An Investigation into the Role of Protein Kinases in T lymphocyte Migration

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Award date:
2009

Awarding institution:
University of Bath

An Investigation into the Role of Protein Kinases in T lymphocyte Migration

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for the degree of Ph.D.

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March 2009

Table of Contents

Table of Figures

Chapter 1: Introduction

Chapter 2: Materials and Methods

Results I: The role of PI3K and downstream kinases in human T lymphocyte migration

Results II: The role of PI3K in murine Th17 cell polarisation and migration

Final Conclusions and Future Work

Acknowledgments

Firstly, I would like to thank my supervisors Professor Stephen Ward, Dr Tom Crabbe and Dr Louise Jopling for all the support, advice and knowledge they have given over the course of this PhD. The guidance you provided has been invaluable and I am sincerely grateful.

Secondly, I would like to thank all members of the Ward, Welham and MacKenzie labs who have helped me during my time in Bath. Without your support and friendship, this would have been a lot harder. Thank you also to the staff of UCB in Cambridge who went out of their way to help me while I was there. Special thanks must go to David Medina-Tato and Laura Smith for their support, friendship and for being a sounding-board when I needed it most.

I would also like to thank my parents. From the beginning you have always been supportive of the choices I've made. Without you, I would never have gotten this far and I want you to know how much I appreciate that.

Finally, thank you Gen. Your love, support and encouragement have been invaluable. Even through the difficult times you have stood by me and that means everything.

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Webb, A., Johnson, A., Fortunato, M., Platt, A., Crabbe, T., Christie, M.I., Watt, G.F., Ward, S.G., Jopling, L.A. Evidence for PI-3K-dependent migration of Th17-polarized cells in response to CCR2 and CCR6 agonists. J Leukoc Biol. (2008) 84(4):1202-12.

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Abstract

The migration of T lymphocytes is a vital component of the immune system, with roles in immunosurveillance and inflammation. The role of Phosphoinositide 3-kinase within T lymphocyte migration is unclear, with some evidence that it may be a disposable signal. Here, using Staphylococcal Enterotoxin B activated peripheral blood mononuclear cells and the T cell line CEM cells, the role of Phosphoinositide 3-kinase and its downstream kinases was investigated. CCL22 mediated CEM cell migration and CXCL12 mediated peripheral blood mononuclear cell migration were shown to be independent of Phosphoinositide 3-kinase using several different broad-spectrum Phosphoinositide 3-kinase inhibitors. However, these cells were Akt-dependent, as demonstrated by incubation with the Akt inhibitor Akti-1/2. Differences in the effect of the inhibitors on Akt activity were discovered, indicating that either Akt can be activated in the absence of Phosphoinositide 3-kinase, or differences exist regarding the relative abundance of each protein within the cell.

Th17 cells are a subtype of the T helper cell family and have been shown to be involved in inflammation and immune diseases. Mouse splenocytes were polarised to a Th17 phenotype and analysed for the surface expression of chemokine receptors. CCR2, CCR6 and CCR9 were shown to be expressed on Th17 cells and upregulated under Th17 polarising conditions. However, only CCR2 and CCR6 induced migration of Th17 cells. This migration was sensitive to Phosphoinositide 3-kinase and Akt inhibitors. This data reveals a model for the migration of Th17 cells to areas of inflammation, and sheds light on the role of Phosphoinositide 3-kinase during this process.

Abbreviations

AC	Adenylyl cyclase
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
AP-1	Activator protein-1
APC	Antigen presenting cell
APKC	Atypical PKC
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
Chemokine	Chemotactic cytokine
Class I α	Phosphoinositide 3-kinase class I α
Class I β	Phosphoinositide 3-kinase class I β
Class I γ	Phosphoinositide 3-kinase class I γ
Class I δ	Phosphoinositide 3-kinase class I δ
Class II α	Phosphoinositide 3-kinase class II α
Class II β	Phosphoinositide 3-kinase class II β
Class II γ	Phosphoinositide 3-kinase class II γ
cPKC	Conventional PKC
CTL	Cytotoxic T-lymphocytes
CTLA4	Cytotoxic T lymphocytes antigen-4
DAG	Diacylglycerol
DC	Dendritic cells
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescent reagent
ERK	Extracellular regulated kinase
F-actin	Filamentous actin
FITC	Fluorescein isothiocyanate
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	Gunanosine Triphosphate
HRP	Horse radish peroxidase
ICAM-1	Intracellular adhesion molecule-1
ICOS	Inducible costimulatory receptor
IL	Interleukin
IL-2	Interleukin-2
IP_3	Inositol triphosphate
JAK	Janus Kinase
JNK	c-jun-NH2 terminal kinase
LAT	Linker for activation in T cells
LPS	Lipopolysaccharides
LY294002	Specific PI3K inhibitor
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatability complex
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
MTOC	Microtubule-organizing center
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NK	Natural-Killer [cell]
NPKC	Novel PKC
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood derived lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PDK-1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIK	Phosphatidylinositol kinase domain
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptors

PI	Phosphatidylinositol(3)phosphate
PIP2	Phosphatidylinositol(3,4)bisphosphate
PIP3	Phosphatidylinositol(3,4,5)trisphosphate
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
PX	Phox homology
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho-associated coiled-coil forming kinase
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SEB	Staphylococcal Enterotoxin B
SDS	Sodium dodecyl sulphate
SH2	Src homology 2 domain
SHIP	SH2-containing inositol phosphatase
SiRNA	Short interfering RNA
SLC	Secondary lymphoid chemokine
SoS	Son of Sevenless
STAT	Signal Transducer and Activator of Transcription
TAPP	Tandem PH-domain-containing protein.
TCR	T-cell receptor
TLR	Toll-like receptor
Treg	Regulatory T cell
V	volts
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen -4
Wortmannin	PI3K inhibitor
ZAP-70	Z –chain associate protein kinase

Chapter 1: Introduction

Chapter 1: Introduction

1.1 The Immune System

The immune system is an intricate and finely controlled collection of mechanisms within an organism that maintains health by limiting infection and disease. It can detect a wide variety of agents, from bacteria and viruses to helminths, while recognising an organisms own healthy cells. This ability to distinguish between self and non-self is one of the most important aspects of the immune system. In mammals, the immune system is divided into two branches, termed innate and acquired immunity. These systems, while separate, must interact closely for the proper operation of the immune system. The innate immune system comprises those aspects of the immune system that operate in a generic and non-specific manner. This system provides immediate protection for the host, and 'buys time' for the host while the acquired immune system is activated.

1.1.1 Innate Immunity

The innate immune system is thought to be an evolutionary older system than the acquired immune system and is the main immune system in plants and insects. The first line of protection is the barrier provided by skin, pulmonary and gut epithelia. This mechanical barrier works by preventing access to the organism. As this barrier cannot be completely sealed, other defence mechanisms such as mucus, secreted by the respiratory and gastrointestinal systems, and coughing serve to entrap and expel pathogens.

If this barrier is breached, the secondary line of defence is inflammation. Blood vessels near to the infected tissue alter, resulting in edema and the recruitment of leukocytes to the infected tissue. The leukocytes then recognise foreign pathogens within the tissue, before undergoing phagocytosis and degrading the pathogens. The recognition of self and non-self within the innate immune system does not work by recognising every possible antigen, as in the acquired immune system, instead it recognises molecules shared by groups of related pathogens that are essential for the survival of those pathogens and are not

found on the surface of mammalian cells. These molecules are known as pathogen-associated molecular patterns (PAMPS) (Medzhitov and Janeway, Jr., 1997) and include LPS from the gram-negative cell wall, peptidoglycan and lipotechoic acids from the gram-positive cell wall, the sugar mannose, bacterial and viral unmethylated CpG Deoxyribonucleic acid (DNA), bacterial flagellin, the amino acid *N*-formylmethionine found in bacterial proteins, double-stranded and single-stranded ribonucleic acid (RNA) from viruses, and glucans from fungal cell walls. In addition, unique molecules displayed on stressed, injured, infected, or transformed human cells also act as PAMPS. PAMPS are recognised by pattern-recognition receptors (PRRs), of which there are two types; endocytic PRRs, found on the surface of phagocytes and promote the attachment of microorganisms to phagocytes leading to their subsequent engulfment and destruction; and signalling PRRs, an example of which are Toll-like receptors (TLRs). There are many different TLRs, which are responsible for binding different molecules on the surface of pathogens. Binding of PAMPS to different TLRs results in the transcription and translation of different inflammatory cytokines, thus tailoring the immune response to the pathogen.

1.1.1.1 Leukocytes involved in innate immunity

Several cell types are involved in innate immunity, including natural killer (NK) cells, Neutrophils and Monocytes / Marophages.

NK cells are not part of the inflammatory response, but are a major component of the innate immune system as they are involved in the rejection of tumours and cells infected with viruses. NK cells are activated by receptors on their surface termed Fc receptors. These bind to the Fc portion of antibodies bound to infected / abnormal cells. Once activated, NK cells degranulate, releasing proteins such as perforin and proteases such as granzymes. Perforin forms holes in the membrane of the targeted cells, allowing the proteases access. The proteases then induce apoptosis of the cell.

Neutrophils are a polymorphonuclear cell that normally circulates through the blood. In response to signals such as interleukin (IL)-8 and interferon (IFN)γ,

they migrate from the blood through tissue to the site of infection. Having recognised PAMPs through their surface TLRs, neutrophils phagocytose pathogens, degrading them using reactive oxygen species and hydrolytic enzymes. In addition, neutrophils can undergo degranulation, releasing several different types of molecules.

Macrophages, the differentiated form of monocytes, can be tissue based (such as Microglia in neural tissue and Osteoclasts in bone) or circulate through the blood stream where they migrate to the site of infections in response to signals from damaged tissue. Once at the site, they release a series of chemicals to attract other cells to the site of infection, and also engulf and degrade pathogens through phagocytosis.

1.1.2 Acquired Immunity

Acquired immunity (also known as adaptive immunity) is found in vertebrates and provides the host with the ability to recognise and remember specific pathogens. Therefore, the host can mount stronger challenges each time the pathogen is encountered. It is described by Janeway as "the optimum host defence system" (Janeway and Medzhitov, 2002) although this has also been disputed (Hedrick, 2004). The acquired immune system can be split into two systems, the humoral system (mediated mainly by B cells) and the cell mediated system (mediated mainly by T cells).

The humoral response is mediated by secreted antibodies, produced by cells in the B cell lineage known as plasma cells. These antibodies bind to antigens on the surface of pathogens, flagging them for destruction. Antibodies consist of two large heavy chains and two small light chains. There are several different types of heavy chain, which determine the isotype of the antibody. At the end of the antibody is a region known as the hypervariable region. This binds to a target on a pathogen known as the antigen. Antibodies can be very diverse, and allow the immune system to recognise a wide variety of pathogens.

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 (DCs) play a leading role, with immature DCs 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 DCs 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 acquired immune system is very complex and as such is highly regulated. This complexity is only possible due to the array of specialist cells that make up the system, including B cells and T cells.

1.2 B Cells

B cells mature within the bone marrow where they express an antibody molecule on their membrane known as the B cell receptor (Matthias and Rolink, 2005). When this antibody encounters a new antigen the antigen is internalised and processed. A fragment of the antigen is then presented to a subtype of T cells, known as T helper cells, in complex with a major histocompatability complex (MHC) molecule. If the complex is not recognised, cytokines are produced by the T helper cells which cause the B cell to divide rapidly and differentiate into either a memory B cell or a plasma cell (Fuentes-Panana et al., 2004). A memory B cell has a longer life span than the naïve B cell but expresses the same antibody to protect against repeat infection (McHeyzer-Williams 2005). Plasma cells no longer express the membrane bound antibody; instead they secrete it in large volumes to target the antigen (Shapiro-Shelef and Calame, 2005).

1.3 T cells

T cells, also known as T lymphocytes, are the focus of this piece of work. They form a major component of the immune system and can be diverse in their

actions. T lymphocytes are derived from bone marrow haemopoetic stem cells that enter and populate the thymus. T cells are classified by the heterodimer which comprises their T Cell Receptor (TCR), this may either be the most common and studied T cell, α/β TCR, or the γ/δ TCR which makes up between 1-5 % of peripheral blood T cells (Hayday, 2000). Although, γ/δ T cells make up a relatively small subgroup, they have a distinct receptor profile, and have been suggested to play a role in immune defence against infection, in particular in the gut. They have also been shown to be an important producer of IL-17 (Roark et al., 2008a). Following TCR rearrangement there is a massive IL-7 driven expansion and differentiation of T lymphocytes into $CD4^+/CD8^+$ double positive thymocytes. Through positive and negative selection, cells are selected for either the $CD4^+$ or $CD8^+$ lineage. Positive selection selects for cells that are able to recognise and bind to a MHC complex while negative selection selects for those cells which recognise self antigens. These naïve T lymphocytes then leave the thumus and recirculate through the blood, homing to secondary lymphoid organs such as the lymph nodes. Inside these, the T lymphocytes screen antigen presenting cells (APCs), such as dendritic cells and macrophages, for peptide/MHC complexes. Those that recognise peptide/MHC molecules are activated and will divide into effector T lymphocytes. Each T lymphocyte that is activated by a particular antigen gives rise to a clonal population of cells all expressing the same TCR. This process is referred to as clonal selection. Activated T lymphocytes usually leave the secondary lymphoid organs to accumulate at sites of inflammation or other effector sites. Importantly, some T lymphocytes also become memory cells, which are long-lived antigen-specific T lymphocytes that become activated during a secondary immune response to a pathogen already encountered. They retain the TCR affinity of the originally activated T lymphocyte and are used to act later as effector cells.

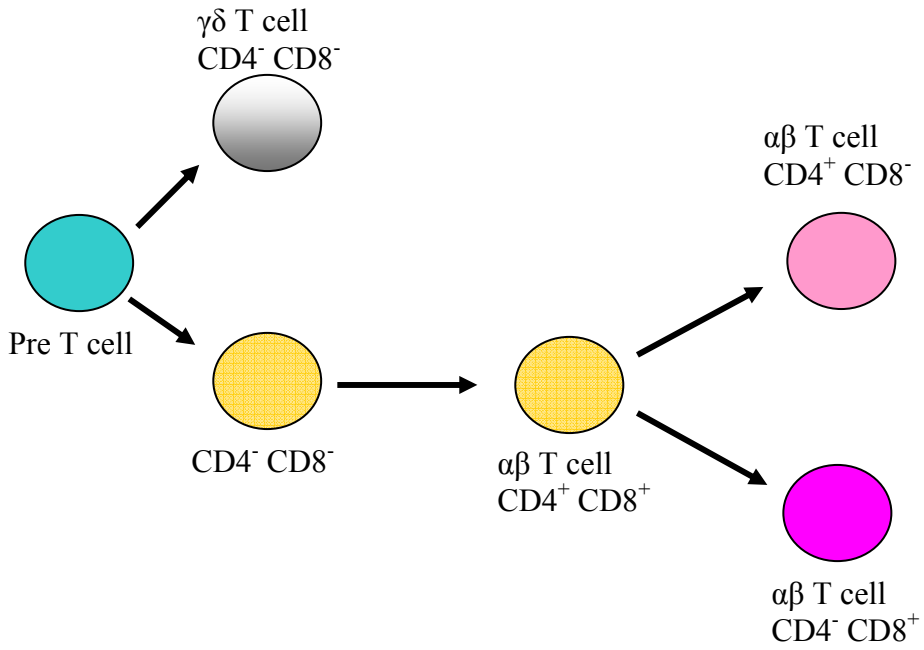

Figure 1.1: Lineage decisions within T cell development. Pre T cells enter the thymus and undergo a series of lineage decisions, including TCR type and whether to become $CD4^+$ T helper cells or $CD8^+$ Killer Cells. Adapted from (Rothenberg et al., 2008)

1.3.1 T lymphocyte activation

Naïve T lymphocyte activation requires two separate signals to be accomplished. Firstly the TCR interacts with specific MHC/peptide complexes presented by APCs. It is largely the CD3 and ζ-chains within the TCR complex that are responsible for transducing signals into the cells via immunosuppressor tyrosine-based activation motifs (ITAMS) (Kane et al., 2000; Sun et al., 2001). These conserved motifs act as tyrosine kinase substrates and once phosphorylated, as binding sites for other kinases. In this way, the TCR complex is coupled to a wide variety of kinases and adaptor proteins that act to initiate signals that induce T lymphocyte activation. CD4, found on the surface of the T lymphocyte, is important for stabilising cell-cell interactions so that the T lymphocyte can interact with the APC. Further stabilisation occurs through adhesion molecules such as leukocyte function associated antigen-1 (LFA-1) which binds intracellular adhesion molecule-1 (ICAM-1) on the APC.

Optimal T cell activation requires a second co-stimulatory signal; this ensures amplification of the TCR signal and prevents the T lymphocyte 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 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 (thought to be mainly p110δ (Okkenhaug et al., 2002)) 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 lymphocyte 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, cytotoxic T lymphocytes antigen-4 (CTLA-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).

Once activated, T lymphocytes differentiate into effector cells. $CD4^+$ T lymphocytes differentiate into Th1, Th2, Th17 or Treg cells, while $CD8^+$ cells differentiate into cytotoxic T lymphocytes.

1.3.2 Differentiation of $CD8^+$ T lymphocytes

Cytotoxic T cells act to kill infected cells and thereby help to prevent the spread of viral and bacterial pathogens. Cytotoxic T cells recognise antigens bound to MHC class 1 molecules presented to them by DCs and all nucleated cells after priming.

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).

1.3.3 Differentiation of $CD4^+$ T lymphocytes

$CD4^+$ T lymphocytes differentiate into a wide variety of T lymphocyte subsets. T helper cells play an important role in establishing and maximising the capabilities of the immune system. There are three different subtypes of T helper cell, known as Th1, Th2 and Th17 depending on the cytokines they produce, and the type of immune system they promote. Another type of $CD4^+$ T lymphocyte is the regulatory T lymphocyte (Treg), which acts to suppress the immune system.

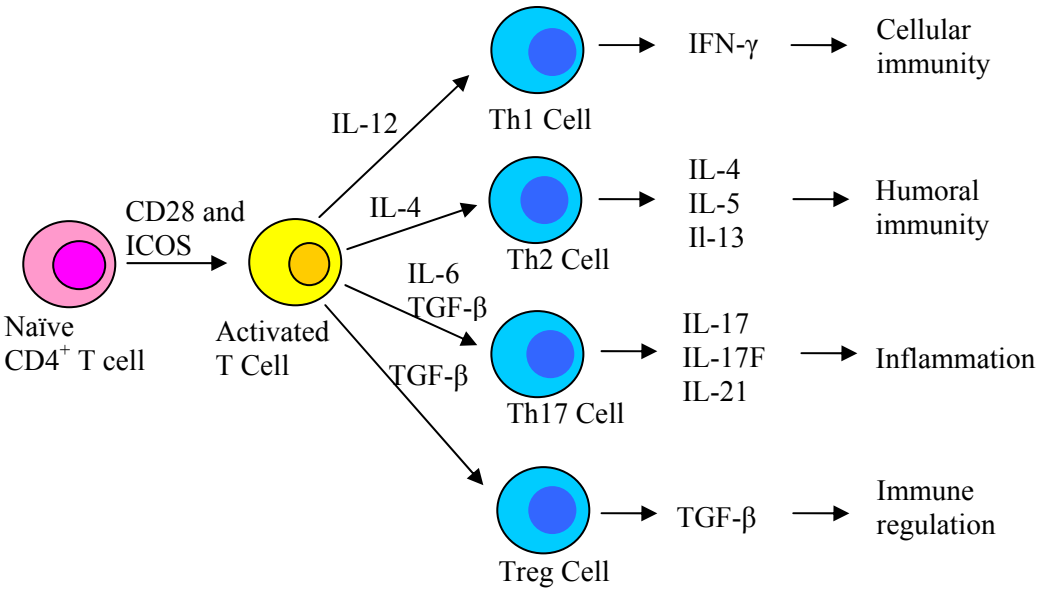

Figure 1.2 Overview of CD4$^+$ cell differentiation. Naïve CD4$^+$ T lymphocytes are activated by binding through the TCR. The presence of cytokines determines the differentiation pathway of the cells, with IL-12 inducing Th1 polarisation, IL-4 inducing Th2 polarisation, IL-6 (and TGF-β) inducing Th17 polarisation, and TGF-β inducting Treg polarisation. Once polarised, the cells secrete multiple cytokines and play a vital role within the immune system. Adapted from (Dong, 2008)

1.3.3.1 Regulatory T lymphocytes

Regulatory T lymphocytes (Tregs), sometimes referred to as suppressor T lymphocytes, have been shown to play a role in the suppression of T cell responses and autoimmunity (Sakaguchi, 2000). Tregs can be divided into two categories, known as 'natural' Treg cells and 'induced' Treg cells (Wraith et al., 2004). Natural Tregs originate in the thymus from the same precursors as other T helper cells and express a transcription factor known as FoxP3 (Fontenot et al., 2003). In mice this marker appears to be limited to T cells with regulatory function, thus acting as a specific marker. Although in humans *FOXP3* is expressed more widely than a subpopulation of T cells, studies have shown that humans and mice whose *FOXP3*/*Foxp3* gene is defective, succumb to diseases with characteristics of autoimmunity (Wildin et al., 2001). These cells can also be further divided based on their expression of the IL-2 receptor CD25 (Nicolson et al., 2006). Induced Tregs are Tregs that can be induced from naïve T lymphocytes in the periphery. These can also express FoxP3, although not all

do. Indeed, one study identified cells that had potent regulatory actions despite being FoxP3$^-$ (Vieira et al., 2004).

Tregs have been shown to be generated in response to the protein TGF-β both *in vitro* and *in vivo* (Chen et al., 2003a; Peng et al., 2004), which leads to the induction of FoxP3. The suppressive activity of Tregs relies on their prior activation by the TCR, in a similar fashion to other CD4$^+$ T lymphocytes. Once activated, the cells mediate their suppressive function through a variety of methods including production of IL-10 (Suri-Payer and Cantor, 2001) and TGF-β (Nakamura et al., 2001), expression of the inhibitory receptor CTLA-4 (Read et al., 2006) or by 'mopping-up' IL-2 through expression of the high-affinity IL-2 receptor CD25. This would prevent effector T cells from proliferating and differentiating (Pandiyan et al., 2007). Overall, it seems that Tregs use a variety of different mechanisms and pathways in order to suppress the immune system. It is likely that these systems vary depending on their circumstances and surroundings (Tang and Bluestone, 2008). Interestingly, TGF-β has been shown to be required for both Treg polarisation and Th17 cell polarisation, raising the possibility that these two cell types are very closely linked, in regulation if not function (Wahl, 2007).

1.3.3.2 Th1 cells

The presentation of antigens on APC of the innate immune system is required for naïve T cells to differentiate into the Th1 lineage (Hunter, 2005; Hibbert et al., 2003). The primary driving force behind Th1 differentiation is the cytokine IL-12, which is facilitated by the action IFN-α, 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 report has also eluded to a T-bet independent pathway, although this process has not been not fully elucidated (Way and Wilson, 2004). Signalling through IL-12 potentates IFN-γ production and upregulation of the IL-18 receptor, giving rise to the mature Th1 effector T cell (Yang et al., 1999).

Th1 cells classically produce IFNγ and TNFα. These are cytokines that stimulate the cellular system and lead to the activation of macrophages and NK cells as well as inducing NO production.

1.3.3.3 Th2 cells

The initiation of Th2 cell differentiation is dependent on antigen presentation by APCs, leading to TCR-mediated signalling. In addition, the cytokine IL-4 is required to bind its receptor, leading to IL-4 receptor-mediated STAT6 activation. This in turn causes upregulation of GATA3 transcription, the transcription factor that has been shown to be the master regulator of Th2 cells.

Differentiated Th2 cells produce the cytokines IL-4, IL-5 and IL-13. IL-4 causes the activation of B cells, leading to isotype switching the production of antibodies, as well as the activation of mast cells, while IL-5 is involved in eosinophil differentiation. As such, Th2 cells play a vital role in humoral immune system.

1.3.3.4 Th17 Cells

Th17 cells are characterised by secretion of the pro-inflammatory cytokines IL-17, IL-6 and TNFα (Park et al., 2005). Studies using infectious mouse models have indicated that Th17 cells are involved in antifungal and antibacterial responses (Happel et al., 2003; Ye et al., 2001). In addition, there is considerable evidence for the involvement of Th17 cells in the pathogenesis of several murine models of autoimmune diseases including Experimental Autoimmune Encephalomyelitis (EAE) and collagen-induced arthritis (Komiyama et al., 2006; Nakae et al., 2003).

Th17 cells do not express the Th1 or Th2 lineage-defining transcription factors T-bet or GATA3 respectively (Bettelli et al., 2007; Stockinger and Veldhoen, 2007a). The recent identification of the thymus-specific orphan nuclear RORγt as the key transcription factor that specifies the Th17 lineage was the final step in establishing this T cell population as a unique and distinct $CD4^+$ T cell population (Ivanov et al., 2006). Differentiation of murine Th17 cells from

naïve T cells has been shown to require TGF-β and IL-6 (Veldhoen et al., 2006), and is negatively regulated by the Th1 and Th2 cytokines IFN-γ and IL-4, respectively (Mangan et al., 2006; Stockinger and Veldhoen, 2007b). However, there are important differences in the requirements for differentiation of Th17 cells in humans and mice. For example, despite the critical function of TGF-β in the differentiation of mouse Th17 cells, this cytokine appears dispensable for IL-17 production in human cells (Evans et al., 2007). Additonally, IL-1β is a very effective inducer of IL-17 expression in activated human $CD4^+$ T cells, but only transiently upregulates RORγt. IL-6 is a poor inducer of human Th17 differentation, but in combination with IL-1β, promotes sustained RORγt expression (costa-Rodriguez et al., 2007a). Finally, IL-23 appears to play a role in the differentiation of naïve human T cells toward a Th17 phenotype (Chen et al., 2007; Wilson et al., 2007), while in the mouse it acts on memory T cells and is important in the maintenance of Th17 cells, promoting their responses *in vivo* (Aggarwal et al., 2003).

1.3.3.4.1 IL-17

The IL-17 family of cytokines consists of six cytokines termed IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. Most study has been performed on IL-17A and IL-17F, and these two family members are the most closely related (Kolls and Linden, 2004). Multiple cell types including $CD4^+$ αβ T cells, γδ T cells, NK cells, and neutrophils have been shown to produce IL-17A and IL-17F (Roark et al., 2008b). Studies on IL-17 have shown that it induces the mobilisation, recruitment and activation of neutrophils and triggers the production of pro-inflammatory cytokines and chemokines by a broad range of cellular targets including epithelial cells, endothelial cells and macrophages (Weaver et al., 2007).

1.3.3.5 Reciprocal inhibition

The specific circumstances that surround the generation of Th1, Th2 or Th17 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); this model also extends to Th17 cells. Both IL-4 and IFNγ have been shown to negatively regulate the polarisation of Th17 cells. Interestingly, IL-2, important for the proliferation of other T lymphocyte subsets, has also been shown to inhibit Th17 cell polarisation. (Laurence et al., 2007)

1.4 Cell migration

In order for the immune system to function efficiently, white blood cells or leukocytes need to be able to circulate not just through the blood and lymphatic system but also move into and through tissue. This allows any tissue based infections to be detected and fought. To induce cells to leave the blood and enter tissue or to move towards sites of infection, several cell types such as endothelial cells, fibroblasts and leukocytes can produce proteins called chemokines in response to stimuli such as bacteria, pro-inflammatory cytokines and viruses. The members of the chemokine family of chemoattractants determine leukocyte localisation and consequently control their multiple functions in innate and acquired immunity. Chemokines can regulate leukocyte entry into the circulation, emigration from blood, positioning in local tissue microenvironments and departure from the tissues. Besides influencing cellular movement and positioning, chemokines can rapidly induce alterations in leukocyte adhesive properties (by modulating integrins), regulate proliferation and differentiation, apoptosis, cytokine release and degranulation.

1.4.1 Migration - The Physiological Role of Chemokines

In order for the immune system to function efficiently, white blood cells or leukocytes need to be able to circulate not just through the blood and lymphatic system but also move into and through tissue (Goldsby et al., 2000). For example, during immunosurveillance T cells traffic into regional lymphoid organs such as lymph nodes and the gut-associated Peyer's patch. In addition,

as part of the immune response, cells are required to migrate from the blood and into the infected tissue. 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. Briefly, interactions mediated by surface adhesion molecules cause leukocytes to tether to and then roll along the endothelium. Chemoattractants, such as chemokines presented on the endothelial cell surfaces by glycosaminoglycan's (GAGs), trigger increases in integrin affinity and avidity thereby inducing firmer adhesion of the leukocytes to the endothelial cells. The leukocytes can then transmigrate through the vascular wall into the extravascular tissue where they are then further guided by chemokines and other chemoattractants into specific tissue environments.

1.4.2 Types of Migration

Cells of the immune system are capable of migration in several defined ways. The term chemotaxis refers to directed migration of a cell towards a soluble chemotactic source, i.e. up the chemokine gradient. Chemofugetaxis describes 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 is thought to contribute to thymic emigration (Poznansky et al., 2000).

In vivo, chemokines exist not only as soluble peptides, but can also be surface bound. Haptotaxis describes the directional movement of a cell towards a chemotactic gradient but on an immobilised substrate, while haptorepulsion describes migration away from bound chemokine.

Finally, some cells migrate in the absence of chemokine and therefore a chemokine gradient. This is known as chemokinesis, where cells may spontaneously generate cytoplasmic projections and retractions and display migratory properties, yet this movement of the cell is without any directionality.

1.5 The processes involved in directed cell migration

Migration of any cell is made up several distinct processes. Firstly, the cells must sense the chemoattractant gradient and undergo polarisation, causing extension of protrusions or pseudopodia in the direction of migration. These pseudopodia then bind to the substratum, for instance the extracellular matrix, stabilising the cell. The cell then moves forward by contracting the cell body and releasing attachment sites at the rear.

1.5.1 Extension of pseudopodia

During random migration or chemokinesis, cytoplasm projections extend and contract over the surface of the cell. Following chemoattractant binding, 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.

1.5.2 Polarisation

An early event within leukocyte migration is the polarisation 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). Polarisation can also be referred to in terms of the positioning of the nucleus and reorientation of the microtubule-organizing centre and Golgi apparatus towards the leading edge. Within directional sensing, it is important that once a cell detects a shallow chemoatttractant 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 subcellular localisation of F-actin and assembled myosin II, this allows rapid responses to chemoattractant gradients (Chung et al., 2001a).

One of the first polarised responses in many leukocytes is the accumulation of PtdIns(3,4,5)P_3 (PIP3) at the leading edge of the cell. This is caused by the activation of PI3K at the leading edge, and subsequent signalling through Rho GTPase that results in further accumulation of PIP3 at the leading edge and activation of PTEN at the sides and back of the cell. This results in the degradation of PIP3. The accumulation of PIP3 results in the recruitment of PH-domain containing proteins at the leading edge of the cell.

1.5.3 Protusion and adhesion formation

Several different types of pseudopods exist. Lamellipodia are large, broad protusions that act as the motor which pulls the cell forward during cell migration. Filopodia however, are spike-like protusions which may instead act as sensors with which to explore the extracellular environment.

These protusions are the result of actin polymerisation. 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 (Fig. 1.3). During migration the generation of a new filament is focussed 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, where they inhibit the dephosphoylation 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., 2004a).

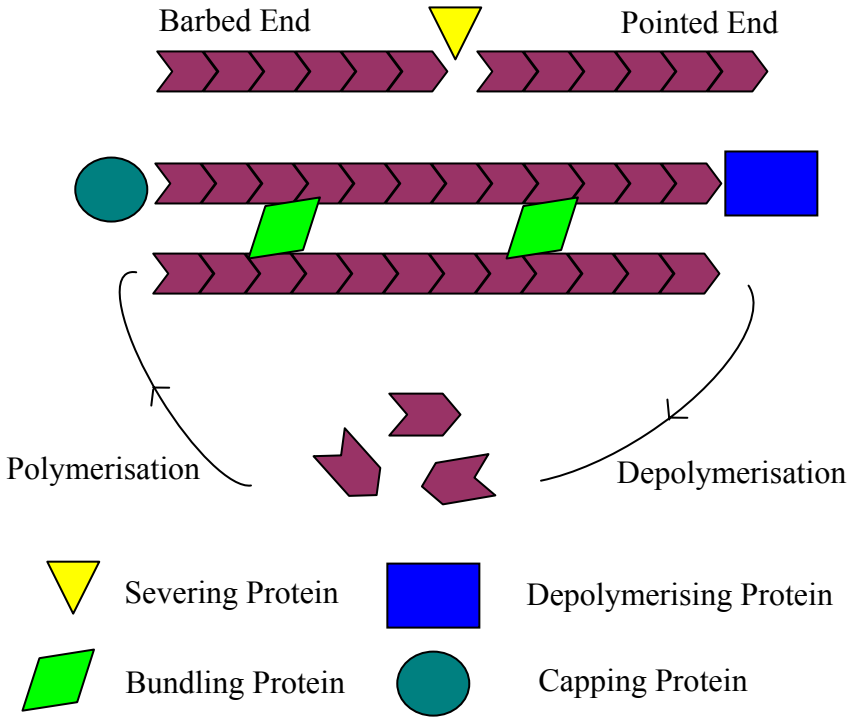

Figure 1.3 Actin filaments: structure and dynamics. Actin monomers known as G-actin bind ATP and assemble much more rapidly at the 'barbed end' compared to the 'pointed end'. During treadmilling, the polymerisation rate and depolymeriztion rate are equal thus filament length is maintained at a constant. Several proteins are known to bind to actin; capping proteins may bind to the barbed end preventing addition of further momomers, while some as demonstrated are known as crosslinking proteins, allowing several filaments to bind together. Adapted from (Revenu et al., 2004b)

Several other steps are also involved in migration. The leading edge must adhere to the underlying substratum; otherwise they will retreat back into the cell. Adherence is mediated by integrin family receptors. These are heterodimeric receptors composed of non-covalently associated α and β subunits, including α4 integrins such as $\alpha_4\beta_1$ (VLA-4) and β2 integrins such as $\alpha_L\beta_2$. Chemoattractants signal to activate or functionally upregulate integrins from their normal low affinity state. Once activated, they preferentially localise to the leading edge of the cell where they await binding by ligands. Once bound to their ligands, they undergo conformational change and clustering, resulting in intracellular signalling and regulation of focal adhesion site formation.

1.5.4 Release of adhesion

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).

