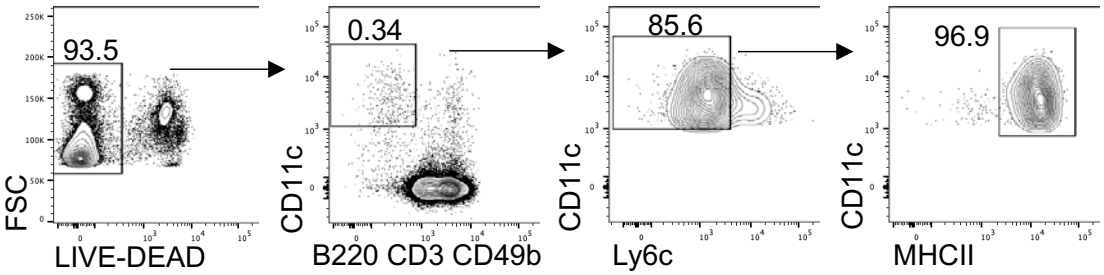
As described in chapter 4, OX40L upregulation on DC in response to Lm-2W1S follows specific pattern, occurring early in the response and being partially dependent on early IFNγ production. We hypothesised that due to the rapid IFNγ production post AS01 immunisation, there was high probability that similar mechanisms could be involved in the OX40L upregulation on DC in draining LNs.

We first aimed to track OX40L expression on DC in draining LNs, contralateral LNs and spleen of WT mice, assessing it at 24, 48 and 72 hrs post immunisation with OVA-2W1S/AS01 followed by the overnight culture, like in the case of the Lm-2W1S model (Figure 6.11 A). Assessment of contralateral LNs and spleen ensured for thorough investigation of AS01 induced effects. Additionally, we used identical gating strategy to identify DC in draining LNs, characterising them by the expression of $CD11c^{+}$, $Ly6C^{low}$, $MHCII^{+}$ (Figure 6.11 B) and untreated WT mice were used to set accurate gates to assess OX40L at individual time points. Flow cytometric analyses revealed AS01 induced upregulation of OX40L on DC which reached a peak expression on LN and splenic DC at 24 hrs post immunisation with AS01 (Figure 6.11 C, 6.12 A, B, C), as compared to the untreated controls. Following the initial upregulation, the proportion of DC expressing OX40L gradually decreased at 48 hrs and was maintained at approximately 10% and 8% at 72 hrs in draining and contralateral LNs, respectively (Figure 6.12 A, B). In the spleen, the decline in OX40L on DC was less prominent but still evident reaching baseline levels at 72 hrs post immunisation with AS01 (Figure 6.12 C).

A)


Intramuscular injections
24, 48 and 72 hr
Draining LNs cultured
18-24 hrs
Analysis
20µg OVA-2W1S + 1/50HD AS01

B)


93.5
FSC
LIVE-DEAD
0.34
CD11c
B220 CD3 CD49b
85.6
CD11c
Ly6c
96.9
CD11c
MHCII

C)

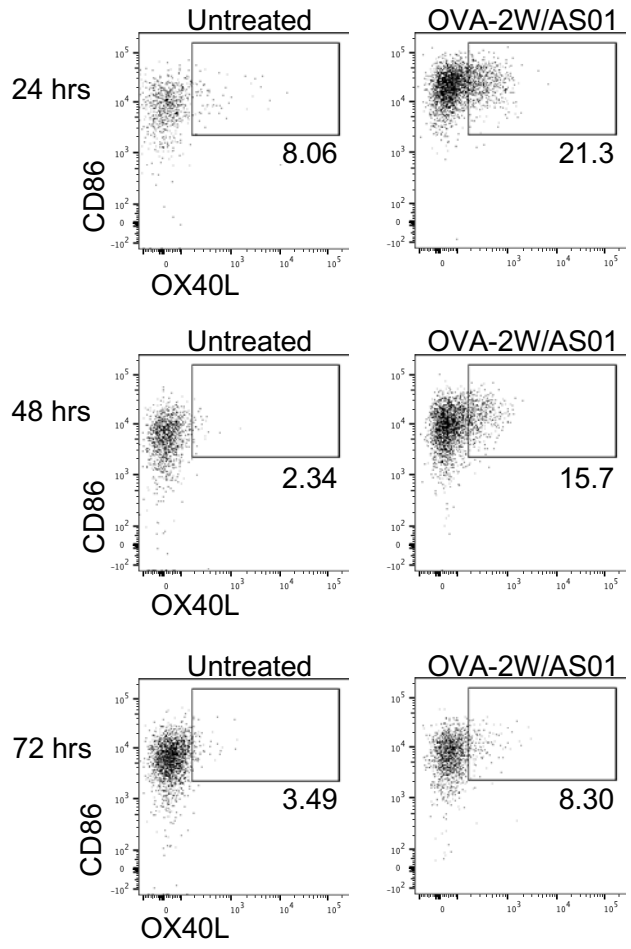

Untreated
OVA-2W/AS01
24 hrs
8.06
21.3
CD86
OX40L
Untreated
OVA-2W/AS01
48 hrs
2.34
15.7
CD86
OX40L
Untreated
OVA-2W/AS01
72 hrs
3.49
8.30
CD86
OX40L

Figure 6. 11 Tracking OX40L expression on DC in draining LNs post intramuscular immunisation with AS01 adjuvant

To investigate the effects of AS01 on the expression of OX40L on DC, mice were injected intramuscularly with 20 µg OVA-2W/AS01, sacrificed 24hrs post immunisation and the pool of iliac and inguinal LNs were cultured overnight. (**A**) Experimental design. (**B**) Gating strategy used to identify DC in draining LNs ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, MHCII). **(C)** Representative flow cytometry plots showing OX40L expression on DC in draining LNs (iliac and inguinal combined) at 24, 48 and 72 hrs post post immunization with OVA-2W/AS01. Values on flow cytometry plots represent percentages.

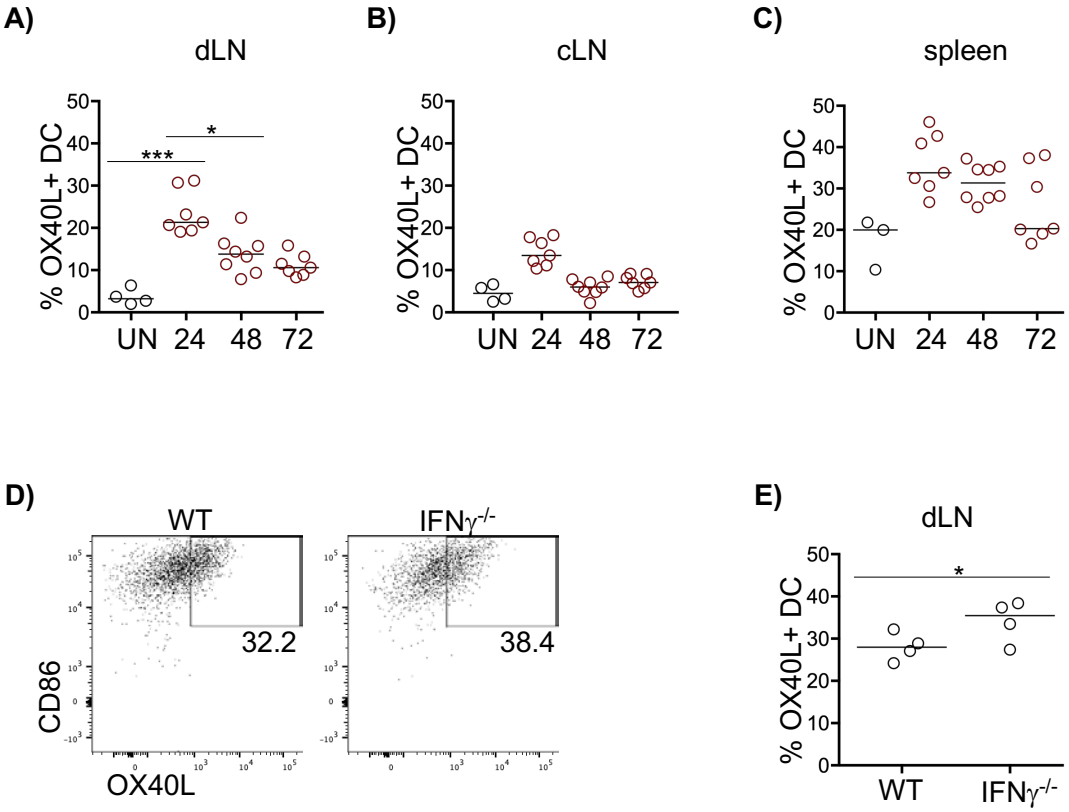

A)
dLN
% OX40L+ DC
***
*
UN 24 48 72
B)
cLN
% OX40L+ DC
UN 24 48 72
C)
spleen
% OX40L+ DC
UN 24 48 72
D)
WT
IFNγ-/-
32.2
38.4
CD86
OX40L
E)
dLN
% OX40L+ DC
*
WT
IFNγ-/-

Figure 6. 12 AS01 upregulates OX40L expression on DC in draining LNs

(**A**) Percentages of DC expressing OX40L in dLNs at 24, 48 and 72 hrs post post immunization with OVA-2W/AS01. (**B**) Percentages of DC expressing OX40L in contralateral, cLNs at 24, 48 and 72 hrs post post immunization with OVA-2W/AS01. (**C**) Percentages of DC expressing OX40L in spleen at 24, 48 and 72 hrs post post immunization with OVA-2W/AS01. (**D**) Representative flow cytometry plots, showing DC ($B220^{-}CD3^{-}CD49b^{-}$, CD11c+, $Ly6C^{low}$, MHCII). expressioin of OX40L in dLN at 24 post post immunization with OVA-2W/AS01 in WT and $IFN\gamma^{-/-}$ mice. (**E**) Percentages of DC expressing OX40L in dLN at 24 post post immunization with OVA-2W/AS01 in WT and $IFN\gamma^{-/-}$ mice. Data in **A-C** are pooled from two independent experiments, n=4 UN; n=7 24 hrs; n=8 48 hrs and n= 7 72 hrs. Data in **D** represents one experiment, n=4. Bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *p≤0.05, **p≤0.01, ***p≤0.001.

Interestingly, identical analysis performed in IFN$\gamma^{-/-}$ mice showed no significant differences between WT mice with regards to the OX40L expression on DC at 24 hrs post immunisation with AS01 (Figure 6.12 D, E). Here we provided novel data on AS01 induced upregulation of OX40L on DC, showing the importance and efficiency of AS01 adjuvant in stimulating costimulatory signals as previously shown for CD86, CD50 and CD40 (A. M. Didierlaurent et al., 2014). To investigate whether these signals were required for potent T cell responses, we decided to repeat our previous experiments using AS01 model combined with our conditional knockout mice targeting specifically OX40L on DC *in vivo*. The CD11c^{Cre} x OX40L$^{f/f}$ mice alongside the CD11c^{Cre} only controls and OX40$^{-/-}$ negative control mice were immunised with OVA-2W1S/AS01 and the responses assessed in draining iliac and inguinal LNs 7 days later (Figure 6.13 A). Gating on CD3^{+}CD4^{+}CD44^{high} T cells, 2W1S-specific responses were measured using MHCII Tetramers (Figure 6.13 B, C). Moreover, we assessed T-bet versus CXCR5 expression as a mean of distinguishing between effector T cells (T-bet^{+}CXCR5^{-}) and T follicular helper cells (T-bet^{-}CXCR5^{+}) (Figure 6.13 D). Analyses unveiled a definite OX40 dependency of 2W1S specific responses post AS01 immunisation, represented by a significant reduction in the number of 2W1S-specific CD4 T cells in both iliac and inguinal LNs in OX40$^{-/-}$, compared to the CD11c^{Cre} only control mice (Figure 6.13 C; 6.14 A, E). Interestingly, initial analyses of the AS01 induced 2W1S-specific CD4 T cell responses in CD11c^{Cre} x OX40L$^{f/f}$ mice showed that they were not significantly affected by the lack of OX40L on DC (Figure 6.13 C; 6.14 A, E). However, further assessment of T-bet expression showed partial dependency of the effector T cells on OX40L provision by DC in CD11c^{Cre} x OX40L$^{f/f}$ mice. Although not statistically significant, the effect observed was reflected in reduction of T-bet^{+} T cells (Figure 6.13 D; 6.14 B, C, F, G)

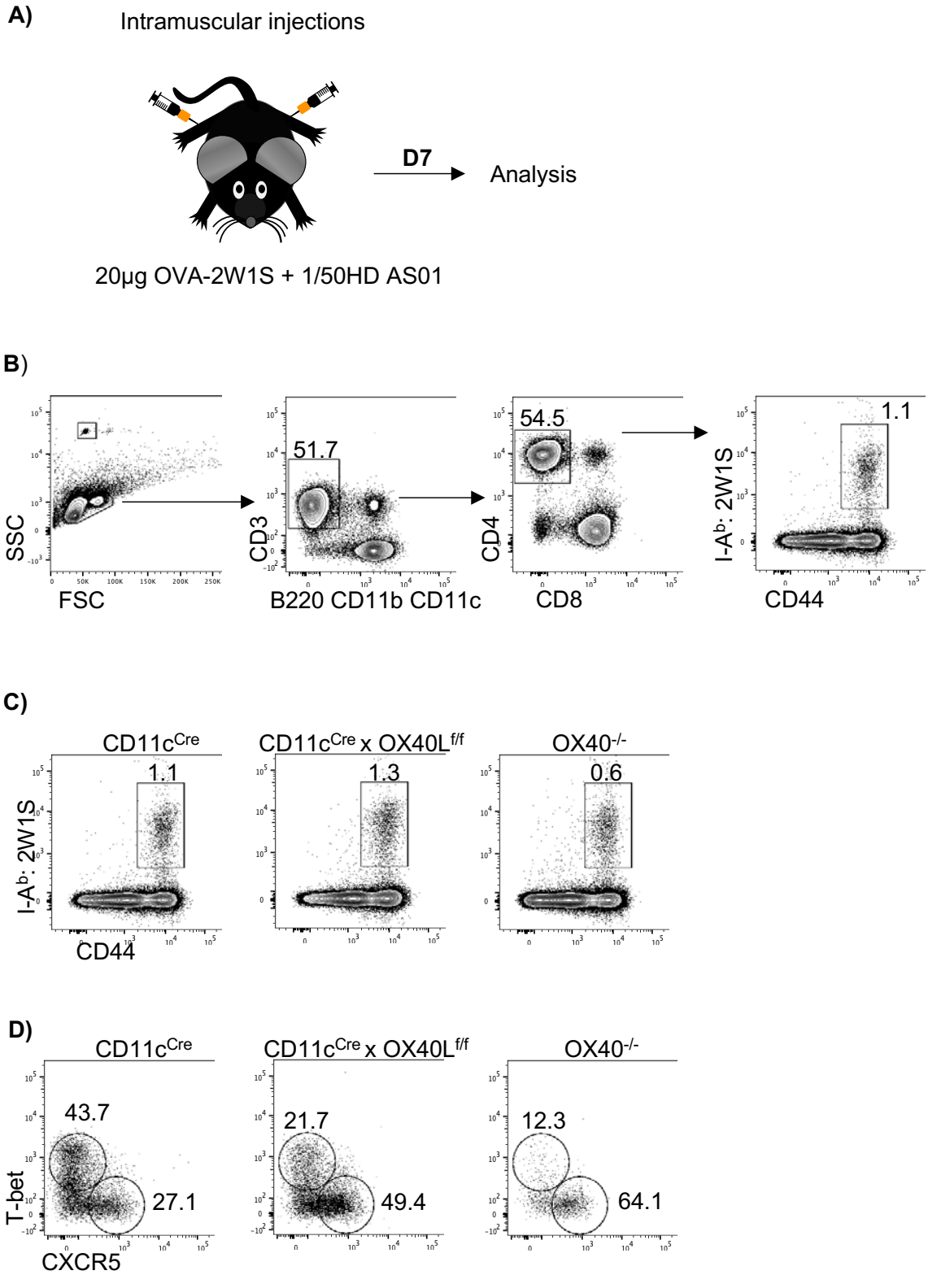

A)
Intramuscular injections
D7
Analysis
20µg OVA-2W1S + 1/50HD AS01
B)
SSC
FSC
51.7
CD3
B220 CD11b CD11c
54.5
CD4
CD8
1.1
I-Ab: 2W1S
CD44
C)
CD11cCre
1.1
I-Ab: 2W1S
CD44
CD11cCre x OX40Lf/f
1.3
OX40-/-
0.6
D)
CD11cCre
43.7
27.1
T-bet
CXCR5
CD11cCre x OX40Lf/f
21.7
49.4
OX40-/-
12.3
64.1

Figure 6. 13 AS01 induced 2W1S-specific CD4 T cell responses require OX40 and are moderately affected by the lack of OX40L on DC

The $CD11c^{cre}$, $CD11c^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice were immunised with OVA-2W1S/AS01 and sacrificed at D7 when the responses in both iliac and inguinal dLNs were assessed. (**A**) Experimental design (**B**) Gating strategy used for identification and expression of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^{+}CD4^{+}B220^{-}CD11b^{-}CD11c^{-}$) in iliac LNs. (**C**) Expression of specific $CD44^{hi}$ CD4 T cells in iliac dLNs of $CD11c^{cre}$, $CD11c^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice. (**D**) T-bet versus CXCR5 by 2W1S-specific $CD44^{hi}$ CD4 T cells in iliac dLNs of $CD11c^{cre}$, $CD11c^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice. Representative flow cytometry plots for inguinal dLNs can be found in Appendix Figure 8.10 Values on flow cytometry plots represent percentages.

However, unlike the results obtained in our previous investigations, AS01 responses in $CD11c^{Cre}$ x $OX40L^{f/f}$ mice did not recapitulate the defect in the total absence of OX40 signals, as the effector T cell responses post OVA-2W1S/AS01 immunisations were further abrogated in $OX40^{-/-}$ mice in both iliac and inguinal LNs. These analyses gave strong indication for other possible providers of OX40L *in vivo*. Moreover, AS01 induced generation of Tfh cells was heavily impaired by the ablation of OX40 signals as shown by the significant decrease in the total number on $CXCR5^{+}$ cells in iliac and inguinal draining LNs in $OX40^{-/-}$ mice (Figure 6.14 D, H). These results are particularly interesting as were not previously observed in the Lm-2W1S model and could indicate the possible B cell-specific provision of OX40L.

Altogether, data presented in this chapter outlined mechanisms of AS01 function, primarily its importance in promoting innate IFNγ responses and their subsequent role in adaptive immunity as well as the role in upregulation of OX40L on DC which proved to be moderately involved in antigen specific CD4 T cell responses.

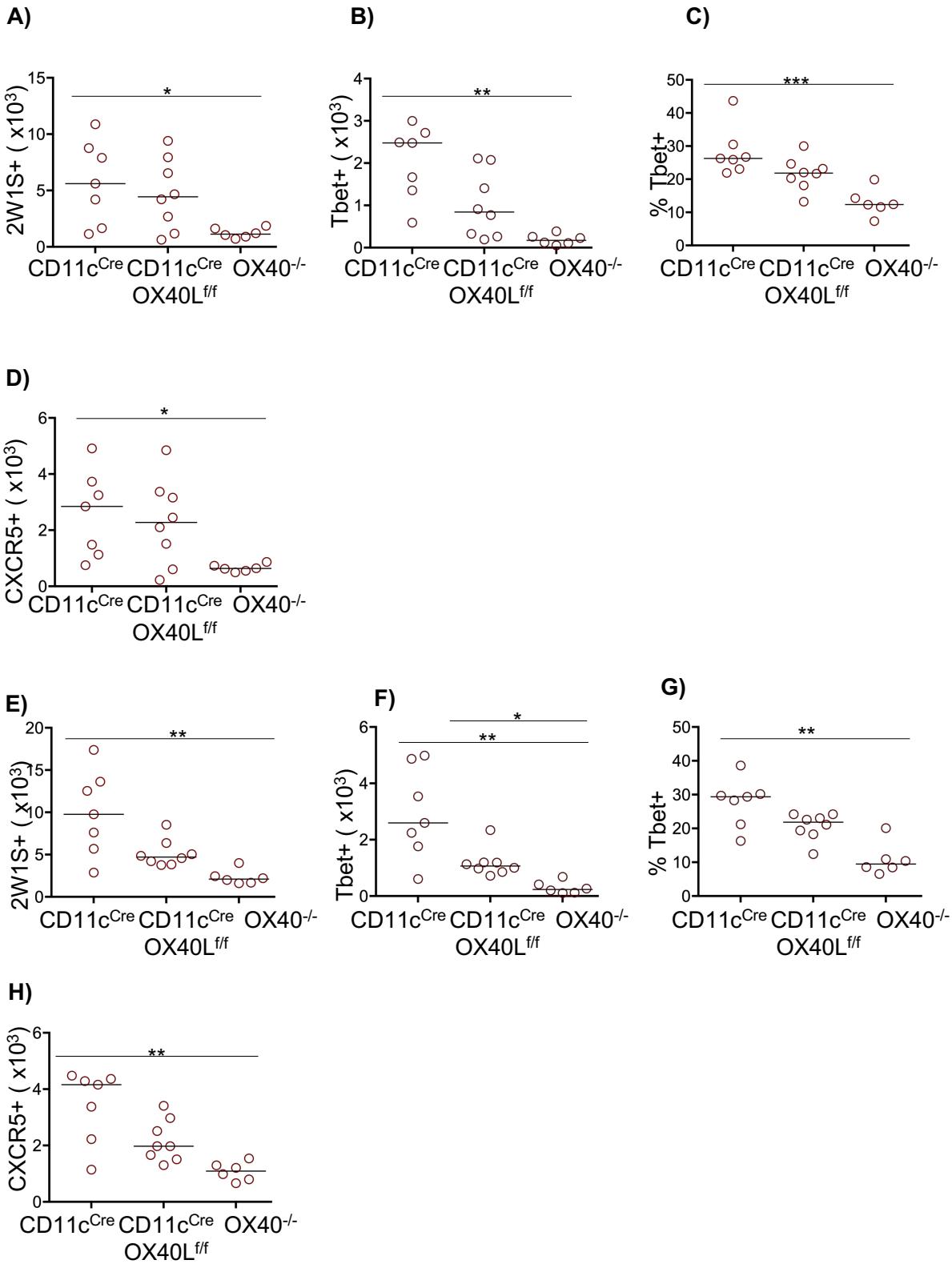

A)
B)
C)
D)
E)
F)
G)
H)
2W1S+ (x10^3)
Tbet+ (x10^3)
% Tbet+
CXCR5+ (x10^3)
CD11c^Cre OX40L^f/f
CD11c^Cre
OX40^-/-
*
**
***

Figure 6. 14 Expression of OX40L by DC is partially required for efficient AS01 derived effector Th1 responses

(**A, E**) Enumeration of 2W1S-specific $CD44^{hi}$ CD4 T cells $CD11c^{cre}$, $CD11c^{cre}$ x $OX40L^{f/f}$ and $OX40^{-/-}$ mice. (**B, F**) Enumeration of T-bet^{+} 2W1S-specific $CD44^{hi}$ CD4 T cells in iliac and inguinal LNs (**C, G**) Percentage of 2W1S-specific CD4 T cells expressing T-bet in iliac and inguinal LNs. (**D, H**) Enumeration of $CXCR5^{+}$ 2W1S-specific $CD44^{hi}$ CD4 T cells in iliac and inguinal LNs. Data are pooled from two independent experiments, n=7 $CD11c^{cre}$, n=8 $CD11c^{cre}$ x $OX40L^{f/f}$ and n=6 $OX40^{-/-}$. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Kruskal-Wallis one-way ANOVA with post hoc Dunn's test: *p≤0.05, **p≤0.01, ***p≤0.001.

6.3 Discussion

The initiation of an immune response to an infection is mediated through extensive collaboration between innate and adaptive immune cells. Both groups of cells possess specific recognition mechanisms to discriminate self from pathogens and initiate a chain of organised events leading up to the formation of long-term immunity. Vaccines mimic an infection to artificially induce protective immunity through stimulation of different parts of immune system. Modern adjuvanted vaccines are the most effective, exhibiting increased immunogenicity and speed as well as having minimal side effects. However, most importantly, the different types of adjuvants contained within these vaccines can modulate and promote specific phenotypes of T cell responses and antibody titers required to fight the infection.

As presented in Chapter 3, part of this PhD project has been devoted to investigating the mechanisms of action of GSK licensed adjuvant AS01 in the context of Th1 immunity. In this chapter we have focused on trying to better understand how the adjuvants impact innate cells to enhance the adaptive responses and specifically investigate the aspects of control of costimulatory ligand provision. AS01 belongs to a class of novel and recently introduced combination adjuvants which have been shown to promote immunity through synergistic action of two or more components. In the case of AS01, these are the MPL and QS-21 which together induce potent antigen-specific CD4 T cell responses *in vivo*, as we have previously shown in Chapter 3 and others have already published (Leroux-Roels et al., 2013; Vandepapelière et al., 2008).

The saponin QS-21 has been known to have agonistic function in activation of NLRP3 inflammasome, promoting a NLRP3-Asc-caspase1 dependent release of IL-1β and IL-18 (Marty-Roix et al., 2016). Contrarily, MPL has been shown to have reduced inflammatory properties but can induce production of IL-12 (Salkowski et al., 1997). The NLRP3 inflammasome activation can be mediated by many pathways, but in the AS01 system it is most probably a result of TLR4 signalling. TLR4 is one of the most studied PRRs, which is

predominantly expressed on the myeloid lineage of immune cells, including macrophages and dendritic cells (Ketloy et al., 2008; Lizundia et al., 2008; Rehli, 2002; Vaure and Liu, 2014). Moreover, in mice, the *Tlr4* transcripts have been found in many tissues, including lungs, heart and muscle (Qureshi et al., 1999). Both components of AS01 signal through the TLR4 receptor, as their stimulatory effects were reduced in $TLR4^{-/-}$ mice (Marty-Roix et al., 2016; Pouliot et al., 2014).

The AS01 system relies on rapid activation of monocytes and other resident cells within the muscle and the draining LNs to induce TLR4 signalling and the subsequent release of proinflammatory cytokines like IL-1β, IL-6, TNFα which have been shown to be present at both sites within 3 hrs (A. M. Didierlaurent et al., 2014). In a recent study, these properties of AS01 have been identified as essential for the rapid production of IFNγ observed within the first 6 hrs of AS01 administration (Coccia et al., 2017). Our data presented in this chapter, supported the recent findings, demonstrating early production of IFNγ in WT mice in response to OVA-2W1/AS01 at 6 hrs post immunisation. Although, the original study showed the response to be specific only to the iliac draining LNs, as in our previous investigations, we have shown that inguinal LNs also displayed a significant increase in IFNγ production.

6.3.1 Early IFNγ responses post immunisation with AS01

The combined effects of IL-1β, IL-18, IL-6 and IL-12 have a significant effect on the type of cells becoming activated and acquiring their effector states. Consistent with the recently published study, we have shown that NK cells and T cells were the two dominant producers of IFNγ at the 6 hrs post OVA-2W1S/AS01 immunisation. NK cell activation is highly influenced by the secretion of IL-12 and IL-18 (Bellora et al., 2012; Metzger et al., 1997). IL-18 has been recognised as a key IFNγ inducing factor and its mature form is mostly secreted by activated macrophages (Okamura et al., 1995). When cocultured *in vitro* with, $CD16^{+}$ or $CD16^{-}$ macrophages and LPS, resting NK cells have been shown to express increased levels

of CCR7 and IFNγ, which were abrogated with the addition of anti-IL18 monoclonal antibody (Bellora et al., 2012). Alongside IL-18 production, IL-12 is also critical for the induction of the early IFNγ response, thereby promoting Th1 polarising environment for the differentiation of CD4 T cells (Scharton-Kersten et al., 1995; Seder et al., 1993). Moreover, NK cell activation and their effector functions can also be impaired by IL-12 signalling (Metzger et al., 1997).

The other two proinflammatory cytokines, IL-6 and IL-1β, have been identified as both T cell and DC activation stimulants. In particular, IL-1β enhances the differentiation of monocytes into conventional DC and M0 (Schenk et al., 2014; Yakimchuk et al., 2011). For IL-6, the reports a more varying, with some suggesting a stimulatory role for IL-6 in DC maturation, while others presenting *in vivo* data on IL-6 mediated suppression of DC maturation (S.-J. Park et al., 2004; Su et al., 2008). Both cytokines promote CD4 T cell differentiation, with IL-6 inducing Th17 phenotype following immunisation with IFA (Bettelli et al., 2006; Korn et al., 2008). IL-1β has also been shown to induce CD8 T cell activation and function (Ben-Sasson et al., 2013). Similarly, IL-6 and IL-1β have been shown to suppress Tregs function by inhibiting TGFβ (Bettelli et al., 2006; Ikeda et al., 2014). Together, these data are consistent with a model in which combined action of the two immunostimulant components of AS01 induces the release of IL-12, IL-6, IL-1β and IL-18 which collectively drive early IFNγ production from NK cells.

In the recent study, the use of NKp46DTA mice enabled assessment of the NK cell specific contribution to the IFNγ production, which showed a significant reduction in the proportion of IFNγ produced by NK cells as opposed to other cells (Coccia et al., 2017). Those analyses however were flawed since both ILC1 and for instance NK cells can express NKp46 (Spits et al., 2013; Yu et al., 2011). Hence inducible deletion of NKp46 would affect both NK cells as well as ILC1 population. We therefore performed different type of analyses, including more surface and transcription factor markers which revealed that ILC1 indeed attributed to a small

proportion of the NK1.1+ IFNγ producing cells. This data was used to support another PhD student's paper on migratory properties of ILC subsets in peripheral LNs (Dutton et al., 2019). The observed significant increase in the population of cells expressing cell surface markers indicative of iNKT cell population, was assessed using $CD1d^{-/-}$ mice. Those analyses disproved original assumptions of their possible phenotype. This was consistent with the previous data, presenting the iNKT cells to account for approximately less than 10% of IFNγ producing cells following immunisation with AS01 (Coccia et al., 2017). We showed that those cells also had a low expression of EOMES, with a transient expression of CD8 markers, implying that they could indeed be CD8 T cells upregulating expression of NK1.1 as previously shown (Assarsson et al., 2000). Moreover, other study identified a population of $NK1.1^{+}CD4^{+}$ effector T cells following infection with *Plasmodium yoelli*, which responded in MHCII restricted but CD1d independent manner (Wikenheiser et al., 2018). Overall, more detailed analyses were needed to provided better understanding with regards to the phenotype of this population, however considering that these cells are not dominant IFNγ producers, their phenotype was not explored any further.

During the direct detection of IFNγ production, a large T cell population based on CD3 expression was identified, which was surprising, yet further analyses demonstrated that they expressed a phenotype characteristic for central memory CD8 T cells. The previously described report showed that these cells expressed CD44, CD69 and CD25, associated with activation post antigen encounter (Budd et al., 1987; Kim et al., 2001; Ziegler et al., 1994). We have observed similar results, with respect to the CD44 staining, but have additionally characterised them on the basis of CD62L expression, showing a significant dominance of the $CD62L^{high}$ population. The CD62L expression can be used to identify central memory population which are able to traffic into lymph nodes because of the elevated expression of the homing chemokine receptor CCR7 (Sallusto et al., 1999; Wherry et al., 2003). These cells

were also shown to have strong proliferative potential and were mostly involved in recall responses to an antigen (Wherry et al., 2003).

