Investigating the Molecular Mechanisms of CD4 T Cell Persistence at Inflamed Peripheral Tissues

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Abstract

CD4 T cells play an important role in the initiation and maintenance of inflammation in numerous inflammatory diseases. Rheumatoid arthritis (RA) is one such autoimmune inflammatory condition where inflammation of the joint occurs. CD4 T cells are one of the key cells in RA pathogenesis due to their ability to activate or influence other cells in the joint including B cells, macrophages and osteoclasts, which collectively lead to joint destruction. The recruitment and function of CD4 T cells at inflamed tissues has been studied extensively. However, the signals that regulate CD4 T cell accumulation and persistence at peripheral inflamed sites are poorly understood.

In this study, a novel *in vivo* model of inflammation was designed in the murine ear pinnae to study the signals which regulate CD4 T helper 1 (Th1) cell persistence at inflamed tissues. Congenically marked, *in vitro* polarised CD4 Th1 cells were adoptively transferred directly into inflamed or non-inflamed ear pinnae and their persistence and survival were studied using flow cytometry. Higher numbers of CD4 Th1 cells were found at the inflamed as compared to the non-inflamed site.

Intravital microscopy was used to further study the behaviour of these cells. Th1 cells were found to be more mobile in inflamed compared to non-inflamed ear pinna. To investigate the molecular mechanism of this, either the ear pinnae or the T cells themselves were manipulated. Introducing cognate antigen at the inflamed site did not alter the number of recovered T cells, nor did the T cells proliferate at the site.

Next, the survival of persistent CD4 Th1 cells was examined by investigating their expression of active caspases. Lower proportion of Th1 cells recovered from inflamed tissues were found to express active caspases compared to those from a non-inflamed site. Together these data suggest that local T cell activation is not required for persistence but rather, the increase in T cells at inflamed sites may be due to a combination of persistence and survival signals.

The sphingolipid sphingosine-1-phosphate (S1P) has been implicated in driving both egress of T cells out of secondary lymphoid organs and their survival. To investigate whether S1P affects Th1 cell persistence and/or survival at inflamed tissues, Th1 cells were treated with S1PR agonists or antagonists, prior to transfer. Fewer Th1 cells were recovered from the inflammatory site of mice injected with antagonist treated cells. Additionally, S1PR agonism was sufficient to induce Th1 cell persistence at non-inflamed tissues.

A trend towards increased expression of active caspases was also found in S1PR antagonist treated T cells recovered from inflamed ear pinnae compared to untreated controls. Finally, elevated levels of the S1P metabolising enzyme, SPHK1, was found in human RA joints compared to OA joints.

In sum, I propose a novel function for S1P and its receptors in regulating the persistence of activated CD4 Th1 cells at inflamed tissue sites. Moreover, targeting S1P and its receptors at peripheral inflamed tissues could provide a novel target for the development of more effective anti-inflammatory therapeutics.

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Talk Presentations

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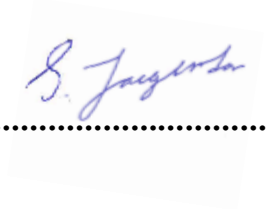
Author's Declaration

I declare that this thesis is the result of my own work, except where explicit reference is made to the contribution of others. No part of this thesis was submitted for any other degree at the University of Glasgow or any other institution.

Signature:  .....

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Abbreviations

7TM	Seven transmembrane
ABC	ATP-binding cassette
ACAD	Activated cell autonomous death
ACPA	Anti-citrullinated peptide antibody
AIA	Antigen induced arthritis
AICD	Activation induced cell death
AMP	Anti-microbial peptides
ANOVA	Analysis of variance
APAF	Apoptotic-protease-activating factor
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
BAK	BCL-2 antagonist/killer
BAL	Broncheo-alveolar lavage
BAX	BCL-2 associated X protein
Bcl	B cell lymphoma
BCR	B cell receptor
BIM	BCL-2 interacting mediator of cell death
C1P	Ceramide-1-phosphate

CAM	Cell adhesion molecule
CD	Cluster of differentiation
CFA	Complete freund's adjuvant
CIA	Collagen induced arthritis
CKR	Chemokine receptor
CLA	Cutaneous leukocyte antigen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A2
CTLA-4	Cytotoxic-T-lymphocyte associated protein-4
DAB	3,3' – Diaminobenzidine
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DR	Death receptor
DSS	Dextran sodium sulphate
DTH	Delayed type hypersensitivity

ECM	Extra-cellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESL	E-selectin ligand
FACS	Fluorescence-activated cell sorting
FASL	FAS ligand
FLICA	Fluorescent inhibitor of caspases
Fox	Forkhead box
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GlyCAM	Glycosylation-dependent cell adhesion molecule
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HDL	High-densty lipoprotein
HEV	High endothelial venule
HIV	Human immuno-deficiency virus
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
HSV	Herpes simplex virus

IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ID	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IMID	Immune-mediated inflammatory diseases
KLF	Kruppel-like factor
LEC	Lymphatic endothelial cell
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor
MACS	Magnetic-activated cell sorting
MADCAM	Mucosal vascular addressin cell-adhesion molecule
MEST	Mouse ear swelling test
MHC	Major histocompatibility class
MS	Multiple sclerosis

MTX	Methotrexate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-κB
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain
NOS	Nitric oxide synthase
OA	Osteoarthritis
Ova	Ovalbumin
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PKC	Protein kinase C
PMA	Phopbol-myristate acetate
PMN	Polymorphonuclear leukocyte

PRR	Pattern recognition receptor
PSGL	P-selectin glycoprotein ligand
PTX	Pertussis toxin
PUMA	p53-upregulated modulator of apoptosis
PUVA	Psolarin and ultraviolet A
qRT-PCR	Quantitative-reverse transcription PCR
RA	Rheumatoid arthritis
RBC	Red blood cell
RF	Rheumatoid factor
RIPK	Receptor-interacting protein kinase
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RORγ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RPMI	Rosswell park memorial institute medium
RT	Room temperature
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SEM	Standard error of mean

siRNA	Small-interfering RNA
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
SMase	Sphingomyelinase
SPHK	Sphingosine kinase
SPNS	Spinster homolog
STAT	Signal transducer and activator of transcription
T-PER	Tissue-protein extraction reagent
T1D	Type-1-diabetes
TBST	Tris-buffered saline+tween
TCR	T cell receptor
Tg	Transgenic
TGF-β	Transforming growth factor-β
TLO	Tertiary lymphoid organ
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
VEGF	Vascular-endothelial growth factor
YFP	Yellow fluorescent protein

1 Introduction

1.1 Overview

Immune-mediated inflammatory diseases (IMID) are one of the primary causes of significant morbidity and mortality in the western world. The term IMID is used to describe a group of diseases that share common inflammatory pathways. They include diseases such as rheumatoid arthritis (RA), psoriasis and multiple sclerosis (MS) amongst others (1).

IMIDs affect 5-7% of the population (2). They exhibit linked genetic susceptibilities which play a crucial role in disease development. Moreover, environmental precipitants such as smoking, infection and trauma are also shared. Commonly, individual patients present with multiple IMIDs (3).

The aetiology of most IMIDs is unknown, however, dysregulated cytokine networks have been identified as central to disease pathogenesis (4). CD4 T lymphocytes, often considered the orchestrator of immune responses play essential roles in the regulation of these cytokine networks at inflamed sites (5). These T cells accumulate in large numbers at effected tissues. Indeed, T cell accumulation is considered a molecular hallmark of multiple IMIDs (6-8).

Recent studies have investigated CD4 T cell activation, function, migration and death at peripheral inflamed tissues in an effort to understand their roles in disease pathogenesis (9-11). Processes involved in the trafficking of CD4 T cells to and from diseased tissues offers an attractive avenue for the development of novel anti-inflammatory therapeutics.

As part of this, recruitment of T cells to inflamed tissues has been extensively studied (12-17). Tissue persistence of these cells, however, remains a critical but poorly studied area of research. Considering the destructive influence of persistent CD4 T cells at tissues, targeting them remains a prime candidate for the development of new therapies.

This research project was undertaken with the aim to identify novel signals regulating pathogenic CD4 T cell persistence at inflamed tissues. This introduction will review the fundamental scientific knowledge which underlies the immunological basis of CD4 T cell persistence at peripheral inflamed tissues.

1.2 Inflammation

Inflammation is a complex and protective biological response of tissues to infection, injury or other harmful stimuli (18). Upon stimulation, tissues commence a cascade of events including vascular alteration, inflammatory mediator release and immune cell activation/infiltration. Through these mechanisms, inflammation aids in the clearance of the initial stimulus and restores homeostasis (19).

1.2.1 History of inflammation research

Aspects of inflammation were discovered as far back as 5th century BC. The Greek philosopher Hippocrates described inflammation as an essential component of wound healing. He also introduced the term *edema* or swelling, which is commonly associated with inflammation. Around 30 BC, Celsus, a Roman encyclopaedist described four of the five cardinal signs of inflammation. The Latin terms *calor* (heat), *dolor* (pain), *rubor* (redness) and *tumor* (swelling) are still in use today to describe inflammation. Virchow, a German pathologist, later added the fifth, loss of function (20).

The 18th and 19th centuries saw the advent of empirical research and with that the cellular and molecular details of inflammation began to emerge. Hunter, first described angiogenesis in wound healing in the late 18th century. Dutrochet described white blood cell adhesion and Cohnheim demonstrated changes in blood vessels including vascular leakage. Metchnikoff and Ehrlich, often considered the founders of immunology, demonstrated white blood cell (leukocyte) accumulation in inflamed tissues. Metchnikoff also described phagocytes and phagocytosis (21).

Technological advancements in the 20th and 21st centuries allowed for rapid progress in inflammation research. The application of genetic and molecular biology approaches in immunology led to the discovery of inflammatory mediators. Cytokines, chemokines, cell adhesion molecules and some of their *in vivo* functions were elucidated using these powerful tools (18). Despite such progress, treatments for many inflammatory diseases remain mostly disease modifying rather than curative. Moreover, molecular mechanisms underlying

multiple inflammatory diseases are as yet unclear. Hence there exists an unmet need for fundamental inflammation research. In the following sections, the current understanding of inflammation is reviewed.

1.2.2 Acute inflammation

Acute inflammation is triggered by infection or tissue damage. Pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs) released by pathogens or damaged cells respectively are then recognised by innate immune cells (22). PAMPs are a set of conserved molecular patterns expressed on microbes (23, 24). One of the best known PAMPs is bacterial lipopolysaccharide (LPS) found on gram-negative bacteria. DAMPs are signals released by cells upon breakdown of homeostasis such as cell death (25-27). Some of the best-known DAMPs include nucleic acids (DNA/RNA) and high mobility group box 1 (HMGB1) proteins (28). These molecules carry out crucial functions in intact cells, but act as danger signals upon release from necrotic cells (28).

PAMPs and DAMPs are recognised by pattern recognition receptors (PRRs) on tissue resident innate immune cells (29). PRRs include Toll-like receptors (TLRs), NOD (nucleotide-binding oligomerisation-domain binding protein)-like receptors (NLRs) and RIG-I like receptors (RLRs) (29). LPS and HMGB1 both trigger TLR4 and TLR2 respectively whereas RLRs recognise various nucleic acids (29-31). The recognition of PAMPs/DAMPs by PRRs leads to the production of inflammatory mediators such as cytokines, chemokines, adhesion molecules and lipid mediators (29).

Inflammatory mediators elicit local inflammatory exudates. Vascular permeability is increased, allowing selective entry of leukocytes, red blood cells (RBCs) and plasma proteins into inflamed tissues (32). Selectivity is conferred by modulation of gap junction space and adhesion molecule expression on vascular endothelial cells (33). Adhesion molecules interact with their receptors on leukocytes leading to extravasation (34, 35). Chemokines released at inflamed tissues, also aid tissue entry of vascular leukocytes by engaging their chemokine receptors (35, 36).

Neutrophils, which are polymorphonuclear leukocytes (PMNs), make up the majority of early leukocyte populations in inflamed tissues (37). Upon entry, neutrophils undergo activation either by direct interaction with the pathogen or by the local inflammatory cytokine milieu produced by tissue resident cells. Neutrophils engage targets by releasing toxic molecules such as reactive oxygen and nitrogen species (ROS), (NOS), proteinases, elastases and cathepsins (38). While highly potent, these molecules also cause significant off-target damage, often leading to the general pain associated with inflammation (39).

At the end of a neutrophil response, monocytes and macrophages are recruited (40). This is marked by a change of lipid mediator composition at the inflamed tissue. Pro-inflammatory prostaglandins are replaced by anti-inflammatory lipoxins. This drives the resolution phase of inflammation mediated by monocytes and macrophages (41). Monocytes remove apoptotic/necrotic cell debris and begin the tissue remodelling process (42). Macrophages carry out further tissue repairs and produce amongst others, two potent anti-inflammatory mediators transforming growth factor-β (TGF-β) and interleukin -10 (IL-10) (43, 44). Together these processes reduce inflammation and restore tissue homeostasis (45, 46).

1.2.3 Acute inflammatory diseases

The most common form of acute inflammation is resolving bacterial/viral infection or local tissue damage/wounds. In these cases, the process of acute inflammation restores tissue homeostasis (47). However, acute respiratory distress syndrome (ARDS) and sepsis are examples of non-resolving acute inflammatory diseases.

1.2.3.1 Acute respiratory distress syndrome

ARDS is characterised by the sustained accumulation of PMNs within alveolar compartments of the lung (48). ARDS occurs in adults usually after traumatic injury or bacterial pneumonia (49, 50). In infants, ARDS is most common following premature birth (51). Although mechanisms for ARDS development is poorly understood, fluids from ARDS lungs demonstrate hallmarks of aberrant acute inflammation (52). Bronchoalveolar fluids from ARDS patients contain

large numbers of PMNs, fibrin and complement components which are discussed in the “mediators of inflammation” section of this introduction. Collectively, this causes severely restricted gas exchange in the lungs. The majority of ARDS patients die of acute respiratory failure. Some patients resolve disease completely, while others suffer from pulmonary fibrosis (53). No effective therapy for ARDS exists. Patients are only provided with mechanical ventilation and fluids to help them recover (54).

1.2.3.2 Sepsis

Sepsis occurs primarily in immunocompromised individuals. It is most commonly triggered by bacterial infection of the lungs, although infections of other organs, both viral and bacterial can also cause sepsis (55). Like ARDS, mortality rates following sepsis are high. In sepsis, patient blood plasma contains large amounts of pro-inflammatory mediators such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-6 (56, 57). Cytokines are discussed in the “cytokines” section of this chapter.

Moreover, vascular endothelium express high levels of adhesion molecules as do infiltrating leukocytes (58). The complement and coagulation cascades are activated and tissue macrophages show excessively activated phenotypes (59). Overall, sepsis is a systemically dysregulated acute immune response. Like ARDS, causes of sepsis are unknown and no effective therapy exists.

1.2.4 Chronic inflammation

Chronic inflammation ensues once acute inflammation fails to eliminate pathogenesis (60). Lymphocytes, plasma cells and macrophages replace the primarily monocytic/neutrophilic infiltrates at acutely inflamed tissues (61). If these cells fail to control pathogenesis, persistent, long term, inflammation develops, potentially leading to the formation of tertiary lymphoid organs (TLOs) or granulomas (62). Triggers for chronic inflammation include persistent bacterial/viral infections, autoimmunity and foreign bodies (63-65).

The chronic inflammatory response to each individual trigger can be diverse. Whereas an anti-bacterial response in the lung might attract a certain subset of

leukocytes and lymphocytes, an autoimmune inflammatory skin lesion could be targeted by an entirely different subset of immune cells (66). Finally, foreign bodies as well as some pathogens often trigger granulomatous inflammation due to unsuccessful phagocytosis by macrophages (67).

1.2.5 Chronic inflammatory diseases

1.2.5.1 Psoriasis

Psoriasis is an autoimmune inflammatory skin disease characterised by epidermal keratinocyte hyperplasia (68). This aberrant proliferation of skin cells is mediated by accumulation of specific subtypes of inflammatory T helper 17 cells (Th17), macrophages and dendritic cells (DCs) (69-71). These cells produce pro-inflammatory cytokines such as IL-1, IL-6, TNFα, IL-17 and IL-22 which perpetuate disease pathogenesis (72).

Causes of psoriasis remain incompletely understood. However, there is a genetic association of psoriasis with T cells and MHC-II expressing cells (73). Treatments for psoriasis include immunosuppressants such as methotrexate (74). More severe psoriasis is often treated with biologics such as anti-TNFα (infliximab) (75). However, as with most chronic inflammatory disease, there are currently no drugs that cure the disease.

1.2.5.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease. It primarily manifests in the small joints of the hands and feet. In RA, synovial membranes of the joint are inflamed. Persistent inflammation leads to significant bone and cartilage damage which is mediated in part by dysregulated osteoclast proliferation (76).

Like psoriasis, the aetiology of RA is yet to be established. However, certain human leukocyte antigen (HLA) genes are negatively implicated (77, 78). These genes are critical in regulating antigen presentation to T cells (79). Moreover, co-stimulation molecules such as CD28 and CD40 as well as lymphocyte activation associated gene PTPN22 are also negatively implicated (80). Hence,

activated adaptive immune cells and their products play a key role in RA pathophysiology.

One of the hallmarks of RA is the presence of autoantibodies against the Fc portion of IgG known as rheumatoid factor (RF) in patient blood plasma (81, 82). Autoantibodies against citrullinated peptides (ACPAs) are another diagnostic marker for RA (83). While RF and ACPA implicate B/plasma cells in RA pathogenesis, aberrant accumulation of subsets of CD4 T cells such as Th1 and Th17 cells are also observed at RA synovia (84). Moreover, therapies targeted against T cell co-stimulation are effective in diminishing synovial inflammation (85, 86). Evidence suggests that synovial T cells contribute to disease by producing pro-inflammatory cytokines IFNγ and IL-17 (87-89).

Pro-inflammatory cytokines in general contribute significantly to RA pathophysiology. IL-1, IL-6 and TNFα produced by innate immune cells play major roles in disease progression (90). This is highlighted by the fact that the most effective therapy for RA targets TNFα (91). Despite its efficacy, not all patients respond to TNFα therapies.

Hence, there exists an unmet need for the development of novel anti-inflammatory therapeutics. Targeting master regulatory cells of cytokine networks, such as the CD4 T cell could provide new avenues for drug development.

Relevance of this section to thesis aims:

- Accumulation of leukocytes, and in particular T cells, is central to the pathogenesis of multiple inflammatory diseases as discussed in this section

- Our knowledge of the signals regulating T cell accumulation in inflamed tissues remains incomplete

- Discovery of novel signals of T cell accumulation at inflamed tissues could aid in the development of novel and more effective therapies for IMIDs

1.3 Mediators of inflammation

PAMPs and DAMPs are inducers of inflammation. Recognition of PAMPs and DAMPs by cells results in complex signalling cascades leading to the production of diverse classes of inflammatory mediators (92). These mediators have numerous effects including increasing vascular permeability. Furthermore, many regulate leukocyte recruitment (93, 94). Inflammatory mediators can be divided into seven groups based on their function: Vasoactive amines and peptides, complement components, proteolytic enzymes, cytokines, chemokines, cell adhesion molecules (CAMs) and lipid mediators (61, 95). A brief overview of inflammatory mediators will be provided in this section with emphasis on cytokines, chemokines and lipid mediators.

1.3.1 Vasoactive amines and peptides

Histamine and serotonin are vasoactive amines. They are the products of platelet and mast cell degranulation (96, 97). Vasoactive amines regulate vascular permeability (98). This is mediated by context dependent dilation or constriction of blood vessels by histamine and serotonin (99).

In addition, vasoactive peptides also play a critical role in controlling vasodilation. This family of small peptides is strongly regulated by the coagulation cascade protein Factor XII also known as Hageman factor (100-102). Factor XII processes and activates inactive compounds in extracellular fluids such as kinin and other fibrin degradation products (103, 104). These activated peptides then either act directly on the vasculature or trigger histamine and serotonin release by mast cells and platelets (105, 106). Factor XII importantly, controls the production of another peptide, Bradikynin (107). Bradikynin on top of being a vasodilator is a potent pro-algesic (108).

1.3.2 Complement components

Complement proteins C3a, C4a and C5a are produced in response to inflammatory stimuli (109, 110). This group of proteins are together known as anaphylotoxins (110). They are potent inducers of mast cell degranulation. Furthermore, anaphylotoxins promote granulocyte recruitment to inflamed tissues (111).

1.3.3 Proteolytic enzymes

Matrix metalloproteinases, elastin and cathepsins form a few of the multitude of proteolytic enzymes that mediate inflammation. These proteins function at all stages of inflammation. They facilitate leukocyte migration at inflamed tissues as well as aid in tissue remodelling and repair at the end of an inflammatory response (112, 113).

1.3.4 Cytokines

Cytokines are a family of small proteins important in cell-cell communication. They are key mediators of both acute and chronic inflammation. At inflamed tissues, cytokines are produced by a host of cells including endothelial cells, macrophages and neutrophils (114). At chronic sites of inflammation, such as RA joints, cytokines produced by osteoclasts and T cells also have critical implications for disease pathogenesis (90). Cytokines can be divided into three groups based on their function: Growth/survival factors, inflammatory cytokines and regulatory cytokines. These have been summarised in Table 1.

Group	Members	Target Cell(s)	Effect(s)
Growth Factors	IL-2	B and T cells	Proliferation and activation
	IL-3	Stem cells	Haematopoietic precursor proliferation and differentiation
	IL-4	B, T cells and macrophages	B, T cell proliferation and enhances MHC II expression

	IL-5	Eosinophils, B cells	Proliferation and maturation, stimulates IgA and IgM production
	IL-7	B and T cells	B, T cell growth and survival factor
	IL-9	T cells	Differentiation and proliferation
	IL-13	B cells and monocytes	IgE class switching and MHC class II expression
	IL-15	NK and T cells	NK cell development and maturation, memory T cell maintenance
	IL-21	NK and T cells	NK cell proliferation and T cell differentiation
	G-CSF	Bone marrow stem cells	Granulocyte production

	GM-CSF	Stem cells	Granulocyte, monocyte, eosinophil differentiation
	M-CSF	Stem cells	Monocyte activation and differentiation
Inflammatory Cytokines	IL-1α	Various cell types	Pyrogenic, proliferation and differentiation of cells
	IL-1β	Various cell types	Cell proliferation, differentiation and apoptosis
	IL-6	B cells and plasma cells	Differentiation, IgG production
	IL-11	B cells	Differentiation, induction of acute phase proteins
	IL-17	Keratinocytes, fibroblasts and macrophages	Pro-inflammatory cytokine production and cell proliferation

	IL-18	NK and T cells	T and NK cell differentiation and proliferation
	IL-25	Eosinophils and T cells	Eosinophil proliferation and regulation of T helper 17 cell cytokine production
	IL-23	T cells	Pro-inflammatory T cell differentiation
	IL-31	Various cell types	Cell proliferation and homeostasis, recruitment of leukocytes to inflamed sites
	IL-33	Mast cells, ILCs and T cells	Stimulates pro-inflammatory type 2 cytokine secretion
	IL-36	DCs and T cells	DC, T cell activation and pro-inflammatory cytokine

			production
	TNFα	Macrophages	Phagocytosis, endotoxic shock
	Type I IFNs	Various cell types	Anti-viral
	IFNγ	Various cell types	Macrophage activation, phagocytosis, increased MHC I and II expression
Regulatory Cytokines	IL-10	B cells, macrophages	Inhibits cytokine production and cellular activation, anti-inflammatory
	IL-12	NK and T cells	T and NK cell activation and regulation
	IL-19	Various cell types	Anti-inflammatory
	IL-20	Various cell types	Anti-inflammatory
	IL-22	Epithelial cells, hepatocytes and	Host defence at mucosal surfaces and

		keratinocytes	tissue repair
	IL-24	Various cell types	Wound healing, cell survival and proliferation
	IL-26	Various cell types	Anti-inflammatory, bactericidal
	IL-27	T cells	Promotes regulatory cell type differentiation and IL-10 production
	IL-28	Keratinocytes and melanocytes	Anti-viral
	IL-29	Keratinocytes and melanocytes	Anti-viral
	IL-35	Various cell types	Anti-inflammatory
	TGF-β	Various cell types	Inhibit cell proliferation, promote wound healing

Table 1 Cytokines, their Targets and Effects

Cytokines can have distinct and often antagonistic effects on cells. This is in part due to the diverse nature of cytokine receptors and their overlapping intracellular signalling pathways (114). The most targeted pro-inflammatory cytokines in inflammatory diseases include tumour necrosis factor α (TNFα), interleukin-1 (IL-1) family and interleukin-6 (IL-6) (114). Interestingly, these cytokines signal through structurally conserved type I cytokine receptors unlike most other cytokine receptors, which are structurally divergent (114, 115).

1.3.4.1 TNFα

TNFα is one of the most important cytokines in inflammation and immune regulation. Primary producers of TNFα are macrophages, although various other cell types also produce it at different stages of an inflammatory response (115). TNFα activates the production of other cytokines and acute phase proteins by various cells (115). It also promotes expression and activation of adhesion molecules which allow cell trafficking into inflamed tissues (116, 117). TNFα has roles in promoting cell survival, proliferation and apoptosis at inflamed tissues (115). Moreover, TNFα has roles in maintaining endothelial function and lipid metabolism (118, 119). Inhibiting TNFα using monoclonal antibodies has proven efficacious in multiple inflammatory diseases such as RA and psoriasis (1). This further signifies the central role of TNFα in mediating inflammation.

1.3.4.2 IL-1 family

The IL-1 family of cytokines includes 11 members (120). The most potent pro-inflammatory cytokines of this family are IL-1α and IL-1β (121). Production of these cytokines occurs downstream of PRRs triggered by PAMPs/DAMPs (122, 123). IL-1 is produced by numerous cells including, but not limited to, macrophages, monocytes and DCs (124). IL-1 is an endogenous pyrogen and is potently anti-viral (123, 125). Like TNFα, IL-1 promotes production of other cytokines and acute phase proteins (126). Likewise, IL-1 receptor antagonists such as anakinra and canakinumab are used therapeutically in multiple inflammatory disorders including RA and Muckle-Wells syndrome respectively (127-130).

1.3.4.3 IL-6

IL-6 is another potent inflammatory cytokine. Unlike TNF and IL-1, IL-6 is produced by a wider variety of cells including endothelial cells, bone marrow cells and fibroblasts as well as monocytes and macrophages (131). IL-6 is important in regulating haematopoiesis (132). IL-6 also controls adaptive immune responses by skewing T cell activation towards T helper 17 and inhibiting T regulatory cell phenotypes (133, 134). Similar to IL-1 and TNF, IL-6 plays an important role in the acute phase response (135, 136). Finally, monoclonal antibodies targeting IL-6 are also in use to treat RA and juvenile idiopathic arthritis (137, 138). Efficacy in other inflammatory diseases is currently being evaluated (139).

1.3.4.4 Cytokines in inflammatory diseases

Aberrant cytokine signalling contributes to disease pathogenesis in many inflammatory disorders (140). IL-1, IL-6 and TNFα levels are elevated in RA, type 1 diabetes (T1D), psoriasis and systemic lupus erythematosus (SLE) (141-145). In RA, TNFα plays a key role in triggering local inflammation and chemokine expression (146, 147). Downstream of TNFα, lipid mediators and adhesion molecule expression is dysregulated. This leads to tissue oedema and immune cell infiltration (148). Although, understood to a lesser extent, IL-1 and IL-6 play similar roles in inflammatory disorders. The efficacy of targeting these inflammatory cytokines as therapeutics provides strong evidence for their role in inflammatory disease pathogenesis (1).

1.3.5 Chemokines

Chemokines, also known as chemotactic cytokines, are primary regulators of cellular migration. Pro-inflammatory chemokines produced at inflamed tissues attract leukocytes (149). An elegant example of this is the chemokine induced expression of β2 integrin-lymphocyte function-associated antigen (LFA-1) in leukocytes. This promotes the arrest, rolling, diapedesis and extravasation of leukocytes from the vasculature into inflamed tissues (150). Although, chemokines are named and described for their chemotactic functions, they also

carry out a myriad of other functions. Chemokines have been noted for their role in haematopoiesis, angiogenesis and cell survival (151).

1.3.5.1 Chemokine characterisation

Chemokines are characterised by the presence of three or four conserved cysteine residues. They are subdivided into four groups based on their N-terminal cysteine group positioning. The four groups are called C-C, C-X-C, C, and C-X3-C chemokines (152). The majority of chemokines belong to the C-X-C and C-C groups of chemokines (153). Chemokines can also be functionally divided into inflammatory or homeostatic, although a few perform dual roles (150). Table 2 contains a list of the chemokines, their receptors and their main function.

1.3.5.2 Chemokine receptors

Chemokines signal by binding to a family of seven transmembrane helix family of receptors (7TM) known as G-protein coupled receptors (GPCRs) (154, 155). Expression of chemokine receptors (CKRs) is temporally and spatially variable on a wide variety of cell types (155). Tissue specific leukocyte entry and exit is in part controlled by this variability in chemokine and CKR expression. Moreover, chemokines play a critical role in adhesion molecule expression which also regulate selective tissue entry/exit of leukocytes (36).

To complicate matters further, CKRs bind their ligands with promiscuity (156). Promiscuity amongst CKRs is thought to be an evolutionary adaptation to allow redundancy in the system (157). In contrast, knocking out certain CKRs in animals leads to embryonic lethality. This demonstrates the fundamental importance of some chemokines in the developmental process (158). To date, 20 signalling CKRs have been described (159). Interestingly, four atypical chemokine receptors (ACKRs) have also been described. These receptors are incapable of binding to GPCRs and are therefore considered chemokine scavengers (160). ACKRs are important in dampening immune responses by removing chemokines from the surrounding environment (114).

1.3.5.3 Chemokines in inflammatory diseases

Like cytokines, elevated chemokine and CKR levels contribute to pathogenesis of many inflammatory disorders. CXCL8, the prototypical inflammatory chemokine, is elevated in RA synovial fluid as well as in the colons of ulcerative colitis patients (161, 162). T cell recruitment into inflamed RA synovia is also associated with elevated levels of CCL2,3 and 5 (10). In psoriasis, the CKR CXCR3 is responsible for the infiltration of pathogenic T cells into psoriatic lesions and contributing to disease pathogenesis (163). Despite strong evidence of the role of chemokines in perpetuating inflammatory disorders, no chemokine mediated anti-inflammatory therapeutics currently exist. In part, this is due to the difficulty in targeting individual chemokines/CKRs due to their promiscuity (164). Multiple chemokine/CKR targeted therapeutics are, however, currently in clinical trials (164).

Group	Chemokine	Receptor	Main Function
CC	CCL1	CCR8,11	Th2 cell and Treg trafficking
	CCL2	CCR2	Inflammatory monocyte trafficking
	CCL3	CCR1,5	Macrophage and NK cell migration T cell-DC interactions
	CCL4	CCR5	
	CCL5	CCR1,3,4,5	
	CCL6	CCR1,2,3	Myeloid cell differentiation, CNS homeostasis
	CCL7	CCR1,2,3	Monocyte

			mobilization
	CCL8	CCR1,2,5,8,11	Th2 response, skin homing
	CCL9	CCR1	DC migration, Osteoclast activation
	CCL10	CCR1	
	CCL11	CCR3	Eosinophil and basophil migration
	CCL12	CCR2	Inflammatory monocyte trafficking
	CCL13	CCR1,2,3,11	Th2 response
	CCL14	CCR1	Monocyte activation, Monocyte and Neutrophil chemotaxis
	CCL15	CCR1,3	
	CCL16	CCR1	
	CCL17	CCR4	Th2 responses, Th2 cell migration, Treg lung and skin homing
	CCL18	CCR6,8	Th2 response, marker of alternatively activated macrophages, skin

			homing
	CCL19	CCR7,11	T cell and DC homing to LN
	CCL20	CCR6	Th17 responses, B cell and DC homing to gut-associated lymphoid tissue
	CCL21	CCR7,11	T cell and DC homing to LN
	CCL22	CCR4	Th2 response, Th2 cell migration, Treg migration
	CCL23	CCR1	Unknown
	CCL24	CCR3	Eosinophil and basophil migration
	CCL25	CCR9,11	T cell homing to gut, thymocyte migration
	CCL26	CCR3	Eosinophil and basophil migration
	CCL27	CCR2,3,1	T cell homing to skin
	CCL28	CCR3,10	T cell and IgA plasma cell

			homing to mucosa
CXC	CXCL1	CXCR2	Neutrophil trafficking
	CXCL2	CXCR2	
	CXCL3	CXCR2	
	CXCL4	CXCR3	Procoagulant
	CXCL5	CXCR1,2	Neutrophil trafficking
	CXCL6	CXCR1,2	
	CXCL7	CXCR2	
	CXCL8	CXCR1,2	
	CXCL9	CXCR3	Th1 response, Th1, CD8, NK cell trafficking
	CXCL10	CXCR3	
	CXCL11	CXCR3	
	CXCL12	CXCR4,7	Bone marrow homing
	CXCL13	CXCR3,5	B cell and Tfh positioning LN
	CXCL14	CXCR4	Macrophage skin homing (human)
	CXCL15	Unknown	Unknown

	CXCL16	CXCR6	NKT and ILC migration and survival
	CXCL17	CXCR8	Monocyte/DC chemoattractant
C	XCL1	XCR1	Cross-presentation by $CD8^+$ DCs
	XCL2	XCR1	
CX3C	CX3CL1	CX3CR1	NK, monocyte, and T cell migration

Table 2 Chemokines, Receptors and Functions

1.3.6 Cell adhesion molecules (CAMs)

Cell adhesion molecules are cell surface proteins that allow cells to "adhere" to other cells or extracellular matrix (ECM). CAMs further enable entry and exit of leukocytes to/from circulation into tissues. There are four families of CAMs. They include the Immunoglobulin (Ig) superfamily CAMs, cadherins, integrins and selectins (165).

Inflammatory insult leads to the upregulation of CAMs such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin on vascular endothelial cells (166, 167). Likewise, activated leukocytes express ligands such as the integrins $\alpha L\beta 2$ (LFA-1) and $\alpha 4\beta 1$ (VLA-4) which bind to ICAM-1 and VCAM-1 respectively (168). Leukocytes also express E-selectin ligand-1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1) which interact with E and P-selectins respectively (169).

Expression of CAMs are temporally and spatially co-ordinated during inflammation to enable leukocyte rolling, adhesion and diapedesis into tissues. Moreover, specific CAMs such as the integrins $\alpha E\beta 7$ (CD103), $\alpha 1\beta 1$ (VLA-1) and

α2β1 (VLA-2) enable leukocyte retention at inflamed tissues (170). Clinically efficacious anti-inflammatory drugs such as Natalizumab currently target integrins α4β1 and α4β7 in multiple sclerosis and Chron's disease. Moreover, drugs targeting the integrin αEβ7 are also undergoing trials in MS and IBD, further highlighting the importance of CAMs in regulating inflammation (171).

1.3.7 Lipid mediators

Historically, two classes of lipid mediators have been described: eicosanoids and platelet activating factors (PAFs) (172). Both mediators are generated by the degradation of cell membrane phospholipids. The enzyme phospholipase A2 breaks down phosphatidylcholine on cell membranes into two components: arachidonic acid and lysophosphatidic acid. These form the precursors of the two classes of lipid mediators respectively (172).

1.3.7.1 Eicosanoids

Eicosanoids are generated when arachidonic acid is metabolised either by cyclooxygenase enzymes to form thromboxanes and prostaglandins. Alternatively, arachidonic acid can also be metabolised by lipoxygenase enzymes to generate leukotrienes and lipoxins. Interestingly, the two subgroups of eicosanoids have opposing effects. Prostaglandins and thromboxanes are pyrogenic, hyperalgesic and cause vasodilation. Hence, they are potently pro-inflammatory. In contrast, leukotrienes and lipoxins promote tissue repair and resolution of inflammation. Therefore, they are anti-inflammatory (173).

1.3.7.2 Platelet activating factors

PAFs on the other hand are generated from the other component of phosphatidylcholine metabolism: lysophosphatidic acid. Lysophosphatidic acid is acetylated to generate PAFs. PAF was originally described as a single phosphoglycerylether molecule. More recently however, PAFs are seen as a family of ether like molecules that carry out similar functions. PAFs bind to and signal through PAF receptors (PAFRs) which are GPCRs, like CKRs. It is unsurprising, therefore, that PAFs contribute to vascular permeability regulation, leukocyte recruitment and platelet activation, functions that chemokines are also accredited with (173).

1.3.8 Sphingolipids: A new family of lipid mediators

While Eicosanoids and PAFs are classical lipid mediators, sphingolipids have recently been identified as a new class of inflammatory lipid mediators. Sphingolipids are a class of lipids that are characterised by their eighteen-carbon amino-alcohol backbones (174). This backbone is shared amongst all members of the family and modification to this structure gives rise to the diverse family of sphingolipids. Sphingolipids are generated by the metabolism of sphingomyelin which is another component of the cell membrane (175).

1.3.8.1 Sphingolipid metabolism: the sphingomyelin cycle

The breakdown of sphingomyelin is regulated by enzymes known as sphingomyelinase (SMase), this pathway is summarised in Figure 1.3.1 (176). There are three different isoforms of SMase: Acid SMase, neutral SMase and secretory SMase. Acid SMase are found in acidic intracellular lysosomes. Neutral SMase are cell membrane bound and are ubiquitously expressed in mammalian cells. Secretory SMase is found in the Golgi secretory pathway (174, 177).

SMase isoforms are activated by a variety of stimuli including growth factors, inflammatory cytokines and cell stress (178-180). Activated SMase breakdown sphingomyelin to ceramide (177, 181). Ceramide is then either phosphorylated to make ceramide 1-phosphate (C1P) or broken down by ceramidase to make sphingosine (182, 183). Sphingosine itself can then be phosphorylated by either of two sphingosine kinases (SPHK1 or SPHK2) to make sphingosine 1-phosphate (S1P) (184, 185). S1P is then dephosphorylated by sphingosine phosphatase or degraded completely by S1P lyase (186, 187). Finally, sphingomyelin is re-synthesised from sphingosine to ceramide by ceramide synthase and then ceramide to sphingomyelin by sphingomyelin synthase (188, 189).

Sphingolipid formation and degradation are intimately connected as demonstrated in Figure 1.3.1. It is therefore unsurprising that their functions can also be reciprocal. For example, increased ceramide results in a concomitant decrease in S1P and is associated with increased cellular apoptosis. In contrast increased S1P and a concomitant decrease in ceramide is associated with increased survival (190). Hence, ceramide is considered a pro-apoptotic

molecule whereas S1P is considered anti-apoptotic (191). Despite this intimate connection, sphingolipids are produced at varied locations in different cell types. They can also be functionally distinct. In this section, the three best studied sphingolipids ceramide, C1P and S1P will be explored with a detailed assessment of S1P.

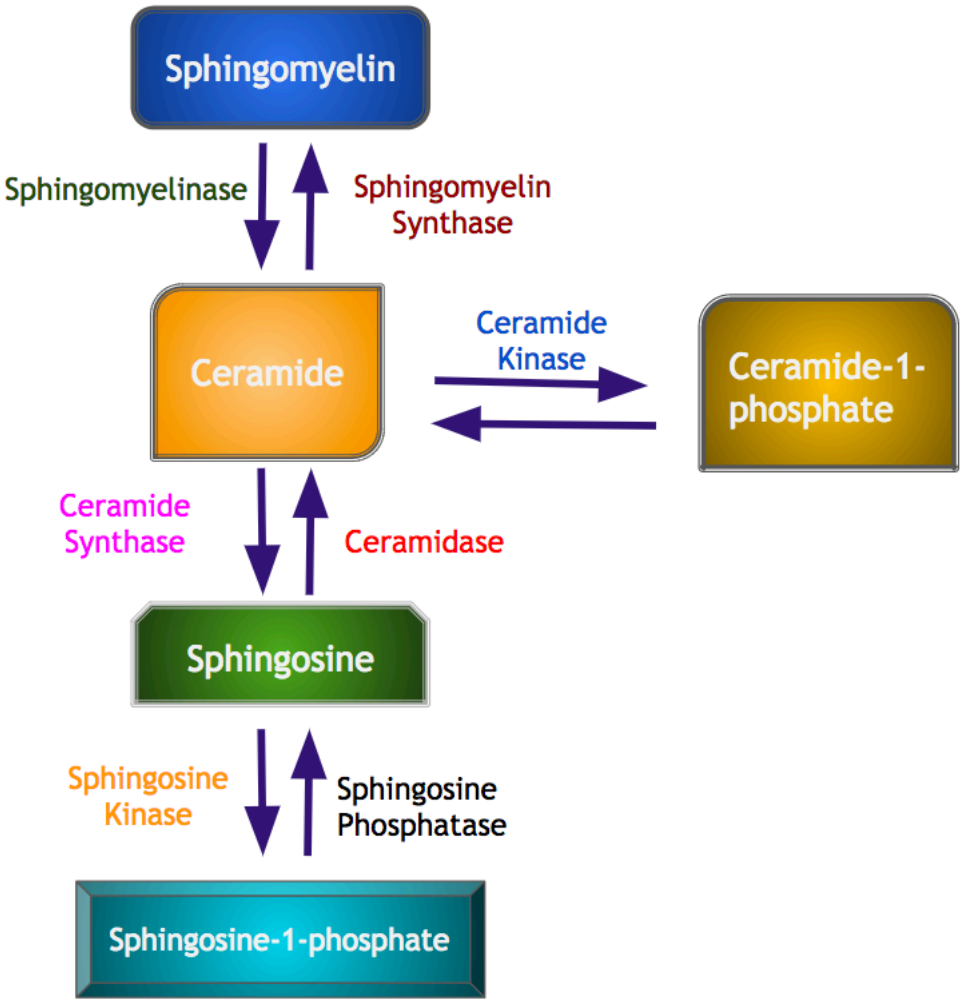

Figure 1.3.1 The sphingomyelin cycle
Diagram adapted from (192)

1.3.8.2 Ceramide

Ceramide typically forms 16-24 carbon long fatty acid chains that are strongly hydrophobic physiologically (193). It is usually synthesised at mitochondrial membranes due to the activity of the SMase breaking down sphingomyelin at such locations (194). Ceramide regulates a wide variety of signalling pathways via interaction with protein kinases and phosphatases (193). Studies have shown that ceramide forms lipid raft like structures at the cell membrane to promote signalling complex formation (195). Moreover, some proteins have ceramide

binding domains such as protein kinase C (PKC) (196). Ceramide is thought to regulate PKC mediated signalling by directly binding to PKC (196).

1.3.8.3 Ceramide in inflammation

Ceramide production is often regulated by TNF in inflammatory tissues (197). TNF mediated ceramide can activate the pleiotropic transcription factor nuclear factor-κB (NF-κB) (198, 199). NF-κB are a family of transcription factors that control the expression of many inflammatory cytokines, chemokines and lipid mediators (199, 200). These include the classical pro-inflammatory cytokines IL-1β, IL-6 and IL-8 (199). NF-κB also induces the expression of inflammatory lipid mediator cyclooxygenase 2 (COX-2) enzyme which subsequently triggers the expression of prostaglandin E2 (PGE-2) (201). Ceramide can also upregulate the transcription factor CCAAT/enhancer binding proteins (c/EBP) (202). Like NF-κB, c/EBP can also induce the production of inflammatory cytokines such as TNF and IL-1β (203).

1.3.8.4 Ceramide 1-phosphate

C1P is primarily found in the cytoplasm or localised in the perinuclear space (204). The intracellular localisation allows C1P to play important roles in regulating intracellular signalling pathways. C1P has been shown to regulate the eicosanoid metabolism pathway (204).

1.3.8.5 C1P in inflammation

C1P is the least studied member of the sphingolipid family. Indeed, since the discovery of the ceramide kinase enzyme, many functions ascribed to ceramide have been re-assigned as functions of C1P (205). C1P was found to directly interact with Ca^{2+} dependent phospholipid binding domain on cytosolic phospholipase A2 ($cPLA_2$) (206). This resulted in the production of PGE-2 and its downstream effects (207). Moreover, C1P was found to cause mast cell degranulation in a Ca^{2+} dependent manner (208). Inhibiting ceramide kinase was found to reverse this effect (209).