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 cysteine motif. The majority of the known chemokines (around 43 in humans) reside within two major classes, the CXC chemokines (in which the two amino terminal cysteine residues are separated by a single amino acid residue) and the CC chemokines (in which two amino terminal cysteine residues are adjacent). There are also two other minor classes, the CX3C (three conserved amino acids separate the cysteines) and C (containing one cysteine) chemokine families. Chemokines can also be divided based upon their function. Some chemokines, such as CXCL12, are mainly homeostatic, meaning they are present at all times and have responsibility for directing leukocytes during immunosurveillance. Others, such as CCL5 which recruits neutrophils, are inflammatory. These are upregulated during infection or cell damage and are important for innate and adaptive immunity. However, the division of chemokines into these two camps is not categorical, with some chemokines falling into both camps. All chemokines exert their effects through chemokine receptors, part of the G-protein coupled receptor (GPCR) family.

1.6.2 G-protein coupled receptors

G-protein coupled receptors, or GPCRs, comprise a large family of transmembrane receptors that function to convert extracellular signals into intracellular signals. In addition to chemokines, GPCR's can bind a wide range of ligands including neurotransmitters and hormones. 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). GPCRs are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices resulting in three intracellular loops. The extracellular parts of the receptor can be glycosylated and contain two highly-conserved cysteine residues that form disulfide bonds to stabilize the receptor structure. (Allen et al., 2007).

To allow the transmission of a signal, the receptor must go through several processes. First, it must bind the ligand. This binding then initiates a conformational change within the receptor, activating it. Finally, this conformational change results in the activation or inhibition of secondary messengers within the cell.

The linking of the activated receptor to 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. In the resting state, the G-protein is inactive, not associated with the receptor and has a molecule of GDP bound to the α subunit. When the receptor binds its ligand, a conformational change in the receptor allows it to now bind the G-protein, which causes the displacement of the GDP molecule, replacing it with a GTP molecule. This exchange leads to the removal of the hydrophobic pocket to which G-βγ binds, thus reducing the affinity of Gα-GTP and G-βγ and resulting in the dissociation of the Gα-GTP from the Gβγ complex (Lambright et al., 1994).

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; G_s, $G_{i/o}$, G_q, $G_{12/13}$. 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 Ca^{2+} 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 increases its activity (Suire et al., 2002). Activation of class IA isoforms is thought to be via the Gαi 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).

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 desensitisation.

Desensitisation 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, 2004b). The extent of receptor desensitisation can vary depending on the cellular system, from attenuation to complete termination of the signal (Ferguson, 2001b; Aramori et al., 1997).

Internalisation or sequestration of GPCRs is another mechanism in which signalling and function through GPCRs is controlled. In comparison to desensitisation 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, 2001a; Cheng et al., 2000; Kristiansen, 2004a).

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 resensitises 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 β_2-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 we must decipher which 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).

1.6.6 Chemokine receptor expression

Although 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.

The expression profile of receptors on T lymphocytes is indicative of functionally distinct T lymphocytes subsets. Naïve 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 naïve population is observed between the blood and lymph nodes, which is aided by the expression of CCR7 and L-selectin (Sallusto et al., 1999).

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., 1998b; Bonecchi et al., 1998b). 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., 1996). CCR1 has been shown to be expressed on Th1 while CCL11 and CCR3 are preferentially expressed on Th2. There are conflicting reported 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., 1998a; Bonecchi et al., 1998a). 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 The Role of Phosphoinositide 3-kinase(s) in T Lymphocyte Migration.

The co-ordinated and directional trafficking of T lymphocytes in lymphoid and peripheral tissues is a key process in immunosurveillance and immune responses. This involves a multi-step adhesion cascade of selectin- or integrin-(and their corresponding vascular ligands) supported rolling, chemokine-mediated integrin activation and firm integrin-mediated adhesion to the microvasculature endothelium followed by transmigration through the vessel wall and further migration in extravascular tissue. For a cell to migrate to a chemoattractant source it must be polarised which means that the molecular processes at the front (leading edge) and the back (uropod) of a moving cell are different. Establishing and maintaining cell polarity in response to extracellular stimuli appear to be mediated by a set of interlinked positive-feedback loops involving phosphoinositide 3-kinase (PI3Ks), Rho family GTPases, integrins, microtubules and vesicular transport. The relative contributions of these various signals depends on the cell type and the specific stimulus. These intracellular signals result in reorganisation of the cytoskeleton and cell adhesion causing the cells to send out pseudopodia and crawl up the chemoattractant gradient. PI3K-dependent signalling events have previously been demonstrated in several cell systems to contribute to several aspects of the migratory machinery including gradient sensing, signal amplification, actin reorganisation and hence cell motility.

1.8 The PI3K Family

1.8.1 Phosphoinoitide 3-kinase

Phosphoinoitide 3-kinase (PI3K) mediates the formation of D-3 phosphoinositide lipids by transferring the terminal phosphate of ATP to the D-3 position of the inositol head group of phosphoinositide lipids (Fig. 1.4).

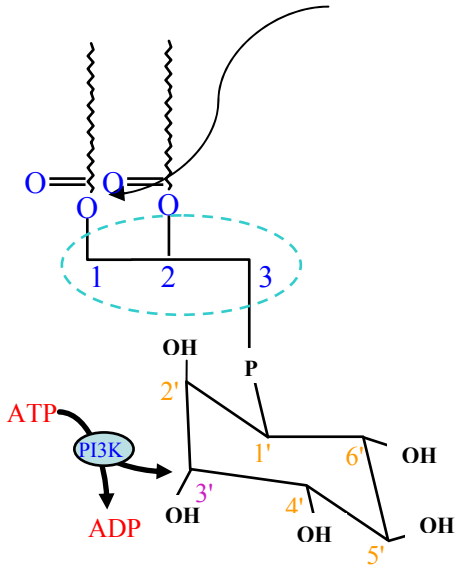

Figure 1.4 Transfer of terminal phosphate of ATP to D-3 position of the inositol head group of phosphoinositide lipids. Adapted from (Vanhaesebroeck and Alessi, 2000)

PI3K can thus potentially produce three products: phosphatidylinositol (3)-phosphate [PtdIns(3)P], phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] (Fig. 1.5) (DuBois et al., 1998; Vanhaesebroeck and Waterfield, 1999).

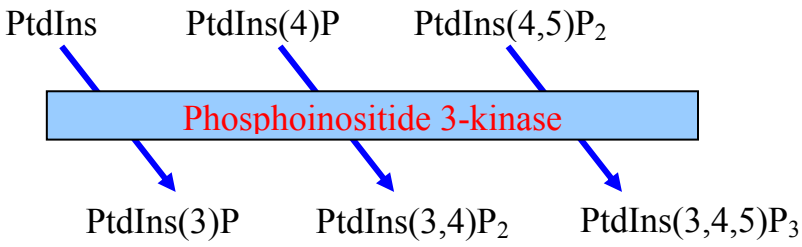

Figure 1.5 Formation of phosphoinositide lipids by PI3K.

PI(3)P is constitutively present in eukaryotic cells and its levels are largely unaltered upon cellular stimulation. In contrast, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of receptors suggesting a second messenger function. There are multiple isoforms of PI3K (Fig. 1.6) in mammalian cells and these are subdivided into three classes on the basis of the *in vitro* lipid substrate specificity, structure and likely mode of regulation (Koyasu, 2003a).

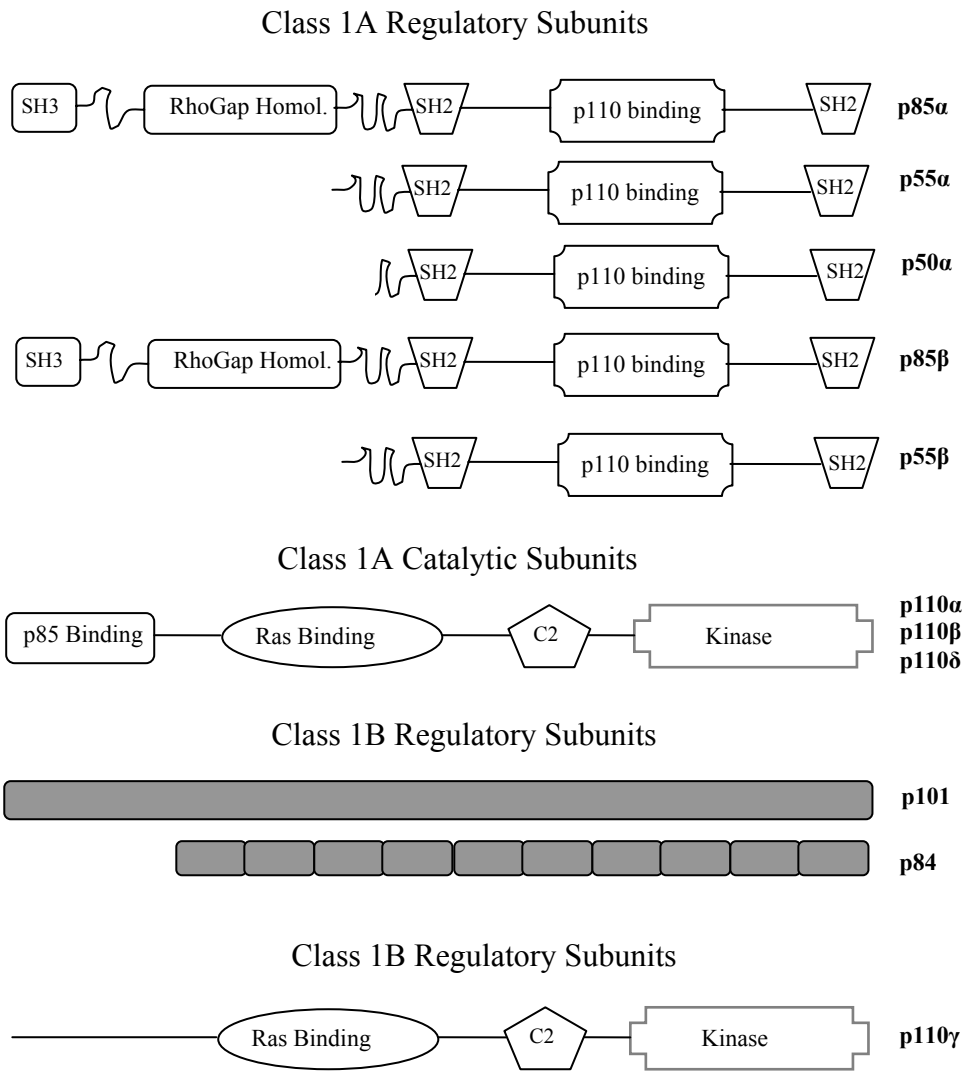

Figure 1.6 Structural characteristics of the PI3K family. Adapted from (Koyasu, 2003b)

1.8.1.1 Class I

The Class I PI3K phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, resulting in the formation of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 *in vitro*, whereas PtdIns(4,5)P_2 is the main substrate *in vivo*. They also interact with Ras and form heterodimeric complexes with adaptor proteins that link them to different upstream signalling events. The prototypical class IA PI3K is a heterodimer consisting of the 85kDa regulatory subunit (responsible for protein-protein interactions either via protein tyrosine phosphate-binding SH2 domains or SH3 domains and/or proline-rich regions) and a catalytic 110kDa subunit. Mammals have three genes for the class IA p110 subunits, which encode p110α, p110β and p110δ. The existence of multiple isoforms of both components, which do not appear to preferentially associate, means that there is considerable scope for specific variation between tissues and with association to different receptor tyrosine kinases. The class IB PI3K is stimulated by G protein βγ subunits and does not interact with the SH2-containing adaptors that bind class IA PI3K. Instead, the first identified member of this family, p110γ, associates with a unique p101 adaptor molecule.

1.8.1.2 Class II and Class III

Class II PI3Ks are large proteins that have a catalytic domain that is 45-50% homologous to Class I PI3K. Class II PI3K also have a C-terminal region that has homology to the C2 domains that mediate calcium/lipid binding in classical protein kinase C isoforms (Cantrell, 2001). Class II PI3K preferentially phosphorylate PtdIns and PtdIns(4)P *in vitro*, whereas the Class III PI3K utilise only PI as a substrate. The mammalian homologue of Class III PI3K is likely to be the main source of PtdIns(3)P.

1.8.2 Phosphatases

The PI3K family mediate various biological effects through the action of the D-3 phosphorylated phosphoinositide second messengers. The levels of the second messenger are regulated by PTEN (phosphatase and tensin homolog) and SHIP (Src-homology-2 containing inositide 5'-phosphatase) that can specifically

hydrolyse the D-3 and D-5 phosphate groups respectively from the lipid products and thus control the level of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

1.8.3 Downstream targets and functions

A number of proteins have been identified that directly bind PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 via pleckstrin homology (PH) domains, which are lipid binding domains, including protein kinase B (PKB/Akt), PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK-1), Bruton's tyrosine kinase, various PLC isoforms and exchange factors for the ADP-ribosylation factor family of GTP-binding proteins. Moreover, the D-3 phosphoinositide lipids have been linked to the triggering of a diverse array of cellular responses including cell survival, mitogenesis, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide production as well as actin polymerisation and chemotaxis.

1.9 Class I PI3Ks and T lymphocyte Migration.

Most of what we understand about biochemical events in T cells moving toward a chemoattractant gradient has been derived from study of chemokine signal transduction**.** Chemokines mediate at least two chemokine-mediated processes, namely chemokine-mediated integrin activation to mediate cell adhesion and chemokine-induced chemotaxis (that is likely to be integrin independent). PI3K activation seems to be a signalling event shared by most chemokine receptors expressed on T cells. Signalling via CXCR4 has been studied in most detail and SDF-1/CXCL12 triggers a transient PTX-sensitive accumulation of PI(3,4,5)P_3 in the Jurkat leukemic cell line. Similar lipid accumulation in murine and human T lymphocytes stimulated with ELC/CCL19 and SLC/CCL21 has also been reported. The use of PI3K inhibitors has also revealed that *in vitro* chemotaxis across synthetic membranes of PBLs and NK cells in response to CXCL12 and several other chemokines such as RANTES/CCL5, can be severely attenuated by PI3K inhibitors. During migration and chemotaxis, there are at least two chemokine-mediated processes operating, namely chemokine-mediated integrin activation to mediate cell adhesion and chemokine-induced chemotaxis (that is likely to be integrin independent). There is some evidence

that enhanced integrin mobility in chemokine-stimulated lymphocytes is dependent on PI3K activation. Generally however, rapid integrin activation on rolling lymphocytes does not depend on PI3K activation processes.

Chemokine interaction with GPCRs on lymphocytes has been shown to depend predominantly on Gi proteins. This has led to the assumption that these receptors are coupled to the βγ-dependent p110γ isoform. Indeed, this does appear to be the case, although several chemokine receptors can additionally activate other PI3K isoforms. Significant progress has been made in resolving the confusion concerning the specific role of PI3K in lymphocyte migration. This has been made possible by availability of mice deficient in the p110γ catalytic isoform as well as analysis of mice expressing a mutant, catalytically inactive class 1A p110δ isoform. The *in vitro* migration of p110γ-deficient $CD4^+$ and $CD8^+$ T cells to CCL19 and CXCL12 and CCL21 is significantly decreased compared to cells from wild type mice. In contrast, T cell responses were largely unaffected by p110δ deficiency. Hence, in settings where T cell migration required PI3K activation, the p110γ isoform appears to be the predominant isoform required. This correlates with observations that p110γ selective inhibitors reduce numbers of $CD4^+$ memory T cells in models of systemic lupus. Interestingly, B cell migration to chemokines was not significantly affected by p110γ deficiency, thus implicating involvement of other PI3K isoforms or signalling pathways in B cell migration. In this regard, analysis of p110δ deficient B cells showed a defect in B cell chemotaxis to CXCL13, whilst responses to CCR7 and CXCR4 ligands were less affected. Adoptive transfer experiments with B cells expressing inactive p110δ revealed diminished CXCR5-mediated homing to Peyer's patches and splenic white pulp cords. The ability of p110δ to function downstream of chemokines receptors in lymphocyte chemotactic responses is consistent with the finding that a broad spectrum loss-of-function mutant that disrupts all class 1A catalytic isoforms, reduced chemotactic responses of leukemic T cells to CXCL12. Together, these data indicate that individual lymphoid chemokine receptors have differing dependence on PI3K-dependent signals for achieving ordered migration.

1.10 Downstream Effectors of PI3K

The production of PIP_2 and PIP_3 by PI3K leads to the activation of several different signalling pathways and proteins. A number of these proteins can trace their activation back to a master kinase called protein dependent kinase 1 or PDK-1. Activation of this protein can lead to phosphorylation of many other proteins including Akt, Glycogen synthase kinase-3 and Protein Kinase Cδ.

1.10.1 PDK-1

PDK-1 is a 63 kDa Ser / Thr kinase ubiquitously expressed in human tissue. It is recruited to the membrane by its PH domain which binds $PI(3,4,5)P_3$ and $PI(3,4)P_2$, where it has been shown to phosphorylate many protein kinases including Akt and protein kinase C (PKC) (Toker and Newton, 2000b). Its regulation is still unclear, and was originally thought to be constitutively active however recent evidence suggests its activity to be dependent on substrate conformation and subcellular location, providing a good mechanism for discriminating between one subset of targets over another (Collins et al., 2005). As PDK-1 controls the activation of a large number of proteins (Fig. 1.7), it is known as a master kinase, crucial for the functioning of large numbers of cellular pathways (Lawlor et al., 2002). For a long time after its discovery, it was thought that the ability of PDK-1 to phosphorylate its substrates was dependent on PI3K and the production of phospholipids. However, there is now some evidence that PDK-1 can function independently of PI3K with some substrates, notably PKC (Sonnenburg et al., 2001).

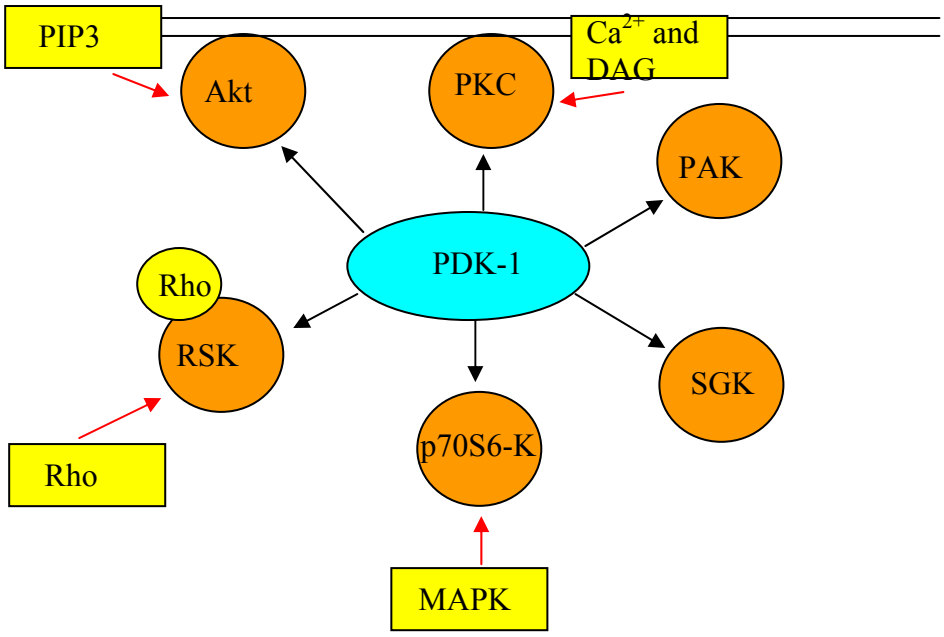

Figure 1.7 Schematic showing Protein Kinase Substrates of PDK-1. Structures in yellow represent the activating mechanisms which induce a conformational switch in the substrate allowing subsequent PDK-1 phosphorylation of the activation loop. Examples of this switch include: phosphorylation of p70S6-K and p90RSK by MAPK (and possibly other pathways comprising TOR [target of rapamycin] and PKC); binding of the small GTPase Rho to PRK; PtdIns-3,4,5-P_3 binding to the PH domain of Akt/PKB; and Ca^{2+} and DAG binding of newly synthesised PKC. (adapted from Toker and Newton, 2000)

1.10.2 Akt/PKB

Akt (also known as protein kinase B or PKB (Burgering and Coffer, 1995)) is a serine threonine kinase that is recruited to the membrane by its PH domain which binds PI(3,4,5)P_3 and PI(3,4)P_2. At the membrane it is phosphorylated and activated by the protein kinase: protein dependent kinase-1 (PDK-1) which is also recruited to the membrane by its PH domain. Phosphorylation of Akt occurs at two sites - Thr308 and Ser473 in the kinase activation loop, and hydrophobic region of the C-terminal regulatory domain (Toker and Newton, 2000b). PDK-1 is known to be responsible for the phosphorylation of Thr308 but the kinase responsible for the Ser473 phosphorylation remains elusive, although evidence now points to the mTor-RICTOR complex (Hresko and

Mueckler, 2005). Akt activity can be modulated by phosphatases such as PTEN and SHIP which reduce the levels of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ preventing Akt from localising to the membrane (Stambolic et al., 1998). Akt has been shown to play a role in various signalling pathways including cell growth, nutrient metabolism, proliferation and survival. In the mould *Dictyostelium* and in some mammalian cells an essential role in chemotaxis has also been demonstrated (Meili et al., 1999).

1.10.3 Glycogen Synthase Kinase-3

Glycogen synthase kinase-3 or GSK-3 is a serine / threonine kinase that was first isolated as the enzyme responsible for insulin stimulated glycogen synthase inactivation (Doble and Woodgett, 2003). However, a much broader role for GSK-3 has recently been discovered, with effects on cell morphology, transcription factor regulation, protein synthesis and Wnt signalling (Ferkey and Kimelman, 2000). There are two closely related isoforms of GSK-3, named GSK-3α and GSK-3β and both are ubiquitously expressed, although can have differing roles within the cell.

GKS-3 is rare among protein kinases in that it is constitutively active and is inactivated by phosphorylation in response to signals (Fig. 1.8). Phosphorylation occurs at Ser21 in GSK-3α and Ser9 in GSK-3β and is catalysed by Akt in response to PI3K activation, mitogen activated protein kinase-activated protein kinase 1 (MAPKAP-K1 or RSK) in response to growth factor stimulation, or by p70 s6 kinase-1 (Ohteki et al., 2000). Phosphorylation creates a primed pseudo-substrate that binds intramolecularly to the kinase pocket, blocking it and preventing phosphorylation of other substrates. This method of inactivation also gives insight into the required state of substrates before they can be phosphorylated. GSK-3, unlike many other kinases, has a preference for primed, pre-phosphorylated substrates (Doble and Woodgett, 2003). This pre-phosphorylation is not required but greatly increases the efficiency of phosphorylation and is thought to work by binding a pocket within GSK-3, optimising the orientation of the kinase domains and placing the

substrate at the correct position within the catalytic groove for phosphorylation to occur.

Another method of GSK-3 inactivation is via the Wnt pathway. The Wnts are a family of secreted protein ligands that can influence cell growth, differentiation, migration and fate, with at least 19 Wnts in mammals (Prunier et al., 2004). One of the pathways regulated by Wnt molecules is known as the Wnt/β catenin pathway and results in gene activation (Akiyama, 2000). In unstimulated cells GSK-3 is associated with axin, is active and can phosphorylate β catenin, targeting it for ubiquitination and degradation in the proteosome. When Wnt binds to its receptor on the cell surface it causes phosphorylation of a protein called dishevelled, releasing the protein FRAT. FRAT can then bind GSK-3, dissassociating it from axin, and effectively inactivating it. The Wnt pathway appears to be insulated from PI3K dependent GSK-3 inactivation although how this insulation occurs is not yet clear (Dominguez and Green, 2001).

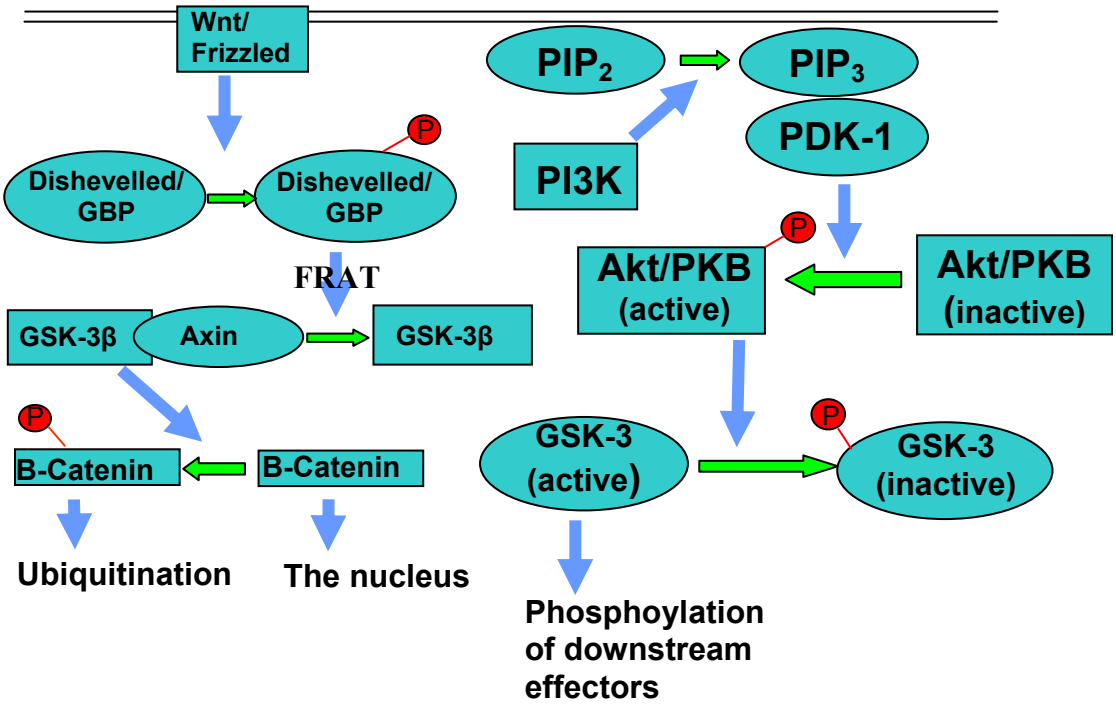

Figure 1.8: Cellular pathways for inactivating GSK-3. GSK-3 inactivation can result from Wnt activation (left panel) leading to the ubiquitination of β-catenin or Akt activation (right panel) resulting in the inactivation of several downstream pathways.

1.10.4 Protein Kinase C Delta

Protein Kinase C Delta or PKCδ is a member of the protein kinase C family of serine threonine kinases. The PKC family can be divided into three subgroups called conventional, novel and atypical and are divided based on their structure and function. Conventional isoforms are sensitive to DAG and are Ca^{2+} responsive through their C2 domain. Novel isoforms, such as PKCδ, are DAG sensitive but not Ca^{2+} responsive as their C2 domains lack the coordinating residues; instead they form a phosphotyrosine binding domain. Atypical isoforms are both DAG and Ca^{2+} insensitive and instead are regulated through a PB1 domain which can bind proteins containing a PC motif (Poole et al., 2004).

PKCδ is targeted to the membrane and activated through binding DAG through its C1 domain. In addition to this, phosphorylation at several sites including Tyr525 and Thr505 are thought to be important in activation and enzyme stability. It has been reported that phosphorylation of Thr505, which occurs via the master kinase PDK-1, is not a prerequisite for enzyme activity as previously thought (Stempka et al., 1997), but can contribute to enzyme stability and so increasing levels of phosphorylation of the downstream targets. Phosphorylation of Thr505 by PDK-1 is thought to occur independently of PI3K in some cases, giving PKCδ a PI3K independent role (Sonnenburg et al., 2001). PKCδ is ubiquitously expressed and has been implicated in many cellular processes including cell cycle progression, gene regulation and fibroblast contractility and motility, giving it a role in cell migration (Cai et al., 2005).

1.10.5 Guanine nucleotide binding proteins: the Ras superfamily of small GTPases

Guanosine Triphosphateses (GTPases) are a 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 can be found in two forms, in their active state they are bound to GTP and following hydrolysis of GTP, while in their inactive state, they are 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 dissassociation 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 and 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 they can insert into the plasma membrane where they interact with GEFs and thus become active (Robbe et al., 2003).

1.10.6. Ras Family

Ras Sarcoma oncoproteins is a 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 guanyl releasing factor) and RasGRP (Ras guanyl 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.

1.10.7 Mitogen-activated protein kinase

The mitogen-activated protein kinases (MAPK) pathway, is comprised of many different subfamiles, the best known of these are ERK (extracellular signal-regulated kinase), JNK (c-jun-NH_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 (Fig. 1.9). 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).

1.10.8 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).

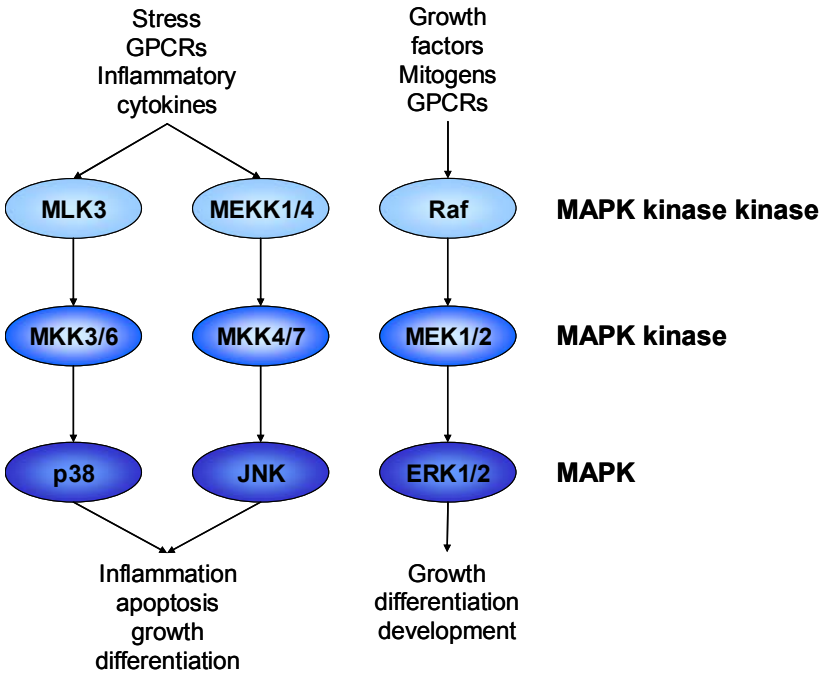

Figure 1.9 Schematic representation of the structure of MAPK pathways. Each MAP kinase family member; p38, JNK and ERK1/2 is activated by successive activation of a MAPK kinase kinase and MAPK kinase.

1.10.9 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 transendothethial 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.

1.10.10 Ras Homologous Family

Ras Homologous (Rho) family have also been shown to be key regulators of chemokine mediated signalling that induce actin organisation, 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, through control of GEF and GAP and regulation of cellular location and expression levels.

1.10.10.1 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 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, and mediating uropod retraction, an essential role in migrating leukocytes. In comparison, the polymerisation of actin and production of lamellipodia and filopodia by Rac and Cdc42 is provided at the front or leading edge of the cell.

1.10.10.2 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 processes, 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 RasGRP, 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 This demonstrates a requirement in the neutrophils for gradient sensing and orientation (Sun et al., 2004).

1.10.11 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-organising 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 polymerisation (Millard et al., 2004).

1.11 Aims of the Study

Chemotaxis of T lymphocytes is a critical step of immune surveillance and the immune response. Indeed, altered levels of chemotaxis have been observed in a range of diseases including multiple sclerosis, arthritis and psoriasis. As such, it is vital that the signalling pathways involved in chemotaxis are well characterised, enabling the discovery of possible future drug targets. PI3K was previously thought to have a central role in chemotaxis. However, previous work performed within the laboratory revealed that PI3K may be a dispensable signal (Cronshaw et al., 2004), and that its importance is determined by chemoattractant, cell type and/or differentiation state (Ward, 2006).

In addition, recently published work has revealed the existence of another subtype of T helper cells. These cells, known as Th17 cells, had been shown to be involved in several autoimmune diseases. However, no work had been performed to determine the chemokine receptor expression profile of Th17 cells, or the signalling pathways that mediated chemotaxis.

Therefore, the aims of the project were as follows:

- Using pharmacological tools, gain more insight into the role of PI3K in T lymphocyte chemotaxis by exploring the role of downstream kinases in cell migration.
- Generate Th17 cells and determine what chemokine receptors are expressed on the cell surface
- Determine the role of PI3K, if any, in the migration of Th17 cells.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell Isolation and Culture Materials

Cell culture media, RPMI-1640, foetal bovine serum, penicillin and streptomycin, L-glutamine, HEPES, trypan blue, 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) and Nycoprep 1.077A were purchased from Axis-Shield (Cambridgeshire, UK). Tween-20, Staphylococcal Enterotoxin B, 2-Mercaproethanol and recombinant hTGF-β1 were purchased from Sigma-Aldrich (Gillingham, UK) and Dynalbeads® CD3/CD28 T cell expander beads from Invitrogen (Paisley, UK). Recombinant mIL-1β, IL-2, IL-6 and TNF-α were purchased from Peprotech (London, UK). $CD4^+$ and pan-T cell isolation kits and LS columns were purchased from Miltenyi Biotech (UK). Recombinant mIL-23 and anti-IFN-γ Ab (clone no:37895) were purchased from R&D systems (Abingdon, UK). Anti-IL-4 Ab (clone no:11HB11) was produced in-house at UCB (Slough, 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) and anti-phospho Akt^{Ser473} (catalogue no: 9271) were purchased from Cell Signalling Technologies (NEB, Hitchin, UK). Anti-CD4-TC (clone no:RM4-5), anti-IFNγ-FITC and anti-IFNγ-AF610 (clone no:XMG1.2) were purchased from Invitrogen (Paisley, UK). Goat polyclonal pan Akt (catalogue no: sc1618) was obtained from Santa Cruz Biotechnologies (Santa Cruz, USA). Anti-CCR5-PE (clone no:C34-3448) and anti-IL-17-PE (clone no:TC11-18H10.1) were purchased from BD Biosciences (Oxford, UK). Monoclonal Anti-human CXCR4-fluorescein (clone 12G5), Mouse IgG_1 isotype control (clone 11711), Anti-CCR3-FITC (clone no:83101), anti-CCR6-FITC (clone no:140706) and anti-CCR9-FITC (clone no:242503)

were purchased from R&D systems (UK). Anti-CCR7-PE (clone no:EBI-1) was purchased from e-Bioscience (San Diego, USA). Anti-CCR2 (clone no:MC-21) was a kind gift from Professor Matthias Mack (University of Regensburg, Germany). Anti-Rat IgG-FITC was purchased from Sigma-Aldrich. All other isotype antibodies were purchased from Invitrogen or BD Biosciences. Secondary Antibodies for immunoblotting were purchased from DAKO (Glostrup, Denmark).

