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An Investigation into the Role of PI3K Isoforms in Human T Lymphocyte Migration

A thesis submitted by

Laura D. Smith

for the degree of Ph.D.
University of Bath
Department of Pharmacy and Pharmacology
September 2007

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Ph.D.
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Publications

Papers


Book chapter:

Abstracts presented at:


Abstract
The activation of PI3K seems to be a signalling event initiated by many chemokine receptors that are expressed on T cells; however, the role of PI3K within T cell migration it has not been thoroughly elucidated. The use of knockout murine models has been invaluable in assigning function to certain isoforms within this family, yet there is potential for other family members to initiate compensatory mechanisms and therefore unequivocal isoform function assignment is problematic. Furthermore, whether the information generated through the use of murine models will provide an accurate reflection of the responses generated within human physiology is unclear.

Within these studies pharmacological and siRNA strategies were undertaken to assess the contribution of individual PI3K isoforms within human T cell migration. The use of broad spectrum PI3K inhibitors demonstrated a role for PI3K within CXCL12 mediated migration of freshly isolated T cells. Following CXCL12 stimulation, isoform selective inhibitors against the class I family furthered this observation by revealing a dominant role for PI3Ky in the migratory response and PI3K/PKB signalling (as assessed by S6 phosphorylation). Utilizing a non-viral method (termed Nucleofection) to deliver siRNA into human T cells requires maintenance of cells ex vivo for 3 days and although targeting siRNA reduces mRNA levels of PI3K class ly, class II α and β, curiously this silencing has no effect on the CXCL12-mediated migratory response. However, it is observed that ex vivo culture of T cells in the presence or absence of IL-2 develop a PI3K independent migratory response, while maintaining their dependence on PI3K for random migration.

The observations within this study suggest limitations for the delivery of siRNA via Nucleofection into human T cells. Furthermore, this study suggests a dominant role for PI3K class ly within the migratory responses of freshly isolated T cells, however, the dependence on PI3K is context-dependent and varies depending on the ex vivo maintenance and/or activation state of the cells.
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Abbreviations

5-LO  5-Lipoxygenase
AC    Adenylyl cyclase
ADP   Adenosine Diphosphate
ANOVA Analysis of Variance
AOP-CCL5 Amino-oxpentane-CCL5
AP-1   Activator protein-1
APC    Antigen presenting cell
APKC   Atypical PKC
Arp2/3 Actin-related protein2/3
ATP    Adenosine Triphosphate
BSA    Bovine serum albumin
C2     C2 domain
C5a    Complement Component 5
Ca\textsuperscript{2+} Calcium ions
cAMP   Cyclic adenosine monophosphate
Chemokine Chemotactic cytokine
Class I α Phosphoinositol 3-kinase class I α
Class I β Phosphoinositol 3-kinase class I β
Class I γ Phosphoinositol 3-kinase class I γ
Class I δ Phosphoinositol 3-kinase class I δ
Class II α Phosphoinositol 3-kinase class II α
Class II β Phosphoinositol 3-kinase class II β
Class II γ Phosphoinositol 3-kinase class II γ
COPD   Chronic obstructive pulmonary disease
cPKC   Conventional PKC
CRAC   Calcium release activated channels
CTL    Cytotoxic T-lymphocytes
CTLA4  Cytotoxic T lymphocytes antigen-4
DAG    Diacylglycerol
DAPP-1 Dual adaptor for phosphotyrosine and 3-phosphoinositides
DARC   Duffy Antigen receptor for chemokines
DC     Dendritic cells

XIV
DMSO  Dimethylsulphoxide
ECL   Enhanced Chemiluminescent reagent
ERK  Extracellular regulated kinase
F-actin Filamentous actin
FITC Fluorescein isothiocyanate
fMLP Formyl-methionyl-leucyl-phenylalanine
FYVE Fab1, YOTB, Vac1 and EEA1 domain
GAP  GTPase-activating proteins
GDI  Guanine nucleotide dissociation inhibitor
GDP  Guanosine diphosphate
GEF  Guanine nucleotide exchange factor
GFP  Green fluorescent protein
GM-CSF Granulocyte-macrophage colony-stimulating factor
GPCR G-protein coupled receptor
GRK  G-protein coupled receptor kinase
GTP  Guanosine Triphosphate
HRP  Horse radish peroxidase
ICAM-1 Intercellular adhesion molecule-1
ICL  Intracellular loop
ICOS Inducible costimulatory receptor
IL   Interleukin
IL-2 Interleukin-2
IP$_3$ Inositol triphosphate
ITAM Immunoreceptor tyrosine-based activation motifs
JAK  Janus Kinase
JNK  c-jun-NH2 terminal kinase
LAT  Linker for activation in T cells
LTA4H Leukotriene A4 hydrolase
LTB4 Leukotriene B4
LY294002 Specific PI3K inhibitor
MAPK Mitogen-activated protein kinase
MHC  Major histocompatibility complex
mRNA Messenger Ribonucleic Acid
MS   Multiple Sclerosis

XV
MTOC  Microtubule-organizing center
mTOR  Mammalian target of rapamycin
NFAT  Nuclear factor of activated T cells
NNY-CCL5 N-nonanoyl-CCL5
NPKC  Novel PKC
PAMP  Pathogen-associated molecular patterns
PBL  Peripheral blood derived lymphocytes
PBMC  Peripheral Blood Mononuclear Cells
PBS  Phosphate buffered saline
PDE  Phosphodiesterase
PDK-1  Phosphoinositide-dependent kinase-1
PECAM  Platelet-endothelial cell adhesion molecule
PH  Pleckstrin homology
PHA  Phytohemagglutinin
PI3K  Phosphoinositide 3-kinase
PIK  Phosphatidylinositol kinase domain
PKB  Protein kinase B
PKC  Protein kinase C
PLC  Phospholipase C
PMA  Phorbol myristate acetate
PMN  Polymorphonuclear leukocytes
PP2A  Protein-serine/threonine phosphatase 2 A
PRR  Pattern recognition receptors
PS  Phosphatidylserine
PSC-CCL5 L-Thia-Pro², L-α-cyclohexyl-Gly³NN-CCL5
PSC-Rantes [L-thiazolidine-4-carboxyl2,cyclohexyl-Gly3]NNY-Rantes
PtdIns(3)P  Phosphatidylinositol(3)phosphate
PtdIns(3,4)P2  Phosphatidylinositol(3,4)bisphosphate
PtdIns(3,4,5)P3  Phosphatidylinositol(3,4,5)trisphosphate
PTEN  Phosphatase and tensin homologue deleted from chromosome 10
PX  Phox homology
PTX  Pertussis toxin
RA  Rheumatoid Arthritis
Ras-BD  Ras binding domain
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ras-GRF</td>
<td>Ras-Guanine nucleotide releasing factor</td>
</tr>
<tr>
<td>Ras-GRP</td>
<td>Ras-Guanine nucleotide releasing protein</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil forming kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal Enterotoxin B</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol phosphatase</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SLC</td>
<td>Secondary lymphoid chemokine</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2-containing Leukocyte protein of 76 kDa</td>
</tr>
<tr>
<td>SoS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>STIM1</td>
<td>Stromal interaction molecule 1</td>
</tr>
<tr>
<td>TAPP</td>
<td>Tandem PH-domain-containing protein.</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-R</td>
<td>Tumour necrosis factor-receptor</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>Tyk</td>
<td>Tyrosine Kinase</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen -4</td>
</tr>
<tr>
<td>Vps34</td>
<td>Vacuolar protein sorting 34</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Z -chain associate protein kinase</td>
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CHAPTER 1:
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Introduction

1.1 The immune system

The immune system is a finely controlled system and functions to maintain the health of an organism by limiting infection and disease. In mammals this process is coordinated via two distinct branches of the immune system, termed innate and acquired immunity. These seemingly discrete systems initiate very different immunological profiles however, interaction is essential for initiation of the acquired response. The innate immune system is triggered rapidly after infection with a pathogen; this response can act to limit the infection while the slower adaptive response is activated. It is important to note, that identification of self and non-self is the primary hurdle within the immunological response.

1.1.1 Innate Immunity

The first response to infection is initiated by the innate immune system, this may occur in several forms, the simplest being barrier protection provided by skin, pulmonary and gut epithelia. If breached the specialised cells of the innate immune system, monocytes and macrophages, neutrophils and dendritic cells (DC) begin to clear the infection. Within innate immunity, detection of self or non-self is determined by the binding of a region of the pathogen known as PAMP (pathogen-associated molecular patterns) (Medzhitov and Janeway, Jr., 1997) to PRRs (pattern recognition receptors) such as Toll-like receptors, which recognise the conserved sequences shared among pathogens but not the host. Binding of different antigens gives rise to activation of different Toll-like receptors, thus initiating a variety of responses such as the production of reactive oxygen species and nitrogen intermediates.

These cells possess a fixed repertoire of non-specific inherited receptors, in which further infections will initiate the same innate immunological response. In contrast, cells of the adaptive immune response, such as T and B lymphocytes, respond in a highly specific manner against the pathogen, showing immunological specificity and memory. The primary infection which initiates adaptive immune response will be
slow, however, subsequent infections will be more rapid due to pools of memory cells.

1.1.2 Adaptive Immunity

Adaptive or acquired immunity is split into humoral (B cell mediated) and cell mediated (T cell mediated) responses. Humoral responses involve soluble antibodies generated by plasma cells, while cell mediated responses are specialised into many sub-groups, which can physically bind foreign molecules.

Innate immunity can act as the trigger for activation of the acquired immune response by inducing the production of pro-inflammatory cytokines, resulting in further leukocytes being recruited to the site of inflammation. Dendritic cells play a leading role, with immature DC binding conserved sequences within the pathogen, initiating maturation and expression of TLR (Vestweber, 2003) and co-stimulatory molecules, such as CD80/CD86 (Akira et al., 2001). During T cell activation, mature DC migrate to the lymph nodes and present the processed antigen (bound to the major histocompatibility molecule) to naïve T lymphocytes, while a second co-stimulatory signal is given via binding of B7 to CD28 (Banchereau and Steinman, 1998; Reis e Sousa, 2001).

The complexity of the immune system is only achieved by the dynamic and evolving array of specialised cells which make up this system. Central to this is the fine control achieved within the cellular migration and response. This control is accomplished via up and down regulation of specific receptors expressed on these cells, with T lymphocytes exemplifying this process exquisitely.

1.2 T lymphocytes

The work presented within this thesis has been performed primarily on human T lymphocytes. In order to provide a background of this cellular system, a detailed presentation of the physiological life cycle of the T cell, including the activation of these cells will be performed.
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T cells are derived from bone marrow haematopoietic stem cells that enter and populate the thymus. At the point of entry, it is not yet determined what sub-group of T cells these immature cells (thymocytes) will form, in order for this to be determined, cells undergo multiple stages of selection (figure 1.1).

**Figure 1.1: Development of γδ and αβ T cells within the thymus.** Immature T cells enter the thymus and must undergo 2 main decisions within their lineage. The first is which TCR will be displayed on the surface, the common αβ or less common γδ (only 5 %). The second decision is between CD4+ T helper cell and CD8+ killer cell.

The circulating pool of naïve T lymphocytes pass through their final phase of development in the thymus, this is initiated by TCR rearrangement. T cells are classified by the heterodimer which comprises their TCR, this may either be the most common and studied T cell, α/β TCR or the γδ TCR which makes up between 1-5 % of peripheral blood T cells (Hayday, 2000). Although, γδ T cells make up a relative small subgroup, they have a distinct receptor profile, and have been suggested to play a role in immune defence against infection, in particular the gut (Glatzel et al., 2002). Following TCR rearrangement there is a massive IL-7 driven expansion and differentiation into CD4+CD8+ double positive thymocytes. Through positive and negative selection, cells are selected for either the CD4+ or CD8+ lineage. Negative selection determines whether the T cell recognises self antigens. If cells recognise self antigens with high affinity, these cells receive signals to undergo apoptosis. This process ensures that any autoreactive T cells do not enter the circulation, which may result in disease. Positive selection determines how capable the T cell is at recognising and binding the MHC complex. If cells show high affinity for this process, the cells receive survival signals. This selection process ensures that only single positive cells proceed to exit the thymus.
Within this process, large numbers of thymocytes undergo cell death, leading to comparatively few lymphocytes forming the circulating naïve T cell pool.

1.2.1 T cell activation

Once the T cells have exited the thymus, these naïve cells undertake an immunosurveillance role, circulating through the lymph. Only upon encountering an antigen will these cells activate and mature into the corresponding effector cell. Naïve T cells require 2 signals in order to activate. This activation is a tightly controlled process, requiring interaction between the T cell receptor (TCR) and its ligand, but also binding and activation of co-receptors and orchestrated reorganisation of the cytoskeleton, ensuring full activation of the T cell (see figure 1.2).

**Antigen-presenting cell**

**T cell**

**Figure 1.2 Model of T cell activation.** Complete activation of the T cell requires 2 signals. The first signal is generated through antigen stimulation which is initiated through the APC which presents the antigen bound to the major histocompatibility complex (MHC). It is this complex that has the capacity to bind the TCR/CD3 complex on the T cell. The second co-stimulatory signal is via the T cell receptor CD28, binding to its complementary ligand B7.

**Two signals are required in T cell activation**

The initial signal required for T cell activation, is generated by the ligation of the TCR with the MHC complex of the antigen presenting cell, (e.g. a dendritic cell). However, as the TCR alone is not capable of transmitting the extracellular signal
into a response, a complementary molecule is required, this is provided via the association of CD3 molecules (Kuhns et al., 2006).

The TCR is comprised of αβ subunits that specifically recognise and bind the peptide/MHC (figure 1.3). The signals that are generated from this interaction, that subsequently lead to the activation of the T cell, are dependent on protein tyrosine kinases, namely Fyn, Lck, ZAP-70 and ITK. Transmittal of the antigen induced signal is dependent upon the CD3 molecules, which contain multiple immunoreceptor tyrosine-based activation motifs (ITAMs) (Kane et al., 2000; Sun et al., 2001).

![Zeta Chains](image)

**Figure 1.3 The T cell receptor complex.** The TCR is comprised of the αβ subunits which signal through the association with CD3 and the ζ-chain accessory molecules. The CD4 receptor aids in stabilizing the TCR association with the peptide/MHC complex.

These conserved motifs found in CD3γ, δ and ε cytoplasmic domains, as well as the TCR ζ chain, act as tyrosine kinase substrates that bind other kinases, thus propagating the signal. Although the mechanism of signal transduction from ligation of the TCR, through to the phosphorylation of the ITAM motifs is not fully elucidated, it has been proposed that the TCR undergoes a conformational change that is relayed to the CD3 interacting subunits (Kjer-Nielsen et al., 2003; Krogsgaard et al., 2003). It has been proposed that the initial phosphorylation of the ITAM motif is primarily due to the Src family kinase Lck, this kinase interacts with the CD4 or CD8 cytoplasmic domain and following binding of the TCR/MHC, Lck is brought into
close proximity phosphorylating the ITAM motifs. The ITAM motifs now have the capacity to bind and phosphorylate ζ-chain associate protein kinase (ZAP-70), which in turn acts to recruit and activate adaptor proteins such as SH2-containing Leukocyte Protein of 76 kDa (SLP-76) and Linder for activation in T cells (LAT). The adaptor proteins can bind many different intracellular molecules, thus bringing them into close proximity to each other, and facilitating the propagation of the signalling response. The culmination of these signals is an increase in the activity of transcription factors, such as activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NFκB and thereby increasing expression of new proteins, such as CD25 and IL-2.

Optimal T cell activation requires a second co-stimulatory signal; this ensures amplification of the TCR signal and prevents the T cell from becoming anergic. This involves the interaction of the co-stimulatory molecule CD28 with the B7 family of proteins CD80 (B7.1) and CD86 (B7.2) on the APC. This interaction facilitates the production of IL-2 transcription, CD25 expression, entry into the cell cycle and enhanced cell survival. As with the TCR, CD28 lacks direct enzymatic activity and therefore is presumed to signal through protein tyrosine kinases, such as Lck and ITK. Furthermore the phosphorylation of CD28 by Lck, is essential in the recruitment of the p85 regulatory subunit of PI3K, which in turn recruits the catalytic p110 domains (α, β or δ) leading to PI3K activation. Further to this, is the discovery of the CD28 family member ICOS (inducible costimulatory receptor), a receptor which unlike the constitutively present CD28, is upregulated following T cell activation and functions to maintain T cell responses (Coyle et al., 2000; Hutloff et al., 1999).

T cell activation is also negatively regulated, in order to limit the response of the T cell. This inhibitory pathway is initiated by the third member of the CD28 family, CTLA-4 (cytotoxic T lymphocytes antigen-4) which is upregulated upon T cell activation (Thompson and Allison, 1997). Ligation of CTLA-4 terminates T cell activation, thus avoiding the detrimental overstimulation of the system (Sansom and Walker, 2006).
1.2.2 Differentiation of Effector cells

Following activation, the naïve T cells differentiate into effector T cells. CD4+ into Th1 or Th2 subtypes, and CD8+ into cytotoxic T cells. The process involved in this differentiation is described below.

Differentiation of CD8+ T lymphocytes

Differentiated CD8+ or cytotoxic T cells are an essential part of the adaptive immunity, recognising antigens presented on DC bound to MHC class I molecules. They are an important branch of the immune system which recognize and eliminate viral and bacterial pathogens (Maggi et al., 1997).

Following initial activation of the CD8+ T cells, the second phase of maturation begins. This involves immense proliferation, normally lasting for about 5-8 days, and can lead to an increase of more than 10,000 fold (Badovinac and Harty, 2002). Following proliferation, the now differentiated effector T cells are capable of migrating to the site of infection (Weninger et al., 2001). The final phase of the CD8+ lifecycle is the initiation and maintenance of memory cells. This has been shown to be consistently 5-10 % of the peak detected CD8+ levels within the response (Kaech et al., 2002).

Differentiation of CD4+ T lymphocytes

Since the discovery of CD4+ T helper cells, there have been significant developments in our understanding of the divergence of CD4+ T lymphocytes into their respective subsets. These studies have more thoroughly detailed, not only the conditions surrounding the development of the most prominent Th1 and Th2 subgroups, but have further highlighted the importance of regulatory T cells or Tregs and the newly discovered Th17 cells. Figure 1.4 illustrates the selection of these distinct classes. The role that cytokines play within this system cannot be underestimated, as it is clear that the divergence of Th1 and Th2 lineages, and thus there response in both normal and pathological conditions, is dependent on the cytokine environment that the naïve cell is exposed to.
Polarised Th1 or Th2 subsets

Driving of naive CD4\(^+\) precursors into the Th1 lineage, is initiated through presentation of antigens on APC of the innate immune system (Hunter, 2005; Hibbert et al., 2003). Importantly, it is not only one driving force behind the production of the Th1 subset, but rather a combination of cytokines. It has been well documented that the primary driving force behind Th1 differentiation is the cytokine IL-12, which is facilitated by the action IFN-\(\gamma\), plus IL-18 (Robinson et al., 1997) and IL-23 (Oppmann et al., 2000). Activation of the transcription factor T-bet, has been shown to be a ‘master’ regulator of Th1 cells (Mullen et al., 2001). A recent report has also elucidated to T-bet independent pathway although this process has not been not fully elucidated (Way and Wilson, 2004). Signalling through IL-12 potentates IFN-\(\gamma\) production and upregulation of the IL-18 receptor, giving rise to the mature Th1 effector T cell (Yang et al., 1999).

Th2 cells originate from the same precursor as Th1 cells, yet show an independent mechanism for generation. Their activation is a coordinated response between the
TCR and IL-4 stimulating STAT6, which in turn upregulates the master regulator in the differentiation of Th2 cells, GATA-3 (Ouyang et al., 2000).

Reciprocal inhibition of Th1 and Th2 cells
The specific circumstances that surround the generation of Th1 or Th2 cells, means that not only do cells of that lineage have the ability to stimulate further growth and proliferation, but this is reinforced by cross regulation of the opposing sub group (Romagnani, 2006). In this regard, generation of a typical Th1 cytokine such as IFN-γ, or IL-12 inhibits the production of Th2 cells and the corresponding humoral responses (Kips et al., 1996; Lack et al., 1996; Manetti et al., 1993; Parronchi et al., 1992). Conversely, the production of Th2 cytokines such as IL-4 inhibits Th1 development and activation (Skapenko et al., 2004; Ghoreschi et al., 2003).

1.2.3 Memory Generation of CD4+ and CD8+ T cells
Following antigen presentation, both CD4+ and CD8+ cells produce memory T lymphocytes which are further subdivided into central memory and effector memory cells. Memory T cells form part of the immunological memory pool, thus on a repeated exposure to the same antigen, the cellular response will be rapid and specific. To enable this response, central memory T cells are maintained primarily within lymphoid circulation. It has been shown that circulation of these cells is similar to naïve cells, migrating through the lymph node which is mediated by the presence of the CCR7 receptor. Effector memory T cells have the capacity to enter peripheral tissues, for this task they will specifically express the receptors required for migration into the tissue which first encountered the pathogen (Campbell et al., 2001). For example, if a pathogen was encountered in the gut, there is an increased chance the effector memory cell will recirculate through that tissue. This is facilitated as the lymph node homing receptor, CCR7, is downregulated and the α4β7 homing receptors are upregulated, facilitating entry into the gut and allowing the pathogen to be neutralised (Sallusto et al., 1999; Kupper and Fuhlbrigge, 2004; Butcher et al., 1999). This feature of effector memory T cells, suggests they may play a role in the first line of defence upon a second exposure of the antigen. Upon binding of the specific antigen, memory T cells rapidly proliferate, regenerating effector T cells which can respond to the antigen (Sallusto et al., 2004).
1.2.4 Th17
A recently characterised third subset of CD4+ T cells termed Th17 has been identified. Derived from the same precursor as Th1 and Th2, they are defined through their ability to secrete IL-17 (Langrish et al., 2005). This subset is distinct from either Th1 or Th2 and has its own unique master regulator, RORγt (Ivanov et al., 2006). The action of IL-6 and TGFβ on the naïve T cell pool are essential in the generation of these IL-17 producing cells (Veldhoen et al., 2006). IL-17 is a proinflammatory cytokine which has been demonstrated to induce the production of other proinflammatory cytokines such as IL-6, IL-8 and CCL2 (Aggarwal and Gurney, 2002). The identification of this subfamily has interesting implications, as diseases are reassessed and a role for the Th17 subtype is identified. These investigations are in the early stages of development but it has already been noted that high levels of IL-17 have been identified within autoimmune diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis, suggesting a role for this lineage in autoimmune disease (Mangan et al., 2006; Komiyama et al., 2006).

1.2.5 Regulatory T cells
In addition to effector T cells, CD4+ T cell can also differentiate into regulatory T cells. These adaptive regulatory T cells or Tregs, are produced from the same precursor as Th1, Th2 and Th17 cells and have been shown to play a role in the suppression of T cell responses and autoimmunity (Sakaguchi, 2000). These cells mature in parallel to effector T cells, and as such migrate to sources of inflammation, acting to suppress T cell driven inflammation, as pathogen induced antigens are resolved (Fantini et al., 2004; Wakkach et al., 2003). Natural Tregs have also been identified which develop from distinct T cell lineage, these cells act preferentially in secondary lymphoid tissue, suppressing effector T cell activation before it begins (Weaver et al., 2006).

1.3 Migration - The Physiological Role of Chemokines
An essential component to the immune response is the ability of immune cells to be recruited from the circulating system to areas of functional requirement. For
example, during immunosurveillance T cells traffic into regional lymphoid organs such as lymph nodes and the gut-associated Peyer's patch. Furthermore, during the immune response cell undergo activation and differentiation, allowing them to migrate into sites infection or injury in response to a large number of chemoattractants. The process by which cells pass from the blood into a selected region is known as extravasation, and is an essential component of immunosurveillance and the immune response (figure 1.5). Briefly, lymphocytes within the blood initiate interactions with the endothelial cells of the lumen, generating the characteristic rolling effect. Binding of chemoattractants, such as chemokines, which are bound to the surface of the lumen via glycosaminoglycans (GAGs), mediate integrin activation and firm adhesion. This adhesion and slowing of the cell, allows the lymphocyte to migrate across the endothelium. This process must be tightly regulated as entry of immune cells into healthy tissue can be destructive.

Under the normal flow of blood, immune cells are rushing through the blood vessels, therefore in order for cells to cross the endothelium to the required areas, cells must undergo a complex series of steps which will be described below.
1.3.1 Tethering and reversible rolling

Due to the fast flowing nature of blood flow, the initial interactions of the immune cell with endothelium are transient, these rapid and transient interactions allow the cell to ‘tether and roll’ along the endothelium, acting to slow down the speed of the passing cell. Tethering and rolling is mediated by specialized lectins e.g. selectins and leukocyte integrins sharing an α4 subunit e.g. VLA-4 (Very late antigen-4) and mucosal homing receptor α4β7. L-selectin on the surface of leukocytes is perfect for this process as it can form rapid weak interactions with mucins on the endothelium (Lasky et al., 1992; Grabovsky et al., 2000). Although not involved in the initial tethering of the cell, LFA-1 optimises the L-selectin binding decreasing the velocity at which lymphocytes pass over endothelium (Salas et al., 2002; Kadono et al., 2002).

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**Figure 1.5 Multi-step model of leukocyte extravasation.** This process is a tightly control cascade of events. Lymphocytes in the blood initiate interaction with endothelial cells via sequential selectin-integrin interactions, mediating the characteristic lymphocyte rolling. Upon binding their endothelial-displayed chemokine ligands, chemokine receptors signal to mediate integrin activation. Following activation, integrins bind their Ig superfamily ligands on the endothelium, mediating firm adhesion, and the lymphocyte can migrate across the endothelium (Adapted from (Pribila et al., 2004)).
1.3.2 Activation and stable adhesion

The deceleration of the cell allows for or increases the chance of sufficient lymphocyte activation leading to firm integrin-mediated arrest (Grabovsky et al., 2000; Campbell et al., 1998). Studies have shown that knocking out these leukocyte specific integrin genes leads to phenotypes such as impaired leukocyte recruitment and inflammatory responses, T cell proliferation defects and deficits in gut-associated lymphocytes.

Firm arrest on vascular endothelium depends on modulation of integrin avidity to ligand. Most integrins are present on the cell surface but in an OFF state when the cell is resting, however, upon activation the integrins are activated, allowing binding of the receptor to its complementary ligand (Hynes, 2002). This activation of the stimulated by chemokines that are attached to the endothelium. VLA-4 is the major vascular ligand for vascular cell adhesion molecule 1 (VCAM-1). Grabovsky and co-workers showed that immobilised chemokines can augment reversible VLA-4 mediated tethering and rolling on VCAM-1, before independent firm integrin mediated arrest on the endothelial ligand (Grabovsky et al., 2000).

The arrest of circulating lymphocytes is essential before the cell exits the circulation to the site of inflammation. The participation of the lymphocyte chemoattractant receptor in conversion from rolling to firm adhesion, has been evaluated and confirmed in 

1.3.3 Transmigration

Following firm adhesion is the process of diapedesis, in which the cell crosses the endothelial membrane (Petri and Bixel, 2006). Compared to other steps in the extravasaion process, relatively little is known about the precise mechanism under which these cells cross the endothelium. For many years this process was thought to occur purely through a paracellular mechanism, i.e. between two adjacent
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endothelial cells (as shown in above in figure 1.5). However, it has now become clear that leukocytes may also cross the endothelial membrane transcellularly or through an individual endothelial cell (Vestweber, 2003; Engelhardt and Wolburg, 2004). Carmen and Springer have shown that in vitro, monocytes can cross through the endothelium both para- and trans- cellulary (Carman and Springer, 2004; Muller et al., 1993).

Although the molecular basis for diapedesis is yet to be fully elucidated, several molecules have been identified within this process. Following arrest of the leukocyte, it begins to crawl along the endothelium and within minutes begins to cross. Platelet-endothelial cell adhesion molecule (PECAM)-1 has been shown to be involved during this process by the use of anti-PECAM-1 blocking antibodies. These studies suggest that PECAM-1 is involved not in the crawling of the cell to the junction, but in the actual crossing of the membrane. Removal of the blocking anti-body allows the leukocytes to cross the endothelium (Bird et al., 1993).

Interestingly, PECAM-1 knockout mice do not show a compromised immunological response, suggesting a PECAM-1 independent pathway. This maybe through other pathways such as ICAM-2 (Schenkel et al., 2006; Duncan et al., 1999; Huang et al., 2006).

1.4 Types of chemoattractants and methods of migration

1.4.1 Chemoattractants

Chemoattractants are substances that can induce directed cell migration or chemotaxis within motile cells. There are several different types of chemoattractant, the most notable is the chemokine family, which has been described in detail within this work. Some important chemokines and other chemoattractants responsible for the migration of immune cells are described below.

In order to provide effective immunesurveillance, T cells are required to migrate into and out of secondary lymphoid organs; two receptors that have been demonstrated to play essential roles in this process are CCR7 and sphingosine-1-phosphate, S1P
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(respectively). CCL19 and 21 are chemokines that bind the receptor CCR7. CCR7 is found not only on naïve cells but also on central memory T cells and more recently on natural killer and γδ T cells, indicating a broader range of cells that may utilize these chemokines for migration (Sallusto and Mackay, 2004).

Sphingosine-1-phosphate (S1P) is a lipid that has been shown to play an essential role in the migration of T cells out of the thymus. Consistent with S1Ps role in this process, mature single positive T cells have been demonstrated to exhibit the S1P receptor on the surface of the cell, facilitating the lymphocyte egress, while, S1P deficient T cells fail to exit the thymus. Interestingly, following antigen presentation, the responding T cell downregulates the S1P receptor (for up to 4 divisions), allowing for sustained stimulation of T cells within the lymph node (Sallusto and Mackay, 2004).

Following trauma or stimulation by bacterial peptides or immune complexes one of the early events of inflammation is the production of leukotrienes (Funk, 2001). Of particular importance is leukotriene B4 (LTB4), a potent mediator of neutrophil chemotaxis and stimulator of leukocyte adhesion to endothelial cells. Like chemokines, LTB4, exerts its actions through GPCRs, namely B-LT1 and B-LT2, with expression of B-LT1 restricted to leukocytes (Kamohara et al., 2000; Tager and Luster, 2003). LTB4 has been implicated in several inflammatory disorders, and increased levels have been recorded in samples from murine models of COPD, asthma and RA (Crooks et al., 2000; Montuschi and Barnes, 2002). Concurrent with this observation, administration of LTB4 antagonists in models of RA, demonstrate a decrease in inflammation and immune cell recruitment into subsynovial connective tissue (Griffiths et al., 1995).

The complement system, activated in response to a pathogen, represents an effective mechanism in the innate immune system. Furthermore, its activation has been implemented in the pathogenesis of acute and chronic inflammatory disorders such as MS, RA and asthma (Ffrench-Constant, 1994; Hawlisch et al., 2004). C5a has a diverse array of cellular functions from oxidative burst in neutrophils (Mollnes et al., 2002), to modulation of cytokine expression (Laudes et al., 2002). However,
it is also known to be a potent chemoattractant molecule in neutrophils (nM range), monocytes and macrophages (Marder et al., 1985).

1.4.2 Types of Migration

Cells of the immune system are capable of migration in several defined ways. The term chemotaxis is the directed migration of a cell towards a soluble chemotactic gradient, similarly, haptotaxis also describes the directional movement of a cell towards a chemotactic gradient but on an immobilized substrate. Chemokinesis involves the random movement of cell that is independent of any chemotactic gradient. Therefore, cells may spontaneously generate cytoplasmic projections and retractions and display migratory properties, yet this movement of the cell is without any directionality (figure 1.6). Chemofugetaxis or fugetaxis, are terms used to describe the active movement of a cell away from a chemokine source, this phenomenon was first described following the observation that high (but not low) concentrations of CXCL12 could induce chemofugetaxis in a sub-population of T cells and it is thought to contribute to thymic emigration (Poznansky et al., 2000).
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1.5 The processes involved in directed cell migration

Migration towards an extracellular chemoattractant gradient requires 3 distinct features of a cell including: extension of pseudopodia, gradient sensing and polarization and directional movement.

1.5.1 Extension of pseudopodia

During random migration or chemokinesis, cytoplasm projections are extending and contracting over the surface of the cell. Following stimulation (e.g. chemokine stimulation), the formation of projections or pseudopodia are focused towards the front or leading edge of the cell due to the accumulation of signalling molecules such as PtdIns(3,4,5)P$_3$. This transition and re-arrangement of intracellular signalling molecules is an essential element in the initiation of chemotaxis.

Figure 1.6 Methods of migration. Several methods of migration exist for leukocytes. A) Chemotaxis involves the migration of a cell towards the source of the gradient within a soluble environment. B) Chemokinesis occurs through random movement of a cell although no directionality is observed. C) As seen in chemotaxis, haptotaxis exhibits directional migration towards a concentration gradient, however, the chemokine is bound to the surface of the cell which the cell then crawls over. D) Chemofugetaxis occurs at high concentrations of chemokine, which in contrast to chemo- and hapto- taxis the cell moves away from the source of the chemokine. The gray arrow denotes direction of migration and the intensity of the yellow corresponds to the concentration of chemokine.
1.5.2 Polarization

An early event within leukocyte migration is the polarization of the cell, this involves the production of a ‘front’ or leading edge and ‘rear edge’ or uropod in leukocytes (del Pozo et al., 1995). Within directional sensing, it is important that that once a cell detects a shallow chemoattractant gradient, this is amplified within the cell. Once a chemoattractant gradient has been established, (for as little as 2% between the anterior and posterior of the cell), reorganisation of the internal environment is triggered. This reorganisation includes a change in the distribution of F-actin, from throughout the cell, to a concentration within the leading edge of the cell; resulting in the polarised shape (Parent and Devreotes, 1999; Howard and Oresajo, 1985; Coates et al., 1992; Chung et al., 2001b). Leukocytes do possess some intrinsic cell polarity in which there is differential sub cellular localization of F-actin and assembled myosin II, allowing rapid responses to chemoattractant gradients (Chung et al., 2001b).

Within murine models of neutrophils chemotaxis, phosphoinositide 3-kinase or PI3K (a family of lipids capable of phosphorylating the inositol ring on membrane bound phosphoinositides) has been shown to contribute to gradient sensing. Binding of chemokines to their receptors results in the rapid activation of this family leading to the accumulation of PI3K products in particular PtdIns(3,4,5)P$_3$ at the plasma membrane (Iglesias and Levchenko, 2002). Accumulation of PtdIns(3,4,5)P$_3$ acts to recruit PH-domain contain proteins to the leading edge of the cell which aid in driving downstream signalling events (Bokoch, 1995; Servant et al., 2000). The PI3K family, its regulation and role in chemotaxis is discussed in greater detail within section 1.8.2.

Actin polymerisation within motile cells is of fundamental importance and is continually being polymerised and depolymerised within the cell, allowing for a dynamic environment. Actin’s functional unit is known as globular actin or G-actin, and it is the polymerisation of these intrinsically polarised units (by the action of ATP), that once bound together generate filamentous actin (F-actin). The building of new F-actin is performed by the polymerisation at one end of the filament, known as the ‘barbed end’ while depolymerisation occurs at the opposing or pointed end (figure 1.7). During migration the generation of a new filament is focused at the
leading edge of the cell and pushes the plasma membrane forward, resulting in a protrusion of the cell. The continued growth of F-actin continues until halted by the binding of a capping protein on the barbed end of the filament. This capping procedure acts to either maintain or stabilize the filament or promote depolymerisation. These capping proteins can be located on the pointed end, in which they inhibit the dephosphorylation of the filament, or at the barbed end, ensuring that no further G-actin monomers are added. In a process termed as treadmilling, the phosphorylation and dephosphorylation rate are comparatively equal, thus ensuring the maintenance of the current filament length (Vicente-Manzanares et al., 2005; Revenu et al., 2004).

**Figure 1.7 Actin filaments: structure and dynamics.** Actin monomers known as G-actin bind ATP and assemble much more rapidly at the 'barbed end' compared to the 'pointed end'. During treadmilling, the polymerization rate and depolymerization rate are equal thus filament length is maintained at a constant. Several proteins are known to bind to actin; capping proteins may bind to the barbed end preventing addition of further monomers, while some as demonstrated are known as crosslinking proteins, allowing several filaments to bind together.
In order to maintain motility, cells must release the adhesions at the back of the cell and retract the uropod, which is dependent on the motor activity of myosin II. In myosin II null cells retraction of the uropod is defective thus resulting in ineffective chemotaxis (Clow and McNally, 1999). The most conclusive evidence that PI3K plays a role in chemotaxis, has come from the utilization of PI3K inhibitors such as wortmannin and LY294002, and the study of PI3Kγ knockout mice. Studies indicate that mice which are PI3Kγ−/−, have an impaired ability for mounting an immune response, in particular the function of neutrophils and macrophages is severely impaired both in vitro and in vivo (Li et al., 2000).

The role of PI3K in chemotaxis or directed cell migration has been thoroughly established and of particular importance is the role that this molecule plays in the migration of cells within the immune system (Ferguson et al., 2007; Hirsch et al., 2000). The clinical implications of developing an inhibitor against isoforms within the PI3K family have a potential therapeutic benefit in diseases as diverse as autoimmune disease, cancer and asthma. While it is clear that the PI3K family plays a pivotal role in the chemotaxis of neutrophils, the contribution of PI3K within T cell chemotaxis is less clear.

Broad spectrum PI3K inhibitors notably LY294002 and wortmannin have highlighted a role for this family within migration, although these compounds are unable to adequately discriminate between individual members of this family. If a specific and directed therapy against the PI3K family is to be developed, the role and relative contribution to each function must be dissected out. The data generated thus far has demonstrated a certain level of context dependence, in that the role that the PI3K family plays within chemotaxis depends on the cell under investigation and or the priming before chemokine stimulation.

Chemokines induce migration through G-protein mediated interactions which have been attributed to the Gai subfamily, this has highlighted a role for PI3K class Iγ which has been shown to lie downstream of βγ subunits. Chemokines also have the potential to activate other members of the PI3K family, with their contribution to optimal migratory responses being evaluated within the Jurkat T cell line, however
the contribution of these isoforms within the migratory process of primary human T cells has yet to be determined.