1.3.8.6 Sphingosine 1-phosphate

S1P is the most widely studied member of the sphingolipid family. S1P is produced by one of two enzymes SPHK1 or SPHK2 (210). Both enzymes are either found in the cytosol or bound to the nuclear membrane (211). S1P can have both autocrine and paracrine effects on cells (205). Intracellular S1P can regulate calcium response pathways via modulation of phospholipase C/inositol 1,4,5 tri-phosphate pathways (212). S1P also affects cell proliferation and survival by regulating B cell lymphoma-2 (Bcl-2) family members (213, 214).

Paracrine effects are possible due to the extracellular export of S1P by ATP-binding cassette (ABC) or spinster homolog 2 (Spns2) transporters (215, 216). Blood plasma constitutively carries high levels of S1P (217). This S1P is produced by vascular endothelial cells, RBCs or platelets which are the most potent producers of S1P (217). S1P concentrations are typically very low in interstitial fluids. Therefore, S1P forms a strictly regulated gradient in mammalian systems (217). This gradient is critical for the regulation of leukocyte migration and will be explored in further detail in the "T cell trafficking" section of this thesis (218).

Paracrine functions of S1P is mediated via five S1P receptors (S1PR1-S1PR5) (219-221). These are all 7TM GPCRs. The S1P GPCRs can couple to multiple different heterotrimeric G proteins and thus they can signal through multiple signalling pathways (212). Once S1PRs are ligand bound and signalling has occurred, S1PRs are internalised. Internalised S1PRs either undergo lysosomal degradation or are recycled back to the cell surface (212, 222, 223). Like chemokines, temporal and spatial expression of S1PRs are critical in determining the responses the ligand elicits on the target cell (205, 224). In homeostatic conditions, S1PR1-S1PR3 are widely expressed in various tissues and cell types (225). S1PR4 expression is restricted primarily to lymphoid and haematopoietic cells while S1PR5 is mainly found in the brain and the skin (226-229).

S1P is constitutively expressed in almost all mammalian cell types as part of the plasma membrane turnover (230). However, under inflammatory conditions, multiple stimuli upregulate S1P production. TNF-α, TGF-β and growth factors including platelet derived growth factor (PDGF) and vascular endothelial growth

factor (VEGF) have so far been identified to positively regulate S1P production (231). All of these factors increase S1P production by regulating SPHK1 expression levels (231).

In contrast, very little is known about external factors that downregulate S1P levels at the end of inflammatory events. It is known that phosphatases and ceramidases play an important role in regulating levels of S1P (174). However, external regulation of phosphatase and ceramidase enzymes in the sphingolipid metabolism pathway (Figure 1.3.1) has not been yet been demonstrated (232).

Known negative regulators of S1P include DNA damage detection, high-density lipoproteins (HDLs) and ceramide (231, 233-235). DNA damage downregulates SPHK1 expression, thus directly abrogating S1P production (235). In contrast, HDL regulates plasma S1P concentrations by controlling the extracellular transport and bioavailability of S1P to other cells (236). Ceramide is a well-known negative regulator of S1P (177, 190). Increased levels of intracellular ceramide, itself regulated by exogenous stimuli such as FAS ligand and oxidative stress, leads to increased S1P degradation and ceramide production (237). Regulators of S1P and its functions are succinctly summarised in

Figure 1.3.2.

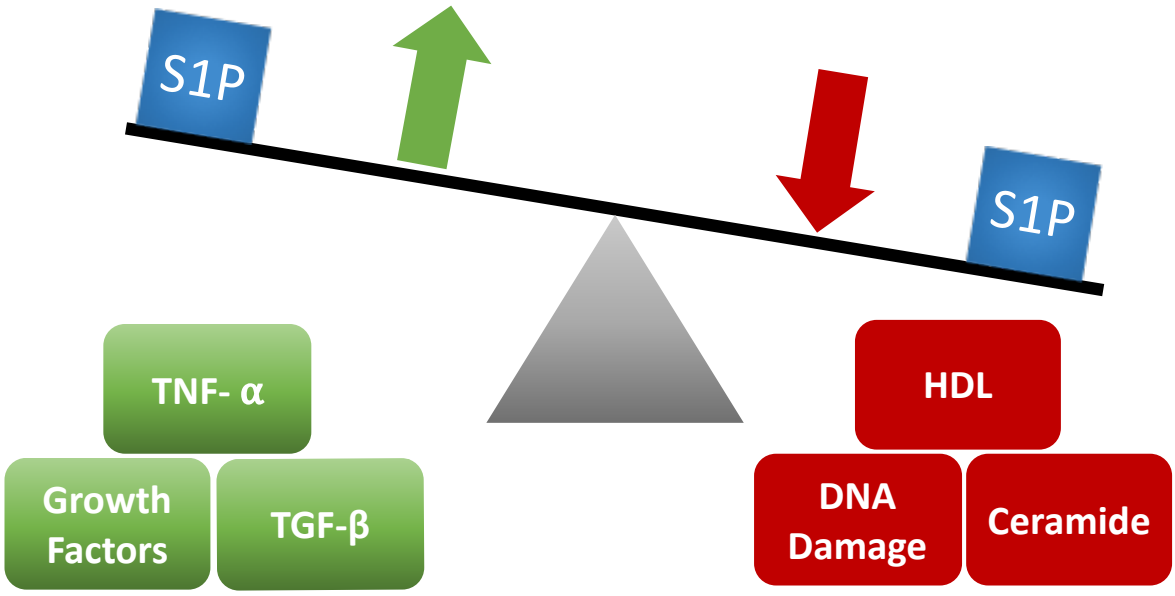

Figure 1.3.2 Regulation of S1P
S1P levels are regulated by a variety of factors. Inflammatory cytokines and growth factors such as TNF-α, TGF-β, VEGF and PDGF promote the production of S1P. In contrast, DNA damage, high-density lipoproteins (HDLs) and ceramide negatively regulate S1P levels.

1.3.8.7 S1P receptor modulators

Functional aspects of S1P signalling were elucidated in large parts by studying the effect of S1PR modulators. These include S1PR pan agonists and S1PR pan antagonists as well as selective agonists and antagonists for individual S1P receptors. Here three S1PR modulators will be described very briefly.

The best known S1PR modulator is FTY720. It is derived from a fungal protein and is a functional antagonist of S1PR1,3,4 and 5 (238). FTY720 binding with S1PRs causes receptor internalisation and their subsequent degradation (222). Essentially this makes the cell insensitive to S1P signals mediated via any of the aforementioned receptors. FTY720 has also been clinically approved for use in MS which is an autoimmune neuro-inflammatory disease (238). S1PR1 antagonism is believed to cause the arrest of auto-reactive T cells at SLOs (239). This results in a reduction of pathogenic T cell migration to the central nervous system, leading to disease abrogation (238).

W146 is an S1PR1 selective antagonist. It is a synthetically derived small molecule inhibitor. W146 binds to and blocks S1PR1 mediated signalling (240). Finally, SEW2871 is an S1PR1 selective agonist. It is also a synthetically derived small molecule inhibitor. SEW2871 binding to S1PR1 causes receptor internalisation and signalling followed by receptor recycling to the cell surface (223, 241). Unlike FTY720, neither of these drugs have been approved in the clinic. They have primarily been used as research tools to elucidate functions of S1P and S1PRs (240, 241).

S1PR modulators were primarily used in lymphocyte migration studies. T cell trafficking section of this thesis describes some of these studies.

1.3.8.8 S1P in inflammation

S1P is rapidly emerging as a critical mediator of inflammation. The primary role of S1P in inflammation control is to regulate the trafficking of immune cells to and from inflamed tissues (242). S1P controls the dwell time and egress of activated lymphocytes from SLOs (243). Modulating S1PR signalling via FTY720 was found to induce tissue retention of CD4 T cells in mice (244). The same study reported that increased S1P levels at inflamed tissues reduce T cell egress

from such sites (244). Impaired T cell egress from inflamed peripheral tissues has been associated with exacerbation of inflammation (245). Thus, S1P controls the adaptive immune response to an inflammatory insult.

Likewise, S1P regulates migration and endocytosis of DCs as well as the recruitment and chemotaxis of macrophages to inflamed tissues (246, 247). S1P further effects monocyte activation and monocyte mediated TNFα production (248). S1P, therefore also plays a key role in innate immune responses to inflammatory insults.

S1P also regulates mast cell mediated inflammatory responses. Ligand binding to mast cell IgE receptors leads to S1P production in a SPHK2 mediated manner (249, 250). Animals with abrogated SPHK2 expression have reduced mast cell degranulation, cytokine and eicosanoid production (251, 252). S1P produced by mast cells is also transported out of the cell to elicit autocrine and paracrine effects (253). Mast cells express both S1PR1 and S1PR2. S1P binding to mast cell S1PR1 leads to mast cell migration. In contrast, S1P binding to mast cell S1PR2 leads to mast cell degranulation (224).

1.3.8.9 S1P in airway inflammation

Mast cells are key contributors to airway inflammation (254). Therefore, it is unsurprising that S1P levels were found to be elevated in the bronchoalveolar lavage of asthmatics, which is an airway inflammatory disease (255). In mouse models of airway inflammation, FTY720 administration was found to reduce Th1 and Th2 cell infiltration in the airway and the animals had abrogated disease pathogenesis (256, 257). Inhibition of SPHK1 was also shown to reduce airway inflammation as well as S1P concentration in bronchoalveolar lavage (258).

S1P is involved in regulating TNF mediated responses in L929 fibroblasts and A549 lung epithelial cells. In these cell lines, SPHK1 was found to regulate TNF mediated COX-2 and PGE2 production (259). Similarly, in LPS stimulated macrophages, SPHK1 was found to regulate COX-2 production (260). S1P was also found to directly stimulate arachidonic acid production in A549 lung epithelial cells (261).

1.3.8.10 S1P in autoimmune inflammatory diseases

Inflammatory bowel diseases (IBD) incorporate ulcerative colitis and Crohn's diseases. These are both inflammatory diseases of the intestinal mucosa (262). Disease pathogenesis is usually mediated by dysregulated production of inflammatory cytokines and dysregulated T cell infiltration (262, 263). In animal models of colitis, FTY720 administration was found to significantly dampen intestinal inflammation. This was associated with an FTY720 mediated decrease in inflammatory cytokine production and a concomitant increase in regulatory cytokine production (264). In another study with SPHK1-/- animals, intestinal inflammation was found to be significantly reduced compared to wild type animals treated with an inflammatory chemical (DSS) (265). Finally, animals with colitis treated with oral SPHK1 inhibitors showed substantially reduced intestinal inflammation and inflammatory cytokine production (266).

A role for S1P has also been suggested in RA pathophysiology. S1P levels were elevated in RA joints compared to osteoarthritic (OA) joints (267). Moreover, SPHK1 and SPHK2 expression was found to be increased in RA synovial tissues (268, 269). Most interestingly, in a mouse model of joint inflammation, inhibiting SPHK1 was found to reduce disease severity, articular inflammation and joint damage (270, 271).

On top of this, S1P plays a key role in lymphocyte trafficking (272). Many inflammatory diseases have dysregulated lymphocyte trafficking patterns (273-276). Therefore, one of the primary mechanism of S1P action in inflammatory diseases is by regulating lymphocyte trafficking (277). This will be explored in greater detail in the "T cell trafficking" section.

Relevance of this section to thesis aims:

- T cell activation, trafficking and function is regulated by inflammatory mediators

- S1P and chemokines in particular, are critical for T cell trafficking

- Are there novel chemokine pathways that influence T cell persistence at inflamed tissues?

- Does S1P also control egress or persistence of T cells at peripheral inflamed tissues?

- S1P targeted drugs (FTY720) are already approved for use in MS. Could targeting S1P prove an effective therapeutic in other inflammatory diseases?

1.4 Innate and adaptive immunity in inflammation control

The inflammatory response is initiated once host cells are activated by infection or injury. A specialised family of innate immune cells known as antigen presenting cells (APCs) such as DCs pick up fragments of pathogen or damaged tissue and migrate to downstream draining lymph nodes. Present in the lymph nodes are naïve cells of the adaptive immune system (T lymphocytes). Professional APCs process and present these epitopes known as antigens on MHC II to these T cells (278, 279).

T and B cells (another adaptive lymphocyte), express unique receptors which are specific for a single antigen. Once, an APC carrying that antigen, encounters a T cell, the lymphocyte undergoes activation and clonal proliferation (279). In contrast, B cells primarily sense soluble antigen directly using their B cell receptor (BCR) (280). B cells can also however, be activated by APCs carrying antigen (281).

Activated or effector T cells exit the lymph node and home to inflamed tissues to carry out effector functions (276, 282). Effector B cells in contrast, produce class switched and affinity matured antibodies which are carried in blood plasma to inflamed tissue sites (283). The majority of effector T/B cells undergo apoptotic cell death at the end of a response (284). A few of these cells however, become memory T/B cells and persist long term in the host (285). These long-lasting memory cells remain primed for rapid response in the case of a secondary infection by the same pathogen (285).

While the functions of these cells are strictly regulated, in many inflammatory diseases functional regulation is altered or lost leading to pathogenesis (279). In this section, the contribution of some of these innate and adaptive immune cells at inflamed tissues will be explored.

1.4.1 Neutrophils

Neutrophils form the immune system's primary line of defence. They constitute the largest proportion of leukocytes in the mammalian immune system (286). In

homeostatic conditions, neutrophils circulate in a resting state which ensures that toxic neutrophilic granules are locked away (287). Pathogenic insult or injury, leads to rapid neutrophil priming, recruitment and activation (288).

Neutrophil priming occurs either by encountering PAMPs/DAMPs directly or via cytokine mediated manner. This leads to rapid upregulation of cell surface adhesion molecules which allows neutrophils to extravasate into tissues (288). Once in tissue, neutrophils migrate towards the pathogenic insult by sensing chemokine gradients such as CXCL8 (289).

At an infected site, neutrophils can release antimicrobial peptides (AMPs), phagocytose or form neutrophil extracellular traps (NETs) to kill the pathogen (290-292). AMPs are small proteins that kill pathogens by forming pores in their membranes (293). Phagocytosed pathogens are killed by ROS found inside neutrophil intracellular phagosomes (294). NETs consist of a meshwork of chromatin fibres that are dotted with immobilised microbicidal molecules derived from neutrophil granules. NETs function by trapping and immobilising pathogens followed by their degradation by the microbicidal granules (295). Neutrophils are also adept at producing pro-inflammatory cytokines including TNFα, IL-1β, IL-6 and lipid mediators (296, 297). This stimulates other cells such as endothelial cells and macrophages to carry out their effector function (298, 299).

1.4.1.1 Role of neutrophils in inflammatory disease

Neutrophils play a critical and positive role in acute infectious inflammation. Indeed, in human diseases of reduced blood neutrophils (severe congenital neutropenia), a lack of neutrophil recruitment to infected tissues leads to severe immunodeficiency (300). Likewise, in animal models of acute infection, systemic neutrophil depletion increased disease severity (301). However, the role of neutrophils in chronic inflammatory diseases remains controversial.

1.4.1.2 Neutrophils in psoriasis

One of the hallmarks of psoriasis is neutrophil accumulation in inflamed dermis and epidermis of the skin (302). This leads to the formation of psoriatic lesions (302). Here, neutrophils get stuck in a positive feedback loop. Activated

neutrophils produce inflammatory cytokines, chemokines and other mediators which activate local macrophages and keratinocytes (303, 304). These cells produce more neutrophil chemoattractants and activators or undergo hyper proliferation (303, 305).

The importance of neutrophils in maintaining psoriasis has been highlighted in animal studies where neutrophil depletion was found to alleviate symptoms in flaky skin mice (306). Moreover, pan selectin inhibitors, which reduced neutrophil recruitment to inflamed skin were found to be efficacious in murine models of psoriatic skin inflammation as well as in human psoriasis (307, 308). Finally, current anti-psoriatic therapies exert their effects, at least in part, through neutrophils (309).

1.4.1.3 Neutrophils in RA

The most abundant immune cell found in RA synovial fluid are neutrophils (310). Moreover, neutrophils are found in large numbers in the pannus/cartilage interface where most joint destruction occurs (311). Here, aggregated immunoglobulins activate neutrophil degranulation which leads to the production of ROS (311). Increased ROS production mediated oxidative stress and inflammatory cytokine production is directly implicated in RA pathogenesis (312).

Mouse models of joint inflammation implicate neutrophils in disease further. K/BxN mice develop symmetrical joint swelling, pannus formation, synovial hyperplasia and cartilage destruction. Serum transfer from K/BxN mice to other mice leads to similar joint inflammation. Neutrophil depleted mice however, develop no diseases pathogenesis upon serum transfer. In this model, the inflammatory lipid mediator leukotriene B4 (LTB4) has been identified as the key arthritogenic molecule (313).

In Antigen induced arthritis (AIA) and collagen induced arthritis (CIA), blocking neutrophil recruitment via CXCR1/2 or complement C5a receptor blockade abrogated joint pathology respectively (314). Lastly, as in psoriasis, first line RA therapy methotrexate (MTX) reduces neutrophil chemotaxis and ROS production. MTX also increases neutrophil apoptosis (315).

1.4.2 Macrophages

The term macrophage translates as “big eaters” in Greek. Metchnikoff was the first person to describe the macrophage based on its primary function: eating or phagocytosis (316). Since discovery, macrophages were found to be much more heterogeneous with functions as diverse as metabolism control, wound healing, infection control and bone remodelling (317). Macrophages are found in almost all tissues of an adult mammal (318). Mouse macrophages are usually identified by their cell surface expression of CD64 and/or F4-80 along with CD11b in mice and CD11b and CD68 in humans (317, 319, 320).

Despite significant research, macrophage development remains a topic of debate. The most accepted system of macrophage ontogeny defines macrophages as the most mature form of mononuclear phagocytes (monocytes) (321). Monocytes originate in the bone marrow and circulate in the vascular system. Some migrate into tissues and mature into tissue resident macrophages. Others are recruited rapidly upon tissue damage and mature into activated “M1” or alternatively activated “M2” macrophages based on the type of insult and local cytokine environment (321, 322).

Inflammatory M1 macrophages are best known for their role in pathogen/apoptotic cell uptake and killing through nitric oxides. These macrophages also activate adaptive immune responses by presenting antigen to naïve T and B cells after migration to SLOs. In contrast, M2 macrophages are accredited with anti-parasitic and regulatory functions (323). A third group of macrophages have recently been identified as wound healing macrophages. They promote tissue repair (324).

M1 Macrophages are the primary producers of pro-inflammatory cytokines TNFα, IL-1β and IL-6 amongst a myriad of other pro-inflammatory cytokines. Through these mechanisms macrophages control acute inflammatory insults. In contrast, M2 macrophages are potent producers of the anti-inflammatory cytokines TGFβ and IL-10 which dampen immune responses (325). Dysregulation of macrophage activation and their mediators play key roles in development and persistence of many inflammatory diseases (326).

1.4.2.1 Macrophages in psoriasis

The role of monocytes and macrophages in psoriatic inflammation is not well characterised. However, enrichment of inflammatory M1 macrophages have been identified in human psoriatic lesions (327). These macrophages were also found in other human inflammatory skin diseases such as atopic dermatitis (328). In two different mouse models of psoriasis-like disease, depletion of macrophages expressing F4-80 and CD11b were found to resolve skin inflammation (70, 329).

In another model, polarisation of inflammatory macrophages to an M2 phenotype was found to abrogate allergic skin inflammation. In the absence of M2 macrophages however, inflammation persisted (330). Interestingly, despite psoriasis being a disease of non-resolving skin inflammation, to date, no studies have investigated a role for wound healing macrophages in regulating psoriasis. These studies suggest that a skewed macrophage phenotype at inflamed skin contributes to disease pathogenesis.

1.4.2.2 Macrophages in RA

Macrophages play a very important role in RA pathogenesis. They are the primary producers of joint destructive pro-inflammatory cytokines (331). These cytokines activate chondrocytes and osteoclasts leading to cartilage damage and bone erosion (332). Macrophage numbers in the synovial lining can be used as a predictive biomarker for disease severity (333). Indeed, the hallmark of early RA is macrophage accumulation at synovial lining (334).

Disease flares in RA strongly correlate with increased M1 macrophage enrichment in the joint. In contrast, quiescent phases of disease display an increase in M2 macrophages in the synovium (335). At an inflamed joint, macrophages have been shown to induce angiogenesis and hypoxia (336, 337). Macrophage growth factors granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF) are also found abundantly in inflamed joint tissues (335).

Critically, most clinically effective biologics were shown to reduce macrophage trafficking to joints (338). Finally, in multiple models of inflammatory joint

diseases in mice as well as human trials, systemic depletion of macrophages using agents such as liposomal clodronate abrogated joint inflammation (339). These studies suggest that macrophages play a key role in mediating local joint pathology in RA.

1.4.3 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells of the innate immune system. Their primary role is to shape adaptive immune responses by the activation of T and B cells (340, 341). DCs were first described by Steinman and Cohn as cells possessing dendritic morphology (342, 343). Like macrophages, DCs are found in almost all mammalian tissues. DCs are identified by their cell surface expression of CD11c and MHC II molecules (340).

Subsets of DCs are often defined by combinations of additional cell surface receptor expression including CD11b, CD103, CD207 and CD8α. CD103 DCs are often described as migratory tissue DCs. They are the primary cell type that activate T and B cells in the SLOs. In contrast, CD8α DCs are known for their ability to cross present antigen to CD8 T cells. CD207 expressing DCs are also known as Langerhans cells. One of their specialised functions include the induction of regulatory T cell differentiation. Functional differences between subsets remain controversial however, primarily due to the plastic nature of DCs. Dendritic cell ontogeny and function is exhaustively reviewed by Merad et al. and should be read for more in depth information (340).

DCs develop in the bone marrow from either the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP) (344). These differentiated but immature, DCs constantly sense their surroundings and either rest at tissue sites or circulate in the vasculature (340). Once DCs encounter pathogenic stimuli, usually through their PRRs, they undergo maturation which includes both phenotypic and morphological changes. Mature DCs process and present antigen on peptide:MHC complexes (341). They also express co-stimulatory molecules CD80/CD86 and CKR CCR7 (345, 346). They further produce pro-inflammatory cytokines which direct T cell polarisation (347, 348). These combined changes allow DCs to migrate to SLOs and present antigen to T and B cells.

DCs play a critical role in inflammation control by selectively driving T cell responses. DC activating stimuli as well as location allow DCs to imprint tissue homing signature on T cells (349). Furthermore, DC mediated co-stimulation and cytokines are the primary regulators of Th1/Th2/Th17/Treg differentiation (350). It is therefore unsurprising that DCs have a central role in inflammatory disease pathogenesis.

1.4.3.1 Dendritic cells in psoriasis

DC mediated activation of adaptive immune responses occur at very early stages of psoriasis (351). Since this breach of tolerance phase occurs prior to clinical manifestations, it is difficult to study and thus poorly understood in all human inflammatory diseases. Nonetheless, roles for skin resident dermal DCs and recruited plasmacytoid DCs (pDCs) have been described in maintaining psoriatic inflammation (351, 352). Psoriatic lesions display the presence of both Th1 and Th17 cells at different phases of disease (353, 354). This is believed to be mediated by the local cytokine environment in which DCs are activated prior to T cell activation (71). Moreover, the local inflammatory environment favours the skewing of Th17 responses with abundant presence of IL-23, IL-12 and TNF (72).

DCs in psoriatic lesions also contribute to inflammation by their cytokine production. pDCs are primary producers of type I IFNs in psoriasis which acts on dermal DCs to stimulate their production of IL-12 (355). Moreover, DCs produce TNF and IL-1β to add to the local inflammatory microenvironment (356). Local DC-T cell interactions in ectopic lymphoid tissue like aggregates further support T cell activation and persistence in psoriatic plaques (357). This is particularly important since studies in mice have suggested that local populations of tissue resident DCs and memory T cells alone can initiate disease (358).

Lastly, multiple effective therapeutics in psoriasis are found to disrupt DC-T cell interactions. Psolarin and ultraviolet A (PUVA) treatment reduces chronic plaque psoriasis by depleting lesional DCs and epidermal T cells (359). Efalizumab is a monoclonal antibody targeting LFA-1 on DCs. Efalizumab disrupts interaction of DCs with intercellular adhesion molecule-1 (ICAM-1) expressing T cells. This results in disease abrogation via decreased numbers of CD11c+ DCs in lesions

(360). Finally, alefacept, a fusion protein that depletes CD2 expressing DCs and T cells, is also effective in psoriasis (361).

1.4.3.2 Dendritic cells in RA

DCs play a very similar role in RA as in psoriasis. DCs are important in breach of tolerance and disease initiation as demonstrated in multiple animal studies (362-364). Inflamed synovial microenvironment allows activated DCs to constantly prime polarised and inflammatory Th1/Th17 cells which maintain pathogenesis (365, 366). DCs themselves contribute to joint destruction by producing pro-inflammatory cytokines in synovial tissue (367). Finally, as in psoriasis, DCs play a key role in the formation of ectopic lymphoid aggregates near the synovium which maintains chronic disease (368).

RA therapies reduce disease severity rather than cure disease. Anti-TNF and abatacept are two examples that act by inhibiting DC and T cell functions (369, 370). One avenue of research being explored in RA include engineering DCs to tolerise activated T cells or drive regulatory instead of inflammatory T cell polarisation pathways. If successful, this avenue has the potential to yield a cure for multiple inflammatory diseases (371, 372).

1.4.4 B cells

B cells are adaptive immune cells of the lymphoid lineage whose primary function is the production of antibodies. B cells develop and mature in the bone marrow. Here, they undergo selection and training processes which allow them to discriminate self-antigen from foreign antigen. B cells express cell surface receptors called the B cell receptor (BCR). They use this receptor to detect antigen both during maturation and during a pathogenic insult (373).

Once mature, B cells travel to SLOs where they remain until they encounter their cognate antigen. Antigen recognition through the BCR activates B cells to undergo clonal proliferation and produce antibodies. B cells are also professional APCs and potent cytokine producers. Certain types of antigens known as T-cell dependent antigens, require help from T cells to activate B cells. In these cases, T and B cells specific for the same antigen meet in designated areas of SLOs. In this area, known as the germinal centre, specialised CD4 T cells known as

follicular T helper cells (Tfh) provide co-stimulation and cytokine mediated signals to B cells (373).

These signals allow B cells to undergo proliferation, affinity maturation and somatic hypermutation to produce affinity matured and class switched antibodies. Some B cells become long lived plasma cells which constantly produce low levels of antibodies. Memory B cells are also produced. These cells enter circulation and constantly survey the periphery to provide a rapid response to secondary insult from the same pathogen (373).

B cells and their products are involved in the initiation and maintenance of multiple inflammatory diseases. The function of B cells in psoriasis and RA are briefly explored in the next sections:

1.4.4.1 B cells in psoriasis

The role of B cells in psoriatic inflammation remains controversial. In human studies, CD19+ B cell levels were found to be elevated in human psoriatic lesions (374). Moreover, increased levels of the cytokine IL-21 was found in psoriatic lesions. IL-21 is produced by Tfh cells to activate B cells in the germinal centre. This suggests a role for B cells in the formation of ectopic lymphoid tissues (375, 376). Interestingly, B cells were found to produce IL-10 in psoriatic lesions, suggesting they may play an anti-inflammatory role (377).

This was supported when depletion of CD20+ B cells by Rituximab therapy in RA or SLE patients was found to cause psoriatic inflammation in genetically non-susceptible people (378). Animal studies further support this finding. CD19 deficient animals were found to have increased skin inflammation in an imiquimod-driven model of psoriatic inflammation (377). This was directly associated with the depletion of IL-10 producing B10 B cells. Adoptive transfer of these cells ameliorated disease (377). However, no antibody mediated pathways have been investigated in the pathogenesis of psoriasis. Hence, while B cells may play a role in psoriasis, they do not seem to be a major contributor to disease pathogenesis.

1.4.4.2 B cells in RA

B cells play a more prominent role in RA disease pathogenesis. In animal models of RA, B cell deficiency was found to abrogate disease (379, 380). B cells are thought to contribute to RA pathogenesis primarily by the production of autoantibodies. Autoantibodies against citrullinated peptides (ACPA), type II collagen (CII) and Fc portion of IgG known as rheumatoid factor (RF) are all major indicators of RA (381). Moreover, antibodies to CII has a direct effect on joint pathology by modifying collagen fibril synthesis (382, 383). Antibodies recognising CII also form immune complexes in the joint activating multiple pro-inflammatory pathways including complement activation and cytokine production by innate cells (384).

Evidence indicates that B cells may play a role in antigen presentation to T cells in the synovium. ACPA specific B cells have recently been found to be enriched in RA joints (385). Likewise, large presence of citrullinated peptides in RA joints has also been reported (386). This leads to the possibility that B cells might be interacting with ACPA specific T cells in the synovial environment, contributing to pathology (368). Rituximab therapy was also found to be efficacious in RA suggesting a prominent role for B cells in disease pathogenesis (387).

1.4.5 T cells

T cells are adaptive immune cells of the lymphoid lineage. T cells develop in the bone marrow and later migrate and mature in the thymus. Here they diverge into two separate lineages, CD4 and CD8. T cells express unique receptors on their cell surface named the T cell receptor (TCR). The TCR comprises of an α and a β chain. T cells also express CD3 and either CD4 or CD8 co-receptors (388).

During thymic development, random gene rearrangement events allows each T cell to express a unique TCR. The TCR recognises a single peptide antigen in the context of self MHC molecules. This system allows for T cells to recognise up to 10^{18} unique antigen specificities. T cells also undergo selection processes in the thymus which eliminate highly self-reactive cells. Despite this, some self-reactive T cells remain and in many cases, are partly responsible for the onset of autoimmunity (388).

Once educated, naïve T cells circulate through the blood and lymphatics and SLOs (389). This process only stops once the T cell encounters its cognate antigen. Upon recognition of antigen, and co-stimulation signals from the APC, T cells undergo clonal proliferation and maturation (390). These effector T cells then migrate to inflamed or infected tissues where they carry out their effector functions (12, 391-393).

Finally, upon pathogen clearance, the majority of effector T cells undergo apoptosis (284). A small minority of cells however, undergo transcriptional changes and become long lived memory cells (5).

In this section, the various cell types will be briefly explored with a focus on CD4 T cells and their role in mediating inflammatory diseases.

1.4.5.1 CD8 T cells

CD8 T cells are known as cytotoxic T cells. They regulate immune responses by killing damaged, infected or cancerous cells. Naïve CD8 T cells recognise antigen in the context of MHC I presentation by APCs. This recognition leads to clonal proliferation of CD8 T cells. These cells then migrate to tissue sites where they recognise their cognate antigen again on MHC I molecules which are expressed on all nucleated cells. Recognition triggers CD8 T cells to kill by forming pores using perforin and releasing toxic granzyme molecules inside the target cell (388).

1.4.5.2 CD4 T cells

CD4 T cells are often known as master regulators of the immune system. They primarily regulate immune responses via cytokine production (394). Upon antigen recognition via MHCII presentation by APCs, naïve CD4 T cells differentiate into one of five major subsets (395-399). The differentiation of T cells into subsets is regulated by cytokines in the immediate environment, usually produced by the APC (400). Table 3 below summarises the T cell subsets, cytokines needed for their differentiation, key transcription factors, CKR expression and effector cytokines. Proliferated CD4 T cells migrate to infected or inflamed tissues where they produce their effector cytokines. Subsets of

effector T cells also preferentially express sets of CKRs which allow them to home to specific tissue sites (14, 393, 401).

T cell subset	Key cytokines required for differentiation	Transcription factor required for differentiation	Cytokine profile	Chemokine receptor expression
Th1	IL-18 IL-12	T-bet STAT4	IFNγ TNFα	CCR5, CXCR3
Th2	IL-4 IL-2	GATA3 STAT6	IL-4 IL-5 IL-13	CCR4, CCR3
Th17	TGF-β IL-6 IL-21	RORγT STAT3	IL-17 IL-22	CCR6
Tfh	IL-6 IL-21	Bcl6 STAT3	IL-21	CXCR5
Treg	TGF-β	Foxp3	TGF-β IL-10	CCR4,5,6,8

Table 3 CD4 T cell subsets

1.4.5.3 Memory T cells

Memory T cells (CD4 or CD8) either remain at tissue sites or circulate through the vasculature, tissues and SLOs (5). This allows memory cells to come into rapid contact with recurring pathogens (402). However, unlike naïve T cells which require multiple signals for activation and proliferation, memory T cells have a much lower threshold of activation (403). Upon activation, memory T cells carry out their effector functions both more quickly and with a greater magnitude (404). In the case of CD8 memory T cells, this usually leads to faster and more effective cytotoxicity (405). CD4 memory T cells rapidly produce large quantities of their effector cytokines (404).

Like CD4 T cells, subclasses of memory T cells also exist (5). Memory T cells are subdivided based on their migration patterns. Tissue resting memory cells are

aptly termed tissue resident memory cells (Trm cells) while, circulating memory cells in SLOs or tissues are known as central memory T cells (Tcm cells) or effector memory T cells respectively (Tem cells). These cells are defined by their cell surface receptor expression (5). Table 4 summarises the memory cell subsets, their transcriptional profile and cell surface marker expression.

Memory cell subset	Cell surface marker expression	Tissue location
Tissue resident memory T-cells (Trm)	CD103+, CD69+	Non-circulating, resident in peripheral tissues
Effector memory T-cells (Tem)	CD62L lo, CCR7 lo	Circulating and found in both SLOs and in peripheral tissues
Central memory T-cells (Tcm)	CD62L hi, CCR7+	Found primarily at SLOs, mobilised upon infection

Table 4 Memory T cell subsets

1.4.5.4 Role of CD4 T cells in infection and inflammatory disease

CD4 T cells carry out critical functions in the control of infection and inflammation. Tfh cells provide help to B cells to produce class switched antibodies which directly aid in infection control (399). Th1 cell production of IFNγ primes macrophages for increased phagocytosis, increased pathogen killing and increased antigen presentation (325). IFNγ also has anti-viral effects by reducing viral replication and activating NK cells which are potent killers of virally infected cells (406).

Th2 cells on the other hand help in parasitic infection control. Th2 cytokines, IL-4, IL-5 and IL-13 activate basophils, eosinophils and mast cells. They carry out multiple functions such as degranulation which are anti-parasitic. Finally, Tregs are potent producers of the anti-inflammatory cytokines IL-10 and TGFβ. These

cytokines dampen immune responses by reducing inflammatory mediator production as well as activating pro-resolving pathways (407).

While CD4 T cells are crucial in controlling infection and inflammation, dysregulation of CD4 T cell function causes some of the most debilitating and difficult to treat inflammatory conditions. Various functions of CD4 T cell subsets have been implicated in chronic inflammatory diseases.

1.4.5.5 CD4 T cells in Psoriasis

Psoriasis is an autoimmune inflammatory disease of the skin. Psoriatic lesions demonstrate significant hyperplasia of keratinocytes (68). For this reason, psoriasis was initially considered a disease of skin cell dysfunction. However, genetic analysis of psoriatic patients soon implicated adaptive immune cells and specifically CD4 T cells (73).

Subsequently, subsets of CD4 T cells were found to accumulate in psoriatic skin lesions (7, 408). Moreover, Th1 and Th17 cell cytokines IFNγ and IL-17 levels were found to be elevated in both psoriatic lesions as well as in peripheral blood of psoriasis patients (353, 354). Interestingly, IFNγ was shown to increase Th17 cell migration into murine inflamed skin lesions (409). Th17 cell cytokines IL-23 and IL-21 were found to directly cause keratinocyte hyperplasia (410). This provided key evidence that psoriasis was mediated by a combination of Th1 and Th17 cells.

Further evidence in the role of CD4 T cells in psoriasis pathogenesis comes from studying the efficacy of psoriasis therapeutics. T cell co-stimulation blocker anti-CTLA4 Ig is efficacious in psoriasis (411). Recently developed monoclonal antibody against the adhesion molecule LFA-1 was also highly successful. This antibody interferes with T cell-DC interactions but also stops effector T cell migration into psoriatic lesions (1, 360).

1.4.5.6 CD4 T cells in RA

RA is an autoimmune disease characterised by articular destruction. However, joint destruction occurs months or even years downstream of the breakdown of immunological tolerance. It is this critical event, that leads to the development

of auto-reactive CD4 T cells which ultimately orchestrate RA pathogenesis (363) . Large numbers of CD4 T cells accumulate at RA synovial tissues throughout disease (84).

Moreover, in multiple mouse inflammatory joint disease models, CD4 T cells are essential for disease initiation, progression and pathogenesis (412, 413). Likewise, CD4 T cell depletion using multiple mechanisms abrogated joint pathology in mice (414, 415). In humans, CD4 T cell co-stimulation blockade was also found to be efficacious (370). Therefore, evidence strongly implicates CD4 T cells in RA pathology.

Unlike infectious diseases where homogeneous CD4 T cell subsets often regulate disease; multiple T cell subsets are implicated in RA pathophysiology at different disease stages (416). Analysis of early RA joints demonstrates an abundance of Th2 and Th17 cytokines. Established disease joints however demonstrate aggregates of IFNγ producing Th1 cells (417). These T cell cytokines enable constant activation of tissue resident macrophages, stromal cells, osteoblasts and chondrocytes leading ultimately to joint destruction (90).

While accumulation of T cells at inflamed tissues is not unusual, RA joints exhibit large numbers of non-joint specific T cells such as influenza specific T cells (418). Moreover, a joint homing chemokine profile has not been identified. Some studies have implicated type I IFNs in aiding T cell persistence via decreased apoptosis at RA joints (419, 420). Others have identified the chemokines CXCL12/CXCR4 as important in T cell retention (84, 421).

Psoriasis and RA are two examples of inflammatory diseases where trafficking of T cells remains a critical but poorly studied area. Considering the destructive influence of effector T cells in these tissues, targeting T cell trafficking pathways remains an attractive area for anti-inflammatory therapeutics. This project aims to use reductionist models to elucidate mechanisms of T cell persistence at inflamed tissues. In the next section, an overview of current understanding of T cell trafficking will be explored.

Relevance of this section to thesis aims:

- CD4 T cells play critical regulatory role in inflammatory disease pathogenesis

- Do innate immune cells at inflamed sites directly or indirectly influence the persistence of activated CD4 T cells?

- Do APCs influence the persistence of CD4 T cells at inflamed sites?

- Does the activation status of the T cell influence its persistence at inflamed sites?

1.5 T cell trafficking

In a naïve host, only a few thousand T cells are specific for a given antigen (422). Naïve T cells therefore, constantly circulate through SLOs using blood and lymphatic vasculature to encounter its cognate antigen (389). Upon antigen encounter, effector T cells proliferate and migrate out of SLOs and enter inflamed tissues (390). Here, they carry out their effector function and either egress back into circulation or undergo apoptosis. In the long term, some cells also develop into memory cells (403).

The processes of naïve circulation, activation, effector cell LN egress, tissue entry, apoptosis and tissue egress are strictly controlled. In this section, these aspects of T cell trafficking will be explored in the context of inflammation.

1.5.1 Naïve T cell circulation

Naïve lymphocyte recirculation between blood and SLOs was first demonstrated as far back as the 1960s (423). Circulating naïve T cells express CD62L, a lymph node homing molecule (424). CD62L interacts with glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) expressed by cells of the high endothelial venules of SLOs (424). This interaction enables T cells to enter LNs through high endothelial venules (HEVs). Naïve T cells express high levels of the CKR CCR7, which is often considered a LN homing CKR (424). Upon LN entry, CCL19/21 levels guide T cell motility, migration and positioning (425, 426). These naïve T cells spend 6-12 hours surveying a LN for antigen. If no antigen is detected, naïve T cells migrate through cortical or medullary sinuses of the LN and exit through efferent lymphatic vessels (427, 428).

T cell exit from LNs is regulated by S1PR1 and its ligand S1P (239, 243). Lymphatic endothelial cells (LECs) produce S1P in efferent lymphatic vessels (429). T cells expressing S1PR1 migrate towards increased S1P gradients in LECs which guides them out of the LN (430). In most cases, these T cells travel to the downstream draining lymph node and carry out the same process, eventually exiting back into the bloodstream.

1.5.2 T cell activation and effector cell LN egress

Along with CCR7, naïve T cells also express high levels of S1PR1 (431). Upon LN entry and survey, if a T cell detects their cognate antigen, some immediate changes take effect to alter its migration pattern. T cells upregulate CD69, a cell surface C-type lectin protein (432). CD69 is a negative regulator of S1PR1 (433). Therefore, activated T cells rapidly downmodulate their S1PR1 expression (434). This ensures increased T cell dwell time in LNs to allow them to undergo clonal proliferation. These T cells also express increased levels of CCR7 to further aid LN retention (243). Sustained CCR7 signals however, desensitise the CKR (435).

At this stage, the now effector T cells are ready for LN exit. They rapidly upregulate S1PR1 on their cell surface and downregulate CCR7 (243). This releases T cells from a retention phenotype to an egress phenotype. These T cells start randomly moving in the LN until they encounter a lymphatic sinus where they can sense S1P (436, 437). This promotes egress of effector T cells from LNs. Studies using the S1PR1 functional antagonist FTY720, demonstrated that T cells "log jam" at lymphatic sinuses and fail to exit LNs in the absence of S1P signals (272, 438, 439). S1P concentration is low in LNs, gradually increases in lymphatic vessels and is greatest in blood vessels (430, 440). Effector T cells use this gradient to circulate between SLOs and vasculature (430).

1.5.3 Effector T cell tissue entry

Effector T cells display tissue tropism (441). Tropism refers to the tendency of a T cell to preferentially migrate to a particular tissue site for example, the skin over others such as the gut. This tissue tropism is determined by selective expression of tissue homing receptors upon T cell activation (13, 15, 349). Which tissue-homing receptors are expressed on T cells are determined by the environment of the lymph node where they were activated. Moreover, the location of the migratory DCs which activates the T cell also plays a role in determining T cell tissue tropism (441).

Tissue homing receptors primarily include cell adhesion molecules and CKRs (441). In humans, two such adhesion molecules, integrin $\alpha 4\beta 7$ and cutaneous

leukocyte antigen (CLA), have been found to direct effector T cells to the gut and the skin respectively (13, 15, 16, 442). Inflamed vascular endothelium adjacent to these tissue sites express elevated levels of mucosal vascular addressin cell-adhesion molecule 1 (MADCAM-1) and E-selectin or P-selectin which are ligands for $\alpha4\beta7$ and CLA respectively (13, 15, 16, 442).

Chemokines and CKRs are likewise necessary for T cell tissue tropism to the gut and skin. $\alpha4\beta7$ expressing T cells also selectively express the CKR CCR9 (443). CCL25, the ligand for CCR9 is expressed almost exclusively in the small intestinal lamina propria (17). Similarly, CKRs CCR4 and CCR10 are considered skin homing (444, 445). Most CLA expressing CD4 T cells also express CCR4 (445). Moreover, CCR4 ligand CCL27 is exclusively expressed by skin resident keratinocytes (14, 444). Similar to adhesion molecules, chemokines are upregulated at inflamed tissues, allowing tissue specific effector T cell entry into the affected tissue. Therefore, adhesion molecules and CKRs synergistically regulate effector T cell tissue entry at inflamed sites.

It is however, important to note, that many tissue specific effector T cells can be found at other tissue locations such as the lung or the brain (446). The tissue "post-code" system is therefore not absolute. Additionally, inflamed tissues are generally a permeable environment for circulating leukocytes (149). As such, many non-specific effector and even naïve T cells can be found at profoundly inflamed tissues (447, 448).

