Sphingosine 1-phosphate: Regulation of Receptor Signalling and Cross-talk with Chemokines

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Abstract

The generation and execution of adaptive immune responses require fine control of the migration of lymphocytes in homeostasis and inflammation. Recently it has been shown that certain chemokines and the lipid sphingosine 1-phosphate interact to regulate egress of resting T cells from lymph nodes. As T cells are exposed simultaneously to many chemokines and S1P at other points in their life-cycle, other instances of cross-talk are likely. How S1P receptor signalling changes following T cell activation, and how S1P may modulate cellular responses to chemokines before and after this transition, are important questions.

S1P pre-treatment enhanced the chemotaxis of Jurkat T cells towards CXCL12. This was a specific effect as S1P did not affect the amount of cell surface CXCR4 or certain signalling responses downstream of chemokine stimulation. Unexpectedly, this effect was opposite to that observed with primary resting cells, where S1P treatment suppressed migration towards CXCL12. The chemokine-induced phosphorylation of Akt was unaffected, suggesting a PI3K-independent effect. In contrast, S1P enhanced the migration of activated T cells towards the inflammatory chemokine CXCL10.

Using an S1P receptor 1-selective agonist it was shown that receptor signalling in T cells was lost transiently after activation. This was demonstrated, using siRNA, to be partly dependent on the initial upregulation of CD69. The relevance of modulation of S1PR1 signalling was explored further *in vivo*. The S1P receptor super-agonist FTY720 sequestered activated alloreactive cells in a peripheral lymph node, suggesting that their egress was dependent on S1P receptor signalling.

These results show that S1P is a key regulator of T cell migration at three different stages of their life-cycle: before activation, in their response to homeostatic chemokines; following activation, in their ability to egress lymphatic tissue; and finally in the responses of differentiated effector cells to inflammatory chemokines.

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Abbreviations

(d)NTP	(Deoxy)nucleotide triphosphate
(p)APC	(Professional) antigen-presenting cell
ABC	Adenosine triphosphate-binding cassette
Akt	(Also known as protein kinase B)
AP-1	Activator protein-1
BCA	Bicinchoninic acid
BCR	B cell receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CLSM	Confocal laser-scanning microscopy
DAPI	4,6-diamidino-2-phenylindole dihydrochloride
DEPC	Diethylpyrocarbonate
DOCK2	Dedicator of cytokinesis-2
EDG	Endothelial differentiation gene
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
fMLP	N-Formylmethionyl-leucyl-phenylalanine
FSC	Forward-scatter
GPCR	G protein-coupled receptor
Gy	Gray (SI unit of absorbed radiation)
HDAC	Histone deacetylase
HEV	High endothelial venule

i.p. / i.v.	Intraperitoneal / intravenous
ICAM-1	Intercellular adhesion molecule -1
IFN-α/β	Interferon-alpha/beta
IFN-γ	Interferon-gamma
IgE / IgG	Immunoglobulin E / Immunoglobulin G
IL-(x)	Interleukin-(x)
K_d	Dissociation constant
KLF2	Kruppel-like factor-2
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte-function associated antigen -1
LN	Lymph node
LPP	Lipid phosphate phosphatase
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule-1
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C

PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase (enzyme); also reverse transcription (process)
RT-PCR	Real-time polymerase chain reaction
S1P	Sphingosine 1-phosphate
S1PR(x)	S1P receptor (x)
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SK(x)	Sphingosine kinase (x)
SPP	Sphingosine phosphate phosphatase
SSC	Side-scatter
TBST	Tris-buffered saline solution + 0.1% Tween 20
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNFR(x)	Tumour-necrosis factor receptor (x)
TNF-α	Tumour-necrosis factor-alpha
Tris	Tris(hydroxymethyl)aminoethane
UTR	Untranslated region

VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
WASP	Wiskott-Aldrich syndrome protein

Chapter 1. General Introduction

1.1 Immune System

The immune system of mammals evolved to protect the host from disease caused by infection with micro-organisms (such as bacteria and viruses) and certain larger, multi-cellular ones (for example helminths). In general terms, this is achieved by structures and systems that: prevent or inhibit entry of the pathogen into the host, inhibit its metabolism and / or replication, effect its clearance, or in some cases result in the maintenance of an equilibrium between the pathogen and host that allows for their continued survival.

Two main subsystems are often distinguished: innate and adaptive immunity. Whereas the innate system is relatively non-specific to the pathogen, the adaptive is highly so. The innate system does not form memory of the pathogen; the adaptive can (Medzhitov and Janeway, 2000).

1.1.1 Innate Immunity

Cellular and acellular components are present that function to either prevent invasion by pathogens or inhibit their colonisation on outer surfaces of the host. The skin and mucosal epithelia form a tight cellular barrier that retards entry of micro-organisms. Small proteins called defensins, produced by epithelial cells coat these surfaces and have direct anti-microbial function (Ganz and Lehrer, 1995). In the respiratory tract, motile cilia move mucus, and any associated micro-organisms, upwards towards the mouth and therefore inhibit their colonisation of the lung (Vareille *et al.* 2011).

Innate immune cells are distributed widely in peripheral tissues and act as sentinels. They respond to detection of structural components of pathogens (pathogen-associated molecular patterns (PAMPs)) that are often very highly conserved between classes of microbe. PAMPs are recognised by pattern-recognition receptors (PRRs) expressed by certain types of immune cell; they can be found on the cell surface, on the surface of internal membranes and in the cytosol (Matzinger, 1998). A large and important class of PRRs are the Toll-like receptors (TLRs): there are more than 10 distinct types (O'Neill, 2008). TLR 3, expressed on the luminal surface of endosomes, binds double-stranded RNA (produced during the replication of some viruses) (Gauzzi *et al.*, 2010). TLR 4, expressed on the cell surface, acts in concert with other proteins to bind lipopolysaccharide (a part of Gram-negative bacteria) (Lu *et al.*, 2008). Innate immune cells can be also be activated by endogeneous danger signals (Erridge, 2010); these are only available to bind their cognate receptors under conditions of cell stress and are therefore indirect markers of infection. Signalling of TLRs and other danger receptors on innate immune cells results in inflammation, a process characterised by activation of vascular and immune cells that signal with each other and coordinate the generation, perpetuation and resolution of an immune response to clear the infection (Janeway and Medzhitov, 2002).

The effector cells of innate immunity include neutrophils, eosinophils, basophils, mast cells, macrophages and natural killer (NK) cells. Attracted by proteins produced early at the acutely inflamed site, neutrophils are usually one of the first cells to be recruited (Summers *et al.*, 2010). Once activated through any one of a number of different receptors, including those that can bind

components of pathogens (such as carbohydrate structures), they can phagocytose microbes, generate a cytotoxic respiratory burst, and release cytotoxic granules (amongst other roles) (Wright *et al.*, 2010). Macrophages, particularly after activation by cytokines such as interferon-γ, can ingest and kill bacteria within phagolysosomes (Rees, 2010). NK cells provide early protection against viral infection. They are sensitive to changes in host cells caused by viral infection (specifically, for example, a reduction in the expression of class I proteins of the major histocompatibility complex). They can release perforin granules on activation and therefore mediate cytolysis of virally-infected cells; their production of interferon-γ promotes macrophage activity and can influence the type of adaptive response that might develop (Lunemann *et al.*, 2009). An important acellular component of innate immunity consists of the complement proteins. This protein cascade can either be directly activated by pathogens (on their surface) or indirectly via other immune-recognition mechanisms. Complement proteins can coat pathogens (opsonisation), and thereby signal their destruction by complement-receptor bearing immune cells, or in some cases they can mediate direct cytolysis (Ricklin *et al.*, 2010).

1.1.2 Adaptive Immunity

The adaptive immune system is distinct from the innate in two important respects: a high degree of specificity against the pathogen is developed during the course of the response (beyond that provided by the relatively limited range of germline-encoded receptors), and the ability to form memory. In the case of a primary response to a pathogen, the effector cells of the adaptive system are slow to appear when compared with the innate system. This is because of the requirement that specialised cells (which are part of the innate immune system) must process and present components of pathogens to lymphocytes, in order to select those capable of recognising the pathogen. These naïve cells (which have not been previously triggered through their specific receptor) then proliferate and differentiate to generate large numbers of effector and memory cells (Medzhitov and Janeway, 2000).

1.1.2.1 Antigen Presentation

Most mammalian cells 'present' antigen, but only specialised cells, professional antigen-presenting cells (pAPC), are able to initiate primary adaptive immune responses (Banchereau *et al.*, 2000; Lanzavecchia and Sallusto, 2001). Dendritic cells are a type of pAPC, and different subsets of DC are widespread in the body.

Activation of pAPC through signalling of innate immune receptors triggers enhanced uptake of pathogens, their processing and finally the presentation of pathogen components on the cell surface (Mellman and Steinman, 2001). A wide range of structures can be presented, including whole proteins, peptides, and lipid or carbohydrate-conjugated versions of these. Best understood is the presentation of small peptides (typically between 8 and 24 amino acids in length) within a pocket of proteins of the major histocompatibility complex (MHC). The MHC (the human leukocyte antigens, HLA, in humans; in mice they are the H2 proteins) is a large, highly polymorphic and complex gene cluster in mammals. The MHC consists of different gene subsets: MHC classes I and II consist of three genes each, and the proteins they encode are directly involved in the presentation of peptides to T lymphocytes (Kelley *et al.*, 2004). Broadly, MHC class I molecules present peptides derived from the cytoplasm (Peaper and Cresswell, 2008); MHC class II molecules are loaded with peptides present in the lumen of endosomes (Rocha and Neefjes, 2008). Generally, only professional antigen-presenting cells express MHC class II, whereas most cells express class I. Professional APC can therefore present peptides derived from structures that they have phagocytosed (Savina and Amigorena, 2007).

Once they have been activated and taken up potentially foreign material, pAPC acquire the ability to migrate to lymphoid tissue. This tissue is structured for ensuring optimal interaction between pAPC and the rare lymphocytes that can recognise the presented peptides (Randolph *et al.*, 2005). Activated pAPC also upregulate co-stimulatory molecules (such as CD80 and CD86) and produce polarising cytokines (such as interleukin-12 (IL-12)) (Greenwald *et al.*, 2005). All of these factors are necessary to ensure productive interaction with T lymphocytes and therefore generation of an adaptive response.

1.1.2.2 T Lymphocytes

T lymphocytes are central to adaptive immunity: they act directly as immune effectors, for example by mediating cytolytic activity; they also coordinate the activity of other immune cells (such as macrophages and B lymphocytes). T lymphocytes originate from common lymphoid precursors. Their precursors seed the thymus and then undergo several stages of differentiation there (Miosge and Zamoyska, 2007). It is during this process that the unique T cell receptors (TCRs) that are characteristic of the mature cell population are generated. These TCR sequences are not encoded in the germline but are produced by a random process of gene recombination and nucleotide addition that operates on TCR variable region genes and occurs in each cell during development. A highly diverse pool of TCR in the mature T lymphocyte population is generated (an estimated 10^{18} unique receptors are possible (Blom *et al.*, 1999; Medzhitov and Janeway, 2000). The TCR is the antigen-specific receptor that binds particular peptide - MHC complexes and controls proliferation of naïve cells and effector function of differentiated ones.

Resting T lymphocytes are often described according to their expression of particular molecules, which generally indicate the types of activity they can perform. For example, some T lymphocytes express CD4 and others CD8. The former interacts with MHC class II molecules (Konig *et al.*, 1996) and as a consequence CD4 T lymphocytes are largely restricted to antigen-specific interaction with professional antigen-presenting cells. CD4 T lymphocytes can be triggered by pAPC to produce cytokines such as interferon-gamma (IFN-γ), which promotes early innate activity (Kasten *et al.*, 2010) (such macrophage anti-microbial activity); they also interact with MHC class II-bearing B lymphocytes and provide essential signals for the generation of certain antibody responses (Nutt and Tarlinton, 2011). CD8 interacts with MHC class I, so CD8 T lymphocytes can engage with the much larger pool of MHC class I-expressing cells (Jones *et al.*, 1998). CD8 cytotoxic T lymphocytes (CTLs) contain perforin and can mediate direct cytotoxicity. They recognise virally-infected cells through TCR engagement of viral peptide-MHC class I complexes on the cell surface, which triggers the release of cytotoxic granules (Berke, 1994).

It has already been stated that as part of their maturation process pAPC migrate into lymphoid tissue, where they present pathogen-derived peptides on their

surface. Naïve T cells continuously recirculate in the body through the blood and lymphoid tissue, and it is within the T cell area of lymph nodes that pAPC and T lymphocytes interact (Sallusto and Lanzavecchia, 2000). If a naive T cell engages, through its TCR, a mature pAPC bearing a complementary peptide-MHC complex, the T cell stops migrating (Dustin *et al.*, 1997). A stable immune synapse develops between the T cell and APC. This comprises a varied cluster of molecules (such as adhesion proteins) in addition to the TCR / peptide-MHC complex, which likely play an important role in control of synapse stability (Huppa and Davis, 2003). An array of signalling events, centred around the CD3 complex of proteins that are associated with the TCR, trigger T cell proliferation and subsequent differentiation of daughter cells into particular subtypes. The activation of naïve T cells is absolutely dependent upon the provision of co-stimulation by pAPC: CD80 and 86 engage CD28 expressed on the T cell. This requirement for CD28 co-stimulation is due to negative regulation of TCR signalling by phosphatase and tensin homolog (PTEN) in naïve T cells; this is often not the case for memory T cells (Buckler *et al.*, 2006). Effector cells leave the lymph node and move to the site of inflammation where they mediate their function including direct cytotoxicity. Central memory cells recirculate in the organism and their role is to facilitate rapid secondary responses to that antigen (Sallusto *et al.*, 1999).

There are a number of lineages of CD4 effector T cells: the classical ones being the Th1 and Th2 subsets, although the Th3, Treg and Th17 (amongst others) are now broadly accepted. The Th1 subset of CD4 T cells is characterised by high production of the cytokine IFN-γ and cell-mediated immunity. The Th2 subset is characterised by high production of the cytokines IL-4, -5 and -13, and is associated with antibody-mediated responses, including immunoglobulin E (IgE)-associated allergy and anti-helminth immunity (Dong and Flavell, 2001).

1.1.2.3 B Lymphocytes

Like T cells, B lymphocytes originate from common lymphoid precursors; they complete their development however in the bone marrow (Hardy and Hayakawa, 2001). Also akin to T lymphocytes, all mature B cells possess an antigen-specific receptor: the B cell receptor (BCR). The precise sequence is generated in a process of gene rearrangement, recombination and mutation similar, but not identical, to that which occurs in the case of the TCR (Borghesi and Milcarek, 2006). The mature B cell population thus contains cells bearing BCRs with a very large range of specificities (up to 10^{14} unique immunoglobulin sequences) (Medzhitov and Janeway, 2000). Importantly, the BCR is not restricted to binding structures in the context of MHC proteins. The BCR can be secreted (without the membrane-spanning domain) by a type of differentiated B cell, the plasma cell, and is known in that form as antibody (Oracki *et al.*, 2010). Antibodies mediate protection through three main mechanisms: they bind and neutralise particles (such as viruses, preventing their binding to entry receptors (Corti *et al.*, 2011)), they bind structures on the surface of pathogens and thereby target them for removal by FcR-bearing (Nimmerjahn and Ravetch, 2007) immune cells (such as macrophages), and they can trigger complement activation on pathogen surfaces (Ricklin *et al.*, 2010).

The B1 subset of B cells produces natural antibody that binds carbohydrates not present in mammals, providing constitutive protection against certain classes of bacteria. B2 B cells function as antigen-presenting cells and can differentiate into antibody-producing or memory cells (Hardy, 2006; Montecino-Rodriguez and Dorshkind, 2006). Binding of the BCR to antigen triggers its efficient internalisation, processing and presentation of linked peptides (in an MHC class II-restricted fashion) to T cells. CD4 T cells recognising these peptides provide essential co-stimulatory signals (such as CD40L) for the proliferation and differentiation of these B cells. This occurs within specialised regions of lymphoid tissue known as germinal centres. Further gene rearrangement can occur resulting in production of antibody of different isotypes, a process known as class switching. Gene point mutation also occurs within the expressed immunoglobulin locus of germinal centre B cells, and through a T cell-driven selection mechanism (affinity maturation), high affinity BCR (and thus antibody) against antigen can be generated (McHeyzer-Williams *et al.*, 2001).

1.2 Lymphocyte Migration

1.2.1 Cell Biology

The movement of cells, guided by chemical gradients, evolved early in the evolution of life. The type of movement, and the mechanism by which it is achieved, is different between prokaryotic and eukaryotic cells. Within eukaryota, from amoebae to human leukocytes, the general mechanisms are remarkably conserved (Swanson and Taylor, 1982).

Leukocytes move following what is termed a common amoeboid program; there are small differences in behaviour and underlying mechanism between cell subsets. For example, three sections of a migrating lymphocyte can be easily discerned: a leading edge, mid-region and uropod. Migrating monocytes are exceptional in that they have no noticeable uropod (Friedl and Weigelin, 2008). Lymphocytes are highly dynamic; they can change rapidly from moving quickly (characterised by a few, weak adhesive interactions and high actin turnover) to absolute arrest (tight integrin-mediated adhesion and low actin turnover). They can also adopt a state of limited movement that may, for instance, be used during scanning of antigen-presenting cells (Friedl *et al.*, 1998; Wolf *et al.*, 2003; Lammermann *et al.*, 2008).

The front portion of a migrating leukocyte is termed the leading edge. Rapidly forming and degrading filamentous actin networks occurring in this region underlie the cell extensions (pseudopods and lamellipods) into the environment (Pollard and Borisy, 2003). Membrane is continuously endocytosed and then preferentially exocytosed at the leading edge to facilitate this process (Traynor and Kay, 2007). Dendritic actin polymerisation is associated spatially and temporally with extension of the cell at the leading edge, but is not the only mechanism driving it. Detachment of cytoskeletal support from the membrane at the leading edge would lead to membrane blebbing under hydrostatic pressure of the cytosol (Cunningham, 1995; Charras *et al.*, 2005). It has been postulated that actin polymerisation in the bleb follows its formation, perhaps stabilising it (Charras *et al.*, 2006).

Integration of signals received from the environment at the leading edge guide directional migratory behaviour (Negulescu *et al.*, 1996; Wei *et al.*, 1999; Stanley *et al.*, 2008). In steep gradients of chemoattractant (substances to

which cells are attracted), pseudopodia are projected directly up the gradient of attractant (Gerisch and Keller, 1981). Evidence suggests that Phosphatidylinositol 3-kinase (PI3K) is a central controller of signalling activity at the leading edge (Franca-Koh *et al.*, 2007; Marone *et al.*, 2008). G protein-coupled receptor (GPCR) signalling causes recruitment of the PI3Kγ subunit to the membrane, and local generation of phosphatidylinositol (3,4,5) triphosphate (PIP_3) (Parent *et al.*, 1998; Janetopoulos and Devreotes, 2001). Other types of receptor, for example the TCR, also couple to PI3K and thereby induce cell migration (in the case of ζ chain receptors via the PI3Kδ subunit (Rommel *et al.*, 2007)). An array of docking proteins couple PIP_3 (in the plasma membrane) to actin-binding effectors. For example, Rac, via Wiskott-Aldrich syndrome protein (WASP)- family proteins, couples to the Arp2/3 complex that causes branching of actin filaments. Rac activity is central to cell migration; Rac-negative cells are largely unable to migrate (Nombela-Arrieta *et al.*, 2004; Goley *et al.*, 2006; Stambolic and Woodgett, 2006). Cofilin also seems to play an important role regarding actin polymerisation at the leading edge (Svitkina and Borisy, 1999; DesMarais *et al.*, 2004).

Spontaneous formation of pseudopods may be more important in weaker gradients of chemoattractant (Bosgraaf and Van Haastert, 2009). PIP_3 and Rac are constitutively active at a low level in migrating cells, and this probably drives the process. There is a very high rate of actin turnover and random pseudopod formation in PTEN-negative cells (Iijima and Devreotes, 2002). Differences in signal strength transduced by receptors in each pseudopod could underlie differential rates and positions of pseudopod growth and retraction, thereby biasing the rate and direction of movement (Andrew and Insall, 2007).

Cell migration can be triggered through pathways other than PI3K signalling. p38 mitogen-activated protein kinase (MAPK) and dedicator of cytokinesis 2 (DOCK2) seem to have important roles (Hoeller and Kay, 2007), as they also link to Rac and can therefore influence actin filament dynamics. End-stage chemoattractants (which guide cells towards a particular source and migration end-point) activate p38 MAPK and inhibit PI3K. PI3K activity is not required for chemotaxis of neutrophils to N-formylmethionyl-leucyl-phenylalanine (fMLP) (Heit *et al.*, 2008), whereas it is necessary for migration to CXCL8 (Knall *et al.*,

1997). This seems to offer an explanation for how prioritisation of end-stage over intermediate chemoattractants is achieved.

The mid-region of a migrating leukocyte contains the nucleus. It is held immobile by crosslinked actin filaments around the nucleus, and is carried forward with the cell (Laevsky and Knecht, 2003; Friedl and Weigelin, 2008). The cell is anchored to the matrix at the rear, the uropod, with intercellular adhesion molecule-1 (ICAM-1) and -3, and β1 integrin having important roles. The uropod is relatively richer in mitochondria, which likely power the contraction of this part of the cell as it moves forward. The enzyme PTEN, excluded from the leading edge, dephosphorylates various effectors of actin polymerisation in the mid-region and uropod (Funamoto *et al.*, 2002; Heit *et al.*, 2008).

1.2.2 Chemokine Signalling

1.2.2.1 Chemokines

Chemokines are a family of endogeneous compounds in mammals that direct the migration of immune and other cell types. They are small proteins, mostly between 7 and 14 kDa in size, and they are related in sequence. CXCL8 was the first to be cloned and characterised (Baggiolini *et al.*, 1994). The crystal structure of CXCL8 has been resolved and shows the presence of a Greek-key domain that is characteristic of the family.

Chemokines have traditionally been classified with reference to the N terminal cysteine residues that are common to the family. C chemokines have one N-terminal cysteine (there is another some distance away in the primary sequence), CC chemokines have two adjacent cysteines, CXC chemokines have one intervening non-cysteine amino acid, and the CX_3C chemokines have three such separating residues. The CXC chemokines can themselves be further sorted into those that contain ELR in their primary sequence (including CXCLs 1, 2, 3, 5 and 6) and are mostly neutrophil chemoattractants, and those that do not (Van Coillie *et al.*, 1999; Zlotnik and Yoshie, 2000). CX_3CL1 and CXCL16 are exceptional chemokines, as they can exist in both a soluble form and as a tethered molecule on endothelial cells (functioning as an adhesion molecule) (Schwarz *et al.*, 2010; van der Voort *et al.*, 2010).

In the context of the immune system, chemokines have two main functions: to direct the migration of immune cells (Stein and Nombela-Arrieta, 2005), and trigger the activation of adhesion molecules called integrins (Shamri *et al.*, 2005). Chemokines coordinate the movement of immune cells so that they are placed appropriately for their function at any particular time, a crucial function for effective immunity. They can be produced by a great number of different cell types, and may be either constitutively produced or induced by some stimulus. Macrophages and endothelial cells produce chemokines (for example, CXCL8) on receipt of danger signals (for example in response to ligation of TLRs) and this is associated with recruitment of innate effector cells. Lymphatic endothelium and lymph node stroma constitutively produce chemokines (CCL19 and 21) that control the recirculation of naïve T cells and recruit mature DC to lymphoid tissue (Bromley *et al.*, 2008).

Many chemokines bind with low affinity to glycosaminoglycans (GAGs) that are located in the extracellular matrix and on the surface of cells. This interaction is important for the establishment of solid-phase gradients for migrating cells to follow (Patel *et al.*, 2001). Chemokines that are bound to a solid substrate strictly induce cell haptotaxis (or –kinesis, if non-directional); in soluble form, they induce chemotaxis (-kinesis) (Carter, 1967). The nature of the interaction with glycosaminoglycans has been examined in detail for CXCL12 (Amara *et al.*, 1999; Lortat-Jacob *et al.*, 2002). Changes in the structure of GAGs, for example during inflammation, are known to affect the presentation of chemokine ligands to their receptors (Proudfoot *et al.*, 2001; Turnbull *et al.*, 2001; Proudfoot *et al.*, 2003).

1.2.2.2 Chemokine Receptors

Chemokine receptors belong to the G-protein-coupled receptor superfamily. Their nomenclature is derived from that of their ligands: CC chemokines (e.g. CCL5) bind receptors denoted CCR (CCL5 can bind CCRs 1, 2 and 5); CXC chemokines (e.g. CXCL12) bind CXCR (CXCR4). Many chemokine receptors couple to the Gαi G protein, and effect cell migration in part due to Gβγ -driven activation of PI3K (Brock *et al.*, 2003). There is evidence that certain receptors can co-opt the other signalling pathways. CXCR4 is able to utilise some of the TCR signalling domains within the CD3 complex (Rossi and Zlotnik, 2000; Kumar *et al.*, 2006).

Immune cell migration in mammals is mainly controlled by regulation of expression of chemokines and their receptors (Rabin *et al.*, 1999). Some chemokines, such as CXCL12, are produced constitutively. Bone marrow stromal cells and lymphatic endothelial cells produce CXCL12, inducing homing of memory B cells to the bone marrow and contributing to the recirculation of resting T cells (which express the cognate receptor CXCR4) (Karin, 2010). Other chemokines, such as CCL5 and CXCL10, are produced during inflammation. The receptors for these ligands, CCR5 and CXCR3 respectively, are upregulated on the activation of T cells (Loetscher *et al.*, 1998; Nakajima *et al.*, 2002). Differentiated, effector T cells therefore have a chemokine receptor profile that facilitates their migration to inflamed sites in the periphery (Campbell *et al.*, 2003). Effector T helper cell subsets have characteristic profiles of chemokine receptor expression (Siveke and Hamann, 1998). Th1 cells typically express CCR5 (Loetscher *et al.*, 1998) and CXCR3, whereas Th2 cells express CCRs 3, 4 and 8 (Sallusto *et al.*, 1997; D'Ambrosio *et al.*, 1998; Sallusto *et al.*, 1998).

1.2.3 Regulation of Lymphocyte Migration by Chemokines

1.2.3.1 In Homeostasis

Resting naive T cells express high levels of CCR7. CCL19 and 21, the ligands for CCR7, are produced by lymphatic tissues (Forster *et al.*, 2008): high endothelial cells (HEC) produce CCL21 (and can present CCL19 that has been transcytosed from inside the lymph node), and fibroblastic reticular cells CCL19 and 21. Professional antigen-presenting cells do not constitutively express CCR7, but it is upregulated on maturation (Randolph *et al.*, 2005). The consequence is that large numbers of naïve T cells traffic through individual lymph nodes, where they can scan the surface of resident mature pAPC for foreign antigen. A T cell will typically spend between 6 and 24 hours within a node under non-inflammatory conditions before egress. The majority of resting T cells egress by squeezing into the cortical sinusoids and are then carried under flow into the efferent lymphatics (Beauchemin *et al.*, 2007; Grigorova *et al.*, 2010).

T cells enter lymph nodes via high endothelial venules (HEVs). L-selectin, expressed at high levels on the surface of T cells, interacts with surface carbohydrate (e.g. PNAd and GlyCAM-1) on HEC (Galkina *et al.*, 2007) and causes the T cells to roll along the endothelium. Interactions between ICAM-1 on the endothelium and lymphocyte function-associated antigen -1 (LFA-1) (an integrin) on the T cell cause cell arrest (Warnock *et al.*, 1998). CCR7 signalling is necessary for cell entry into the node: monocytes express L-selectin and ICAM-1, but not CCR7, and cannot enter via HEV. Chemokine receptor signalling triggers changes in integrin conformation, enhancing adhesion and promoting endothelial diapedesis of the cell into the node parenchyma (Miyasaka and Tanaka, 2004).

The microstructure of the lymph node is highly specialised to promote effective immunosurveillance. Movement of cells within lymph nodes, from their entry till exit, occurs along stromal cell networks (Bajenoff *et al.*, 2006). Fibroblastic reticular cells (FRCs) form a scaffold along which lymphocytes can migrate. CCL19 and CCL21 are bound to the reticular network and appear to induce haptokinetic migratory behaviour. DC associate with the reticular network (Gretz *et al.*, 1997; Link *et al.*, 2007), promoting the interaction of lymphocytes and APC.

1.2.3.2 In Inflammation

1.2.3.2.1 Within Secondary Lymphoid Tissue

In the early stages of an immune response, innate immune cells (activated by danger signals) secrete pro-inflammatory cytokines such as Tumour necrosis factor-alpha (TNF-α) and IL-6. These diffuse from their site of production and influence the behaviour of multiple cell types in a paracrine fashion. They also enter the tissue lymphatics, which drain the cytokines into local lymph nodes. There they induce lymph node shutdown, a non-specific block in lymphocyte egress. Interferon-γ within the node causes downregulation of CCL21 and CXCL13, and the relocalisation of resident DC and T cells. This might promote development of an adaptive response (Mueller *et al.*, 2007).

Lymph node activation is marked by upregulation of ICAM-1 and increased presentation of CCR7 ligands by HEV (Forster *et al.*, 1999). CXCL9 and CXCL10 are newly presented and cause recruitment of NK cells and activated T cells into the node. Chemokines such as CCL21, produced by stromal cells and mature DC, increase the general motility of resting T cells within the node (Stachowiak *et al.*, 2006; Woolf *et al.*, 2007; Worbs *et al.*, 2007). Prolonged signalling through the TCR results in cell activation, a complete loss in responsiveness to chemotactic factors, and cessation of migration (Dustin *et al.*, 1997). The TCR-driven accumulation of PIP_3 in the cell membrane results in activation of the mammalian target of rapamycin (mTOR) pathway and ultimately a loss in expression of kruppel-like factor 2 (KLF2). This transcription factor is a master regulator of multiple adhesion molecules, chemokine receptors and others, including CD62L, CCR7 and Sphingosine 1-phosphate receptor 1 (S1PR1) (Finlay and Cantrell, 2010).

Chemokine signalling plays additional roles in promoting interactions between other cell types in inflamed lymph nodes. CD8 T cell activation requires help from both professional antigen presenting cells and CD4 T cells. DC – CD4 T cell conjugates can produce ligands for CCR5, and attract CD8 T cells that express the receptor. The germinal centre reaction and development of high affinity antibody is similarly CD4 T cell-dependent. A subset of CD4 T cells differentiates after activation to become follicular helper cells. These cells downregulate CCR7 and upregulate the receptor CXCR5 - which binds the follicular chemokine CXCL13 (von Andrian and Mempel, 2003; Cyster, 2005).

1.2.3.2.2 Homing to the Periphery

As T cells differentiate following activation, they upregulate inflammatory chemokine receptors, lipid receptors and various types of adhesion molecule. Some of these are common to all differentiated T cells, whereas other molecules are specific to certain subsets, and confer destination specificity to the cell migration program. The precise chemokine receptor profile of effector T cells is imprinted during the priming interaction with dendritic cells. Cells activated within gut-associated lymphoid tissue upregulate the chemokine receptor CCR9 (which guides migration to the small intestine), whereas skin-homing T cells express CCRs 4 and 10. The ligands for these receptors are produced at particular sites: CCL25, the ligand for CCR9, is expressed by endothelial cells in the small intestine (Kunkel and Butcher, 2002); CCL17, the ligand for CCR4, by endothelial cells of skin but not intestinal venules (Reiss *et al.*, 2001).

Adhesion molecules act in concert with chemokines to control effector cell migration to specific sites in the periphery. Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) is expressed by vascular endothelial cells within the gut, and binds the $\alpha 4\beta 7$ integrin expressed on a particular subset of T lymphocytes (Berlin *et al.*, 1993). T cells activated within skin-draining lymph nodes upregulate cutaneous lymphocyte antigen (CLA), a carbohydrate-modified form of PSGL-1 that interacts with E-selectin (Fuhlbrigge *et al.*, 1997).

Ingress of effector T cells into peripheral tissue proceeds in a broadly similar way to lymph node entry via HEV. A carbohydrate - selectin tethering interaction induces cell rolling along the endothelium. The lymphocyte can then sample for chemokines and complementary adhesion molecules on the endothelium. ICAM-1 and (in inflamed tissue) Vascular cell adhesion molecule -1 (VCAM-1) on endothelial cells interact with LFA-1 and Very late antigen -4 (VLA-4) on lymphocytes (Ley *et al.*, 2007). Inflammatory chemokines bound to surface GAGs engage their cognate receptors on the migrating cells, driving changes in integrin affinity and avidity. This causes cell arrest and induces endothelial diapedesis. Adhesion to laminin in the basement membrane is mediated by the $\alpha 6\beta 1$ integrin (Dangerfield *et al.*, 2002). Early in the inflammatory process neutrophils move through areas of reduced laminin and collagen density (Steadman *et al.*, 1997). Matrix metalloproteinases and serine proteases

produced by leukocytes and activated in their immediate surroundings break down the laminin and collagen mesh (El-Shabrawi *et al.*, 2004; Young *et al.*, 2007). The cumulative effect of matrix degradation by different immigrating cell types over time creates paths of least resistance and therefore preferred tissue entry points. Inside the tissue, the bulk of cell migration occurs along tracks close to the vasculature. The $\alpha 6\beta 1$ and $\alpha 4\beta 1$ integrins play important roles in anchoring the cell uropod, while chemokine gradients provide appropriate directional cues (Friedl and Weigelin, 2008).

1.3 The Sphingosine 1-phosphate Signalling Axis

1.3.1 S1P

Sphingosine 1-phosphate (S1P) is a 379 Da bioactive lipid. It is amphipathic: consisting of a polar headgroup and a long saturated aliphatic tail (Figure 1). It has a wide range of effects on different cell types in multiple organ systems, mediated via both cell surface and intracellular receptors. Cellular responses are modulated through changes in the subcellular localisation of S1P, its import and export from the cell, protein carrier binding and changes in receptor expression by target cells (Rosen *et al.*, 2009).

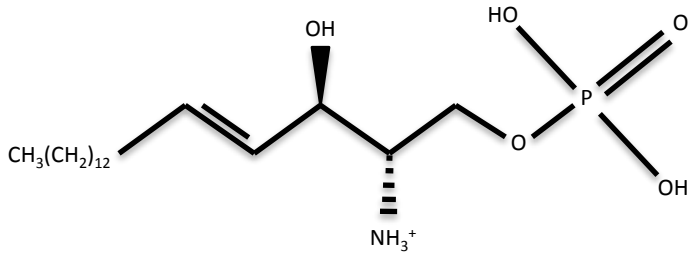


Figure 1. Sphingosine 1-phosphate.
The chemical structure of sphingosine 1-phosphate.

1.3.1.1 Generation

Sphingosine 1-phosphate is formed on phosphorylation of the primary hydroxyl group of its immediate precursor sphingosine, an 18 carbon aliphatic amino alcohol. The phosphorylation reaction is catalysed by one of the isoenzymes sphingosine kinases 1 and 2 (SK1 & 2). Most sphingosine is derived from the breakdown of the membrane component sphingomyelin, which is enriched in the lipid rafts of eukaryotic cell membranes (Rivera *et al.*, 2008). Degradation of sphingomyelin yields ceramide, which can be interconverted with sphingosine. *De novo* synthesis of S1P begins with the condensation of serine and palmitoyl CoA. This is catalysed by serine palmitoyltransferase in the endoplasmic reticulum. A series of further enzyme-catalysed steps result in the generation of ceramides of various lengths (Kihara *et al.*, 2007; Tani *et al.*, 2007; Lynch and Macdonald, 2008; Takabe *et al.*, 2008).

Sphingosine kinases 1 and 2 are homologous and consist of five conserved domains (Pyne *et al.*, 2009). They do however display different catalytic properties, subcellular locations, tissue distribution and temporal expression patterns. SK1 prefers D-erythro sphingosine as a substrate, whereas SK2 phosphorylates a wider range of substrates (including the sphingosine analogue FTY720 (Kharel *et al.*, 2005)). Single sphingosine kinase knockout (KO) mice appear largely normal and their blood S1P concentrations are near normal, indicating functional redundancy. Double KO mice do not survive beyond embryonic stage because of multiple developmental defects, notably in the vasculature (Maceyka *et al.*, 2005; Mizugishi *et al.*, 2005; Takabe *et al.*, 2008).

Numerous growth factors, cytokines, hormones, various GPCR ligands and lysophospholipids can induce SK1 activity (Hait *et al.*, 2006; Taha *et al.*, 2006; Takabe *et al.*, 2008). The pro-inflammatory effect of TNF-α on endothelium is absolutely dependent on SK1 activity (Xia *et al.*, 2002; Limaye, 2008). SK1 is phosphorylated (seemingly by extracellular signal-related kinase (ERK) 1/2 (Pitson *et al.*, 2003)) and moves to the plasma membrane on activation (Young *et al.*, 2003; Stahelin *et al.*, 2005). The relative activity of the sphingosine kinases and S1P degrading enzymes is an important determinant of cell fate. Ceramide and sphingosine promote apoptosis, whereas S1P suppresses apoptosis and causes cell proliferation (Olivera *et al.*, 1999; Goetzl *et al.*, 2000; Chalfant and Spiegel, 2005).

1.3.1.2 Degradation

In vivo, the half-life of S1P is short, estimated in mice to be around 15 minutes (Venkataraman *et al.*, 2008). S1P can be cleared either by dephosphorylation, a reversible process, or irreversible splitting of the molecule. Enzymes of the lipid phosphate phosphatase (LPP) family (three isoforms have been described: LPP1-3) dephosphorylate S1P to sphingosine. They have relatively broad specificity, acting on other lysophospholipids including ceramide 1-phosphate. LPP activity can be detected on the cell surface, and it is known that ecto-LPP can regulate signalling of S1P through its surface receptors (Brindley *et al.*, 2002). Sphingosine 1-phosphate phosphatases 1 and 2 (SPPs 1 and 2) perform the same function, but are specific to sphingoid bases. These enzymes are located intracellularly; extracellular substrate accesses the enzyme via adenosine triphosphate-binding cassette (ABC) transporters. Sphingosine is very hydrophobic and can cross the membrane much more easily than S1P; dephosphorylation is an efficient mechanism of clearance of S1P from the extracellular space (Peest *et al.*, 2008; Pyne *et al.*, 2009).

S1P lyase (SPL) degrades its substrate irreversibly to phosphoethanolamine and hexadecanal (Bandhuvula and Saba, 2007). The enzyme is very widely expressed in mice and humans, and is upregulated on inflammation. It functions intracellularly and is resident on the ER membrane with the active site facing the cytosol (Ikeda *et al.*, 2004; Bandhuvula and Saba, 2007). Knockout mice do not survive beyond weaning, with multiple problems observable in the heart, lungs and bone. Disturbances of lipid metabolism and accumulation of very high levels of S1P likely underlie the observed pathology (Bandhuvula and Saba, 2007). S1P lyase expression is absent in platelets (Yatomi *et al.*, 1997) and erythrocytes (Hanel *et al.*, 2007), the latter being the major site of synthesis in the body (Ito *et al.*, 2007). Expression is higher in the thymus and secondary lymphoid tissue. The maintenance of low levels of S1P in those compartments is required for normal lymphocyte recirculation, as demonstrated by inhibition of S1P lyase using 2-acetyl-4 tetrahydroxybutylimidazole (THI) (Schwab *et al.*, 2005).

1.3.1.3 Transport

Most of the S1P in blood is associated with erythrocytes, which act as a reservoir for the compound (Hanel *et al.*, 2007; Bode *et al.*, 2010). In serum, the majority of S1P is associated with lipoproteins (particularly HDL) and albumin. The interaction with such carriers has been shown to influence the action of the ligand (Murata *et al.*, 2000). Binding to serum proteins means that S1P can be carried far from its site of synthesis in blood.

S1P cannot cross lipid bilayers unassisted, due to its amphipathic character. ABC transporters drive active transport. In platelets and astrocytes ABCA7 and 1 (respectively) have a major role (Sato *et al.*, 2007), and in mast cells ABCC1 (Mitra *et al.*, 2006). An ATP-dependent transporter for S1P has been identified in rat erythrocytes (Kobayashi *et al.*, 2009). Erythrocytes isolated from plasma do not release S1P: HDL and serum albumin trigger its export (Bode *et al.*, 2010).

Transmembrane transport of S1P is necessary for binding to cell surface receptors, on the same, neighbouring or distant cells. Chemotaxis to platelet-derived growth factor (PDGF) is dependent on cellular expression of both SK and S1PR1 (Rosenfeldt *et al.*, 2001). PDGF signalling causes a membrane-proximal local increase in S1P production. The S1P is then exported and binds S1PR1 on the extracellular surface to induce migration (Hobson *et al.*, 2001).