Previous studies have highlighted a potential role for memory CD8 T cells in rapid early cytokine production. Past study investigated the pool of endogenous B8R vaccina virus peptide and OVA-specific CD8 populations and found that unprimed mice averaged approximately 1,070 and 170 of each type of cells, respectively (Haluszczak et al., 2009). These were identified using tetramers and included both $CD44^{high}$ and $CD44^{low}$ populations. Interestingly, in *in vitro* settings, following an overnight culture with IL-2, IL-12 and IL-18, splenocytes isolated from unprimed animals showed that B8R-specific $CD44^{high}$ CD8 T cells had an increased expression of IFNγ (Haluszczak et al., 2009). Further evidence for the presence of memory CD8 T cells able to provide early cytokine expression in unimmunised animals was also reported by Lee et al., 2013. This study identified named 'virtual memory' (VM) cells, to distinguish them from the homeostatic populations found in previous investigation. These cells again expressed all memory phenotype markers, including CD62L, but were described as poor IFNγ producers following *in vitro* stimulation with OVA. This result was however skewed by the use of true memory (TM) cells as a comparison. These TCR CD8 T cells, were purified, transferred and primed with Lm-OVA before being detected 30 to 60 days later. Considering the time allowed for their development and expansion it was not surprising that they performed better at in OVA induced IFNγ responses. Nevertheless, at 5 hrs post of *in vitro* stimulation, approximately 30% of VM cells isolated from unprimed animals were shown to express IFNγ in response to OVA (J.-Y. Lee et al., 2013). Together these reports are consistent with our data that a population of memory CD8 T cells can rapidly produce IFNγ *in vivo* within hrs of AS01 immunisation. Particularly, the rapid secretion of IL-18 and IL-12 post administration of AS01 could indeed promote activation of those cells. Further studies are required to fully investigate this phenomenon with improved staining panels, including tetramers. Similarly, to our previous investigations in Chapters 3 and 4, we

have utilised GS mice which enabled assessment of the early AS01 induced IFNγ responses with no further *ex vivo* manipulations, setting a foundation for the following experiments on adaptive immune responses. Once again, these IFNγ reporter mice proved to be a highly valuable, as they fully supported the results obtained in experiments with WT mice. The rapid production of IFNγ was observed at 6 hrs post AS01 immunisation in both iliac and inguinal draining LNs and moreover, the phenotype of the IFNγ producing cells matched the previously described data.

6.3.2 Early IFNγ supports Th1 CD4 T cell responses

The innate production of IFNγ creates polarising environment required for differentiation of CD4 T helper cells into a fully functional Th1 subset capable of controlling the infection (Mosmann and Coffman, 1989). Additionally, also the level of IFNγ induced during a response can affect the course of T cell differentiation (Scott, 1991).

Expression of IFNγ is induced following activation of Stat4 transcription factor which is in turn controlled by IL-12 signals, as shown by reduced Th1 differentiation in Stat4 deficient mice (Bacon et al., 1995; Kaplan et al., 1996). The produced IFNγ activates another transcription factor Stat1, which leads to an upregulation of T-bet expression and subsequent reinforcement of the Th1 phenotype as well as IFNγ production (Krause et al., 2006; Lighvani et al., 2001). The sources of polarising IFNγ in many infections include NK cells and CD8 T cells which promote Th1 development (Das et al., 2001; Scharton and Scott, 1993). We have observed similar pattern in Lm-2W1S model described in Chapter 4 as well as in this investigation following immunisation with the AS01 adjuvant.

In comparison to AS01, other adjuvants including Alum, and oil-in-water adjuvants like MF59 (Novartis) and AS03 (GSK) show limited bias towards the Th1 immunity (Krause et al., 2006; Lighvani et al., 2001). At 4 hrs post immunisation with AS01, genes associated with IFNγ

signalling were upregulated, including Stat1 pathway, while responses post second dose at D14 showed increased number of polyfunctional IFNγ, IL-2 and TNFα producing CD4 T cells (Coccia et al., 2017). Furthermore, tests for RTS,S/AS01 vaccine in rhesus monkeys showed elevated antibody titers and a strong Th1 profile as opposed to other adjuvants (Stewart et al., 2007). In particular comparisons of the vaccine efficacy between RTS,S/AS01 and RTS,S/AS02 in human trials showed significantly higher numbers of CSP-specific CD4 T cells and increased IFNγ production (Kester et al., 2009).

We investigated the AS01 induced T cell responses using GS mice which were assessed 6 hrs post secondary (boost) immunisation with OVA-2W1S/AS01 or OVA-2W1S/PBS at D7. This method enabled assessment of innate and adaptive immune responses, which clearly demonstrated a significant increase in both responses in mice which received 2 doses of AS01. This emphasised the requirement for administrating booster vaccines, which has already been implemented in human trials with AS01 (RTS,S Clinical Trials Partnership, 2015).

Our findings showed that AS01 booster resulted in increased production of total IFNγ with NK cells and T cells once again accounting for majority of it. The increase in the NK derived IFNγ production post second dose of AS01 might be simply a result of an enhanced production of the inflammatory cytokines, however some reports suggested that NK cell activation by cytokines could favour generation of memory NK cells. For instance, preactivated NK cells, labelled with CFSE and transferred into a CFSE negative host, not only had higher proliferation rates but also produced much higher levels of IFNγ upon restimulation with IL-12 and IL-15 (Gillard et al., 2011). Consistently, during vaccinia virus challenge, T cell and B cell deficient mice receiving hepatic memory NK cells from vaccinia primed animals were more protected against secondary exposure (Gillard et al., 2011). Similar observations were made with regards to human trials, where adoptive transfer of memory NK cells significantly improved condition of patients suffering with leukemia (Romee et al., 2016). In the AS01

model, it would be beneficial to assess the NK cell responses post second boost at the later timepoints, in particular their localization within the LNs or other organs, for instance the liver. With the use of MHCII tetramers we were able to also assess the number of 2W1S-specific CD4 T cells and their production of IFNγ. Interestingly, the secondary boost with AS01 had minor effect on the number of the 2W1S-specific CD4 T cells, as compared to mice immunised with only one dose at D0. However, the booster dose had definitely improved the effector function of those cells showing increased IFNγ production.

In human studies, a multi-parametric modelling study which compared data from controlled phase II trials of a cohort of 291 participants immunised with HBs antigen and different adjuvants, $AS01_B$, $AS01_E$, AS02 , AS03 and AS04, found that any significant changes in the IFNγ and interferon-inducible protein 10 levels were only observed after the second dose received at D30 (Burny et al., 2017). There were, however, some participants immunised with $AS01_B$ who showed much higher IFNγ responses at 24 hrs post initial dose. At day 31, they observed a up to 3-fold increase in the magnitude of IFNγ responses and levels of the IP-10 as well as an increase in IL-6 production post immunisation with $AS01_B$ and $AS01_E$ as oppose to other adjuvants. This was further verified by a strong association found between the AS01 and AS03 treatments and upregulation of Stat1, IRF1, MX1 and CXCL10 genes and the overall CD4 T cell responses.

6.3.3 Early immune cell activation and how it drives robust adaptive immune responses

The main goal of vaccinations is the establishment of immunological memory, which can be mediated by T cells but also B cells which are responsible for generation of antibody responses. In thymus-dependent development of GCs, B cells rely on interaction with T cells which is mediated by the CD40 signalling, resulting in class switching and antibody production (Foy et al., 1993). This is specifically facilitated by the Tfh population of CD4 T cells, as shown

by the absence of GCs and lower IgG antibody titers in Bcl6 deficient mice (Hollister et al., 2013). However, in the absence of T cells, thymus-independent type II antigens, like polysaccharides can act directly on B cells engaging with their BCR and inducing formation of GC-like structures (de Vinuesa et al., 2000; Obukhanych and Nussenzweig, 2006). Previous reports have shown that T cell-independent GC formation is still highly conditioned on CD40 signalling but not the T cell derived provision of its ligand (Gaspal et al., 2006).

Adjuvanted vaccinations with virus-like particles and polyICLC enhanced Th1 responses which led to an increased number of Tfh cells and B cell class switching (Martins et al., 2016). In the same experiments, mice lacking CD4 T cells failed to mount IgG responses post vaccinations suggesting they were T cell dependant.

We have previously shown that AS01 induced high numbers of antigen-specific CD4 T cells which, can be phenotyped as effector T cells expressing T-bet and Tfh cells expressing CXCR5. Therefore, there an increased likelihood of them providing help to B cells and inducing formation of GCs. In this chapter we performed analyses of B cell responses post immunisation with phycoerythrin (PE) and AS01. The fluorescent nature of the PE antigen has been described in the past as a highly useful tool for identification of antigen-binding B cells (Hayakawa et al., 1987). We have shown that AS01 induced PE-specific B cell responses and GC formation in WT mice. In contrast, TCR$\alpha^{-/-}$ mice, deficient in T cells showed abrogation of those responses, suggesting a T cell dependent mechanism of AS01-induced B cell activation. With the respect to the early IFNγ production being required in generation of adaptive responses post immunisation with AS01, we hypothesised that it could also be involved in generating PE-specific B cells responses. Previous research have shown that IFNγR expression on B cells was not required for generating primary responses and GC formation following treatment with foreign T cell-dependent antigens, however was crucial for controlling autoimmune antigens (Domeier et al., 2016). Similarly, in the AS01 model, IFNγR$^{-/-}$ mice showed no changes in antibody titres (Coccia et al., 2017). Consistently, we also found

that IFNγ$^{-/-}$ mice generated efficient B cell responses post immunisation with AS01, showing no requirement for early IFNγ production.

Finally, in this chapter we have also assessed the role of early IFNγ signals in upregulation of costimulatory OX40L signalling and its provisional requirements for effector CD4 T cells responses. We have previously demonstrated that DC OX40L was upregulated at 24 hrs post infection with Lm-2W1S, when the majority of innate IFNγ was detected. Similarly, we showed that early IFNγ signals were important for optimal upregulation of OX40L in *in vivo* and *in vitro* settings. In this investigation, immunisation with AS01 led to upregulation of OX40L on DC in draining LNs at 24 hrs. Interestingly and unlike in the Lm-2W1S model, when testing the responses in IFNγ$^{-/-}$ mice, we observed no significant role of the early IFNγ in upregulation of OX40L on DC. This suggested that other signals, like for instance IL-12, IL-18 and TNFα could be involved in its upregulation. All of those cytokines are being produced in the early responses to AS01 and some of them particularly IL-18 have already been shown to induce OX40L expression (Maxwell et al., 2006). One way of testing this would involve blocking IL-18 or IL-12 signals in IFNγ$^{-/-}$ mice and assess the OX40L expression on DC post immunisation with AS01.

In Chapter 3, we have shown that efficient CD4 T cell responses post immunisation with AS01 were OX40 dependent but have not determined which cellular provider was pivotal. Here we have used CD11c^{Cre} x OX40L$^{f/f}$ mice to assess whether, similarly to the Lm-2W1S data, DC OX40L provision played a significant role in generation of effector CD4 T cell responses. However, considering its induction on DC at 24 hrs, our data showed that requirement for DC OX40L in AS01 induced responses was partial, implying that there could be another cellular provider, for instance B cells. Further experiments, assessing individual OX40L conditional knockout mice, like Mb1^{Cre} x OX40L$^{f/f}$ mice at both primary and boost or memory stages would provide better understanding of the costimulatory requirements of T cells in responses to AS01 adjuvant.

6.3.4 Summary

Overall this chapter we have developed the mechanism of action of GSK licensed AS01 adjuvant further. We showed that early IFNγ was required for the effector T cell response, but not the B cell GC response. We enhanced the understanding of phenotype of the cells involved in early IFNγ production. Finally, we revealed that OX40L expression on DC was important, however other cells could also likely contribute.

CHAPTER 7: GENERAL DISCUSSION

7.1 Discussion

Throughout the years of immunological research, our understanding of immune responses has greatly improved, however it has also become more complex. We have learnt that although T and B cells provide long term protection against reoccurring infections, their action is often dependent on the critical elements of the innate immune system, like the important ability to sense invading pathogens. The two immune systems are therefore no longer described as working independently and are viewed as complementary and integral.

The CD4 T cell activation is one of the best examples of the cooperation between the innate and adaptive immune systems. The outcome of this highly complex and dynamic process is determined by several factors like the initial interaction with APC and signal strength, costimulatory and coinhibitory molecules as well as the cytokine profile. Collectively, these factors act together to activate and direct naive T cells to generate highly efficient antigen specific responses. However, the contribution of individual factors as well as mechanisms regulating their actions still remain unclear.

One of the big challenges in studying T cell responses has been knowing the time frame of the response as well as the limited access to precise methods of tracking antigen-specific T cells *in vivo*, which has hampered our understanding of the mechanisms controlling generation and function of these cells. The development of MHCII tetramers has transformed our research, allowing to identify and track antigen specific CD4 T cells over time, including detection of endogenous populations and their subsequent development through the phases of the immune response. Moreover, this has provided a reliable method for assessing of all the previously mentioned driving forces responsible for induction of specific T cell mediated immunity.

Immune responses generated against the 2W1S peptide incorporated into an infectious agent, have been amongst the most studied due to the large endogenous population of T

cells capable of recognising this peptide (Pepper et al., 2010; Rowe et al., 2012). Tracking of this stable 2W1S specific population with a half-life of 40 days enabled detailed *in vivo* assessment of mechanism involved in all phases of T cell responses including formation of memory population. Unfortunately, further advances are needed to fully understand the role of costimulatory and coinhibitory pathways in developing these specific CD4 T cell responses. Additionally, understanding of the exact time and duration of their costimulatory and coinhibitory effects as well as studying the cellular provision of these signals would be particularly useful for the development of modern immunotherapies which aim to manipulate various signalling pathways at different stages of the immune response to either promote or impede T cell function.

Costimulation represents more than just an augmentation of the initial CD4 T cell-APC interaction through increasing TCR avidity. It involves a number of highly regulated signalling pathways which work synergistically alongside the TCR-pMHCII complex to activate T cells. Initial models of costimulation were based almost entirely on the concept of cytokine derived signals which would enhance T cell activation, however later it was discovered that specific ligand-receptor binding was instead essential for T cell activation. Some of the receptors, like CD28 are constitutively expressed, being involved in the initial binding and stabilisation of the TCR-pMHCII complex, however past reports also showed that in the absence of CD28 receptor, mice were still able to develop immune responses against some viruses. Identification of CD28 prompt investigations into other possible genes involved in T cell costimulation, concluding in a discovery of multiple families of genes.

Other costimulatory pathways like signalling through ICOS and members of the TFNRSF become induced after the primary costimulation and are thought to be particularly important for the function of the differentiated T cells like effector, memory or Tfh cells (Gramaglia et al., 1998; A Hutloff et al., 1999). For this reason, investigating the role of the latter signals has proven much more difficult due to their transient expression. In particular, the regulation and control of costimulatory and coinhibitory molecule expression have not been properly

addressed yet it appears crucial for the precise understanding of the processes involved in T cell activation, differentiation and survival.

This PhD project focused on the role of one particular costimulatory molecule, namely OX40 which has been known as a primary enhancer of CD4 T cell activation and survival. Following the proposed hypotheses, its role was explored across different microenvironments using three different models generating immune responses against both pathogens and non-living immunostimulants (testing hypothesis 1). In addition, the mechanisms regulating its costimulatory potential on the basis of OX40L expression were assessed following the infection with intracellular pathogen Lm-2W1S and immunisation with AS01 adjuvant (testing hypothesis 3 and 4). Furthermore, due to a very limited number of previous studies, this investigation aimed to dissect the cellular providers of OX40L *in vivo*, utilising novel OX40L conditional knockout mice to target specific cell type provision of this ligand (testing hypothesis 2).

OX40 signalling acts as a secondary costimulatory immune checkpoint, being expressed later in the response. The CD4 T cell requirement for OX40 signals refers mainly to the expansion of effector and memory populations.

When investigating the costimulatory requirements of CD4 T cells, it is crucial to consider the type and the nature of the response. In acute infections, like *L. monocytogenes*, CD4 T cell response is shaped into the 3 known phases of expansion, contraction and memory formation. The expansion of the low frequency antigen specific T cells is highly OX40 dependent with the $OX40^{-/-}$ mice showing fewer primary effector T cells (Gramaglia et al., 1998). The conventional TCR transgenic studies suggested that this was due to decrease in the CD4 T cell survival in the absence of OX40 signals, implying that the signals were important for maintaining T cells, particularly effector cells, through the late stages of the expansion phase, yet not many *in vivo* studies have been conducted (Rogers et al., 2001; Song et al., 2005; Soroosh et al., 2014). As mentioned, many times before, the TCR transgenic studies are characterised by a very large frequency of the antigen specific cells

transferred into a mouse, which can skew their overall response, hence we used the *in vivo* Lm-2W1S model to generate an accurate representation of the CD4 T cell responses. We presented a strong evidence that OX40 signals were not only important for the maintenance of effector T cells but also their expansion and differentiation (Figure 7.1). As enhanced T cell division and survival have been considered to be the dominant features of the OX40 signalling, it was even more striking that the lack of this molecule had such a profound effect on the function of effector T cells, specifically their ability to make IFNγ in response to Lm-2W1S. This would argue that this signalling pathway is most likely to be crucial and not redundant for the generation of optimal effector CD4 T cell responses, supporting hypothesis 1 (Marriott et al., 2014; Soroosh et al., 2007). Similarly, in chronic viral infections which follow a completely a different response pattern, where the constant antigen stimulation maintained over longer periods of time ensures the appropriate control of pathogen's replication, OX40 signals have also proven to be vital for effector T cells (Boettler et al., 2012).

Nevertheless, effector CD4 T cells in other types of infections, in particular intestinal pathogens have not yet been fully investigated and could follow differential requirement for OX40 signals. We showed that in chronic responses to STM-2W1S, the lack of OX40 did have an impact on the overall magnitude of the response, however production of IFNγ by the existing/ surviving effector T cells appeared to be maintained. This once again emphasised the need for more infection specific studies to understand the nature of costimulatory signals and indeed their regulation.

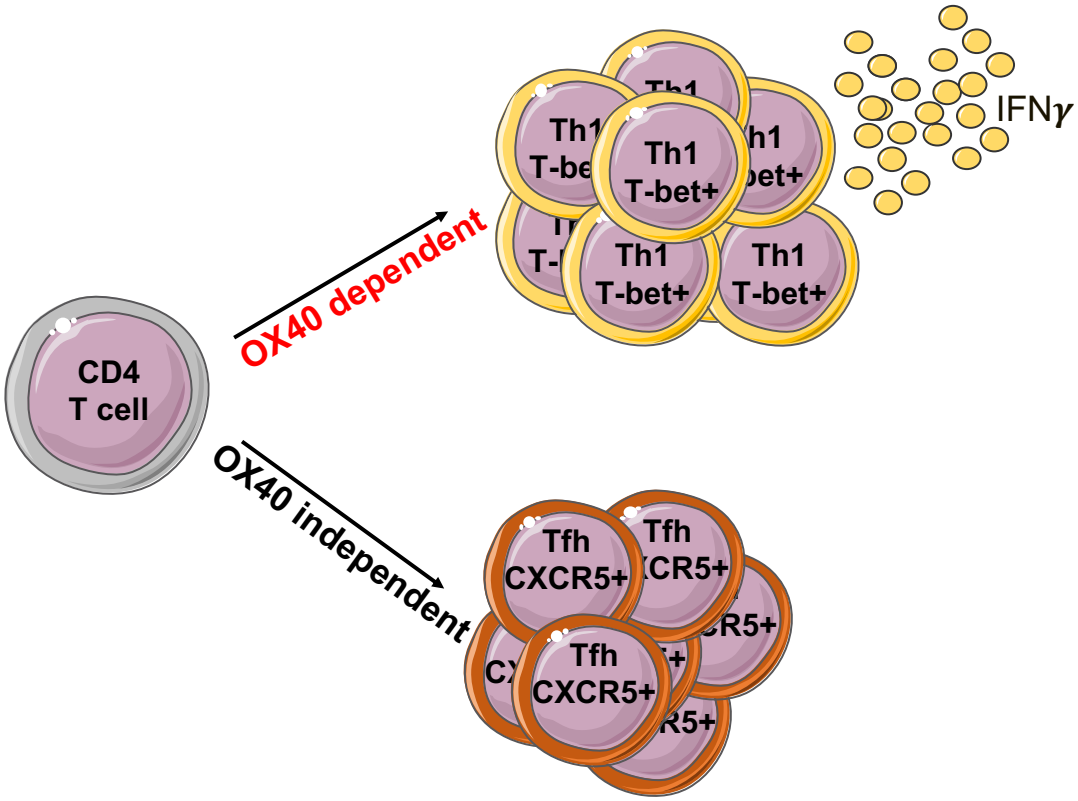


Figure 7. 1 Requirements for OX40 signals in Th1 responses.

Different infectious diseases are associated with various innate immune responses which ultimately lead to T cell and B cell activation. The same innate signals could be involved in the regulation of inducible costimulatory signals, as we have seen in responses to Lm-2W1S where early production of IFNγ was required for the optimal expression of OX40L on DC yet had no effect in responses to adjuvanted vaccines. Hence, mechanisms underpinning the costimulatory potential of individual pathways are as important as the direct effect of each pathway on the augmentation of T cell responses.

Elucidating mechanisms involved in the generation of effector T cells and their subsequent survival is vital to the design and production of more efficient vaccines. With the majority of pathogens constantly evolving, modern vaccines can no longer rely on the stable antigens generating high affinity antibodies, instead include non-living immunostimulants which primarily act to enhance innate signals and thereby induce better T cell responses

(Didierlaurent et al., 2017). Mechanisms of action of many vaccine adjuvants have not been fully understood, partially because majority of studies were based on the empirical approaches. Nowadays, the emphasis on more advanced understanding of immunological and molecular processes requires more precise approaches which could improve the development of more effective vaccine strategies. We sought to use the detailed mechanistic insight from the Lm-2W1S model to investigate how AS01 influenced the CD4 T cell responses and whether early IFNγ, previously identified as major innate signals induced by AS01, could be equally required for the optimal OX40L on DC (Coccia et al., 2017). Our work with AS01 adjuvant has demonstrated the enhancing effects on antigen specific CD4 T cell responses which were also highly dependent on OX40 signals. These results correlated with previously established adjuvant-like properties of immunisations with agonistic OX40 at the same time highlighting the crucial role of this pathway in generation of effector T cell responses. Further investigations into the importance of OX40 pathway could be key to refining the mode of action of AS01 adjuvant, however they should specifically take into consideration the regulation of OX40L expression post immunisation with AS01 and other innate signals influencing it, since the rapid production of IFNγ was proved redundant. For instance, IL-18 which has been shown to be produced after the immunisation with AS01 could be one of the possible signals required for the optimal expression of OX40L on DC.

Expression of OX40L is highly transient and not restricted to a specific cells type. With many potential cellular providers of the ligand, studying its function has been particularly difficult. Moreover, studies were limited to few models, mostly assessing the effect of total ablation of OX40L. Development of more advanced methods, like Cre-flox technology provided more sophisticated way of assessing cell specific deletion of genes. We have utilised this method to systematically dissect *in vivo* provision of costimulatory signal that is critical post CD28 signalling. All together our data revealed the importance of early IFNγ signals in the upregulation of OX40L on DC post Lm-2W1S infection, which were in turn critical sources of

OX40L required for optimal CD4 T cell responses (Figure 7.2). This has fully supported hypothesis 3 stated prior the investigation as well as revised hypothesis 2, narrowing the cellular sources of OX40L to one specific cell type.

With the results not being as clear in the AS01 model, our data once again highlighted the importance of assessing costimulatory requirements across different responses. In the AS01 model, early IFNγ was redundant for normal expression of OX40L on DC and similarly DC provision of OX40L in CD4 T cell responses was only partially required (Figure 7.3). This therefore provided an interesting new concept of an alternative OX40L provision or an idea of sequential provision in response to adjuvanted vaccines, despite the previously established role of DC in generating AS01 induced responses (Arnaud M Didierlaurent et al., 2014). Although these findings fully supported the hypothesis 4 stated previously, a further assessment specifically of B cell OX40L could provide more clear data, especially when taking into consideration its importance in Tfh function.

Overall, this thesis represents just a tip of an iceberg of potential roles of OX40 signalling in promoting different effector CD4 T cell responses. Mechanisms behind the regulation of its ligand still need to be uncovered and could provide a valuable information about the function of innate immunity in enhancing T cell activation, however, similarly signals involved in the subsequent shutdown of the OX40 pathway would equally improve our definition of T cell costimulation.

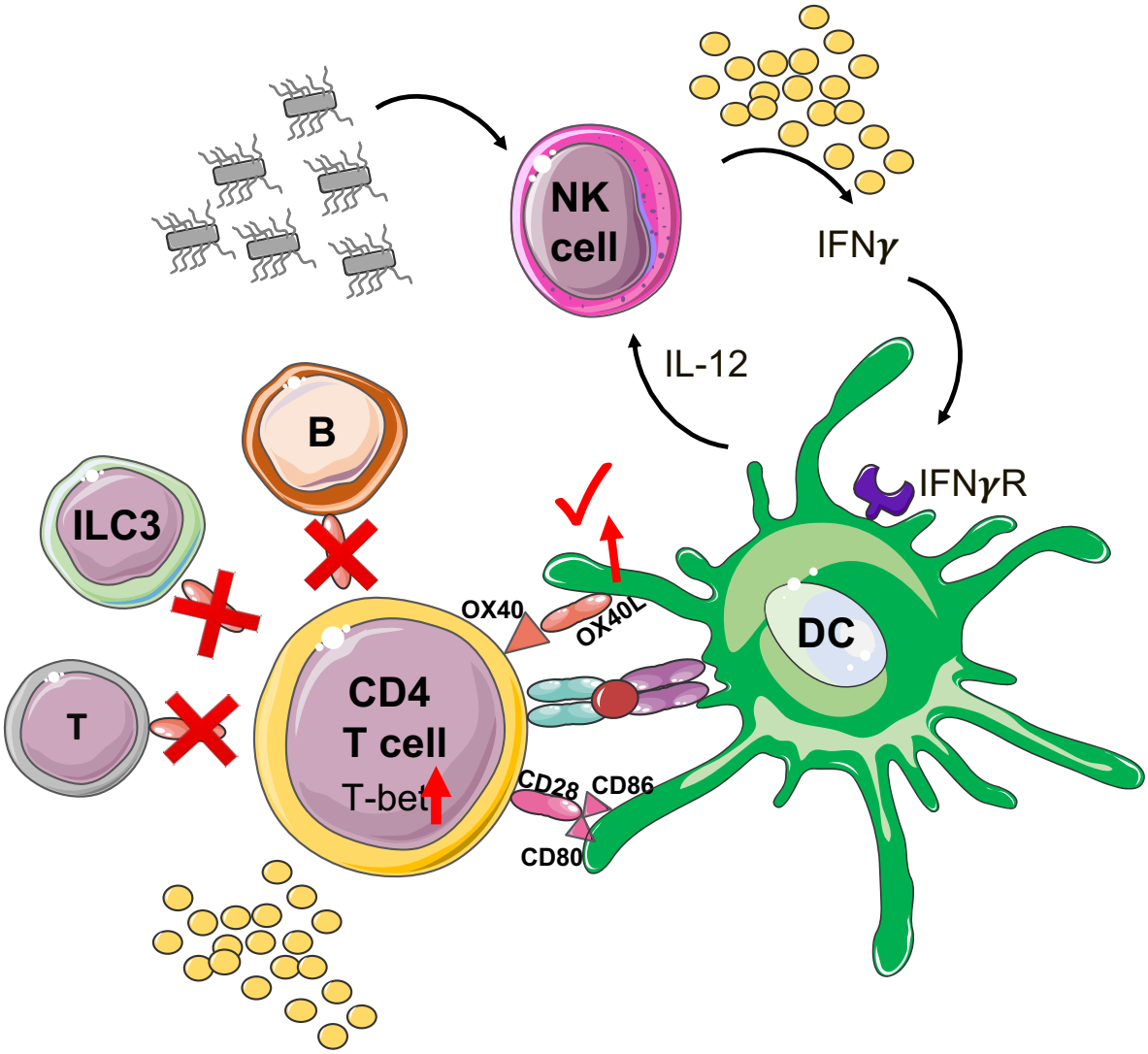


Figure 7. 2 Graphical summary of key findings in the investigation, outlining novel aspects of OX40-OX40L regulation and defining DC as specific cellular sources of OX40L in vivo required for generation of effector CD4 T cell responses in response to Lm-2W1S.

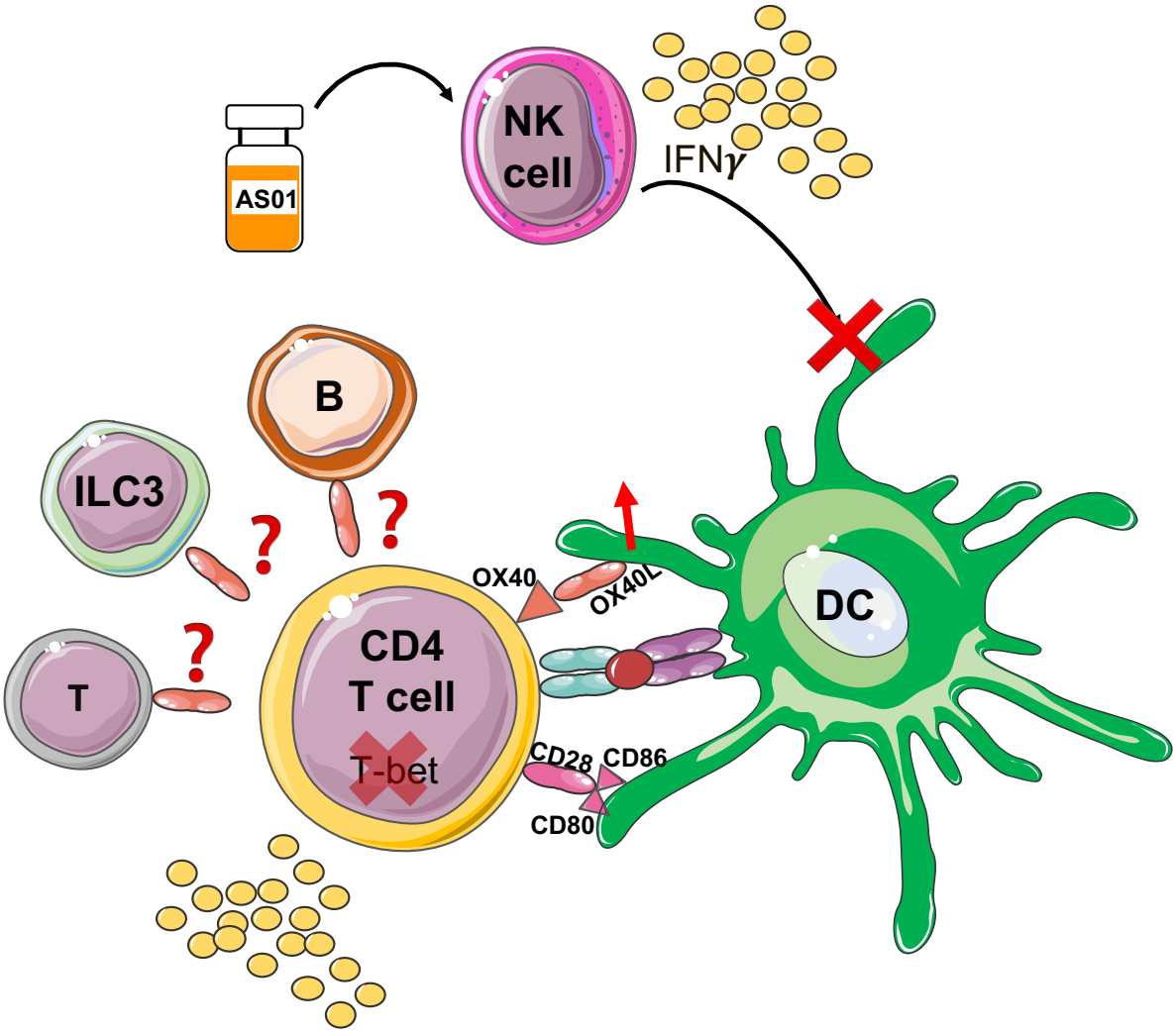


Figure 7. 3 Graphical summary of key findings from the investigation, outlining the regulation of OX40-OX40L pathway following immunisation with AS01 adjuvant and defining potential cellular sources of OX40L required for efficient CD4 T cell responses.