1.5.4 Tissue egress

Effector and memory CD4 and CD8 T cells produce cytokines and kill infected cells at inflamed tissues respectively (449). At the end of an effector response, T cells leave inflamed tissues via afferent lymphatics into tissue draining LNs (450, 451). Failed exit of effector T cells has been associated with persistent tissue inflammation (245). A couple of studies have identified CCR7/CCL21 as well as S1PR1/S1P as regulators of T cell tissue exit (244, 452).

Brown et al. demonstrated that fewer $CCR7^{-/-}$ T cells exited tissues compared to wild type cells in acute but not chronic inflammation (453). Additionally, $CCR7^{-/-}$

T cells persisted at acutely inflamed lungs, epithelial tissues and skin (452, 454, 455).

Overexpression of S1PR1 on CD8 T cells was found to increase their tissue exit from multiple tissues including lungs, kidney, skin and salivary gland (456). Furthermore, CD69 deficient CD8 T cells failed to persist at HSV infected skin tissue. When these same cells were treated with an S1PR1 agonist however, their skin retention was restored (433). In contrast, two studies on CD4 T cells demonstrated that at acutely inflamed tissues, S1PR1 signals were responsible for reduced T cell egress from tissue sites (244). At chronically inflamed tissues however, S1PR1 and S1P was found to once again promote T cell tissue egress (453).

1.5.5 T cell death

Following tissue egress or indeed while still in tissue, most effector T cells undergo programmed cell death (PCD) (284). PCD can be triggered via one of three mechanisms: Autophagy, necroptosis or apoptosis.

1.5.5.1 Autophagy

Macroautophagy (referred to as autophagy) is a catabolic cellular process that is important in protein and intracellular organelle turnover as well as cytoplasmic renewal (457). Autophagy occurs in autophagosomes which are double membraned vesicles with cytoplasmic material inside. Autophagy is regulated by a host of autophagy related genes (ATG) (457).

In T cells, autophagy has been reported to promote both apoptosis and survival (458). Two recent studies have demonstrated that effector CD4 T cells undergo cell death via autophagy in the absence of FADD, caspase-8 or Irgm-1 proteins (459, 460). In contrast, decreased number of thymocytes and peripheral T cells were found in $ATG5^{-/-}$ mice. Furthermore, increased numbers of $ATG5^{-/-}$ T cells underwent apoptosis on TCR stimulation than WT T cells (461). Inhibition of autophagy in effector Th2 cells was also found to increase T cell death (462).

1.5.5.2 Necroptosis

Necroptosis is a programmed form of necrosis (463). It shares characteristics with both necrosis (unprogrammed cell death) and apoptosis. Necroptotic cells undergo nuclear condensation and organelle swelling like necrotic cells. However, the programmable nature of this death makes it similar to apoptosis. Molecular characterisation of necroptosis identified the use of two proteins receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) as the hallmark of necroptotic cell death (464).

Evidence for necroptosis in T cells is minimal. One study by Ch'en et al. demonstrated that caspase-8 deficient effector T cells undergo cell death using RIPK1 and RIPK3 mediated pathways. Moreover, they displayed typical necroptotic morphology (465). More recently, another study by Kesarwani et al. found that tumour specific CD8 T cells undergo TCR stimulation mediated necroptosis. Pre-treating T cells with necroptosis inhibitors increased CD8 T cell tumour persistence (466).

1.5.5.3 Apoptosis

Apoptotic cell death is the best studied and most common form of effector T cell death. Apoptotic cell death can occur using multiple mechanisms which can be largely divided between extrinsic apoptotic pathways and intrinsic apoptotic pathways (284). T cells undergo activated cell autonomous death (ACAD) which occurs in the absence of survival signals by the intrinsic apoptotic pathway (467). Activation induced cell death (AICD) may also be triggered in effector cells with TCR restimultation in the absence of secondary signals (468). This is mediated by the extrinsic apoptotic pathway.

A set of proteins, often termed "the executioners of death" are caspases. Caspases are produced by cells as inactive zymogens that must be activated for effector function (469). There are two classes of caspases: initiator caspases (caspases 1,2,4,5,8,9,10, 11 and 12) and effector caspases (caspases 3,6,7 and 14) (470, 471). Initiator caspases undergo activation by external stimuli and form large multi-protein complexes where effector caspases are activated. Effector caspases disrupt various cellular processes such as actin polymerization and

nuclear lamin formation. This causes the characteristic chromatin condensation and nuclear shrinkage (470, 471). Figure 1.5.1 provides a simple overview of both the intrinsic death pathway.

1.5.5.3.1 Extrinsic apoptotic pathway

Extrinsic pathway signals emanate from extracellular signals. These include TNF, CD95L or FAS ligand (FASL) and TNF-related apoptosis inducing ligand (TRAIL). They interact with their respective death receptors which include TNFR1, CD95 or FAS, TRAILR1 and TRAILR2 (468, 472, 473). Death receptor triggering results in the formation of death-inducing signalling complex (DISC). In the case of FASL-FAS pathway, complex molecular interactions in the DISC results in the activation of initiator caspases 8 and 10 (474, 475). These caspases then activate effector caspases 3,6 and 7 which initiate apoptosis (284, 476).

Alternatively, caspase 8 can also activate the pro-apoptotic functions of the mitochondria by cleaving the BCL-2 (B cell melanoma-2) family protein BID (BH3 interacting domain-death agonist) to tBID (truncated BID). tBID causes the aggregation of pro-apoptotic proteins BAX (BCL-2 associated X protein) or BAK (BCL-2 antagonist/killer) to the mitochondria. This causes the release of cytochrome c from the mitochondria. Cytochrome c directs the formation of another death complex known as the apoptosome. In this complex, molecular interactions result in the activation of caspase 9. Caspase 9 then activates effector caspases 3,6 and 7 which initiates apoptosis (284, 476, 477).

AICD is an example of extrinsic cell death in T cells. AICD is triggered in T cells by the ligation of FASL, TNFR1 and TRAILR. Most activated T cells express these receptors after undergoing several rounds of clonal proliferation (284). Interestingly however, T regulatory cells are resistant to FASL mediated apoptosis (478).

1.5.5.3.2 Intrinsic apoptotic pathway

The intrinsic pathway of apoptosis is triggered by a myriad of stimuli including TCR stimulation, DNA damage and cytokine deprivation. These stimuli instigate the permeabilisation of the mitochondrial membrane which results in the release of cytochrome c (479). Cytochrome c, in conjunction with APAF1, (apoptotic-

protease-activating factor 1) found in the cytoplasm, form the apoptosome. Apoptosome formation results in activation of caspase 9 and the downstream activation of caspases 3,6 and 7 as stated previously (471).

ACAD is an example of intrinsic cell death in T cells. Activated T cells deprived of cytokines, produce BCL-2 family members BIM (BCL-2 interacting mediator of cell death) and PUMA (p53-upregulated modulator of apoptosis). These are both pro-apoptotic molecules that bind to, and inhibit anti-apoptotic molecules BCL-2 and BCL-XL. BCL-2 and BCL-XL themselves inhibit accumulation of pro-apoptotic BAX or BAK to the mitochondria, thus maintaining mitochondrial membrane integrity. Once BCL-2 and BCL-XL are inhibited however, BAX or BAK actively accumulate at the mitochondria and induce the release of cytochrome c and its downstream effects (284, 476).

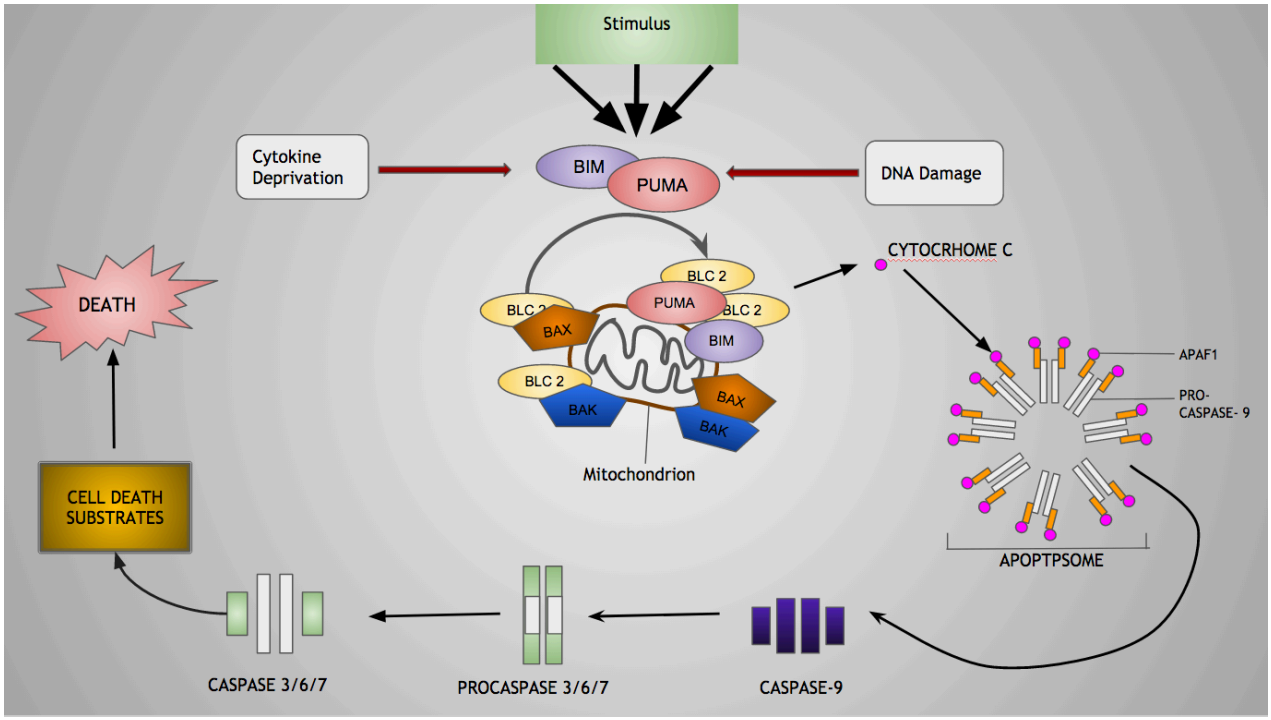

Figure 1.5.1 The intrinsic apoptotic pathway
Diagram adapted from Life and death in peripheral T cells (284)

1.5.6 T cell retention

To enable effector T cells to carry out their functions, they must persist at tissue sites following their recruitment and prior to their egress or death. This phenomenon is referred to as their retention. Retention of T cells remains, arguably, the least understood area of the T cell trafficking pathway. Current

models suggest that retention of T cells at tissue sites is mediated by a combination of three factors. T cells respond to positive cues which keep them at inflamed tissues (9, 170, 421, 480, 481). In addition, T cells downregulate egress receptors slowing down their exit (244, 245, 432, 433, 453). Finally, T cells alter their metabolic programming to enable prolonged survival (482, 483).

One of the earliest identified retention signals for T cells was the TCR-Peptide MHC interaction. APCs at sites of infection/inflammation interact with effector T cells with cognate antigen/TCR, forcing their arrest and accumulation at peripheral tissues (392).

Another positive retention cue is the cytokine TGF-β. TGF-β stimulates the expression of the integrin αEβ7, also known as CD103, on CD8 and CD4 T cells (484). This enables CD8 and CD4 Trm cells to bind to E-cadherin expressed by epithelial cells, leading to their retention in peripheral tissues including skin, small intestine and brain (485-488). Likewise, CD25+ FoxP3+ regulatory CD4 T cells also express CD103 for their retention at peripheral tissues (481).

Multiple chemokine-chemokine receptor pairings have also been found to provide tissue retention signals to T cells. CX3CL1 was found to be necessary for the retention of effector CD4 T cells in the skin in a model of atopic dermatitis (480). In human rheumatoid synovium, CXCL12 and CXCR4 interactions have been described as crucial for the retention and accumulation of T cells (421).

Chemokines have also been found to increase the survival of T cells at peripheral inflamed tissues. Indeed, CXCL12 was found to increase the survival of tumour infiltrating T cells as well as increase their chemotaxis (489, 490). Likewise, IL-15 is necessary for the survival and retention of CD8 Trm cells at peripheral tissues (491). Expression of the aryl hydrocarbon receptor (AHR) has also been implicated in aiding survival mediated persistence of T cells at tissue sites (492). Recently, TRM cells have also been found to alter their metabolic programming and depend on fatty acid uptake and metabolism for their survival and persistence in tissues (483).

Egress of T cells out of tissues is regulated by the receptors CCR7 and S1PR1 as discussed previously (239, 451, 452). The expression of both S1PR1 and CCR7 is

under the regulation of the transcription factor KLF2 (450, 456, 485). Moreover, CD69 is another molecule which regulates the expression of S1PR1 (433, 434). Both CD8 TRM cells and CD4 TRM cells have been found to express high levels of CD69 and low levels of KLF2 (456, 493). Indeed, while long term tissue resident cells may need increased chemotactic cues as well as decreased egress cues, a reduction in egress cues alone could be enough to increase the dwell time of early effector T cell populations. This would enable pathogen elimination without causing long term inflammation induced tissue damage. The dynamic regulation of S1PR1 and CCR7 expression makes them the perfect molecules to facilitate early retention of T cells in tissues.

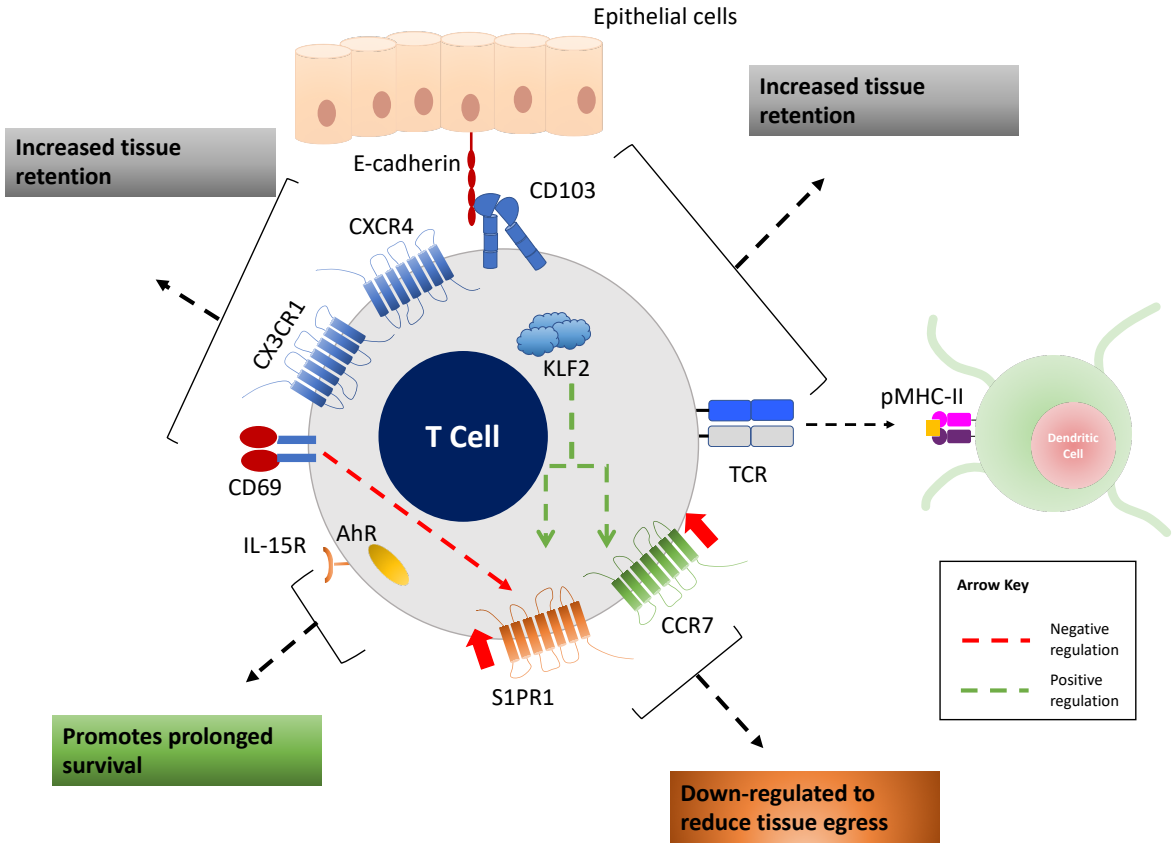

Figure 1.5.2 Regulation of T cell retention at peripheral tissues
T cell retention is regulated by positive retention cues which include TCR-pMHC, CD103 and chemokines. T cells also receive specialised survival signals such as IL-15, AhR ligands and fatty acids which enables their prolonged survival in tissues. Finally, T cells downregulate egress receptors CCR7 and S1PR1. S1PR1 and CCR7 expression is regulated by KLF2. CD69 is also a negative regulator of S1PR1 expression.

Relevance of this section to thesis aims:

- T cell persistence at inflamed tissues could be regulated at multiple checkpoints of the T cell trafficking pathway

- Is activated T cell persistence due to decreased egress or increased retention signals at inflamed tissues?

- Could increased activated T cell persistence be linked with their improved survival at inflamed tissues?

- Could the behavioural patterns of activated T cells at inflamed sites influence their persistence? i.e. their interaction with other immune cells or the availability of localised soluble mediators.

1.6 Aims of the project

Effector CD4 T cells accumulate at inflamed tissues in large numbers. As demonstrated in psoriasis and RA, accumulated CD4 T cells contribute to disease pathology. Moreover, removal of these cells from such sites abrogates disease. Increased T cell accumulation can be controlled at multiple points of the T cell trafficking pathway. Most studies have targeted T cell activation, inhibiting LN egress as well as inhibiting tissue entry of effector CD4 T cells.

In contrast, little is known about effector T cells after entry at inflamed peripheral tissues. Signals that allow CD4 T cells to persist at inflamed tissues are poorly understood. Likewise, few studies have investigated regulation of effector T cell egress from inflamed tissue sites.

This study aims to identify novel mechanisms of CD4 T cell persistence at inflamed tissues. Understanding these mechanisms will permit improved targeting of CD4 T cell mediated therapies at inflamed tissues. To achieve these goals, the aims in the project were set out as follows:

1. Establish a model inflamed tissue where effector CD4 T cells persist.

2. Identify which signals promote CD4 T cell persistence at inflamed tissue.

3. Identify the effect these signals have on CD4 T cell behaviour.

4. Identify whether similar control mechanisms exist in established human inflammatory disease.

2 Materials and Methods

2.1 Animals

All animals were housed at the University of Glasgow central research facility in accordance with UK home office guidelines. Animals were caged appropriately and allowed food and water *ad libitum*. All procedures undertaken were approved under Prof. Paul Garside's home office project license (60/4368). During this project, five different strains of animals were used:

2.1.1 C57BL/6

Male C57BL/6 mice were purchased from Harlan laboratories UK (currently ENVIGO) at 6 weeks of age. Mice were rested for 7 days upon arrival prior to commencement of any procedures. All C57BL/6 mice were used within the ages of 6-10 weeks as recipients in adoptive transfer experiments.

2.1.2 CD45.1 OT-II

CD45.1 OT-II T cell receptor (TCR) transgenic (Tg) mice (494) were bred in house. Most T cells in OT-II mice express a TCR which exclusively recognises ovalbumin (OVA) peptide 323-339. OT-II mice in my lab also express CD45.1 on all leukocytes. CD45 is a pan-leukocyte tyrosine phosphatase. There are two alleles of CD45, CD45.1 and CD45.2 They are both functionally identical. This enables the identification of cells transferred from a donor to a host with different congenic backgrounds (i.e. transfer cells from an OT-II CD45.1 host to a C57BL/6 CD45.2 donor) by flow cytometry. T cells from male OT-II mice were used between 6-10 weeks of age as donors for polarisation and adoptive transfer experiments.

2.1.3 hCD2DsRed x CD45.1 OT-II (OT-II DsRed)

hCD2DsRed mice were originally gifted by D Kioussis and A Patel, National Institute of Medical Research, London and bred in house at the University of Glasgow (495). hCD2DsRed mice have a DsRed fluorescent reporter tag attached to the CD2 promoter. This allows T cells to fluoresce red when excited with 554nm wavelength of light. Some NK cells and lymphoid tissue inducer (LTi) cells also express DsRed in these animals. hCD2DsRed mice were crossed with CD45.1

OT-II mice to make OT-II DsRed mice. This ensured that most DsRed T cells also expressed a TCR specific for ovalbumin peptide 323-339 as described above.

2.1.4 LysMGFP

LysMGFP mice (496) were originally gifted by Sussan Noursargh, William Harvey Research Institute, London, and bred in house at the University of Glasgow. LysMGFP mice have a green fluorescent protein (GFP) tag attached to mouse lysozyme M protein. Monocytes, neutrophils and some macrophages in these mice express GFP. LysMGFP mice were used as recipients for some intravital microscopy experiments.

2.1.5 CD11cYFP

CD11cYFP mice (497) were bred in house and used as recipients for intravital microscopy experiments. These mice have a yellow fluorescent protein (YFP) tag attached to the CD11c protein. This enables most dendritic cells and some macrophages and monocytes to fluoresce yellow when excited with 500nm wavelength of light. CD11cYFP mice were used as recipients for some intravital microscopy experiments.

2.2 Induction of inflammation

2.2.1 LPS model

Animals were first anaesthetised by isoflurane inhalation prior to LPS injection. Inflammation was then induced by intradermal injection of 10μg of bacterial lipopolysaccharide (LPS) from Escherichia *coli* (E.*coli*) strain 0111:B4 (Sigma-Aldrich) in a final volume of 10μl in sterile phosphate buffered saline (PBS)(Gibco). Intradermal injection was carried out in the ear pinnae of mice to allow the development of an inflamed tissue site in the mouse skin.

2.2.2 Chronic inflammatory model – using inert polybeads

The beads model is akin to a delayed type (DTH) inflammation in mouse ear pinnae. To achieve this, mice were injected subcutaneously with OVA emulsified in complete Freund's adjuvant (CFA). 100μg of a OVA/CFA was injected

subcutaneously into the scruff of mice in a final volume of 100μl in PBS. Following a rest period of 7-9 days, 25μg of OVA conjugated to polystyrene beads was injected intradermally into the ear pinnae of anaesthetised mice in a final volume of 10μl in PBS.

2.2.3 Preparation of Beads

Polybeads (Polysciences Inc.) were made up using the standard manufacturers guidelines. Briefly, 500μl of beads (2.6% w/v) (12.5mg) were washed twice in polylink wash buffer then resuspended in 0.17ml polylink coupling buffer. 20μl of a 200mg/ml EDAC solution was then added to the coupling buffer and beads mix. Subsequently, 2mg of OVA made up in 1ml of PBS was added to the same tube incubated overnight at room temperature (RT) in a shaker. The next day, the beads were washed twice, making sure to collect and store the supernatant for free protein measurement. The beads were finally resuspended in 0.4ml of storage buffer and stored at 4° C until required. The supernatant was measured for free protein using a nanodrop and the total protein bound to the beads was calculated accordingly.

2.3 T cell polarisation

2.3.1 Tissue harvesting

OT-II and C57BL/6 mice were euthanised by cervical dislocation or CO_2 administration. Lymph nodes (OT-II) and spleen (OT-II and C57BL/6) were collected in PBS and stored on ice at all times. Tissues from OT-II mice and C57BL/6 mice were used for T cell isolation and antigen presenting cell (APC) fraction preparation respectively.

2.3.2 Tissue processing

Collected tissue was prepared into a single cell suspension by disrupting them through a 40μm sieve (Greiner Bio-One) using the rubbery side of the plunger of a 3ml syringe (BD Biosciences) in 1ml of PBS. The cells were washed through using sterile PBS and collected in a 50ml falcon tube (Corning). Cells were then centrifuged at 400x G for 5 minutes at 4°C to form a pellet. The supernatant was poured off and the pellet resuspended in either 1ml/10 LNs MACS buffer (1% FCS,

2mM EDTA in HBSS) or 0.5ml/spleen red blood cell (RBC) lysis buffer (eBioscience).

Splenocytes were incubated at 4°C for 2 mins before washing thoroughly with PBS. The splenocytes were then resuspended in 5ml of complete RPMI (cRPMI) (10% FCS, 5% Pen/Strep, 5% L-glutamine) and stored at 4ºC for later use.

2.3.3 CD4 T cell isolation

CD4 T cells were isolated by negative selection from single cell suspension of the LNs and spleens from OT-II mice. To do this, a CD4 isolation kit was used from STEMCELL technologies (Vancouver, Canada) according to their guidelines. Briefly, cells were transferred from 50ml falcon to a 5ml polystyrene tube (Corning). 50μl of rat serum (STEMCELL technologies) was added to the tube per ml of cell suspension. Subsequently, 50μl of the isolation antibody cocktail was added to the tube per ml of cell suspension and incubated for 10 minutes at RT.

Next, 75μl of RapidSphere beads were added per ml of cell suspension and mixed thoroughly before incubating at RT for 2.5 mins. These magnetic beads bind to all cells with antibody bound to them. The tube was then topped up to 2.5, 5 or 10ml based on the number of LNs harvested with MACS buffer. The tube was then inserted into a Big Easy magnet and incubated at RT for 2.5 minutes allowing beads bound cells to separate from untouched CD4 T cells. Finally, the magnet was inverted rapidly with the tube inside to allow the unbound CD4 T cells to flow out into a 50ml falcon tube.

2.3.4 APC fraction preparation

The beads bound cells from the CD4 T cell isolation were washed and mixed in with the splenocytes prepared earlier in cRPMI and centrifuged and resuspended in 10mls of cRPMI (APC fraction). 500μl of Mitomycin C (Sigma-Aldrich) prepared at 1mg/ml with sterile PBS was then added to the APC fraction and all cells incubated for 1hr at 37°C with intermittent mixing. Mitomycin C interferes with DNA, RNA and protein synthesis and therefore restricts APC proliferation or cytokine production. Mitomycin C treated APCs were then washed 3 times in cRPMI before using them for T cell polarisation.

2.3.5 CD4 T cell polarisation

Isolated T cells were cultured with mitomycin C treated APCs at a ratio of $0.5x10^6$ T cells:$2.5x10^6$ APC ratio in cRPMI. These cells were cultured in Th1 polarising conditions. For Th1 polarisation, cultured cells were supplemented with 1μg/ml Ova $Peptide_{323-339}$ (Sigma-Aldrich), 2-4μg/ml anti-IL4 (BioXcell) and 10ng/ml IL-12 (R&D systems). Cells were cultured in a T25 flask in 50ml volume for 3 days at 37°C and 5% CO_2.

2.4 T cell treatments

For some experiments, CD4 T cells were treated with certain compounds before their adoptive transfer. Polarised CD4 T cells were treated with Pertussis Toxin (100ng/ml) (Sigma-Aldrich), FTY720 (Sigma-Aldrich) (0.5μg/ml), SEW2871 (R&D Systems) (5μg/ml), W146 (R&D Systems) (5μg/ml) or vehicle for 60 mins (Pertussis Toxin) or 90 mins in cRPMI at 37°C and 5% CO_2. Once treated, T cells were washed thoroughly in PBS three times. The cells were then resuspended in PBS in preparation for adoptive transfer.

2.5 T cell fluorescent labelling

For proliferation experiments, T cells were fluorescently labelled before adoptive transfer. After polarisation, T cells were incubated with cell tracker blue (Thermo-Fischer scientific) at 5μM for 15 mins at 37° C in PBS. The cells were shaken at 5 min intervals. Following staining, the dye was quenched by mixing with 1/5 volume of FCS and incubating for 5 mins. The cells were then washed in cRPMI twice to remove any excess dye and resuspended in PBS for adoptive transfer.

2.6 T cell adoptive transfer

T cells were adoptively transferred intradermally into inflamed or non-inflamed ear pinnae. This transfer was carried out for subsequent enumeration of cells by flow cytometry or imaging by intravital microscopy.

For flow cytometry enumeration, polarised cell culture was washed and resuspended in MACS buffer in a 5ml polystyrene tube in a total volume of 5-

10ml. The tube was then placed in the Big Easy magnet for 2.5 mins to remove all beads. The beads free cell suspension was poured into a 50ml falcon and resuspended in a known volume of sterile PBS and counted. 2-3x10^6 cells were then adoptively transferred into mouse ears.

For intravital microscopy, similar steps were carried out, however, a much lower number of cells, 2-300x10^3 were transferred in very small volume (2-4μl) at shallow depths in the ear pinnae. This facilitated the visualisation of cells with an intravital microscope.

2.7 Tissue digestion for flow cytometry

Mouse ears and superficial cervical draining lymph nodes were harvested into empty 6 well plates or submerged in PBS respectively. The ears were then cut into small pieces and digested using 2mg/ml Collagenase IV (Sigma-Aldrich), 2mg/ml hyaluronidase (Sigma-Aldrich) and 100 Units/ml DNase I (Invitrogen) prepared in PBS (Gibco) and incubated at 37ºC for 40 min at 180 RPM in a rotating incubator in a total volume of 2 ml. Following incubation, a single cell suspension of the ears was prepared with a gentleMACS dissociator (Miltenyi Biotec) using a gentleMACS C tube (Miltenyi Biotec). The cells were then counted using a haemocytometer and dead cells were excluded via trypan blue staining. Samples were stained for flow cytometry as described in section 2.8.

Draining lymph nodes and spleens were disrupted into a single cell suspension in a petri dish between two pieces of 40μm nitex. The cells were then counted and stained for flow cytometry using antibodies as described in section 2.8.

2.8 Flow cytometry

Multiple types of flow cytometry staining were undertaken as part of this project. These included extracellular, intracellular and FLICA staining:

2.8.1 Extracellular staining

Single cell suspensions of cells were added to a 96 well round bottomed plate. The cells were incubated with a fixable viability dye (eBioscience) diluted in PBS for 20 mins at 4ºC. The cells were subsequently incubated with FC block (24G2

grown in house and mouse serum) for 20 mins at RT. Flow cytometry antibodies were then added at the appropriate concentration (section 2.8.6) and the cells were incubated for a further 20 mins at RT. Finally, cells were washed in FACS buffer and transferred through 40μm nitex into polystyrene FACS tubes. The samples were acquired on a Macsquant analyser (Miltenyi Biotec).

2.8.2 Intracellular staining

Intracellular staining was carried out following staining with extracellular antibodies using the BD bioscience intracellular staining kit. Samples were fixed and permeabilised using the fixation/permeabilisation buffer, incubated at RT for 30 mins, then washed using a perm/wash buffer. Intracellular antibodies were diluted in perm/wash buffer and then added to the samples. Samples were incubated at RT for 45 mins. The cells were then washed, transferred through nitex and acquired as stated previously.

2.8.3 FLICA staining

Cell death was assayed using a fluorescent inhibitor of caspase assay kit (FLICA) (Life technologies). After extracellular staining, samples were resuspended in 100μl cRPMI, then incubated with FLICA reagent diluted in PBS. Cells were cultured for 60 mins at 37°C with 5% CO_2. The cells were then washed, transferred through nitex and acquired as stated previously.

2.8.4 Data analysis

Flow cytometry data was analysed using Flowjo (Treestar) version 10.0 and graphs were plotted on Graphpad PRISM for statistical analysis.

2.8.5 Gating strategy

Representative flow cytometry gating strategies are outlined below for mouse ear tissue and lymph nodes. All subsequent analyses were carried out on the gated transferred OT-II CD4 T cells.

2.8.5.1 Ear pinnae

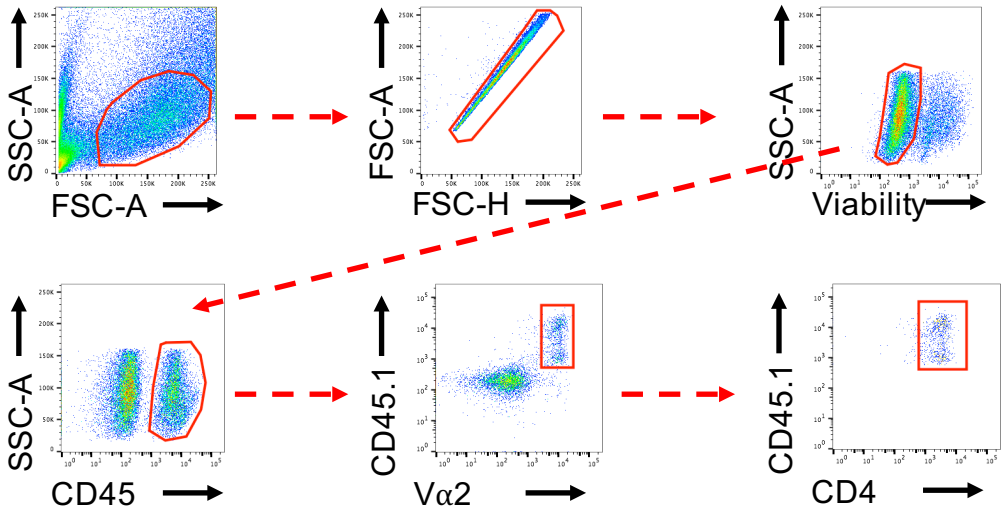

Figure 2.8.1 Gating strategy for the analysis of transferred CD4 T cells in ear pinnae
Cells were first selected based on their forward and side scatter. Doublets were excluded by using FSC-A and FSC-H. Dead cells were then excluded and immune cells were identified based on their expression of CD45. Finally, transferred OT-II T cells were identified by selecting for the cells expressing both CD45.1, Vα2 and CD4. Total cell numbers were determined by back calculating from these plots.

2.8.5.2 Selection of transferred OT-II T cells

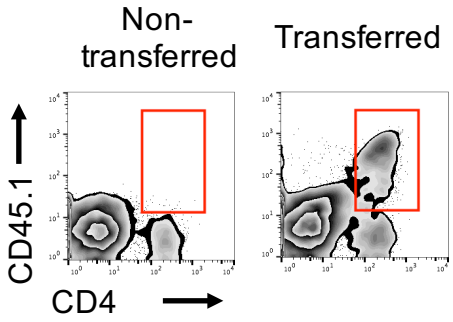

Figure 2.8.2 Gating strategy for the selection of transferred OT-II T cells
Transferred cells were identified by their expression of CD45.1, CD4 and/or Vα2. Gates for transferred cells were set on samples from animal tissue where no OT-II T cells were transferred. This enabled the clear identification of transferred OT-II T cells.

2.8.5.3 Lymph nodes

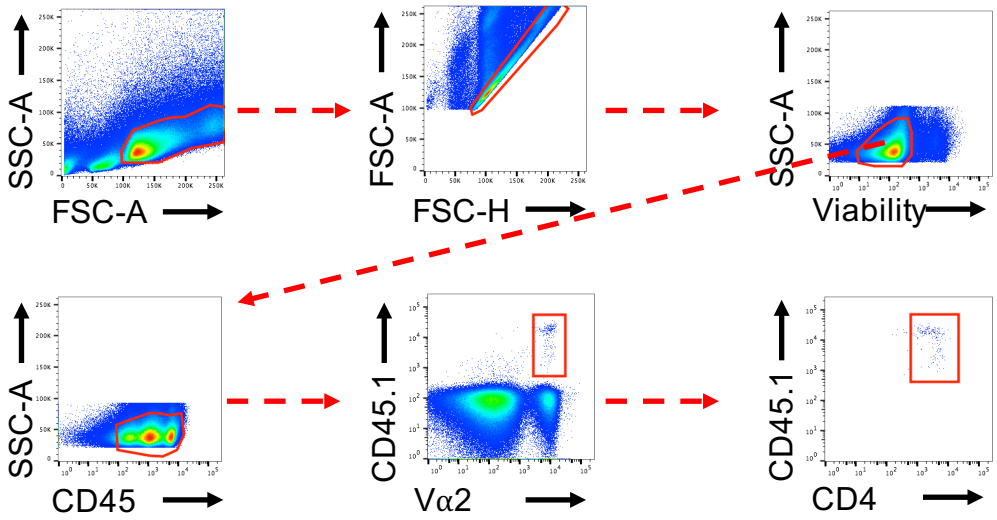

Figure 2.8.3 Gating strategy for the analysis of transferred CD4 T cells in lymph nodes
Cells were first selected based on their forward and side scatter. Doublets were excluded by using FSC-A and FSC-H. Dead cells were then excluded and immune cells were identified based on their expression of CD45. Finally, transferred OT-II T cells were identified by selecting for the cells expressing both CD45.1, Vα2 and CD4. Total cell numbers were determined by back calculating from these plots.

2.8.6 Antibody list

Cells analysed using flow cytometry studies were co-stained with the following antibodies in various combinations and conjugated to various fluorophores:

Antibody	Clone	Dilution	Company
CD45.1	A20	1/200	eBioscience
CD4	RM4-5	1/400	eBioscience
Vα2	B20.1	1/200	BD bioscience
MHC II	M5/114.15.2	1/800	eBioscience
CD64	X54-5/7.1	1/200	Biolegend

CD8α	53-6.7	1/200	eBioscience
CD103	M290	1/200	BD horizon
Ly6G	1A8	1/200	BD bioscience
CD69	H1.2F3	1/200	BD bioscience
S1PR1	713412	5μl/sample	RnD systems
IFNγ	XMG1.2	1/200	Biolegend
CD44	IM7	1/200	eBioscience
CCR4	2G12	2μl/sample	Biolegend
CCR5	HM-CCR5	1/200	Biolegend
CX3CR1	SA011F11	1/400	Biolegend

Table 5 List of antibodies used for flow cytometry
Table details information about the antibody target proteins, clones, dilutions used at and the companies from which they were purchased.

2.9 Chemokine array

The chemokine array was carried out using a chemokine profiler kit (RnD systems). Mouse ear tissue was digested according to section 2.7 and then counted. Cells were lysed in recommended lysis buffer with complete ultra mini protease inhibitors (Roche). Once added, cells were lysed for 30 mins in a shaking incubator at 4ºC. After lysis, cells were centrifuged and the supernatants were extracted for further processing.

Blots from the kit were activated and blocked and cell lysate samples were prepared with buffers from the kit as described in the manual. A detection antibody cocktail was added to the samples and incubated for 1 hour at RT.

The activation/blocking buffer was aspirated from the blots and antibody+sample mix were added to 2 separate blots on the plate provided in the kit. The blots were then incubated overnight on a rocking shaker at 4ºC.

Next, membranes were washed 3 times in the wash buffer provided for 10 mins each on a rocking platform at RT. Streptavidin-HRP was prepared according to the manual and added to the blots and incubated for 30mins at RT in a rocker.

The membranes were then washed 3x as before and then carefully blot dried. They were then placed on plastic sheets and 1 ml of chemi reagent mix was added per blot as described in the manual. Another piece of plastic was placed on top and air bubbles ironed out. The blots were then incubated with the chemi reagent mix for 1 min. The blots were dried by very gently blotting blue roll on them. The top was then covered with another plastic sheet and imaged on a C-Digit blot scanner (LI-COR bioscience) machine. Different exposure times were used to get the optimum image.

The blot images were subsequently analysed using HLImage++ software. This software can differentiate the intensity of the dots and normalise it to give value for each chemokine assayed.

2.10 Intravital microscopy

2.10.1 Animal preparation

Animals were anaesthetised using 10mg/kg ketaset mix administered intra-peritoneally and maintained with low volume isoflurane inhalation using a gas mask. The ear was immobilised on a stand using veterinary grade glue and the core temperature of the animal maintained at 37ºC using a heat mat.

2.10.2 Imaging

Microscopy was carried out using a Zeiss LSM 7MP system equipped with 20×/1.0 NA water-immersion objective lens (Zeiss UK, Cambridge, UK) and a tunable Titanium: sapphire solid- state two-photon excitation source (Chameleon Ultra II; Coherent Laser Group, Glasgow, UK) and optical parametric oscillator (OPO;

Coherent Laser Group). Videos were acquired in 15-30 min intervals at an X-Y pixel resolution of 512x512 with 1.5µm increments in Z stack.

2.10.3 Image analysis

Videos were analysed in Volocity version 6 after correction for tissue drift using second harmonic as the anchor. Individual cells were defined as objects and tracked manually in 3D. The generated values were used to calculate the velocity, meandering index and displacement of T cells.

2.11 Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were performed on excised mouse ear tissue to determine the concentration of cytokines and chemokines. Prior to performing the ELISA, tissue was processed and protein concentration was measured via the bicinchoninic acid (BCA) assay.

2.11.1 Tissue processing

Mouse ears were collected and cut into small pieces in a 1.5ml eppendorf in 200-500µl of tissue protein extraction reagent (T-PER) (Thermo Fisher scientific) in the presence of protease inhibitors complete ultra mini (Roche). Once cut, the tissue was homogenised by a motorised mortar and pestle into a fine mix. The eppendorfs were then centrifuged at 10,000x G for 5 mins. The supernatants were then collected and stored at -80ºC for further analysis.

2.11.2 BCA assay

BCA assays were performed to determine protein concentration of ear homogenates. This enabled normalisation of ELISAs by loading the same amount of protein sample in each well for ELISA measurement. To perform the BCA assay an assay kit was used from BIO-RAD laboratories following the manufacturer's guidelines. Briefly, a 1mg/ml BSA was prepared in water as a stock solution. The BCA solution was diluted 1 in 5 as instructed in distilled water (BCA mix). A 6 point, 1:2 serial dilution of the BSA stock solution was made using the BCA mix, ranging from 12µg/ml to 2µg/ml in a 96 well plate in duplicate. These samples are used to generate a standard curve. 1µl of each homogenate sample was then

added to 1ml of the BCA mix. Duplicates of this sample was added to wells in the 96 well plate. The plate was then read in sunrise ELISA reader (Tecan) to determine the optical density of the samples.

Using the standard curve, the protein concentration of each homogenate sample was determined and normalised appropriately for future ELISAs.

2.11.3 CCL5 ELISA

The CCL5 ELISA kit was purchased from RnD technologies as a duoset ELISA kit. Manufacturer's guidelines were followed to perform the ELISA. Briefly, diluted capture antibody was coated on a 96 well microtiter plate and incubated overnight at RT. The plates were then washed 3 times and blot dried thoroughly. The plates were then blocked for non-specific binding by incubating with reagent diluent for 1 hour at RT. Plates were washed and dried again and appropriately diluted samples or standards were added to the appropriate wells. The plates were sealed with a plate sealer and incubated at RT for 2 hours.

The plates were once again washed and dried before adding appropriately diluted detection antibody. The plates were then sealed again and incubated at RT for 2 hours. The plates were once again washed and dried and appropriately diluted streptavidin-HRP was added to each well and incubated in the dark for 20 mins at RT. Following a further washing and drying step, substrate solution was added to each well and the plate incubated in the dark for 20mins at RT. Finally, the reaction was stopped by adding a stop solution to each well.

The colour development was quantified using a sunrise ELISA reader (Tecan) at 450nm with wavelength correction at 540nm.

2.11.4 IL-7 and FASL ELISA

IL-7 and FASL ELISAs were carried out using quantikine ELISA kits from RnD technologies. Manufacturer's guidelines were followed to perform the ELISA. Briefly, 50μl of assay diluent was added to each well along with 50μl of appropriately diluted sample, standard or control. The plate was sealed and incubated for 2 hours at RT on a plate shaker. Wells were washed using the

provided wash buffer 5 times and the plate thoroughly blot dried. 100μl of provided conjugate was added to each well. The plates were sealed and incubated for 2 hours at RT on a plate shaker. The plate was washed and dried as before and 100μl of substrate solution was added to each well and incubated at RT for 30 mins in the dark. Finally, 100μl of stop solution was added to each well.

Colour development was quantified using a sunrise ELISA reader (Tecan) at 450nm with wavelength correction at 540nm.

2.11.5 TNF ELISA

Mouse TNF OptELA ELISA kit was purchased from BD bioscience. Manufacturer's guidelines were followed to perform the ELISA. Briefly, a 96 well microtiter plate was coated with 100μl of capture antibody, sealed and incubated overnight at 4ºC. Wells were washed 3 times with recommended wash buffer and blot dried thoroughly the next morning. The plates were then blocked with 200μl of assay diluent and incubated at RT for 1 hour. After another round of washing and drying, 100μl of appropriately diluted standard, sample or control was added into appropriate wells. The plate was sealed and incubated at RT for 1 hour. The plates were washed and dried again and 100μl of working detector reagent (detection antibody + streptavidin-HRP) was added to each well. The plate was sealed and incubated in the dark for 1 hour at RT. The plates were washed and dried as before and 100μl of substrate solution was added to each well and incubated at RT for 30 mins in the dark. 50μl of stop solution was added to each well to stop the reaction.