1.3.2 S1P Receptors

The receptors for S1P, denoted S1PR1 - 5, are heterotrimeric G protein coupled receptors, with seven transmembrane domains (Rosen and Goetzl, 2005). Sequences within the endothelial differentiation gene (EDG) gene family (that code for the S1PR) have amino acid identities in the range 25-52%. The affinity of S1P for these receptors (dissociation constant (K_d)) is mostly in the single-digit nanomolar range (Rosen *et al.*, 2008). Several S1PR types are expressed on immune cells. S1PR1 is expressed widely, whereas the others are more limited in their distribution. S1PR4 is expressed by T lymphocytes (Wang *et al.*, 2005), S1PR5 by NK cells (Jenne *et al.*, 2009) and S1PR3 by DC (Czeloth *et al.*, 2005) (the latter two receptors play important roles in control of cell migration). Binding of agonist to the receptor can trigger a multitude of signalling pathways, some common to several of these receptors and others unique (Pyne and Pyne, 2000; Speigel and Milstein, 2000; Sanchez and Hla, 2004). The signaling biology of the receptors is complicated by the fact that multiple receptor subtypes are often expressed on the same cell and certain individual receptor subtypes can be split into subpopulations based upon the coupled heterotrimeric G proteins: α_i, α_q or $\alpha_{12/13}$ (Ishii *et al.*, 2004; Rosen *et al.*, 2008). Additionally, S1PR and Lysophosphatidic acid receptors can form homo- and heterodimers (Van Brocklyn *et al.*, 2002; Zaslavsky *et al.*, 2006).

S1PR1-5 are responsible for mediating many of the effects of S1P on cell biology including proliferation, cell migration, invasion, angiogenesis, and vascular maturation, actin cytoskeleton rearrangement and adherens junction assembly (Pyne and Pyne, 2000; Spiegel and Milstein, 2000). S1P can also act intracellularly and independently of S1PR1-5 to enhance cell proliferation and suppress apoptosis (Van Brocklyn *et al.*, 1998; Maceyka *et al.*, 2002). SK2 can physically associate with, and S1P can suppress the activity of, histone deacetylases 1 and 2 (HDACs 1 & 2) in the nucleus (Hait *et al.*, 2009).

1.3.2.1 S1PR1

S1PR1 is expressed by a wide variety of tissues in mice and humans. S1PR1-KO mice die between E12.5 – E14.5 due to severe haemorrhage resulting from a defect in vascular stabilisation (Liu *et al.*, 2000). Conditional gene deletion and experiments using selective receptor agonists show that the receptor has additional roles in angiogenesis, immune cell trafficking, regulation of vascular tone and endothelial barrier function (Garcia *et al.*, 2001; Matloubian *et al.*, 2004; Singleton *et al.*, 2005). It is the most highly expressed S1P receptor on lymphocytes. It seems to be exclusively responsible for mediating the effects of S1P on T cell trafficking, as shown in multiple experiments using S1PR KO mice and selective receptor agonists (Sanna *et al.*, 2004). Natural and functionally similar S1PR1 agonists are also sufficient for the maintenance and enhancement of endothelial integrity (Rosen *et al.*, 2007; Limaye, 2008). S1PR1 signalling can influence activity of members of the Rho GTPase family and therefore cytoskeletal organisation, adherens and tight junction assembly, and focal adhesion (McVerry and Garcia, 2005).

Human S1PR1 is 382 amino acids long and has a molecular weight of 42 kDa. Mutagenesis studies indicate that S1P binds the receptor with the phosphate group on the outer surface (a relatively hydrophilic environment) and the hydrophobic fatty acid chain located between the transmembrane helices (Fujiwara *et al.*, 2007). S1P may need to dissociate entirely from its carrier in order to dock onto the receptor. It might even insert in the outer leaflet of the plasma membrane first, and then bind the receptor in *cis*. Ligand-bound S1PR1 couples to the G protein Gαi alone, with complete inhibition of S1PR1-mediated responses by pertussis toxin (Lee *et al.*, 1996). Agonism of this receptor has been shown, in many different cell types, to cause an increase in MAPK activity, decrease in adenylate cyclase activity (Lee *et al.*, 1998; Okamoto *et al.*, 1998; Zondag *et al.*, 1998), increase in phospholipase C (PLC) activity, increase in Rac activation and increase in PI3K activity (Okamoto *et al.*, 1998; Pyne and Pyne, 2000).

Surface expression of S1PR1 is modulated at the transcriptional and post-transcriptional levels. The most important regulator of gene transcription in T cells is the protein KLF2 (Bai *et al.*, 2007). KLF2 expression is potently downregulated on T cell activation. Plasma-membrane S1PR1 is internalised

into endosomal compartments on receptor activation (Liu *et al.*, 1999). The G protein receptor kinase GRK2, activated by receptor signalling, phosphorylates the C-terminal tail of S1PR1. β-arrestin binds to the phosphorylated motif and facilitates assembly of clathrin-coated pits (Kihara *et al.*, 2007). S1P and phorbol 12-myristate 13-acetate (PMA; which activates protein kinase C) stimulate the phosphorylation of residues on the tail of S1PR1 via distinct mechanisms and are associated with different pathways leading to loss of the receptor from the cell surface (Watterson *et al.*, 2002). S1PR1 can also undergo polyubiquitinylation upon internalisation, the extent of which is dependent on the type and concentration of agonist (Oo *et al.*, 2007). The poorly ubiquitinylated receptor recycles from the late endosomal compartment back to the plasma membrane whereas the highly ubiquitinylated receptor is degraded in lysosomes (Rosen *et al.*, 2008). Lastly, activation of T cells via the TCR induces translocation of S1PR1 from the plasma membrane to the nuclear envelope; there the receptor can effect changes in gene transcription (Goetzl *et al.*, 2007; Liao *et al.*, 2007).

1.3.3 The Vascular Gradient of S1P

1.3.3.1 Compartmentalisation

A vascular gradient of S1P contributes to control of T cell recirculation through the blood, secondary lymphoid tissue and lymphatic vasculature (Figure 2). The amount of S1P in these compartments is determined by the quantity and activity of S1P synthetic and degrading enzymes. Most measurements place the concentration of S1P in human serum in the single unit micromolar range, with some variation in reported values (Hanel *et al.*, 2007; Hla *et al.*, 2008). In serum, it is mostly bound to carriers such as high-density lipoprotein and albumin (Murata *et al.*, 2000). Erythrocytes express sphingosine kinases, lack S1P lyase, and are the primary source of S1P in blood (Pappu *et al.*, 2007). They can buffer its concentration there (Hanel *et al.*, 2007), and also pass S1P to other cell types including endothelial cells (Bode *et al.*, 2010). The vascular endothelium also contributes to S1P in blood. Shear flow stimulates increased synthesis and secretion of S1P by vascular endothelial cells (Pappu *et al.*, 2007; Venkataraman *et al.*, 2008).

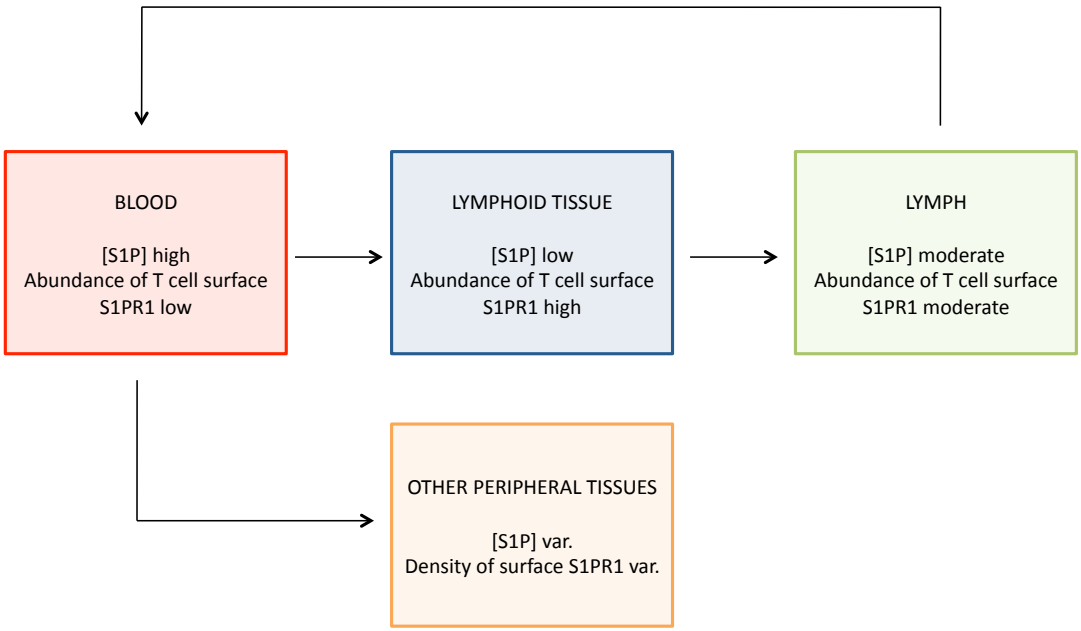


Figure 2. The compartmentalisation of S1P in the lymphatic and vascular systems. The concentration of S1P in blood is in the single unit micromolar range. Chronic stimulation with ligand results in internalisation of S1PR1 from the surface of T cells located in that compartment. High levels of S1P lyase activity underpin a very low concentration of S1P in lymphoid tissues under normal conditions. S1PR1 can recycle back to the surface of resting T cells that have recently ingressed. S1P, originating from lymph, is sampled by T cells located at exit structures in lymphatic tissue, and is required for their efficient egress. Under normal conditions the concentration of S1P in peripheral tissues is low, but it can increase due to upregulation of synthetic enzyme activity during inflammation.

Lymph contains approximately one quarter the level of S1P in blood (Schwab *et al.*, 2005; Pappu *et al.*, 2007). Lymphatic endothelial cells are the source of S1P in lymph; expression of the enzyme by lymphatic endothelium is required for normal lymphocyte egress from lymph nodes (LNs) and Peyer's patches (Pham *et al.*, 2010). Neural crest-derived perivascular cells provide the S1P required for egress of T cells from the thymus (Zachariah and Cyster, 2010).

S1P is present at very low levels in primary and secondary lymphoid tissue, and indeed many peripheral tissues under normal conditions. The amounts of S1P in the thymus, lymph nodes and spleen of mice are approximately 20, 40 and 150 ng per g of tissue respectively (Edsall and Spiegel, 1999). There is a high level of S1P lyase activity in non-inflamed lymphoid tissues, and this is required for normal lymphocyte recirculation (as shown by inhibition of the enzyme (Schwab *et al.*, 2005)).

1.3.3.2 S1P Signalling in Homeostatic Lymphocyte Trafficking

S1PR1 becomes expressed as thymocytes undergo their final stages of differentiation. This confers upon them a capacity to egress into the high S1P environment of the blood. The egress of resting naïve T cells from lymph nodes is similarly dependent upon signalling of S1PR1. However, in the case of recirculating T cells, surface expression of the receptor is cyclically modulated by homologous desensitisation, rather than by transcription.

The high concentration of S1P in blood means that any S1PR1 present on the T cell surface is quickly internalised to endosomes, with a consequent loss in sensitivity to that ligand. On entering secondary lymphocyte tissue, where the concentration of S1P is kept low, S1PR1 that is currently sequestered recycles back onto the T cell surface (Lo *et al.*, 2005). This allows lymph node -resident lymphocytes to sample S1P at exit structures (primarily the cortical sinuses). Intravital imaging of LN egress structures shows cortical sinus probing by T cells. Local S1PR1 signalling in the probing pseudopod probably stabilises it and repolarises the cell for egress (Grigorova *et al.*, 2009). S1PR1 signalling antagonises that mediated by CCR7, which promotes retention of T cells within the node, thus allowing the cells to leave (Dustin and Chakraborty, 2008; Pham *et al.*, 2008). The requirement for S1PR1 for T cell egress is supported by multiple experiments. For example, agonist–induced downmodulation of S1PR1 on lymphocytes results in their retention in lymphoid tissue, presumably as it leaves them unable to transduce egress signals and conditional S1PR1 knockout T cells cannot egress lymph nodes (Matloubian *et al.*, 2004).

Injection of labelled T cells into a mouse shows that they are capable of moving from HEV entry points into cortical sinuses in less than 30 minutes, indicating rapid re-acquisition of sensitivity to S1P. These cells (a fraction of all cells that enter the node) probably move directly between closely apposed entry and exit structures, rather than migrating through the parenchyma that would necessarily extend their lymph node residence time (Grigorova *et al.*, 2010). Unactivated T cells typically transit a murine lymph node in 6-10 hours (Tomura *et al.*, 2008).

The lymphocyte-extrinsic model of T cell egress postulates an important role for S1PR1 on endothelial cells. Agonism of S1PR1 on endothelial cells enhances cell-cell contacts and thus potentially reduces the ability of lymphocytes to traverse the egress barrier (Wei *et al.*, 2005). This seems to be mediated

through S1P enhancement of the interaction between focal adhesion and adherens junction proteins (Sun *et al.*, 2009). S1PR1 antagonists promote, rather than block, T cell egress in vivo. This would be consistent with a predicted reduction in lymphatic endothelial barrier integrity at lymph node exit sites and a higher rate of T cell egress (Sanna *et al.*, 2006).

1.3.3.3 S1P Signalling in Inflammation

1.3.3.3.1 Within Secondary Lymphoid Tissue

Inflammation in peripheral tissues is associated with production of pro-inflammatory cytokines such as TNF-α and IL-6, and the maturation of antigen-presenting cells that have been activated through danger receptors (Janeway and Medzhitov, 2002). These components drain via the afferent lymphatics to the local lymphatic tissue and activate the node for the initiation of an adaptive response. There is often a transient block in lymphocyte egress from an activated node called lymph node shutdown. TNF-α, IFN-γ and interferon-alpha (IFN-α) have all been implicated as causal factors in this process (Young, 1999; Seabrook *et al.*, 2005), and it is probable that they act in concert. An important role for IFN-α is clearly demonstrated by the fact that IFNAR1-null lymphocytes are less efficiently sequestered in activated lymph nodes than wild-type cells. It has now been shown that IFN-α signalling triggers a complex cascade of events that regulate egress signal transduction by S1PR1 (Shiow *et al.*, 2006).

CD69 is a type II transmembrane protein and C-type lectin of 23 kDa (Lopez-Cabrera *et al.*, 1993; Testi *et al.*, 1994). It is an early activation marker of a number of immune cell types, notably T cells. Several stimuli cause the upregulation of CD69 on the surface of T cells; treatment of resting T cells with IFN-α/β will cause a significant amount of protein to appear on the cell surface within 1 hour (Shiow *et al.*, 2006). CD69 protein is present in the cytoplasm of resting T cells (Risso *et al.*, 1991), and this pool can be rapidly trafficked to the plasma membrane. Various stimuli, such as stimulation through the TCR, will also dramatically upregulate transcription and *de novo* protein synthesis (Ziegler *et al.*, 1994).

CD69 physically associates with S1PR1 on the cell surface. Domain-swapping experiments show that the intracellular membrane proximal domain and transmembrane domain of CD69 are necessary for this interaction. Mutagenesis studies show that S1PR1 membrane helix 4 is crucial (Bankovich *et al.*, 2010). Importantly, the interaction of these two proteins is associated with a loss in the capacity for S1PR1 to signal (Shiow *et al.*, 2006). The half-life of the ligand-bound CD69 - S1PR1 complex is significantly longer than that of ligand bound S1PR1 alone, and CD69 overexpression is associated with a

greater rate of degradation of S1PR1. So, protein-protein interaction might induce a conformational change in S1PR1 more akin to its ligand-bound state, thus enhancing desensitisation of the receptor (Bankovich *et al.*, 2010). The net effect is that T cells expressing significant levels of CD69 cannot respond to S1P at lymph node exit structures and egress into the cortical sinuses. In animals stimulated with polyinosinic:polycytidylic acid (poly I:C), which stimulates IFN-α production and results in strong induction of CD69 on lymph node resident T cells, cortical sinusoids appear collapsed under intravital microscopy (Grigorova *et al.*, 2010). This is consistent with a block in access of T cells to these structures.

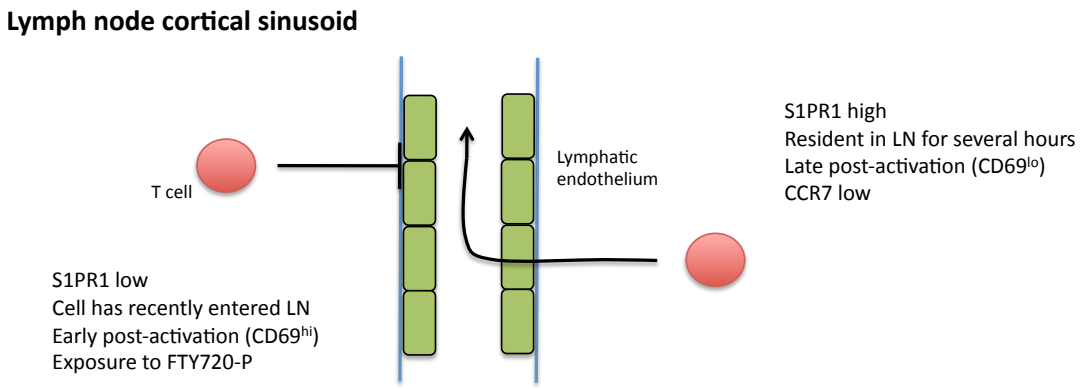


Figure 3. Regulation of T cell egress from the lymph node cortex.
In homeostatic conditions, resting T cells that have recently entered a lymph node have low levels of S1PR1 on the cell surface. Internalised receptor moves back to the cell surface in the low S1P environment of an uninflamed lymph node (this can be blocked by administration of the S1PR1 superagonist FTY720-phosphate (FTY720-P), which downregulates the receptor). Re-acquisition of S1PR1 surface expression allows T cells to receive exit signals by sampling S1P inside cortical sinusoids. In conditions of inflammation, innate immune cytokines (such as interferon-alpha/beta) trigger upregulation of CD69 on the surface of lymph node-resident T cells. CD69 interacts with S1PR1 protein, preventing transduction of signals required for cell egress. Loss of CD69 expression by T cells relieves this inhibition, allowing the cells to leave.

Innate cytokines therefore play a crucial role in the generation of a lymph node environment best suited for lymphocyte retention and antigen scanning. In the event of a rare productive interaction between a T cell and a professional antigen-presenting cell, S1PR1 transcription is reduced as KLF2 is ubiquitinated and degraded (Carlson *et al.*, 2006). The specific lymphocyte clone thus remains in an environment optimised for proliferation and control of differentiation of daughter cells. With KLF2 expression restored in differentiated cells, S1PR1 re-appears on the cell surface. Both antigen-specific effector and

memory T cells therefore acquire the ability to leave the lymph node (Matloubian *et al.*, 2004).

In addition to its role in regulation of T lymphocyte trafficking, S1P can also act alongside other mediators to influence T cell differentiation and B cell responses within lymphoid tissue. In particular, there is some evidence for a role for S1P in the generation of regulatory T cells. In an *in vitro* model, S1P acted similarly to IL-23 in its ability to promote CD3/CD28-stimulated $CD4^+$ T cells incubated with Transforming growth factor–beta (TGF-β), IL-1 and IL-6 to differentiate into Th17 cells (Liao *et al.*, 2007). Constitutive S1PR1 signaling in B cells seems to promote B cell localisation in the marginal zone of spleen, in opposition to the effect of the follicular-homing chemokine CXCL13 (Cinamon *et al.*, 2004; Cinamon *et al.*, 2008). The effect is continual B cell shuttling of antigen from the marginal zone to follicles, perhaps a mechanism to transport antigen from the blood to follicular dendritic cells. This is an essential requirement for the generation of T-dependent B cell responses. The essential role for S1P in high affinity B cell mediated immunity is further demonstrated by the fact that S1PR1 on immunoglobulin G (IgG)-secreting plasma cells is required for their egress from the spleen into the blood, and ultimately residence in the bone marrow (Kabashima *et al.*, 2006).

1.3.3.3.2 In Peripheral Tissues

The amount of S1P is low in uninflamed peripheral tissues, but increases during acute inflammation (Graler *et al.*, 2005). TNF-α, VEGF and histamine open paracellular gaps between vascular endothelial cells, disrupting junctions and focal adhesion complexes (Wang and Dudek, 2009). S1P, bound primarily to high-density lipoproteins and albumin in blood, can enter the tissue and bind S1P receptors on tissue-infiltrating leukocytes and other cells. Inflammatory cytokines such as TNF-α upregulate sphingosine kinase 1 in multiple cell types (Taha *et al.*, 2006) and therefore raise the intra- and extracellular levels of S1P at the inflamed site. Furthermore, platelets release S1P when activated by thrombin, and mast cells when triggered by FcεR cross-linking (Jolly *et al.*, 2002; Jolly *et al.*, 2004).

The increased level of S1P in inflamed tissue effects both innate and adaptive cells. For example, stimulation of S1PR1 and 2 on mast cells induce cell chemotaxis and degranulation respectively (Olivera *et al.*, 2007). S1P significantly enhances the endocytic capacity of mature DC (Maeda *et al.*, 2007), and can promote their chemotaxis (Czeloth *et al.*, 2005). Tissue S1P might also act later in the immune response, when antigen-specific T cells have trafficked to the inflamed site. Agonism of S1PR1 inhibits movement of T cells across lymphatic endothelium. This is dependent on the interaction of LFA-1 and VLA-4 on T cells with their ligands ICAM-1 and VCAM-1 on the basal surface of the endothelium (Ledgerwood *et al.*, 2008). In this way, S1P would act to restrain exit of effector T cells from the inflamed site and thereby enhance protective immunity.

1.4 Signalling Cross-talk

1.4.1 Between S1P and Chemokine Receptors

Both S1P receptors and chemokine receptors belong to the G protein-coupled receptor superfamily. In many cases they utilise shared G protein-coupled signalling pathways, for example both S1PR1 and many chemokine receptors (such as CXCR4) couple to the Gαi G protein and activate PI3K through the Gβγ subunit (Cyster, 2005). If the receptors are activated either simultaneously, or sufficiently close in time, crosstalk between the two signalling axes might be expected.

It has already been explained that crosstalk between the CCR7 and S1PR1 signalling pathways determines T cell egress decision-making at lymph node exit structures. Resting naïve and differentiated effector T cells express both S1PR1 and CCR7. S1PR1 (which promotes cell egress) is ligated by S1P accessed by pseudopodia that probe the inside of cortical sinusoids. CCL19 and CCL21, the ligands of CCR7, are produced by stromal cells within the node and promote cell retention. CCR7 and S1PR1 provide opposing chemotactic cues and the signals are integrated to determine cell behaviour. Consistent with this model, downmodulation of surface S1PR1 allowed CCR7-deficient T cells to egress more efficiently than wild-type T cells, and treatment with pertussin toxin to inactivate Gαi-dependent signalling of CCR7 restored egress of S1PR1-deficient T cells (Pham *et al.*, 2008).

S1P can modulate T cell chemotaxis to other chemokines. The *in vitro* transendothelial migration of splenic T cells towards CXCL12 was enhanced by S1P, and peripheral lymph node -derived cells required S1PR signalling to migrate in response to the chemokine (Yopp *et al.*, 2005). Others have demonstrated a dose-dependent effect of S1P on responses to chemokines. Pre-treatment of $CD4^+$ T cells with 10-100 nM S1P resulted in increased chemotaxis towards CCL5 and CCL21, whereas pre-treatment with 300 nM to 3 μM S1P strongly suppressed migration (Graeler *et al.*, 2002).

Cross-talk between S1PR and chemokine receptors has also been observed in non-lymphocyte immune cell subsets. S1PR1 overexpression in haematopoietic stem cells caused a reduction in surface CXCR4 and potent inhibition of ligand-induced chemotaxis and signalling responses (Ryser *et al.*, 2008). Others have

shown that S1P receptor signalling can support CXCR4-dependent migration of endothelial progenitor cells (Walter *et al.*, 2007) and haematopoietic stem cells (Whetton *et al.*, 2003). S1P reduced neutrophil chemotaxis in response to CXCL8 or fMLP (Kawa *et al.*, 1997). Interestingly, S1PR3 was found to heterodimerise with CXCR1, the receptor for CXCL8 (Rahaman *et al.*, 2006).

1.4.2 Involving Non- G protein-coupled Receptors

Crosstalk can occur between pathways of completely different types, for example between GPCR and receptor tyrosine kinases. In lymphocytes, interaction between chemokine or S1P signalling and that of the TCR, inflammatory cytokines, and growth factors has been described.

The chemokine receptor CXCR4 shows cooperative interaction with the T cell receptor. Chemokine binding to CXCR4 resulted in physical association with the TCR, in a PI3K -dependent manner. This allows CXCR4 to utilise the ITAM chains of the CD3 complex to signal, in addition to the coupled G proteins. Simultaneous triggering of the TCR and chemokine receptor resulted in synergistic enhancement of activator protein-1 (AP-1) transcriptional activity, CD69 expression, and IL-10 and IL-12 secretion (Nanki and Lipsky, 2000; Kumar *et al.*, 2006).

TNF-α, an important inflammatory cytokine, mediates many of its effects in an S1P-dependent manner. S1P is a co-factor for TRAF2 and is crucial for Tumour necrosis factor receptor (TNFR) coupling to NF-κB activation (Alvarez *et al.*, 2010). TNF-α -mediated activation of sphingosine kinase is also important for protection of endothelial cells from apoptosis (Xia *et al.*, 1999). S1PR signalling is absolutely required for PDGF-induced chemotaxis. PDGF (and similarly VEGF) trigger the activation and translocation of SK1 to the plasma membrane, and inside out signalling of S1P through S1PR1 (Hobson *et al.*, 2001; Rosenfeldt *et al.*, 2001). TGF-β and S1PR signalling are co-dependent in some cell types, where both are expressed (Xin *et al.*, 2004). In human keratinocytes, Smads 2 and 3 are phosphorylated in response to S1P or TGF-β stimulation, and this response is absolutely dependent on the presence of receptors for both compounds (Sauer *et al.*, 2004). Similarly, the presence of TGFβRII and S1PR3 is necessary for FTY720-induced Smad phosphorylation in renal mesangial cells (Xin *et al.*, 2006). It has been speculated that a S1PR3 - TGFβRII complex might form on the cell surface.

1.5 Aims of this Study

The overall aim of this investigation was to further our knowledge of the ways in which S1P regulates T cell migration. Existing work points to two main areas of interest. First, there is some evidence to indicate that S1P can modulate T cell responses to chemokines. This is however limited and mechanistic data is lacking. The second aspect concerns the regulation of T cell egress from lymph nodes. While it is known that S1P provides a necessary signal for cell exit under non-inflamed conditions, the situation after T cell activation is less well understood. In particular, there are outstanding questions over the dynamics of S1P signalling after cell activation, and the extent to which activated T cells show the same dependence as resting cells on S1P for exit from lymph nodes.

The objectives of this study were:

- To see whether S1P can affect the migration of T cells towards a prototypical homeostatic chemokine
- To investigate the mechanism behind the regulation of this chemokine response by S1P
- To describe the changes in S1PR1 signalling that occur after stimulation of T cells through their antigen-specific receptor
- To identify the way in which S1PR1 signalling is controlled during T cell activation
- To assess *in vivo* the relevance of modulation of S1P signalling in activated T cells

Chapter 2. General Materials and Methods

2.1 General Laboratory Procedure

All work was carried out with Good Laboratory Practice and in a manner compliant with the Newcastle University Safety policy. Prior to use of laboratory reagents, appropriate COSHH and BIOCOSHH forms were read, understood and signed.

Qualified staff provided training for the purpose of ensuring safe and efficient use of equipment and reagents. In particular, work with the Gamma Cell Irradiator was undertaken in compliance with the University Local Rules regarding radiation protection; these included and expanded upon relevant legal requirements.

2.2 Tissue Culture

2.2.1 Cells

2.2.1.1 Cell Lines

All of the cell lines used in this study were grown from stocks that had been stored in liquid nitrogen. They had been previously screened for the presence of mycoplasma (MycoAlert Mycoplasma Detection Kit, Lonza, Switzerland), and shown to be negative.

Cells were recovered from cryostocks as follows. Cryovials were removed from the liquid nitrogen store and their contents thawed rapidly in a 37 °C waterbath. The cells were washed in an excess of phosphate-buffered saline (PBS; Sigma-Aldrich, USA) then resuspended at an appropriate density in relevant medium. They were cultured for at least one week before use in experiments.

2.2.1.1.1 Jurkat T Cells

The Jurkat T cell line (ATCC TIB-152) was established from peripheral blood T cells taken from a 14 year-old human male suffering from acute T cell leukaemia. The cells have a lymphoblast morphology and grow in suspension, with a doubling time of approximately 48 hours. They are pseudodiploid, with a modal chromosome number of 46.

2.2.1.1.2 Molt 4 T Cells

The Molt 4 T cell line (ATCC CRL-1582) was derived from cells taken from a 19 year-old human male suffering a relapse of acute lymphoblastic leukaemia. The cells have a lymphoblast morphology and grow in suspension, with a doubling time of approximately 24 hours. They are hypertetraploid, with a modal chromosome number of 95.

2.2.1.2 Primary T Cells

Human peripheral blood and its derivatives were used in this study. Trained persons performed phlebotomy, and informed consent was gained in each instance of donation by healthy volunteers. Peripheral blood samples (volumes up to 20 ml) were dispensed into plastic tubes and immediately mixed with 100 U/ml heparin (Sigma-Aldrich, USA) to inhibit coagulation.

Leukocyte apheresis cones were procured from the Newcastle-upon-Tyne Donor Centre of the NHS Blood and Transplant Service. Briefly, these were prepared by the NHSBT as follows. Peripheral blood was separated into fractions of plasma, platelets, leukocytes and erythrocytes by centrifugation. Most of the platelets were retained for clinical use, and many of the red cells and leukocytes returned to the donor. The leukocytes that remained in the centrifugation cone were made available for research. Only cones prepared on the same day were used.

T cells were isolated from either fresh peripheral blood samples or leukocyte apheresis cones. RosetteSep human T cell negative selection cocktail (StemCell Technologies, France) was mixed with peripheral blood (50 μl per ml respectively) or apheresis product (75 μl per ml) and left for 20 minutes at room temperature. Blood or apheresis product (now diluted one part to three parts PBS + 2 % foetal bovine serum (FBS; Lonza, Switzerland)) was layered at room temperature over Lympholyte-H (Cedarlane Laboratories Ltd, Canada), and then centrifuged at 800 g for 20 minutes. The T cells at the density-medium – plasma interface were transferred to a separate tube and washed twice with PBS + 2 % FBS before use.

2.2.2 Culture Method

Cell culture was performed with sterile technique inside Class II Microbiological Safety cabinets.

2.2.2.1 Cell Counting

The density of cells in suspension was determined using a phase haemocytometer (Fisher Scientific, UK). 20 μl of cell suspension was applied to the slide underneath a coverslip. The number of cells within the 5 x 5 grid was counted using an inverted microscope at 200x magnification. This was multiplied by $1x10^4$ to give an estimate of the number of cells per ml of suspension.

Viable cell counts were determined using the Trypan Blue-exclusion method. Equal volumes of cell suspension and 0.4 % Trypan Blue solution (Sigma-Aldrich, USA) were mixed thoroughly, and the non-blue cells enumerated using the haemocytometer. The cell density was calculated as before, accounting for the additional 2x dilution step.

2.2.2.2 Cell Maintenance

Cells were cultured at 37 °C in a humidified atmosphere adjusted to contain 5 % CO_2. They were grown in plastic tissue culture plates of 6, 24 or 96 well format, or cultured in vented 25 cm^2 or 75 cm^2 flasks (Greiner Bio-one, Germany).

Both the Jurkat T cell and Molt 4 T cell lines were grown in complete RPMI 1640 medium. This comprised RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 2 mM L-Glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all Sigma-Aldrich) and 10 % FBS (Lonza, Switzerland). Serum-free RPMI 1640 medium was comprised as complete medium with the exception that the FBS was replaced with 0.5 % fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich). Cells that had been previously cultured in serum-containing medium were washed once in PBS before resuspension in serum-free conditions. Primary human T cells were cultured in X-VIVO 15 medium (Lonza, Switzerland). This was a chemically-defined serum-free medium that can support the survival of resting T cells and the proliferation of activated cells. The manufacturer confirmed that the medium did not contain sphingosine 1-phosphate, sphingosine or lysophosphatidic acid.

Jurkat T cells and Molt 4 T cells were maintained during routine culture at between $1x10^5$ and $1x10^6$ cells per ml. The cells were placed into fresh medium at least every 3 days. Resting human T cells were kept at a density of between $1x10^6$ and $1x10^7$ per ml in X-VIVO 15 medium. They were adjusted to a density of $1x10^6$ cells per ml before activation.

2.3 Reconstitution of S1P Receptor Ligands

2.3.1 S1P

D-*erythro*-sphingosine 1-phosphate (Sigma-Aldrich) was dissolved in methanol at 1 mg/ml by heating to 65 ºC until the solution was clear and colourless. The S1P was divided into 0.1 mg aliquots in glass vials then dried with a stream of nitrogen gas for long-term storage at -80 ºC.

Before *in vitro* use the dried S1P was dissolved in 0.01 M NaOH at 1 mg/ml by heating to 80 ºC until the solution was clear and colourless. The S1P was then diluted into PBS + 0.5 % fatty acid-free BSA (Sigma-Aldrich) to a concentration of 125 µM S1P then aliquotted and used immediately or stored at -20 ºC for up to one month.

2.3.2 SEW2871

SEW2871 (Sigma-Aldrich) was dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich) at a concentration of 2 mg/ml (≡ 4.54 mM). DMSO was added to the solid compound, and the product vial sealed. It was heated to 37 ºC in a waterbath and repeatedly vortexed until the solution was clear and colourless. The solution was then aliquotted into glass vials and stored at -20 ºC in the dark (for up to three months).

Before use, the solution was brought to 37 ºC in a waterbath and vortexed to ensure that the SEW2871 was completely dissolved. The ligand was further diluted in RPMI + 0.5 % fatty acid-free BSA, unless otherwise specified.

2.4 Migration Assays

2.4.1 General Principle

Cell migration can be measured using a two-chamber assay, similar to that developed by Boyden (Boyden, 1962). Bare transwell permeable supports (Corning Life Sciences, polycarbonate-type; Sigma-Aldrich, USA) are placed into tissue culture plates, splitting each well into two compartments separated by a permeable membrane. The insert membrane is about 10 μm thick and covered densely with pores. Whereas chemoattractants and similar factors can easily diffuse across the membrane, cells may or may not be able to cross, depending upon the pore size selected.

Cells suspended in medium are placed into the upper compartment, and medium with or without added factors into the lower compartment. The plate is left at 37 °C in an atmosphere of 5 % CO_2 for a specified length of time (typically two to three hours). The number of cells that have entered the lower compartment after that time is determined, providing a measure of their migratory response. By placement of chemoattractant in the lower compartment alone, directional chemotactic responses up a gradient of chemokine can be studied. Alternatively, by placement of a specific stimulus in both compartments, the chemokinetic, non-directional effect on cell motility can be examined.

2.4.2 Specific Experimental Parameters

Before each experiment the bottom and sides of each lower compartment were blocked for 1 hour with PBS + 1 % BSA.

Lymphocyte and lymphocyte cell lines migrated across bare membranes. Resting primary human T cells migrated through membranes with 3 μm diameter pores, while the Jurkat and Molt 4 T cell lines, and primary lymphoblasts, migrated through 5 μm pores. All migration assays were performed in a base medium of RPMI 1640 + L-Glutamine + Penicillin + Streptomycin + 0.5 % fatty acid-free bovine serum albumin. Both 24 well format and 96 well format transwell systems were used. The total volumes of medium added to the upper and lower compartments, for the 24 and 96 well format inserts, were: 100 and 600 µl, and 75 and 235 µl respectively.

Migration was measured after 2 hours incubation at 37 °C, 5 % CO_2, unless otherwise stated. The inserts were removed and cells extracted from the lower compartments by pipetting of 500 μl or 200 μl (24 well format or 96 well format chambers respectively) of cell suspension from each compartment into separate tubes. A defined number of CountBright Absolute Counting Beads (Invitrogen, UK) was added to each tube, and absolute cell numbers determined using a flow cytometer.

2.5 Immunofluorescent Techniques

Fluorescence is the phenomenon in which a substance absorbs light at a particular wavelength and then rapidly emits light at a slightly longer wavelength. Fluorochromes are functional groups that are fluorescent; they can be linked to molecules and larger structures in order to make them detectable with instruments configured to detect fluorescence.

The immunofluorescent technique involves labelling a cell sample with fluorochrome-conjugated antibodies so that the quantity of fluorescence associated with a particular cell can be correlated to the abundance of a specific antigen. Intact cells can simply be incubated with antibodies to label surface antigens, or they can be permeabilised to allow access of antibodies to intracellular targets.

2.5.1 Flow Cytometry

The flow cytometer measures the optical properties of single cells as they pass through the functional core of the instrument. Allied with immunofluorescent staining, it can be used to measure the expression level of multiple surface and intracellular proteins by individual cells in a population. Proliferation of cells can be measured over time by labelling the starting population with a fluorescent dye (such as carboxyfluorescein succinimidyl ester (CFSE)).

The input to the instrument is a single-cell suspension placed under pressure and focussed into a stream inside the flow cell. Laser light, focussed on each cell as it passes through the compartment, is scattered or absorbed by the sample. This light is detected by very sensitive components called photomultiplier tubes (PMT). One PMT lies in line with the laser light, and its output yields a forward-scatter (FSC) value; another is located perpendicular to the light source and yields a value for side-scatter (SSC). These measurements give some indication of the gross properties of individual cells; FSC and SSC values are loosely positively correlated with size and granularity respectively. The flow cytometer usually contains additional lasers that excite fluorochromes or fluorescent molecules inside or tagged onto the surface of cells in the sample. Photomultiplier tubes, situated perpendicular to the excitation lasers, detect the fluorescently-emitted light. These detectors are shielded with optical band-pass filters that permit the transmission of light of only a narrow range of

wavelengths. Each PMT is therefore capable of detecting light emitted from one or a few different fluorescent molecules. Table 1 details the fluorochromes used in this study and the corresponding flow cytometer settings.

Wavelength of excitation laser light (nm)	Fluorescent species	Band-pass filter characteristics – mid-point and width of transmission interval (nm)
488	FITC	530 / 30
	CFSE	530 / 30
	RPE	585 / 40
633	APC	660 / 20

Table 1. Fluorochromes and associated flow cytometer settings used in this investigation. Antibodies tagged with three different fluorochromes were used in this study. In some experiments, cells were labelled with the dye CFSE so that their division could be followed. This table shows the wavelengths of the excitation lasers, and the characteristics of the band-pass filters, that were used with respect to each fluorescent species.

2.5.1.1 Compensation

After excitation, fluorochromes and fluorescent dyes emit light over a range of wavelengths. The exact profile of the relative quantity of light emitted at different wavelengths by a particular fluorescent molecule is known as its emission spectrum.

Multiple dyes and fluorochromes have overlapping emission spectra, and can be picked up by a single detector. In situations where multicolour labelling is used, it is necessary to subtract the contribution of emission of one fluorescent species to the signal in detectors other than the primary detector. This ensures that the size of the signal from any one detector corresponds only to the quantity of light emitted from one particular fluorochrome or dye. Compensation beads, labelled with each of the fluorochromes used in any individual experiment, are now typically used to facilitate this compensation process.

The problem described can be avoided entirely by using fluorochromes and dyes with emission spectra that do not overlap. In this study, for example, cells dyed with CFSE were also labelled with a CD69-specific antibody conjugated to APC. These fluorescent molecules have emission maxima of approximately 515 nm and 660 nm respectively, and there is negligible spectral overlap.

2.5.1.2 Instrument and Software

A FACSCanto II flow cytometer (BD Biosciences) was used for data acquisition. The output data was analysed with either FlowJo 7.6 (Treestar, USA) or FACSDiva 6.1.3 software (BD Biosciences, USA).

2.5.1.3 Method of Labelling Surface Proteins

Typically, between 100 000 and 500 000 cells were stained in individual 12 x 75 mm plastic test tubes. All steps were performed with ice-cold buffers and cells were kept on ice during the staining process and before data acquisition. Unless otherwise specified, the dot plots and histograms in this thesis represent at least 10 000 events.

After treatment or culture, cells were washed twice with PBS + 1 % BSA by sequential resuspension in 2 ml buffer and centrifugation at 500 g for 5 minutes. Cells were stained with primary antibody for between 20 minutes and 1 hour on ice. In some cases, cells were then stained with a secondary antibody (for coupling to a fluorochrome) for 1 hour on ice. Cells were washed three times with PBS + 1 % BSA then resuspended usually in the same buffer, in a volume of between 200 μl and 400 μl. Unless otherwise stated, samples were stained with an antigen-specific antibody and separately with a species and isotype-matched antibody, coupled to the same fluorochrome, as a control.

2.5.2 Confocal Microscopy

The confocal microscope can be used to generate detailed three-dimensional images of microscopic structures. Used in conjunction with immunofluorescent labelling, it is a powerful tool for measuring the expression level and determining the localisation of specific proteins within or on the surface of individual cells.

The microscope functions as follows. Laser light is focussed onto a small region (the focal volume) within the sample. Light reflected from, or emitted by (in the case of fluorescence), that precise region is allowed to reach the detector (usually a photomultiplier tube). Light originating from outside that particular volume is blocked. In the case of fluorescence microscopy, band-pass filters are placed in front of detectors so that only fluorescently-emitted light is received. The signal from a detector is used to generate a single image unit – a pixel – corresponding to the original focal volume. So, with multicolour labelling and appropriate detectors, many units of information can be gathered from an individual focal volume. By scanning across (x and y axes) and through (z axis) a specimen, and acquiring each pixel of information, an image can be assembled.

2.5.2.1 Instrument and Software

Data was captured with a TCS SP II AOBS UV confocal microscope (Leica Microsystems, Germany). Samples were illuminated with a 488 nm argon laser for excitation of the FITC fluorochrome, and with a 405 nm diode laser for the DNA stain 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Analysis was performed with LCS software (also Leica Microsystems).