The role of knock-out and knock-in murine models have been invaluable in assigning function to specific isoforms within this family (Thomas et al., 2005). Throughout these studies, the focus was on the effect of these isoforms within human T cells, enabling data obtained to reflect the role of these molecules in the human physiological setting, particularly migratory responses.

Within the murine neutrophil model, chemotactic responses have been demonstrated to be dependent on PI3K class Iγ, yet within T cells there are mixed reports, with some data suggesting a central role for the PI3K family within T cell chemotaxis, while others have shown that contribution may only be required for optimal chemotactic responses. In a situation where T cells might rely on alternative pathways for migration, the molecular mechanisms underlying this process are starting to be explored. A candidate for PI3K independent cell migration is the Rac GEF DOCK-2, as mice lacking DOCK-2 show a decrease within the chemotactic responses towards several chemokines (Nombela-Arrieta et al., 2004; Reif et al., 2004).
1.6 Chemokines and Chemokine Receptors

1.6.1 Nomenclature and Structural characteristics of Chemokines

Chemokines are highly conserved 8-10 kDa proteins characterized by a 4 cystein motif. Dependent on their primary amino acid structure chemokines can be divided into 4 sub-families C, CC, CXC, or CX3C depending upon the number of amino acids separating the first two cystein residues at the NH2 terminal (Wells et al., 1998). A further structural sub classification of the CXC chemokine family can be made depending on the presence of a tripeptide motif ELR in the NH2 terminus Glu-Leu-Arg (ELR)+ and Glu-Leu-Arg (ELR)-. CXC chemokines classified as ELR+ are, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 with these chemokines being known to be strong chemoattractants for neutrophil polymorphonuclear leukocytes (PMN) into inflamed tissues.

Further to the structural classification, chemokines may also be classified according to their function and expression. Inflammatory or inducible chemokines encompass those that are upregulated upon injury or disease and are therefore important components of the innate and adaptive immunity. Homeostatic or constitutive chemokines are present even during a healthy or disease and injury free state and as such are responsible for orchestrating the traffic and homing of leukocytes during their immune surveillance role (figure 1.8). Discussed within this work are some of the main concepts within chemokine signalling and function as well as some of the most recent discoveries within this field.
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Figure 1.8 Classification of human chemokines into functional subfamilies.
Chemokines can be classified according to their function. Homeostatic chemokines are responsible for orchestrating the traffic and homing of leukocytes during their immune surveillance role, while inflammatory chemokines are important components of the innate and adaptive immunity (Adapted from Moser et al., 2004).

Chemokines may be classified according to their structure or function, yet common to all chemokines is the family of receptors through which they exert their biological function, the family of G-protein coupled receptors.

1.6.2 G-protein coupled receptors

G-protein coupled receptors or GPCRs constitute a group of large and diverse receptors capable of binding not only chemokines but also hormones and lipid mediators. These cell surface receptors couple to intracellular heterotrimeric G-proteins which regulate downstream effectors such as adenylyl cyclase, phospholipases, protein kinases and ion channels (Armbruster and Roth, 2005). As shown in figure 1.9, GPCRs are characterised by a unique structure, a hydrophobic core of seven transmembrane spanning domains creating three intracellular loops, an amino terminus on the outside of the cell and a carboxyl terminus on the inner surface (Allen et al., 2007).

Taking the ‘simplistic’ view within GPCR signalling, a monomeric GPCR binds a ligand on the N-terminus side of the receptor, this binding of the ligand induces a
conformational change within the receptor which is transduced through the 7TM domains allowing for interaction and activation of the G-proteins (Armbruster and Roth, 2005). However, it has recently become apparent that the signalling through GPCRs is more complex, with these receptors combining to form not only homodimers but also heterodimers (Breitwieser, 2004). For example, in T cells the CCR5 chemokine receptor has been shown to homodimerise, with the dimerisation specifically dependent on two residues in transmembrane 1 and 4. Mutation of these residues does not give rise to a dimer and importantly leads to lack of signalling through CCR5 (Hernanz-Falcon et al., 2004). Biochemical and functional evidence has also highlighted the heterodimerisation of CCR5 with CCR2. These heterodimers signal and traffic differently from the component receptors. Mellado also showed that following stimulation by both CCL2 and CCL5, (agonists for CCR2 and CCR5), these receptors heterodimerise resulting in an increased sensitivity of the receptor complex, leading to a 10 % decrease in the concentration of agonist required to produce the same chemotactic and calcium responses (Mellado et al., 2001).

The functional relevance of this dimerisation may be important in several areas of T cell biology. For example, during the extravasation of T cells, chemokines are bound to the surface of the endothelium and binding to their respective chemokine receptors may induce the characteristic rolling of the cell (figure 1.5). Dimerisation of receptors may not only decrease the required threshold for activation and arrest, but provide an increased sensitivity and specificity within the system (Rodriguez-Frade et al., 2001).

These results suggest that chemokines can act synergistically to facilitate responses even to low concentrations of chemokines, but this also means that they could suppress some couplings. Although heterodimerisation has been shown for certain receptors, this cannot be a generalised for all chemokine receptors and it most probably shows that certain chemokine receptors can couple to only a few other receptors therefore each case must be evaluated independently. The apparent homo- and hetero-dimerisation of these receptors only adds to the complexity of chemokine signalling and functional response.
In order to transmit an extracellular signal into a meaningful signal and function within the cell, the ligand and receptor must undergo a 3 step response. This response encompasses binding of the ligand or agonist, activation of the receptor and initiation or inhibition of second messengers within the cell (figure 1.9).

Figure 1.9 A schematic depicting a GPCR and the interaction of CXCL12 with its receptor CXCR4. (A) A 2D representation of an unfolded GPCR within the cell membrane, highlighting the N- and C-terminals as well as the intracellular loops (ICL), as discussed later ICL2 is essential for the signalling of GPCRs. (B) CXCR4 is shown with the seven helices represented as cylinders, which are connected by the surface and cytoplasmic loops. (B, left panel) indicates the receptor and ligand separately prior to any interaction between the two. (B, middle panel) indicates the interaction between CXCL12 with the N-terminal segment of the receptor. The contact region is in blue. (B, right panel) shows the N-terminal region of CXCL12 bound in groove at the top of the helices (orange). Binding of the N-terminal region results in activation of the receptor, which is depicted in right panel by the change in conformation of the receptor helices compared with the middle panel (Crump et al., 1997).
The binding of a ligand on the extracellular portion of the receptor, initiates a conformational change within the receptor thus activating it. The linking of the activated receptor and the second messenger signalling cascade is provided by a heterotrimeric guanine nucleotide binding protein (G-protein) on the inner leaflet of the cell membrane (Neer, 1995). These G-proteins function as molecular switches within the cell and are comprised of an α, β, and γ subunit. Binding of the heterotrimeric G-protein with the receptor initiates the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α subunit. This exchange leads to the removal of the hydrophobic pocket of which G-βγ binds thus reducing the affinity of Ga-GTP and G-βγ resulting in the dissociation of the Ga-GTP from the Gβγ complex (Lambright et al., 1994). The importance of the second intracellular loop in facilitating the flow of signal into the cell, has been highlighted by the discovery of ‘atypical’ chemokine receptors (discussed later).

The free G-βγ subunits are now available to activate or inhibit downstream signalling molecules. Termination of these effects which occurs rapidly within the cell, is a result of GTP being hydrolysed back to GDP through the innate GTPase activity of the α subunit allowing reassociation of the α, βγ complex (Hamm, 1998; Sprang, 1997).

1.6.3 G-α and G-βγ interaction with effectors

Activation of specific downstream effectors is achieved through the variability of G-proteins. G-proteins are divided into four families based on the similarity of their α subunits; Gs, Gi0, Gq, G12/13. Historically, it was assumed that it was only the G-α subunit that triggered intracellular signalling through well defined pathways such as adenylate cyclase and phospholipase C. However, it is now clearly established that the G-βγ complex, couples to and triggers the activation of signalling cascades such as PLCβ2 and β3 (Katz et al., 1992), ACs (Tang and Gilman, 1991), β-adrenergic receptor kinase, PI3K (Stephens et al., 1994; Tang and Downes, 1997), components of the MAPK cascade (Inglese et al., 1995), and K+ and Ca2+ channels (Cabrera-Vera et al., 2003).
Class IB is the only class of PI3K shown to be activated by the dissociated G-βγ subunits, with the regulatory subunit p101, sensitising the catalytic subunit to G-βγ (Stephens et al., 1997). It is further proposed that p110γ is activated via interaction with GTP-bound Ras which via allosteric mechanism or altered orientation of p110γ in respect to its substrate further increase its activity (Suire et al., 2002). Activation of class IA isoforms is thought to be via the Gai activation of Src family kinases, (Ma et al., 2000). However although it has been shown that class II isoforms lie downstream of chemokine receptors their activation mechanism has yet to be fully elucidated (Curnock et al., 2002).

In conclusion, in order for chemokines to exert an effect within the cell, the signal must be controlled at several levels. This is performed by each receptor binding a specific number of ligands and combination of G proteins, with these G-proteins only triggering specific signal transductions cascades within the cell. Amplification of this particular signal is ensured, as following dissociation of the G-protein complex, the receptor/ligand coupling is then free to bind the next G-protein. Furthermore, although deactivation of the functional G-proteins is rapid, the active functional complex is stable long enough to ensure the triggering and amplification of the second messengers.

1.6.4 Regulation of GPCR signalling

The availability of GPCRs on the cell surface is a tightly controlled process. The initial step is the synthesis of the receptor in the endoplasmic reticulum, in which the GPCR is then packaged for transport to the surface. The mature glycosylated receptor can now interact with its specific ligand. One notable aspect of GPCR signalling is the sensitivity of the cell following stimulation of the GPCR via its ligands. This sensitivity or negative regulation is often termed desensitization.

Desensitization of the receptor can occur rapidly after exposure to stimuli, reducing the responsiveness of the receptor to further stimulation. It occurs due to the action of G protein-coupled receptor kinases (GRKs) which phosphorylate the receptor following agonist induced conformational changes, this facilitates the binding of the inhibitory β-arrestin proteins that uncouple the receptor from the G-proteins (Vroon
et al., 2006; Pao and Benovic, 2002). Uncoupling can also occur via second messenger dependent kinases e.g. protein kinase A and C. In contrast to GRK mediated uncoupling, this method has the capacity to desensitise any receptor in the presence or absence of ligand binding, it is often referred to as heterologous desensitisation and mediates a generalised cellular hyporesponsiveness (Kristiansen, 2004). The extent of receptor desensitization can vary depending on the cellular system, from attenuation to complete termination of the signal (Ferguson, 2001; Aramori et al., 1997).

Internalization or sequestration of GPCRs is another mechanism in which signalling and function through GPCRs is controlled. In comparison to desensitization, this process takes longer, occurring over several minutes (Luttrell and Lefkowitz, 2002). However, it can be dependent on β-arrestins. Following chemokine exposure the CCR5, CXCR1 and CXCR4 receptors have all been identified to undergo sequestration via β-arrestins, this may also be coupled to associated proteins such as clathrin which promotes the sequestration process (Ferguson, 2001; Cheng et al., 2000; Kristiansen, 2004).

Once the GPCR has been sequestered it undergoes one of two mechanisms; receptor recycling or degradation. For recycling back to the plasma membrane, the receptor undergoes dephosphorylation and removal of β-arrestin which resensitizes the receptor to the ligand. The time course for this process is dependent on the ligand/receptor interaction; however the kinetics behind this process can be adjusted with the use of modified chemokines. This technique has been demonstrated to increase the time the receptor is sequestered within the cell before recycling (Pastore et al., 2003). The alternative is degradation of the receptor via the lysosomal degradation pathway, this has been highlighted as a process for β2-adrenergic and CXCR4 receptor degradation (Wojcikiewicz, 2004).
1.6.5 Chemokine Receptors

The action of chemokines on their receptors can elicit a plethora of cellular responses. In order to fully appreciate the importance of specific ligand / receptor interactions it must deciphered what response can be assigned to each interaction. This goal is made all the more challenging by the promiscuous nature of chemokines, as it has been shown that individual chemokines may activate several receptors and each receptor may be activated by a number of different chemokines, indicating redundancy and versatility within the system (Ward and Westwick, 1998). Shown in Figure 1.10 is a summary of the chemokine receptors and the ligands which activate them, due to this promiscuity there has been difficulties when assigning function to receptor-ligand interactions.

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**Figure 1.10 Chemokine receptors and ligands.** Chemokines are divided into subclasses based on the spacing of the N-terminal cysteine residues. The receptors for the α (or CXC) subclass are shown in blue, the receptors for the β (or CC) subclass are in red and the receptors for the minor subclasses (C and CX3C) are in green. The pairing of chemokines to their receptors has been carried out principally by receptor-binding assays, and has identified receptors that are specific, in that they bind a single ligand, or shared, in that they bind more than one ligand (Adapted from Johnson et al., 2004).
1.6.6 Atypical chemokine receptors

The control of the inflammatory process relies on the intricate control of each stage of the process. Chemokine receptors have long been thought to play a role in the recruitment of inflammatory cells to the site of injury or infection. However, the discovery of 'atypical' chemokine receptors with their apparent non-signalling or silent properties, introduces an exciting field within chemokine receptor function, and raises the question of what role these abundant receptors play within the regulation of inflammation.

These modified GPCRs have been termed 'chemokine interceptors' for internalizing receptors. These receptors differ from their counterparts as they show a mutation in the second intracellular loop. Typical chemokine receptors display the normal 'DRYLAIVHA' motif which has been shown to be a key mediator in the coupling to downstream signalling. Yet these atypical chemokine receptors have an alteration in this critical motif (figure 1.11). This subtle change leads to a silent receptor, in that no signalling or functional response following chemokine binding is observed (Nibbs et al., 1997; Haraldsen and Rot, 2006).

D6 and DARC (Duffy Antigen Receptor for Chemokines) are two of the most studied examples of these 'atypical' receptors. These investigations have lead to the belief that the primary role for these silent receptors is for chemokine sequestration and degradation, thus providing a mechanism for removing the chemokine from the inflamed site. By undertaking this role, these so called silent receptors play a crucial role in regulating chemokine bioavailability and as such have an influence over the inflammatory process (Comerford and Nibbs, 2005; Nibs et al., 2003). Although these receptors do not signal and are therefore excluded from the systematic nomenclature of chemokine receptors, they do play an important role within the control of the chemokine availability and will therefore be discussed in more detail.

D6

The atypical chemokine receptor D6, shows no signalling, calcium flux or chemotactic response following binding of a chemokine, yet is indispensable in the
resolution of inflammation (Fra et al., 2003). In vivo studies have demonstrated that a deletion of this receptor can result in susceptibility to skin cancer and demonstrates signs of a disease similar to the human condition psoriasis. The presence of abnormalities in the skin, correlates with the abundance of the D6 receptor in the skin (Nibbs et al., 1997).

![Diagram](image)

**Figure 1.11 Schematic of the chemokine 'Interceptors'.** Silent or atypical chemokine receptors display a mutation in the 'DRYLAIV' motif of the second intracellular loop and are unable to induce a signalling or functional response. This alteration suggests a possible role in the sequestration of chemokines.

The D6 receptor shows structural similarities to other chemokine receptors and can bind at least 13 CC chemokines (Nibbs et al., 1997), all of which are inflammatory, interestingly D6 cannot bind constitutive chemokines (Fra et al., 2003; Bonecchi et al., 2004). The D6 scavenging receptor retains its ability to associate with β-arrestin and internalize in a clathrin-coated pit dependent mechanism. However, it varies from other chemokine receptors as it is constitutively active, performing the internalisation function even in the absence of a ligand (Galliera et al., 2004; Weber et al., 2004). As well as the modified ‘DRYLAIVHA’ to the ‘DKYLEIVHA’ motif, D6 also has replaced an aspartic acid residue to an asparagine in the second transmembrane domain, both have been shown to be important the G-protein dependent signalling (Mantovani et al., 2001).
DARC
As with D6 the traditional ‘DRYLAIVHA’ signalling motif within GPCRs second intracellular loop is altered, in this case to ‘LGH’ and as such the receptor is unable to signal. DARC and D6 are both considered to be silent receptors, however, it is important to realise the distinct differences between them. As discussed above, D6 acts to internalise the chemokine, this is followed by degradation of the chemokine by lysosomes within the cell. In contrast, chemokines bound to and internalised by DARC, maintain their full biological activity.

The maintenance of biological function of the chemokines is essential to the role that DARC plays within the immune system. DARC may act as either a sink or a transporter to both CC and CXC chemokines. DARC is located on erythrocytes as well as vascular endothelial, on erythrocytes, it acts as a chemokine sink maintaining the presence of chemokines within the circulation, chemokines can then be released when required, moreover, this can aid in the prevention of desensitization in leukocytes. On vascular endothelial, the DARC receptor can act as a transporter of chemokines from the extracellular space onto the lumen of the vasculator. This process allows the chemokine to be bound to the lumen, instead of being released into the lumen to act as a free chemokine which may have crossed between the endothelial cells.

PPR1
In contrast to D6 and DARC, the PPR1 receptor is unique in that it binds homeostatic chemokines CCL19, 21 and 25. As with all ‘interceptors’ it demonstrates a mutation in the second intracellular loop, in which the characteristic ‘DRYLAIVHA’ motif is altered to ‘DRYVAVTKV’ (Murphy, 2002).

Upon first investigation this receptor was termed CCR11, as it was thought that the chemokines CCL2, CCL8 and CCL13 bound to this receptor and induced chemotactic responses. However, further investigation into this receptor determined that this was in fact an atypical or silent receptor and the term CCR11 was withdrawn (Schweickart et al., 2000).
CXCR7

The recently identified CXCR7 (RDC-1) chemokine receptor is intriguing. Research into its function is still in its infancy and there are contradictions in the possible mechanisms of action or even lack of action. It is due to these contradictions that the CXCR7 receptor shall be mentioned within this section, although further investigations are required to identify if this is indeed an atypical receptor.

Initial observation identified this receptor on isolated T cells, and noted that following blockade of either the CXCR4 or CXCR7 receptors, chemotaxis towards CXCL12 was diminished (Balabanian et al., 2005). This suggested a possible role for this receptor in the chemotactic responses of T cells and its subsequent renaming from RDC-1 to CXCR7. However, observations in murine and tumour cell lines have demonstrated that signalling through CXCR7 does not induce calcium flux or chemotactic responses (Burns et al., 2006). It is interesting to observe that within a zebra fish study, the role of CXCR4 and CXCR7 have also been shown to play complementary roles in cell migration. Here the leading edge of the cell is shown to contain high levels of CXCR4, with the trailing edge containing CXCR7. This apparent spatial distinction allows for the same cell to respond differently towards the same chemokine depending on the spatial restriction of the receptors. It is possible that in this scenario, CXCR7 may act to sequester CXCL12, decreasing the available chemokine and preventing binding to CXCR4, furthermore the affinity of CXCR7 is 10 X greater than that of CXCR4, which would favour this theory (Dambly-Chaudiere et al., 2007). It has also been shown that not only CXCL12 binds CXCR7 but also the inflammatory chemokine CXCL11 (Burns et al., 2006).

Due to the initial observed role of CXCR7 in T cell chemotaxis is unclear how this information may relate in the T cell, but it brings an interesting angle to what was at one time thought to be a one chemokine, one receptor relationship. The results from investigations into CXCR7 are awaited with anticipation.
1.6.7 Chemokine receptor expression

So far we have briefly alluded to the role that chemokine receptors play in the life of the T cell. Although we have demonstrated that the cells can be categorised by the CD4 or CD8 receptor and the cytokine expression profile, these cells may also be characterised by the chemokine receptors that are present on the cell surface (figure 1.12).

The expression profile of receptors on T lymphocytes is indicative of functionally distinct T lymphocytes subsets. Naive T lymphocytes have a limited number of receptors expressed on the cell surface; the most notable are the CXCR4 and CCR7 chemokine receptors. Recirculation of this naive population is observed between the blood and lymph nodes, which is aided by the expression of CCR7 and L-selectin (Sallusto et al., 1999).

![Diagram showing chemokine receptor expression on Th1 and Th2 lymphocytes.](image)

**Figure 1.12 Example of Chemokine receptor expression on Th1 and Th2 lymphocytes.**

Following activation and differentiation of the T cell, the number of receptors on the cell surface are upregulated, this allows migration towards an increased number of chemokines. Yet the chemokine receptors that are upregulated are specific for the T cell subgroup. For example, the CXCR3 chemokine receptor has been shown to be expressed at much higher levels on Th1 lineage than Th2 (Sallusto et al., 1998; Bonecchi et al., 1998). Two of the ligands for CXCR3, CXCL9 and CXCL10 have been shown to be IFN-γ inducible and as Th1 cells produce IFN-γ this correlates well with the observed CXCR3 expression (Loetscher et al., 1996a). CCR1 has been shown to be expressed on Th1 while CCR11 and CCR3 are preferentially
expressed on Th2. There are conflicting reports about the expression profile of CCR5 on T cells, in some cases expression was found on both Th1 and Th2 and in others higher in Th1 (Sallusto et al., 1998; Bonecchi et al., 1998). Interestingly this discrepancy may be explained by the activation state of the cell, as well as culture conditions, with CD3/CD28 down regulating CCR5, whereas culture with IL-2 increases expression. It is important to note that these receptor profiles are not definitive, expression of CCR3 on Th1 and CCR5 on Th2 have been recorded.

1.7 Chemokines in human pathophysiology
A characteristic of inflammation is the increase in the number of chemokines and subsequently the number of immune cells that infiltrate the target site. An increase in the infiltration of immune cells is also prevalent when disregulation of this system occurs, this disregulation may lead to autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Chrons disease, and cardiovascular disease.

Many reviews have described in detail the role of chemokines within disease states and provided a summary on the current clinical status of small molecule antagonists (Medina-Tato et al., 2006; Ruffini et al., 2007; Szekanecz et al., 2006). This work focuses primarily on the chemokine CXCL12, and as such an overview of both this chemokine and its receptors CXCR4 and CXCR7 shall be discussed. In addition, investigations were also performed into the role of CCL5 and its derivatives and we shall discuss this chemokine in the context of HIV.

1.7.1 CXCL12 and its receptors
CXCL12 was first cloned in a mouse bone marrow stromal cell line, PA6 and due to alternative splicing of the same gene (located on chromosome 10q), can be found in one of two forms, CXCL12α or CXCL12β, (the former being more abundant) (Shirozu et al., 1995; Nagasawa et al., 1994). Unusually for cytokines it is highly conserved between mouse and human (> 90 %). The action of CXCL12 has been found to be a highly potent chemotactic agent within leukocyte populations (Oberlin et al., 1996; Bleul et al., 1996)
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CXCR4

The CXCR4 receptor was isolated from a human blood monocyte cDNA library and was known as the orphan receptor LESTR, consisting of 352 amino acids (Loetscher et al., 1994). As with all chemokine receptors it is seven-transmembrane G-protein coupled receptor and has been shown to induce chemotactic responses in immune cells, such as monocytes, pre-B cells and T lymphocytes (Bleul et al., 1996; Loetscher et al., 1994). CXCR4 belongs to the CXC or α -chemokine family, in which the NH₂ terminal cystein residues are separated by one amino acid residue (Aiuti et al., 1997).

CXCR7

Chemokines are highly promiscuous with many chemokines capable of binding more than one receptor and each receptor capable of binding more than one chemokine. Early indications suggested that the CXCL12/CXCR4 axis was an exception to this, as it was considered that CXCL12 and CXCR4 only bound each other. This observation was intriguing as it allowed assignment of signalling pathways to be categorically attributed to this signalling axis without any interference. However, recent studies have elucidated that the orphan receptor RDC-1, can also bind CXCL12 and CXCL11 with high affinity. These investigations are only in their infancy, yet due to the signalling and functional responses which are initiated through this receptor, it has lead to the reassigning of the orphan receptor RDC-1, to be subsequently renamed CXCR7. This recent addition to the chemokine/chemokine receptor family only aids to highlight the ever increasing complexity of this system.

Study of the CXCL12/CXCR4 axis has implemented this system in several areas of development. Murine systems have been the most widely studied, with mice lacking CXCR4 resulting in defects of organ vascularisation, with most mice dying in utero or perinatally (Tachibana et al., 1998). Furthermore, the CXCR4 receptor was the first GPCR to be shown to be involved in neuronal cell migration, with disruption in of CXCR4 gene in mice leading to foetal lethality in homozygous mutants (Zou et al., 1998).
CXCL12-/- mice showed defects in cardiac tissue, ventricular septum formation, B cell lymphopoiesis, bone marrow myelopoiesis, formation of tissue elements of bone marrow or any types of blood cells derived from bone marrow (Nagasawa et al., 1996). All of the above indicate that CXCR4 is essential in development and is not just limited to the haemotopoietic system.

CXCL12 and its receptor CXCR4 have been identified to play a role in several disease states. Rheumatoid Arthritis is a chronic condition characterised by inflammation of the joints. It is thought to occur due to accumulation of CD4+ memory T cells within the affected joint. Nanki and colleagues found CXCR4 expression on CD4+ memory T cells was enhanced and CXCL12 was expressed within the synovium. This observation coupled to the antiapoptotic effects induced by CXCL12 in migrating CD4+ cells to the synovium, suggests a role for the CXCL12/CXCR4 axis in the accumulation of CD4+ memory T cells in rheumatoid arthritis (Nanki et al., 2000). It has also been shown that CXCR4 is expressed on 23 different types of cancer from haematopoietic origin to breast, prostate, pancreatic, and lung (Balkwill, 2004).

1.7.2 CCR5 and CCL5

A huge advancement in knowledge about HIV came with the discovery that a link existed between HIV and the chemokine field. It was observed that entry of HIV into T cells is mediated through CD4 and two co-receptors CCR5 and CXCR4. This observation was of clinical importance, as with this knowledge, agents that block the entry of this virus could be developed. Individuals which are homozygous for a null CCR5 allele, not only have a decreased risk of infection with the HIV virus, but also demonstrate that inactivation of this receptor, does not lead to pathological characteristics and individuals display normal cellular responses (Samson et al., 1996). This observation suggested that targeting and modulating the function of this receptor would have limited pathological effects. To further this, individuals which are heterozygous for the CCR5 allele show slower progression of the disease (renzana-Seisdedos and Parmentier, 2006).
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Taken together, these observations all heightened the excitement around a potential CCL5 antagonist as a prophylactic treatment for HIV. Research started by modifying CCL5 in order to generate a molecule which was highly specific, showed lower agonist activity, but displayed longer sequestration of the receptor. These modifications would ensure limited exposure of the functional receptor at the cell surface, decreasing the likelihood of HIV binding. Simmons et al demonstrated that the N-terminally modified chemokine AOP-CCL5 was capable of blocking the R5 tropic HIV entry in several different cell types (Simmons et al., 1997). This molecule resulted in agonist activity which was assessed via the dimerisation of the receptor, measurement of calcium flux and intracellular signalling (Rodriguez-Frade et al., 1999). As expected with an agonist, it induced internalisation of the receptor, however, it was interesting as it also internalised the receptor for increased periods of time compared to the parent CCL5 chemokine. In the drive to develop HIV inhibitors of even higher potency, several other ligands were developed, including PSC-CCL5. This ligand had increased potency against CCR5 sequestering it for longer periods of time (Pastore et al., 2003; Sabbe et al., 2001).

The functional and biochemical effects generated by CCL5 within human T cells has been contradictory. Some studies demonstrate functional responses in freshly isolated T cells, while others have displayed a response profile which indicates that activation of the cell is required (Bleul et al., 1997; Loetscher et al., 1996b; Turner et al., 1995). Within this study we re-investigate the role of CCL5 and its derivatives on T cell responses and clarify the functional responses.

1.8 Chemokine Signal Transduction Pathways

The binding of a chemokine and subsequent activation of G-proteins initiates a variety of signalling pathways that result in functional responses. Discussed below are some important downstream signalling cascades that may be triggered.
Phosphoinositide Metabolism

1.8.1 Phospholipase C/Protein Kinase C cascade

The Phospholipase C/Protein Kinase C (PLC/PKC) cascade has been demonstrated to be activated downstream of chemokine receptors (figure 1.13). The role PLC plays, like PI3K, is critical in the modulation of phosphoinositides at the plasma membrane. PLC isoforms (β, γ, δ, and the relatively newly identified ε, ζ and η) are a family of membrane bound enzymes that utilize PtdIns(4,5)P₂ as its substrate (Harden and Sondek, 2006; Katan, 2005). Following activation of PLC, PtdIns(4,5)P₂ is hydrolysed into 2 second messengers; Diacylglycerol (DAG) and inositol triphosphate (IP₃) (Cicchetti et al., 2002).

The production of IP₃ results in mobilisation of intracellular calcium mobilisation via interaction of IP₃ with its receptor. Following the rise of intracellular calcium, Ca²⁺ ions from the extracellular space enter the cell through calcium release-activated channels (CRAC) channels (Fomina et al., 2000; Luik et al., 2006; Luik and Lewis, 2007). The activation and opening of these channels is thought to be directly related to the reduction of calcium within the intracellular stores (such as the endoplasmic reticulum). The components which facilitate this process are known as STIM1 (stromal interaction molecule 1) located within the ER, which functions as the calcium sensor and ORAI1 a functional component of the CRAC channel (Feske et al., 2006; Roos et al., 2005). It is postulated that the depletion of calcium from intracellular stores, triggers a redistribution of STIM1 from a diffuse distribution to a focal point near the plasma membrane. This redistribution facilitates the interaction with the CRAC channel and refilling of the intracellular stores (Luik et al., 2006). DAG being highly lipophilic remains within the membrane recruiting PKC from the cytoplasm to the cell membrane, directly activating cPKC and nPKC.
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Figure 1.13 PLC/PKC cascade. Upon T cell receptor (TCR) engagement, Lck (a member of the Src family of protein tyrosine kinases) phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the cytoplasmic domains of the chains of the CD3 complex. Subsequently, ZAP-70 (a member of the Syk family of kinases) is recruited by its Src homology-2 (SH2) domains, binding to the phosphorylated ITAM sites. Activated ZAP-70 propagates signal transduction through the phosphorylation of downstream targets including the adapter molecules LAT and SLP-76. These adapters, or following chemokine stimulation the dissociated Gβγ subunits, in turn, facilitate phospholipase C-gamma 1 (PLC-γ1) activation, resulting in the cleavage of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers calcium mobilization, which leads to activation of nuclear factor of activated T cells (NF-AT). DAG activates Ras-GRP and protein kinase C theta (PKC-θ), which in turn leads to activation of the Ras-mitogen-activated protein kinase (Ras-MAPK) and nuclear factor kappa-B (NF-kB) pathways respectively.
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PLCβ is the most abundant PLC isoform within immune cells and a role for this isoform in neutrophils has been implemented in many cellular physiological functions, such as superoxide formation (Rhee and Bae, 1997; Wu et al., 2000), regulation of protein kinases, and calcium efflux. Although no role has been shown for PLC in neutrophil chemotaxis, the role of PLC in the chemotaxis of Th2 cells has been identified (Li et al., 2000; Cronshaw et al., 2006). This observation has been furthered recently with the use of murine models. It was observed that PLCβ plays a prominent role in T cell chemotaxis, and was dependent specifically on the production of IP₃ and the subsequent rise in intracellular calcium and not the activation of PKC (Bach et al., 2007). These studies highlight the distinct differences that are observed following chemokine stimulation in neutrophils and T lymphocytes.

The PKC family of serine/threonine protein kinases is composed of has 9 members, split into 3 subgroups, determined on their structure and function (Parker and Murray-Rust, 2004). Conventional PKC (cPKC) formed of α, β, γ show DAG sensitive calcium responsiveness. Novel PKCs δ, ε, η, θ are DAG sensitive and calcium insensitive while atypical PKCs ζ, λ have altered C1 domains and are not DAG or calcium sensitive (table 1).
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Class Isoforms Requirements for activation
---
cPKC α, β, γ ○ Ca\textsuperscript{2+}-dependent ○ Activated by DAG in presence of PS
nPKC δ, ε, η, θ ○ Ca\textsuperscript{2+}-insensitive ○ Activated by DAG in presence of PS
αPKC ζ, θ ○ Ca\textsuperscript{2+}-insensitive ○ Do not respond to DAG ○ Regulated by PS

In vitro PKC can be activated independently of cell surface receptors with the use of phorbol esters such as PMA, (PMA cannot distinguish between the different PKC isoforms) resulting in a strong, robust activation of the signalling cascade (Kazanietz et al., 2000).

**Table 1:** The PKC family and how they are activated. Activation of cPKC and nPKC have been shown to be activated or have interaction with DAG, in which DAG or Phorbol esters (see information box) act as hydrophobic anchors to recruit the PKC isoforms. PKC activation requires a membrane factor in the form of phosphatidylserine (PS), and it is the presence of DAG that results in an increased affinity for PS. The initial recruitment of cPKC is calcium sensitive and therefore helped by IP\textsubscript{3} induced calcium release aiding cPKCs affinity for PS, however, no equivalent step has been shown for nPKC. This process leads to the activation of nPKC and cPKC. αPKC are activated at least in part via interaction with the Cdc42-GTP-Par6 complex. Optimum activation of all PKCs requires phosphorylation of their activation loops (Parekh et al., 2000; Newton, 2003) a process catalysed by phosphoinositide-dependent kinase 1 (PDK1), which is recruited to the membrane by PIP3. Once membrane bound, the open and activated PKC is capable of phosphorylating its substrates.

1.8.2 Phosphoinositide 3-kinase

Phosphoinositide 3-kinase or PI3K is a term used to describe a family lipids which are characterized by their ability to catalyse the phosphorylation of the inositol ring on membrane bound phosphoinositide lipids (figure 1.14). It is these lipid, membrane bound products that are key mediators of intracellular signalling. The activation of PI3K has been shown to be regulated by several extracellular signals, such as chemokines, cytokines and co-stimulatory molecules (Astoul et al., 2001).
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Figure 1.14 Phosphorylation of the inositol ring of PtdIns(4,5)P2 converting it to PtdIns(3,4,5)P3 via the action of PI3K.

The PI3K family can be subdivided into three main classes, according to their varying in vivo substrates and structural characteristics (figure 1.15). The class I PI3Ks are further subdivided into 2 groups, Class IA family are heterodimers comprised of a catalytic 110 kDa protein which is encoded by 3 genes p110α, p110β, and p110δ binding a regulatory adaptor protein known as p85. Activation of class IA isoforms is ultimately dependent on activation of receptor or cytosolic tyrosine kinases. It was the initial identification and cloning of a class IA isoform that highlighted the role of this family of kinases and as such is the best studied class within the PI3K family.

Class IB is comprised of only one member, p110γ. P110γ also forms a heterodimers but varies from the class IA isoforms as it lacks the N-terminus p85 binding site (Foster et al., 2003), binding instead p101 an adaptor subunit unique to p110. Class IB also differs from Class IA as it is regulated via G-protein coupled receptors. In vitro class IA and IB isoforms have been shown to catalyse PtdIns, PtdIns(4)P and PtdIns(4,5)P2 into their respective products, however, in vivo their preferred substrate is PtdIns(4,5)P2 which is converted into PtdIns(3,4,5)P3 (Vanhaesebroeck and Waterfield, 1999; Fruman and Cantley, 2002). Class III is comprised of one member the human homologue of the yeast vesicular protein-sorting protein, Vps34 (vacuolar protein sorting 34), which both in vitro and in vivo only catalyzes PtdIns and is thought to play a role in intracellular trafficking events (Yan and Backer, 2007).
Figure 1.15 The PI3K family. Class I PI3Ks are heterodimeric enzymes consisting of a regulatory and a catalytic subunit. The class IA catalytic subunit is comprised of an N-terminal p85 binding domain that binds the p85 regulatory subunit, a Ras binding domain that mediates activation by the small GTPase Ras, a C2 domain and Phosphatidylinositol kinase homology (PIK) domain. The class IB PI3K catalytic subunit p110γ is similar in structure to the class IA catalytic subunits, but lacks the p85 binding domain, instead associating with a p101 regulatory subunit. The p101 regulatory subunit facilitates the interaction between p110γ and the βγ subunits of the heterotrimeric G proteins that are activated by G-protein-coupled receptors. Class II PI3K are comprised of 3 members and are similar to class IB but contain additional PX and C2 domains at the C-terminal. Class III is comprised of the single member Vps34. (Taken from (Engelman et al., 2006; Okkenhaug and Vanhaesebroeck, 2003)).

Class II PI3Ks are encoded for by three separate genes, PI3K Class II α, β, and γ. PI3K Class II α and β are ubiquitously expressed, while class II γ is located almost exclusively in the liver. The isoforms that comprise this family can, at least in vitro, bind phospholipids in a calcium independent manner via a C-terminal domain (figure 1.15). An interesting aspect of the class II PI3Ks is that they show distinct lipid specificity compared to Class I and Class III PI3K enzymes utilizing, in vitro, PtdIns to PtdIns(3)P and PtdIns(4)P to PtdIns(3,4)P₂ (Vanhaesebroeck and Waterfield, 1999). Class II isoforms are therefore likely to elicit accumulation of lipids with a profile distinct from that stimulated by class I and III PI3Ks. The PI3K family has been implemented in an abundance of biological process, such as cell survival, proliferation and cell migration.