The colour development was quantified using a sunrise ELISA reader (Tecan) at 450nm with wavelength correction at 570nm.

2.12 Mouse tissue histology and fluorescence microscopy

2.12.1 Tissue harvesting and processing

Mouse ears were collected and fixed in neutral buffered formalin between two pieces of filter paper ensuring the tissue remained flat to keep its structure. The tissue was then embedded in paraffin and cut using a cryotome at 10μm thick sections and embedded on superfrost plus glass slides. The slides were stored at 4ºC until further processing.

2.12.2 Staining for fluorescence microscopy

Stored tissue was first deparaffinised by submerging in xylene and then hydrated through decreasing grades of alcohol and into water. After washing twice in Tris-buffered saline + 0.05% tween (TBST), peroxidase activity was blocked by submerging slides in 0.5% hydrogen peroxide in methanol for 30 mins at RT. The antigens were then retrieved by boiling the slides in antigen retrieval buffer (abcam) for 8 mins. The sections were washed twice in TBST and blocked with 2.5% horse serum for 30 mins at RT. The tissue was further blocked with an avidin/biotin kit (Vector Labs) for 30 mins at RT. Rabbit polyclonal anti-SPHK1 (2.5ug/ml) (abcam) or rabbit IgG (2.5ug/ml) (abcam) was then added to the sections and incubated overnight in a dehumidified chamber at 4ºC. The sections were subsequently washed and stained with anti-rabbit biotin (1-200) (Vector Labs) for 30 mins. The sections were finally stained with Streptavidin-PE (1-200) (Vector Labs) and mounted with prolong gold anti-fade mountant with DAPI.

2.12.3 Imaging and analysis

Slides were imaged using cell observer SD (Zeiss). Ear sections from individual animals were imaged using a tile scan and analysed using Imagej software (NIH). Three equally sized areas were randomly selected per section and the number of total and SPHK1+ cells manually counted, and percentages determined.

2.12.4 Histology staining

For histology staining, ear section slides were deparaffinised and hydrated as before. The sections were then dipped in haematoxylin and washed in running water. The sections were then dipped in eosin, dehydrated through graded alcohols and xylene and mounted with DPX mounting media. The slides were then allowed to dry overnight.

2.12.5 Imaging of histology sections

Stained sections were imaged using an Olympus BX 41 microscope attached with a DP 25 camera with axiovision software at 10X or 20X.

2.13 Human tissue immunohistochemistry

2.13.1 Patient samples

Synovial tissue specimens were obtained from RA and OA patients at the time of arthroscopic biopsy or total joint replacement surgeries at Glasgow Royal Infirmary (Glasgow, U.K.). All RA and OA patients fulfilled the diagnostic criteria for RA and OA classification respectively, and written consent form was obtained from all subjects. All procedures received Ethics Approval (West of Scotland Research Ethical Committee Approval: 11/S0704/7). Collected tissue was preserved in 10% formalin, embedded in paraffin and cut using a cryotome at 5μm thick sections.

2.13.2 Immunohistochemical staining

Collected tissue was deparaffinised by xylene submersion and hydrated through decreasing grades of alcohol and into water. The sections were washed twice in TBST and the peroxidase activity was blocked by submerging in 0.5% hydrogen peroxide in methanol for 30 mins. The antigens were then retrieved by boiling the slides in antigen retrieval buffer (abcam) for 8 mins. The sections were then blocked with 2.5% horse serum for 30mins at RT. The tissue was then further blocked with an avidin/biotin kit (Vector Labs) for 30 mins at RT. 200μl of Rabbit polyclonal anti-SPHK1 (2.5ug/ml) (abcam) or Rabbit IgG (2.5ug/ml) (abcam) was then added to the sections and incubated overnight in a dehumidified chamber

at 4ºC. The sections were subsequently washed and stained with anti-rabbit biotin (1:200) (Vector Labs) in 2.5% horse and human serum for 30 mins at RT. Sections were washed again and avidin/biotin complex (Vector Labs) was added and incubated for 30 mins. Finally, the sections were washed and dried before 3,3' – diaminobenzidine (DAB) (Vector Labs) was added to the sections. The sections were allowed to develop for 2 mins before washing, dehydration and mounting in DPX mountant solution.

2.14 Statistical analysis

All data shown were analysed using Graphpad Prism version 6. Error bars represent standard error of mean (SEM). One-way ANOVA statistical analysis tests (more than 2 groups), Mann-Whitney test (2 groups) or Student's T-test (2 groups) were carried out for all the data shown with Tukey's multiple comparison tests (ANOVA only) to compare the differences between groups. * Denotes p values of <0.05, ** $p<0.01$ *** $p<0.001$. **** $p<0.0001$. Blank or n.s. denotes not significant.

3 Establishment of a model inflamed site to test CD4 T cell persistence in mouse ear pinnae

3.1 Introduction

CD4 T cells play a critical role in the initiation, maintenance and resolution of inflammation in numerous diseases. Immunological dogma dictates that antigen specific CD4 T cells proliferate and migrate to inflamed tissues. These T cells then carry out their effector function. Most effector T cells subsequently undergo apoptosis, while some turn into memory cells (284).

Evidence from several inflammatory conditions demonstrate that activated T cells may also accumulate at inflamed tissues (7, 9, 84, 421, 498). Accumulation of CD4 T cells at inflamed tissues is associated with increased disease pathogenesis (245). The migration, persistence, survival, proliferation, retention and egress of T cells are therefore key checkpoints in controlling T cell numbers at inflamed tissues. A loss of regulation of these signals may lead to aberrant and long-term accumulation of T cells at such sites.

3.1.1 Migration and Accumulation

There have been numerous studies, using various models to identify and understand migration and accumulation signals of T cells at non-lymphoid tissues. Some of the earlier studies identified a critical role for selectins in effector T cell entry into inflamed tissues (499). Analysis of endothelial cells on inflamed skin has revealed abundant expression of P- and E-selectin. Furthermore, Th1 cells were found to express their ligands P-selectin glycoprotein ligand 1 (PSGL-1). These discoveries together suggested that selectin mediated mechanisms were critical for migration of T cells to inflamed skin (12).

Later, a plethora of chemokines were identified that guided migration of specific subsets of CD4 T cells (Th1 vs. Th2) to home to particular tissues (393). Analysis of Th1 associated diseases such as RA and MS revealed an important role for the Th1 chemokine receptors CCR5, CXCR3 and their ligands in effector T cell accumulation in affected tissues (401, 500). In contrast, Th2 cells were found to preferentially express a different set of chemokine receptors. CCR3, CCR4, CCR8 and CXCR4 were all identified as Th2 guiding chemokines (393, 501, 502). The interaction between CCR3 on Th2 cells and its ligand Eotaxin (CCL11) was

further identified as crucial in the migration and accumulation of these cells in the allergic airway (503, 504).

Teleologically, the idea that the migration and accumulation of antigen specific T cells to inflamed tissues is initiated and maintained by cognate antigen and local tissue signature was attractive to many in the field. Hence their importance was investigated in several studies. Using a localised subcutaneous IFA injection and antigen specific T cell adoptive transfer model, Reinhardt et al. demonstrated that effector CD4 T cells preferentially home to, and accumulate in, antigen depot sites (392).

Other studies identified that T cells primed in skin draining lymph nodes were preferentially recalled to the infected skin site. In contrast, T cells that were primed in the Peyer's patches returned to the gut to carry out their effector function (505, 506). More recently, there has been a focus on the role of integrins in tissue homing of T cells. Conrad et al. elegantly demonstrated that the integrin $\alpha 1\beta 1$ was crucial for the accumulation of Th1 cells in a psoriasis model of skin inflammation (7). Furthermore, another study found a critical role for αv integrins in mediating CD4 T cell motility at inflamed tissues by multi-photon microscopy (391). Finally, the integrin CD11a was shown to be critical in regulating CD8 T cell accumulation in an adipose tissue inflammation model (507).

3.1.2 Survival, Proliferation and Egress

On top of increased migration and accumulation in inflamed tissues, studies have also suggested that inflamed tissues may provide local survival, proliferation and reduced egress signals to activated T cells (9, 244, 508). This increases their tissue dwell time, effector response time and perhaps their likelihood to develop into Tissue resident memory T cells (Trm).

One study found that T cells in the RA synovium were less susceptible to apoptotic cell death and this increased survival was mediated by T cell interaction with fibroblasts in an integrin-ligand mediated manner (419). Piling et al. also demonstrated that T cells at chronically inflamed tissues receive survival cues from stromal cells via interferon β (420).

The local proliferation of effector CD4 T cells was demonstrated in a DTH model of inflammation. Here effector T cell accumulation was largely associated with tissue proliferation and decreased egress from local inflamed tissue which was mediated by CCR7 (9). Gomez et al. demonstrated in another study that disrupting CCR7 mediated egress of T cells from inflamed tissues led to aggravated local tissue inflammation (245). Lastly, the signalling sphingolipid S1P was found to cause tissue retention of naïve T cells at inflamed tissue sites in an integrin mediated manner (244).

3.1.3 Aims of this chapter

These elegant studies clearly demonstrate that accumulation of T cells at inflamed tissues is a multi-step process, regulated by several redundant factors. The studies also however, highlight the difficulty in separating one factor from another. For instance, do the chemokine receptors that signal migration of T cells into inflamed tissues also cause their long-term retention? Additionally, chemokines such as CCR7, that are responsible for tissue egress may further act as retention signals by desensitizing or downregulating their receptors.

The activation and polarisation state of the T cell likewise, may have a major role in their mechanism of retention. Chemokine receptors are differentially expressed on distinctive T cell subsets. Thus, one may expect early inflamed tissues to recruit and retain Th1 or Th2 cells but later on attract regulatory T cells to help resolve the inflammation.

In this chapter, a novel tissue adoptive transfer model was established to differentiate migration, persistence, retention and egress signals of CD4 Th1 cells at inflamed tissue sites. This model was utilised in subsequent chapters, to specifically identify new signals which cause prolonged persistence of CD4 Th1 cells.

3.2 Results

3.2.1 Cellular profile of inflamed vs. non-inflamed ear pinnae

An easily manipulatable inflamed tissue was required to study the fundamental mechanisms of CD4 T cell persistence. Requirements for such a site included non-invasive access to the tissue, allowing simple manipulations of the site such as the application of inflammatory stimuli and adoptive transfer of cells. For enumeration purposes, the tissue had to be relatively simple to process for both flow cytometric analysis and intravital microscopy.

Furthermore, the model had to be relatively short to negate both the recirculation of transferred cells and the effects of endogenous T cell recruitment to such sites. Acknowledging this, LPS, an endotoxin from the gram-negative bacteria E.*coli* was injected intradermally (ID) in the ear pinnae of mice to elicit acute inflammation.

ID LPS administration in the skin leads to rapid oedematous lesion formation, increased vascular permeability followed by haemorrhagic necrosis within the space of 24 hours (509). LPS also activates TLR 4 which induces transcription of MyD88. This leads to production of inflammatory mediators such as TNFα and IL-1β (510).

To establish that LPS administration induced skin inflammation in mouse ear pinnae, LPS or saline was injected ID into the ear pinnae of mice. 24 hours later the animals were euthanised and their ears were assessed for inflammatory changes (Figure 3.2.1A). LPS challenged ear pinnae underwent significantly increased swelling compared to PBS challenged ears, suggesting oedema formation (Figure 3.2.1B). Visually, LPS inflamed ears developed red colouration, signifying an increase in local vascular permeability and perhaps haemorrhage of blood vessels and red blood cell infiltration (Figure 3.2.1C).

Finally, histology was performed on LPS inflamed and saline mouse ears by sectioning them and staining them with haematoxylin and eosin. Haematoxylin typically stains cell nuclei with a blue colouration. Eosin is a non-specific protein stain, and it often stains red blood cells pink or red (511). LPS inflamed ears

displayed substantial infiltration of red blood cells and an overall increase in cellularity. The increase in ear pinnae thickness was also evident in LPS administered ears (Figure 3.2.1C).

While histology and morphology are important in determining the state of a tissue, flow cytometry allows for a more rigorous approach in understanding the cellular changes that take place at the inflamed mouse skin. Moreover, it was important to determine the cellular composition of the inflamed skin to speculate and target potential signals that may influence CD4 T cell persistence.

Hence, to determine the cellular changes that take place at LPS inflamed skin, mice were injected with LPS or saline ID as before. 24 hours later, the animals were euthanised and their ears collected and processed for flow cytometry. Inflamed ears were found to contain significantly reduced percentage of macrophages and CD8α DCs without affecting their total number (Figure 3.2.2A,B). This decrease in percentage could be due to the influx of other cells such as CD103 DCs and neutrophils. Significant increases were found in both the percentage and total numbers of these cells at inflamed tissues (Figure 3.2.2B,C).

In summary, the data demonstrates that 24 hours post LPS administration, the mouse ear has undergone significant changes in its morphology and cellular content, suggesting the tissue has undergone a profound inflammatory response. The data also suggests that using LPS as an inflammatory stimulus in the mouse skin may be suitable to model an inflamed site to study molecular signals provided to T cells at inflamed tissues to cause their persistence.

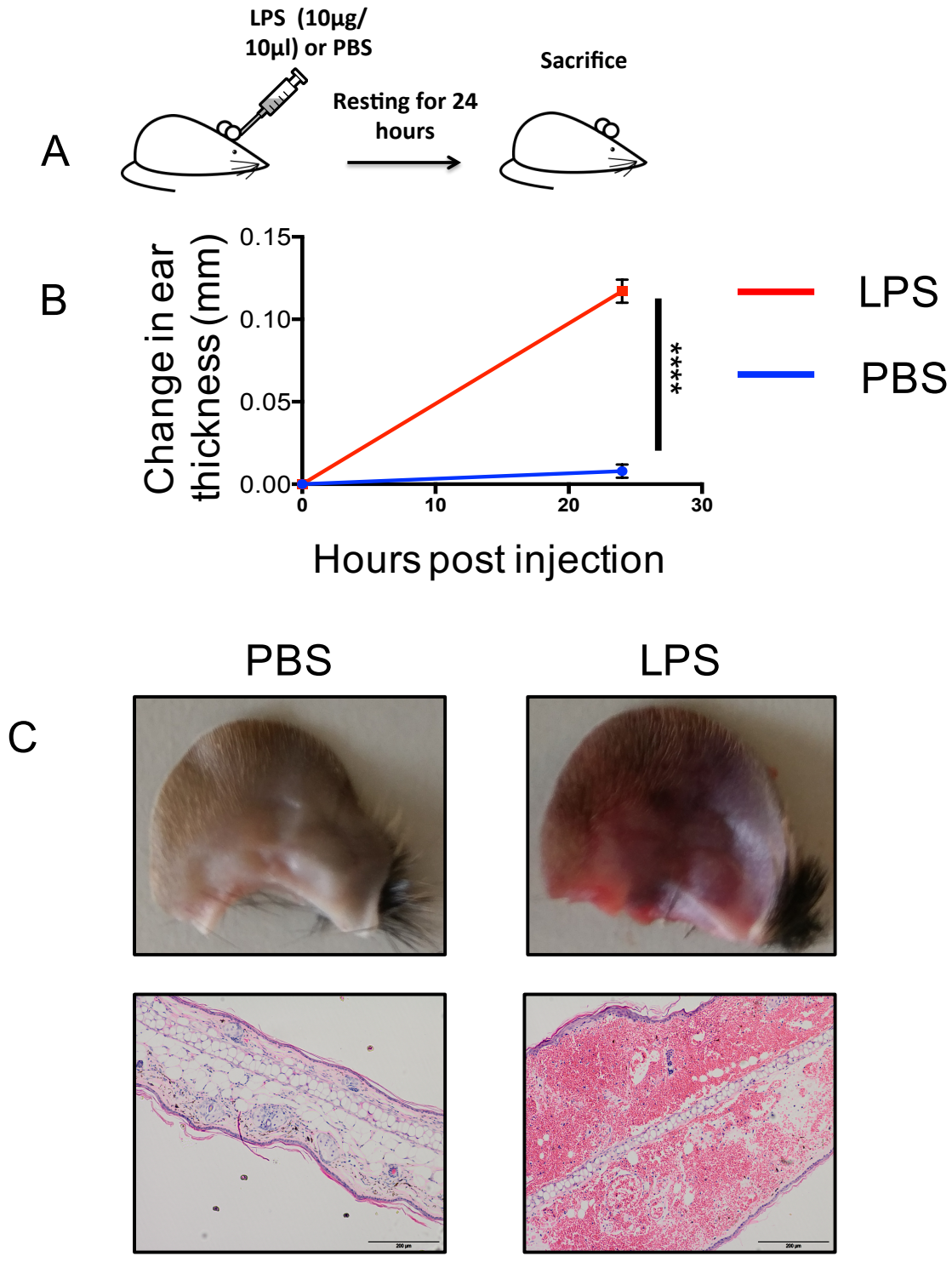

Figure 3.2.1 Inflamed tissues undergo substantial cellular and morphological changes
Ear thickness of age matched C57BL/6 mice at 6 weeks of age were measured prior to challenge with LPS (10µg/10µl) or PBS in their right ear pinnae. 24 hours later, the ears were measured again. Animals were then, euthanised and their ears harvested, photographed and processed for histology. The tissue was then stained with haematoxylin and eosin and mounted in DPX medium before imaging using a standard light microscope. Images shown are representative of 1 experiment with 5 animals per group. Image magnification: 20x. Scale bar in image shows 200µm. Error bars in graph represent SEM. Statistical differences between groups were determined by carrying out an unpaired Student's T-test in Graphpad PRISM. * denotes a p value of <0.05, ** <0.01. *** <0.001 and **** <0.0001 (A) Schematic of inflamed ear pinnae model. (B) Graph demonstrates change in ear thickness 24h after LPS or PBS administration compared with thickness measured prior to substance administration (time zero). (C) Representative photograph and H&E stained sections of LPS and PBS challenged ear pinnae.

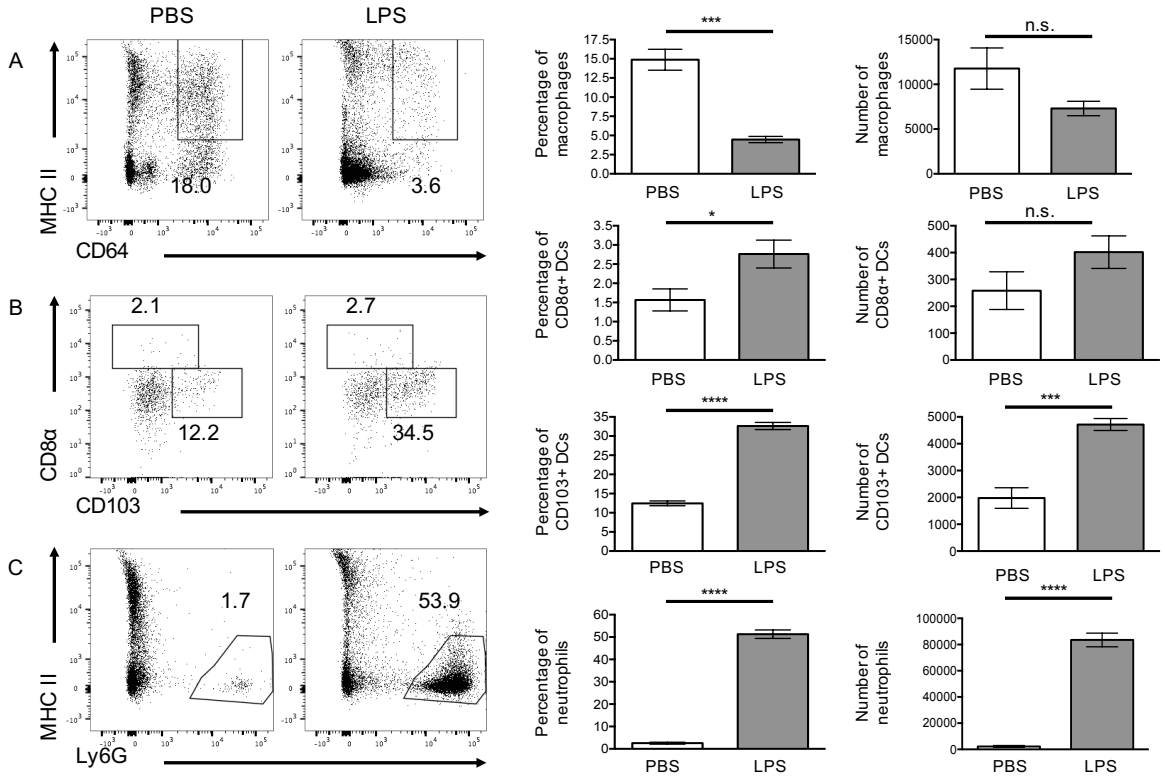

Figure 3.2.2 Increased numbers of APCs and neutrophils are found at inflamed tissue sites
Age matched C57BL/6 mice were challenged with 10μg/10μl of LPS or 10ul of PBS in their right ear pinnae. 24 hours later, animals were euthanised and their ears were harvested, processed and stained for FACS. Data is representative of 2 independent experiments with 4 animals in each group. FACS plots were pre-gated on live CD45+ lymphocytes. Error bars represent standard error of mean. Statistical differences were determined by carrying out an unpaired Student's T test in Graphpad prism. * denotes a p value of <0.05, ** <0.01. *** <0.001 and **** <0.0001

3.2.2 Detectable populations of T cells can be recovered from mouse ears and dLNs following adoptive transfer

Upon establishing the potent inflammatory environment created by the administration of LPS in mouse ear pinnae, it was necessary to optimise both the dose of LPS as well as the number of T cells to transfer into the tissue. This was critical to reduce the use of reagents, animals and severity of the procedure.

To test the recovery of T cells from ear skin and draining lymph nodes, 0.5, 1 or 3 million *in vitro* polarised CD4 Th1 cells were adoptively transferred ID in the ear pinnae of mice. 24 hours later, the animals were euthanised and their ears and draining lymph nodes were harvested and the proportion of transferred cells recovered were enumerated by flow cytometry (Figure 3.2.3).

Populations of transferred T cells, signified by their co-expression of CD45.1 and Vα2 were observed in all three groups (Figure 3.2.3A). The best recovery of cells

was observed in the 3 million cell group with up to 44.5% of CD4 T cells representing transferred cells from the ear pinnae and up to 0.30% in the draining lymph node. Likewise, there was a lower, but modest recovery of transferred cells in the 1 million group with up to 10.8% of CD4 T cells representing transferred cells from the ear pinnae and 0.11% in the dLN. The populations recovered from the 0.5 million group however was quite modest from both the ear pinnae and dLN (4.41% and 0.078% respectively) (Figure 3.2.3A).

Next, it was important to check whether higher doses of LPS improved recovery of T cells from the ear tissue or dLN. To test this, mouse ear pinnae were challenged with either 10μg or 20μg of LPS. 24 hours later, 3 million CD4 Th1 cells were adoptively transferred ID into the ear pinnae. 24 hours after that, the animals were euthanised and their ear pinnae and dLN were harvested and the number of T cells recovered from the tissues enumerated by flow cytometry.

No significant differences were observed in the recovery of transferred CD4 Th1 cells from mouse ears treated with either 10μg or 20μg of LPS. Moreover, the recovery of transferred cells from the draining lymph node was also not affected (Figure 3.2.3B)

Hence, 10μg of LPS and greater than 1 million T cells per animal was chosen as the optimal conditions to test CD4 Th1 cell persistence. These values are used henceforth in all experiments in this thesis.

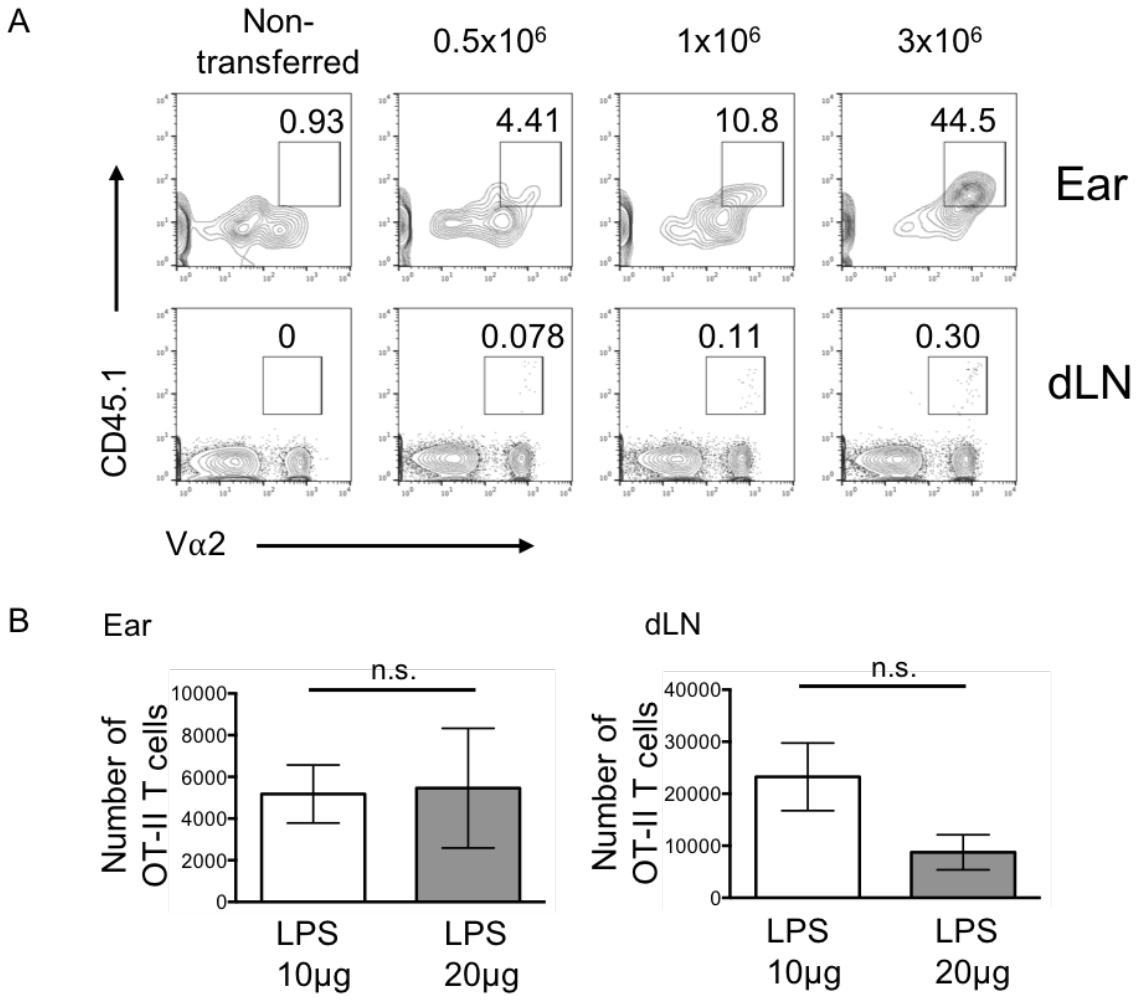

Figure 3.2.3 Transferring between 1 and 3.0e6 cells allows recovery of detectable population of CD4 Th1 cells from tissues and no difference in T cell recovery is observed between 10 and 20ug LPS administration

A: 0, 0.5, 1 or 3.0e6 polarised CD4 Th1 cells were adoptively transferred into the ear pinnae of age matched C57BL/6 mice. 24 hours later, animals were euthanised and their ears and dLN were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots show representative examples from one experiment with 3 animals in each group.

B: Age matched C57BL/6 mice were challenged with 10 or 20μg of LPS in their ear pinnae. 24 hours later 3.0e6 CD4 Th1 cells were adoptively transferred into their right ear pinnae. 24 hours later, animals were euthanised and their ears and draining lymph nodes were harvested, processed and stained for FACS as above. Graphs show combined data from 1 experiment with 4 (LPS 20μG) or 5 (LPS 10μG) animals in each group. Statistical differences were determined by carrying out an unpaired student's T-test. N.s. denotes not significant.

3.2.3 Greater number of CD4 Th1 cells are recovered from inflamed tissue sites

Accumulation of CD4 T cells at inflamed tissues has been documented in numerous inflammatory diseases (84, 408, 498, 512-514). However, accumulation of T cells comprises of distinct steps including recruitment, persistence, retention and egress of cells to/from inflamed sites. While studies have been undertaken to address each of these steps, persistence specific signals for CD4 T cells at inflamed tissues are poorly studied.

Hence, an intradermal tissue adoptive transfer model was designed to specifically address the question of T cell persistence at inflamed tissues. To do this, animals were injected with LPS intradermally in their ear pinnae. Following the development of inflammation, activated CD4 Th1 cells were adoptively transferred directly into the inflamed tissue. Direct injection of T cells negated confounding recruitment signals.

Th1 cells were used since activated rather than naïve T cells are usually found at inflamed tissues (515). Moreover, Th1 rather than Th2 or 17 cells have been documented to persist at inflamed RA tissues (417). Therefore, OT-II CD4 T cells were polarised to Th1 phenotype by culturing them in the presence of ovalbumin peptide (323-339), IL-12 and anti-IL-4 antibody in the presence of mitomycin C treated splenocytes to act as antigen presenting cells.

OT-II mice have a transgenic TCR which specifically recognises ovalbumin at peptides 323-339 (494). The majority of the CD4 T cells in OT-II mice carry this transgenic TCR. Mitomycin C cross links DNA and causes cell cycle arrest of splenocytes (516). While this allows antigen presentation, it stops splenocytes from proliferating. IL-12 promotes naïve CD4 T cells to polarise to a Th1 phenotype. Finally, anti-IL-4 antibody was used to ensure a pure population of Th1 cells were present post culture.

Activated T cells were assessed for their polarisation into a Th1 phenotype prior to adoptive transfer. Th1 cells were treated with phorbol-myristate acetate (PMA) and Ionomycin in the presence of brefeldin A. PMA is an activator of

protein kinase C (PKC) and Ionomycin is a calcium ionophore (517). Brefeldin A is a Golgi transport inhibitor (518). Together, these compounds increase the production of cytokines (PMA/Ionomycin) and allows their intracellular detection (brefeldin A) by flow cytometry (519).

Expression of CD44 and IFNγ by polarised T cells was assessed to ensure that CD4 T cells were of the Th1 phenotype. CD44 is a cell surface glycoprotein and cell adhesion molecule that is often associated with antigen experienced and activated CD4 T cells (520). IFNγ is a cytokine that is classically associated with Th1 cell polarisation (521). T cells in the presence or absence of stimulation were universally found to express CD44. However as expected, only PMA/Ionomycin treated cells demonstrated a substantial production of IFNγ (Figure 3.2.4). The data therefore demonstrates, that the polarisation protocol has produced a pool of activated and antigen specific CD4 Th1 cells.

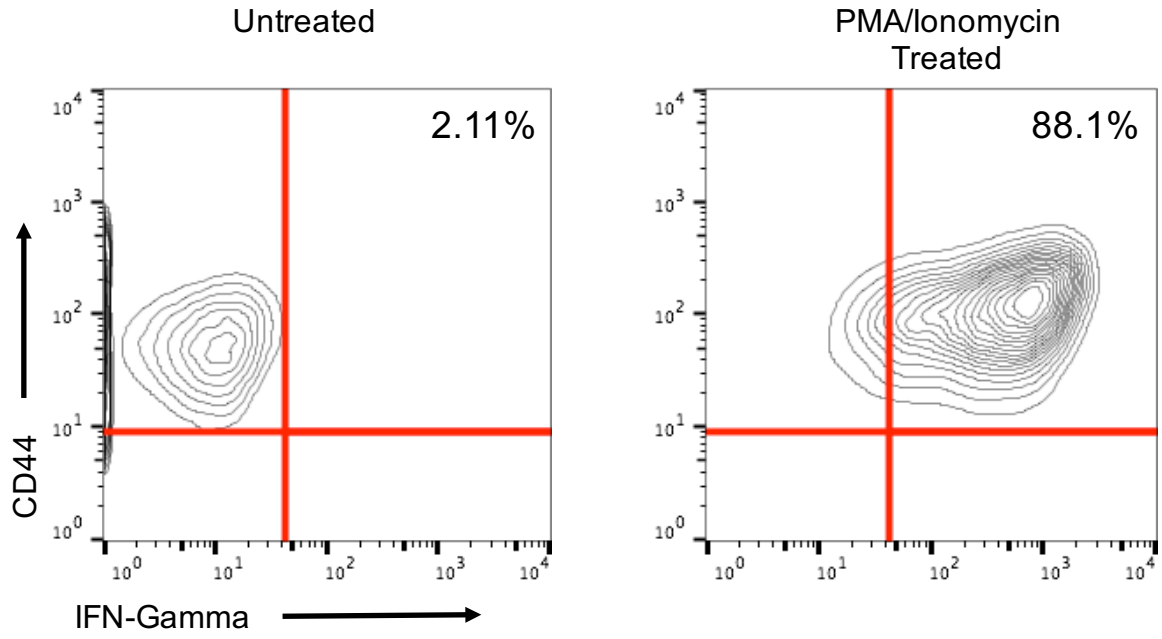

Figure 3.2.4 Most polarised Th1 cells express CD44 and produce IFNγ
CD4 T cells were isolated from the lymph nodes and spleens of CD45.1 OT-II mice using a STEMCELL technologies CD4 T cell isolation kit. The cells were then cultured with IL-12, anti-IL-4 and ovalbumin peptide$_{323-339}$ in the presence of mitomycin C treated splenocytes for 72 hours at 37°C with 5% CO_2. Thereafter, cells were stimulated with or without PMA and Ionomycin with brefeldin A for 4 hours. The cells were subsequently stained with fluorescently labelled antibodies against CD4, CD44 and intracellular IFNγ for FACS. Cells were initially gated on live CD4+ lymphocytes and subsequently analysed for their expression of IFNγ and CD44. Number shows the percentage of plotted cells in top right-hand quadrant. The plots shown are representative of 3 independent experiments.

Next, these polarised Th1 cells were adoptively transferred intradermally into an LPS inflamed or saline treated ear pinnae. The animals were allowed to rest for 12 or 24 hours before they were euthanised and their ear tissue and draining lymph nodes were harvested, assessed and enumerated at both of these time points.

Significantly greater proportions and numbers of transferred Th1 cells were recovered from inflamed ear pinnae when compared to non-inflamed ear pinnae, 12 hours after their transfer (Figure 3.2.5A). However, no significant differences were found in the transferred T cell numbers between the ear draining lymph nodes of inflamed or resting ears 12 hours after CD4 Th1 cell transfer (Figure 3.2.5B). Although a trend towards decreased number of Th1 cells in the draining lymph nodes of inflamed ears was observed, this did not reach significance (Figure 3.2.5).

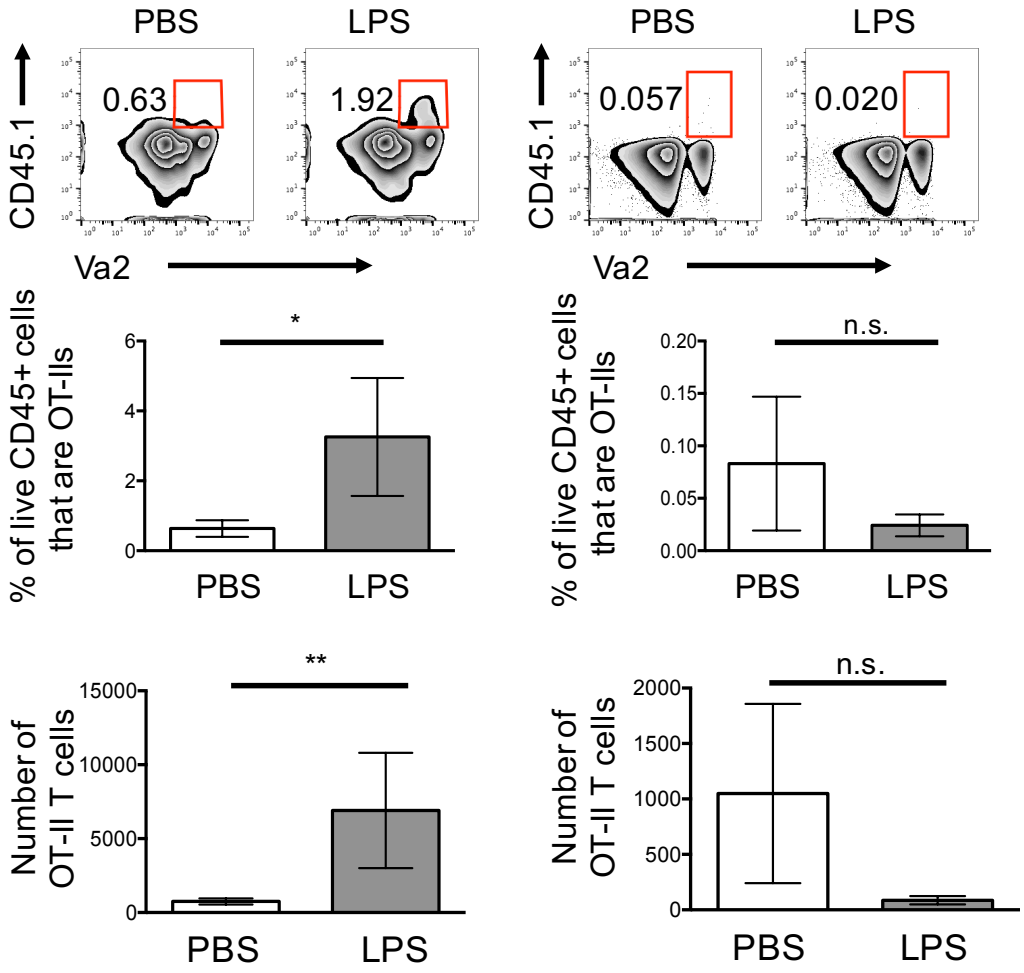

Figure 3.2.5 Greater Numbers of CD4 Th1 cells are recovered from inflamed tissues 12 hours after adoptive transfer
Age matched C57BL/6 mice were challenged with LPS (10μg/10μl) or PBS (10μl) in their right ear pinnae. 24 hours later 2.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 12 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. Percentages are representative of 2 independent experiments with 11 (PBS) or 7 (LPS) animals in each group. Number graphs are combined data from 2 independent experiments with 11 (PBS) or 7 (LPS) animals per group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a Mann-Whitney test in Graphpad prism. * denotes a p value of <0.05, ** <0.01. n.s. denotes not significant.

At 24 hours post transfer, significantly greater proportions and numbers of transferred T cells were again recovered from inflamed ears compared to resting ears (Figure 3.2.6A). Likewise, mimicking the 12 hour results, no differences were observed in the number of transferred T cells recovered from the draining lymph nodes of inflamed ear pinnae compared to resting ear pinnae (Figure 3.2.6B). A similar trend of decreased transferred T cell recovery from inflamed ear draining lymph nodes was observed.

In all, the data indicates that LPS inflammation generates signals which cause persistence of CD4 Th1 cells early after insult in mouse skin tissues. Increased T cell persistence however, is not due to reduced egress of cells to ear draining lymph nodes.

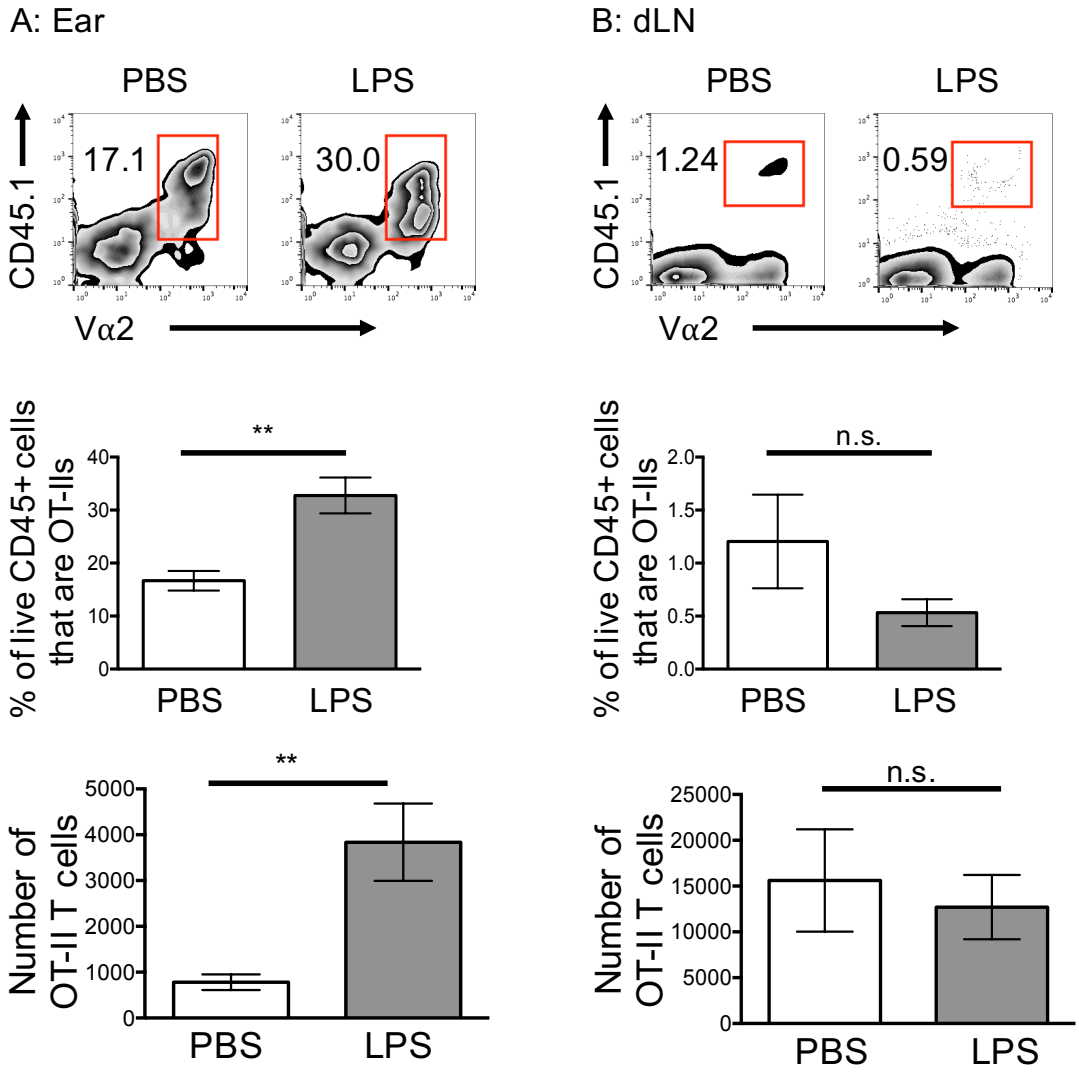

Figure 3.2.6 Greater Numbers of CD4 Th1 cells are recovered from inflamed tissues 24 hours after adoptive transfer

Age matched C57BL/6 mice were challenged with LPS (10μg/10μl) or PBS (10μl) in their right ear pinnae. 24 hours later 5.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots and percentage plots are representative of 3 independent experiments with 12 animals in each group. Number graphs are combined data from 3 independent experiments with 12 animals per group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a Mann-Whitney test in Graphpad prism. ** <0.01. n.s. denotes not significant.

3.2.4 Transferred T cells do not circulate within 24 hours

Earlier in this chapter it was stated that one of the requirements for a model to study T cell persistence was to minimise T cell circulation. This was important because systemic circulation of ID transferred T cells would complicate the enumeration of persistent T cells. The number of T cells recovered from the inflamed tissue, in that case, may represent cells that had left the tissue and returned prior to their enumeration.

To investigate whether circulation of T cells occurred in this model, mice were challenged with PBS or LPS in their right ear pinnae. 24 hours later 2 million CD4 Th1 cells were transferred into the same ear pinnae. 24 hours following the transfer, the animals were euthanised and their left ear pinnae (contra-lateral to the injected ear) as well as their spleen were harvested and enumerated by flow cytometry.