2.5.2.2 Method of Immunolabelling

Cytospins were prepared for staining as follows. Suspension cells were washed with cold PBS + 1 % BSA. 50 – 100 000 cells were cytospun at 1000 rpm for 3 minutes (Shandon Cytospin 3, Thermo Scientific, USA) onto charged glass slides (Superfrost Plus, VWR) and left to air-dry. The cells were fixed in methanol at -20 ºC for 10 minutes and air-dried. The section was circumscribed with a hydrophobic barrier pen (Delimiting Pen, Dako, Denmark).

The cells were blocked for 1 hour with PBS + 1 % BSA. They were stained overnight, in a humidified box at 4 ºC, with primary antibody diluted into PBS + 1 % BSA. The cells were washed twice for 5 minutes with PBS then stained with a fluorescently-conjugated secondary antibody (diluted into PBS + 1 % BSA) for three hours. The cells were washed three times for 5 minutes with PBS. The sections were counterstained with 1 μM DAPI for 2 minutes and washed once more with PBS. They were then mounted with fluorescent mounting medium (Dako) and coverslip. Slides were stored in the dark at 4 °C and examined within two weeks with the confocal microscope.

2.6 Western Blotting

2.6.1 Preparation of Whole Cell Lysates

Cells were treated as described then washed in cold PBS and lysed with a buffer containing protease and phosphatase inhibitors (CelLytic M (Sigma Aldrich, USA)), Complete Protease Inhibitor Cocktail (Roche), Halt Phosphatase Inhibitor Cocktail (Pierce, Thermo Scientific, USA). As a guide, approximately 30 μl buffer was used per million cells to be lysed.

2.6.2 Protein Quantification

Protein concentrations were determined using a bicinchoninic Acid (BCA) assay kit (Pierce, Thermo Scientific, USA). This is based upon the Biuret test, in which peptide bonds reduce Cu^{2+} ions to Cu^{1+} ions in an alkaline solution. Two molecules of BCA then chelate each Cu^{1+} ion, forming a purple reaction product. A spectrophotometer can therefore be used to correlate the intensity of absorbance of light at a specific wavelength (562 nm) and the abundance of peptide bonds in a sample of defined volume.

Equal volumes of unknown samples were assayed in duplicate along with the standards (typically 0, 125, 250, 500, 1000, 2000 and 4000 μg/ml BSA) for each experiment. 10 μl of samples and standards were mixed with BCA working reagent in a 96 well plate. After 20 minutes incubation at room temperature absorbances were measured using a spectrophotometer. A linear regression was performed with the standard data, and sample concentrations estimated by interpolation. A representative standard curve is shown in Figure 4.

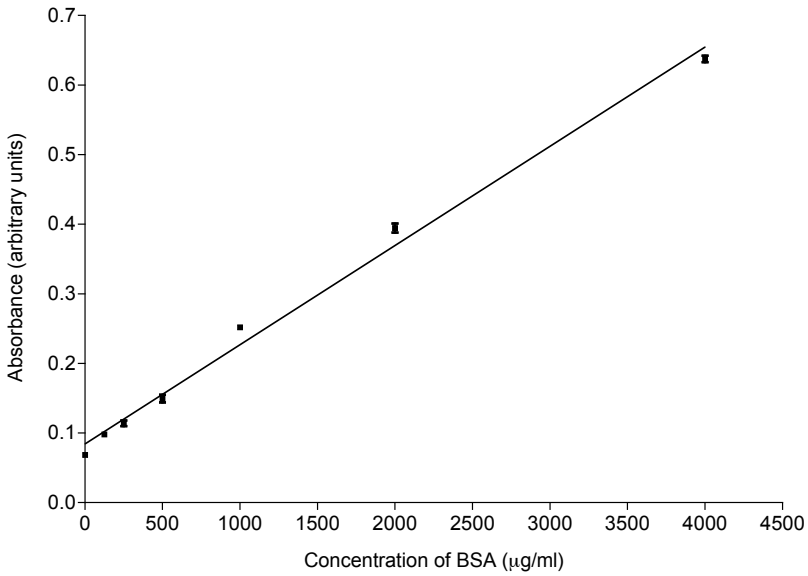


Figure 4. BCA standard curve.
Bovine serum albumin was dissolved in PBS at 125, 250, 500, 1000, 2000 and 4000 μg/ml. 10 μl of each standard and PBS alone were assayed in duplicate. The absorbances at each concentration have been plotted and a best-fit line (least-squares algorithm) is shown. Points are means ± s.e.m.

2.6.3 SDS-PAGE

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) is a process in which proteins contained within a sample are separated according to their size as they move through a small pore matrix of polyacrylamide. This concept is based on the technique described by Laemmli (Laemmli, 1970). The sample is first denatured with a reducing agent in the presence of the anionic detergent SDS. The reducing agent breaks disulphide bonds holding together the higher-order structure of the proteins, and the polypeptide chain becomes associated with SDS molecules. The number of bound SDS molecules is proportional to the length of the polypeptide chain, therefore the magnitude of the electrical charge is approximately proportional to protein mass. By applying an electric field across the polyacrylamide gel, proteins are induced to migrate from the sample well through the gel towards the anode. Smaller proteins move relatively faster than larger proteins, leading to separation of the individual protein components.

2.6.3.1 Specific Experimental Parameters

Generally, between 10 and 40 μg protein were loaded per lane of a 0.75 mm thick 12 % resolving polyacrylamide gel. Samples were mixed with 5 parts lysate to one part 6x SDS sample loading buffer (300 mM Tris(hydroxymethyl)aminoethane (Tris).Cl (pH 6.8), 600 mM diothiothreitol, 12 % (w/v) SDS, 0.6 % bromophenol blue, 60 % glycerol (all Sigma-Aldrich)), heated to 99 °C for 10 min, then loaded onto the gel.

10 μl Prestained Protein Marker, Broad Range (New England Biolabs) was loaded into one well of each gel prior to electrophoresis. The proteins contained in the mixture were covalently-conjugated to a blue dye. The apparent molecular weight of the proteins in the ladder were 175, 80, 58, 46, 30, 25, 17 and 7 kDa.

Composition of Polyacrylamide Gels

4 % stacking gel	**12 % resolving gel**
1.8 ml 30 % (w/v) acrylamide / bis-acrylamide solution 5.0 ml stacking gel buffer (0.25 M Tris.HCl, 0.2 % (w/v) SDS, pH 6.8) 4.0 ml water 50 μl 10 % (w/v) ammonium persulphate solution 10 μl N,N,N,N – tetramethylethylenediamine	7.5 ml 30 % (w/v) acrylamide / bis-acrylamide solution 9.0 ml resolving gel buffer (0.75 M Tris.HCl, 0.2 % (w/v) SDS, pH 8.8) 2.0 ml water 180 μl 10 % (w/v) ammonium persulphate solution 18 μl N,N,N,N - tetramethylethylenediamine

Proteins were separated using a mini vertical electrophoresis unit (SE260, GE Life Sciences). The proteins were separated in a discontinuous buffer system, with a Tris-Glycine electrophoresis buffer (0.025 M Tris.Cl, 0.192 M Glycine, 0.1 % (w/v) SDS, pH 8.3). Running conditions were: constant current of 20 mA per gel, for between one and two hours at room temperature.

2.6.4 Wet Electroblotting

Proteins were transferred from the polyacrylamide gel to polyvinylidene fluoride (PVDF) membrane by wet electroblotting in a tank transfer unit (TE22, GE Life Sciences). The transfer buffer was comprised 0.025 M Tris, 0.192 M Glycine, 20 % methanol. Running conditions were either 250 mA constant current for 90 minutes, or 30 V constant voltage overnight, both with water cooling.

2.6.5 Immunoblotting

The PVDF membrane was typically blocked: for 1 hour in 5 % (w/v) milk powder (Marvel, Premier Foods) in Tris-buffered saline solution + 1% Tween 20 (TBST) for non-phospho-protein targets; for 1 hour in 5 % (w/v) essentially immunoglobulin-free bovine serum albumin (Sigma-Aldrich) in TBST for phospho-protein targets. TBST was Tris-buffered saline solution (20 mM Tris.HCl, 137 mM NaCl, pH 7.6) supplemented with 0.1 % v/v Tween-20 (Sigma-Aldrich).

The membrane was rinsed in TBST then incubated with primary antibody, diluted into blocking buffer, for 3 hours at room temperature or overnight at 4 °C. The membrane was washed three times for 10 minutes with TBST.

The membrane was incubated for 1 hour with secondary antibody (specific for the species of the primary antibody, and coupled to horseradish peroxidase) diluted into blocking buffer. After four washes of 10 minutes with TBST, the bound secondary antibody was detected by enhanced chemiluminescence (Pierce SuperSignal West Pico Substrate and Kodak film).

2.6.5.1 Reprobing Western Blots

Stripping is the process in which primary and secondary antibodies are detached from the PVDF membrane. The PVDF membrane may be re-probed for a second target without interference from antibodies bound in the first round of blotting. Here, the membrane was stripped by incubation of the membrane with a low pH buffer (0.2 M Glycine, 0.1 % (w/v) SDS, 1 % Tween-20, pH 2.2; all Sigma-Aldrich).

After the first chemiluminescent reaction, the membrane was washed three times with TBST for 10 minutes then placed into an excess of stripping buffer. The membrane was incubated with this buffer for a total of 1 hour, replacing

with fresh buffer every 15 minutes. The membrane was then washed three times 10 minutes with TBST before re-probing.

2.7 Measurement of Gene Expression

Gene expression involves first transcription of genomic DNA to messenger ribonucleic acid (mRNA), then translation of mRNA into protein. Some expressed genes are not translated; RNA is the functional product. This includes certain ribosomal RNA (rRNA) such as 18S. The abundance of a specific mRNA species in a cell usually, but not always, gives a good indication of the amount of corresponding protein. It is important to note that other regulatory mechanisms, such as those controlling protein turnover, can result in a significant disparity between the quantity of observed protein and abundance of its coding mRNA. Nevertheless, measurement of the abundance of specific mRNAs is used to compare gene expression between different types of cells and before and after application of various stimuli to them.

2.7.1 RNA Isolation

In this study, total RNA was extracted from cells using TRI Reagent (Sigma-Aldrich). The sample (tissue or cell pellet) is first homogenised in a mixture of phenol and guanidine thiocyanate. The latter compound helps to completely denature the released proteins. These include, importantly, nucleases that might otherwise degrade the exposed nucleic acids. The RNA, DNA and protein components are then separated from each other by addition of chloroform. The RNA can be extracted, washed and then resuspended for use in various reactions.

In detail, the preparation of RNA proceeded as follows. Typically, 2 - $5x10^6$ cells per prep. were washed with cold PBS then pelleted. Each sample was homogenised in 1 ml TRI Reagent. The homogenate was then incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added and the components mixed. After 2 to 3 minutes incubation at room temperature the sample was centrifuged at 12 000 g for 15 minutes at 4 °C. The aqueous phase containing the RNA was extracted to a separate tube and the remainder discarded. The RNA was then precipitated with 0.5 ml of isopropanol, incubating the mixture for 10 minutes at room temperature. The RNA was pelleted by centrifugation at 12

000 g for 10 minutes at 4 °C, then washed with 75 % ethanol (7500 g for 5 minutes at 4 °C). Finally, the RNA was resuspended in water (typically 50 μl per sample). RNA samples were aliquotted and stored at -80 °C.

The water used to resuspend the RNA was previously treated with diethylpyrocarbonate (DEPC). Briefly, DEPC was added to deionised (>18 MΩ) distilled water to a final concentration of 0.1 %, and the mixture incubated at room temperature for 1 hour. The DEPC was then removed by autoclaving, yielding a supply of water free of RNase activity.

Every RNA preparation was examined by spectrophotometry (Figure 5) using a Nanodrop instrument (Thermo Scientific). Only those with a ratio of absorbances at 260 nm and 280 nm close to 2, and a relatively low absorbance at 230 nm (indicative of a pure preparation) were used in further experiments.

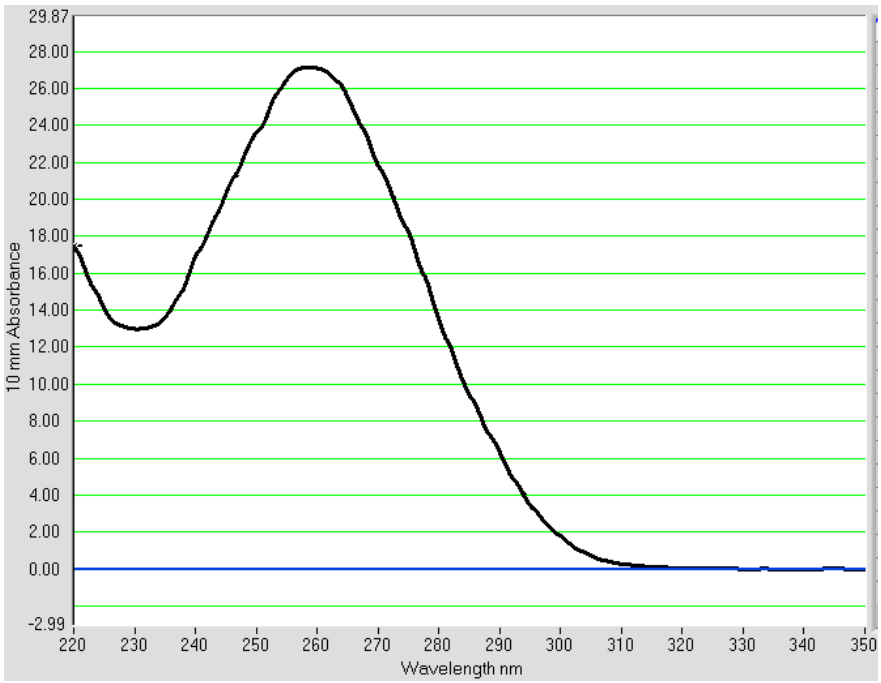


Figure 5. A typical absorbance spectrum of an RNA sample.
$5x10^6$ Molt 4 T cells were washed in cold PBS then pelleted. Total RNA was prepared following the method previously described. 2 μl of the sample was taken and measured on the Nanodrop spectrophotometer. The absorbance spectrum is shown in the figure. The ratio of the absorbances at 260 nm and 280 nm, and at 260 nm and 230 nm, are 2.00 and 2.09 respectively.

2.7.2 cDNA Synthesis

In order for the relative quantities of specific RNA species to be measured, they must first be copied to form complementary DNA (cDNA). Reverse transcriptases can copy RNA to cDNA; these enzymes are present in a number of viruses that employ reverse transcription as part of their replication cycle in host cells. Reverse transcriptase (RT) is strictly an RNA-dependent DNA polymerase. It binds an RNA template and catalyses the formation of a complementary single-stranded DNA molecule. RT usually also have ribonuclease H (RNase H) activity and degrade the RNA template as the DNA strand in synthesised.

Here, the enzyme SuperScript III (Invitrogen) was used. It is an engineered version of Moloney Murine Leukaemia Virus reverse transcriptase. The cDNA synthesis reactions were primed with random hexamers. This is a very non-specific priming method, facilitating the copying of almost all RNA species present, including non-polyadenylated RNA.

cDNA synthesis reactions were performed in thin-walled polymerase chain reaction (PCR) tubes (VWR) placed into a G-Storm GS482 Thermocycler (Labtech International, UK). A typical reaction proceeded as follows. 1 μg RNA was mixed with 500 ng random hexamers (Invitrogen, USA) and 10 mM deoxynucleotide triphosphates (dNTPs) (Rovalab, Germany) in a 13 μl reaction volume at 65 °C for 7 minutes. The following components were then added: 4 μl 5x first strand buffer, 1 μl 0.1 M dithiothreitol, 1 μl (100 U) SuperScript III enzyme (all Invitrogen). The tube was incubated for 5 minutes at 25 °C, then the temperature raised to 50 °C for 1 hour. Finally, the enzyme was inactivated at 70 °C for 15 minutes. The cDNA was aliquotted and stored at -80 °C before use.

2.7.3 Real-time PCR

2.7.3.1 Principles of PCR

Polymerase chain reaction is an enzyme-catalysed process in which sections of DNA are amplified geometrically to yield many copies of the original sequence. A heat-stable DNA-dependent DNA polymerase (such as *Taq* polymerase) elongates from oligonucleotide primers complementary to short sequences on the sense and anti-sense strands at either end of the target DNA sequence.

The use of a heat-stable polymerase in a thermal cycling process is fundamental to the reaction. In the first step, complementary DNA strands in which the target sequence is located are separated at high temperature (generally around 95 °C for 15 seconds). The reaction mixture is then cooled (to a temperature dependent upon the primer sequences and composition of the reaction mixture, but typically in the range 40 - 60 °C) for 1 minute and the oligonucleotide primers anneal to their specific sequences at either end of the target. The temperature of the reaction mixture is then raised (usually to temperatures in the region of 72 °C, and for 1 – 2 minutes for amplicons up to 2 kb) and the polymerase catalyses the addition of complementary dNTPs to the elongating strands in the 5’ to 3’ direction. The thermal cycling process is then repeated, usually for between 20 and 40 cycles. This results in geometric amplification of the sequence bounded by the forward and reverse primers, with the number of copies equal to 2^n, where n is the number of cycles.

Careful primer design is necessary to achieve an optimal PCR reaction. The length and specificity of the primers, their annealing temperature, and their affinity for each other must all be considered. There are software-based packages such as OligoPerfect (Invitrogen) that can assist with this.

2.7.3.2 Real-time PCR

Real-time polymerase chain reaction (RT-PCR) is an adaptation of basic PCR. In RT-PCR the progress of the reaction is followed as it proceeds, allowing the (relative or absolute) quantity of copies of a particular target at the start of the reaction to be calculated. Slightly different reaction components and measuring equipment to those used in standard PCR are required. RT-PCR stands in contrast to end-point PCR; in the latter process a specific sequence is amplified for a set number of cycles and the quantity of copies determined at the end of the reaction.

2.7.3.2.1 TaqMan Gene Expression Assays

TaqMan Gene Expression Assays (Applied Biosystems, USA) were used in this investigation. TaqMan MGB fluorescent probes, specific for the target sequence, were added to each reaction mixture at the start.

Briefly, the TaqMan technology functions as follows. The fluorescent probes have the reporter fluorescent dye 6-FAM at their 5' end, and the quencher MGB at their 3' end. An intact probe does not fluoresce due to the proximity of the reporter and quencher that are anchored to the sequence. During each cycle, when the reaction mixture cools after the denaturation step, the probe is able to hybridise with its target sequence. The number of probes that bind in any one reaction is proportional to the number of copies of target sequence. During the following elongation step, the polymerase encounters the bound probe and the 5' nuclease activity of the enzyme separates the reporter from the quencher. The reporter becomes fluorescent. The detection instrument excites the free reporter molecules and measures the fluorescently-emitted light. The amount of fluorescence is proportional to the number of copies of the target sequence, assuming the other components (such as the probes and enzyme) are not limiting.

A typical RT-PCR reaction was comprised: 1 μl TaqMan Gene Expression Assay (primer and probe mix; Applied Biosystems), 9 μl cDNA (50 ng equivalent of RNA), and 10 μl TaqMan Universal PCR Master Mix (including polymerase and dNTPs; Applied Biosystems). For the 18S control, 9 μl cDNA (diluted 5000x to 0.01 ng equivalent of RNA) was used. Control reactions without cDNA template were run for each assay, with no amplification observed.

The reactions took place in 96 tube format PCR plates (VWR) placed into an ABI Prism 7000 Sequence Detection System (Applied Biosystems) instrument, which recorded the fluorescence of each reaction tube after each thermal cycle. The following program was used: one cycle; 2 minutes at 50 °C, then 10 minutes at 95 °C; forty cycles, 15 seconds at 95 °C, then 1 minute at 60 °C.

2.7.3.2.2 Relative Quantitation of Gene Expression

Experiments to compare gene expression between samples were designed and analysed following the instructions set out in the *Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR* (Applied Biosystems).

Inventoried TaqMan Gene Expression Assays (Applied Biosystems) were used for the quantitation of expression of S1PR1 (ID: Hs00173499_m1), S1PR3 (Hs01019574_m1) and 18S (Hs99999901_s1). The primers and probes for S1PR1 and S1PR3 were exon-spanning, so as to avoid interference from any contaminating genomic DNA. 18S was used as the endogeneous control.

Equal quantities of cDNA from the samples to be compared were run, in triplicate, using the same reaction conditions. The relative quantities of the target cDNA sequence in the samples were inferred from their corresponding amplification curves: specifically, the number of cycles taken for ΔR_n to reach a common threshold. This was set where geometric amplification of the target sequence was seen to begin.

Data was expressed in the $2^{-\Delta Ct}$ format, where $\Delta C_t = C_t$ [target] – C_t [reference]. The C_t values used in this equation were the mean of the three corresponding raw output values.

The uncertainty in the value of ΔC_t was calculated as follows:

$s_{delta} = \sqrt{(s_{target}^2 + s_{reference}^2)}$; where s_{delta} is the standard deviation of the ΔCt value, s_{target} is the standard deviation of the C_t [target] value and $s_{reference}$ is the standard deviation of the C_t [reference] value.

2.8 Statistics

For the real-time PCR data, differences between groups were tested for significance using Rest 2008 software (Corbett Research, UK). Else, the Student's two-tailed *t* test for unpaired data was used to compare groups.

Statistical analysis and graphing of data was performed with Prism 4.0c software (GraphPad Software, USA).

P values less than or equal to 0.05 were considered significant (*), $P \leq 0.01$ highly significant (**), and $P \leq 0.001$ very highly significant (***).

Chapter 3. Modulation by S1P of T Cell Responses to CXCL12

Modulation by S1P of T cell Responses to CXCL12

3.1 Introduction

Chemokines are small proteins that control immune cell migration around the body. Some are produced constitutively and function in homeostatic processes, such as the regulation of lymphocyte recirculation. Others cause the migration of immune effectors to inflamed sites and are produced on inflammatory signalling in certain cell types (Rossi and Zlotnik, 2000). More recently, it has become clear that the endogeneous lysophospholipid sphingosine 1-phosphate is another important player in regulation of leukocyte migration *in vivo* (Spiegel and Milstien, 2011). G protein-coupled receptors for both chemokines and S1P are present on T cells, and T cells can be exposed to one or both substances simultaneously during their life cycle. Published work has lent support to the hypothesis that the chemokine and S1P signalling pathways may interact in determining the precise nature of leukocyte migration. For example, signalling of both the S1P receptor S1PR1 and the chemokine receptor CCR7 influence egress decision making of T cells at lymph node exit sites (Pham *et al.*, 2008).

The family of receptors for S1P are denoted S1PR1-5. Splenic T cells express predominantly S1PR1 and S1PR4, and much lower levels of S1PR3 and S1PR5 (Graeler and Goetzl, 2002). Although S1PR3 is expressed at a very low level by resting T cells, it is upregulated on activation of splenic T cells (Graeler and Goetzl, 2002), albeit still to a low level, and is expressed strongly by the Jurkat activated T cell line (Jin *et al.*, 2003). S1PR4 does not appear to have any role in control of T cell migration, but can alter T cell proliferation and cytokine secretion (Wang *et al.*, 2005). S1PR1 is more highly expressed than S1PR4 and S1P has a greater affinity for the former (K_d = 8 nM and 63 nM respectively) (Lee *et al.*, 1998; Van Brocklyn *et al.*, 2000). S1PR1 is required for T cell egress from lymph nodes and agonism of the receptor can induce the chemotaxis of resting T cells (Matloubian *et al.*, 2004). S1PR1, similarly to many other GPCR, is desensitised, by receptor internalisation, on chronic exposure to its ligand S1P (Oo *et al.*, 2007).

This property of the receptor is an important determinant of the *in vivo* effects of S1P. The effect of the vascular gradient of S1P is to induce cyclical modulation of surface S1PR1 on T cells as they transit through the various lymphoid compartments, and thereby changes in the responsiveness of T cells to

signalling through the receptor (Lo *et al.*, 2005). The re-acquisition of surface S1PR1 on T cells in the S1P low environment of lymphatic tissue is essential for their capacity to egress. Abrogation of surface S1PR1 expression using FTY720, a super-agonist of the receptor, blocks T cell egress (Matloubian *et al.*, 2004). Inside resting lymph nodes, T cells located close to cortical sinuses probe the insides of the structures and sample S1P there (Grigorova *et al.*, 2009). Ligands for the entry receptor CCR7, CCL19 and 21, are expressed abundantly by antigen-presenting and stromal cells in the lymph node cortex (Luther *et al.*, 2000; Vissers *et al.*, 2001). These act to retain lymphocytes within the node. Signalling of S1PR1 seems to antagonise such retention signals thereby promoting egress (Pham *et al.*, 2008).

Recent evidence has shown that S1P can modulate chemotaxis of T cells and other leukocyte subsets to many other chemokines. S1P has been shown to reduce chemotaxis of neutrophils towards the end-stage chemoattractants CXCL8 and fMLP (Kawa *et al.*, 1997). S1P enhanced the migration of splenic T cells towards CXCL12 and was required for the movement of peripheral lymph node derived cells towards the same chemokine (Yopp *et al.*, 2005). Dose-dependent effects of S1P have also been reported. Whereas pre-treatment of $CD4^+$ T cells with 10-100 nM S1P resulted in increased chemotaxis of $CD4^+$ T cells towards CCL5 and CCL21, 300 nM to 3 μM S1P strongly suppressed migration towards them (Graeler *et al.*, 2002). Although such studies clearly indicate an effect of S1P on migration towards certain chemokines, they have not examined in detail the effect of S1P on responses of T cells to a particular chemokine.

CXCL12 is a prototypical homeostatic chemokine. It is pleiotropic with very well established roles in immune homeostasis, inflammation and cancer (Karin, 2010). It is constitutively expressed and found in a number of compartments alongside S1P. CXCL12 along with CCL19 and CCL21 is present on the luminal surface of high endothelial venules under basal conditions and they act in concert to direct movement of resting T cells into secondary lymphoid tissue. CXCL12 seems to sensitise T cells to CCR7 signalling, augmenting CCR7 responses including ERK phosphorylation and actin polymerisation (Bai *et al.*, 2009). CXCL12 appears to play a role in the homing of T cells to multiple tissues under basal and inflamed conditions. CXCL12 promotes LFA-1

interaction with ICAM-1 (Campbell *et al.*, 1998), and $\alpha 4\beta 7$ interaction with MAdCAM-1 and fibronectin (Wright *et al.*, 2002). In addition to these effects on migration, there is also abundant evidence for CXCR4 -mediated effects on T cell activation (Molon *et al.*, 2005). CXCR4 utilises the TCR ITAM domains for signal transduction. It can also act synergistically with T cell activation to enhance cytokine secretion (Kumar *et al.*, 2006).

In this study, bare transwell assays were used to investigate the effect of S1P on T cell chemotaxis. This meant that the effect of S1P on endothelial barrier permeability did not need to be considered. Signalling assays were also used to probe the effect of S1P treatment on multiple pathways downstream of CXCR4 ligation. Pre-treatment with a high concentration of S1P (and the S1PR1 selective agonist SEW2871) enhanced the migration of Jurkat T cells towards the homeostatic chemokine CXCL12. This appeared to be a highly selective effect. It was not due to an S1P-mediated effect on surface abundance of a receptor for this chemokine, CXCR4. Nor did it correlate with other ligand-induced responses to CXCL12 including the magnitude of ERK 1/2 phosphorylation and inhibition of adenylate cyclase activity. Interestingly, S1P pre-treatment did not affect migration of Molt 4 T cells (which have a different S1P receptor profile) towards the same chemokine.

The aims of this chapter were as follows:

- To examine the responses of Jurkat T cells to S1P
- To determine whether or not the migration of Jurkat T cells towards CXCL12 was affected by S1P pre-treatment
- To investigate how signalling downstream of CXCR4 was modified by S1P
- To compare the effects of S1P on Jurkat T cell and Molt 4 T cell responses to CXCL12

3.2 Materials and Methods

Agarose gel electrophoresis

For analysis of RNA quality, the RNA was first heated to 65 ºC for 5 minutes. 1 μg total RNA per lane was then separated by electrophoresis in a 1 % agarose gel in Tris – boric acid – EDTA buffer (pH 8.3).

For analysis of PCR products, half of a 25 μl reaction (starting with a quantity of cDNA equivalent to 100 ng RNA input into the RT reaction) was run per lane. DNA was separated in a 1 % agarose gel in Tris - boric acid – EDTA buffer.

All gels were pre-stained with ethidium bromide (0.5 μg/ml). They were visualised under UV illumination after electrophoresis. Images were captured using an AlphaImager system (Alpha Innotech, USA).

cAMP assays

In each experiment, cells were stimulated with the specified concentration of forskolin (Sigma Aldrich) with or without the specified receptor ligand for 30 minutes at 37 ºC in 5 % CO_2. Cyclic adenosine monophosphate (cAMP) was detected using a colorimetric competitive-binding assay kit (R&D Systems). Briefly, the procedure was as follows. After stimulation, the cells were washed in cold PBS then pelleted. cAMP was released by lysis in the supplied acidic buffer then three freeze-thaw cycles in liquid nitrogen and a 37 ºC waterbath. Samples were mixed with known quantities of horseradish peroxidase-conjugated cAMP, and the non-conjugated cAMP originating from the sample left to compete for binding to a capture antibody. After washing, enzyme activity was measured using the catalysed conversion of a specific substrate to a product absorbing light at 450 nm (measured on a Dynex MFX instrument). A standard curve was run for each assay using known concentrations of unconjugated cAMP as the competitor. The concentration of cAMP in each experimental sample was inferred from the curve by interpolation, using the corresponding absorbance values.

PCR and Real time PCR

RNA was prepared from cell pellets using TRI reagent (Sigma Aldrich). cDNA was synthesised using SuperScript III Reverse Transcriptase and random hexamers as primers (Invitrogen).

Standard end-point PCR. A 570 bp sequence within the human GAPDH gene was amplified from cDNA (a volume equivalent to 100 ng RNA input into the prior RT reaction) using TaqMan Universal PCR mastermix (Applied Biosystems). The thermocycling parameters were: 10 minutes at 95 ºC (1 cycle); 30 seconds at 95 ºC, 1 minute at 65 ºC, 30 seconds at 72 ºC (35 cycles). The primer sequences were:

GAPDH forward: 5' ACCCAGAAGACTGTGGATGG 3'

GAPDH reverse: 5' AGGGGTCTACATGGCAACTG 3'.

Real-time PCR. Expression of S1PR1 and S1PR3 were determined semi-quantitatively using specific TaqMan Gene Expression assays (Hs00173499_m1 and Hs01019574_m1 respectively), with 18S as the reference (Hs99999901_s1; all Applied Biosystems). The amplifications were run on an ABI Prism 7000 instrument (Applied Biosystems).

Phospho-kinase array

Cells were treated as described, washed in cold PBS then lysed. The protein content of lysates was measured by BCA assay. Equal quantities of total protein were assayed in parallel using a Proteome Profiler Phospho-kinase Array kit (R&D Systems). This used nitrocellulose membranes covered with an array of capture antibodies against various protein targets. Lysates were washed over the membranes and proteins left to bind overnight. Proteins bound to the array membrane were then detected by immunoenzymatic labelling and enhanced chemiluminescence (recorded onto X-ray film). The film was scanned and the intensities of the dots on the array (mean gray value in a region of constant area) measured using Photoshop CS3 v10 software (Adobe Systems). The signal corresponding to each protein was normalised to the intensity of the appropriate positive control spot on each membrane section.

Flow cytometry

For flow cytometry, Jurkat and Molt 4 T cells were stained with various fluorochrome-conjugated monoclonal antibodies or appropriate isotype-matched control antibodies. The specific antibodies used were: S1PR1 (218713; R&D Systems), CXCR4 (12G5; R&D Systems). Data was acquired on a FACSCanto II instrument (BD) and analysis performed using FACSDiva (BD) and FlowJo (Treestar) software.

For immunofluorescence confocal microscopy, Jurkat T cells were blocked for 1 hour with PBS + 1% BSA, before sequential staining with anti-human S1PR3 antibody (SC22210; Santa Cruz Biotechnology), fluorescein-conjugated anti-goat IgG (F2016, Sigma Aldrich), and DAPI (Sigma Aldrich). Images were captured with a Leica TCS SP2UV confocal microscope and analysed with Leica LCS Lite software.

Western blotting and ELISA

Proteins were separated by SDS-PAGE and transferred to PVDF membrane as previously described. The primary antibodies used for immunoblotting were: pERK 1/2 [T202/Y204] (SC16982; Santa Cruz Biotechnology), ERK 1 [pan] (MK12; BD Biosciences), phospho-Akt [Ser473] (D9E; Cell Signaling Technology), Akt [pan] (C68E7; Cell Signaling Technology), α-tubulin (B-5-1-2; Sigma Aldrich), S1PR1 (H-60; Santa Cruz Biotechnology), phosphorylated lymphocyte-specific protein tyrosine kinase (pLck) [Y394] (SC101728; Santa Cruz Biotechnology), Lck [pan] (SC28882; Santa Cruz Biotechnology). The secondary antibodies were: horseradish peroxidase-conjugated anti-rabbit IgG (A6154; Sigma Aldrich), anti-mouse IgG (A9044; Sigma Aldrich) and anti-goat IgG (A5420; Sigma Aldrich).

Phospho-ERK 1 [T202/Y204] / 2 [T185/Y187] and ERK 1/2 [pan] were also detected using a cell-based enzyme-linked immunosorbent assay (ELISA) (R&D Systems). 100 000 Jurkat T cells were added to each well of a 96 well-format plate (coated beforehand with 10 μg/ml poly-lysine in PBS (Poly-L-Lysine hydrobromide; Sigma Aldrich) for 30 minutes) and left to attach for 1 hour. The cells were then treated as desired before fixation with 8 % formaldehyde. Phospho-ERK 1 / 2 and ERK 1 / 2 were detected by double immunoenzymatic labelling. The relative quantities of these proteins were deduced from the spectrally distinct fluorescence of substrates associated with each target. Data was acquired on a Dynex MFX instrument operating with excitation and emission wavelengths of 540 nm and 600 nm (phospho-ERK 1 / 2) and 360 nm and 450 nm (ERK 1 / 2).

Chemotaxis assays

Migration of Jurkat and Molt 4 T cells was measured using transwell chambers (polycarbonate, 5 μm pore size; Corning Costar, Sigma Aldrich). Assays were

over 2 hours (unless otherwise stated), after which cells were extracted from the lower chamber and enumerated by flow cytometry.

Statistics

Prism 4.0 (GraphPad Software, USA) was used to analyse data. Statistical comparisons between groups were made using the Student's two-tailed T test for unpaired data. P values > 0.05 were considered not significant (n.s.), ≤ 0.05 significant (*), ≤ 0.01 highly significant (**), ≤ 0.001 very highly significant (***).

3.3 Results

Primary resting T cells express S1PR1 and respond to S1P in chemotaxis assays (Graeler and Goetzl, 2002). To see whether Jurkat T cells had a similar phenotype, they were analysed for expression of S1PR1 and S1PR3 proteins by immunofluorescence. A monoclonal antibody against S1PR1 stained serum-starved Jurkat T cells only slightly (Figure 6). Using confocal microscopy, they were shown to express significant levels of S1PR3, with staining predominantly on the perimeter of cells and outside the nuclear compartment (indicated by DAPI staining) (Figure 7). Treatment of cells with 1 μM S1P resulted in an increase in the amount of S1PR3 staining in the nucleus with time (Figure 8). This is consistent with ligand-induced internalisation of the receptor into that compartment.

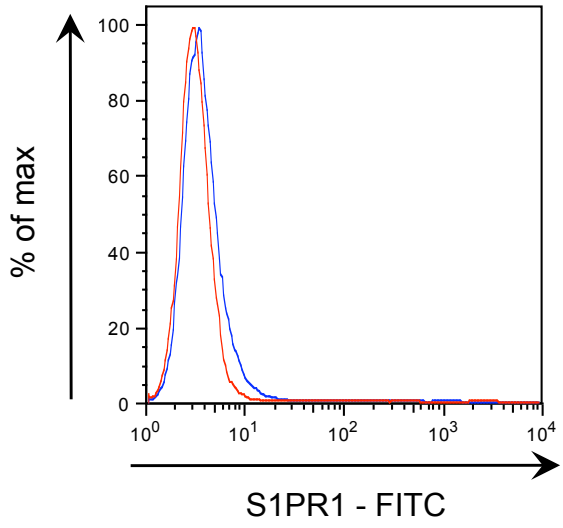


Figure 6. Jurkat T cells stained poorly for surface S1PR1.
Jurkat T cells were serum-starved overnight then stained with an S1PR1-specific monoclonal antibody (blue) or a fluorescently-labelled isotype-matched control monoclonal antibody (red). Analysis was by flow cytometry. Data are representative of three independent experiments.

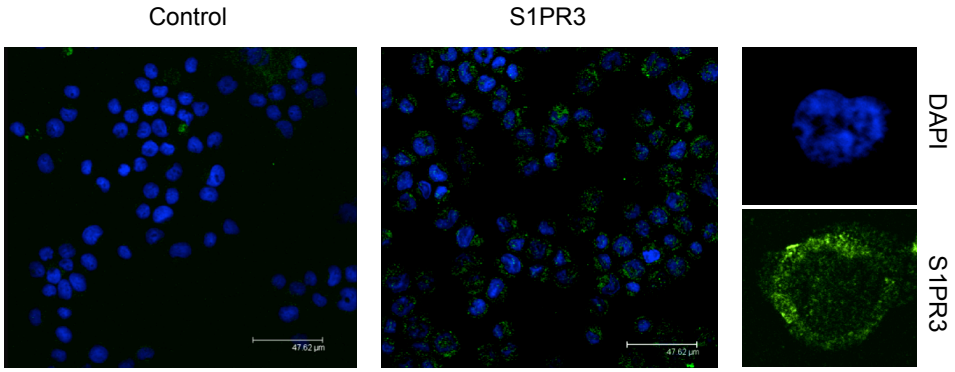


Figure 7. Jurkat T cells expressed S1PR3 on their surface.
Jurkat T cells were serum-starved overnight, then cytospins prepared. They were stained with a polyclonal antibody against S1PR3 and fluorescent secondary antibody (green). Cells were further stained with DAPI (blue) then visualised by confocal laser-scanning microscopy (CLSM). First panel, no primary antibody; second panel, S1PR3 polyclonal antibody; third and fourth panels, representative images (mid-point of cell on z-axis) of a single cell – upper image DAPI alone, lower image S1PR3 alone. Data are representative of three independent experiments.

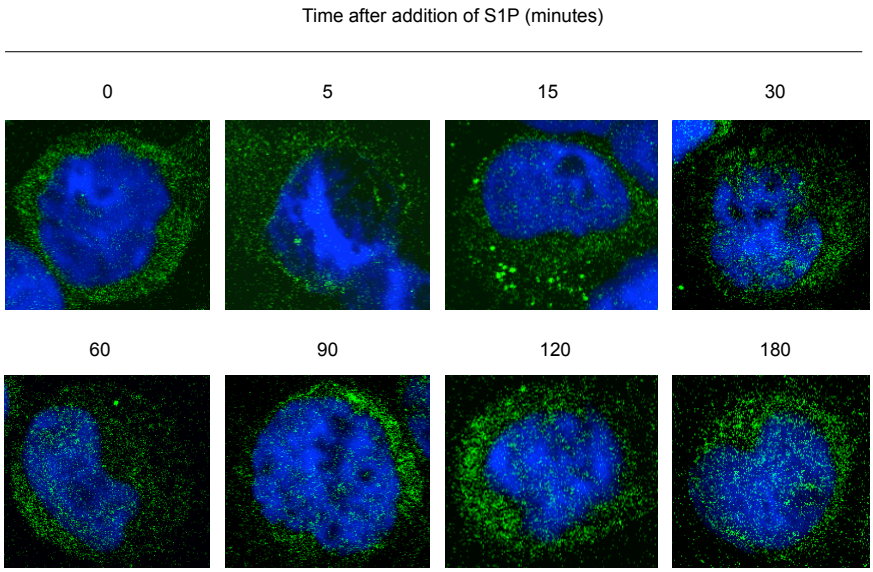


Figure 8. Ligand-induced internalisation of S1PR3.
Jurkat T cells were serum-starved overnight then incubated for various times with 1 μM S1P. They were rapidly cooled, cytospins prepared, and these stained for S1PR3, and with DAPI, as before. Imaging was by CLSM. Images shown were taken at the mid-point on the z-axis, as determined by DAPI staining. Data are representative of two independent experiments.

The response of Jurkat T cells to S1P was investigated further. Cells were stimulated for various times with 1 μM S1P and the extent of phosphorylation of ERK 1 / 2 determined, either by cell-based ELISA or western blotting. As expected, an early spike in the quantity of phospho-ERK 1 / 2 was followed by a gradual reduction over time (to within 25 % of background 30 minutes after ligand addition) (Figure 9). Jurkat T cells also migrated towards S1P in bare transwell assays, most efficiently at a ligand concentration of 10 nM (Figure 10). This is close to the K_d of S1P for S1PR1 of 8 nM (Lee *et al.*, 1998). Chequerboard analysis indicated that the migration was chemokinetic rather than chemotactic in nature. Whereas the response to the known chemotactic stimulus CXCL12 was greatly reduced with the presence of the ligand in the upper as well as the lower compartment, this was not the case for S1P (Figure 11).