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Formation of phosphoinositide lipids by PI3K

Within mammalian cells, PtdIns(3)P occurs constitutively in cells, whereas PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ are found in abundance in stimulated cells, this differing expression profile suggests a role for PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ as intracellular mediators (figure 1.16). The activation of PI3K leads to transient accumulation of these lipid products at the plasma membrane, which act to recruit proteins via their PH domains, such as protein kinase B (PKB), this recruitment leads to modification of their activity or subcellular localization triggering a cascade of signal transduction throughout the cell culminating in functional responses.
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Figure 1.16 Pathways following formation of phosphoinositide lipids by PI3K.
Chemokine ligation of GPCRs stimulates Class IB PI3K through the actions of Gβγ subunits and the GTPase Ras. This activation leads to the rapid accumulation of PtdIns(3,4,5)P₃ and indirectly PtdIns(3,4)P₂ in the plasma membrane, these 2 lipids then coordinate the regulation of several effector proteins by binding directly to the PH domains. Direct effectors of this pathway are highlighted: Btk, Vav1, Dock2, PDK1 and AKT.
1.8.3 Regulation of PI3K - SHIP and PTEN

Of central importance to the tight control of PI3K are the lipid phosphatases SHIP (SH2-containing inositol phosphatase) and the tumour suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10) (Rohrschneider et al., 2000; Cantley and Neel, 1999; Astoul et al., 2001). These molecules modulate PI3K signalling by dephosphorylating the products of PI3K. PTEN is a 3-phosphoinositide phosphatase converting PtdIns(3,4,5)P$_3$ to PtdIns(4,5)P$_2$ while SHIP is a 5-phosphoinositide phosphatase converting PtdIns(3,4,5)P$_3$ to PtdIns(3,4)P$_2$. These regulatory components are essential for several reasons. The PI3K lipid products are essential component in many signalling cascades and functional responses, therefore the PI3K regulators ensure the signal propagation is generated without overstimulation of the system, and the location of these molecules is essential for their function, as highlighted in figure 1.17. PI3K has been shown to be involved in the migratory responses of certain cell types. Within resting cells, PI3K is cytoplasmic, generating little product within the cell, this is aided by the location of PTEN at the plasma membrane. Following stimulation, PI3K is translocated to the leading edge of the cell while PTEN is delocalised from this area, promoting the generation and maintenance of lipid products at the leading edge. Furthermore, PTEN localises at the sides and rear of the cell acting to dephosphorylate PI3K products, further maintaining the polarised shape.
Figure 1.17 Regulation of PTEN and PI3K induces cell polarization in response to a chemoattractant signal. A) In un-stimulated cells, class I PI3K is mainly cytoplasmic, whereas PTEN is localized at the plasma membrane. B) When cells sense the chemoattractant signal, a signalling pathway yet to be identified promotes the rapid PI3K translocation to the leading edge facing the high chemoattractant concentration and the delocalization of PTEN from the leading edge. C) PtdIns(3,4,5)P3 recruits and activates at the leading edge Rho GEF proteins and other PH domain-containing proteins, the activity of these proteins is important to stimulate the actin polymerization necessary for cell motility (adapted from (Merlot and Firtel, 2003)).
1.8.4 Assessing the role of PI3K

There are several methods which can be utilised in order to assess the role of a particular gene. Here we shall describe the techniques which can be used and highlight the advantages and disadvantages of using these methods. The tools used to elucidate gene function can be thought of in terms of a spectrum. At one end of the spectrum are antibodies/inhibitors used to block function, these molecules are utilized following expression of the protein and modify the effects of the protein via binding. At the other end of the spectrum is the use of knockout/knockin animal models, where the protein is not transcribed and therefore the protein is not formed. Between these two extremes is the recent development of RNA interference which provides alternative to these methods, in which the specific isoform of interest is transcribed but degraded before generation of the protein.

1.8.4.1 Pharmacological intervention

Investigations into the PI3K family and the contribution towards leukocyte navigation mechanisms, has been achieved with the use of pharmacological inhibitors. The most widely used PI3K inhibitors are Wortmannin and LY294002, these are two chemically unrelated compounds that act as irreversible and reversible inhibitors of the ATP binding site (respectively). These broad spectrum class I PI3K inhibitors that have been invaluable in implementing a role for PI3K in the chemotactic responses of leukocytes. Pre-treatment of murine T and B lymphocytes or freshly isolated human T cells with LY294002 or wortmannin show a requirement for PI3K in optimal chemotaxis towards lymphoid chemokines (Reif et al., 2004; Sotsios et al., 1999). Wortmannin has been shown to decrease the number of human neutrophils which polarise and undergo locomotion induced by chemotactic peptides (Niggli and Keller, 1997), while the use of the specific class I δ inhibitor IC87114, suggests a link between p110δ and murine and human neutrophil tethering and chemotaxis (Sadhu et al., 2003; Puri et al., 2004). Other work has previously shown that activation of PI3K by the chemokine CCL22 is a dispensable signal for the chemotaxis of Th2 cells, suggesting that the contribution of PI3K in chemotaxis may be determined by the chemokine/receptor coupling (Cronshaw et al., 2004). However, the use of these inhibitors is limited as they target the whole
PI3K family and not individual isoforms. This leads to many cellular processes being attributed to the PI3K family, but confusion over which isoforms contribute to a particular cellular function. Curiously, investigations into the specificity of LY294002 and wortmannin, have suggested that they may in fact have little specificity for the class II isoforms leading to question if the biological importance of these family members may have previously been underestimated within these models.

A recent and exciting development in the field of PI3K research has been the development of isoform specific inhibitors. Although in the early stages of research these inhibitors are already starting to contribute to our knowledge on isoform specific function. PI-103 has been noted as PI3K class Iα inhibitor and has demonstrated a role for PI3Kα in insulin signalling (Knight et al., 2006). IC87114 and AS605240 the PI3K δ and γ selective inhibitors highlighted a role for these isoforms in neutrophils trafficking (Puri et al., 2004; Camps et al., 2005).

The advancement in the specificity of PI3K inhibitors allows the dissection of the roles of the PI3K family members. A recent study highlighted that the imidazopyridines (e.g. PI-103) preferentially targets PI3Kα, the chromones (e.g. TGX-121) preferentially target PI3Kβ/PI3Kδ, quinazolinone purine inhibitors (e.g. IC87114) selectively target of PI3Kδ, and the thiazolidinediones (AS605240) preferentially target PI3Kγ (Table 2).
### Table 2: IC<sub>50</sub> values (µM) for isoform-discriminating PI3K inhibitors

Values are based on in vitro assays of inhibitor activity against purified protein activity (Knight et al., 2006; Camps et al., 2005; Domin et al., 1997).

<table>
<thead>
<tr>
<th></th>
<th>Class I PI3K Isoforms</th>
<th>Class II Isoforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>PI-103</td>
<td>0.008</td>
<td>0.088</td>
</tr>
<tr>
<td>TGX-121</td>
<td>&gt;5</td>
<td>0.05</td>
</tr>
<tr>
<td>IC87114</td>
<td>&gt;200</td>
<td>16</td>
</tr>
<tr>
<td>AS605240</td>
<td>0.06</td>
<td>0.27</td>
</tr>
<tr>
<td>LY294002</td>
<td>0.72</td>
<td>0.31</td>
</tr>
</tbody>
</table>

1.8.4.2 Genetic approaches

It cannot be underestimated the contribution that knockout and knockin murine models have played in furthering our knowledge and elucidating the function of particular isoforms within a given family, the PI3K family is no exception (reviewed figure 1.18). There are 2 possible ways in assessing this information, firstly, the use of knockout models in which the isoform or gene of interested is target and not expressed within the cell, secondly is the kinase dead or knock in models, in which point mutations within the gene of interest are generated, resulting in the transcription of the isoform and production of protein, yet it provides no functional signals. On a cautionary note, there is difficulty in interpreting isoform specific data from knockout models due to compensatory mechanisms in protein expression of other PI3K isoforms or subunits. Generation of kinase dead or knockin models have overcome this by maintaining protein stoichiometry. Reviewed below is the data generated by the use of these 2 models and the functional roles that can be attributed to these isoforms.
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<table>
<thead>
<tr>
<th>Targeted Subunit</th>
<th>Genetic Approach</th>
<th>Viability</th>
<th>Immunological phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110α</td>
<td>KO</td>
<td>Embryonic lethal</td>
<td>Not applicable</td>
<td>(Bi et al., 1999)</td>
</tr>
<tr>
<td>p110β</td>
<td>KO</td>
<td>Embryonic lethal</td>
<td>Not applicable</td>
<td>(Bi et al., 2002)</td>
</tr>
<tr>
<td>p110δ</td>
<td>KO</td>
<td>Viable</td>
<td>Decrease in neutrophils tethering and trafficking across inflamed venules. B cell numbers and proliferation decreased.</td>
<td>(Puri et al., 2004; Jou et al., 2002; Clayton et al., 2002)</td>
</tr>
<tr>
<td>p110δ</td>
<td>KI</td>
<td>Viable</td>
<td>Decrease in neutrophils chemotaxis and PIP3 production. B cell numbers and proliferation decreased. Decrease in T cell proliferation.</td>
<td>(Okkenhaug et al., 2002)</td>
</tr>
<tr>
<td>p110γ</td>
<td>KO</td>
<td>Viable</td>
<td>B cells not affected. Impaired in vivo &amp; in vitro chemotaxis of neutrophils and macrophages. Decrease in proliferation of T cells.</td>
<td>(Hirsch et al., 2000; Li et al., 2000; Rodriguez-Borlado et al., 2003)</td>
</tr>
<tr>
<td>p110γ</td>
<td>KI</td>
<td>Viable</td>
<td>Decrease of in vivo &amp; in vitro chemotaxis</td>
<td>(Patrucco et al., 2004)</td>
</tr>
<tr>
<td>Class II α</td>
<td>Not yet generated</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td>Class II β</td>
<td>KO</td>
<td>Viable</td>
<td>Wound healing in the epidermis unaffected</td>
<td>(Harada et al., 2005)</td>
</tr>
<tr>
<td>Class III</td>
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<td>Not applicable</td>
<td>Not applicable</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.18 Phenotypes of mice that have PI3K catalytic subunits genetically targeted (Adapted from (Vanhaesebroeck et al., 2005)).

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Data from p110γ knockin models both *in vivo* and *in vitro* show chemotaxis is severely affected (Patrucco et al., 2004). Further studies have indicated that the class I γ knockout model demonstrates reduced infiltration of immune cells such as neutrophils and macrophages into the peritoneal cavity following E.coli induced peritonitis compared to WT controls (Li et al., 2000; Hirsch et al., 2000; Sasaki et al., 2000). This data is supported by *in vitro* observations in which neutrophil chemotaxis towards C5a, fMLP and IL-8 is decreased (Hirsch et al., 2000).

It has also been reported that when using knockout animal models there is the possibility of functional redundancy between distinct PI3K isoforms (Vanhaesebroeck and Waterfield, 1999), while the issue of species variation also generates drawbacks when studying this system. It is therefore important, when possible, to consider the effect of PI3K isoforms in human cells. The use of siRNA technology to specifically target isoforms will be an invaluable tool, not only within the elucidation of gene function, but in the application of human cells.

**1.8.4.3 RNA interference**

One of the problems within knockout models is the issue of functional redundancy between the given isoforms, in that, by completely knocking out a particular gene other isoforms within this family compensate for this and functionality may be artificially driven through other members of the family. This may be overcome with the use of siRNA technology, as knockdown is 80 – 90 % of normal levels, allowing background function of the selected isoforms, thus compensatory mechanisms by other isoforms within the same family may not be activated.

RNA inhibition is a naturally occurring process by which double stranded RNA molecules induce the degradation of the corresponding mRNA, thus inhibiting mRNA translation. Gene silencing of target messenger RNA, termed RNA interference (RNAi), has been used within research for many years, being exploited initially in plants with post transcriptional gene silencing, followed in the late 1990s in invertebrates such as *C. elegans*. However, it was the switch into the mammalian system which posed the biggest problem, as introduction of long double stranded RNAs into mammalian cells initiate an interferon response leading to
global protein inhibition (Bass, 2001). In 2001, this was overcome with the use of small double stranded RNA molecules (21-23 base pairs) (figure 1.19) which were utilized to decrease target mRNA levels correlating to a decrease in protein expression of up to 90 % (Gresch et al., 2004).

Figure 1.19 Schematic representation of siRNAs mediating complementary mRNA degradation. Long dsRNA are introduced into the cell and are cleaved by enzymes known as Dicer, into siRNAs of 21-23 nucleotides in length. These siRNAs are then incorporated into RISC complex where the two strands are unwound, allowing one strand to bind to complementary mRNA within the cells. The mRNA is then cleaved and degraded by nucleases, with the RISC free to bind further siRNAs. Abbreviations dsRNA, double stranded RNA, siRNA, short interfering RNA, RISC, RNA-induced silencing complex. (Adapted from (Sioud, 2004)).

RNA interference is based on the principle that only targeted genes are downregulated. Most studies performed using siRNA, focused on the target gene and several additional genes, yet recent data suggests that care must be taken as off target effects may be seen. Off target effects are genes within the cell of interest which may show similar sequence homology to the target gene and as such are
targeted indirectly by the introduction of the siRNA. Jackson et al. showed that following a genome-wide analysis of the efficacy and specificity of siRNAs, off target effects are seen and that both the sense and anti-sense strand may contribute to mRNA silencing thus enforcing the need for well designed siRNAs. Reducing the effects of off target mechanisms and interferon responses can be achieved by the use of selective, potent siRNAs, reducing the amount of siRNA required for the same knockdown of the target gene thus reducing the possibility of off target effects.

Several methods of delivery can be used to facilitate the entry of siRNA into a cell in vitro. Lipid based carriers and viruses have been utilized with varying success rates, however, a novel non-viral technique termed Nucleofection has also been developed. This technique is based on electroporation and has been shown to have high transfection rates in hard to transfect primary cells and cell lines. In vivo, one of the current obstacles in delivery of siRNA is targeting the tissue/cell of interest. Unless a siRNA can be designed to specifically target a tissue/cell of interest, systemic delivery will only increase off target effects and result in detrimental knockdown in other tissues. Currently, where possible, direct application of the siRNA onto the tissue of interest is reducing off target effects, the drawback is this restricts the number of tissues that can be targeted by this technology. Direct application of siRNAs onto mucosal membranes have been shown to be particularly susceptible to uptake of siRNA explaining why the lung has shown some of the most successful applications of siRNA technology in vivo.

1.8.5 PI3K and its Downstream Effectors

As discussed, ligation of chemokine receptors leads to the activation of the PI3K family. This activation has been linked to the triggering of a diverse array of cellular responses, such as cell survival, mitogenesis, membrane trafficking, glucose transport, membrane ruffling, superoxide production as well as actin polymerisation and chemotaxis (figure 1.20). A number of cellular proteins have been identified that bind the products of PI3K via their lipid binding domains (known as pleckstrin homology (PH) domains), and some of these will be considered here.
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Figure 1.20 Downstream targets and functions of PI3K. Phosphatidylinositol (3,4,5)-trisphosphate \([\text{PtdIns}(3,4,5)P_3]\) recruits proteins that contain a pleckstrin homology (PH) domain to the plasma membrane, these direct effectors of PtdIns(3,4,5)P_3 include protein kinase B (PKB), phosphoinositide-dependent kinase 1 (PDK1), guanine nucleotide exchange factors (GEFs), protein tyrosine kinases of the Tec family and phospholipase Cy (PLC-y). These signalling cascades subsequently activate protein kinases such as MAPK, PKC, GSK3, mTOR and small GTPases and many more. These integrated signalling cascades have roles in secretion, movement, adhesion, contraction and apoptosis.

1.8.5.1 Protein Kinase B

One of the core molecules phosphorylated by the activation of PI3K is the serine/threonine kinase, Protein Kinase B (PKB, also known as AKT (Burgering and Coffer, 1995)) which is recruited to the plasma membrane via its PH domain by PtdIns(3,4,5)P_3. It has 3 known members PKBa, PKBβ and PKBγ which display close homology within their kinase domains to the protein kinases A, G and C and thus collectively form the AGC kinase family (Manning and Cantley, 2007). Although the exact mechanism of activation has not yet been fully elucidated, the events that are thought to occur are discussed here and depicted in figure 1.21.
Figure 1.21 The proposed mechanism of activation of PKB/AKT and its downstream effectors. PTO for figure legend.
Figure 1.21 The proposed mechanism of activation of PKB/AKT and its downstream effectors. A) 1) The production of PtdIns(3,4,5)P$_3$ by the action of PI3K at the plasma membrane results in the recruitment of PKB via its PH domain. 2) This binding allows a conformational change in PKB facilitating its phosphorylation by PDK1 at position Thr308 on the kinase domain. Phosphorylation by the rictor-mTOR complex at the Ser473 site, in the hydrophobic domain completes this activation. 3) The now active PKB translocates into the cell. B) Supporting the role of PKB/AKT as a core signalling molecule is the array of downstream effectors that are regulated by the action of this kinase. Displayed are an example of these effectors and the subsequent cellular processes that are mediated through these interactions. (Arrows indicate activation and blocking arrows indicate inhibition. Taken from (Manning and Cantley, 2007)). C) The signal transduction pathway linking AKT to S6 signalling. Biochemical studies have shown that activated Akt phosphorylates TSC2, part of the TSC1/TSC2 protein complex leading to its inactivation. TSC1/TSC2 displays a GTPase activating property for the Rheb GTPase and activated Rheb activates mTOR, leading to the subsequent phosphorylation of S6 through the action of S6 kinase.
Phosphorylation of PKB occurs via two key sites, Thr308 within the kinase activation loop and Ser473 in the hydrophobic region of the C-terminal regulatory domain (Toker and Newton, 2000). Modulation of PKB activation is dependent on active PI3K which produces PtdIns(3,4,5)P$_3$ at the plasma membrane, resulting in the recruitment of PKB via its PH domain. Once at the membrane it is suggested that PDK-1 (Phosphoinositide-dependent kinase-1) mediates the phosphorylation of the Thr308, while the rictor-mTOR complex directly phosphorylates PKB on the Ser473 in the hydrophobic region of PKB (Sarbassov et al., 2005). Both of these residues must be phosphorylated to induce the full activity of PKB. Following full activation, PKB is released into the cytoplasm where it translocates through the cell to the site of action. The regulation of PKB activation is controlled by the dephosphorylation of PtdIns(3,4,5)P$_3$ by the cellular phosphatase SHIP. The decreased levels of PtdIns(3,4,5)P$_3$ at the membrane lead to PKB remaining inactive in the cytoplasm (Stambolic et al., 1998).

Chemokines have been shown to activate the PI3K family, and the subsequent phosphorylation of PKB is therefore a downstream biochemical signal generated from this stimulation. Within biochemical studies, the phosphorylation of PKB and its subsequent activation can be utilized via immunoblotting as in indirect method to assess PI3K activation.

The activation of PKB has been demonstrated to phosphorylate a number of downstream proteins, particularly those involved in the regulation of glucose metabolism and cell survival such as GSK-3 (Wymann et al., 2003). Further downstream pathways include mTOR which is discussed in further detail below. A clear role for PKB within chemotaxis has emerged in the mould Dictyostelium however the role PKB plays within mammalian cell chemotaxis is still being investigated (Meili et al., 1999).

1.8.5.2 S6

S6 kinase is a component of the PI3K / mTOR pathway. PI3K has been shown to be a crucial regulator of S6 kinase, which in turn phosphorylates the ribosomal S6
protein, by measuring the phosphorylated form of the S6 protein, we have an indirect method of measuring PI3K activity.

S6 phosphorylation has important implications in chemotaxis and also has been found to be the rate limiting step in protein synthesis, allowing cells to pass through G1 (Richardson et al., 2004). Additionally, S6 kinases are under the tight control of the mammalian target of Rapamycin (mTOR). In fibroblasts, activated mTOR has been found to be enriched within the actin arc, (formation of which has been linked to migration), suggesting that the PI3K / mTOR pathway is important chemotaxis (Berven et al., 2004). Neutrophil chemotaxis and chemokinesis induced by GM-CSF (granulocyte-macrophage colony-stimulating factor) is prevented by pre-treatment with the immuno-suppressant rapamycin; rapamycin specifically binds to and inhibits mTOR (Gomez-Cambronero, 2003) suggesting this pathway plays a critical role within both chemotaxis and chemokinesis. Although a clear role for mTOR has been observed in neutrophils, the role that this molecule plays in T cell chemotaxis has not been elucidated.

1.8.6 Tyrosine kinases

1.8.6.1 The JAK / STAT pathway

It has been well documented, via the use of mutant cell lines and knock-out mice, that the cytokine family can induce the activation of the JAK/STAT pathway (Janus kinase / signalling transducer and activator of transcription). However, it was not until the mid-nineties that a G-protein coupled receptor (GPCR) was shown to induce this activation and since this time several reports have suggested chemokines may also activate this pathway (Marrero et al., 1995; Soriano et al., 2003).

The JAK family, are non-receptor tyrosine kinases which are comprised of four members, JAK1, JAK2, JAK3 and Tyk2 (Tyrosine kinase 2). Each member includes 2 kinase domains known as JAK homology (JH) domains, or JH1 and JH2, the first of these domains is thought to infer kinase activity, although both are required for full activation. The remaining domains JH3-7 are thought to aid
receptor binding (Schindler, 1999). STATs are monomeric, cytosolic proteins which are recruited to, and activated at the plasma membrane, acting as the substrate for JAK family members. Their family comprises of 7 members, STAT1,2,3,4,5a,5b and 6, which can be specifically recruited depending on the activated JAK proteins.

Signalling via chemokine receptors has been suggested to activate two different pathways, firstly via heteromeric G-proteins and secondly the Jak/STAT pathway. Mueller and Strange used pertussis toxin (PTX) a Gi inhibitor and AG490 to inhibit JAK2 and showed that the pre-incubation with either inhibitor followed by CCL3 activation has no effect on the other pathway, suggesting for the first time that chemokine receptors could signal via Gi-protein and non-Gi-protein pathways (Mueller and Strange, 2004).

Compared to cytokine signal transduction the characterisation of the chemokine activated JAK/STAT pathway is less well established, however, several reports have begun to elucidate this pathway. Activation is initiated via binding of a chemokine such as CCL2 to its receptor CCR2, triggering receptor dimerization and binding of specific Jaks to the receptor. The subsequent activation of JAK is rapid and promotes tyrosine phosphorylation of the receptor (Schindler, 1999). Signalling molecules, such as the STAT family members which contain Src homology 2 (SH2) domains are then recruited to the plasma membrane. Subsequent activation by receptor bound Jaks via phosphorylation of a single tyrosine residue is followed by the release, homodimerisation and translocation to the nucleus. In the nucleus STATs can bind promoter elements which regulate gene expression.

The first chemokine characterised to signal through this pathway was CCL2 (MCP1) binding to its receptor CCR2, resulting in the dimerisation of CCR2 and the subsequent binding of Jak2 facilitating the activation of STAT 3 (Mellado et al., 1998). Following this, several chemokines have been shown to signal through the Jak/STAT pathway. In the human progenitor cell line (CTS) CXCL12 was shown to binds to its receptor CXCR4 and induce the tyrosine phosphorylation of Jak1, Jak2 and TYK2, leading to the activation of STAT2 and STAT4 (Zhang et al., 2001). CCL5 induces the rapid phosphorylation of Jak2 and Jak3 via CCR5 in the T cell
line, PM1. CCL5 and CCL3 (macrophage inflammatory protein -1α) have also been shown to activate STAT1 and STAT3 in the Jurkat cell line.

In contrast to previous reports, Moriguchi et al. use genetic approaches to suggest that signalling of CXCL12 through the receptor CXCR4, is independent of Jak2 and Jak3 (Moriguchi et al., 2005). They attribute this discrepancy to the use of pharmacological tools which are not potent Jak inhibitors and show low specificity between the Jak family (Changelian et al., 2003). Therefore there is a cautionary note, as although the role of Jaks could not be ruled out completely, their role in chemokine signalling may not be as assured as first thought.

1.8.6.2 Tec kinases
The Tec family are non-receptor tyrosine kinases which are found within T cells and provide an essential signalling cascade. The family is comprised of 5 members; Tec, Btk, Itk, Rlk and Bmx but only Itk, Rlk and Tec are expressed in T cells. In recent years the role of Itk or IL-2 inducible T cell kinase has generated interest due to its role in T cell development, T cell effector function, actin regulation and cell adhesion (Berg et al., 2005).

Activation of these family members is downstream of several different receptors, the TCR, chemokine receptors and cytokine receptors (figure 1.22). In the case of ITK, the inactive form is cytoplasmic and in a folded state due to intramolecular bonding, prohibiting the binding of ITKs natural ligands. ITK is recruited via its PH domain to the plasma membrane by the action of PI3K generating PtdIns(3,4,5)P3. Phosphorylation by Lck initiates full activation of Itk by subsequent autophosphorylation, through scaffolding proteins such as SLP-76, ZAP-70 and LAT. PLCγ and Itk are brought into close proximity allowing Itk to phosphorylate and activate PLCγ (the action of PLC on its downstream effectors is discussed above) (Qi and August, 2007).
Figure 1.22 Signalling through Tec kinases. Binding of ligands to receptors such as the TCR, cytokine receptors and chemokine receptors trigger activation of Tec kinases. Itk, Rlk and Tec are expressed within T cells and each regulate downstream signalling cascade. RTK, receptor tyrosine kinases; TLR, Toll-like receptors (Taken from (Gomez-Rodriguez et al., 2007)).

TCR stimulation elicits responses such as proliferation and cytokine production. Murine T cells deficient in ItkRlk have further highlighted roles for these kinases downstream of the TCR, as these cells display diminished responses, including decreased IL-2 production and T cell proliferation, these responses are most critically observed in the double knockouts however, single knockout of Itk or Rlk generate moderate and minimal effects respectively (Schaeffer et al., 1999).

Further to the observations, Tec kinases are also activated downstream of chemokine receptors. Fischer et al demonstrated that Itk is recruited to the membrane in a PI3K and pertussis toxin sensitive manner following stimulation with the chemokine CXCL12 (Fischer et al., 2004). This data is extended with the observation that CXCL12 mediated chemotaxis is decreased in Itk deficient T cells.
1.8.7 Guanine nucleotide binding proteins: the Ras superfamily of small GTPases

Guanosine Triphosphataeses (GTPases) are another family of molecular players that are shown to be activated downstream of PI3K. Once activated, these molecules relay extracellular signals to downstream effectors, producing a number of cellular responses, in particular cytoskeletal and membrane rearrangements required for cell movement (Hawkins et al., 2006). The Ras superfamily of small GTPases are divided into 5 families, Ras, Rho, Rab, Ran and Arf based on their sequence and functionality. Here we shall discuss some of the important members of these families. These monomeric G-proteins are known as the molecular switches of the cell and are seen in many signalling cascades. GTPases and can be found in two forms, in their active state they are bound to GTP and following hydrolsis of GTP, in their inactive state, bound to GDP. Both forms show similar conformations but have significant differences in their ability to recognise specific domains (Shields et al., 2000).

To fully appreciate the role of GTPases, it is essential to understand how these molecules are regulated. In the case of the GTPase family this is through the regulating properties of guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). GTPases contain a low level of intrinsic GTPase function converting GTP to GDP, however, this is further controlled by GEFs and GAPs. Rho and Rab GTPases also have a further level of regulation; guanine nucleotide dissociation inhibitors (GDI) (DerMardirossian and Bokoch, 2005). For all GTPases, activation via GEFs can facilitate the conversion of GDP to GTP thus activating the GTPases. In their active state GTPases can now bind to and activate downstream effectors. GAPs complete the cycle by increasing the intrinsic GTPase activity, allowing rapid conversion back to the GDP bound inactive state. For Rho and Rab subfamilies the GTPases are cytosolic and bound to GDIs preventing the action of GEFs, therefore maintaining the GTPases inactive state (Bishop and Hall, 2000; Boguski and McCormick, 1993). Following dissociation from the GDI, Rho GTPases insert into the plasma membrane, interact with GEFs and are subsequently activated (Robbe et al., 2003).
1.8.7.1 Ras Family

Ras Sarcoma oncoproteins is family comprised of Rap, Ras, Ral and Rheb, which can be activated by GEFs, of which the 3 main classes are Sos (Son of Sevenless), Ras-GRF (Ras guanine nucleotide releasing factor) and Ras-GRP (Ras guanine nucleotide releasing protein). The best characterised pathway within this family is the activation of the Ras GTPases and the subsequent MAPK signalling cascade, which shall now be discussed.

Mitogen-activated protein kinase

The mitogen-activated protein kinases (MAPK) pathway, is comprised many different subfamilies, the best known of these are ERK (extracellular signal-regulated kinase), JNK (c-jun-NH\textsubscript{2}-terminal kinase) and the p38 MAP kinases, all of which play a role in signal transduction from the plasma membrane to the nucleus. Although in resting cells MAPKs largely reside in the cytoplasm, following activation these molecules have the capacity to phosphorylate nuclear substrates such as protein kinases, cell cycle regulators and transcription factors. The activation of MAPKs follows a cascade of subsequent phosphorylation via protein kinases, namely MAPK kinase kinase and MAPK kinase (figure 1.23). There are numerous different kinases involved at each tier of this process, with each being differentially regulated through G-proteins, scaffolds, adaptors, substrates and regulator proteins. These subfamilies have been implemented in cellular functions such as cell proliferation, differentiation, development, the inflammatory response and apoptosis (Weston and Davis, 2007).

ERK

ERK is the classical MAPK and is the most well known member of this family. Its activation is in response to stimulation via receptor tyrosine kinases, GPCRs, cytokine receptors and integrins. This stimulates the recruitment of the small GTPase Ras, which in turn activates the serine/threonine kinase Raf, a MAPK kinase kinase triggering this signalling cascade. Phosphorylation of ERK 1/2 is catalysed by the upstream kinases MEK 1 or MEK 2, (MAPK kinase). Upon activation ERK1/2 homodimerize, and translocate to the nucleus where they activate additional kinases and transcription factors (Kolch, 2005).
The action of CXCL12 on the receptor CXCR4, has been shown to activate the MAP kinase pathway, in particular the ERK 1/2 response. The involvement of this pathway in chemotactic responses has been provided by the use of the MEK1/2 pharmacological inhibitor PD098059, which has been shown to partially inhibit CXCL12 stimulated chemotaxis (Sotsios et al., 1999).

Figure 1.23 Schematic representation of the structure of MAPK pathways. Each MAP kinase family member; p38, JNK and ERK1/2 is activated by successive activation of a MAPK kinase kinase and MAPK kinase.

Rap1
Rap1 is the closest homolog to the Ras GTPase and over recent years has gained increasing interest due to its role in cell adhesion. It has been shown that chemokines in particular CXCL12 and SLC (secondary lymphoid chemokine) can induce Rap1 mediated signals that regulate integrin activation and transendothelial migration (Shimonaka et al., 2003). Furthermore, murine models and human T cells have been shown to be dependent on Rap1 for chemokine and PMA induced LFA-1 activation, an integrin essential for the transmigration of cells across endothelium (Ghandour et al., 2007 in press).
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1.8.7.2 Ras Homologous Family

Ras Homologous (Rho) family have also been shown to be key regulators of chemokine mediated signalling that induce actin organization, cell cycle and gene expression. This family is comprised of over 20 members the most well studied being Rho, Rac and CDC42 which in regard to actin reorganisation, result in distinct filamentous actin structures (Wennerberg et al., 2005). In order to ensure efficient signalling and control, these GTPases are regulated at multiple points through the signalling cascade, control through GEF and GAP, regulation of cellular location and expression levels (figure 1.24).

Rho

The activation of Rho to promote stress fibre formation and focal adhesion assembly has been found to be dependent on activation of both Rho kinase/ROCK (Rho-associated coiled-coil forming kinase) a serine/threonine protein kinase and mDia. Phosphorylation of the myosin light-chain phosphatase via Rho inhibits dephosphorylation of the myosin light chains and increasing myosin II activity and stress-fibre formation and contractility. Activation of mDia allows binding to the barbed ends of the actin filament enhancing elongation and blocking binding of capping proteins (Riento and Ridley, 2003; Watanabe et al., 1999). The action of Rho and ROCK at the rear of the cell regulates the assembly of contractile actin and myosin. This has been demonstrated to play a key role in both actin assembly and contractility, mediating uropod retraction, an essential role in migrating leukocytes. In comparison the polymerization of actin and production of lamellipodia and filopodia by Rac and Cdc42 is provided at the front or leading edge of the cell.

Rac

Rac1 has been demonstrated to be involved in lamellipodium extension, following the ligation of chemokine receptors; inhibition of Rac prohibits cell migration. Further to these actin dependent process, Rac has also been shown to be involved in translational activation, protein synthesis and cell survival (Etienne-Manneville and Hall, 2002). In resting cells Rac is cytoplasmic, yet following activation by GEFs (which are the same as for the Ras family, Sos, Ras-GRF and Ras-GRP)
they are located to the leading edge of the cell and critical in the formation of lamellipodium (Kraynov et al., 2000). Although observations are context dependent, it has been observed in macrophages that constitutively active Rac has the potential to inhibit cellular migration. This process is through lamellipodia extension over the entirety of the cell, instead of focused at the leading edge, demonstrating a requirement in the neutrophils for gradient sensing and orientation (Sun et al., 2004).

**Figure 1.24 Control of actin filament assembly by Rho GTPases.** The signal transduction pathways linking Rho, Rac and Cdc42 to the formation of spatially defined actin filaments. Activation of Rho promotes acin polymerization through the activation of mDia, a forming-containing protein that binds to the barbed ends of filaments promoting the linear elongation. The activation of ROCK phosphorylates and inactivates myosin light-chain phosphatase, leading to an increase in myosin II activity, which cross-links actin filaments generating a contractile force at the rear of the cell. Rac and Cdc42 are located at the leading edge of the cell mediating their action through WASP or Sra-1 respectively. Both WASP and Sra-1 act on Arp2/3 which interacts with the sides of pre-existing filaments to generate a branched network.

**Cdc42**

During migration Cdc42 can be located at the leading edge and also at the Golgi apparatus, where it is thought that it enables the generation of cell polarity. This polarity is thought to be generated via Cdc42s reorientation of the MTOC (microtubule-organizing centre) and the Golgi in the orientation of migration. This rearrangement of the intracellular environment may lead to cellular polarity through the control of secretory and endocytic transport to the leading edge, thus maintaining the forward protrusions (Raftopoulou and Hall, 2004). Furthermore this GTPase is also required for the promotion of directed production of actin
microspikes and filopodium formation at the leading edge of the cell. The activation of Cdc42 triggers activation of WASP (Wiskott-Aldrich syndrome protein), in turn activating Arp2/3 (actin-related protein 2/3) a signalling molecule known to promote actin polymerization (Millard et al., 2004).
1.9 Aims of the Study

PI3K plays a central role within a myriad of signalling cascades and subsequent physiological process; as such the dysregulation of this system leads to many disease states. Infiltration of immune cells into areas of disease is a characteristic process within the inflammation setting and as such elucidating the molecules that govern this migration is an essential process that may provide effective therapeutic targets. Instrumental in this process is not only identifying the families involved but also the specific isoforms of which these families are comprised.

Thus far the role of PI3K within T cell migration has not been thoroughly elucidated; furthermore the functions of specific isoforms within this family have not been clarified. It was hypothesed that individual PI3K isoforms may play a role within human T cell migration.

Thus the aims of the project were as follows:

- Utilize pharmacological tools to explore the contribution of the PI3K family within freshly isolated and previously activated T cell migration and signalling.
- Dissect out the functions of individual isoforms with the use of isoform specific inhibitors as well as RNAi strategies.
- Optimise the delivery of PI3K isoform specific siRNAs utilizing a novel non-viral technique termed Nucleofection.
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Materials & Methods

2.1 Materials

2.1.1 Cell Isolation and Culture Materials

Cell culture media, RPMI-1640, foetal bovine serum, penicillin and streptomycin, trypsin blue, and phosphate buffer saline (without Ca$^{2+}$ and Mg$^{2+}$) and falcon tubes were purchased from Gibco® (Paisley, UK). Lymphoprep (Ficoll-paque 1.077 g/ml density) was purchased from Axis-Shield (Cambridgeshire, UK). Sodium citrate, Tween-20, Staphylococcal enterotoxin B and Phytohemagglutinin were purchased from Sigma-Aldrich (Gillingham, UK) and Dynalbeads® CD3/CD28 T cell expander beads from Invitrogen (UK). CD3+, CD4+, CD8+ isolation kits and LS columns were purchased from Miltenyi Biotec (UK). Recombinant human IL-2 was purchased from Chemicon (Hampshire, UK). All plastics were obtained from Nunc (UK).

2.1.2 Antibodies

Rabbit polyclonal anti-phospho-S6 ribosomal protein$^{Ser235/236}$ antibody (catalogue no: 2211), S6 Ribosomal Protein (5G10) Rabbit Monoclonal Antibody (catalogue no: 2217) and anti-phospho PKB$^{Ser473}$ (catalogue no: 9271) were purchased from Cell Signalling Technologies (UK). Goat polyclonal pan PKB (catalogue no: sc1618) was obtained from Santa Cruz Biotechnologies (USA). Fluorescein Isothiocyanate (FITC)-conjugated mouse Anti-human CD3 FITC and PI3K class II beta antibody were purchased from BD Biosciences (UK). Monoclonal Anti-human CXCR3-fluorescein (clone 49801, catalogue no: FAB160F), Monoclonal Anti-human CXCR4-fluorescein (clone 12G5, catalogue no: FAB170F), Mouse IgG$_2$A isotype control carboxyfluorescein (CFS) conjugated (clone 20102, catalogue no: IC003F) and Mouse IgG$_1$ isotype control (clone 11711, catalogue no: IC002F) were purchased from R&D systems (UK).

2.1.3 Chemokines

Human recombinant CXCL9 (catalogue no: 392-MG), CXCL10 (catalogue no: 266-NP), CXCL11 (catalogue no: 672-IT), CXCL12 (catalogue no: 350-NS), were
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purchased from R&D Systems (Abingdon, U.K.). CCL5, AOP-CCL5, and PSC-CCL5 were a kind gift from Oliver Hartley, Universite de Geneve.

2.1.4 Assay systems and kits
MACS dead cell removal kit (catalogue no: 130-090-101) was purchased from Miltenyl Biotech (UK). Chemo Tx® System was purchased from Neuro Probe (Gaithersburg, USA). Human T cell Nucleofector™ Kit was purchased from Amaxa Biosystems (Cologne, Germany). Lipofectamine 2000 was obtained from Invitrogen (UK). QiAshredder kit and the RNeasy mini kit were purchased from Qiagen (UK). Quantitative PCR probes for PI3K class I α (catalogue no: Hs00180679), β (catalogue no: Hs00178872), δ (catalogue no: Hs00192399), γ (catalogue no: Hs00176916), PI3KCl2α (catalogue no: Hs00153223), PI3KC2β probes (catalogue no: Hs00153248), class III (catalogue no: Hs00176908), TaqMan® Reverse Transcriptase Reagents and TaqMan® Universal PCR Master Mix were purchased from Applied Biosystems, (Warrington, UK). Molecular weight Markers were purchased from Bio-Rad (UK). The Enhanced chemiluminescence detection kit and X-OMAT film were purchased from Amersham International (UK). The nitrocellulose membrane, 0.45 µM pore was obtained from BDH, (UK). The filter paper used in immunoblotting was obtained from Whatman (Maidstone, UK). Marvel was purchased from the local supermarket.