No transferred cells were recovered from the contralateral ear of either the PBS or LPS challenged ear pinnae (Figure 3.2.7A). Likewise, no transferred T cells were detectable in the spleen of either PBS or LPS challenged ear pinnae (Figure 3.2.7B). These data indicate that 24 hours after ID adoptive transfer of T cells in the ear pinnae, cells do not undergo systemic circulation.

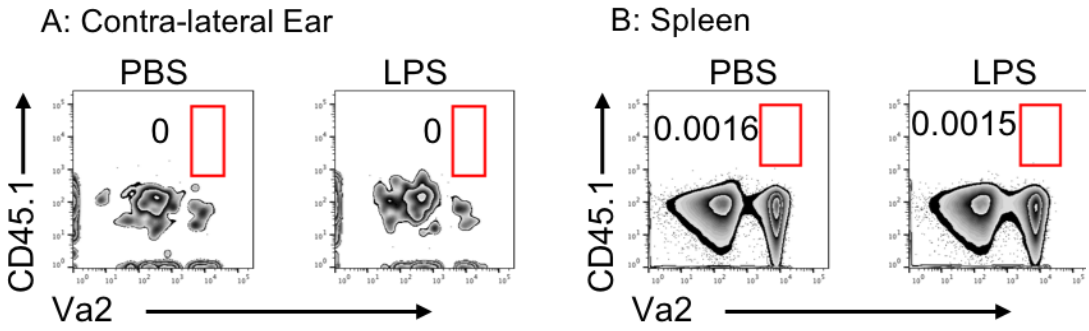

Figure 3.2.7 Few transferred T cells are recovered from peripheral tissues of mice within 24 hours of adoptive transfer
Age matched C57BL/6 mice were challenged with LPS (10μg/10μl) or PBS (10μl) in their right ear pinnae. 24 hours later 2.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots are representative of 1 experiment with 4 animals in each group.

3.2.5 T cells recovered from inflamed tissues are functionally distinct from those at resting sites

Following the finding that activated CD4 T cells persist at LPS inflamed ear pinnae, it was necessary to perform some functional and phenotypic analysis of the recovered cells. One of the primary functions of activated CD4 T cells at inflamed tissues is to produce effector cytokines (449). As discussed previously, Th1 cells produce IFNγ. Therefore, to assess the function of persisting CD4 T cells, their IFNγ production was measured.

Polarised Th1 cells were transferred into LPS or saline treated ear pinnae. 24 hours later animals were euthanised and their ears and draining lymph nodes were harvested. After tissue processing, single cell suspensions were cultured in the presence of a cell stimulation cocktail containing PMA/Ionomycin and brefeldin A for 4 hours, following which cells were stained for extra and intracellular flow cytometry. A very small percentage of transferred Th1 cells recovered from inflamed tissues produced IFNγ when compared to those recovered from non-inflamed tissues (Figure 3.2.8A). In the draining lymph nodes however, no differences were observed between the two groups (Figure 3.2.8B).

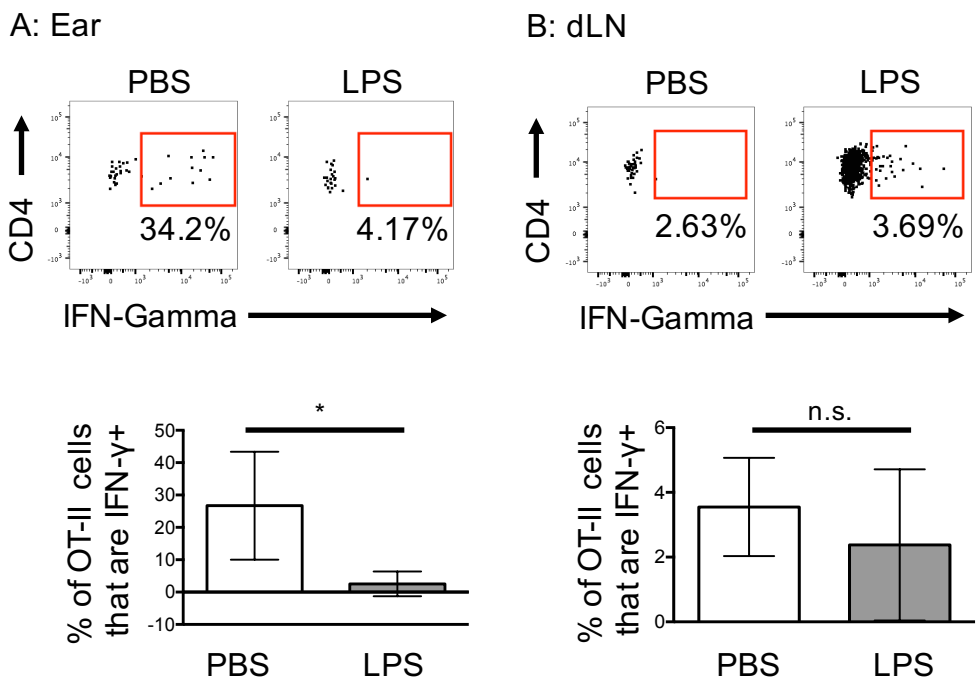

Figure 3.2.8 Significantly lower proportion of CD4 Th1 cells produce IFNγ at inflamed tissue site
Age matched C57BL/6 mice were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for extra and intracellular FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and IFNγ. Lymphocytes were initially gated on live CD45+, CD4+, CD45.1+ cells and subsequently analysed for their expression of IFNγ. Data shown is from one experiment with 5 animals in each group. Error bars represent standard error of mean. Statistical differences were determined by carrying out an unpaired Student's T-test in Graphpad prism. * denotes a p value of <0.05, n.s. denotes not significant.

Additionally, activated T cells express CD69. CD69 is a C-Type lectin receptor which has often been associated with early lymphocyte activation (522). CD69 expression is also associated with Trm cells (523). Thus, expression of CD69 by Th1 cells was assessed both before and after transfer.

Before transfer, typically greater than 95% of the CD4 T cells in culture were found to express CD69 (Figure 3.2.9A). However, 24 hours after transfer, only around half the cells were expressing CD69 (Figure 3.2.9A). No differences were observed in CD69 expression between inflamed and non-inflamed ears (Figure 3.2.9B).

In contrast, significantly lower proportion of Th1 cells recovered from lymph nodes draining inflamed ears expressed CD69 when compared to cells recovered from resting draining lymph nodes (Figure 3.2.9B). Altogether, the data indicates

that persisting Th1 cells differ in their cytokine potential at inflamed vs. resting tissue sites. Furthermore, differences in CD69 expression may also play a role in the persistence or drainage of T cells to/from inflamed tissues.

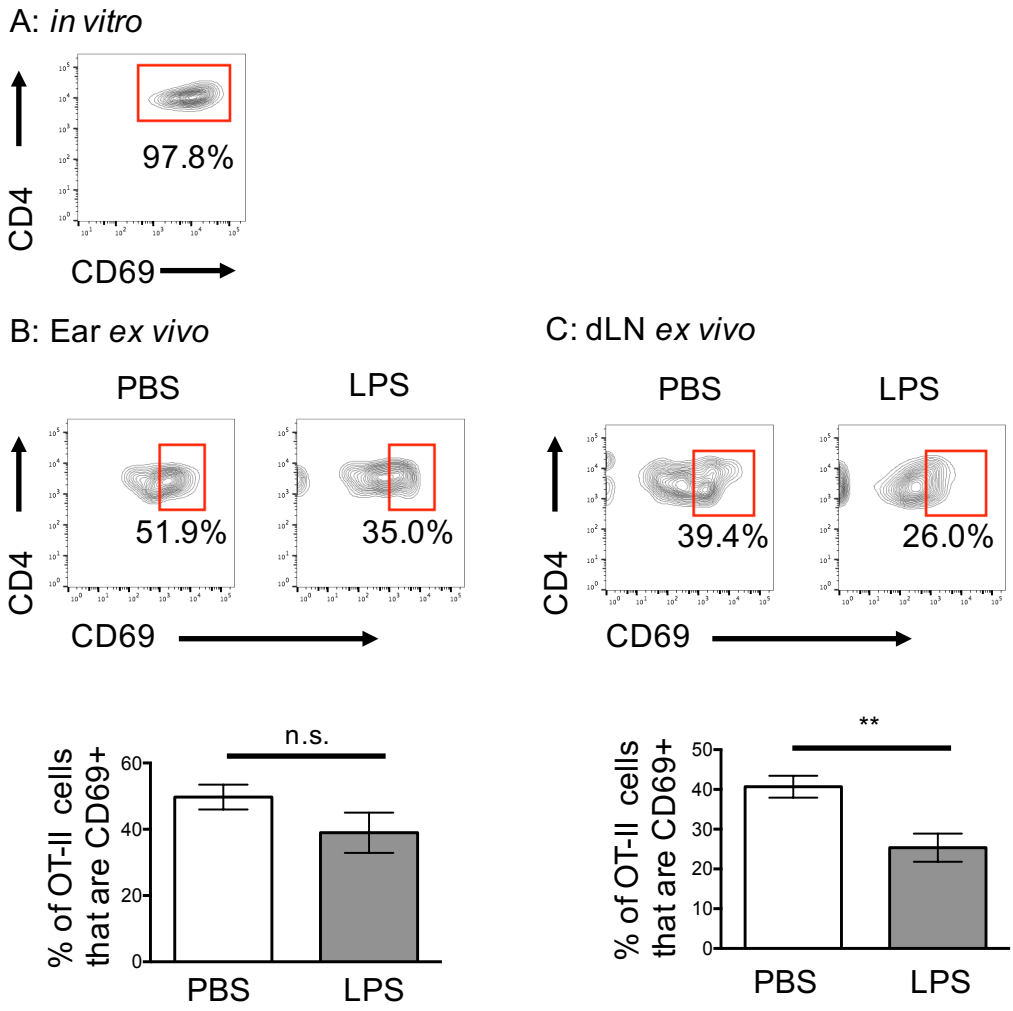

Figure 3.2.9 CD4 Th1 cells express CD69 in vitro but no difference was observed in T cell CD69 expression ex vivo between inflamed and resting tissues
A: CD4 T cells were isolated from the lymph nodes and spleens of CD45.1 OT-II mice using a STEMCELL technologies CD4 T cell isolation kit. The cells were then cultured with IL-12, anti-IL-4 and ovalbumin peptide$_{323-339}$ in the presence of mitomycin C treated splenocytes for 72 hours at 37°C with 5% CO_2. The cells were subsequently stained with fluorescently labelled antibodies against CD4 and CD69 for FACS. Cells were initially gated on single, lymphocytes and subsequently gated for their expression of CD69 and CD4. The plot shown is representative of 3 independent experiments.

B,C: Age matched C57BL/6 mice were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (B) and draining lymph nodes (C) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and CD69. Lymphocytes were initially gated on live CD45+, CD4+, CD45.1+ cells and subsequently analysed for their expression of CD69. Data shown is combined from 4 experiments with 22 animals in each group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a Mann-Whitney test in Graphpad prism. ** denotes a p value of <0.01, n.s. denotes not significant.

3.2.6 Naïve CD4 T cells are retained at inflamed tissue sites

T cells differ significantly in their form and function based on their activation and polarisation status. This allows naïve T cells to traffic through secondary lymphoid organs and re-circulate in the blood stream (389). It also allows effector T cells to traffic to target tissues (12, 14). Therefore, testing whether naïve T cells also persist at inflamed skin could provide clues as to the mechanism of effector T cell persistence.

To test this, congenically labelled naïve T cells were transferred into ear pinnae treated with LPS or saline 24 hours previously. 24 hours after the transfer of cells, animals were euthanised and their ears and draining lymph nodes, which drain the ear, were analysed to enumerate the persistence and drainage of transferred cells.

Significantly greater proportion and number of transferred CD4 T cells were recovered from inflamed ear pinnae compared to non-inflamed ear pinnae (Figure 3.2.10A). Additionally, the draining lymph nodes of inflamed ear pinnae contained significantly lower number of transferred T cells than the draining lymph node of the resting ear (Figure 3.2.10B).

Together these data indicate that LPS inflamed skin tissues cause the retention rather than persistence of naïve T cells.

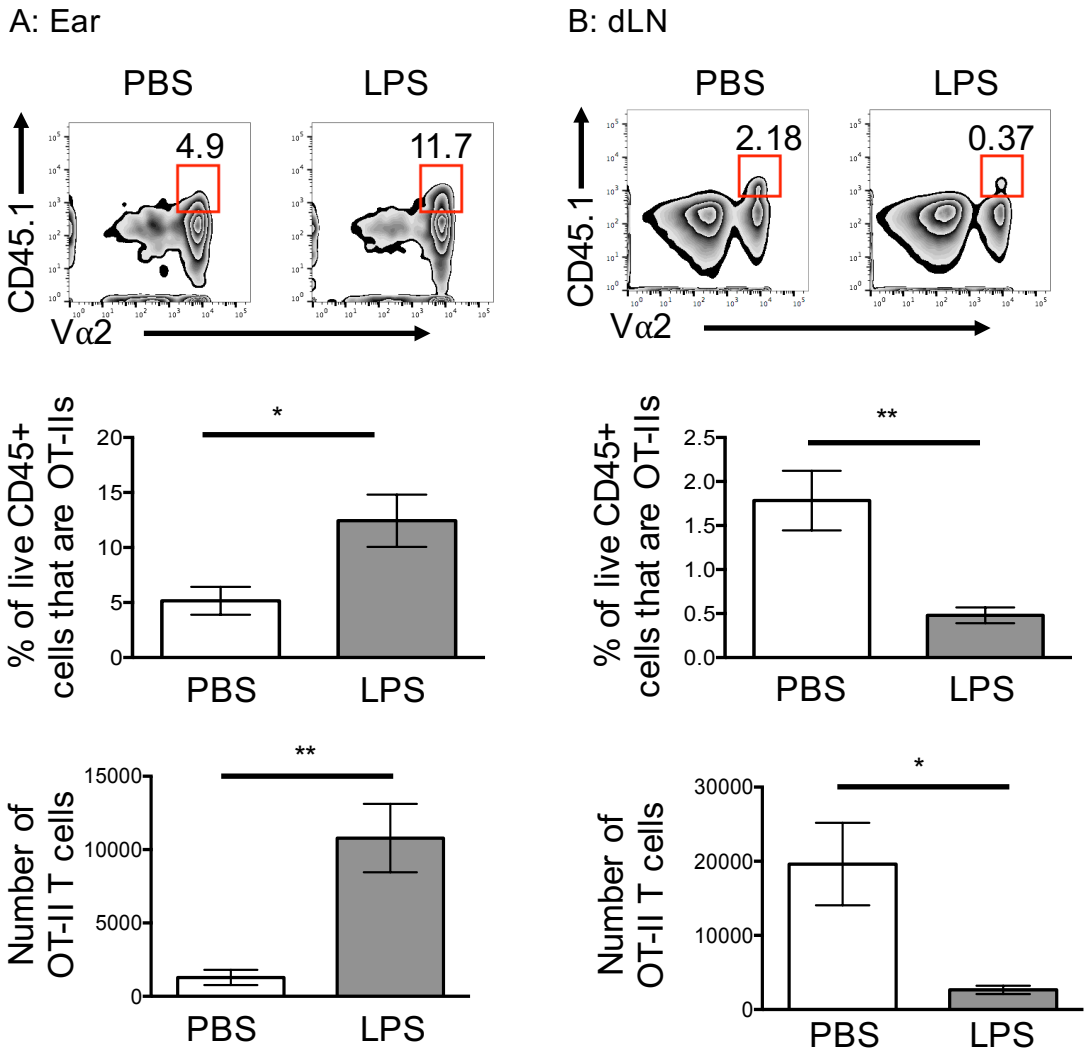

Figure 3.2.10 Greater Number of naïve CD4 T cells are recovered from inflamed tissues 24 hours after adoptive transfer
Age matched C57BL/6 mice were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 freshly isolated CD4 T cells from OT-II mice were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. Data is representative of 1 independent experiment with 5 animals in each group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a Mann-Whitney test in Graphpad prism. * denotes a p value of <0.05, ** <0.01.

3.2.7 Polarisation state of CD4 T cells does not alter their persistence at inflamed tissues

The migration and retention of T cells at peripheral tissues is often dictated by their specific expression profile of chemokines and cell adhesion molecules (14, 36, 393, 502). Th1 cells are known to express chemokines such as CCR5 and 9 while Th2 cells predominantly express CCR3 and 4, guiding them to peripheral tissue sites (393, 502). Moreover, polarisation of T cells also influences their death and survival mechanisms (483, 524, 525). Hence, understanding whether the polarisation state of the cell contributes to their persistence in the tissue could enlighten a path towards understanding the mechanism of Th1 cell persistence at inflamed tissues.

To investigate this, OT-II T cells were activated with ovalbumin peptide in the absence of an IL-12 polarising signal to generate non-polarised but activated T cells (referred to as Th0 cells henceforth). These cells were then adoptively transferred ID into LPS or PBS challenged ear pinnae as before. 24 hours later, the animals were euthanised and their tissues were harvested and enumerated by flow cytometry.

Greater numbers of CD4 Th0 cells were recovered from LPS inflamed ear pinnae compared to resting tissues (Figure 3.2.11A). However, there were no differences observed in the proportion of transferred cells recovered from the tissue. Furthermore, no differences were observed in either the proportion or the number of transferred cells in the ear draining lymph nodes (Figure 3.2.11B).

It is important to note that the total number transferred cells recovered from peripheral tissues and draining lymph nodes were far lower than the ones recovered from experiments where either Th1 or naïve cells were transferred.

Altogether, the data indicates that polarisation state of the T cell may play a role in the persistence of T cells at inflamed tissues.

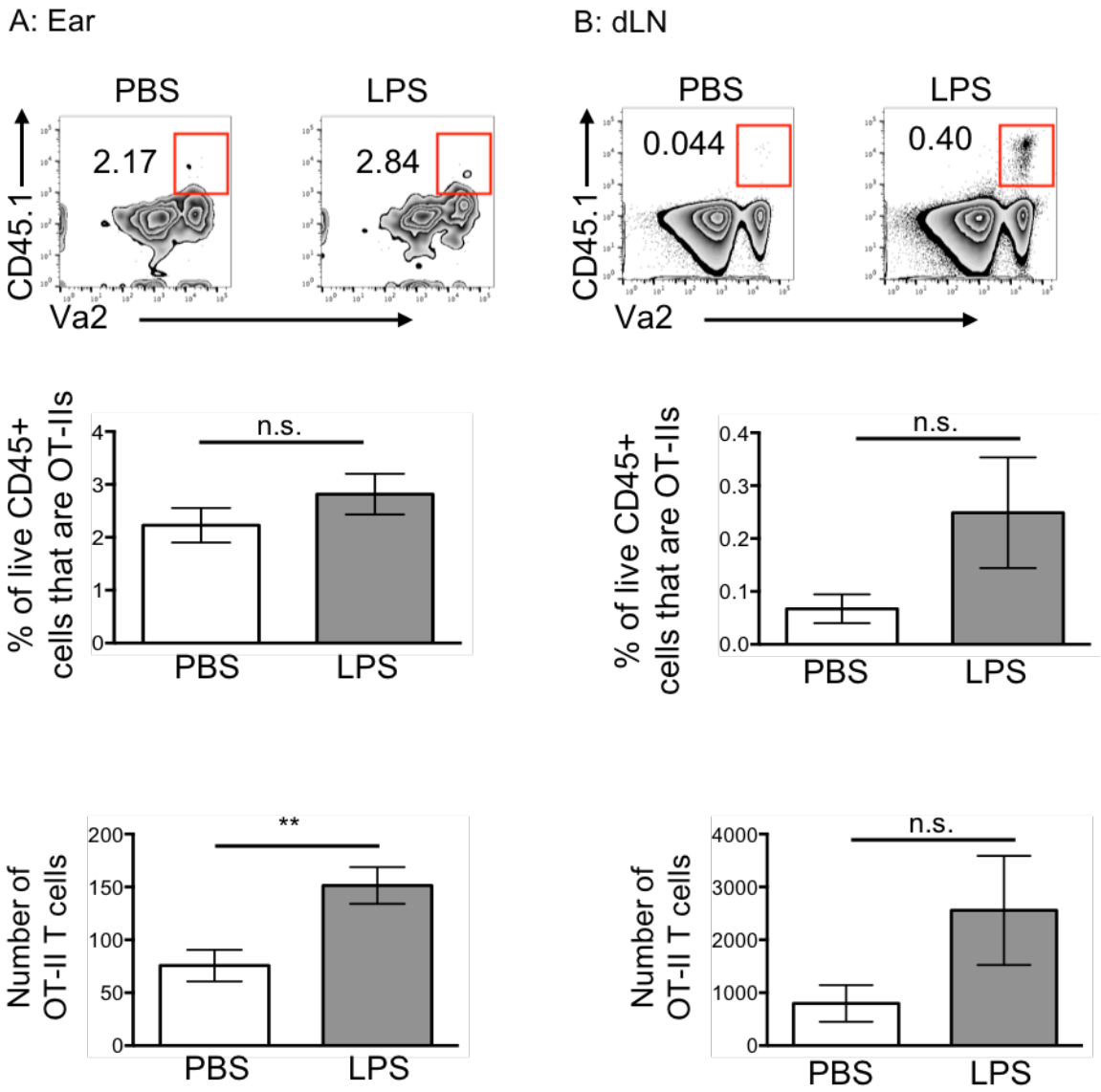

Figure 3.2.11 Greater number, but not proportion of Th0 cells are recovered from inflamed peripheral tissues

Age matched C57BL/6 mice were challenged with LPS (10μg/10μl) or PBS (10μl) in their right ear pinnae. 24 hours later 3.0e6 polarised CD4 Th0 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots and percentage plots are representative of 2 independent experiments with 9 animals in each group. Number graphs are combined data from 2 independent experiments with 9 animals per group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a Mann-Whitney test in Graphpad prism. ** <0.01. n.s. denotes not significant.

3.2.8 Type of inflammation plays a role in T cell persistence

Chronically inflamed tissues often differ from acute sites of inflammation. Chronic inflammation is not only defined by its period of persistence but also by the involvement of adaptive immune cells (61). Crucially, most human inflammatory diseases are examples of chronically inflamed tissues such as an RA joint and a psoriatic skin lesion (1).

Previous studies have demonstrated differential regulation of cellular accumulation based on the inflammatory stimuli (244, 453). Thus, a model of persistent T cell mediated skin inflammation was designed to investigate this question. In this model, animals were immunised subcutaneously with ovalbumin emulsified in CFA. CFA consists of heat killed mycobacterial particles. It is a potent activator of immune responses. The emulsified solution forms a granulomatous depot at the site of challenge which drives persistent antigen presentation and antigen specific T/B cell activation in the draining lymph nodes (526).

7 days post immunisation, animals were challenged with PBS, inert polybeads or polybeads conjugated with ovalbumin intra-dermally in the ear pinnae. The beads were designed to persist at the site of injection for a prolonged period of time. Persistent antigen at peripheral tissues is known to elicit delayed type hypersensitivity responses in previously immunised hosts (9).

Ear inflammation was allowed to persist for 9 days. Inflammation was measured by a mouse ear swelling test (MEST) throughout the period of inflammation. Mouse ear thickness was found to be significantly increased in animals challenged with ovalbumin conjugated beads compared to beads alone or PBS challenged animals (Figure 3.2.12A).

CD4 Th1 cells were then adoptively transferred ID in the previously challenged ear pinnae. 24 hours after transfer, animals were euthanised and their ear pinnae and draining lymph nodes were harvested and stained for flow cytometric analysis. No significant differences were observed in the proportion or number of transferred cells recovered from either the resting compared to the inflamed ear pinnae (Figure 3.2.12B).

Interestingly however, significantly greater proportion and number of transferred T cells were recovered from the draining lymph node of mice challenged in the ear pinnae with ovalbumin conjugated beads compared with either beads alone or PBS (Figure 3.2.12C).

Finally, differences in the number of endogenous CD4 T cells were enumerated to determine whether T cell mediated inflammation had taken place. Significantly greater numbers of endogenous CD4 T cells were recovered from the ear pinnae of animals challenged with ovalbumin conjugated beads compared to either beads alone or PBS. No significant differences were observed in the number of endogenous CD4 T cells between the groups in the draining lymph nodes (Figure 3.2.12D).

These data demonstrate that T cell persistence at inflamed tissues is dependent on the type of inflammation. While acute bacterial inflammation promotes persistence of Th1 cells, chronic hypersensitive inflammation does not.

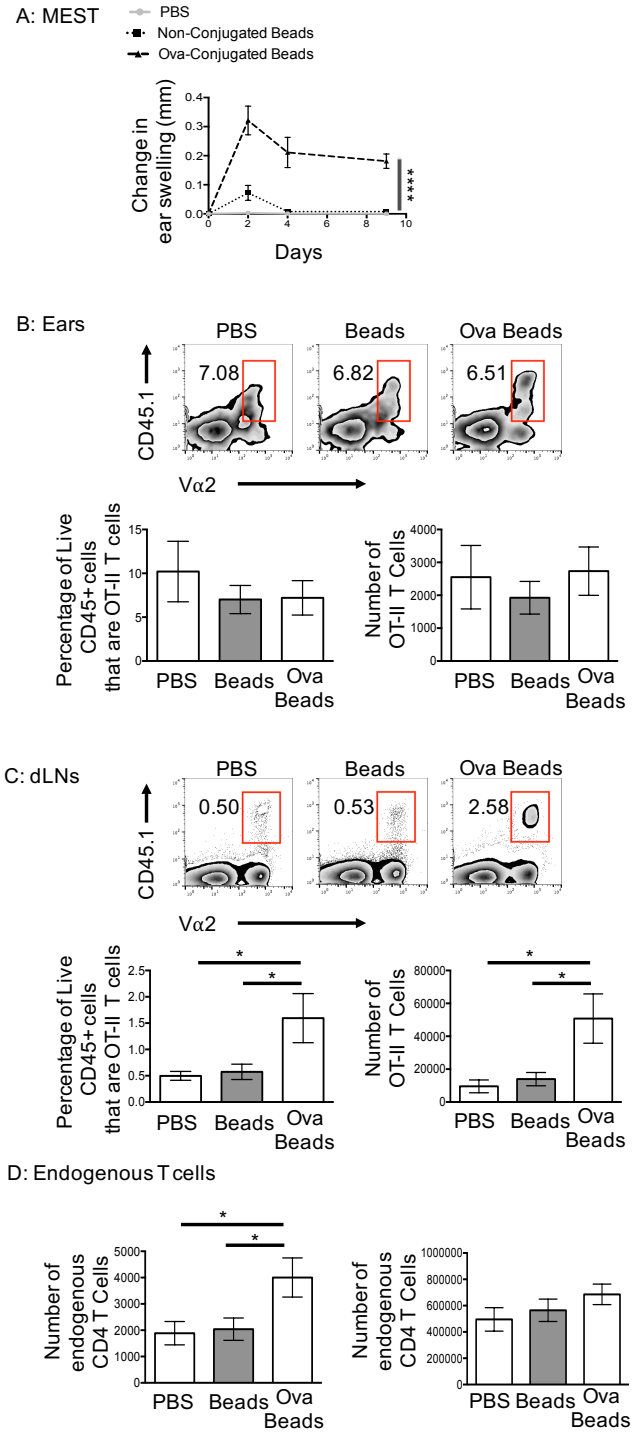

Figure 3.2.12 CD4 Th1 cells do not persist at chronically inflamed tissues
Age matched C57BL/6 mice were challenged with Ovalbumin emulsified in CFA (100μg/100μl) subcutaneously. 7 days later, PBS, beads alone or ovalbumin conjugated beads were injected intradermally in their right ear pinna. Ear swelling was measured using digital callipers over the next 9 days (A). 2.0e6 CD4 Th1 cells were then adoptively transferred into the same ear pinnae. 24 hours later, animals were euthanised and their ear pinnae or draining lymph nodes were harvested, analysed by flow cytometry and the number of transferred (B,C) or endogenous (D) CD4 T cells enumerated. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. Representative FACS plots are shown from 1 of 2 independent experiments. Graphs show combined data from 2 independent experiments with 10 animals per group. Error bars represent standard deviation. Statistical differences were determined by carrying out a One-way ANOVA and Tukey's multiple comparisons test in Graphpad PRISM. * denotes a p value of <0.05.

3.2.9 Cellular profile of chronically inflamed tissues

The interesting finding that Th1 cells did not persist in antigen mediated delayed type hypersensitivity inflamed tissue led to the hypothesis that LPS inflamed and DTH inflamed tissues were different from each other. Thus, the myeloid cell profile of the chronically inflamed tissue was also characterised.

Mice were challenged subcutaneously with Ova emulsified in CFA. 7 days later, PBS or ovalbumin conjugated beads were injected intradermally to induce inflammation. 9 days later, OT-II Th1 cells were transferred into these same ears.

Mice injected with Ova-beads had significantly greater numbers of macrophages, CD103 DCs, CD11b DCs, CD8α DCs and neutrophils compared to PBS challenged mice. No differences were observed in the proportions of macrophages or CD8α DCs but reduced proportions of CD103 and CD11b DCs were found at ova-beads challenged ears compared to PBS challenged ears. The proportion of neutrophils were also substantially increased in Ova-beads challenged ears. These data indicate that antigen-mediated chronic DTH inflammation causes significant infiltration of multiple myeloid cell populations (Figure 3.2.13).

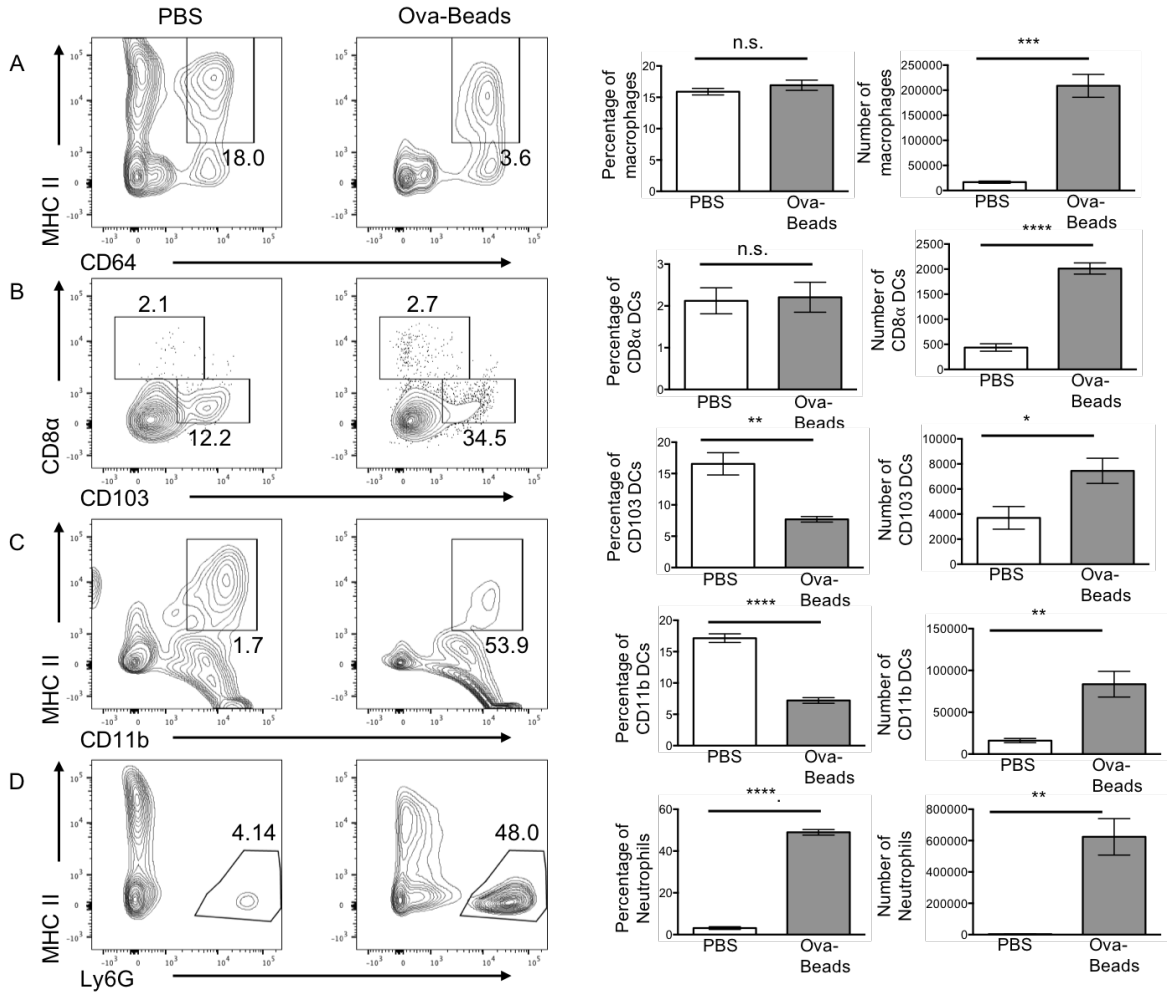

Figure 3.2.13 Chronically inflamed tissues have increased proportions and numbers of APCs and neutrophils compared to non-inflamed tissues

Age matched C57BL/6 mice were challenged with Ovalbumin emulsified in CFA (100μg/100μl) subcutaneously. 7 days later, PBS or ovalbumin conjugated beads (Ova-beads) were injected intradermally in their right ear pinna. 9 days later, animals were euthanised and their ears were harvested, processed and stained for FACS. Data is representative of 2 independent experiments with 4 animals in each group. FACS plots were pre-gated on live CD45+ lymphocytes. Error bars represent standard error of mean. Statistical differences were determined by carrying out an unpaired Student's T test in Graphpad prism. * denotes a p value of <0.05, ** <0.01. *** <0.001 and **** <0.0001. n.s. denotes not significant.

3.3 Discussion

Immune cell trafficking to and from inflamed tissues is a critical process for the mammalian immune system. It enables CD4 T cells to be at the right place at the right time. Naïve T cells circulate through SLOs until they encounter their cognate antigen (389). In contrast, effector T cells migrate from SLOs to inflamed peripheral tissues, upon activation (276). Once at inflamed tissues, CD4 T cells can help clear pathogen and resolve inflammation. However, in chronic inflammatory diseases, CD4 T cells can accumulate at affected tissues (7, 84, 421). This can prolong and exacerbate inflammation, leading to tissue damage. In this chapter, an inflamed tissue model was developed to study effector CD4 T cell persistence. Understanding T cell persistence may allow us to develop therapies which reduce inflammation in chronic diseases or increase persistence in vaccine settings.

3.3.1 LPS elicits substantial tissue inflammation

To set up a short-term tissue inflammation model, LPS was administered intradermally in the ear pinnae of mice. LPS was chosen as an inflammatory stimuli due to its ability to induce rapid acute inflammation (527). Rapid inflammation was necessary to enable the study of early stages of inflammatory responses.

LPS injection in the ear pinnae caused significant ear swelling 24 hours after administration. In addition, histology showed that inflamed ear pinnae had significant RBC extravasation into the tissue. Both of these findings are supported by previous studies and confirmed that LPS caused early and significant inflammation in mouse skin.

LPS administration also caused substantial cellular changes. Significantly greater numbers of neutrophils and CD103+ DCs were found in inflamed ear pinnae. Neutrophil influx into early inflamed tissues is well documented (527). Early neutrophil influx is guided by the chemokine CXCL8 (528, 529). LPS mediated upregulation of CXCL8 production has been reported in multiple studies (530, 531). Likewise, the recruitment of CD103+ DCs into inflamed or damaged tissues at early time points is well documented in the literature (532).

In sum, injection of LPS in the mouse ear pinnae caused a significant development of inflammation, 24 hours post administration. This model tissue site may form a suitable site to study the persistence of CD4 T cell persistence.

3.3.2 Tissue inflammation promotes T cell persistence

To develop a model of T cell persistence at the newly designed inflamed tissue, polarised CD4 Th1 cells were adoptively transferred intradermally into the pre-treated ear pinnae. Direct transfer of cells into the inflamed tissue was a key point in the design of the model and played a significant role in the choice of tissue site. Direct transfer enabled me to exclude compounding recruitment signals. Hence, any effects observed would be as a direct result of signals which the cells received following their artificial recruitment into the tissue. The ear pinnae was a suitable site for such adoptive transfers (244). Likewise, Th1 cells were chosen due to their reported accumulation at, and role in, early stages of tissue inflammatory diseases such as RA and psoriasis (7, 66, 69, 413).

A greater proportion and number of transferred cells were recovered from inflamed ear pinnae compared to resting ear pinnae, 12 and 24 hours after T cell transfer. In contrast, no differences were observed in the numbers of transferred cells at lymph nodes draining the inflamed or resting ear pinnae. While these results established that greater numbers of T cells remained at inflamed tissues, they also confirmed that this was not due to decreased T cell egress to draining lymph nodes. This is a key point as previous studies have demonstrated a role for CCR7 in controlling T cell accumulation at inflamed tissues by regulating their tissue egress (451, 452). Hence, my model indicates that mechanisms other than CCR7 mediated retention also play a role in the regulation of peripheral T cell accumulation.

3.3.3 T cells at inflamed tissues are functionally distinct

Transferred T cells recovered from resting or inflamed tissues were analysed for their production of IFNγ. A significantly greater proportion of T cells recovered from resting tissues were found to produce IFNγ, compared to T cells from inflamed tissues. This finding was surprising since effector T cells normally carry out their function at inflamed tissues (441). IFNγ plays a critical role in

activating macrophages to induce pathogen killing (533, 534). In contrast, IFNγ can increase pathogenesis in chronic inflammatory diseases by aberrant and prolonged activation of innate immune cells which in turn causes tissue damage (535).

One explanation for this could be, that Th1 cells were artificially placed in a tissue site where they did not receive the correct signals. Therefore, their production of IFNγ was not a directed response. Studies have previously shown that CD4 T cells undergoing AICD often produce large amounts of IFNγ. Moreover, studies have also reported the necessity of IFNγ for the induction of apoptosis (536, 537). This suggests that IFNγ producing Th1 cells at non-inflamed tissues may be undergoing apoptosis.

T cell expression of CD69, an early lymphocyte activation marker was also analysed prior to transfer and after recovery from tissues. CD69 has previously been described as a lectin that promotes memory T cell persistence and retention in peripheral tissues (523). Almost all Th1 cells were found to be CD69 positive prior to transfer. Following recovery from tissue however, no differences were observed in the proportion of T cells expressing CD69. This result suggests that the activation state of the T cell may dictate the mechanism of its persistence. I.e. effector T cells use a different signal for tissue persistence than naïve or memory T cells.

3.3.4 Activation state of T cell and inflammatory stimuli affects T cell persistence

T cells differ significantly in their function and receptor expression based on their state of activation (538, 539). Naïve T cells express high levels of CCR7 to enable them to home to SLOs (345). Effector T cells in contrast express tissue specific CKRs such as CCR5 (Th1) or CCR4 (Th2) (393, 401, 504). This enables them to traffic to inflamed or infected tissues. To understand whether persistence of T cells at LPS inflamed tissue was subtype specific, naïve T cells or T cells stimulated with peptide alone (Th0) were transferred into inflamed or resting ear pinnae. Greater numbers of naïve and Th0 T cells were recovered from inflamed tissues compared to resting tissues, phenocopying the Th1 experiments. In contrast to the Th1 experiments, reduced number of transferred

naïve T cells were recovered from inflamed dLNs compared to resting ones. No differences were observed in the recovery of Th0 cells between resting and inflamed dLNs. This result indicated that naïve T cell persistence at inflamed tissues was due to decreased egress to draining lymph nodes, unlike Th1 and Th0 cell persistence. Studies by Debes et al. and Bromley et al. have previously indicated a role for CCR7 in controlling T cell retention at peripheral tissues (451, 452). Naïve and effector T cells have different CCR7 expression profiles. Thus, changes in CCR7 ligand CCL19/21 at inflamed tissues could differentially regulate naïve and effector T cell retention.

Inflamed tissues also differ significantly in terms of their morphology and cellular content. Whereas acute inflammation is driven primarily by innate immune cells and their cytokines, chronic inflammation is often driven by adaptive immune cells (61). Hence, I tested whether Th1 cells persist in a longer-term inflammation mediated by adaptive immune cells. No differences were found in the tissue persistence of Th1 cells at inflamed or resting tissues.

Intriguingly, however, a greater proportion and number of OT-II cells were recovered from draining lymph nodes of chronically inflamed ears. This could be due to one of two reasons: Inflammation was induced using ova attached to polybeads, hence it was an antigen mediated system. Antigen could have drained to the local draining lymph node where ova specific transferred OT-II Th1 cells accumulated in response to antigen. Antigen specific T cell accumulation is well documented (9).

Alternatively, T cell egress at chronically inflamed tissues could be controlled differently to that of acutely inflamed tissues. This idea was explored by Brown et al. who demonstrated that T cell exit from acute and chronically inflamed tissues are differentially regulated (453). The study demonstrated that CCR7 plays an important role in T cell egress from acute but not chronically inflamed tissues. Thus, the authors suggested that other mechanisms may play a role in regulating T cell egress at chronic tissues. Investigating whether Th1 cells persist at chronically inflamed sites with a non-antigen specific inflammation, i.e. BSA-specific T cells or inflammation induced by non-ova antigen, may reveal which mechanism might be at play here.

I also investigated the myeloid cell population in the Ova-beads inflamed tissue to check whether differences in myeloid cells could explain the lack of T cell persistence in this model. Despite a significantly prolonged inflammatory time point and a different type of insult, the myeloid compartment looked similar between LPS injected and Ova-beads injected tissues. The only difference observed was the increased number of CD64+ macrophages in the ova-beads tissues. Further investigations with a macrophage depleted ova-beads inflamed tissue could reveal whether they have a role in reducing T cell numbers at inflamed tissues.

In conclusion, a model inflamed tissue site was developed in this chapter where CD4 Th1 cells persist. In the next chapter, both the tissue site and the cells are manipulated to dissect the signals which cause Th1 cell persistence at LPS inflamed tissue sites.

4 Investigating the signals which cause CD4 T cell persistence at inflamed tissues

4.1 Introduction

T cell entry into tissue sites is predominantly controlled by the varied expression of chemokines and cell adhesion molecules (CAMs) such as selectins and integrins (540). Once at a tissue site however, the signals which result in T cell persistence is not only regulated by the varied expression of chemokines and CAMs, but also antigen bearing APCs (541-545). Together, these molecules regulate the entry, movement and exit of leukocytes to, within and from inflamed tissues (149).

Inflamed tissues further differ in their expression of survival and apoptotic signals. These include varied expression of death signals as well as cytokines and chemokines which promote and/or suppress cell survival and proliferation (525, 546). Naturally, survival, proliferation and death signals also have an impact on tissue behaviour and exit of persistent leukocytes.

In this introduction, these distinctive yet complementary processes are briefly explored as potential mechanisms of T cell persistence. Evidence in the literature is explored in context of the observed phenotype of T cell persistence at inflamed tissues as described in the previous chapter.

4.1.1 Antigen mediated T cell persistence

Naïve T cells circulate through secondary lymphoid organs. Here, they encounter antigen bearing APCs. The interaction between an antigen bearing APC and its cognate T cell results in arrest and persistence of the T cell in the SLO (545, 547). It is believed this reduction in T cell velocity enables appropriate scanning and subsequent activation of T cells (548).

At inflamed peripheral tissues, effector T cells likewise interact with antigen bearing APCs (549). A study by Reinhardt et al. demonstrated that this interaction also results in the increase of T cell persistence at the inflamed tissue site. However, unlike in the SLO, where antigen encounter results in T cell proliferation, no such proliferation was observed following antigen encounter at tissue site (392).