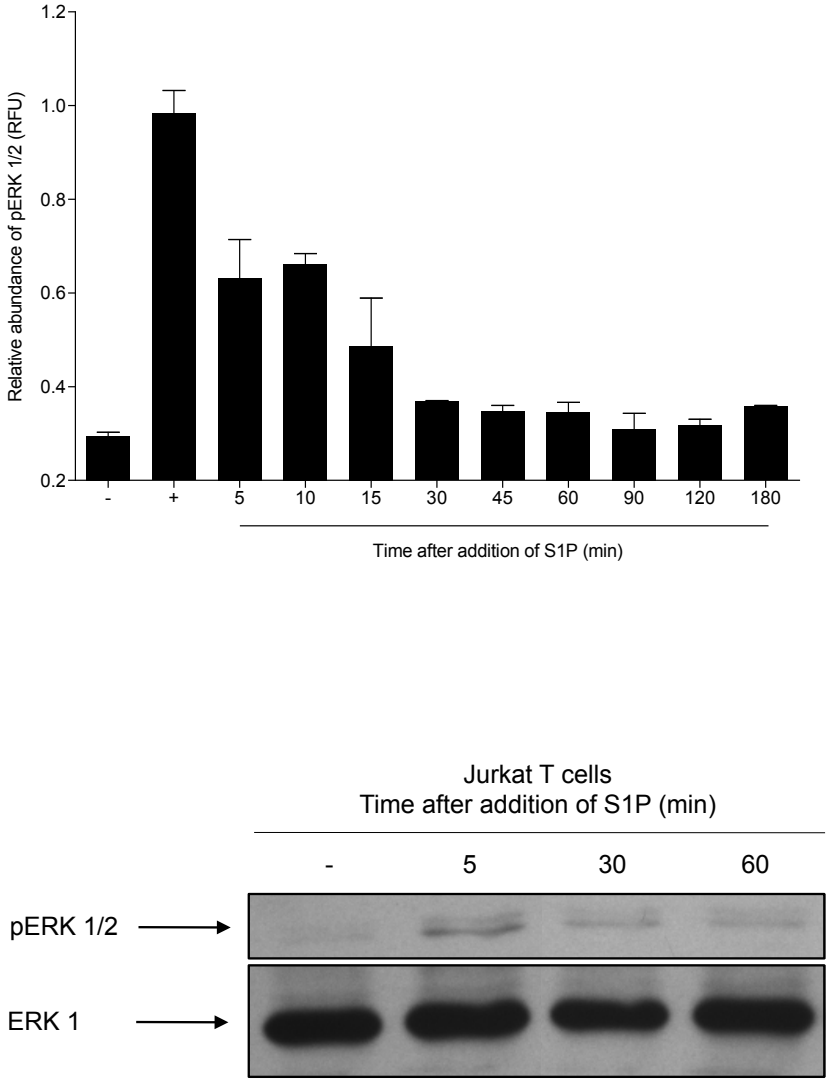


Figure 9. Phosphorylation of ERK 1 / 2 following stimulation of Jurkat T cells with S1P.

Upper panel, Jurkat T cells were serum-starved overnight then stimulated for various times with 1 μM S1P. -, stimulation with vehicle for 5 minutes; +, stimulation with 100 nM CXCL12 for 5 minutes. Phospho-ERK 1 (T202/Y204) / phospho-ERK 2 (T185/Y187) and ERK 1 / 2 (pan) were detected by cell-based ELISA. The fluorescence signals corresponding to phospho-ERK 1 / 2 have been normalised to those of total ERK 1 / 2 at each time point. Graph shows means ± range of duplicate determinations. Data are from one experiment. Lower panel, Jurkat T cells were stimulated either with vehicle for 5 minutes (-) or 1 μM S1P for various times. Cells were lysed and phospho-ERK 1 (T202/Y204) / phospho-ERK2 (T185/Y187) and ERK 1 detected by western blotting. Data are representative of two independent experiments.

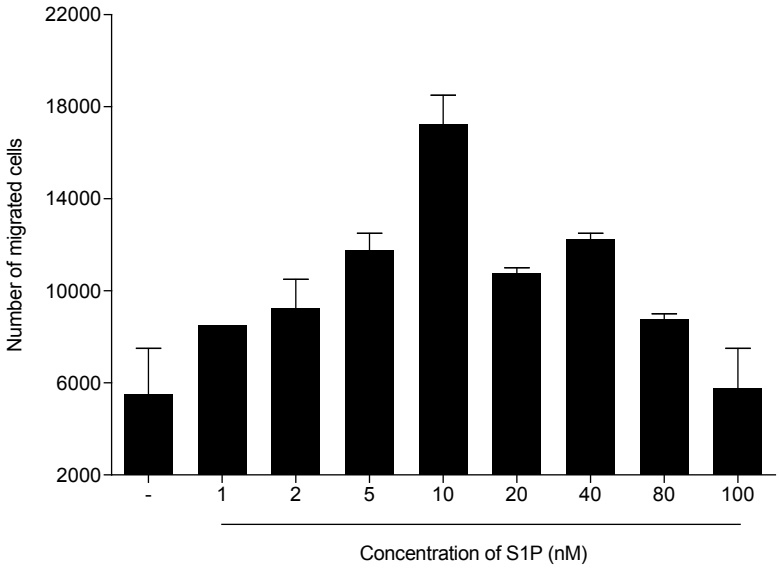


Figure 10. The efficiency of Jurkat T cell migration towards S1P was concentration-dependent.
Jurkat T cells (500 000 per well) were placed into bare transwell migration assays and left to migrate for 3 hours towards various concentrations of S1P or vehicle (-). Cells in the lower compartment were enumerated by flow cytometry. Graph shows means ± range of duplicate determinations. Data are from a single experiment.

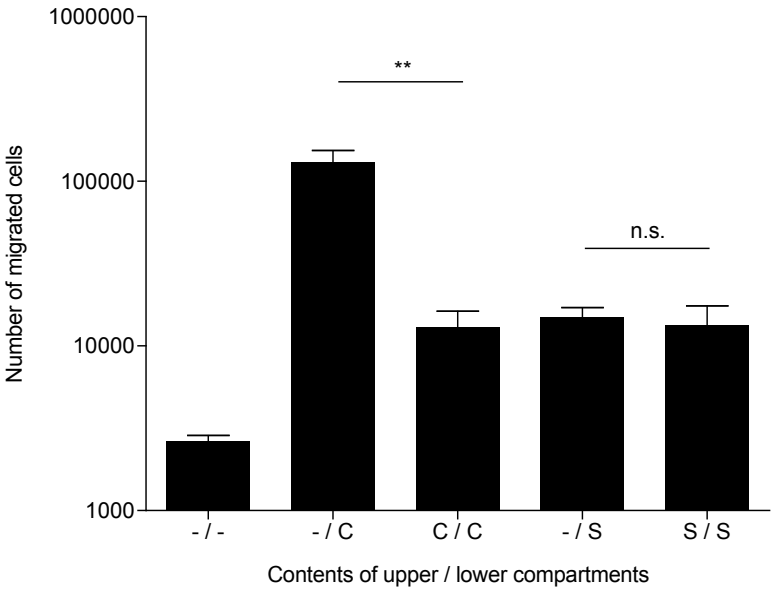


Figure 11. Chequerboard analysis of S1P-induced Jurkat T cell migration.
Jurkat T cells (500 000 per well) were left to migrate for 3 hours with 10 nM S1P (S) or 6.25 nM CXCL12 (C) either in the lower compartment alone, or both the upper and lower compartments. Cells in the lower compartment were enumerated by flow cytometry. Graph shows means ± s.e.m. Data are representative of two independent experiments.

The next step was to test whether treatment with S1P would affect cell migration towards a chemokine. Jurkat T cells were shown to express high levels of CXCR4 by immunofluorescence flow cytometry (Figure 12). Consistent with this, they migrated efficiently towards a ligand for the receptor, the homeostatic chemokine CXCL12. Pre-treatment of Jurkat T cells with 1 μM S1P for 1 hour immediately before the assay significantly increased the efficiency of chemotaxis (Figure 13, upper panel). When S1P was substituted with the S1PR1-specific agonist SEW2871, a similar effect was observed (Figure 13, lower panel). So, although flow cytometric analysis indicated that there was a very low level of S1PR1 on the cell surface, it was clearly sufficiently abundant to exert a strong effect on this chemotactic response.

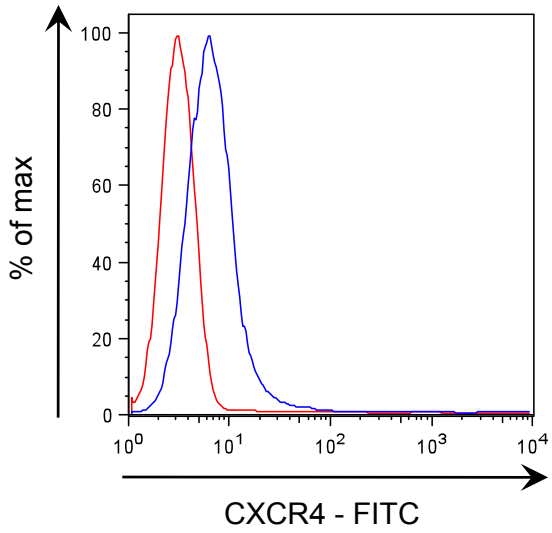


Figure 12. Jurkat T cells expressed high levels of CXCR4 on their surface.
Jurkat T cells were stained with a fluorescently-labelled monoclonal antibody against CXCR4 (blue) or isotype-matched control antibody (red), then analysed by flow cytometry. Data are representative of three independent experiments.

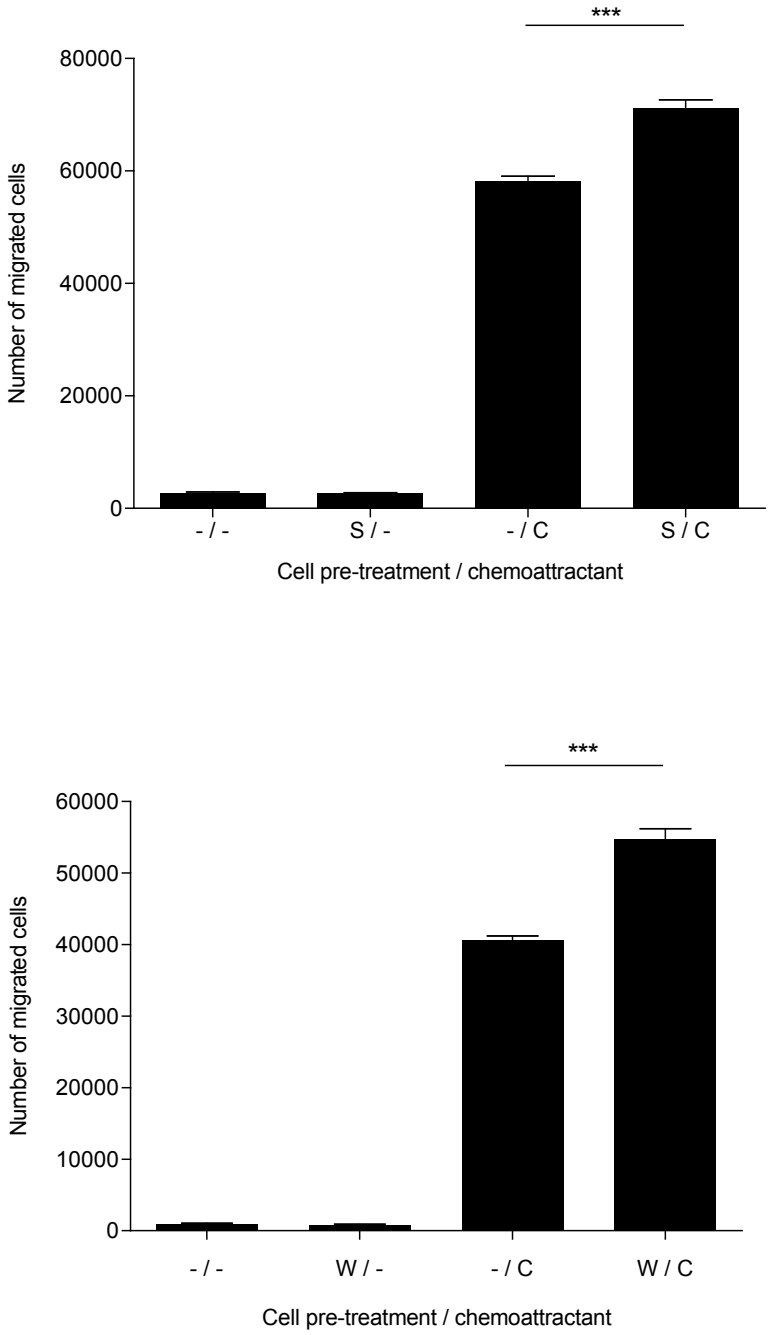


Figure 13. Pre-treatment of Jurkat T cells with either S1P or SEW2871 enhanced their migration towards CXCL12.
Upper panel, Jurkat T cells (100 000 per well) pre-treated with vehicle (-) or 1 μM S1P (S) for 1 hour were left to migrate for 2 hours towards 6.25 nM CXCL12 (C) or vehicle (-) in the lower compartment. Graph shows means ± s.e.m. Lower panel, as before, except that S1P was substituted with 10 μM SEW2871 (W). Graph shows means ± s.e.m. Cells in the lower compartment were enumerated by flow cytometry. Upper panel representative of three independent experiments, the lower representative of two.

One obvious explanation for the increased migration associated with S1P treatment would be increase in the amount of CXCR4 on the cell surface. However, treatment of Jurkat T cells with S1P for one hour did not change the intensity of staining for that receptor (Figure 14) (nor did two or three hours incubation with S1P (data not shown)).

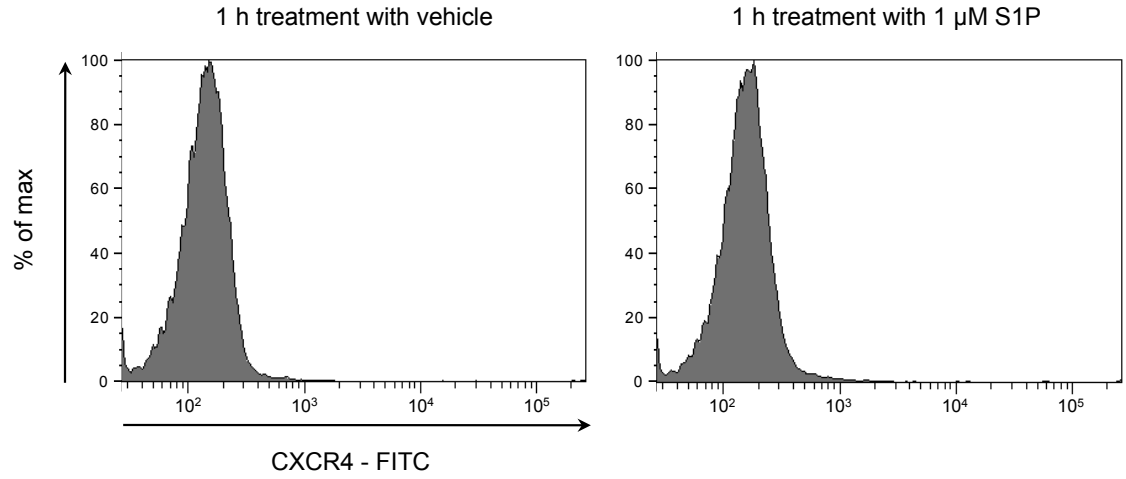


Figure 14. Treatment of Jurkat T cells with S1P did not change the amount of CXCR4 on the cell surface.
Jurkat T cells were treated for 1 hour with vehicle or 1 μM S1P then stained with a fluorescently-labelled monoclonal antibody against CXCR4. Analysis was by flow cytometry. Data are representative of three independent experiments.

Ligand-bound CXCR4 couples to the heterotrimeric G protein Gαi and inhibits the activity of membrane-resident adenylate cyclase (Busillo and Benovic, 2007). Forskolin is commonly used to activate adenylate cyclase in assays designed to measure Gαi coupling to that enzyme. To test whether S1P affected signal transduction downstream of CXCR4, Jurkat T cells were treated for 1 hour with S1P or vehicle, then stimulated with CXCL12 and forskolin. S1P did not affect the magnitude of reduction of cAMP levels caused by stimulation with CXCL12 (Figure 15). Similarly, ERK 1 / 2 phosphorylation following stimulation of cells with CXCL12 was unaffected by S1P (Figure 17).

These data suggested that the effect of S1P pre-treatment on CXCR4 signalling was highly selective. PI3K signalling and the enrichment of PIP_3 at the leading edge of a migrating cell is usually required for chemotaxis. The kinase Akt is phosphorylated and activated on binding PIP_3 at the plasma membrane. To further elucidate the mechanism of the S1P-induced effect on Jurkat T cell migration, its ability to modulate phosphorylation of Akt was tested. Jurkat T cells were incubated with S1P or vehicle for 1 hour, then stimulated with CXCL12 for 10 minutes. Phosphorylated Akt and total Akt were subsequently detected in whole cell lysates by western blotting. Similar levels of phospho-Akt were observed whether or not the cells were pre-treated with S1P (Figure 16). Unusually, stimulation of untreated cells with CXCL12 did not result in any noticeable increase in phosphorylated protein. This is probably related to the reported deficiency in PTEN activity in Jurkat T cells (Shan *et al.*, 2000).

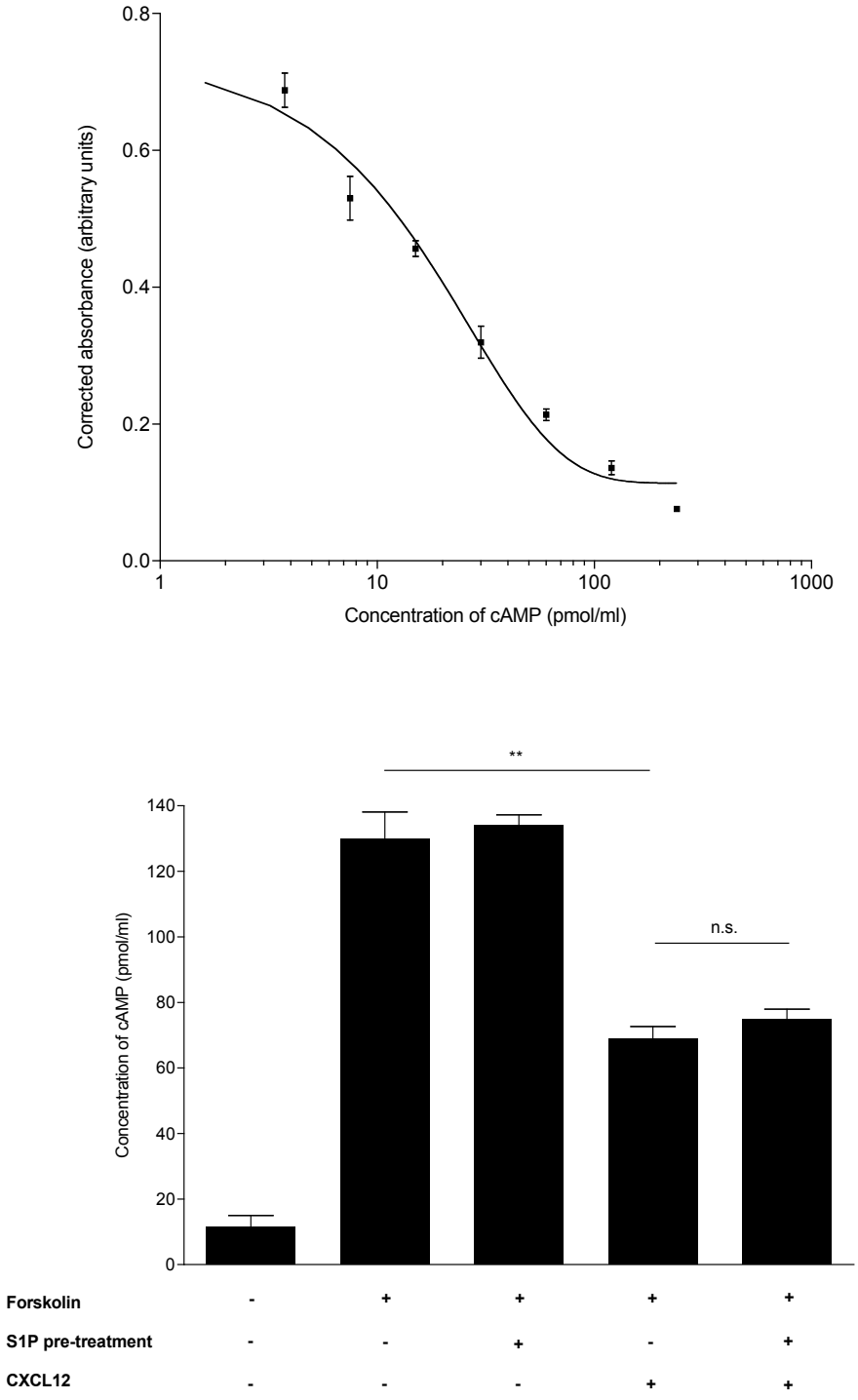


Figure 15. Pre-treatment of Jurkat T cells with S1P did not affect the magnitude of the CXCL12-stimulated inhibition of adenylate cyclase activity.
Upper panel, cAMP in solution at 240 pmol/ml was serially diluted and the set assayed using a competitive ELISA. A four-parameter logistic curve has been fitted to the data. Graph shows means ± s.e.m. of duplicate determinations. A standard curve was prepared for each experiment. Lower panel, equal numbers of Jurkat T cells were pre-treated with 1 μM S1P or vehicle for 1 hour, then stimulated with either 50 μM forskolin and vehicle or 50 μM forskolin and 6.25 nM CXCL12. Graph shows means ± s.e.m. Data are representative of two independent experiments.

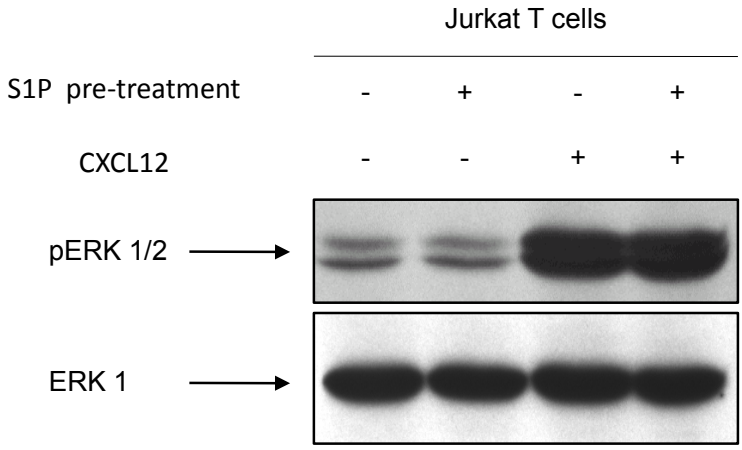


Figure 17. Phosphorylation of ERK 1 / 2 following stimulation of Jurkat T cells with CXCL12 was not affected by S1P.
Jurkat T cells were incubated with vehicle or 1 μM S1P for 1 hour, then stimulated with vehicle or 6.25 nM CXCL12 for 5 minutes. Phospho-ERK 1 (T202/Y204) / phospho-ERK2 (T185/Y187) and ERK 1 were detected in whole cell lysates by western blotting. Data are representative of three independent experiments.

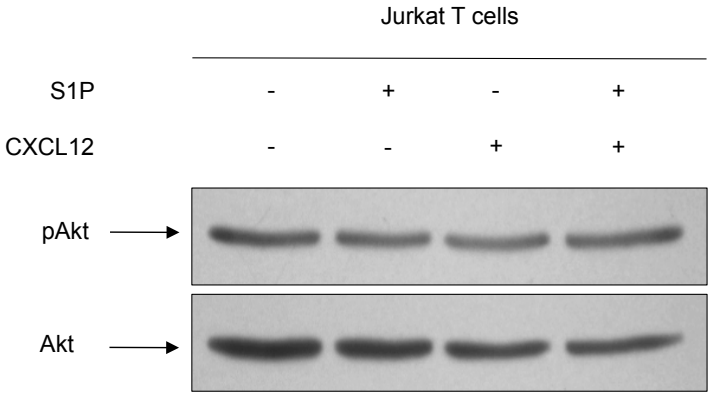


Figure 16. Pre-incubation of Jurkat T cells with S1P did not affect intracellular levels of phospho-Akt.
Jurkat T cells were treated with 1 μM S1P or vehicle for 1 hour, then stimulated with 6.25 nM CXCL12 or vehicle for 10 minutes. The cells were lysed and phospho-Akt (Ser473) and Akt (pan) detected by western blotting. Representative of two independent experiments.

Because none of the signalling pathways assayed so far appeared to be affected by S1P, a phospho-kinase array was used to screen a large number of potential targets. Jurkat T cells were incubated for 1 hour with S1P or vehicle then stimulated with CXCL12 or vehicle for 5 minutes. Whole cell lysates were prepared and probed using a sandwich-type immunoarray. The results of one experiment are shown. The graph shows that several proteins were most highly phosphorylated in the condition of S1P pre-treatment followed by CXCL12-stimulation of Jurkat T cells (Figure 18).

Phosphorylation of Lck was more than two-fold higher in cells that had been pre-treated with S1P then stimulated with CXCL12, compared with cells that were pre-treated with vehicle control but similarly stimulated with chemokine. Additionally, Lck activity is known to be required for chemotaxis towards CXCL12 (Inngjerdingen *et al.*, 2002). This protein was therefore selected for further investigation. To better understand the dynamics of Lck phosphorylation after receptor ligation, Jurkat T cells were first stimulated with CXCL12 for various times and cell lysates prepared. Western blotting for phospho-Lck (Y394) and total Lck showed that the phosphorylation response was biphasic, with most phospho-protein one minute after addition of chemokine, and another peak apparent at 10 minutes. The one-minute time-point was chosen to test the effect of pre-incubation of Jurkat T cells with S1P. In two of three independent experiments treatment with S1P was associated with a small increase in phosphorylation of Lck after CXCL12 stimulation (Figure 19). This was not however consistent or statistically significant when analysed by densitometry.

Normalised signal intensity
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-S1P -CXCL12
+S1P -CXCL12
-S1P +CXCL12
+S1P +CXCL12
p38alpha
ERK1/2
JNK pan
GSK-3alpha/beta
MEK1/2
MSK1/2
AMPKalpha1
Akt
TOR
CREB
HSP27
AMPKalpha2
beta-catenin
Src
Lyn
Lck
STAT2
STAT5a
Fyn
Yes
Fgr
STAT3
STAT5b
Hck
Chk-2
FAK
STAT6
STAT5a/b
p53
Akt
p53
p70 S6 kinase
p53
p27
Paxillin
p70 S6 kinase
RSK1/2/3
p27
PLCgamma-1
p70 S6 kinase
RSK1/2
c-Jun
Pyk-2
STAT1
STAT4
eNOS

Figure 18. A phospho-kinase array was used to screen for an effect of S1P on CXCL12 signalling.
Jurkat T cells were pre-treated with either 1 μM S1P or vehicle for 1 hour, then stimulated with 6.25 nM CXCL12 or vehicle for 5 minutes. Cell lysates were prepared and samples containing equal masses of protein assayed using a sandwich immunoarray. ECL signals were recorded onto light-sensitive film, which was then scanned into a computer. Protein target signal intensities were normalised to that of the positive control on each array part. Graph shows means of duplicate determinations in one experiment. Experiment was performed twice with similar results.

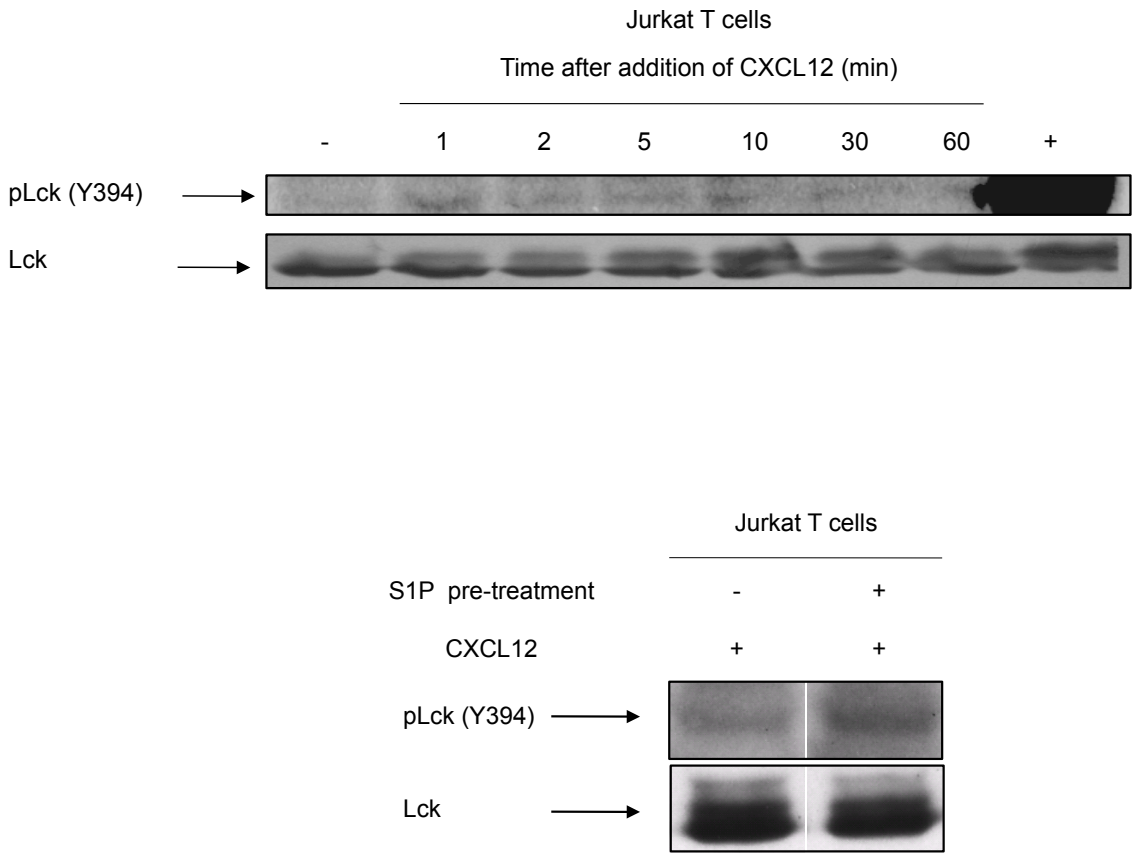


Figure 19. The effect of pre-treatment of Jurkat T cells with S1P on CXCL12-stimulated phosphorylation of Lck.
Upper panel, Jurkat T cells were stimulated for various times with 6.25 nM CXCL12, or for 10 minutes with pervanadate (+) or vehicle (-). The cells were lysed and phospho-Lck (Y394) and Lck detected by western blotting. Data is from one experiment. Lower panel, Jurkat T cells were incubated for 1 hour with 1 μM S1P or vehicle then stimulated for 1 minute with 6.25 nM CXCL12. The cells were rapidly lysed and phospho-Lck (Y394) and Lck detected by western blotting as before. Three independent experiments were performed.

These data showed that S1P, acting through S1PR1, significantly enhanced the migration of Jurkat T cells towards CXCL12. The other CXCL12 signalling responses examined here were not affected by S1P pre-treatment. To develop the study further, it was extended to include the Molt 4 T cell line. As earlier experiments had shown poor staining of S1PR1 protein on the surface of Jurkat T cells, the expression of S1PR1 (and S1PR3) was subsequently measured by semi-quantitative real-time PCR.

First however, the method of RNA preparation and real-time PCR assays were validated. Total RNA was isolated from cell pellets as detailed in the Methods. A sample of RNA was heated to disrupt secondary structure and separated by electrophoresis. Sharp bands corresponding to the small (18S) and large (28S) ribosomal RNAs can be seen in (Figure 20, upper panel), indicating little degradation. Another sample of RNA was reverse transcribed to cDNA, and the latter used in a test amplification of a portion of the GAPDH coding sequence (Figure 20, lower panel). The efficiency of amplification of S1PR1, S1PR3, and the reference gene 18S, using TaqMan Gene Expression Assays were individually tested across a 10^3 – fold range of cDNA quantities. Efficiencies were within 10 % of maximum for all genes: Figure 21 is an example efficiency curve, generated using a TaqMan Gene Expression Assay for S1PR1.

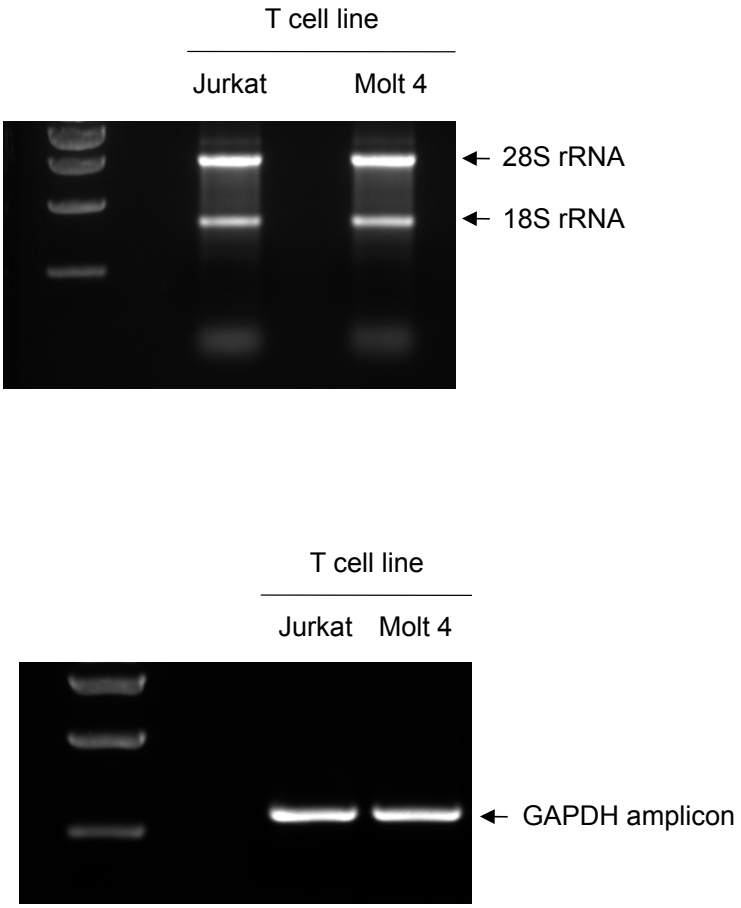


Figure 20. Quality assessment of isolated RNA and synthesised cDNA pool.
RNA was isolated from Jurkat and Molt 4 T cells. Upper panel, 1 μg samples of RNA were heated to 65 °C for 5 minutes then separated by agarose gel electrophoresis. The gel was pre-stained with ethidium bromide so that the nucleic acid could be visualised under UV illumination. Lower panel, RNA samples were reverse transcribed to generate a pool of cDNA. A 570 bp sequence within the GAPDH gene was amplified by PCR using specific primers. The PCR products were separated by agarose gel electrophoresis and the gel imaged under UV illumination. Data are from a single experiment.

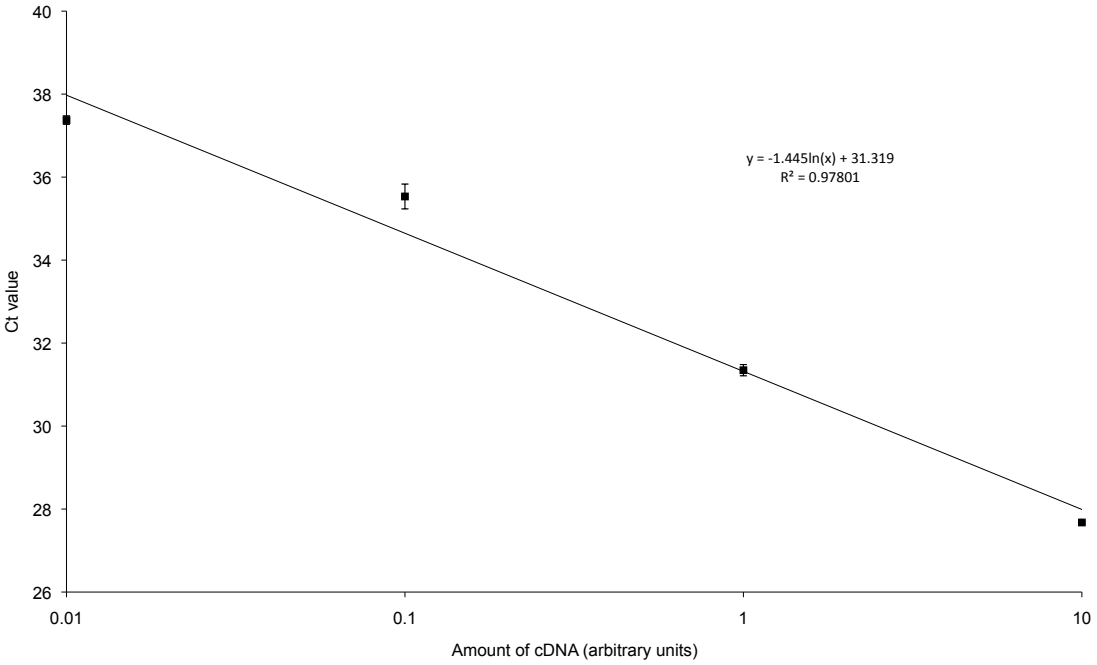


Figure 21. The S1PR1 target was amplified efficiently by real-time PCR over a range of different starting quantities of cDNA.
RNA isolated from Molt 4 T cells was reverse transcribed to cDNA. The preparation was serially diluted and those samples amplified by semi-quantitative real-time PCR using an exon-spanning S1PR1-specific TaqMan Gene Expression Assay. Graph shows means ± s.d. Data are from a single experiment.

The expression of S1PR1 and S1PR3 in the Jurkat and Molt 4 T cell lines were compared. S1PR1 expression was more than three times higher in Molt 4 than Jurkat T cells. Levels of S1PR3 were similar. Using a polyclonal antibody against S1PR1 (different to that used earlier to stain for surface receptor), Molt 4 T cells were shown to express more protein in whole cell lysates than Jurkat T cells (Figure 22).

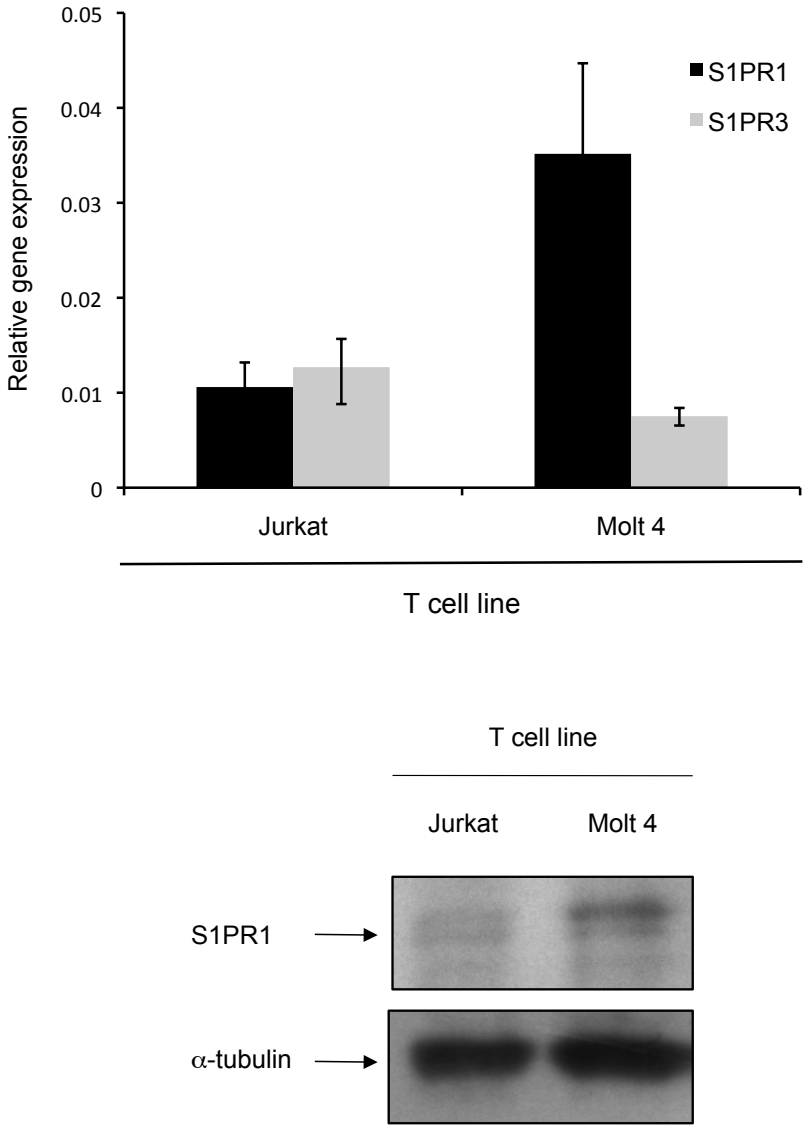


Figure 22. Molt 4 T cells expressed more S1PR1 than Jurkat T cells.
Upper panel, cDNA was prepared from Jurkat and Molt 4 T cells. Expression of S1PR1 and S1PR3 were determined by semi-quantitative real-time PCR. 18S was the reference gene. Graph shows means ± s.d. Lower panel, S1PR1 protein was detected in Jurkat and Molt 4 T cell lysates by western blotting. The PVDF membrane was stripped and probed for α-tubulin to show equal protein-loading. Data are representative of two independent experiments.