2.1.3 Chemokines

Human recombinant CXCL12 (catalogue no: 350-NS) and mouse recombinant CCL25 were purchased from R&D Systems (Abingdon, U.K.). All other chemokines were purchased from Peprotech (London, U.K.).

2.1.4 Assay systems and kits

Chemo Tx® System was purchased from Neuro Probe (Gaithersburg, USA). 24-well Transwell® chemotaxis plates were purchased from Corning (New York, USA). 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. Omnia® Lysate Assay for Akt/PKB was purchased from Invitrogen. Human Human Phospho-MAPK Array Kit was purchased from R&D systems limited.

2.1.5 Inhibitors

LY294002 and Wortmannin were purchased from Sigma (Poole, UK). Akti-1/2 and Rottlerin were purchased from Calbiochem (Gibbstown, USA). IC87114 (patent WO 0181346) and ZSTK474 (patent WO 037812) has been described elsewhere and was synthesised solely for this study.

2.1.6 Mice

DO11.10 mice (John Radcliffe Hospital, Oxford, UK), carrying a transgenic TCR specific for the $OVA_{323–339}$ peptide, BALB/c mice (Charles River

Laboratories, Kent, UK), D910A / D910A mice (a kind gift from Dr Klaus Okkenhaug, Babraham Institute, UK) and PKCδ knockout mice were kept under barrier conditions. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Cell Types and Culture Conditions

All human cell lines and primary cells were routinely maintained in RPMI 1640 medium supplemented with antibiotics and 10 % (v/v) foetal bovine. Cells were cultured every 2-3 days as required and maintained at 37 °C and 5 % CO_2. Murine cells were routinely maintained in RPMI 1640 medium supplemented with penicillin (10 u/ml), streptomycin (10μg/ml), L-Glutamine (2mM), HEPES buffer (25mM), 0.00035% v/v 2-mercaptoethanol and 10% v/v foetal calf serum (FBS), and new medium was added on day 3 post culture. Cells were maintained at 37 °C and 5 % CO_2. Prior to experimental procedures, cell viability was determined using trypan blue to stain any dying cells.

2.2.1 CEM Cells

CEM cells were obtained from The European Collection of Cell Cultures (ECACC). The cells were maintained in 175cm^2 flasks in RMPI 1640 medium supplemented with 10 % foetal calf serum (FBS), 10 % non essential amino acids, 10 u/ml penicillin and 10 μg/ml streptomycin (complete medium). Cells were grown to a maximum of 2 x 10^6 cells / ml and diluted with fresh complete medium to 5 x 10^5 / ml cells every two to three days. Cells were maintained in a humidified incubator at 37 °C and 5 % CO_2.

2.2.2 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 1500 rpm at room temperature for 30 minutes with the brake off. Following centrifugation, the interface layer between the plasma and the lymphoprep, as seen in Fig 2.1, which contains the lymphocytes and monocytes was swiftly removed by aspiration.

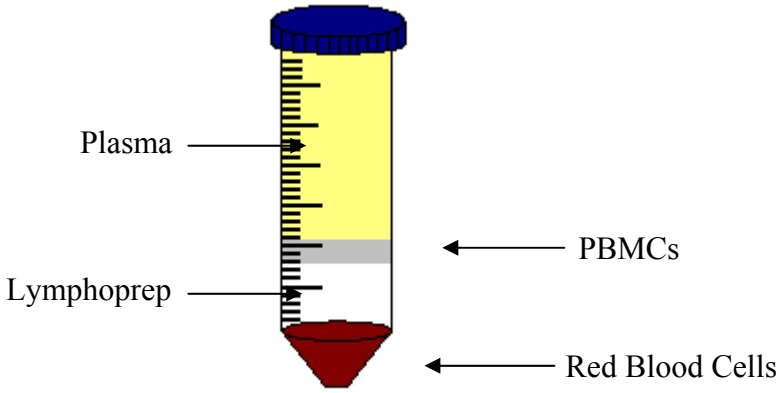

Figure 2.1 Diagram of PBMC separation with Lymphoprep after centrifugation. The diagram is representative of the separated cell layers observed after whole blood undergoes centrifugation with Lymphoprep.

2.2.4 Ex-vivo activation and clonal expansion of T lymphocytes

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

2.2.5 Isolation of $CD4^+$ T lymphocytes from murine splenocytes.

DO11.10 mice were humanely culled using a schedule 1 method and their spleens removed. The spleen was disaggregated using a cell strainer with a 40µm pore size and the resulting cell mixture was resuspended in 10mls complete medium. Cells were centrifuged at 1800rpm for 5 minutes before being resuspended in 2mls red blood cell lysis buffer and incubated at room temperature for 5 minutes with occasional shaking. Cells were washed twice in complete medium, and the cell pellet resuspended in 10mls complete medium. Cells were checked for viability and counted using a haemocytometer after mixing 20µl of cell suspension with 20µl of Trypan Blue. Dead cells were

stained blue due to the uptake of trypan blue. Cell viability was always above 90%.

Cells were counted and resuspended at 1 x 10^7 cells / 40µl MACS buffer (PBS, 0.5% w/v Bovine Serum Albumin (BSA) and 2mM EDTA). 10µl of the Biotin-Antibody Cocktail from the MACS $CD4^+$ T cell isolation kit was added / 1 x 10^7 cells and incubated on ice for 10 minutes. 30µl of MACS buffer and 20µl Anti-Biotin Microbeads from the MACS $CD4^+$ T cell isolation kit were added / 1 x 10^7 cells and incubated on ice for a further 15 minutes. Cells were washed once in MACS buffer then resuspended in 500µl MACS buffer and applied to a MACS LS column. The column was washed through with MACS buffer and the resulting effluent collected. This represents the enriched $CD4^+$ T cells. Cells were washed twice and resuspended at 1 x 10^6 / ml in complete medium.

For PKC$\delta^{-/-}$ C57BL/6 mouse spleens, cells were counted and resuspended at 1 x 10^7 cells / 40µl MACS buffer (PBS, 0.5% w/v Bovine Serum Albumin (BSA) and 2mM EDTA). 10µl of the Biotin-Antibody Cocktail from the MACS Pan T cell isolation kit was added / 1 x 10^7 cells and incubated on ice for 10 minutes. 30µl of MACS buffer and 20µl Anti-Biotin Microbeads from the MACS $CD4^+$ T cell isolation kit were added / 1 x 10^7 cells and incubated on ice for a further 15 minutes. Cells were washed once in MACS buffer then resuspended in 500µl MACS buffer and applied to a MACS LS column. The column was washed through with MACS buffer and the resulting effluent collected. This represents the enriched pan T cells. Cells were washed twice and resuspended at 1 x 10^6 / ml in complete medium.

2.2.6 Generation of APC's

Balb/c mice were humanely culled using a schedule 1 method and their spleens removed. The spleen was disaggregated using a cell strainer with a 5µm pore size and the resulting cell mixture was resuspended in 10mls complete medium. Cells were centrifuged at 1800rpm for 5 min before being resuspended in 2mls red blood cell lysis buffer and incubated at room temperature for 5 minutes with occasional shaking. Cells were washed twice in complete medium, and the cell

pellet resuspended in 10mls complete medium. Cells were checked for viability and counted using a haemocytometer after mixing 20µl of cell suspension with 20µl of Trypan Blue. Cell viability was always above 90%. Cells were counted and resuspended at 5 x 10^7 / ml in complete medium and 50µg / ml mytomycin C added. Cells were then incubated at 37°C for 30 minutes, washed four times in complete medium and resuspended at 5 x 10^6 / ml in complete medium.

2.2.7 Polarisation of Th17 cells.

Equal volumes of $CD4^+$ T cells and APC's were co-cultured, with resulting final volumes of 5 x 10^5 / ml and 2.5 x 10^6 / ml respectively. The co-culture was divided into two and 200ng / ml Ovalbumin $peptide_{323-339}$ and 10ng / ml IL-2 were added to one batch and incubated at 37°C in an atmosphere of 5% CO_2. These cells were known as Th0. 200ng / ml Ovalbumin $peptide_{323-339}$, 1ng / ml TGF-β1, 20ng / ml IL-6, 10ng / ml IL-1β, 10ng / ml TNF-α, 10ng / ml IL-23, 10µg / ml anti-Interferon gamma (IFN-γ) antibody and 10µg / ml anti-IL-4 antibody were added to the other batch and incubated at 37°C in an atmosphere of 5% CO_2. These cells were known as Th17 cells. After 3 days 10ng / ml IL-2 was added to both the Th0 and Th17 cells. On day 7, 35ml of cells were carefully layered on 15ml nycoprep 1.077A (Axis Shield) and centrifuged at 1500 rpm at room temperature for 20 minutes with the brake off. Healthy cells were collected from the interface and used.

2.2.8 Freezing/Thawing of cells

For storage, 10 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 Immunoblotting

2.3.1 Sample Generation

1-2 x 10^6 activated T lymphocytes or 5 x 10^6 Th17 cells per point were washed twice in RPMI 1640 and incubated at 37 °C in serum free RPMI for 60 minutes. If required, cells were incubated with inhibitors diluted in RMPI 1640 for the final 30 minutes of this period. Cells were left unstimulated or stimulated with chemokines at the required concentrations diluted in RPMI 1640. 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 twenty minutes, followed by centrifugation at 14,000 rpm for 10 minutes. The supernatants (containing protein) were removed and diluted with 5x loading buffer (10 % SDS, 50 % glycerol, 200 mM Tris HCl pH 6.8, Bromophenol blue), heated to 95 °C for 5 minutes and stored at -20 °C until used.

2.3.2 Protein Separation, Membrane Transfer and Visualisation

Solubilised proteins were electrophoresed on a one dimensional 7.5% or 10 % sodium dodecyl sulphate - polyacrylamide gel (SDS-PAGE). This was carried out using the Bio-Rad Mini Protean II system (Biorad Labs, UK). 20µl of sample was loaded into a stacking gel and run at 80 volts (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 % H_20, 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 % non-fat milk), with slight agitation and rinsed twice for 5 minutes in TBS-Tween. The membrane

was incubated in the specified 1° antibody dilution 1:500 - 1:1000, in TBS-Tween supplemented with 0.01% sodium azide and 5 % non-fat 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 horseradish peroxidase (HRP) diluted 1:10,000 in block buffer, for 60 minutes at room temperature before being washed in TBS-Tween three times for 5 minutes. Visualisation 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.

2.3.3 Membrane Stripping and reprobing

The membrane was rehydrated in TBS for 10 minutes and then placed in 50 ml of stripping buffer (100 mM 2 Mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCL pH 6.7) to remove bound antibody; 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, and re-probed with the relevant primary antibody and the immunoblotting procedure is carried out as described above.

2.4 Detection of Protein Phosphorylation Levels using the Phospho-array

2.4.1 Sample Generation

Analysis of protein activation was performed using a Human Phospho-MAPK Array Kit (R&D Systems). 1 x 10^7 cells were washed twice in RPMI 1640 and incubated at 37 °C in serum-free RPMI for 60 minutes. If required, cells were incubated with inhibitors diluted in RMPI 1640 for the final 30 minutes of this period. Cells were left unstimulated or stimulated with chemokines at the required concentrations diluted in RPMI 1640. Stimulations were terminated by aspiration of the supernatant followed by the addition of Lysis Buffer (supplied with the kit) and incubated at 4°C for 30min, shaking. Cell debris was pelletted by centrifugation at 14000rpm for 10 minutes and the supernatant transferred to a clean tube and frozen at -20°C until required.

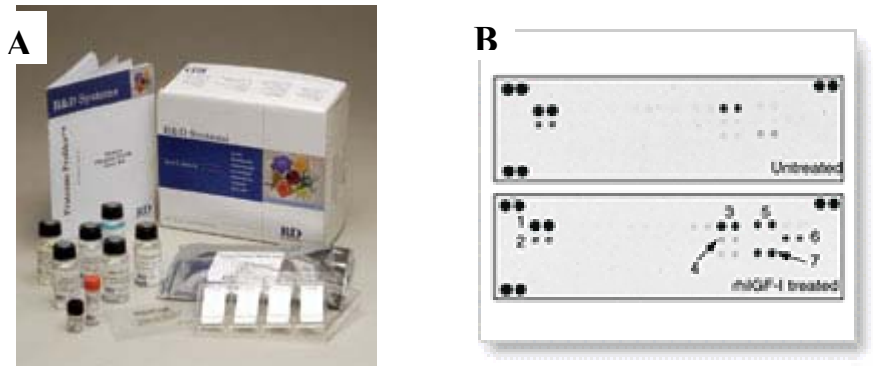

Figure 2.2: An R&D systems Human Phospho-MAPK Array Kit A: The contents of the kit, showing the different buffers provided (left) and the four membranes in their tray (bottom right). B: A representative diagram of a developed membrane. The black dots are where an antibody has bound protein, resulting in the production of light following incubation with ECL.

2.4.2 Determination of Protein Concentration

Lysates were thawed and protein concentration determined using a BCA Protein Assay (Pierce). 25µl of lysate or known concentrations of BSA were added to a 96 well plate along with 200µl of working reagent (supplied with protein assay kit) and mixed thoroughly for 30 seconds before being incubated at 37°C for 30 minutes. The plate was cooled to room temperature and absorbance at 562nm read on a plate reader. The protein concentrations were calculated by linear regression from the standard curve using GraphPad Prism.

2.4.3 Visualisation of Protein Phosphorylation Levels.

The array was incubated for one hour in 1.5mls of Array Buffer 1, which was then removed and 1.5mls of Array Buffer 1 containing 250µl of lysate added. The array was incubated overnight at 4°C, rocking. The array was washed three times in 20ml wash buffer before 1.5ml of Array Buffer 2/3 containing 15µl of Detection Antibody Cocktail Concentrate was added and the array incubated for two hours at room temperature on a rocking platform shaker. Array was washed three times in 20ml wash buffer and 1.5 ml of Streptavidin-HRP diluted 1:2000 in Array Buffer 2/3 added and incubated for 30 minutes at room temperature on a rocking platform shaker. Array was washed three times in 20ml wash buffer

and exposed to 3mls of ECL chemiluminescent reagent for 1 minute before being exposed to Kodak X-OMAT film for 2-10 minutes.

2.4.4 Densitometric Analysis

Densitometric analysis was performed to analyse these assays. Optical densitometry was measured using the Adobe® Photoshop and values were expressed as average pixel density for each spot.

2.5 Protein Kinase Activity Assays

2.5.1 Extraction of proteins from cells.

1-2 x 10^6 activated T lymphocytes or 5 x 10^6 Th17 cells per point were washed twice in RPMI 1640 and incubated at 37 °C in serum free RPMI for 60 minutes. If required, cells were incubated with inhibitors diluted in RMPI 1640 for the final 30 minutes of this period. Cells were left unstimulated or stimulated with chemokines at the required concentrations diluted in RPMI 1640. Stimulations were terminated by aspiration of the supernatant followed by the addition of ice cold PBS and subsequent centrifugation at 1500rpm for 5 minutes. The PBS was removed by aspiration and the cell pellet resuspended in 100µl of ice cold *"Cell Extraction Buffer"* provided with the assay kit. The cells were rotated at 4˚C for 30 minutes before centrifugation at 13000rpm for 20 minutes, also at 4˚C. The clarified cell extracts were then transferred to clean tubes in preparation for the kinase assay.

2.5.2 Preparation and Performance of the Kinase Assay.

Protein concentration was determined as described in section 2.5.2. Substrate peptide, ATP solution, DTT solution, kinase reaction buffer and kinase inhibitor cocktail were prepared as per the instruction in the product manual, then 5µl of each was mixed with 15µl of dH_2O and incubated for 5 minutes at reaction temperature. 5µl (15µg) of cell lysate was added in duplicate to a 96 well plate, the reaction mixture added and well mixed. Readings were taken using a fluorescent plate reader (λex 360/λem 485) every 30 seconds for 60 minutes.

Results were plotted as change in fluorescence intensity over time, corresponding to rate of phosphorylation by target kinase.

2.6 Flow Cytometry

2.6.1 Detection of extracellular ligands

Cells were washed twice in RPMI 1640 and counted before being resuspended at 2 x 10^6 / ml in FACS buffer (PBS, 0.25% w/v BSA). 1ml of cells were added to FACS tubes, centrifuged at 1500 rpm for 5 minutes and the resulting cell pellet resuspended in 100µl of 10µg / ml antibody and incubated at 4°C for 30 minutes in the dark. Cells were washed twice in 1ml of FACS buffer and resuspended in 500µl FACS buffer. Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software or BD FACSCanto II using BD FACS Diva software.

2.6.2 Detection of intracellular ligands

Cells were stimulated with 50ng / ml PMA, 500ng / ml Ionomycin and 4µl / 6mls of GolgiStop (BD Biosciences) for 4 hours at 37°C in an atmosphere of 5% CO_2. Cells were carefully layered on 15ml nycoprep 1.077A (Axis Shield) and centrifuged at 1500 rpm at room temperature for 20 minutes with the brake off. Healthy cells were collected from the interface, washed in phosphate-buffered saline (PBS) and counted before being resuspended at 1 x 10^6 / ml in Extracellular Staining Buffer (PBS, 1% FBS and 0.9% w/v Sodium Azide) and 1ml added to each FACS tube. Cells were centrifuged at 1500 rpm for 5 minutes, resuspended in 100µl of 10µg / ml antibody in Extracellular Staining Buffer and incubated at 4°C for 30 minutes in the dark. Cells were washed twice and resuspended in 1ml of Extracellular Staining Buffer before 250µl of Fixation/ Permeabilisation Solution (BD Biosciences) was added and the cells were incubated on ice for 20 minutes in the dark. Cells were then washed twice in Perm/Wash buffer (BD Biosciences) and resuspended in 100µl of 10µg / ml antibody in Perm/Wash Buffer and incubated on ice for 30 minutes in the dark. Cells were washed twice in Perm/Wash Buffer and resuspended in 500µl Extracellular Staining Buffer. Data were acquired on a Beckman Coulter EPICS

XL machine using EXPO32 ADC software or BD FACSCanto II using BD FACS Diva software.

2.7 Cell Migration Assays

2.7.1 Neuroprobe Chemotaxis.

Cells were washed twice in chemotaxis medium (RPMI 1640 w/o phenol red, 0.25% w/v BSA) before resuspension at 1 x 10^7 / ml. Cells were incubated for 60 minutes at 37˚C in the presence of inhibitors if required. Neuroprobe ChemoTX® chambers (Neuroprobe) were blocked with 29µl of chemotaxis medium / well and incubated for 30 minutes at 37°C before 29µl of the appropriate chemokine in chemotaxis medium was added and the 5µm membrane placed carefully on top. 25µl of cells were placed on the membrane and the cells incubated for 3 hours at 37°C in an atmosphere of 5% CO_2 while migrating. The filter was then discarded and the cell suspension transferred to a FACS tube containing 250µl of Extracellular Staining Buffer and 50µl Flowcount Beads (Beckman Coulter). Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software or BD FACSCanto II using BD FACS Diva software.

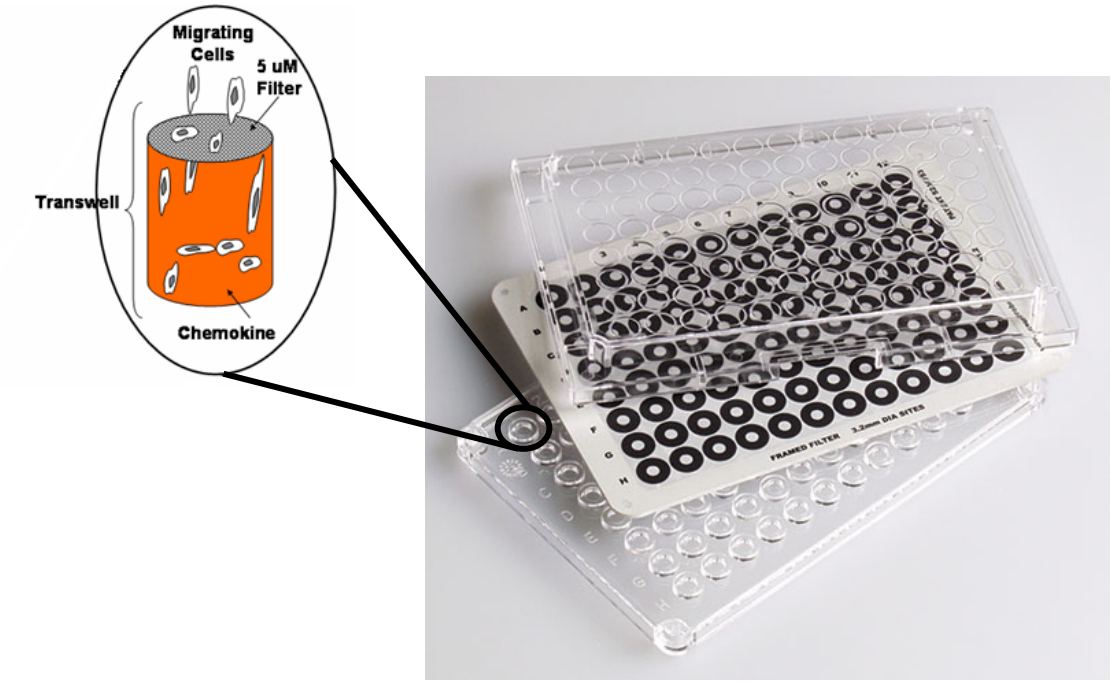

Figure 2.3: Diagram of a Neuroprobe Chemotaxis Plate including a cartoon of how it works.

2.7.2 Transwell Chemotaxis

Cells were washed twice in chemotaxis medium (RPMI 1640 w/o phenol red, 0.25% w/v BSA) before resuspension at 1 x 10^7 / ml. Cells were incubated for 60 minutes at 37˚C in the presence of inhibitors if required. Transwell Chemotaxis Plates (Costar) were blocked in 600µl chemotaxis medium / well for 30 minutes at 37°C before 600µl of the appropriate chemokine in chemotaxis medium was added and the 5µm membrane placed on top. 100µl of the cell suspension was placed on top of the filter and the cells allowed to migrate for 3 hours at 37°C in an atmosphere of 5% CO_2. The filter was then discarded and cells from the bottom well of four wells were pooled, washed in complete medium and resuspended in 1ml of chemotaxis medium. When required, 100µl was removed for use in an ELISpot assay and the rest were stimulated for 3 hours with 50ng / ml PMA, 500ng / ml Ionomycin and 0.66µl / ml of GolgiStop at 37°C in an atmosphere of 5% CO_2. Cells were washed and resuspended in 1ml of Extracellular Staining Buffer, transferred to a FACS tube and pelletted. Cells were then resuspended in 100µl of 10µg / ml extracellular antibody and incubated at 4°C for 30 minutes in the dark, before being washed

and resuspended in 400µl of 4% paraformaldehyde solution and incubated at 4°C for 15 minutes in the dark. Cells were washed three times and resuspended in 500µl of extracellular staining buffer and stored at 4°C overnight. The next day, cells were spun down, resuspended in 500µl of Perm/Wash Buffer and incubated at 4°C for 15 minutes in the dark. Cells were spun down and resuspended in 100µl of 10µg / ml intracellular antibody in Perm/Wash Buffer and incubated on ice for 30 minutes in the dark. Cells were then washed twice in Perm/Wash Buffer and resuspended in 450µl of Extracellular Staining Buffer and 50µl Flowcount Beads (Beckman Coulter). Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software.

2.8 Secreted Cytokine Detection

2.8.1 Luminex Cytokine Detection

Cytokine specific capture antibodies against IL-17, IFN-γ, TNF-α, IL-4, IL-5, and IL-10 were bound to fluorescently distinct LiquiChip Carboxy Beads following manufacturers instructions, mixed together and diluted 1:1000 in reagent buffer (PBS, 1% BSA). 50µl of bead mix was combined with 50µl of cell culture supernatant in a 96 well filter plate and incubated at room temperature for two hours, shaking. The beads were washed twice with 100µl of wash buffer (PBS, 0.05% v/v Tween-20) and 50µl of cytokine-specific biotinilated antibody diluted 1:100 in reagent buffer was added. The plate was incubated in the dark for one hour, shaking and then washed twice with 100µl wash buffer. 50µl of Streptavidin-HRP (1:1000 dilution in reagent buffer) was added and the plate incubated at room temperature for a further 30 minutes in the dark. The plate was washed twice with wash buffer and the beads resuspended in 100µl of wash buffer. The mean fluorescent intensity (MFI) of each distinct bead type was acquired using a Luminex^{100}IS machine and analysed using Developer Workbench Software (Qiagen). The cytokine concentration was estimated using a standard curve generated from known concentrations of each cytokine.

2.9 Cell Death Assay

Healthy cells were resuspended at 2x 10^6 / ml in wash medium and incubated with kinase inhibitors for the required period of time. 100µl of cells were mixed with 100µl of Trypan blue and 20µl was placed on a haemocytometer. The number of dead cells (those that had taken up the dye and thus were stained blue) were then counted under a microscope.

2.10 Statistical analysis

Statistical analysis was performed using students' t-test or 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 4.0 software.

CHAPTER 3: RESULTS I – THE ROLE OF PI3K AND DOWNSTREAM KINASES IN T LYMPHOCYTE MIGRATION

Results I: The role of PI3K and downstream kinases in human T lymphocyte migration

3.1 Background

Phosphoinositide 3-Kinase (PI3K) is a lipid kinase important in several intracellular pathways including cell growth, proliferation, survival and motility. Indeed, PI3K has been shown to be essential in several models of chemotaxis including *Dictyostelium* (Sasaki et al., 2007; Funamoto et al., 2001) and neutrophils (Puri et al., 2005; Sasaki et al., 2000). Several studies in *Dictyostelium* have been performed, imaging the location of PI3K and the 5' phosphatase PTEN in migrating cells (Janetopoulos et al., 2005; Huang et al., 2003). These studies showed that in an unstimulated resting cell, class I PI3K was found mainly in the cytosol while PTEN was localised to the membrane. However, upon sensing a chemoattractant gradient, PTEN dissociates from the membrane while PI3K localises to the leading edge of the cell, where it produces $PI(3,4,5)P_3$ (Merlot and Firtel, 2003), recruiting and activating Rho GEF proteins and PH domain containing proteins such as Akt, setting up a signalling cascade resulting in chemotaxis. Studies of T lymphocyte migration involving the broad spectrum PI3K inhibitors LY294002 and Wortmannin, or using knockouts of the P110δ or P110γ genes in mice (Reif et al., 2004) have shown that there is impaired movement in cells, a loss of gradient sensing and a general impaired ability to mount an immune response. In humans however, there is some evidence that PI3K is a dispensable signal (Cronshaw et al., 2004), and that its importance is determined by chemoattractant, cell type and/or differentiation state (Ward, 2006). Activation of PI3K results in the subsequent activation of a number of protein kinases within the cell (Cantley, 2002). The role of these has not been fully explored in chemokine-induced human T lymphocyte migration and may help to shed further light on the role of PI3K. This section of the study aims to gain more insight into the role of PI3K in T lymphocyte migration, using both T lymphocyte cell lines and primary peripheral blood mononuclear cells (PBMCs), and to determine whether any of the downstream kinases also play a role in migration.

3.2 Activation of PI3K in T lymphocytes by chemokines

Direct measurement of PI3K activation via assessment of 3'-phosphoinositide lipid generation is technically formidable, so a surrogate readout is often used. Akt, or Protein Kinase B, is a protein kinase that resides within the cell cytoplasm. Activation of PI3K leads to the formation of Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) in the cell membrane, allowing Akt to bind to the membrane via its PH domain. Once there, it is phosphorylated at Thr308 by the protein kinase PDK-1, and subsequently by an unknown protein, thought to be mTOR-RICTOR (Sarbassov et al., 2005), at position Ser473. This dual phosphorylation allows a conformational change in the protein causing activation of Akt. Flow cytometry was used to confirm the presence of chemokine receptors on the surface of the T lymphocyte cell line CEM cells, previously shown by work carried out within the Ward group to express two chemokine receptors, only one of which induced PI3K dependent migration (Cronshaw et al., 2004), and on human PBMCs. Staining of CEM cells with FITC-tagged anti-CXCR4 antibodies and PE-tagged anti-CCR4 antibodies revealed the presence of both receptors on the cell surface, with 45% of cells expressing CXCR4 and 59% of cells expressing CCR4 (Fig. 3.1*A*-*B*). CCR4 has previously been shown to be expressed only in very small quantities on PBMCs (Yamamoto et al., 2000), and so was not tested for in the PBMC populations, however staining for CXCR4 revealed that 62% of cells expressed the receptor (Fig. 3.1*C*). Many chemokine receptors show promiscuity for ligands, binding several different chemokines. To date, CXCR4 has only been shown to have a single ligand, known as CXCL12 or SDF, while CCR4 binds a range of chemokines including CCL22 (MDC), CCL3 (MIP-1) and CCL17 (TARC). While some evidence suggests that there may be an alternative receptor for CCL22 (Proost et al., 2006), it is generally accepted that CCL22 is a specific ligand for CCR4. Therefore, CXCL12 and CCL22 were used to stimulate CXCR4 and CCR4 respectively in these assays.

Using an antibody specific for Akt phosphorylated at Ser473, the effect of stimulating the cells with different concentrations of the chemokines was explored. Incubation of CEM cells with increasing concentrations of CXCL12

(Fig. 3.1*D*) or CCL22 (Fig. 3.1*E*) induced the dose dependent phosphorylation of Akt, indicating that PI3K is activated upon chemokine / chemokine receptor interactions. Incubation of Day 10 SEB activated PBMCs with CXCL12 showed the same result (Fig. 3.1*F*). To confirm that this effect was PI3K dependent, a broad-spectrum inhibitor of PI3K, LY294002, was used. LY294002 is a commonly used inhibitor of PI3K that works by binding to the ATP-binding site of PI3K. Pre-incubation of either cell type with 10μM LY294002 for 30 minutes prior to stimulation with chemokines significantly reduced phosphorylation of Akt (Fig. 3.1*D*-*F*), confirming the activation of PI3K by chemokines. Interestingly, LY294002 was never able to fully abrogate CXCL12-incuced phosphorylation of Ser473. This latent phosphorylation could be due to the MAPK-family of proteins, who are also able to activate the mTOR-RICTOR complex.

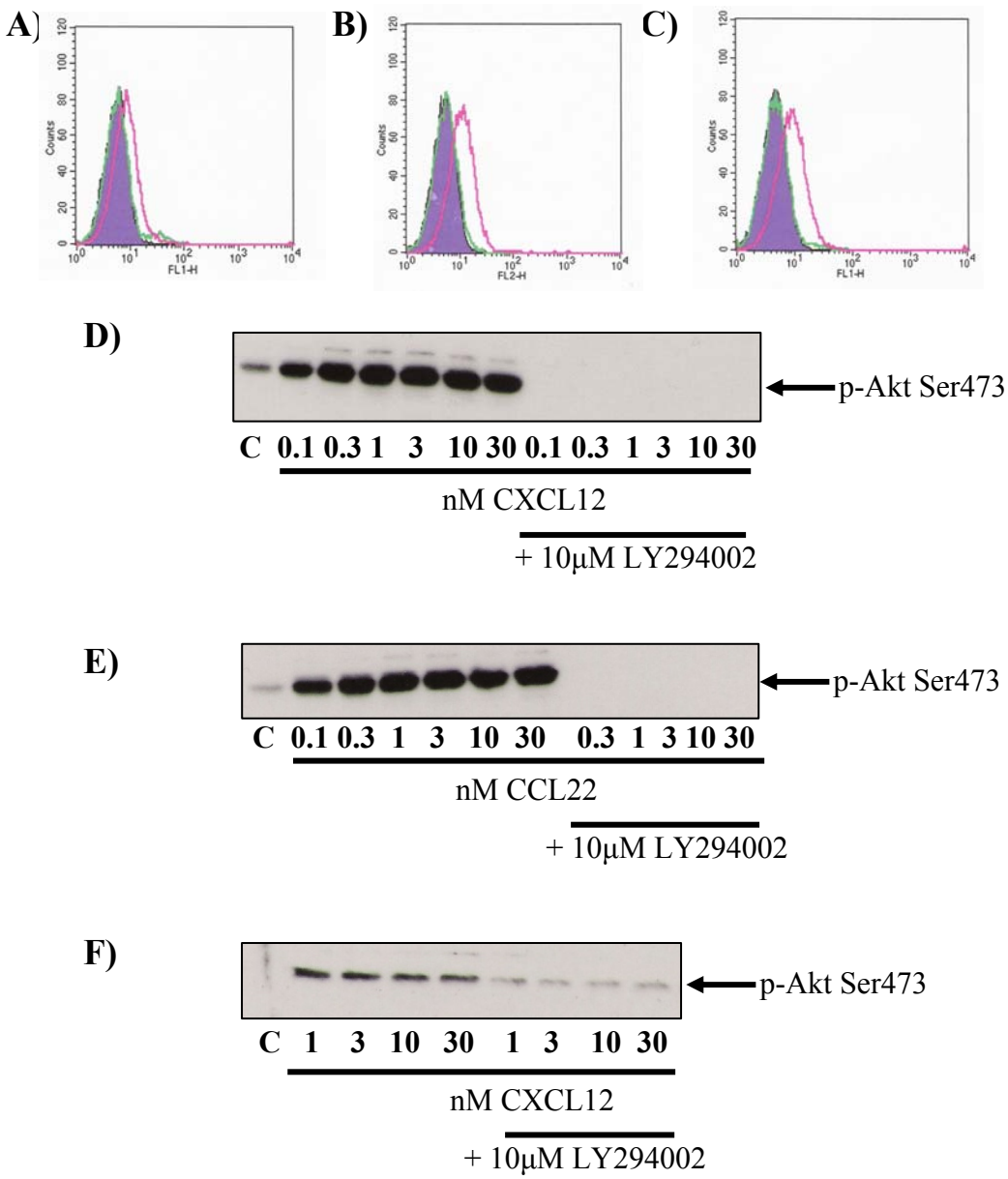

Figure 3.1 Activation of PI3K by stimulation of PBMCs and CEM cells through CXCR4 and CCR4. Flow cytometry was used to determine the expression of CXCR4 (*A*) or CCR4 (*B*) on CEM cells and CXCR4 on SEB activated PBMCs (*C*). Cells were washed into FACS buffer at 2 x 10^6 / ml, 1ml added to a FACS tubes and incubated with 100μl of fluorescently tagged antibody. After 30 minutes, cells were washed and data acquired on a BD FACSCanto II machine. The green line indicates the isotype control antibody, while the red line shows binding of the antibody in question. Immunoblotting was used to determine whether binding of chemokines to CXCR4 (*D*) or CCR4 (*E*) on CEM cells or CXCR4 (*F*) on SEB activated PBMCs. WCLs of the various cell types were generated as described in *Materials and Methods*. All data are representative of at least three separate experiments.