APPENDIX AND REFERENCES

Figure 8.1

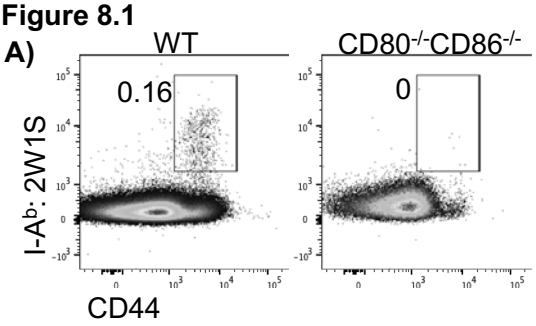


Figure 8.2

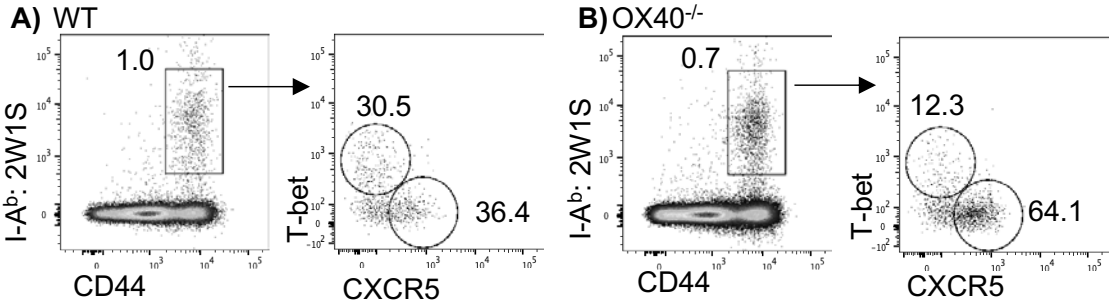


Figure 8.3

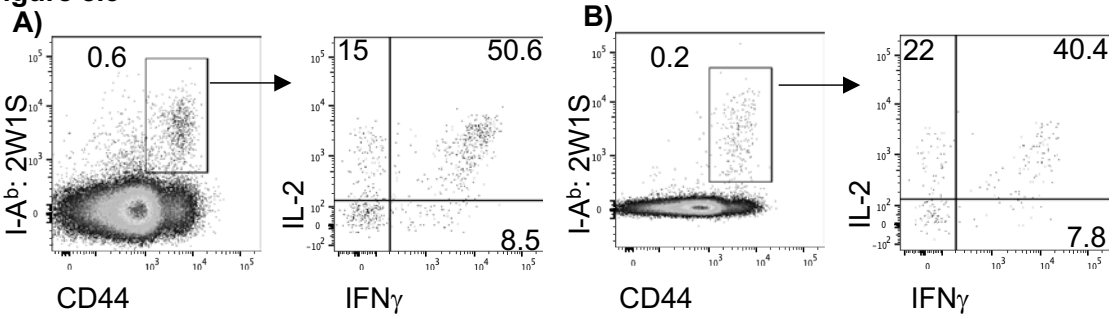
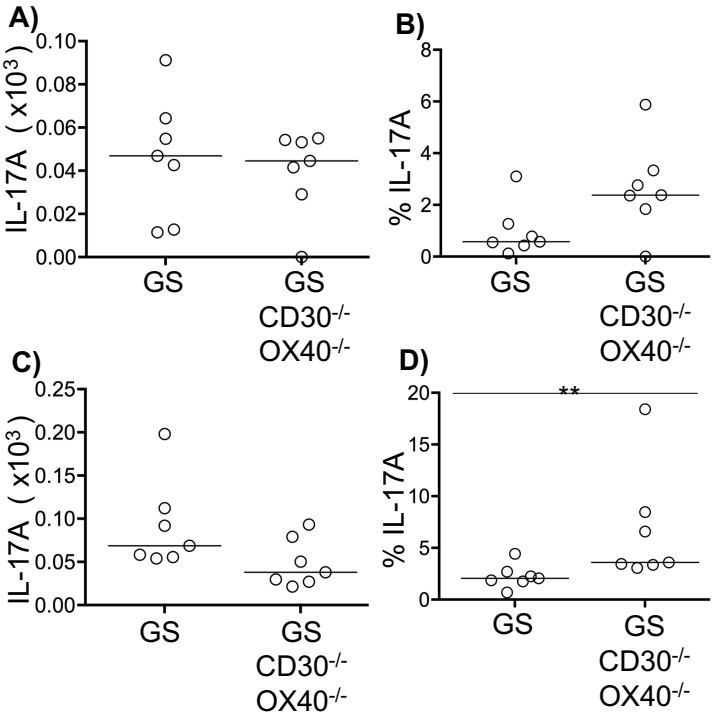


Figure 8.4



8. Appendix

Figure 8. 1 AS01 drives a robust CD4 T cell response to OVA-2W1S in draining LNs in a CD80 and CD86 dependent manner

(**A**) Gating strategy showing identification of 2W1S-specific $CD44^{hi}$ CD4 T cells in iliac dLNs of immunised WT and $CD80^{-/-}86^{-/-}$ mice.

Figure 8. 2 CD4 T cell responses to OVA-2W1S in draining LNs at D7 post immunisation are OX40 dependent

(**A**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells and expression of T-bet versus CXCR5 in iliac dLNs of WT mice. (**B**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells and expression of T-bet versus CXCR5 in iliac dLNs of $OX40^{-/-}$ mice.

Figure 8. 3 OX40 signalling is required for optimal functional CD4 T cell responses elicited by AS01

(**A**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells and their expression of IL-2 and IFNγ in inguinal dLNs of WT mice. (**B**) Identification of 2W1S-specific $CD44^{hi}$ CD4 T cells and their expression of IL-2 and IFNγ in inguinal dLNs of $OX40^{-/-}$ mice.

Figure 8. 4 IL-17 responses following infection with STM-2W1S

(**A, B)** Enumeration and percentages of IL-17A (hNGFR) on 2W1S-specific $CD44^{hi}$ CD4 T cells in mLN of GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice. (**C, D)** Enumeration and percentages of IL-17A (hNGFR) on 2W1S-specific $CD44^{hi}$ CD4 T cells in colon of GS and GS $CD30^{-/-}$ x $OX40^{-/-}$ reporter mice. Data pooled from two independent experiments, n=7. Values on flow cytometry plots represent percentages, bars on scatter plots represents the median. Statistical significance was tested using an unpaired, non-parametric, Mann-Whitney two tailed T test: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 8.5

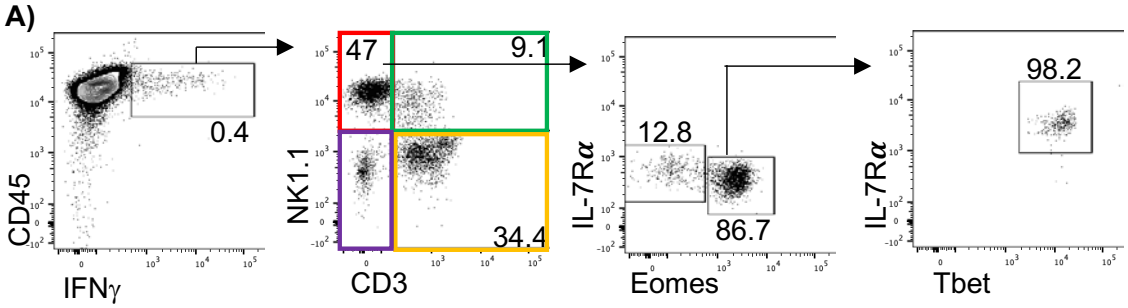


Figure 8.6

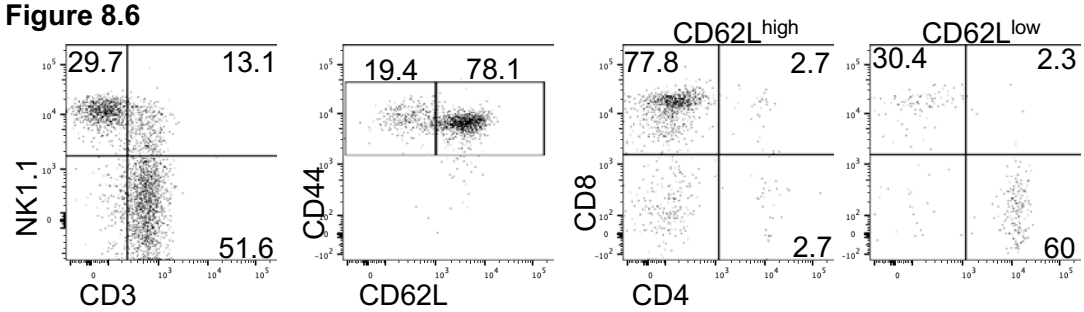


Figure 8.7

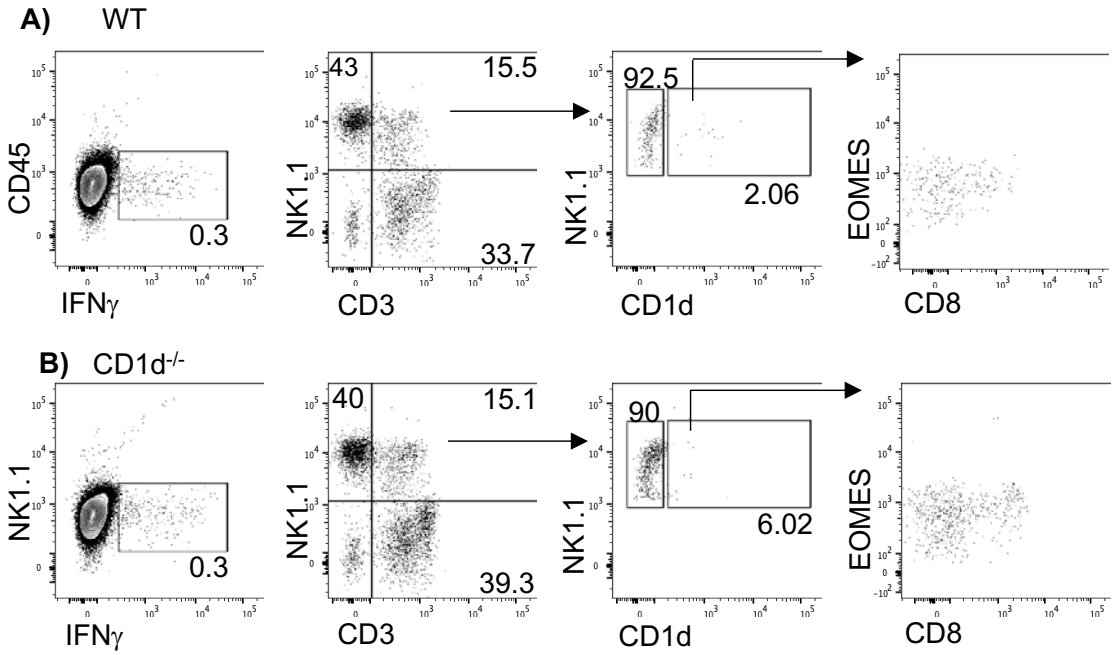


Figure 8.8

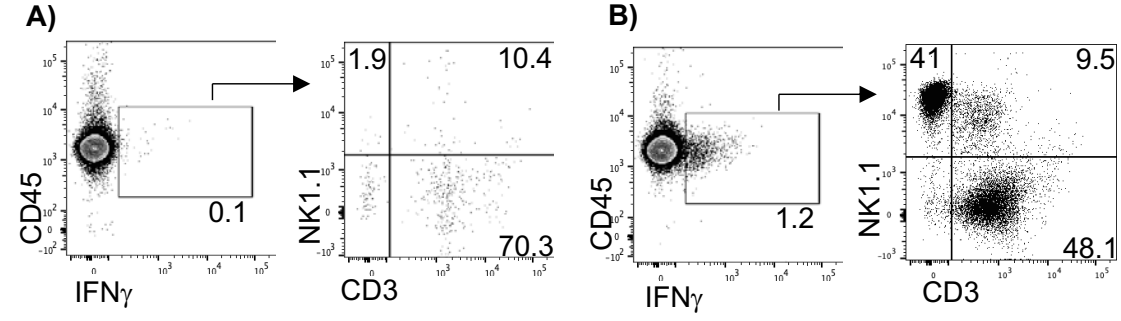


Figure 8. 5 AS01 immunisation have an effect of different cell subsets

(**A**) Flow cytometry plots showing gating strategy used to identify different $CD45^{+}IFN\gamma^{+}$ cell subsets (NK cells (NK) - RED, putative iNKT cells (NK1.1+ CD3+) - GREEN, T cells (T) - YELLOW, other cells (Other) - PURPLE) in inguinal dLNs. These plots also show identification of ILC population within the NK cell subset.

Figure 8. 6 Phenotyping CD3 T cells found in dLNs at 6 hrs post immunisation with AS01

(**A**) Gating strategy and representative flow cytometry plots showing proportion of $CD62L^{+}$ and $CD62L^{-}$ T cells present at 6 hrs post immunisation with OVA-2W1S/AS01 and their expression of CD4 and CD8.

Figure 8. 7 The $NK1.1^{+}CD3^{+}$ population of $IFN\gamma^{+}$ cells cannot be identified as putative iNKT cells

(**C, D)** Representative flow cytometry plots showing IFNγ production in inguinal dLN of WT and CD1d-/- mice 6 hrs post immunization with OVA-2W/AS01.

Figure 8. 8 Assessment of IFNγ production induced by AS01 using IFNγ reporter mice

(**B**) Representative flow cytometry plots showing eYFP expression in WT mice following the intramuscular injections and the gating strategy used to identify different cell subsets in iliac LNs. (**C**) Representative flow cytometry plots showing eYFP expression GS mice following the intramuscular injections and the gating strategy used to identify different cell subsets in inguinal LNs.

Figure 8.9

A)

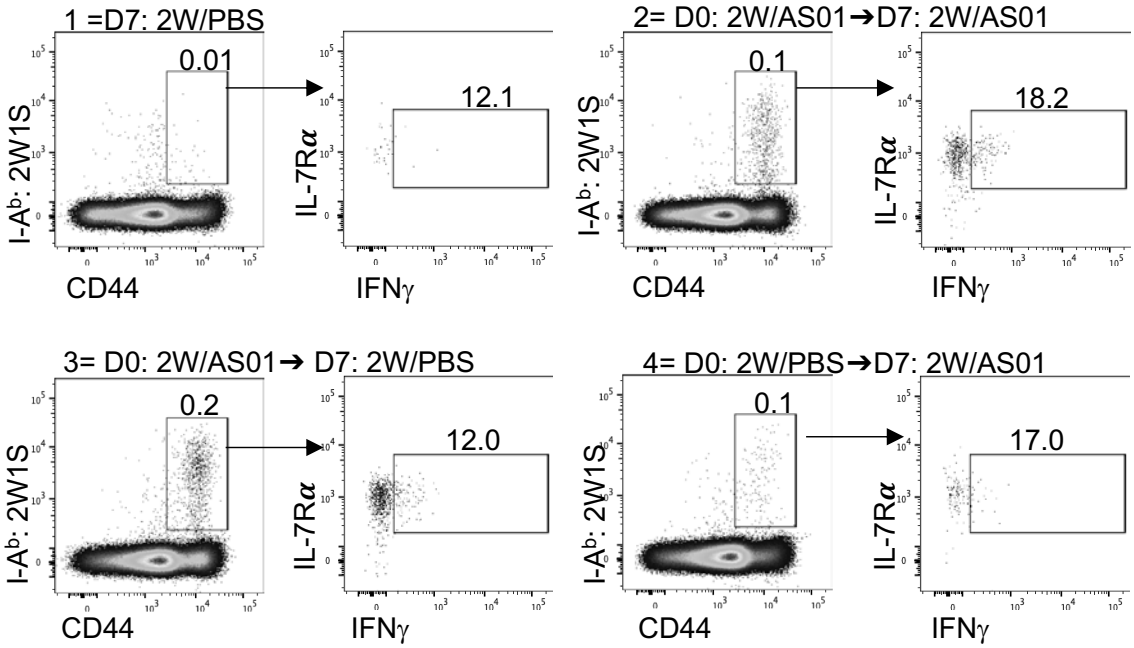


Figure 8.10

A)

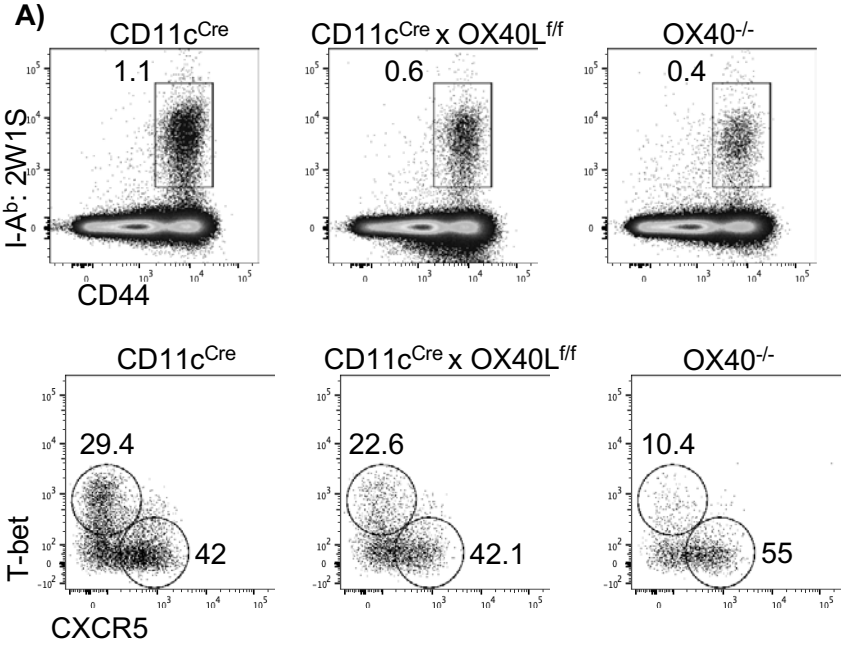


Figure 8. 9 AS01 promotes IFNγ production by responding CD4 T cells in the draining LNs

(**A**) Representative flow cytometry plots identifying 2W1S-specific CD4 T cells ($CD3^+CD4^+B220^-CD11b^-CD11c^-$) and showing eYFP (IFNγ) expression in inguinal dLNs for the tested experimental conditions.

Figure 8. 10 AS01 induced 2W1S-specific CD4 T cell responses require OX40 and are moderately affected by the lack of OX40L on DC

B) Gating strategy used for identification and expression of 2W1S-specific $CD44^{hi}$ CD4 T cells ($CD3^+CD4^+B220^-CD11b^-CD11c^-$) in inguinal LNs.

9. References

Abed, N.S., Chace, J.H., Cowdery, J.S., 1994. T cell-independent and T cell-dependent B cell activation increases IFN-gamma R expression and renders B cells sensitive to IFN-gamma-mediated inhibition. J. Immunol. 153, 3369–3377.

Aggarwal, B.B., Eessalu, T.E., Hass, P.E., 1985. Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. Nature.

Aichele, P., Zinke, J., Grode, L., Schwendener, R.A., Kaufmann, S.H.E., Seiler, P., 2003. Macrophages of the Splenic Marginal Zone Are Essential for Trapping of Blood-Borne Particulate Antigen but Dispensable for Induction of Specific T Cell Responses. The Journal of Immunology. https://doi.org/10.4049/jimmunol.171.3.1148

Aizawa, S., Nakano, H., Ishida, T., Horie, R., Nagai, M., Ito, K., Yagita, H., Okumura, K., Inoue, J., Watanabe, T., 1997. Tumor necrosis factor receptor-associated factor (TRAF) 5 and TRAF2 are involved in CD30-mediated NFkappaB activation. The Journal of biological chemistry. https://doi.org/10.1074/jbc.272.4.2042

Ajdary, S., Riazi-Rad, F., Jafari-Shakib, R., Mohebbali, M., 2006. Soluble CD26/CD30 levels in visceral leishmaniasis: Markers of disease activity. Clinical and Experimental Immunology. https://doi.org/10.1111/j.1365-2249.2006.03105.x

Akiba, H., Miyahira, Y., Atsuta, M., Takeda, K., Nohara, C., Futagawa, T., Matsuda, H., Aoki, T., Yagita, H., Okumura, K., 2000. Critical Contribution of Ox40 Ligand to T Helper Cell Type 2 Differentiation in Experimental Leishmaniasis. Journal of Experimental Medicine. https://doi.org/10.1084/jem.191.2.375

Akiba, H., Oshima, H., Takeda, K., Atsuta, M., Nakano, H., Nakajima, A., Nohara, C., Yagita, H., Okumura, K., 1999. CD28-Independent Costimulation of T Cells by OX40 Ligand and CD70 on Activated B Cells. The Journal of Immunology 162, 7058–7066.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801. https://doi.org/10.1016/j.cell.2006.02.015

Allen, C.D.C., Ansel, K.M., Low, C., Lesley, R., Tamamura, H., Fujii, N., Cyster, J.G., 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nature Immunology. https://doi.org/10.1038/ni1100

Alpuche Aranda, C.M., Swanson, J.A., Loomis, W.P., Miller, S.I., 2006. Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.89.21.10079

Amakawa, R., Hakem, A., Kundig, T.M., Matsuyama, T., Simard, J.J.L., Timms, E., Wakeham, A., Mittruecker, H.W., Griesser, H., Takimoto, H., Schmits, R., Shahinian, A., Ohashi, P.S., Penninger, J.M., Mak, T.W., 1996. Impaired Negative Selection of T Cells in Hodgkin's Disease Antigen CD30-Deficient Mice. Cell. https://doi.org/10.1016/S0092-8674(00)81031-4

Anderson, M.S., Su, M.A., 2011. Aire and T cell development. Current opinion in immunology 23, 198–206. https://doi.org/10.1016/j.coi.2010.11.007

Andersson, A., Dai, W.J., Di Santo, J.P., Brombacher, F., 1998. Early IFN-gamma production and innate immunity during Listeria monocytogenes infection in the absence of NK cells. J. Immunol. 161, 5600–5606.

Ansell, S.M., Horwitz, S.M., Engert, A., Khan, K.D., Lin, T., Strair, R., Keler, T., Graziano, R., Blanset, D., Yellin, M., Fischkoff, S., Assad, A., Borchmann, P., 2007. Phase I/II study of an anti-CD30 monoclonal antibody (MDX-060) in Hodgkin's lymphoma and anaplastic large-cell lymphoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. https://doi.org/10.1200/JCO.2006.07.8972

Apostólico, J. de S., Lunardelli, V.A.S., Coirada, F.C., Boscardin, S.B., Rosa, D.S., 2016. Adjuvants: Classification, Modus Operandi, and Licensing. J Immunol Res 2016. https://doi.org/10.1155/2016/1459394

Artis, D., Spits, H., 2015. The biology of innate lymphoid cells. Nature 517, 293–301. https://doi.org/10.1038/nature14189

Aruffo, A., Seed, B., 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.84.23.8573

Assarsson, E., Kambayashi, T., Sandberg, J.K., Hong, S., Taniguchi, M., Van Kaer, L., Ljunggren, H.G., Chambers, B.J., 2000. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. Journal of Immunology.

Autengruber, A., Gereke, M., Hansen, G., Hennig, C., Bruder, D., 2012. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. European Journal of Microbiology and Immunology. https://doi.org/10.1556/EuJMI.2.2012.2.3

Babior, B.M., Kipnes, R.S., Curnutte, J.T., 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52, 741–744. https://doi.org/10.1172/JCI107236

Bacon, C.M., McVicar, D.W., Ortaldo, J.R., Rees, R.C., O'Shea, J.J., Johnston, J.A., 1995. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. Journal of Experimental Medicine 181, 399–404. https://doi.org/10.1084/jem.181.1.399

Baerenwaldt, A., von Burg, N., Kreuzaler, M., Sitte, S., Horvath, E., Peter, A., Voehringer, D., Rolink, A.G., Finke, D., 2016. Flt3 Ligand Regulates the Development of Innate Lymphoid Cells in Fetal and Adult Mice. J.I. 196, 2561–2571. https://doi.org/10.4049/jimmunol.1501380

Bairwa, M., Rajput, M., Khanna, P., Rohilla, R., Verma, R., Chawla, S., 2012. Malaria vaccine: A bright prospect for elimination of malaria. Human Vaccines and Immunotherapeutics 8, 819–822. https://doi.org/10.4161/hv.20145

Balasubramanian, R., Im, J., Lee, J.-S., Jeon, H.J., Mogeni, O.D., Kim, J.H., Rakotozandrindrainy, R., Baker, S., Marks, F., 2018. The global burden and epidemiology of invasive non-typhoidal Salmonella infections . Human Vaccines & Immunotherapeutics. https://doi.org/10.1080/21645515.2018.1504717

Ballesteros-Tato, A., León, B., Graf, B.A., Moquin, A., Adams, P.S., Lund, F.E., Randall, T.D., 2012. Interleukin-2 Inhibits Germinal Center Formation by Limiting T Follicular Helper Cell Differentiation. Immunity. https://doi.org/10.1016/j.immuni.2012.02.012

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature. https://doi.org/10.1038/32588

Bancroft, G.J., 1993. The role of natural killer cells in innate resistance to infection. Current Opinion in Immunology 5, 503–510. https://doi.org/10.1016/0952-7915(93)90030-V

Barbulescu, K., Becker, C., Schlaak, J.F., Schmitt, E., Meyer zum Büschenfelde, K.H., Neurath, M.F., 1998. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-gamma promoter in primary CD4+ T lymphocytes. Journal of immunology (Baltimore, Md. : 1950).

Barr, T.A., Brown, S., Ryan, G., Zhao, J., Gray, D., 2007. TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. European Journal of Immunology. https://doi.org/10.1002/eji.200636483

Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Rüssmann, H., Hardt, W.-D., 2003. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infection and immunity.

Baumgartner, C.K., Malherbe, L.P., 2010. Regulation of CD4 T-cell receptor diversity by vaccine adjuvants. Immunology 130, 16–22. https://doi.org/10.1111/j.1365-2567.2010.03265.x

Baumjohann, D., Preite, S., Reboldi, A., Ronchi, F., Ansel, K.M., Lanzavecchia, A., Sallusto, F., 2013. Persistent Antigen and Germinal Center B Cells Sustain T Follicular Helper Cell Responses and Phenotype. Immunity. https://doi.org/10.1016/j.immuni.2012.11.020

Bauquet, A.T., Jin, H., Paterson, A.M., Mitsdoerffer, M., Ho, I.C., Sharpe, A.H., Kuchroo, V.K., 2009. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nature Immunology. https://doi.org/10.1038/ni.1690

Bauvois, B., Nguyen, J., Tang, R., Billard, C., Kolb, J.-P., 2009. Types I and II interferons upregulate the costimulatory CD80 molecule in monocytes via interferon regulatory factor-1. Biochem. Pharmacol. 78, 514–522. https://doi.org/10.1016/j.bcp.2009.05.005

Bekiaris, V., Gaspal, F., Kim, M.Y., Withers, D.R., Sweet, C., Anderson, G., Lane, P.J.L., 2009a. Synergistic OX40 and CD30 signals sustain CD8+T cells during antigenic challenge. European Journal of Immunology 39, 2120–2125. https://doi.org/10.1002/eji.200939424

Bekiaris, V., Gaspal, F., Kim, M.Y., Withers, D.R., Sweet, C., Anderson, G., Lane, P.J.L., 2009b. Synergistic OX40 and CD30 signals sustain CD8+T cells during antigenic challenge. European Journal of Immunology 39, 2120–2125. https://doi.org/10.1002/eji.200939424

Bell, J.C., Sonnenberg, N., Editors, G., Abraham, N., Veillette, A., 1991. The lymphocyte-specific tyrosine protein kinase p56lck. Cancer Investigation. https://doi.org/10.3109/07357909109084644

Bella, S.D., Gennaro, M., Vaccari, M., Ferraris, C., Nicola, S., Riva, A., Clerici, M., Greco, M., Villa, M.L., 2003. Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br J Cancer 89, 1463–1472. https://doi.org/10.1038/sj.bjc.6601243

Bellora, F., Castriconi, R., Doni, A., Cantoni, C., Moretta, L., Mantovani, A., Moretta, A., Bottino, C., 2012. M-CSF induces the expression of a membrane-bound form of IL-18 in a subset of human monocytes differentiating in vitro toward macrophages. European Journal of Immunology 42, 1618–1626. https://doi.org/10.1002/eji.201142173

Bénaroch, P., Yilla, M., Raposo, G., Ito, K., Miwa, K., Geuze, H.J., Ploegh, H.L., 1995. How MHC class II molecules reach the endocytic pathway. The EMBO journal.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. PNAS 98, 13681–13686. https://doi.org/10.1073/pnas.251194298

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., Ochs, H.D., 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nature genetics 27, 20–1. https://doi.org/10.1038/83713

Ben-Sasson, S.Z., Wang, K., Cohen, J., Paul, W.E., 2013. IL-1β Strikingly Enhances Antigen-Driven CD4 and CD8 T-Cell Responses. Cold Spring Harb Symp Quant Biol 78, 117–124. https://doi.org/10.1101/sqb.2013.78.021246

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Diversity Is Generated by Gene Rearrangements.