Similar to Jurkat T cells, Molt 4 T cells expressed surface CXCR4 (Figure 24). Pre-treatment with S1P did not, however, affect their migration towards CXCL12 (Figure 23). This might point to an important role for the quantity of S1PR1 in determining the ability of S1P to modulate cellular responses to chemokines.

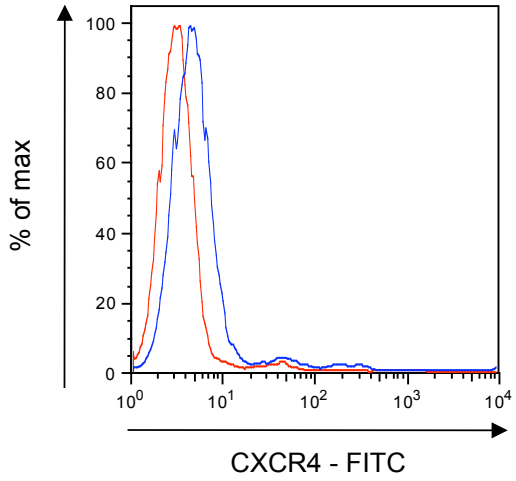


Figure 24. Molt 4 T cells expressed CXCR4.
Molt 4 T cells were stained with a fluorescently-labelled monoclonal antibody against CXCR4 (blue) or an isotype-matched control (red), then analysed by flow cytometry. Data are representative of three independent experiments.

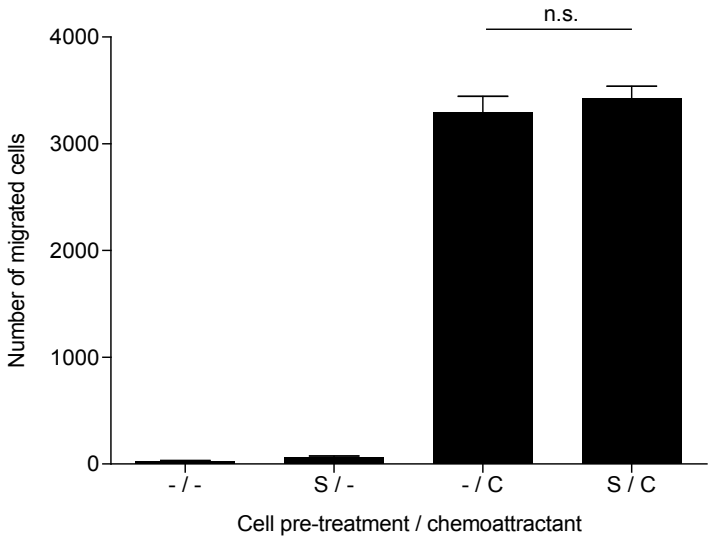


Figure 23. The efficiency of chemotaxis of Molt 4 T cells towards CXCL12 was unaffected by S1P.
Molt 4 T cells were incubated for 1 hour with 1 μM S1P (S) or vehicle (-), then left to migrate for 2 hours towards either 6.25 nM CXCL12 (C) or vehicle (-). Migrated cells in the lower compartment were enumerated by flow cytometry. Data are representative of two independent experiments.

3.4 Discussion

The role of chemokines in directing T cell migration in basal conditions, and in inflammation, is well established. CXCL12 for example is produced constitutively and contributes to homeostatic functions such as the entry of T cells into lymph nodes (Bai *et al.*, 2009). More recently it has been shown that S1P signalling is required for T cell egress from lymphatic tissue (Matloubian *et al.*, 2004). Here, using functional assays of cell migration and receptor signalling, the effect of the endogeneous lysophospholipid S1P on T cell responses to the chemokine CXCL12 were studied. These findings support a model in which S1P and chemokines do not solely have separate functions, but one where there is crosstalk between their signalling pathways. The increased complexity of regulation might allow for finer control of T cell migration in different immune compartments and immune states.

For the experiment represented in Figure 8, Jurkat T cells were treated with 1 μM S1P for various times and then cytospins prepared and stained for S1PR3. The preparations were examined using confocal laser-scanning microscopy. The data suggest that S1PR3 was internalised after treatment of serum-starved cells with S1P. However, an accompanying time-course of treatment of cells with vehicle was not included, and therefore spontaneous recycling of the receptor with time cannot be ruled out. Also, distortions of the cell membrane caused by cytospinning render this method of analysis of receptor recycling unreliable. Others have shown internalisation of S1PR3 from the surface of receptor-transfectants following treatment of cells with S1P. In their experiment they treated the cells with 1 μM S1P for 30 minutes, and showed using surface receptor staining and flow cytometry that about 60 % of the receptor was lost from the cell surface (Jongsma *et al.* 2009).

Jurkat T cells expressed S1PR1 and S1PR3 at a similar level, measured by real-time PCR. However, while S1PR3 protein was easily labelled, the monoclonal antibody against S1PR1 used here stained the surface of Jurkat T cells poorly. The internalisation of this receptor on application of ligand to intact cells could therefore not be studied directly. Others have reported similarly poor staining of S1PR1 (M. Graeler and E. Goetzl, personal communication). Most of the published work on the quantitative aspects of S1PR1 expression has either involved use of real-time PCR or the protein has been examined *in situ* by using

epitope-tagged receptor constructs (FLAG; Matloubian *et al.*, 2004) or polyclonal antibodies (Lo *et al.*, 2005). The weak staining of S1PR1 on Jurkat T cells probably implies a low amount of receptor on the cell surface. Nevertheless, the S1PR1-specific agonist SEW2871 strongly enhanced cell migration towards CXCL12, indicating that the receptor was at a functionally significant level. Used at a concentration of 10 μM, SEW2871 is highly selective for S1PR1 (Gonzalez-Cabrera *et al.*, 2007). These results support a model in which S1PR1 signalling is important in the modulation of lymphocyte migration.

S1PR1, like S1PR3 and many other GPCR, undergoes homologous desensitisation on ligand binding, by internalisation of the receptor from the cell surface and protein degradation (Liu *et al.*, 1999; Oo *et al.*, 2007). S1PR1 is unusual in its sensitivity to, and speed of internalisation (Schwab *et al.*, 2005; Grigorova *et al.*, 2009). After 1 hour of stimulation with 1 μM S1P, little S1PR1 probably remains on the surface of Jurkat T cells. A reciprocal relationship in surface expression of S1PR1 and CXCR4 would provide a simple explanation for the observed enhancement in migration towards CXCL12 after S1P treatment. It has been reported that overexpression of S1PR1 in haematopoietic progenitor cells resulted in suppression of their migration towards CXCL12, and that this was associated with a 1.8 fold decrease in CXCR4 expression (Ryser *et al.*, 2008). CXCR4 can be held in intracellular stores, and moved rapidly to the surface of lymphocytes on PMA stimulation (Forster *et al.*, 1998). Conceivably, CXCR4 could be translocated quickly from intracellular stores to the plasma membrane following downregulation of S1PR1. In these experiments, however, S1P treatment did not affect the intensity of staining of surface CXCR4, suggesting that an upregulation of the receptor does not occur.

Heterodimerisation of chemokine receptors can subtly alter their signalling responses. CCR5/CCR2 dimers associate with Gα11 rather than Gαi, and this biases towards signal-induced changes in cell adhesion rather than chemotaxis (Mellado *et al.*, 2001). In neutrophils, S1PR3 heterodimerises with the chemokine receptor CXCR1 (Rahaman *et al.*, 2006). As there is a high degree of homology between the S1P receptors, it is possible that CXCR4 and S1PR1 could complex on the surface of Jurkat T cells. The observation that S1P treatment did not affect CXCL12-mediated inhibition of adenylate cyclase suggests that G protein coupling to CXCR4 was not significantly altered.

Nevertheless, it remains a possibility that heterodimerisation of S1PR1 and CXCR4 may have a significant role in the effect of S1P on migration. Analysis of protein-protein interaction on the cell surface, using flow cytometry (and fluorescence resonance energy transfer between immunolabelled receptors) and co-immunoprecipitation, is needed to answer this question.

Jurkat T cells are deficient in expression of the enzyme PTEN, which dephosphorylates PIP_3 in the plasma membrane (Shan *et al.*, 2000). High basal levels of PIP_3 probably explain the constitutive phosphorylation of Akt at S473 seen here. The observation that phosphorylation was not further increased on stimulation with CXCL12 was however unexpected. Jurkat T cells migrate efficiently towards CXCL12, and this presumably involves enrichment of PIP_3 at the leading edge of cells. The formation of 'signalling patches' in the plasma membrane, to which pleckstrin homology domain-containing proteins are bound, has been reported as a mechanism for the sensitisation of chemotactic responses (Postma *et al.*, 2004). Although S1P treatment did not cause any detectable change in the amount of phospho-Akt, modulation of signalling downstream of PIP_3 cannot be ruled out.

Lck was flagged as a protein of interest following the phospho-kinase array screen. The kinase is known to be phosphorylated and activated on stimulation of Jurkat T cells with CXCL12, and is necessary for chemotaxis towards that ligand (Inngjerdingen *et al.*, 2002). Lck has also been implicated as having a significant role in the cooperative action of the TCR and CXCR4 (Kumar *et al.*, 2006). Phosphorylation of Lck at the activatory residue Y394 following chemokine stimulation was very variable, and no consistent effect of S1P treatment on this response was observed. A similar lack of reproducibility in assays to probe for phospho-Lck has been reported by others (Oreste Acuto, personal communication). Recent work has cast doubt on whether phosphorylation at Y394 is even a good marker of active kinase. Activated Lck is normally present in resting T cells, and TCR signalling does not increase the amount of it. Receptor signalling may licence existing pre-activated Lck to phosphorylate its substrates – perhaps through changes in conformation or localisation of the latter (Nika *et al.*, 2010).

Pre-treatment of Molt 4 T cells with S1P did not increase their migration towards CXCL12. The fact that their expression of S1PR1 was more than three times

higher than that of Jurkat T cells is probably significant. As previously stated, overexpression of S1PR1 has been associated with suppression, not enhancement, of migration towards CXCL12 (Ryser *et al.*, 2008). Analysis of the dynamics of S1PR1 and CXCR4 expression on the cell surface after S1P treatment will help resolve this question.

Chapter 4. Regulation of S1PR1 Signalling in Primary T cells and S1P-mediated Effects on Cell Chemotaxis

Regulation of S1PR1 Signalling in Primary T cells and S1P-mediated Effects on Cell Chemotaxis

4.1 Introduction

In the course of its lifetime, a T cell may adopt one of a number of different migration programmes. They are initiated on receipt of signals from the environment and ensure appropriate cell movement for implementation of function at a particular stage. Altered migratory behaviour is associated with changes in the complement of cell surface and intracellular molecules, including chemokine receptors and S1PR1. Resting T cells express chemokine receptors such as CCR7 and CXCR4 whose ligands are expressed constitutively by lymphatic tissues, and which direct cell recirculation (Bai *et al*., 2009). Receptors such as CXCR3 and CCR5 bind ligands that are expressed at inflamed sites in the periphery and direct the trafficking of effector T cells to those locations.

During immunosurveillance, under normal conditions, T cells continuously recirculate through lymphoid organs. Entry to lymph nodes is driven primarily by CCR7, and exit by antagonistic S1PR1 signalling at egress structures (Pham *et al.*, 2008). The mean residence time of T cells in a particular lymphoid compartment is usually between 6 and 12 h (von Andrian and Mempel, 2003). Recycling of S1PR1 to the cell surface is quite rapid after T cell entry, causing a return of sensitivity to egress signals within 20 minutes (Grigorova *et al.*, 2010). The time it takes for randomly moving T cells to encounter egress structures, and the relative strengths of CCR7 and S1PR1 -mediated signals transduced there, are probably the primary determinants of cell residence time.

In a primary adaptive immune response, naïve T cells are activated within lymphoid tissue by interaction with antigen presenting cells. We know that the naïve T cells are arrested by interaction with the pAPC, and this presumably prevents them from accessing egress structures. Naïve T cells can form stable conjugates with DC bearing cognate peptide-MHC for up to 12 hours (Mempel *et al.*, 2004). TCR triggering of T cells also causes a complete loss in sensitivity to S1P, and therefore susceptibility to lymph node egress signals. 24 hours after activation with CD3/CD28 antibodies, mouse splenic T cells responded negligibly to S1P in chemotaxis assays. This was associated with a complete loss of S1PR1 protein expression (Graeler and Goetzl, 2002). A similar loss in

S1PR1 gene expression and S1P-mediated chemotaxis one day after activation has been observed *in vivo*, in the context of antigen-specific activation (Matloubian *et al.*, 2004). The differentiated daughter cells of the original activated parent leave typically between 3 and 5 days after activation (Reinhardt *et al.*, 2001).

Lymph node shutdown is a phenomenon triggered during certain immune responses and is characterised by a transient and profound reduction in T cell egress from lymphatic tissue (Korngold *et al.*, 1983; Shiow *et al.*, 2006). T cells are retained regardless of their specificity. It is speculated that this is a mechanism for increasing the probability of a productive adaptive immune response. An increase in the number of T cells scanning mature pAPC within the node clearly increases the chance of a rare specific T cell being activated. Previous *in vivo* work has implicated interferon α/β signalling, acting via upregulation of CD69 on T cells, as critical for egress blockade (Shiow *et al.*, 2006).

CD69 interacts directly with S1PR1, inactivating signalling of the receptor and promoting its internalisation and degradation (Bankovich *et al.*, 2010). T cells that enter an inflamed lymph node go from showing no or negligible surface expression of CD69 to clearly detectable levels within an hour of entry (Shiow *et al.*, 2006; Grigorova *et al.*, 2010). This is important, as modelling implies that half of the T cells entering a typical lymph node will contact cortical sinuses within 2 hours of entry (Grigorova *et al.*, 2010). Rapid upregulation of CD69 is therefore required in order to block S1PR1-mediated egress of a large proportion of recently ingressed cells.

Although published work shows a role for CD69 in regulation of T cell egress from inflamed lymph nodes, it has remained unclear whether CD69 plays an important role in the context of cell activation through the antigen-specific receptor. T cell activation by antigens or mitogens has been known for some time to cause rapid and potent upregulation of CD69 on the cell surface, usually peaking at 24 – 48 h, and that it can persist at detectable levels for six days (Hara *et al.*, 1986). Stimulation of peripheral blood T cells with anti-CD3 mAb resulted in expression of CD69 within 2 hours (Testi *et al.*, 1989), with the antigen detectable two days later. PMA stimulation induced detectable levels of CD69 at 3 hours, with maximum surface protein 12 hours after activation

(Lopez-Cabrera *et al.*, 1993). These studies indicate that CD69 is present soon after cell activation and could potentially have a role in regulation of S1PR1 signalling in this context.

Here, anti-CD3/28 beads have been used to activate human peripheral blood T cells. CD69 was rapidly upregulated to very high levels and then diluted on cell division. $CD69^{hi}$ T cells, 24 hours after activation, did not respond to stimulation with the S1PR1-specific agonist SEW2871 by phosphorylation of Akt. Three days after activation, when the majority of cells were $CD69^{lo}$, a signalling response was observed. In addition, S1P significantly enhanced the migration of these T cells towards a prototypical inflammatory chemokine. Attenuation of CD69 expression on 24 hour -activated T cells using specific small interfering ribonucleic acid (siRNA) increased their response to S1PR1 stimulation. These observations support a model in which CD69 plays an important role in control of S1PR1 signalling in T cells activated through CD3 and CD28.

The aims of this chapter were as follows:

To study the dynamics of CD69 and S1PR1 expression following activation of T cells through CD3 and CD28

To probe for simultaneous changes in the ability of S1PR1 to signal

To identify whether there was a quantitative relationship between the surface expression of CD69 and the signalling capacity of S1PR1

To study the effect of S1P receptor stimulation on the migration of resting and activated primary T cells

4.2 Materials and Methods

Receptor ligands

D-erythro sphingosine 1-phosphate (S1P) and SEW2871 were purchased from Enzo Life Sciences; CXCL10 and CXCL12 from R&D Systems.

Cell isolation, culture and activation

Total $CD3^+$ T cells were derived from human peripheral blood or platelet-depleted leukocyte cones. They were isolated using an erythrocyte-rosetting negative-selection kit (Stem Cell Technologies) and density-gradient centrifugation (Lympholyte H, Cedarlane Laboratories). T cells were cultured in X-VIVO 15 medium (Lonza) and activated with Dynabeads Human T Activator CD3/CD28 (Invitrogen), at a ratio of one bead per cell and a starting culture density of 1×10^6 cells per ml.

Antibodies, cell labelling and flow cytometry

T cells were labelled for 5 minutes at 37 °C with 1 μM CFSE (Sigma Aldrich) in PBS containing 0.1 % (v/v) FBS. They were chilled rapidly on ice then washed with cold RF10 before use.

T cells were stained with various fluorochrome-conjugated monoclonal antibodies or appropriate isotype-matched control antibodies. The specific antibodies used were: S1PR1 (218713; R&D Systems), CXCR4 (12G5; R&D Systems), CD3 (CD3-12; AbD Serotec), CD69 (FN50; BD Pharmingen), CXCR3 (49801; R&D Systems). Data was acquired on a FACSCanto II instrument (BD) and analysis performed using FACSDiva (BD) and FlowJo (Treestar) software.

Real time PCR

RNA was prepared from cell pellets using TRI Reagent (Sigma). cDNA was synthesised using SuperScript III Reverse Transcriptase and random hexamers as primers (Invitrogen). Expression of S1PR1 was measured semi-quantitatively using specific TaqMan Gene Expression assays (Hs00173499_m1), with 18S as the reference (Hs99999901_s1; both Applied Biosystems). The amplifications were run on an ABI Prism 7000 instrument (Applied Biosystems).

Protein assays

Proteins were subject to SDS-PAGE and transferred to PVDF membrane as previously described. The primary antibodies used for immunoblotting were: phospho-Akt [Ser473] (D9E; Cell Signaling Technology) and Akt [pan] (C68E7; Cell Signaling Technology). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG (A6154; Sigma Aldrich).

Phospho-Akt [Ser473] and Akt [pan] were also quantified using a cell-based ELISA (R&D Systems). Approximately 10 000 T cells were added to each well of a 96 well-format plate (coated with 10 μg/ml poly-lysine (Sigma Aldrich) for 30 minutes) and left to attach for 1 hour. The cells were then treated as desired before fixation with 8 % formaldehyde. Phospho-Akt and total Akt were detected by double immunoenzymatic labelling. The relative quantities of these proteins were deduced from the spectrally distinct fluorescence of substrates associated with each target. The detector was a Dynex MFX instrument operating with excitation and emission wavelengths of 540 nm and 600 nm (phospho-Akt) and 360 nm and 450 nm (Akt) respectively.

Gene knockdown

Resting T cells were transfected with 100 pmol siRNA per million cells by electroporation (Nucleofector I instrument; Amaxa, Lonza). For CD69 knockdown, an equimolar mixture of three 21 nucleotide duplexes (MISSION siRNA; Sigma Aldrich) were used. The single strand sequences were:

5’ GAGUUAGAUGUUGGUACUA 3’

5’ CUACUCUUGCUGUCAUUGA 3’

5’ CUCUCAUUGCCUUAUCAGU 3’

Separately, as a control, cells were transfected with an equal mass of MISSION siRNA Universal Negative Control 1 (Sigma Aldrich).

The cells were rested for 5 hours after transfection then activated with Dynabeads Human T Activator CD3/CD28 at a ratio of 1 bead per T cell.

In some cases, siRNA transfected cells were sorted 24 h after activation. This was done using a FACSAria II (BD) instrument, using the forward and side scatter characteristics of the input cells.

Chemotaxis assays

Resting or three day-activated T cells migrated across 96 well-format 3 or 5 μm polycarbonate filters (Corning Costar) respectively for 2 h at 37 °C in 5 % CO_2. The total volumes added to the upper and lower compartments were 75 and 235 μl respectively. The base medium for the assays was FBS-free RPMI 1640 with 0.5 % fatty acid-free BSA (Sigma Aldrich). On completion of the assay, migrated cells were mixed with a known number of Countbright beads (Invitrogen) and enumerated by flow cytometry.

Statistics

Prism 4.0c (GraphPad Software) was used for all statistical analyses. Comparisons between groups were made using the two-tailed Student's *t* test for unpaired data. P values less than or equal to 0.05 were considered significant (*), $P \leq 0.01$ highly significant (**), and $P \leq 0.001$ very highly significant (***).

4.3 Results

For these experiments, T cells were isolated either from fresh peripheral blood or leukocyte-enriched cones. The T cells were negatively selected using an erythrocyte-rosetting antibody cocktail and density gradient centrifugation. Purity was checked by staining a sample of the isolated cells with a fluorescently-conjugated monoclonal antibody against CD3. Figure 25 shows a typical scatter plot and fluorescence histogram: here, 98 % of the cells in the marked FSC / SSC gate expressed CD3. Purity was consistently above 95 % over several experiments.

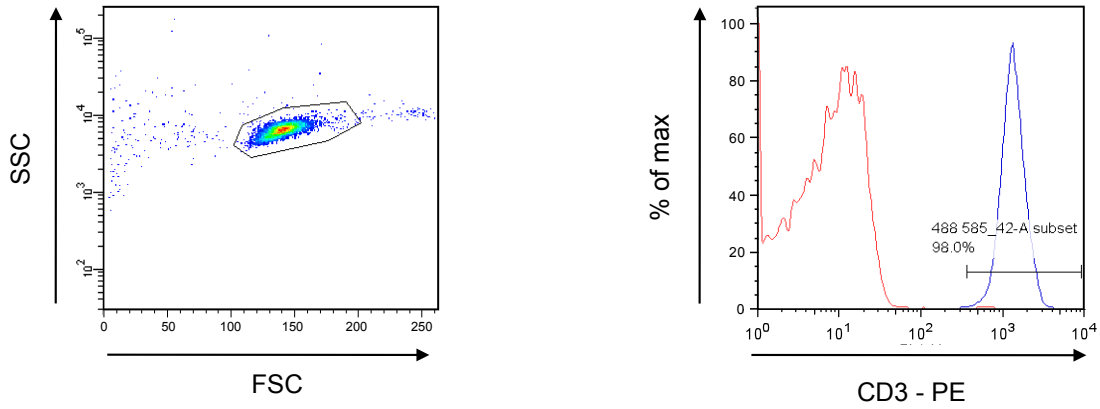


Figure 25. Negative selection was used to isolate highly pure populations of human resting T cells.
Peripheral blood or leukocyte-enriched cones were mixed with an erythrocyte-rosetting negative selection cocktail of antibodies against antigens present on non-CD3+ T lymphocyte blood cell subsets. T cells were separated from rosetted cells and other components of blood in a density gradient centrifugation process. Purified cells were stained with an anti-CD3 monoclonal antibody and analysed by flow cytometry. Left panel: representative FSC/SSC dot plot. Right panel: cells in gate shown in left panel represented. Isotype control stained cells red, CD3 monoclonal antibody stained cells blue (represents approx. 9500 events). Routine control experiment.

Peripheral blood T cells were rested overnight in serum-free conditions and then tested for expression of S1PR1. There was no excess staining with a monoclonal antibody against S1PR1 over that of the isotype-matched control antibody (Figure 26).

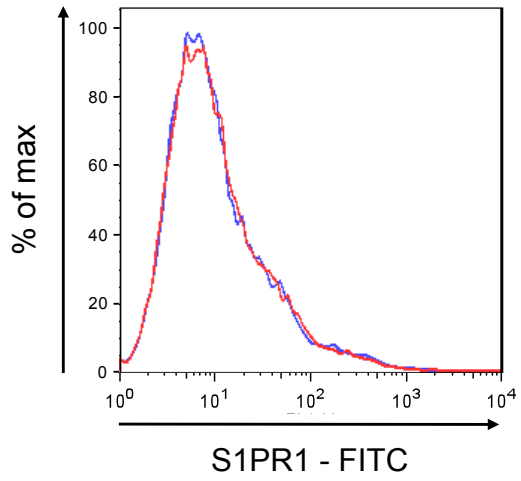


Figure 26. Resting T cells showed no surface staining with a monoclonal antibody against S1PR1.
Resting T cells were stained with a fluorescent monoclonal antibody against S1PR1 (blue) or isotype-matched control antibody (red), then analysed by flow cytometry. Data are representative of three independent experiments.

As Jurkat T cells did also not stain to any significant extent with the same antibody (Chapter 3), but did respond to selective agonism of S1PR1 in various signalling assays, protein expression was not discounted. Indeed, RT-PCR showed expression of S1PR1 in resting T cells. Gene expression was significantly lower in T cells that had been activated with CD3/28 beads, at both one day and three days after their addition to culture (Figure 27).

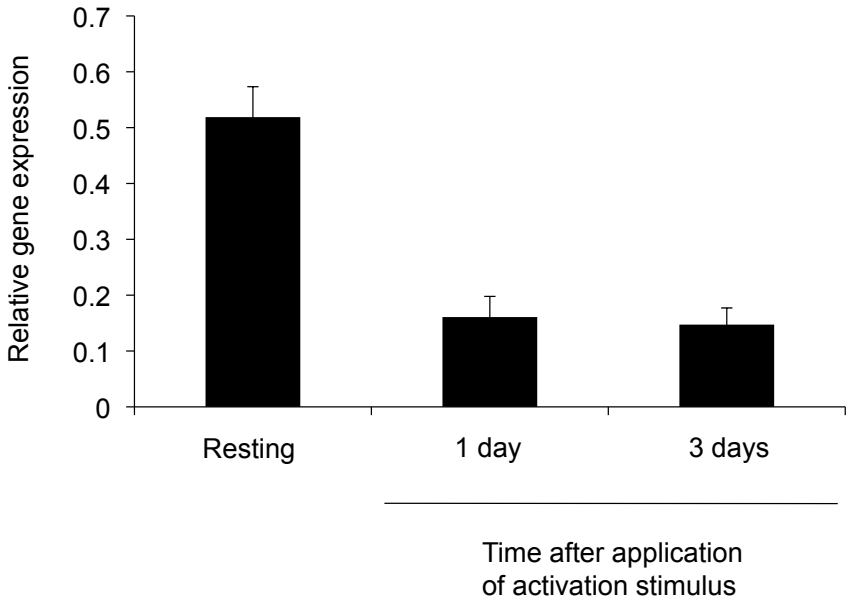


Figure 27. S1PR1 was expressed by resting T cells and downregulated on activation of them with CD3/28 beads.
T cells were left at rest or activated for one or three days with beads coated with agonist antibodies specific for CD3 and CD28. Total RNA was isolated from the cell populations, which was then reverse transcribed to generate cDNA. The relative expression of S1PR1 was determined by semi-quantitative RT-PCR; 18S was the reference gene. Data are representative of two independent experiments.

CD69, induced on the cell surface by type I interferons, is known to be an important regulator of S1PR1 (Shiow *et al*., 2006). Before investigation of the dynamics of CD69 expression after T cell activation by CD3/28 beads, it was first established whether or not freshly isolated peripheral blood T cells showed any surface expression of that protein. Immunofluorescence flow cytometry was used to show that only a small proportion of such T cells showed expression of CD69 on the cell surface (here, just over 1 % stained positive for CD69 (Figure 28)).

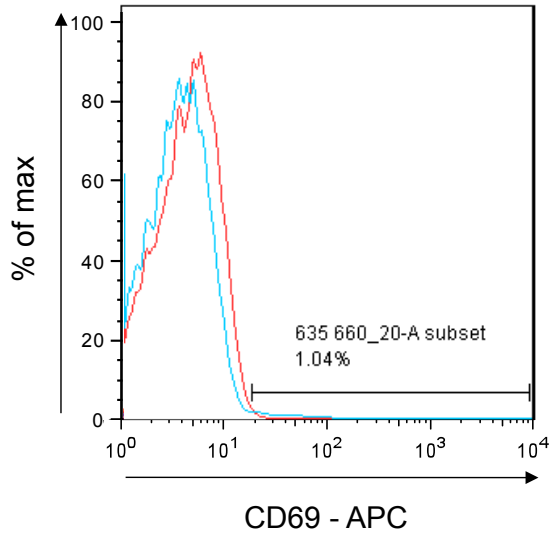


Figure 28. A low proportion of T cells isolated from peripheral blood stained positively for CD69.
T cells isolated from peripheral blood were stained immediately with a fluorescently-conjugated monoclonal antibody against CD69 (blue) or isotype-matched control antibody (red), and analysed by flow cytometry. Data are representative of three independent experiments.

Next, samples of resting T cells were activated with CD3/28 beads for various times, and the kinetics of upregulation of CD69 on the cell surface studied by immunofluorescence flow cytometry (Figure 29). After 4 hours a significant proportion of cells were $CD69^{+}$, and by 24 hours almost all cells expressed the protein, and at a high level. The median fluorescence intensity values for the experiment shown were: resting, 93; 1 h, 100; 2 h, 140; 4 h, 1039; 8 h, 1886; 16 h, 4475; 24 h, 21 839.

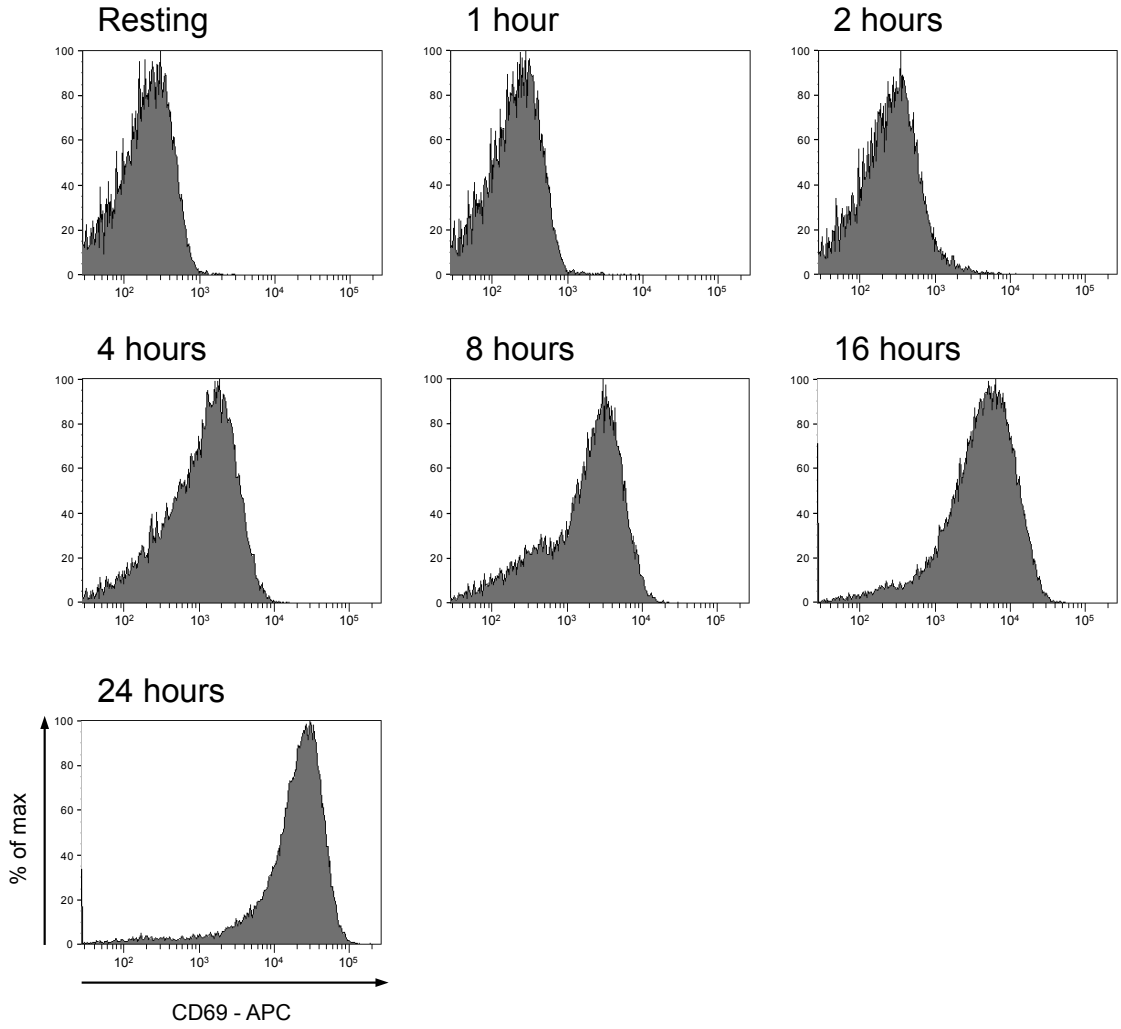


Figure 29. T cells activated with CD3 / 28 beads progressively upregulated CD69 over the course of 24 hours.
T cells were left unactivated or stimulated for various times with CD3/28 beads then stained with a fluorescent monoclonal against CD69 and analysed by flow cytometry.

CD69 does probably not play an important role in regulation of cell motility in the few hours immediately after activation. *In vivo*, adhesive interactions between T cells and professional antigen presenting cells centred on the immune synapse are most important in mediating lymphocyte arrest in this time window. Our primary goal in this study was to understand in greater detail the dynamics of CD69 expression in the days following activation, when it may be more important. To do this, T cells were labelled with CFSE, activated with CD3/28 beads, and then stained at various times for CD69. Dye labelling was used so that in addition to following CD69 expression with time, any correlation with cell division could be noted.

T cells are known to respond to a number of stimuli, including various types of cell stress, by upregulating CD69. It was therefore necessary to test whether loading the cells with CFSE had any effect on CD69 expression. In the cases of resting, one day –activated and six day-activated T cells, CFSE loading had no significant effect on measured protein expression (Figure 30).

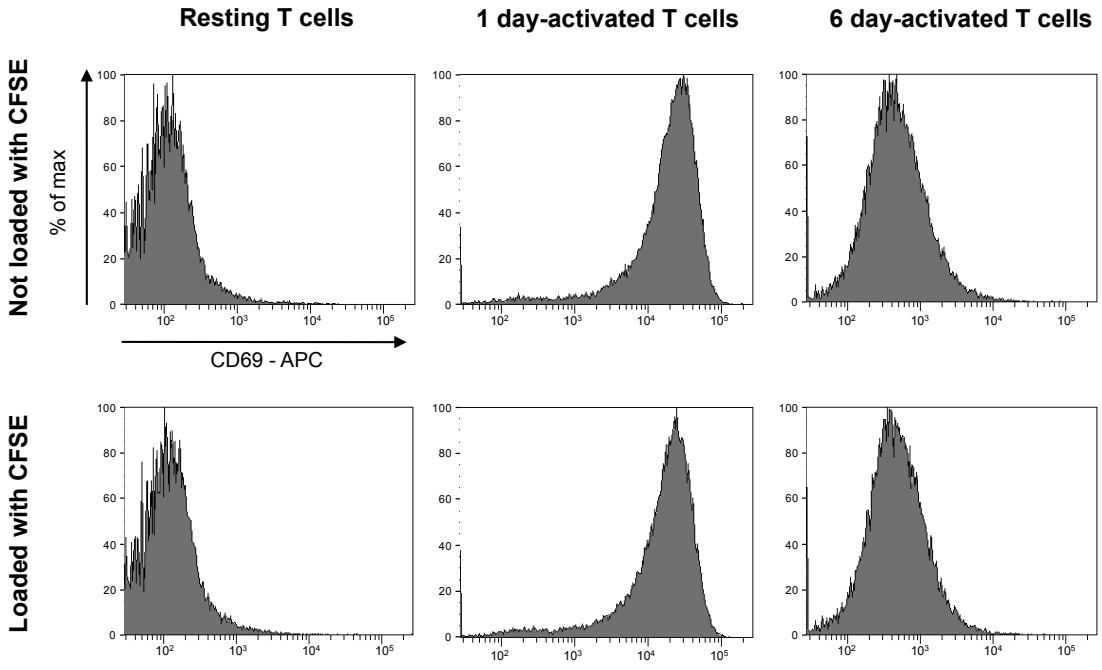


Figure 30. Loading with CFSE did not cause CD69 to be expressed by resting T cells or affect the upregulation of CD69 following cell activation.
T cells were loaded with carboxyfluorescein succinimidyl ester (CFSE) or not then left overnight. They were then either left unactivated or stimulated with CD3/28 beads for one or six days. The cells were stained with a fluorescently-conjugated monoclonal antibody against CD69 and analysed by flow cytometry.

T cells were loaded with CFSE and then either left to rest or activated with CD3/28 beads. Samples of resting T cells and 1 day, 3 day and 6 day -activated T cells were analysed for expression of CD69 by flow cytometry (Figure 31). Very few T cells in the resting population expressed CD69, whereas more than 95 % of the cells stained strongly for CD69 24 hours after activation. The amount of CD69 on the cell surface decreased with time. The majority of T cells at day 3 still expressed CD69 (86.2%), whereas by day 6 $CD69^{+}$ cells were in the minority (33.8%).

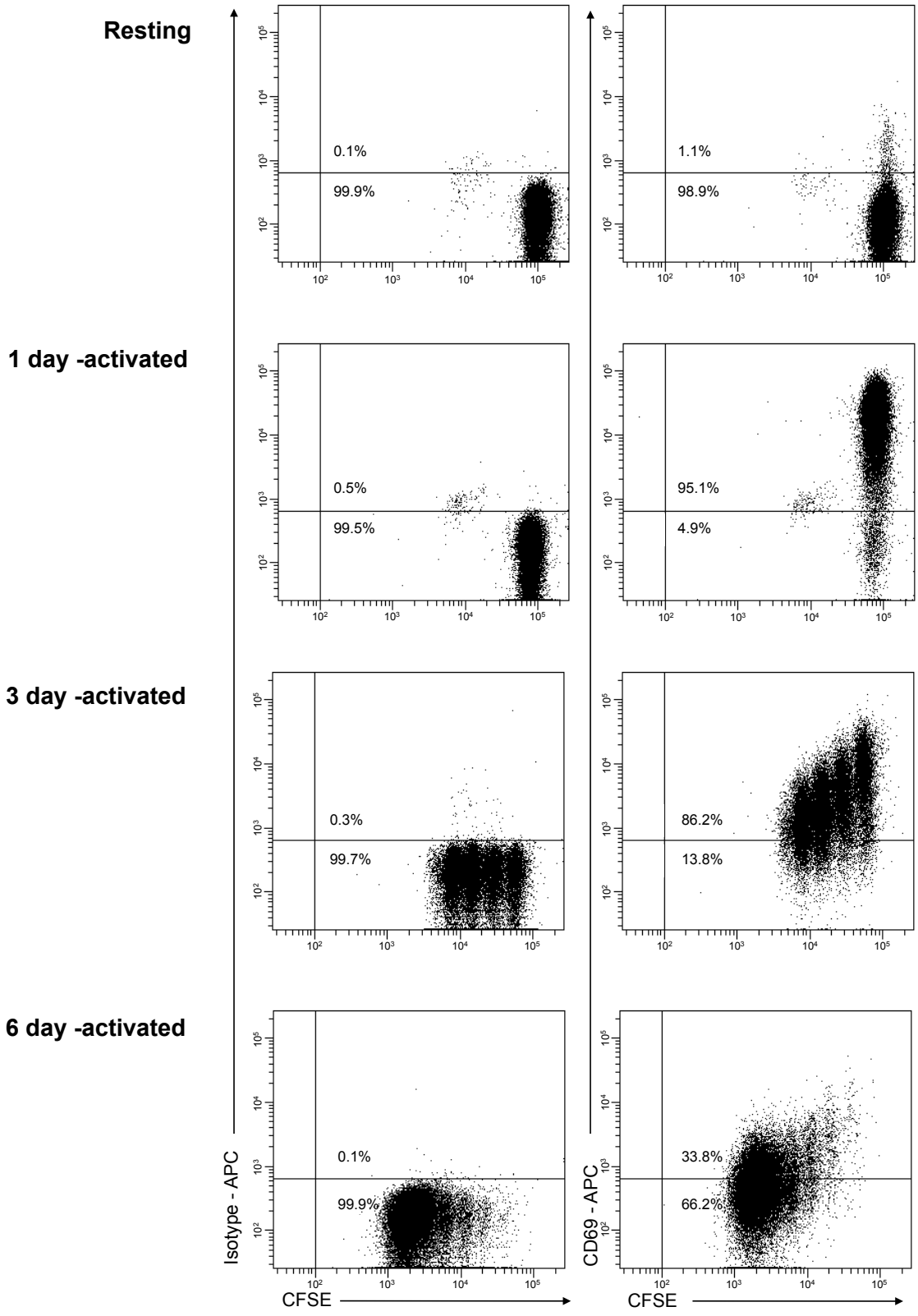


Figure 31. CD69 was rapidly and strongly upregulated on T cell activation and decreased with cell division.
Resting T lymphocytes were loaded with CFSE and either left unactivated or activated with CD3/28 beads for one, three, or six days. The cells were then stained with a fluorescent monoclonal antibody against surface CD69 and analysed by flow cytometry. Data are representative of two independent experiments.

Using the CFSE fluorescence data, a quantitative analysis was performed to compare the mean fluorescence intensity of CD69 staining between the generations of three day -activated T cells (Figure 32). The mean fluorescence intensity of CD69 staining decreased by approximately one half with each successive generation.