3.3 Characterisation of PI3K inhibitors by Immunoblotting

Although LY294002 is a well established PI3K inhibitor, questions have recently been raised about its specificity. A paper by Gharbi *et al*, (Gharbi et al., 2007) investigated the specificity of several commonly used PI3K inhibitors including LY294002 and the original PI3K inhibitor, Wortmannin. The study revealed that in addition to it's inhibitory effects on PI3K, LY294002 also inhibited the kinase casein kinase 2 (CK2), GSK-3β and p97/VCP, an ATPase, at concentrations similar to that used to inhibit PI3K. To control against this, wortmannin and a new PI3K inhibitor known as ZSTK474 were also used to investigate the role of PI3K in chemotaxis. ZSTK474 is an *s*-triazine derivative that possesses greater selectivity and potency compared to LY294002 (Yaguchi et al., 2006). This compound has been shown to inhibit all of the class I PI3K isoforms, and does so in an ATP competitive manner (Kong and Yamori, 2007). Using the phosphorylation of Akt at Ser473 as a marker of PI3K activation, immunoblotting was used to investigate the effects of the three inhibitors on PI3K activation and to determine the optimum concentrations and incubation times of the inhibitors for use in later studies. These experiments were performed in both CEM cells and SEB activated PBMCs in order to reveal any difference in the efficacy of the inhibitors on these cells.

Incubation of day 10-12 SEB activated PBMCs with LY294002 for 30 minutes, prior to stimulation with 10nM of the CXCR4 ligand CXCL12, revealed that inhibition of phosphorylation occurs at 10-30μM (Fig. 3.2*A*). When 10μM of LY294002 was incubated with the cells for varying time points, 15-30 minutes was shown to be the best time point. When these experiments were repeated with Wortmannin, a concentration of 300nM was shown to be most effective (Fig. 3.2*B*). However this concentration was slightly higher than expected, and may have off-target effects at this concentration. Theorising that this result could be due to the short incubation time of the inhibitor (15 minutes), a concentration of 100nM was used to determine the best incubation time. Results showed that inhibition of phosphorylation could be achieved, if the compound was incubated for a longer time-point of 30 to 60 minutes. Therefore incubation of 100nM wortmannin for 30 minutes was used in future experiments. In the original

publication of ZSTK474 it was shown that it was more potent that LY294002 (IC_{50} of 37nM for ZSTK474 and 790nM for LY294002) (Yaguchi et al., 2006), however when used in these cells, the compound was shown to be of a similar potency to LY294002, with 10μM inhibiting phosphorylation of Akt (Fig. 3.2*C*). When this concentration was investigated for optimum incubation times, it proved difficult to get reliable reproducible results. Generally, inhibition of Akt phosphorylation occurred almost immediately, showing reduction after 1-2 minutes, and then wore off before regaining effect after 15-30 minutes. The reasons for this are unknown and were not reported in other publications. To ensure complete penetration of the cell by ZSTK474, 30 minutes was used as the standard incubation time.

Incubation of CCL22-stimulated CEM cells with the PI3K inhibitors, while revealing some differences in incubation times, showed general similarities in the inhibitor profiles. LY294002 inhibited phosphorylation of Akt at Ser473 at a concentration of 10μM, and after 15-30 minutes incubation (Fig. 3.3*A*). This was slightly more effective than in PBMCs, although not greatly so, and was unexpected. CEM cells lack the 3'phosphatase PTEN (Sakai et al., 1998), a phosphatase responsible for the removal of phosphates from the D3 position on phosphoinositides, thereby opposing the activity of phosphoinositide 3-kinase (PI3K). This causes continuous and sustained activation of PI3K within the cells, and is the likely reason for the cells being leukemic. It was thought that this sustained activation would result in a requirement for increased concentration of inhibitors; however this is not the case for LY294002. Wortmannin inhibited phosphorylation at the same concentration in CEM cells as in PBMCs (Fig. 3.3*B*). Again, this concentration was quite high and could be due to the short incubation time (15 minutes). Indeed, when used at 100nM, wortmannin showed complete inhibition of Akt after 30-60 minutes incubation. ZSTK474 was again less efficacious than expected, requiring a concentration of 10μM to inhibit phosphorylation (Fig. 3.3*C*). However, the kinetics of this inhibitor was much easier to discern in CEM cells, with complete inhibition occurring after 30 minutes.

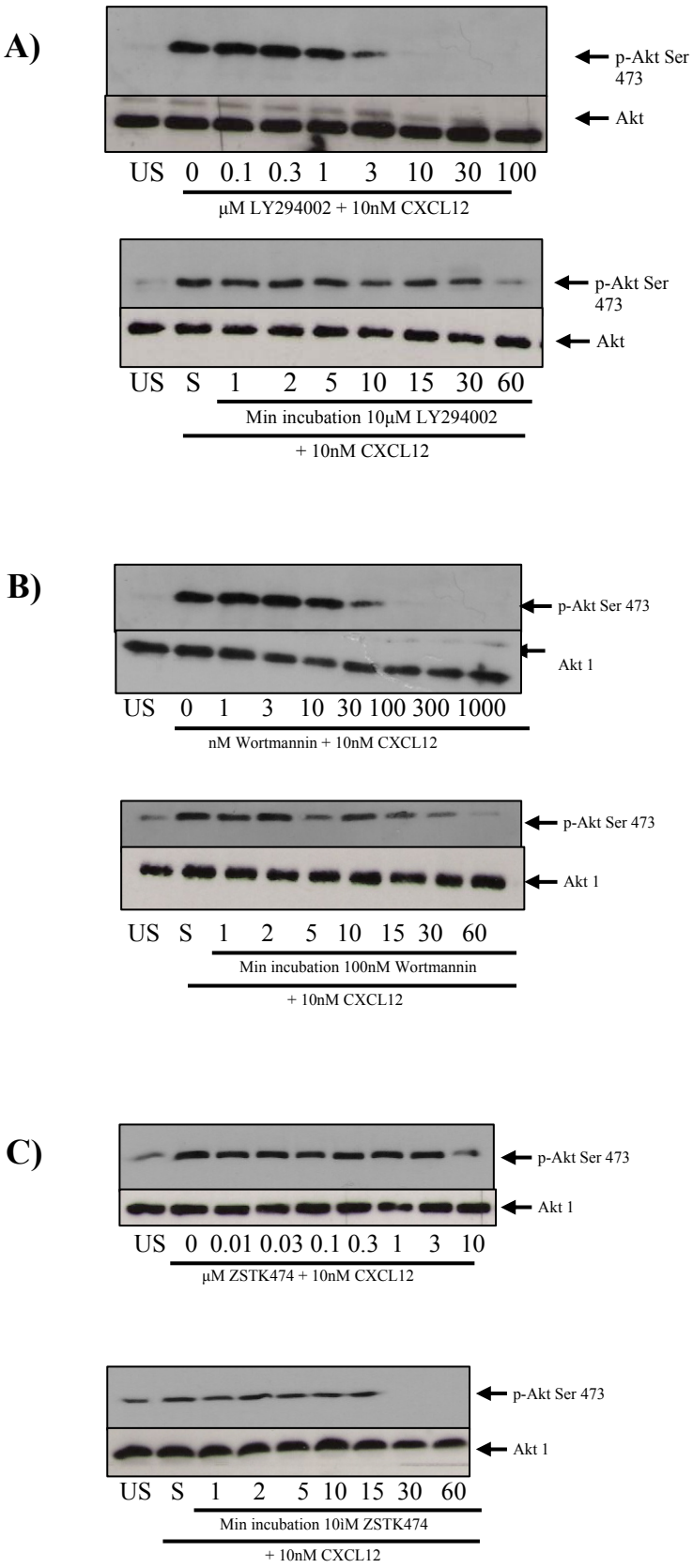

Figure 3.2 The effect of PI3K inhibitors on Akt phosphorylation
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Figure 3.2 The effect of PI3K inhibitors on Akt phosphorylation. Day 10-12 SEB activated PBMCs were incubated with LY294002 (*A*) Wortmannin (*B*) or ZSTK474 (*C*) at the times and contentrations shown, then stimulated with 10nM CXCL12 for 5 min. Cells were subsequently lysed as described in *Materials and Methods*, and lysates (1 x 10^6 cells per lane) resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, and immunoblotted with a phospho-specific Akt antibody with affinity for the active Ser473 Phosphorylated form of Akt, and protein was visulaised with ECL. The blots were stripped and reprobed with pan-Akt antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments. US: unstimulated, S: stimulated.

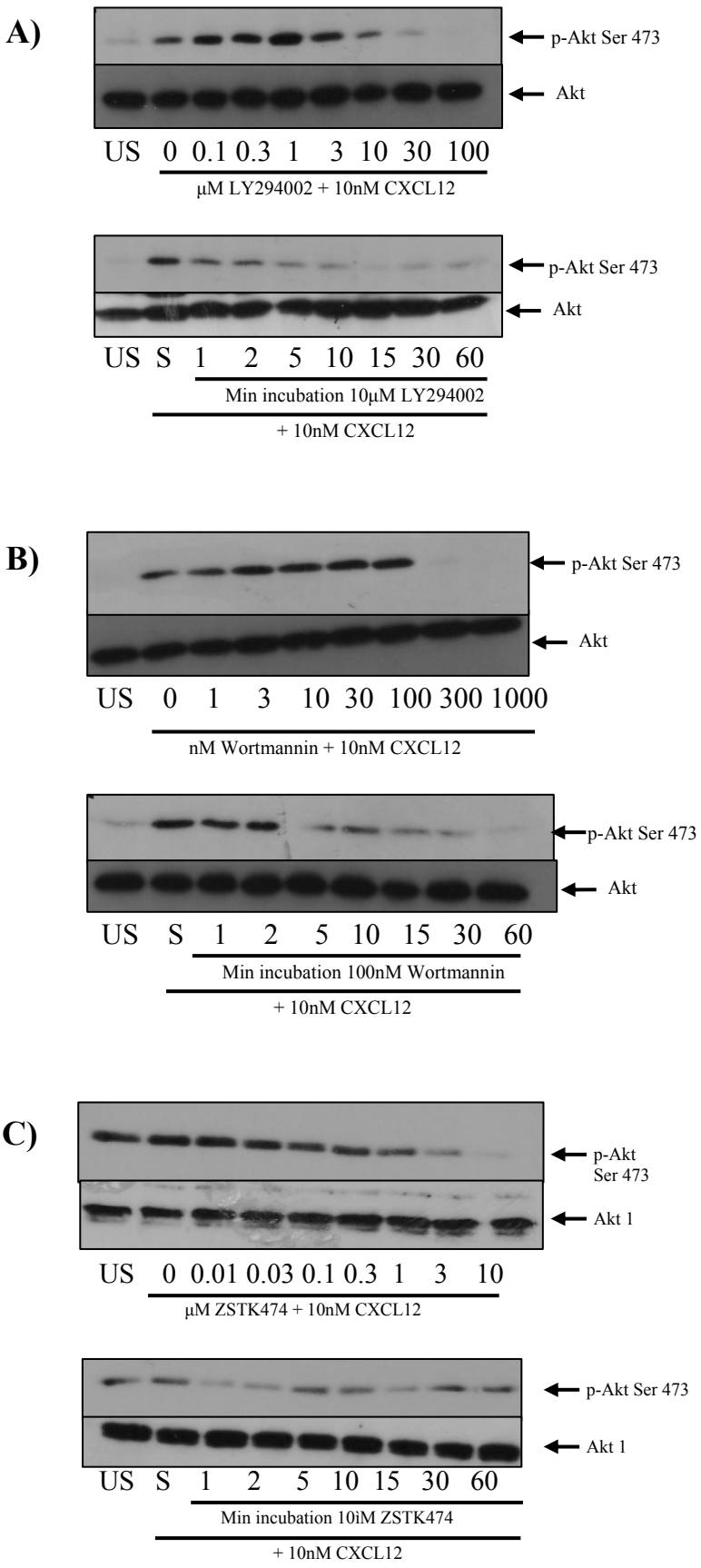

Figure 3.3 The effect of PI3K inhibitors on Akt phosphorylation.
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Figure 3.3 The effect of PI3K inhibitors on Akt phosphorylation. CEM cells were incubated with LY294002 (*A*) Wortmannin (*B*) or ZSTK474 (*C*) at the times and contentrations shown, then stimulated with 10nM CXCL12 for 5 min. Cells were subsequently lysed as described in *Materials and Methods*, and lysates (1 x 10^6 cells per lane) resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, and immunoblotted with a phospho-specific Akt antibody with affinity for the active Ser473 phosphorylated form of Akt, and protein was visulaised with ECL. The blots were stripped and reprobed with pan-Akt antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments. US: unstimulated, S: stimulated.

3.4 Effect of PI3K inhibition on T lymphocyte chemotaxis

Once the kinetics of the PI3K inhibitors and their efficacy in inhibiting PI3K had been determined, the effects of LY294002, Wortmannin and ZSTK474 on chemokine induced migration of T Lymphocytes was investigated. Studies using the T lymphocyte cell line, CEM cells, revealed that increasing concentrations of the CCR4 agonist CCL22 induced dose-dependent migration of these cells in chemotaxis assays, with 10nM CCL22 effecting greatest migration. When cells were pre-incubated with 10μM LY294002 for 30 minutes, a concentration previously shown to result in PI3K inhibition, no effect on cell migration was observed (Fig. 3.4*A*). Exposure of cells to the CXCR4 agonist CXCL12 also resulted in dose-dependent migration, with 10nM CXCL12 also inducing maximal response. Interestingly, when these cells were incubated with 10μM LY294002 for 30 minutes prior to stimulation with CXCL12, migration was partially inhibited (Fig. 3.4*A*). The basal migration of these cells, migration that occurs in the absence of chemokine, was unaffected, indicating that it was the directional sensing of these cells and not their motility that was impeded.

Similar results were seen when using Wortmannin (Fig. 3.4*B*) and ZSTK474 (Fig. 3.4*C*). In each case the pre-incubation with the inhibitor, at 100nM and 10μM respectively, at doses previously shown to inhibit the action of PI3K had no effect on CCL22-stimulated migration. However, when the cells were stimulated with CXCL12, inhibition of PI3K resulted in a significant decrease in cell migration, although not complete inhibition. Again, no effect on basal cell migration was observed. Interestingly, pre-incubation with ZSTK474, a PI3K inhibitor resported to be more specific than LY294002 and Wortmannin showed less effect on migration, although still significantly inhibited it, possibly indicating that at least some of the inhibition of migration could result from off-target effects.

Day 10 SEB activated PBMCs were also employed to determine whether PI3K has a role in T lymphocyte migration. Stimulation of the cells with increasing concentrations of CXCL12 resulted in dose-dependent migration of the cells

(Fig. 3.5). This migration showed a different profile to CEM cell migration, with maximal migration occurring at 3nM CXCL12. When the cells were pre-incubated with LY294002 (Fig 3.5*A*), wortmannin (Fig 3.5*B*) or ZSTK474 (Fig 3.5*C*), no effect was seen on cell migration, a result different to that observed with CEM cells, where the PI3K inhibitors inhibited CXCR4 mediated migration. This result indicates a difference in CXCR4 mediated signalling pathways between CEM cells and activated PBMCs. As PBMCs do not express CCR4, experiments to determine the effect of PI3K inhibition on CCL22-stimulated migration were not performed.

To determine whether the lack of efficacy of LY294002, wortmannin and ZSTK474 in CCL22 mediated migration of CEM cells and CXCL12 mediated migration of SEB activated PBMCs was due to the concentrations of inhibitor used, higher concentrations of the compounds were tried, including at concentrations where the inhibitors were no longer specific. CEM cells were incubated with a range of concentrations from 0.3μM to 100μM of the three PI3K inhibitors for 30 minutes, and then assayed for their ability to migrate to 1nM CCL22. Analysis of the results revealed that no significant inhibition of migration was seen at concentrations of 30μM for LY294002 and ZSTK474 and 10μM for wortmannin; concentrations much higher than normal for these compounds (Fig 3.6*A*). Inhibition was seen at 100μM, but this is likely explained by the high concentrations of DMSO that the inhibitors were dissolved in killing the cells. As expected, a normal inhibitory profile was observed for CEM cells migrating to CXCL12 (Fig 3.6*B*), already shown to be sensitive to PI3K inhibitors. When this experiment was repeated using SEB activated PBMCs migrating to CXCL12, no inhibition was seen at concentrations of 30μM for LY294002 and ZSTK474 and 10μM for wortmannin (Fig 3.6*C*). Again, inhibition was observed at 100μM and was likely due to the high concentrations of DMSO.

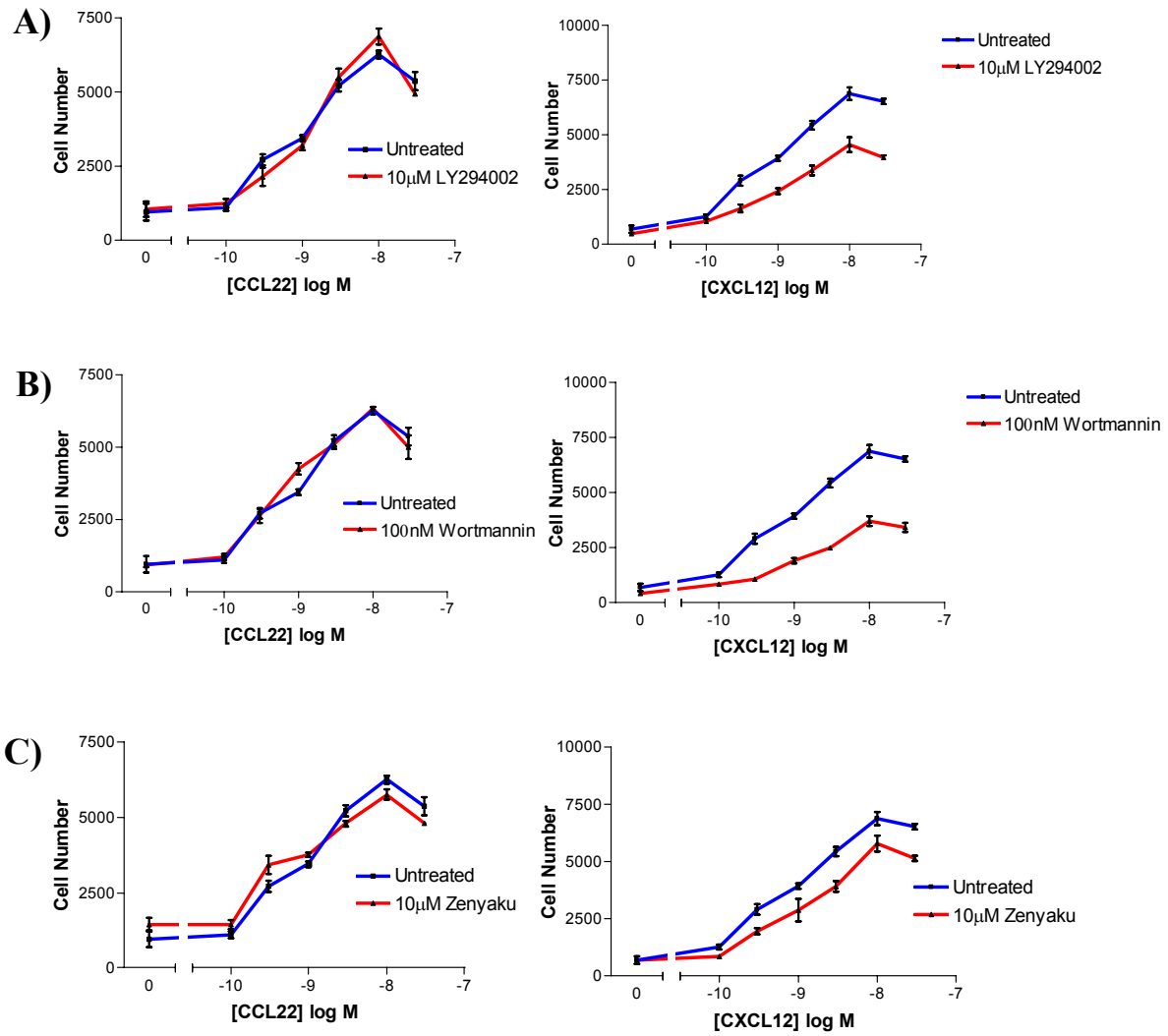

Figure 3.4 The effect of broad-spectrum PI3K inhibitors on CCL22 and CXCL12 mediated CEM cell migration. CEM cells were cultured as described in *Materials and Methods* before being treated with LY294002 for 30 min (*A*), Wortmannin for 20 min (*B*) or ZSTK474 (*C*) for 30 min. Cells were placed in Neuroprobe chemotaxis chambers containing the indicated concentrations of CCL22 or CXCL12 and allowed to migrate for 2 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=4.

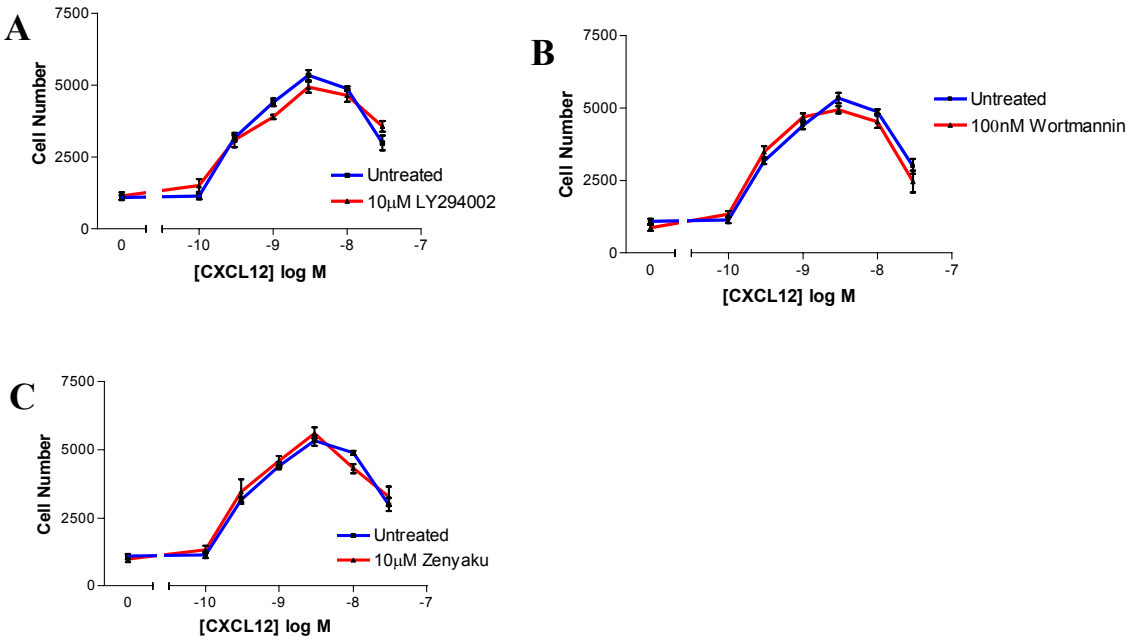

Figure 3.5 The effect of broad-spectrum PI3K inhibitors on CXCL12 mediated T lymphocyte migration. Day 10-12 SEB activated PBMCs were cultured as described in *Materials and Methods* before being treated with LY294002 for 30 min (*A*), Wortmannin for 20 min (*B*) or ZSTK474 (*C*) for 30 min. Cells were placed in Neuroprobe chemotaxis chambers the indicated various concentrations of CXCL12 and allowed to migrate for 3 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=4.

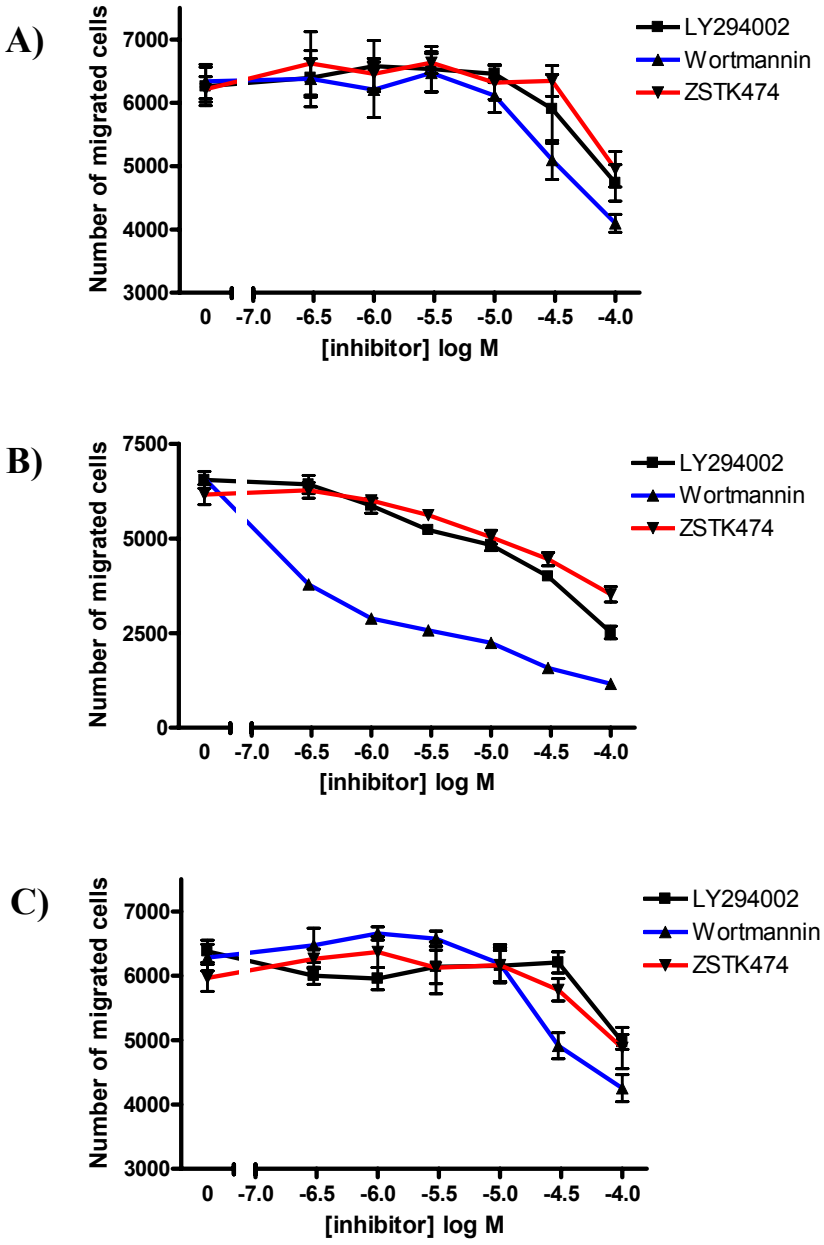

Figure 3.6 The effect of PI3K inhibitors on cell migration. A migration assay was used to determine whether high doses of PI3K inhibitors result in inhibition of chemotaxis. CEM Cells (*A,B*) or Day 10-12 SEB activated PBMCs (*C*) were cultured as described in *Materials and Methods* before being treated with PI3K inhibitors at the indicated doses for 30 minutes (LY294002 and ZSTK474) or 20 minutes (wortmannin). Cells were placed in Neuroprobe chemotaxis chambers containing various concentrations of CCL22 (*A*) or CXCL12 (*B,C*) and allowed to migrate for 3 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=4.

3.5 Characterisation of the Akt inhibitor Akti-1/2

To confirm the differing requirements for PI3K in CEM cell and T lymphocyte migration mediated by the chemokine receptors CCR2 and CXCR4, the role of Akt in mediating migration was investigated. As Akt lies downstream of PI3K and is thought to be the main effector of PI3K activation, the role of Akt within the cell is assumed to be the same as PI3K and thus the phenotype of any inhibition of Akt should mimic PI3K inhibition. Traditionally, inhibition of Akt was very difficult, as most inhibitors had many off-target effects, especially with similar kinases, such as the MAPK family.

A new type of Akt inhibitor has been synthesised (Zhao et al., 2005b), with a different mechanism of action to other Akt inhibitors. Although not yet fully determined, it does not act through the ATP binding pocket, and instead is though to bind the hinge region of the protein between the active site and the PH domain. Once bound, the inhibitor prevents the kinase from unfolding upon binding to the phospholipid membrane, and thus preventing the active site from binding to downstream kinases. Previous studies have revealed that although Akti- 1/2 does not bind to or inhibit PDK-1 or the mTOR-RICTOR complex; it does prevent the phosphorylation of Akt at sites Ser473 and Thr308 (Barnett et al., 2005). This allows easy determination of Akt inhibition by analysis of phosphorylation at these sites.

To test the efficacy of this inhibitor, as well as determine the optimum concentration and incubation time points for the inhibitor, CEM cells and SEB activated PBMCs were pre-incubated with Akti- 1/2 before stimulation with 10nM CXCL12 for five minutes. Incubation of CEM cells with increasing concentrations of the inhibitor revealed that complete inhibition of Ser473 phosphorylation occurred at 1-10μM after incubation for 30 minutes (Fig. 3.7*A*). When the cells were incubated with 10μM Akti- 1/2, incubation times of 1 minute were shown to be sufficient to abrogate Ser473 phosphorylation. Similarly, when activated PBMCs were incubated with Akti-1/2, it was revealed that concentrations of 1μM and incubation times of one minute were sufficient to inhibit Akt, showing that the inhibitor has strong efficacy (Fig. 3.7*B*).

A phosphor-array assay was used to help confirm the specificity of this inhibitor. This assay works on similar principles to immunoblotting, but tests the phosphorylation state of many different kinases at the same time. A phosphor-array against members of the MAPK family was used, as this family was the most likely to be affected by inhibitors of Akt. SEB activated PBMCs were incubated with 10μM Akti- 1/2 for 30 minutes prior to stimulation with 10nM CXCL12 for 5 minutes. Alongside this, cells were incubated with DMSO at the same quantity as in the inhibitor, to act as a vehicle control prior to stimulation with 10nM CXCL12 for 5 minutes. The cells were lysed and the resulting lysates incubated with the array which contained phosphor-specific antibodies pre-bound to nitrocellulose membrane. Subsequent analysis of the spots revealed that of the 17 kinases tested, the only ones that were significantly inhibited by the Akti-1/2 inhibitor were Akt1, Akt2, Akt3, GSK-3α and GSK-3β (Fig. 3.8). It was expected that phosphorylation levels of GSK-3 would also be affected, as these kinases lie downstream of Akt. It was interesting that Akt3 was also inhibited, as the Akti-1/2 inhibitor has not previously been shown to bind to this protein, and this result should be noted when interpreting future results. The inhibitor showed no effect on the MAP kinases, proving the specificity of this inhibitor. Levels of the total Akt proteins were not affected, nor HSP27 (a control protein), indicating that the inhibitor has no detrimental effects on the cell by causing immediate cell death. To confirm this result using more rigorous assays, its effects on cell death were investigated using a Typan blue assay.

Day 10 SEB activated PBMCs, and CEM cells were incubated with 10μM Akti-1/2 for various time points up to four hours in RPMI 1640. Cells were then washed twice in media, and stained with Trypan Blue. Trypan Blue is a blue dye that cannot pass through the intact cell membrane, therefore only cells with a permeated membrane, e.g. those who are dead or dying, will be stained blue. The cells were placed in a haemocytometer, and the number of healthy cells counted. No statistically significant difference was seen between cells treated with the inhibitor, and those left untreated, for either CEM cells (Fig. 3.9*A*) or PBMCs (Fig. 3.9*B*). This result indicates that the inhibitor has no effect on cell survival for the incubation times used.

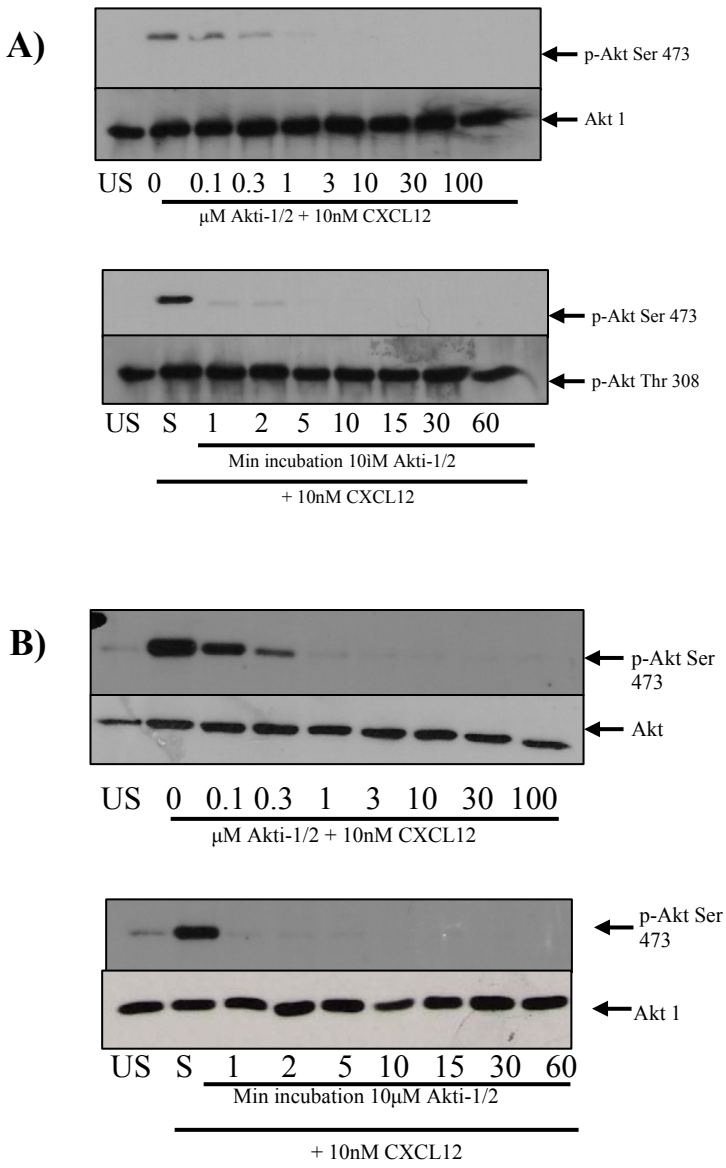

Figure 3.7 The effect of Akt inhibition on Akt phosphorylation. Day 10-12 SEB activated PBMCs (*A*) or CEM cells (*B*) were incubated with Akti-1/2, then stimulated with 10nM CXCL12 for 5 min. Cells were subsequently lysed as described in *Materials and Methods*, and lysates (1 x 10^6 cells per lane) resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, and immunoblotted with a phospho-specific Akt antibody with affinity for the active Ser473 Phosphorylated form of Akt, and protein was visulaised with ECL. The blots were stripped and reprobed with pan Akt antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments.