2.1.5 Inhibitors and siRNAs
SiGLO RISC-free siRNA (catalogue no: D-001600-01-05) and targeting siRNAs for PI3Kα (catalogue no:D005271 -05,-06,-07 and -08), PI3Kβ (catalogue no: D005272 -05,-06,-07 and -08), PI3Kδ (catalogue no: D005273 -01, -02, -03 and -04), PI3Kγ (catalogue no: D005274 -01, -02, -03 and -04), PI3KCIIα (catalogue no: D006771-01, -02, -03 and -04), PI3KCIIβ (catalogue no: D006772-01, -02, -03 and -04), class III PtdIns 3-kinase (catalogue no: D005250-05, -06, -07, -08) were purchased from Dharmaco (Cramlington, UK). Ly294002 and Wortmannin were purchased from Sigma (Poole, UK), PI-103 (patent WO01083456), TGX-121 (patent WO0153266), IC87114 (patent WO 0181346) and AS605240 (patent
WO2004007491) have been described elsewhere and were synthesised solely for this study.

2.2 Cell Types and Culture Conditions

The focus of this work was to further characterise the signalling and functions downstream of chemokine receptors in human T cells. The models used were freshly isolated CD3⁺, CD4⁺ and CD8⁺ T cells and *in vitro* activated human T cells. Furthermore, HeLa cells were utilized to aid validation of the siRNA used within this study. All cell lines and primary cells were routinely maintained in their respective media supplemented with antibiotics, 10 % (v/v) foetal bovine serum and additionally non-essential amino acids for the HeLa cell line, as described in detail below. Cells were cultured every 2-3 days as required and maintained at 37 °C and 5 % CO₂. Prior to experimental procedures, cell viability was determined using trypan blue to stain any dying cells.

2.2.1 HeLa Cells

HeLa cells were obtained from Cancer Research, UK and maintained in 175cm² tissue culture flasks in DMEM F12 medium supplemented with 10 % foetal calf serum (FBS), 10 % non essential amino acids, 10 u/ml penicillin and 10 µg/ml streptomycin (complete media). Cells were grown to a maximum of 1.5 x 10⁶ cells and diluted with fresh complete medium to 5 x 10⁵ cells every three days. Cells were plated at 5 x 10⁵ / well in a 12-well dish 24 hours prior to siRNA knockdown in complete media minus antibiotics. HeLas were maintained in a humidified incubator at 37 °C and 5 % CO₂.

2.2.2 Freshly isolated T lymphocytes and activated T lymphocytes

Routinely within this study freshly isolated human CD3⁺, CD4⁺, CD8⁺ T cells were obtained from PBMCs, cultured *in vitro* and utilized in experimental procedures for up to 72 hours. Alternatively, PBMCs were activated *in vitro* using bacterial superantigens, lectins or antibodies and cultured for up to 14 days. Experimental procedures were performed between 6-14 days post-activation on CD3⁺, CD4⁺ or CD8⁺ T cells.
2.2.3 Isolation of PBMCs by density gradient centrifugation

Blood was collected from healthy volunteers in heparinised syringes (500 U/50 ml) and diluted 1:1 with RPMI 1640 medium. 35 ml of the blood/RPMI mix was then carefully layered over 15 ml of lymphoprep in a 50 ml falcon tube before being centrifuged at 1,500 rpm at room temperature for 30 mins with the brake off. Following centrifugation, the PBMCs as seen in Fig 2.1, which contains the lymphocytes and monocytes was swiftly removed (as described previously (Cronshaw et al., 2004)). Following removal of the PBMCs the protocol proceeded for either isolation of CD3+, CD4+, CD8+ cells or in vitro activation, see protocols below.

![Figure 2.1 Diagram of PBMC separation with Lymphoprep after centrifugation.](image)

The diagram is representative of the separated cell layers observed after centrifugation with Lymphoprep.

2.2.4 Ex-vivo activation and clonal expansion of T lymphocytes

Activation of T cells and their subsequent expansion in vitro mimics the in vivo activation of a T cell in response to antigen presentation. Within this study, the contribution of the PI3K family was investigated in previously activated (in vitro) human T lymphocytes. Described here are the methods of in vitro activation used within this study. Phytohemagglutinin (PHA) is the most commonly used lectin T cell activator of human cells; it functions by acting as a crosslinker of glycoproteins on the surface of the T cells inducing activation within the cell. Staphylococcal Enterotoxin B (SEB) is a potent superantigen, binding to class II MHC molecules
expressed on professional APC in the PBMC population and acting as a linker for subsequently binding the TCR, inducing robust activation of the cell. Finally, CD3/CD28 antibodies immobilized on beads mimic the stimulation of T cells by APC by binding the TCR and the co-stimulatory molecule CD28. As this method of T cell activation is the closest to the in vivo activation this provides the most physiologically relevant in vitro T cell activation method.

Freshly isolated PBMCs were washed 3 times in RPMI-1640 and re-suspended at 1 x 10^6 /ml RPMI complete media. Mononuclear cells were activated with Phytohemagglutinin (PHA) 5 μg/ml, Staphylococcal Enterotoxin B (SEB) 1 μg/ml or CD3/CD28 antibodies immobilized on beads (at a ratio of 1 bead per cell). Following activation T-lymphocytes were expanded by addition of IL-2 every 2-3 days (20 ng/ml) extra complete media was added when required. Cells were maintained up to a maximum of 14 days.

2.2.5 Isolation and culture of CD3^+, CD4^+, CD8^+ T lymphocytes

An alternative model to the activated T cell system was the utilization of freshly isolated T cells. This model was assessed for its potential use Nucleofection delivery of siRNA into T cells to determine the role of PI3K class II isoforms in chemotaxis. Furthermore, the role of individual PI3K isoforms were tested after obtaining isoform PI3K specific inhibitors.

CD3^+, CD4^+, CD8^+ T lymphocytes were purified using the Pan, CD4^+ or CD8^+ T Cell Isolation Kits (Miltenyi Biotec GmbH). This system is an indirect magnetic labelling system, in which untouched cells pass through the magnetic column allowing for purified unlabelled cells to be utilized within experimental procedures. For CD3^+ purification, PBMCs are incubated with a cocktail of biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A. For CD4^+ purification PBMCs are incubated with biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γδ, and Glycophorin A. For CD8^+ purification PBMCs are incubated with biotin-conjugated antibodies against CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γδ, and Glycophorin A. Following
incubation with these antibodies cells are subsequently magnetically labelled with Anti-Biotin MicroBeads for depletion allowing for a pure population.

The protocol was followed as per manufacturers instructions. Briefly, freshly isolated PBMCs were washed 3 times in MACS buffer (phosphate buffered saline pH 7.2, 0.5 % BSA and 2 mM EDTA), resuspended in 40 μl of buffer with 10 μl of specific Biotin-Antibody Cocktail per 10⁷ total cells and incubated at 4 ºC for 10 minutes. 30 μl of buffer plus 20 μl of Anti-Biotin MicroBeads per 10⁷ total cells were added and incubated at 4 ºC for a further 15 minutes. Cells were washed once in MACS buffer and resuspended in 500 μl. T cells were negatively selected using an LS MACS Separator column. Total cells were applied to the column and the column washed 3 times with 3 ml buffer (see fig 2.2). The eluted, enriched T cell fraction was collected, washed in RPMI and resuspended in RPMI plus 0.1 % BSA for use in biochemical and functional assays or resuspended in complete RPMI supplemented with or without IL-2 (20 ng / ml) and cultured for up to 3 days.

![Diagram of CD3', CD4' or CD8' T lymphocyte enrichment by magnetic separation.](image)

2.2.6 Evaluation of T lymphocyte population purity

In order to verify that the purification procedure was successful flow cytometry analysis of the percentage of cells expressing CD3', CD4', CD8' was carried out as described in the flow cytometry section. Purity was always > 95%.
2.2.7 Freezing/Thawing of cells

For storage, 1 x 10^6 cells/ml in exponential growth were re-suspended in freeze medium containing 10 % dimethylsulphoxide (DMSO), and 90 % foetal calf serum. The cell suspension was transferred to cryotubes (1 ml/tube), cooled overnight at 1 °C/minute in ethanol to -80 °C and transferred to liquid nitrogen tanks for long-term storage. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted for one minute in a 37 °C water bath, washed once in RPMI and re-suspended in 50 mls complete medium and cultured as stated previously.

2.3 Target specific knockdown of RNA expression using RNA interference

Within this study, siRNA was transfected into both primary cells and cell lines in the interest of determining specific isoform function and siRNA validation. The siRNA is delivered into the cell of interest, then targets and degrades complementary mRNA within the cell. The decrease in mRNA can be quantified using qPCR, while the functional significance that this knockdown had was assessed primarily through chemotactic assays.

Lipid based delivery was used to transfect HeLa cells. The HeLa cell line was utilized to provide a ‘high throughput’ screen for siRNA validation (compared to cultured human T cells) which was essential for the large number of siRNAs being tested against each expressed PI3K isoform. HeLas were ideal for this function as they provide high transfection efficiency while maintaining cell viability and strong RNA quality, which are essential components when detecting subtle differences in target gene knockdown. In comparison to the lipid based methods used in HeLa transfections, primary human T cells are notoriously difficult to transfect and therefore although lipid and adenoviral methods were assessed, our focus in T cells was siRNA delivery via Nucleofection.

Nucleofection is a non-viral technique that is based on the use of electrical parameters, much like electroporation, and the use of cell-type specific nucleofection solutions. Within this study freshly isolated and previously activated T cells a human T cells were used (fig 2.3). Following 72 hours of recovery, the RNA
was extracted from the cells, and quantified using the Bioanalyzer. Following reverse transcription, qPCR was used to analyze the levels of RNA in test samples compared to controls.

<table>
<thead>
<tr>
<th>Freshly isolated T cells</th>
<th>Previously activated T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong></td>
<td>Freshly isolated CD3+ cells, Nucleofected with targeting siRNA</td>
</tr>
<tr>
<td><strong>Day 3</strong></td>
<td>Functional assays performed on viable cells</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>PBMCs isolated, stimulated and expanded <em>in vitro</em> for 6 days</td>
</tr>
<tr>
<td><strong>Day 6</strong></td>
<td>Previously activated T cells, Nucleofected with targeting siRNA</td>
</tr>
<tr>
<td><strong>Day 9</strong></td>
<td>Functional assays performed on viable cells</td>
</tr>
</tbody>
</table>

Figure 2.3: A comparison of timelines in freshly isolated vs. previously stimulated T cells.

### 2.3.1 Lipid based transfection of the HeLa cell line

SiRNA was serially diluted in Opti-MEM® I media minus antibiotics (see figures for final concentrations), Oligofectamine™ Reagent was mixed and diluted into Opti-MEM® I media minus antibiotic, both were incubated at room temperature for 10 minutes. SiRNA and Oligofectamine™ dilutions were mixed and incubated at room temperature for a further 15 minutes. Cells were washed once in medium without serum. 800 μl media minus serum and antibiotics were added to the cells. Drop wise the siRNA and Oligofectamine™ Reagent was added to each well. Cells were incubated for 4 hours at 37 °C and 5 % CO₂. Following incubation growth medium containing three times serum was added to the cells. Cells were incubated for 24 hours, lysed and total RNA was isolated using Qiagen RNeasy kit and analysed via Quantitative-PCR, (as described below).
2.3.2 Lipid based transfection of previously activated human T cells

Previously activated human T cells (1 x 10^6) were alloquoted into 12 well plates. Cy3-tagged siRNA was diluted in complete media minus antibiotics, lipofectamine™ 2000 was mixed and diluted into complete media minus antibiotic; both were incubated at room temperature for 15 minutes. SiRNA and Lipofectamine™ 2000 dilutions were mixed and incubated at room temperature for a further 15 minutes. 620 µl media was added per well and 230 µl of lipofectamine™ 2000 and siRNA dilution was added to each well drop wise. Cells were incubated for 24 hours, and analysed by light and fluorescence microscopy.

2.3.3 Nucleofection of freshly isolated and previously activated human T lymphocytes

Freshly isolated CD3+ T lymphocytes or day 6 activated T lymphocytes were Nucleofected as per manufacturer's instructions. Briefly 5 x 10^6 cells per sample were centrifuged at 1500 rpm, room temperature, for 5 minutes. All supernatant was removed and the pellet re-suspended in 100 µl Human T Cell Nucleofector™ Solution / 5 x10^6 cells (fig 2.4). The sample was mixed (100 µl) with 4 µM siRNA and transferred to an Amaxa certified cuvette, and nucleofected on program V-24 for freshly isolated T lymphocytes and T-20 or T-23 for activated T lymphocytes. Samples were removed and cultured in 2 ml RPMI 1640 at 37 °C 5 % CO₂. After 6 hours the media was changed and supplemented with IL-2 (20 units /ml). 72 hours after nucleofection dead cells were removed using a dead cell removal kit (Miltony Biotech) and the viable cells were used in functional studies.
2.3.4 Adenoviral delivery of shRNAs

During the course of these investigations, we had the opportunity to test the effectiveness of adenoviral delivery of shRNA. In contrast to the siRNA we used, shRNA is introduced into the cell and then must be transcribed and cleaved in order to produce the functional siRNA. Under normal circumstances this method of delivery would be ineffective due to the lack of expression of the receptor which facilitates the delivery of the virus into the T cell. However, this system was unique as a chimeric adenovirus was created that uses a receptor which is expressed on T cells.

PBMCs were isolated from whole blood as previously described, and activated with PHA (1 μg/ml). Twenty-four hours post activation; T lymphocytes were seeded in 24 well plates and infected or co-infected with the adenoviruses (multiplicity of infection 5000). Single control infections were performed for an empty vector control and with shRNA encoding for eGFP or CD46. Co-infections using shRNA CD46 and the gene of interest PLC Gamma V1, V2 or V3 were performed. On day 5, 6 and 7 FACS analysis was performed on cells infected with the shRNA for eGFP to determine infection efficiency. RNA was isolated from each sample on day 7 (as detailed below) and analysed via quantitative PCR to determine knockdown of the gene of interest.
2.4 Determination of mRNA and protein knockdown

To determine how effective the siRNA had been at targeting the isoform of interest, levels of mRNA of the target gene were compared to control levels in each cell model. Total cellular RNA was isolated from the cell and quantified via the 2100 Bioanalyser. Reverse transcription generates the cDNA that will be utilized as the template for qPCR analysis. QPCR is a multi-step process in which probes which bind a fluorescent reporter dye and quencher bind to complementary cDNA within the reaction mixture. While intact the fluorescence is suppressed by the close proximity of the quencher. During replication of the cDNA the polymerase cleaves the fluorescence reporter from the probes which are hybridized to the target, any non-specific amplification is not detected. This results in an increase in fluorescence which can be detected and analysed.

Determining the knockdown at the RNA level, is a strong indication of the effect of the siRNA, but further validation can be obtained by assessing the expression of the protein at the time of functional studies. Within these investigations we used immunoblotting and flow cytometry to determine the expression levels of the protein of interest.

2.4.1 RNA extraction and Reverse Transcription

Total cellular RNA was isolated from the cells using RNeasy mini kit (Qiagen Ltd) according to the manufacturer’s instructions, (all buffers used are as supplied). Briefly, $5 \times 10^6$ activated T-lymphocytes were lysed in RLT buffer, and homogenised with the QIAshredder™ column. 70 % ethanol was mixed with the lysate and applied to an RNeasy mini column. Contaminants were removed via washing with Buffer RW1 and RPE. RNA was double eluted in 30 µl RNase free water. The concentration of RNA was determined on a 2100 Bioanalyzer using the RNA 6000 LabChip kit described in fig 2.5 (Agilent Technologies).
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Figure 2.5: Examples of the RNA 6000 Nano Series II kit (left panel) and LabChip (right panel) used within this study. Samples are prepared as described above and the concentration of RNA within a given sample is determined by loading samples carefully into the chip. Pressure within the system allows movement of samples through channels that are etched onto the chip. The process separates nucleic acid fragments based on their size as they are driven through the channels electrophoretically. This method can be used to detect RNA concentration as well as purity, with the advantage that detection and analysis can be performed within the same chip. Analysis was performed on the 2100 Bioanalyser.

RNA was reverse transcribed in the presence of TaqMan RT buffer (10 X), MgCl₂, 25 mM, dNTP 25000 µM each, required primers 50 µM, RNase inhibitor 20 units/µl, RT enzyme 50 units/µl, RNase. Reactions were performed in a Biometra UNO II thermocycler (Biometra, Göttingen, Germany). 25 °C for 10 minutes, 48 °C for 30 minutes, followed by 5 minutes at 95 °C, samples were stored at 4 °C prior to Q-PCR.

2.4.2 Quantitative-PCR

For each 25µl of TaqMan reaction 40 ng cDNA was mixed with 12.5 µl of 2X TaqMan Universal PCR Master Mix, 1.25 µl of primer probe mix plus 6.25 µl H₂O and briefly centrifuged. The reactions were carried out using the following parameters: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 seconds, 60 °C for 1 min. TaqMan data was analysed using the Sequence Detector Software (SDS version 2.1). Relative levels of expression were determined by comparison against a standard curve of known concentration, values are then normalised against the house keeping gene GAPDH. This protocol was followed for each PI3K probe, (Class I α, β, and δ, class II α, β, and γ, class III).
2.5 Determination of protein expression

Immunoblotting and flow cytometry are techniques which can be utilized to facilitate the detection of a protein of interest. As these techniques are also used in the analysis of signalling pathways and functional studies, these topics will be discussed in greater detail within that section (see below), and an overview of the technique will be presented here. Immunoblotting uses whole cell lysates which are then analysed for the expression of a particular protein using electrophoresis to separate the proteins according to their size. The transfer of these proteins onto a nitrocellulose membrane facilitates their detection by incubating this membrane in the presence of antibodies against the protein of interest which can be detected by the use of HRP-secondary antibody. Flow cytometry has an advantage over immunoblotting as fewer cells need to be used in order to quantify the amount of protein within a given sample. This technique again uses whole cell lysates but differs in that antibodies can be added directly to the sample without the need for protein separation. With the use of fluorescently labelled antibodies, the sample is passed through a laser which can detect and record the fluorescence.

2.6 Assessment of T cell Signalling and Functionality

In order to characterize T cell signalling and functionality and furthermore to assess the role of PI3K and the individual isoforms which make up this family we utilized several methods. Signalling downstream of chemokine receptors and the involvement of PI3K isoforms was assessed utilizing immunoblotting and flow cytometry. Functional responses were assessed using the chemotactic assay following utilization of broad spectrum PI3K inhibitors as well as isoform specific inhibitors and target specific siRNAs. Cytokine release was assessed following delivery of target specific siRNAs.

2.7 Immunoblotting

Immunoblotting or Western blotting is a technique that facilitates the detection of a protein of interest or the phosphorylated form of that protein. Within this study, cells were stimulated with numerous chemokines; this stimulation triggers downstream signalling events, characterized by the phosphorylation of proteins within that
pathway. By lysing the cells, the mixture of proteins contained with the cell can be extracted. The whole cell lysates are then dispensed into wells within a polyacrylamide gel, upon applying an electrical current across the gel, the proteins separate according to their molecular weight, smaller proteins migrating the furthest. These separated proteins are then transferred onto a nitrocellulose membrane which can be ‘probed’ using an antibody against the protein of interest. Following incubation and binding of the primary antibody to the protein of interest, a secondary antibody is applied which is coupled to a horseradish peroxidase tag. Upon delivering a chemiluminescent agent there is a reaction which produces luminescence that is in relation to the amount of protein detected. This luminescence is detected using a sensitive photographic film, allowing for visualization of the protein of interest.

Immunoblotting was utilized within this study to allow visualization of the phosphorylation of PKB and S6. This phosphorylation was observed as a readout of chemokine stimulation (CXCL12, CCL5, AOP-CCL5 and PSC-CCL5) in freshly isolated, cultured or activated T cells. Furthermore, to determine the involvement of PI3K isoforms within this pathway, we studied the effect of PI3K inhibition on this response.

2.7.1 Sample Generation – Stimulation of cells and collection of whole cell extracts

2 x 10^6 T lymphocytes or activated T lymphocytes per point were washed twice in RPMI 1640 and incubated at 37 °C in serum free RPMI for 60 minutes. Cells were or were not stimulated with CXCL12 (1-100 nM) diluted in RPMI 0.1 % BSA. Stimulations were terminated by aspiration of the supernatant followed by the addition of ice cold lysis buffer (50 mM Tris-HCL pH 7.5, 150 mM sodium chloride, 1 % Nonidet P40, 10 % Glycerol, 5 mM EDTA, 1 mM sodium vanadate, 1 mM sodium molybdate, 10 mM sodium fluoride, 40 μg/ml PMSF, 0.7 μg/ml Pepstatin A, 10 μg/ml Aprotinin, 10 μg/ml leupeptin, 10 μg/ml soyabean trypsin inhibitor). Lysates were rotated at 4 °C for fifteen minutes, followed by centrifugation at 14,000 rpm. The supernatants (containing protein) were diluted with 5x loading buffer (10 %
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SDS, 50 % glycerol, 200 mM Tris HCl pH 6.8, Bromophenol blue) heated to 95 °C for 5 minutes and stored at -20 °C.

2.7.2 Electrophoretic separation, transfer and immunoblotting of cellular proteins

Solubilised proteins were electrophoresed on a one dimensional 10 % sodium dodecyl sulphate - polyacrylamide gel (SDS-PAGE). This was carried out using the Bio-Rad Mini Protean II system (Biorad Labs, UK). Samples were loaded into a stacking gel and run at 80 V in running buffer containing 25 mM Tris base, 192 mM Glycine and 0.1 % (w/v) SDS. Upon reaching the resolving gel samples were electrophoresed at 180 V. The proteins were transferred by electroblotting for 60 minutes at 40 mV onto nitrocellulose membrane 0.45 μM soaked in semi-dry transfer buffer (70 % H₂O, 20 % methanol, 10 % blot buffer (39 mM Glycine, 48 mM Tris base and 0.0375 % SDS). Membranes were incubated for 60 minutes at room temperature in block buffer, (Tris Buffered Saline (20 mM Tris-HCl pH 7.6, 150 mM sodium chloride) with 0.1 %Tween (TBS-Tween) containing 5 % milk), with slight agitation and rinsed three times for 5 minutes in TBS-Tween. The membrane was incubated in the specified 1° antibody dilution 1:1000, in TBS-Tween supplemented with 0.01% sodium azide and 5 % milk, overnight at 4 °C with slight agitation. The membrane was washed three times for 5 minutes in TBS-Tween, and incubated in the 2° antibody coupled to horse radish peroxidase (HRP) diluted 1:10,000 in block buffer, for 60 minutes at room temperature and washed in TBS-Tween three times for 5 minutes. Visualization of the proteins was performed by incubating the membrane in 5 ml of Enhanced Chemiluminescent reagent (ECL), for one minute and exposing to Kodak X-OMAT film.

2.7.3 Membrane Stripping and reprobing

In order to verify whether the samples were equally loaded the membranes were stripped of antibody and reprobed with a pan antibody that detects absolute levels of protein. Firstly, the membrane was rehydrated in TBS for 10 minutes and then placed in 50 ml of stripping buffer to remove bound antibody, (100 mM 2 Mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCL pH 6.7) and incubated at 60 °C for
30 min. After extensive washing with TBS-Tween, the membrane was incubated for 60 minutes at room temperature in block buffer washed three times in TBS-Tween, blots were reprobed with a different primary antibody and the immunoblotting procedure is carried out as described above.

2.8 In Vitro cell migration Assay

The ChemoTx® System is a disposable 96-well format migration assay. The system is set up with the appropriate ligands in the lower wells, over which a 5 μm pore size filter is placed, through which the cells migrate. This filter is designed to mirror the 96 well plate structure beneath, in that, surrounding each well is a hydrophobic material that focuses the cell suspension directly over the ligand below, eliminating the need for the upper chambers (see fig 2.6). Following the incubation time, the migrated cells are counted.
Chemotaxis assays were performed in 96-well chemotaxis chambers (Neuro Probe, USA) with polycarbonate membranes (5 μm pore size). The lower chambers were filled with 29 μl of chemokine diluted in RPMI-1640, 0.1 % BSA and carefully overlaid with the polycarbonate membrane. Cells were washed twice in RPMI-1640, and re-suspended in RPMI-1640, 0.1 % BSA at 3.2 x 10^6 cells/ml. 25 μl of the cell suspension was loaded on top of the filter.

Migration was performed at 37 °C, 5 % CO₂ for 180 minutes, after which non-migrated cells on the top of the filter were rinsed off with PBS. Following centrifugation (1,500 rpm, 10 mins) the filter was removed, and the migrated cells were re-suspended in 300 μl PBS, 0.1 % BSA. Analysis of migration was performed using FACS Vantage (BD Biosciences, San Jose, CA), samples were analysed for 60 seconds. Data is expressed as cell number.
2.9 Human Cytokine Assay

The Meso scale cytokine assays are based on the principles of the ELISA, in that the system is designed to detect the presence of proteins within a given sample. We utilized this technique to detect levels of Th1/Th2 cytokines following nucleofection of PI3K targeting and non-targeting siRNAs into freshly isolated T cells. The MSD MULTI-SPOT® plate used in this investigation is a 96-well format, each well contains 10 different spots that correspond to 10 different cytokines, thus allowing for up to 10 cytokines to be detected within each sample/well (see figure 2.7).

![Schematic of 4 wells of a MSD MULTI-SPOT plate. Each well contains 10 spots each with a particular capture antibody.](image)

For this technique to work successfully each spot within a well is comprised of capture antibodies for a particular cytokine and it is important to note, that each spot within a well works independently of every other spot and is optimised for that particular cytokine.

Human cytokine assays were performed in a 96 well format, with a 10-spot layout. (All reagents are provided within the assay). The plate was blocked for 1 hour in
0.1 % (w/v) blocker B solution, washed 3X with PBS plus 0.05 % Tween. Undiluted supernatants or calibrator solutions, 10,000, 2500, 625, 156, 39, 9.8, 2.4 and 0 pg/ml (25 µl) were dispensed, the plate sealed and incubated for 2 hours with shaking (900 rpm) room temperature. Twenty-five µl of antibody detection solution (1.0 µg/ml) was dispensed into each well, the plate sealed and incubated at room temperature for 2 hours with shaking (900 rpm). The plate was washed 3X with PBS plus 0.05 % Tween and 150 µl of 2X read buffer was added to each well of the plate, and analysed immediately on the SECTOR™ Imager.

### 2.10 Flow Cytometry

Flow cytometric analysis is a highly sensitive technique used to count, examine and sort single cells with the use of a laser. Within this study this technique was utilized in several different ways. This technique was used to count the number of migrating cells following a chemotactic assay, to determine purity of cell populations or expression of cell surface receptors, and finally this technique was used as an alternative to western blotting by using antibodies against phosphorylated forms of proteins within a lysed cell population.

The sample being analysed is directed into a stream of fluid that passes through a number of detectors which can analyse the properties of the particles that pass through it, such as size, and density. With the use of fluorescently labelled antibodies surface receptors, or intracellular proteins can be detected and the expression levels compared to other samples. For example, following inhibition with a PI3K isoform specific inhibitor, phosphorylation of downstream effectors can be determined and compared to control samples to observe if this inhibition has an effect on downstream signalling.

### 2.10.1 Assessing cell number following chemotaxis

Following migration of cells during chemokinesis and chemotaxis, the cells are located within the lower chambers of a 96 well in vitro migration assay system. The number of cells that have migrated are analysed by resuspending the cells in 400 µl of PBS and counting using the FACS Vantage (BD Biosciences, San Jose, CA).
Samples are analysed for 60 seconds, with each point performed in at least triplicate. Data is expressed as cell number.

2.10.2 Determining expression of cell surface receptors

Freshly isolated T lymphocytes and activated T lymphocytes were washed twice in PBS 0.1 % BSA and resuspended in 100 µl. 10 µl of FITC-conjugated anti-human antibodies against the receptor of interest or the respective immunoglobulin isotype control was added and incubated on ice and darkness for 30 mins. Cells were washed twice in PBS 0.1 % BSA and resuspended in 400 µl for analysis. This technique can also be utilized to determine the purity of cell populations by analysing expression of a receptor that is present on only the sub-population of interest (e.g. CD3+ T cells).

2.10.3 Determining expression or phosphorylation levels of Intracellular proteins

2 x 10^6 T lymphocytes or activated T lymphocytes were incubated at 37 °C in serum free RPMI for 60 minutes were stimulated with or without CXCL12 (1-100 nM) in RPMI 0.1 % BSA. Stimulations were terminated and the cells fixed by aspiration of the supernatant and addition of 2 % formaldehyde in PBS, for 10 min, at 37 °C followed by 1 min. on ice. Cells were washed in PBS and permeabilised by the addition of 90 % methanol at −20 °C, while slowly vortexing, followed by incubation at 4 °C for 30 min. Cells were washed, resuspended in 100 µl of PBS 0.1 % BSA, and blocked for 10 min. at room temperature. Primary antibody or isotype control was added (200 ng antibody in PBS) and incubated at room temperature for 30 min. Cells were washed twice and incubated in 100 µl FITC-conjugated secondary antibody dilution (1 in 25 dilution) at room temperature for 30 min. Cells were washed twice and re-suspended in 500 µl PBS, for flow cytometric analysis using a Becton Dickinson FACS Vantage flow cytometer and analysed using CellQuest software.
2.11 Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) with Dunnets correction where necessary. These calculations were carried out to test for statistical significance following chemotactic or basal migration assays. A statistical cut-off of P<0.05 was used. These calculations were performed using the GraphPad Prism version 3.0 software.
CHAPTER 3: RESULTS
CHAPTER 3: RESULTS, SECTION I

Characterisation of CXCL12 & CCL5
Chapter 3 - Results, Section I

Results, Section I

T cells are an integral player within both immune surveillance and the immune response to injury and disease. It is therefore crucial to characterise not only the chemokines that trigger circulating T cells to enter a specific tissue, but also the cascade of intracellular signalling events that they govern.

The chemokines that govern the migration of T cells into specific tissues, can be divided into two distinct subsets, homeostatic and inflammatory (Moser et al., 2004). Homeostatic or constitutive chemokines are present during a healthy or disease free state and as such are responsible for orchestrating the trafficking and homing of leukocytes, during their immune surveillance role. Inflammatory or inducible chemokines, encompass those that are upregulated upon injury or disease and are therefore important components of the innate and adaptive immune system (Kashiwazaki et al., 2003).

In order to fully elucidate the role of PI3K in T cell migration, a panel of chemokines were examined. Initial studies began by reinvestigating the role of the chemokine CCL5. These investigations were undertaken due to the contradictory results obtained for not only the expression profile of CCR5 but the subsequent response to in vitro stimulation.

To facilitate the tight control during long term immunosurveillance, only a limited number of chemokine receptors are expressed on the cell surface, (responding to chemokines such as CXCL12, CXCL13 (Okada et al., 2002), CCL2 (Palframan et al., 2001), and CCL19 (Baekkevold et al., 2001)). During an inflammatory response, certain receptors are upregulated e.g. CXCR3. However, the expression profile of CCR5 and its in vitro functions are not yet fully elucidated. Some reports state only activated T cells migrate to CCL5, while other investigations observe migration in freshly isolated T cells (Turner et al., 1995; Bleul et al., 1997; Loetscher et al., 1996b).

Clinical interest surrounding CCR5 and its ligand CCL5, has intensified following the observation that CCR5 acts as a co-receptor for HIV entry and that CCL5 could act as a weak antagonist, by blocking the binding of HIV (Capoulade-Metay et al.,...
This focused work in this field into developing antagonists based on CCL5, which may block the entry of HIV into T cells.

### 3.1 Introduction to the study

The aim of this study was to re-investigate the role of the chemokine CCL5 and clarify its functional response in both freshly isolated and previously activated T cells. To achieve this, the chemotactic and biochemical responses towards CCL5 and its derivatives PSC-CCL5 and AOP-CCL5 were investigated. These responses were compared against the chemokine CXCL12, which is known to be expressed in both freshly isolated and activated T cells. CCL5 has been reported to bind CCR1, CCR3 and CCR5 and the expression of all 3 of these receptors was monitored over a 14 day period (Murphy, 2002; Capoulade-Metay et al., 2006).

CCL5 derivatives were generated through the truncation of CCL5. The amino terminal was truncated and modified with a methionine to produce Met-CCL5, and subsequent modifications generated AOP-CCL5 (Amino-oxpentane-CCL5), NNY-CCL5 (N-nonanoyl-CCL5) and finally the most potent PSC-CCL5 (L-Thia-Pro², L-α-cyclohexyl-Gly³NN-CCLS) (Notohamiprodjo et al., 2006). These derivatives were produced with the aim of developing a clinically successful inhibitor of HIV entry. It has been suggested that signalling downstream of these CCL5 derivatives, may be altered compared to CCL5 itself this was assessed by monitoring downstream signalling events (Vila-Coro et al., 1999).

Although the exact mechanism of action for the derivatives of CCL5 are not known, it is clear that they result in the rapid downregulation of CCR5 and inhibit the recycling of the internalised receptor, leading to prolonged and profound receptor sequestration. Through this action the level of CCR5 at the surface of the cell is reduced and the HIV virus is unable bind (Mack et al., 1998; Sabbe et al., 2001).

### 3.2 Characterisation of CCL5 and its derivatives

To establish the role of CCL5 in freshly isolated T cells, CCL5-mediated chemotaxis was performed over a range of concentrations. Biochemical studies focused on the
phosphorylation of S6, following stimulation with CCL5 and its derivatives AOP- and PSC-CCL5.

The ribosomal S6 protein is a downstream component of the PI3K/mTOR pathway and its subsequent phosphorylation has been observed following chemokine stimulation. PI3K is a crucial mediator of S6 kinase, which is responsible for the phosphorylation of the ribosomal S6 protein, therefore phosphorylation of S6 can be used as an indirect readout of PI3K activity. Strong biochemical and chemotactic responses have been reported following stimulation with CXCL12, in both in freshly isolated and activated T lymphocytes (Reif et al., 2004). For this reason, CXCL12 was utilized as a comparison within chemotactic and biochemical analysis.

3.2.1 CCL5 fails to induce chemotaxis in freshly isolated CD3+ T cells

Figure 3.1 (left panel), demonstrates the chemotactic response and biochemical signalling of freshly isolated T lymphocytes, following stimulation with CCL5, its derivatives and CXCL12. Flow cytometry was used established the surface expression of the receptors CCR1, CCR3 and CCR5.

No detectable surface expression of either CCR1, 3, or 5 was observed (figure 3.1a left panel) and increasing concentrations of CCL5 (up to 100 nM) did not induce a significant chemotactic response within freshly isolated CD3+ cells (figure 3.1c left panel). Furthermore, CCL5 and its derivatives PSC-CCL5 and AOP-CCL5 (100 nM) induced no detectable phosphorylation of S6, while pre-incubation with LY294002 had no effect on this response (figure 3.1b left panel). In comparison, CXCL12 induces a chemotactic response in freshly isolated CD3+ T cells that is concentration dependent. Stimulation with CXCL12 induces phosphorylation of S6 which is sensitive to inhibition with 10 μM LY294002 (figure 3.1b & c left panel).
3.2.2 CCL5 and PSC-CCL5 fail to induce chemotaxis in day 6 PHA activated T cells

In day 6 PHA activated T cells, the surface expression of CCR1 and CCR3 remained undetectable, yet interestingly, following activation and culture, an increase of CCR5 was observed (figure 3.1A right panel). The increase in detectable surface expression of CCR5, did not translate into a functional or biochemical response, as increasing concentrations of CCL5 and its derivative PSC-CCL5, failed to induce a significant chemotactic response or detectable phosphorylation of S6 (figure 3.1B & C right panel). CXCL12 induced chemotaxis in a concentration dependent manner, and phosphorylation of S6 was inhibited by 10 μM LY294002 (figure 3.1B & B right panel).

3.2.3 CCL5 and its derivatives initiate chemotactic and biochemical responses within day 10 and day 14 PHA activated T cells

Following expression of CCR5 at day 6, no further increase in expression was observed. Expression of CCR1 and CCR3 were not observed (figure 3.2A). In contrast to day 6 T cells, day 10 and day 14 activated T lymphocytes, stimulated with CCL5 and its derivatives, induced strong phosphorylation of S6. Furthermore, this induction was PI3K dependent, as the phosphorylation of S6 was inhibited by pre-incubation with 10 μM LY294002 (figure 3.2B).