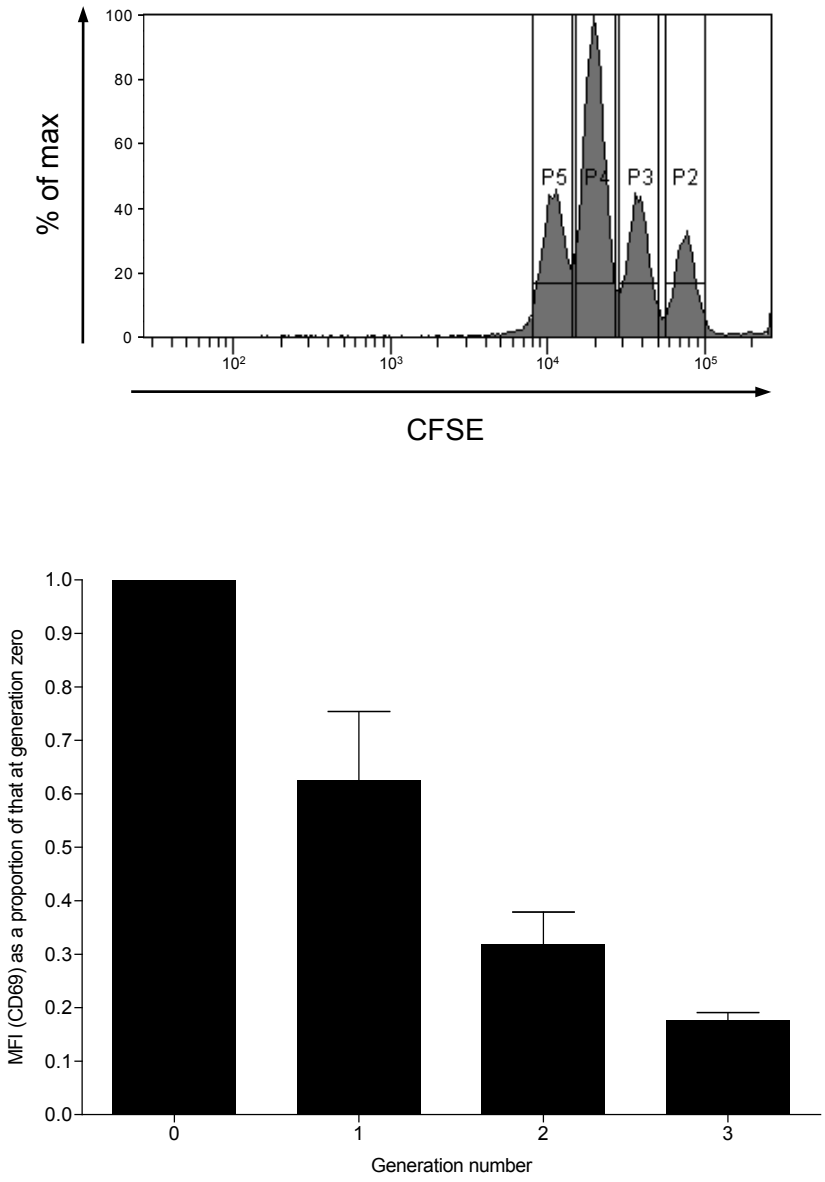


Figure 32. The decrease in surface CD69 protein was closely associated with cell division. T cells were loaded with CFSE and activated with CD3/28 beads for three days. They were stained with a monoclonal antibody against CD69, or isotype-matched control, and analysed by flow cytometry. Upper panel: distinct generations of T cells were identified by their CFSE-mediated fluorescence, and gates established using this fluorescent parameter. Lower panel: the mean fluorescence intensity of specific CD69 staining of each generation was calculated by subtracting the mean fluorescence intensity associated with the isotype-matched control antibody from that with the CD69-specific monoclonal antibody. Graph shows means ± s.e.m. Data are representative of two independent experiments.

Previous studies have implicated CD69 as a negative regulator of both S1PR1 expression and signalling capacity (Shiow *et al.*, 2006; Bankovich *et al.*, 2010). Although surface S1PR1 expression could not be detected by immunofluorescence staining, it was possible to test the ability of S1PR1 to signal. This was done by stimulating T cells with the S1PR1-specific agonist SEW2871 and then subsequently measuring the extent of Akt phosphorylation, a downstream consequence of receptor signalling.

T cells were first left at rest or activated for one, three or six days with CD3/28 beads. They were then stimulated with SEW2871 for 10 minutes and lysed. Phosphorylated (at serine 473) Akt and total Akt were detected in the cell lysates by western blotting (Figure 33, upper panel). Data from three independent experiments were analysed by densitometry, and expressed in the form of a stimulation index. The stimulation index refers to the proportional increase in amount of phospho-Akt caused by ligand stimulation, normalised to the quantity of total Akt. Resting T cells responded strongly to addition of the S1PR1 agonist, whereas no such response by one day-activated cells could be detected. Stimulation with SEW2871 caused significant Akt phosphorylation in day 3 and day 6 -activated cells, and to a similar extent of that in resting cells (Figure 33, lower panel).

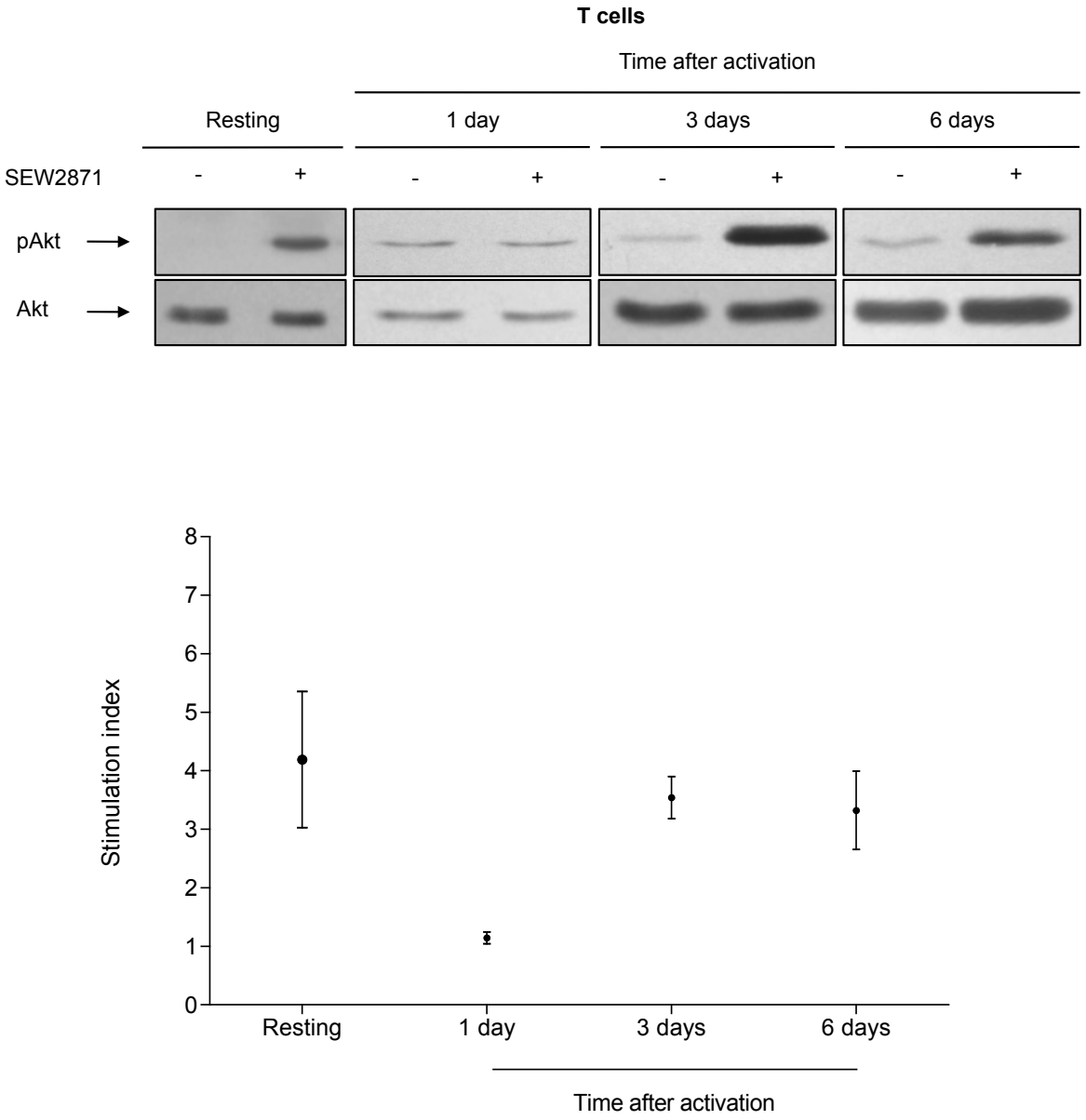
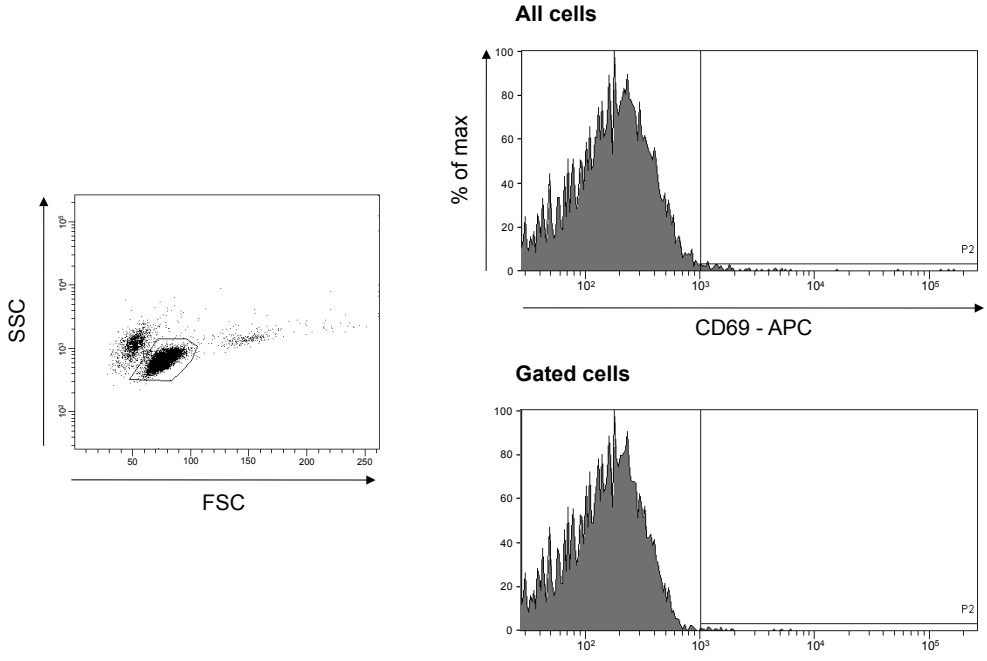


Figure 33. The response of T cells to stimulation with an S1PR1 agonist was lost 24 hours after activation but returned by day 3.
T cells were either left unactivated or stimulated for 1, 3 or 6 days with CD3/28 beads. The beads were removed from cell cultures and the cells rested for 3 hours. The cells were then stimulated for 10 minutes with 10 μM SEW2871, or vehicle, then lysed. Upper panel: pAkt (Ser473) and Akt were detected in total cell lysates by western blotting. Lower panel: graphical representation of densitometry performed on immunoblots. The stimulation index represents the proportional increase in pAkt on stimulation with SEW2871, normalised to the total quantity of Akt. Graph shows means ± s.e.m.; *n* = 3. Data are representative of three independent experiments.

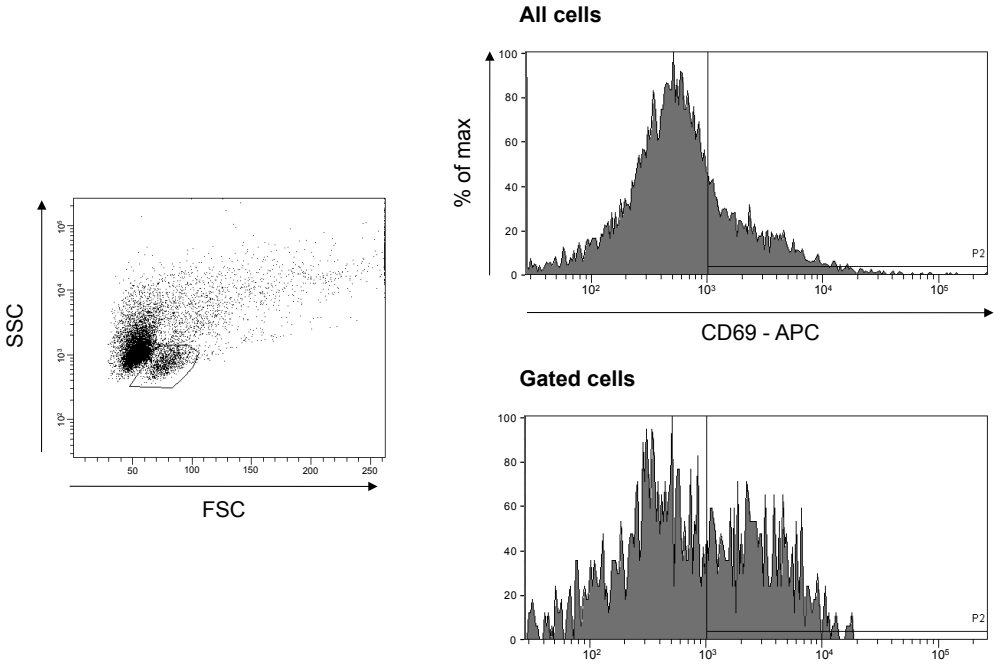
The observation that three-day activated cells responded strongly to stimulation with SEW2871 suggested that S1PR1 signalling could occur in cells expressing low levels of CD69.

To explore this further, siRNA was used to attenuate the upregulation of CD69 that follows T cell activation. T cells were transfected with negative control siRNA, with CD69-specific siRNA, or subjected to the transfection procedure without any siRNA, and left for six hours. The siRNA-transfected cells were then activated with CD3/28 beads for 24 hours. T cells transfected with CD69-specific siRNA stained 56 % less intensely for CD69 than control- transfected cells (Figure 34).

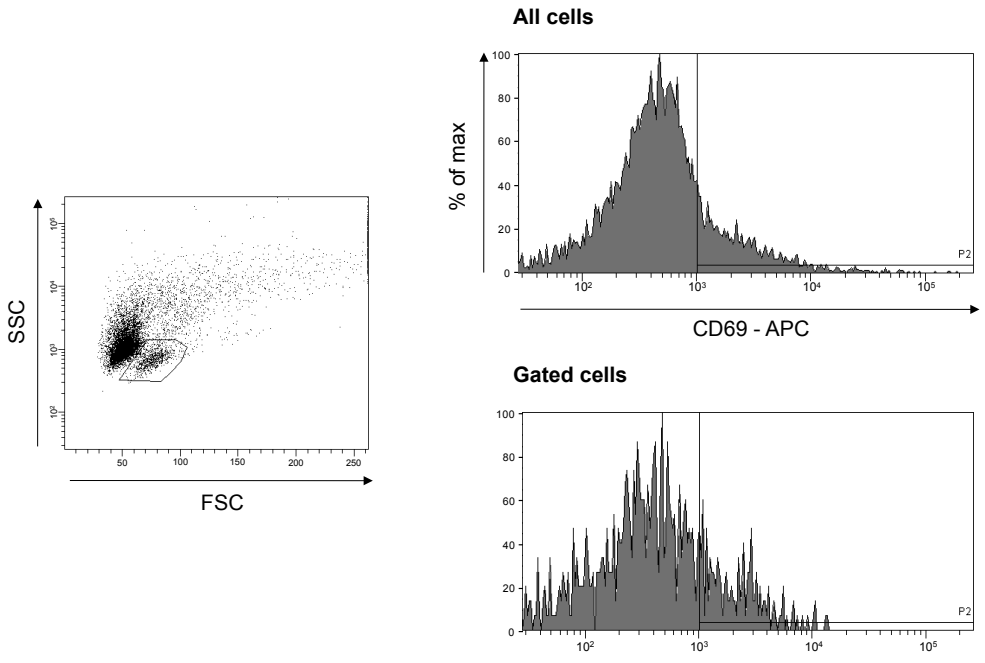
Resting T cells; no siRNA



Activated T cells; control siRNA



Activated T cells; CD69 siRNA



Population	Frequency of $CD69^+$ cells (%)		Mean fluorescence intensity (CD69)	
	All cells	Gated cells	Gated cells	Gated $CD69^+$ cells
Resting; no siRNA	0.8	0.2	-	-
Activated; control siRNA	24.8	37.0	1575	3770
Activated; CD69 siRNA	17.9	17.7	684	2787

Figure 34. Pre-loading T cells with CD69-specific siRNA reduced, but did not abolish, upregulation of CD69 following cell activation.
T cells were isolated then rested overnight. They were split into three groups: one was not transfected with siRNA and left unactivated (first panel); the second was transfected with negative control siRNA (second panel) and then the cells activated with CD3/28 beads; the third group was transfected with CD69 siRNA (third panel) and the cells activated with CD3/28 beads. 24 hours after activation the beads were removed and samples of the cells stained with a monoclonal antibody against CD69. The cells were analysed by flow cytometry. The frequency of CD69-positive cells in each population and the mean fluorescence intensity of CD69 staining is shown in the table (fourth panel). Data are from a single experiment. Histograms relating to all cells represent approx. 10 000 events, those relating to gated cells approximately 9500, 1500 and 1000 events in the first, second and third parts respectively.

The responses of the control siRNA -loaded and CD69 siRNA -loaded activated T cells to stimulation with an S1PR1-specific agonist were tested as previously described. Similar levels of phospho-Akt were detected in lysates of control and CD69 siRNA -transfected cells, whether ligand stimulated or not (Figure 35).

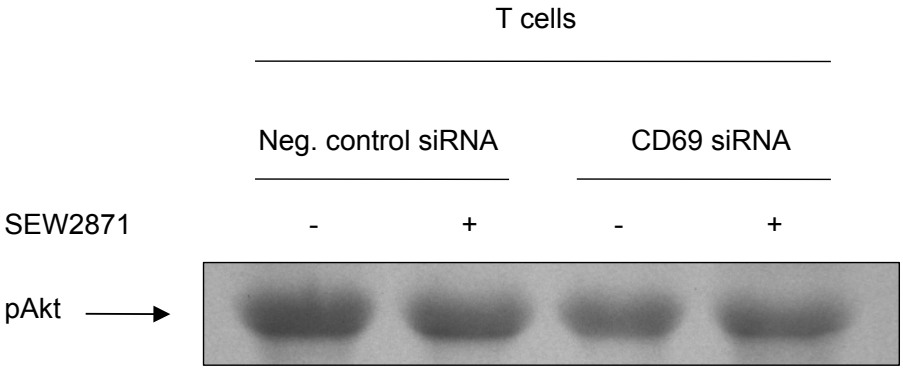
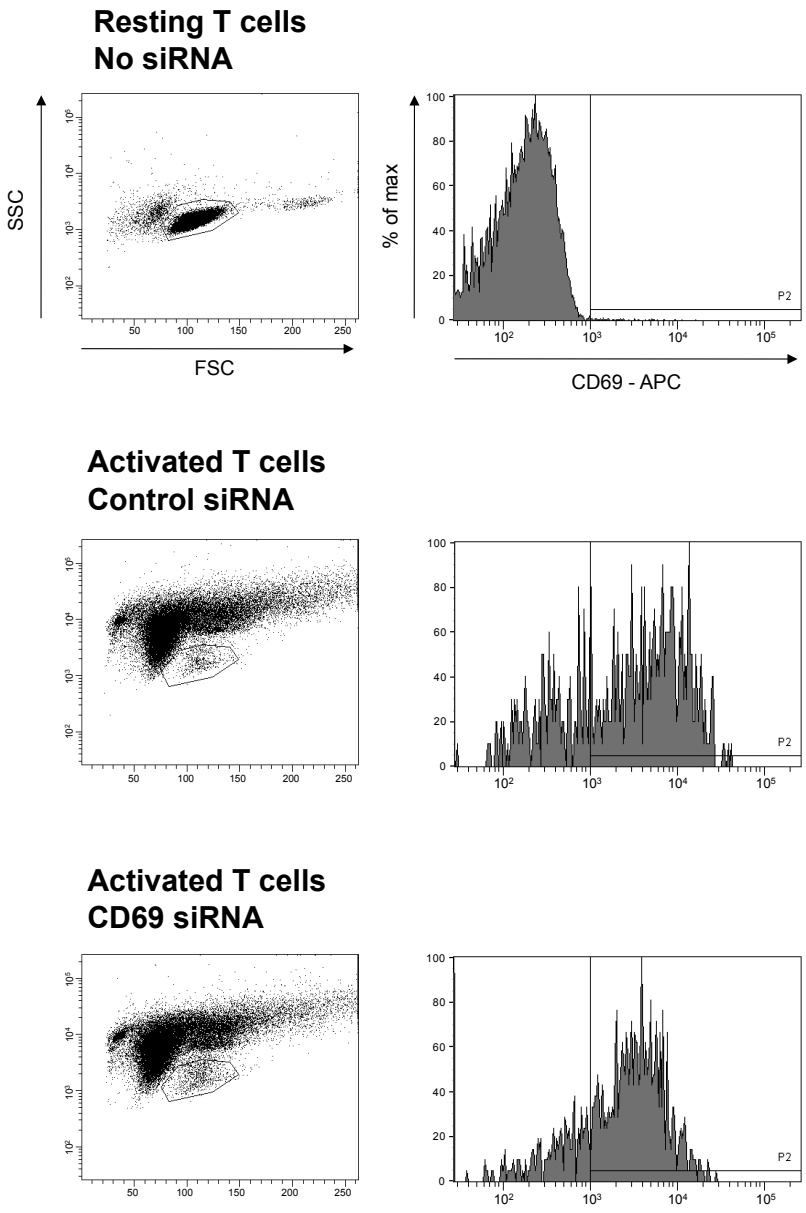


Figure 35. High levels of phospho-Akt were present in both unstimulated and SEW2871-stimulated siRNA-transfected cells.
Samples of the siRNA-transfected cell populations were washed and rested for 3 hours with the CD3/28 beads removed. The cells were stimulated for 10 minutes with 10 μM SEW2871 or vehicle, then lysed. Equal quantities of protein were separated by SDS-PAGE and pAkt detected by western blotting. Data are from a single experiment.

The high level of phosphorylated Akt detected in the unstimulated cell lysates was probably a consequence of the stress induced in those cells by electroporation. To reduce the amount of phosphorylated Akt in the unstimulated lysates, a strategy was developed to remove the highly stressed (probably pre-apoptotic) cells.

T cells were transfected with siRNA then activated, as before. 24 hours after activation a subset of the cells were sorted out of the population according to their forward and side scatter characteristics (marked gate, Figure 36). The activated T cells loaded with CD69 -specific siRNA stained 34 % less intensely for CD69 than the control cells (table, Figure 36).



Population	Percentage of CD69$^+$ cells	Mean fluorescence intensity (CD69)	
		Whole cell population	CD69$^+$ cells
Resting T cells	0.2	101	-
Activated T cells, control siRNA	67.4	5480	7971
Activated T cells, CD69 siRNA	74.6	3622	4726

Figure 36. CD69 and control siRNA -loaded activated T cells were sorted based on their FSC / SSC characteristics and analysed for CD69 expression.
T cells were either left unactivated or transfected with siRNA as before then activated with CD3/28 beads for approximately 24 hours. Samples of the cells were taken and sorted based on their FSC and SSC characteristics. They were also stained with a monoclonal antibody against CD69, and analysed by flow cytometry. Data are representative of three independent experiments. Histograms relating to all cells represent approx. 30 000 events; those relating to gated cells 26 000, 608 and 931 events in first, second and third parts respectively.

The ability of the sorted cells to respond to stimulation with SEW2871 was then tested. A more sensitive assay for phospho-Akt, a cell-based ELISA, was used. The cells were seeded into a 96 -well plate coated with poly-lysine and rested for 3 hours. They were then stimulated as before, for 10 minutes with either 10 μM SEW2871 or vehicle. The cells were fixed with formaldehyde and the relative amounts of pAkt and Akt in the samples determined. Treatment with SEW2871 resulted in significantly greater phosphorylation of Akt in CD69 siRNA-transfected cells than control cells. The basal level of phosphorylation of Akt in both cell populations was very similar (Figure 37).

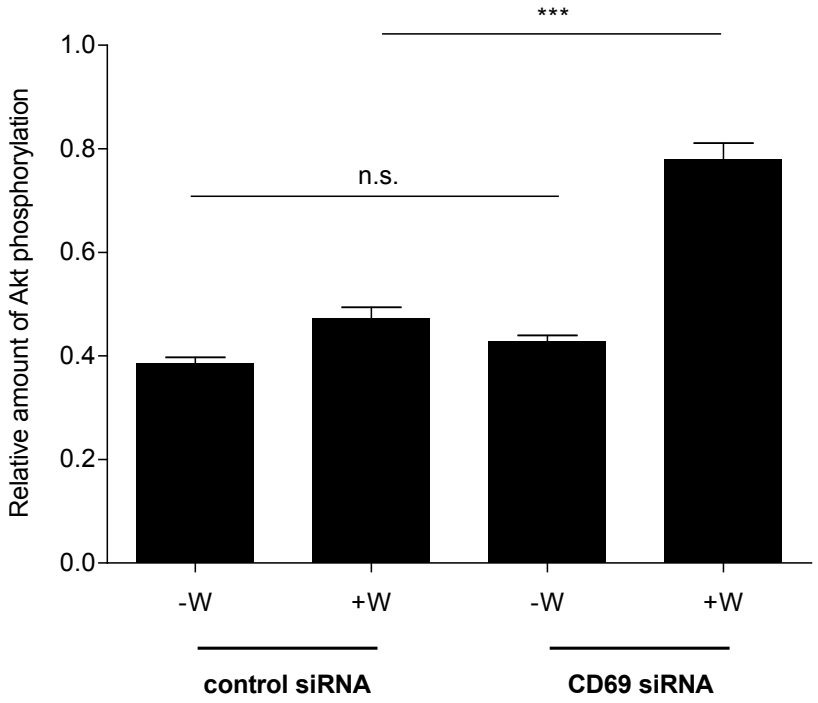


Figure 37. A comparison of the responses of CD69 siRNA-transfected and control siRNA-transfected activated T cells to stimulation with an S1PR1 -specific agonist.
A flow cytometer / cell sorter was used to isolate the desired subsets of T cells based on their FSC and SSC characteristics (detailed in Figure 36). The sorted cells were washed and placed into serum-free medium. They were left to adhere to the bottom of poly-lysine coated plastic wells and rested for 3 hours. They were stimulated with either 10 μM SEW2871 (+W) or vehicle (-W) for 10 minutes then fixed with formaldehyde. pAkt (S473) and total Akt were detected using a cell-based ELISA. The relative amounts of pAkt in the samples were calculated by normalising the fluorescent signal value corresponding to pAkt to that of total Akt in each experimental well. Graph shows means ± s.e.m., *n* = 3. Data are representative of three independent experiments.

These data showed that the capacity for S1PR1 signalling to occur in T cells was transiently lost during cell activation through CD3 and CD28, and that this was in part due to the upregulation of CD69. Three days after activation T cells became again responsive to stimulation of S1PR1.

As resting and activated T cells express different sets of chemokine receptors, the next step was to compare modulation of their chemotactic responses by S1P. Resting T cells were shown to express CXCR4 by flow cytometry (Figure 38). Pre-treatment of the cells with either S1P or the S1PR1-specific agonist SEW2871 for one hour potently suppressed their subsequent migration towards CXCL12. In both cases this was highly significant (Figure 39).

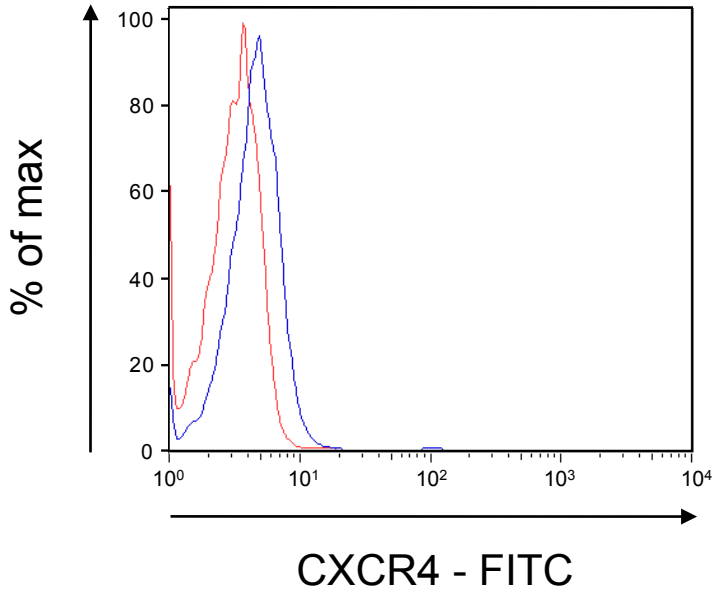


Figure 38. Resting T cells expressed CXCR4.
Resting T cells were isolated and stained either with a fluorescently-conjugated monoclonal antibody against CXCR4 (blue) or the appropriate isotype-matched control (red). Cells were analysed by flow cytometry. Data are representative of three independent experiments.

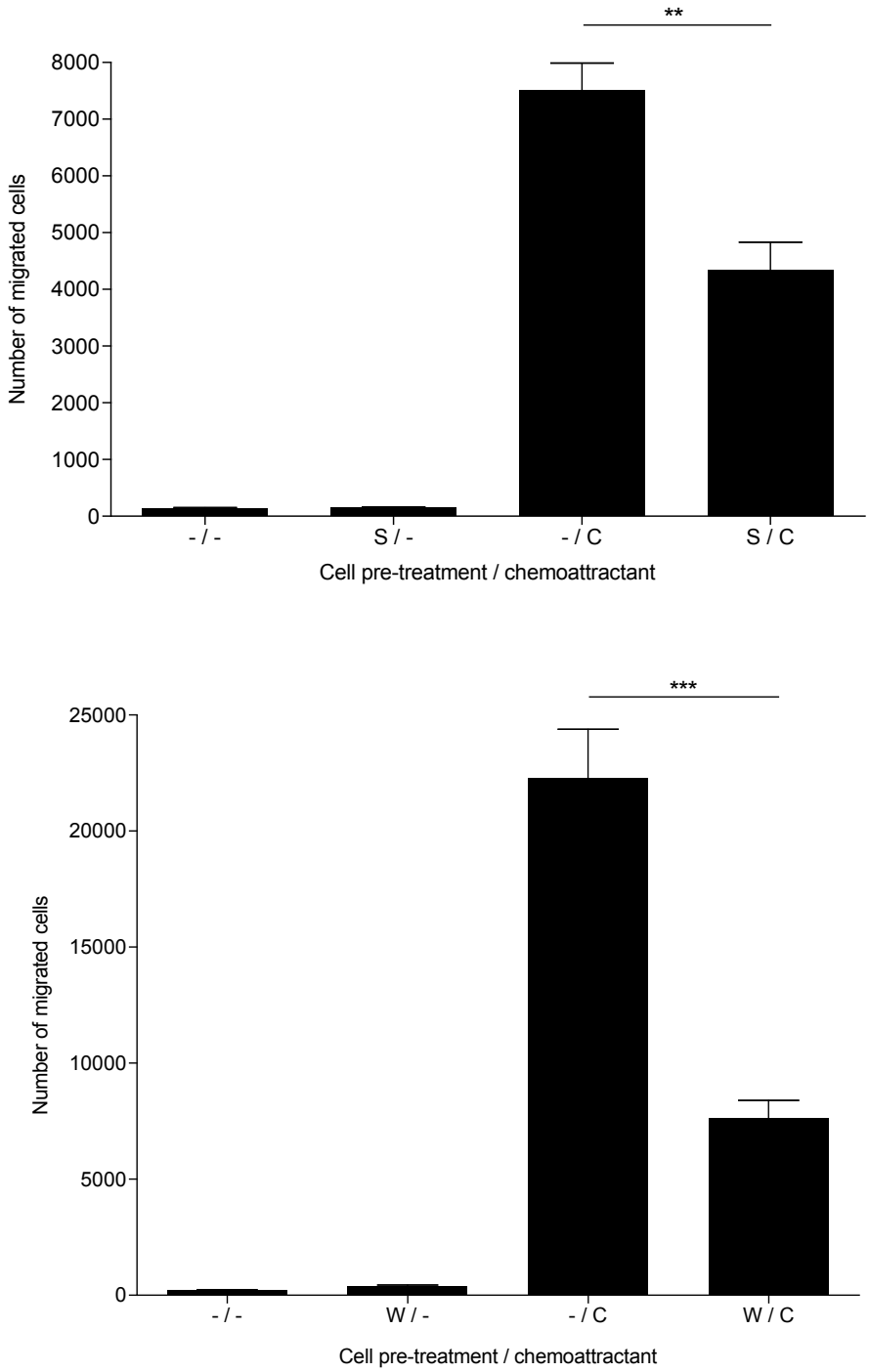


Figure 39. Pre-treatment with S1P or SEW2871 potently inhibited the migration of resting T cells towards CXCL12.
Upper panel; Resting T cells were isolated and either treated for 1 hour with 1 μM S1P (S) or vehicle (-), then left to migrate for 2 hours towards either 6.25 nM CXCL12 (C) or vehicle (-). Cells were enumerated by flow cytometry. Lower panel, same as upper panel except SEW2871 (W) was substituted for S1P. Graph shows means ± s.e.m. Data are representative of three independent experiments.

The migration of T cells towards many chemokines, including CXCL12, is dependent upon PI3K -catalysed formation of PIP_3 at the leading edge of migrating cells (Curnock *et al.*, 2002). As Akt contains a pleckstrin homology domain and binds to PIP_3, phosphorylation of this kinase was used in a test for any effect of S1P on this signalling pathway. T cells were pre-treated for 1 hour with either 1 μM S1P or vehicle then stimulated with CXCL12 for 5 minutes and lysed. Similar amounts of phosphorylated Akt (S473) were observed in the lysates of CXCL12 -stimulated cells, whether or not the cells were pre-treated with S1P (Figure 40).

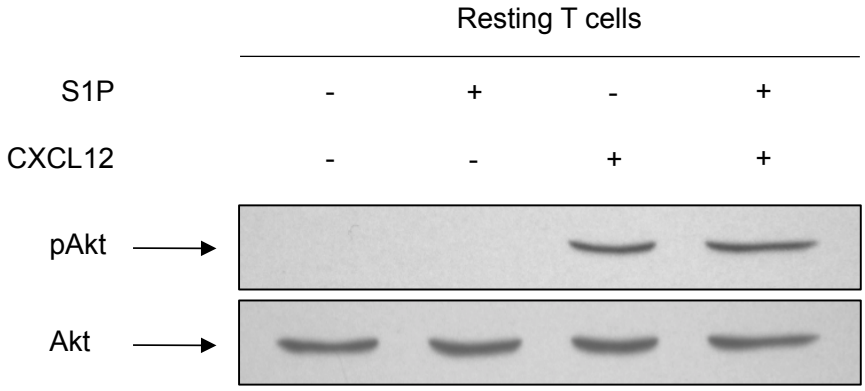


Figure 40. S1P pre-treatment did not affect the extent of Akt phosphorylation caused by stimulation with CXCL12.
Resting T cells were incubated for 1 hour with either 1 μM S1P or vehicle, and then stimulated either with 6.25 nM CXCL12 or vehicle for 5 minutes. Whole cell lysates were prepared and phospho-Akt (Ser473) and total Akt detected in them by western blotting. Data are representative of two independent experiments.

As previously stated, activated T cells express a different profile of chemokine receptors to resting cells, and consequently have different homing preferences. CXCL10 is a prototypical inflammatory chemokine expressed early at inflamed sites in the periphery, and binds the chemokine receptor CXCR3. Three days after activation with CD3/28 beads, T cells expressed high levels of this receptor (Figure 41).

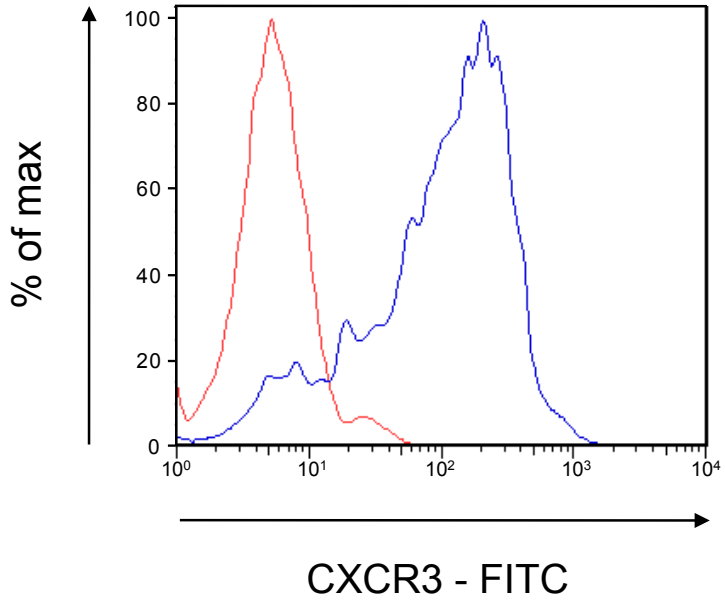


Figure 41. Three day-activated T cells expressed high levels of the chemokine receptor CXCR3.
T cells were activated with CD3/28 beads for three days and then the beads removed. The cells were stained either with a fluorescently-conjugated monoclonal antibody against CXCR3 (blue; representing approx. 5000 events) or an appropriately matched isotype-control antibody (red), and analysed by flow cytometry. Data are representative of two independent experiments.

Interestingly, S1P pre-treatment significantly enhanced the migration of three day-activated T cells towards CXCL10 (Figure 42). Thus, although S1P did effect the migration of both resting and activated T cells, it had opposing effects on them.

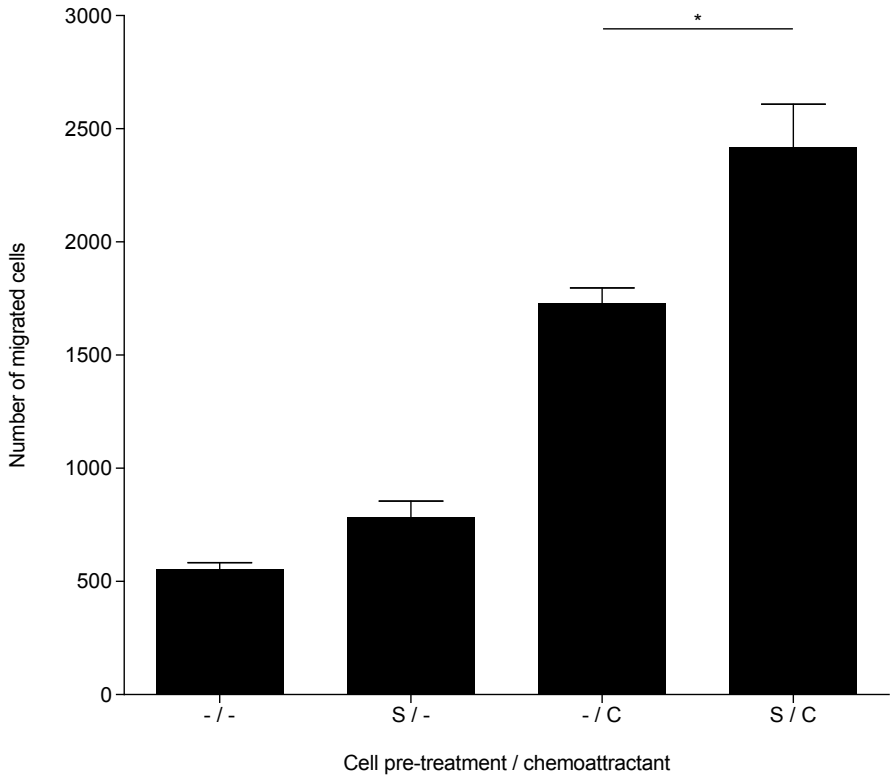


Figure 42. Pre-treatment with S1P enhanced the migration of three day-activated T cells towards CXCL10.
T cells were activated for three days with CD3/28 beads and the beads removed. The cells were rested for 3 hours, then treated for 1 hour either with 1 μM S1P (S) or vehicle (-). The cells were then left to migrate for 2 hours towards either 100 nM CXCL10 (C) or vehicle (-). Migrated cells were enumerated by flow cytometry. Graph shows means ± s.e.m. Data are representative of two experiments.

4.4 Discussion

The current model of regulation of egress of T cells from lymphoid tissue encompasses two aspects: the situation in homeostatic conditions and in non-specific lymph node inflammation. The relationship between the dynamics of CD69 expression on activation of T cells through their antigen-specific receptor and changes in S1PR1 signalling have not previously been studied in detail. Here, various approaches including flow cytometry, RNA interference and phospho-protein assays have been used to address this question. These findings support a model of regulation of S1PR1 signalling in CD3/28 activated T cells in which CD69 has a major role. Rapid upregulation of CD69 is induced on cell activation, reaching very high levels by 24 hours. Cells do not respond through S1PR1 at this time. Surface CD69 is then diluted on cell division, and the ability of S1PR1 to signal returns after three days in daughter T cells.