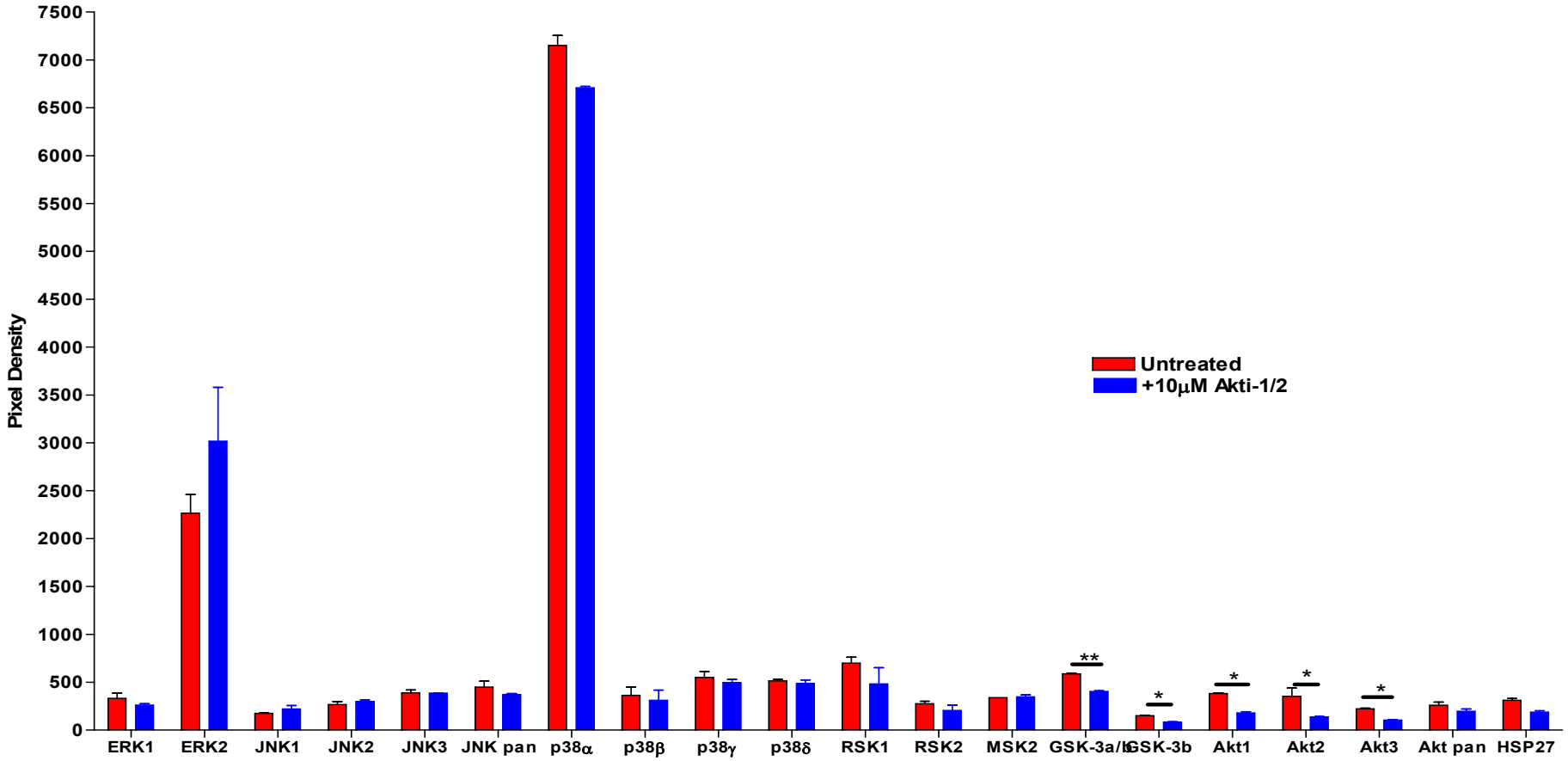

Figure 3.8 The Inhibition of MAPK-family Kinases by Akti-1/2
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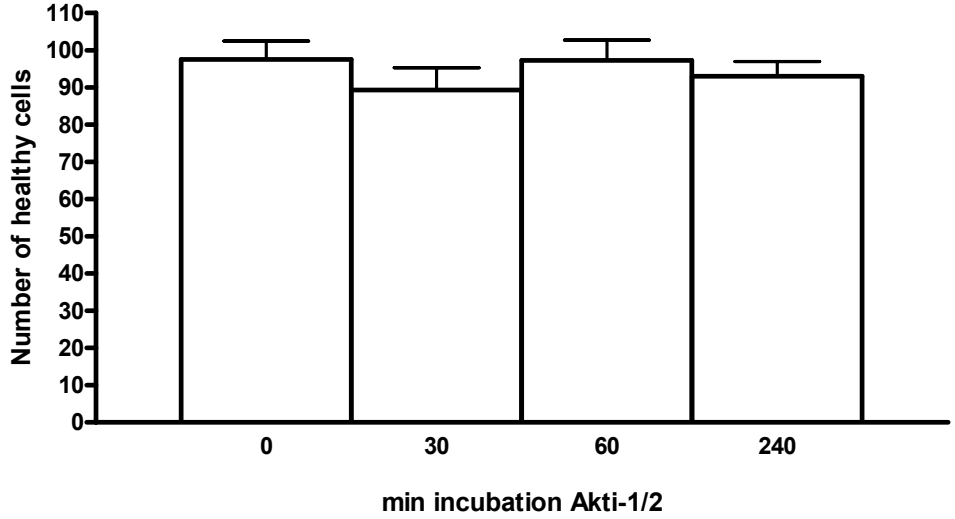
Figure 3.8 The inhibition of MAPK-family Kinases by Akti-1/2. A phospho-array assay was used to determine the off target effects, if any, of Akti-1/2 on the MAPK family. Day 11 SEB activated PBMCs were cultured as described in *Materials and Methods*. On day 11 of culture, after 24hrs in the absence of IL-2, cells were incubated with 10μM Akti-1/2 or DMSO (control) for 30 minutes prior to 5 minutes stimulation with CXCL12. The cells were then lysed and the lysate incubated with the nitrocellulose membrane as described in *Materials and Methods.* Unbound cell lysate was washed off, and the membrane targeted with HRP-tagged secondary antibody. ECL was used to visualise the proteins. The developed films were scanned onto a computer, and the pixel density of the spots was calculated using Adobe® Photoshop. All antibodies shown are phospho-antibodies with the exception of those labelled "pan" and the control protein, HSP27. Results are expressed as Mean ± SEM, n=3. Students t-test, * $p<0.05$, ** $p<0.01$.

(A)



(B)

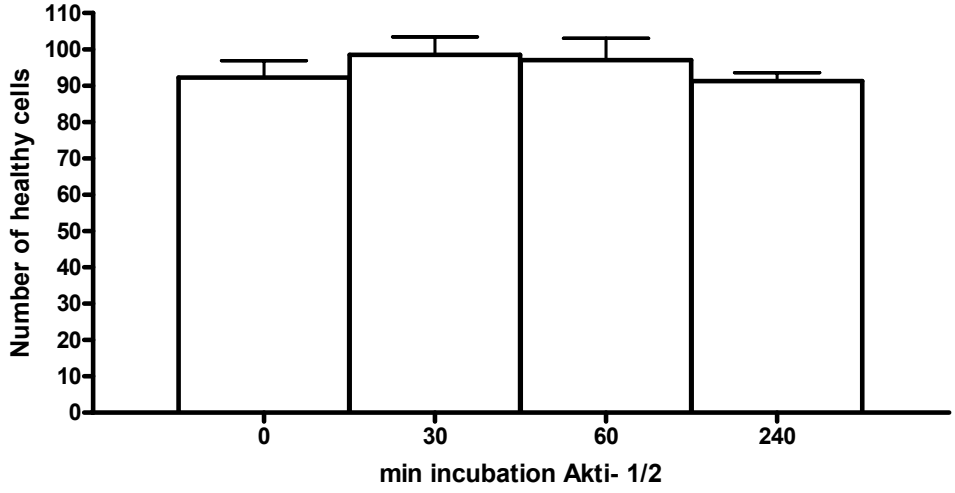


Figure 3.9 The effect of Akti- 1/2 on cell death. A cell death assay using Trypan Blue was utilised to determine the effect of Akti-1/2 incubation over a prolonged period of time on cell mortality. CEM cells (*A*) or Day 10-11 SEB activated PBMCs (*B*) were incubated with Akti-1/2 for 30, 60 or 240 minutes, or not exposed to the inhibitor, before 50µL of cells were mixed with 50µL of Trypan Blue. The number of stained (dead) cells was counted using a haemocytometer as described in *Materials and Methods*. Results are expressed as Mean ± SEM, n=5.

3.6 The Role of Akt in T lymphocyte chemotaxis

To investigate the role of Akt in T lymphocyte migration, and confirm the differing requirements for Akt between SEB activated PBMCs and CEM cells, both types of cells were preincubated with the inhibitor prior to their use in a chemotaxis assay.

Cells were pretreated with varying concentrations of Akti-1/2 from 0.3μM to 30μM for 30 minutes. The cells were then washed in RPMI 1640 before their addition to a Neuroprobe Chemotaxis plate. Four different concentrations of chemokine were used in the assay as well as a control. Each concentration of inhibitor was run in triplicate against each concentration of chemokine, and each experiment was repeated a minimum of four times. After the cells had migrated for three hours, the number of migrated cells was assessed using Flow Cytometry and FlowCount beads.

Analysis of the results revealed that pre-incubation with the inhibitor significantly inhibited the migration of CEM cells (Fig. 3.10). Interestingly, this occurred for both CXCL12 and CCL22 mediated migration, and not just for CXCL12 as with PI3K inhibition. The number of cells that migrated to 10nM of the chemokines (the maximum migration) was used to determine the percentage maximum migration for each inhibitor. This information was then used to construct an IC_{50} graph for each chemokine. IC_{50} is a measure of the concentration of inhibitor needed to inhibit 50% of the response, and is used as a measure of the inhibitors potency. CCL22 mediated migration of CEM cells was inhibited with an IC_{50} of 7.3μM (Fig. 3.10*A*), while CXCL12 mediated migration was inhibited with an IC_{50} of 9.15μM (Fig 3.10*B*). These values, while not particularly potent, reveal that this inhibitor causes significant inhibition of migration.

Using Akti-1/2 in T lymphocyte migration revealed that Akt has an important role in CXCL12 mediated migration. Pre-incubation of day 10-12 SEB activated PBMCs with varying concentrations of the inhibitor resulted in significant dose-dependent inhibition of migration, with 30μM of the inhibitor almost

completely abrogating the migratory response (Fig 3.11). Using the maximal migration concentration of 1μM CXCL12 to plot an IC_{50} graph revealed that Akti-1/2 inhibited migration of PBMCs with an IC_{50} of 0.86μM, indicating that Akti-1/2 is more potent at inhibiting T lymphocyte migration than CEM cell migration.

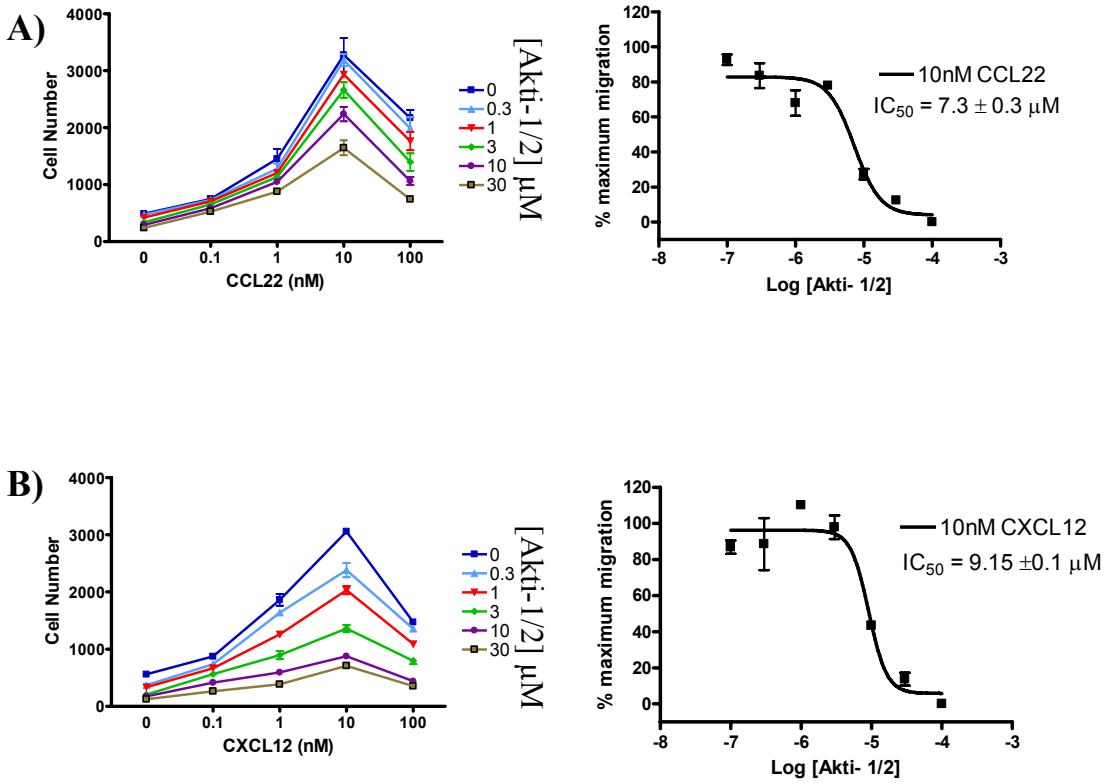


Figure 3.10 The effect of the broad-spectrum Akt inhibitor Akti- 1/2 on CCL22 and CXCL12 mediated CEM cell migration. Chemotaxis assays were used to determine the role of Akti-1/2 in CEM cell migration. CEM cells were cultured as described in *Materials and Methods* before being treated with Akti- 1/2 for 30 min. Cells were placed in Neuroprobe chemotaxis chambers containing various concentrations of CCL22 (*A*) or CXCL12 (*B*) and allowed to migrate for 2 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=3.

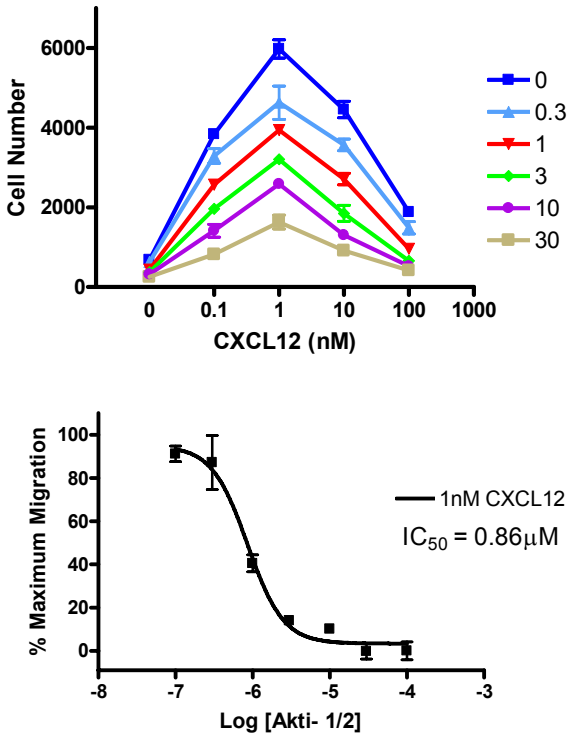


Figure 3.11 The effect of the broad-spectrum Akt inhibitor Akti-1/2 on CXCL12 mediated T lymphocyte migration. A chemotaxis assay was used to determine the role of Akti-1/2 in T lymphocyte migration. Day 10-12 SEB activated PBMCs were cultured as described in *Materials and Methods* before being treated with Akti- 1/2 for 30 min. Cells were placed in Neuroprobe chemotaxis chambers containing various concentrations of CXCL12 and allowed to migrate for 3 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=3.

3.7 The effect of PI3K and Akt inhibitors on Akt activity

The surprising result that PI3K inhibitors had no effect on CCL22 mediated CEM cell migration or CXCL12 mediated T lymphocyte migration, while inhibition of Akt, a kinase downstream of PI3K, abrogated chemotaxis, required further investigation. It was theorised that this result could be explained if, although both PI3K inhibitors and Akt inhibitors have been shown to have similar effects of Akt phosphorylation, they had differing effects on Akt activity. To test this, a system that directly measured Akt activity, as opposed to Akt phosphorylation, was utilised. The Omnia® Lysate Assay system uses fluorophores attached to substrate peptides specific for Akt. Upon phosphorylation of the peptide by Akt, a change in fluorescence occurs, allowing measurement of Akt activity.

Measurement of Akt activity in CCL22-stimulated CEM cells incubated with LY294002 revealed that only high concentrations of the compound resulted in inhibition (Fig 3.12*A*). These concentrations were greater than those used in the chemotaxis assays, and may have off-target effects. This result was the same for Wortmannin (Fig 3.12*B*) and ZSTK474 (Fig 3.12*C*) as well. Interestingly, Akti-1/2 was more potent at inhibiting Akt activity than PI3K inhibitors, inhibiting Akt at concentrations similar to those used in chemotaxis assays, and at concentrations where the inhibitor is believed to be specific (Fig 3.12*D*). When this experiment was repeated for CEM cells stimulated with 10nM CXCL12, which induced migration that was inhibited by PI3K inhibitors, the results revealed that all of the inhibitors reduced Akt activity in a statistically-significant way, at concentrations similar to those used in the migration assays. Indeed, LY294002 reduced Akt activity at concentrations as low as 1μM (Fig 3.13*A*), while Wortmannin reduced Akt activity at 100nM (Fig 3.13*B*) and ZSTK474 at 1μM (Fig 3.13*C*). Akti-1/2 was slightly more potent against CXCL12-stimulated cells compared to CCL22-stimulated cells, with significant inhibition beginning to occur at 1μM (Fig 3.13*D*).

Analysis of Akt activity in CXCL12-stimulated SEB activated PBMCs revealed that while the levels of Akt activity are lower in unstimulated cells and higher in

stimulated cells when compared to CEM cells (Fig 3.14), the effect of PI3K and Akt inhibitors on Akt activity was similar to CCL22-stimulated CEM cells, the other PI3K-independent migration model. Only incubation with high concentrations of LY294002 (Fig 3.14*A*), Wortmannin (Fig 3.14*B*) and ZSTK474 (Fig 3.14*C*) resulted in a reduction in Akt activity, at concentrations higher than those used in chemotaxis assays. Akt-1/2 significantly reduced Akt activity at concentrations from 1μM, a concentration where the compound is still selective and similar to those that caused inhibition of migration.

These results help to explain the differences between the inhibitors and their effects of cell migration, and also demonstrate a difference between Akt phosphorylation and Akt activity, revealing that Akt phosphorylation is not necessarily the best way of measuring Akt activity.

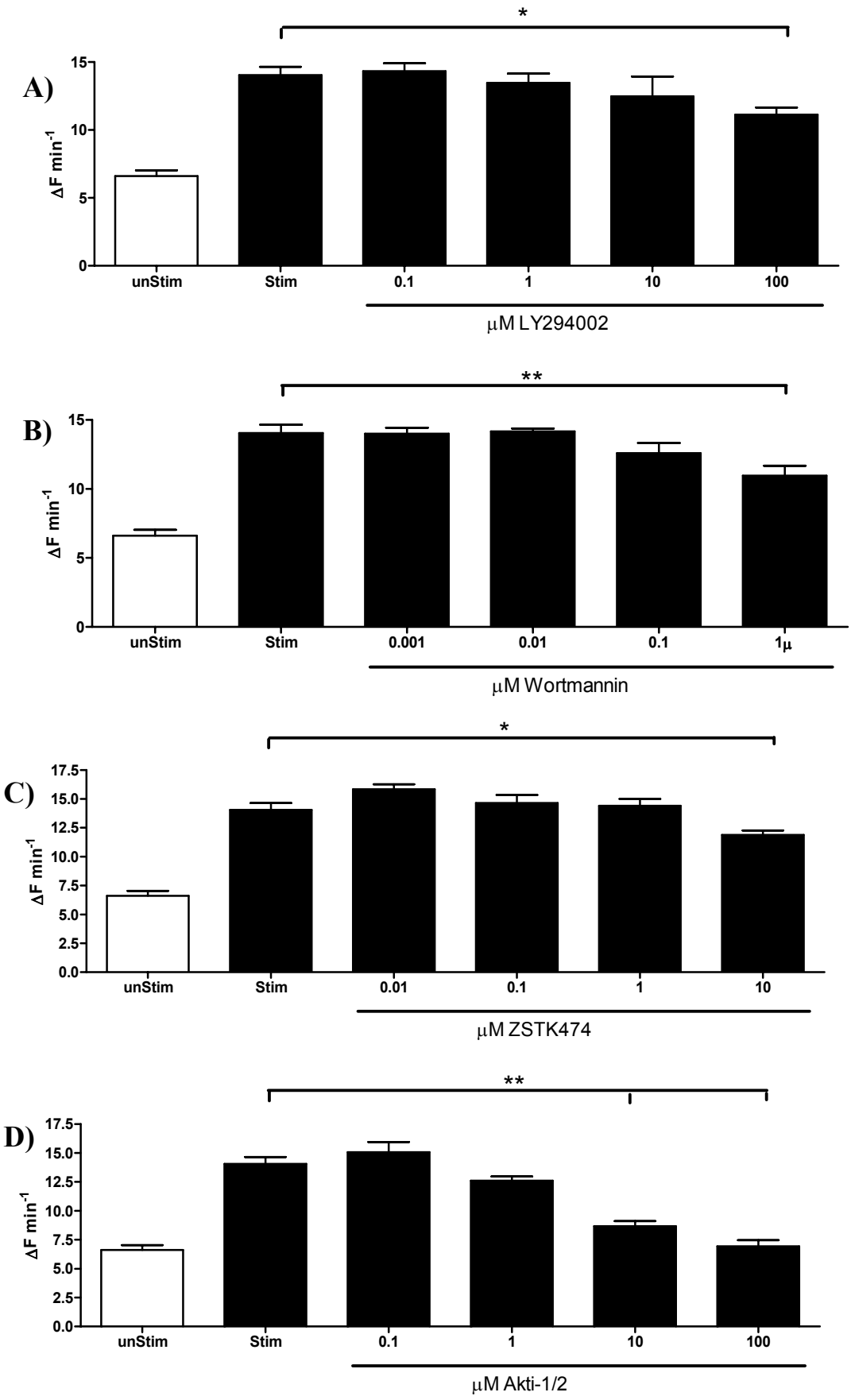


Figure 3.12 The effect of PI3K and Akt inhibitors on Akt activity of CEM cells.

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Figure 3.12 The effect of PI3K and Akt inhibitors on Akt activity of CEM cells. An Akt activity assay was used to determine the efficacy of Akt and PI3K inhibitors in Akt inhibition. CEM cells were incubated with LY294002 (*A*) Wortmannin (*B*) ZSTK474 (*C*) or Akti-1/2 (*D*) for 30 min then stimulated with 10nM CCL22 for 5 min and lysed. Resulting Akt activity was assessed using Akt activity assay as described in *Materials and Methods*. Results shown as change in the rate of phosphorylation, which corresponds to change in the rate of flourescence of target peptides by Akt. Results are expressed as mean ± SEM, n=4. One-way ANOVA * $p<0.05$, ** $p<0.01$. Stim, Stimulated

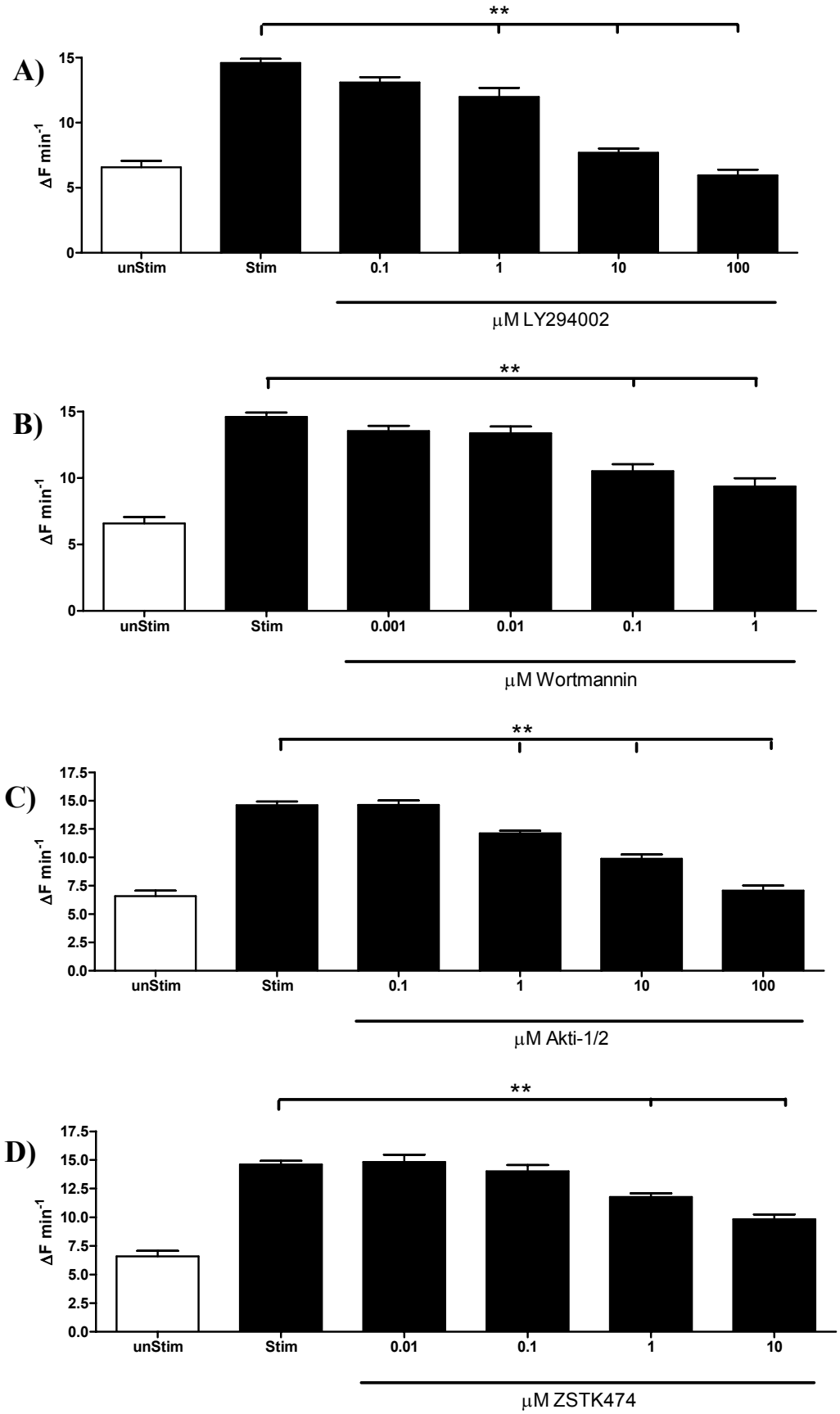


Figure 3.13 The effect of PI3K and Akt inhibitors on Akt activity of CEM cells. Please see the next page for legend

Figure 3.13 The effect of PI3K and Akt inhibitors on Akt activity of CEM cells. An Akt activity assay was used to determine the efficacy of Akt and PI3K inhibitors in Akt inhibition. CEM cells were incubated with LY294002 (*A*) Wortmannin (*B*) ZSTK474 (*C*) or Akti-1/2 (*D*) for 30 min then stimulated with 10nM CXCL12 for 5 min and lysed. Resulting Akt activity was assessed using Akt activity assay as described in *Materials and Methods*. Results shown as change in the rate of phosphorylation, which corresponds to change in the rate of flourescence of target peptides by Akt. Results are expressed as mean ± SEM, n=4. One-way ANOVA ** $p<0.01$. Stim, Stimulated

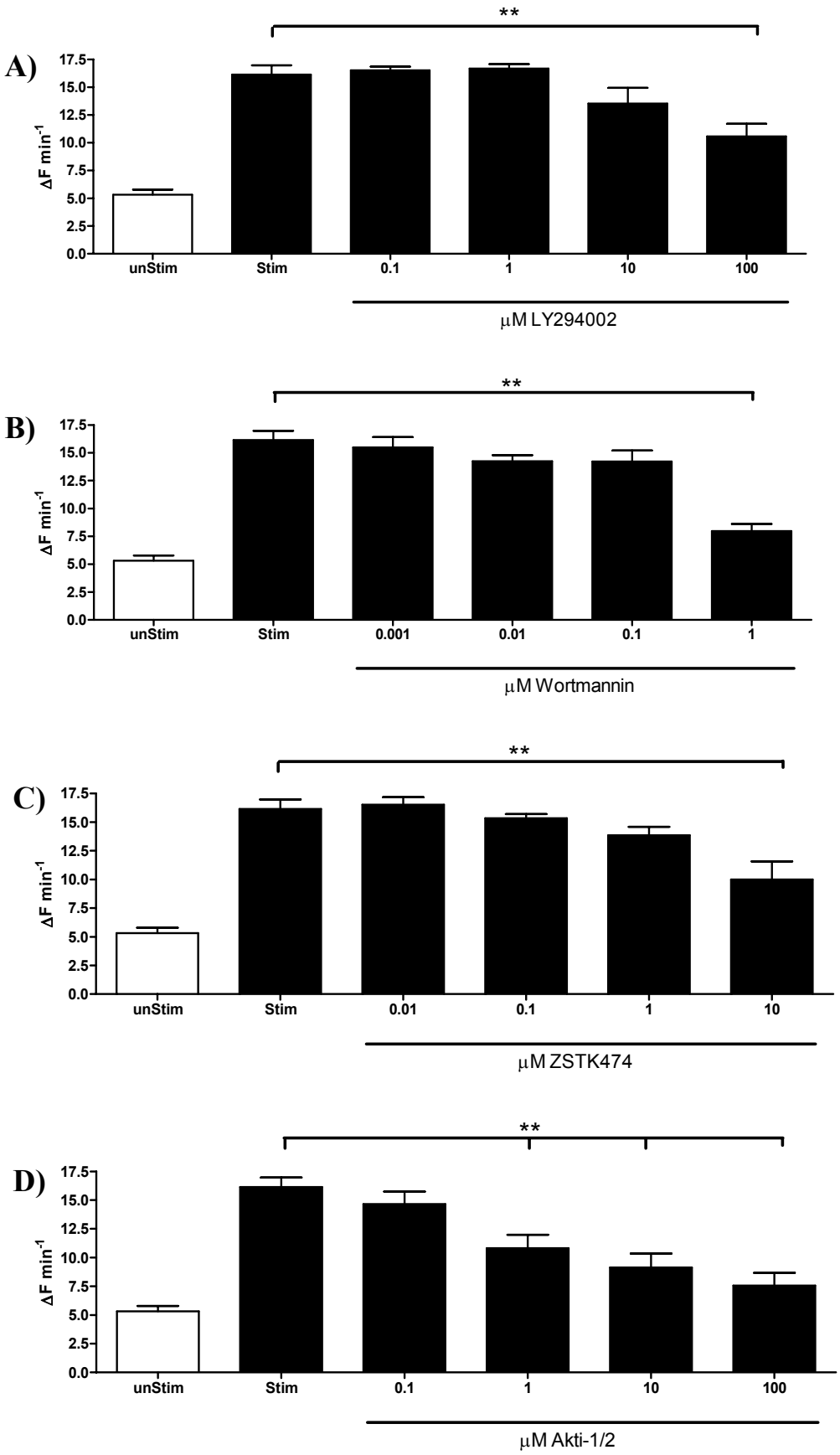


Figure 3.14 The effect of PI3K and Akt inhibitors on Akt activity of PBMCs. Please see the next page for legend

Figure 3.14 The effect of PI3K and Akt inhibitors on Akt activity of PBMCs. An Akt activity assay was used to determine the efficacy of Akt and PI3K inhibitors in Akt inhibition. PBMCs were incubated with LY294002 (*A*) Wortmannin (*B*) ZSTK474 (*C*) or Akti-1/2 (*D*) for 30 min then stimulated with 10nM CCL22 for 5 min and lysed. Resulting Akt activity was assessed using Akt activity assay as described in *Materials and Methods*. Results shown as change in the rate of phosphorylation, which corresponds to change in the rate of flourescence of target peptides by Akt. Results are expressed as mean ± SEM, n=4. One-way ANOVA ** $p<0.01$. Stim, Stimulated

3.8 The role of GSK-3 in T lymphocyte migration

GSK-3 is a protein kinase shown to be phosphorylated by Akt at Ser9. GSK-3 is unusual among protein kinases in that in its unphosphorylated state it is active, and it's phosphorylation that causes it to become inactive. GSK-3 has a role in inhibiting glycogen synthase and thus leading to an increase in glucose, essential for energy production and a process that is important in cell migration. Therefore, GSK-3 was investigated to determine whether it was involved in cell migration, and to help confirm a role for Akt.

In 2003, the inhibitor 6-Bromoindirubin-30-oxime (BIO) was released (Meijer et al., 2003a). This inhibitor showed much stronger selectivity for GSK-3 than classic inhibitors for GSK-3 such as lithium, Staurosporine or Flavopiridol and for this reason was used to investigate the role of GSK-3 in cell migration. As phosphorylation of GSK-3 by Akt leads to a loss of GSK-3 activity, use of GSK-3 inhibitors can be said to mimic the actions of Akt. As such, it also makes a good tool to investigate the role of Akt and its effect on GSK-3 in migration.