Berg, R.E., Crossley, E., Murray, S., Forman, J., 2005. Relative contributions of NK and CD8 T cells to IFN-gamma mediated innate immune protection against Listeria monocytogenes. Journal of immunology (Baltimore, Md. : 1950). https://doi.org/10.4049/jimmunol.175.3.1751

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic

effector TH17 and regulatory T cells. Nature 441, 235–238. https://doi.org/10.1038/nature04753
Bettelli, E., Sullivan, B., Szabo, S.J., Sobel, R.A., Glimcher, L.H., Kuchroo, V.K., 2004. Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20031819
Bhardwaj, V., Kanagawa, O., Swanson, P.E., Unanue, E.R., 1998. Chronic Listeria infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression. J. Immunol. 160, 376–384.
Bhatt, G., Maddocks, K., Christian, B., 2016. CD30 and CD30-Targeted Therapies in Hodgkin Lymphoma and Other B cell Lymphomas. Current Hematologic Malignancy Reports. https://doi.org/10.1007/s11899-016-0345-y
Billiau, A., Matthys, P., 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. J. Leukoc. Biol. 70, 849–860.
Biswas, P., Cozzi-Lepri, A., Delfanti, F., Galli, A., Colangeli, V., Moioli, M.C., Scarchilli, A., Abrescia, N., Vigevani, G., D'Arminio-Monforte, A., Novati, R., Lazzarin, A., 2006. Significant link between sCD30 changes and HIV viremia in patients treated with HAART. Journal of Medical Virology. https://doi.org/10.1002/jmv.20733
Bodmer, J.L., Schneider, P., Tschopp, J., 2002. The molecular architecture of the TNF superfamily. Trends in Biochemical Sciences. https://doi.org/10.1016/S0968-0004(01)01995-8
Boettler, T., Choi, Y.S., Salek-Ardakani, S., Cheng, Y., Moeckel, F., Croft, M., Crotty, S., von Herrath, M., 2013. Exogenous OX40 Stimulation during Lymphocytic Choriomeningitis Virus Infection Impairs Follicular Th Cell Differentiation and Diverts CD4 T Cells into the Effector Lineage by Upregulating Blimp-1. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1300013
Boettler, T., Moeckel, F., Cheng, Y., Heeg, M., Salek-Ardakani, S., Crotty, S., Croft, M., von Herrath, M.G., 2012. OX40 facilitates control of a persistent virus infection. PLoS Pathog. 8, e1002913. https://doi.org/10.1371/journal.ppat.1002913
Borghesi, L., Hsu, L.-Y., Miller, J.P., Anderson, M., Herzenberg, Leonard, Herzenberg, Leonore, Schlissel, M.S., Allman, D., Gerstein, R.M., 2004. B Lineage–specific Regulation of V(D)J Recombinase Activity Is Established in Common Lymphoid Progenitors. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20031800
Bourgeois, C., Rocha, B., Tanchot, C., 2002. A Role for CD40 Expression on CD8+ T Cells in the Generation of CD8+ T Cell Memory. Science 297, 2060–2063. https://doi.org/10.1126/science.1072615
Bowen, M.A., Lee, R.K., Miragliotta, G., Nam, S.Y., Podack, E.R., 1996. Structure and expression of murine CD30 and its role in cytokine production. Journal of immunology (Baltimore, Md. : 1950).
Bravo-Blas, A., Utriainen, L., Clay, S.L., Kästele, V., Cerovic, V., Cunningham, A.F., Henderson, I.R., Wall, D.M., Milling, S.W.F., 2018. Salmonella enterica Serovar Typhimurium Travels to Mesenteric Lymph Nodes Both with Host Cells and Autonomously. The Journal of Immunology ji1701254. https://doi.org/10.4049/jimmunol.1701254
Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., Förster, R., 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. The Journal of experimental medicine 192, 1545–52. https://doi.org/10.1084/jem.192.11.1545
Bretscher, P.A., Wei, G., Menon, J.N., Ohmann-Bielefeldt, H., 1992. Establishment of Stable, Cell-Mediated Immunity That Makes "Susceptible" Mice Resistant to Leishmania major. Science. https://doi.org/10.1126/science.1636090

Brewer, J.M., Conacher, M., Hunter, C.A., Mohrs, M., Brombacher, F., Alexander, J., 1999. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. J. Immunol. 163, 6448–6454.

Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C.A., Zollinger, M., Lynch, K.R., 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. Journal of Biological Chemistry. https://doi.org/10.1074/jbc.C200176200

Brinkmann, V., Pinschewer, D.D., Feng, L., Chen, S., 2001. FTY720: Altered lymphocyte traffic results in allograft protection. Transplantation. https://doi.org/10.1097/00007890-200109150-00002

Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundalia, C., Lane, P., 1999. CD4 T cell traffic control: In vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. European Journal of Immunology. https://doi.org/10.1002/(SICI)1521-4141(199905)29:05<1610::AID-IMMU1610>3.0.CO;2-8

Brunet, J.-F., Denizot, F., Luciani, M.-F., Roux-Dosseto, M., Suzan, M., Mattei, M.-G., Golstein, P., 1987. A new member of the immunoglobulin superfamily—CTLA-4. Nature. https://doi.org/10.1038/328267a0

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., Ramsdell, F., 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nature genetics 27, 68–73. https://doi.org/10.1038/83784

Buchmeier, N.A., Schreiber, R.D., 1985. Requirement of endogenous interferon-gamma production for resolution of Listeria monocytogenes infection. Proceedings of the National Academy of Sciences of the United States of America. https://doi.org/10.1073/pnas.82.21.7404

Budd, R.C., Cerottini, J.C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R.C., MacDonald, H.R., 1987. Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. J. Immunol. 138, 3120–3129.

Burnet, F., 1959. The clonal selection theory of acquired immunity. Vanderbilt University Press, Nashville, Tenn.

Burny, W., Callegaro, A., Bechtold, V., Clement, F., Delhaye, S., Fissette, L., Janssens, M., Leroux-Roels, G., Marchant, A., van den Berg, R.A., Garçon, N., van der Most, R., Didierlaurent, A.M., ECR-002 Study Group, 2017. Different Adjuvants Induce Common Innate Pathways That Are Associated with Enhanced Adaptive Responses against a Model Antigen in Humans. Front Immunol 8, 943. https://doi.org/10.3389/fimmu.2017.00943

Bushar, N.D., Corbo, E., Schmidt, M., Maltzman, J.S., Farber, D.L., 2010. Ablation of SLP-76 signaling after T cell priming generates memory CD4 T cells impaired in steady-state and cytokine-driven homeostasis. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.0908126107

Byun, M., Ma, C.S., Akçay, A., Pedergnana, V., Palendira, U., Myoung, J., Avery, D.T., Liu, Y., Abhyankar, A., Lorenzo, L., Schmidt, M., Lim, H.K., Cassar, O., Migaud, M., Rozenberg, F., Canpolat, N., Aydoğan, G., Fleckenstein, B., Bustamante, J., Picard, C., Gessain, A., Jouanguy, E., Cesarman, E., Olivier, M., Gros, P., Abel, L., Croft, M., Tangye, S.G., Casanova, J.-L., 2013. Inherited human OX40 deficiency underlying classic Kaposi sarcoma of childhood. The Journal of Experimental Medicine 210, 1743–1759. https://doi.org/10.1084/jem.20130592

Calderhead, D.M., Buhlmann, J.E., van den Eertwegh, A.J., Claassen, E., Noelle, R.J., Fell, H.P., 1993. Cloning of mouse Ox40: a T cell activation marker that may mediate T-B cell interactions. Journal of immunology (Baltimore, Md. : 1950).

Cantin, E., Tanamachi, B., Openshaw, H., Mann, J., Clarke, K., 1999. Gamma interferon (IFN-gamma) receptor null-mutant mice are more susceptible to herpes simplex virus type 1 infection than IFN-gamma ligand null-mutant mice. J. Virol. 73, 5196–5200.

Cantor, H., Boyse, E.A., 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly+ cells in the generation of killer activity. The Journal of experimental medicine. https://doi.org/10.1084/jem.141.6.1390

Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., Demengeot, J., 2003. Regulatory T Cells Selectively Express Toll-like Receptors and Are Activated by Lipopolysaccharide. The Journal of Experimental Medicine 197, 403–411. https://doi.org/10.1084/jem.20021633

Carlson, C.M., Endrizzi, B.T., Wu, J., Ding, X., Weinreich, M.A., Walsh, E.R., Wani, M.A., Lingrel, J.B., Hogquist, K.A., Jameson, S.C., 2006. Kruppel-like factor 2 regulates thymocyte and T-cell migration. Nature. https://doi.org/10.1038/nature04882

Carnaud, C., Lee, D., Donnars, O., Park, S.H., Beavis, A., Koezuka, Y., Bendelac, A., 1999. Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. Journal of immunology.

Carrington, P.E., Sandu, C., Wei, Yufeng, Hill, J.M., Morisawa, G., Huang, T., Gavathiotis, E., Wei, Yu, Werner, M.H., 2006. The Structure of FADD and Its Mode of Interaction with Procaspase-8. Molecular Cell. https://doi.org/10.1016/j.molcel.2006.04.018

Carside, P., Ingulli, E., Merica, R.R., Johnson, J.C., Noelle, R.J., Jenkins, M.K., 1998. Visualization of Specific B and T Lymphocyte Interacti ~ ns in the Lymph Node 281, 1994–1997. https://doi.org/10.1126/science.281.5373.96

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.72.9.3666

Carthon, B.C., Wolchok, J.D., Yuan, J., Kamat, A., Ng Tang, D.S., Sun, J., Ku, G., Troncoso, P., Logothetis, C.J., Allison, J.P., Sharma, P., 2010. Preoperative CTLA-4 blockade: Tolerability and immune monitoring in the setting of a presurgical clinical trial. Clinical Cancer Research. https://doi.org/10.1158/1078-0432.CCR-10-0569

Casella, C.R., Mitchell, T.C., 2008. Putting endotoxin to work for us: Monophosphoryl lipid a as a safe and effective vaccine adjuvant. Cellular and Molecular Life Sciences. https://doi.org/10.1007/s00018-008-8228-6

Casrouge, A., Beaudoing, E., Dalle, S., Pannetier, C., Kanellopoulos, J., Kourilsky, P., 2000. Size Estimate of the TCR Repertoire of Naive Mouse Splenocytes. The Journal of Immunology. https://doi.org/10.4049/jimmunol.164.11.5782

Cassell, D.J., Schwartz, R.H., 1994. A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation. The Journal of experimental medicine. https://doi.org/10.1016/B978-0-12-810441-5.00023-3

Castellanos, J.G., Woo, V., Viladomiu, M., Putzel, G., Lima, S., Diehl, G.E., Marderstein, A.R., Gandara, J., Perez, A.R., Withers, D.R., Targan, S.R., Shih, D.Q., Scherl, E.J., Longman, R.S., 2018. Microbiota-Induced TNF-like Ligand 1A Drives Group 3 Innate Lymphoid Cell-Mediated Barrier Protection and Intestinal T Cell Activation during Colitis. Immunity 49, 1077-1089.e5. https://doi.org/10.1016/j.immuni.2018.10.014

Caton, M.L., Smith-Raska, M.R., Reizis, B., 2007. Notch–RBP-J signaling controls the homeostasis of CD8− dendritic cells in the spleen. J Exp Med 204, 1653–1664. https://doi.org/10.1084/jem.20062648

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. The Journal of experimental medicine 180, 1263–1272. https://doi.org/10.1084/jem.180.4.1263

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G., 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J. Exp. Med. 184, 747–752.

Celli, S., Garcia, Z., Beuneu, H., Bousso, P., 2008. Decoding the dynamics of T cell-dendritic cell interactions in vivo. Immunological reviews 221, 182–7. https://doi.org/10.1111/j.1600-065X.2008.00588.x

Cerottini, J.C., Nordin, A.A., Brunner, K.T., 1970. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature. https://doi.org/10.1038/2281308a0

Chaplin, D.D., 2010. Overview of the immune response. Journal of Allergy and Clinical Immunology. https://doi.org/10.1016/j.jaci.2009.12.980

Chaudhry, A., Das, S.R., Hussain, A., Mayor, S., George, A., Bal, V., Jameel, S., Rath, S., 2005. The Nef Protein of HIV-1 Induces Loss of Cell Surface Costimulatory Molecules CD80 and CD86 in APCs. The Journal of Immunology 175, 4566–4574. https://doi.org/10.4049/jimmunol.175.7.4566

Chaux, P., Moutet, M., Faivre, J., Martin, F., Martin, M., 1996. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation. Lab. Invest. 74, 975–983.

Chen, J., Liao, M.Y., Gao, X.L., Zhong, Q., Tang, T.T., Yu, X., Liao, Y.H., Cheng, X., 2013. IL-17A induces pro-inflammatory cytokines production in macrophages via MAPKinases, NF-κB and AP-1. Cellular Physiology and Biochemistry. https://doi.org/10.1159/000354525

Chen, W., Jin, W., Hardegen, N., Lei, K., Li, L., Marinos, N., McGrady, G., Wahl, S.M., 2003. Conversion of Peripheral CD4 + CD25 − Naive T Cells to CD4 + CD25 + Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20030152

Chen, Y.-T., Su, Y.-C., Chang, M.-L., Tsai, P.-F., Kung, J.T., 2017. Low-Level MHC Class II Expression Leads to Suboptimal Th Cell Response, Increased Autoaggression, and Heightened Cytokine Inducibility. J. Immunol. 198, 1928–1943. https://doi.org/10.4049/jimmunol.1600967

Chen, Z.M., Jenkins, M.K., 1999. Clonal expansion of antigen-specific CD4 T cells following infection with Salmonella typhimurium is similar in susceptible (Ity(s)) and resistant (Ity(r)) BALB/c mice. Infection and Immunity.

Chien, Y., Meyer, C., Bonneville, M., 2014. γδ T Cells: First Line of Defense and Beyond. Annual Review of Immunology 32, 121–155. https://doi.org/10.1146/annurev-immunol-032713-120216

Chlibek, R., Bayas, J.M., Collins, H., de la Pinta, M.L.R., Ledent, E., Mols, J.F., Heineman, T.C., 2013. Safety and Immunogenicity of an AS01-adjuvanted Varicella-zoster Virus Subunit Candidate Vaccine Against Herpes Zoster in Adults >=50 Years of Age. Journal of Infectious Diseases. https://doi.org/10.1093/infdis/jit365

Chlibek, R., Pauksens, K., Rombo, L., Rijckevorsel, G.V., Richardus, J.H., Plassmann, G., Schwarz, T.F., Catteau, G., Lal, H., Heineman, T.C., 2016. Long-term immunogenicity and safety of an investigational herpes zoster subunit vaccine in older adults. Vaccine 34, 863–868. https://doi.org/10.1016/j.vaccine.2015.09.073

Choi, B.K., Asai, T., Vinay, D.S., Kim, Y.H., Kwon, B.S., 2006. 4-1BB-mediated amelioration of experimental autoimmune uveoretinitis is caused by indoleamine 2,3-dioxygenase-dependent mechanisms. Cytokine 34, 233–242. https://doi.org/10.1016/j.cyto.2006.04.008

Choi, Y.S., Eto, D., Yang, J.A., Lao, C., Crotty, S., 2013. Cutting Edge: STAT1 Is Required for IL-6-Mediated Bcl6 Induction for Early Follicular Helper Cell Differentiation. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1203032

Choi, Y.S., Kageyama, R., Eto, D., Escobar, T.C., Johnston, R.J., Monticelli, L., Lao, C., Crotty, S., 2011. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity. https://doi.org/10.1016/j.immuni.2011.03.023

Chong, J., Jones, P., Spelman, D., Leder, K., Cheng, A.C., 2017. Overwhelming post-splenectomy sepsis in patients with asplenia and hyposplenia: a retrospective cohort study. Epidemiology and Infection 145, 397–400. https://doi.org/10.1017/S0950268816002405

Chung, J.Y., Park, Y.C., Ye, H., Wu, H., 2002. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. Journal of cell science 115, 679–688.

Coccia, M., Collignon, C., Hervé, C., Chalon, A., Welsby, I., Detienne, S., Van Helden, M.J., Dutta, S., Genito, C.J., Waters, N.C., Van Deun, K., Smilde, A.K., Van Den Berg, R.A., Franco, D., Bourguignon, P., Morel, S., Garçon, N., Lambrecht, B.N., Goriely, S., Van Der Most, R., Didierlaurent, A.M., 2017. Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFNγ response promoting vaccine immunogenicity. npj Vaccines. https://doi.org/10.1038/s41541-017-0027-3

Coffman, L., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone . I . Definition according to profiles of lymphokine activities and secreted proteins. Journal of immunology 136, 2348–57.

Cohen, J.A., Barkhof, F., Comi, G., Hartung, H.-P., Khatri, B.O., Montalban, X., Pelletier, J., Capra, R., Gallo, P., Izquierdo, G., Tiel-Wilck, K., de Vera, A., Jin, J., Stites, T., Wu, S., Aradhye, S., Kappos, L., 2010. Oral Fingolimod or Intramuscular Interferon for Relapsing Multiple Sclerosis. New England Journal of Medicine. https://doi.org/10.1056/NEJMoa0907839

Collins, A. V., Brodie, D.W., Gilbert, R.J.C., Iaboni, A., Manso-Sancho, R., Walse, B., Stuart, D.I., Van Der Merwe, P.A., Davis, S.J., 2002. The interaction properties of costimulatory molecules revisited. Immunity. https://doi.org/10.1016/S1074-7613(02)00362-X

Cooper, M.A., Elliott, J.M., Keyel, P.A., Yang, L., Carrero, J.A., Yokoyama, W.M., 2009. Cytokine-induced memory-like natural killer cells. Proc. Natl. Acad. Sci. U.S.A. 106, 1915–1919. https://doi.org/10.1073/pnas.0813192106

Corry, D.B., Folkesson, H.G., Warnock, M.L., Erle, D.J., Matthay, M.A., Wiener-Kronish, J.P., Locksley, R.M., 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. The Journal of experimental medicine. https://doi.org/10.1084/jem.183.1.109

Cortini, A., Ellinghaus, U., Malik, T.H., Cunninghame Graham, D.S., Botto, M., Vyse, T.J., 2017. B cell OX40L supports T follicular helper cell development and contributes to SLE pathogenesis. Annals of the rheumatic diseases. https://doi.org/10.1136/annrheumdis-2017-211499

Cose, S., Brammer, C., Khanna, K.M., Masopust, D., Lefrançois, L., 2006. Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. European Journal of Immunology. https://doi.org/10.1002/eji.200535539

Coyle, A.J., Le Gros, G., Bertrand, C., Tsuyuki, S., Heusser, C.H., Kopf, M., Anderson, G.P., 1995. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. American journal of respiratory cell and molecular biology. https://doi.org/10.1165/ajrcmb.13.1.7598937

Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A., Gosselin, M., Owen, L.R., Rudd, C.E., Gutierrez-Ramos, J.C., 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity. https://doi.org/10.1016/S1074-7613(00)00011-X

Croft, M., 2010. Control of Immunity by the TNFR-Related Molecule OX40 (CD134). Annual Review of Immunology. https://doi.org/10.1146/annurev-immunol-030409-101243
Croft, M., Duncan, D.D., Swain, S.L., 1992. Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. Journal of Experimental Medicine 176, 1431–1437. https://doi.org/10.1084/jem.176.5.1431
Croft, M, Duncan, D.D., Swain, S.L., 1992. Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.176.5.1431
Crozat, K., Guiton, R., Contreras, V., Feuillet, V., Dutertre, C.-A., Ventre, E., Vu Manh, T.-P., Baranek, T., Storset, A.K., Marvel, J., Boudinot, P., Hosmalin, A., Schwartz-Cornil, I., Dalod, M., 2010. The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8α $^{+}$ dendritic cells. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20100223
Cua, D.J., Sherlock, J., Chen, Y., Murphy, C. a, Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. a, Gorman, D., Kastelein, R. a, Sedgwick, J.D., 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421, 744–748. https://doi.org/10.1038/nature01355
Cunningham, A.J., Lafferty, K.J., 1977. A Simple, Conservative Explanation of the H-2 Restriction of Interactions Between Lymphocytes. Scandinavian Journal of Immunology 6, 1–6. https://doi.org/10.1111/j.1365-3083.1977.tb00317.x
Curiel, R.E., Garcia, C.S., Rottschafer, S., Bosco, M.C., Espinoza-Delgado, I., 1999. Enhanced B7-2 Gene Expression by Interferon-γ in Human Monocytic Cells Is Controlled Through Transcriptional and Posttranscriptional Mechanisms. Blood 94, 1782–1789.
Curti, B.D., Kovacsovics-Bankowski, M., Morris, N., Walker, E., Chisholm, L., Floyd, K., Walker, J., Gonzalez, I., Meeuwsen, T., Fox, B.A., Moudgil, T., Miller, W., Haley, D., Coffey, T., Fisher, B., Delanty-Miller, L., Rymarchyk, N., Kelly, T., Crocenzi, T., Bernstein, E., Sanborn, R., Urba, W.J., Weinberg, A.D., 2013. OX40 is a potent immune-stimulating target in late-stage cancer patients. Cancer Research. https://doi.org/10.1158/0008-5472.CAN-12-4174
Curtis, B.M., Scharnowske, S., Watson, a J., 1992. Sequence and expression of a membrane-associated C-type lectin that exhibits CD4-independent binding of human immunodeficiency virus envelope glycoprotein gp120. Proceedings of the National Academy of Sciences of the United States of America 89, 8356–60. https://doi.org/10.1073/pnas.89.17.8356
Curtsinger, J.M., Lins, D.C., Mescher, M.F., 2003. Signal 3 Determines Tolerance versus Full Activation of Naive CD8 T Cells: Dissociating Proliferation and Development of Effector Function. The Journal of Experimental Medicine J. Exp. Med. https://doi.org/10.1084/jem.20021910
Curtsinger, J.M., Schmidt, C.S., Mondino, a, Lins, D.C., Kedl, R.M., Jenkins, M.K., Mescher, M.F., 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. Journal of immunology (Baltimore, Md. : 1950) 162, 3256–3262.
Cyster, J.C., 1999. Chemokines and cell migration in secondary lymphoid organs. Science. https://doi.org/10.1126/science.286.5447.2098
Dai, Z., Li, Q., Wang, Y., Diggs, L., Gao, G., 2004. CD4 CD25 regulatory T cells suppress allograft rejection mediated by memory CD8 T cells via a CD30-dependent mechanism. Journal of Clinical 113, 2–9. https://doi.org/10.1172/JCI200419727.Introduction
Dalsgaard, K., 1974. Saponin adjuvants - III. Isolation of a substance from Quillaja saponaria molina with adjuvant activity in foot-and-mouth disease vaccines. Archiv für die gesamte Virusforschung. https://doi.org/10.1007/BF01240612

Dalton, D.K., Pitts-Meek, S., Keshav, S., Figari, I.S., Bradley, A., Stewart, T.A., 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. Science. https://doi.org/10.1126/science.8456300

Dannull, J., Nair, S., Su, Z., Boczkowski, D., DeBeck, C., Yang, B., Gilboa, E., Vieweg, J., 2005. Enhancing the immunostimulatory function of dendritic cells by transfection with mRNA encoding OX40 ligand. Blood 105, 3206–3213. https://doi.org/10.1182/blood-2004-10-3944

Darnell, J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421. https://doi.org/10.1126/science.8197455

Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W.B., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., Roederer, M., Seder, R.A., 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nature Medicine. https://doi.org/10.1038/nm1592

Das, G., Sheridan, S., Janeway, C.A., 2001. The source of early IFN-gamma that plays a role in Th1 priming. J. Immunol. 167, 2004–2010. https://doi.org/10.4049/jimmunol.167.4.2004

Davis, M.M., Bjorkman, P.J., 1988. T-cell antigen receptor genes and T-cell recognition. Nature. https://doi.org/10.1038/334395a0

de Vinuesa, C.G., Cook, M.C., Ball, J., Drew, M., Sunners, Y., Cascalho, M., Wabl, M., Klaus, G.G.B., MacLennan, I.C.M., 2000. Germinal Centers without T Cells. J Exp Med 191, 485–494.

Debes, G.F., Arnold, C.N., Young, A.J., Krautwald, S., Lipp, M., Hay, J.B., Butcher, E.C., 2005. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nature Immunology. https://doi.org/10.1038/ni1238

Del Prete, G., De Carli, M., D'Elios, M.M., Daniel, K.C., Almerigogna, F., Alderson, M., Smith, C. a, Thomas, E., Romagnani, S., 1995. CD30-mediated signaling promotes the development of human T helper type 2-like T cells. The Journal of experimental medicine.

Delfs, M.W., Furukawa, Y., Mitchell, R.N., Lichtman, A.H., 2001. CD8+T cell subsets TC1 and TC2 cause different histopathologic forms of murine cardiac allograft rejection. Transplantation. https://doi.org/10.1097/00007890-200103150-00005

den Haan, J.M.M., Bevan, M.J., 2002. Constitutive versus activation-dependent cross-presentation of immune complexes by CD8 (+) and CD8 (−) dendritic cells in vivo. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20020295

Dendouga, N., Fochesato, M., Lockman, L., Mossman, S., Giannini, S.L., 2012. Cell-mediated immune responses to a varicella-zoster virus glycoprotein E vaccine using both a TLR agonist and QS21 in mice. Vaccine 30, 3126–3135. https://doi.org/10.1016/j.vaccine.2012.01.088

Deng, T., Suo, C., Chang, J., Yang, R., Li, J., Cai, T., Qiu, J., 2019. ILC3-derived OX40L is essential for homeostasis of intestinal Tregs in immunodeficient mice. Cellular & Molecular Immunology 1. https://doi.org/10.1038/s41423-019-0200-x

Denis, M., 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. 132, 150–157.

DeYoung, a L., Duramad, O., Winoto, a, 2000. The TNF receptor family member CD30 is not essential for negative selection. Journal of immunology (Baltimore, Md. : 1950).

Di, C., Lin, X., Zhang, Y., Zhong, W., Yuan, Y., Zhou, T., Liu, J., Xia, Z., 2015. Basophil-associated OX40 ligand participates in the initiation of Th2 responses during airway inflammation. Journal of Biological Chemistry. https://doi.org/10.1074/jbc.M115.642637

Didierlaurent, A. M., Collignon, C., Bourguignon, P., Wouters, S., Fierens, K., Fochesato, M., Dendouga, N., Langlet, C., Malissen, B., Lambrecht, B.N., Garcon, N., Van Mechelen,

M., Morel, S., 2014. Enhancement of Adaptive Immunity by the Human Vaccine Adjuvant AS01 Depends on Activated Dendritic Cells. The Journal of Immunology 193, 1920–1930. https://doi.org/10.4049/jimmunol.1400948

Didierlaurent, Arnaud M, Collignon, C., Bourguignon, P., Wouters, S., Fierens, K., Fochesato, M., Dendouga, N., Langlet, C., Malissen, B., Lambrecht, B.N., Garçon, N., Van Mechelen, M., Morel, S., 2014. Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. Journal of immunology (Baltimore, Md. : 1950) 193, 1920–30. https://doi.org/10.4049/jimmunol.1400948

Didierlaurent, A.M., Laupèze, B., Di Pasquale, A., Hergli, N., Collignon, C., Garçon, N., 2017. Adjuvant system AS01: helping to overcome the challenges of modern vaccines. Expert Review of Vaccines 16, 55–63. https://doi.org/10.1080/14760584.2016.1213632

Diez-Domingo, J., Weinke, T., Garcia de Lomas, J., Meyer, C.U., Bertrand, I., Eymin, C., Thomas, S., Sadorge, C., 2015. Comparison of intramuscular and subcutaneous administration of a herpes zoster live-attenuated vaccine in adults aged ≥50 years: a randomised non-inferiority clinical trial. Vaccine 33, 789–795. https://doi.org/10.1016/j.vaccine.2014.12.024

Doherty, P.C., Hou, S., Tripp, R.A., 1994. CD8+ T-cell memory to viruses. Current Opinion in Immunology. https://doi.org/10.1016/0952-7915(94)90139-2

Doherty, P.C., Zinkernagel, R.M., 1975. A biological role for the major histocompatibility antigens. Lancet (London, England). https://doi.org/10.1016/S0140-6736(75)92610-0

Domeier, P.P., Chodisetti, S.B., Soni, C., Schell, S.L., Elias, M.J., Wong, E.B., Cooper, T.K., Kitamura, D., Rahman, Z.S.M., 2016. IFN-γ receptor and STAT1 signaling in B cells are central to spontaneous germinal center formation and autoimmunity. J. Exp. Med. 213, 715–732. https://doi.org/10.1084/jem.20151722

Duckett, C.S., Thompson, C.B., 1997. CD30-dependent degradation of TRAF2: Implications for negative regulation of TRAF signaling and the control of cell survival. Genes and Development. https://doi.org/10.1101/gad.11.21.2810

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Chae, G.P., Steinman, R.M., Nussenzweig, M.C., 2007. Differential antigen processing by dendritic cell subsets in vivo. Science. https://doi.org/10.1126/science.1136080

Dunn, P.L., North, R.J., 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infection and Immunity 59, 2892–2900.