The exact mechanism of antigen mediated peripheral T cell persistence is not well understood. One possibility is that MHC-TCR interactions are sufficient to retain T cells, however, due to the low affinity of this interaction, it is unlikely to be true (550). The more attractive idea is that antigen encounter induces increased adhesion molecule expression on T cells, resulting in their increased persistence. Two studies by Andreasen et al. and Ray et al. report high expression of integrins on T cells at peripherally inflamed tissues, suggesting that this could be true (551-553).

4.1.2 Control of T cell apoptosis

Naïve T cells encounter antigen followed by their activation and clonal proliferation at SLOs (390). Effector T cells subsequently migrate to inflamed tissues to carry out their effector function (441). The final stage of an immune response is the contraction and memory phase. Here, the majority of activated and effector T cells undergo apoptotic cell death at tissue sites or develop into long lasting memory cells (403).

Apoptotic cell death occurs via one of two pathways. Cell extrinsic pathways such as AICD or cell intrinsic pathways including, ACAD (284). Cellular apoptosis in both cases is primarily executed by proteolytic enzymes known as caspases (469). Caspases are initially produced as inactive zymogens. Later, these caspases undergo proteolytic processing leading to their activation and ultimately, to cell death (469).

Caspase activation is a complex and multi-step process. Essentially, receptor triggering leads to a signalling cascade followed by the formation of a caspase initiation complex (554). In this complex, precursor initiator caspases are activated. These initiator caspases then activate effector caspases including, caspase-3, caspase-6 and caspase-7 (554).

Effector caspases interfere with actin and nuclear lamins which maintain cell and nuclear morphology respectively. This leads to nuclear shrinkage, blebbing, cell fragmentation and the formation of apoptotic bodies (470, 471). Apoptotic cells further express extracellular phosphatidyl serine which triggers their phagocytosis by professional phagocytes (555).

4.1.3 Extrinsic and intrinsic executioners of death at inflamed tissues

AICD on activated T cells is triggered by ligation of cell surface death receptors. Ligands include TNF, FASL and TRAIL. Activated T cells express TNFR1, FAS, TRAILR1/TRAILR2 and death receptors (DR) 3 and 6 to interact with these ligands (284). Some, or all of these ligands have been found across multiple inflamed tissues (546).

Controversially, some of these death signals also generate pro-survival effects on T cells. TNF-α was found to interfere with FAS signalling and increase T cell survival (508). Moreover, DR3 signalling directly increased T cell accumulation and resistance to apoptosis (556, 557)

ACAD, in contrast is initiated by TCR stimulation, DNA damage, ER stress and cytokine deprivation (467). Previous studies have demonstrated that TCR stimulation of activated T cells, without co-stimulatory signals, leads to increased apoptosis (524). Moreover, lack of cytokines such as IL-2, IL-4 and IL-7 substantially increased T cell apoptosis (525).

Interestingly, increased levels of IL-7 has been reported in multiple inflamed tissues including RA joints (558, 559). This suggests that T cell accumulation at inflamed sites might in part be regulated by decreased T cell apoptosis.

4.1.4 Chemokines as regulators of T cell survival, apoptosis and chemotaxis

Chemokines are chemotactic cytokines whose main function is controlling cellular migration (151). Chemokines also regulate cell proliferation, apoptosis and survival (560, 561). Chemokines signal through a multitude of G-protein coupled receptors known as chemokine receptors (154, 155). The differential effects of chemokines on cells is a consequence of distinct temporal and spatial expression of chemokine receptors (155). Moreover, chemokines display promiscuity for their receptors. This further enhances their functional repertoire (157).

Effector T cells home to inflamed tissues, guided by chemokines (36). Additionally, differentially polarised T cells display distinct chemokine receptor expression (393, 502). Migration of Th1 cells to inflamed tissues is mediated by a myriad of inflammatory chemokines. CCR4, CCR5, CCR9, CXCR3, CXCR4 and CX3CR1 are but a few of the Th1 chemokine receptors responsible for migration to inflamed tissues (393, 502).

Additionally, signalling through some of these chemokine receptors has aided cell survival or apoptosis. CCL5-CCR5 interactions were found to increase apoptosis of human T cell lines via the ACAD pathway (562). CCL5 was found to aid survival of human macrophages (563, 564). In contrast, CXCL12-CXCR4 signals were initially described as anti-apoptotic in neurons but were later found to be pro-apoptotic in effector CD4 T cells (565, 566).

CCL19/21 binding to CCR7 can also trigger effector T cell apoptosis in SLOs (567). In contrast, *in vitro* studies, revealed that CCR7 signals can aid survival of CD4 T cells (568). Exact mechanisms of antagonistic effects of chemokine ligands and receptors have not been elucidated. Receptor promiscuity and specific inflammatory settings may explain some of the observed discrepancies.

4.1.5 Aims of this chapter

This introduction highlights that T cell accumulation at tissue sites is regulated by multiple overlapping factors. Cytokines, chemokines, survival and apoptotic factors are intricately intertwined in this process.

Greater numbers of transferred CD4 Th1 cells were recovered from the inflamed skin model established in the previous chapter. The aim for this chapter was to identify the signals which enable Th1 cells to persist at inflamed tissues compared to non-inflamed tissues.

As part of this, cytokine, chemokine and pro-apoptotic factor production at tissue sites were compared. Furthermore, chemokine receptor and pro-apoptotic molecule expression on Th1 cells were investigated. Together, the data in this chapter aims to identify novel signals which cause CD4 T cell persistence at inflamed tissue sites.

4.2 Results

4.2.1 Cognate antigen does not alter the persistence of CD4 Th1 cells at inflamed tissues

Migrational arrest and prolonged persistence of antigen specific T cells in response to cognate antigen on APCs is well documented. Studies have demonstrated that both naïve and effector T cells undergo antigen mediated arrest at SLOs and peripheral tissues (541-545).

Hence, investigations were undertaken to determine whether cognate antigen mediated interactions altered the persistence of Th1 cells at LPS inflamed peripheral tissues. Mouse ears were inflamed by the administration of LPS in the presence or absence of heat-aggregated ovalbumin (HAO). HAO rather than soluble ovalbumin was used to ensure prolonged localised persistence of ovalbumin at the tissue site.

In vitro polarised OT-II Th1 cells specific for ovalbumin were then transferred ID into the inflamed or resting ear pinnae, 24 hours after induction of inflammation. The animals were then allowed to rest for a further 24 hours prior to their euthanasia. Treated ear pinnae and their downstream draining lymph nodes were harvested and processed for flow cytometric analysis.

Significantly greater numbers, but not proportion of transferred cells were recovered from LPS inflamed ear pinnae compared to resting ear pinnae as seen in previous experiments. HAO administration at inflamed ears did not further increase Th1 cell persistence. However, number of cells recovered from inflamed, HAO treated ear pinnae were not significantly higher than those recovered from resting ear pinnae. HAO administration at non-inflamed ear pinnae resulted in no significant changes in transferred T cell recovery (Figure 4.2.1A).

Likewise, HAO administration did not alter the recovery of T cells from draining lymph nodes draining either inflamed or resting ear pinnae. No significant differences were observed in either the proportions or numbers of recovered OT-II T cells from draining lymph nodes between any groups (Figure 4.2.1B). In

summary, these data suggest that at acute LPS inflamed peripheral tissues, presence of cognate antigen does not alter T cell persistence.

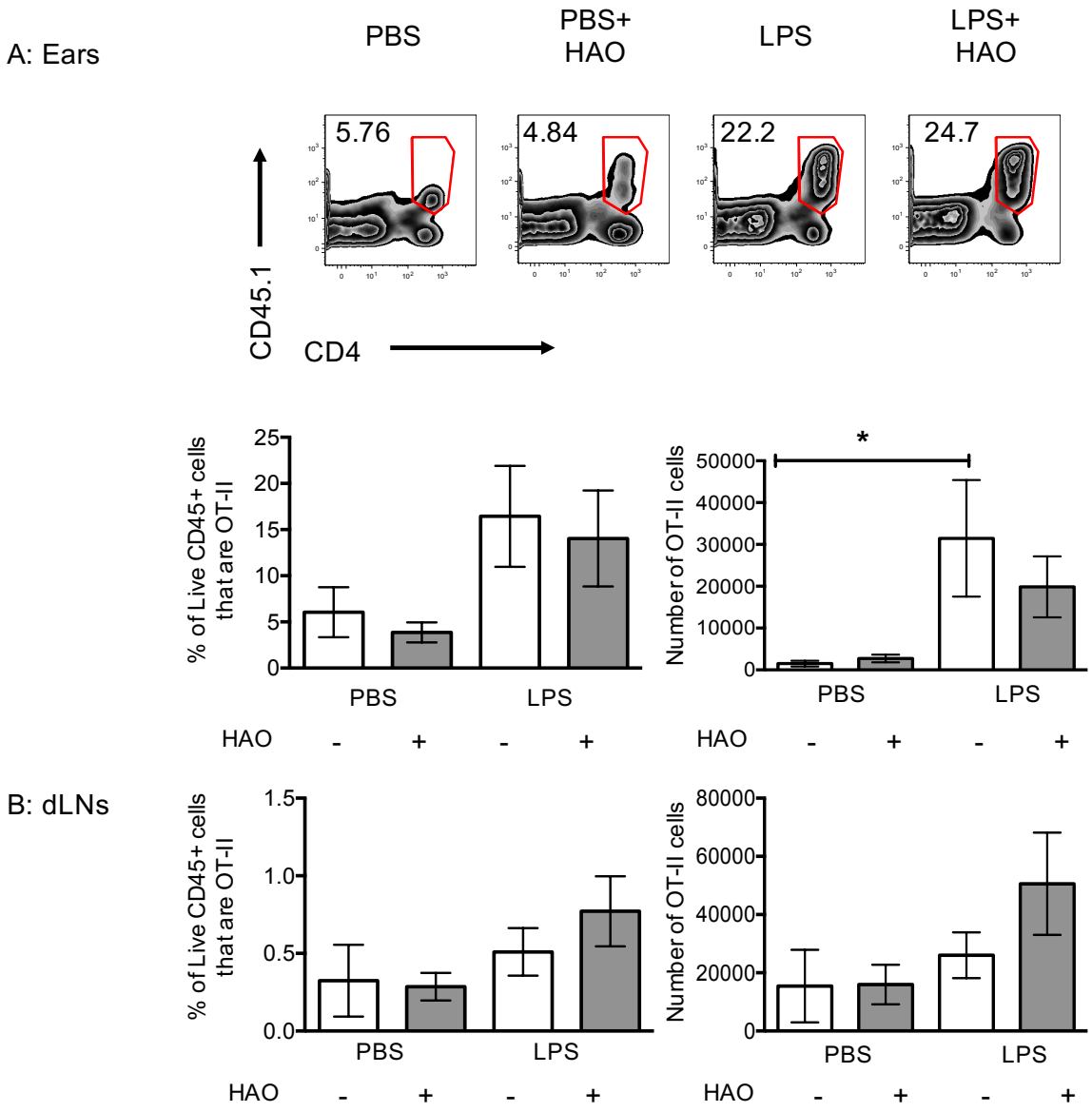

Figure 4.2.1 Cognate antigen does not alter the persistence of CD4 Th1 cells at LPS inflamed peripheral tissues

Age matched C57BL/6 mice were challenged with LPS (10μg/10μl) or PBS (10μl) in their right ear pinnae with or without HAO (20μg/10μl). 24 hours later 5.0e6 polarised CD4 Th1 cells were transferred into the same ear pinna. 24 hours after that animals were euthanised and their right ear pinna (A) and draining lymph nodes (B) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4 and CD45.1. Lymphocytes were initially gated on live CD45 cells and subsequently analysed for their expression of CD4 and CD45.1. FACS plots are representative of 2 independent experiments with ≥ 3 animals in each group. Percentage and number graphs are combined data from 2 independent experiments with 8 animals per group. Error bars represent standard error of mean. Statistical differences were determined by carrying out a One-way ANOVA and Tukey's multiple comparison's test in Graphpad prism. * <0.05.

4.2.2 CD4 Th1 cells exhibit altered behaviour at inflamed tissues

Intravital multiphoton microscopy allows for imaging and analysis of cellular behaviour deep within tissues of living animals (569). Analysing parameters such as velocity, displacement and interaction of cells within tissue can illuminate novel behavioural mechanisms of T cell persistence at inflamed sites.

To study such behaviours, fluorescent reporter animals were necessary. Th1 cells were polarised from OT-II mice that expressed an endogenous DsRed fluorescent protein within their T cells. These T cells were then transferred into LPS or saline treated ears of CD11cYFP mice. CD11cYFP mice endogenously express the fluorescent protein YFP within CD11c+ cells, which is classically expressed on dendritic cells (497).

The second harmonic signal (blue) demarcates tissue structural complexes, such as collagen. It is often useful for the identification of landmarks within tissues such as lymphatic or blood vessels which are hollow and do not generate second harmonic signals.

To understand the initial behaviours that may result in T cell persistence 24-hours post transfer, animals were imaged for 4-5 hours after T cell transfer. Animals were anaesthetised and restrained on a temperature-controlled stage and time-series movies of cells in the ear were acquired.

OT-II T cells displayed limited motility in resting ear pinnae with low velocity and displacement. In contrast, OT-II T cells in inflamed ears demonstrated higher motility as well as increased displacement rate (Figure 4.2.2A-D). Movies and still images further confirm that T cells were localised in similar locations either within or near collagen fibres in tissues (Figure 4.2.2E,F)(Video 1,2). The increased motility and displacement of T cells at inflamed ears are similar to that of T cells in LNs (570). This suggests that T cells at inflamed tissues are not restricted by tissue matrix. Highly motile behaviour also indicates the lack of cellular interactions. Consequently, no direct and prolonged interactions were observed with DCs. In contrast, the immobile Th1 cells at the saline treated ear pinnae looked similar to cells undergoing apoptosis (571). This suggested that the T cells were undergoing apoptotic death at non-inflamed sites.

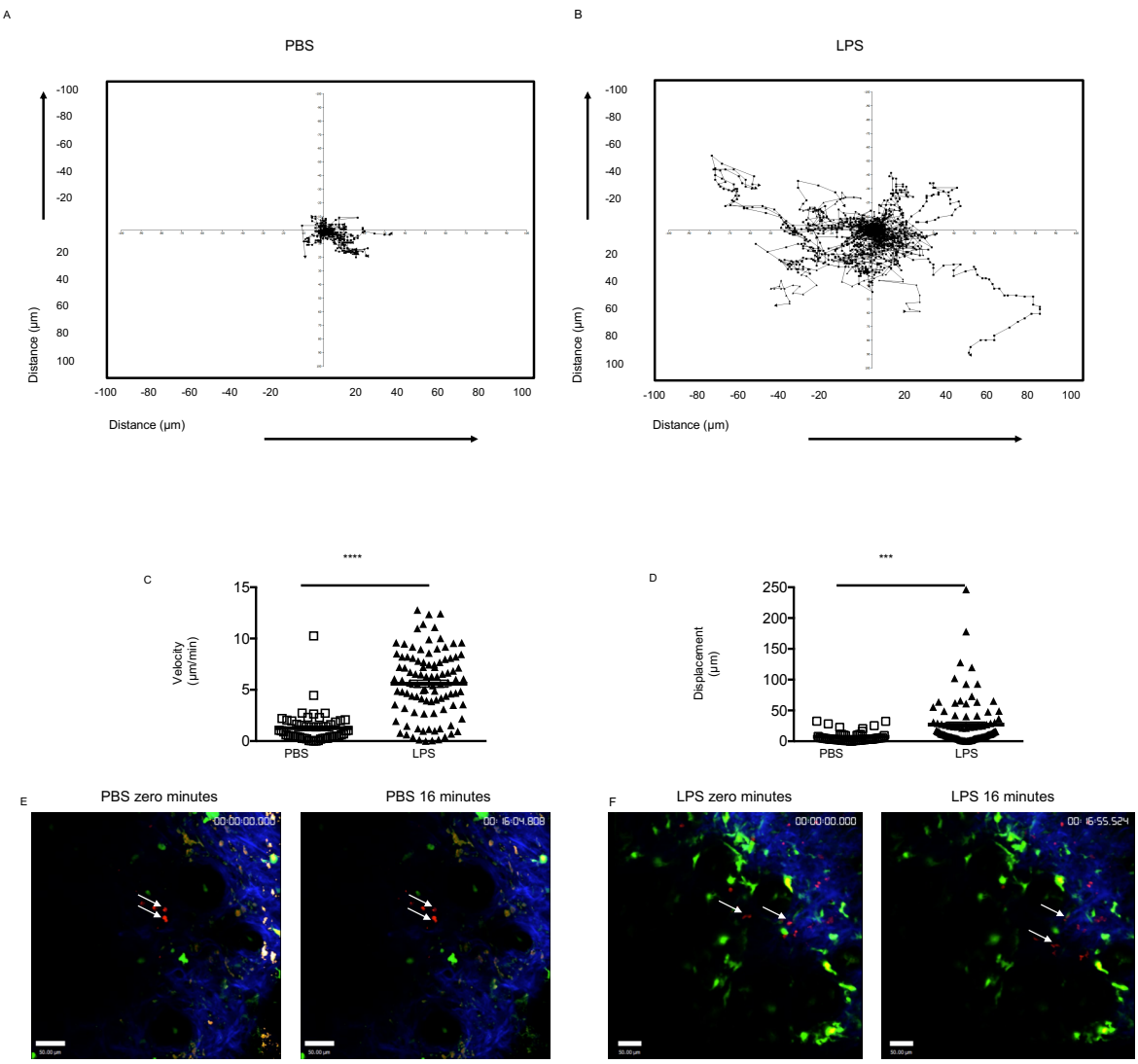

Figure 4.2.2 CD4 Th1 cells travel greater distances at a greater velocity in inflamed tissues compared to resting sites

CD11cYFP mice were challenged with either PBS or LPS. 24 hours later, ~2.0e3 polarised Th1 cells expressing DsRed were transferred into the injection site at a shallow depth, in a small volume. The animals were allowed to rest for 4-5 hours before the ears were imaged using a Zeiss LSM 7MP microscope. Images were acquired using a 20x/1.0NA water immersion objective lens. Images were then analysed using volocity software and DsRed positive cells were individually tracked manually, generating their velocity and displacement rates. (A,B) X-Y plots show the distance of T cell tracks and are representative plots from 1 of 3 individual mice per treatment group. (C,D) Velocity and displacement graphs show combined values from 3 individual mice per group. (E,F) Representative stills from 1 of 3 individual mice per treatment group, illustrating distance travelled by T cells from time zero, indicated by arrows. Each data point represents a DsRed T cell and bars represent mean. In the movies, green cells = CD11c+ cells, blue = second harmonic signal and red cells = CD4 Th1 cells. Arrows demarcate the position of the same T cell at different time points, indicating their movement or lack thereof. Statistical differences between groups were assessed by carrying out a Mann-Whtiney test using Graphpad Prism *** denotes p value of < 0.001 and **** < 0.0001.

4.2.3 CD4 Th1 cells survive better at inflamed tissues

My observation of greater Th1 cell recovery from inflamed ear tissue, without a concomitant decrease in the dLN, suggested that T cell proliferation or survival might be taking place at inflamed tissues. At non-inflamed tissues however, T cells were immobile and produced increased IFNγ. Decreased motility as well as increased IFNγ production have both been previously associated with apoptotic T cells (536, 571). Therefore, the survival and proliferation of Th1 cells in ear tissue was evaluated.

To assess cellular proliferation, T cells were labelled with cell tracker blue. Cell tracker blue is a fluorescent dye that is equally divided into daughter cells upon cell division. Subsequent enumeration of cells within each fluorescent peak determined the proportion of proliferating cells.

To evaluate apoptosis, T cells recovered *ex vivo* from ear tissue were cultured in the presence of a fluorescent inhibitor of caspases (FLICA) molecule. The FLICA molecule undergoes an enzymatic reaction when it is covalently bound to any active caspase 3 or 7. Apoptotic cells express caspases 3 and 7, accordingly FLICA labels apoptotic cells with a green colour (fluorescein) (572, 573). Assessment of the proportion of FLICA bound cells enabled the enumeration of cells at early stages of apoptosis.

Polarised OT-II Th1 cells labelled with cell tracker blue were thus transferred into LPS inflamed or saline treated mouse ears. 24 hours later, the animals were euthanised and their ears were harvested and processed. Single cell suspensions of cells were stained with flow cytometry antibodies and then cultured with the FLICA reagent for 1 hour. Subsequently, cell survival and proliferation were assessed.

A significantly greater proportion of Th1 cells recovered from non-inflamed tissues were found to express FLICA compared to cells recovered from inflamed tissues (Figure 4.2.3A,B). In contrast, Th1 cells did not undergo any proliferation at either the inflamed or non-inflamed tissue site (Figure 4.2.3C). Together, these data suggest that increased survival signals at inflamed tissues regulate the persistence of Th1 cells.

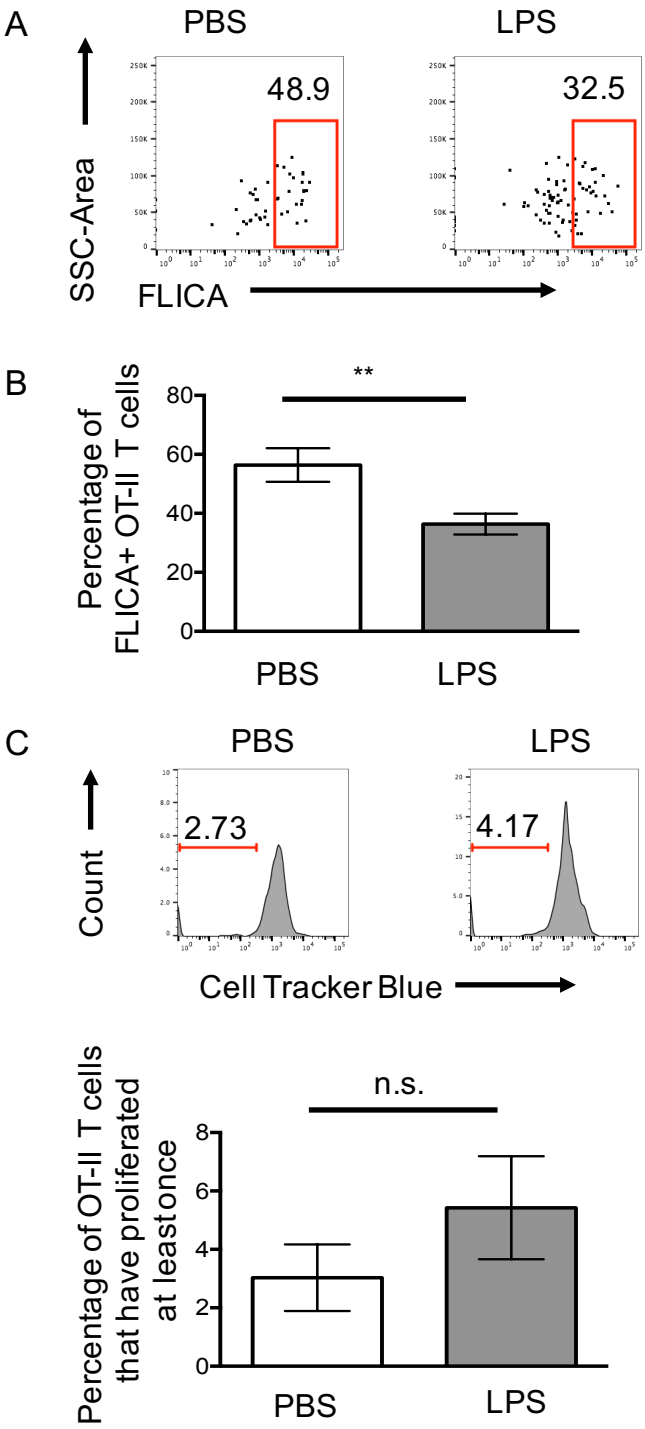

Figure 4.2.3 Fewer CD4 Th1 cells undergo apoptotic death at inflamed tissues but no differences are observed in T cell proliferation at tissue sites

Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS (1μg/10μl) or PBS in their right ear pinnae. 24 hours later 2.0e6 OT-II T_H1 cells were labelled with cell tracker blue and transferred into the animals' same ear pinnae. 24 hours after the transfer, mice were euthanised and their ears were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+, CD45.1+, Vα2+ cells and the expression of active caspase 3 and 7 was analysed by measuring their expression of FLICA by incubating cells using a FLICA kit (life technologies). FLICA FACS plots are representative of 3 independent experiments, Cell tracker blue is representative of 2 experiments. FLICA graph shows combined data from 3 independent experiments with 10 animals in PBS and 11 animals in LPS groups. Error bars represent SEM. Statistical differences between groups were assessed by carrying out an unpaired Student's T test using Graphpad Prism ** denotes p value of <0.001.

4.2.4 Increased survival of Th1 cells is not mediated by differences in IL-7, TNFα or FAS-Ligand expression at tissue site

Greater recovery of T cells at tissue sites might be regulated by increased survival signals or decreased apoptotic signals. TNFα and IL-7 are pro-inflammatory cytokines abundantly present at inflamed tissues (558, 574). Studies have suggested a role for TNFα in rescuing T cells from apoptotic death at inflamed tissues (508). Likewise, studies have reported IL-7 as a survival signal for T cells at inflamed sites (525).

In contrast, FASL is a transmembrane protein of the TNF family that induces caspase mediated apoptosis. FASL is found in both transmembrane and soluble forms. Interaction of FASL with its receptor FAS on T cells leads to cell death (575). Previous reports have suggested increased FASL expression at TLR 4-agonist mediated inflamed tissues (576).

To assess whether TNFα, IL-7 or FASL expression is differentially regulated at inflamed tissues, LPS or saline treated mouse ears were harvested 24 hours post challenge. The ear tissue was homogenised, proteins extracted and concentration measured. Enzyme-linked immunosorbent assays (ELISA) were carried out by loading equal amount of protein for each animal and determining expression levels of the cytokines and FASL.

No significant differences were observed in the production of IL-7 or TNFα between inflamed or resting tissues (Figure 4.2.4A,B). Likewise, no difference was observed in FASL expression between tissues (Figure 4.2.4C). In summary, these data suggest that survival of Th1 cells at inflamed tissues is not directly mediated by differences in TNFα, IL-7 or FASL expression. However, this does not rule out the possibility that signals at inflamed tissues makes cells more sensitive to signals from these proteins.

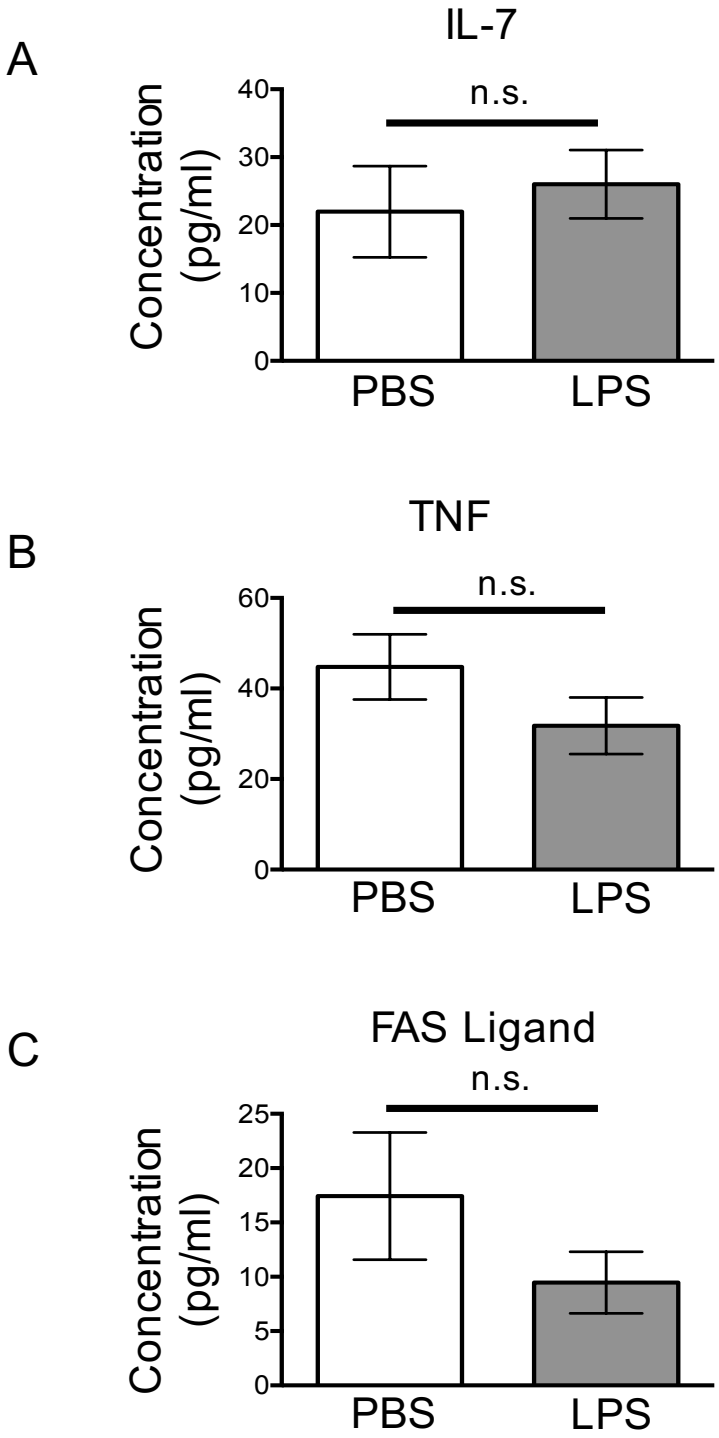

Figure 4.2.4 Survival of Th1 cells is not mediated by differences in IL-7, FASL or TNFα
Age matched C57BL/6 mice were treated with LPS or PBS in their right ear pinnae. 24 hours later, animals were euthanised and their ears were harvested. The tissue was then homogenised in tissue protein extraction buffer (T-PER) using a tissue homogeniser. The protein concentration was measured using a standard BCA assay. Equal amounts of protein were then loaded into wells in duplicate. Concentration of cytokines and FASL was measured against a standard curve by following manufacturers' guidelines as described in materials and methods. IL-7 and FASL ELISAs were quantikine ELISA kits from R&D systems and TNF ELISA was a Mouse TNF ELISA set from BD Biosciences. Graphs show data from one experiment with 4 (TNF) or 5 (IL-7 &FAS-L) animals per group. Error bars represent SEM. Statistical differences between groups were assessed by carrying out an unpaired Student's T test in Graphpad PRISM. n.s. denotes not significant.

4.2.5 CCL5 is upregulated at inflamed tissues

Another potential source of T cell survival signals are chemokines. As discussed in section 4.1.4, multiple chemokines have been reported to function in promoting T cell survival. Since no direct differences were found in cell survival/apoptotic signals, chemokine expression at tissues and chemokine receptor expression on Th1 cells were examined.

To evaluate chemokine expression levels, a proteome profiler array was undertaken. This is a multiplex antibody based protein array that simultaneously detects the expression of 25 different chemokines. They included CCL21, CX3CL1, CXCL12, CCL4 and CCL5 among others. For chemokine receptor expression on T cells, polarised OT-II Th1 cells were stained with anti- CCR4, CCR5 and CX3CR1 antibodies. These chemokine receptors and ligands have been associated with Th1 cell migration and survival in various studies (14, 393, 502, 513).

To assess chemokine expression at tissue sites, mouse ear pinnae were injected with PBS or saline. 24 hours later, animals were euthanised and their ears were harvested, processed and tissue lysates extracted. The lysates were then mixed with detection antibodies and incubated with a membrane pre-coated with capture antibodies.

Finally, the blot was developed using a chemiluminescent reagent to give spots in duplicate for each chemokine assayed. Mean pixel density of these spots were evaluated and relative differences between inflamed and resting ears was established. The graph displays fold change in mean pixel density of Inflamed ears compared to resting ears. Overall, 13 chemokines were upregulated and 12 downregulated at LPS inflamed mouse ears (Figure 4.2.5A). Only CCL5 underwent a greater than 1-fold increase and was therefore considered substantial (Figure 4.2.5A).

Polarised OT-II Th1 cells were also assessed for their expression of CCR4, CCR5 and CX3CR1. Almost all Th1 cells were found to express CCR5 (Figure 4.2.5B). A large majority of Th1 cells were also found to express CCR4 (Figure 4.2.5B). In

contrast, only a small population of polarised Th1 cells were found to express CX3CR1 (Figure 4.2.5B).

Since CCL5 was substantially upregulated and its cognate receptor CCR5 was expressed in all polarised T cells, a further CCL5 ELISA was carried out. This was to confirm whether CCL5 upregulation was statistically significant. CCL5 was found to be significantly upregulated at inflamed tissues (Figure 4.2.5C). In combination, the data suggests that CCL5-CCR5 interactions may play a role in regulating Th1 cell persistence at inflamed tissue sites.

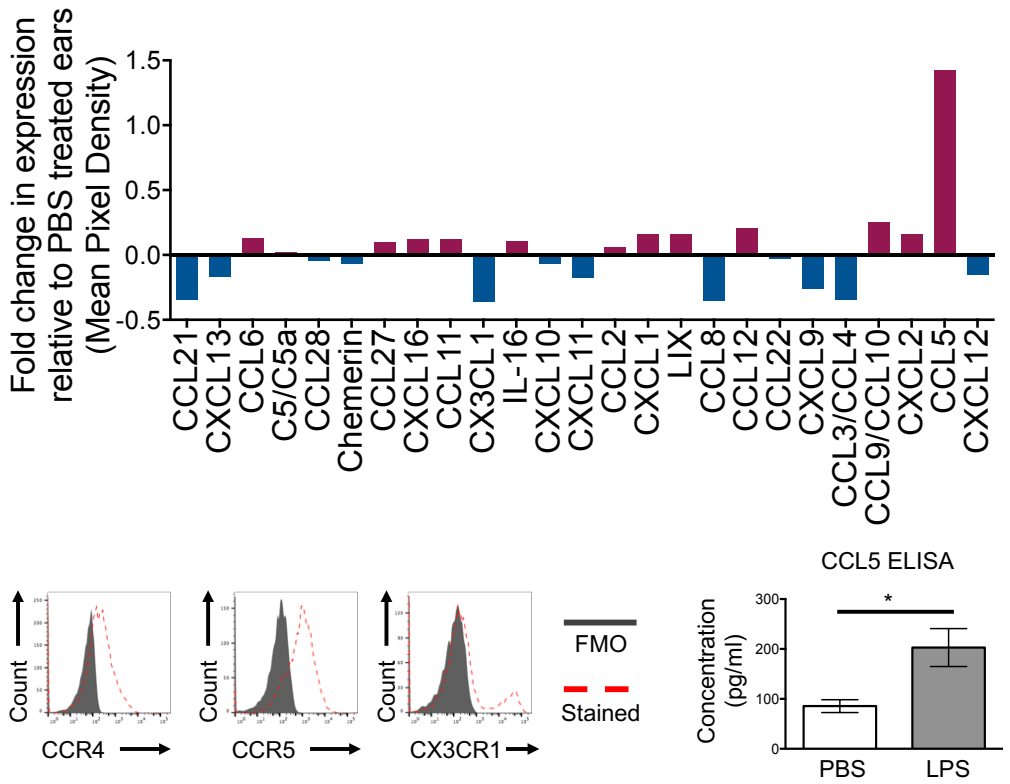

Figure 4.2.5 CCL5 is increased at inflamed tissues
Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS or PBS in their right ear pinnae. 24 hours later, animals were euthanised and their ears harvested and tissue lysates prepared. The lysates were then blotted using a proteome profiler mouse chemokine array kit (A). Fold change was calculated by normalising the pixel density according to proteome profiler array kit instructions and then subtracting pixel density of PBS group values from LPS group values and dividing by the PBS value ((PBS-LPS)/PBS). For flow cytometry, polarised and negatively selected Th1 cells were stained with or without anti-mouse CCR5, CCR4 and CX3CR1 antibodies. Cells are pre-gated on CD4+ Lymphocytes. (B). CCL5 ELISA was carried out on protein extracted from LPS or PBS treated ear pinnae. The protein concentration was measured using a standard BCA assay. Equal amounts of protein were loaded into wells in duplicate and concentration of CCL5 was measured against a standard curve using standard manufacturers' guidelines as described in materials and methods. CCL5 ELISA was a duoset ELISA kit from R&D systems (C). Proteome profiler data shown is from 1 experiment with 5 animals per group. CCL5 ELISA is data from 1 experiment with 5 animals per group and the FACS plots are representative data from 2 independent experiments. Error bars represent SEM. Statistical differences between groups were assessed by carrying out an unpaired Student's T test using Graphpad Prism * denotes p value of <0.05

4.2.6 G-protein coupled receptor signalling mediates Th1 cell persistence at inflamed sites

G-protein coupled receptors (GPCRs) are a family of seven transmembrane helix proteins that are critical in cell signalling. Chemokine receptors are GPCRs. Chemokines, signalling through GPCRs have been shown to upregulate both pro-apoptotic and anti-apoptotic factors in cells (562-564). Chemokines are also more classically known for their role in cellular trafficking (155).

To disrupt GPCR signalling, polarised Th1 cells were treated with pertussis toxin prior to transfer. Pertussis toxin (PTX) is an exotoxin produced by Bordetella *pertussis (577)*. Upon binding the GPCR, PTX is internalised where it catalyses the ADP-rybosylation of the αi subunits of the G protein. This rybosylation prevents G-proteins from associating with GPCRs at the cell surface, disrupting intracellular signalling (578).

PTX or vehicle treated Th1 cells were transferred into LPS or saline treated mouse ear pinnae. 24 hours after transfer, the animals were euthanised and their ears and dLNs were harvested, stained for flow cytometry and numbers of transferred cells enumerated (Figure 4.2.6A).

PTX treatment did not alter the number of T cells recovered from resting tissue sites. In contrast, PTX treatment significantly reduced the number of cells recovered from inflamed tissues (Figure 4.2.6B). PTX treatment however, did not alter the number of transferred cells recovered from the draining lymph nodes of either inflamed or resting ear pinnae (Figure 4.2.6C). These data suggest that GPCR signalling is essential for Th1 cell persistence at inflamed tissues.

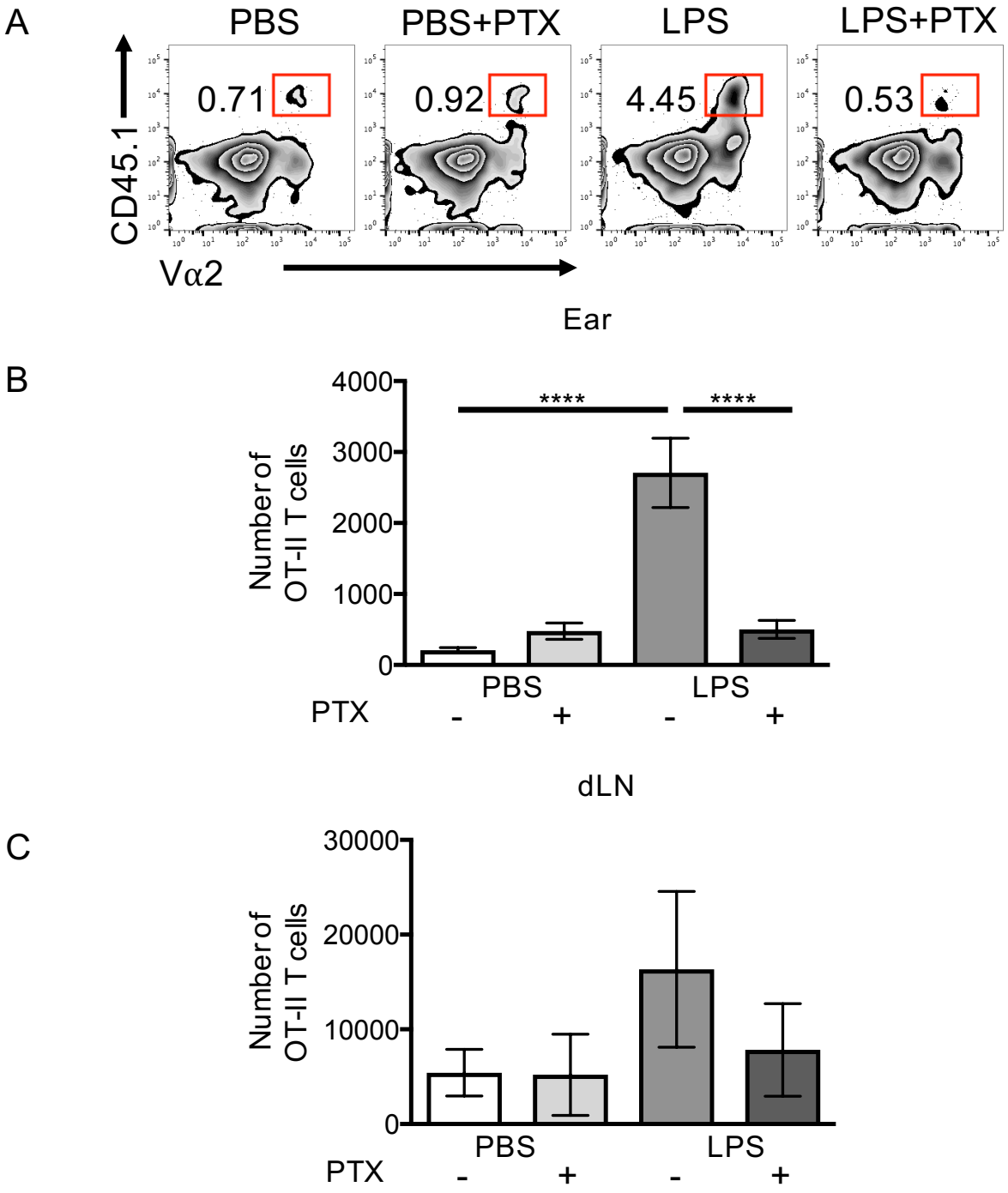

Figure 4.2.6 Fewer Th1 cells are recovered from inflamed sites after PTX treatment
Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 pertussis toxin (100ng/ml) or vehicle treated OT-II T_H1 cells were transferred into the same ear pinnae of animals. 24 hours after the transfer, mice were euthanised and their ears and dLN were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots are representative of 3 independent experiments. Graphs show combined data from 3 independent experiments with 9 (PBS and PBS+PTx) or 7 (LPS and LPS+FTY720) animals per group. Error bars represent SEM. Statistical differences between groups were assessed by carrying out a One-way ANOVA and Tukey's multiple comparisons test using Graphpad Prism **** denotes p value <0.0001.

4.3 Discussion

Effector T cell death is a critical step in the regulation of an immune response. It enables the dampening of inflammation and restoration of tissue homeostasis (579). At inflamed tissues several factors including cytokines, lipid mediators and chemokines regulate the decision for T cell survival or death (580-582). In chronically inflamed tissues however, these factors are dysregulated. This leads to prolonged persistence of T cells which drives local inflammation and tissue destruction (419, 583). In this chapter, the factors that maintain the persistence of Th1 cells at inflamed tissues were investigated.

4.3.1 T cell persistence is not antigen mediated

Previous studies have suggested that antigen-bearing APC and cognate T cell interactions play a role in central and peripheral T cell persistence (541-545). Hence, whether the presence of cognate antigen altered T cell persistence in the LPS inflamed tissue model was investigated. The results suggested that presence of cognate antigen at the tissue site does not alter T cell persistence.

While this result contradicts previous studies, it is in some ways unsurprising. In my model system, animals were not previously primed for a specific antigen as done by Reinhardt et al. and Rosenbaum et al (392, 549). Moreover, while HAO was injected intradermally, its persistence in tissue for 24 hours was not determined. Therefore, whether cognate antigen was present at the tissue site at the time of T cell transfer and beyond cannot be confirmed.

To overcome these technical limitations and address this question more comprehensively, animals could be primed with OVA in the presence of an adjuvant 7 days prior to induction of localised ear inflammation by LPS and HAO followed by intradermal T cell transfer. Presence of OVA at the tissue site should also be confirmed by fluorescent labelling.