The strong upregulation of CD69 over the course of 24 hours after activation with CD3/28 beads was expected and is broadly consistent with that reported by others (Risso *et al.*, 1991). Significant expression of CD69 was observed at 4 hours. This is consistent with the idea that there is a pre-synthesised intracellular pool of CD69 that can be translocated from inside the cell to the cell surface on receipt of various stimuli (Risso *et al.*, 1991). It is known that CD3/28 stimulation is a potent inducer of CD69 transcription, with evidence of binding sites in the 5' untranslated region (UTR) of CD69 for NFkB, Egr and AP-1 transcription factors (Lopez-Cabrera *et al.*, 1995). It has been previously reported that CD3 and CD28 can act synergistically to induce very high levels of CD69 (Secchierio *et al.*, 2000). CD69 is known to interact directly with S1PR1 on the cell surface (Shiow *et al.*, 2006) and will trigger degradation of S1PR1 when overexpressed (Bankovich *et al.*, 2010). It is a possibility that the very high levels of CD69 induced by CD3/28 stimulation are at least in part responsible for the rapid degradation of S1PR1 protein that is seen in the first day after activation (Graeler and Goetzl, 2002).

Published work has shown that CD69 can appear at a very low level on T cells just one hour after entry into inflamed lymph nodes, with progressive upregulation over three hours (Grigorova *et al.*, 2010). The authors propose a model in which it is implied that the quantity of CD69 on the cell surface after two hours is sufficient to block T cell egress. The exact point at which CD3/28

activated T cells lost responsiveness to stimulation through S1PR1 was not addressed here. Intracellular levels of pAkt were significantly increased one hour after addition of activation beads (data not shown) and this masked any effect of stimulation with SEW2871. CD69-mediated regulation of S1PR1 signalling immediately after activation through CD3/28 is probably not important. In this instance, CD3 signalling triggers complete cell arrest (Dustin *et al.*, 1997) and the cells are probably prevented from reaching exit structures in the first place.

CD69 expression on the cell surface is at its height around 24 hours after activation, and S1PR1 signalling is blocked at this time. CD69 expression then decreased as the cells divided. Although the vast majority of three-day activated T cells were CD69-positive, they nevertheless responded to stimulation with the S1PR1 –specific agonist. The strength of their response, measured as the amount of Akt phosphorylation, was unexpected. It is shown here that S1PR1 gene expression is reduced significantly on cell activation, and remains low three days later. With evidence for complete loss of protein 24 hours after activation (Graeler and Goetzl, 2002), only low levels of S1PR1 could conceivably return to the cell surface by day three. The lack of a reliable monoclonal antibody against S1PR1 precludes the resolution of this matter. The data here are consistent with a report which showed that *in vivo* activated T cells re-acquire at least some responsiveness to S1P by day three, although not to the extent of resting cells (Matloubian *et al.*, 2004). In this report, the magnitude of the response to stimulation with SEW2871 was similar six days after activation, by which time two-thirds of the cells were CD69-negative. Published work shows that effector and memory T cells begin to appear in the circulation three days after cell activation in a primary response, and are widely disseminated in the periphery, including in other lymphoid tissues by day five (Matloubian *et al.*, 2004; Miller *et al.*, 2004).

Considered as a whole, there was a large degree of variation in CD69 expression in three day -activated T cells. Part of this is attributable to the mixture of cells of different generations. The mean fluorescence intensity of CD69 staining decreased by approximately one half with each generation across four distinct generations. CD69 gene expression is only transiently enhanced by cell activation, with little mRNA detectable 24 hours after PMA

stimulation of peripheral blood lymphocytes (Lopez-Cabrera *et al.*, 1993). It is probable that there is little neosynthesis of CD69 after 24 hours, and that existing protein simply splits equally between daughters on cell division.

That there might be differential responsiveness to stimulation of S1PR1 of different generations of cells at a single time point was not investigated. The mean fluorescence intensity of CD69 staining of all cells at three days was approximately one-fifth of that at 24 hours. More than half of the cells at three days belonged to generations two and three, and their mean fluorescence intensity (MFI) (of CD69) was only one-tenth that at 24 hours. It seems probable that the later generations of cells were more responsive to stimulation of S1PR1 than the earlier ones, simply by virtue of their lower mean expression of CD69. Cells that have not proliferated and differentiated would not possess the capacity to exert their appropriate functions once they had left the lymphoid compartment, and it would make sense for these to be retained inside the node. Effector and memory T cells observed outside the node have invariably undergone multiple rounds of cell division (Miller *et al.*, 2004).

siRNA was used to attenuate the extent of upregulation of CD69 after T cell activation through CD3 and CD28. While the percentage of CD69-positive cells was not altered greatly, the amount of CD69 protein on the cell surface was reduced – and this resulted in a measurable increase in the amount of Akt phosphorylation caused by stimulation with SEW2871. One interpretation of this data is that below a certain threshold of CD69 expression, all S1PR1 protein is not inactivated and receptor signalling can be triggered. Alternatively the attenuated upregulation of CD69 may interfere with the ability of that protein to trigger efficient degradation of S1PR1. Measurement of the amounts of S1PR1 protein both on the cell surface and inside the cell is needed in this experiment to clarify this matter.

The suppression of resting primary T cell migration towards CXCL12 by S1P was unexpected, as it had been previously observed that Jurkat T cell migration towards this chemokine was enhanced (Figure 13). The reason for this is not clear, although one report has shown cell-type and S1P concentration – dependent differences in the ability of S1P to modulate migration of mouse T cells towards CXCL12. It was shown that for peripheral lymph node -derived T cells, lower concentrations of S1P, in the 10 – 100 nM range, enhanced

chemotaxis, whereas 1 μM S1P (the concentration used in this study) had a slightly inhibitory effect. The inhibitory effect was not seen with splenic T cells. FTY720 inhibited migration of mouse PLN T cells towards CXCL12 (Yopp *et al.*, 2005). Low concentrations of S1P probably induce persistent S1P receptor signalling, whereas high concentrations of S1P (or FTY720) would be expected to downregulate the receptor from the cell surface. Perhaps differences between Jurkat and primary T cells in surface expression, and/or the dynamics of recycling, of S1PR1 underlie the disparity observed.

The opposing effects of S1P pre-treatment on T cell migration towards CXCL12 and CXCL10 might be related to differences in types of Gαi G proteins coupled to their cognate receptors – CXCR4 and CXCR3 respectively. Efficient migration towards CXCL12 is dependent on the presence of both the Gαi2 and Gαi3 proteins, whereas migration mediated through CXCR3 is enhanced by downregulation of Gαi3. Additionally, Gαi3-deficient T cells did not show S1P-mediated inhibition of CXCL12, rather an enhancement (Jin *et al.*, 2008).

Chapter 5. S1P Receptor-dependent Regulation of Egress of Activated T Cells from Lymph Nodes

S1P Receptor-dependent Regulation of Egress of Activated T Cells from Lymph Nodes

5.1 Introduction

The recirculation of resting T cells under normal conditions is controlled by homeostatic chemokines and S1P, broadly controlling lymph node entry and exit respectively. A vascular gradient of S1P (with high levels of S1P in blood, intermediate levels in lymph and very low levels in lymphoid tissue (Hla *et al.*, 2008)) induces a characteristic pattern of recycling of S1PR1 between the cell surface and endosomes as a T cell recirculates (Lo *et al.*, 2005). Inside the low S1P environment of uninflamed lymph nodes, S1PR1 recycles quickly from endosomes back to the surface of T cells and they become competent to egress soon after entry (Matloubian *et al.*, 2004; Grigorova *et al.*, 2010).

In inflamed lymph nodes, T cell exit is blocked for up to several days. Interferon-α/β signalling causes upregulation of CD69, and this protein interacts with S1PR1 on the cell surface, causing the receptor to be internalised and degraded (Shiow *et al.*, 2006; Bankovich *et al.*, 2010; Grigorova *et al.*, 2010). The block in egress contributes to a non-specific accumulation of T cells, increases the probability of productive interactions between mature antigen-presenting cells and T cells expressing the appropriate antigen-specific receptor, and thereby promotes adaptive immunity.

In contrast to the situation outlined above, a number of significant questions remain regarding the control of egress from lymph nodes of T cells activated through their antigen-specific receptor. T cells are known to arrest on stimulation through their antigen-specific receptor (Dustin *et al.*, 1997). This occurs alongside an initial rapid upregulation of CD69 on the cell surface following T cell receptor stimulation, subsequently downregulated with cell division. Simultaneously, the capacity for S1PR1 to signal in T cells is first lost following activation, then re-acquired after about three days (Chapter Four). These changes correlate with the appearance of activated T cells in the circulation about three days after the initial priming event in lymphatic tissue (Matloubian *et al.*, 2004). Nevertheless, it is still not entirely clear to what extent activated T cells require S1P signalling to egress lymph nodes. According to the existing model, S1P signalling through S1PR1 is required to antagonise CCR7-mediated retention signals. But while resting naïve T cells are $CCR7^{hi}$, cell

activation downregulates this receptor (although it becomes re-expressed on central memory T cells (Sallusto *et al.*, 1999)). Published work has shown that T cells genetically deficient in CCR7 are poorly sequestered in lymph nodes by FTY720 treatment, suggesting a limited requirement for S1P signalling for them to leave (Pham *et al.*, 2008).

Much of our knowledge regarding the contribution of S1PR1 signalling to promoting egress of resting T cells from lymphatic tissue comes from studies utilising the compound FTY720. FTY720 is in fact a pro-drug, and is rapidly phosphorylated *in vivo* by sphingosine kinase 2 (Kihara and Igarashi, 2008) to form the biologically active compound. Structurally similar to S1P, FTY720-P binds all the S1P receptors except S1PR2 (Brinkmann *et al.*, 2002). Most importantly, it is a 'super-agonist' of S1PR1. In a $\gamma(^{35}S)$ guanosine triphosphate assay, the EC_{50} of FTY720-P for S1PR1 was 0.14 nM, compared with 0.9 nM for S1P (Hogenauer *et al.*, 2008). *In vitro*, seconds of incubation with FTY720-P caused significant internalisation of S1PR1 over the course of several hours (Graler and Goetzl, 2004), and it is much more potent than S1P at causing ubiquitination and degradation of S1PR1 (Oo *et al.*, 2007). FTY720-P therefore behaves as a functional antagonist of S1PR1 on lymphocytes *in vivo*, downregulating the receptor and completely desensitising the cells to S1P-mediated egress signals in lymph nodes. Treatment of mice with FTY720 caused lymphocytes to become sequestered in lymph nodes, packed next to sinus-lining endothelium and unable to exit. This preceded and caused a profound drop in blood lymphocyte numbers (Mandala *et al.*, 2002).

Daily treatment of mice, rats, dogs and monkeys with FTY720 significantly prolonged allograft survival (Brinkmann *et al.*, 2001). FTY720 was even trialled as an anti-rejection therapy in humans, although significant side-effects (including bradycardia, hypertension and macula oedema) and comparable efficacy to existing therapies meant that its use did not proceed beyond the trial stage (Budde *et al.*, 2002; Tedesco-Silva *et al.*, 2005; Salvadori *et al.*, 2006). Impressive results have been reported in a phase III trial for multiple sclerosis (Kappos *et al.*, 2010). Because FTY720 treatment resulted in a dramatically reduced number of allograft-infiltrating T cells in animal models, and that this correlated with sequestration of T cells in lymphoid tissue, it was initially assumed that FTY720 could block egress of activated T cells from lymph nodes

(Chiba *et al.*, 1998). Consistent with this, FTY720 appeared to work therapeutically, rather than just prophylactically, to block immune responses. For example, in rats, FTY720 administered 3 to 4 days after engraftment prolonged graft survival (Xu *et al.*, 1998). Furthermore, Pinschewer *et al.* showed that FTY720 could block the homing of LCMV-specific TCR transgenic effector T cells out of lymph nodes into skin in a delayed-type hypersensitivity reaction model (Pinschewer *et al.*, 2000).

Recent data however has cast doubt on the existing explanation for these findings. These studies suggested that disruption of T cell recirculation by FTY720, with a consequent impairment of immune priming, might be the primary mechanism of its immunosuppressive effect (Xie *et al.*, 2003; Nakashima *et al.*, 2008). Here, a novel approach has been used to clarify whether or not the egress of newly formed activated T cells from lymph nodes is dependent on S1P signalling (and can therefore be blocked by FTY720 treatment). By injecting fully MHC-mismatched cells into the mouse footpad, a specific alloimmune response was generated. Enzyme-linked immunosorbent spot (ELISPOT) was used to measure the frequency of alloreactive cells in various immune compartments after six days. The response was modulated with daily administration of FTY720-P, delayed for 48 hours after footpad injection to exclude any effect of the drug on movement of sensitising material to the draining popliteal node.

Sensitisation with allogeneic cells injected into the footpad resulted in an increase, measured after six days, in the frequency of alloreactive cells in the draining popliteal lymph node and other lymphoid compartments. This confirmed the generation of a specific alloimmune response. Treatment of unimmunised mice with FTY720-P resulted in depletion of T cells from their peripheral blood, confirming biological activity of the drug. Importantly, FTY720-P treatment of immunised animals resulted in an increase in the frequency of alloreactive cells within the popliteal node measured at the day six time point. This was correlated with a higher percentage of CXCR3-positive cells in the activated lymph nodes of drug-treated mice, consistent with a blockade in egress of activated T cells. These findings suggest that the egress of (at least a proportion of) activated T cells from lymph nodes is dependent on intrinsic S1P receptor signalling.

The aims of this chapter were as follows:

- To design a suitable mouse model of alloimmunity
- To optimise a method for measurement of the frequency of alloreactive cells in lymphoid tissue
- To study the effect of treatment with FTY720-P on the egress of activated alloreactive cells from lymph nodes

5.2 Materials and Methods

Reagents

FTY720 (S) Phosphate (also referred to here as FTY720-P and FTY-P) was purchased from Cambridge Bioscience (Cambridge, UK). It was dissolved in ethanol at 1 mg/ml then aliquotted for storage at -20 °C. 1 mg/ml FTY720-P was diluted further in sterile water + 2 % β-cyclodextrin (Sigma-Aldrich) to 100 μg/ml, and then mixed thoroughly, on the day of use.

Animals and procedures

Female mice of the BALB/c and C57BL/6 strains were purchased from Charles River (Margate, UK). They were maintained under pathogen-free conditions and used at between six and eight weeks of age. All animal procedures were done in accordance with UK Home Office and EU Institutional Guidelines and within the parameters of current personal and project licences.

Work with live mice was performed by trained staff at the Comparative Biology Centre (Medical School, Newcastle University, UK). Blood samples were taken either via the tail vein while alive, or by cardiac puncture during terminal anaesthesia. Lymph nodes and spleens were removed within 30 minutes of death. Unilateral mouse footpad injections (subcutaneous) were performed by Professor John Robinson (Institute of Cellular Medicine, Newcastle University, UK). Each injection comprised 5 million splenocytes suspended in 25 μl RPMI 1640 medium.

Mice were injected either intraperitoneally (i.p.) or intravenously (i.v.) with 100 μl 100 μg/ml FTY720-P as required.

Isolation of cells from lymph nodes and peripheral blood

Popliteal lymph nodes, mesenteric lymph nodes, spleens and blood samples were removed from dead mice six days after injection of their footpads with allogeneic splenocytes. Cell suspensions were prepared from lymph nodes and spleens by pressing them through 70 μm cell strainers into RPMI 1640 medium. In the case of splenocyte preparations, the cells were layered over Histopaque-1083 (Sigma-Aldrich) and separated by density-gradient centrifugation. The leukocytes were isolated from the interface and the rest discarded. Splenocytes and lymph node-derived cells were washed twice with RPMI 1640 medium before use.

Blood samples were taken either by cardiac puncture or dropwise from a puncture wound in the tail vein. Samples were immediately mixed *ex vivo* with 100 U / ml heparin to prevent coagulation. Erythrocytes were lysed by resuspension of cells in 1 ml of Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich) per sample, and incubation for 1 minute at room temperature. Remaining cells were washed twice with RPMI 1640 medium before use.

Isolation of T cells from splenocytes

Total $CD3^+$ T cells were isolated from splenocyte preparations using a negative-selection kit (EasySep Mouse T Cell Enrichment Kit; StemCell Technologies, Grenoble, France). The purity of the cells was verified by flow cytometry.

Irradiation of cells

In some experiments cells were exposed to 20 Gray (Gy) γ-radiation before use. This was done using a high-activity sealed-source Gamma Cell Irradiator (Nordion, Ottowa, Canada), with an effective exposure of sample containers to the ^{60}Co source of 3.02 Gy per minute. Samples were exposed for a total of 6 minutes and 37 seconds in each instance.

ELISPOT assay

Cells were cultured in 96-well format Immobilon MultiScreen-P plates (Millipore, Watford, UK). The plates were first activated and coated with a capture rat anti-mouse IFN-γ antibody (AN18; Mabtech, SE-131 28 Nacka Strand, Sweden) diluted into carbonate-bicarbonate buffer (Sigma-Aldrich) overnight at 4 °C. The plates were then washed twice with PBS and blocked with RPMI 1640 + 10 % FBS + 2 mM L-Glutamine + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin

(Sigma-Aldrich) for one hour at room temperature. Before use in cell culture, the plates were washed once further with PBS.

Unless otherwise stated, the recall mixed leukocyte reaction assays were constituted as follows in each well of the 96-well format plate. 10 000 C57BL/6 – derived cells (from blood, lymph node or spleen) were mixed with 200 000 BALB/c splenocytes in a total volume of 200 μl RPMI 1640 + 10 % FBS + 50 μM β-mercaptoethanol + 2 mM L-Glutamine + 100 U/ml Penicillin + 0.1 mg/ml Streptomycin (Sigma-Aldrich). The cells were cultured for approximately 18 hours before being discarded. In some cases cells were stimulated with 5 μg/ml concanavalin A (Sigma-Aldrich) as a positive control.

ELISPOT assays were developed as follows. The cells were discarded and plates washed six times with PBS. The biotinylated IFN-γ detection antibody (R4-6A2; Mabtech), diluted in PBS, was applied and the plates left overnight at 4 °C. The detection antibody was discarded and the plates washed six times with PBS. The streptavidin-alkaline phosphatase conjugate (Mabtech) was diluted in PBS and applied for one hour to the plates at room temperature. They were washed another six times with PBS. 50 μl of BCIP/NBT liquid substrate system for membranes (Sigma-Aldrich) was added to each well. Development was for 5 – 10 minutes at room temperature, and the reaction was stopped by removal of substrate and thorough washing with tap water.

Spots were enumerated using an ELISPOT reader (AiD, Strassberg, Germany).

Flow cytometry

Splenocytes and lymph node-derived cells were stained with fluorescently-conjugated monoclonal antibodies or isotype-matched controls. The specific antibodies used were: CXCR3 (220803; R&D Systems) and CD3 (KT3; AbD Serotec). Data was acquired on a FACSCanto II instrument (BD Biosciences) and analysis performed using FACSDiva 6.1.3 (BD Biosciences) and FlowJo 7.6 (Treestar) software.

Statistics

Prism 4.0c (GraphPad Software, USA) was used for all statistical analyses. Comparisons between groups were made using the Students' *t* test for unpaired data. P values ≤ 0.05 were considered significant (*), ≤ 0.01 highly significant (**), and ≤ 0.001 very highly significant (***).

5.3 Results

In this study, alloimmune responses were generated *in vivo* by subcutaneous injection of BALB/c splenocytes into the footpads of C57BL/6 mice. Prior to those experiments however, the mixed leukocyte reaction between C57BL/6 T cells and BALB/c splenocytes was studied *ex vivo.* T cells were isolated by negative selection from the splenocytes of C57BL/6 mice. Analysis of the cells after selection showed that the population of T cells was of very high purity (Figure 43).

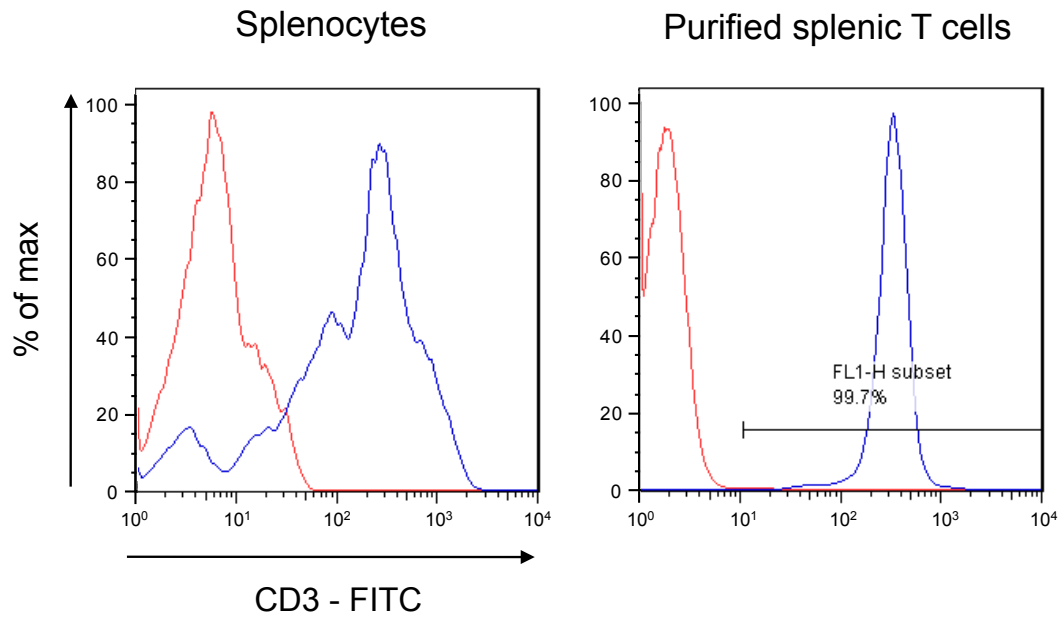


Figure 43. T cells were purified from whole splenocytes.
Spleens were removed from C57BL/6 mice and disrupted to release splenocytes. The splenocytes were mixed with a cocktail of antibodies against non-T cell markers, which were then coupled to magnetic particles. T cells were separated from the population by negative selection using a magnet. Cell samples before and after selection were stained with a fluorescently-labelled monoclonal antibody against CD3 (blue) or appropriate isotype-matched control (red), and analysed by flow cytometry.

Using a constant number of BALB/c splenocytes as stimulators, a panel of mixed leukocyte reactions (MLRs) with different numbers of C57BL/6 T cell responders were set up. In this experiment, some of the stimulators were irradiated before the assay (to inhibit any cytokine production by them) and others not. The number of IFN-γ spot- forming cells in each well after 18 hours of co-culture was determined by ELISPOT. Figure 44 shows the appearance of experiment wells at the end-point of a typical (unspecified) assay. Figure 45 (upper panel) shows the results of the various MLRs.

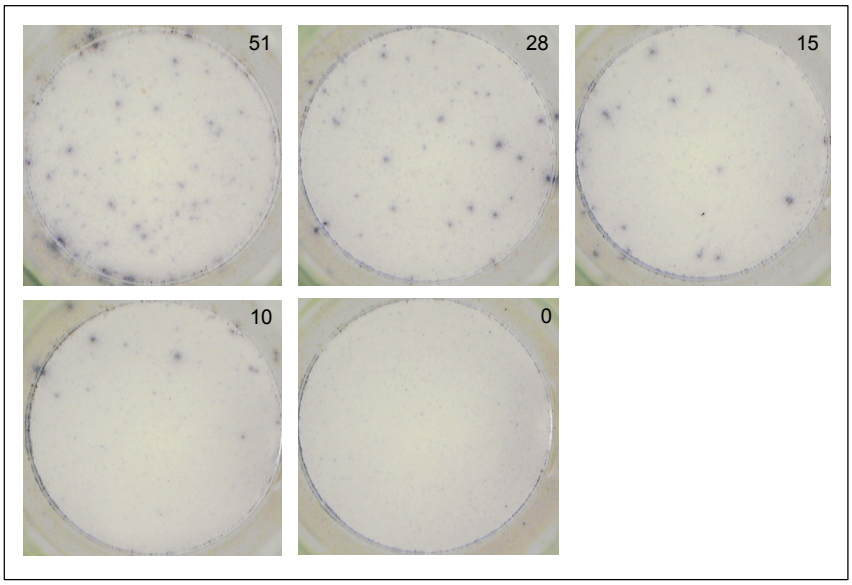


Figure 44. The number of IFN- γ spot-forming cells in a cell sample was determined using the ELISPOT technique.
Samples containing potentially IFN- γ producing and non-producing cells were placed into the wells of a 96-well plate, in which the bottom of each well was coated with IFN- γ capture antibody adsorbed to PVDF membrane. The cells were cultured for 18 hours then removed. An IFN- γ detection antibody coupled to enzyme-catalysed production of an insoluble precipitate was used to detect bound cytokine. The wells were imaged with a CCD-camera and individual spots counted by specialist software.

As expected, very few IFN-γ spot-forming cells were found among stimulators (irradiated or not) or responders cultured alone. The T cell mitogen concanavalin A, added to stimulators or responders alone, or a mixture of the two, resulted in a very high number of IFN-γ producing cells. With the number of BALB/c splenocytes kept constant in each reaction, the number of IFN-γ spot-forming cells detected was linearly correlated with variation in the number of C57BL/6 T cells (Figure 45 (lower panel)). With unirradiated stimulators and 10 000 C57BL/6 T cells in the reaction, the mean number of spot-forming cells was 7.67 (Standard error of the mean (SEM): 1.20). Alternatively expressed, 1 in 1300 cells was stimulated to produce sufficient IFN-γ to be detected in this assay. Irradiation of the stimulators reduced the number of IFN-γ spot-forming cells detected by approximately one half, compared with the equivalent reaction with unirradiated cells. With 10 000 C57BL/6 T cells in the reaction, the mean number of spot-forming cells was 3.67 (SEM: 0.33), equivalent to 1 in 2730 cells.

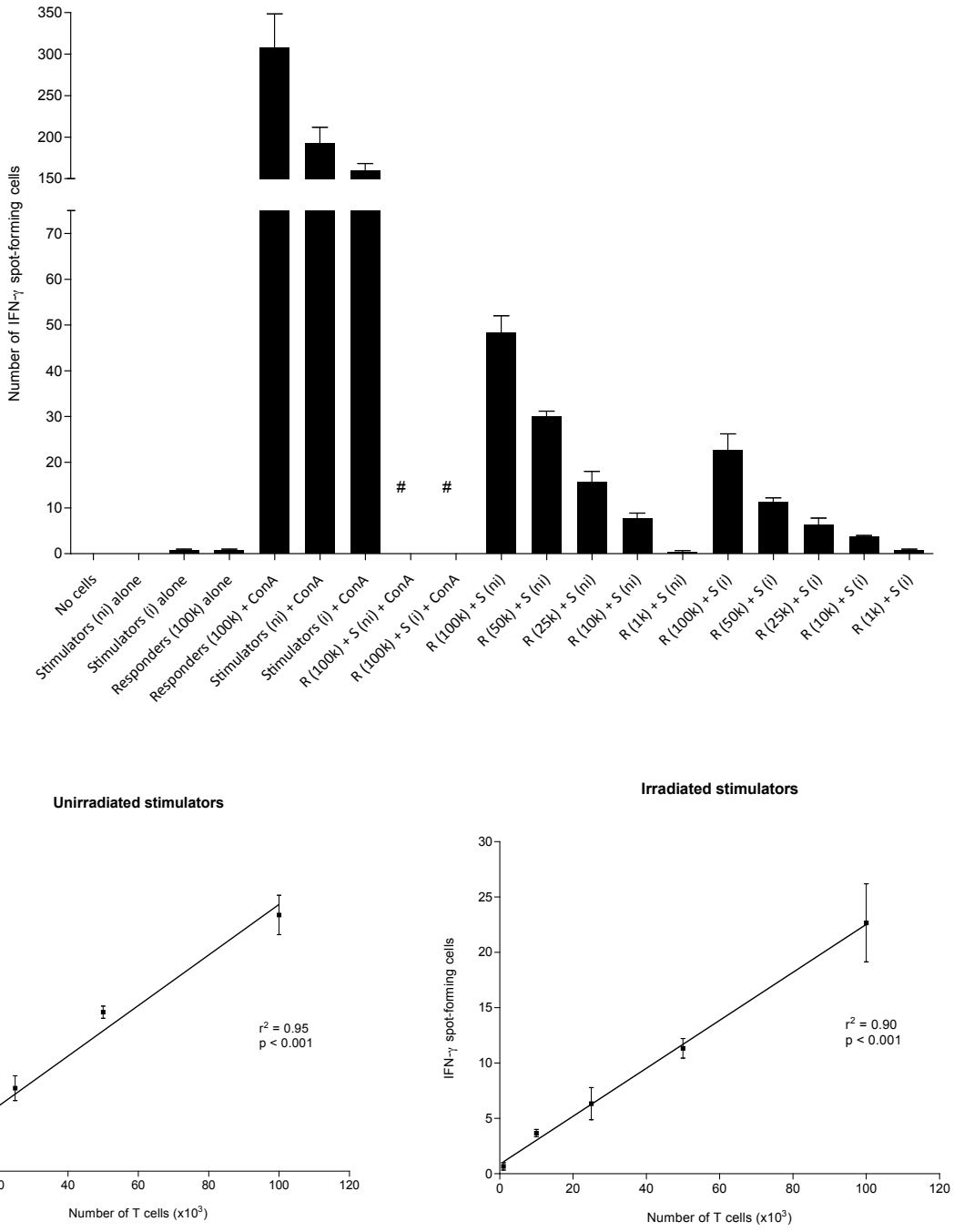


Figure 45. Measurements of the number of IFN- γ spot-forming cells resulting from various mixed leukocyte reactions.
Upper panel, splenocytes were isolated from BALB/c mice and splenic T cells (purified by negative selection) from C57BL/6 mice. The BALB/c cells were used as stimulators (S) and the C57BL/6 T cells as responders (R) in mixed leukocyte reactions. The cells were (co)cultured for 18 hours before being removed and the number of IFN- γ spot-forming cells in each well determined by ELISPOT. Where present, 200 000 stimulators were used per well. Where present, the number of responders was varied from 100 000 (100k) to 1000 (1k) per well. The stimulators were either irradiated (i) or not (ni). In some cases cells were stimulated with 5 μg/ml concanavalin A (ConA). #, spots were too numerous to be counted reliably. Data shows means ± s.e.m. (triplicate determinations). Lower panel, separate linear regression analyses of data with unirradiated and irradiated stimulators.

$5x10^6$ BALB/c splenocytes were injected subcutaneously into the left hind footpad of each of five C57BL/6 mice. Six days later, the mice were killed and the lateral and contralateral popliteal lymph nodes (PLN) removed (Figure 46). The number of cells derived from the draining (lateral) popliteal lymph nodes was approximately four times that from the contralateral nodes (mean cell counts: $2.01x10^6$ and $0.49x10^6$ respectively) (Figure 47). This was consistent with the generation of a localised immune response.

Figure 46. Excision of a popliteal lymph node.
A mouse was killed and an incision made in the skin of the hind limb, running from the foot to the abdomen. The skin was peeled back and secured with pins. The popliteal node, located behind the knee, was separated from the body using forceps placed behind the node and scissors to cut any restraining tissue. This was done aseptically in a category II laminar flow cabinet with an ethanol-rinsed mouse dissection kit.

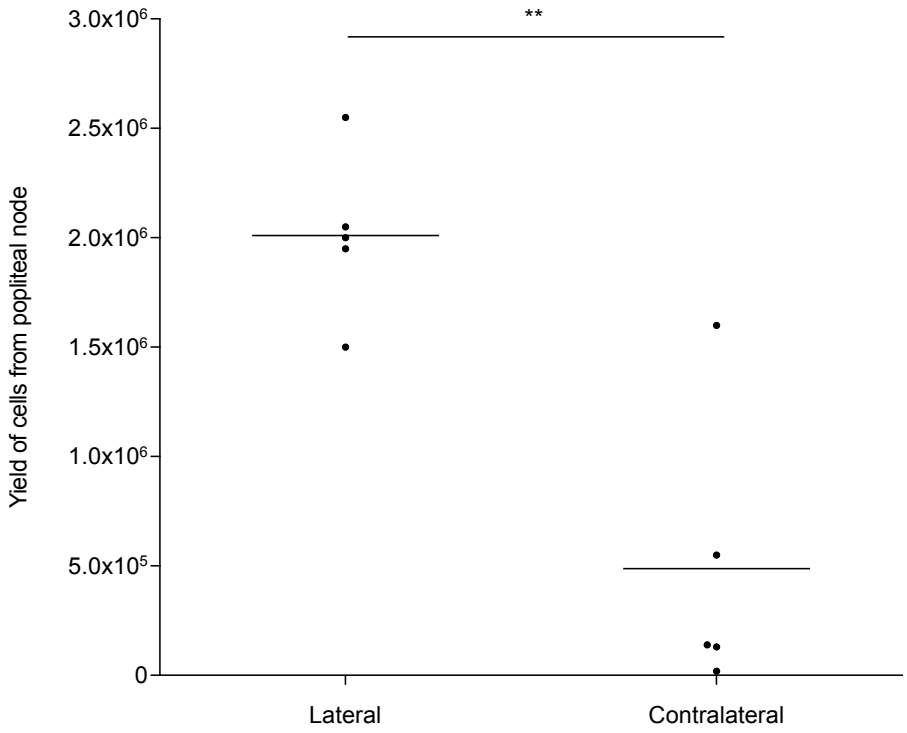


Figure 47. Injection of BALB/c splenocytes into single footpads of C57BL/6 mice resulted in higher cellularity of the draining lateral than contralateral popliteal lymph nodes.
Five C57BL/6 mice were each injected with $5x10^6$ BALB/c splenocytes into their left hind footpad. Six days later the mice were euthanased and the lateral and contralateral popliteal lymph nodes removed. The nodes were disrupted and the freed cells counted using a haemocytometer. Points represent individual mice; horizontal lines the means.

Next, three mice were sensitised as before and the draining popliteal nodes removed after six days. The lymph node-derived cells were cultured with BALB/c stimulators for 18 hours and IFN-γ spot-forming cells detected by ELISPOT. The results for the three mice are shown separately in Figure 48. Expressed as frequencies, the responses of the three mice were very similar: mean 3.15, range 3.06 - 3.21 IFN-γ spot-forming cells per 1000 PLN –derived cells.

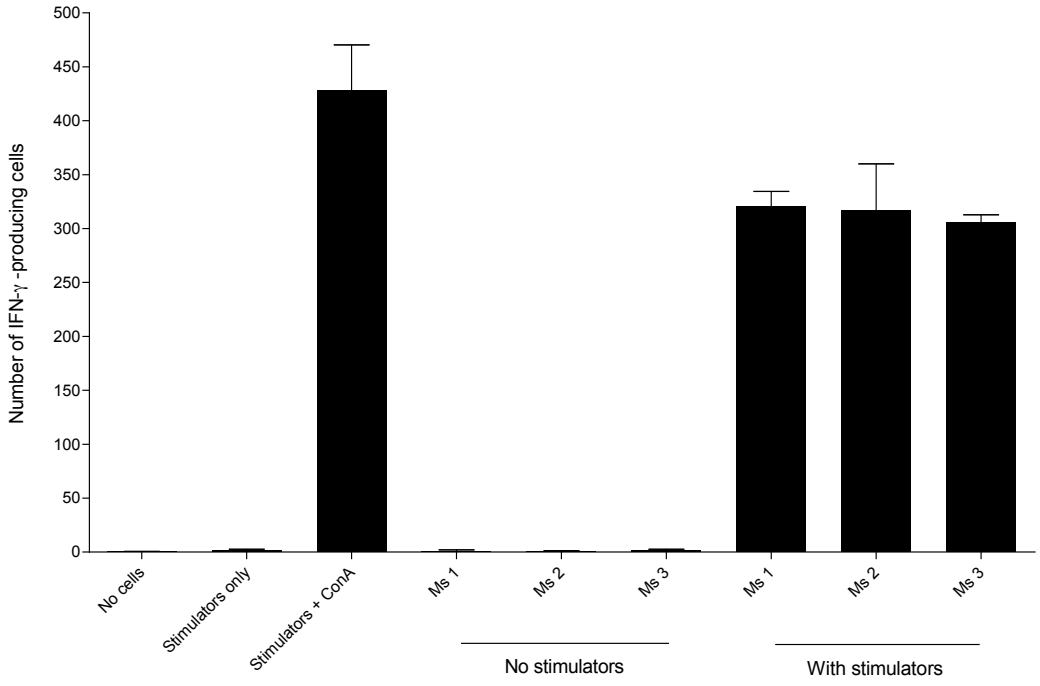


Figure 48. The number of popliteal lymph node -derived cells that produced IFN-γ on recall stimulation was determined by ELISPOT.
Three C57BL/6 mice (Ms 1 – 3) were each injected with $5x10^6$ BALB/c splenocytes into their left hind footpad. After six days the mice were killed and the lateral popliteal nodes removed. The popliteal lymph node-derived cells were either seeded alone (100 000 per well) or co-cultured with BALB/c splenocytes (200 000 per well) in a mixed leukocyte reaction. In some cases stimulators were cultured alone or with 5 μg/ml concanavalin A (ConA). After 18 hours the cells were removed and the number of IFN-γ -producing cells that had been present in each well determined by ELISPOT. Graph shows means ± s.e.m. (triplicate determinations).

To confirm that the increase in frequency of IFN-γ spot-forming cells was a consequence of a specific alloimmune response, and not a consequence of non-specific inflammation, a further experiment was performed. Three mice had allogeneic cells injected into a footpad, and a control mouse received syngeneic cells. After six days, the mice were killed and draining popliteal nodes, mesenteric nodes, spleens and blood samples taken. ELISPOT was used as before to detect IFN-γ spot-forming cells resulting from the mixed leukocyte reaction. The data are now expressed as frequencies to aid comparison between experiments. The frequencies of IFN-γ spot-forming cells were higher, in all compartments examined, in the allosensitised mice than in the mouse that

received syngeneic cells (Figure 49). Interestingly, the frequencies of responding cells were similar in the draining popliteal nodes, spleens and blood, but lower in the mesenteric nodes. This probably indicates that alloactivated T cells have emigrated from the popliteal lymph nodes into the blood compartment.

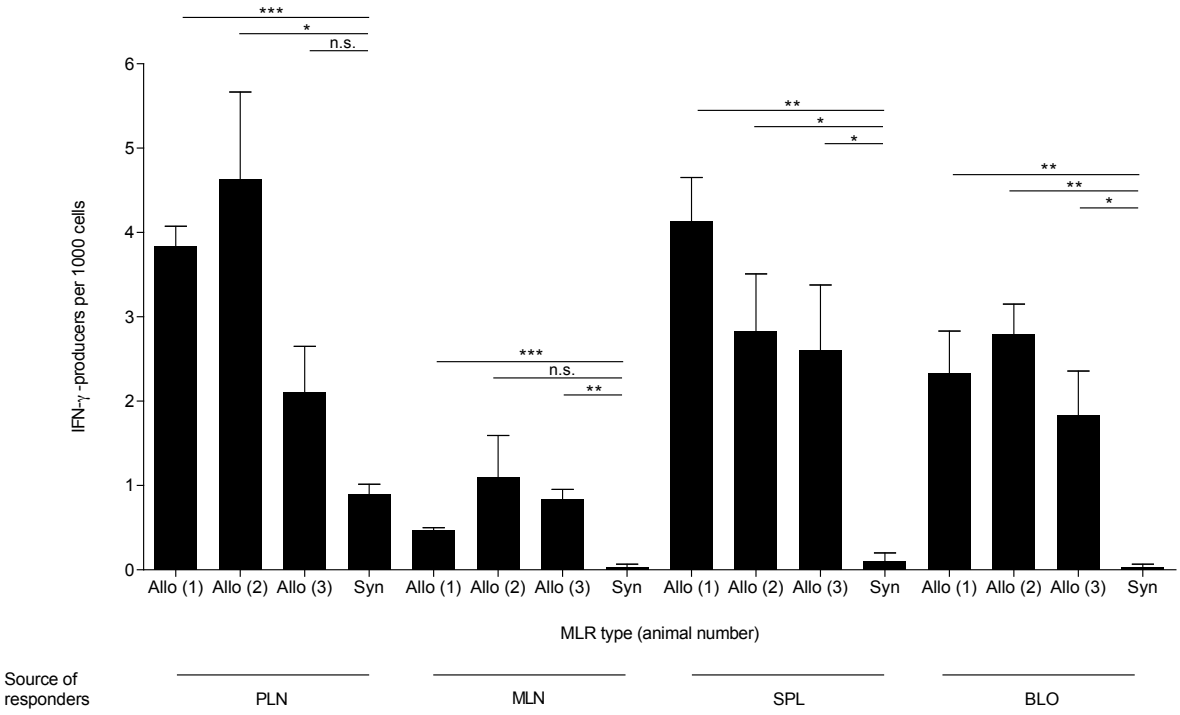


Figure 49. Injection of allogeneic cells into the footpad resulted in a higher frequency of IFN-γ spot-forming alloreactive cells in several immune compartments than sensitisation with syngeneic cells.
Three C57BL/6 mice were each injected with $5x10^6$ BALB/c splenocytes into their left hind footpad. One mouse was injected with $5x10^6$ C57BL/6 splenocytes. Six days later the mice were killed and the draining popliteal lymph node (PLN), mesenteric nodes (MLN), spleen (SPL) and sample of peripheral blood (BLO) taken from each animal. 10 000 of the spleen, lymph node or peripheral blood-derived cells were co-cultured with 200 000 BALB/c splenocytes in mixed leukocyte reactions. After 18 hours of culture the number of IFN-γ spot-forming cells per well was measured by ELISPOT. The data are expressed here, and in similar subsequent figures, as frequencies. Graph shows means ± s.e.m. (triplicate determinations).