To test the effect of BIO on GSK-3 inhibition, concentrations of the inhibitor from 0.3μM to 30μM were incubated with CEM cells for either 30 or 60 minutes. The effect on GSK-3 was assessed using beta-catenin. As the inhibitor prevents phosphorylation of GSK-3, direct assessment of its activity is difficult to determine. However, GSK-3 is also involved in a different pathway to the PI3K/Akt/GSK-3 pathway. This pathway, the Wnt/beta-catenin pathway, is involved in gene transcription. GSK-3, bound to APC (adenomatosis polyposis coli), becomes complexed with beta-catenin and Axin (a scaffolding protein). This complex increases the activity of GSK-3, resulting in an increase in the phosphorylation of beta-catenin. When beta-catenin is phosphorylated, it becomes targeted for degradation through the ubiquitination pathway. Therefore, inhibition of GSK-3, through the actions of BIO, prevents the phosphorylation and subsequent degradation of beta-catenin, leading to an increase in the levels of beta-catenin. This increase can be measured using immuno-blotting and an antibody that binds beta-catenin, providing a method for determining BIO's activity. This assay revealed that BIO inhibits GSK-3

and prevents the subsequent phosphorylation of beta-catenin, at concentrations of 3μM and 10μM, although this effect is less strong at 30μM (Fig 3.15). The optimal incubation time was 30 minutes, to prevent toxicity on the cells.

The effect of BIO and subsequent inactivation of GSK-3 on T lymphocyte migration was investigated using both CEM cells, and day 10-12 SEB activated PBMCs. Interestingly, although not unexpectedly as Akt activation and therefore GSK-3 inhibition is required for migration, incubation of BIO actually increased levels of migrating CEM cells to CCR4 in a concentration-dependent fashion, with 10μM of the compound providing maximal response (Fig 3.16). As BIO mimics the effect of Akt activation, this increase in migratory levels underscores the importance of Akt in migration.

One explanation for the increase in the number of migrating cells could be that GSK-3 has a role in chemokinesis (random cell movement) rather than chemotaxis (directional cell migration). In the migration assay, the increase in migrating cells was significant only during chemotaxis, i.e. when chemokine was present, and not during chemokinesis. However to confirm this, a chequerboard analysis was performed. This analysis uses a standard chemotaxis assay, but with varying concentrations of chemokine in both the upper and lower chambers. In those wells where the concentration of chemokine in both the upper and lower wells is the same, then there can be no chemokine gradient, and any resulting movement in cells to the bottom chamber must be due to an increase in chemokinesis.

Analysis of the results from this assay using CCL22-stimulated CEM cells revealed that in the absence of BIO, CEM cells migrate normally to CCL22 (vertical yellow bar) and that some of the CCL22 induced cell movement is chemokinetic (diagonal yellow bar), although to a much lesser extent than chemotaxis, as determined by comparing cell numbers (Fig 3.17). When this experiment was repeated in the presence of 1μM BIO, the number of cells that migrated was significantly increased, but the number of cells that underwent chemokinesis stayed the same, indicating that GSK-3, and therefore Akt, are involved in the directional movement of cells.

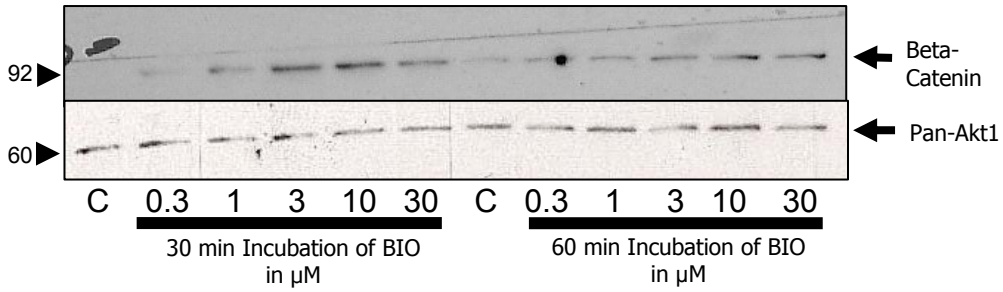


Figure 3.15 The effect of BIO on intercellular beta-catenin. Immunoblotting was used to determine the optimum concentration and incubation time of BIO in CEM cells. CEM cells were incubated with the GSK-3 inhibitor BIO at the times and contentrations shown, then lysed as described in *Materials and Methods*. Lysates (1 x 10^6 cells per lane) were resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, immunoblotted with a anti-beta-catenin antibody and protein was visulaised with ECL. The blots were stripped and reprobed with pan Akt antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments.

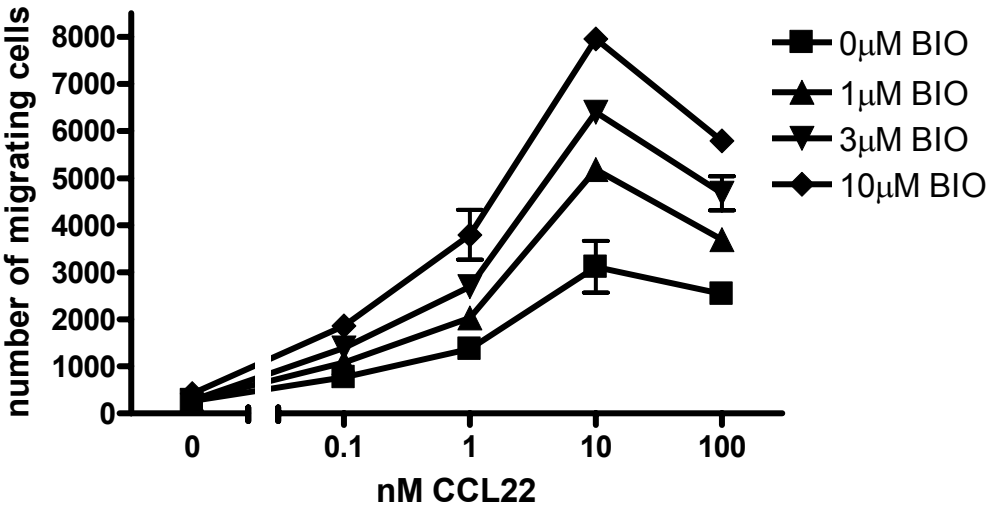


Figure 3.16 The effect of the GSK-3 inhibitor BIO on CCL22 mediated CEM cell migration. A chemotaxis assay was used to determine the role of GSK-3 in CEM cell migration. Rested CEM cells were cultured as described in *Materials and Methods* before being treated with GSK-3 for 30 min. Cells were placed in Neuroprobe chemotaxis chambers containing various concentrations of CCL22 and allowed to migrate for 2 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=3.

		Top Well CCL22			
		0nM	**0.1nM**	**1nM**	**10nM**
Bottom Well	**0nM**	4577	4207	3802	2540
CCL22	**0.1nM**	15157	7690	4358	1256
	1nM	30010	19710	16390	1237
	10nM	20603	20097	16820	15343

		Top Well CCL22 + 1uM BIO			
		0nM	**0.1nM**	**1nM**	**10nM**
Bottom Well	**0nM**	3130	3950	2397	1982
CCL22	**0.1nM**	27230	8300	5057	4417
"+ 1uM BIO"	**1nM**	51803	46657	17426	9783
	10nM	30883	19290	17897	14590

Table 3.1 The effect of BIO on cell chemokinesis. Two chequerboard analyses were performed to determine whether the increase in cell migration after incubation of cells with BIO is a result of an increase in cell chemokinesis. Rested CEM cells were cultured as described in *Materials and Methods* before pre-treatment with 1μM BIO for 30 min (*lower table*) or left untreated (*upper table*) for 30 min. Cells were placed in Neuroprobe chemotaxis chambers containing various concentrations of CCL22 in both upper and lower chambers and allowed to migrate for 2 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as number of migrating cells. Data are representative of four separate experiments.

3.9 The role of PKCδ in migration

Previous studies within the Ward laboratory revealed a role for a calcium independent, diacylglycerol (DAG) dependent protein kinase in CCR4 mediated migration of CEM cells (Cronshaw et al., 2006). Protein Kinase C delta (PKCδ) is a serine/threonine kinase that plays a role in growth regulation and tissue remodelling. It is one of the novel isoforms of PKC which are activated by DAG and lipid binding alone, as opposed to conventional isoforms which require the presence of Ca^{2+} in addition to DAG and lipid binding, and atypical isoforms which are activated solely by phosphorylation. As PKCδ fits the criteria, and has previously been implicated in cell migration (Fanning et al., 2005b; Iwabu et al., 2004b), it was investigated regarding its role in T lymphocyte chemotaxis.

In addition to PKCδ's dependence on PI3K for activation through its production of PIP_3, which is subsequently broken down by phospholipase C to form DAG; essential for activation, it should also be noted that most PKC's require phosphorylation at the activation loop in order to prime and stabilise the kinase in an active form. PDK-1 has been shown to be responsible for phosphorylation of PKCδ at Thr505 on the activation loop (Steinberg, 2004; Le Good et al., 1998). Although there is some debate as to whether this phosphorylation is important in activation of the kinase, or just for its subsequent stabilisation (Stempka et al., 1997b), it does appear that PI3K, via the downstream kinase PDK-1, is involved in PKCδ activation. Therefore, a role for PKCδ in chemotaxis could also help to confirm a role for PI3K.

To confirm whether PKCδ is present in T cells and activated during chemotaxis, CEM cells were stimulated with the chemokine CCL22 for 2 minutes. The cells were then lysed and immunoblotted with an antibody that recognises Thr505, the residue phosphorylated by PDK-1. Analysis of the result revealed that phosphorylation of Thr505 occurs after stimulation with 0.3nM and 1nM CCL22 (Fig 3.17). To confirm whether this result was PI3K dependent, cells were also pretreated with 10μM LY294002 prior to stimulation. Interestingly,

although LY294002 did partially reduce phosphorylation, it was not fully abrogated.

Rottlerin is marketed as a PKCδ-selective inhibitor that uncouples mitochondria, decreasing cellular ATP and thus indirectly blocking PKCδ. To test whether PKCδ was involved in chemotaxis of CEM cells, Rottlerin was used in a chemotaxis assay. CEM cells were pre-incubated with several different concentrations of Rottlerin, and then allowed to migrate to 1nM CCL22. Analysis of the results revealed that Rottlerin significantly inhibited chemotaxis in a dose dependent manner, indicating that PKCδ was important in migration (Fig. 3.18).

Subsequent to this result, several papers were published questioning the selectivity of Rottlerin towards PKCδ (Soltoff, 2007), and indicating that it may in fact target other kinases (Alonso et al., 2008). As there are currently no other selective inhibitors of PKCδ, an alternative method had to be used to determine the role of PKCδ in chemotaxis. Research performed within the lab has demonstrated the limitations of siRNA for migration assays of suspension cells (Smith et al., 2007) and so this system was not used. Therefore, PKCδ null mice were employed to determine the role of PKCδ in migration.

Spleens from both PKCδ$^{-/-}$ and wildtype C57Bl/6 mice were disaggregated and the red blood cells lysed. T cells were negatively selected from the remaining population of cells using a MACS murine T cell sorting kit. The murine PBMCs were cultured in ConA and IL-2 for 3 days to activate them, prior to their use. An antibody was not available to test the expression of CCR4 on these cells, but previous studies had already identified CCR4 as being present on murine T cells (Youn et al., 1997). The cells were placed in a chemotaxis assay with varying concentrations of murine CCR4 and after 3 hours, the number of migrated cells was counted using flow cytometry. Analysis of the results revealed that CCL22 caused the splenocytes to migrate but that significantly fewer cells from the knockout mice migrated to both chemokines (Fig. 3.19). As the only difference between the cells is the lack of PKCδ, this result reveals a role for PKCδ in T

lymphocyte migration. Interestingly, migration was not completely abrogated, indicating the presence of a non PKC delta-dependent pathway.

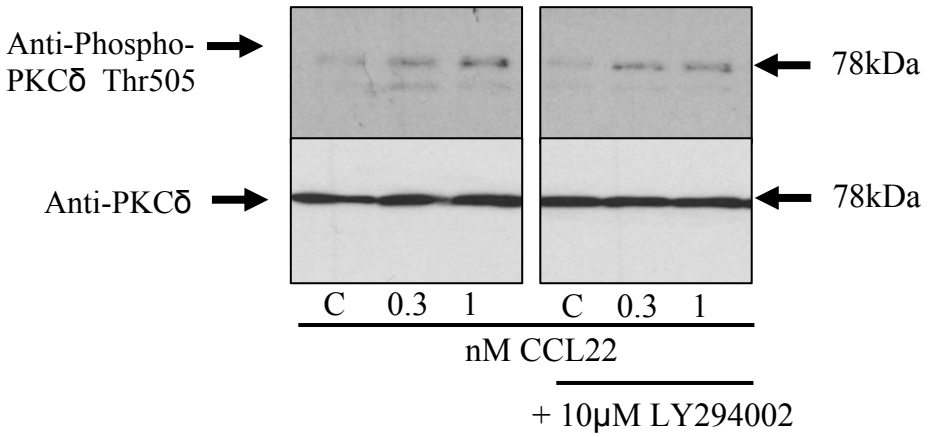


Figure 3.17 The effect of CCL22 stimulation on PKCδ phosphorylation. Immunoblotting was used to determine whether stimulation with CCL22 results in phosphorylation of PKC delta at Thr505, and the role of PI3K in this process. CEM cells were incubated with the LY294002 for 30 minutes at the contentration shown, then lysed as described in *Materials and Methods*. Lysates (2.5×10^6 cells per lane) were resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, blotted with a phospho-specific PKCδ antibody with affinity for the active Thr505 phosphorylated form of PKCδ, and protein was visulaised with ECL.. The blots were stripped and reprobed with pan PKCδ antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments.

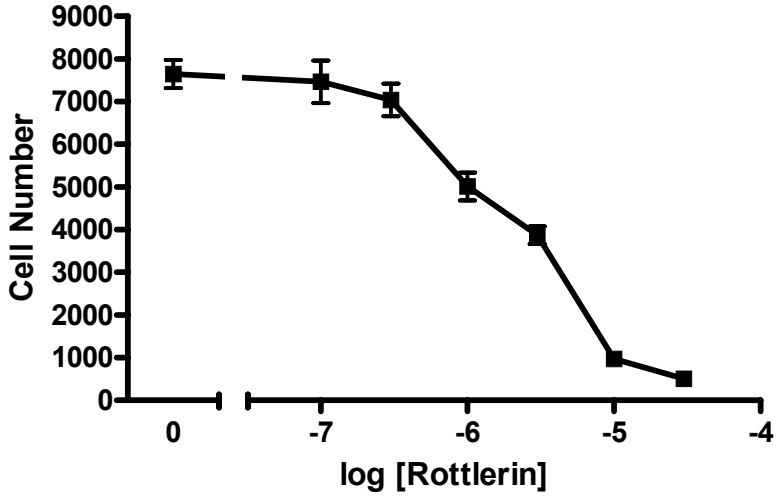


Figure 3.18 The effect of the PKC delta inhibitor Rottlerin on CCL22 mediated CEM cell migration. A migration assay was used to determine whether PKCδ is important in CEM cell migration. CEM cells were cultured as described in *Materials and Methods* before being treated with Rottlerin for 30 min at the concentrations shown. Cells were placed in Neuroprobe chemotaxis chambers containing 1nM CCL22 and allowed to migrate for 2 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=3.

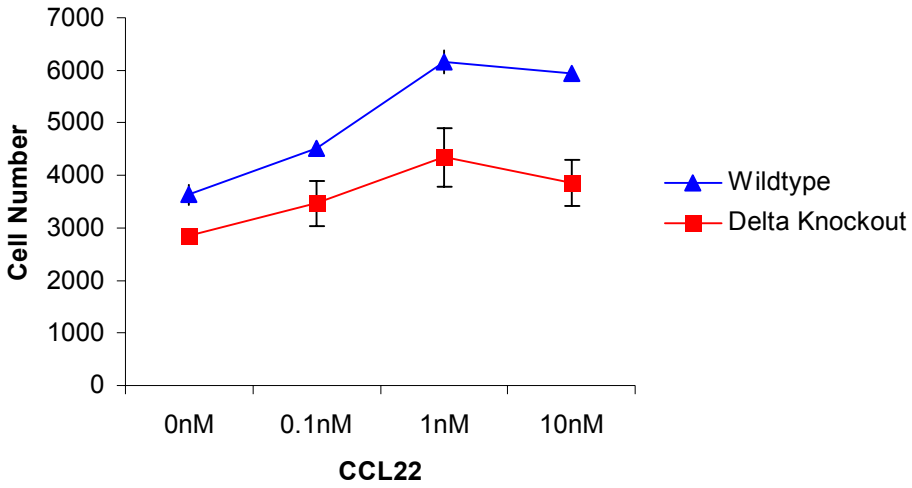


Figure 3.19 The ability of wildtype and PKCδ$^{-/-}$ murine splenocytes to migrate to CCL22. A migration assay was used to determine whether PKCδ is important in the migration of murine splenocytes. Murine splenocytes were disaggregated, sorted and cultured as described in *Materials and Methods.* The cells were starved of IL-2 for 24 hours prior to use. Wildtype and knockout cells were placed in Neuroprobe chemotaxis chambers containing varying concentrations of CCL22 and allowed to migrate for 3 hours. Migrated cells were then counted using FlowCount Beads on a BD FACSCanto II using BD FACS DiVa software. Results are expressed as mean ± SEM, n=4.

3.10 Summary

- Using the T lymphocyte cell line CEM cells and SEB activated lymphocytes; this study has shown that stimulation by the chemokines CXCL12 and CCL22 results in activation of PI3K.
- In addition, stimulation with these chemokines results in robust migratory responses in both cell types. However, CCL22 mediated migration of CEM cells and CXCL12 mediated migration of PBMCs are unaffected by inhibition of PI3K using a panel of PI3K inhibitors.
- Using an inhibitory compound specific for Akt, studies revealed that Akt was critical for migration of both cell types to CXCL12 and for CEM cell migration to CCL22. The different effects on migration between Akt and PI3K inhibitors were shown to be as a result of their differing effects on Akt activity.
- GSK-3, a protein downstream of PI3K in several signalling pathways was shown to be also involved in migration. Inhibition of GSK-3 resulted in an increase in the number of migrating cells, and this was shown to be because of an increase in chemotaxis and not chemokinesis.
- Another protein thought to be downstream of PI3K signalling, PKCδ, which previous studies indicated had a role in migration, was shown to be activated by CCL22. Using murine splenocytes, a role for PKCδ in the migration of PBMCs was observed.

3.11 Discussion

The aim of this part of the study was to gain insight into the role of Phosphoinositide 3-Kinase (PI3K), and other kinases shown to be downstream of PI3K in several signalling pathways, in T lymphocyte migration. This involved investigating several different inhibitors for their effects on cell migration, including inhibitors of PI3K, Akt and GSK-3. However, before this work could be carried out, the expression of chemokine receptors on T lymphocytes, as well as the optimum concentration of PI3K inhibitors had to be determined.

3.11.1 The contribution of PI3K to CEM cells and PBMC chemotaxis.

The dependence of PI3K in the role of directed cell migration has been well established (Hannigan et al., 2004; Barber and Welch, 2006; Sasaki and Firtel, 2006), although previous studies have revealed that PI3K was a dispensable signal during CEM cell and activated PBMC migration (Cronshaw et al., 2004; Smith et al., 2007). These studies had been performed using the broad spectrum inhibitors of PI3K; LY294002 and Wortmannin, which have been shown to have off target effects, including mTOR, CK2 and polio-like kinase. (Gharbi et al., 2007; Liu et al., 2005). Recently, a PI3K inhibitor that inhibits all PI3K isoforms, but shows no activity against protein kinases has been developed (Yaguchi et al., 2006). This compound, ZSTK474, was used alongside LY294002 and Wortmannin to determine the role of PI3K in T lymphocyte migration. This revealed that although all three inhibitors reduced Akt phosphorylation, a surrogate marker for PI3K activity, they failed to inhibit migration of CEM cells to CCL22 or SEB activated PBMCs to CXCL12. Interestingly, CEM cell migration to CXCL12 was dependent on PI3K. This demonstrates a difference in chemokine receptor signalling pathways within a cell, as well as a difference between the same chemokine receptor in different cells.

Several other studies have also noted PI3K independence during chemotaxis. Smit *et al.* (Smit et al., 2003a) have showed that CXCR3-mediated migration of

T cells was regulated through a phospholipase C-dependent pathway as opposed to a PI3K/Akt pathway. In this study, the authors suggested that migration could still be mediated through a PI3K class II isoform, however LY294002 has been shown to inhibit this class of PI3K making this explanation unlikely. Another study (Lacalle et al., 2004) showed that PTEN, the phosphatase that converts PIP3 into PIP2, regulates motility but not directionality in chemotaxis. This indicates a role for PI3K in migration but not directed migration.

SEB activated PBMCs are cultured in IL-2 prior to use. Culture in IL-2 has been shown to lead to a loss of the requirement for PI3K during chemotaxis while maintaining the requirement during chemokinesis (Smit et al., 2003b; Smith et al., 2007). Therefore the lack of PI3K-dependent migration may be a result of culture in IL-2 as opposed to a fundamentally different pathway. Further work exploring the migration of PBMCs using other chemokine receptors would be needed to confirm this.

3.11.1.1 If not PI3K then what?

If PI3K is not responsible for the migration of these cells, what is? One candidate for the role is the Rac-specific Guanine Nucleotide Exchange Factor DOCK-2. Mice that lack the gene for DOCK-2 show a lack of migration of lymphocytes to several chemokines, while PI3K/Akt signalling was unaffected (Fukui et al., 2001). In addition, studies using these mice revealed that T lymphocytes can still mount a chemotactic response through p110γ in the absence of DOCK-2. Interestingly, in neutrophils, where PI3K has been shown to play a vital role in migration, DOCK-2 is not present (Nishihara et al., 1999).

Another possibility is Phospholipase C (PLC). A study in 2007 used mice lacking the PLCβ isoforms of PLC to demonstrate significantly impaired T lymphocyte migration. This corresponds with another study (Cronshaw et al., 2006), which also showed the importance of PLC; however the two studies differed on the importance of calcium mobilisation during this process.

Finally, a recent paper has implicated PKCθ as an important kinase in PI3K-independent chemotaxis. Using a PKCθ-specific inhibitor, they discovered that PKCθ was vital for migration in the PI3K-independent model of migration using CXCL12-mediated chemotaxis of IL-2 cultured T lymphocytes (Shahabi et al., 2008).

3.11.2 A role for Akt in T lymphocyte migration

Akt has long been described as a kinase downstream of PI3K. Phosphorylation of Akt is often used as a marker of PI3K activity, and many publications make reference to the PI3K / Akt pathway. To confirm the PI3K independence of CCR4-mediated CEM cell migration and CXCR4-mediated PBMC migration, an inhibitor against the two most common isoforms of Akt; Akt1 and Akt2, was used (Lindsley et al., 2005; Zhao et al., 2005b). Use of this inhibitor revealed that chemotaxis in all three models of cell migration was significantly reduced. To confirm that the loss of migration was not due to cell death, a trypan blue cell death assay was performed that showed the inhibitor had no effect on cell mortality during the incubation period.

One possible explanation for this effect is that Akti-1/2 is having off target effects. To test this, Akti-1/2 was screened against a panel of kinases and was shown to have no effect. However, a paper has recently been published showing that this inhibitor does have the ability to potentiate protease-activated factor 1 (PAR-1). While this protein is only found in platelets, and to a lesser extent on myocytes and neurones (Macfarlane et al., 2001), and so is unlikely to explain this result in T lymphocytes, it does raise the question of what other off-target effects there are. As the inhibitor is still new, little independent work has been undertaken to confirm its selectivity, but this is something that will need to be completed before this result can be fully confirmed.

Another explanation for this result could be that Akt is being activated independently of PI3K. A paper published in 2001 (Sonnenburg et al., 2001) revealed that PDK-1, the kinase shown to phosphorylate Akt at Thr308, phosphorylated PKCα independently of PI3K and PIP3. However, this paper

also specifically mentions that Akt phosphorylation was inhibited during this process. Another paper looked at the activity of PDK-1 and Akt in nematode worms. Using gain-of-function and loss-of-function mutants, they showed the interdependence of the nematode homologues of PDK-1 and Akt, which could be active even in the absence of AGE-1, the nematode homologue for PI3K (Gami et al., 2006). However, aside from these two articles, there is no mention of PI3K-independent Akt activation. Therefore, other explanations for this result were sought.

3.11.2.1 Differences in effect on Akt activity

It was theorised that this result could be explained by differences in the effect of the inhibitors on Akt activity. While Akt activity had already been measured through phosphorylation, a more quantitative system that directly measured Akt activity was utilised. This system, which measures Akt's ability to phosphorylate a target peptide, revealed significant differences in the ability of the PI3K inhibitors and the Akt inhibitor to affect Akt activity. This can also be seen in the Immunoblots showing phosphorylation of Akt. The Akt inhibitor sharply decreased Akt activity as well as quickly and potently inhibiting Akt phosphorylation in all three models of chemotaxis. In the PI3K-dependent CXCL12 stimulation of CEM cells, the effects of the PI3K inhibitors were similar to those of the Akt inhibitor, but in CCL22 stimulated CEM cells, and CXCL12 stimulated PBMCs, incubation with PI3K inhibitors only had a mild effect on Akt activity, while the Akt inhibitor completely abrogated it.

These results give rise to two possible explanations. Firstly, as mentioned above, Akt could be activated independently of PI3K, yet play a vital role in the migration of T lymphocytes. The second option could be a difference in the quantity of each kinase within the cell (Fig. 3.20). If Akt is a pinch-point in the signalling cascade, then an inhibitor that targeted here would have a much greater effect than one targeted at a kinase that was present in much greater quantities. Further work will have to be performed to test this hypothesis. The effect of PI3K inhibitors on PI3K activity in these systems should be investigated to determine whether PI3K is still active. In addition, using siRNA

or a similar system, PI3K should be inactivated in a PI3K independent model of chemotaxis. If the Akt inhibitor still had an effect, it would be a good indication that Akt is activated by kinases other than PI3K. Either way, this data demonstrates the importance of using Akt inhibitors alongside PI3K inhibitors when investigating the PI3K/Akt pathway.

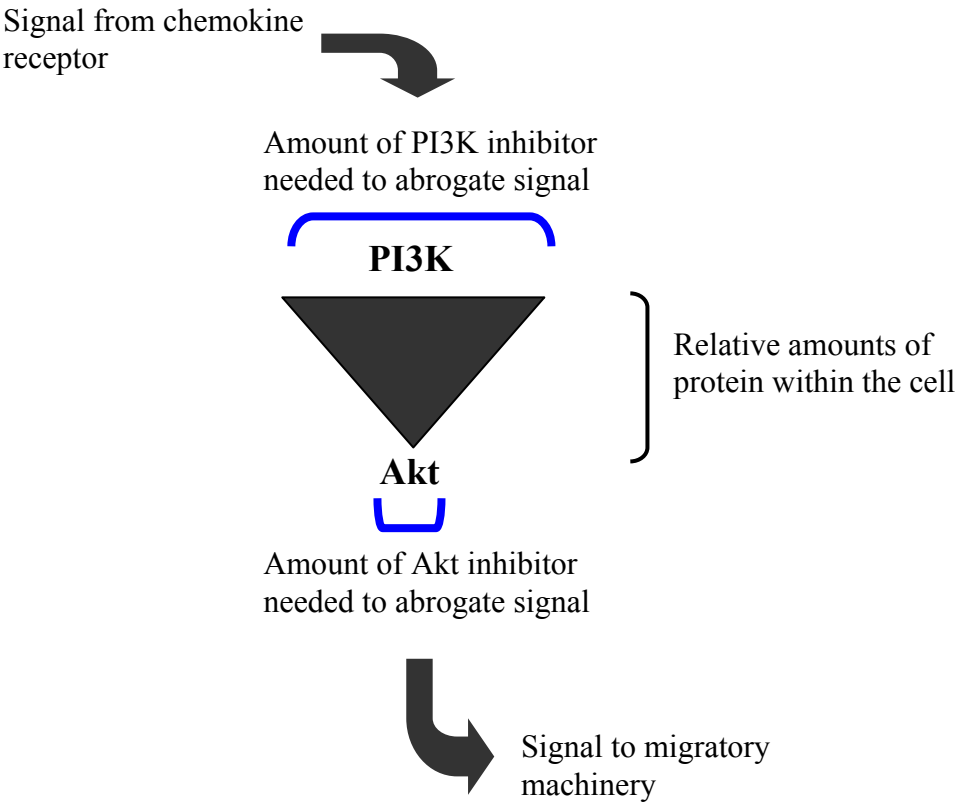


Figure 3.20 Schematic diagram showing proposed pinch-point hypothesis. If an imbalance exists between the amount of PI3K and Akt within a cell, then a much greater concentration of inhibitor is required to achieve the same effect a small amount of inhibitor would have on a different target.

3.11.3 The role of GSK-3 in T lymphocyte chemotaxis

GSK-3 was the first physiological target of PKB to be identified and is a key player in PKB signalling. It is unusual in that phosphorylation by Akt inactivates GSK-3 (Woodgett, 2005). To investigate the role of GSK-3 within T lymphocytes, a specific inhibitor of GSK-3, BIO, was used (Meijer et al., 2003b). Inhibition of GSK-3, via BIO, should have the same effect as if Akt

had been activated. It was therefore theorised that incubation with BIO would promote chemotaxis.

Using the CCL22-mediated chemotaxis of CEM cells model, it was shown that incubation with the GSK-3 inhibitor significantly promoted chemotaxis. This result is not without precedent. Studies using fibroblasts from mice that lack a functional GSK-3β isoform show increased wound healing, accelerated wound closure and increased fibrogenic responses (Kapoor et al., 2008).

One of the ways that GSK-3 could mediate its inhibitory effect on migration is through NFAT. NFAT (nuclear factor of activated T cells) is a transcription factor implicated in the migration of cells (Yoeli-Lerner et al., 2005). In T lymphocytes, GSK3β phosphorylates serine motifs on NFATc1, inhibiting DNA binding and promoting its nuclear export (Beals et al., 1997). Therefore, the inactivation of GSK-3 by Akt allows NFAT access to the nucleus, where it can promote transcription and aid migration (Chow et al., 2008).

GSK-3 is also important in cell polarity. Cells must undergo highly polarised reorganisation of cytoskeletal elements prior to directional migration (Gundersen et al., 2004). One of these re-arrangements is that the microtubule system and the MTOC move to the front of the cell, resulting in the transport of structural proteins to the leading edge of the cell. This system depends on the inactivation of GSK-3. When GSK-3 is phosphorylated, it is unable to phosphorylate the tumour-suppressor gene product Apc. This allows Apc to associate with the microtubules and regulate microtubule dynamics and therefore polarisation (Etienne-Manneville and Hall, 2003).

3.11.4 A role for PKCδ in T lymphocyte migration.

Previous studies within the laboratory had identified a PLC-dependent, Ca^{2+}-dependent protein that was important in CEM cell migration (Cronshaw et al., 2006). Protein Kinase C Delta (PKCδ) is a member of the novel class of PKC isoforms. This class require DAG, but not Ca^{2+} for activation. In addition, PKCδ has been shown to be phosphorylated at Thr505 by PDK-1, and while this is not

a prerequisite for activation (Stempka et al., 1997a); it does indicate a role for PI3K in this process.

CCL22 mediated stimulation of CEM cells resulted in phosphorylation of PKCδ at Thr505, indicating that it is involved in migration. Rottlerin, an inhibitor for PKCδ, since shown to be not very specific, inhibited migration in a dose-dependent manner. Due to a lack of alternative pharmacological tools, mice lacking PKCδ were used to confirm a role for PKCδ in T lymphocyte migration. Migration of murine splenocytes to CCL22 revealed that the PKCδ null cells underwent significantly less migration than the wildtype mice, showing that PKCδ is indeed involved in migration.

Other studies have shown similar results. Studies have shown that PKCδ is important for the migration of smooth muscle under mechanical stress (Li et al., 2003). In addition, CCL15, the ligand for CCR1 and CCR3 which have been shown to be expressed on neutrophils, monocytes and lymphocytes, have been shown to induce migration of human osteogenic sarcoma cells through a PLC / PKCδ dependent process (Ko et al., 2002).

3.12 Conclusions

In this part of the study, I have demonstrated that CCR4-mediated migration of CEM cells and CXCR4-mediated migration of PBMCs occurs even in the presence of PI3K inhibitors. However, CXCR4-mediated migration of CEM cells was unable to migrate in the presence of PI3K inhibitors. This demonstrates a difference in receptor signalling pathways within a cell type, as well as a difference in signalling pathways for the same chemokine receptor in different cell types. This was achieved using the standard broad-spectrum inhibitors of PI3K; LY294002 and Wortmannin, as well as a new, more specific, inhibitor of PI3K; ZSTK474.

Subsequent to this, I determined that inhibition of Akt using a novel, specific inhibitory compound abrogated chemotaxis in all models – regardless of whether they had previously been shown to be PI3K-independent. This was

found to be due to a difference in the ability of PI3K inhibitors and Akt inhibitors to inhibit Akt activity. There are two possible explanations for this result. Firstly, that Akt is activated independently of PI3K, through PKC and / or PDK-1. Secondly, it could be due to a difference in the relative amounts of each protein within the cell. Therefore a little Akti-1/2 could have a far greater effect than larger concentrations of the PI3K inhibitors. Further research would have to be performed to determine if this is the case. This data then demonstrates the importance of using both PI3K and Akt inhibitors before ascribing any response to the PI3K / Akt pathway.

In this part of the study it was also determined that GSK-3 and PKCδ play an important role in T lymphocyte migration. Inhibition of GSK-3 with the compound BIO resulted in an increase in the migration of CEM cells to CCL22. GSK-3 is phosphorylated by Akt and inactivated, so inhibition by BIO mimics Akt activation. In addition, PKCδ was shown to be activated upon chemokine binding, and using PKCδ null mice, shown to be important in the migration of murine splenocytes to CCL22.

CHAPTER 4: RESULTS II - THE ROLE OF PI3K IN MURINE TH17 CELL POLARISATION AND MIGRATION

Results II: The role of PI3K in murine Th17 cell polarisation and migration

4.1 Background

$CD4^+$ T helper cells are important in the regulation and function of the adaptive immune system and have classically been characterised as either Th1 or Th2 subsets depending on their cytokine production profile (Mosmann et al., 2005). Th1 cells, which produce IFNγ, IL-2 and TNF-α, are associated with defence against intracellular pathogens (cell-mediated immunity), while Th2 cells, which produce IL-4, IL-5, IL-10 and IL-13, mediate defense against extracellular parasites (humoral immunity) (Sher and Coffman, 1992; Abbas et al., 1996). Recently, a novel T helper cell subtype has been described, Th17, and is characterised by IL-17, IL-6 and TNF production (Harrington et al., 2005; Park et al., 2005). It is now understood that these cells form a separate lineage from Th1 and Th2 cells, one that bridges the innate and adaptive immune systems.