4.3.2 T cell motility is mediated by soluble factors

Intravital microscopy of adoptively transferred Th1 cells demonstrated that they were more motile at inflamed tissues compared to resting tissues. This finding is supported by studies which demonstrate that T cells have increased motility at

inflamed tissues (391). This pattern of motility is known as a Lévy walk (584). A Lévy walk allows effector T cells to efficiently scan a peripheral tissue site to find their target cell/area (584, 585).

Multiple factors are responsible for regulating T cell behaviour at tissues including intrinsic programming, physical and chemical guidance cues (391, 585). Intriguingly, soluble mediators have been shown to increase T cell motility rather than provide directional movement *in vivo* (425). This could suggest that the increased motility observed in our studies are due to increased soluble mediators at inflamed tissues. This hypothesis is further explored in the next chapter.

Finally, a study by the Bousso group demonstrated that apoptotic T cells lose their motility prior to caspase 3 expression and death (571). This finding strongly suggests that the non-motile Th1 cells observed in resting tissues were undergoing apoptotic death. Ultimately though, it is difficult to conclude that Th1 cells at the non-inflamed tissues are apoptotic without *in vivo* reporter of apoptosis.

4.3.3 Th1 cell persistence is regulated by inhibition of apoptosis at inflamed tissues

Decreased motility of T cells at non-inflamed tissues was an indication that they were undergoing apoptotic death. To confirm this, an *ex vivo* caspase assay was carried out. The assay demonstrated that greater number of transferred T cells at resting tissues were indeed expressing active caspases compared to T cells at inflamed tissues.

Effector T cells are pre-programmed to undergo apoptosis (586). However, at inflamed tissues several anti-apoptotic signals have been shown to prolong effector T cell survival (587). Elevated levels of the cytokine IL-7 has been reported in RA synovial fluid (581, 588). Moreover, TNF, a cytokine commonly elevated in multiple inflammatory disorders can have both pro and anti-apoptotic effects on T cells (508, 589). FASL ligation to FAS is a common mechanism for induction of T cell death at peripheral tissues (590).

Levels of these death/survival cytokines were assayed to determine whether they were responsible for T cell persistence at LPS inflamed tissues. No differences were observed in the levels of any of these cytokines 24hours after LPS administration. The 24-hour time point investigated may account for the discrepancies between my findings and previous studies.

Copeland et al. demonstrated that TNFα levels peaked in mouse blood plasma 4 hours after systemic endotoxin administration (527). In another study by Calil et al., TNFα levels peaked in mouse paws 3 hours after local LPS administration (510). In contrast, induction of acute inflammation in mouse lungs was found to cause a peak in TNFα levels 24 hours post local carrageenan administration (591). These studies demonstrate that TNFα levels at inflamed tissues is regulated by both the inflammatory stimuli as well as the tissue location. LPS mediated TNFα production most likely peaks very early following stimuli (510, 527).

Similarly, FASL levels were found to be elevated 3 days after a virus induced acute inflammatory response in murine vaginal tissue (592). Analyses targeting the expression of IL-7 at acutely inflamed tissues have not been undertaken. However, IL-7 is believed to be an early mediator of inflammation in RA (593).

4.3.4 Chemokines as potential regulators of T cell death

Chemokines are another class of mediators that have roles in cell survival and migration. CXCL12-CXCR4 interactions have previously been reported to modulate T cell survival (84, 566). CCL5 has also been reported in promoting and inhibiting cellular apoptosis (562-564). Moreover, spatial and temporal changes in chemokine ligand expression is well documented during inflammatory responses (155). A chemokine array was performed to analyse relative differences in chemokine ligand expression. Surprisingly, only CCL5 levels were substantially altered 24 hours after LPS administration.

Similar to the cytokine levels, chemokine expression found was a snapshot of the 24-hour time point. Multiple studies reveal a substantial increase in CXCL8 levels after LPS challenge which was not observed in our case (594). Analysing

chemokine expression by other methods such as RNA-seq might provide more robust results.

4.3.5 GPCR signals are necessary for Th1 cell persistence

Chemokine receptors are GPCRs (154, 155). To analyse whether T cell persistence was mediated via chemokines, Th1 cells were treated with pertussis toxin prior to their adoptive transfer. Pertussis toxin inhibits GPCR signalling (577, 578, 595). Lower numbers of PTX treated cells were recovered from inflamed tissues than untreated cells from inflamed tissues. This result clearly demonstrated that T cell persistence was directly mediated by GPCR signals.

This finding contradicts with multiple studies which have demonstrated that PTX treatment of T cells resulted in their accumulation at peripheral tissues rather than their disappearance (448, 596). This phenomenon is thought to be CCR7 mediated as CCL19/21 guide T cell egress from peripheral tissues (451, 452). The inflammation state as well as T cell polarisation differs in my experiments from these studies and this may account for the observed discrepancy.

The Bromley et al. study examined the egress of Th2 cells from lungs with the use of CCR7 overexpression transgenic model system. Using this system they demonstrated that Th2 cells require CCR7 to leave the lungs and accumulate in the mediastinal lymph nodes, 36 hours after intratracheal instillation. In contrast, Debes et al. adoptively transferred naïve T cells treated with or without pertussis toxin intradermally into mouse footpad. They demonstrated that PTX inhibited naïve T cell egress from the footpad and that this egress was CCR7 mediated. My studies investigated the migration of Th1 cells from an acutely inflamed mouse skin tissue. Hence, both my cell polarisation state and tissue site and state were different from these two studies (451, 452).

Other studies have suggested roles for S1PRs in regulating T cell accumulation and egress from peripheral tissues (244). S1PRs are also GPCRs which are PTX sensitive. In the next chapter, the role S1P plays in T cell persistence is investigated.

5 Sphingosine-1-Phosphate regulates Th1 cell persistence at inflamed tissues

5.1 Introduction

Sphingosine-1-phosphate (S1P) is a bioactive lysosphingolipid. Sphingosine, the precursor to S1P is produced by the sequential degradation of cell membrane glycosphingolipids and sphingomyelin to make ceramide (176, 597). Ceramide is then hydrolysed to sphingosine which is phosphorylated by one of two kinases SPHK1 and SPHK2 to produce S1P (598). Studies have shown that $SPHK^{-/-}$ leads to embryonic lethality in mice emphasising the physiological importance of S1P (599).

S1P is primarily produced in intracellular lysosomes of RBCs, platelets and endothelial cells (600-603). Subsequently, it is either used/degraded intracellularly or transported out of the cell (604-607). Five S1P receptors have been identified to date (S1PR1-5) (226). These are all high-affinity GPCRs (226). The varied cellular expression and signalling of each individual receptor yields the pleiotropic and antagonistic effects of S1P (226).

5.1.1 Heterogeneous roles of S1P

S1P plays an important role in vascular maintenance. Signalling via S1P receptors 1-3 co-operatively regulates vascular angiogenesis during embryogenesis (608, 609). The intra-extracellular S1P transporter spns2 is also critical in this process (608). Postnatally, S1P regulates vascular integrity. This was demonstrated when mice lacking plasma S1P exhibited vascular leakage under homeostatic conditions (610).

Interestingly, systemic pertussis toxin administration in wild type mice induced similar vascular leakage. Treatment with S1PR1 agonists restored vascular integrity (610). Finally, S1P regulates vascular smooth muscle tone. Signalling via S1PR2 was found to be crucial in maintaining smooth muscle contractility (611, 612).

S1P is likewise crucial in embryonic neurogenesis. It was found that defects in either S1P biosynthesis or S1PR1 led to neural tube closure and thus embryonic lethality (599). In adult nervous systems, multiple S1PRs are expressed across several cell types including neurons and microglia. Roles for S1P has been

described in neurotransmitter release, microglial proliferation and pain pathophysiology (613-616).

In contrast S1P plays a negative role in cancer. S1P metabolism is dysregulated in several tumours (617). Furthermore, S1P was found to promote tumour angiogenesis, proliferation, aid in metastasis and invasion as well as resist tumour cell death (617-622). Cancer therefore, is a hallmark of the pleiotropic and antagonistic effects of S1P on its targets.

5.1.2 S1P in cell survival and apoptosis

Many studies have investigated the role S1P plays in regulating cellular survival and apoptosis. One study in human hepatic fibroblasts found that S1P induced apoptosis in a receptor independent manner while increasing survival in a receptor dependent manner (623). Likewise, a few studies have suggested that ceramide, an intracellular pre-cursor to S1P induces pro-apoptotic signals.

In contrast, S1P itself is pro-survival (624). The mechanism for S1P mediated survival remained elusive until recently, when Rutherford et al. demonstrated that S1P suppresses the pro-apoptotic molecule bim and promotes upregulation of pro-survival Mcl-1 in lung fibroblasts to aid in cell survival (625).

Very recently, Mendoza et al. demonstrated for the first time, a role for S1P in regulating T cell survival. They found that knocking out the intra-extracellular S1P transporter SPNS2 led to increased death of naïve T cells. They further demonstrated that S1PR1 KO animals also had an increased number of apoptotic naïve T cells in LNs. Finally, they elegantly demonstrated that S1PR1 is essential for the maintenance of mitochondrial integrity in naïve T cells (626).

Whether these varying mechanisms of S1P mediated cell survival are spatially and temporally conserved across cell types remains to be determined.

5.1.3 S1P is a key regulator of cellular trafficking

S1P levels across organ systems is strictly regulated in homeostatic conditions. Micromolar and nanomolar levels of S1P are found in the blood and lymphatic vasculature respectively. In contrast, interstitial fluid has either undetectable or

sub-nanomolar levels of S1P (217). The maintenance of this gradient is critical, as cells vary their S1PR expression to traffic between organs and vasculature (205, 224). T and B cells sense the S1P gradient via S1PR1 to traffic between SLOs and circulation (218). Likewise, S1P gradients regulate NK cell and haematopoietic stem cell mobilisation via S1PR5 and S1PR1 respectively, between the bone marrow and the circulation (627).

T cell trafficking is an elegant example of how S1P, S1PR1 and its regulators are critical in inflammatory conditions. Naïve T cells in SLOs express high levels of S1PR1 and high levels of CCR7 (424, 431). This leads to T cell retention due to increased gradients of CCL19/21 interacting with CCR7 in SLOs (425, 426).

Antigen exposure increases CD69 expression on T cells (432). CD69 is a negative regulator of S1PR1. Multiple studies have shown that CD69 directly interacts with, and downregulates S1PR1 expression on cell surface. This downregulation of S1PR1 allows T cells to dwell in SLOs while undergoing clonal expansion (432-434).

Once fully activated, effector T cells downregulate CCR7 and upregulate S1PR1 (243). T cells expressing S1PR1 migrate towards higher S1P gradients in efferent lymphatic vessels, eventually leading to their egress to peripheral tissues (436, 437). The S1PR1 expression profile of T cells in tissues is not well defined.

KLF2 is a transcription factor that regulates S1PR1 expression on T cells (628). High levels of KLF2 has been associated with high S1PR1 expression (456). Studies have found that early effector cells at tissue sites are KLF2 high, but over time KLF2 expression is lost (629).

Furthermore, several studies have suggested that inflamed tissues have elevated S1P levels. Ledgerwood et al. found that S1P levels were significantly increased at alloantigen or adenovirus inflamed mouse skin (244). Increased S1P levels were reported in joints of CIA mice as well as in the synovial fluid of RA patients (271, 630).

Interestingly, Ledgerwood et al. demonstrated that migration of CD4 T cells across lymphatic, but not blood endothelium was inhibited from inflamed mouse

skin, regulated by S1PR1 (244). Thus, S1P-S1PR1 signalling regulates lymphocyte trafficking in homeostatic and inflammatory conditions.

5.1.4 S1P in cytoskeletal rearrangement

Cells use cytoskeletal machinery such as actin and myosin to migrate (631). It is therefore unsurprising that S1P elicits most of its physiological effects via regulation of the cytoskeleton. S1PR signalling directly activates the RHO family of GTPases which regulate actin and myosin assembly (632). This has been shown to have varying effects on distinct cell types.

In endothelial cells, S1P was found to enhance chemotaxis and regulate vascular barrier integrity mediated via cytoskeletal rearrangements (633, 634). S1P mediated control of vascular smooth muscle tone is also facilitated via Rho-kinase activation (611, 635).

In neuronal cells, S1P modulates neuronal outgrowth by controlling RAC activation and thus neuron morphology (636). In contrast, S1P potently inhibits melanoma cell migration by preventing actin nucleation and pseudopod formation (637). In glioblastoma cells however, S1P promotes tumour cell migration and metastasis mediated via distinct S1PR and Rho GTPase activation (638).

Finally, the molecular mechanisms of S1P mediated immune cell migration is poorly understood (632). In human DCs, S1P was found to enhance chemotaxis by increasing actin polymerisation (639). S1P robustly stimulates NK cell motility in a PI3-kinase dependent manner (640). In contrast, S1P was found to inhibit mast cell migration (641).

5.1.5 Modulators of S1PRs

S1P is difficult to detect *in vivo* (430). Moreover, a S1P blocking antibody was only made available recently (642). Therefore, functions of S1P have primarily been elucidated by studying the effects pharmacological modulators elicit on S1PRs. FTY720 was the first described S1PR modulator (438). It was later discovered that FTY720 was a functional antagonist of S1PR1,3,4 and 5 but had no effect on S1PR2 (222).

Later, receptor selective agonists and antagonists were synthesised. These included FTY720-P, an S1PR1,3,4 and 5 agonist, SEW2871, an S1PR1 selective agonist and W146, an S1PR1 selective antagonist (223, 240, 643). All of these S1PR modulators have played key roles in the current understanding of S1P biology.

5.1.6 Aims of this chapter

Greater recovery of CD4 Th1 cells from inflamed tissues was found to be GPCR sensitive (chapter 4). S1PRs are members of the GPCR family and regulators of T cell survival and trafficking. The aims for this chapter were to investigate whether S1PR signalling was responsible for the observed T cell persistence phenotype at inflamed tissues.

S1P signalling also modulates cell survival and behaviour. Therefore, whether S1PR signalling modulated Th1 cell behaviour and survival was also investigated. Finally, a human inflammatory disease was investigated for the presence of elevated S1P levels, which may contribute to T cell persistence at inflamed tissues.

5.2 Results

5.2.1 S1PR signalling is critical for Th1 cell persistence

S1P is a pleiotropic signalling sphingolipid (644). It plays diverse roles in cellular communication. Studies have reported a role for S1P in regulating T cell trafficking and survival (243, 626). Interestingly, S1P receptors are also GPCRs, and sensitive to pertussis toxin mediated signalling disruption (219-221). Hence, to identify whether S1PR signalling contributes to the persistence of effector Th1 cells at inflamed tissues, T cells were treated with an S1PR pan functional antagonist, FTY720.

FTY720 or vehicle treated Th1 cells were transferred into inflamed or resting ear pinnae. 24 hours later, animals were euthanised and their ears and dLNs were harvested, stained for flow cytometry and the transferred cells enumerated. Just like PTX treatment, significantly lower numbers of cells were recovered from inflamed tissues following pre-treatment with FTY720 (Figure 5.2.1A).

FTY720 treatment did not alter the number of cells recovered from non-inflamed tissues (Figure 5.2.1A). No differences were observed between the draining lymph nodes of inflamed or resting ear pinnae irrespective of FTY720 treatment (Figure 5.2.1B). In sum, these data suggest that persistence of Th1 cells at inflamed tissues is mediated by PTX sensitive S1PR signalling.

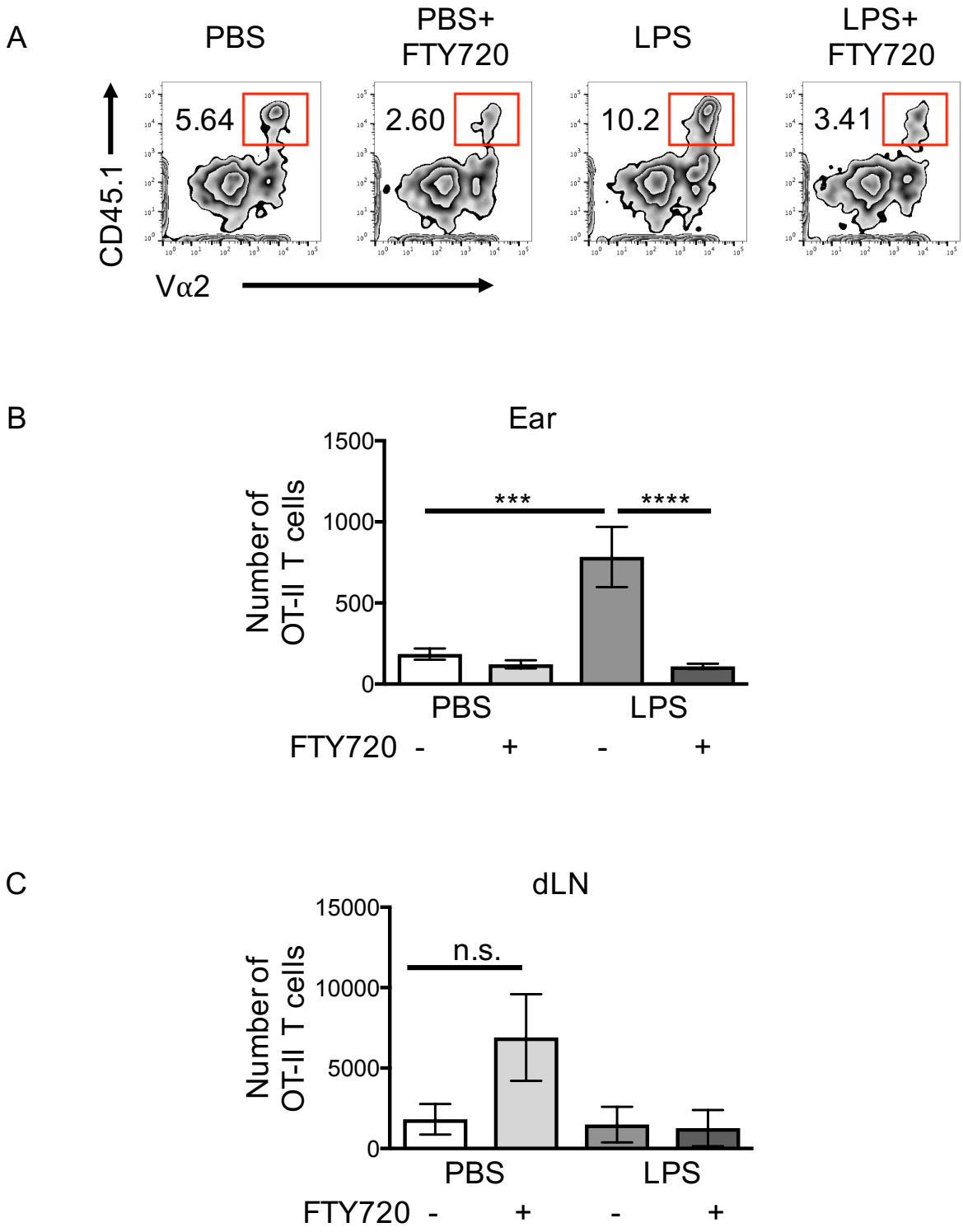

Figure 5.2.1 Fewer Th1 cells are found at inflamed sites after FTY720 treatment
Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 FTY720 or vehicle treated OT-II Th1 cells were transferred into the same ear pinnae of animals. 24 hours after the transfer, mice were euthanised and their ears (A,B) and dLN (C) were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots are representative of 3 independent experiments. Graphs show combined data from 2 independent experiments of 11 or 9 (LPS) animals per group. Error bars represent SEM. Statistical differences between groups were assessed by carrying out a One-way ANOVA and Tukey's multiple comparisons test using Graphpad Prism *** denotes p value of < 0.001 and **** < 0.0001. n.s. denotes not significant.

5.2.2 S1PR signalling regulates T cell behaviour at inflamed tissues

S1P has roles in cytoskeletal rearrangement in various cell types including immune cells (632). S1P regulates RHO GTPases via S1PR signalling (632). RHO GTPases are critical in actin and myosin assembly which regulates cellular motility (631). In the previous chapter Th1 cells were found to be more motile at inflamed tissues.

To investigate whether S1PR signalling was regulating this behaviour, FTY720 or vehicle treated Th1 cells were transferred into LPS inflamed ear pinnae of LysMGFP mice. Similar to the experiments in (Figure 4.2.2) Th1 cells were transferred in a small volume at shallow depths to keep tissue localisation of the T cells as consistent as possible. LysMGFP mice have a GFP tag associated with the lysozyme gene (496). This labels neutrophils in these mice, bright green in colour. Monocytes and macrophages also express a lower intensity of GFP.

4-5 hours later, animals were anaesthetised and restrained on a temperature controlled stage. The ear of the animal was then imaged and time-series movies were captured (video 3, 4).

The movies were analysed and CD4 T cells tracked to assess their velocity and displacement (Figure 5.2.2A, B). FTY720 treated Th1 cells had substantially reduced velocity compared to vehicle treated cells at inflamed sites (Figure 5.2.2C). Arrows in the still images and videos demonstrate reduced movement of T cells when treated with FTY720 compared to vehicle treatment. The videos and still images also demonstrate T cells travelling within or near collagen fibre networks (Figure 5.2.2 E,F)(video 3,4), suggesting similar tissue localisation of T cells compared to T cells in (Figure 4.2.2). Hence, the behaviour of FTY720 treated T cells was similar to T cells at resting tissues. FTY720 treated Th1 cells also exhibited decreased displacement (Figure 5.2.2D), however this was to a lesser extent than cells at resting tissues (Figure 4.2.2). This suggests that factors other than an S1P signal may also contribute to the overall behaviour of T cells leading to their persistence.

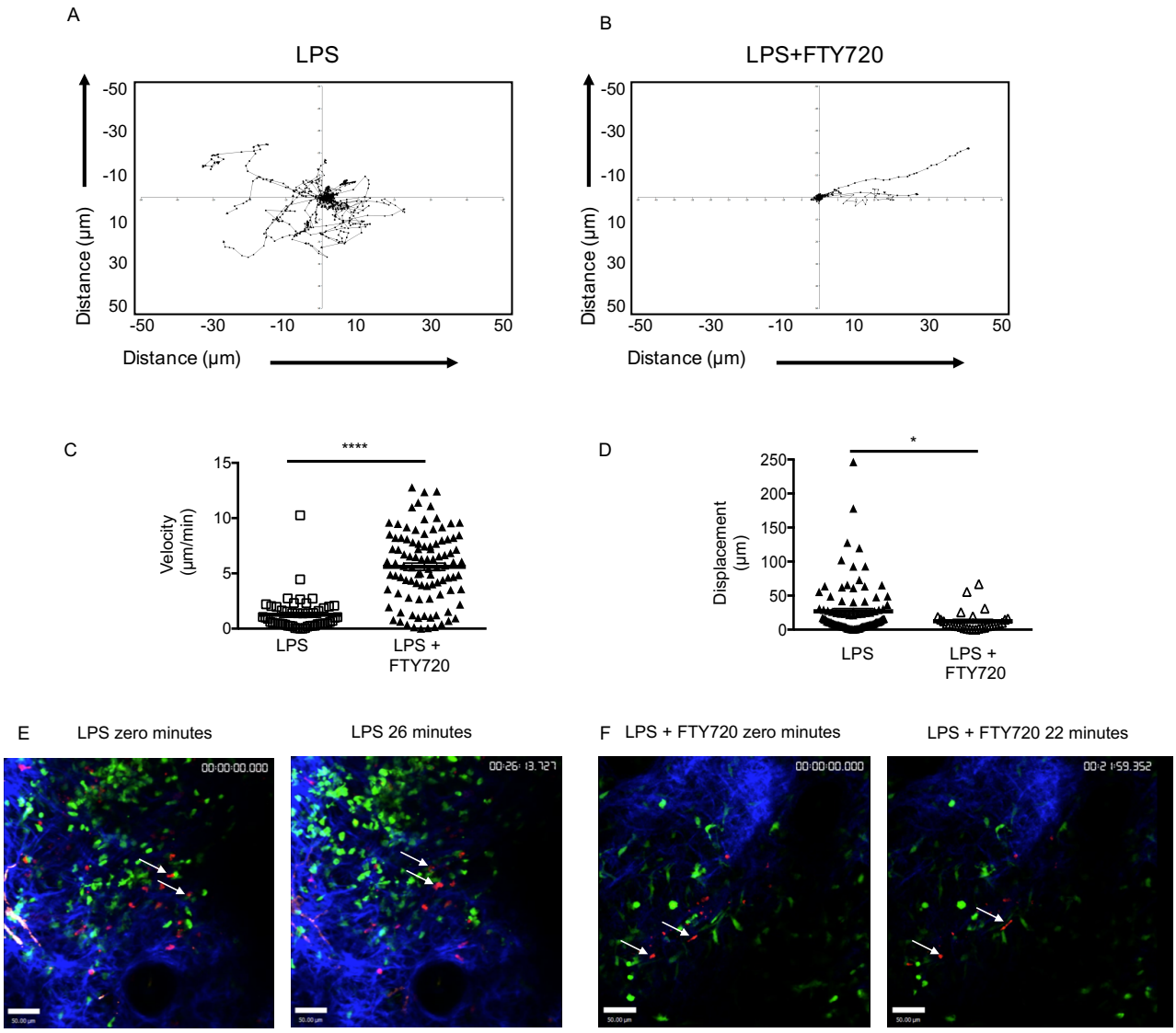

Figure 5.2.2 FTY720 treated Th1 cells travel reduced distances and at a lower velocity compared to untreated cells at inflamed tissues

LysMGFP mice were challenged with either PBS or LPS. 24 hours later, ~2.0e3 polarised Th1 cells treated with or without FTY720 expressing DsRed were transferred into the injection site at a shallow depth, in a small volume. The animals were rested for 4-5 hours before the ears were imaged by a Zeiss LSM 7MP microscope. Images were acquired using a 20x/1.0NA water immersion objective lens. Images were then analysed using volocity software and DsRed positive cells were manually tracked individually, generating their velocity and displacement rates. (A,B) X-Y plots show distance of T cell tracks and are representative plots from 1 of 3 individual mice per treatment group. (C,D) Velocity and displacement graphs show combined values from 3 individual mice. (E,F) Representative stills from 1 of 3 individual mice per treatment group, illustrating distance travelled by T cells from time zero, indicated by arrows. Each data point on graphs (C,D) represents a DsRed T cell and bars represent mean. In the movies, green cells = LysM+ cells (neutrophils/monocytes), blue = second harmonic signal and red cells = CD4 Th1 cells. Arrows demarcate the position of the same T cell at different time points, indicating their movement or lack thereof. Statistical differences between groups were assessed by carrying out a Mann-Whtiney test using Graphpad Prism * denotes p value of <0.05 and **** < 0.0001.

5.2.3 S1P mediated Th1 cell survival at inflamed tissues remains inconclusive

The effect of S1P on naïve T cell survival at SLOs has only very recently been determined by Mendoza et al. as discussed in section 5.1.2. However, a role for S1P in supporting effector T cell survival at inflamed tissues has not been studied.

Hence, to assess whether S1P was increasing Th1 cell survival at inflamed tissues, S1PR signalling was disrupted via FTY720 and Th1 cell survival assayed. FTY720 or vehicle treated Th1 cells were transferred into inflamed or resting ear pinnae. 24 hours later, animals were euthanised and their ears and dLNs were harvested, stained with flow cytometry antibodies and incubated with the FLICA reagent for an hour. The cells were then analysed and the proportion of OT-II cells that expressed active caspases 3 and 7 was determined via positive FLICA staining (Figure 5.2.3A).

A trend to fewer apoptotic cells were found in T cells recovered from inflamed compared with non-inflamed tissues (Figure 5.2.3B), contradicting the results in Figure 4.2.3. Furthermore, FTY720 treatment somewhat reversed Th1 cell survival at inflamed tissues without reaching statistical significance (Figure 5.2.3B). Taken together, this data remains inconclusive, due to it directly contradicting the results of section 4.2.3 which will be discussed further in the discussion of this chapter below.

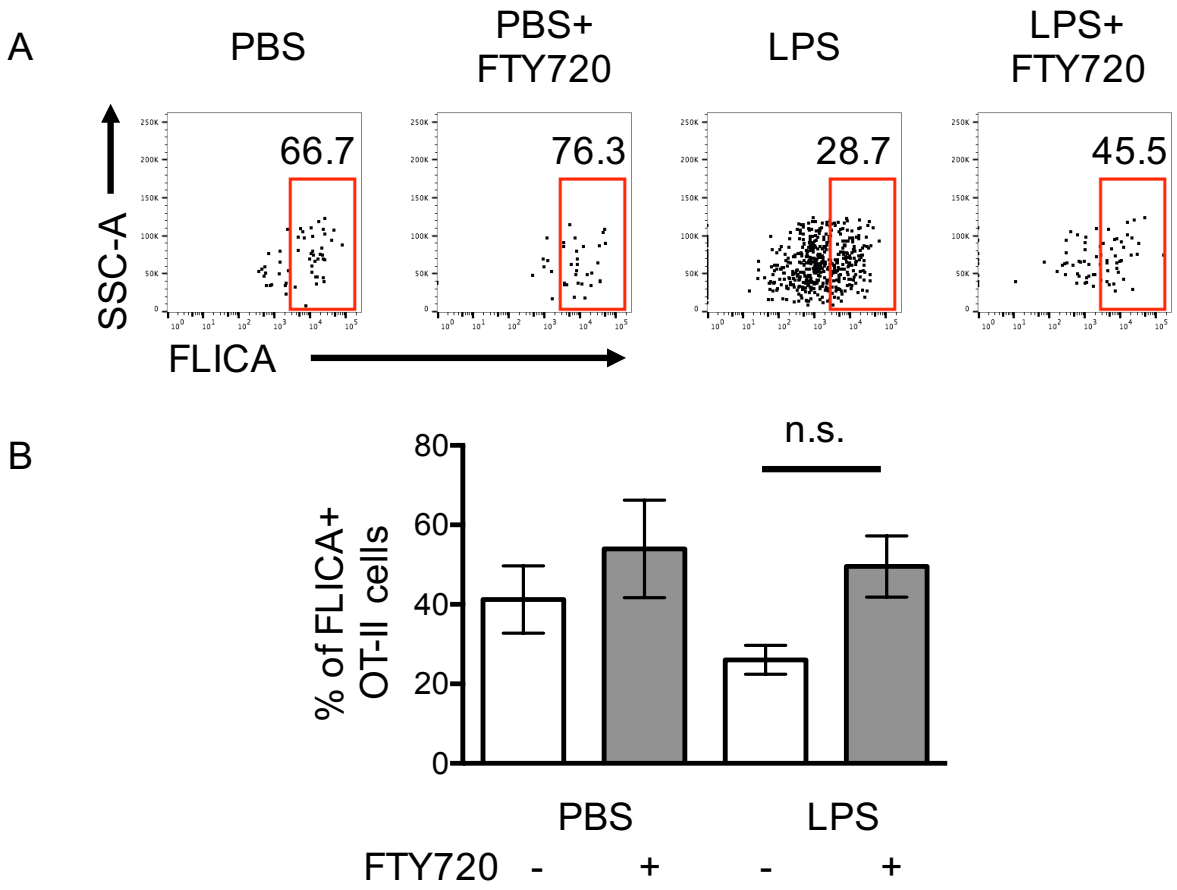

Figure 5.2.3 Increased number of T cells at inflamed tissues is not due to increased survival mediated via S1P receptors
Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 OT-II Th1 cells treated with or without FTY720 were transferred into the same ear pinnae of animals. 24 hours later, mice were euthanised and their ears were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+, CD45.1+, Vα2+ cells and the expression of active caspase 3 and 7 was analysed by measuring their expression of FLICA by incubating cells using a FLICA kit (life technologies). FACS plots are representative of 3 independent experiments. Graphs show combined data from 2 independent experiments of 7 or 6 (LPS+FTY720) animals per group. Error bars represent SEM. Statistical differences between groups were assessed by carrying out a One-way ANOVA and Tukey's multiple comparisons test using Graphpad Prism. n.s. denotes not significant.

5.2.4 S1P signal is necessary and sufficient for CD4 Th1 cell persistence at inflamed tissues

To date, five S1P receptors have been identified (S1PR1-5) (226). The specific role of S1P is determined by the cellular and temporal expression of these S1PRs (205, 224). S1PR signalling has resulted in effects as distinct as cellular proliferation, death, migration, retention and egress (644). These effects have been observed in diverse cell types including immune cells, neurons, tumour cells and endothelial cells (644). Likewise, the S1P analogue FTY720, used in previous studies has distinct effects on multiple S1PRs (438).

To determine whether S1PR1 specific agonism was responsible for Th1 cell persistence at inflamed tissues, S1PR1 selective agonist or antagonist were used. Prior to transfer, Th1 polarised OT-II T cells were treated with either W146, SEW2871 or vehicle. W146 is a S1PR1 selective antagonist (240). SEW2871 is a S1PR1 selective agonist (223). Treated cells were transferred into inflamed or resting ear pinnae. 24 hours later, the animals were euthanised and their ears and dLNs were harvested, stained for flow cytometry and the transferred cells enumerated (Figure 5.2.4A).

Significantly lower numbers of Th1 cells were recovered from inflamed tissues when pre-treated with W146 just as with FTY720 (Figure 5.2.4B). W146 treatment however, did not affect Th1 cell recovery from non-inflamed tissues (Figure 5.2.4B). In contrast, agonism of S1PR1 via SEW2871 treatment resulted in a significant increase of Th1 cell recovery from non-inflamed tissues compared to vehicle treated cells (Figure 5.2.4B).

Surprisingly however, no significant increase was observed at inflamed tissues when T cells were pre-treated with SEW2871 compared with vehicle treated cells at resting sites (Figure 5.2.4B). As in previous experiments, no significant differences were observed between the lymph nodes draining either inflamed or resting ears, irrespective of T cell pre-treatment (Figure 5.2.4C). In sum, these data demonstrate that S1PR1 specific signals were responsible for T cell persistence at inflamed tissues. Moreover, they suggest that FTY720 was acting as an antagonist of S1PR1 in previous experiments.

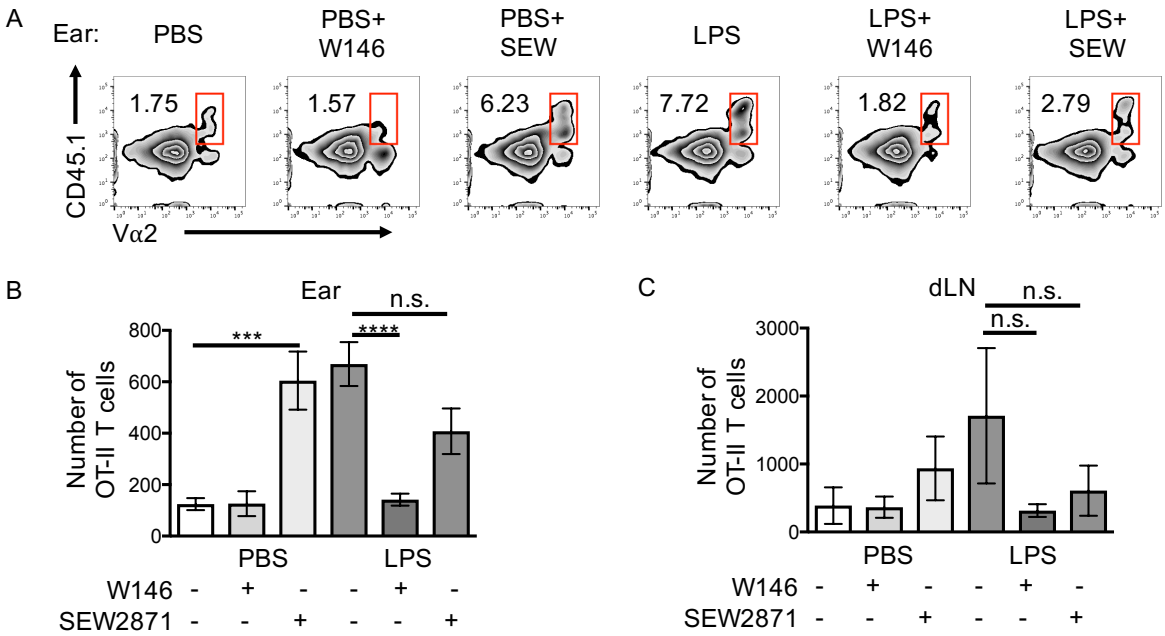

Figure 5.2.4 S1PR1 selective agonism results in increased CD4 Th1 cell recovery from tissue sites
Age matched C57BL/6 mice at 6 weeks of age were challenged with LPS or PBS in their right ear pinnae. 24 hours later 2.0e6 SEW2871, W146 or vehicle treated OT-II Th1 cells were transferred into the same ear pinnae of animals. 24 hours later, mice were euthanised and their ears and dLN were harvested, processed and stained for FACS. Cells were stained with a viability dye followed by fluorescently labelled antibodies against CD45, CD4, CD45.1 and Vα2. Lymphocytes were initially gated on live CD45+, CD4+ cells and subsequently analysed for their expression of CD45.1 and Vα2. FACS plots are representative of 3 independent experiments. Graphs show combined data from 2 independent experiments of 6 (LPS, LPS+ SEW2871, PBS+ SEW2871, PBS+W146) or 7 (PBS, LPS+ W146) animals per group. Statistical differences between groups were assessed by carrying out a One-way ANOVA and Tukey's multiple comparisons test using Graphpad Prism *** denotes p value of <0.001, ** and **** < 0.0001. n.s. denotes not significant.

5.2.5 Th1 cells express S1PR1 prior to transfer but downregulate it at inflamed tissues

S1PR1 expression on T cells is dynamic and temporally regulated. Naïve T cells in circulation express little surface S1PR1, but are S1PR1hi once in SLOs (389). Early effector T cells are S1PR1hi but down modulate its expression once at tissue sites for a few days (629). Measuring surface S1PR1 expression is challenging. Sub nano molar levels of S1P causes receptor internalisation and degradation of S1PR1 (212, 222, 223).

Indeed, local S1P gradient dynamics have been elucidated by measuring cell surface expression of S1PR1 (431, 440, 602). Previous data suggested that signals through S1PR1 were responsible for Th1 cell persistence at inflamed tissues. Thus, it was important to examine the relative expression of S1PR1 on Th1 cells both *in vitro* and *ex vivo*.

To determine whether Th1 cells expressed S1PR1 *in vitro*, polarised OT-II T cells were stained with anti-S1PR1 antibody and analysed on a flow cytometer. Roughly a quarter of Th1 cells were found to express surface S1PR1 (Figure 5.2.5A). Intracellular staining with the same S1PR1 antibody revealed that a large majority of cells expressed S1PR1 intracellularly (Figure 5.2.5B).

Next, *ex vivo* surface and intracellular expression of S1PR1 by Th1 cells recovered from ear tissue was assessed. Polarised OT-II Th1 cells were transferred into inflamed or resting mouse ear pinnae. 24 hours later, ear tissue was harvested, stained for flow cytometry and cell surface and intracellular S1PR1 expression determined. No surface S1PR1 expression was detected from tissue recovered Th1 cells, possibly due to digestion (Figure 5.2.5C). Intriguingly however, Th1 cells were found to have reduced intracellular S1PR1 expression at inflamed ear tissues compared to T cells at resting tissues (Figure 5.2.5D,E). In sum these data suggest that Th1 cells express S1PR1 at transfer and are therefore responsive to S1P signals. Moreover, a reduced intracellular expression at inflamed ears may be attributed to increased S1P and thus increased S1PR1 degradation at the site.

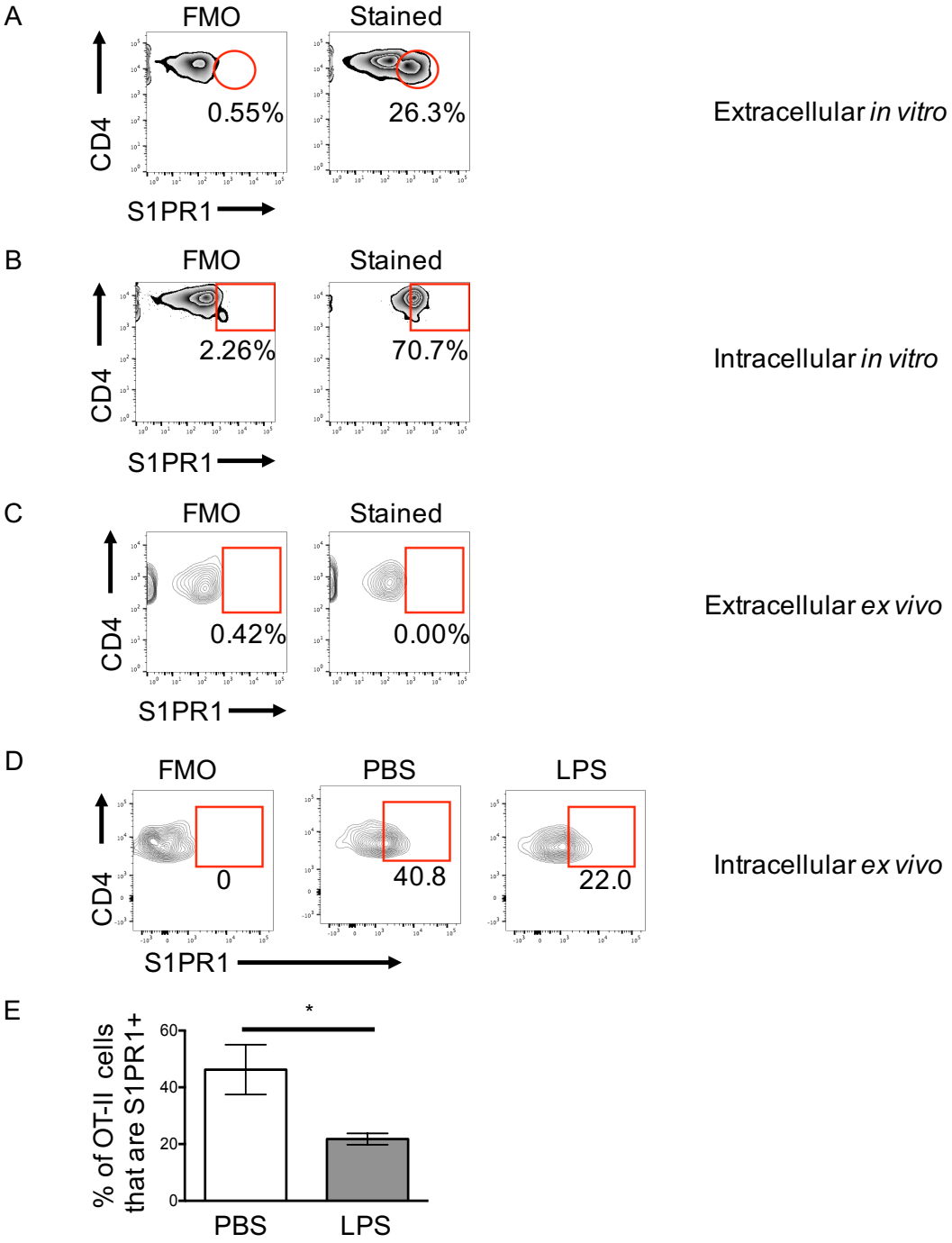

Figure 5.2.5 Th1 cells express S1PR1 before transfer but downregulate it at inflamed tissues
CD4 T cells were isolated from the lymph nodes and spleens of CD45.1 OT-II mice using a STEMCELL technologies CD4 T cell isolation kit. The cells were then cultured with IL-12, anti-IL-4 and ovalbumin peptide$_{323-339}$ in the presence of mitomycin C treated splenocytes for 72 hours at 37°C with 5% CO_2. Thereafter, cells were stained with anti-CD4 with or without anti-S1PR1 antibodies (A). Cells were then fixed and permeabilised with a BD cytofix/perm kit and stained with or without anti-S1PR1 antibody (B). Animals were treated with LPS or saline in their ear pinnae before 2.0e6 CD4 Th1 cells were transferred into the ear pinnae. 24 hours later animals were euthanised and their ears harvested and stained for flow cytometry with a cocktail of antibodies including anti-CD4 and anti-S1PR1 extracellularly (C). In another experiment, cells were fixed and permeabilised using a BD cytofix/perm kit and stained with anti-S1PR1 antibody intracellularly (D,E). *In vitro* experiments were pre-gated on live, single lymphocytes and are representative of 2 independent experiments. *Ex vivo* plots are pre-gated on live, single CD45+ lymphocytes and are representative of two (C) or one experiment (D,E) respectively with 5 animals in each group. Statistical differences were determined by carrying out an unpaired Student's T test. Error bars represent SEM. * denotes P value of <0.05.