Dupasquier, M., Stoitzner, P., van Oudenaren, A., Romani, N., Leenen, P.J.M., 2004. Macrophages and dendritic cells constitute a major subpopulation of cells in the mouse dermis. J. Invest. Dermatol. 123, 876–879. https://doi.org/10.1111/j.0022-202X.2004.23427.x

Dutton, E.E., Gajdasik, D.W., Willis, C., Fiancette, R., Bishop, E.L., Camelo, A., Sleeman, M.A., Coccia, M., Didierlaurent, A.M., Tomura, M., Pilataxi, F., Morehouse, C.A., Carlesso, G., Withers, D.R., 2019. Peripheral lymph nodes contain migratory and resident innate lymphoid cell populations. Science Immunology 4, eaau8082. https://doi.org/10.1126/sciimmunol.aau8082

Eberl, G., Littman, D.R., 2003. The role of the nuclear hormone receptor RORγt in the development of lymph nodes and Peyer's patches. Immunological Reviews. https://doi.org/10.1034/j.1600-065X.2003.00074.x

Eberl, G., Marmon, S., Sunshine, M.-J., Rennert, P.D., Choi, Y., Littman, D.R., 2004. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nature immunology 5, 64–73. https://doi.org/10.1038/ni1022

Edelson, B.T., Bradstreet, T.R., Hildner, K., Carrero, J.A., Frederick, K.E., Kc, W., Belizaire, R., Aoshi, T., Schreiber, R.D., Miller, M.J., Murphy, T.L., Unanue, E.R., Murphy, K.M.,

2011. CD8α+ Dendritic Cells Are an Obligate Cellular Entry Point for Productive Infection by Listeria monocytogenes. Immunity 35, 236–248. https://doi.org/10.1016/j.immuni.2011.06.012
Eisenbarth, S.C., Colegio, O.R., O'Connor, W., Sutterwala, F.S., Flavell, R.A., 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453, 1122–1126. https://doi.org/10.1038/nature06939
Elgueta, R., Benson, M.J., Vries, V.C.D., Wasiuk, A., Guo, Y., Noelle, R.J., 2013. Molecular mechanism and function of CD40/CD40L engagement in the Immune System 229, 1–31. https://doi.org/10.1111/j.1600-065X.2009.00782.x.Molecular
Ertelt, J.M., Johanns, T.M., Rowe, J.H., Way, S.S., Lai, J.C., McLachlan, J.B., 2009. Selective Priming and Expansion of Antigen-Specific Foxp3-CD4+ T Cells during Listeria monocytogenes Infection. The Journal of Immunology 182, 3032–3038. https://doi.org/10.4049/jimmunol.0803402
Fazou, C., Yang, H., McMichael, A.J., Callan, M.F.C., 2001. Epitope Specificity of Clonally Expanded Populations of CD8+ T Cells Found Within the Joints of Patients with Inflammatory Arthritis. Arthritis and Rheumatism. https://doi.org/10.1002/1529-0131(200109)44:9<2038::AID-ART353>3.0.CO;2-1
Fehniger, T.A., Caligiuri, M.A., 2001. Interleukin 15: Biology and relevance to human disease. Blood. https://doi.org/10.1182/blood.V97.1.14
Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., Chambon, P., 1996. Ligand-activated site-specific recombination in mice. Proceedings of the National Academy of Sciences of the United States of America.
Finotto, S., Neurath, M.F., Glickman, J.N., Qin, S., Lehr, H.A., Green, F.H.Y., Ackerman, K., Haley, K., Galle, P.R., Szabo, S.J., Drazen, J.M., Sanctis, G.T.D., Glimcher, L.H., 2002. Development of Spontaneous Airway Changes Consistent with Human Asthma in Mice Lacking T-bet. Science 295, 336–338. https://doi.org/10.1126/science.1065544
Flynn, S., Toellner, K.-M., Raykundalia, C., Goodall, M., Lane, P., 1998. CD4 T Cell Cytokine Differentiation: The B Cell Activation Molecule, OX40 Ligand, Instructs CD4 T Cells to Express Interleukin 4 and Upregulates Expression of the Chemokine Receptor, Blr-1. J Exp Med 188, 297–304.
Fontenot, J.D., Gavin, M.A., Rudensky, A.Y., 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology 4, 330–6. https://doi.org/10.1038/ni904
Ford, M.L., Koehn, B.H., Wagener, M.E., Jiang, W., Gangappa, S., Pearson, T.C., Larsen, C.P., 2007. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. The Journal of experimental medicine. https://doi.org/10.1084/jem.20062319
Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., Lipp, M., 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. https://doi.org/10.1016/S0092-8674(00)80059-8
Foti, M., Granucci, F., Pelizzola, M., Beretta, O., Ricciardi-Castagnoli, P., 2006. Dendritic cells in pathogen recognition and induction of immune responses: a functional genomics approach. J. Leukoc. Biol. 79, 913–916. https://doi.org/10.1189/jlb.1005547
Foy, T.M., Shepherd, D.M., Durie, F.H., Aruffo, A., Ledbetter, J.A., Noelle, R.J., 1993. In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. Journal of Experimental Medicine 178, 1567–1575. https://doi.org/10.1084/jem.178.5.1567
Fu, S., Zhang, N., Yopp, A.C., Chen, Dongmei, Mao, M., Chen, Dan, Zhang, H., Ding, Y., Bromberg, J.S., 2004. TGF-β induces Foxp3 + T-regulatory cells from CD4 + CD25 -

precursors. American Journal of Transplantation 4, 1614–1627. https://doi.org/10.1111/j.1600-6143.2004.00566.x
Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., Okumoto, T., 1994. Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. The Journal of antibiotics. https://doi.org/10.7164/antibiotics.47.208
Garçon, N., Vaughn, D.W., Didierlaurent, A.M., 2012. Development and evaluation of AS03, an Adjuvant System containing α-tocopherol and squalene in an oil-in-water emulsion. Expert Rev Vaccines 11, 349–366. https://doi.org/10.1586/erv.11.192
Gaspal, F., Bekiaris, V., Kim, M.-Y., Withers, D.R., Bobat, S., MacLennan, I.C.M., Anderson, G., Lane, P.J., Cunningham, A.F., 2008. Critical synergy of CD30 and OX40 signals in CD4 T cell homeostasis and Th1 immunity to Salmonella. Journal of immunology (Baltimore, Md. : 1950).
Gaspal, F., Withers, D., Saini, M., Bekiaris, V., McConnell, F.M., White, A., Khan, M., Yagita, H., Walker, L.S.K., Anderson, G., Lane, P.J.L., 2011. Abrogation of CD30 and OX40 signals prevents autoimmune disease in FoxP3-deficient mice. The Journal of experimental medicine 208, 1579–84. https://doi.org/10.1084/jem.20101484
Gaspal, F.M.C., Kim, M.-Y., McConnell, F.M., Raykundalia, C., Bekiaris, V., Lane, P.J.L., 2005. Mice Deficient in OX40 and CD30 Signals Lack Memory Antibody Responses because of Deficient CD4 T Cell Memory. The Journal of Immunology 174, 3891–3896. https://doi.org/10.4049/jimmunol.174.7.3891
Gaspal, F.M.C., McConnell, F.M., Kim, M.-Y., Gray, D., Kosco-Vilbois, M.H., Raykundalia, C.R., Botto, M., Lane, P.J.L., 2006. The generation of thymus-independent germinal centers depends on CD40 but not on CD154, the T cell-derived CD40-ligand. Eur. J. Immunol. 36, 1665–1673. https://doi.org/10.1002/eji.200535339
Gavillet, B.M., Eberhardt, C.S., Auderset, F., Castellino, F., Seubert, A., Tregoning, J.S., Lambert, P.-H., Gregorio, E. de, Giudice, G.D., Siegrist, C.-A., 2015. MF59 Mediates Its B Cell Adjuvanticity by Promoting T Follicular Helper Cells and Thus Germinal Center Responses in Adult and Early Life. The Journal of Immunology 194, 4836–4845. https://doi.org/10.4049/jimmunol.1402071
Gehad, A., Al-Banna, N.A., Vaci, M., Issekutz, A.C., Mohan, K., Latta, M., Issekutz, T.B., 2012. Differing Requirements for CCR4, E-Selectin, and 4 1 for the Migration of Memory CD4 and Activated T Cells to Dermal Inflammation. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1102315
Geijtenbeek, T.B.H., Torensma, R., Van Vliet, S.J., Van Duijnhoven, G.C.F., Adema, G.J., Van Kooyk, Y., Figdor, C.G., 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell. https://doi.org/10.1016/S0092-8674(00)80693-5
Germain, R.N., 2002. T-cell development and the CD4–CD8 lineage decision. Nature Reviews Immunology 2, 309–322. https://doi.org/10.1038/nri798
Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G., Trinchieri, G., 2002. Reciprocal Activating Interaction between Natural Killer Cells and Dendritic Cells. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20010938
Gilfillan, M.C., Noel, P.J., Podack, E.R., Reiner, S.L., Thompson, C.B., 1998. Expression of the costimulatory receptor CD30 is regulated by both CD28 and cytokines. J Immunol.
Gillard, G.O., Bivas-Benita, M., Hovav, A.-H., Grandpre, L.E., Panas, M.W., Seaman, M.S., Haynes, B.F., Letvin, N.L., 2011. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. PLoS Pathog. 7, e1002141. https://doi.org/10.1371/journal.ppat.1002141
Glenny, a T., Südmersen, H.J., 1921. Notes on the Production of Immunity to Diphtheria Toxin. The Journal of hygiene 20, 176–220. https://doi.org/10.1017/S0022172400033945

Glenny, A.T., Pope, C.G., Waddington, H., Wallace, U., 1926. Immunological notes. XVII–XXIV. The Journal of Pathology and Bacteriology 29, 31–40. https://doi.org/10.1002/path.1700290106
Gmünder, H., Lesslauer, W., 1984. A 45-kDa human T-cell membrane glycoprotein functions in the regulation of cell proliferative responses. European Journal of Biochemistry.
Godfrey, W.R., 2004. Identification of a human OX-40 ligand, a costimulator of CD4+ T cells with homology to tumor necrosis factor. Journal of Experimental Medicine. https://doi.org/10.1084/jem.180.2.757
Goldrath, a W., Bevan, M.J., 1999. Selecting and maintaining a diverse T-cell repertoire. Nature 402, 255–262. https://doi.org/10.1038/46218
Goldszmid, R.S., Caspar, P., Rivollier, A., White, S., Dzutsev, A., Hieny, S., Kelsall, B., Trinchieri, G., Sher, A., 2012. NK Cell-Derived Interferon-γ Orchestrates Cellular Dynamics and the Differentiation of Monocytes into Dendritic Cells at the Site of Infection. Immunity. https://doi.org/10.1016/j.immuni.2012.03.026
Gordon, S.M., Chaix, J., Rupp, L.J., Wu, J., Madera, S., Sun, J.C., Lindsten, T., Reiner, S.L., 2012. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. Immunity. https://doi.org/10.1016/j.immuni.2011.11.016
Gormley, P.D., Powell-Richards, A.O.R., Azuara-Blanco, A., Donoso, L.A., Dua, H.S., 1998. Lymphocyte subsets in conjunctival mucosa-associated-lymphoid-tissue after exposure to retinal-S-antigen. International Ophthalmology. https://doi.org/10.1023/A:1006191022900
Gough, M.J., Killeen, N., Weinberg, A.D., 2012. Targeting macrophages in the tumour environment to enhance the efficacy of αOX40 therapy. Immunology 136, 437–447. https://doi.org/10.1111/j.1365-2567.2012.03600.x
Gowans, J.L., 1957. The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. Br J Exp Pathol 38, 67–78.
Gowans, J.L., Knight, E.J., 1964. The route of re-circulation of lymphocytes in the rat. Proceedings of the Royal Society of London. Series B, Biological sciences. https://doi.org/10.1098/rspb.1964.0001
Graham, S.M., Molyneux, E.M., Walsh, A.L., Cheesbrough, J.S., Molyneux, M.E., Hart, C.A., 2000. Nontyphoidal Salmonella infections of children in tropical Africa. Pediatric Infectious Disease Journal. https://doi.org/10.1097/00006454-200012000-00016
Gramaglia, I., Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., Croft, M., 2000. The OX40 Costimulatory Receptor Determines the Development of CD4 Memory by Regulating Primary Clonal Expansion. The Journal of Immunology 165, 3043–3050. https://doi.org/10.4049/jimmunol.165.6.3043
Gramaglia, I, Jember, A., Pippig, S.D., Weinberg, A.D., Killeen, N., Croft, M., 2000. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. Journal of immunology (Baltimore, Md. : 1950) 165, 3043–50.
Gramaglia, I., Weinberg, A., Lemon, M., Croft, M., 1998. OX40 Ligand: A Potent Costimulatory Molecule for Sustaining Primary CD4 T Cell Responses1. Journal of Immunology 161, 6510–7.
Granger, S.W., Ware, C.F., 2001. Turning on LIGHT. Journal of Clinical Investigation. https://doi.org/10.1172/JCI14651
Gravestein, L. a, Borst, J., 1998. Tumor necrosis factor receptor family members in the immune system. Seminars in immunology 10, 423–34. https://doi.org/10.1006/smim.1998.0144
Gray, D., Matzinger, P., 1991. T cell memory is short-lived in the absence of antigen. The Journal of experimental medicine 174, 969–74.
Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., Scheurich, P., 1995. The transmembrane

form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell. https://doi.org/10.1016/0092-8674(95)90192-2
Gretz, J.E., Anderson, A.O., Shaw, S., 1997. Cords, channels, corridors and conduits: Critical architectural elements facilitating cell interactions in the lymph node cortex. Immunological Reviews. https://doi.org/10.1111/j.1600-065X.1997.tb00955.x
Grewal, I.S., Xu, J., Flavell, R.A., 1995. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. Nature. https://doi.org/10.1038/378617a0
Griffin, A.J., McSorley, S.J., 2011. Development of protective immunity to *Salmonella*, a mucosal pathogen with a systemic agenda. Mucosal Immunology 4, 371–382. https://doi.org/10.1038/mi.2011.2
Groom, A.C., Schmidt, E.E., MacDonald, I.C., 1991. Microcirculatory pathways and blood flow in spleen: new insights from washout kinetics, corrosion casts, and quantitative intravital videomicroscopy. Scanning Microsc.
Gross, J. a, Callas, E., Allison, J.P., 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. Journal of immunology (Baltimore, Md. : 1950) 149, 380–388.
Grun, J.L., Maurer, P.H., 1989. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. Cell. Immunol. 121, 134–145.
Grusby, M.J., Johnson, R.S., Papaioannou, V.E., Glimcher, L.H., 1991. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science 253, 1417–1420. https://doi.org/10.1126/science.1910207
Gu, H., Marth, J.D., Orban, P.C., Mossmann, H., Rajewsky, K., 1994. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science. https://doi.org/10.1126/science.8016642
Gu, H., Zou, Y.R., Rajewsky, K., 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell. https://doi.org/10.1016/0092-8674(93)90644-6
Guilliams, M., Henri, S., Tamoutounour, S., Ardouin, L., Schwartz-Cornil, I., Dalod, M., Malissen, B., 2010. From skin dendritic cells to a simplified classification of human and mouse dendritic cell subsets. European Journal of Immunology. https://doi.org/10.1002/eji.201040498
Guo, Y., Sun, X., Shibata, K., Yamada, H., Muta, H., Podack, E.R., Yoshikai, Y., 2013. CD30 is required for activation of a unique subset of interleukin-17A-producing γδ T cells in innate immunity against Mycobacterium bovis Bacillus Calmette-Guerin infection. Infection and Immunity. https://doi.org/10.1128/IAI.00887-13
Gupta, R.K., 1998. Aluminum compounds as vaccine adjuvants. Advanced Drug Delivery Reviews 32, 155–172. https://doi.org/10.1016/S0169-409X(98)00008-8
Gupta, R.K., Rost, B.E., Relyveld, E., Siber, G.R., 1995. Adjuvant properties of aluminum and calcium compounds. Pharmaceutical biotechnology 6, 229–48.
Ha, J., Bingaman, A.W., Durham, M.M., Pearson, T.C., Larsen, C.P., 2001. Aggressive skin allograft rejection in CD28-/- mice independent of the CD40/CD40L costimulatory pathway. Transplant Immunology. https://doi.org/10.1016/S0966-3274(01)00043-0
Hakamada-Taguchi, R., Kato, T., Ushijima, H., Murakami, M., Uede, T., Nariuchi, H., 1998. Expression and co-stimulatory function of B7-2 on murine CD4+ T cells. Eur. J. Immunol. 28, 865–873. https://doi.org/10.1002/(SICI)1521-4141(199803)28:03<865::AID-IMMU865>3.0.CO;2-T
Halim, T.Y.F., Rana, B.M.J., Walker, J.A., Kerscher, B., Knolle, M.D., Jolin, H.E., Serrao, E.M., Haim-Vilmovsky, L., Teichmann, S.A., Rodewald, H.-R., Botto, M., Vyse, T.J., Fallon, P.G., Li, Z., Withers, D.R., McKenzie, A.N.J., 2018. Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells. Immunity 48, 1195-1207.e6. https://doi.org/10.1016/j.immuni.2018.05.003

Haluszczak, C., Akue, A.D., Hamilton, S.E., Johnson, L.D.S., Pujanauski, L., Teodorovic, L., Jameson, S.C., Kedl, R.M., 2009. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. J Exp Med 206, 435–448. https://doi.org/10.1084/jem.20081829

Haranaka, K., Satomi, N., Sakurai, A., 1984. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumors in nude mice. Int J Cancer.

Harding, F. a, McArthur, J.G., Gross, J. a, Raulet, D.H., Allison, J.P., 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 356, 607–609. https://doi.org/10.1038/356607a0

Harper, K., Balzano, C., Rouvier, E., Mattéi, M.G., Luciani, M.F., Golstein, P., 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. Journal of immunology (Baltimore, Md. : 1950).

Harrell, Maria.I., Iritani, B.M., Ruddell, A., 2008. Lymph Node Mapping in the Mouse. J Immunol Methods 332, 170–174. https://doi.org/10.1016/j.jim.2007.11.012

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., Weaver, C.T., 2005. Interleukin 17–producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nature Immunology 6, 1123–1132. https://doi.org/10.1038/ni1254

Hart, D.N., 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90, 3245–3287.

Hataye, J., Moon, J.J., Khoruts, A., Reilly, C., Jenkins, M.K., 2006. Naïve and memory CD4+ T cell survival controlled by clonal abundance. Science. https://doi.org/10.1126/science.1124228

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., Nussenzweig, M.C., 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J. Exp. Med. 194, 769–779.

Hayakawa, K., Ishii, R., Yamasaki, K., Kishimoto, T., Hardy, R.R., 1987. Isolation of high-affinity memory B cells: phycoerythrin as a probe for antigen-binding cells. Proc. Natl. Acad. Sci. U.S.A. 84, 1379–1383. https://doi.org/10.1073/pnas.84.5.1379

He, Y.W., Deftos, M.L., Ojala, E.W., Bevan, M.J., 1998. RORγt, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. Immunity. https://doi.org/10.1016/S1074-7613(00)80645-7

Hedberg, C.W., 2011. Foodborne illness acquired in the United States. Emerging Infectious Diseases. https://doi.org/10.3201/eid1707.110019

Hedrick, S.M., Cohen, D.I., Nielsen, E.A., Davis, M.M., 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature. https://doi.org/10.1038/308149a0

Heffner, C.S., Herbert Pratt, C., Babiuk, R.P., Sharma, Y., Rockwood, S.F., Donahue, L.R., Eppig, J.T., Murray, S.A., 2012. Supporting conditional mouse mutagenesis with a comprehensive cre characterization resource. Nat Commun 3, 1218. https://doi.org/10.1038/ncomms2186

Hepworth, M.R., Monticelli, L.A., Fung, T.C., Ziegler, C.G.K., Grunberg, S., Sinha, R., Mantegazza, A.R., Ma, H.-L., Crawford, A., Angelosanto, J.M., Wherry, E.J., Koni, P.A., Bushman, F.D., Elson, C.O., Eberl, G., Artis, D., Sonnenberg, G.F., 2013. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature 498, 113–117. https://doi.org/10.1038/nature12240

Hepworth, M.R., Sonnenberg, G.F., 2014a. Regulation of the adaptive immune system by innate lymphoid cells. Current opinion in immunology 27, 75–82. https://doi.org/10.1016/j.coi.2014.01.013

Hepworth, M.R., Sonnenberg, G.F., 2014b. Regulation of the adaptive immune system by innate lymphoid cells. Current opinion in immunology 27, 75–82. https://doi.org/10.1016/j.coi.2014.01.013

Herman, A.E., Freeman, G.J., Mathis, D., Benoist, C., 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J. Exp. Med. 199, 1479–1489. https://doi.org/10.1084/jem.20040179

Hess, J., Ladel, C., Miko, D., Kaufmann, S.H.E., 1996. Salmonella typhimurium aroA- Infection in Gene-Targeted immunodeficient Mice. Journal of immunology (Baltimore, Md. : 1950).

Higginbotham, J.N., Lin, T.L., Pruett, S.B., 1992. Effect of macrophage activation on killing of Listeria monocytogenes. Roles of reactive oxygen or nitrogen intermediates, rate of phagocytosis, and retention of bacteria in endosomes. Clin Exp Immunol 88, 492–498.

Hirano, T., Kikuchi, T., Tode, N., Santoso, A., Yamada, M., Mitsuhashi, Y., Komatsu, R., Kawabe, T., Tanimoto, T., Ishii, N., Tanaka, Y., Nishimura, H., Nukiwa, T., Watanabe, A., Ichinose, M., 2016. OX40 ligand newly expressed on bronchiolar progenitors mediates influenza infection and further exacerbates pneumonia. EMBO Mol Med 8, 422–436. https://doi.org/10.15252/emmm.201506154

Hiromatsu, K., Yoshikai, Y., Matsuzaki, G., Ohga, S., Muramori, K., Matsumoto, K., Bluestone, J.A., Nomoto, K., 1992. A protective role of gamma/delta T cells in primary infection with Listeria monocytogenes in mice. Journal of Experimental Medicine 175, 49–56. https://doi.org/10.1084/jem.175.1.49

Hobeika, E., Thiemann, S., Storch, B., Jumaa, H., Nielsen, P.J., Pelanda, R., Reth, M., 2006. Testing gene function early in the B cell lineage in mb1-cre mice. PNAS 103, 13789–13794. https://doi.org/10.1073/pnas.0605944103

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., Akerley, W., van den Eertwegh, A.J.M., Lutzky, J., Lorigan, P., Vaubel, J.M., Linette, G.P., Hogg, D., Ottensmeier, C.H., Lebbé, C., Peschel, C., Quirt, I., Clark, J.I., Wolchok, J.D., Weber, J.S., Tian, J., Yellin, M.J., Nichol, G.M., Hoos, A., Urba, W.J., 2010. Improved survival with ipilimumab in patients with metastatic melanoma. The New England journal of medicine. https://doi.org/10.1056/NEJMoa1003466

Hoffmann, J. a, 2003. The immune response of Drosophila. Nature 426, 33–38. https://doi.org/10.1038/nature02021

Hollister, K., Kusam, S., Wu, H., Clegg, N., Mondal, A., Sawant, D.V., Dent, A.L., 2013. Insights into the role of Bcl6 in follicular Th cells using a new conditional mutant mouse model. J. Immunol. 191, 3705–3711. https://doi.org/10.4049/jimmunol.1300378

Holt, M.P., Punkosdy, G.A., Glass, D.D., Shevach, E.M., 2017. TCR Signaling and CD28/CTLA-4 Signaling Cooperatively Modulate T Regulatory Cell Homeostasis. J. Immunol. 198, 1503–1511. https://doi.org/10.4049/jimmunol.1601670

Hong, G.U., Lim, J.Y., Kim, N.G., Shin, J.H., Ro, J.Y., 2015. IgE and IgA produced by OX40-OX40L or CD40-CD40L interaction in B cells-mast cells re-activate FcεRI or FcαRI on mast cells in mouse allergic asthma. European Journal of Pharmacology. https://doi.org/10.1016/j.ejphar.2015.02.023

Hori, S., Nomura, T., Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061. https://doi.org/10.1126/science.1079490

Howland, K.C., Ausubel, L.J., London, C.A., Abbas, A.K., 2000. The Roles of CD28 and CD40 Ligand in T Cell Activation and Tolerance. The Journal of Immunology 164, 4465–4470. https://doi.org/10.4049/jimmunol.164.9.4465

Hsieh, C. S., Macatonia, S.E., O'Garra, A., Murphy, K.M., 1993. Pathogen-induced Th1 phenotype development in CD4+ alpha beta-TCR transgenic T cells is macrophage dependent. Int. Immunol. 5, 371–382. https://doi.org/10.1093/intimm/5.4.371

Hsieh, C S, Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., Murphy, K.M., 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science (New York, N.Y.) 260, 547–9. https://doi.org/10.1126/science.8097338

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, R., Aguet, M., 1993. Immune response in mice that lack the interferon-gamma receptor. Science 259, 1742–1745. https://doi.org/10.1126/science.8456301

Humann, J., Lenz, L.L., 2010. Activation of naïve NK cells in response to Listeria monocytogenes requires IL-18 and contact with infected dendritic cells1. J Immunol 184, 5172–5178. https://doi.org/10.4049/jimmunol.0903759

Humphreys, I.R., Edwards, L., Walzl, G., Rae, A.J., Dougan, G., Hill, S., Hussell, T., 2003. OX40 ligation on activated T cells enhances the control of Cryptococcus neoformans and reduces pulmonary eosinophilia. Journal of immunology (Baltimore, Md. : 1950). https://doi.org/10.4049/jimmunol.170.12.6125

Hutchison, S., Benson, R.A., Gibson, V.B., Pollock, A.H., Garside, P., Brewer, J.M., 2011. Antigen depot is not required for alum adjuvanticity. The FASEB Journal 26, 1272–1279. https://doi.org/10.1096/fj.11-184556

Hutloff, A, Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., Kroczek, R.A., 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397, 263–266. https://doi.org/10.1038/16717

Hutloff, Andreas, Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., Kroczek, R.A., 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. https://doi.org/10.1038/16717

Ikeda, S., Saijo, S., Murayama, M.A., Shimizu, K., Akitsu, A., Iwakura, Y., 2014. Excess IL-1 signaling enhances the development of Th17 cells by downregulating TGF-β-induced Foxp3 expression. J. Immunol. 192, 1449–1458. https://doi.org/10.4049/jimmunol.1300387

Imura, A., Hori, T., Imada, K., Ishikawa, T., Tanaka, Y., Maeda, M., Imamura, S., Uchiyama, T., 1996. The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. The Journal of experimental medicine 183, 2185–95.

Inglis, J.J., Criado, G., Medghalchi, M., Andrews, M., Sandison, A., Feldmann, M., Williams, R.O., 2007. Collagen-induced arthritis in C57BL/6 mice is associated with a robust and sustained T-cell response to type II collagen. Arthritis Res Ther 9, R113. https://doi.org/10.1186/ar2319

Ishii, N., Ndhlovu, L.C., Murata, K., Sato, T., Kamanaka, M., Sugamura, K., 2003. OX40 (CD134) and OX40 ligand interaction plays an adjuvant role during in vivo Th2 responses. European Journal of Immunology 33, 2372–2381. https://doi.org/10.1002/eji.200324031

Ito, T., Wang, Y.-H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X.-F., Yao, Z., Cao, W., Liu, Y.-J., 2005a. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20051135

Ito, T., Wang, Y.-H., Duramad, O., Hori, T., Delespesse, G.J., Watanabe, N., Qin, F.X.-F., Yao, Z., Cao, W., Liu, Y.-J., 2005b. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20051135

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., Littman, D.R., 2006. The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+T Helper Cells. Cell. https://doi.org/10.1016/j.cell.2006.07.035

Jacobsen, M., Cepok, S., Quak, E., Happel, M., Gaber, R., Ziegler, A., Schock, S., Oertel, W.H., Sommer, N., Hemmer, B., 2002. Oligoclonal expansion of memory CD8+ T cells

in cerebrospinal fluid from multiple sclerosis patients. Brain. https://doi.org/10.1093/brain/awf059
Jahan, N., Talat, H., Curry, W.T., 2018. Agonist OX40 immunotherapy improves survival in glioma-bearing mice and is complementary with vaccination with irradiated GM-CSF-expressing tumor cells. Neuro-oncology 20, 44–54. https://doi.org/10.1093/neuonc/nox125
Janeway, C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197–216. https://doi.org/10.1146/annurev.immunol.20.083001.084359
Jelley-Gibbs, D.M., Strutt, T.M., McKinstry, K.K., Swain, S.L., 2008. Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. Immunology and cell biology 86, 343–52. https://doi.org/10.1038/icb.2008.13
Jember, A.G.-H., Zuberi, R., Liu, F.-T., Croft, M., 2002. Development of Allergic Inflammation in a Murine Model of Asthma Is Dependent on the Costimulatory Receptor Ox40. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.193.3.387
Jenkins, M.K., Ashwell, J.D., Schwartz, R.H., 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. J. Immunol. 140, 3324–3330.
Jenkins, M.K., Khoruts, A., Ingulli, E., Mueller, D.L., McSorley, S.J., Reinhardt, R.L., Itano, A., Pape, K.A., 2001. In vivo activation of antigen-specific CD4 T cells. Annual review of immunology. https://doi.org/10.1146/annurev.immunol.19.1.23
Jenkins, M.K., Schwartz, R.H., 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. The Journal of Experimental Medicine. https://doi.org/3029267
Jenkins, S.J., Perona-Wright, G., Worsley, A.G.F., Ishii, N., MacDonald, A.S., 2007. Dendritic Cell Expression of OX40 Ligand Acts as a Costimulatory, Not Polarizing, Signal for Optimal Th2 Priming and Memory Induction In Vivo. The Journal of Immunology 179, 3515–3523. https://doi.org/10.4049/jimmunol.179.6.3515
Jones, T.F., Ingram, L.A., Cieslak, P.R., Vugia, D.J., Tobin-D'Angelo, M., Hurd, S., Medus, C., Cronquist, A., Angulo, F.J., 2008. Salmonellosis outcomes differ substantially by serotype. J. Infect. Dis. 198, 109–114. https://doi.org/10.1086/588823
Jordan, M.S., Boesteanu, A., Reed, a J., Petrone, a L., Holenbeck, a E., Lerman, M. a, Naji, A., Caton, a J., 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nature immunology. https://doi.org/10.1038/86302
June, C.H., Ledbetter, J.A., Linsley, P.S., Thompson, C.B., 1990. Role of the CD28 receptor in T-cell activation. Immunology Today. https://doi.org/10.1016/0167-5699(90)90085-N
Jung, C., Hugot, J.-P., Barreau, F., 2010. Peyer's Patches: The Immune Sensors of the Intestine. International Journal of Inflammation. https://doi.org/10.4061/2010/823710
Jung, S., Unutmaz, D., Wong, P., Sano, G.-I., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E.G., Littman, D.R., Lang, R.A., 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity 17, 211–220.
Kallies, A., Hawkins, E.D., Belz, G.T., Metcalf, D., Hommel, M., Corcoran, L.M., Hodgkin, P.D., Nutt, S.L., 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. Nature Immunology. https://doi.org/10.1038/ni1321
Kang, S.-J., Liang, H.-E., Reizis, B., Locksley, R.M., 2008. Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells. Immunity 29, 819–833. https://doi.org/10.1016/j.immuni.2008.09.017
Kaplan, M.H., Sun, Y.L., Hoey, T., Grusby, M.J., 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 382, 174–177. https://doi.org/10.1038/382174a0
Karandikar, N.J., 1996. CTLA-4: a negative regulator of autoimmune disease. Journal of Experimental Medicine 184, 783–788. https://doi.org/10.1084/jem.184.2.783

Karulf, M., Kelly, A., Weinberg, A.D., Gold, J.A., 2010. OX40 Ligand Regulates Inflammation and Mortality in the Innate Immune Response to Sepsis. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1000404

Kashala, O., Amador, R., Valero, M. V., Moreno, A., Barbosa, A., Nickel, B., Daubenberger, C.A., Guzman, F., Pluschke, G., Patarroyo, M.E., 2002. Safety, tolerability and immunogenicity of new formulations of the Plasmodium falciparum malaria peptide vaccine SPf66 combined with the immunological adjuvant QS-21. Vaccine 20, 2263–2277. https://doi.org/10.1016/S0264-410X(02)00115-9

Kashiwakura, J., Yokoi, H., Saito, H., Okayama, Y., 2004. T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. Journal of immunology (Baltimore, Md. : 1950) 173, 5247–57.

Kaslow, D.C., Biernaux, S., 2015. RTS,S: Toward a first landmark on the Malaria Vaccine Technology Roadmap. Vaccine, Malaria Vaccines 2015 33, 7425–7432. https://doi.org/10.1016/j.vaccine.2015.09.061

Katayama, S., Oda, K., Ohgitani, T., Hirahara, T., Shimizu, Y., 1999. Influence of antigenic forms and adjuvants on the IgG subclass antibody response to Aujeszky's disease virus in mice. Vaccine. https://doi.org/10.1016/S0264-410X(98)00499-X

Kato, H., Kojima, H., Ishii, N., Hase, H., Imai, Y., Fujibayashi, T., Sugamura, K., Kobata, T., 2004. Essential role of OX40L on B cells in persistent alloantibody production following repeated alloimmunizations. J. Clin. Immunol. 24, 237–248. https://doi.org/10.1023/B:JOCI.0000025445.21894.e5

Kawahata, K., Misaki, Y., Yamauchi, M., Tsunekawa, S., Setoguchi, K., Miyazaki, J. -i., Yamamoto, K., 2002. Generation of CD4+CD25+ Regulatory T Cells from Autoreactive T Cells Simultaneously with Their Negative Selection in the Thymus and from Nonautoreactive T Cells by Endogenous TCR Expression. The Journal of Immunology. https://doi.org/10.4049/jimmunol.168.9.4399

Kawai, K., Shahinian, A., Mak, T.W., Ohashi, P.S., 1996. Skin allograft rejection in CD28-deficient mice. Transplantation 61, 352–5.

Kearney, E.R., Pape, K.A., Loh, D.Y., Jenkins, M.K., 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity. https://doi.org/10.1016/1074-7613(94)90084-1

Keersmaecker, B.D., Heirman, C., Corthals, J., Empsen, C., Grunsven, L.A. van, Allard, S.D., Pen, J., Lacor, P., Thielemans, K., Aerts, J.L., 2011. The combination of 4-1BBL and CD40L strongly enhances the capacity of dendritic cells to stimulate HIV-specific T cell responses. Journal of Leukocyte Biology 89, 989–999. https://doi.org/10.1189/jlb.0810466

Kensil, C.R., Kammer, R., 2005. QS-21: a water-soluble triterpene glycoside adjuvant. Expert Opinion on Investigational Drugs. https://doi.org/10.1517/13543784.7.9.1475

Kensil, C.R., Patel, U., Lennick, M., Marciani, D., 1991. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria molina cortex. The journal of Immunology.

Keppler, S.J., Rosenits, K., Koegl, T., Vucikuja, S., Aichele, P., 2012. Signal 3 Cytokines as Modulators of Primary Immune Responses during Infections: The Interplay of Type I IFN and IL-12 in CD8 T Cell Responses. PLoS ONE 7, e40865. https://doi.org/10.1371/journal.pone.0040865

Kester, K.E., Cummings, J.F., Ofori-Anyinam, O., Ockenhouse, C.F., Krzych, U., Moris, P., Schwenk, R., Nielsen, R.A., Debebe, Z., Pinelis, E., Juompan, L., Williams, J., Dowler, M., Stewart, V.A., Wirtz, R.A., Dubois, M.-C., Lievens, M., Cohen, J., Ballou, W.R., Heppner, D.G., RTS,S Vaccine Evaluation Group, 2009. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J. Infect. Dis. 200, 337–346. https://doi.org/10.1086/600120

Ketloy, C., Engering, A., Srichairatanakul, U., Limsalakpetch, A., Yongvanitchit, K., Pichyangkul, S., Ruxrungtham, K., 2008. Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. Veterinary Immunology and Immunopathology 125, 18–30. https://doi.org/10.1016/j.vetimm.2008.05.001

Khoruts, A., Mondino, A., Pape, K.A., Reiner, S.L., Jenkins, M.K., 1998. A Natural Immunological Adjuvant Enhances T Cell Clonal Expansion through a CD28-dependent, Interleukin (IL)-2–independent Mechanism. J Exp Med 187, 225–236.