Upon differentiation, polarised Th1 and Th2 $CD4^+$ T cells have been shown to preferentially express different chemokine receptors. Typically, CXCR3, CXCR6 and CCR5 are preferentially expressed on Th1 cells (Qin et al., 1998), with CCR3, CCR4, CCR8 and the PGD2 receptor CRTH2, expressed on Th2 cells (Sallusto et al., 1998c; Nagata et al., 1999). This differential chemokine receptor expression can be used to characterise the different T helper subsets, and is thought to be important in the specific and discrete homing of these cells, which could explain specific roles for these cells in inflammatory diseases. For example, IFNγ, a Th1 cytokine, induces the release of ligands for CXCR3, a receptor that is upregulated on Th1 cells. In a similar fashion, CCL17 levels are increased in skin diseases such as atopic dermatitis (Saeki and Tamaki, 2006), recruiting Th2 cells via CCR4 to these sites of cutaneous inflammation (Campbell et al., 1999).

Th17 cells have been implicated in several mouse models of autoimmune diseases such as EAE and collagen-induced arthritis. These diseases have also had chemokine receptors implicated in their pathogenesis, while several isoform

specific chemokine inhibitors are being developed to target these diseases. Several papers have recently been published, identifying CCR2 and CCR6 as being present on human Th17 cells, however no studies have investigated chemokine receptor expression on murine Th17 cells, crucial for the modelling of these diseases, nor the role of protein and lipid kinases in Th17 cell migration. Using Th17 cells derived from murine DO11.10 splenocytes, this section of the study aimed to gain more insight into the chemokine receptor expression of murine Th17 cells, and the kinases that govern chemokine induced migration in these cells.

4.2 Generation of Th17 cells

Several papers have been published investigating the optimum conditions for generation of Th17 cells from murine splenocytes. Work performed by Andrew Johnson at UCB, Cambridge, and based on the original study by Veldhoen *et al* (Veldhoen et al., 2006) produced a protocol based on splenocytes from DO11.10 mice. The DO11.10 strain of mice express a transgene for a TCR clonotype, specific for the chicken Ovalbumin epitope 323-339 ($OVA_{323-339}$) (Murphy et al., 1990). The vast majority of peripheral DO11.10 T cells express the transgenic TCR. $OVA_{323-339}$ is presented to T cells by the major histocompatibility complex (MHC) class II molecule I-A^d, which preferentially activates $CD4^+$ T cells. Therefore co-culture with non-proliferative APCs and $OVA_{323-339}$ activates DO11.10 $CD4^+$ T cells *in vitro,* in an antigen specific manner.

In addition to activation by Ovalbumin peptide, generation of Th17 cells requires polarisation by cytokines including TGF-β, the pro-inflammatory cytokines IL-6, IL-1β and TNFα, IL-23 to maintain the Th17 phenotype of memory $CD4^+$ T cells, as well as antibodies neutralising the cytokines IFN-γ and IL-4. CD4 enriched DO11.10 T cells were cultured with Ova Peptide, supplemented with cytokines and antibodies and incubated for 7 days. Th17 cell proportions were compared to non-polarised cultures (Th0) using 3-colour intracellular flow cytometry. Cells were stimulated with PMA and Ionomycin to stimulate cytokine production before being stained against extracellular CD4,

fixed, permiabilised and stained against intracellular IFN-γ and IL-17 (Fig. 4.1*A*+*B*). Although some reports have described a small proportion of IL-17$^+$ IFN-γ$^+$ cells, (Suryani and Sutton, 2007) most papers, and this study, define Th17 cells as CD4$^+$, IL-17$^+$, IFN-γ$^-$ cells. Under Th17 polarising conditions, the proportion of IL-17$^+$ IFN-γ$^-$ cells was significantly elevated (16.9%±0.9, n=12) compared with nonpolarised (Th0) cultures (3.4%±0.3, n=12) (Fig. 4.1*C*).

The Luminex assay is a multiplex system that can measure the concentration of several different cytokines in a single sample. Distinct microspheres are conjugated to antibodies specific for the cytokines of interest. These spheres are incubated with cell culture supernatant, and then bound cytokines are detected with a secondary biotinylated antibody. PE-streptavadin is incubated with the microspheres, and then the mean fluorescent intensity (MFI) of each distinct microsphere type is detected using the Luminex machine. The MFI is then compared to a standard curve of known cytokine concentrations to determine the concentration of cytokines in the sample. Using this system, analysis of secreted cytokines within the cell culture supernatants revealed a significantly higher concentration of IL-17 in Th17-polarised conditions (7401 pg/ml±1723 pg/ml, n=4) compared with nonpolarised conditions (230 pg/ml±36 pg/ml, n=4) (Fig. 4.1*D*). In addition, levels of IFN-γ were significantly lower under Th17 polarising conditions (73 pg/ml±5 pg/ml, n=4) compared with nonpolarising conditions (737 pg/ml±179 pg/ml, n=5).

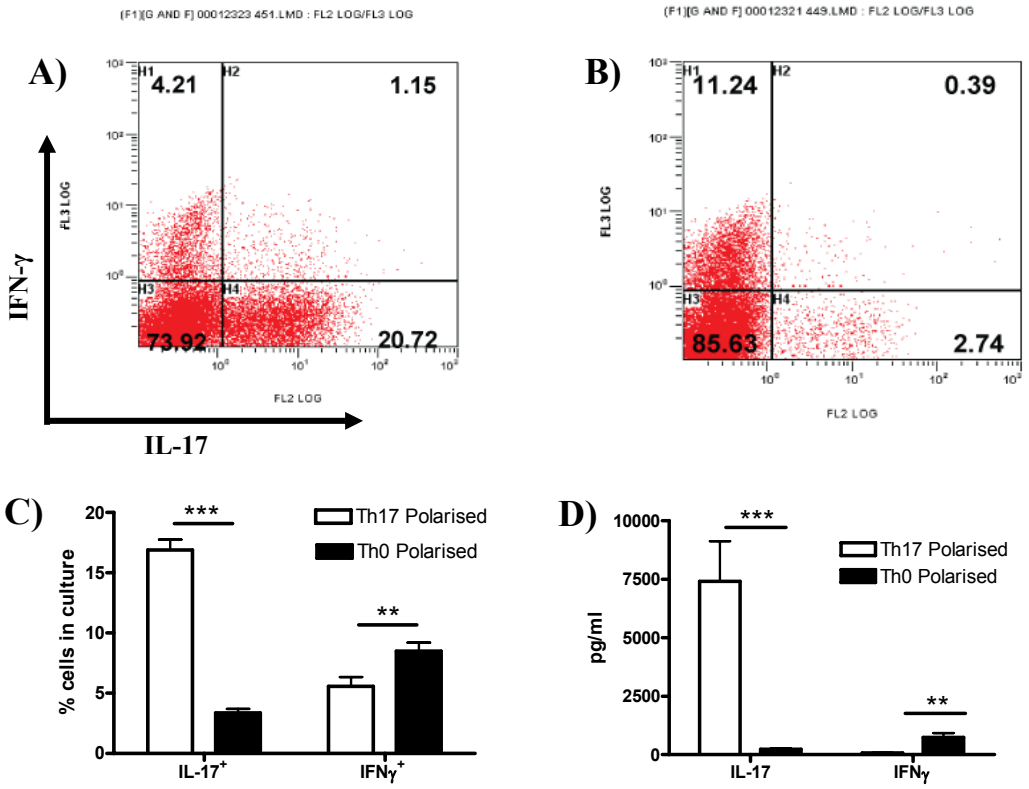


Figure 4.1 Phenotype of CD4$^+$ T cells cultured under Th17 or Th0 polarising conditions. CD4$^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions or Th0 non-polarising conditions. On day 7 post-isolation, cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiStop, and then stained with antibodies against CD4, IL-17 and IFNγ to determine the proportion of Th17 cells. Representative flow cytometry plots from cells cultured under Th17 polarising conditions (*A*) or non-polarised Th0 cells (*B*) are shown. Mean intracellular flow cytometry data from eight separate experiments is also shown (n=8; *C*). Cell culture supernatant from cells cultured for seven days was analysed using Luminex, as described in M*aterials and Methods,* to determine the concentrations of soluble IL-17 and IFN-γ (n=4; *D*). Results are expressed as mean ± SEM ** p<0.01, *** p<0.001. FL2 and FL3; Fluorescence 2 and Fluorescence 3 respectively.

4.3 Expression of chemokine receptors on the surface of Th17 polarised cells

Other subsets of CD4$^+$ T cells, such as Th1 cells and Th2 cells, have been shown to express specific chemokine receptors on their surface. These receptors have a distinct role in targeting the cells towards inflammation and have been identified as possible drug targets. In addition, they act as useful markers for the identification of these cells. While the surface expression of chemokine

receptors is well documented in Th1 and Th2 cells, and the expression of chemokine receptors on human Th17 cells is also becoming clearer, their expression on murine Th17 cells has yet to be investigated. The impact of Th17 polarising conditions on chemokine receptor surface expression was investigated by staining cells with antibodies against the CC chemokine receptors CCR2, CCR3, CCR5, CCR6, CCR7 and CCR9 (Fig. 4.2*A*) and the CXC chemokine receptors CXCR2, CXCR3, CXCR4 and CXCR5 (Fig. 4.2*B*).

The whole cell population was stained to identify the impact of Th17 polarising versus non-polarising culture conditions on the proportion of cells expressing these receptors. No significant difference between non-polarised Th0 cells and cells cultured under Th17 polarising conditions was observed in the proportions of cells expressing the CC chemokine receptors CCR3, CCR5 or CCR7 or the CXC chemokine receptor CXCR5. However, the percentage of cells expressing CCR2, CCR6, CCR9, CXCR2 and CXCR4 was significantly increased on cells cultured under Th17 polarising conditions compared to non-polarised cells. As the proportions of CXCR2 and CXCR4 were low (3% and 5% respectively), it was decided not to pursue these further as the low levels of expression would make it difficult to assess any impact on functional readouts. Interestingly, the percentage of cells expressing the CXCR3 receptor was significantly decreased under Th17 polarising conditions when compared to nonpolarising conditions (31% and 72% respectively).

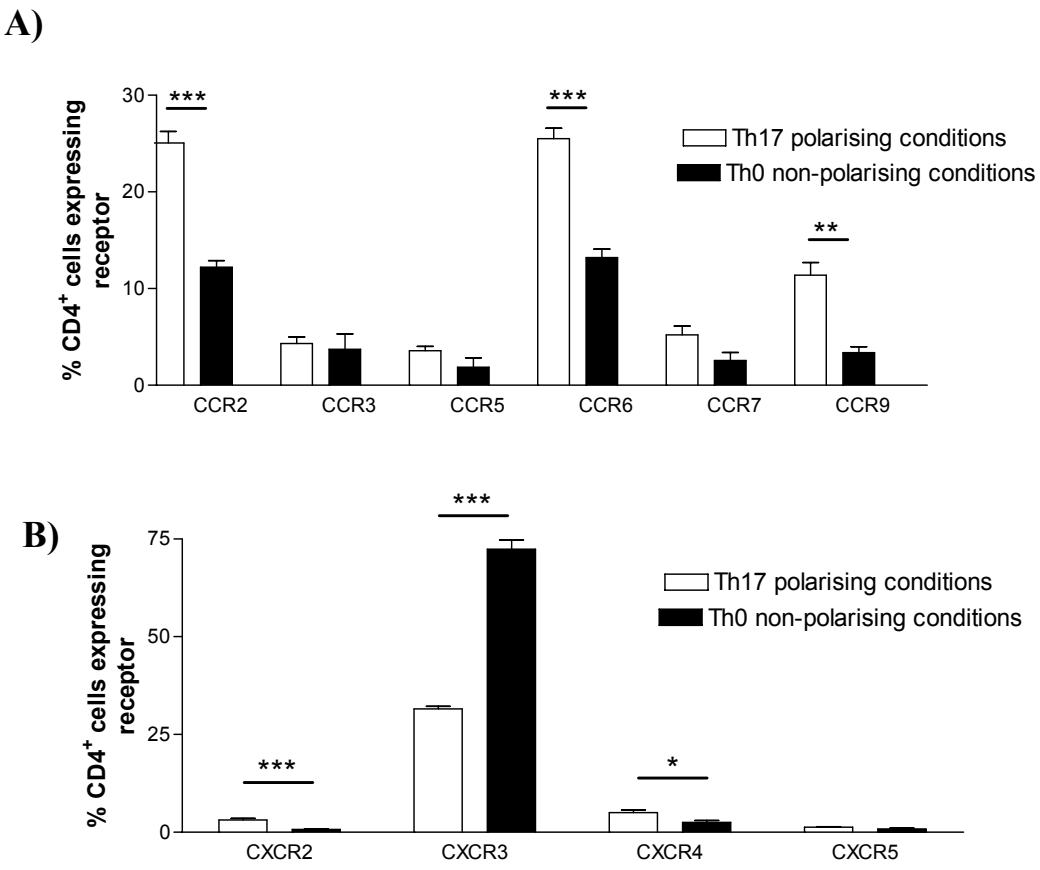


Figure 4.2 Surface expression profile of chemokine receptors on CD4$^+$ T cells cultured under Th17 or Th0 polarising conditions. Extracellular flow cytometry was used to determine the expression of CC chemokine receptors (*A*) and CXC chemokine receptors (*B*) on the surface of CD4+ splenocytes cultured under Th17 and Th0 polarising conditions. CD4$^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions or Th0 non-polarising conditions as outlined in *Materials and Methods*. On day 7 post-isolation, cells were aliquoted into FACS tubes and stained with fluorescently conjugated anti-CC and anti-CXC chemokine receptor antibodies. Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software. Results are expressed as mean ± SEM. n=4, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.4 The functional responsiveness of CCR2, CCR6 and CCR9 on Th17-polarised cells

Although increased expression of chemokine receptors was observed on cells cultured under Th17 polarising conditions, further study was required to determine whether these receptors were biochemically functional. Migration assays were used to determine whether chemokine binding could induce migration of the cells. The ligands CCL20 and CCL25 are specific chemokines for CCR6 and CCR9 respectively, and were used in this assay. CCR2 lacks a specific chemokine and so two chemokines, CCL2 (binds CCR2 and CCR4) and CCL12 (binds CCR2 and CCR5) were used. Th17-polarised cells (Fig. 4.3*A*) and non-polarised cells (Fig. 4.3*B*) were incubated for 3 hours in a chemotaxis assay with varying concentrations of CCL2, CCL12, CCL20 and CCL25. Migrated cells were counted on a flow cytometer using fluorescent beads to quantify the number of migrated cells. Concentration-dependent migration of cells occurred in response to CCL2, CCL12, and CCL20 for nonpolarised and Th17-polarised cells, while CCL25 only induced migration of Th17-polarized cells, albeit with less efficacy than the other agonists. Migration of nonpolarised cells in response to CCL25 was not observed.

Other methods of determining whether chemokine receptors are functional include activation of Akt and loss of surface receptor expression. This biochemical read-out was therefore used to explore whether CCR2, CCR6 and CCR9 detected on Th17 cells were biochemically functional. Direct measurement of PI3K products in primary cells is technically formidable, so phosphorylation of proteins downstream of PI3K, such as Akt, is often used as a surrogate readout of PI3K signalling output. Stimulation of cells cultured under Th17 polarising conditions with ligands for CCR2 (CCL2), CCR6 (CCL20) and CCR9 (CCL25) resulted in the concentration-dependent phosphorylation of residues Ser473 and Thr308 on Akt, with maximal phosphorylation occurring at 1nM CCL25 and CCL20 and 0.1nM CCL2 (Fig. 4.4*A)*.

Chemokine receptors undergo constitutive trafficking to and from the plasma membrane under basal conditions, leading to degradation or recycling to the cell

surface, which have been shown to be greatly enhanced upon ligand binding to functional receptors (Neel et al., 2005a). The ability of CCL2, CCL20 and CCL25 to induce the loss of surface expression of their receptors was therefore investigated. Cells cultured under Th17-polarising conditions were stimulated with the chemokines at the concentration shown to induce maximal chemotaxis. After stimulation for between 1 minute and 60 minutes, cells were washed into ice-cold FACS buffer to prevent receptor recycling, then stained for the chemokine receptors and analysed using flow cytometry. Stimulation of cells cultured under Th17 polarising conditions with CCL2, CCL20, and CCL25 (Fig. 4.4*B*) induced a loss of surface expression of their respective receptors in a time-dependent manner. Stimulation with CCL2 resulted in a 63.2%±5.8 loss of surface expression of CCR2, while CCL25, stimulation caused a 56%±3 reduction in surface expression of CCR9. Interestingly, incubation of Th17-polarised cells with CCL20 only resulted in a 42%±3.3 loss of surface expression of CCR6.

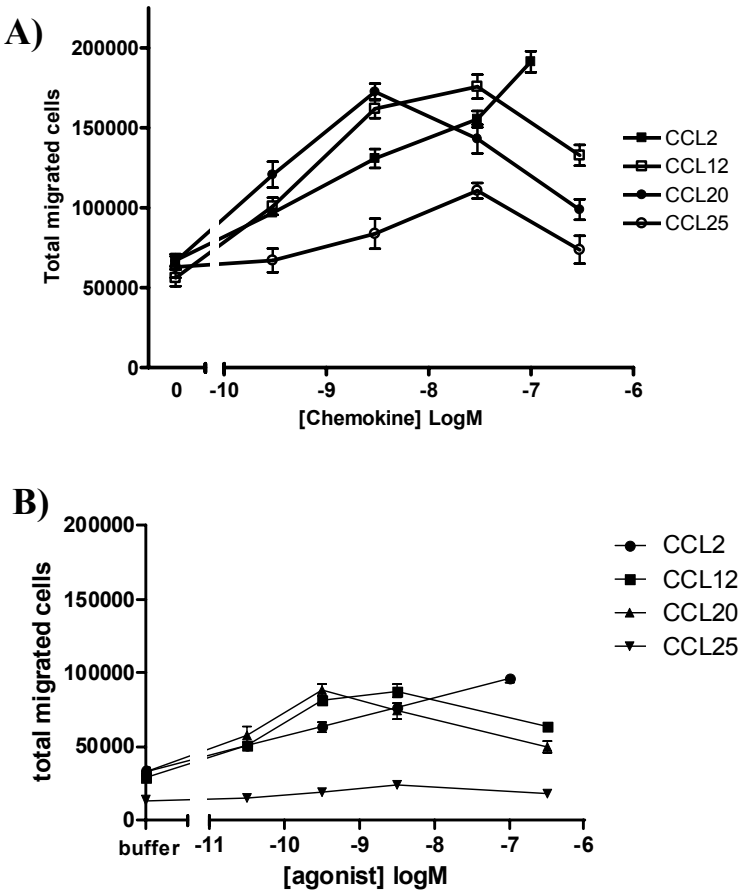


Figure 4.3 Chemokine induced migration of CD4$^+$ T lymphocytes cultured under Th17 or Th0 polarising conditions. Chemotaxis assays were used to determine whether cells cultured under Th17 or Th0 polarising conditions migrated to chemokines that bind CCR2, CCR6 and CCR9. CD4$^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions (*A*) or Th0 polarising conditions (*B*) were placed in the upper chamber of a 24 –well Transwell chemotaxis plate above lower chambers containing varying amounts of CCL2, CCL12, CCL20 or CCL25 as described in *Materials and Methods*. Migration across a 5μm pore membrane was determined after 3 hours at 37^{o}C. Fluorescent beads were added to samples prior to data acquisition to quantify number of migrated cells. Results are expressed as mean ± SEM, n=4.

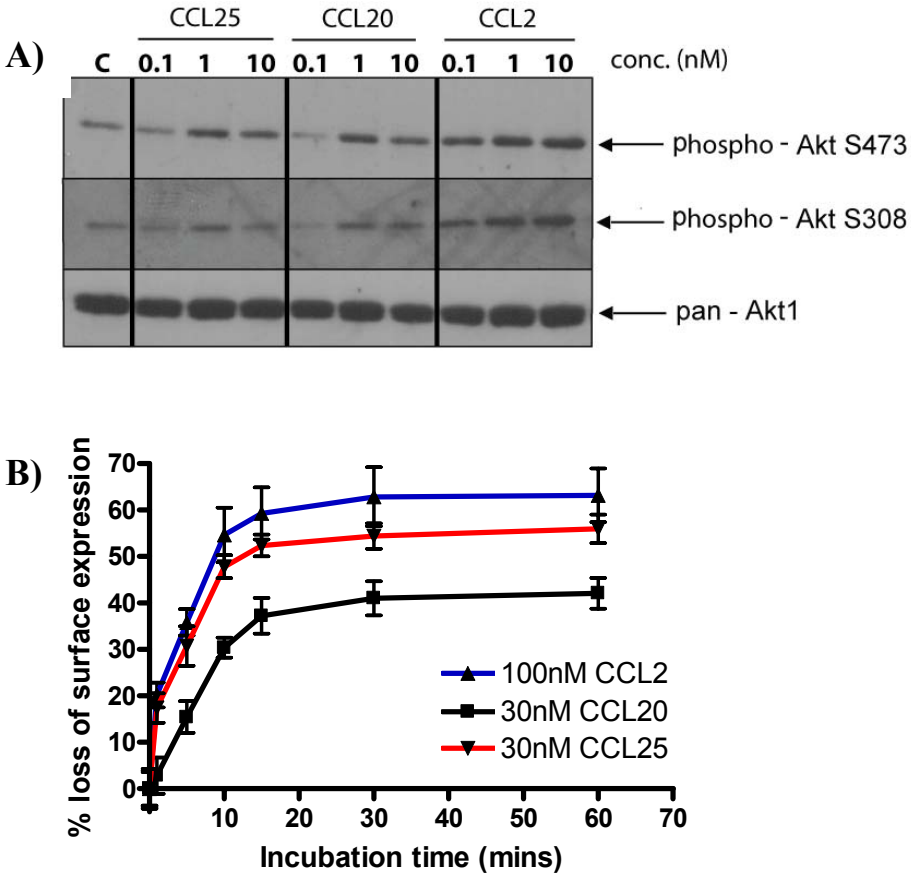


Figure 4.4 Interactions between chemokine receptors and their ligands in Th17 polarised CD4$^+$ splenocytes induce phosphorylation of Akt and loss of surface receptor expression. Immunoblotting and flow cytometry were used to determine whether CCR2, CCR6 and CCR9 detected on Th17 polarised cells were biochemically functional. CD4$^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions for seven days, then stimulated with CCL25, CCL20 or CCL2 at the concentrations indicated (*A*). Cells were subsequently lysed as described in *Materials and Methods*, and lysates (1.6 x 10^6 cells per lane) resolved by SDS-PAGE gel, transferred to nitrocelluose membranes, and immunoblotted with a phospho-specific Akt antibody with affinity for the active Ser473 phosphorylated form of Akt or a phospho-specific Akt antibody with affinity for the active Thr308 phosphorylated form of Akt, and protein was visulaised with ECL. The blots were stripped and reprobed with pan-Akt antibody to verify equal loading and efficiency of protein transfer. Data are representative of at least three separate experiments.
1 x 10^6 cells cultured under Th17 polarising conditions were stimulated with CCL2, CCL20 or CCL25 (*B*) at stated concentrations and incubation periods at 37˚C, washed into ice-cold PBS, and then stained at 4˚C with fluorescently conjugated anti-chemokine receptor antibodies as described in *Materials and Methods*. Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software. Results are expressed as mean ± SEM, n=4. C, Control.

4.5 The role of PI3K and Akt in chemokine receptor signal transduction on Th17-polarised cells

The result from Fig. 4.4*A* revealed that Akt is phosphorylated upon stimulation of Th17-polrised cells by chemokines. This result indicated that PI3K and Akt are involved in the signal transduction of chemokine receptors on Th17-polarised cells. However, in the previous chapter it was seen that phosphorylation of Akt does not necessarily correspond to Akt activity. To confirm that Akt is activated by chemokines in this system, and that PI3K is involved, the Omnia® Lysate Assay system was used. The Omnia® Lysate Assay system uses fluorophores attached to substrate peptides specific for Akt. Upon phosphorylation of the peptide by Akt, a change in the rate of fluorescence occurs. This change in fluorescence corresponds to a change in the rate of phosphorylation by Akt, allowing measurement of Akt activity.

Measurement of Akt activity in Th17-polarised splenocytes revealed that stimulation with 10nM CCL2 significantly increased the rate of phosphorylation by Akt. Pre-incubation of CCL2-stimulated cells with LY294002 significantly reduced the rate of phosphorylation by Akt from 13.8±0.5 to 8.5±0.5 (Fig. 4.5*A*, n=4) at 10μM. Similarly, pre-incubation with the Akt inhibitor Akti-1/2 also reduced Akt activity in this system, from 15.2±0.6 to 7±0.7 (Fig. 4.5*B,* n=4) at 10μM.

Stimulation of cells with 10nM CCL20 also significantly increased Akt activity. This activity was decreased by LY294002 or Akti-1/2 in a comparable fashion to CCL2 stimulation, with 10μM LY294002 reducing activity from 15.0±0.7 to 8.7±0.7 (Fig. 4.5*C*, n=4) while 10μM Akti-1/2 reduced activity from 13.9±0.8 to 8.7±0.3 (Fig. 4.5*D,* n=4).

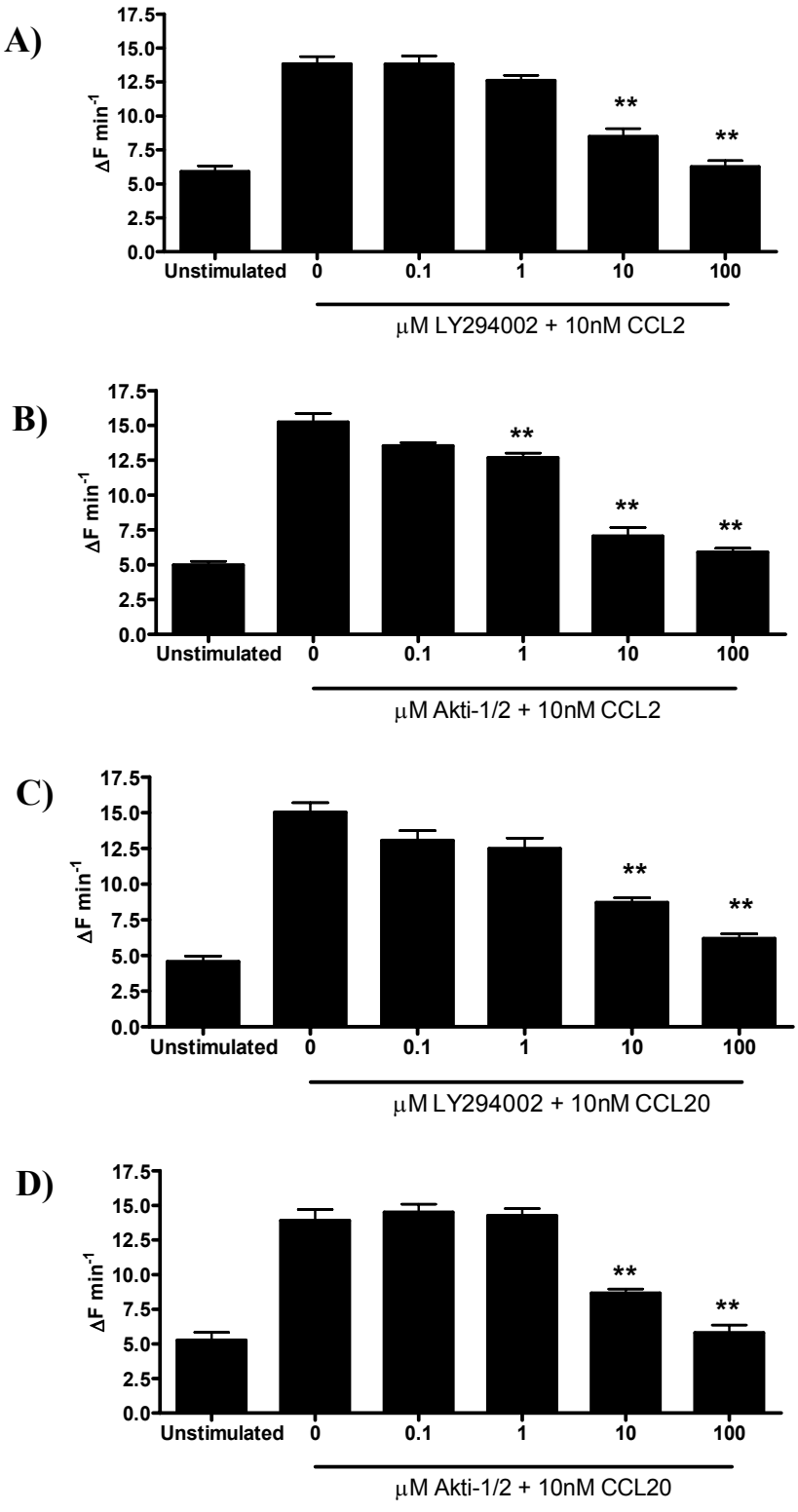


Figure 4.5 Activation of Akt is induced by chemokine receptor – ligand interactions in Th17 polarised CD4$^+$ splenocytes and is sensitive to inhibition of PI3K. Please see next page for the legend.

Figure 4.5 Activation of Akt is induced by chemokine receptor – ligand interactions in Th17 polarised $CD4^+$ splenocytes and is sensitive to inhibition of PI3K. An Akt activity assay was used to determine whether stimulation of CCR2 or CCR6 causes and increase in Akt activity, and whether this was a PI3K dependent process. $CD4^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions for seven days, then incubated with LY294002 (*A*,*C*) or Akti-1/2 (B,*D*) for 30 min then stimulated with 10nM CCL2 (*A*,*B*) or 10nM CCL20 (*C*,*D*) for 5 min and lysed. Resulting Akt activity was assessed using Akt activity assay as described in *Materials and Methods*. Results shown as change in the rate of flourescence, which corresponds to change in the rate of phosphorylation of target peptides by Akt. Results are expressed as mean ± SEM, n=4. One-way ANOVA ** $p<0.01$.

4.6 Chemokine-induced migration of Th17 cells

Having shown that cells polarised under Th17-polarising conditions show upregulated surface chemokine receptor expression of CCR2, CCR6 and CCR9, and that ligands for these receptors induce PI3K / Akt dependent migration, the ability of these agonists to induce migration of Th17 cells was investigated. As no specific marker for Th17 cells existed with which to isolate and purify the cells without killing them, an alternative method to identify migrated Th17 cells was used. $CD4^+$ splenocytes cultured under Th17-polarising conditions, or nonpolarising conditions, were suspended in chemotaxis media and incubated in a transwell chemotaxis chamber with varying concentrations of chemokine. After three hours, cells were recovered from the bottom of the chamber and stimulated for three hours with 50 ng/ml PMA, 500 ng/ml Ionomycin and 0.66 µl/ml of GolgiStop to induce intracellular cytokine production and prevent subsequent exocytosis of the cytokines. The cells were then incubated with a fluorescent extracellular antibody against CD4, then fixed with paraformaldehyde and stored at 4ºC overnight. The cells were then permeated and incubated with fluorescent intracellular antibodies against IL-17 and IFNγ. Known quantities of fluorescent beads were added to the sample prior to data acquisition using a flow cytometer. This allowed the number of migrated cells to be counted. In addition, the antibody staining allowed the proportion of Th17 and $IFN\gamma^+$ cells within the sample to be assessed. Using these two pieces of data, the number of Th17 and $IFN\gamma^+$ cells that migrated could then be calculated.

Analysis of the results revealed significant concentration-dependent migratory responses to CCL2, CCL12 and CCL20, with 100nM CCL2 (Fig. 4.6.1*A*) 30nM CCL12 (Fig. 4.6.1*B*) and 3nM CCL20 (Fig. 4.6.1*C*) causing maximal migration. These results, and the migratory profiles, are similar to those observed for total Th17-polarised cell migration. Interestingly, CCL25 elicited no significant migratory response in Th17 cells, indicating that although increases in surface expression of CCR9 were observed in Th17-polarised cell populations, the increases were unlikely to be on Th17 cells. None of the agonists examined caused significant migration of $IFN\gamma^+$ cells, indicating that the increase in CCR9

expression is on the approximately 74% of cells that are neither IL-17$^+$ or IFNγ$^+$. Significant changes in the proportions of Th17 cells in culture after migration were also observed. Migration to CCL2 (Fig. 4.6.2*A*), CCL12 (Fig 4.6.2*B*) and CCL20 (Fig 4.6.2*C*) all resulted in significantly higher proportions of Th17 cells in the post-migration culture than the pre-migration culture. This indicated that in addition to the cells migrating expressing CCR2 and CCR6, they also migrate in preference to other cells expressing those receptors. No change in Th17 cell proportions was observed after migration to CCL20 (Fig. 4.6.2*B*)

To confirm the finding that CCR9 is not expressed on Th17 cells, 3-colour flow cytometry was used. Th17-polarised cells were labelled for the chemokine receptor as well as IL-17 and IFNγ. Due to a lack of commercially available antibodies with different fluorophores, we were unable to stain for CD4 and the chemokine receptor at the same time. However, previous results indicated that all IL-17$^+$ cells were CD4$^+$ as well, and so this was thought to be acceptable. The IFNγ$^-$ population was selected for, then the proportion of cells that expressed either the chemokine receptor, IL-17 or both were analysed. The data revealed that although CCR2 was expressed on 25% of Th17 polarised cells (Fig. 4.2*A*), only 11.9% ± 0.4% of IL-17$^+$ cells coexpressed CCR2 (representative plot Fig 4.7*A*). CCR6 was also expressed on 25% of total cells within polarised Th17 cultures (Fig. 4.2*A*), and only 10.9% ± 0.5% of IL-17$^+$ cells coexpressed CCR6 (representative plot Fig 4.7*B*). Contrary to what was expected, a similar proportion of Th17 cells expressed CCR9 as CCR2 and CCR6, with 11.3% ± 0.8% of Th17 cells coexpressing the receptor (representative plot Fig 4.7*C*). This indicates that the lack of migration of Th17 cells to CCL25 is not due to a lack of receptor expression on Th17 cells. Overall, these data suggest that surface expression of the chemokine receptors examined does not fully define the Th17 population, and would not be suitable as a marker for Th17 cells. The relatively low level of chemokine receptor expression on Th17 cells, coupled with a lack of commercially available antibodies conjugated to an appropriate range of fluorophores, prevented further investigation into the coexpression of CCR2, CCR6 and CCR9 on Th17 cells.