In this study, the phosphorylated derivative of FTY720 – which is the bioactive compound - was administered directly to the mice in order to modulate cell trafficking. Before use in this model, the activity of the drug was tested. FTY720-P was administered i.p. or i.v. to mice each day for two days and the T cell count in peripheral blood measured on the third day. As expected, and irrespective of the route of administration, FTY720-P caused a very large decrease in the number of T cells in the peripheral blood (Figure 50). In one experiment, the absolute $CD3^+$ cell counts were: no drug, $8.14x10^6$ per ml; i.p. FTY720-P, $0.46x10^6$ per ml; i.v. FTY720-P, $0.36\ x10^6$ per ml. In further experiments, the drug was administered via the i.p. route.

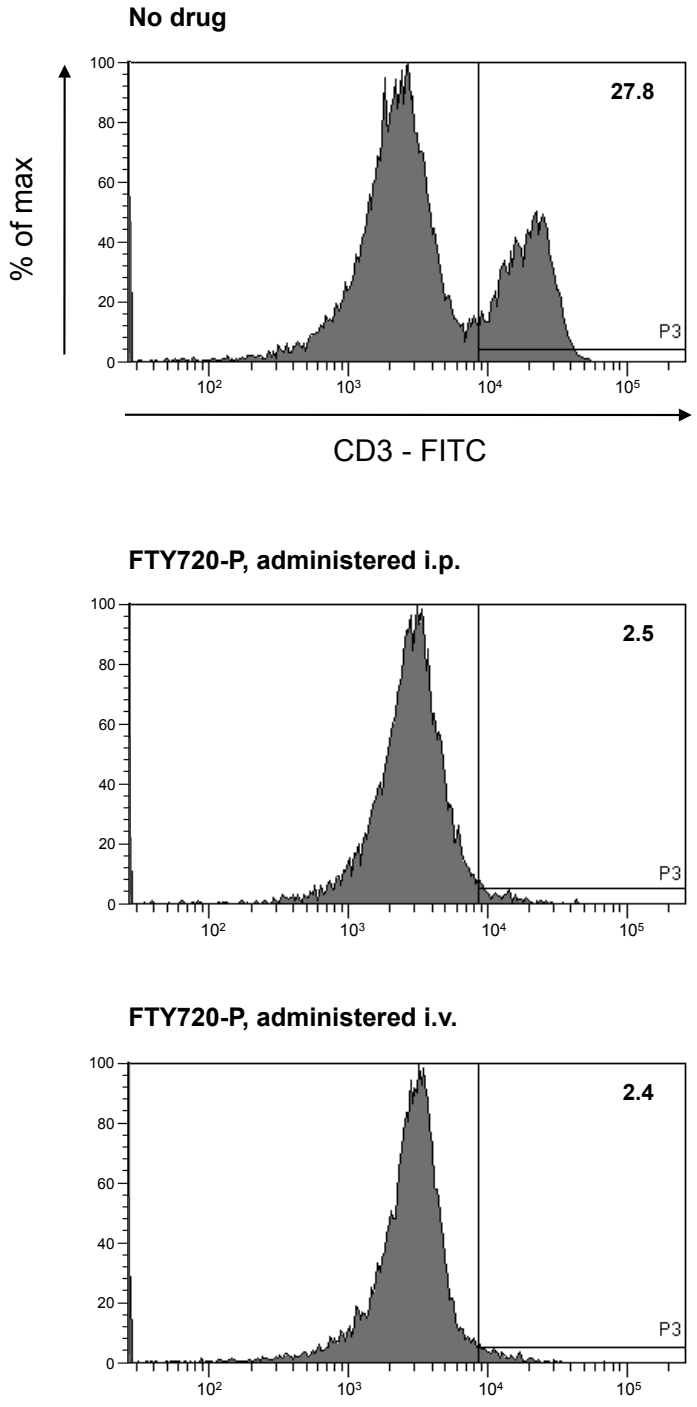


Figure 50. Treatment of mice with FTY720-P either i.v. or i.p. resulted in almost complete depletion of T cells from peripheral blood.
Three C57BL/6 mice were treated as follows. One was left untreated; the second injected i.p. with 100 μl of 100 μg/ml FTY720-P each day for two days; the third injected i.v. with 100 μl of 100 μg/ml FTY720-P each day for two days. On the third day 50 μl blood samples were taken from the mice via the tail vein. The blood samples were stained with a fluorescently conjugated anti-mouse CD3 monoclonal antibody. CD3-positive cells were enumerated by flow cytometry. Numbers shown in the figure are the percentage of cells derived from peripheral blood staining positive for CD3. Data is from one experiment.

Adaptive immune responses begin with the transport of antigen to a lymph node where it is presented by specialised antigen-presenting cells to T cells. As there is some evidence to suggest that FTY720-P may interfere with the early steps in this process, in our protocol administration of the drug was delayed for two days after footpad injection. So, to see whether FTY720-P could block egress of activated alloreactive cells from the popliteal node, C57BL/6 mice were first sensitised by footpad injection with BALB/c splenocytes, as before, on day zero. The mice received i.p. injections either with FTY720-P or control each day from day two to day five and were killed on day six. The draining popliteal lymph nodes were removed, and the cells derived from them reacted with BALB/c splenocytes to determine the number of alloreactive cells by IFN-γ ELISPOT.

Interestingly, while FTY720-P had no effect on the total number of cells within the activated popliteal lymph nodes (Figure 51, upper panel), it did cause a significant increase in the frequency of alloreactive cells there (mean frequencies of 15.0 (drug-treated) and 7.9 (control treated) per 1000 PLN-derived cells respectively; Figure 51, lower panel).

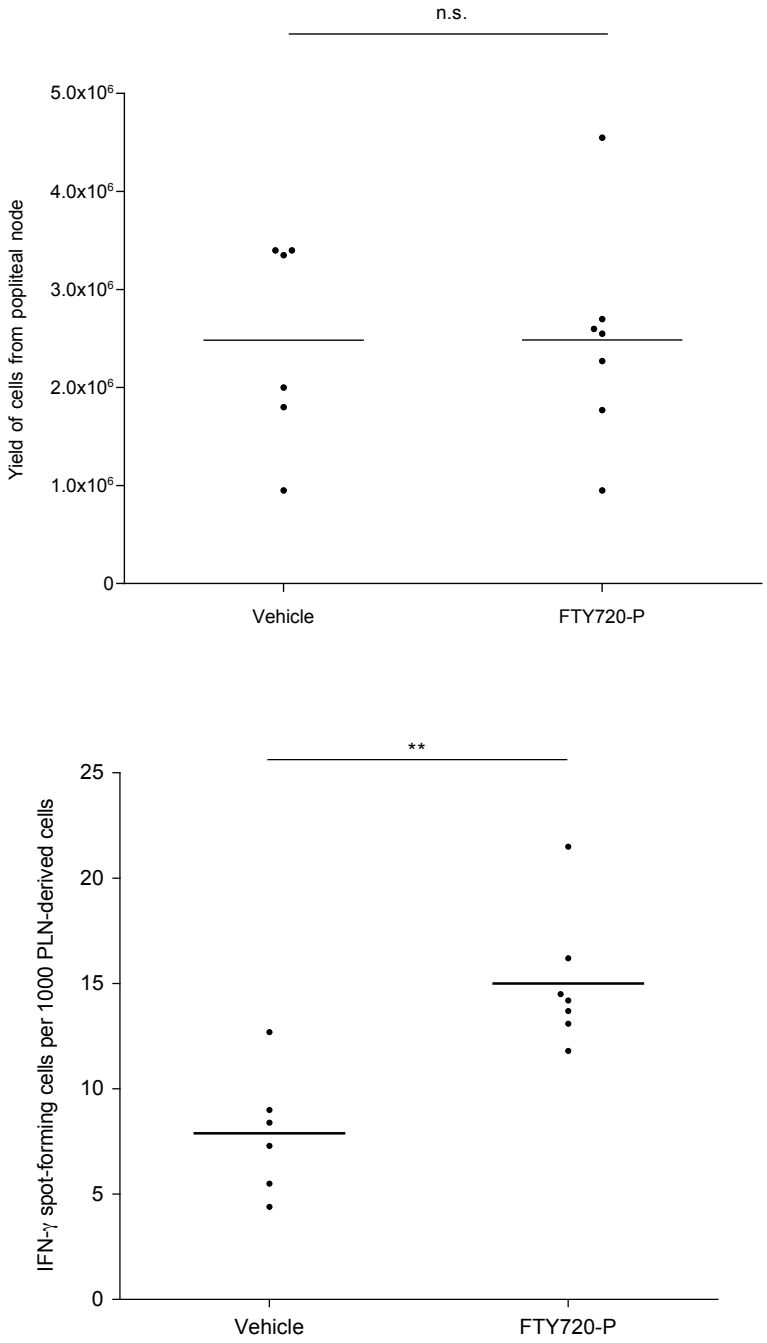


Figure 51. FTY720-P treatment increased the frequency of alloreactive cells in the popliteal lymph node.
Day 0: $5x10^6$ BALB/c splenocytes were injected into the hind left footpads of C57BL/6 mice. Days 2-5: mice were injected daily i.p. with 100 μl of 100 μg/ml FTY720-P or vehicle. Day 6: Mice were killed, and the draining popliteal nodes removed. Upper panel; the total number of cells derived from each node was measured using a haemocytometer. Lower panel; popliteal node-derived cells were co-cultured with BALB/c splenocytes. The frequency of IFN-γ spot-forming cells was determined by ELISPOT. Points represent individual mice; 6 mice treated with vehicle, 7 with FTY720-P. Horizontal lines are means.

These results indicated that FTY720 could sequester activated, alloreactive, cells within the draining lymph node. To confirm that these cells were activated T cells, cells from the draining popliteal nodes of FTY720-P and control–treated animals were stained with a CXCR3-specific monoclonal antibody. As expected, FTY720-P treatment resulted in an almost doubling of the percentage of CXCR3-positive cells in the draining lymph node (Figure 52; FTY720-P -treated: 18.9 %, SEM: 2.56; control-treated: 9.83 %, SEM: 0.491).

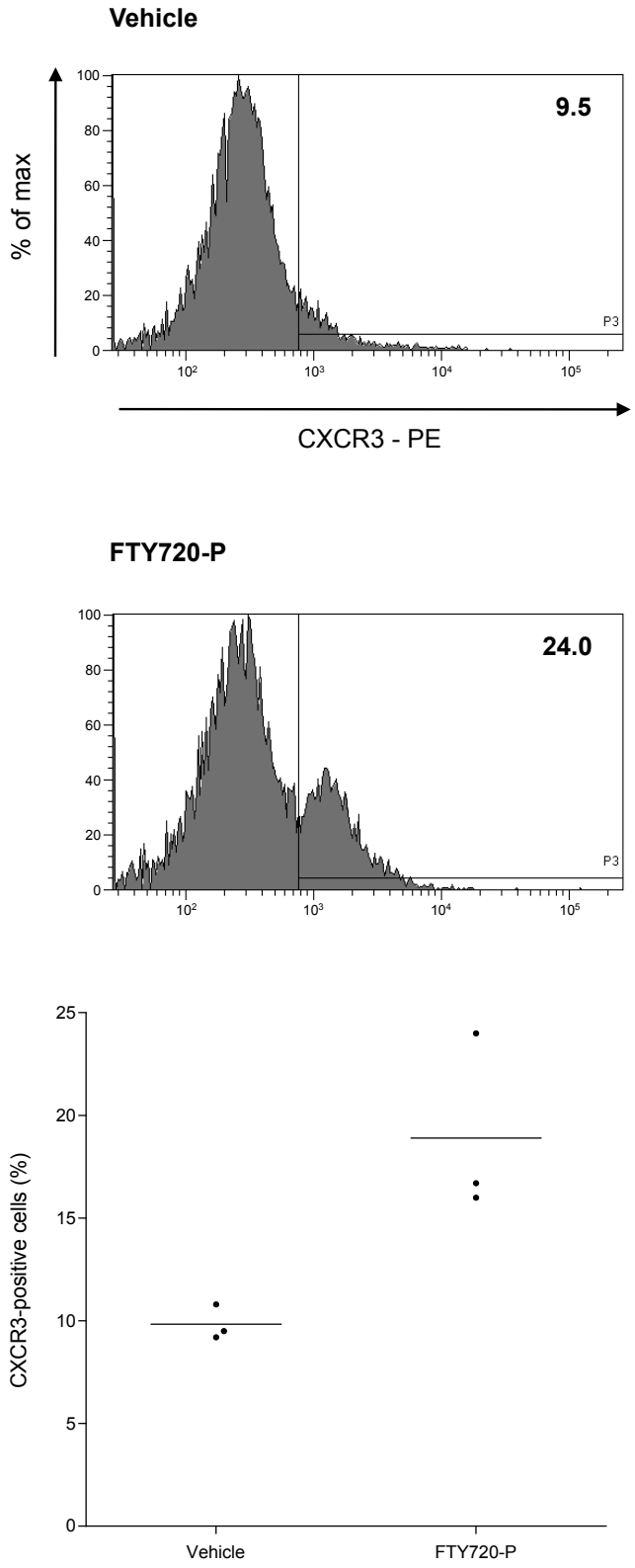


Figure 52. FTY720-P treatment of allosensitised mice caused the retention of CXCR3-positive cells within the draining popliteal node.
Popliteal lymph node -derived cells were taken from three vehicle-treated and three FTY720-P -treated mice used in the previous experiment. The cells were stained with a fluorescently conjugated anti-mouse CXCR3 monoclonal antibody, and analysed by flow cytometry. Upper panel; representative staining of cells from one control mouse and one drug-treated mouse. Numbers are the percentage of CXCR3-positive cells. Lower panel; percentages of cells derived from the draining popliteal lymph nodes of three control and three drug-treated mice that were CXCR3-positive. Data points are individual mice, horizontal lines are means of each group.

5.4 Discussion

Here, an *in vivo* model of alloimmunity has been used to address several outstanding questions over the control of egress of antigen-activated T cells from lymphoid tissue. An alloantigen challenge model was used to test for dependence of activated T cells on S1P receptor signalling for egress from an activated lymph node. The findings support a model in which recently activated T cells are able to leave an inflamed lymph node while non-specific cells remain sequestered there. This presumably allows for early trafficking of effector T cells to the periphery and immune protection, whilst maintaining capacity inside the lymph node for further immune priming and maturation of the adaptive immune response.

Cultured ELISPOT specific for IFN-γ was used in this study to estimate the frequency of alloreactive cells in various compartments. This was because alloimmune responses are commonly associated with a dominance of Th1 type immunity, associated with high levels of IFN-γ production (Strom *et al.*, 1996). The finding here that approximately 0.1 % of splenic T cells from 'naïve' C57BL/6 mice reacted with BALB/c splenocytes by production of IFN-γ is broadly similar with results reported by others (Felix *et al.*, 2007). Nevertheless, it is probable that the frequencies reported here are an underestimate of the true numbers of alloreactive cells *in vivo*, either because of the existence of alloreactive cells that did not produce IFN-γ, or those producing levels that were below the limit of detection of the assay.

In the first set of *in vivo* experiments, mice were primed with BALB/c splenocytes injected into one hind footpad. After six days, the cellularity of the draining popliteal lymph node and contralateral node from each mouse were measured. As expected, there was a significant difference between them. There was a mean of approximately $2x10^6$ cells in the draining node, four times that in the contralateral one. In a similar study, in which the same number of BALB/c splenocytes were injected into the footpads of C57BL/6 mice, the authors reported slightly higher counts in the popliteal node than those recorded here (mean cell counts of about $3x10^6$ in the draining node), although they looked seven days after footpad injection (Funeshima-Fuji *et al.*, 2008). Whether the disparity can be attributed to the different experiment endpoint, or other factors, could be resolved by further study of the dynamics of node inflammation after

footpad injection. Nevertheless, the results here are consistent with inflammation of the draining node, and an accumulation of lymphocytes caused by a non-specific block in lymphocyte egress. Direct presentation of intact allo-MHC on BALB/c splenocytes to C57BL/6 T cells within the node itself was probably the most important method of immune priming in this situation. This is because there is a much higher frequency of precursor T cells that will be activated by intact MHC complexes on allogeneic cells than processed peptides derived from allo-MHC (indirect presentation by host antigen-presenting cells). High levels of IFN-α produced by T cells (and probably also by NK cells) will then trigger upregulation of CD69 on ingressing T cells, resulting in a block in their egress (Shiow *et al.*, 2006).

Priming with BALB/c splenocytes resulted in an increase in the frequency of alloresponders (measured six days after injection) in not just the draining popliteal lymph node, but also the spleen, mesenteric nodes and blood. This implies the release of alloreactive T cells, originally activated in the popliteal node, throughout the mice. Interestingly, the number of alloreactive cells in the mesenteric nodes was much lower than the spleen and blood. This probably reflects the low number of specific central memory cells able to enter that compartment, whereas the blood and spleen probably contained a mixture of memory and effector T cells. In a study in which mice were challenged with OVA antigen intradermally in the neck and ear, antigen-specific cells were observed in distal lymph nodes as early as day five (Miller *et al.*, 2004). These data therefore suggest that by day six after injection of alloimmune cells into the footpad, specific cells are egressing the draining node, whilst inflammation there is still ongoing.

Published work has shown that FTY720 can influence the earliest stages involved in the generation of adaptive immune responses. Using an antigen challenge model, Xie *et al.* showed that if FTY720 is administered prior to injection of antigen into the footpad, then the number of activated T cells in the draining lymph node, measured three days after priming, was significantly reduced (Xie *et al.*, 2003). They suggest that this effect is mediated, in part, by a defective recirculation of naïve T cells triggered by drug treatment. This attenuates the accumulation of potentially reactive naïve T cells in the local lymph node, thereby impairing the efficiency of the adaptive response.

Therefore, to minimise the influence of FTY720 on immune priming in this model, FTY720-P treatment was delayed for two days after footpad injection. By this time, it would be expected that direct presentation will have occurred and T cells will be proliferating in the node. Few T cells would be expected to be leaving however at this stage.

FTY720-P treatment increased the frequency of alloactivated cells in the draining lymph node six days after footpad injection. This clearly implies that at least some of the alloactivated T cells capable of leaving up to that time point were dependent on S1P receptor signalling for egress. The results of our study bolster the conclusions of Habicht *et al.* who showed that effector memory T cells could be trapped in regional lymphoid tissue by FTY720 treatment. They also used an alloimmune model, although they adoptively transferred TCR-transgenic allospecific T cells along with the allograft, and then followed how the distribution of those cells was affected by FTY720 (Habicht *et al.*, 2006). Here, ELISPOT has been used to show that activated alloreactive cells (originating from the recipient animal) are similarly retained in lymph nodes by FTY720-P treatment.

Other groups have suggested that antigen-activated T cells are not dependent on S1P receptor signalling for cell egress from lymph nodes. It has been known for some time that whereas FTY720 treatment efficiently depletes naïve T cells from peripheral sites, and sequesters them in LN, antigen-experienced cells are not so affected (Hofmann *et al.*, 2006). Furthermore, using an *in vivo* model of contact hypersensitivity, Nakashima *et al.* showed that FTY720 did not significantly suppress the delayed-type hypersensitivity reaction if administered during the elicitation phase (Nakashima *et al.*, 2008). It is possible however to reconcile these apparently contradictory sets of results. Regarding the delayed type hypersensitivity model, the generation of effector memory cells in the sensitisation phase probably explains their results. Pre-existing effector memory T cells do not efficiently enter lymph nodes, and are not therefore sequestered there by FTY720 treatment delivered during the elicitation stage.

That antigen-activated T cells were indeed being retained by FTY720-P treatment was confirmed by CXCR3 staining. However, as both effector memory and central memory T cells express this receptor (Groom and Luster, 2011), it is not possible to be certain whether there was any bias in retention of

one of these subsets. Central memory cells do express CCR7 and it seems reasonable to suppose that this may confer a stronger requirement for S1PR1 signalling to facilitate their egress (compared with effector memory and effector cells). T cells genetically deficient in CCR7 are less efficiently retained in lymph nodes by FTY720 treatment than wild type cells (Pham *et al.*, 2008). Analysis of the retained lymphocytes for effector and central memory markers (including CD45RA, CD45RO and CCR7) is clearly needed to resolve this question.

Chapter 6. General Discussion

General Discussion

6.1 Summary

Effective T cell-mediated adaptive immunity depends on the orchestration of a complex series of cell movements. Chemokines produced either constitutively, or induced for example in response to inflammation, play an important role in many cases. Recent work has indicated that the endogeneous lipid sphingosine 1-phosphate and the chemokine ligands for CCR7, CCL19 and 21, act in a complementary way to direct T cell recirculation under homeostatic conditions. In this thesis, the role of S1P in control of T cell trafficking was explored in further detail. At the start of the investigation, one objective was to look for potential cross-talk between the S1P signalling axis and that of a prototypical chemokine. Secondary to this, the aim was to understand mechanistically any observed effects on chemokine function. Another objective was to observe and explain the regulation of S1P signalling after T cell activation, and to test *in vivo* the dependence on S1P for the egress of antigen-activated T cells from lymphoid tissue.

The data presented in this thesis show cross-talk between the S1P and chemokine signalling pathways. They show that S1P can potently affect the ability of both resting and activated T cells to migrate towards homeostatic and inflammatory chemokines. This furthers our understanding of the ways in which T cell migration can be cross-regulated by multiple factors that they may encounter *in vivo*. It was also shown here that T cell activation is followed by changes in the capacity of S1P to signal through its receptor S1PR1. This was demonstrated to be a result of changes both in S1PR1 gene expression and in expression of the transmembrane protein CD69. This is biologically significant as the egress of activated T cells from lymph nodes is dependent on S1P receptor signalling. These results contribute to our understanding of a fundamental aspect of adaptive immunity: how the egress of activated T cells, primed in lymph nodes during adaptive immune responses, is regulated.

6.2 Cross-talk between S1P and Chemokines

Changes in the expression of chemokines in various immune compartments, and in the expression of chemokine receptors on T cells, can only partly explain the fine control of cell migration *in vivo*. It has become clear that there is much cross-regulation and integration of different signals that allows the migration of cells to be precisely controlled depending on the immune status at any particular time and at any specific site in the body. Foxman *et al.* have described a 'chemotactic field' in which cells receive multiple inputs at each stage as they migrate (for example from vascular endothelium to their final destination at some site in peripheral tissues (Foxman *et al.*, 1997)).

Cross-regulation of chemokine responses is extensively described in the literature. An example of this is the cross-inhibition of migration and adhesion responses induced by CXCL12 and CCL5 (Vaday *et al.*, 2001; Hecht *et al.*, 2003). This occurs via a process known as heterologous desensitisation, in which G protein-coupled receptors (other than the type directly stimulated) are internalised. Cross-phosphorylation of chemokine receptors by protein kinase C, and a pathway involving phospholipase C activity (Didsbury *et al.*, 1991; Ali *et al.*, 1999) are involved in the process. Evidence has been found that points to a specific role for the protein kinase C -epsilon (PKCε) isoform in the ability of CXCR1 and CCR5 to cross-desensitise each other on receptor signalling (Nasser *et al.*, 2005). fMLP (a bacterial product and end-stage chemoattractant) can stimulate the cross-phosphorylation of the C5aR and the IL-8 receptor CXCR1, but is itself resistant to this regulation (Richardson *et al.*, 1995). It is thought that this kind of regulation is necessary for the prioritisation of chemotactic cues. Responses to chemokines are also affected by the non-chemotactic factors. TNF-α augments the pro-adhesive action of CXCL12, but suppresses the migratory response to the chemokine (Franitza *et al.*, 2000). It has been suggested that this might be important for anchoring T cells at inflamed sites where they may then execute their effector function.

A drive to understand the mechanism of action of the immunosuppressive drug FTY720 led to the discovery that the lysophospholipid S1P plays, at the least, an important role in the homeostatic trafficking of T cells. Pham *et al.* showed that S1P functions specifically in T cells at egress sites in lymph nodes. S1PR1 signalling was shown to inhibit Gαi signalling, predominantly that of CCR7, to

promote cell entry into cortical sinuses (Pham *et al.*, 2008). Thus the S1P / S1PR1 signalling axis was demonstrated to interact directly with that of chemokines in the control of T cell recirculation. The ubiquity of chemokines and S1P in many different compartments *in vivo* means that there are other opportunities for crosstalk between the two signalling pathways. These include S1PR1 and the other prominent homeostatic chemokine CXCL12, and also inflammatory chemokines such as CXCL10.

A number of preliminary studies showed that S1P could indeed modulate responses to chemokines other than the CCR7 ligands (Graeler *et al.*, 2002; Yopp *et al.*, 2005). They did not however offer any mechanistic insight into how this was mediated. In Chapter 3 the ability of S1P to modulate T cell chemotaxis towards the homeostatic chemokine CXCL12 was tested using the Jurkat human T cell line. A systematic approach was then applied to address the question of a potential mechanism for any observed effect. Pre-treatment with 1 μM S1P for one hour enhanced the migration of Jurkat T cells towards CXCL12. Use of a receptor-specific agonist showed that the effect was mediated through S1PR1. The enhancement of migration could not be explained simply with reference to heterologous desensitisation of the chemokine receptor, as this typically results in the suppression rather than enhancement of chemokine responses. Investigation into the mechanism behind the effect ruled out S1P - driven changes in levels of CXCR4, coupling to adenylate cyclase via the Gαi G protein, or other pathways including the phosphorylation of ERK 1 / 2 as possible explanations.

In Chapter 4, S1P was shown to potently suppress the migration of resting primary T cells towards CXCL12. This is opposite to the effect of S1P on the migration of Jurkat T cells towards the same chemokine, pointing to the unsuitability of this, and perhaps all cell lines, for studies of this kind. Although the mechanism for the effect on primary cells was not sought here, the phenomenon is analogous to that caused by other GPCR agonists and might be a result of heterologous desensitisation of CXCR4 (Hecht *et al.*, 2003). Differences in the complement of proteins involved in chemokine receptor signalling, S1P receptor signalling, or both presumably account for the disparity in the effects seen in Jurkat versus primary T cells.

The inhibition of migration of resting primary T cells towards CXCL12 fits with the report of Pham *et al.* which stated that S1P signalling inhibited that of Gαi-coupled chemokine receptors (Pham *et al.*, 2008). Both CCR7, to which they refer, and CXCR4 (a receptor for CXCL12), are Gαi coupled receptors that direct entry of T cells into lymph nodes (Bai *et al.*, 2009). CXCL12 is produced constitutively at a significant level by endothelial cells and stromal cells in the parafollicular areas of lymph nodes, is presented on high endothelial venules (Karin, 2010), and probably functions alongside CCL19 and CCL21 as a retention signal in lymph nodes.

S1P pre-treatment enhanced, rather than suppressed, T cell migration towards the inflammatory chemokine CXCL10. A subset of activated T cells begin expressing the receptor for this chemokine, CXCR3, after priming in lymph nodes (Groom and Luster, 2011). CXCL10 is important for the trafficking of effector T cells into many inflamed tissues including the CNS (Zhang *et al.*, 2008), allografts (Agostini *et al.*, 2001) and inflamed liver (Zeremski *et al.*, 2007). The high level of S1P in blood may therefore act to 'sensitise' activated T cells that have recently left the lymph node to CXCL10. This would promote adaptive immunity by increasing the efficiency of migration of effector T cells to inflamed sites. Increased levels of sphingosine 1-phosphate in inflamed tissues, caused by vascular leakage (Dvorak, 2010) and increased export from cells (such as mast cells; Mitra *et al.*, 2006), may conceivably also enhance cell migration along interstitial gradients of inflammatory chemokines.

6.3 Regulation of S1PR1 Signalling After T Cell Activation

An adaptive immune response begins with the presentation of processed antigen by mature specialised antigen-presenting cells to lymphocytes. This occurs within the specialised microenvironment of lymphoid tissue. Activation of the innate immune system precedes the adaptive response, and prepares lymphoid tissue to increase the probability of a productive adaptive immune response. One way this is achieved is via simultaneously increasing recruitment of lymphocytes to inflamed lymph nodes and also blocking their egress, sequestering them there. This block in egress is known as lymph node shutdown. Early work implicated prostaglandin E2 and complement activation as important mediators of the effect (Hay and Hobbs, 1977; Hopkins *et al.*, 1981). However, without a complete model to describe lymphocyte, particularly T cell, recirculation under normal conditions their data could not be placed in adequate context.

As has been previously detailed, a crucial role for S1PR1 signalling in control of egress of resting T cells from lymph nodes under normal conditions has been demonstrated (Matloubian *et al.*, 2004). Subsequently, Pham *et al.* explained the role of S1PR1, showing that CCR7 and S1PR1 form an antagonistic axis, promoting entry and retention, and the exit of T cells respectively (Pham *et al.*, 2008). By inference, lymph node shutdown was presumably therefore dependent on a loss in the capacity for S1P to signal through S1PR1 (expressed on T cells) and permit cell egress. A potential regulator of S1PR1 was identified by Chu *et al.*, who performed a genetic screen in Jurkat T cells and identified a negative regulatory interaction between CD69 and S1PR1 (Chu *et al.*, 2003). It was then shown that interferon α/β signalling is crucial for the block in egress of T cells from lymph nodes, providing the link between the innate response and lymph node shutdown (Shiow *et al.*, 2006). They demonstrated that the phenomenon was T cell intrinsic and was caused by IFN-α/β dependent upregulation of CD69, resulting in downregulation and inactivation of surface S1PR1. CD69 was found to interact directly with S1PR1 protein, perhaps altering the conformation of the receptor, and utilising existing regulatory pathways of S1PR1 expression to downregulate the protein (Bankovich *et al.*, 2010). Experiments using *in vivo* imaging techniques showed that T cell entry into inflamed nodes was associated with very rapid upregulation

of CD69, to a level sufficient to block entry into lymphatic sinuses (Grigorova *et al.*, 2010).

In productive adaptive immune responses, a small number of sequestered T cells are primed through interaction with mature antigen-presenting cells bearing cognate antigen. These cells, and their daughters that result from cell division, are known to be temporarily retained in lymph nodes as they differentiate into effector and memory cells. Until now it has been unclear whether S1PR1 signalling in, and by extension egress capability of, antigen-activated T cells is regulated differently to that of non-specific cells in inflamed lymph nodes. In Chapter 4, the regulation of S1PR1 signalling in activated T cells *in vitro* was investigated. T cells were stimulated with CD3 / CD28 beads to mimic activation by a mature professional antigen-presenting cell presenting peptide specific for the T cell receptor. The S1PR1-specific agonist SEW2871 was then used to probe for the capacity of S1PR1 to signal in the cells at various time points after activation.

Using SEW2871, resting T cells were shown to respond to stimulation of S1PR1. T cell activation through CD3 and CD28 resulted in a complete loss in the capacity of S1PR1 to signal 24 hours after activation. By day 3, receptor signalling was restored. These changes in the capacity of T cells to receive signals through S1PR1 mirror the *in vivo* migration of T cells before and after activation. Resting T cells can enter and exit lymph nodes quickly under homeostatic conditions. T cell activation results in complete arrest of T cells within lymph nodes, and it is not until approximately three days after the initial activation event that activated T cells begin leaving the node and enter the circulation (Matloubian *et al.*, 2004).

Graeler and Goetzl showed that activation of mouse splenic T cells was associated with a significant decrease in S1PR1 gene expression (Graeler and Goetzl, 2002). Similarly, here it is shown that stimulation through CD3 and CD28 resulted in a significant decrease in S1PR1 gene expression in human T cells, measured 24 hours later. This decrease was sustained for three days. Reasoning that this did not adequately explain the rapid and complete loss of S1PR1 signalling one day after activation, the dynamics of CD69 expression were also investigated. CD69 was upregulated to the cell surface very rapidly and to a high level after stimulation through CD3 and CD28. Levels were

highest approximately 24 hours after activation when ability of S1PR1 to signal was completely lost. Between one and six days after activation CD69 protein was progressively downregulated with cell division. These results showed a strong negative correlation between CD69 expression and S1PR1 signalling capacity in T cells. To confirm whether CD69 did indeed regulate S1PR1 signalling in activated T cells, siRNA was used to attenuate the upregulation of CD69 following T cell activation. Preloading T cells with CD69-specific siRNA, but not non-specific siRNA, restored S1PR1 signalling in 24 hour-activated T cells. This confirmed CD69 as an important regulator of this receptor in the context of T cell activation.

This work supports a model in which CD69 expression plays an important role in the regulation of S1PR1 signalling in antigen-activated T cells. It seems that the upregulation of CD69 is necessary for the rapid loss in S1PR1 signalling following activation, which cannot be achieved through the reduction in gene expression alone. The CFSE dilution experiment showed that in proliferating T cells, surface CD69 decreased by approximately one half with each cell division. This would be consistent with transient burst of neosynthesis of CD69 protein following T cell activation, followed by splitting of existing protein between daughter cells as the cells divide. This means that, on average, cells that have divided more times express lower levels of CD69. *In vivo*, this probably translates into an increase in the probability of egress of a particular cell from a lymph node the more times that it has divided.

6.4 S1P Receptor-dependent Egress of Activated T Cells from Lymph Nodes

In Chapter Four, it was shown that S1PR1 signalling is modulated after T cell activation. Changes in both the expression of the receptor and in surface expression of the known interaction partner CD69 were demonstrated to be important. As this work was performed entirely *in vitro* however, the physiological significance remained open to doubt. Specifically, an outstanding controversy in the literature is the extent to which, *in vivo*, the egress of activated T cells is dependent on S1PR1 signalling in T cells. T cell activation consists of a complex array of cellular events and produces daughter cells expressing a different complement of proteins including those involved in adhesion and migration. This raises the possibility that the egress of activated T cells is regulated differently to that of resting T cells.

The work detailed in Chapter Five complements that in the previous chapter by showing that S1P receptor signalling is indeed necessary for the egress of activated T cells from lymph nodes. An alloimmune response was generated in the popliteal lymph node of a mouse by transfer of fully MHC-mismatched splenocytes into the footpad. Cultured ELISPOT was used to determine the frequency of alloactivated cells within the popliteal node six days after footpad injection. Treatment with the S1P receptor superagonist FTY720-P (which downregulates S1P receptors and abrogates receptor signalling) following footpad injection resulted in an increase in the frequency of alloactivated cells within the draining lymph node. The fraction of cells that were $CXCR3^{+}$ (indicative of activated T cells) in the node at day six was also increased by FTY720-P treatment. Alloactivated cells were thus found to be sequestered in a lymph node by FTY720-P, suggesting that their egress was dependent on S1P receptor signalling. These data are consistent with those of another report showing that the release of antigen-activated CD4 T cells into the blood was S1P-dependent (Xie *et al.*, 2003). In their study however, adoptively transferred antigen-specific cells were used and they counted activated T cells once they had exited into the peripheral blood. Here, natively produced alloactivated cells have been enumerated in the local lymph node.

6.5 Future Work

There are a number of questions arising from this work that provide a sound basis for further investigation.

A number of different approaches were used to try, unsuccessfully, to identify the mechanism by which S1P enhanced chemotaxis of Jurkat T cells towards CXCL12. Subsequently, experimentation with primary cells showed that S1P suppressed the migration of resting T cells towards the same chemokine. As has been already stated, heterologous desensitisation of CXCR4 resulting from prior S1PR1 signalling might account for this effect. The mechanism of desensitisation usually entails phosphorylation of the intracellular tail of the receptor, and internalisation of the protein from the plasma membrane. Immunofluorescence flow cytometry could be used to detect any change in the abundance of surface CXCR4 following S1P treatment. If a reduction in surface receptor is seen, this work could be followed up by examination of phosphorylation of the receptor tail by western blotting. Hecht *et al.* showed that CCL5 inhibited CXCR4 signalling independently of receptor levels, but that Ca^{2+} mobilisation, and ERK and Protein tyrosine kinase 2 phosphorylation triggered by receptor stimulation were suppressed (Hecht *et al.*, 2003). These pathways could also be examined. Chemokines do not simply trigger chemotaxis, but also signal changes in the conformation of integrins (Laudanna *et al.*, 2002) and affect cell adhesion to matrix or other cells. A flow cytometry-based adhesion assay could be used to determine the effect of S1P pre-treatment on the ability of CXCL12 to trigger changes in integrin conformation and capacity for high affinity binding to their ligand (for example VLA-4 on activated T cells and VCAM-1 (O'Boyle *et al.*, 2009)). This could also be investigated using microfluidic apparatus that can be used to create a more accurate model of cell flow within capillaries.

Cross-talk between S1P and chemokines *in vivo* could be better modelled using transendothelial migration assays. The bare transwell assays used here simply rely upon cell squeezing through pores in a plastic membrane. Transendothelial work allows one to assess the capacity of cells to respond to presented chemokine and form the required adhesive interactions for paracellular migration. These experiments could be done by coating the transwell membrane with suitable cells such as the human endothelial cell line EA.hy926

(Edgell *et al.*, 1983). These can be cultured on the membrane till confluent, and then migration assays performed as before (Newton *et al.*, 2009). By including S1P in the upper compartment and combining this with T cell pre-treatment, this would constitute a better representation of the *in vivo* situation of cellular migration from the vascular compartment across an endothelial barrier into tissue.

The reasons behind the different effects of S1P on migration towards homeostatic and inflammatory chemokines (CXCL12 and CXCL10 respectively) were not addressed. Hints at a potential mechanism have emerged from studies examining the precise Gαi subunit usage of these chemokine receptors. CXCR3-mediated signalling is Gαi2-dependent and enhanced in the absence of Gαi3, indicating a potential antagonistic role for the latter (Thompson *et al.*, 2007). Chemotaxis towards CXCL12 shows dependence on both Gαi subunits. Interestingly, although the total magnitude of the response is reduced, in Gαi3 knockout cells the effect of S1P is to enhance rather than suppress chemotaxis towards CXCL12 (Jin *et al.*, 2008). These data therefore indicate that differences in Gαi subunit usage might underpin the different effects of S1P on responses to different chemokines. The work of Jin *et al.* (which used mouse T cells and a slightly different experimental protocol) could be followed up with primary human resting T cells using the protocol outlined in this thesis. siRNA could be used to downmodulate the Gαi3 protein. The effect of S1P pre-treatment (with 1 μM ligand for 1 hour) on migration of these cells towards CXCL12 could then be tested.

While strong staining of HEK 293 S1PR1 transfectants (data not shown) could be achieved with mouse monoclonal 218713 (R&D Systems) against S1PR1, primary T cells stained very weakly with the same antibody. Another group has been able to achieve significant staining of S1PR1 using a self-produced affinity-purified anti-serum (Lo *et al.*, 2005). With a highly specific antibody for the receptor, it would be possible to expand on the work in Chapter Four, and study the change in the abundance of surface S1PR1 after T cell activation. Shiow *et al.* showed that not only does CD69 inhibit S1PR1 signalling, but that it results in downregulation of the protein from the cell surface (Shiow *et al.*, 2006). The very high levels of CD69 induced by T cell activation may even drive significant degradation of S1PR1 (seen in CD69 S1PR1 double transfectants

(Bankovich *et al.*, 2010)) and thus contribute to the significant reduction in total protein seen 24 hours after cell activation (Graeler and Goetzl, 2002). This could be tested by blocking the upregulation of CD69 on T cell activation with specific siRNA, using the method detailed in Chapter Four.

There is scope to expand work with the *in vivo* model described in Chapter Five. The limitation of the work here is that the ELISPOT assay does not allow the phenotype of the alloactivated cells to be studied in any detail. This is a significant deficiency as adaptive immune responses typically result in the generation of a heterogeneous population of activated T cells. There are short-lived effectors and longer-lived memory cells, and the latter can be further categorised as central memory or effector memory cells. An outstanding question in the literature concerns whether these different types of T cell have differential lymph node egress requirements. Like CD69, CCR7 expression is progressively downregulated with T cell division, and this is correlated with simultaneous upregulation of S1PR1 (Pham *et al.*, 2008). Central memory cells, which have a shorter division history, express higher levels of CCR7 than effector T cells (Sallusto and Lanzavecchia, 2009). Because of this, central memory cells might be expected to exhibit relatively stronger dependence on S1P signalling for egress from lymph nodes – and might leave on average later in the response. This question could be addressed using an IFN-γ cytokine secretion assay (Miltenyi Biotech) instead of ELISPOT to identify alloactivated cells inside lymph nodes. This would allow simultaneous phenotyping of the cytokine-secreting cells, such as distinguishing central memory (CCR7+ CD62L+ CD44+) from effector memory and short-lived effector cells (CCR7- CD62L- CD44+). The end-point of the experiment could also be varied from day three to day six, so that the dynamics of cell egress over that period could be studied.

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Publications Arising from this Study

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Published papers:

SWAN, D. J., KIRBY J. A. & ALI S. (2010) Vascular biology: the role of sphingosine 1-phosphate in both the resting state and inflammation. *J Cell Mol Med*, 14, 2211-22.

Published abstracts:

SWAN, D. J., KIRBY J. A. & ALI S. (2010) Modulation of T lymphocyte responses to chemokines by sphingosine 1-phosphate. *Immunology*, 131, 73.

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Oral presentations:

Sphingosine 1-phosphate: roles in the modulation of T lymphocyte trafficking. Institute of Cellular Medicine Research Day, June 2010 and Immunology North East Annual Symposium, June 2010.