Khoshnan, a, Tindell, C., Laux, I., Bae, D., Bennett, B., Nel, a E., 2000. The NF-kappa B cascade is important in Bcl-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. Journal of immunology (Baltimore, Md. : 1950). https://doi.org/10.4049/jimmunol.165.4.1743

Kim, C., Wilson, T., Fischer, K.F., Williams, M.A., 2013. Sustained interactions between T cell receptors and antigens promote the differentiation of CD4+memory T cells. Immunity. https://doi.org/10.1016/j.immuni.2013.08.033

Kim, H.P., Kelly, J., Leonard, W.J., 2001. The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. Immunity 15, 159–172.

Kim, M., Bekiaris, V., McConnell, F.M., Gaspal, F.M.C., Raykundalia, C., Lane, P.J.L., 2005. OX40 Signals during Priming on Dendritic Cells Inhibit CD4 T Cell Proliferation: IL-4 Switches off OX40 Signals Enabling Rapid Proliferation of Th2 Effectors. The Journal of Immunology 174, 1433–1437. https://doi.org/10.4049/jimmunol.174.3.1433

Kim, M.-Y., Anderson, G., White, A., Jenkinson, E., Arlt, W., Martensson, I.-L., Erlandsson, L., Lane, P.J.L., 2005. OX40 ligand and CD30 ligand are expressed on adult but not neonatal CD4+CD3- inducer cells: evidence that IL-7 signals regulate CD30 ligand but not OX40 ligand expression. Journal of immunology (Baltimore, Md. : 1950) 174, 6686–91.

Kim, M.-Y., Gaspal, F.M.C., Wiggett, H.E., McConnell, F.M., Gulbranson-Judge, A., Raykundalia, C., Walker, L.S.K., Goodall, M.D., Lane, P.J.L., 2003. CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. Immunity 18, 643–54.

Kim, M.-Y., Toellner, K.-M., White, A., McConnell, F.M., Gaspal, F.M.C., Parnell, S.M., Jenkinson, E., Anderson, G., Lane, P.J.L., 2006. Neonatal and Adult CD4+CD3− Cells Share Similar Gene Expression Profile, and Neonatal Cells Up-Regulate OX40 Ligand in Response to TL1A (TNFSF15). The Journal of Immunology 177, 3074–3081. https://doi.org/10.4049/jimmunol.177.5.3074

Kim, Y.H., Seo, S.K., Choi, B.K., Kang, W.J., Kim, C.H., Lee, S.K., Kwon, B.S., 2005. 4-1BB costimulation enhances HSV-1-specific CD8+ T cell responses by the induction of CD11c+CD8+ T cells. Cell. Immunol. 238, 76–86. https://doi.org/10.1016/j.cellimm.2006.01.004

King, C., Tangye, S.G., Mackay, C.R., 2008. T Follicular Helper (TFH) Cells in Normal and Dysregulated Immune Responses. Annual Review of Immunology 26, 741–766. https://doi.org/10.1146/annurev.immunol.26.021607.090344

Kisielow, P., Hirst, J.A., Shiku, H., Beverley, P.C.L., Hoffmann, M.K., Boyse, E.A., Oettgen, H.F., 1975. Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes of the mouse. Nature. https://doi.org/10.1038/253219a0

Kitamura, N., Murata, S., Ueki, T., Mekata, E., Reilly, R.T., Jaffee, E.M., Tani, T., 2009. OX40 costimulation can abrogate Foxp3+regulatory T cell-mediated suppression of antitumor immunity. International Journal of Cancer. https://doi.org/10.1002/ijc.24435

Kolstad, A.M., Rodriguiz, R.M., Kim, C.J., Hale, L.P., 2012. Effect of Pain Management on Immunization Efficacy in Mice. J Am Assoc Lab Anim Sci 51, 448–457.

Kool, M., Soullié, T., van Nimwegen, M., Willart, M.A.M., Muskens, F., Jung, S., Hoogsteden, H.C., Hammad, H., Lambrecht, B.N., 2008. Alum adjuvant boosts adaptive immunity

by inducing uric acid and activating inflammatory dendritic cells. J. Exp. Med. 205, 869–882. https://doi.org/10.1084/jem.20071087
Korn, T., Mitsdoerffer, M., Croxford, A.L., Awasthi, A., Dardalhon, V.A., Galileos, G., Vollmar, P., Stritesky, G.L., Kaplan, M.H., Waisman, A., Kuchroo, V.K., Oukka, M., 2008. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. PNAS 105, 18460–18465. https://doi.org/10.1073/pnas.0809850105
Korthuis, R.J., 2011. Anatomy of Skeletal Muscle and Its Vascular Supply. Morgan & Claypool Life Sciences.
Krammer, P.H., Arnold, R., Lavrik, I.N., 2007. Life and death in peripheral T cells. Nature Reviews in Immunology. https://doi.org/10.1038/nri2115
Krause, C.D., He, W., Kotenko, S., Pestka, S., 2006. Modulation of the activation of Stat1 by the interferon-γ receptor complex. Cell Research 16, 113. https://doi.org/10.1038/sj.cr.7310015
Kündig, T.M., Shahinian, A., Kawai, K., Mittrücker, H.W., Sebzda, E., Bachmann, M.F., Mak, T.W., Ohashi, P.S., 1996. Duration of TCR stimulation determines costimulatory requirement of T cells. Immunity. https://doi.org/10.1016/S1074-7613(00)80308-8
Kwok, W.W., Tan, V., Gillette, L., Littell, C.T., Soltis, M.A., LaFond, R.B., Yang, J., James, E.A., DeLong, J.H., 2012. Frequency of Epitope-Specific Naive CD4+ T Cells Correlates with Immunodominance in the Human Memory Repertoire. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1102190
Lafage-Pochitaloff, M., Costello, R., Couez, D., Simonetti, J., Mannoni, P., Mawas, C., Olive, D., 1990. Human CD28 and CTLA-4 Ig superfamily genes are located on chromosome 2 at bands q33-q34. Immunogenetics 31, 198–201.
Lafferty, K.J., Andrus, L., Prowse, S.J., 1980. Role of Lymphokine and Antigen in the Control of Specific T Cell Responses. Immunological Reviews. https://doi.org/10.1111/j.1600-065X.1980.tb00325.x
Lal, H., Cunningham, A.L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S.-J., Levin, M.J., McElhaney, J.E., Poder, A., Puig-Barberà, J., Vesikari, T., Watanabe, D., Weckx, L., Zahaf, T., Heineman, T.C., 2015. Efficacy of an Adjuvanted Herpes Zoster Subunit Vaccine in Older Adults. New England Journal of Medicine 372, 2087–2096. https://doi.org/10.1056/NEJMoa1501184
Lametschwandtner, G., Biedermann, T., Schwärzler, C., Günther, C., Kund, J., Fassl, S., Hinteregger, S., Carballido-Perrig, N., Szabo, S.J., Glimcher, L.H., Carballido, J.M., 2004. Sustained T-bet expression confers polarized human TH2 cells with TH1-like cytokine production and migratory capacities. Journal of Allergy and Clinical Immunology. https://doi.org/10.1016/j.jaci.2004.02.004
Landsverk, O.J.B., Bakke, O., Gregers, T.F., 2009. MHC II and the endocytic pathway: Regulation by invariant chain. Scandinavian Journal of Immunology. https://doi.org/10.1111/j.1365-3083.2009.02301.x
Langrish, C.L., 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. Journal of Experimental Medicine 201, 233–240. https://doi.org/10.1084/jem.20041257
Lanzavecchia, A., Sallusto, F., 2005. Understanding the generation and function of memory T cell subsets. Current opinion in immunology 17, 326–32. https://doi.org/10.1016/j.coi.2005.04.010
Lara-Tejero, M., Pamer, E.G., 2004. T cell responses to Listeria monocytogenes. Curr. Opin. Microbiol. 7, 45–50. https://doi.org/10.1016/j.mib.2003.12.002
Lathrop, S.K., Huddleston, C.A., Dullforce, P.A., Montfort, M.J., Weinberg, A.D., Parker, D.C., 2004. A Signal through OX40 (CD134) Allows Anergic, Autoreactive T Cells to Acquire Effector Cell Functions. The Journal of Immunology. https://doi.org/10.4049/jimmunol.172.11.6735

Lau, L.L., Jamieson, B.D., Somasundaram, T., Ahmed, R., 1994. Cytotoxic T-cell memory without antigen. Nature. https://doi.org/10.1038/369648a0
Leach, D.R., Krummel, M.F., Allison, J.P., 1996. Enhancement of antitumor immunity by CTLA-4 blockade. Science. https://doi.org/10.1126/science.271.5256.1734
Lee, B.O., Haynes, L., Eaton, S.M., Swain, S.L., Randall, T.D., 2002. The Biological Outcome of CD40 Signaling Is Dependent on the Duration of CD40 Ligand Expression: Reciprocal Regulation by Interleukin (IL)-4 and IL-12. Journal of Experimental Medicine. https://doi.org/10.1084/jem.20020845
Lee, J.-Y., Hamilton, S.E., Akue, A.D., Hogquist, K.A., Jameson, S.C., 2013. Virtual memory CD8 T cells display unique functional properties. Proc Natl Acad Sci U S A 110, 13498–13503. https://doi.org/10.1073/pnas.1307572110
Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., Magnuson, M.A., Boothby, M., 2010. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. Immunity. https://doi.org/10.1016/j.immuni.2010.06.002
Lee, S.H., Carrero, J.A., Uppaluri, R., White, J.M., Archambault, J.M., Lai, K.S., Chan, S.R., Sheehan, K.C.F., Unanue, E.R., Schreiber, R.D., 2013. Identifying the Initiating Events of Anti-Listeria Responses Using Mice with Conditional Loss of IFN-γ Receptor Subunit 1 (IFNGR1). The Journal of Immunology 191, 4223–4234. https://doi.org/10.4049/jimmunol.1300910
Lee, S.K., Silva, D.G., Martin, J.L., Pratama, A., Hu, X., Chang, P.-P., Walters, G., Vinuesa, C.G., 2012. Interferon-γ Excess Leads to Pathogenic Accumulation of Follicular Helper T Cells and Germinal Centers. Immunity 37, 880–892. https://doi.org/10.1016/j.immuni.2012.10.010
LeibundGut-Landmann, S., Weidner, K., Hilbi, H., Oxenius, A., 2011. Nonhematopoietic Cells Are Key Players in Innate Control of Bacterial Airway Infection. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1003565
Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. Cell. https://doi.org/10.1007/978-94-007-7696-8_9
Leroux-Roels, G., Marchant, A., Levy, J., Van Damme, P., Schwarz, T.F., Horsmans, Y., Jilg, W., Kremsner, P.G., Haelterman, E., Clément, F., Gabor, J.J., Esen, M., Hens, A., Carletti, I., Fissette, L., Tavares Da Silva, F., Burny, W., Janssens, M., Moris, P., Didierlaurent, A.M., Van Der Most, R., Garçon, N., Van Belle, P., Van Mechelen, M., 2016. Impact of adjuvants on CD4+ T cell and B cell responses to a protein antigen vaccine: Results from a phase II, randomized, multicenter trial. Clinical Immunology 169, 16–27. https://doi.org/10.1016/j.clim.2016.05.007
Leroux-Roels, I., Forgus, S., De Boever, F., Clement, F., Demoitié, M.A., Mettens, P., Moris, P., Ledent, E., Leroux-Roels, G., Ofori-Anyinam, O., Jongert, E., Bourguignon, P., Janssens, M., Vastiau, I., Vinals, C., Cohen, J., Ballou, W.R., 2013. Improved CD4+ T cell responses to Mycobacterium tuberculosis in PPD-negative adults by M72/AS01 as compared to the M72/AS02 and Mtb72F/AS02 tuberculosis candidate vaccine formulations: A randomized trial. Vaccine. https://doi.org/10.1016/j.vaccine.2012.05.035
Li, J., Lamine Mbow, M., Sun, L., Li, L., Yang, G., Griswold, D.E., Schantz, A., Shealy, D.J., Goletz, T.J., Wan, J., Peritt, D., 2004. Induction of dendritic cell maturation by IL-18. Cellular Immunology 227, 103–108. https://doi.org/10.1016/j.cellimm.2004.02.002
Li, J., Yin, Q., Wu, H., 2013. Structural Basis of Signal Transduction in the TNF Receptor Superfamily. Adv Immunol 119, 135–153. https://doi.org/10.1016/B978-0-12-407707-2.00005-9
Li, N., van Unen, V., Höllt, T., Thompson, A., van Bergen, J., Pezzotti, N., Eisemann, E., Vilanova, A., Chuva de Sousa Lopes, S.M., Lelieveldt, B.P.F., Koning, F., 2018. Mass

cytometry reveals innate lymphoid cell differentiation pathways in the human fetal intestine. J Exp Med 215, 1383–1396. https://doi.org/10.1084/jem.20171934
Lighvani, A.A., Frucht, D.M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B.D., Nguyen, B.V., Gadina, M., Sher, A., Paul, W.E., O'Shea, J.J., 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc. Natl. Acad. Sci. U.S.A. 98, 15137–15142. https://doi.org/10.1073/pnas.261570598
Lim, W., Gee, K., Mishra, S., Kumar, A., 2005. Regulation of B7.1 Costimulatory Molecule Is Mediated by the IFN Regulatory Factor-7 through the Activation of JNK in Lipopolysaccharide-Stimulated Human Monocytic Cells. The Journal of Immunology 175, 5690–5700. https://doi.org/10.4049/jimmunol.175.9.5690
Lin, Y., Roberts, T.J., Sriram, V., Cho, S., Brutkiewicz, R.R., 2003. Myeloid marker expression on antiviral CD8+ T cells following an acute virus infection. Eur. J. Immunol. 33, 2736–2743. https://doi.org/10.1002/eji.200324087
Linsley, P.S., Clark, E.A., Ledbetter, J.A., 1990. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proceedings of the National Academy of Sciences of the United States of America 87, 5031–5. https://doi.org/10.1073/pnas.87.13.5031
Linterman, M.A., Rigby, R.J., Wong, R.K., Yu, D., Brink, R., Cannons, J.L., Schwartzberg, P.L., Cook, M.C., Walters, G.D., Vinuesa, C.G., 2009. Follicular helper T cells are required for systemic autoimmunity. J. Exp. Med. 206, 561–576. https://doi.org/10.1084/jem.20081886
Linton, P.-J., Bautista, B., Biederman, E., Bradley, E.S., Harbertson, J., Kondrack, R.M., Padrick, R.C., Bradley, L.M., 2003. Costimulation via OX40L Expressed by B Cells Is Sufficient to Determine the Extent of Primary CD4 Cell Expansion and Th2 Cytokine Secretion In Vivo. J Exp Med 197, 875–883. https://doi.org/10.1084/jem.20021290
Liu, S.K., Fang, N., Koretzky, G.A., McGlade, C.J., 1999. The hematopoietic-specific adaptor protein Gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. Current Biology. https://doi.org/10.1016/S0960-9822(99)80017-7
Lizundia, R., Sauter, K.-S., Taylor, G., Werling, D., 2008. Host species-specific usage of the TLR4-LPS receptor complex. Innate Immun 14, 223–231. https://doi.org/10.1177/1753425908095957
Locksley, R.M., Killeen, N., Lenardo, M.J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104, 487–501. https://doi.org/10.1016/s0092-8674(01)00237-9
Loschko, J., Schreiber, H.A., Rieke, G.J., Esterházy, D., Meredith, M.M., Pedicord, V.A., Yao, K.-H., Caballero, S., Pamer, E.G., Mucida, D., Nussenzweig, M.C., 2016. Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. Journal of Experimental Medicine 213, 517–534. https://doi.org/10.1084/jem.20160062
Lugo-Villarino, G., Glimcher, L.H., Possemato, R., Maldonado-Lopez, R., Penaranda, C., 2003. T-bet is required for optimal production of IFN- and antigen-specific T cell activation by dendritic cells. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.1332767100
Ma, D.Y., Clark, E. a, 2010. The role of CD40 and CD40L in Dendritic Cells. Semin Immunol. 2009 21, 265–272. https://doi.org/10.1016/j.smim.2009.05.010.The
Mackaness, G.B., 1962. CELLULAR RESISTANCE TO INFECTION. J Exp Med 116, 381–406.
Mackley, E.C., Houston, S., Marriott, C.L., Halford, E.E., Lucas, B., Cerovic, V., Filbey, K.J., Maizels, R.M., Hepworth, M.R., Sonnenberg, G.F., Milling, S., Withers, D.R., 2015. CCR7-dependent trafficking of RORγ+ ILCs creates a unique microenvironment within mucosal draining lymph nodes. Nature Communications. https://doi.org/10.1038/ncomms6862
MacLennan, I.C., Gulbranson-Judge, A., Toellner, K.M., Casamayor-Palleja, M., Chan, E., Sze, D.M., Luther, S.A., Orbea, H.A., 1997. The changing preference of T and B cells

for partners as T-dependent antibody responses develop. Immunological reviews 156, 53–66.
Magri, G., Miyajima, M., Bascones, S., Mortha, A., Puga, I., Cassis, L., Barra, C.M., Comerma, L., Chudnovskiy, A., Gentile, M., Llige, D., Cols, M., Serrano, S., Aróstegui, J.I., Juan, M., Yagüe, J., Merad, M., Fagarasan, S., Cerutti, A., 2014. Innate lymphoid cells integrate stromal and immune signals to enhance antibody production by splenic marginal zone B cells. Nat Immunol 15, 354–364. https://doi.org/10.1038/ni.2830
Malek, T.R., Yu, A., Vincek, V., Scibelli, P., Kong, L., 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. Immunity. https://doi.org/S1074761302003679 [pii]
Malherbe, L., Mark, L., Fazilleau, N., McHeyzer-Williams, L.J., McHeyzer-Williams, M.G., 2008. Vaccine adjuvants alter TCR-based selection thresholds. Immunity 28, 698–709. https://doi.org/10.1016/j.immuni.2008.03.014
Manel, N., Unutmaz, D., Littman, D.R., 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nature immunology 9, 641–649. https://doi.org/10.1038/ni.1610
Mann, J.D., Higgins, G.M., 1950. Lymphocytes in thoracic duct intestinal and hepatic lymph. Blood 5, 177–90.
Månsson, R., Hultquist, A., Luc, S., Yang, L., Anderson, K., Kharazi, S., Al-Hashmi, S., Liuba, K., Thorén, L., Adolfsson, J., Buza-Vidas, N., Qian, H., Soneji, S., Enver, T., Sigvardsson, M., Jacobsen, S.E.W., 2007. Molecular Evidence for Hierarchical Transcriptional Lineage Priming in Fetal and Adult Stem Cells and Multipotent Progenitors. Immunity. https://doi.org/10.1016/j.immuni.2007.02.013
Marinelarena, A., Bhattacharya, P., Kumar, P., Maker, A.V., Prabhakar, B.S., 2018. Identification of a Novel OX40L + Dendritic Cell Subset That Selectively Expands Regulatory T cells. Scientific Reports 8, 14940. https://doi.org/10.1038/s41598-018-33307-z
Marriott, C.L., Mackley, E.C., Ferreira, C., Veldhoen, M., Yagita, H., Withers, D.R., 2014. OX40 controls effector CD4+ T-cell expansion, not follicular T helper cell generation in acute Listeria infection. European journal of immunology 44, 2437–47. https://doi.org/10.1002/eji.201344211
Martins, K.A.O., Cooper, C.L., Stronsky, S.M., Norris, S.L.W., Kwilas, S.A., Steffens, J.T., Benko, J.G., van Tongeren, S.A., Bavari, S., 2016. Adjuvant-enhanced CD4 T Cell Responses are Critical to Durable Vaccine Immunity. EBioMedicine 3, 67–78. https://doi.org/10.1016/j.ebiom.2015.11.041
Marty-Roix, R., Vladimer, G.I., Pouliot, K., Weng, D., Buglione-Corbett, R., West, K., MacMicking, J.D., Chee, J.D., Wang, S., Lu, S., Lien, E., 2016. Identification of QS-21 as an Inflammasome-activating Molecular Component of Saponin Adjuvants. J. Biol. Chem. 291, 1123–1136. https://doi.org/10.1074/jbc.M115.683011
Masopust, D., Schenkel, J.M., 2013. The integration of T cell migration, differentiation and function. Nature Reviews Immunology 13, 309–320. https://doi.org/10.1038/nri3442
Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., Cyster, J.G., 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature. https://doi.org/10.1038/nature02284
Matthews, N., 1979. Tumour-necrosis factor from the rabbit. III. Relationship to interferons. British Journal of Cancer. https://doi.org/10.1038/bjc.1979.218
Maxwell, J.R., Yadav, R., Rossi, R.J., Ruby, C.E., Weinberg, A.D., Aguila, H.L., Vella, A.T., 2006. IL-18 bridges innate and adaptive immunity through IFN-gamma and the CD134 pathway. Journal of immunology (Baltimore, Md.: 1950) 177, 234–45. https://doi.org/10.4049/jimmunol.177.1.234
Mazzucchelli, R.I., Warming, S., Lawrence, S.M., Ishii, M., Abshari, M., Washington, A.V., Feigenbaum, L., Warner, A.C., Sims, D.J., Li, W.Q., Hixon, J.A., Gray, D.H.D., Rich,

B.E., Morrow, M., Anver, M.R., Cherry, J., Naf, D., Sternberg, L.R., McVicar, D.W., Farr, A.G., Germain, R.N., Rogers, K., Jenkins, N.A., Copeland, N.G., Durum, S.K., 2009. Visualization and identification of IL-7 producing cells in reporter mice. PLoS ONE. https://doi.org/10.1371/journal.pone.0007637

McAdam, A.J., Chang, T.T., Lumelsky, A.E., Greenfield, E.A., Boussiotis, V.A., Duke-Cohan, J.S., Chernova, T., Malenkovich, N., Jabs, C., Kuchroo, V.K., Ling, V., Collins, M., Sharpe, A.H., Freeman, G.J., 2000. Mouse Inducible Costimulatory Molecule (ICOS) Expression Is Enhanced by CD28 Costimulation and Regulates Differentiation of CD4+ T Cells. The Journal of Immunology. https://doi.org/10.4049/jimmunol.165.9.5035

McBlane, J.F., van Gent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M., Oettinger, M.A., 1995. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell. https://doi.org/10.1016/0092-8674(95)90116-7

McKeage, K., Romanowski, B., 2011. AS04-adjuvanted human papillomavirus (HPV) types 16 and 18 vaccine (Cervarix®): A review of its use in the prevention of premalignant cervical lesions and cervical cancer causally related to certain oncogenic HPV types. Drugs. https://doi.org/10.2165/11206820-000000000-00000

McKee, A.S., Munks, M.W., MacLeod, M.K.L., Fleenor, C.J., Van Rooijen, N., Kappler, J.W., Marrack, P., 2009. Alum Induces Innate Immune Responses through Macrophage and Mast Cell Sensors, But These Sensors Are Not Required for Alum to Act As an Adjuvant for Specific Immunity. The Journal of Immunology. https://doi.org/10.4049/jimmunol.0900164

Medzhitov, R., Janeway, C., 2000. Innate immunity. The New England journal of medicine 343, 338–44. https://doi.org/10.1056/NEJM200008033430506

Melo-Gonzalez, F., Hepworth, M.R., 2017. Functional and phenotypic heterogeneity of group 3 innate lymphoid cells. Immunology 150, 265–275. https://doi.org/10.1111/imm.12697

Merad, M., Helft, J., Sathe, P., Miller, J., Mortha, A., 2013. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. Annual Review of Immunology. https://doi.org/10.1146/annurev-immunol-020711-074950

Metzger, D.W., McNutt, R.M., Collins, J.T., Buchanan, J.M., Van Cleave, V.H., Dunnick, W.A., 1997. Interleukin-12 acts as an adjuvant for humoral immunity through interferon-gamma-dependent and -independent mechanisms. Eur. J. Immunol. 27, 1958–1965. https://doi.org/10.1002/eji.1830270820

Micallef, M.J., Tanimoto, T., Kohno, K., Ikeda, M., Kurimoto, M., 1997. Interleukin 18 induces the sequential activation of natural killer cells and cytotoxic T lymphocytes to protect syngeneic mice from transplantation with Meth A sarcoma. Cancer Research.

Miles, J.J., Douek, D.C., Price, D.A., 2011. Bias in the αB T-cell repertoire: Implications for disease pathogenesis and vaccination. Immunology and Cell Biology. https://doi.org/10.1038/icb.2010.139

Miller, L.H., Saul, A., Mahanty, S., 2005. Revisiting Freund's incomplete adjuvant for vaccines in the developing world. Trends in Parasitology 21, 412–414. https://doi.org/10.1016/j.pt.2005.07.005

Miller, M.A., Skeen, M.J., Ziegler, H.K., 1995. Nonviable bacterial antigens administered with IL-12 generate antigen-specific T cell responses and protective immunity against Listeria monocytogenes. J Immunol.

Mitchell, L.M., Brzoza-Lewis, K.L., Henry, C.J., Grayson, J.M., Westcott, M.M., Hiltbold, E.M., 2011. Distinct responses of splenic dendritic cell subsets to infection with Listeria monocytogenes: maturation phenotype, level of infection, and T cell priming capacity ex vivo. Cell. Immunol. 268, 79–86. https://doi.org/10.1016/j.cellimm.2011.03.001

Miura, S., Ohtani, K., Numata, N., Niki, M., Ohbo, K., Ina, Y., Gojobori, T., Tanaka, Y., Tozawa, H., Nakamura, M., 1991. Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator p40tax. Molecular and cellular biology. https://doi.org/10.1128/MCB.11.3.1313

Molina, T.J., Kishihara, K., Siderovskid, D.P., Van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.U., Veillette, A., Davidson, D., Mak, T.W., 1992. Profound block in thymocyte development in mice lacking p56lck. Nature. https://doi.org/10.1038/357161a0

Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., Jenkins, M.K., 2007. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity 27, 203–13. https://doi.org/10.1016/j.immuni.2007.07.007

Morris, D.H., Bullock, F.D., 1919. The importance of the spleen in resistance to infection. Annals of surgery 70, 513–21.

Mosca, F., Tritto, E., Muzzi, A., Monaci, E., Bagnoli, F., Iavarone, C., O'Hagan, D., Rappuoli, R., De Gregorio, E., 2008. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci U S A 105, 10501–10506. https://doi.org/10.1073/pnas.0804699105

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. The Journal of Immunology 136, 2348–2357.

Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. Annual Review of Immunology 7, 145–173. https://doi.org/10.1146/annurev.iy.07.040189.001045

Mosmann, T.R., Li, L., Sad, S., 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. Seminars in Immunology. https://doi.org/10.1006/smim.1997.0065

Mosyak, L., Zaller, D.M., Wiley, D.C., 1998. The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. Immunity. https://doi.org/10.1016/S1074-7613(00)80620-2

Mueller, D.L., Jenkins, M.K., Schwartz, R.H., 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. J. Immunol. 142, 2617–2628.

Munks, M.W., Mourich, D. V., Mittler, R.S., Weinberg, A.D., Hill, A.B., 2004. 4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine. Immunology. https://doi.org/10.1111/j.1365-2567.2004.01917.x

Murata, K., Ishii, N., Takano, H., Miura, S., Ndhlovu, L.C., Nose, M., Noda, T., Sugamura, K., 2000a. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. The Journal of experimental medicine 191, 365–74.