Surprisingly, although CCL5 does not induce a significant chemotactic response, PSC-CCL5 is more potent, giving rise to a small increase in chemotactic response on day 10 and by day 14 a robust and significant response is observed that is comparable to that of CXCL12 (figure 3.2C right & left panels).
Figure 3.1 Freshly isolated and Day 6 activated T lymphocytes migrate in a CXCL12-dependent but CCR5 independent manner. PTO for figure legend.
Figure 3.1 Freshly isolated and Day 6 activated T lymphocytes migrate in a CXCL12-dependent but CCR5 independent manner. (A) Surface expression profiles of CCR1, 3 and 5 are indicated. The respective immunoglobulin isotype control is shown. Briefly, 1 x 10^6 cells were washed in PBS and incubated with the respective PE conjugated antibody at 4 °C, cells were washed in PBS analysed by flow cytometry. (B) Day 10 activated T lymphocytes were treated with either 10 nM or 100 nM CXCL12, CCL5, PSC-CCL5 or AOP-CCL5 for the times indicated. Protein lysates were generate and resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-phospho S6 antibody. The blots were stripped and reprobed with anti-S6 antibody to verify equal loading and efficiency of protein transfer. (C) Cells were treated with either 10 nM CXCL12, 100 nM CCL5, PSC-CCL5 or AOP-CCL5 for 10 mins, as control or in the presence of 10 μM LY294002 (30 mins pre-incubation). Protein lysates were generate and analysed as described above. (D) CXCL12, CCL5 or PSC-CCL5 mediated chemotactic responses of freshly isolated and day 6 activated T lymphocytes. Cells (8 x 10^4 / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing varying concentrations of CXCL12 (circle) CCL5 (black square) or PSC-CCL5 (white square). Chemotaxis across a 5 μm pore size membrane was determined after 3 hour incubation at 37 °C in 5 % CO_2 as described in Materials and Methods. The data are derived from a single experiment with quadruplicate replicates that is representative of three other experiments. Data are expressed as mean ± SD. Data were analyzed by ANOVA and Dunnetts correction where required (, p < 0.05).
Figure 3.2 Day 10 & 14 activated T lymphocytes migrate in a CXCL12 and CCR5-dependent manner. (A) Surface expression profiles of CCR1, CCR3 and CCR5 are indicated. The respective immunoglobulin isotype control is shown. Briefly, $1 \times 10^6$ cells were washed in PBS and incubated with the respective PE conjugated antibody at $4 \, ^\circ C$; cells were washed in PBS analysed by FACS. (B) Day 10 or 14 activated T lymphocytes were treated with either 10 nM CXCL12, 100 nM CCL5, PSC-CCL5 or AOP-CCL5 for 10 mins as control or in the presence of 10 μM LY294002 (30 mins pre-incubation). Protein lysates were generate and resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-phospho S6 antibody. The blots were stripped and reprobed with anti-S6 antibody to verify equal loading and efficiency of protein transfer. (C) CXCL12, CCL5 or PSC-CCL5 mediated chemotactic responses of day 10 & 14 activated T lymphocytes. Cells ($8 \times 10^4 / 25 \, \mu l$) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing varying concentrations of CXCL12 (circle) CCL5 (black square) or PSC-CCL5 (white square). Chemotaxis across a 5 μm pore size membrane was determined after 3 hour incubation at $37 \, ^\circ C$ in 5 % CO$_2$ as described in Materials and Methods. The data are derived from a single experiment with quadruplicate replicates that is representative of three other experiments. Data are expressed as mean ± SD. Data were analyzed by ANOVA and Dunnetts correction where required (*, $p < 0.05$).
3.3 Summary

In characterising the role of CXCL12, CCL5 and its derivatives in freshly isolated T cells and previously activated T cells, the following observations were made:

- Freshly isolated T lymphocytes do not express CCR1, CCR3 or CCR5 and are unable to chemotax towards CCL5. Furthermore, CCL5 and its derivatives do not induce phosphorylation of the S6 ribosomal protein.
- Freshly isolated T cells show concentration dependent CXCL12-mediated chemotaxis and CXCL12 dependent phosphorylation of S6 is PI3K dependent.
- Day 6 activated T lymphocytes express CCR5 but not CCR1 and CCR3. This expression does not translate this into a chemotactic or biochemical response.
- In day 6 activated T cells, CXCL12 induces a concentration dependent chemotactic response and phosphorylation of S6 is PI3K dependent.
- Day 10 and 14 activated T lymphocytes chemotax towards PSC-CCL5 and to a limited extent CCL5.
- By day 10-14, activated T cells stimulated by CXCL12, CCL5 and its derivatives PSC-CCL5 and AOP-CCL5 signal through S6, this signalling is dependent on PI3K.

These investigations were performed in order to further characterise the ligands that induce migratory responses in freshly isolated and activated T cells. This panel of ligands would then be used to assess the role of PI3K within this process.

Although by day 10 - 14, CCL5 did induce small chemotactic responses, these responses were superseded by the induction seen with PSC-CCL5. This compound was generated in the search for a therapeutically viable CCL5 derivative. The aim was to generate a molecule that would potently bind the receptor but demonstrate no or lower agonist potential before sequestering the receptor within the cell. However, as seen within these investigations it induces strong chemotactic responses within T cells. It is clear this is not an ideal compound and ensures this product will not be clinically developed.
In conjunction with other studies it may be proposed that it is the presence of IL-2 within the culture that induces the expression of CCR5 and the subsequent functional responses. This suggests an essential role for this cytokine within inflammatory settings in order to induce the full migratory response. These investigations support the view that CCR5 expression is upregulated over time, and that chemotactic and signalling responses are only demonstrated 10 – 14 days post activation/culture. Unfortunately this chemotactic response is not statistically significant and CCL5 was not used further within these investigations.
CHAPTER 3: RESULTS, SECTION II

Exploring the role of PI3K in the migration of freshly isolated T cells
Chapter 3 - Results, Section II

Results - Section II

With the established role for CXCL12-mediated migratory responses in the freshly isolated T cell model; the aim within these investigations was to elucidate the role of individual PI3K family members.

Freshly isolated T cells mimic the migration that occurs during continual immunosurveillance of the body. This system is highly specialized, therefore described here is an overview of how the components of the peripheral T cell pool are generated and subsequently maintained.

In the long term, T cell numbers within the immune system are steadily maintained (Jameson, 2002). However, for this pool of cells to respond adequately during times of injury or disease, cell numbers must be capable of identifying specific antigens, in order to address the immune requirements at that precise moment. To perform this task adequately, this pool must be comprised of several subsets of cells. The most abundant class of T cells in peripheral blood are α/β T cells, comprised of CD4+ and CD8+ T cells, these are further divided into 3 types of functionally diverse cells; naïve, effector and memory.

Following positive and negative selection, naïve CD4+ and CD8+ cells exit the thymus, and circulate the lymph and peripheral tissues as naïve cells, where their role is one of immunosurveillance. Following stimulation by an appropriate antigen, these naïve CD4+ and CD8+ cells undergo activation and expansion, resulting in the mature effector cell capable of eliminating the antigen. By their nature a large proportion of these cells undergo apoptosis, however, those that do survive differentiate into circulating memory T cells (Mahajan et al., 2005).

In a healthy individuals, the pool of T cells that are found within the peripheral blood, are a mixture of primarily naïve and memory cells with a small number of effector T cells; it is from these healthy individuals that peripheral CD3+ T cells are isolated. As the majority of these cells are naïve or memory cells, they express a limited repertoire of receptors on the cell surface and consequently respond to a restricted number of chemokines. It is from this pool of cells that the investigations within this study will be drawn.
Investigations into the role of PI3K in the freshly isolated T cell model, was addressed through the use of the PI3K broad spectrum inhibitors LY294002 and wortmannin. This was assessed using the phosphorylation of PKB and CXCL12 mediated chemotaxis as biochemical and functional readouts. This work was furthered by highlighting the role of specific PI3K isoforms in biochemical and chemotactic responses, following the utilization of isoform specific inhibitors.

3.4 Freshly isolated T cells demonstrate a dependence on PI3K for their chemotactic response

Within this investigation, the role of PI3K in the chemotactic response of freshly isolated T cells was addressed. To evaluate this thoroughly, dose responses to CXCL12 were performed and the effect of LY294002 and wortmannin were assessed against the peak chemotactic response. Biochemical assays were utilized to determine coupling of the receptor to intracellular signalling events. The signalling molecule PKB is dependent on the activation of PI3K for its activation and subsequent propagation of the signalling cascade. The close relationship between PKB and PI3K enabled the phosphorylation of PKB to be utilized as an indirect method to assess PI3K activity.

The CXCR4 chemokine receptor is constitutively expressed on naïve T cells and ligation of this receptor is a pivotal axis within the immune system. Regulation of this coupling is essential in the homing/trafficking of cells such as neutrophils (Link, 2005), T cells (Butcher et al., 1999) and stem cells (Molyneaux et al., 2003) and has been implicated in roles such as organogenesis, angiogenesis and tumorigenesis (Bagri and Tessier-Lavigne, 2002; Ratajczak et al., 2006; Jin et al., 2006). A role for PI3K has been demonstrated within T cell chemotactic responses (Reif et al., 2004).

CXCL12 provides a strong, robust chemotactic signal in freshly isolated T cells (figure 3.3A left panel). All isoforms except class II γ are expressed at the RNA level within freshly isolated T cells (figure 3.3E). Following pre-treatment with the PI3K inhibitor LY294002, CXCL12-mediated chemotaxis in freshly isolated T lymphocytes, is inhibited in a concentration dependent manner (figure 3.3B). 10 μM
LY294002 decreased the CXCL12-mediated chemotactic response significantly, while 30 μM almost completely abrogated this response. Furthermore, pre-incubation with LY294002 also exhibited a significant concentration dependent decrease in basal migration. The effect on both CXCL12-mediated chemotaxis and on basal migration was corroborated with wortmannin (figure 3.3C). At the biochemical level, PI3K also inhibited the phosphorylation of PKB in a concentration dependent manner, suggesting that CXCL12 intracellular signalling events are coupled through PI3K (figure 3.3D).

To determine if the response was driven by a particular subset of T cells, CD4+ and CD8+ T lymphocytes were isolated and the biochemical and functional responses assessed (figure 3.4). CD4+ and CD8+ T cells both displayed strong chemotactic responses. Following pre-incubation with LY294002, CD4+ and CD8+ T cells were inhibited in a concentration dependent manner, with 3 μM producing significant decreases in CXCL12-mediated chemotaxis.
Figure 3.3 CXCL12-mediated chemotactic responses in freshly isolated CD3$^+$ T lymphocytes are PI3K dependent. PTO for figure legend.
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Figure 3.3 CXCL12-mediated chemotactic responses in freshly isolated CD3 T lymphocytes are PI3K dependent. (A) Effect of increasing concentrations of CXCL12 on the chemotactic responses of freshly isolated CD3 T lymphocytes, in the presence of LY294002 (B) and Wortmannin (C). Cells were pre-incubated with either vehicle, LY294002 (3-30 μM) or Wortmannin (30-300 nM) for 30 mins (left panels) and expressed as number of cells migrating. Cells (8 x 10⁴/25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXC12 as described in Materials and Methods. The percentage inhibition by LY294002 or Wortmannin for basal (black lines) and stimulated (red lines) is depicted in B & C right handside panels. The data are derived from a single experiment with quadruplicate replicates that is representative of four (LY294002) or two (Wortmannin) other experiments. Data were analysed using ANOVA with Dunnet's correction where required. (Data from all donors were further analysed using ANOVA with repeated measures followed by Dunnet's correction. Significance was observed at each concentration of inhibitor for both stimulated and basal levels, P < 0.05). D) Aliquots of T cells (2 x 10⁶ cells/ml) were left untreated or stimulated in parallel for 2 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser⁴⁷³-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer. Data are expressed as mean ± SD. (E) Expression of class I, class II and class III PI3K isoforms in freshly isolated T lymphocytes. T lymphocytes were isolated from whole blood using negative selection as described in Materials and methods. Total human RNA (5 x 10⁶ cells) was isolated using the Qiagen RNeasy kit and DNA was obtained by RT-PCR followed by quantitative PCR as described in Materials and methods. Levels of individual PI3K mRNA are normalised to levels of standards which are an equal mixture of human brain/spleen/thymus RNAs.
Figure 3.4 CXCL12-mediated chemotaxis of CD4+ and CD8+ freshly isolated T lymphocytes are sensitive to LY294002 inhibition. (A) Effect of increasing concentrations of CXCL12 on the chemotactic responses of freshly isolated CD4+ and CD8+ T lymphocytes, and in the presence of LY294002 (B & C). Cells were pre-incubated with either vehicle or LY294002 (3-30 μM) for 30 mins. Cells (8x10^5 cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5% CO₂ as described in Materials and Methods. The percentage inhibition by LY294002 for basal (black lines) and stimulated (blue or red lines) is depicted in the right panels. The data is taken from a single experiment in quadruplicate and is representative of 3 individual donors, data above were analyzed by ANOVA with Dunnett's correction where required (* P < 0.05). Data are expressed as mean ± SD. (N.B. Data from 3 donors were further analysed using ANOVA with repeated measures followed by Dunnett's correction. Both CD4 and CD8 exhibited significance at each concentration of inhibitor, P < 0.05).
3.5 The use of Pharmacological tools to explore the roles of individual PI3K in T cell responses

Phosphatidylinositol-regulated signalling pathways, can be used as an indirect method by which to measure PI3K activity, one such example is PI3K/mTOR, in which PI3K has been shown to be a crucial mediator of S6 kinase (Sasaki et al., 2000). S6 kinase phosphorylates the ribosomal S6 protein, by measuring this phosphorylation, with and without specific PI3K isoform inhibition, provides an indirect method to measure the contribution that each isoform may play within this signalling cascade. Although a role for S6 phosphorylation in the migration of T cells has not been established, S6 phosphorylation has implications in chemotaxis of other cell types. S6 kinases are under the tight control of the mammalian target of Rapamycin (mTOR), which is enriched within the actin arc and formation of which has been linked to migration (Berven et al., 2004). Neutrophil chemotaxis and chemokinesis induced by GM-CSF (granulocyte-macrophage colony-stimulating factor) is prevented by pre-treatment with the immuno-suppressant rapamycin, which specifically binds to and inhibits mTOR, suggesting this pathway plays a critical role within both chemotaxis and chemokinesis (Gomez-Cambronero, 2003). Rapamycin, the broad-spectrum PI3K inhibitor LY294002 and class I isoform selective inhibitors, were utilized to assess the effect on the phosphorylation of S6 following CXCL12 stimulation (figure 3.5).

3.5.1 PI3K isoform selective inhibitors

The advancement in the selectivity of PI3K inhibitors, allows the dissection of the role of the PI3K family. A recent study highlighted that the imidazopyridines (e.g. PI-103) preferentially targets PI3Kα, the chromones (e.g. TGX-121) preferentially target PI3Kβ/PI3Kδ, quinazolinone purine inhibitors (e.g. IC87114) selectively target PI3Kδ, and the thiazolidinediones (AS605240) preferentially target PI3Kγ.
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3.5.2 PI3K-dependent phosphorylation of S6

Phosphorylation of the S6 ribosomal subunit is dependent on PI3K activity and can be measured via a flow cytometry based assay. Following stimulation with CXCL12 the phosphorylation of S6 increases. Figure 3.5 A-F demonstrates the effect of LY294002, Rapamycin and isoform selective inhibitors on the phosphorylation of S6 following CXCL12 stimulation. Figure 3.5 G demonstrates (in a graphical form) the phosphorylation of S6 with and without selective inhibition. This data was used to calculate the mean fluorescence for each treatment, which is demonstrated within figure 3.5 A – F.

Pre-treatment with LY294002 inhibits the phosphorylation of S6 in a concentration dependent manner, although residual phosphorylation is still observed (figure 3.5A). The dual class I α/γ inhibitor AS605240 produced the most robust effects on inhibition (0.3 - 3 µM), although phosphorylation of S6 was not completely inhibited (3 µM), suggesting contribution of other PI3K isoforms (figure 3.5F). The class I β/δ inhibitor, TGX-121, had no effect on the phosphorylation of S6 even at 500 nM, which is higher than its known selectivity range (figure 3.5D). Inhibition with IC87114 (δ selective) exhibited a modest decrease in phosphorylation, and at 30 µM exhibited a statistically significant decrease (figure 3.5E).

The α selective inhibitor (PI-103) produced a concentration dependent decrease in S6 phosphorylation, with 100 and 300 nM producing significant responses (figure 3.5C). However, PI-103 also inhibits mTOR with a similar IC_{50} to that for PI3Kα (20-100 nM), therefore as S6 lies downstream of both mTOR and PI3K, the relative contribution of mTOR and PI3Kα cannot be dissected out. However, inhibition with Rapamycin, an inhibitor of mTOR, demonstrates a significant decrease in the phosphorylation of S6, suggesting at least in part, the result obtained with PI-103 may be due to an effect on mTOR (figure 3.5B).

3.5.3 The role of class I PI3K isoforms in CXCL12-mediated chemotaxis

The use of classical pharmacological PI3K inhibitors, such as LY294002, has highlighted the importance of PI3K in CXCR4-mediated chemotaxis of freshly
isolated T lymphocytes. However, these inhibitors can only imply a role for the class I PI3K family as a whole. The use of knockout models in particular p110y knockouts, have been invaluable in implementing this isoform within the chemokine mediated chemotactic response, yet the role of other class I isoforms within this response is yet to be fully elucidated (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000; Thomas et al., 2005). In order to clarify the specific PI3K isoforms that are involved in T cell chemotaxis, class I isoform specific inhibitors were utilised.

In freshly isolated T cells, increasing concentrations of the chemokine CXCL12 were used to build a profile of the chemotactic response. The effect of increasing concentrations of specific class I PI3K isoforms upon this response were assessed (figure 3.6). The CXCL12-mediated chemotactic response displayed the typical bell shaped curve associated with chemokine induced chemotactic responses, with a peak response seen between 10-30 nM depending on the donor (figure 3.6A-D).

The class I alpha inhibitor, PI-103 shows 5-10-fold selectivity for a over δ and β isoforms respectively (table 1). At 30 nM PI-103 showed no significant effect on the CXCL12-mediated chemotactic response, yet, there is a trend to see an increased chemotactic responsiveness at 10-30 nM CXCL12, this was replicated for each donor tested. Although there was a decreasing trend for chemotaxis following inhibition with 300 nM PI-103, this effect was not significant. No effect on basal migration was observed even at the highest concentration (figure 3.6A). Inhibition of class l|3/5 PI3K with the inhibitor TGX-121 demonstrates around 100-fold selectivity against PI3K a/y, and showed little effect on the CXCL12-mediated chemotactic response at concentrations ranging from 5 - 500 nM (figure 3.6B).

IC87114 displays around 100-fold selectivity against PI3K δ vs. β isoforms and >1000 fold over α and γ. Concentrations of the class I delta PI3K inhibitor IC87114, up to 10 μM, showed no effect at the lowest concentrations of CXCL12-mediated chemotaxis (1-10 nM figure 3.6C). Interestingly, when inhibiting at 10-30 μM IC87114 (a concentration which in non-selective between PI3K isoforms) there was a concentration dependent decrease in chemotactic response which was significant at 30 μM. No effect on basal migration was observed. Similarly, PI-103 impaired
cell migration at concentrations where it would be expected to target PI3Kγ activity, in addition to the primary isoform target (table 1).

The dual class I γ/α inhibitor AS605240 displays 10-fold selectivity against PI3Kγ over α but 40-fold selectivity against β/δ. A concentration dependent decrease in CXCL12-mediated chemotactic responses was observed from 0.3 – 10 μM AS605240. A concentration dependent decrease in basal migration was observed within these studies (figure 3.6D & E).
Chapter 3 - Results, Section II

Figure 3.5 CXCL12-stimulated PI3K-dependent S6 phosphorylation is inhibited by PI-103 and AS605240 inhibitors. Freshly isolated T lymphocytes were pre-incubated in the presence of (A) 3-30 μM LY294002, (B) 100 nM Rapamycin, (C) 0.03-0.3 μM PI-103, (D) 0.005-0.5 μM TGX-121, (E) 3-30 μM IC87114, (F) 0.3-3 μM AS605240 for 30 mins. T lymphocytes (1 x 10⁶) were then either left untreated or stimulated with CXCL12 (10 nM) for 10 mins. Cells were fixed, permeabilised and stained for phosphorylated S6 as described in materials and methods. Cells were analysed by Flow cytometry. Data is from a single donor, and is representative of 4 others. Statistical analysis was performed using ANOVA with Dunnetts correction (* p < 0.05) when required. Further analysis using ANOVA with repeated measures and Dunnett's correction was performed. The significance indicated above is representative of what was observed with further analysis. Data are expressed as mean ± SD. (G) Example of histogram overlays generated using software on the FACS Vantage following flow cytometry analysis. Figures A-F were generated through the analysis of the mean fluorescence of the histograms presented in figure G.
Figure 3.6 Effect of isoform specific inhibitors on CXCL12 mediated chemotactic responses of freshly isolated T cells. Freshly isolated T lymphocytes were pre-incubated in the presence of (A) 0.03-1 μM PI-103, (B) 0.005-0.5 μM TGX-121, (C) 1-30 μM IC87114 or (D) 0.3-10 μM AS605240 for 30 mins. E) Effect of AS605240 on basal. T lymphocytes (8 x 10^4/25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing varying concentrations of CXCL12 as described in Materials and Methods. Chemotaxis across a 5 μm pore size membrane was determined after 3 hour incubation at 37 °C in 5% CO₂. The data (A-E) are derived from a single experiment performed in triplicate that is representative of three other experiments. Data are expressed as mean ± SD. F) For ease of viewing, statistical analysis for 3 identical experiments have been analysed using ANOVA with Dunnets correction where required. The table indicates the concentration or concentrations of inhibitor which show statistical significance (P<0.05) at each concentration of CXCL12. If no concentrations of inhibitor were significant this is indicated by NS, not significant.
3.6 Summary

By investigating the role of PI3K in freshly isolated T cells the following observations were made:

- CXCL12-mediated chemotaxis of freshly isolated T lymphocytes is PI3K dependent.
- CD4+ and CD8+ T lymphocyte subsets chemotax towards CXCL12 and are sensitive to increasing concentrations of LY294002 inhibition.
- PI3K isoform specific inhibition suggests a role for class I γ in freshly isolated T cell chemotaxis.
- Signalling through S6 is dependent on PI3K class I γ.

In this section of work it has been demonstrated that freshly isolated T cells are dependent on PI3K isoforms for both CXCL12-mediated migration, chemokinesis and signalling through PKB. This data is in support of other studies which have also identified PI3K as having a role within this system (Butcher et al., 1999). In furthering these observations, CD3+ T cells were subdivided into CD4+ and CD8+ T cells and the investigation repeated to determine if this response was primary dependent on one of these subgroups. The data suggests that both CD4+ and CD8+ T cells generate robust CXCL12-mediated chemotactic responses that are dependent on PI3K.

The use of class I PI3K isoform selective inhibitors demonstrated a role for PI3K γ in CXCL12-mediated phosphorylation of S6. Significant decreases in phosphorylation were observed at all concentrations of inhibition. At non-selective concentrations, IC87114 (δ selective) significantly inhibited phosphorylation, although no role for β/δ was established when using any concentration of the β/δ selective inhibitor. PI-103 (α selective) showed significant decrease of S6 phosphorylation at 100-300 nM, yet within this inhibition may be due to inhibition of mTOR.

Within the migratory response, limited roles for α and β were observed. At the highest concentrations of IC87114 there is inhibition of migration, but at this concentration the inhibitor loses its selectivity. The use of isoform selective
inhibitors identified a predominant role for PI3Kγ within migration and chemokinesis. However, residual migration was still observed suggesting an alternate isoform within the PI3K family or an alternate pathway may play a role within this response.
CHAPTER 3:
RESULTS, SECTION III

Investigating the role of PI3K in the migration of cultured / activated T cells
Chapter 3 - Results, Section III

Results, Section III
The use of broad spectrum PI3K inhibitors LY294002 and wortmannin has revealed a role for the PI3K family within migration of freshly isolated human T cells. With the use of isoform specific inhibitors a dominant role for the PI3Ky isoform has been established. However, 2 lines of evidence suggest additional signalling events are required to facilitate optimal migratory responses; first, following inhibition with LY294002 and AS605240 residual chemotaxis is still observed; second, \textit{ex vivo} cultured and activated T cells are insensitive to broad spectrum PI3K inhibitors (Smit et al., 2003). The possibility that class II α and β PI3K isoforms, which both exhibit resistance to LY294002 and wortmannin (Domin et al., 1997; Arcaro et al., 1998), may contribute to this response was therefore investigated. PI-103 has inhibitory effects on PI3K class IIβ (which is resistant to LY294002). However, as some inhibitory effects on cell migration are observed and pharmacological analysis of this isoform is limited, (as there are no class II specific inhibitors), an siRNA strategy was adopted to further characterise the role of PI3K isoforms in human T cell migration.

This work focuses on both the freshly isolated and \textit{in vitro} activated T cell, therefore a brief overview of the generation of these cells and the advantages that this system affords shall be discussed. In a physiological setting naive or memory T cells are performing an immunosurveillance role, when such cells encounter an antigen, presented by an APC the T cell subsequently undergoes activation leading to proliferation, release cytokines, chemokines and upregulation of the receptor repertoire on the surface of the cell. This upregulation allows biochemical and functional responses towards a myriad of chemokines. The \textit{in vivo} activation of T cells is a complex mechanism, therefore, the discovery that bacterial antigens and lectins can replicate this activation \textit{in vitro}, by mimicking some of these events, initiated a huge growth in this field of research, leading to 1,000s of publications utilizing this tool of investigation.

Activation of T cells and their subsequent expansion \textit{in vitro}, mimics the \textit{in vivo} activation of a T cell in response to antigen presentation and is the closest comparable model of human T cell activation. The advantage of using this \textit{in vitro} model is two-fold. Firstly, investigations can be performed on mature T cells which
participate in responses to injury or disease, secondly, this activation leads to upregulation of receptors on the surface of the cell, allowing for greater investigation of the downstream signals that are generated through a plethora of chemokine receptors. This allows for greater investigation within the in vitro system, and for these investigations provides a greater insight into the role of PI3K downstream of these receptors.

Common in vitro methods of activation include; Staphylococcal enterotoxin B (SEB), Phytohemagglutinin (PHA), Concanavalin A (Con A), Pokeweed mitogen (PWM). Activation of T cells can also be performed with CD3/CD28 stimulation which is the closest physiologically relevant activation method. These methods of activation are independent of antigen stimulation and circumvent the normal T cell activation mechanism, allowing for strong and robust activation, proliferation and release of pro-inflammatory cytokines and chemokines (Krakauer et al., 2005).

This body of work will be discussed in 3 parts; the first presents the expression profile of PI3K in human T cells. Part 2 stresses the importance of strong siRNA design, and illustrates the effectiveness, potency and selectivity of the siRNAs used within this study. Finally, part 3 reports in detail the optimisation of Nucleofection and effect of ex vivo culture and activation.

3.7 Expression levels of PI3K isoforms following activation

The aim of this experiment is to determine which PI3K isoforms are present within activated T cells and the expression profile throughout the life cycle of the cell. Both PHA and CD3/CD28 activated T cells were analysed for PI3K expression to allow comparison throughout the spectrum of in vitro activation methods.

Figure 3.7 illustrates the expression profile of each individual PI3K isoform for both PHA and CD3/CD28 activated T cells as determined by qPCR. The cells are monitored over a 5 – 13 day time course, as activated cells can be first used within experimental procedures (e.g. Nucleofection) on day 6 and can be cultured and used in experimental procedures up to day 13. As expected class II γ which is expressed predominantly in hepatocytes, is not expressed in activated T cells,
however all other PI3K isoforms were present. Of particular note is the different expression profiles of each isoform following either PHA or CD3/CD28 activation, (with the exception of class I β which shows low levels following both types of activation). Up to a week after activation, levels of each isoform in PHA and CD3/CD28 activation are very similar, however, after this time expression in PHA activated cells increases rapidly. In comparison, levels in CD3/CD28 activated cells remain relatively constant.
Figure 3.7 mRNA expression levels for PI3K isoforms in activated human T cells. Expression of PI3K isoforms. (A) Class I alpha, (B) class I beta, (C) class I delta, (D) class I gamma, (E) class II alpha, (F) class II beta and (G) class III. PHA activated T lymphocytes were obtained from PBMCs isolated from whole blood and stimulated with PHA (72 hours, 5 μg/ml). RPMI-1640 media supplemented with 10 % FCS and IL-2 (20 units/ml) was added every 2 days to expand the PHA activated cells. Total human RNA (5 x 10^6 cells) was isolated using the Qiagen RNeasy kit. RNA quantity was determined on a 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 LabChip kit. cDNA was obtained by RT-PCR followed by quantitative PCR as described in Materials and Methods. Ratio of expression is relative to standards.
3.8 siRNA validation

When designing an experiment that incorporates the use of siRNA, it has been thoroughly established that the most critical factor is ensuring an effective and specific siRNA. The field of siRNA design is growing rapidly, and many studies have focused purely on investigating the ‘rules’ surrounding solid siRNA design; giving an invaluable insight into the generation of these tools. At this time, due to the complex nature of the system, theoretical knowledge of siRNA design goes only part way in identifying strong \textit{in vitro} siRNA candidates, and full validation within experimental setting is still an essential tool.

The ability to induce a decrease in target mRNA and yet show specificity for not only that gene but a particular isoforms within that family, determines the effectiveness of that siRNA. Any effects on other isoforms or proteins, which are known as ‘off-target effects’, limit the strength of data that can be obtained by utilizing that siRNA.

Within this study each of the expressed PI3K isoforms, were targeted by four different siRNAs and evaluated for their ability to decrease/target that particular isoform. The most effective siRNAs were subsequently assessed for their off-target effects, this was determined through quantification of the other PI3K isoforms. This method was established to identify the effective siRNAs. Furthermore, titration of individual siRNAs was used to highlight the differing potencies that can be achieved even within the same siRNA pool.

3.8.1 RNA quality

Validation of the PI3K targeting siRNA was initially performed in the previously validated PHA activated T cell model. This system was utilized to ensure consistency would be maintained throughout the experimental design, for example, cell type, expression of PI3K, concentrations of siRNA to be used and the delivery method. Before quantification of the target gene can occur, levels of total RNA within the samples are assessed via an RNA 6000 labchip. Figure 3.8 exemplifies the readout which is obtained from which the quantity and quality of RNA can be assessed. An example of good quality RNA can be seen in the left panel generated
from a control sample of $5 \times 10^6$ T cells. This readout shows 2 sharp peaks from which the area under the curve is used to calculate the RNA concentration. The two peaks correspond to the 28s and 18s ribosomal peaks within the RNA sample. The middle panel is the RNA from $5 \times 10^6$ T cells that have been nucleofected with non-targeting siRNA, within this sample no clear peaks can be seen and the RNA concentration cannot be determined. Doubling the concentration to $1 \times 10^7$ cells increases the concentration of RNA to a level which can be analysed on this system, yet the quality of RNA obtained was not sufficient to ensure confidence that subtle differences within the targeting effects of the siRNA could be determined. In order to generate reliable and accurate results an alternative model to validate the PI3K siRNAs was established.

The classic immortalized HeLa cell line is routinely used and has been shown to undergo efficient transfection of both plasmid and siRNA. Importantly for this particular study, HeLas demonstrate high transfection efficiency, with little effect on cell viability, RNA yield or quality, all of which were identified as hurdles in the activated T cell model (figure 3.8). This ensured a comparatively 'high throughput' screen compared to cultured human T cells, which was essential for the large number of siRNAs being tested against each expressed PI3K isoform.

Figure 3.9A illustrates the expression of PI3K isoforms with the HeLa cell line. As expected class I and class II $\gamma$ isoforms were not present, and as class II $\gamma$ is not expressed in human T cells this posed no constraints on future experiments. Due to the lack of class I $\gamma$ expression, no validation of the targeting siRNAs could be performed, and therefore future experiments within human T cells used all 4 targeting siRNAs. All other PI3K isoforms were expressed in HeLas with comparative levels of class I $\alpha$ and class II$\beta$ at 3 or 4 times that of the housekeeping gene GAPDH. PI3K class I $\beta$, class II $\alpha$ and class III were all expressed at around 20 times that of GAPDH. This confirmed that each of the isoforms present could be targeted for siRNA validation.
3.8.2 siRNA effectiveness

Four siRNA sequences against each of the isoforms present were tested within this system. The class I alpha isoform was successfully targeted by 2 of the 4 siRNAs, the other 2 siRNAs induced only minimal knockdown of mRNA when compared to a non-targeting siRNA (figure 3.9B top left panel). Each of the 4 siRNA sequences tested against class I beta, delta, class II alpha, beta and class III produced strong mRNA silencing of the targeted isoform compared to a non-targeting siRNA (figure 3.9B).

As discussed previously, residual chemotactic responses had been identified even following the inhibition of class I PI3K isoforms or using knockout models in combination with class I isoform specific inhibitors. This suggested a role for either an alternative pathway or utilization of other isoforms within the PI3K family within the chemotactic response. Therefore within this present study the primary objective was to assess the contribution of class II PI3K family members. In order to achieve this goal the two most effective siRNAs against class II α and β were further assessed for potency and ‘off-target’ effects to ensure effectiveness and selectivity. SiRNA concentrations were titrated to 0.1 nM and compared at each concentration to control levels of the target isoform and to non-targeting siRNA (figure 3.10 left panels). As a comparison, identical studies were also performed for class I beta and delta. Initially the study was designed to incorporate the comparison between all PI3K isoforms; this was to provide an overview of the siRNAs available against the whole PI3K family not just for work within this study, but within the lab as a whole. However, during the validation studies of siRNA in activated T lymphocytes, all the siRNA against class I alpha was exhausted. As the focus for this study was the class II isoforms no additional class I α siRNA or specific probes were purchased.

Increasing the concentration of all of the siRNAs from 0.1, 1 to 25 nM produced a concentration dependent decrease in the quantity of knockdown seen within each targeted isoform (figure 3.10 left panels). Delivery of non-targeting siRNA did have a minimal effect on isoform expression, although these differences were not
concentration dependent and they were seen at even at the lowest concentrations of siRNA, suggesting no effect on specific targets.

SiRNAs specific for class I β suggest that although both sequences were effective in reducing the mRNA levels, sequence 5 shows the highest potency, as it produced a greater knockdown at lower concentrations. The lowest concentration of siRNA tested against class I δ (0.1 nM) was unable to produce a knockdown of the targeted isoform. Strong knockdown of class I δ was achieved with 1 nM of sequence 4 siRNA while sequence 2 only gave this level of potency by 25 nM. The class II alpha siRNAs gave strong and comparable concentration dependent knockdown of the target isoform even at 0.1 nM. Class II β siRNAs were comparatively less potent than α, with sequence 1 showing no effective response until 1 nM, although potencies of sequence 1 and 3 were comparable at this concentration.

Figure 3.10 right panels, demonstrates the 'off-target' effects of the siRNAs which will be used within this study. Here 25 nM target specific siRNA was used (the highest concentration tested within this system) to assess the effect not only on the target isoform but against other PI3K isoforms. Delivery of class I β siRNAs induced strong knockdown of the class I β isoform compared to control and non-targeting siRNA, with no decrease in the other PI3K isoforms. This target specificity was observed following delivery of class I δ, class II α or β, all of which demonstrated a decrease in target specific RNA and no decrease in any other PI3K isoforms tested. It is however interesting to note, that following delivery of either class I β or class II α siRNA there was a slight increase in the RNA levels of class I delta. This trend was observed for each sequence of siRNA and over the triplicate experiments.
Figure 3.8 Validation of siRNA is ineffective in activated T lymphocytes. PHA activated T lymphocytes (5 x 10^6 cells) were left untreated or nucleofected with 4 μM of PI3K Class I alpha siRNA (siRNA 5). 72 hours post treatment dead cells were removed and cells lysed and homogenised. Contaminants were removed and the RNA isolated. The concentration and quality of the RNA was determined on a 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 LabChip kit. 5 x 10^6 cells in the control sample (left panel) was compared to 5 x 10^6 nucleofected cells (middle panel) and 1 x 10^7 nucleofected cells. Data is representative of 2 individual donors analysed in triplicate.
Figure 3.9 Validation of siRNAs in HeLa cells. Target specific knockdown of PI3K class I alpha, beta, delta, class II alpha, beta and class III in HeLa cells. Briefly, HeLa cells were transfected using Oligofectamine for 24 hours, and total cellular RNA was isolated using the Qiagen RNeasy kit according to manufacturer's instructions. The concentration and quality of the RNA was determined on a 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 LabChip kit. RNA was reverse transcribed using a Biometra UNO II thermocycler. Quantitative PCR was performed and analysed using Sequence Detector Software (SDS version 2.1). Data was obtained in triplicate. mRNA levels are expressed as mean ± SD.
Concentration dependent target specific knockdown of PI3K class I beta (A, left panel), delta (B, left panel), class II alpha (C, left panel), beta (D, left panel) in HeLa cells. PI3K isoform off target effects monitored following delivery of 25 nM siRNA PI3K class I beta (A, right panel), delta (B, right panel), class II alpha (C, right panel), beta (D, right panel) and class III (E, right panel) in HeLa cells. Briefly HeLa cells were transfected using Oligofectamine for 24 hours, and total cellular RNA was isolated using the Qiagen RNeasy kit according to manufacturer’s instructions. The concentration and quality of the RNA was determined on a 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 LabChip kit. RNA was reverse transcribed using a Biometra UNO II thermocycler. Quantitative PCR was performed and analysed using Sequence Detector Software (SDS version 2.1). Data was obtained in triplicate. mRNA levels are expressed as mean ± SD.
3.9 Lipid based delivery of DNA and siRNA into activated T lymphocytes is ineffective.

Before embarking on the optimisation of a new transfection method (Nucleofection), the efficiency of other methods of siRNA delivery were evaluated. Delivery of DNA such as plasmids, have been successfully transfected into cell lines using a variety of techniques; including viral methods such as lenti- and adeno- viral, or non-viral methods such as lipofection and electroporation. Initially, there was no capacity to test viral methods of delivery, therefore lipid based systems were tested. This technique is advantageous as it enables high transfection efficiencies coupled with low toxicity.

Within these investigations, delivery of GFP-encoding plasmids and the smaller Cy3 tagged siRNAs into day 6 activated human T cells were compared. Figure 3.11A upper panel, demonstrates the effect using a lipid based methods to introduce a GFP encoding plasmid (open blue histogram). This figure illustrates no increase in fluorescence (this would be indicated with a shift in the histogram to the right) when compared to control samples which had no plasmid (filled purple histogram), control sample mixed with plasmid but no lipid carrier (open pink histogram) or transfected with an empty vector (open green histogram).