5.2.6 Inflamed tissues express increased levels of SPHK1, the enzyme that generates S1P

Sphingolipids are difficult to measure reliably. Due to a critical role for S1P in membrane sphingolipid metabolism, intracellular S1P is abundantly produced by various cell types. This makes even mass spectrometry results difficult to interpret (430). Instead, kinases that either help produce or degrade S1P are often used to measure tissue S1P levels (645). Sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2) are the final two kinases responsible for metabolising sphingosine into sphingosine-1-phosphate (210).

To measure S1P levels at inflamed tissues, animals were treated with LPS or saline in their ear pinnae to induce inflammation. 24 hours later, animals were euthanised and their ears harvested and fixed in neutral buffered formalin. The tissue was subsequently sectioned into 10μm thick sections and stained with human and mouse cross reactive anti-SPHK1 or isotype control antibodies. Secondary and tertiary antibodies were used to add fluorescent tags to the tissues to allow visualisation on a fluorescent microscope.

The images were then analysed and the number of total cells and cells expressing SPHK1 manually counted in three randomly selected areas per section using ImageJ software. The percentages of cells expressing SPHK1 were then calculated. A significantly greater proportion of cells at inflamed tissues were found to express SPHK1 (Figure 5.2.6A, B). These data suggest that increased S1P may be produced at inflamed ear pinnae. Taken together with the T cell persistence data, there is strong evidence suggesting that dysregulated S1P production at inflamed tissues contributes to increased CD4 Th1 cell persistence at such sites.

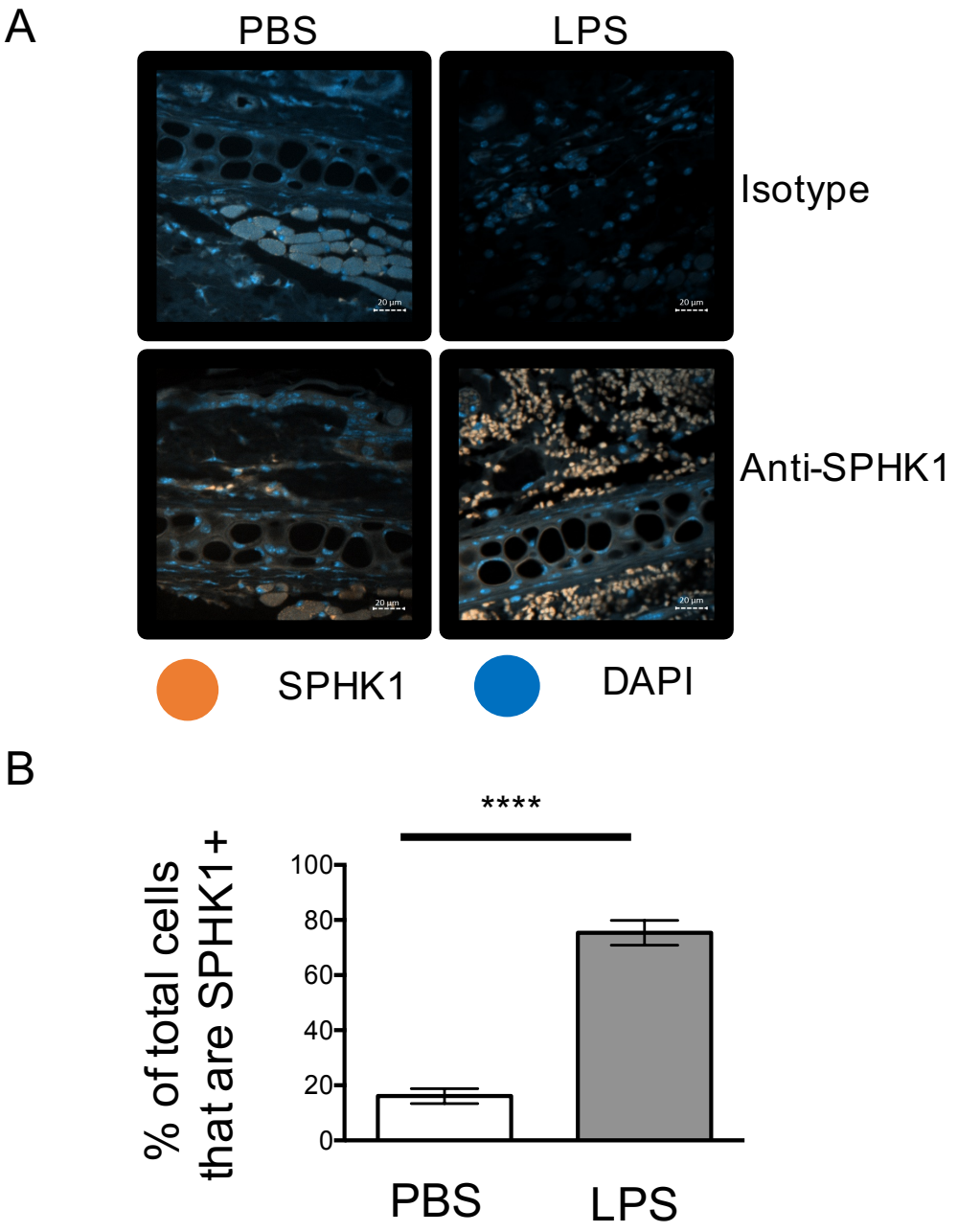

Figure 5.2.6 Inflamed mouse ears contain a significantly greater number of cells expressing SPHK1

Age matched C57BL/6 animals were injected with LPS or saline in their ear pinnae. 24 hours later, animals were euthanised, ears harvested and fixed in neutral buffered formalin. Ear tissue was mounted in paraffin and cut into 10um thick sections. The cut sections were mounted on a slide and stained with rabbit anti human/mouse-SPHK1 or rabbit IgG (Isotype control). Sections were then stained with anti-rabbit biotin, followed by streptavidin-PE. Sections were mounted in prolong gold with DAPI and imaged on a spinning disk confocal microscope. Images were acquired using uniform settings at similar locations. Images were acquired at 20x magnification. Error bar shows 20um. Three uniform areas were randomly selected in each tile section for quantification. Number of SPHK1 positive dots were manually counted using imagej software. Images are representative from one experiment with 5 animals per group. 2 randomly selected sections were quantified from serially sectioned mouse ears. Error bars represent SEM. Statistical differences were analysed by carrying out an unpaired Student's T test. **** denotes P value of <0.0001.

Molecular pathways are difficult to study in human disease settings. Therefore, aspects from animal models of inflammation need to be tested in humans to elucidate whether similar mechanisms may be present in human disease settings. Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory condition where human joint synovia are inflamed (76). One of the hallmarks of RA is the accumulation of large numbers of CD4 T cells in synovial tissues (84). The role of S1P in regulating accumulation of T cells at human RA joints remains unexplored.

To assess whether S1P is increased at RA joints, a collaborative project was undertaken with Dr. Aziza Elmesmari and Prof. Iain McInnes, rheumatologists at Gartnavel hospital, Glasgow. SPHK1 levels were measured in biopsies from RA patients or OA patients. OA is a form of less-inflammatory arthritis, often associated with age related degeneration of joint cartilage. Furthermore, minimal accumulation of CD4 T cells have been observed in primary OA (646).

Biopsy sections were fixed in formalin and cut into 5μm thick sections before staining with the same anti-SPHK1 antibody. Using appropriate secondary, tertiary antibodies and Diaminobenzidine (DAB), colour was developed in positively stained areas for visualisation on a light microscope fitted with a camera module.

The images were then semi-quantified by Dr. Aziza Elmesmari. Three representative fields per slide were selected and graded on a scale of 0-4 by two blinded observers on two independent occasions. The scores were later combined and statistical analysis performed to determine differences between groups. Blinded observers consistently scored RA tissues higher for their expression of SPHK1 compared to OA tissues (Figure 5.2.7A,B). These data indicate that inflamed RA synovial tissues may produce increased S1P. Keeping previous conclusions in mind, increased S1P levels in synovial tissues may contribute to CD4 T cell accumulation in RA.

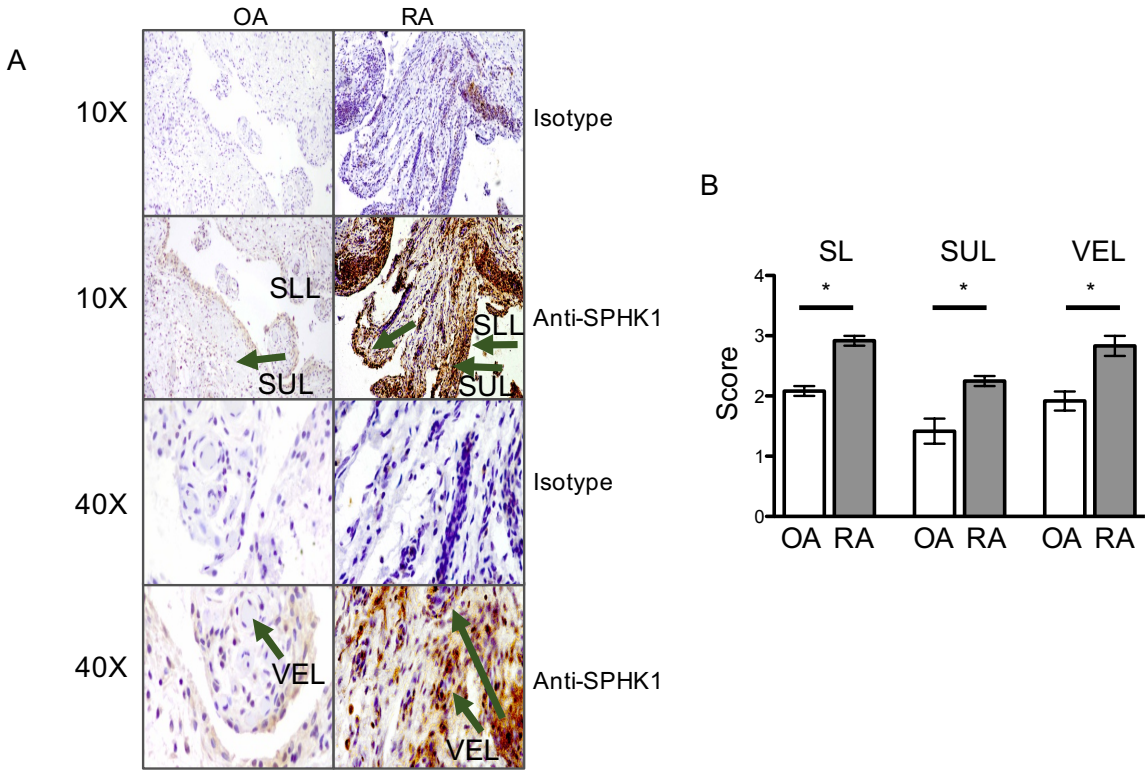

Figure 5.2.7 Human RA joints express increased SPHK1
Human RA or OA biopsy samples were fixed and embedded in paraffin. 5μm thick sections were cut and mounted on slides. The slides were then stained with anti-human/mouse SPHK1 or rabbit IgG (Isotype control). Appropriate biotinylated secondary antibody and an ABC kit were used for colour development before DAB was used to form positive brown deposits. Sections were imaged on a light microscope with a camera attachment. Images were scored on a scale of 0-4 by two blinded observers on two independent occasions. 0 = no staining, 1 = 0-25%, 2 = 25-50%, 3 = 50-75%, 4= 75%-100% staining. Image is representative of 4 independent biopsy samples. Synovial lining layer (SLL), sub lining layer (SUL) and vascular endothelial layers (VEL) of tissue were scored individually and a kruskal wallis test was used to calculate statistical differences between OA and RA in each layer. The data was then combined in graph above. Error bars represent SEM. * denotes P value <0.05.

5.3 Discussion

Sphingosine-1-phosphate is a pleiotropic sphingolipid responsible for numerous cellular processes including survival, proliferation and migration (176). S1PRs are GPCRs and sensitive to PTX mediated inhibition of signalling (647). In this chapter, the role of S1PR signals in the persistence of CD4 Th1 cells was investigated.

5.3.1 S1PR1 signals are necessary and sufficient for Th1 cell persistence at inflamed tissues

Following on from the last chapter where T cell persistence was found to be pertussis toxin sensitive, the role of S1PRs, another PTX sensitive GPCR was examined. Th1 cells were treated with either FTY720, W146 or SEW2871 prior to their adoptive transfer into inflamed or resting tissue sites. These are S1PR pan antagonist, S1PR1 specific antagonist and agonist respectively.

Lower numbers of Th1 cells were recovered from inflamed tissues when treated with S1PR antagonists, phenocopying the pertussis toxin treatment experiments. These results suggested that the pertussis toxin sensitive persistence of T cells is mediated via S1PR1 signals. These results contradict a study by Ledgerwood et al. where naïve T cells treated with FTY720 were found to undergo reduced egress to draining lymph nodes from peripheral tissue sites (244). The authors suggested that the lower number of T cell recovery from draining lymph nodes suggested tissue retention mediated via FTY720. Ledgerwood et al. also demonstrate that S1P treated T cells failed to migrate to lymph nodes but rather are retained at tissue sites.

The difference in the polarisation state of my T cells (activated Th1 in my experiments versus naïve T cells in Ledgerwood et al. experiments) as well as their trafficking from inflamed rather than resting tissues could explain the differences observed. It is also important to note that Ledgerwood et al. did not enumerate the number of T cells at the tissue site but rather inferred their presence by enumerating the draining lymph nodes. In contrast, I have enumerated both the tissue site and the draining lymph nodes. I believe that this demonstrates a more robust experimental approach. Finally, FTY720 is a

functional antagonist of S1PRs. The Ledgerwood study in contrast, suggests it may be functioning as an agonist. This makes their results difficult to interpret, since FTY720s function as an S1PR pan functional antagonist is well documented (222, 438, 439, 643).

Another study by Brown et al. demonstrated that Th1 cell egress from chronically inflamed tissues is mediated via S1P signals (453). Systemic FTY720 treatment of animals prior to adoptive transfer of T cells at tissue sites reduced the subsequent recovery from tissue draining lymph nodes 12 hours after adoptive transfer. This study once again differs from my experiments in several key methods. Systemic FTY720 treatment of animals can have effects on multiple cell types and therefore the effect on T cell egress could be indirect. Moreover, chronically inflamed tissues may differ significantly from acutely inflamed tissues, both in their cellular and molecular contents (61). Thus, S1P levels may be different depending on the type of inflammatory stimulus used. Indeed, as shown in chapter 1, my investigations using a DTH inflammatory model failed to demonstrate Th1 cell persistence. This may suggest that S1P mediated T cell persistence is inflammation specific. Thus, it would be informative to identify the mechanism(s) of S1P gradient dysregulation at inflamed tissues.

5.3.2 S1PR1 signals may regulate T cell survival

Since LPS inflammation mediated T cell persistence was found to increase cell survival at the tissue, similar investigations were undertaken to determine whether S1PR1 signals were regulating T cell survival directly. Intravital microscopy revealed a marked decrease in T cell motility at inflamed tissue following FTY720 treatment, indicating that they may be undergoing apoptosis.

S1P mediated cellular motility has been explored in some studies. As mentioned previously, S1P was found to increase NK cell motility as well as DC chemotaxis (639, 640). In contrast S1P signals were found to inhibit mast cell migration (641). In contrast, S1PR1 deficiency was found to reduce CD8 T cell velocity in lymph nodes (648). S1PR agonism via FTY720-P was also found to reduce CD4 T cell motility in explanted lymph nodes (645). Due to the conflicting nature of these studies, it remains uncertain whether S1P's effects on cellular motility is

differential based on the tissue type and/or the cell polarisation state. These results are confounded by the fact that S1P acts both as a survival and migration signal (239, 626). Dissecting these two pathways may indeed reveal the role differential cellular motility plays in regulating survival or migration.

Active caspase expression was also measured on FTY720 treated T cells. However, the results proved inconclusive due to no survival phenotype observed in vehicle treated T cells at inflamed tissues as found previously. One reason for this could be the prolonged tissue processing necessary for the FTY720 treated set of experiments due to the increased number of experimental groups.

A role for S1P in regulating cell survival has been widely studied in various cell types (623-626). Studies into the molecular mechanisms revealed that inhibition of caspase activation played a key role (625, 626). Hence it was rational to undertake a caspase assay.

Despite my inconclusive results, a very recent study by Mendoza et al. elegantly demonstrated for the first time that naïve T cells require S1PR1 signals to maintain mitochondrial integrity and thus survival (626). They measured levels of several mitochondrial proteins in S1PR1 deficient T cells and found them to be lower than their S1PR1 sufficient controls. Moreover, Mendoza et al. performed RNA-seq analysis on the T cells to demonstrate differential expression of survival and apoptotic genes in S1PR1 deficient T cells. The differences observed in this study were small and thus suggest that a different assay for Th1 cell survival at tissues such as RNA-seq may have revealed significant differences in my case.

5.3.3 S1P levels are dysregulated at inflamed tissues

S1P levels are low in peripheral tissue fluids and high in blood and lymphatic vessels (217). Therefore, increased levels of S1P in my inflamed tissue model may further indicate its role in supporting early Th1 cell survival in tissues. S1P however is difficult to measure. S1P mRNA is constitutively expressed due to its role in membrane sphingolipid synthesis pathway. Intracellular S1P is also made by almost all cells making mass spec results difficult to interpret (430).

The final step of the S1P synthesis pathway involves the phosphorylation of sphingosine to S1P by either SPHK1 or SPHK2 (210). Multiple studies have demonstrated that SPHK1 regulates inflammatory S1P production whereas SPHK2 seems to control homeostatic S1P production (649). Hence, SPHK1 expression was analysed by immunofluorescent microscopy in LPS inflamed mouse ears. The results demonstrated that a significantly greater proportion of cells expressed SPHK1 in inflamed mouse tissues compared to resting ones.

Interestingly, a lot of the SPHK1 expressing cells were not nucleated. Paired with histology results, where large regions of blood vessel haemorrhage and blood components were found in tissue, this result strongly suggests that increased SPHK1 expression was due to blood borne cellular infiltration. Red blood cells and platelets are the most potent producers of S1P (217). Likewise, increased vascular leakage at inflamed tissues is well documented (61). Thus, I propose that LPS inflammation causes increased vascular leakage resulting in increased local S1P levels. This increased local S1P regulates the survival and/or migration of T cells at inflamed tissues. Future studies, either inhibiting vascular leakage or transferring activated platelets to produce S1P at local tissue could be used in lieu of LPS to confirm this hypothesis.

Another way to enumerate relative differences in S1P levels is measuring the expression levels of S1PR1 on T cells (431, 440, 602). This method relies on the exquisite sub-nanomolar sensitivity of S1P to S1PR1. T cells exposed to very low concentrations of S1P potently downmodulate their receptor expression (440). One caveat to this method however, is that the differentiation state of the cell may also alter the S1PR1 expression (430). Nonetheless, relative differences between S1PR1 expression levels were investigated in transferred Th1 cells at inflamed versus resting tissues. *Ex vivo* staining of extracellular S1PR1 expression proved inconclusive possibly due to tissue digestion processes. However, intracellular staining demonstrated Th1 cells at inflamed tissues expressed lower S1PR1 levels, suggesting a relative increase in local tissue S1P level.

Finally, studies by various groups have demonstrated that acutely inflamed mouse skin and gut have increased local S1P levels (244, 645). S1P levels were also elevated in human inflammatory diseases. Examination of bronchoalveolar lavages from asthma patients revealed increased S1P concentrations compared

to healthy controls (650). Synovial fluids of RA joints also contain elevated levels of S1P compared to OA joints (267). These studies correlate well with my data which demonstrates that human RA joints express elevated levels of SPHK1 compared to OA joints. Interestingly, most of these human inflamed tissues also promote prolonged persistence of T cells. Inhibiting local T cell persistence by local administration of S1PR1 antagonists may provide an interesting new therapeutic approach in tissue inflammatory disease.

6 General Discussion and Future Directions

6.1 General Discussion

Timely and efficient trafficking of T cells to and from inflamed tissues is a key step in controlling an inflammatory response. It enables the movement of the appropriate subsets of effector cells to inflamed tissues. Here, effector CD4 T cells participate in pathogen clearance, tissue repair and memory formation (276, 403). However, in chronic diseases multiple steps of the trafficking pathway may be dysregulated. This causes pathogenic accumulation of CD4 T cells at inflamed peripheral tissues (7, 84, 421, 513, 514).

In this thesis, the signals that affect the persistence of CD4 T cells at inflamed tissues were investigated. A reductionist inflamed tissue model was designed to study signals which cause tissue persistence rather than recruitment of CD4 T cells. Increased understanding of these signals may allow novel therapeutic intervention to diminish T cell accumulation in inflammatory peripheral tissue diseases such as RA and psoriasis. In contrast, enhancing T cell persistence may aid in the development of novel vaccine candidates by enhancing memory cell formation for infectious diseases at the site of infection, such as in HIV (651).

My studies have identified that LPS inflammation in mouse ear tissue increases local persistence of CD4 Th1 cells. Accumulation of T cells at inflamed tissues is a well-documented phenomenon (7, 84, 421, 498, 513, 514, 553). A myriad of studies have identified specific chemokines, CAMs, survival factors and lipid mediators as regulators of T cell accumulation at peripheral tissues (84, 244, 421, 481, 525).

Some of the early studies identified a role for chemokines in the attraction and accumulation of effector T cells at peripheral inflamed tissues. Campbell et al. first described the role of CCR4 in the chemoattraction of T cells to cutaneous sites of inflammation (14, 445). Likewise, Zabel et al. discovered the crucial role of CCR9 in T cell trafficking to the intestine (652).

Chemokines however, not only act as migration cues but often have other functions. For example, the chemokine receptor CXCR4 has been reported to cause T cell accumulation in the rheumatoid synovium by both increased survival and chemotaxis mechanisms (6, 84, 421). Th1 cells were found to chemotax

towards RA synovial fluid. In addition, Vlahakis et al. found, in a separate study, that CXCR4 signalling confers CD4 T cells resistant to apoptosis (561). Similarly, CX3CL1-CX3CR1 interactions have been reported to induce both T cell survival and retention in models of atopic dermatitis (480). Thus, accumulation of T cells at inflamed tissues could be a combination of multiple factors such as increased migration and increased survival, perhaps delivered by the same molecule.

I further demonstrated that Th1 cells that persisted in the tissue neither proliferated *in situ* nor migrated to the dLN at greater numbers than from resting tissues. Interestingly, intravital microscopy revealed that T cells at inflamed tissues remained highly motile while those at the resting tissue were immotile. These findings led me to hypothesise that persistent T cells received signals at inflamed tissues that rendered them resistant to apoptosis.

Caspase assays demonstrated that a lower proportion of T cells expressed active caspases at inflamed tissues compared to T cells at resting tissues, supporting my hypothesis. Akkoc et al. reported that IFNγ high T cells from atopic dermatitis patients expressed increased caspases and underwent apoptotic cell death (536). They further found that these cells expressed increased FAS and TNF receptors and that T cell death was mediated via FAS (536). In light of this study as well as my caspase and microscopy results, the increased production of IFNγ by T cells at resting tissues found in (Figure 3.2.8) may suggest that T cells were undergoing activation induced cell death.

Hence, levels of FAS ligand, TNF and IL-7 were measured at inflamed and resting tissues. These molecules have all been identified to play a role in promoting T cell death or survival (525, 527, 575, 589). However, no differences were observed in their levels. This does not, however, rule out a role for these compounds in T cell survival. It is plausible that T cells at inflamed tissues alter their expression of FAS, TNF or IL-7 receptors, rendering them more or less sensitive to the same level of signal. It would, therefore be important to measure levels of receptor expression on T cells to definitively rule out a role for these compounds in T cell survival at inflamed tissues.

As discussed previously, chemokines could also mediate T cell survival at inflamed tissues. Moreover, a recent study by Mendoza et al. demonstrated a novel role for the lipid mediator S1P in supporting naïve T cell survival (626). Since both chemokines and S1PRs signal through GPCRs, I inhibited GPCR signalling by treating T cells with either pertussis toxin or FTY720 which are a pan GPCR and a pan S1PR inhibitor respectively.

Treatment with either compound led to the disappearance of T cells from inflamed tissues without affecting their numbers at resting tissues or dLNs. This provided the first piece of evidence that S1P signalling was critical for Th1 cell persistence at inflamed tissues. This result however, could be interpreted in multiple ways.

Studies have demonstrated a role for CCR7 and S1PR1 in controlling the egress of T cells from both SLOs and peripheral tissues (282). In SLOs, CCR7 and S1PR1 were found to have linked but antagonistic roles in controlling T cell egress. Pham et al. demonstrated that CCR7 signals favour retention of naïve T cells while S1PR1 signals favoured their egress via efferent lymphatics, in SLOs (243). In peripheral tissues, both S1PR1 and CCR7 signals promote the egress of naïve T cells via afferent lymphatics (453).

This suggests that disruption of pan GPCR signalling should lead to T cell retention at peripheral tissues, whereas disruption of S1PR specific signalling should be compensated by the presence of CCR7 signalling to enable T cell egress. Indeed Brown et al. demonstrate these results when they found that pertussis toxin treated splenocytes were retained at peripheral tissues following adoptive transfer (453).

However, the balance of signals alters significantly during inflammation due to changes in both receptor and ligand availability. Activated T cells downregulate CCR7 and upregulate S1PR1 to allow their egress from SLOs (243). At inflamed tissues, little is known about changes in receptor expression, however, increased levels of S1P as well as CCL19/21 has been reported (244). This would suggest a possible levelling of the S1P and chemokine gradient between inflamed tissues and afferent lymphatics, enabling these signals to act as a temporary tissue retention signal.

Supporting this theory, Ledgerwood et al. demonstrated that S1P acts as a retention signal for naïve T cells at acutely inflamed tissues (244). Likewise, Brown et al. found T cell egress to be CCR7 dependent at acute but not chronically inflamed tissues (453). Interestingly, they also found that S1P signals promote T cell egress from chronically inflamed tissues, somewhat contradicting the Ledgerwood study. The differential responses of T cells to S1P signals from tissues suggests that expression of either S1PRs and/or S1P are dynamically regulated based on the length and type of inflammation.

It is also plausible, that S1PR and S1P expression is spatially regulated. Studies by Skon et al. found that S1PR1 signals were less important in tissue retention of CD8 TRM cells in the intestinal epithelium compared to other non-lymphoid tissues (456). Other studies highlighted the importance of the integrin CD103 in the retention of these cells in the intestine (488). Thus, it is possible that CD103 also contributes to effector/memory T cell retention at peripheral tissues in conjunction with CCR7 and S1PR1.

Ledgerwood et al. and Brown et al. interpret their data with the assumption that S1P works as a chemotactic signal for T cells and that T cells migrate from an area of low S1P concentration (peripheral tissues and LN) to an area of high S1P concentration (blood and lymphatics). My studies find that signals through S1PR1 are necessary and sufficient to promote Th1 cell persistence at LPS inflamed peripheral tissues. Some key experimental differences between my studies and those by Ledgerwood et al. and Brown et al. could explain the discrepancies observed. My experiments utilise Th1 cells and an LPS mediated inflamed tissue while they use naïve T cells in adenovirus (Ledgerwood) or CFA (Brown) inflammation.

In addition, both the studies only quantified the number of transferred cells that migrate to the draining lymph node to elucidate what might be happening in the tissue. I believe, this limits the interpretation of their data. However, in light of these studies, it is difficult to definitively rule out a role for either chemokines or S1P in having a role in retention of Th1 cells in my model, despite not observing a difference in Th1 cell numbers in tissue draining lymph nodes.

In addition to its chemotactic properties, S1P is well known for its anti-apoptotic properties across multiple cell types but this has not been investigated for effector T cells (190, 213, 268, 623, 625{Mendoza, 2017 #740, 626). Hence, I hypothesised that increased effector T cell persistence at inflamed tissues was due to an anti-apoptotic role played by S1P. While intravital microscopy data supported my hypothesis, Th1 cells treated with FTY720 had demonstrably reduced motility at inflamed tissues akin to apoptotic cells (571), caspase assays did not reach significance, despite a strong trend towards increased apoptosis in FTY720 treated group (Figure 5.2.3).

This result however, remains inconclusive, since the difference observed in caspase expression between T cells transferred into resting or inflamed tissues in previous experiments were not present in these experiments. As demonstrated by Mendoza et al. measuring apoptosis of T cells can be tricky. They used markers of mitochondrial integrity to identify a role for S1P in T cell survival (626). Perhaps using an assay similar to this would generate more replicable results.

Interestingly, while Mendoza et al. speculate the effect of S1P on naïve T cell survival is direct, they do not rule out an indirect mechanism of S1P mediated T cell survival. S1PR signalling has been shown to activate STAT3 which has roles in the expression of Bcl-2 and Bcl-xl, both anti-apoptotic molecules (619, 653). Moreover, IL-7/IL-7R signalling, the quintessential T cell survival signal, also activates the Akt signalling pathway used by S1P signalling (654). This suggests a potential synergy between IL-7 and S1P signals in facilitating T cell survival. This may also be applicable to effector T cells which are somewhat sensitive to IL-7 signalling and in some cases use oxidative phosphorylation and the mitochondrial pathway for their energy generation (482).

Another important factor to consider is the recent body of evidence that suggests a role for FTY720 in inducing cellular apoptosis in a caspase independent manner in multiple cell types (655). Although this has not yet been investigated specifically in T cells, it is possible that FTY720 treatment is working in a similar manner to induce apoptosis of T cells in my experiments.

Finally, for S1P to elicit its effects on T cells at peripheral tissues, be that to induce survival or chemotaxis or both, it has to be present at the tissue. Measurement of S1P remains a challenge due to its constitutive intracellular expression and role in cell membrane formation (430). Therefore, I measured the expression of one of the enzymes that phosphorylates sphingosine to S1P, SPHK1. Substantially increased SPHK1 expression at LPS inflamed mouse tissues as well as human rheumatoid synovial tissues suggested that S1P levels may be elevated at such sites. This is supported by mounting evidence that indicate S1P levels are elevated at inflamed tissues.

Raised levels of S1P was found in the synovial fluid of RA patients (267). Likewise, BAL fluid from asthma patients, intestinal IBD samples, and animal models of intestinal inflammation all demonstrated increased S1P (255, 645). Despite the growing evidence for a critical role for S1P in regulating inflamed tissue immunopathology, little is known about how S1P levels are elevated at peripheral tissues.

Studies have identified lymphatic and blood vessel endothelial cells as producers of S1P. Moreover, RBCs and platelets also produce large quantities of S1P (236). H&E staining of inflamed and resting mouse ears demonstrated significant infiltration of blood derived products into inflamed tissues (Figure 3.2.1). Moreover, SPHK1 staining was primarily found in anucleated cells at tissue sites, with very little staining at resting tissues. This leads me to hypothesise that increased S1P levels at acutely inflamed tissues could be a side effect of vascular endothelial leakage. Interestingly, inflammation and in particular LPS is a known inducer of vascular endothelial leakage and could suggest that the effects observed in my model are applicable in other forms of tissue inflammation.

To summarise, I believe my data suggest a novel role for S1P in regulating the persistence of CD4 Th1 cells at peripheral inflamed tissues. The increase in tissue S1P is derived from blood products which leak into LPS inflamed tissue sites. Furthermore, S1P may regulate Th1 cell persistence by having a dual effect of decreased egress as well as increased local survival. Finally, the effect of S1P on Th1 cell survival may not be direct but mediated via secondary survival

signals such as IL-7 or chemokines. This working hypothesis is summarised in Figure 6.1.1 below.

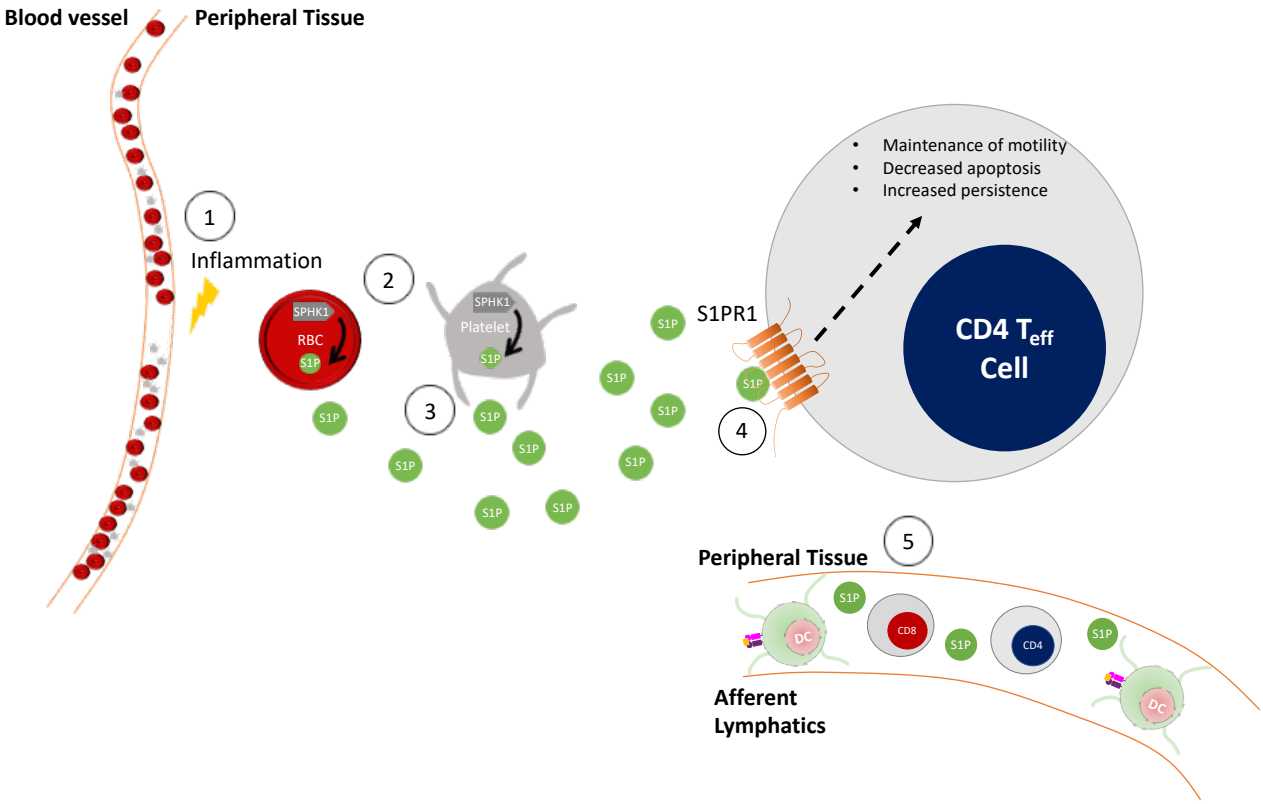

Figure 6.1.1 Working hypothesis on how S1P regulates Th1 cell persistence at inflamed peripheral tissues
1. Inflammation induces vascular damage and leakage of blood products, including RBCs and platelets. 2. Inflammatory stimuli such as LPS also induces activation of SPHKs in these cells, generating large amounts of S1P. 3. RBCs and platelets express S1P transporters allowing extracellular release of S1P. 4. This allows effector T cells to sense increased tissue S1P, increasing their survival. 5. Elevated tissue S1P also eliminates the S1P gradient between tissue sites and lymphatic and blood vasculature, reducing T cell egress. Together, these two factors increase the persistence of effector T cells at acutely inflamed tissues.

6.2 Future studies

6.2.1 Targeting chemokines and integrins in T cell persistence

The key finding of this thesis demonstrates that S1P plays a crucial role in the persistence of CD4 Th1 cells at inflamed tissue sites. However, my studies in section 4 suggest that chemokines could also play a role in T cell persistence. As such, experiments modulating specific chemokine receptors on T cells or their ligands at tissue sites could identify novel chemokine pathways that promote T cell persistence at inflamed tissues.

Indeed, previous studies have demonstrated a role for CCR7 in promoting Th1 cell egress from tissues sites (451, 452). In contrast, APC derived CCL5 was implicated in the retention of memory CD4 T cells at tissue sites (500). The integrin CD103 was directly found to promote regulatory T cell retention in inflamed skin tissue (450). Finally, the integrin VLA-1 was also found to promote CD8 T cell retention in peripheral tissue sites (7, 552).

These studies highlight the multitude of signals which regulate T cell trafficking at tissue sites. Moreover, they demonstrate that the tissue state and differentiation status of the T cell dictates the mechanism of trafficking utilised i.e. inflammatory chemokines, S1P, integrins and others. Developing a comprehensive understanding of how these signals temporally and spatially regulate T cell trafficking may allow for the development of timely and targeted therapies to abrogate tissue inflammation.

6.2.2 Determining S1P mediated T cell survival

While my studies demonstrate that S1P promotes T cell persistence at inflamed tissues, whether this persistence is due to retention or survival is difficult to pinpoint. No differences were found in the numbers of transferred cells in downstream draining lymph nodes or other peripheral organs. Furthermore, T cells were not proliferating at inflamed tissues. This led me to hypothesise that S1P promotes increased T cell survival at the tissue site. Flow cytometry based caspase assays were inconclusive. However, the experiments were compounded by the fact that prolonged processing of the cells *ex vivo* might affect cell viability.

One method to overcome this problem would be to use cells from a transgenic caspase reporter mouse as used by Garrod et al. or by using recently developed caspase probes as demonstrated by Qian et al. (571, 656). This would allow for imaging of T cells *in vivo* and quantifying the number of apoptotic cells in S1P receptor modulated animals (FTY720 treated) versus control animals (vehicle treated). However, this could be difficult to accurately quantify as dead cells may be cleared from the tissue site prior to imaging. Moreover, T cells may be dying in lymphatic vessels while draining from tissue sites, making these cells difficult to image.

Similarly, whole mounted or serially sectioned tissues could be stained with antibodies against active caspases. This method has similar limitations to studying apoptosis *in vivo* as mentioned previously.

Another way to test whether T cell disappearance at tissue sites was due to apoptosis would be to transfer Bcl-2 transgenic T cells. These cells are resistant to apoptosis (657). Thus, any differences in the number of T cells recovered from inflamed vs resting tissue would not be due to apoptosis. This experiment would definitively rule out a role for survival or death in regulating T cell persistence.

Finally, apoptosis could also be assayed by studying the mitochondrial apoptotic pathways. Mendoza et al. very recently discovered that S1P signals are important for naïve T cell survival by studying mitochondrial proteins in S1PR1$^{-/-}$ T cells in secondary lymphoid organs (626). Investigating similar mitochondrial protein expression in my system could potentially illuminate whether S1P signals aid activated Th1 cell survival at inflamed tissues in a similar manner.

6.2.3 Utilising S1PR1$^{-/-}$ T cells

In my experiments, pharmacological agents that target S1PRs were used to modulate S1P signals in T cells. This was achieved by pre-treating activated T cells *in vitro* with S1PR agonists/antagonists prior to their adoptive transfer. This method allowed me to target T cell specific S1P signals and to further study the effects of S1P on T cells specifically at peripheral tissue sites.

The use of pharmacological agents, has some drawbacks. The effect of most of these drugs have been studied *in vivo* and thus how they function *in vitro* is not well known. The length of treatment time and dosage used in my experiments were kept consistent with other published *in vivo* studies where these pharmacological drugs were found to have an effect on T cells (244, 453). However, no studies directly demonstrate how these treatment protocols actually affect S1PR function. Thus, the mechanism of drug action is inconclusive.

Moreover, the mode of action of FTY720 remains controversial. FTY720 has been reported to have both agonistic and antagonistic effects on S1PRs (658-660). Additionally, carryover of drugs cannot be ruled out, despite extensive washing of cells post-treatment. Therefore, the effects observed in my experiments may be due to indirect effects of pharmacological agents on tissue cells following transfer.

Using S1PR1$^{-/-}$ T cells may provide a more robust system to address these questions. However, using such methods poses further challenges for my system. Animals with S1PR1$^{-/-}$ T cells have peripheral lymphopenia (661). Most T cells accumulate in the thymus while some are found in peripheral lymph nodes. While studies suggest that these T cells mature normally, a role for S1P has been described in T cell activation and polarisation (662). Thus, it would be difficult to differentiate whether effects observed in such a system would be due to differences in T cell activation or due to S1PR1 mediated effects at tissue sites. Small interfering RNA (siRNA) knockdown of S1PR1 following OT-II cell polarisation could be used to address these issues (663).

6.2.4 Measuring S1P levels

SPHK1 levels were measured in this thesis as an analogue for S1P. However, S1P levels are regulated not only via SPHKs but also via S1P lyases and phosphatases (430). Additionally, S1P transporters play an important role in regulating levels of extracellular tissue S1P (215, 216, 253, 607). Hence, direct measurement of S1P remains a critical piece of missing evidence in this study.

Substantially elevated levels of S1P has been reported in multiple inflamed peripheral tissues including RA joints (synovial fluid), allergic and asthmatic lungs (BAL), inflamed mouse peritoneum and skin (244, 246, 267, 650). Likewise, SPHK levels were found to be elevated in multiple cancers (664). These studies lend support to my hypothesis that increased S1P at locally inflamed tissue is responsible for T cell persistence.

Measuring tissue S1P directly poses many challenges. Firstly, S1P is produced abundantly intracellularly in almost all cell types due to its role in maintaining cell membranes (430). Thus, mass spectrometry data, the gold standard in S1P measurement, is unlikely to prove a reliable measurement of extracellular S1P. A single S1P ELISA kit is available from echelon biosciences. However, the ELISA sensitivity does not allow for detection at low levels at tissue sites.

Perhaps the best way to measure S1P levels at tissues with any confidence would be to analyse mRNA levels of all the SPHKs, S1P lyases, phosphatases and transporters by qRT-PCR. This analysis would develop a comprehensive picture of S1P regulating proteins at tissue sites. Similar studies have been performed by Karuppuchamy et al. with promising results in murine gut tissues (645).

6.2.5 Who produces S1P at inflamed tissues?

Once elevated levels of S1P can be confirmed, it would be interesting to investigate what cell types are responsible for S1P production at inflamed tissues. Platelets, RBCs and mast cells are potent producers of extracellular S1P (217). Tissue histology of LPS inflamed mouse ears revealed significant blood exudate in inflamed skin tissue (Figure 3.2.1A). This in itself could elevate levels of tissue S1P, due to increased S1P levels in blood (665). Moreover, RBCs and platelets in tissue could undergo further activation by their inflammatory surroundings, elevating levels of tissue S1P.

Studies involving the transfer of activated platelets or RBCs into mouse ear pinnae followed by transfer of T cells to investigate their persistence, could confirm whether platelet or RBC mediated S1P is responsible for T cell persistence. Likewise, challenging platelet depleted animals with LPS and then transferring T cells into their ear pinnae could answer similar questions.

6.3 Concluding remarks

In this study, persistence of CD4 T cells at inflamed tissues was demonstrated using an LPS mediated skin inflammation and tissue adoptive transfer model. Using this system, S1P was found to be responsible for the persistence of activated Th1 cells at inflamed tissues. Moreover, signals through the S1PR1 were found to be both necessary and sufficient for persistence of CD4 T cells at inflamed tissues.

Blocking S1PR signals significantly reduced T cell motility *in vivo* at inflamed tissues. Finally, elevated levels of the S1P producing enzyme SPHK1 was found at inflamed peripheral tissues. Together, my data suggests that increased T cell persistence and motility is due to increased survival of T cells mediated via S1PR signals.

This novel finding that S1P aids effector T cell persistence and, potentially, survival at peripheral inflamed tissues could lead to the development of new therapeutic interventions targeting peripheral tissue inflammatory diseases such as RA and psoriasis.

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