Murata, K., Ishii, N., Takano, H., Miura, S., Ndhlovu, L.C., Nose, M., Noda, T., Sugamura, K., 2000b. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. The Journal of experimental medicine. https://doi.org/10.1084/jem.191.2.365

Murata, K., Nose, M., Ndhlovu, L.C., Sato, T., Sugamura, K., Ishii, N., 2002. Constitutive OX40/OX40 Ligand Interaction Induces Autoimmune-Like Diseases. The Journal of Immunology. https://doi.org/10.4049/jimmunol.169.8.4628

Murphy, C.A., Langrish, C.L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R.A., Sedgwick, J.D., Cua, D.J., 2003. Divergent Pro- and Antiinflammatory Roles for IL-23 and IL-12 in Joint Autoimmune Inflammation. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20030896

Murphy, K.A., Lechner, M.G., Popescu, F.E., Bedi, J., Decker, S.A., Hu, P., Erickson, J.R., O'Sullivan, M.G., Swier, L., Salazar, A.M., Olin, M.R., Epstein, A.L., Ohlfest, J.R., 2012. An in vivo immunotherapy screen of costimulatory molecules identifies Fc-

OX40L as a potent reagent for the treatment of established murine gliomas. Clin. Cancer Res. 18, 4657–4668. https://doi.org/10.1158/1078-0432.CCR-12-0990
Murray, R.A., Siddiqui, M.R., Mendillo, M., Krahenbuhl, J., Kaplan, G., 2007. Mycobacterium leprae inhibits dendritic cell activation and maturation. J. Immunol. 178, 338–344.
Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, N., Luo, L., 2007. A global double-fluorescent cre reporter mouse. Genesis. https://doi.org/10.1002/dvg.20335
Nagata, S., 1997. Apoptosis by death factor. Cell 88, 355–365. https://doi.org/10.1016/S0092-8674(00)81874-7
Nakajima, Y., Asano, K., Mukai, K., Urai, T., Okuwa, M., Sugama, J., Nakatani, T., 2018. Near-Infrared Fluorescence Imaging Directly Visualizes Lymphatic Drainage Pathways and Connections between Superficial and Deep Lymphatic Systems in the Mouse Hindlimb. Scientific Reports 8, 7078. https://doi.org/10.1038/s41598-018-25383-y
Nakamura, T., Lee, R.K., Nam, S.Y., Al-Ramadi, B.K., Koni, P.A., Bottomly, K., Podack, E.R., Flavell, R.A., 1997. Reciprocal regulation of CD30 expression on CD4+ T cells by IL-4 and IFN-gamma. Journal of immunology (Baltimore, Md. : 1950).
Nakamura, Y., Ghaffar, O., Olivenstein, R., Taha, R.A., Soussi-Gounni, A., Zhang, D.-H., Ray, A., Hamid, Q., 1999. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. Journal of Allergy and Clinical Immunology 103, 215–222. https://doi.org/10.1016/S0091-6749(99)70493-8
Nakaya, H.I., Clutterbuck, E., Kazmin, D., Wang, L., Cortese, M., Bosinger, S.E., Patel, N.B., Zak, D.E., Aderem, A., Dong, T., Del Giudice, G., Rappuoli, R., Cerundolo, V., Pollard, A.J., Pulendran, B., Siegrist, C.-A., 2016. Systems biology of immunity to MF59-adjuvanted versus nonadjuvanted trivalent seasonal influenza vaccines in early childhood. Proc Natl Acad Sci USA 113, 1853–1858. https://doi.org/10.1073/pnas.1519690113
Nakayamada, S., Poholek, A.C., Lu, K.T., Takahashi, H., Kato, M., Iwata, S., Hirahara, K., Cannons, J.L., Schwartzberg, P.L., Vahedi, G., Sun, H.-W., Kanno, Y., O'Shea, J.J., 2014. Type I IFN induces binding of STAT1 to Bcl6: divergent roles of STAT family transcription factors in the T follicular helper cell genetic program. J. Immunol. 192, 2156–2166. https://doi.org/10.4049/jimmunol.1300675
Nath, N., Prasad, R., Giri, S., Singh, A.K., Singh, I., 2006. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. Immunology. https://doi.org/10.1111/j.1365-2567.2006.02385.x
Neeland, M.R., Shi, W., Collignon, C., Taubenheim, N., Meeusen, E.N.T., Didierlaurent, A.M., Veer, M.J. de, 2016. The Lymphatic Immune Response Induced by the Adjuvant AS01: A Comparison of Intramuscular and Subcutaneous Immunization Routes. The Journal of Immunology 197, 2704–2714. https://doi.org/10.4049/jimmunol.1600817
Neuenhahn, M., Kerksiek, K.M., Nauerth, M., Suhre, M.H., Schiemann, M., Gebhardt, F.E., Stemberger, C., Panthel, K., Schröder, S., Chakraborty, T., Jung, S., Hochrein, H., Rüssmann, H., Brocker, T., Busch, D.H., 2006. CD8α+ Dendritic Cells Are Required for Efficient Entry of Listeria monocytogenes into the Spleen. Immunity 25, 619–630. https://doi.org/10.1016/j.immuni.2006.07.017
Newman, M.J., Wu, J.Y., Gardner, B.H., Anderson, C.A., Kensil, C.R., Recchia, J., Coughlin, R.T., Powell, M.F., 1997. Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations. Vaccine 15, 1001–1007. https://doi.org/10.1016/S0264-410X(96)00293-9
Nicol, T., Quantock, D.C., Vernon-Roberts, B., 1966. Stimulation of Phagocytosis in Relation to the Mechanism of Action of Adjuvants. Nature 209, 1142. https://doi.org/10.1038/2091142b0

Nilsson, O.R., Kari, L., Steele-Mortimer, O., 2019. Highly reproducible murine model of oral Salmonella infection via inoculated food. bioRxiv 593442. https://doi.org/10.1101/593442

Nishimura, H., Nose, M., Hiai, H., Minato, N., Honjo, T., 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity. https://doi.org/10.1016/S1074-7613(00)80089-8

Nishimura, H., Yajima, T., Muta, H., Podack, E.R., Tani, K., Yoshikai, Y., 2005. A Novel Role of CD30/CD30 Ligand Signaling in the Generation of Long-Lived Memory CD8+ T Cells. The Journal of Immunology. https://doi.org/10.4049/jimmunol.175.7.4627

Nitta, T., Murata, S., Ueno, T., Tanaka, K., Takahama, Y., 2008. Chapter 3 Thymic Microenvironments for T-Cell Repertoire Formation. Advances in Immunology 99, 59–94. https://doi.org/10.1016/S0065-2776(08)00603-2

Nohara, C., Akiba, H., Nakajima, A., Inoue, A., Koh, C.-S., Ohshima, H., Yagita, H., Mizuno, Y., Okumura, K., 2001. Amelioration of Experimental Autoimmune Encephalomyelitis with Anti-OX40 Ligand Monoclonal Antibody: A Critical Role for OX40 Ligand in Migration, But Not Development, of Pathogenic T Cells. The Journal of Immunology 166, 2108–2115. https://doi.org/10.4049/jimmunol.166.3.2108

Nomi, T., Sho, M., Akahori, T., Hamada, K., Kubo, A., Kanehiro, H., Nakamura, S., Enomoto, K., Yagita, H., Azuma, M., Nakajima, Y., 2007. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. https://doi.org/10.1158/1078-0432.CCR-06-2746

Obukhanych, T.V., Nussenzweig, M.C., 2006. T-independent type II immune responses generate memory B cells. J Exp Med 203, 305–310. https://doi.org/10.1084/jem.20052036

O'Connor, R.A., Cambrook, H., Huettner, K., Anderton, S.M., 2013. T-bet is essential for Th1-mediated, but not Th17-mediated, CNS autoimmune disease. European Journal of Immunology. https://doi.org/10.1002/eji.201343689

Offeddu, V., Thathy, V., Marsh, K., Matuschewski, K., 2012. Naturally acquired immune responses against Plasmodium falciparum sporozoites and liver infection. International Journal for Parasitology 42, 535–548. https://doi.org/10.1016/j.ijpara.2012.03.011

O'Hagan, D.T., 2007. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. Expert Review of Vaccines 6, 699–710. https://doi.org/10.1586/14760584.6.5.699

Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., Delespesse, G., 1997. Expression and function of OX40 ligand on human dendritic cells. J. Immunol. 159, 3838–3848.

Ohshima, Y., Yang, L.P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P., Delespesse, G., 1998. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood.

Okabayashi, T., Hanazaki, K., 2008. Overwhelming postsplenectomy infection syndrome in adults - a clinically preventable disease. World journal of gastroenterology 14, 176–9. https://doi.org/10.3748/WJG.14.176

Okamura, H., Kashiwamura, S.I., Tsutsui, H., Yoshimoto, T., Nakanishi, K., 1998. Regulation of interferon-γ production by IL-12 and IL-18. Current Opinion in Immunology. https://doi.org/10.1016/S0952-7915(98)80163-5

Okamura, H., Nagata, K., Komatsu, T., Tanimoto, T., Nukata, Y., Tanabe, F., Akita, K., Torigoe, K., Okura, T., Fukuda, S., 1995. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. Infection and Immunity 63, 3966–3972.

Oliver, A.M., Martin, F., Kearney, J.F., 1997. Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. Journal of Immunology.

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., Kaslow, D.C., Njuguna, P., Marsh, K., Bejon, P., 2016. Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. New England Journal of Medicine 374. https://doi.org/10.1056/NEJMoa1515257

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y.-J., Abrams, J.S., Moore, K.W., Rennick, D., Waal-Malefyt, R. de, Hannum, C., Bazan, J.F., Kastelein, R.A., 2000. Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12. Immunity 13, 715–725. https://doi.org/10.1016/S1074-7613(00)00070-4

Orban, P.C., Chui, D., Marth, J.D., 2006. Tissue- and site-specific DNA recombination in transgenic mice. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.89.15.6861

Pagan, A.J., Pepper, M., Chu, H.H., Green, J.M., Jenkins, M.K., 2012. CD28 Promotes CD4+ T Cell Clonal Expansion during Infection Independently of Its YMNM and PYAP Motifs. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1103231

Pai, S.-Y., Truitt, M.L., Ho, I.-C., 2004. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.0308697100

Paine, a, Kirchner, H., Immenschuh, S., Oelke, M., Blasczyk, R., Eiz-Vesper, B., 2012. IL-2 upregulates CD86 expression on human CD4(+) and CD8(+) T cells. Journal of immunology 188, 1620–1629. https://doi.org/10.4049/jimmunol.1100181

Pamer, E., Cresswell, P., 1998. Mechanisms of MHC Class I – Restricted antigen processing. Annual Review of Immunology. https://doi.org/10.1146/annurev.immunol.16.1.323

Pan, J., Zhang, M., Wang, J., Wang, Q., Xia, D., Sun, W., Zhang, L., Yu, H., Liu, Y., Cao, X., 2004. Interferon-γ is an autocrine mediator for dendritic cell maturation. Immunology Letters 94, 141–151. https://doi.org/10.1016/j.imlet.2004.05.003

Panagioti, E., Boon, L., Arens, R., van der Burg, S.H., 2017. Enforced OX40 Stimulation Empowers Booster Vaccines to Induce Effective CD4+ and CD8+ T Cell Responses against Mouse Cytomegalovirus Infection. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.00144

Pape, K.A., Khoruts, A., Mondino, A., Jenkins, M.K., 1997. Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells. J Immunol 159, 591–598.

Pape, K.A., Taylor, J.J., Maul, R.W., Gearhart, P.J., Jenkins, M.K., 2011. Different B Cell Populations Mediate Early and Late Memory During an Endogenous Immune Response. Science 331, 1203–1207. https://doi.org/10.1126/science.1201730

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.-H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C., 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nature Immunology 6, 1133–1141. https://doi.org/10.1038/ni1261

Park, S.H., Bendelac, A., 2000. CD1-restricted T-cell responses and microbial infection. Nature. https://doi.org/10.1038/35021233

Park, S.-J., Nakagawa, T., Kitamura, H., Atsumi, T., Kamon, H., Sawa, S.-I., Kamimura, D., Ueda, N., Iwakura, Y., Ishihara, K., Murakami, M., Hirano, T., 2004. IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. J. Immunol. 173, 3844–3854. https://doi.org/10.4049/jimmunol.173.6.3844

Park, W.-R., Nakahira, M., Sugimoto, N., Bian, Y., Yashiro-Ohtani, Y., Zhou, X.-Y., Yang, Y.-F., Hamaoka, T., Fujiwara, H., 2004. A mechanism underlying STAT4-mediated up-regulation of IFN-gamma induction inTCR-triggered T cells. International immunology 16, 295–302. https://doi.org/10.1093/intimm/dxh034

Parry, C.M., Thomas, S., Aspinall, E.J., Cooke, R.P.D., Rogerson, S.J., Harries, A.D., Beeching, N.J., 2013. A retrospective study of secondary bacteraemia in hospitalised adults with community acquired non-typhoidal Salmonella gastroenteritis. BMC Infect. Dis. 13, 107. https://doi.org/10.1186/1471-2334-13-107

Parry, R. V, Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S. V, Linsley, P.S., Thompson, C.B., Riley, J.L., 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Molecular and cellular biology. https://doi.org/10.1128/MCB.25.21.9543-9553.2005

Pasquale, A. Di, Preiss, S., Silva, F.T. Da, Garçon, N., 2015. Vaccine Adjuvants: from 1920 to 2015 and Beyond. Vaccines 3, 320–43. https://doi.org/10.3390/vaccines3020320

Paterson, D.J., Jefferies, W.A., Green, J.R., Brandon, M.R., Corthesy, P., Puklavec, M., Williams, A.F., 1987. Antigens of activated rat T lymphocytes including a molecule of 50,000 Mrdetected only on CD4 positive T blasts. Molecular Immunology. https://doi.org/10.1016/0161-5890(87)90122-2

Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.-A., Shen, H., Cereb, N., Yang, S.Y., Lindsten, T., Rossant, J., Hunter, C.A., Reiner, S.L., 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science (New York, N.Y.). https://doi.org/10.1126/science.1090148

Pepper, M., Jenkins, M.K., 2011. Origins of CD4(+) effector and central memory T cells. Nature immunology 12, 467–71.

Pepper, M., Linehan, J.L., Pagán, A.J., Zell, T., Dileepan, T., Cleary, P.P., Jenkins, M.K., 2010. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. Nature immunology 11, 83–9. https://doi.org/10.1038/ni.1826

Pepper, M., Pagán, A.J., Igyártó, B.Z., Taylor, J.J., Jenkins, M.K., 2011. Opposing Signals from the Bcl6 Transcription Factor and the Interleukin-2 Receptor Generate T Helper 1 Central and Effector Memory Cells. Immunity. https://doi.org/10.1016/j.immuni.2011.09.009

Petrovsky, N., Aguilar, J.C., 2004. Vaccine adjuvants: Current state and future trends. Immunology and Cell Biology. https://doi.org/10.1111/j.0818-9641.2004.01272.x

Podack, E.R., Strbo, N., Sotosec, V., Muta, H., 2002. CD30-governor of memory T cells? Ann N Y Acad Sci.

Porcelli, S.A., Modlin, R.L., 2002. THE CD1 SYSTEM: Antigen-Presenting Molecules for T Cell Recognition of Lipids and Glycolipids. Annual Review of Immunology. https://doi.org/10.1146/annurev.immunol.17.1.297

Portnoy, D.A., Auerbuch, V., Glomski, I.J., 2002. The cell biology of Listeria monocytogenes infection: The intersection of bacterial pathogenesis and cell-mediated immunity. Journal of Cell Biology. https://doi.org/10.1083/jcb.200205009

Pouliot, K., Buglione-Corbett, R., Marty-Roix, R., Montminy-Paquette, S., West, K., Wang, S., Lu, S., Lien, E., 2014. Contribution of TLR4 and MyD88 for adjuvant monophosphoryl lipid A (MPLA) activity in a DNA prime–protein boost HIV-1 vaccine. Vaccine 32, 5049–5056. https://doi.org/10.1016/j.vaccine.2014.07.010

Price, A.E., Reinhardt, R.L., Liang, H.E., Locksley, R.M., 2012. Marking and quantifying IL-17A-producing cells in vivo. PLoS ONE. https://doi.org/10.1371/journal.pone.0039750

Purton, J.F., Tan, J.T., Rubinstein, M.P., Kim, D.M., Sprent, J., Surh, C.D., 2007. Antiviral CD4+ memory T cells are IL-15 dependent. The Journal of experimental medicine 204, 951–61. https://doi.org/10.1084/jem.20061805

Qin, H., Wang, L., Feng, T., Elson, C.O., Niyongere, S.A., Lee, S.J., Reynolds, S.L., Weaver, C.T., Roarty, K., Serra, R., Benveniste, E.N., Cong, Y., 2009. TGF- Promotes Th17 Cell Development through Inhibition of SOCS3. The Journal of Immunology. https://doi.org/10.4049/jimmunol.0801986

Qureshi, S.T., Larivière, L., Leveque, G., Clermont, S., Moore, K.J., Gros, P., Malo, D., 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J. Exp. Med. 189, 615–625. https://doi.org/10.1084/jem.189.4.615

Rahman, A.H., Taylor, D.K., Turka, L.A., 2009. The contribution of direct TLR signaling to T cell responses. Immunologic Research. https://doi.org/10.1007/s12026-009-8113-x

Ranson, T., Bregenholt, S., Lehuen, A., Gaillot, O., Leite-de-Moraes, M.C., Herbelin, A., Berche, P., Di Santo, J.P., 2005. Invariant V 14+ NKT Cells Participate in the Early Response to Enteric Listeria monocytogenes Infection. The Journal of Immunology 175, 1137–1144. https://doi.org/10.4049/jimmunol.175.2.1137

Redmond, W.L., Linch, S.N., Kasiewicz, M.J., 2014. Combined Targeting of Costimulatory (OX40) and Coinhibitory (CTLA-4) Pathways Elicits Potent Effector T Cells Capable of Driving Robust Antitumor Immunity. Cancer Immunol Res 2, 142–153. https://doi.org/10.1158/2326-6066.CIR-13-0031-T

Reed, S.G., Orr, M.T., Fox, C.B., 2013. Key roles of adjuvants in modern vaccines. Nature medicine 19, 1597–608. https://doi.org/10.1038/nm.3409

Rehli, M., 2002. Of mice and men: species variations of Toll-like receptor expression. Trends Immunol. 23, 375–378.

Reiner, S.L., Locksley, R.M., 2003. The Regulation of Immunity to Leishmania Major. Annual Review of Immunology. https://doi.org/10.1146/annurev.iy.13.040195.001055

Reinhardt, R.L., Liang, H.-E., Bao, K., Price, A.E., Mohrs, M., Kelly, B.L., Locksley, R.M., 2015. A Novel Model for IFN-γ–Mediated Autoinflammatory Syndromes. J Immunol 194, 2358–2368. https://doi.org/10.4049/jimmunol.1401992

Reinhardt, R.L., Liang, H.-E., Locksley, R.M., 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. Nature Immunology 10, 385–393. https://doi.org/10.1038/ni.1715

Ribi, E., Parker, R., Strain, S.M., Mizuno, Y., Nowotny, A., Von Eschen, K.B., Cantrell, J.L., McLaughlin, C.A., Hwang, K.M., Goren, M.B., 1979. Peptides as requirement for immunotherapy of the guinea-pig line-10 tumor with endotoxins. Cancer Immunology Immunotherapy. https://doi.org/10.1007/BF00205409

Riley, J.L., Blair, P.J., Musser, J.T., Abe, R., Tezuka, K., Tsuji, T., June, C.H., 2001. ICOS Costimulation Requires IL-2 and Can Be Prevented by CTLA-4 Engagement. The Journal of Immunology. https://doi.org/10.4049/jimmunol.166.8.4943

Riteau, N., Radtke, A.J., Shenderov, K., Mittereder, L., Oland, S.D., Hieny, S., Jankovic, D., Sher, A., 2016. –Water-in-oil only adjuvants selectively promote Tfh polarization through a type I interferon and IL-6 dependent pathway. J Immunol 197, 3884–3893. https://doi.org/10.4049/jimmunol.1600883

Roan, F., Stoklasek, T.A., Whalen, E., Molitor, J.A., Bluestone, J.A., Buckner, J.H., Ziegler, S.F., 2016. CD4+ Group 1 Innate Lymphoid Cells (ILC) Form a Functionally Distinct ILC Subset That Is Increased in Systemic Sclerosis. J. Immunol. 196, 2051–2062. https://doi.org/10.4049/jimmunol.1501491

Robey, E., Fowlkes, B.J., 1994. Selective events in T cell development. Annual review of immunology 12, 675–705. https://doi.org/10.1146/annurev.iy.12.040194.003331

Rogers, P.R., Croft, M., 2014. CD28, Ox-40, LFA-1, and CD4 Modulation of Th1/Th2 Differentiation Is Directly Dependent on the Dose of Antigen. The Journal of Immunology. https://doi.org/10.4049/jimmunol.164.6.2955

Rogers, P.R., Song, J., Gramaglia, I., Killeen, N., Croft, M., 2001. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 15, 445–455. https://doi.org/10.1016/S1074-7613(01)00191-1

Roman, F., Clément, F., Dewé, W., Walravens, K., Maes, C., Willekens, J., De Boever, F., Hanon, E., Leroux-Roels, G., 2011. Effect on Cellular and Humoral Immune Responses of the AS03 Adjuvant System in an A/H1N1/2009 Influenza Virus Vaccine

Administered to Adults during Two Randomized Controlled Trials ▿. Clin Vaccine Immunol 18, 835–843. https://doi.org/10.1128/CVI.00480-10

Romee, R., Rosario, M., Berrien-Elliott, M.M., Wagner, J.A., Jewell, B.A., Schappe, T., Leong, J.W., Abdel-Latif, S., Schneider, S.E., Willey, S., Neal, C.C., Yu, L., Oh, S.T., Lee, Y.-S., Mulder, A., Claas, F., Cooper, M.A., Fehniger, T.A., 2016. Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. Sci Transl Med 8, 357ra123. https://doi.org/10.1126/scitranslmed.aaf2341

Ronchese, F., Hausmann, B., 1993. B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.177.3.679

Rottman, J.B., Smith, T., Tonra, J.R., Ganley, K., Bloom, T., Silva, R., Pierce, B., Gutierrez-Ramos, J.C., Ozkaynak, E., Coyle, A.J., 2001. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. Nat. Immunol. 2, 605–611. https://doi.org/10.1038/89750

Rowe, J.H., Ertelt, J.M., Xin, L., Way, S.S., 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature 490, 102–106. https://doi.org/10.1038/nature11462

RTS,S Clinical Trials Partnership, 2015. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: Final results of a phase 3, individually randomised, controlled trial. https://doi.org/10.1016/S0140-6736(15)60721-8

Sad, S., Mosmann, T.R., 1994. Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. Journal of immunology (Baltimore, Md. : 1950) 153, 3514–3522.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. Journal of immunology (Baltimore, Md.: 1950). https://doi.org/10.4049/jimmunol.171.1.417

Sakai, S., Kawamura, I., Okazaki, T., Tsuchiya, K., Uchiyama, R., Mitsuyama, M., 2010. PD-1-PD-L1 pathway impairs T(h)1 immune response in the late stage of infection with Mycobacterium bovis bacillus Calmette-Guérin. Int. Immunol. 22, 915–925. https://doi.org/10.1093/intimm/dxq446

Salek-Ardakani, S., Croft, M., 2006. Regulation of CD4 T cell memory by OX40 (CD134). Vaccine. https://doi.org/10.1016/j.vaccine.2005.07.108

Salek-Ardakani, S., Song, J., Halteman, B.S., Jember, A.G.-H., Akiba, H., Yagita, H., Croft, M., 2003. OX40 (CD134) Controls Memory T Helper 2 Cells that Drive Lung Inflammation. The Journal of Experimental Medicine 198, 315–324. https://doi.org/10.1084/jem.20021937

Salkowski, C.A., Detore, G.R., Vogel, S.N., 1997. Lipopolysaccharide and monophosphoryl lipid A differentially regulate interleukin-12, gamma interferon, and interleukin-10 mRNA production in murine macrophages. Infect. Immun. 65, 3239–3247.

Sallusto, F., Lanzavecchia, A., 2002. The instructive role of dendritic cells on T-cell responses. Arthritis Res 4, S127–S132. https://doi.org/10.1186/ar567

Sallusto, F., Lenig, D., Förster, R., Lipp, M., Lanzavecchia, A., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401, 708–712. https://doi.org/10.1038/44385

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S., Lanzavecchia, A., 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. European Journal of Immunology. https://doi.org/10.1002/(SICI)1521-4141(199809)28:09<2760::AID-IMMU2760>3.0.CO;2-N

Salvatore, G., Cantisani, R., Tavarini, S., Lofano, G., Bagnoli, F., Finco, O., Mancini, F., Rappuoli, R., Nuti, S., Sammicheli, C., Soldaini, E., Monaci, E., De Gregorio, E., Rossi Paccani, S., Bertholet, S., Laera, D., Carrisi, C., 2015. Oil-in-Water Emulsion MF59 Increases Germinal Center B Cell Differentiation and Persistence in Response to Vaccination. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1402604

Samuel, C.E., 2001. Antiviral Actions of Interferons. Clin Microbiol Rev 14, 778–809. https://doi.org/10.1128/CMR.14.4.778-809.2001

Sanchez, A.M., Zhu, J., Huang, X., Yang, Y., 2012. The development and function of memory regulatory T cells after acute viral infections. Journal of immunology (Baltimore, Md. : 1950). https://doi.org/10.4049/jimmunol.1200645

Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Garry Adams, L., Bäumler, A.J., 2001. Animal models of Salmonella infections: Enteritis versus typhoid fever. Microbes and Infection. https://doi.org/10.1016/S1286-4579(01)01495-2

Sauer, B., Henderson, N., 1988. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.85.14.5166

Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I.C., Lennon-Duménil, A.M., Seabra, M.C., Raposo, G., Amigorena, S., 2006. NOX2 Controls Phagosomal pH to Regulate Antigen Processing during Crosspresentation by Dendritic Cells. Cell. https://doi.org/10.1016/j.cell.2006.05.035

Schaible, U.E., Collins, H.L., Kaufmann, S.H.E., 2008. Confrontation between Intracellular Bacteria and the Immune System. https://doi.org/10.1016/s0065-2776(08)60405-8

Scharton, T.M., Scott, P., 1993. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. J. Exp. Med. 178, 567–577. https://doi.org/10.1084/jem.178.2.567

Scharton-Kersten, T., Afonso, L.C., Wysocka, M., Trinchieri, G., Scott, P., 1995. IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis. The Journal of Immunology 154, 5320–5330.

Schenk, M., Fabri, M., Krutzik, S.R., Lee, D.J., Vu, D.M., Sieling, P.A., Montoya, D., Liu, P.T., Modlin, R.L., 2014. Interleukin-1β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells. Immunology 141, 174–180. https://doi.org/10.1111/imm.12167

Schluter, S.F., Bernstein, R.M., Bernstein, H., Marchalonis, J.J., 1999. “Big Bang” emergence of the combinatorial immune system: Editorial. Developmental and Comparative Immunology 107–11. https://doi.org/10.1016/S0145-305X(99)00002-6

Schmidt, E.E., MacDonald, I.C., Groom, A.C., 1993. Comparative aspects of splenic microcirculatory pathways in mammals: the region bordering the white pulp. Scanning Microsc.

Schreiber, H.A., Hulseberg, P.D., Lee, J., Prechl, J., Barta, P., Szlavik, N., Harding, J.S., Fabry, Z., Sandor, M., 2010. Dendritic Cells in Chronic Mycobacterial Granulomas Restrict Local Anti-Bacterial T Cell Response in a Murine Model. PLOS ONE 5, e11453. https://doi.org/10.1371/journal.pone.0011453

Scott, P., 1991. IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J. Immunol. 147, 3149–3155.

Seder, R.A., Gazzinelli, R., Sher, A., Paul, W.E., 1993. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci U S A 90, 10188–10192.

Seiler, P., Aichele, P., Odermatt, B., Hengartner, H., Zinkernagel, R.M., Schwendener, R.A., 1997. Crucial role of marginal zone macrophages and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection. European Journal of Immunology. https://doi.org/10.1002/eji.1830271023

Seubert, A., Monaci, E., Pizza, M., O'Hagan, D.T., Wack, A., 2008. The Adjuvants Aluminum Hydroxide and MF59 Induce Monocyte and Granulocyte Chemoattractants and Enhance Monocyte Differentiation toward Dendritic Cells. The Journal of Immunology 180, 5402–5412. https://doi.org/10.4049/jimmunol.180.8.5402

Shahinian, a, Pfeffer, K., Lee, K.P., Kündig, T.M., Kishihara, K., Wakeham, a, Kawai, K., Ohashi, P.S., Thompson, C.B., Mak, T.W., 1993. Differential T cell costimulatory requirements in CD28-deficient mice. Science (New York, N.Y.) 261, 609–612. https://doi.org/10.1126/science.7688139

Shale, M., Schiering, C., Powrie, F., 2013. CD4+ T-cell subsets in intestinal inflammation. Immunological Reviews. https://doi.org/10.1111/imr.12039

Shanley, D.P., Aw, D., Manley, N.R., Palmer, D.B., 2009. An evolutionary perspective on the mechanisms of immunosenescence. Trends in Immunology. https://doi.org/10.1016/j.it.2009.05.001

Shaw, a S., Chalupny, J., Whitney, J. a, Hammond, C., Amrein, K.E., Kavathas, P., Sefton, B.M., Rose, J.K., 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56lck tyrosine protein kinase. Molecular and cellular biology. https://doi.org/10.1128/MCB.10.5.1853.Updated

Shaw, F.E., Guess, H.A., Roets, J.M., Mohr, F.E., Coleman, P.J., Mandel, E.J., Roehm, R.R., Talley, W.S., Hadler, S.C., 1989. Effect of anatomic injection site, age and smoking on the immune response to hepatitis B vaccination. Vaccine 7, 425–430.

Sheng, K.-C., Day, S., Wright, M.D., Stojanovska, L., Apostolopoulos, V., 2013a. Enhanced Dendritic Cell-Mediated Antigen-Specific CD4+ T Cell Responses: IFN-Gamma Aids TLR Stimulation. Journal of Drug Delivery. https://doi.org/10.1155/2013/516749

Sheng, K.-C., Day, S., Wright, M.D., Stojanovska, L., Apostolopoulos, V., 2013b. Enhanced Dendritic Cell-Mediated Antigen-Specific CD4+ T Cell Responses: IFN-Gamma Aids TLR Stimulation. Journal of Drug Delivery. https://doi.org/10.1155/2013/516749

Shi, Y., Zhu, M., 2013. Medullary thymic epithelial cells, the indispensable player in central tolerance. Science China Life Sciences 56, 392–398. https://doi.org/10.1007/s11427-013-4482-4

Shibahara, I., Saito, R., Zhang, R., Chonan, M., Shoji, T., Kanamori, M., Sonoda, Y., Kumabe, T., Kanehira, M., Kikuchi, T., So, T., Watanabe, T., Takahashi, H., Iwabuchi, E., Tanaka, Y., Shibahara, Y., Sasano, H., Ishii, N., Tominaga, T., 2015. OX40 ligand expressed in glioblastoma modulates adaptive immunity depending on the microenvironment: a clue for successful immunotherapy. Molecular Cancer 14, 41. https://doi.org/10.1186/s12943-015-0307-3

Shiku, H., Kisielow, P., Bean, M. a, Takahashi, T., Boyse, E. a, Oettgen, H.F., Old, L.J., 1975. Expression of T-cell differentiation antigens on effector cells in cell-mediated cytotoxicity in vitro. Evidence for functional heterogeneity related to the surface phenotype of T cells. The Journal of experimental medicine 141, 227–41. https://doi.org/10.1084/jem.141.1.227

Shimozato, O., Takeda, K., Yagita, H., Okumura, K., 1999. Expression of CD30 ligand (CD153) on murine activated T cells. Biochemical and Biophysical Research Communications. https://doi.org/10.1006/bbrc.1999.0336

Shortman, K., Liu, Y.J., 2002. Mouse and human dendritic cell subtypes. Nature Reviews Immunology. https://doi.org/10.1038/nri746

Siddiqui, S., Mistry, V., Doe, C., Stinson, S., Foster, M., Brightling, C., 2010. Airway wall expression of OX40/OX40L and interleukin-4 in asthma. Chest. https://doi.org/10.1378/chest.09-1839

Smith, A.L., Wikstrom, M.E., Fazekas de St Groth, B., 2000. Visualizing T cell competition for peptide/MHC complexes: a specific mechanism to minimize the effect of precursor frequency. Immunity 13, 783–794.