In figure 3.11A lower panel, the cells were transfected via a lipid carrier with a Cy3 tagged siRNA, control cells (filled purple histogram) or cells transfected with a non-targeting siRNA (open green histogram) show no fluorescence. However, an increase in fluorescence is seen in cells transfected with Cy3 tagged siRNA and those just mixed with the Cy3 tagged siRNA (i.e. no lipid carrier). This is further explained in figure 3.11B, in which cells have been mixed with Cy3 tagged siRNA and analysed under a light and fluorescence microscope (upper and lower panel respectively). This figure demonstrates that the siRNA binds to the cell surface and because it has a Cy3 tag, fluoresces even in the absence of transfection into the cell. Thus the ‘expression’ seen following mixing of the siRNA and the cells, can actually be attributed to extracellular binding and is a ‘fake positive’. These results suggest that lipid based delivery of both plasmid and siRNA was met with limited success and should not be carried forward for the delivery of PI3K siRNA. Instead, the novel method of Nucleofection was tested.
Figure 3.11 Lipid based delivery of plasmids or siRNA is ineffective in day 6 PHA activated T lymphocytes. PHA activated T lymphocytes were transfected using lipofectamine 2000, with either GFP encoding plasmids (A, top panel) or Cy3 tagged siRNA (A, bottom panel) (open blue histograms) and analysed using flow cytometry (A) or microscopy (B). These samples were compared to control which where either non-transfected cells (filled purple histograms), non-transfected and mixed with GFP plasmid or Cy3 tagged siRNA (open pink histogram) or transfected with an empty plasmid or non-targeting siRNA (open turquoise histogram). Briefly, plasmid or siRNA were added alone or mixed with lipofectamine and added to cells in RPMI-1640 supplemented with 1 % FCS with no antibiotics, after 4 hours at 37 °C and 5 % CO₂. FCS was added giving a final concentration of 10 %. Cells were incubated for 24 hours, and analysed by flow cytometry or microscopy.
3.10 Delivery of DNA & siRNA into activated human T cells

Previous studies have utilised nucleofection as a technique to deliver siRNA or DNA into primary human cells, such as CD4+ T cells. Within these investigations, activated T cells, which mimic the in vivo development of T cells following antigen presentation, were used. This in vitro activation and maintenance of the T cells, results in the up regulation of chemokine receptors, allowing for a panel of chemokines to be investigated within the chemotactic response.

3.10.1 Optimising the Nucleofection Procedure

Nucleofection is based on electroporation, in which a short electrical pulse creates transient pores within the plasma membrane, allowing siRNA to enter the cell. Within most electroporating systems the electrical pulse can be varied in order to optimise the transfer of DNA/RNA into the cell, yet this cannot be performed with the Nucleofector, as only the program numbers are disclosed. Within the Nucleofector system there is one nucleofector solution for activated T cells and the device has two different Nucleofector programmes which can be used, either T20 or T23. T23 gives higher transfection efficiencies, yet is harsher on the cells, whereas T20 is less harsh but is reported to give lower efficiencies.

Initial studies focused on assessing the efficiency of the nucleofection method to deliver GFP encoding plasmids (figure 3.12). During optimisation of this delivery, the effect of different nucleofector programs (figure 3.12A), increasing concentrations of plasmid (figure 3.12B) and different GFP encoding constructs (figure 3.12C) were evaluated. The experimental procedures mirrored those that would be used for siRNA experiments and could therefore highlight any potential issues that may arise within the system.

In control samples (figure 3.12A upper left panel) less than 1 % of cells were positive for fluorescence, this was comparable to cells nucleofected without a plasmid (mock), however, and larger cell death was observed (10 % and 54 % respectively). The nucleofection device afforded two setting for delivery of DNA or siRNA into activated T lymphocytes. These 2 delivery programs, termed T20 and T23, were assessed for efficiency of transfection, within this system. Both settings
delivered GFP-encoding plasmids into approximately 5% of the nucleofected population, with 85% cell death (figure 3.12B bottom panels). Increasing the concentration of the GFP-encoding plasmid showed limited effect although 2 μg consistently yielded the highest efficiency, while cell death increased with the concentration of DNA, 77, 85 and 90% respectively. Figure 3.12C, illustrates the effect of plasmid sequence on nucleofection efficiency. A panel of GFP- and protein encoding plasmids (GFP, GFP-Arno, -TAPP, -PKB and -CXCR4) were nucleofected into day 6 activated T cells. This study indicated that altering the sequence of the plasmid could not significantly increase the percentage of cells transfected and that nucleofection efficiency was limited within this system. Furthermore, it is clear that nucleofection has a detrimental effect on cell viability.

It was established with the delivery of plasmids into activated T cells (figure 3.12) that cell viability may be a hurdle which needed to be overcome to facilitate a successful protocol. In order to achieve this outcome, it was essential that the protocol was fully optimised, therefore each step of the protocol was scrutinised and tested. The optimisation process that was undertaken is detailed here.

### 3.10.2 Influence of the T cell activating stimuli on survival and functionality

The process began with identifying the best T cell activator for the Nucleofecting protocol. The expression profiles of PHA and CD3/CD28 activated T cells, showed all PI3K isoforms were present over the full time course of the study. Both T cell activation methods were tested for not only cell viability but functional responses in a CXCL12 mediated chemotactic assay (figure 3.13).

Six days post activation with PHA or CD3/CD28, cells were nucleofected and left to recover for 72 hours before proceeding with functional assessment. The viability of cells after this 72 hour recovery period is very low for both PHA and CD3/CD28 activated cells (figure 3.13A left panel). This data suggests that CD3/CD28 activated T cells are more sensitive to the nucleofection process as fewer viable cells are available for functional studies. Furthermore, on some occasions when CD3/CD28 was utilized to activate the cells, there were not enough viable cells to
complete the chemotaxis study. This variability both within PHA and CD3/CD28 activated T cells would suggest that there is also a donor dependent variability within this study.

3.10.3 Effect of T cell age at time of Nucleofection

PHA activated T lymphocytes were expanded in vitro and used in studies up to 12 days following activation, after this time the cells show diminished/altered functional and biochemical responses. The manufacturer’s protocol for nucleofection of activated T cells, states the best time point for nucleofection is day 6. Within these investigations the effect of age on nucleofecting process was assessed. This included nucleofecting activated T cells with non targeting siRNA at day 9, for use in studies 72 hours later. These older T cells were not as robust as day 6 cells, and are more susceptible to cell death resulting in fewer cells to perform functional studies. For this technique, the age of the activated T cell is a critical factor for the viability of the cells.

Figure 3.13B left panel, shows that PHA activated T cells gave a strong chemotactic response to 10 nM CXCL12. Following nucleofection, the cells chemotaxed towards CXCL12 although the response was not as robust. CD3/CD28 activation of T cells showed a strong chemotactic response, although not as robust as PHA activation, and following nucleofection the functional response was impaired (figure 3.13B right panel). Taking into account both the viability and functionality data, these studies suggest that PHA activation gave the most consistent viability and robust functional response and would be used as the T cell activator of choice for these studies.

3.10.4 Effect of the Nucleofection program on functionality

Figure 3.14 shows activated T cells Nucleofected with non targeting siRNA on program T20 or T23, analysis was performed using CXCL12-mediated chemotaxis as a functional readout. These results indicated that nucleofection on either T20 or T23 shows comparable detrimental affects on the CXCL12-mediated chemotactic response.
3.10.5 Targeting siRNAs produce a decrease in corresponding mRNA levels

The design and subsequent potency of an siRNA is difficult to predict within a given system. Therefore during the optimisation of the Nucleofection protocol, it was imperative to test this system with a potent and previously characterised siRNA. This test ensured that the system is effective and results in the subsequent knockdown of a target gene. For this the Phosphodiesterase 7A (PDE7a) was chosen.

Figure 3.15 demonstrates the effect on mRNA levels following the introduction of a non-targeting siRNA, compared to PDE7A siRNA via qPCR analysis. Analysis of the mRNA levels in control samples was compared to levels in samples which have been nucleofected with a non-targeting siRNA; this allows for any alteration in the levels of the RNA due purely to the presence of a siRNA molecule to be assessed. Figure 3.15 shows TaqMan analysis compared to the housekeeping gene GAPDH. There is no significant difference between control and the non-targeting siRNA. Nucleofection on program T20 and T23 with the potent PDE7a siRNA, resulted in a decrease in the levels of PDE7a mRNA when compared to nucleofection of a non-targeting siRNA or control sample (analysis was performed 72 hours post nucleofection). Using this well characterised potent siRNA, it was shown that targeting siRNA could knock-down a specific gene within PHA activated T cells, therefore there is the potential within the system to effectively target PI3K if the siRNAs are effective.

As T23 is considered to be a harsher treatment for the cell (according to manufacturer’s protocol) and RNA knockdown from either T20 or T23 is comparable (figure 3.15) the T20 setting was used in future experiments.

3.10.6 Cellular uptake of Cy3 tagged siRNA

When performing siRNA knockdown experiments, it is essential that the optimum conditions are used in order gain maximum knockdown, while minimising off target
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effects. Cy3 is a fluorophore that can be coupled to siRNA molecules, and following nucleofection into a cell allows visualisation (via flow cytometry or microscopy) of the cellular uptake of the siRNA. To determine the optimum conditions for highest transfection efficiency, increasing concentrations of Cy3 tagged siRNA were introduced into the cells and analysed over 96 hours to ensure the siRNA could be sustained over the time course of the experiment (figure 3.16A). This study allowed for the optimum concentration of siRNA being determined (i.e. highest concentration which is delivered into the cell without resulting in a detrimental effect on the cell). This technique was further validated using flow cytometry to demonstrate the effect of CXCL12-mediated chemotaxis on cells Nucleofected with a Cy3 tagged siRNA (figure 3.16B).

This study indicates that Cy3 tagged siRNA was delivered into activated T lymphocytes in a concentration dependent manner (figure 3.16A). Control cells which have no siRNA are represented by the purple filled histogram, any cells which fall within this region are therefore negative for fluorescence, and contained no siRNA. A shift to the right indicated by all other unfilled histograms suggests an increase in fluorescence within the sample, and transfection with the Cy3 tagged siRNA. The lowest concentration of siRNA 0.1 μM (green line) has been transfected into these cells as early as 2 hours post nucleofection, a concentration dependent response is seen, with 1 (pink line), 4 (green line), and 10 (orange line) μM, each delivering increasing amounts of siRNA into the cells. Over the 96 hour time course, all samples remain positive for increased fluorescence, indicating that the siRNA is still present within the cells at this time point.

It was shown that Cy3 tagged siRNAs can be successfully introduced into activated T cells, yet it must also be ensured that the cells which chemotax contain the siRNA. Figure 3.16B shows that 72 hours post nucleofection with a Cy3 tagged siRNA, viable cells chemotax in a CXCL12 dependent fashion. Moreover, flow cytometry analysis, demonstrates that these cells display a similar profile to those cells of the starting population (cells that contain the siRNA but have not been chemotaxed). This suggests that the chemotaxing cells do contain siRNA.
Figure 3.12 Limited expression of a panel of GFP encoding plasmids in PHA activated T lymphocytes 24 hours post nucleofection. Day 6 PHA activated T cells (5 x 10⁶) cells were re-suspended in T cell specific nucleofecting solution, without nucleofection, Nucleofected without siRNA, Nucleofected with GFP-ARNO on program T20 or T23 (A). Increasing concentrations of GFP-ARNO were Nucleofected on program T20 into day 6 activated T cells, 1 µg (B, left panel), 2 µg (B, middle panel), and 5 µg (B, right panel). (C) A panel of plasmids encoding GFP, GFP-ARNO, GFP-TAPP, GFP-PKB or GFP-CXCR4 were Nucleofected on program T20. Following nucleofection samples were transferred to RPMI-1640 supplemented with 10% FCS and IL-2 and analysed for GFP expression after 24 hours via flow cytometry. Data is representative of 3 individual donors.
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Figure 3.13 The method of T lymphocyte activation and age of the cell at nucleofection can influence viability and functionality. A (left panel) Viability of T cells 3 days post nucleofection comparing PHA and CD3/CD28 activation methods. A (right panel) The viability (72 hours post nucleofection) of PHA activated T cells following nucleofection on either day 6 or day 9. In control or non-nucleofected cells the percentage of dead cells was 10.5%. B) CXCL12-mediated chemotaxis of PHA (left panel) or CD3/CD28 (right panel) T cells following nucleofection of non-targeting siRNA. Briefly, 5 x 10^6 cells were Nucleofected or not with 4 μM non-targeting siRNA, transferred to complete media and after 4 hours resuspended at 1 x 10^6 with the addition of IL-2 (20 ng/ml) at 37°C. Dead cells were removed and following 72 hours of recovery, cells were chemotaxed to 10 nM CXCL12 or assessed for viability (see materials and methods, data is from 3 combined experiments for PHA and CD3/CD28).
Figure 3.14 Delivery of siRNA into activated T lymphocytes via program T20 or T23 demonstrated detrimental effects on CXCL12-mediated chemotactic responses. PHA activated T lymphocytes (5 x 10⁶ cells) were re-suspended in T lymphocyte specific nucleofecting solution (control) or were Nucleofected with non-targeting siRNA on program T20 or T23 as described in Materials and Methods. Following nucleofection samples were transferred to RPMI-1640 and after 4 hours transferred to fresh RPMI-1640 supplemented with 10 % FCS and IL-2 (20 units/ml). Following 72 hours of recovery dead cells were removed and viable cells were chemotaxed to 10 nM CXCL12 see materials and methods. Data is expressed as number of cells (mean ± SD). The data are derived from a single experiment in triplicate that is representative of two other experiments.

Figure 3.15 Target specific knockdown of PDE 7a following nucleofection of a PDE 7a siRNA into day 6 PHA activated T lymphocytes. Activated T lymphocytes (5 x 10⁶ cells) were re-suspended in T lymphocyte specific nucleofecting solution (control) or were Nucleofected with non-targeting or PDE 7a siRNA as described in Materials and Methods. Following nucleofection samples were transferred to RPMI-1640 and after 4 hours transferred to fresh RPMI-1640 supplemented with 10 % FCS and IL-2 (20 units/ml). Total human RNA (5 x 10⁶ cells) was isolated using the Qiagen RNeasy kit, RNA quantity was determined on a 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 LabChip kit. cDNA was obtained by RT-PCR followed by quantitative PCR as described in Materials and Methods. The data are derived from one experiment in triplicate, which is representative of 3 different donors (mean ± SD).
Figure 3.16 Concentration dependent cellular uptake of Cy3 tagged siRNA by PHA activated T lymphocytes. PHA activated T lymphocytes, were analysed i) 2 hours ii) 24 hours iii) 48 hours iv) 72 hours and v) 96 hours post nucleofection. Briefly 5 x 10^6 cells were re-suspended in T lymphocyte specific nucleofecting solution and were or were not Nucleofected with 0.1 (green line), 1 (pink line), 4 (blue line) or 10 µM (orange line) Cy3 tagged siRNA on program T20 as described in Materials and Methods. Following nucleofection samples were transferred to complete media, after four hours cells were resuspended at 1 x 10^6 and cultured in complete media with IL-2 for up to 96 hours. Data is representative of 5 donors. One sample was saved for flow cytometry analysis and one sample of cells (8 x 10^4/25 µl) was placed on the upper membrane of a 96-well chemotaxis plate, above lower chambers containing 10 nM CXCL12. B), Cells Nucleofected with 10 µM Cy3 tagged siRNA were then chemotaxed across a 5 µm pore size membrane after a 3 hour incubation at 37 °C in 5 % CO2 (dark grey line) were compared to the original non-chemotaxed sample (light gray line) and a control non Nucleofected (black filled histogram) sample using flow cytometry. Data is representative of 5 individual donors.
3.11 Characterization of chemotactic responses to a panel of ligands

CXCL12 is a ligand for the CXCR4 receptor, has been found to be a highly potent chemotactic agent within leukocytes and is found not only on activated T cells but also freshly isolated T cells (Loetscher et al., 1994; Bleul et al., 1996). Following activation, T cells upregulate certain receptors on their cell surface, thus allowing them to respond to a greater number of chemotactic agents. Therefore, to create a fuller profile of PHA activated T lymphocyte chemotaxis, a panel of ligands was utilized. Here the migratory responses of activated T cells towards the chemokines CXCL10 and CXCL12 were assessed.

Figure 3.17A compares the dose response curves for CXCL12 (left panel) and CXCL10 (right panel) in control cells and cells Nucleofected with a non-targeting siRNA. Furthermore, expression profiles for CXCR4 and CXCR3 (the receptors for CXCL12 and CXCL10 respectively) before and after nucleofection are examined (B).

CXCL12 induces a concentration dependent chemotactic response, with a peak response at 10 nM. This is also the peak response seen in samples Nucleofected with non-targeting siRNA, yet, functionally the response is diminished, with overall less cells chemotaxing to any given concentration of CXCL12. CXCL10-mediated chemotaxis, gave a strong concentration dependent response with a peak at 10 nM, however, this response was strongly reduced by the effect of nucleofection with a non-targeting siRNA. Interestingly, neither the CXCR4 nor CXCR3 receptors displayed altered expression following nucleofection. It is worth while noting that chemotaxis towards CXCL10 was donor dependent. The data shown in figure 3.17 right panels, is representative of 3 donors, however 6 further donors were studied yet control samples showed no CXCL10-dependent migration.

3.12 Limitations in the use of Nucleofection in activated T cells

Optimisation of the nucleofection procedure for activated T lymphocytes was thorough. However, it was clear that the nucleofection process had detrimental effects on the CXCL12-mediated chemotactic response.
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Figure 3.17C analyses this further by addressing the effect of mock nucleofection, (activated T cells Nucleofected without any siRNA) compared to control cells (left panel) and cells Nucleofected with non-targeting siRNA compared to control (right panel). Following mock nucleofection, the number of viable cells chemotaxing to 10 nM CXCL12 is reduced from 19,000 to 7,000. Following nucleofection with non-targeting siRNA, chemotaxis drops from 17,000 in control to 8,000 in Nucleofected samples. These data suggest that the act of nucleofection with or without siRNA has a detrimental effect on the chemotactic functional readout.
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Figure 3.17 Effect of nucleofection on CXCL12- and CXCL10- mediated chemotactic responses in PHA activated T lymphocytes. CXCL12- (A, left panel) and CXCL10- (A, right panel) mediated chemotactic response of control and Nucleofected PHA activated T lymphocytes, compared to CXCR4 and CXCR3 receptor expression (B). C) CXCL12-mediated chemotaxis of PHA activated T cells following Mock nucleofection (left panel) or nucleofection with a non-targeting siRNA (right panel). Briefly, 5 x 10⁶ cells were Nucleofected or not with 4 µM non-targeting siRNA, transferred to complete media and after 4 hours resuspended at 1 x 10⁶ with the addition of IL-2 (20 ng/ml) at 37 °C. Following 72 hours of recovery dead cells were removed and viable cells were chemotaxed to 10 nM CXCL12. (see figs for details). Chemotactic data is expressed as number of cells. Data is expressed as mean ± SD. The data are derived from a single experiment in triplicate that is representative of two other experiments. A sample of these viable control and Nucleofected T cells were assessed for CXCR4 (B, left and middle panel) and CXCR3 (B, right panel) expression levels (see materials and methods).
3.13 Adenoviral delivery of siRNA into activated T cells is ineffective

With Nucleofection an ineffective tool for transfection of activated T cells, attention turned to a novel adenoviral system for delivery of short hairpin RNA. This technology was unavailable at the beginning of the study, however, had been used successfully within the lab for transfection of primary human fibroblasts and was marketed as a tool for transfection of activated primary T cells.

Before purchasing shRNA against PI3K, the system was tested utilizing a shRNA against PLC γ which had been previously validated with primary human fibroblasts. The protocol used for these studies was defined by the manufacturer as the optimal conditions for activated T cell transfection and if successful would be utilized with PI3K shRNA.

The hallmark receptor utilised by adenovirus to infect a cell is the CAR receptor (Bergelson et al., 1997), (coxsackie/adenovirus receptor), yet T cells do not express this receptor and therefore are not susceptible to infection by the traditional adenovirus. However, certain adenovirals utilize CD46 (MCP, Membrane Cofactor Protein (Nakanishi et al., 1994)) which is expressed on T cells. With this knowledge, a chimeric virus was created that utilised the CD46 receptor and increased its expression on the cell thus allowing for increased rates of transfection. It is these advances in adenoviral delivery that suggested that T cells could in fact benefit from this.

Delivery of the EGFP expressing virus showed that expression of EGFP peaked at day 7 at 40 % (figure 3.18A). Yet following delivery of 3 different siRNA sequences against PLC γ, this did not correlate with a knockdown of mRNA levels of the targeted isoform, when compared to control and mock nucleofections (figure 3.18b). These results suggest that the adenoviral delivery system is ineffective in the transfection of activated T cells.
Figure 3.18 Adenoviral delivery of target specific siRNA into day 7 PHA activated T lymphocytes is ineffective. EGFP expression in PHA activated T lymphocytes with an empty adenoviral vector (A, left panel) or an eGFP encoding adenoviral (A, right panel). Expression of PLC γ in PHA activated T lymphocytes (B). PBMCs were isolated from whole blood and activated with PHA. Twenty-four hours post activation, T lymphocytes were infected with an adenovirus encoding for the eGFP shRNA an empty adenovirus encoding for eGFP alone, CD46 alone, or co-infected with PLC Gamma sequence V1, V2 or V3 with CD46 shRNA (multiplicity of infection 1:5000). Total RNA was isolated using the Quigen RNeasy kit, RNA quantity was determined on a 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 LabChip kit. CDNA was obtained by RT-PCR followed by quantitative PCR as described in Materials and Methods. The data are derived from one experiment in triplicate.
3.14 Optimising Nucleofection of freshly isolated T cells

Following the thorough optimisation of the nucleofection procedure in activated T cells, it was clear that this model could not fulfil the requirements necessary to assess the role of PI3K isoforms.

Although the number of varied receptors on the cell surface is lower on freshly isolated T cells compared to activated cells, CXCL12 is expressed on both populations and provides an insight into the mechanism involved within the migratory processes of the human T cell.

3.14.1 Nucleofection affects Viability but not functionality of freshly isolated T cells

The ultimate problems faced when utilizing the activated T cell model, were issues of viability and subsequent functionality, it was therefore essential to determine the effects that nucleofection would have on freshly isolated T cells. Figures 3.19 & 3.20 address these issues. Although utilizing an alternate cell model, the nucleofection protocols were very similar, differing only in the program from which the cells were Nucleofected on. Figure 3.19A highlights the effect of nucleofection on viability of freshly isolated cells compared to activated cells 72 hours post nucleofection. Freshly isolated cells are more robust within the nucleofection procedure, averaging 35 % viability compared with 9 %. Considering the number of cells that can be isolated from each donor, this is a feasible viability to continue with and functionality was assessed. Although, 72 hours recovery time was recommended before functional studies commence, a time course highlighting the functionality of the cells was established. Twenty-four hours post nucleofection (left panel) demonstrates that both basal and CXCL12 mediated chemotaxis is decreased compared to control samples. This is the case for 48 hours post nucleofection (middle panel). However, resting human T cells for 72 hours post-nucleofection allows for the recovery of a strong CXCL12-mediated chemotactic response that is comparable to the control sample (right panel). Figure 3.19B supports the finding that recovery time at least for freshly isolated cells is an essential component to restore functionality.
3.14.2 Transfection efficiencies in freshly isolated T cells.

To determine the optimum conditions for the highest transfection efficiency, increasing concentrations of Cy3 tagged siRNA, was introduced into the cells and analysed over 72 hours (figure 3.20A). Furthermore, fluorescence microscopy was employed to visualise T cells 72 hours post-nucleofection (figure 3.20B). This technique was validated using flow cytometry, to demonstrate the effect of CXCL12-mediated chemotaxis on cells Nucleofected with a Cy3 tagged siRNA (figure 3.20C).

Cy3 tagged siRNA was delivered into freshly isolated T lymphocytes in a concentration dependent manner (figure 3.20). Control cells without Cy3 tagged siRNA are represented by the filled purple histogram, any cells which fall within this region are therefore negative for fluorescence, and contained no siRNA. A shift to the right indicated by all other unfilled histograms suggests an increase in fluorescence within the sample, and transfection with the Cy3 tagged siRNA. The lowest concentration of siRNA 0.1 μM (green line) showed no transfection of the siRNA at any time point, increasing the concentration of the siRNA resulted in a concentration dependent increase in the amount of siRNA incorporated into the cells, 1 (pink line), 4 (green line), and 10 (orange line) μM. Over the 72 hours 1, 4 and 10 μM all show expression of the Cy3 tagged siRNA.

At 72 hours, viable cells were analysed by confocal microscopy (figure 3.20B). Control cells show no fluorescence (upper panel); while cells transfected with Cy3 tagged siRNA demonstrate that the siRNA has been incorporated into the cell and is maintained within the cytoplasm but not the nucleus of the cell 72 hours post-nucleofection (as assessed by DAPI staining).

A CXCL12-mediated chemotaxis assay followed by flow cytometric analysis (figure 3.20C) shows that the fluorescence profile of these cells is similar to that of cells that just underwent flow cytometric analysis. This expression profile suggests that cells that have undergone nucleofection and incorporated the siRNA, do respond to a chemotactic stimulus, and thus migrate in a CXCL12-dependent manner.
3.15 PI3K targeting siRNA had no effect on CXCL12 induced chemotactic responses.

Thus far, data has been collected and the protocol optimised to ensure that there is confidence in the results obtained in the functional studies. Tests in this freshly isolated T cell model had a favourable outcome, suggesting this may be a successful protocol. Other studies using murine T cell models have demonstrated efficiency. Following this observation, freshly isolated T cells were Nucleofected with class II PI3K isoform specific siRNA, as a comparison, both class I δ and class I γ were tested. The two most potent siRNA (as determined within the HeLa cell line) were selected. However, as HeLas contain no class I γ, all 4 of the siRNAs were tested within the freshly isolated T cell model. 72 hours post nucleofection the number of cells chemotaxing towards CXCL12 was determined. Furthermore, samples of each treatment were analysed by qPCR for the levels of the targeted PI3K isoform, compared to non-targeting siRNA and control levels.

In some donors, delivery of non-targeting siRNA resulted in a decreased chemotactic response compared to control samples (figure 3.21 C, D left panels). However, this effect was not significant. Furthermore, delivery of PI3K target specific siRNA against class I delta, gamma, class II alpha and beta did not produce any significant effects on basal or CXCL12-induced chemotactic responses compared to the responses of cells nucleofected with non-targeting siRNA (A-D left panels).

Knockdown of the PI3K isoform mRNA varied considerably depending on the sequence of the siRNA. Limited knockdown was also seen within some isoforms following delivery of non-targeting siRNA (figure 3.21B, C & D right panels). Class I delta, sequence 3 produced limited knockdown (25 %) while sequence 4 was capable of producing 60 % knockdown (figure 3.21A right panel). However, all 4 siRNAs tested against class I gamma produced knockdown of 50- 60 % (figure 3.21B right panel). Both class II alpha and beta siRNAs produced knockdown of 40-45 % (figure 3.21 C & D right panels respectively).
3.16 Class II β siRNA produced limited knockdown at the protein level

Quantifying the amount of RNA knockdown of a particular isoforms is a good indicator of siRNA effectiveness. However, experience within our lab has highlighted that different quantities of silencing at the RNA level can produce different impacts on protein and functionality dependent on the gene being targeted. It is therefore essential, that the effect or lack of effect seen at the functional level is only assessed within the scope of the subsequently determined protein level. The silencing at the mRNA level was between 50-60 %, however, a limitation when working with human T cells is the high level of cell death over the 3 days of culture (35 % cell viability after nucleofection). Consequently, there were difficulties in recovering enough protein from viable Nucleofected samples, to allow detection by immunoblot. Although attempted many times, only 1 immunoblot was successful (figure 3.22); therefore flow cytometric analysis was also utilized to assess the quantity of protein following knockdown. Figure 3.22A shows 2 samples of cells from a class II β knockdown compared with isotype, control and unstained samples over a 72 hour time course. Although good knockdown at mRNA level is seen for at least 1 siRNA sequence per isoform, the protein data suggests that this was not translated into an effect on the protein levels, as over this time course no knockdown of the class II β isoform was seen. This is supported by western blotting analysis which was performed at 72 hours (figure 3.22B).
Figure 3.19 Nucleofection affects Viability but not functionality of freshly isolated T cells

Freshly isolated T lymphocytes (5 x 10^6 cells) were re-suspended in T lymphocyte specific nucleofecting solution (control) or were Nucleofected with 4 µM non-targeting siRNA as described in Materials and Methods. Following nucleofection samples were transferred to RPMI-1640 and after 4 hours transferred to fresh RPMI-1640 supplemented with 10 % FCS and IL-2 (20 units/ml). A) Viability of freshly isolated cells 72 hours post nucleofection compared to activated T cells. B) Following 24 (left panel), 48 (middle pane) or 72 (right panel) hours of recovery dead cells were removed and viable cells were chemotaxed to 10 nM CXCL12 see materials and methods. Data is expressed as number of cells moving across the filter (mean ± SD). The data are derived from a single experiment in triplicate that is representative of two other donors.
Figure 3.20 Concentration dependent cellular uptake of Cy3 tagged siRNA by freshly isolated T lymphocytes. Pan T lymphocytes were analysed 2 hours (A, top left panel), 24 hours (A, top middle panel) and 72 hours (A, bottom panel) post nucleofection. Briefly 5 x 10^6 cells were re-suspended in T lymphocyte specific nucleofecting solution and were or were not Nucleofected with 0.1 (green line), 1 (pink line), 4 (blue line) or 10 µM (orange line) Cy3 tagged siRNA was delivered into the cell on the nucleofection program V24 as described in Materials and Methods. Following nucleofection samples were transferred to complete media after four hours, cells were resuspended at 1 x 10^6 and cultured in complete media with IL-2 for up to 96 hours. Data was analysed by flow cytometry. B) A sample Nucleofected with 10 µM Cy3 tagged siRNA was analysed by confocal microscopy, with and without DAPI staining. Data is representative of 5 donors. C), Cells Nucleofected with 10 µM Cy3 tagged siRNA were then chemotaxed across a 5 µm pore size membrane after a 3 hour incubation at 37 °C in 5 % CO_2 (dark grey line) and a control non Nucleofected (black filled histogram) sample using flow cytometry. Data is representative of 5 individual donors.
Figure 3.21 legend continues on next page.
Figure 3.21 Class I delta, gamma, class II alpha & beta have no effect on CXCL12-mediated chemotaxis in day 3 T lymphocytes. Freshly isolated T lymphocytes (5 x 10^6 cells) were re-suspended in T lymphocyte specific nucleofecting solution and were left untreated or were Nucleofected with class I delta (A), gamma (B), class II alpha (C), beta (D) siRNAs as described in Materials and Methods. Following nucleofection samples were transferred to RPMI-1640 and after 4 hours transferred to fresh media supplemented with 10 % FCS and IL-2 (20 units/ml). Following 72 hours of recovery, dead cells were removed and viable cells placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 μm pore size membrane was determined after 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. Data is presented as number of cells migrated. mRNA levels are expressed as mean ± SD. Data were analyzed by ANOVA. Total human RNA (5 x 10^6 cells) was isolated using the Qiagen RNasy kit, RNA quantity was determined on a 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 LabChip kit. cDNA was obtained by RT-PCR followed by quantitative PCR as described in Materials and Methods. (class I delta (A, right panel), gamma (B, right panel), class II alpha (C, right panel), beta (D, right panel). The data are derived from one experiment in triplicate, which is representative of 3 different donors.
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Figure 3.22 PI3K Class II Beta Protein is present following target specific knockdown at mRNA level. Freshly isolated T lymphocytes (5 x 10^6 cells) were re-suspended in T lymphocyte specific nucleofecting solution and were left untreated or were Nucleofected with class II beta siRNA. Following nucleofection samples were transferred to RPMI-1640 and after 4 hours transferred to fresh media supplemented with 10 % FCS and IL-2 (20 units/ml). (A) Protein levels of PI3K class II Beta were analysed over a time course of 24 (A, left panel), 72 (A, right panel) hours post nucleofection. At each time point samples were fixed, lysed and incubated with the Class II beta antibody and analysed by flow cytometry. The expression profile of the targeted class II beta isoform (open pink histogram) was compared to control II Beta expression (open orange histogram), the respective isotype control (open green histogram) and unstained cells (closed purple histogram). Western blot analysis was performed at 72 hours (B). Briefly, protein lysates from 3 x 10^6 cells were resolved on an SDS gel. Membranes were immunoblotted with an antibody against II beta. The position of II Beta is indicated. The membrane was stripped and reprobed with an anti-AKT antibody (lower panel) to demonstrate loading (NT, Non-targeting). See appendix for probing conditions.
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3.17 Alternative functional readout

The limitations of looking at the contribution of PI3K isoforms in the migratory responses of T cells is 2 fold. First, due to the culture time necessary following nucleofection, the role of at least class I isoforms cannot be elucidated, due to the switch in sensitivity towards PI3K inhibition; second, although a 50-60% inhibition is observed at the mRNA level, this does not appear to translate into a silencing of the class IIβ targeted isoform. However, an alternative functional readout may require less silencing at the mRNA level, to demonstrate an alteration in functional response. In the search for an alternative readout of functionality, our interest turned to a highly sensitive and high throughput technique which had been recently developed. Like the ELISA (enzyme-linked immunosorbent assay) it is employed to detect the presence of substances within a given sample. This technique was utilized to detect levels of Th1/Th2 cytokines expression with the human T cell samples as an alternative functional readout.

The first investigation (figure 3.23A-I) studied the effects of nucleofecting a non-targeting siRNA into both unstimulated and CD3/CD28 stimulated cells and the effect this had on the production of 9 different cytokines. Unstimulated cells demonstrated low levels of cytokine production, which increased following 24 hours CD3/CD28 stimulation. The level of expression varied depending upon the cytokine under investigation. Following nucleofection with a non-targeting siRNA, the expression of each cytokine decreased, in some unstimulated cells, expression was undetectable (e.g. IL-5). Cells which had been Nucleofected and then stimulated with CD3/CD28, also showed reductions in expression, the extent of which depended on the cytokine of interest.

A further experiment was conducted to investigate the effects of specific PI3K isoforms on CD3/CD28 T cell cytokine production (figure 3.24A-I). Cytokine production following nucleofection of a PI3K targeting siRNA, were compared to those Nucleofected with a non-targeting siRNA. As a control, levels of cytokine production following 24 hours of CD3/CD28 stimulation alone were assessed. Utilizing a non-targeting siRNA induced a detrimental effect on all of the assessed cytokines. Comparing the effect of a non-targeting siRNA or a PI3K targeting siRNA, no effect was seen on IL-1β, IL-4, IL-5, IL-12 and IL-13 cytokine production.
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There was an observed increase in IL-8 production following nucleofection with all PI3K isoforms and this was opposite to the decreasing effects of the non-targeting siRNA. An interesting effect may be seen following targeting of class I delta on the production of IFN-γ and TNF. However, it is difficult to determine and state categorically due to the detrimental effect following introduction of a siRNA molecule.
Figure 3.23 Nucleofection of non-targeting siRNA has a detrimental effect on cytokine production. Supernatant from control or Nucleofected day 3 T lymphocytes stimulated for 24 hours with CD3/CD28 analysed for expression of A) IFN-G, B) IL-1β, C) IL-4, D) IL-5, E) IL-8, F) IL-10, G) IL-12, H) IL-13, I) TNFα. Human cytokine assays were performed in a 96 well format, the plate was blocked for 1 hour in 0.1 % (w/v) blocker B solution, washed 3X with PBS plus 0.05 % Tween. Undiluted supernatants or calibrator solutions were incubated for 2 hours with shaking at room temperature. Antibody detection solution (1.0 µg/ml) was dispensed into each well, the plate sealed and incubated at room temperature for 2 hours with shaking. The plate was washed 3X with PBS plus 0.05 % Tween and 150 µl of 2X read buffer was added to each well of the plate, and analysed immediately on the SECTOR™ Imager.
Assigning PI3K isoform specific involvement in T cell cytokine production is inconclusive following delivery of targeting siRNA into human T cells. Supernatant from control or Nucleofected with PI3K la, β, δ, class II α or β day 3 T lymphocytes stimulated for 24 hours with CD3/CD28 analysed for expression of A) IFN-γ, B) IL-1β, C) IL-4, D) IL-5, E) IL-8, F) IL-10, G) IL-12, H) IL-13, I) TNFα. Human cytokine assays were performed in a 96 well format, the plate was blocked for 1 hour in 0.1 % (w/v) blocker B solution, washed 3X with PBS plus 0.05 % Tween. Undiluted supernatants or calibrator solutions were incubated for 2 hours with shaking at room temperature. Antibody detection solution (1.0 μg/ml) was dispensed into each well, the plate sealed and incubated at room temperature for 2 hours with shaking. The plate was washed 3X with PBS plus 0.05 % Tween and 150 μl of 2X read buffer was added to each well of the plate, and analysed immediately on the SECTOR™ Imager.
3.18 A limited role for PI3K in CXCL12-mediated chemotactic responses of activated human T lymphocytes

T cell activators in vitro can induce activation through a variety of different mechanisms; lectins, by crosslinking of glycoproteins, superantigens by specifically binding and stimulating the TCR independently of antigen, and importantly CD3/CD28 antibody stimulation. Each of these methods provides various levels of stimulation, subsequent T cell ratios, and growth environment. It is therefore essential to address if these different in vitro activation methods result in different biochemically or functionally relevant responses. In order to address this, a panel of T cell activators were studied.

Following activation of T lymphocytes by PHA (Figure 3.25 & 3.26), SEB (Figure 3.27) or CD3/CD28 (figure 3.28), the responses of T lymphocytes were assessed using biochemical readouts and chemotactic assays. The biochemical readout chosen was the effect on the phosphorylation of PKB. PKB is a key serine/threonine kinase that is recruited to the plasma membrane via its PH domain, at which time it is phosphorylated and activated, propagating the downstream signalling cascade. It has been shown to be activated following chemokine stimulation and as the phosphorylation of PKB is dependent on active PI3K it can be utilized as an indirect method to measure PI3K activation. The functional readout within this study was the measurement of the migratory response to the chemokine CXCL12. CXCL12 is a potent inducer of chemotaxis within activated T cells and it is from this response that the effect of PI3K inhibition was measured; in addition the effect on basal migration was also assessed.

3.18.1 PHA activation

Figure 3.25A & B left panel, shows that following preincubation with the PI3K inhibitors LY294002 and wortmannin, PHA activated T cells demonstrate no significant change in the CXCL12-mediated chemotactic response. In contrast, a significant decrease in the basal migration is seen. This result correlates to a percentage inhibition of 55-60 % (figure 3.25A & B right panel). Interestingly, CXCL12-induced PKB phosphorylation was robustly inhibited by both inhibitors in a
concentration dependent manner (figure 3.25C), thus verifying that the CXCL12/CXCR4 axis was biochemically coupled and LY294002 was active.

To ensure that PI3K sensitivity of one T cell subtype was not being masked by the insensitivity of another, PHA activated T lymphocytes were further separated into CD4* and CD8* subtypes (figure 3.26). The CXCL12-mediated chemotactic responses of both CD4* and CD8* are comparable (figure 3.26A), inducing a peak chemotactic response at 10 nM. However, as seen with CD3* T cells, both subsets show no significant decrease in chemotactic response following preincubation with LY294002 (figure 3.26C). CXCL12 optimum chemotactic response could not be inhibited with 30 pM LY294002, yet phosphorylation of PKB was inhibited in a concentration dependent manner (figure 3.26B).

### 3.18.2 SEB activation

Following pre-treatment with either LY294002 or wortmannin, SEB activated T cells do not show a significant decrease in the CXCL12-mediated chemotactic response, compared to control (figure 3.27A & B). Furthermore, both inhibitors produce significant inhibition of basal migration (up to 60 %). This supports the data seen for PHA activation. Phosphorylation of PKB was inhibited in a concentration dependent manner, verifying that the CXCL12/CXCR4 axis was biochemically coupled.

### 3.18.3 CD3/CD28 activation

CD3/CD28 in vitro activation is the most physiologically relevant, and thus it was important to compare both PHA and SEB activation to this method. Preincubation with either PI3K inhibitor, showed no significant decrease in CXCL12-mediated response, interestingly the effect on basal was not as pronounced when compared to either PHA or SEB activation. LY294002 induced a 45% inhibition while wortmannin only induced a maximum of 25 % which was not statistically significant (figure 3.27A & B right panels). Phosphorylation of PKB was inhibited in a concentration dependent manner.
Figure 3.25 CXCL12-mediated PHA activated T lymphocyte chemotaxis is insensitive to PI3K inhibitors. PHA activated T lymphocytes are resistant to increasing concentrations of PI3K inhibitors. Cells were pre-incubated with vehicle (open histograms, (A,B) or in the presence of 3, 10, or 30 μM LY294002 for 30 mins (A, right panel), or 30, 100, or 300 nM Wortmannin for 30 mins (B, right panel), (closed histograms). Cells (8 x 10⁴ cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. The percentage inhibition by Ly294002 or Wortmannin of basal (black lines) and CXCL12-stimulated cell migration (black lines) is depicted in the right panels. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. Data is expressed as mean ± SD. Data were analyzed by ANOVA, and Dunnett's correction where required (.*, p < 0.05). (Data from 3 donors were further analysed using ANOVA with repeated measures followed by Dunnetts correction. Significance was observed at each concentration of inhibitor, P < 0.05). (C) Aliquots of T cells (2 x 10⁶ cells/ml) were left untreated or stimulated in parallel at 37°C with 10 nM CXCL12 for 5 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser⁴⁷³-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (lower panels).
Figure 3.26 CXCL12-mediated chemotaxis of CD4 and CD8 PHA activated T lymphocytes are insensitive to LY294002 inhibition. (A) Effect of increasing concentrations of CXCL12 on the chemotactic responses of CD4+ and CD8+ PHA activated T lymphocytes, and in the presence of LY294002 (B). Cells were pre-incubated with either vehicle (control, open histograms) or LY294002 (3-30 µM, closed histograms) for 30 mins. Cells (8x10^4 cells / 25 µl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 µm pore size membrane was determined after a 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. The percentage inhibition by LY294002 for basal (grey lines) and stimulated (black lines) is depicted in the right panels. The data is taken from a single experiment in quadruplicate and is representative of 3 individual donors. Data is expressed as mean ± SD. Data were analyzed by ANOVA with Dunnett's correction where required (* P < 0.05). (Data from 3 donors were further analysed using ANOVA with repeated measures followed by Dunnett's correction. Significance was observed at each concentration of inhibitor, P < 0.05). (C) Aliquots of T cells (2 x 10^6 cells/ml) were left untreated or stimulated in parallel at 37°C with 10 nM CXCL12 for 5 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser^473-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (lower panels).
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Figure 3.27 CXCL12-mediated chemotaxis of SEB activated T lymphocyte is insensitive to PI3K inhibitors. SEB activated T lymphocytes are resistant to increasing concentrations of PI3K inhibitors. Cells were pre-incubated with vehicle (open histograms, (A,B) or in the presence of 3, 10, or 30 μM LY294002 for 30 mins (A, right panel), or 30, 100, or 300 nM Wortmannin for 30 mins (B, right panel), (closed histograms). Cells (8 x 10⁴ cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. The percentage inhibition by Ly294002 or Wortmannin of basal (grey lines) and CXCL12-stimulated cell migration (black lines) is depicted in the right panels. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. Data is expressed as mean ± SD. Data were analyzed by ANOVA, and Dunnett’s correction where required (*, p < 0.05). The data above is representative of further analysis using ANOVA, repeated measures and Dunnett’s correction. (C) Aliquots of T cells (2 x 10⁶ cells/ml) were left untreated or stimulated in parallel at 37°C with 10 nM CXCL12 for 5 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser473-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (lower panels).
Figure 3.28 CXCL12-mediated CD3/CD28 activated T lymphocyte chemotaxis is insensitive to PI3K inhibitors. CD3/CD28 activated T lymphocytes are resistant to increasing concentrations of PI3K inhibitors. Cells were pre-incubated with vehicle (open histograms, (A,B) or in the presence of 3, 10, or 30 μM LY294002 for 30 mins (A, right panel), or 30, 100, or 300 nM Wortmannin for 30 mins (B, right panel), (closed histograms). Cells (8 x 10^4 cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing 10 nM CXCL12. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. The percentage inhibition by Ly294002 or Wortmannin of basal (grey lines) and CXCL12-stimulated cell migration (black lines) is depicted in the right panels. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. Data is expressed as mean ± SD. Data were analyzed by ANOVA, repeated measures and Dunnetts correction to compare responses in the presence and absence of PI3K inhibitors within CXCL12 stimulated groups (p < 0.05). (C) Aliquots of T cells (2 x 10^6 cells/ml) were left untreated or stimulated in parallel at 37°C with 10 nM CXCL12 for 5 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser473-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer (lower panels).
3.19 CXCR3-mediated chemotaxis is PI3K independent

Upon T cell activation cells proliferate, release cytokines and chemokines and upregulate the receptor repertoire on the surface of the cell. This upregulation allows migration towards a greater number of chemokines. While CXCR4 is expressed both on freshly isolated and on activated cell types, CXCR3 is a receptor that is upregulated following activation. It has 3 known ligands, CXCL9, CXCL10 and CXCL11, all of which have been shown to induce strong chemotactic responses in activated T cells.

In order to assess if the observation of PI3K independent chemotaxis extended to other receptor/ligand interactions, this study investigated if the CXCR3 ligands have a PI3K dependent component in their chemotactic response. Figure 3.29 illustrates the chemotactic responses of PHA activated T lymphocytes to increasing concentrations of the CXCR3 ligands, CXCL9, CXCL10 and CXCL11. Furthermore they demonstrate the effect of increasing concentrations of LY294002 and wortmannin on both the basal and peak chemotactic response for each ligand.

Chemotactic responses towards CXCL9, CXCL10 and CXCL11 demonstrate a concentration dependent response, with peak responses at 30, 1, and 10 nM (figure 3.29A, B, and C left panels respectively). All 3 ligands elicit a strong concentration dependent chemotactic response with a potency of CXCL10>CXCL11>CXCL9. The peak response for each ligand was tested in the presence of increasing concentrations of LY294002 and wortmannin (figure 3.27 middle and right panels). Each response demonstrated limited sensitivity, with 30 μM LY294002 and 300 nM wortmannin, unable to significantly reduce the chemotactic response. As shown previously (figure 3.25) both LY294002 and wortmannin showed a significant decrease in the basal migration. Figure 3.29D demonstrates the percentage inhibition of LY294002 and wortmannin (right and left panels respectively) on basal migration and each of the chemotactic responses elicited by CXCL9, CXCL10 and CXCL11.

The previous experiments using CXCL9, CXCL10, CXCL11 and CXCL12, have focused on the effect on LY294002 and wortmannin inhibition on the peak chemotactic responses of each ligand. However, it was important to ensure that
chemotactic responses are PI3K independent over a concentration range. Figure 3.30 demonstrates a concentration dependent response to each ligand and the effect of 10 μM LY294002 on this response. No significant effect on chemotactic response is observed for any ligand at any concentration. These data further extend the observation that PHA activated T lymphocytes can, under certain conditions, chemotax in a PI3K independent manner.
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Figure 3.29 CXCR3-mediated chemotaxis of PHA activated T lymphocytes is class I PI3K independent. PHA activated T lymphocytes show concentration dependent chemotaxis towards CXCL9, CXCL10 and CXCL11 (a, b and c left panel respectively). CXCR3-mediated chemotaxis of PHA activated T lymphocytes are resistant to increasing concentrations of PI3K inhibitors (a, b, and c, middle and right panel. D, represents the effects of these inhibitors as a percentage inhibition). Cells were or were not pre-incubated in the presence of 3, 10, or 30 μM LY294002 or 30, 100, or 300 nM Wortmannin for 30 mins. Cells (8x10^4 cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing varying concentrations of CXCL9, CXCL10 or CXCL11 as noted in the figure. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5 % CO₂ as described in Materials and Methods. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. Data is expressed as mean ± SD.
Figure 3.30 CXCL9, CXCL10, CXCL11 and CXCL12-mediated chemotaxis of PHA activated T lymphocyte is insensitive to PI3K inhibition. PHA activated T lymphocytes are resistant to 10 μM LY294002 inhibition over a range of chemokine concentrations. Cells were pre-incubated with vehicle (black lines) or in the presence of 10 μM LY294002 for 30 mins (red lines). Cells (8 x 10⁴ cells / 25 μl) were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing different concentrations of chemokines as noted above. Chemotaxis across a 5 μm pore size membrane was determined after a 3 hour incubation at 37 °C in 5% CO₂ as described in Materials and Methods. Data is expressed as mean ± SD. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. Data were analyzed by ANOVA and repeated measures, no concentrations were significant.
3.20 Ex-vivo culture of T lymphocytes determines CXCR4-mediated chemotactic sensitivity to PI3K inhibitors.

The studies presented in section II (figures 3.3 – 3.6) have demonstrated that freshly isolated T cells migrate in a PI3K dependent manner and the use of isoform selective inhibitors suggests a dominant role for PI3Kγ. Interestingly, it has been observed that activated T cells can migrate in a PI3K independent manner. In order to investigate this further, it was essential to determine if this switch in dependence was determined through activation of the T cell or through culture. Surprisingly, 24 hours of in vitro culture without activation (figure 3.31A) was sufficient to render the CXCL12-mediated migratory response PI3K independent. CXCL12 induced a strong chemotactic response that could not be significantly inhibited with up to 30 μM LY294002. This PI3K independent response was maintained over the 72 hour culture period (figure 3.31B & C). Interestingly, basal migration was inhibited in a concentration dependent manner and this was evident up to 72 hours post isolation. Moreover, the biochemical response was inhibited in a concentration dependent manner, resulting in almost complete inhibition of the phosphorylation of PKB.

Utilizing increasing concentrations of a broad spectrum PI3K inhibitor (LY294002), it is shown that the dependence on PI3K within CXCL12-mediated chemotactic response is diminished following ex vivo culture.
Figure 3.31 CXCL12-mediated chemotaxis of cultured T lymphocytes (with or without IL-2) is insensitive to LY294002. PTO for figure Legend.
FIGURE 3.31 CXCL12-mediated chemotaxis of cultured T lymphocytes (with or without IL-2) is insensitive to LY294002. Freshly isolated human T cells were maintained in culture for 24 (A), 48 (B) or 72 hours without IL-2 (C) or D) 72 hours with IL-2 post-isolation. CXCL12-mediated chemotaxis of day 1 (A, left panel), day 2 (B, left panel) and day 3 (C, left panel) are resistant to increasing concentrations of LY294002. Cells were pre-incubated with either vehicle (control, open histobars) or Ly294002 (3-30 μM, solid histobars) for 30 mins. T lymphocytes were placed on the upper membrane of a 96-well chemotaxis plate above lower chambers containing CXCL12 (10 nM), as described in Materials and Methods. Cell migration was determined after 3 hour incubation at 37 °C in 5 % CO₂. The percentage inhibition by LY294002 of basal (black lines) and CXCL12-stimulated cell migration (blue lines) is depicted in the right panels. Data is expressed as mean ± SD. The data is taken from a single experiment in quadruplicates and is representative of 3 individual donors. The above data were analyzed by ANOVA & where appropriate Dunnet's correction (*, p < 0.05). (N.B. Data from 3 donors were further analysed using ANOVA with repeated measures followed by Dunnet's correction. No significance was seen in stimulated migration, in basal migration significance was seen at each concentration of inhibitor, P < 0.05). (E) Aliquots of T cells (without IL-2) (2 x 10⁶ cells/ml) were left untreated or stimulated in parallel at 37°C with 10 nM CXCL12 for 5 mins and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the active Ser⁴⁷³-phosphorylated form of PKB and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer. The data are derived from a single experiment performed in triplicate that is representative of three other experiments.
3.21 Summary
By investigating the role of \textit{in vitro} activation and culture of T cells the following observations were made:

- All PI3K isoforms except class II $\gamma$ are expressed in \textit{in vitro} activated T cells, and the method of activation determines the expression levels within these cells.
- Within HeLa cells, PI3K siRNAs demonstrate a concentration dependent knockdown of target RNA and show limited effects on the other PI3K isoforms tested.
- Nucleofection of siRNA into activated T cells occurs in a concentration dependent manner, with T cells that undergo chemotaxis having incorporated siRNA.
- Activated T cells demonstrate decreased viability and functionality following nucleofection.
- Adenoviral delivery of shRNA is incorporated into 50% of activated T cells by day 7, but no silencing of target RNA is observed.
- PI3K isoform specific mRNA is targeted and decreased by up to 60%, but this does not translate into a knockdown at the protein level. A role for class II isoforms in chemotaxis and PI3K isoforms in cytokine production cannot be ruled out.
- PHA activation - Basal migration is significantly inhibited following inhibition using the broad spectrum inhibitors LY294002 and wortmannin. However, CXCL12 stimulated chemotaxis is not dependent on PI3K for directed cell migration. This observation is extended to the CXCR3 ligands CXCL9, CXCL10 and CXCL11. CXCL12 stimulates a PI3K dependent pathway, demonstrated by the PI3K dependent phosphorylation of PKB.
- The CD4$^+$ and CD8$^+$ subsets that comprise the activated T cells population demonstrate similar trends in PI3K independence.
- The method of T cell activation influences the dependence on PI3K during basal migration.
- Culturing of CD3$^+$ T lymphocytes over a 24-72 hour period results in a loss of PI3K dependent migration, while maintaining PI3K dependent phosphorylation of PKB.
Nucleofection of T cells has a detrimental effect on Th1/Th2 cytokine production.

Within this section of work the difficulties involved within the Nucleofection of both primary and activated T cells were highlighted. The Nucleofection procedure was optimised using the activated T cell model, however, the difficulties in obtaining viable and functionally competent cells demonstrated a limiting factor within the system.

Switching to the freshly isolated T cell model demonstrated successful results, in that greater viability was seen and functional responses could be restored. However, although silencing of the target isoform was achievable this did not correlate into a decrease of protein levels (as assessed by flow cytometry and western blotting).

Interestingly, the use of broad spectrum PI3K inhibitors highlighted a switch in the dependence on PI3K within the migratory response. Freshly isolated T cells migrate in a PI3K dependant manner with PI3K γ playing a pivotal role. Yet ex vivo maintenance of these cells (for as little as 24 hours), renders the migratory process PI3K independent. Basal migration retains the dependence on PI3K although this dependence is not as significant following CD3/CD28 activation.
CHAPTER 4: DISCUSSION
Discussion
The aim of this study was to investigate the role of the PI3K family within T cell migration. To facilitate this process broad spectrum and isoform specific inhibitors were utilized as well as target specific siRNAs delivered by a novel technique termed Nucleofection. Functional and biochemical studies were performed following stimulation with a panel of chemokines.

These investigations began by characterizing the role of CXCL12 in the freshly isolated and activated T cell models. As a comparison to this response, the effect of CCL5 was reinvestigated as confusion surrounded the expression and functional response that this chemokine could evoke. Furthermore, the role of CCL5 derivatives were characterised within T cells as these may have important implications in a clinical setting.

4.1 CCL5 and CCL5 derivatives in a clinical setting
CCL5 is a native CC-chemokine which has the potential to bind 3 receptors, CCR1, CCR3 and CCR5. When a role for the CCR5 receptor was established in the pathogenesis of HIV infection, the interest around this chemokine and its receptor intensified. With increased knowledge about this interaction, the hope emerged of a prophylactic treatment for HIV transfection.

Simmons et al demonstrated that the N-terminally modified chemokine AOP-CCL5 was capable of blocking R5 tropic HIV entry in several different cell types (Simmons et al., 1997). This molecule resulted in agonist activity which was assessed via the dimerisation of the receptor, measurement of calcium flux and intracellular signalling (Rodriguez-Frade et al., 1999). As expected with an agonist it induces internalisation of the receptor, however, it internalised the receptor for increased periods of time compared to the parent CCL5 chemokine. The modification to the chemokine reduced the expression of the receptor on the surface of the cell, decreasing the possibility that the HIV virus binds the receptor.

Redundancy within the CCR5 receptor functions have been observed. Individuals who are homozygous for a 32 base pair deletion of CCR5, express no functional
receptor on the surface of the cell, and show no adverse physiological symptoms (Quillent et al., 1998). This observation highlights the possibility that a modified CCL5 chemokine, that promotes sequestration of the receptor inside the cell, may provide a prophylactic therapy, as sequestration of the receptor should have no negative effects on the patient's health. In the drive to develop potent HIV inhibitors several other ligands were developed, including PSC-CCL5. The aim of these ligands was to generate a profile in which there was limited agonist activity but increased sequestration of the receptor (Pastore et al., 2003; Sabbe et al., 2001).

4.2 Regulation of chemokine receptor expression

Many factors influence the range of chemokine receptors that are expressed on the surface of the cell. The health and age of the donor are contributory factors, as well as the method of in vitro activation and cytokine milieu that surrounds the cell, for example IL-2.

4.2.1 The importance of IL-2 in culture

The β and γ chains of the IL-2 receptor are constitutively expressed in resting blood lymphocytes (Hatakeyama et al., 1989; Takeshita et al., 1992) while the α chain which is required for formation of the high affinity receptor is expressed only after T cell activation (Waldmann, 1991). It has also been shown that the IL-2 treatment of memory cells can induce the expression of this α chain generating the functional receptor (Harel-Bellan et al., 1986; Siegel et al., 1987). The generation of this functional receptor, has been shown to facilitate the upregulation of CC chemokine receptors on the surface of the cell, thus playing a part in the responsiveness of T cells (Loetscher et al., 1996b).

4.3 CCR1 and CCR3 are undetectable in freshly isolated and activated T cells

The expression of CCR1 and CCR3 are considerably lower than that observed for CCR5, with less than 3 % of human T cells within an inflammatory setting expressing these receptors (Bruhl et al., 2001). The expression of CCR1 and
CCR3 was not detected within our investigations and it would therefore be tempting to surmise that all functional or signalling responses generated should be attributed to the CCR5 receptor. However, it cannot be ruled out that there was at least a small contribution from the CCR1 and CCR3 receptors that these investigations did not detect. These low levels of expression even within inflammatory settings may be difficult to detect and combined with the possibility that PHA may have a delaying effect on receptor expression (discussed below), may account for the limited expression profile. With such a limited expression profile it would be good practice to utilize several methods to detect these receptors, such as qPCR and immunoblotting.

4.4 CCR5 is upregulated following T cell activation

It has been shown by others that the receptors CXCR4 and CCR5 are expressed on specific subsets of freshly isolated cells, with CXCR4 being expressed on naïve cells while CCR5 is expressed on memory cells, facilitating the entry into the periphery (Bleul et al., 1997; Wu et al., 1997). However, data shown within this thesis suggests that CCR5 is not expressed on freshly isolated cells but is upregulated following activation and culture. In support of these findings Loetscher et al utilizes northern blot and mRNA analysis to detect CCR5 expression and observes no expression of CCR5 until day 5 (Loetscher et al., 1996b). The differences between our study and Bleul and Wu et al, might be explained in 2 ways; through the specific antibody used to detect this receptor and donor variation.

4.4.1 Is expression of CCR5 donor dependent?

Studying these conflicting reports it is possible that at least some of these results maybe explained through donor variation. Within freshly isolated cell populations CCR5 is only expressed on some memory cells (not naïve cells) and expression of CCR5 may therefore be dependent on the donor's naïve/memory T cell profile. As discussed earlier, due to a mutation within this receptor, a small number of individuals do not express CCR5, providing increased resistance to diseases which depend on this receptor. Furthermore, age can influence the expression of CC
chemokine receptors due to the alteration of the naïve/memory T cell profile (Jaruga et al., 2000).

4.4.2 Activation and IL-2 culture alters receptor expression and functionality

Activation and proliferation of T cells results in the upregulation of surface expressed receptors. This upregulation allows for the cells to chemotax towards a greater array of chemokines. Bleul et al specifically investigated the changes in chemokine receptor expression and correlated this with chemotactic responses. Bleul et al found that PHA activation upregulated the CXCR4 receptor, leading to an increase in CXCL12-mediated responses, while the culturing of these cells in the presence of IL-2 without PHA also had this effect. Furthermore, Bleul et al found that CCR5 was expressed on freshly isolated cells yet this expression did not increase during PHA activation and culture, but expression was increased following culture with IL-2 (Bleul et al., 1997). Data presented within this thesis suggests that following activation and culture in IL-2 expression of CCR5 is increased, however, investigations into the effect of activation or IL-2 alone were not investigated. However these studies would suggest it is an IL-2 dependent component.

4.5 CCL5 fails to induce chemotactic responses in freshly isolated T cells

Work presented within this thesis provides evidence that CCL5 cannot induce chemotactic responses with freshly isolated T cells and this inability to induce chemotaxis is maintained until 10 days post activation. Studies into receptor expression demonstrate that CCR5 is up regulated on the surface of the cell by day 6, but this is not translated into a functional or biochemical response.

This might be explained by receptor density, as it has been reported that optimal functional response through the CCR5 receptor is determined through the density of the receptor on the cell surface (Desmetz et al., 2007; Desmetz et al., 2006). Desmetz et al suggest that although receptor expression does not increase dramatically from day 6 onwards, it may increase enough to surpass the threshold
required for chemotactic responses, with an increase of functional receptor expression at the surface, leading to an increase in migratory responsiveness (Ebert and McColl, 2002).

4.6 CCL5 and PSC-CCL5 induce a chemotactic response in day 10-14 activated T cells

Within our study T cells were activated with PHA and cultured with IL-2 and by day 10-14 an increase in chemotactic response was observed. Biochemical signals were also observed through S6 and were shown to be dependent on PI3K. This observation is supported by other studies that demonstrated no chemotactic responses of freshly isolated T cells, but if cells were cultured in the presence of PHA and IL-2 chemotaxis was noted by day 9 (Ebert and McColl, 2002). Interestingly, Loetscher et al observe that culture with IL-2 alone generated a chemotactic response that was observed from day 4, demonstrating that upregulation of CCR5 was not dependent on blast formation (Loetscher et al., 1996b).

4.6.1 PSC-CCL5 induces a stronger chemotactic response than CCL5

Interestingly we observed that the modified chemokine PSC-CCL5 resulted in a significant chemotactic response in vitro, whereas the CCL5 response was not as robust. This may be due to the modified derivative having an increased affinity for the receptor, facilitating a stronger chemotactic response at lower concentrations. In day 10 and 14 activated T cells, CCL5 induced a small chemotactic response that slowly increased as the concentration of CCL5 increased. The profile of this response is still altered compared to PSC-CCL5, as PSC-CCL5, induced a definitive peak response, whereas CCL5 is more conservative.

It has previously been suggested that phosphorylation of the receptor is enhanced following stimulation with CCL5 derivatives (Vila-Coro et al., 1999). This phosphorylation results in rapid internalisation of the receptor that may explain the strong chemotactic response, as internalisation is a requirement for chemotaxis. However, as the exact mechanism surrounding the internalisation and
sequestration of the receptor has not been elucidated, this cannot be confirmed. Furthermore, although not tested in this investigation, as receptors are sequestered for increasing amount of time following PSC-CCL5 stimulation, it could be presumed that any re-stimulation with any of these ligands would not produce a chemotactic or signalling response (Lederman et al., 2006).

4.7 CCL5 and its derivatives induce similar signalling profiles

It has been suggested that downstream signalling events from CCL5 and its derivatives may differ, this was assessed in these investigations through the phosphorylation of S6 (Vila-Coro et al., 1999). Data presented within this study suggest that in freshly isolated T cells CCL5 and its derivatives induce no phosphorylation of S6, this would be consistent with the findings that no receptor expression, or chemotaxis is observed. By day 6, expression of CCR5 is observed, yet, no biochemical signalling through these receptors was observed. As discussed above, this may be indicative of low receptor density on the surface of the cell.

By day 10 -14, all CCL5 derivatives and CCL5 induced stimulation of S6 at similar levels and this signalling was dependent on PI3K. These results suggest that at least at the level of S6 phosphorylation, signalling events initiated via CCL5 and its derivatives are similar. However, it is worth while noting that only 1 pathway was assessed and it is possible that other signalling cascades are differentially regulated by these modified chemokines. As increased sequestration of the CCR5 receptor is seen with CCL5 derivatives, it is clear that at some level the derivatives affect the intracellular machinery, as to prevent the recycling of the receptor to the membrane. However, this result would suggest that signalling events are at least triggered and that the sequestration of the receptor happens independently of this signalling.

Furthermore, recycling of the CCR5 receptor following stimulation by CCL5 and PSC-CCL5 has been demonstrated to be around 20 mins and 24 hours respectively (Mack et al., 1998). These investigations focused on the stimulation of S6 which occurs 10 mins after stimulation with the chemokines, thus focusing on the acute effects of these compounds on the subsequent signalling events; the kinetics of receptor recycling were not investigated (Lederman et al., 2006).
4.8 Pharmacological investigations into the PI3K family

Following clarification of the functional and biochemical effects of CXCL12 and CCR5, attention focused on utilizing broad spectrum and isoform specific PI3K inhibitors to dissect out the role of class I isoforms in CXCL12 mediated migration and signalling.

4.8.1 The freshly isolated T cell model

The role of the PI3K family (in particular PI3Kγ) in leukocyte chemotaxis has been established primarily in neutrophils, however the contribution of the PI3K family within human T cell responses had not been thoroughly established (Ridley et al., 2003; Hirsch et al., 2000; Wang et al., 2002; Nishio et al., 2007; Hannigan et al., 2002; Ferguson et al., 2007; Ward, 2006) The expression levels within freshly isolated T cells of PI3K isoforms were first established. These observations suggested that all isoforms were expressed with the exception of class II γ, which is primarily expressed in hepatocytes (Wymann and Pirola, 1998).

4.9 Freshly isolated CD3+, CD4+ and CD8+ migrate in a PI3K dependent manner

Within freshly isolated CD3+ cells, a classical bell shaped chemotactic response towards the CXCL12 ligand is observed. Increasing concentrations of both LY294002 and wortmannin against the peak chemotactic response, highlighted a dependence on PI3K during both directed and random migration. Furthermore, investigations into CD4+ and CD8+ T cells demonstrated that both subgroups were dependent on the PI3K family for these responses.

4.9.1 A Dominant role for PI3Kγ in CXCL12-mediated migration

The observation that freshly isolated T cells migrate in a PI3K dependent manner, was exploited with the use of class I isoform selective inhibitors. The use of these inhibitors revealed a dominant role for the class I γ isoform in the signalling through S6 and migratory responses to CXCL12.
As with all pharmacological tools, in order to unequivocally attribute function to a particular isoform, it is essential to establish selectivity for these compounds. The PI3K inhibitors used within this study, exhibit selectivity between their primary target(s) and other members of the family (table 1 & (Knight et al., 2006)). At the lowest concentration used for each inhibitor, in which they display selectivity for that isoform(s), limited effects on the migratory response induced by CXCL12 were observed. Interestingly, the use of the inhibitor AS605240 indicates a dominant role for PI3Ky.

The observation that PI3Ky plays a dominant role within migration, is supported through other studies which have focused on loss-of-function mutants, although it has been determined that a dependence on other class I isoforms is required for optimal response (Curnock et al., 2003). Furthermore, studies in murine models support this observation in both in vivo and in vitro studies (Sasaki et al., 2000). The use of LY294002 highlighted a role for class I PI3K in basal migration and through the use of isoform specific inhibitors, this suggests a predominant role for PI3Ky with little interaction of the other isoforms. These observations suggest PI3Ky plays a role in both the migratory response and the directionality of migration.

4.9.2 A role for PI3Ka?

Within this study the inhibitor PI-103 was utilized to investigate the role of PI3K class I α. This inhibitor is selective for the α member of the PI3K family at concentrations below 100 nM, and at these concentrations the inhibitor has no effect on the migratory responses of freshly isolated T cells (table 1 & (Knight et al., 2006)). However, this inhibitor does not distinguish between PI3K α and mTOR, and while mTOR has been implicated in the migratory response of cells, such as neutrophils and macrophages, to date it has not been identified as a mediator of T cell chemotaxis (Gomez-Cambronero, 2003; Fox et al., 2007; Liu et al., 2006). As PI-103 has no significant effect on chemotaxis, this data would support the notion that mTOR plays little role in these migratory effects. However, pre-incubation with PI-103 did decrease the phosphorylation of S6. As mentioned earlier the phosphorylation of S6 is dependent on the involvement of both PI3K and mTOR signals (Knight et al., 2006). The inhibition of this phosphorylation may therefore be
due to the action on mTOR rather than PI3K class I α. In support of this observation, the use of the inhibitor rapamycin, which targets mTOR, shows a significant decrease in the phosphorylation of S6. The inhibition seen following pre-treatment with rapamycin provides an explanation as to the significant differences seen between S6 phosphorylation and migratory responses.

4.10 Class IA and IB PI3K dependent Chemotaxis

This study would suggest a dominant role for PI3K class Iγ within freshly isolated T cell migration. Other studies have also highlighted a role for class Iγ as well as class IA. Dependence on PI3K for migration has been established within eosinophils, neutrophils, Thp1 and T cells towards both CC and CXC chemokines CCL20, CXCL8, CCL2 and CXCL12 respectively. As seen in this study, the class Iγ isoform has emerged to be of particular importance within migration but also the production of PtdIns(3,4,5)P3, phosphorylation of downstream signalling molecules, and superoxide formation.

An invaluable tool within these investigations has been the p110γ knockout murine models which have focused particular attention on the role of p110γ within neutrophils (Hannigan et al., 2002; Hirsch et al., 2000; Sasaki et al., 2000; Thomas et al., 2005). *In vitro* studies on the p110γ knockout neutrophils, show decreases in chemotactic response of up to 70 % towards fMLP and C5a while *in vivo* models of peritonitis also support this observation (Hannigan et al., 2002; Sasaki et al., 2000). Furthermore, neutrophils from p110γ knockout mice demonstrate no PtdIns(3,4,5)P3 production and have thus suggested a primary role for class IB within the production of PtdIns(3,4,5)P3 in these knockout models (Li et al., 2000). In addition, the PH domain containing protein PKB which is a major downstream effector of PI3K, fails to be phosphorylated (Hirsch et al., 2000).

Contrary to the common belief that class IB is the sole PI3K isoform involved in chemotaxis, several studies have reported the involvement of members of the class IA family. Moreover, a recently published study suggests that it is in fact class IA that is primarily responsible for the chemotactic properties of human myeloid cell line PLB-985, (induced to express a neutrophil-like phenotype). Utilising transient
expression of a dominant negative mutant of either p85α or p110γ, stimulation with fMLP, shows a biphasic PtdIns(3,4,5)P$_3$ response. This is initially governed through class Iγ, with the latter stages and functional responses regulated through class IA (Boulven et al., 2006).

In human neutrophils, although a biphasic response is obtained, the dependence on PI3K isoforms is in fact sequential. The first phase is shown to be p110γ dependent however, the second phase although largely mediated through p110δ, (to some extent β and α), is inextricably dependent on the initial γ dependent phase (Condliffe et al., 2005). Possible explanations for this discrepancy, is the use of a cell model (PLB-985) as opposed to TNF-α primed PMNs, and second, it is possible that following transfection of the dominant negative p110γ vector, residual activation of the class Iγ may be capable of activating class IA.

Supporting the role for class I delta, is the use of a PI3K delta specific inhibitor, IC87114, which facilitates a 60 % reduction in PtdIns(3,4,5)P$_3$ production and a decrease in fMLP stimulated neutrophil migration (Sadhu et al., 2003). This observation was further corroborated using p110δ$^{-/-}$ mice (Puri et al., 2004). Several studies also suggest a role for class IA through the use of protein tyrosine kinase inhibitors, which following chemotactic stimulus resulted in a decrease in PtdIns(3,4,5)P$_3$ production in neutrophils (Ptasznik et al., 1996; Thelen and Didichenko, 1997). Although a role for class IA in neutrophils has been established it would appear at least in T cell migration that this involvement is minimal, as the use of inhibitors at selective concentrations does not show significant reduction in migratory responses. The residual migration is therefore more likely to be driven through an alternate PI3K isoform or another signalling pathway.
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4.11 Targeting of the PI3K family using siRNA strategies

Following pharmacological intervention to determine the role for individual class I isoforms, attention turned to utilizing an siRNA strategy to assess the role of class II isoforms within activated and freshly isolated T cells. This began with validation of the siRNAs and optimization of the delivery technique.

The mechanism through which GPCRs couple to class II PI3K isoforms has not been fully elucidated. However, class II PI3K isoforms have been shown to be activated by several chemokines in leukemic T cell models (Foster et al., 2003; Sotsios and Ward, 2000). In support of a role for these isoforms in migration, class IIβ and its lipid product PtdIns(3,4)P2 have been shown to play important roles in regulating lysophosphatidic acid-induced cell adhesion, actin reorganization and migration (Domin et al., 2005; Maffucci et al., 2005). In this research project, siRNA was utilized to clarify the role, if any, of the class II PI3K isoforms in the migratory response of freshly isolated and in vitro activated T cells.

4.11.1 PI3K isoforms are expressed in activated human T cells

PI3K isoforms are expressed within immune cells (Koyasu, 2003). It has been previously established that the class II γ isoform is not expressed within immune cells but is predominantly expressed within hepatocytes (Wymann and Pirola, 1998). Following in vitro activation of human T cells using PHA and CD3/CD28, qPCR analysis was performed on each of the samples and the absolute levels of these isoforms determined. CD3/CD28 stimulation of T cells provides the most physiologically relevant mode activation. PHA is a lectin which is commonly used to activate T cells in vitro by binding glycoproteins on the cell surface, and provides a robust activation of the cell. Our results support the finding that all PI3K isoforms (class I α,β,δ,γ, class II α, β and class III) except PI3K class II γ are expressed in in vitro activated and cultured T cells. These early experiments were crucial to determine which of the isoforms had the possibility of being targeted within the following studies.
4.12 Evidence that in vitro validation of siRNA is an essential prerequisite for RNA interference studies.

With the advent of RNA interference, came the requirement for strong siRNA design, not only for the siRNA to be target specific and effective at knocking out the gene of interest, but also that it does not affect other genes within the cell and importantly other isoforms within the same family (Pei and Tuschl, 2006). To ensure confidence in the experimental data, the choice of siRNA and the validation of the chosen sequences were thoroughly evaluated. When deciding on which siRNA to deliver, there are 2 schools of thought, the use of either pools of siRNA sequences or single siRNAs.

Pools of siRNA contain several sequences against the same gene. Pools of siRNA provide an advantage as several siRNAs are tested at one time and there is an increased likelihood that at least one of those sequences is potent against the target of interest. Furthermore, this is combined with the idea that the other siRNAs should target this gene with at least some affinity and produce an effect. However, it should be noted that randomly selecting siRNA have silencing activities as low as 62% (Ladunga, 2007). Currently designing an siRNA with no off-target effects is almost impossible and therefore by utilizing several siRNAs, each with different cross-silencing activity, the off-target effects will be diluted over a range of ‘targets’ where as just one siRNA would amplify this effect (Buchholz et al., 2006; Myers and Ferrell, 2005; Jackson et al., 2003).

The alternative to pooled siRNAs and the method chosen within this study, is to deliver one pre-validated siRNA, which shows strong potency for the target of interest. Although this approach may be more time consuming in the short term, long term this approach ensures maximum effect while maintaining limited off target effects (Ladunga, 2007). In order to allow for the off-target effects that are thought to be compounded when utilizing this single sequence method, at least 2 different siRNAs were validated and then taken forward for investigation. This would allow examination of targeted effects, by comparing the results of both siRNAs. If the effect that was observed with one siRNA was specific, the result should be confirmed with the observation from the second validated siRNA.
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The need for strong siRNA design and pre-validation was highlighted within this study when testing the siRNA against class I α PI3K. All siRNAs used within this study are commercially available, and we tested 4 different sequences against each target, and in most cases little difference was seen. Yet 2 of the siRNAs against class I α were ineffective at reducing the target mRNA, highlighting that while computer aided design of these siRNAs suggested these sequences were effective, in fact, experimental testing proved this was not the case.

4.13 Optimization of Nucleofection in activated human T cells is ineffective

Delivery of siRNA into primary cells has proved problematic, yet in 2003 Lai et al utilized a modified electroporation technique termed Nucleofection™ to introduce DNA into primary mouse T cells. Since then an increasing number of groups have used the Nucleofection procedure as an alternative to method to traditional transfection techniques, in both human and rodent T cells (Nakayamada et al., 2007; Jurgens et al., 2006; Goffinet and Keppler, 2006; Keppler et al., 2006). Other primary cells, such as mammalian neurons (Zitelhofer et al., 2007), B cells, dendritic cells and cell lines (Preiss et al., 2007; Boulven et al., 2006) have also benefited from this technique.