Smith, J.B., McIntosh, G.H., Morris, B., 1970. The traffic of cells through tissues: a study of peripheral lymph in sheep. Journal of anatomy 107, 87–100.
So, T., Choi, H., Croft, M., 2011. OX40 Complexes with Phosphoinositide 3-Kinase and Protein Kinase B (PKB) To Augment TCR-Dependent PKB Signaling. The Journal of Immunology. https://doi.org/10.4049/jimmunol.1003156
Soilleux, E.J., Morris, L.S., Leslie, G., Chehimi, J., Luo, Q., Levroney, E., Trowsdale, J., Montaner, L.J., Doms, R.W., Weissman, D., Coleman, N., Lee, B., 2002. Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. Journal of leukocyte biology 71, 445–457.
Soliman, H., Mediavilla-Varela, M., Antonia, S., 2010. Indoleamine 2,3-dioxygenase: is it an immune suppressor? Cancer J 16, 354–359. https://doi.org/10.1097/PPO.0b013e3181eb3343
Song, A.J., Palmiter, R.D., 2018. Detecting and Avoiding Problems When Using the Cre–lox System. Trends in Genetics. https://doi.org/10.1016/j.tig.2017.12.008
Song, J., So, T., Cheng, M., Tang, X., Croft, M., 2005. Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. Immunity 22, 621–631. https://doi.org/10.1016/j.immuni.2005.03.012
Soroosh, P., Ine, S., Sugamura, K., Ishii, N., 2014. OX40-OX40 Ligand Interaction through T Cell-T Cell Contact Contributes to CD4 T Cell Longevity. The Journal of Immunology. https://doi.org/10.4049/jimmunol.176.10.5975
Soroosh, P., Ine, S., Sugamura, K., Ishii, N., 2007. Differential requirements for OX40 signals on generation of effector and central memory CD4+ T cells. J. Immunol. 179, 5014–5023. https://doi.org/10.4049/jimmunol.179.8.5014
Sozzani, S., Sallusto, F., Luini, W., Zhou, D., Piemonti, L., Allavena, P., Van Damme, J., Valitutti, S., Lanzavecchia, A., Mantovani, A., 1995. Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. Journal of Immunology. https://doi.org/10.4049/jimmunol.166.6.4092
Spensieri, F., Siena, E., Borgogni, E., Zedda, L., Cantisani, R., Chiappini, N., Schiavetti, F., Rosa, D., Castellino, F., Montomoli, E., Bodinham, C.L., Lewis, D.J., Medini, D., Bertholet, S., Giudice, G.D., 2016. Early Rise of Blood T Follicular Helper Cell Subsets and Baseline Immunity as Predictors of Persisting Late Functional Antibody Responses to Vaccination in Humans. PLOS ONE 11, e0157066. https://doi.org/10.1371/journal.pone.0157066
Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N.J., Mebius, R.E., Powrie, F., Vivier, E., 2013. Innate lymphoid cells — a proposal for uniform nomenclature. Nature Reviews Immunology 13, 145–149. https://doi.org/10.1038/nri3365
Spits, H., Cupedo, T., 2012. Innate Lymphoid Cells: Emerging Insights in Development, Lineage Relationships, and Function. Annual Review of Immunology. https://doi.org/10.1146/annurev-immunol-020711-075053
Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. Cell. https://doi.org/10.1016/0092-8674(94)90337-9
Srinivasan, M., Wardrop, R.M., Gienapp, I.E., Stuckman, S.S., Whitacre, C.C., Kaumaya, P.T., 2001. A retro-inverso peptide mimic of CD28 encompassing the MYPPPY motif adopts a polyproline type II helix and inhibits encephalitogenic T cells in vitro. Journal of immunology (Baltimore, Md. : 1950) 167, 578–585. https://doi.org/10.4049/jimmunol.167.1.578
Stamper, H.B., Woodruff, J.J., 1976. Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high-endothelial venules. The Journal of experimental medicine. https://doi.org/10.1084/JEM.144.3.828

Stark, G.R., Kerr, I.M., Williams, B.R.G., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264. https://doi.org/10.1146/annurev.biochem.67.1.227

Stein, H., Gerdes, J., Schwab, U., Lemke, H., Mason, D.Y., Ziegler, A., Schienle, W., Diehl, V., 1982. Identification of Hodgkin and sternberg-reed cells as a unique cell type derived from a newly-detected small-cell population. International Journal of Cancer. https://doi.org/10.1002/ijc.2910300411

Steinman, R.M., Cohn, Z.A., 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. The Journal of experimental medicine. https://doi.org/10.1084/jem.137.5.1142

Steinman, R.M., Witmer, M.D., 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc. Natl. Acad. Sci. U.S.A. 75, 5132–5136. https://doi.org/10.1073/pnas.75.10.5132

Stephenson, I., Bugarini, R., Nicholson, K.G., Podda, A., Wood, J.M., Zambon, M.C., Katz, J.M., 2005. Cross-Reactivity to Highly Pathogenic Avian Influenza H5N1 Viruses after Vaccination with Nonadjuvanted and MF59-Adjuvanted Influenza A/Duck/Singapore/97 (H5N3) Vaccine: A Potential Priming Strategy. J Infect Dis 191, 1210–1215. https://doi.org/10.1086/428948

Sternberg, N., Hamilton, D., 1981. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. Journal of Molecular Biology. https://doi.org/10.1016/0022-2836(81)90375-2

Stetson, D.B., Mohrs, M., Mallet-Designe, V., Teyton, L., Locksley, R.M., 2002. Rapid Expansion and IL-4 Expression by Leishmania-Specific Naive Helper T Cells In Vivo. Immunity 17, 191–200. https://doi.org/10.1016/S1074-7613(02)00363-1

Stewart, V.A., McGrath, S.M., Dubois, P.M., Pau, M.G., Mettens, P., Shott, J., Cobb, M., Burge, J.R., Larson, D., Ware, L.A., Demoitie, M.-A., Weverling, G.J., Bayat, B., Custers, J.H.H.V., Dubois, M.-C., Cohen, J., Goudsmit, J., Heppner, D.G., 2007. Priming with an adenovirus 35-circumsporozoite protein (CS) vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to Plasmodium falciparum CS compared to that with either malaria vaccine alone. Infect. Immun. 75, 2283–2290. https://doi.org/10.1128/IAI.01879-06

Stüber, E., Neurath, M., Calderhead, D., Perry Fell, H., Strober, W., 1995. Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. Immunity. https://doi.org/10.1016/1074-7613(95)90031-4

Stüber, E., Strober, W., 1996. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. J. Exp. Med. 183, 979–989. https://doi.org/10.1084/jem.183.3.979

Stüber, E., Von Freier, A., Marinescu, D., Fölsch, U.R., 1998. Involvement of OX40-OX40L interactions in the intestinal manifestations of the murine acute graft-versus-host disease. Gastroenterology 115, 1205–1215.

Su, B., Wang, J., Wang, X., Jin, H., Zhao, G., Ding, Z., Kang, Y., Wang, B., 2008. The effects of IL-6 and TNF-alpha as molecular adjuvants on immune responses to FMDV and maturation of dendritic cells by DNA vaccination. Vaccine 26, 5111–5122. https://doi.org/10.1016/j.vaccine.2008.03.089

Subauste, C.S., de Waal Malefyt, R., Fuh, F., 1998. Role of CD80 (B7.1) and CD86 (B7.2) in the immune response to an intracellular pathogen. Journal of immunology (Baltimore, Md. : 1950) 160, 1831–40.

Sun, J.C., Beilke, J.N., Lanier, L.L., 2009. Adaptive immune features of natural killer cells. Nature. https://doi.org/10.1038/nature07665

Swain, S.L., Hu, H., Huston, G., 1999. Class II-Independent Generation of CD4 Memory T Cells from Effectors. Science 286, 1381–1383. https://doi.org/10.1126/science.286.5443.1381

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. https://doi.org/10.1016/S0092-8674(00)80702-3

Takayama, K., Ribi, E., Cantrell, J.L., 1981. Isolation of a nontoxic lipid a fraction containing tumor regression activity. Cancer Research.

Takeda, I., Ine, S., Killeen, N., Ndhlovu, L.C., Murata, K., Satomi, S., Sugamura, K., Ishii, N., 2004. Distinct Roles for the OX40-OX40 Ligand Interaction in Regulatory and Nonregulatory T Cells. The Journal of Immunology 172, 3580–3589. https://doi.org/10.4049/jimmunol.172.6.3580

Takeda, K., Harada, Y., Watanabe, R., Inutake, Y., Ogawa, S., Onuki, K., Kagaya, S., Tanabe, K., Kishimoto, H., Abe, R., 2008. CD28 stimulation triggers NF-kappaB activation through the CARMA1-PKCtheta-Grb2/Gads axis. International immunology. https://doi.org/10.1093/intimm/dxn108

Takemoto, N., Intlekofer, A.M., Northrup, J.T., Wherry, E.J., Reiner, S.L., 2006. Cutting Edge: IL-12 Inversely Regulates T-bet and Eomesodermin Expression during Pathogen-Induced CD8+ T Cell Differentiation. The Journal of Immunology. https://doi.org/10.4049/jimmunol.177.11.7515

Tang, C., Yamada, H., Shibata, K., Muta, H., Wajjwalku, W., Podack, E.R., Yoshikai, Y., 2008. A Novel Role of CD30L/CD30 Signaling by T-T Cell Interaction in Th1 Response against Mycobacterial Infection. The Journal of Immunology. https://doi.org/10.4049/jimmunol.181.9.6316

Tas, J.M.J., Mesin, L., Pasqual, G., Targ, S., Jacobsen, J.T., Mano, Y.M., Chen, C.S., Weill, J.-C., Reynaud, C.-A., Browne, E.P., Meyer-Hermann, M., Victora, G.D., 2016. Visualizing Antibody Affinity Maturation in Germinal Centers. Science 351, 1048–1054. https://doi.org/10.1126/science.aad3439

Taylor, J.J., Pape, K.A., Jenkins, M.K., 2012. A germinal center–independent pathway generates unswitched memory B cells early in the primary response. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20111696

Thierfelder, W.E., Van Deursen, J.M., Yamamoto, K., Tripp, R.A., Sarawar, S.R., Carson, R.T., Sangster, M.Y., Vignali, D.A.A., Doherty, P.C., Grosveld, G.C., Ihle, J.N., 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature. https://doi.org/10.1038/382171a0

Thompson, B.S., Chilton, P.M., Ward, J.R., Evans, J.T., Mitchell, T.C., 2005. The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. Journal of leukocyte biology 78, 1273–80. https://doi.org/10.1189/jlb.0305172

Thompson, R.H., Dong, H., Kwon, E.D., 2007. Implications of B7-H1 expression in clear cell carcinoma of the kidney for prognostication and therapy, in: Clinical Cancer Research. https://doi.org/10.1158/1078-0432.CCR-06-1868

Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., Sharpe, A.H., 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3, 541–7.

Toennies, H.M., Green, J.M., Arch, R.H., 2004. Expression of CD30 and Ox40 on T lymphocyte subsets is controlled by distinct regulatory mechanisms. Journal of leukocyte biology. https://doi.org/10.1189/jlb.0803401

Tong, N.K.C., Beran, J., Kee, S.A., Miguel, J.L., Sánchez, C., Bayas, J.M., Vilella, A., De Juanes, J.R., Arrazola, P., Calbo-Torrecillas, F., De Novales, E.L., Hamtiaux, V., Lievens, M., Stoffel, M., 2005. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients, in: Kidney International. https://doi.org/10.1111/j.1523-1755.2005.00689.x

Trinchieri, G., 1997. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-γ). Current Opinion in Immunology. https://doi.org/10.1016/S0952-7915(97)80154-9

Tripp, C.S., Gately, M.K., Hakimi, J., Ling, P., Unanue, E.R., 1994. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-γ. The Journal of Immunology.

Tschopp, J., Irmler, M., Thome, M., 1998. Inhibition of Fas death signals by FLIPs. Current Opinion in Immunology 10, 552–558. https://doi.org/10.1016/S0952-7915(98)80223-9

Tubo, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., Jenkins, M.K., 2013. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell. https://doi.org/10.1016/j.cell.2013.04.007

Umeda, K., Sun, X., Guo, Y., Yamada, H., Shibata, K., Yoshikai, Y., 2011. Innate memory phenotype CD4+T cells play a role in early protection against infection by Listeria monocytogenes in a CD30L-dependent manner. Microbiology and Immunology. https://doi.org/10.1111/j.1348-0421.2011.00362.x

Usui, T., Preiss, J.C., Kanno, Y., Yao, Z.J., Bream, J.H., O'Shea, J.J., Strober, W., 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on *IFNG* gene acetylation and transcription. The Journal of Experimental Medicine. https://doi.org/10.1084/jem.20052165

Valzasina, B., Guiducci, C., Dislich, H., Killeen, N., Weinberg, A.D., Colombo, M.P., 2005. Triggering of OX40 (CD134) on CD4+CD25+ T cells blocks their inhibitory activity: A novel regulatory role for OX40 and its comparison with GITR. Blood 105, 2845–2851. https://doi.org/10.1182/blood-2004-07-2959

Van Braeckel, E., Bourguignon, P., Koutsoukos, M., Clement, F., Janssens, M., Carletti, I., Collard, A., Demoitié, M.A., Voss, G., Leroux-Roels, G., McNally, L., 2011. An adjuvanted polyprotein HIV-1 vaccine induces polyfunctional cross-reactive CD4+ T cell responses in seronegative volunteers. Clinical Infectious Diseases. https://doi.org/10.1093/cid/ciq160

Van Der Waaij, D., Berghuis-de Vries, J.M., Lekkerkerk-Van Der Wees, J.E.C., 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. Journal of Hygiene. https://doi.org/10.1017/S0022172400021653

van Furth, R., van Zwet, T.L., Buisman, A.M., van Dissel, J.T., 1994. Anti-tumor necrosis factor antibodies inhibit the influx of granulocytes and monocytes into an inflammatory exudate and enhance the growth of Listeria monocytogenes in various organs. J. Infect. Dis. 170, 234–237. https://doi.org/10.1093/infdis/170.1.234

Van Kaer, L., Parekh, V. V., Wu, L., 2011. Invariant natural killer T cells: Bridging innate and adaptive immunity. Cell and Tissue Research. https://doi.org/10.1007/s00441-010-1023-3

Vandepapelière, P., Horsmans, Y., Moris, P., Van Mechelen, M., Janssens, M., Koutsoukos, M., Van Belle, P., Clement, F., Hanon, E., Wettendorff, M., Garçon, N., Leroux-Roels, G., 2008. Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. Vaccine 26, 1375–1386. https://doi.org/10.1016/j.vaccine.2007.12.038

Vantourout, P., Hayday, A., 2013. Six-of-the-best: unique contributions of γδ T cells to immunology. Nature Reviews Immunology 13, 88–100. https://doi.org/10.1038/nri3384

Vaure, C., Liu, Y., 2014. A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. Front Immunol 5. https://doi.org/10.3389/fimmu.2014.00316

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., Stockinger, B., 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24, 179–89. https://doi.org/10.1016/j.immuni.2006.01.001

Venkatachari, N.J., Majumder, B., Ayyavoo, V., 2007. Human immunodeficiency virus (HIV) type 1 Vpr induces differential regulation of T cell costimulatory molecules: Direct effect of Vpr on T cell activation and immune function. Virology 358, 347–356. https://doi.org/10.1016/j.virol.2006.08.030

Venturi, V., Price, D.A., Douek, D.C., Davenport, M.P., 2008. The molecular basis for public T-cell responses? Nature Reviews Immunology. https://doi.org/10.1038/nri2260

Vinay, D.S., Kim, C.H., Choi, B.K., Kwon, B.S., 2009. Origins and functional basis of regulatory CD11c+CD8+ T cells. Eur. J. Immunol. 39, 1552–1563. https://doi.org/10.1002/eji.200839057

Vogel, K.U., Edelmann, S.L., Jeltsch, K.M., Bertossi, A., Heger, K., Heinz, G.A., Zöller, J., Warth, S.C., Hoefig, K.P., Lohs, C., Neff, F., Kremmer, E., Schick, J., Repsilber, D., Geerlof, A., Blum, H., Wurst, W., Heikenwälder, M., Schmidt-Supprian, M., Heissmeyer, V., 2013. Roquin paralogs 1 and 2 redundantly repress the icos and ox40 costimulator mrnas and control follicular helper t cell differentiation. Immunity. https://doi.org/10.1016/j.immuni.2012.12.004

von Burg, N., Chappaz, S., Baerenwaldt, A., Horvath, E., Bose Dasgupta, S., Ashok, D., Pieters, J., Tacchini-Cottier, F., Rolink, A., Acha-Orbea, H., Finke, D., 2014. Activated group 3 innate lymphoid cells promote T-cell-mediated immune responses. Proceedings of the National Academy of Sciences 111, 12835–12840. https://doi.org/10.1073/pnas.1406908111

Vu, M.D., Xiao, X., Gao, W., Degauque, N., Chen, M., Kroemer, A., Killeen, N., Ishii, N., Li, X.C., 2007. OX40 costimulation turns off Foxp3 + Tregs. Blood 110, 2501–2510. https://doi.org/10.1182/blood-2007-01-070748

Vugia, D.J., Samuel, M., Farley, M.M., Marcus, R., Shiferaw, B., Shallow, S., Smith, K., Angulo, F.J., Emerging Infections Program FoodNet Working Group, 2004. Invasive Salmonella infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. Clin. Infect. Dis. 38 Suppl 3, S149-156. https://doi.org/10.1086/381581

Vuopio-Varkila, J., Nurminen, M., Pyhala, L., Makela, P.H., 1988. Lipopolysaccaride-induced non-specific resistance to systemic Escherichia coli infection in mice. Journal of Medical Microbiology. https://doi.org/10.1099/00222615-25-3-197

Waite, J.C., Leiner, I., Lauer, P., Rae, C.S., Barbet, G., Zheng, H., Portnoy, D.A., Pamer, E.G., Dustin, M.L., 2011. Dynamic Imaging of the Effector Immune Response to Listeria Infection In Vivo. PLOS Pathogens 7, e1001326. https://doi.org/10.1371/journal.ppat.1001326

Walker, L.S., Gulbranson-Judge, A., Flynn, S., Brocker, T., Raykundalia, C., Goodall, M., Förster, R., Lipp, M., Lane, P., 1999. Compromised OX40 function in CD28-deficient mice is linked with failure to develop CXC chemokine receptor 5-positive CD4 cells and germinal centers. J. Exp. Med. 190, 1115–1122. https://doi.org/10.1084/jem.190.8.1115

Wardenburg, J.B., Fu, C., Jackman, J.K., Flotow, H., Wilkinson, S.E., Williams, D.H., Johnson, R., Kong, G., Chan, A.C., Findel, P.R., 1996. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. Journal of Biological Chemistry. https://doi.org/10.1074/jbc.271.33.19641

Watts, C., 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nature Immunology. https://doi.org/10.1038/ni1088

Weaver, T.A., Charafeddine, A.H., Kirk, A.D., 2008. Costimulation blockade: towards clinical application. Frontiers in bioscience : a journal and virtual library. https://doi.org/2829 [pii]

Webb, G., J., 2017. The roles and control of CD4+ effector and regulatory T cells in biliary autoimmunity.

Weinberg, A.D., Rivera, M.-M., Prell, R., Morris, A., Ramstad, T., Vetto, J.T., Urba, W.J., Alvord, G., Bunce, C., Shields, J., 2000. Engagement of the OX-40 Receptor In Vivo Enhances Antitumor Immunity. The Journal of Immunology. https://doi.org/10.4049/jimmunol.164.4.2160

Weinberg, A.D., Wegmann3, K.W., Funatake, C., Whitham, R.H., 1999. Blocking OX-40/OX-40 Ligand Interaction In Vitro and In Vivo Leads to Decreased T Cell Function and Amelioration of Experimental Allergic Encephalomyelitis. The Journal of Immunology 162, 1818–1826.

Weninger, W., Crowley, M.A., Manjunath, N., von Andrian, U.H., 2001. Migratory properties of naive, effector, and memory CD8(+) T cells. The Journal of experimental medicine. https://doi.org/10.1084/JEM.194.7.953

Wherry, E.J., Teichgräber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., Ahmed, R., 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. 4, 225–234. https://doi.org/10.1038/ni889

Whitmire, J.K., Benning, N., Whitton, J.L., 2006. Precursor Frequency, Nonlinear Proliferation, and Functional Maturation of Virus-Specific CD4+ T Cells. The Journal of Immunology. https://doi.org/10.4049/jimmunol.176.5.3028

WHO, 2017. WHO | World Malaria Report 2016. WHO.

WHO, ., 2019. MODULE 2 – Route of administration - WHO Vaccine Safety Basics [WWW Document]. URL https://vaccine-safety-training.org/route-of-administration.html (accessed 6.5.19).

Wikenheiser, D.J., Brown, S.L., Lee, J., Stumhofer, J.S., 2018. NK1.1 Expression Defines a Population of CD4+ Effector T Cells Displaying Th1 and Tfh Cell Properties That Support Early Antibody Production During Plasmodium yoelii Infection. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.02277

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F.D., Byrne, G., McEuen, M., Proll, S., Appleby, M., Brunkow, M.E., 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nature genetics 27, 18–20. https://doi.org/10.1038/83707

Wilkes, D.S., Zheng, Y., Heidler, K.M., Zhang, Z.-D., Webb, T.J., Swanson, K.A., 2014. Flt3-Ligand, IL-4, GM-CSF, and Adherence-Mediated Isolation of Murine Lung Dendritic Cells: Assessment of Isolation Technique on Phenotype and Function. The Journal of Immunology. https://doi.org/10.4049/jimmunol.173.8.4875

Williams, A.P., Peh, C.A., Purcell, A.W., McCluskey, J., Elliott, T., 2002. Optimization of the MHC class I peptide cargo is dependent on tapasin. Immunity. https://doi.org/10.1016/S1074-7613(02)00304-7

Williams, C.A., Murray, S.E., Weinberg, A.D., Parker, D.C., 2007. OX40-Mediated Differentiation to Effector Function Requires IL-2 Receptor Signaling but Not CD28, CD40, IL-12Rβ2, or T-bet. The Journal of Immunology 178, 7694–7702. https://doi.org/10.4049/jimmunol.178.12.7694

Withers, D.R., Gaspal, F.M., Bekiaris, V., McConnell, F.M., Kim, M., Anderson, G., Lane, P.J.L., 2011. OX40 and CD30 signals in CD4(+) T-cell effector and memory function: a distinct role for lymphoid tissue inducer cells in maintaining CD4(+) T-cell memory but not effector function. Immunological reviews 244, 134–48. https://doi.org/10.1111/j.1600-065X.2011.01057.x

Withers, D.R., Gaspal, F.M., Mackley, E.C., Marriott, C.L., Ross, E. a, Desanti, G.E., Roberts, N. a, White, A.J., Flores-Langarica, A., McConnell, F.M., Anderson, G., Lane, P.J.L., 2012. Cutting edge: lymphoid tissue inducer cells maintain memory CD4 T cells within secondary lymphoid tissue. Journal of immunology (Baltimore, Md. : 1950) 189, 2094–8. https://doi.org/10.4049/jimmunol.1201639

Withers, D.R., Jaensson, E., Gaspal, F., McConnell, F.M., Eksteen, B., Anderson, G., Agace, W.W., Lane, P.J.L., 2009. The Survival of Memory CD4+ T Cells within the Gut

Lamina Propria Requires OX40 and CD30 Signals. The Journal of Immunology. https://doi.org/10.4049/jimmunol.0901514
Wu, L.X., La Rose, J., Chen, L., Neale, C., Mak, T., Okkenhaug, K., Wange, R., Rottapel, R., 2005. CD28 Regulates the Translation of Bcl-xL via the Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Pathway. The Journal of Immunology. https://doi.org/10.4049/jimmunol.174.1.180
Wu, T.T., 1970. AN ANALYSIS OF THE SEQUENCES OF THE VARIABLE REGIONS OF BENCE JONES PROTEINS AND MYELOMA LIGHT CHAINS AND THEIR IMPLICATIONS FOR ANTIBODY COMPLEMENTARITY. Journal of Experimental Medicine. https://doi.org/10.1084/jem.132.2.211
Xiao, X., Balasubramanian, S., Liu, W., Chu, X., Wang, H., Taparowsky, E.J., Fu, Y.X., Choi, Y., Walsh, M.C., Li, X.C., 2012. OX40 signaling favors the induction of TH9 cells and airway inflammation. Nature Immunology. https://doi.org/10.1038/ni.2390
Xiao, X., Shi, X., Fan, Y., Wu, C., Zhang, X., Minze, L., Liu, W., Ghobrial, R.M., Lan, P., Li, X.C., 2016. The Costimulatory Receptor OX40 Inhibits Interleukin-17 Expression through Activation of Repressive Chromatin Remodeling Pathways. Immunity. https://doi.org/10.1016/j.immuni.2016.05.013
Xin, L., Jiang, T.T., Chaturvedi, V., Kinder, J.M., Ertelt, J.M., Rowe, J.H., Steinbrecher, K.A., Way, S.S., 2014. Commensal microbes drive intestinal inflammation by IL-17-producing CD4+ T cells through ICOSL and OX40L costimulation in the absence of B7-1 and B7-2. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.1402336111
Xu, J.L., Davis, M.M., 2000. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. Immunity 13, 37–45. https://doi.org/10.1016/S1074-7613(00)00006-6
Yadava, A., Kumar, S., Dvorak, J.A., Milon, G., Miller, L.H., 1996. Trafficking of Plasmodium chabaudi adami-infected erythrocytes within the mouse spleen. Proceedings of the National Academy of Sciences of the United States of America. https://doi.org/10.1073/pnas.93.10.4595
Yakimchuk, K., Roura-Mir, C., Magalhaes, K.G., de Jong, A., Kasmar, A.G., Granter, S.R., Budd, R., Steere, A., Pena-Cruz, V., Kirschning, C., Cheng, T.-Y., Moody, D.B., 2011. Borrelia burgdorferi infection regulates CD1 expression in human cells and tissues via IL1-β. Eur. J. Immunol. 41, 694–705. https://doi.org/10.1002/eji.201040808
Yanagawa, Y., Masubuchi, Y., Chiba, K., 1998. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats, III. Increase in frequency of CD62L-positive T cells in Peyer's patches by FTY720- induced lymphocyte homing. Immunology. https://doi.org/10.1046/j.1365-2567.1998.00639.x
Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S.P., Aleksander, I., Mak, T.W., 1984. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature. https://doi.org/10.1038/308145a0
Yanagihara, S., Komura, E., Nagafune, J., Watarai, H., Yamaguchi, Y., 1998. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. Journal of immunology (Baltimore, Md.: 1950). https://doi.org/10.1002/sd.197
Yang, J.A., Tubo, N.J., Gearhart, M.D., Bardwell, V.J., Jenkins, M.K., 2015. Cutting edge: Bcl6-interacting corepressor contributes to germinal center T follicular helper cell formation and B cell helper function. J. Immunol. 194, 5604–5608. https://doi.org/10.4049/jimmunol.1500201
Yang, S.-S., Fu, L.-S., Chang, C.-S., Yeh, H.-Z., Chen, G.-H., Kao, J.-H., 2006. Changes of soluble CD26 and CD30 levels correlate with response to interferon plus ribavirin therapy in patients with chronic hepatitis C. Journal of gastroenterology and hepatology. https://doi.org/10.1111/j.1440-1746.2006.04677.x

Yang, X.O., Panopoulos, A.D., Nurieva, R., Seon, H.C., Wang, D., Watowich, S.S., Dong, C., 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. Journal of Biological Chemistry. https://doi.org/10.1074/jbc.C600321200

Yang, X.O., Pappu, B.P., Nurieva, R., Akimzhanov, A., Kang, H.S., Chung, Y., Ma, L., Shah, B., Panopoulos, A.D., Schluns, K.S., Watowich, S.S., Tian, Q., Jetten, A.M., Dong, C., 2008. T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. Immunity. https://doi.org/10.1016/j.immuni.2007.11.016

Yen, H.-R., Harris, T.J., Wada, S., Grosso, J.F., Getnet, D., Goldberg, M. V., Liang, K.-L., Bruno, T.C., Pyle, K.J., Chan, S.-L., Anders, R.A., Trimble, C.L., Adler, A.J., Lin, T.-Y., Pardoll, D.M., Huang, C.-T., Drake, C.G., 2009. Tc17 CD8 T Cells: Functional plasticity and subset diversity. The Journal of Immunology. https://doi.org/10.4049/jimmunol.0900368

Yip, H.C., Karulin, A.Y., Tary-Lehmann, M., Hesse, M.D., Radeke, H., Heeger, P.S., Trezza, R.P., Heinzel, F.P., Forsthuber, T., Lehmann, P.V., 1999. Adjuvant-Guided Type-1 and Type-2 Immunity: Infectious/Noninfectious Dichotomy Defines the Class of Response. The Journal of Immunology 162, 3942–3949.

Yoshinaga, S K, Whoriskey, J.S., Khare, S.D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M. a, Kohno, T., Tafuri-Bladt, a, Brankow, D., Campbell, P., Chang, D., Chiu, L., Dai, T., Duncan, G., Elliott, G.S., Hui, a, McCabe, S.M., Scully, S., Shahinian, a, Shaklee, C.L., Van, G., Mak, T.W., Senaldi, G., 1999. T-cell co-stimulation through B7RP-1 and ICOS. Nature 402, 827–832. https://doi.org/10.1038/45582

Yoshinaga, Steven K., Whorlskey, J.S., Khare, S.D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M.A., Kohno, T., Tafuri-Bladt, A., Brankow, D., Campbell, P., Chang, D., Chiu, L., Dal, T., Duncan, G., Elliott, G.S., Hul, A., McCabe, S.M., Scully, S., Shahinlan, A., Shaklee, C.L., Van, G., Mak, T.W., Senaidi, G., 1999. T-cell co-stimulation through B7RP-1 and ICOS. Nature. https://doi.org/10.1038/45582

Yu, J., Mitsui, T., Wei, M., Mao, H., Butchar, J.P., Shah, M.V., Zhang, J., Mishra, A., Alvarez-Breckenridge, C., Liu, X., Liu, S., Yokohama, A., Trotta, R., Marcucci, G., Benson, D.M., Loughran, T.P., Tridandapani, S., Caligiuri, M.A., 2011. NKp46 identifies an NKT cell subset susceptible to leukemic transformation in mouse and human. J Clin Invest 121, 1456–1470. https://doi.org/10.1172/JCI43242

Zaini, J., Andarini, S., Tahara, M., Saijo, Y., Ishii, N., Kawakami, K., Taniguchi, M., Sugamura, K., Nukiwa, T., Kikuchi, T., 2007a. OX40 ligand expressed by DCs costimulates NKT and CD4+Th cell antitumor immunity in mice. Journal of Clinical Investigation. https://doi.org/10.1172/JCI32693

Zaini, J., Andarini, S., Tahara, M., Saijo, Y., Ishii, N., Kawakami, K., Taniguchi, M., Sugamura, K., Nukiwa, T., Kikuchi, T., 2007b. OX40 ligand expressed by DCs costimulates NKT and CD4+Th cell antitumor immunity in mice. Journal of Clinical Investigation. https://doi.org/10.1172/JCI32693

Zammit, D.J., Cauley, L.S., Pham, Q.-M., Lefrançois, L., 2005. Dendritic Cells Maximize the Memory CD8 T Cell Response to Infection. Immunity 22, 561–570. https://doi.org/10.1016/j.immuni.2005.03.005

Zander, R.A., Obeng-Adjei, N., Guthmiller, J.J., Kulu, D.I., Li, J., Ongoiba, A., Traore, B., Crompton, P.D., Butler, N.S., 2015. PD-1 Co-inhibitory and OX40 Co-stimulatory Crosstalk Regulates Helper T Cell Differentiation and Anti-Plasmodium Humoral Immunity. Cell Host Microbe 17, 628–641. https://doi.org/10.1016/j.chom.2015.03.007

Zepp, F., 2010. Principles of vaccine design-Lessons from nature. Vaccine 28 Suppl 3, C14-24. https://doi.org/10.1016/j.vaccine.2010.07.020

Zhang, Z., Zhong, W., Hinrichs, D., Wu, X., Weinberg, A., Hall, M., Spencer, D., Wegmann, K., Rosenbaum, J.T., 2010. Activation of OX40 augments Th17 cytokine expression

and antigen-specific uveitis. American Journal of Pathology. https://doi.org/10.2353/ajpath.2010.100353
Zhu, J., Guo, L., Watson, C.J., Hu-Li, J., Paul, W.E., 2001. Stat6 Is Necessary and Sufficient for IL-4's Role in Th2 Differentiation and Cell Expansion. The Journal of Immunology. https://doi.org/10.4049/jimmunol.166.12.7276
Zhu, J., Min, B., Hu-Li, J., Watson, C.J., Grinberg, A., Wang, Q., Killeen, N., Urban, J.F., Guo, L., Paul, W.E., 2004. Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. Nature Immunology. https://doi.org/10.1038/ni1128
Zhu, J., Yamane, H., Cote-Sierra, J., Guo, L., Paul, W.E., 2006. GATA-3 promotes Th2 responses through three different mechanisms: Induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Research. https://doi.org/10.1038/sj.cr.7310002
Zhuang, Y., Huang, Z., Nishida, J., Brown, M., Zhang, L., Huang, H., 2009. A continuous T-bet expression is required to silence the interleukin-4-producing potential in T helper type 1 cells. Immunology. https://doi.org/10.1111/j.1365-2567.2009.03049.x
Ziegler, S.F., Ramsdell, F., Alderson, M.R., 1994. The activation antigen CD69. Stem Cells 12, 456–465. https://doi.org/10.1002/stem.5530120502
Zingoni, A., Sornasse, T., Cocks, B.G., Tanaka, Y., Santoni, A., Lanier, L.L., 2004. Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions. Journal of immunology (Baltimore, Md. : 1950) 173, 3716–24.
Zinkernagel, R.M., 1974. Restriction by H-2 gene complex of transfer of cell-mediated immunity to Listeria monocytogenes. Nature 251, 230–233. https://doi.org/10.1038/251230a0
Zinkernagel, R.M., Doherty, P.C., 1997. The discovery of MHC restriction. Immunology Today. https://doi.org/10.1016/S0167-5699(97)80008-4
Zinkernagel, R.M., Hengartner, H., 2001. Regulation of the immune response by antigen. Science 293, 251–253. https://doi.org/10.1126/science.1063005
Zubairi, S., Sanos, S.L., Hill, S., Kaye, P.M., 2004. Immunotherapy with OX40L-Fc or anti-CTLA-4 enhances local tissue responses and killing of Leishmania donovani. European Journal of Immunology. https://doi.org/10.1002/eji.200324021