One of the most striking effects seen in our study was the detrimental effect on cell viability, with upwards of 90 % cell death (dependent on the donor), a point that is well reported within these articles (Merkerova et al., 2007). In an attempt to increase the viability of the cells, we investigated the T cell activator and age of the cell at nucleofection. PHA activation gave the highest level of viability compared to CD3/CD28, this may be due to the robust activation signal compared with the more physiologically relevant CD3/CD28 stimulation. This may provide a stronger survival signal that aids viability.
4.13.1 Effect of Age on the in vitro activated T cell

During the optimisation process we tested the optimum age at which the T cells were Nucleofected to produce the highest number of viable cells while demonstrating the greatest functionality. Our results suggest that 5 times more cells survive if the cells are younger. The ex vivo lifecycle of the cell has been investigated with the use of the mitogen PHA and its progression studied through youth to senescence and apoptosis. Jaruga et al have detailed the in vitro T cell growth, and highlighted the important aspects within this cycle. They observed that the largest number of mitotic or actively dividing cells were within the first week following PHA stimulation. Within this population both CD4+ and CD8+ T cells are present, with CD8+ cells predominating at a ratio of 2:1. After this first week there is a slow cessation in cell growth accompanied by an increase in the number of senescent cells, with the ratio of CD8+ to CD4+ being maintained up to day 17. Further culture of these cells, results in the apoptosis of the remaining CD4+ cells leaving the remaining senescent cells CD8+ (Jaruga et al., 2000; Barbano et al., 1988).

Within this study cells were observed over a maximum 12 day time frame in which the CD4+/CD8+ T cell ratio would be expected to remain constant. The only difference between these 2 cultures of cells, is the number of mitotic vs. senescent cells (Jaruga et al., 2000). As we observed an increase in cell death as the age of the T cell increased, this may suggest that during the Nucleofection procedure senescent cells are more susceptible to cell death compared to cultures that contain an increased number of mitotic cells.

4.13.2 Transfection efficiency

Tahvanainen et al used the Nucleofection technique to transfect human unstimulated CD4+ T cells to study T helper cell differentiation. They observed a background of untransfected cells and from this developed a method to enrich the Nucleofected cells (Tahvanainen et al., 2006). Although they are using a slightly different T cell model, it is an interesting observation, as within these studies the results would suggest 100 % transfection efficiency. In order to reach this conclusion we utilized both FACS based assays and confocal microscopy. FACS

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analysis would suggest that at higher concentrations of siRNA (> 4 µM) 100 % of the cells were transfected. When using fluorescent siRNA it is a known problem that these molecules can bind to the surface of the cell and generate ‘false positives’ that cannot be distinguished from true positives. Utilizing confocal microscopy enabled slices to be taken through the cell, and this would suggest that the siRNA did enter the cells. However, we cannot rule out the possibility that some siRNA did bind to the surface of the cell, but our confocal results coupled to the knockdown of mRNA seen, would suggest this is not a problem within our system. Furthermore, as a general rule in order to reduce the amount of fluorescent siRNA that binds to the membrane, Nucleofection is advised as the delivery method of choice.

4.13.3 Decreased Functionality following Nucleofection

Following the full optimisation of the Nucleofection process, chemotactic assays were performed towards the chemokines CXCL12 and CXCL10. Over the concentration range tested, peak chemotactic responses were observed for both ligands in control and Nucleofected samples. This demonstrated that although reduced responses where observed within Nucleofected samples, these responses peaked at the same concentration in control and Nucleofected samples indicating no shift in the concentration dependent response.

However, the process of Nucleofection had a detrimental effect on the chemotactic responses and this was observed more acutely with the CXCL10 response. Interestingly this did not correlate with a decrease in chemokine receptor expression, suggesting that the problem was either a functional defect in the receptor binding the ligand, conformational change within the receptor to facilitate transduction of the signal, or a decreased coupling of the receptor to intracellular signalling events.

Taking into account the low cell viability, reduced function, and quantity of cells that are required and coupled with the economic costs of running these experiments this procedure was not a viable option for the study of activated T cells. Although no article has currently provided evidence for reduced functionality as a direct cause of
Nucleofection, this effect was clearly established with our system. As this technique is relatively new, there is focus in the literature on transfection efficiencies, cell viability and expression of cell surface markers and it will be interesting to see over the next few months and years if our observation of diminished functionality is restricted to this particular cell system or if it is observed within additional cell models.

4.14 Adenoviral delivery of shRNA shows limited success in activated T cells

As an alternative to Nucleofection, adenoviral delivery of shRNA was assessed. The hallmark receptor utilised by adenovirus to infect a cell is the CAR (coxsackie/adenovirus receptor (Bergelson et al., 1997)) receptor, which is strongly expressed in heart, prostate and pancreas with less in liver, brain, colon and small intestine (Shaw et al., 2004). If the CAR receptor is present, the cells are dividing and there is a strong MOI (multiplicity of infection), transfection efficiency should be 80-90 %, yet, as T cells do not express this receptor they are therefore are not susceptible to infection by this system.

However, T cells do express CD46 (MCP, Membrane Cofactor Protein (Nakanishi et al., 1994)) a receptor that can be utilised by subgroup B of the adenovirus family resulting in infection (Roelvink et al., 1998). With this knowledge, a chimeric virus was created commercially that utilised the CD46 receptor and following infection increases the expression of CD46 on the cell surface, thus allowing for increased rates of transfection. These advances in adenoviral delivery seemed to suggest the possibility that T cells could in fact benefit from this new system.

These adenoviral studies were performed in order to assess the level of knockdown which can be achieved within this system. As with the adenovirus which uses the CAR receptor for entry, adenovirus using CD46 should see maximum transfection in dividing cells which have been infected with a high MOI. Within these experiments a very high MOI of 5000 was used following activation of the cells with PHA. Although within our system less than half of the cells were transfected this is comparable to other chimeric adenoviral systems (Schroers et al., 2004). However,
Schroers et al did not look at the knockdown of mRNA (only the expression of GFP) so it is difficult to fully assess how successful their system was. Within this study no mRNA knockdown was achieved, therefore although in theory this technique should be successful, it is ineffective within this activated T cell model.

4.15 Nucleofection to deliver siRNA into freshly isolated T cells

The use of Nucleofection to deliver siRNA into activated T cells had been met with limited success, yet within this study, freshly isolated T cells demonstrated a greater percentage of cell viability, and also following 72 hours recovery, regained comparable chemotactic responses compared to control cells. This functionality and viability was coupled with successful siRNA delivery, assessed via flow cytometry, and knockdown of the targeted PI3K isoforms, assessed by mRNA. Although moderate knockdown of mRNA was achieved no effect was observed on the CXCL12 mediated chemotactic response.

Detection of mRNA knockdown was easily determined using qPCR, however, following nucleofection it was almost impossible to detect protein expression, and this was due to the low protein levels that were obtained following the nucleofection protocol. Therefore several scenarios could account for the lack of effect on the chemotactic response. Firstly, as seen with class I PI3K isoforms, class II may not play a part in the chemotactic response of *ex vivo* maintained cells. Secondly, although mRNA knockdown of up to 60% was observed this may not correlate into a large enough decrease in the functional protein expression, as such, the chemotactic response is maintained through the residual protein levels.

In order to determine protein levels, we first attempted immunoblotting and due to the sensitivity threshold of this technique, this was in the most part ineffective. The most effective way of detecting protein expression was via flow cytometry. However, using this technique, the data suggested that over the 72 hour time course there was no silencing of protein levels. This data suggests that in freshly isolated T cells, the use of PI3K targeting siRNA delivered via Nucleofection is an ineffective method for elucidating function.
4.16 Investigating PI3K independent migration

Curiously following ex vivo culture and or activation it was observed that the CXCL12-mediated migration was PI3K independent. In order to establish a fuller profile of this phenomenon the culture conditions, T cell activator and a panel of chemokines were investigated.

4.16.1 In vitro activation of PBMCs can skew the resultant T cell profile

Although the use of PHA is a commonly used method of in vitro activation, it induces activation and proliferation of specific subsets within PBMC population, resulting in a skewed distribution in the mature T cell culture. In particular, analysis of the expression of CCR7/CD45RA suggested that following PHA activation almost all cells were CCR7+, indicating that cells with effector function had disappeared (Duarte et al., 2002; Chen et al., 2006). This has a negative effect on functionality, with these studies concluding that the resulting pool of activated cells is not representative of the populations seen within in vivo populations. However, in vitro activation induced by CD3/CD28 stimulation closely mirrors the T cell populations that are activated and undergo proliferation in vivo. CD3/CD28 stimulated T cells also maintain a more comparable functionality to that seen in vivo and this is attributed to the subsets of T cells that are being generated.

T cells activated in vitro do not produce an exact physiological activation and by the nature of in vitro experiments we cannot expect it to. However, these experiments provide an invaluable insight into human T cell responses which would otherwise be elusive. During these investigations CD3+, CD4+ and CD8+ subsets, and their chemotactic responses following chemokine stimulation were studied. Although we have not delved further into the more specific subsets that comprise these populations generated via PHA, SEB and CD3/CD28, we have observed trends which highlight these differences at the CD3+, CD4+ and CD8+ level (Duarte et al., 2002; Chen et al., 2006).

Following PHA and CD3/CD28 stimulation it was observed that the expression profile of PI3K isoforms differ following the method of stimulation. PHA routinely expressed higher levels of PI3K isoform expression compared to CD3/CD28, and
therefore suggests that under these conditions PI3K expression is dependent upon the T cell activator.

4.17 CXCL12-mediated migration in activated T cells is resistant to PI3K inhibition

The dependence on PI3K in the role of directed cell migration has been well established (Hannigan et al., 2004; Barber and Welch, 2006; Sasaki and Firtel, 2006; Huang et al., 2003; Comer and Parent, 2006). However there is emerging evidence that some cells can chemotax in a PI3K independent manner (Cronshaw et al., 2004; Huttenlocher, 2005).

Data presented with this thesis suggests that PHA activated cells migrate towards CXCL12 in a PI3K independent manner, while maintaining coupling to PI3K dependent pathways. Subsets of CD3⁺ CD4⁺ and CD8⁺ T cells also displayed this phenomenon. However, as other groups have demonstrated that the method of \textit{in vitro} activation influences the subsequent T cell population (Kilpatrick, 1999; Zamoyska, 2006; Duarte et al., 2002; Chen et al., 2006), we sought to determine if the mechanism of T cell activation affected the dependence on PI3K for migration.

Activation with the superantigen SEB is seen under pathophysiological conditions and has the ability to activate large numbers of T cells (5-20 %) (Li et al., 1999). SEB activation of T cells also generated a population that undergoes PI3K independent migration. CD3/CD28 activated T cells, that have been shown to generate a more physiologically relevant T cell pool (Chen et al., 2006), also displayed this PI3K independent response.

CXCL12 is known to produce migration in both naïve and activated T cells (Butcher et al., 1999). Therefore, although PHA, SEB and CD3/CD28 may generate different T cell pools, the responses and dependence on PI3K isoforms may be conserved with respect to this particular chemokine.
4.17.1 CXCR3-mediated migration is resistant to broad-spectrum PI3K inhibitors

The CXCR4 receptor is expressed within homeostatic and inflammatory conditions. In contrast to CXCR4, CXCR3 is upregulated on T cells following activation, and is therefore only expressed within inflammatory settings, primarily on the Th1 subset (Nakae et al., 2007). This provided an interesting comparison to the constitutively expressed CXCR4, in that we hypothesised that chemokines expressed solely within inflammatory conditions may induce migratory responses through differing signalling pathways than those expressed in homeostatic systems. All 3 CXCR3 ligands, CXCL9, CXCL10, and CXCL11 provided robust chemotactic responses in PHA activated cells. However, these responses were not significantly inhibited by pre-incubation with LY294002. Others studies have also reported this effect in T cells cultured in IL-2 (Smit et al., 2003).
4.17.2 Is CCL5 a poor chemoattractant? - The role of shear flow in chemotactic responses

At the beginning of this study we reinvestigated the role of CCL5 and found that it demonstrated small chemotactic responses within activated T cells. However, the CXCR3 ligands CXCL9, CXCL10 and CXCL11 all demonstrate robust migratory responses. Therefore what determines the strength of the migratory response; is it simply that CCL5 is a poor chemoattractant?

The observation that CCL5 and CXCR3 ligands induce weak and robust migratory responses respectively, is in agreement with other studies which have focused on the chemokine receptors CXCR3 and CCR5. A study by Stanford and Issekutz focused on the migration of radiolabeled lymphoblasts and spleen T cells to cutaneous sites injected with lymphocyte-recruiting agents in rats. They noted that CXCR3 ligands are more potent in vitro than CCL5, interestingly, this observation did not hold true for in vivo investigations (Stanford and Issekutz, 2003). In vitro CCR5 ligands CCL3, CCL4 and CCL5 induced chemotaxis of activated T cells (potencies CCL5>CCL3>CCL4) but could not induce significant chemotactic responses on unactivated spleen T cells (Stanford and Issekutz, 2003). In contrast the CXCR3 ligands, induced strong chemotactic responses in rat lymph node activated T cells and to a lesser extent spleen T cells with a potency of CXCL10>CXCL11>CXCL9. However, during in vivo studies both CXCL10 and CCL5 induced comparable, robust and rapid recruitment of T cells (and to a lesser extent CXCL11, CXCL9, CCL3 and CCL4).

A possible explanation for this apparent difference in in vivo vs. in vitro data may be the immobilization of chemokines on the surface of the cell and the role of shear flow. This theory is supported by the observation that when CXCL12 is presented apically on the surface of endothelial cells in a flow chamber, this stimulates rapid transendothelial migration (Schreiber et al., 2007). As standard in vitro chemotaxis systems do not account for the role of shear flow, the full chemotactic property of CCL5 and possibly other ligands may have been underestimated.
The apparent differences within these in vitro and in vivo observations, highlights how critical it is to assess the migratory responses seen in vitro against in vivo models. While the data that can be drawn from in vitro models, advance our basic understanding, we cannot underestimate the effect other chemokines and the role other cells play in how an organism functions as a whole.

When assessing expression of chemokine receptors in an in vitro setting, it is important that the method of assessment or even the antibody used is considered. This is essential as some groups have observed low levels of CCR5 on the surface of the T cell, while others have demonstrated higher expression levels. So what could account for these discrepancies? Lee et al studied the results of CCR5 expression utilizing 18 different antibodies (Lee et al., 1999b). These antibodies showed differing affinities for the receptor and thus generate apparently different CCR5 expression levels within the same T cell population, highlighting the importance of antibody selection.

Using an antibody which recognised the highest proportion of CCL5, Lee et al further demonstrated that 14.1% of whole T cell populations express CCL5 (Lee et al., 1999a). However, this total population could be further broken down into high and low expressers (memory and naïve respectively). This observation correlates well with a study performed by Schall et al that shows migration of memory T cells (Schall et al., 1990). This observation may explain the findings highlighted within this thesis, as only total CD3+ cells and not subpopulations of T cells were assessed for migratory properties. Thus migratory responses of memory T cells may have been overshadowed by the non-migratory response of the other subpopulations.

4.18 The method of in vitro activation of human T cells determines the PI3K dependence of basal migration

Although this study has established that in vitro activated T cells migrate in a PI3K independent fashion, it is intriguing to observe the effect of PI3K inhibition on basal migration.
Basal migration of PHA activated T cells was inhibited in a concentration dependent manner, with significant inhibition seen following 3 μM LY294002 and 100 nM wortmannin. This observation was further extended to include PHA CD4+ and CD8+ subsets and SEB stimulated populations. However, within CD3/CD28 stimulated T cells this inhibition was less pronounced. Although there was a trend for inhibition this was not significant even at the highest concentrations of either LY294002 or wortmannin.

The observation that LY294002 and wortmannin significantly inhibit basal migration in PHA and SEB but not CD3/CD28 T cell population, is the first observation within this work that functionality may be altered depending on the T cell activator. Furthermore, although 2 out of 3 models show basal migration to be dependent on PI3K, it may be more accurate to conclude that in vivo activated T cells do not significantly depend on PI3K for basal migration, derived from the observation that CD3/CD28 T cells provide a more accurate reflection of the physiological situation.

For these in vitro models, it may be suggested that PHA and SEB activation generates cellular populations where PI3K contributes to the migratory response of the cell but not to the directionality. In contrast the regulation and the directionality of migration in T cells stimulated by CD3/CD28 may be independent of PI3K.

### 4.19 PI3K independent migration

Several studies have investigated the phenomena of PI3K dependent and independent chemotaxis leading to conflicting results. Described below is a selection of studies which have provided further evidence for PI3K independent migration.

In the p110γ knockout models the failure of PIP3 to be generated and thus the failure to recruit signalling molecules to the cell membrane indicates the pivotal role that p110γ plays within the signalling and chemotaxis of these cells, however a small but significant level of residual neutrophil chemotaxis was still present. Further investigations utilising a kinase dead knock-in model of p110δ and in addition the use of either LY294002 or wortmannin ruled out a role for p110δ or the
other class I isoforms within this system (Thomas et al., 2005). Therefore, although these data suggest that p110γ is the major isoform through which neutrophils chemotax, there is evidence that both supports and opposes the role for class IA within the chemotactic response, suggesting the residual response may be provided through another signalling pathway.

This study has also highlighted the complexity surrounding the signalling and functionality involved within T cell migration. Discussed below are the possible mechanisms which may play a role within the switch between PI3K dependent and independent responses.

4.19.1 CXCR4 vs. CXCR7: the chemokine receptor switch

It has been recently determined that CXCL12 mediates chemotaxis of freshly isolated T cells not only through the receptor CXCR4 but also through the newly termed receptor CXCR7 (RDC-1) (Burns et al., 2006; Balabanian et al., 2005). This observation has far reaching implications, as it was thought that the CXCL12/CXCR4 axis was quite unique, in that one receptor (CXCR4) bound only one ligand (CXCL12) and vice versa. From this standpoint, it provided a relatively simplified view of signalling and functionality in a chemokine system which is renowned for its promiscuity (Ward and Westwick, 1998).

This development within the chemokine field may provide a possible explanation as to how the naïve T cell can chemotax in a PI3K dependent manner and activated T cells chemotax independently of class I PI3K. Through these investigations and others, it has been shown that CXCL12 induces chemotaxis and phosphorylation of PKB in freshly isolated T cells in a PI3K dependent manner. These cells have been shown to express both CXCR4 and CXCR7, with blockade of either or both of these receptors resulting in the decrease in chemotaxis (Balabanian et al., 2005).

Within activated cells, the situation is less clear, we have observed CXCL12 induces chemotaxis in a PI3K independent manner while phosphorylation of PKB is maintained as a PI3K dependent mechanism. It is possible that this switch in dependence for PI3K may in fact be due to a switch in the use of these receptors following activation of the cell. For example, CXCR4 could be utilized by naïve cells
in a PI3K dependent manner, while following activation the primary receptor used in the chemotactic response could be CXCR7. This switch may also explain the ability of CXCR7 to bind inflammatory chemokines, as it has already been documented that although CXCR4 can only bind CXCL12, CXCR7 has the capacity to bind CXCL12 and the inflammatory chemokine CXCL11, suggesting a role for this receptor within an activated T cell (inflammatory) setting (Burns et al., 2006).

The situation may not be that clear, as other studies have suggested that the CXCR7 receptor is in fact an atypical receptor that does not induce calcium mobilization, chemotaxis or any signalling function. Instead it is suggested that this receptor acts to sequester available CXCL12. In the zebra fish lateral line, cells have been shown to restrict CXCR4 and CXCR7 to the leading and rear edge respectively, generating an intrinsic compass. Therefore, CXCR4 within the leading edge responds chemotactically to the bound CXCL12 and as the cell passes over the chemokine, the rear of the cell which contains a higher concentration of CXCR7 receptors (and demonstrates higher affinity for CXCL12), binds CXCL12 sequestering it, thus maintaining the polarised shape and molecular distribution (Dambly-Chaudiere et al., 2007).

4.19.2 Chemokine receptors – the molecular switch

It is also possible that the same receptor or receptors are used, yet following activation T cells undergo a molecular switch, in that the chemotactic signal which is generated now depends on an alternate pathway to induce directed cell migration. Discussed below are possible mechanisms (also see figure 4.1).

Dock2 – One molecule which has gained interest over the last few years as a possible player in PI3K-independent migration is DOCK-2. This Rac-specific guanine nucleotide exchange factor has been identified as a candidate, as mice lacking this gene display a significant decrease in CXCL12 (as well as CXCL13, CCL19 and CCL21) induced chemotaxis (Nombela-Arrieta et al., 2004). Interestingly, in cells such as Neutrophils, in which PI3K has been characterised to play a role in the mediation of chemotaxis (Chung et al., 2001a; Devreotes and Janetopoulos, 2003; Ridley et al., 2003), DOCK-2 is not present (Nishihara et al., 1999). Therefore, in T cells which are shown to contain both PI3K and DOCK-2, it
may be the case that these cells chose to preferentially migrate with a DOCK-2 dependent, PI3K independent manner. Although DOCK-2 was the overwhelming driving force behind the chemotaxis of murine T cells, it was also observed that PI3Kγ played a small but significant role within CCL21 mediated chemotaxis, highlighting a context-dependent role for PI3K.

PLCβ – A recent publication highlights a role for PLCβ within the chemotactic responses of T cells. Interestingly, they observed similar PI3K independent chemotactic responses (as were seen in this study) and investigated the possibility of PLC isoforms within this response. Utilizing murine models they highlighted a role for the PLCβ isoform as an alternative mechanism that underlies the chemotactic response in T cells (Bach et al., 2007). Other studies have also highlighted a possible role for PLC in the chemotaxis of Th2 cells, however, in contrast Bach et al demonstrates a dependence on the production of IP₃, while others suggest calcium independent mechanisms (Cronshaw et al., 2006). This data provides an exciting opportunity to address the requirement of human T cells both in the activated and freshly isolated T cell model and compare and contrast these observations. This data suggests that PLCβ may be a strong candidate for the underlying mechanism of PI3K independent chemotaxis.

Collectively these observations suggest that activated human T cells do not depend on PI3K for CXCL9, CXCL10, CXCL11, and CXCL12-mediated chemotaxis. However, care must always be taken when drawing conclusions from in vitro data and making assumptions for the physiological settings.

4.20 The role of in vitro culture on T cell responses

The observation that freshly isolated T cells are dependent on PI3K for their chemotactic responses and in vitro activated T cells migrated in a PI3K independent manner, posed an interesting question, how and when did this switch occur? In order to address this question, freshly isolated T cells were cultured in vitro without activation; furthermore, as IL-2 has been described as playing a role in the inflammatory process and in the upregulation of chemokine receptors, these investigations were carried out in the presence and absence of IL-2. Intriguingly,
following 24 hours of in vitro culture, human T cells modulate their responses in such a way that they lose the dependence on PI3K for their migratory responses, while maintaining the dependence on PI3K for chemokinesis.

In this system it is observed that the dependence on PI3K is altered during culture. As discussed within the activated T cell model, it may be that a switch in dependence is due to a switch in the use of chemokine receptors, either CXCR4 or CXCR7. This may be initiated via IL-2 instead of activation of the cell being responsible for this switch. This is supported by the observation that IL-2 can upregulate CC and CXCR4 on the surface of the cell. However, these investigations showed the functional response was altered after 4 days of IL-2 incubation, and we observed a significant alteration in this dependence with 24 hours. Furthermore, we tested the role of IL-2 within this system and found that even in the absence of this cytokine, cells chemotaxed towards CXCL12 in an independent manner following 24 hours ex vivo maintainence. Therefore these results would favour the opinion that neither IL-2 or activation plays a role in this altered PI3K dependence but if this is the case then what could this switch be dependent on?

4.20.1 Culture Shock

Following isolation cells are routinely transferred into medium for in vitro culture. This culture is performed in an artificial environment, in which cells are maintained in media that is further supplemented depending on the cell under investigation. This culture is routinely performed within a humidified environment with 5 % CO₂.

Due to this artificial system it is therefore possible the components of the media may themselves initiate altered responses within the cell (Berthois et al., 1986). Furthermore, it has been noted that within physiological settings, the amount of reactive oxygen species (ROS) is kept in check by the limited oxygen available, yet within an in vitro system, which has higher levels of oxygen, this leads to higher levels of ROS or ‘culture shock’ which results in a stress on the cells. This stress can possibly lead to alterations in the properties of the cell (Halliwell, 2003; Halliwell, 1997).

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Immune cells may be particularly susceptible to this process. The Redox system has been identified to play a role in the T lymphocyte cell cycle, inducing the apoptosis of cells following resolution of inflammation. Activation of T cells is coupled to an increase in ROS production, although macrophages play a role in countering this response increasing or diminishing T cells' anti-oxidant effects (Buttke and Sandstrom, 1995). Conversely, it has been suggested that cell culture media may contain anti-oxidant properties, with the ions and amino acids necessary for this process being found supplemented in media or added as a part of foetal calf-serum (Faure et al., 2004). The overall net effect of these pro- and anti-oxidants has yet to be fully elucidated, however, it is clear the conditions of in vitro culture have important implications.

Taken together, this data provides evidence for reliance on PI3K in a context dependent setting for both chemotaxis and chemokinesis, generating intriguing possibilities for further in vitro study. To what extent 'culture shock' or the components of the media themselves have within this system is unclear, however, it posses an interesting question as to how we interpret data and the extent to which the functions and signalling we observe are in fact in vitro artefacts.
Figure 4.1 Model of PI3K dependent and independent biochemical signals utilised during CXCL12-mediated T cell migration. It has been shown that freshly isolated T cell migrate in a PI3K dependent manner, primarily through PI3K γ. However, following ex vivo maintainance T cell chemotax through a PI3K independent process. Recent publications have suggested the involvement of PLCβ or DOCK 2 as alternative migratory pathways.
4.21 CXCL12 Signalling in cultured and activated T cells is sensitive to broad spectrum inhibitors

Interestingly, we observed that although CXCL12-mediated migration was independent of PI3K, CXCL12 signalling was biochemically coupled to PI3K (by the measurement of PKB phosphorylation). It has been published that freshly isolated T cells are dependent on PI3K for CXCL12-mediated migration, therefore it is an interesting observation that through culture and/or activation T cells lose their dependence on PI3K, while maintaining their reliance on PI3K for signalling (Butcher et al., 1999; Balabanian et al., 2005).

Activation of class I PI3Ks, in particular class I γ following chemokine stimulation has been suggested to be a requirement for promoting the survival of developing thymocytes and memory T cells. Within the activated T cell model CXCL12 mediated migration is independent of PI3K and yet we still observe a biochemical coupling. This response may be observed due to the activation and promotion of cellular survival and development pathways initiated through ligation of the chemokine receptor (Barber et al., 2006; Okkenhaug et al., 2006)).
4.22 An alternative functional readout

An emerging body of work has highlighted a role for PI3K isoforms within the regulation of cytokine production (Okkenhaug et al., 2006; Okkenhaug et al., 2007). As an alternative functional readout to migratory responses we assessed the role of PI3K isoforms within Th1/Th2 cytokine production by utilizing PI3K targeting siRNA.

4.22.1 A role for class I δ in cytokine production?

It is clear from the literature that the delicate balance between the Th1 vs. Th2 subsets are essential for the ongoing health of an organism, the regulation of this system is therefore of utmost importance. With the myriad of diseases that are encountered from dysregulation of this system a thorough understanding of the mechanisms behind this process is essential if therapeutic strategies are to be developed. An interesting development in the regulation of cytokine production is the dependence on PI3K particularly class Iδ, for the generation of Th1/Th2 cytokines (see figure 4.2) (Okkenhaug et al., 2006). Work presented within this thesis used nucleofection as a tool to deliver PI3K isoform specific siRNAs into freshly isolated cells and measured the Th1/Th2 cytokine expression levels. An ELISA based assay was utilized to determine if the release of Th1/Th2 cytokines of T cells that had been Nucleofected with PI3K isoform specific siRNAs. In order to generate measurable readings cells were stimulated with CD3/CD28 for 24 hours prior to analysis.
Chapter 4 - Discussion

Figure 4.2 A role for class Iδ in Th1 cytokine production of in human T cells?

Metabolism of PtdIns(4,5)P₂ by PLCγ and PI3K. PLCγ hydrolyses PtdIns(4,5)P₂ to yield Inos(1,4,5)P₃ and DAG, both of which function as signalling molecules. Inos(1,4,5)P₃ stimulates the release of Ca²⁺ from the ER into the cytosol, which triggers the nuclear translocation of NFAT. DAG binds to and activates Ras-GRP, which stimulates Ras and the Erk pathway, leading to AP-1-dependent transcription. Ras also binds to p110 and contributes to optimal PI3K activation. DAG binds to and activates PKC. By contrast, PI3K phosphorylates PtdIns(4,5)P₂ at position 3 to produce the membrane phosphoinositol lipid PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ functions as an anchor and cofactor for proteins with PtdIns(3,4,5)P₃-binding PH domains such as Akt, Tec family kinases, and various GEFs and GAPs. Pdk1 is required to co-activate Akt. Akt phosphorylates and inactivates Foxo and GSK3. GSK3 can phosphorylate and inactivate NFAT. Akt stimulates mTOR through Tsc1 and Tsc2. Tec kinases can phosphorylate PLCγ and contribute to its optimal activity. PI3K signalling is antagonised by the Pten phosphoinositide phosphatase, which removes the 3-phosphate, and the SHIP phosphatase, which removes the 5-phosphate. The role of P(3,4)P₂-binding proteins is still unknown. Although PLCγ and PI3K generate mutually exclusive second messenger signalling molecules, several of the pathways activated by these second messengers interact, and the signals are further integrated by the cell to promote gene transcription, cell growth and differentiation. p110δ seems to be the principal PI3K isoform in the context of antigen receptor signaling; however, p110α and p110β are also expressed in lymphocytes but their roles in antigen receptor signalling are unknown. (Taken from (Okkenhaug et al., 2007)).

Recent publications have highlighted a role for the PI3K family within this regulation, in particular that of class Iδ, with evidence suggesting that murine models of inactive class Iδ, produce T cells that are deficient in the production of Th1/Th2 cytokine expression profiles (figure 4.2 (Okkenhaug et al., 2006; Okkenhaug et al., 2007)). A role for class Iδ has also been highlighted in the secretion of TNF and IL-6 in mast cells (Ali et al., 2004). Within this study we observed that the process of Nucleofection had a detrimental effect on cytokine production, therefore it would be
difficult to ascertain what responses have a true functional effect and what are attributed to artifacts of Nucleofection. Drawing any observations from most of the cytokines tested is difficult due to the low levels of expression prior to or following nucleofection. Although at this point within our study we can draw no absolute conclusions, it is interesting to note that following Nucleofection with class Iδ isoform siRNA, we observe a decrease in the expression levels of IFN-γ and TNF-α, where other class IA isoforms fail to induce further effect. This would be in support of class I δ playing a role in the Th1/Th2 balance. Although no role for class II β in cytokine production has been reported, within this study we also observe a decrease in TNF-α and INF-γ and although not as pronounced as class Iδ, this would be interesting to address.

Even though these investigations are in their infancy, it is intriguing that our initial data shows some suggestion that it may mirror observations made in murine systems.
CHAPTER 5: SUMMARY & FUTURE WORK
Summary & Future Work

5.1 Summary
The observations made within this study suggest that freshly isolated T cells migrate towards CXCL12 in a PI3K dependent manner, and this signal is due predominantly to the class I γ isoform. Interestingly, it was also observed that the role of PI3K within migration is context-dependent, varying according to ex vivo maintence and activation state. Preliminary studies may also support a role for class I δ within cytokine production, however, these studies are in their infancy and no conclusions can be drawn upon at this time.

5.2 Future work

5.2.1 Elucidating the molecular mechanisms behind PI3K independent chemotaxis
This work has raised some intriguing observations. One of the main unresolved issues arising from these studies is mechanism behind the apparent switch from PI3K dependent to independent chemotaxis. The data generated suggests this may be observed due to culture conditions rather than triggered through activation, yet it is unclear as to the underlying mechanistic action. The observation that activated cells may chemotax in a PI3K independent manner has been observed in other studies, and recent publication has highlighted a possible role for the PLCβ isoform within the migratory response. It would be interesting to further this research by utilizing PLC isoform specific inhibitors to address this issue not only within the activated T cell model but also following ex vivo culture. Equally it would be interesting to look at other signalling molecules that are possible T cell chemotaxis modulators, such as DOCK2.

5.2.2 Investigating the role of PI3K isoforms in cytokine production
An interesting observation from the cytokine studies suggested PI3K class Iδ plays a role in the cytokine production following CD3/CD28 stimulation. This may be addressed via an alternate method for siRNA delivery which displays a less
detrimental effect on functionality. Furthermore, through the development of isoform selective inhibitors it would be interesting to determine what affect these compounds would have on cytokine production.

5.2.3 Clarifying the functions of the CXCR4 vs. CXCR7 receptors

Given the emerging complexity of the CXCL12/CXCR4 and CXCL12/CXCR7 axis it would be essential to characterise the roles that these 2 receptors play within this system. Initial observations would suggest a role for CXCR7 in chemotactic responses of T cells, thus by utilizing blocking antibodies it would be possible to further elucidate and characterise these receptors. Determining the contribution to migratory and signalling events with this system would provide an invaluable insight into this system. Given the complexity that has been shown to arise from dimerisation of chemokine receptors, it will be important to determine whether these 2 receptors; heterodimerise to alter T cell function, homodimerise to modulate responses that generate independent signals or as synergistic receptors.
CHAPTER 6:
APPENDIX
Appendix

6.1 Buffers and Solutions

6.1.1 Solutions and buffers for SDS-PAGE and western blotting

<table>
<thead>
<tr>
<th>Lysis Buffer</th>
<th>4 X Resolving gel Buffer</th>
<th>4 x Stacking gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % (v/v) Nonidet P-40</td>
<td>1.5 M Trizma base</td>
<td>0.5 M Trizma base</td>
</tr>
<tr>
<td>150 nM NaCl</td>
<td>pH 8.8</td>
<td>pH 6.8</td>
</tr>
<tr>
<td>50 mM Tris pH 7.5</td>
<td>0.4 % (w/v) SDS</td>
<td>0.4 % (w/v) SDS</td>
</tr>
<tr>
<td>5 mM EDTA</td>
<td>MilliQ water</td>
<td>MilliQ water</td>
</tr>
<tr>
<td>10 mM sodium fluoride*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM phenylmethylsulfonyl fluoride*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg ml-1 leupeptin*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µg ml-1 aprotinin*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg ml-1 soybean trypsin inhibitor*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg ml-1 pepstatin A*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM sodium orthovanadate*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM sodium molybdate*</td>
<td></td>
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</tbody>
</table>
| MilliQ water | | |}

NB * denotes added on the day of use
### 6.1.2 Recipes for SDS-PAGE gels

<table>
<thead>
<tr>
<th>SDS-PAGE running buffer</th>
<th>5 x SDS-sample buffer</th>
</tr>
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<tbody>
<tr>
<td>25 mM Trizma base</td>
<td>5 % SDS</td>
</tr>
<tr>
<td>192 mM glycine</td>
<td>50 % glycerol</td>
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<tr>
<td>0.1 % (w/v) SDS</td>
<td>200 mM Tris-HCl pH 6.8</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>MilliQ</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td></td>
<td>5 % 2-mercaptoethanol</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Semi-dry transfer buffer</th>
<th>Tris-buffered saline (TBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39 mM glycine</td>
<td>20 mM Tris-HCl</td>
</tr>
<tr>
<td>48 mM Trizma base</td>
<td>pH 7.5</td>
</tr>
<tr>
<td>0.0375 % SDS</td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td>20 % (v/v) methanol</td>
<td>MilliQ</td>
</tr>
<tr>
<td>MilliQ water</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Tris-buffered saline-Tween (TBST)</th>
<th>Stripping buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBS + 0.1 % (v/v) Tween -20</td>
<td>62.5 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td>pH 6.8</td>
</tr>
<tr>
<td></td>
<td>2 % (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>100 mM 2-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>MilliQ</td>
</tr>
</tbody>
</table>

### Resolving Gels

<table>
<thead>
<tr>
<th>4 X Resolving or Stacking buffer (ml)</th>
<th>Resolving Gels</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 %</td>
<td>5</td>
<td>4 X</td>
</tr>
<tr>
<td>10 %</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>12 %</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

| MilliQ (ml)                           | 9.84           | 6.85         |
| Acrylamide                            | 5.0            | 2.0          |
| APS (10 %) (µl)                       | 150            | 150          |
| TEMED (µl)                            | 15             | 15           |

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### 6.1.3 Immunoblotting conditions for specific primary antibodies

<table>
<thead>
<tr>
<th>Molecule targeted by primary antibody</th>
<th>Primary antibody species</th>
<th>Molecular Weight (kDa)</th>
<th>Blocking buffer&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Primary antibody concentration&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Secondary antibody concentration&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt1</td>
<td>Goat</td>
<td>60</td>
<td>5%</td>
<td>1:1000</td>
<td>1:10000</td>
</tr>
<tr>
<td>S6</td>
<td>Rabbit</td>
<td>32</td>
<td>5%</td>
<td>1:1000</td>
<td>1:10000</td>
</tr>
<tr>
<td>phosphoSer235/236-S6</td>
<td>Rabbit</td>
<td>32</td>
<td>5%</td>
<td>1:1000</td>
<td>1:10000</td>
</tr>
<tr>
<td>phosphoSer473-Akt</td>
<td>Rabbit</td>
<td>60</td>
<td>1%</td>
<td>1:700</td>
<td>1:7000</td>
</tr>
<tr>
<td>PI3K class IIβ</td>
<td>Mouse</td>
<td>165</td>
<td>5%</td>
<td>1:700</td>
<td>1:10000</td>
</tr>
</tbody>
</table>

<sup>1</sup> Expressed as percentage (w/v) non-fat milk diluted w/v in TBST  
<sup>2</sup> Diluted v:v in 0.01% (w/v) sodium azide TBST  
<sup>3</sup> Diluted v:v in 1% (w/v) non-fat milk in TBST
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References


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but not lymphoid, chemokines and is dispensable for lymphocyte homing under physiological conditions. Int. Immunol. 15, 1219-1227.


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