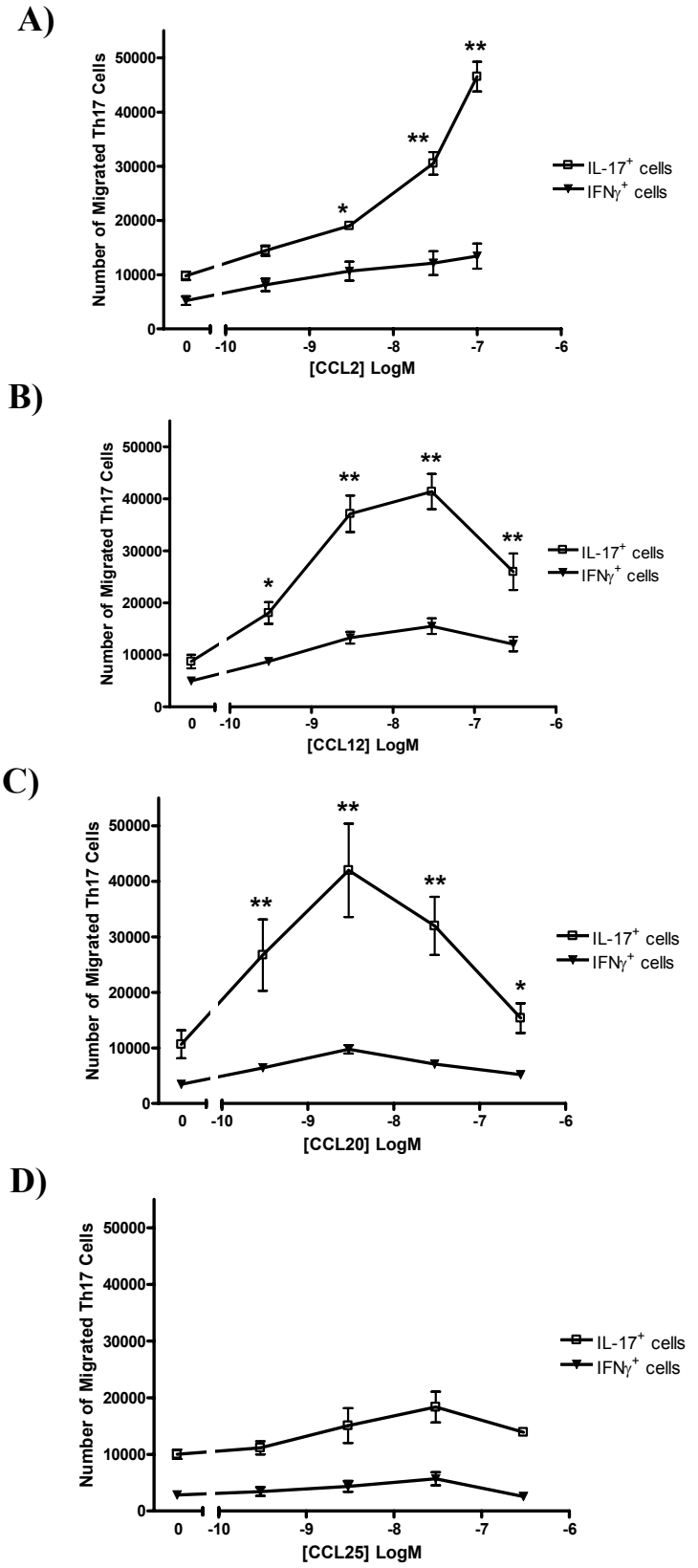


Figure 4.6.1 Number of Th17 cell migrated to CCR2, CCR6 or CCR9 ligands.

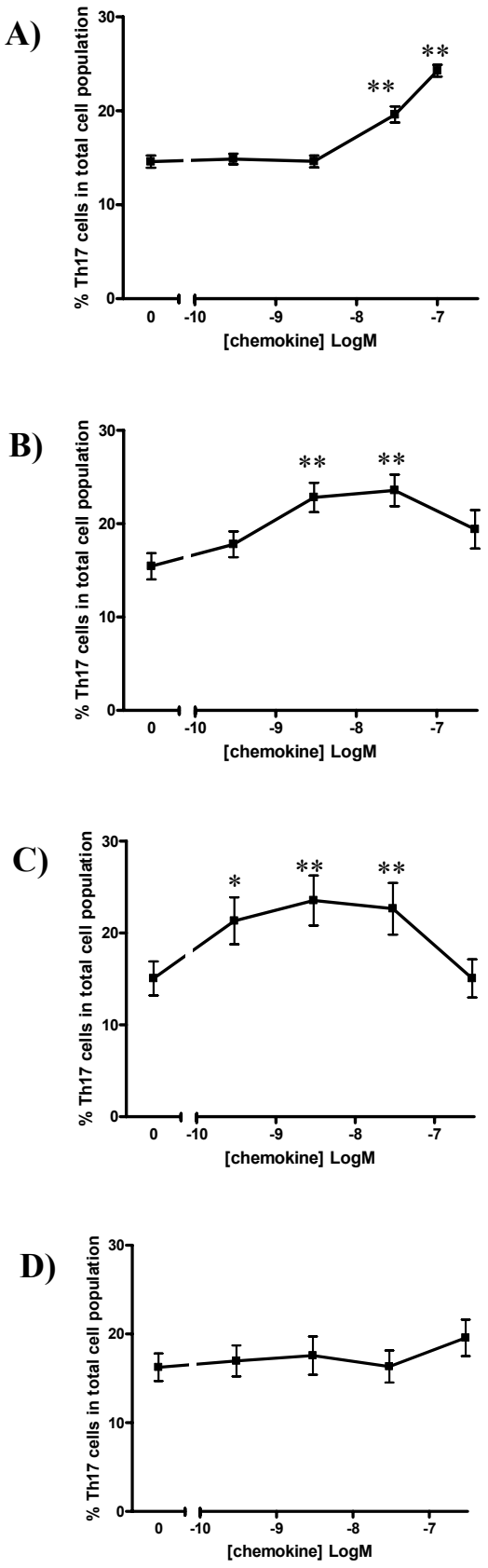


Figure 4.6.2 Proportion of Th17 cell in culture after migration to CCR2, CCR6 or CCR9 ligands.

Figure 4.6.1 Number of Th17 cell migrated to CCR2, CCR6 or CCR9 ligands.

Figure 4.6.2 Proportion of Th17 cell in culture after migration to CCR2, CCR6 or CCR9 ligands.

Chemotaxis assays were used to determine whether Th17 cells (IL-17$^+$) or IFN-γ^+ cells migrated to ligands for CCR2, CCR6 and CCR9. Cells cultured under Th17 polarising conditions were placed in the upper chamber of a 24 –well Transwell chemotaxis plate above lower chambers containing varying amounts of CCL2 (*A*), CCL12 (*B*), CCL20 (*C*) or CCL25 (*D*) as described in *Materials and Methods*. Migration across a 5μm pore membrane was determined after 3 hours at 37^{o}C. Migrated cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiStop, and stained with antibodies against CD4, IL-17 and IFNγ. Fluorescent beads were added to samples prior to data acquisition to quantify number of migrated cells. Results are expressed as mean ± SEM. n≥4. One-way ANOVA, * $p<0.05$, ** $p<0.01$.

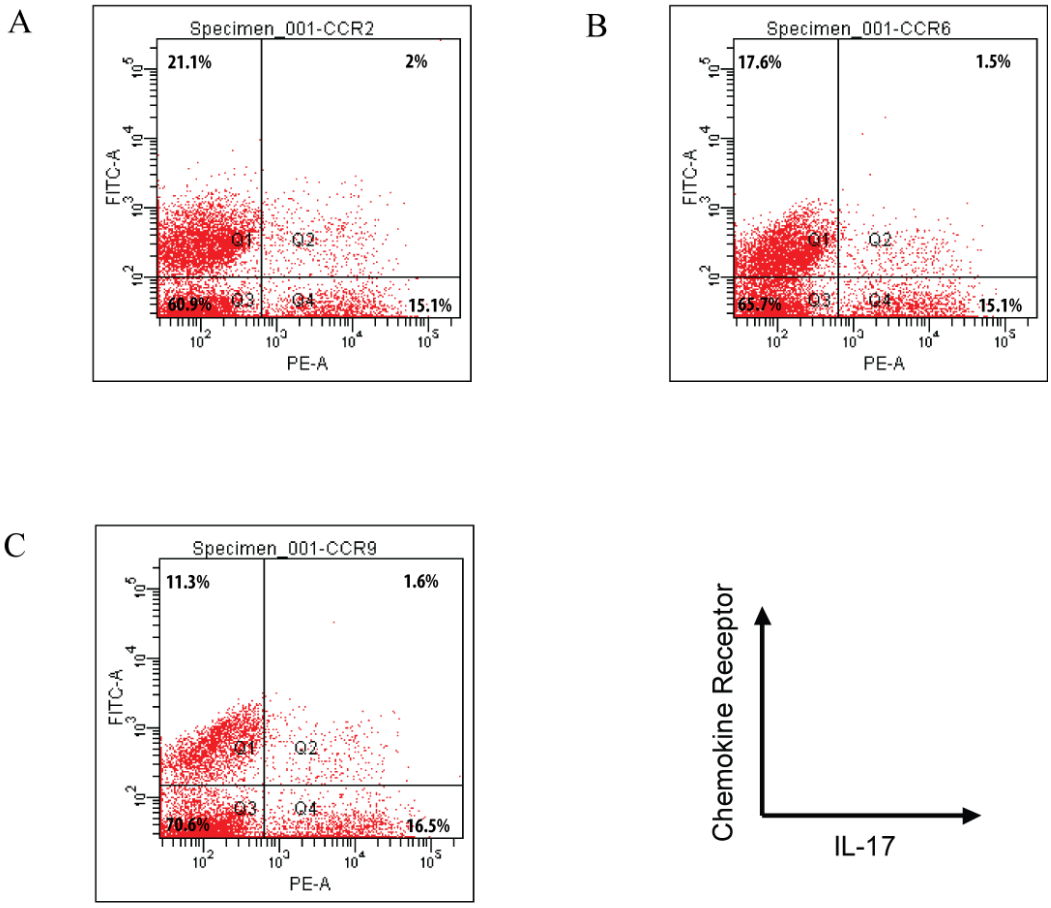
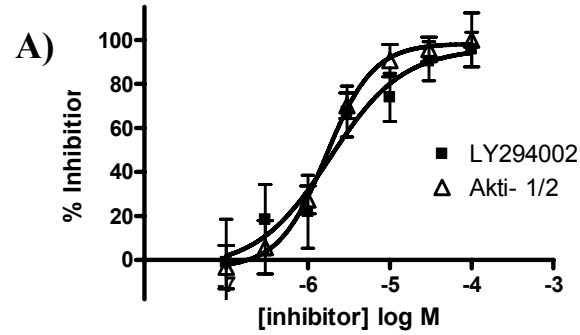


Figure 4.7 Chemokine receptor expression on Th17 cells. Flow cytometry was used to determine the proportion of Th17 cells that express each chemokine receptor. $CD4^+$ DO11.10 splenocytes were cultured under Th17 polarising conditions. On day 7 post-isolation, cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiStop, then stained with antibodies against IFNγ, IL-17 and either CCR2 (*A*), CCR6 (*B*) or CCR9 (*C*). Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software. The proportion of $IL\text{-}17^+$ cells coexpressing CCR2, CCR6 or CCR9 is the value in the top right quadrant (chemokine $receptor^+/IL\text{-}17^+$) calculated as a percentage of the sum of the two right-hand quadrant values (chemokine $receptor^+/IL\text{-}17^+$ and chemokine $receptor^-/IL\text{-}17^+$). Hence, 17% of cells are $IL\text{-}17^+$ (15+2%) in *A*; therefore, 2% of 17% is ~ 11% (see text for further values). Flow cytometry plots and values shown are representative of 3 separate experiments.

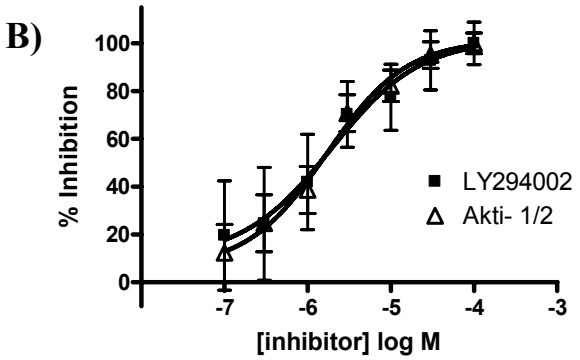
4.7 The role of PI3K / Akt in CCR2 and CCR6 mediated Th17 cell migration

Although PI3K and Akt have been shown to be activated in response to chemokine induced stimulation of Th17 polarised cells, their role in the migration of Th17 cells has yet to be explored. Data from the previous chapter demonstrated that some T cells, albeit human, have differences in their sensitivity to PI3K inhibitors and Akt inhibitors during migration. To determine the role of PI3K and Akt in migration, Th17-polarised cells were pre-incubated with various does of LY294002 or Akti-1/2 for 30 minutes prior to incubation in a Transwell chemotaxis chamber with 100nM CCL2 or 3nM CCL20. These concentrations induced maximal migration in Fig. 4.6, and so would provide a good migration response against which to observe any inhibition. Ligands for CCR9 were not used, as no significant migration of Th17 cells had been observed when using these previously.

The PI3K inhibitor LY294002 significantly inhibited migration of Th17 cells in response to 100nM CCL2 and 3nM CCL20 with pIC_{50} values of 5.73 ± 0.22 (Fig 4.8*A*) and 5.68 ± 0.18 (Fig 4.8*B*) respectively. These data are consistent with the affinity estimates reported for LY294002 in other cell systems (Ito et al., 2007), and also correlates with the Akt activity studies (Fig. 4.5). Interestingly, LY294002 is more potent in this system than was observed using human CEM cells or SEB activated T lymphocytes. Akti-1/2 significantly inhibited the migration of Th17 cells in response to 100nM CCL2 and 3nM CCL20, with pIC_{50} values of 5.75 ± 0.08 (Fig 4.8*A*) and 5.78 ± 0.13 (Fig 4.8*B*) respectively. These results demonstrate that migration of Th17 cells in response to ligands for CCR2 or CCR6 is mediated by PI3K/Akt and is sensitive to inhibitors of these kinases.



	pIC50
LY294002	5.73 ± 0.22
Akti- 1/2	5.75 ± 0.08



	pIC50
LY294002	5.68 ± 0.18
Akti- 1/2	5.78 ± 0.13

Figure 4.8 The effect of PI3K and Akt inhibition on Th17 cell migration. Chemotaxis assays were used to determine whether CCR2 and CCR6 mediated migration of Th17 cells is PI3K / Akt dependent. Cells cultured under Th17 polarising conditions were incubated with LY294002 or Akti-1/2 for 30 minutes at the concentrations shown prior to being placed in the upper chamber of a 24 – well Transwell chemotaxis plate above lower chambers containing 100nM CCL2 (*A*) or 3nM CCL20 (*B*) as described in *Materials and Methods*. Migration across a 5μm pore membrane was determined after 3 hours at 37^{o}C. Migrated cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and 0.66 μl/ml GolgiStop, and stained with antibodies against CD4, IL-17 and IFNγ. Fluorescent beads were added to samples prior to data acquisition to quantify number of migrated cells. Results were transformed using Graphpad Prism to plot the percentage inhibition of migration caused by each inhibitor. This graph was then used to determine the pIC50 of each inhibitor for each chemokine. Results are expressed as mean ± SEM, n=4.

4.8 The role of PI3K in Th17 cell polarisation

Having demonstrated that PI3K is important in the migration of Th17 cells, its role in the polarisation of Th17 cells was also investigated. In 2006, a paper was published (Okkenhaug et al., 2006) that revealed a role for the p110δ isoform of PI3K in the polarisation of murine $CD4^+$ splenocytes into a Th1 or Th2 phenotype. To investigate whether this was true for Th17 cells, another $CD4^+$ cell type, a combined pharmacological and gene targeting strategy was utilised. IC87114 is a p110δ-specific inhibitor developed by ICOS, a subsidiary of Eli Lilly. Studies have shown this inhibitor is specific for p110δ against other PI3K isoforms at the 5μM to 10μM range (Sadhu et al., 2003), making it a very useful compound with which to investigate this isoform.

$CD4^+$ splenocytes from DO11.10 mice were cultured under Th17 polarising conditions. On the indicated day, 10μM IC87114 was added to the culture. On day 7 post cell-isolation, the cells were stained for CD4, IFNγ and IL-17, and the proportion of Th17 cells within culture identified. Analysis of the results revealed that a significant reduction in the proportion of Th17 cells in culture occurred when the inhibitor was added on days one to five after culture, with maximal inhibition occurring when the compound was added on day two (Fig. 4.9). No effect was seen if IC87114 was added at the start of culture, or at the end. Complete abrogation of Th17 cell polarisation was also not observed, as even at maximum inhibition, 10% of cells in culture were Th17 cells.

The surprising result that IC87114 has no effect on Th17 cell polarisation if added at the start of culture was investigated further. It was theorised that this result could be because the inhibitor was degraded after incubation at 37ºC in culture for seven days. To test this theory, splenocytes from mice that lacked the active isoform of p110δ were used. The lack of p110δ in these mice is permanent and so should mimic the addition of IC87114 to Th17 polarisation cultures on day 0. p110δ D910A/D910A mice, a kind donation from Klaus Okkenhaug at the Babraham Institute, have an alanine for aspartic acid substitution in both copies of the gene (Okkenhaug et al., 2002). This substitution lies at the C terminal of the kinase domain and renders the protein

inactive. This kind of mutation has the advantage over deletion mutations in that there is no compensatory expression of other kinases, thus allowing a clear picture of the role of p110δ.

The mice were also on a DO11.10 background so no changes were made to the Th17 polarisation protocol. $CD4^+$ splenocytes from wildtype DO11.10 mice and p110δ D910A/D910A mice were cultured for seven days. The cultures were then analysed by flow cytometry to determine the proportion of Th17 cells within the culture (Fig 4.10). The p110δ D910A/D910A mouse culture contained a significantly lower proportion of Th17 cells than the wildtype culture (11.4% ± 0.5% and 19.4% ± 0.6% respectively). However, similar to the IC87114 incubation, a population of Th17 cells remained.

The culture medium from the two cell cultures was analysed for levels of cytokines to confirm the difference in Th17 cell proportions. IL-17 concentrations were significantly reduced in the D910A mouse culture medium (8900 ± 472.3 pg/ml) compared with medium from the wildtype cells (12770 ± 1153 pg/ml), although levels were generally high (Fig. 4.11*A*). Levels of TNF-α, another cytokine produced by Th17 cells, were also slightly reduced in the D910A cell culture (1763 ± 99.1 pg/ml) compared with the wildtype cell culture (2209 ± 134 pg/ml) (Fig. 4.11*B*). Interestingly, levels of IFNγ and IL-10, cytokines normally associated with Th1 and Th2 cells respectively were significantly reduced in D910A mice compared with wildtype mice. IFNγ concentrations were 11.5 ± 0.8 pg/ml and 3.7 ± 0.6 pg/ml respectively (Fig. 4.11*C*), while IL-10 concentrations were 94.0 ± 8.6 pg/ml and 40.0 ± 5.4 pg/ml respectively (Fig. 4.11*D*). Taken together, these results indicate that the p110δ isoform of PI3K is involved in the polarisation of Th17 cells. However, the percentage reduction in the proportion of Th17 cells in the culture is not as large as seen in Th1 and Th2 cell cultures. This result is borne out by the analysis of cytokines within the Th17 cell cultures. Although there is a slight, albeit significant, reduction in the levels of IL-17 and TNF-α, a far greater reduction is seen in the levels of IFNγ and IL-10, Th1 and Th2 cytokines. These data indicate a different role for p110δ in Th17 generation and Th1 / Th2 generation, with another kinase involved in Th17 polarisation.

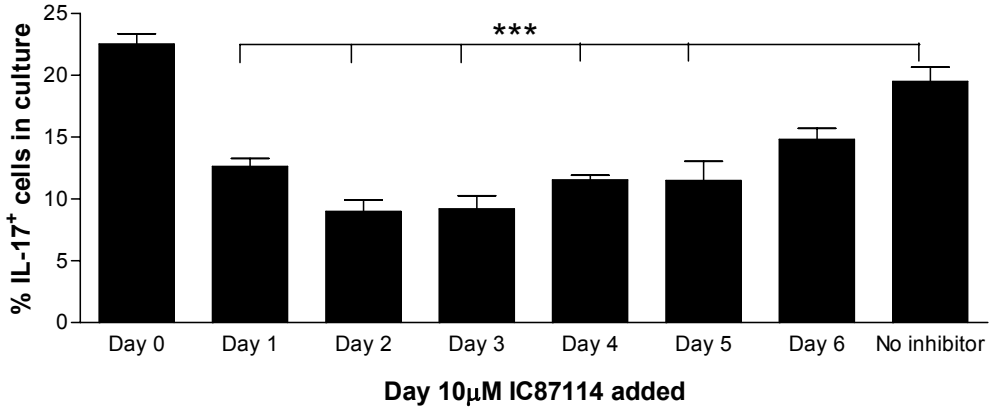


Figure 4.9 The effect of IC87114 on Th17 polarisation. Flow cytometry was used to determine the effect of P110δ inhibition using 10μM IC87114 on the polarisation of Th17 cells from $CD4^+$ splenocytes. $CD4^+$ DO11.10 splenocytes cultured under Th17 polarising conditions were incubated with 10μM IC87114 on the day indicated on the graph until day 7 post-isolation. On day 7, cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiStop, then stained with antibodies against CD4, IL-17 and IFNγ to determine the proportion of Th17 cells. Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software. Results are expressed as mean ± SEM, n=4. One-way ANOVA, *** $p<0.001$.

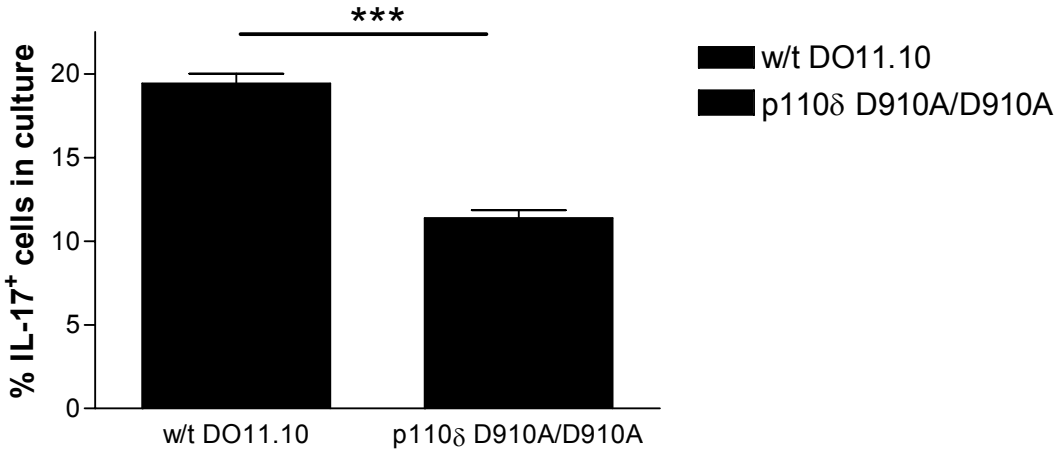


Figure 4.10 The role of P110δ in Th17 polarisation. Flow cytometry was used to determine whether splenocytes from mice lacking a functional P110δ isoform of PI3K are able to successfully polarise to Th17 cells. Wildtype $CD4^+$ DO11.10 splenocytes or $CD4^+$ DO11.10 splenocytes from mice that lack a functional P110δ isoform of PI3K due to a knock-in mutation (p110δ D910A/D910A) were cultured under Th17 polarising conditions as described in *Materials and Methods.* On day 7 post-isolation, cells were stimulated for 3 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiStop, then stained with antibodies against CD4, IL-17 and IFNγ to determine the proportion of Th17 cells. Data were acquired on a Beckman Coulter EPICS XL machine using EXPO32 ADC software. Results are expressed as mean ± SEM, n=8 Students t-test, *** $p<0.001$.

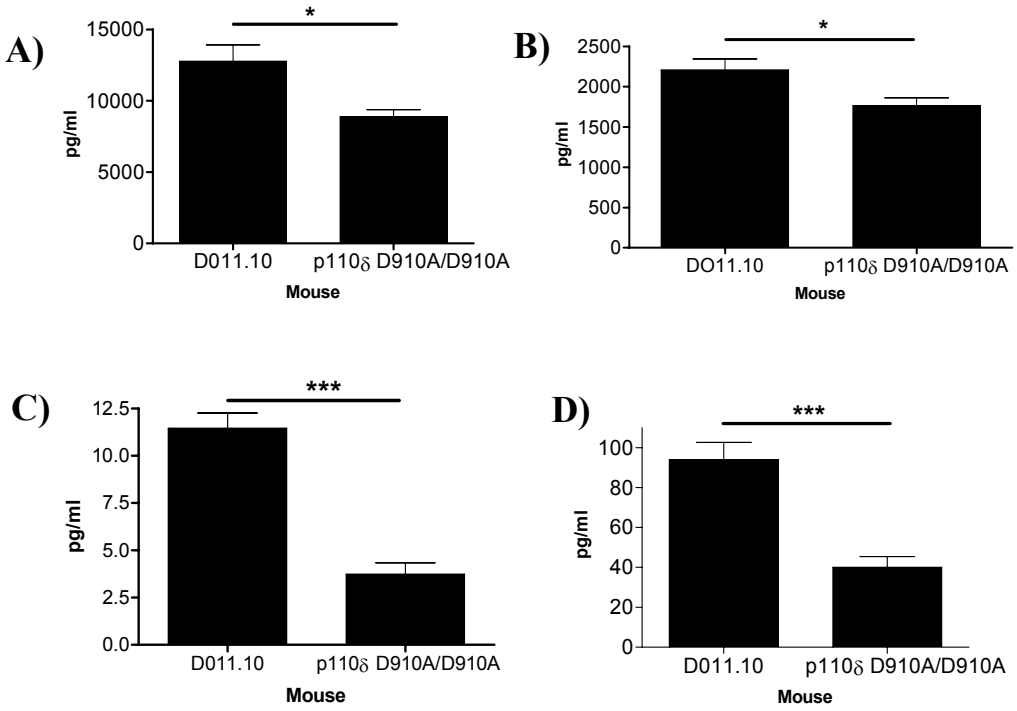


Figure 4.11 Concentrations of cytokines produced by wildtype and P110δ KI CD4$^+$ splenocytes cultured under Th17 polarising conditions. Luminex assays were used to determine the concentrations of IL-17 (*A*), TNF-α (*B*), IFN-γ (*C*) and IL-10 (*D*) produced by CD4$^+$ wildtype splenocytes and CD4$^+$ splenocytes from mice that lack a functional P110δ isoform of PI3K. Wildtype CD4$^+$ DO11.10 splenocytes or CD4$^+$ DO11.10 splenocytes from mice that lack a functional P110δ isoform of PI3K due to a knock-in mutation (p110δ D910A/D910A) were cultured under Th17 polarising conditions as described in *Materials and Methods.* On day 7 post culture, cell culture supernatant from the cultures was analysed using Luminex, as described in M*aterials and Methods,* to determine the concentrations of soluble IL-17, TNF-α, IFN-γ and IL-10. Results are expressed as mean ± SEM, n=8.

4.9 Summary

- Using splenocytes from DO11.10 mice, this study has shown that a significant population of Th17 cells can be generated under the correct polarisation conditions. These cells are defined as $CD4^+$, $IL\text{-}17^+$, $IFN\gamma^-$ and can produce significant concentrations of the cytokine IL-17.
- Analysis of chemokine receptor expression on cells cultured under Th17 polarising conditions revealed that CCR2, CCR6, CCR9, CXCR2 and CXCR4 were upregulated compared to nonpolarised cells, while CXCR3 was downregulated, consistent with its known upregulation on Th1 cells.
- When tested, all three CC chemokine receptors were shown to be biochemically active indicating migration of cells cultured under Th17 polarising conditions, phosphorylation of Akt and loss of surface receptor expression. In addition, ligands to all three receptors induced Akt activation that was dependent on PI3K.
- By isolating Th17 cells post-migration, it was determined that ligands for CCR2 and CCR6 could induce migration of Th17 cells. Interestingly ligands for CCR9 failed to induce the migration of Th17 cells, although expression of the chemokine receptors on the surface of Th17 cells was approximately equal.
- Migration of Th17 cells in response to ligands for CCR2 or CCR6 is mediated by PI3K/Akt and is sensitive to inhibitors of these kinases.
- When the role of the p110δ isoform of PI3K in Th17 cell polarisation was explored, the data revealed that although p110δ is important for Th17 polarisation, a population of Th17 cell remains in the absence of p110δ, and therefore it is possibly not as important as it is for Th1 and Th2 cell differentiation.

4.10 Discussion

The aim of this study was to determine what chemokine receptors are expressed on the surface of murine Th17 cells, and whether migration of Th17 cells through these receptors is dependent on PI3K / Akt. In addition, the role of PI3K in the polarisation of Th17 cells was explored. However, first it was important to polarise Th17 cells from murine splenocytes and confirm that these cells were able to produce IL-17.

4.10.1 $CD4^+$ splenocytes cultured in an inflammatory cytokine milieu polarise to an IL-17 producing phenotype

Previous studies have shown an important role for TGF-β and IL-6 in the polarisation of $CD4^+$ splenocytes to an IL-17 producing phenotype (Veldhoen et al., 2006). In addition, IL-4 and IFNγ have been shown to inhibit the differentiation of Th17 cells (Harrington et al., 2005; Park et al., 2005), while IL-23 is required for the maintenance of pre-existing Th17 populations. Using TGF-β, IL-6 and IL-23, as well as IL-1β and TNFα, and neutralising antibodies towards IL-4 and IFNγ, a population of Th17 cells were cultured from $CD4^+$ murine splenocytes. The Th17 cells were identified using flow cytometry and were defined as $CD4^+$, $IL\text{-}17^+$, $IFN\gamma^-$. Analysis of the cell culture supernatant revealed significantly greater concentrations of IL-17 in the Th17 polarising culture when compared to the nonpolarising cell culture. Interestingly, only 20% of cells within the culture polarised to a Th17 phenotype. While this is consistent with other groups (Veldhoen et al., 2006) it raises the question of the identity of the other cells.

4.10.1.1 Identity of the other cells within Th17-polarising cultures

Th17 polarisation resulted in a significantly higher proportion of Th17 cells when compared to nonpolarising conditions. However, by day 7, a small percentage (approximately 6%) of the population expressed IFNγ and not IL-17. Although differentiation of Th1 cells and Th17 cells was thought to be mutually exclusive, several recent studies have identified populations of cells that share features of Th1 and Th17 cells, including IFNγ expression (Annunziato et al.,

2007; Nakae et al., 2007). In addition, the ability for differentiated cells to alter their cytokine expression profile is not without precedent, as cells that produce both the Th1 cytokine IFNγ and the Th2 cytokine IL-4 have also been reported (Messi et al., 2003). Indeed, a very small but significant population of IL-17$^+$, IFNγ$^+$ cells also exists within the total cell population. The phenotype of the remaining cells is unclear. The presence of anti-IL-4 antibodies within the cell population make it unlikely that Th2 cells would be found within the population. While TGF-β has been shown to induce forkhead box p3 expression, and leads to the polarisation of regulatory T cells (Chen et al., 2003b), IL-6 has been shown to inhibit this process (Dominitzki et al., 2007), making it unlikely that regulatory T cells form a large proportion of the population. It is therefore likely that the remaining population of cells consist of CD4$^+$ cells that are not expressing cytokines, and hence we are unable to categorise them through flow cytometry, or cells that have yet to differentiate to a specific subtype. The inability to differentiate greater proportions of Th17 cells may be due to the failure to separate memory and naive T cell prior to polarisation as some studies where this occurs have lead to large populations of Th17 cells (costa-Rodriguez et al., 2007b).

4.10.2 Surface expression of chemokine receptors on Th17-polarised cells

Several papers have shown that human Th17 cells express chemokine receptors, including CCR2 (Sato et al., 2007), CCR6 (Singh et al., 2008) and CCR4 (costa-Rodriguez et al., 2007b). However chemokine receptor expression on murine Th17 cells had not been investigated (although a paper published subsequently identified CCR6 as present of murine Th17 cells (Hirota et al., 2007).

Studies showed increased expression of CCR2, CCR6, CCR9, CXCR2 and CXCR4 and decreased expression of CXCR3 on the surface of cells cultured under Th17 polarising conditions. CXCR3 is classically associated with Th1 cells, and its decreased expression in this context may reflect the decreased proportion of IFNγ$^+$ cells observed under these culture conditions.

The increased expression of several chemokine receptors on $CD4^+$ T cells maintained under Th17 polarising conditions has several implications for inflammatory diseases. For example, CCR6 is a mucosa homing receptor present on dendritic cells and effector memory T cells (Greaves et al., 1997; Kondo et al., 2007) that has also been implicated in cutaneous, airway and intestinal mucosal immunity (Varona et al., 2001; Cook et al., 2000). Moreover, it has also been implicated in the recruitment of T cells in several inflammatory conditions including airway rheumatoid arthritis (Ruth et al., 2003) and psoriasis (Homey et al., 2000), both conditions in which IL-17 has been shown to play a role (Nakae et al., 2003; Zheng et al., 2007). Recent evidence has revealed that dectin-1-mediated fungal immune recognition preferentially induced Th17 cells in both humans and mice. These cells are characterised by expression of CCR6, a mucosa homing receptor, as well as the skin homing receptor CCR4 (Wilson et al., 2007). Interestingly, the Th17 polarising cytokine IL-23 strongly up-regulates CCL20 expression in Th17 cells (Wilson et al., 2007). This may reflect a positive-feedback loop to ensure sustained recruitment of Th17 effector cells. Alternatively, CCL20 is known to possess direct anti-microbial activity (Hoover et al., 2002). CCL20 also enhances migration of Langerhans cells and immature $CD11b^+$ dendritic cells from peyer's patches as well as memory and effector T cells that home to skin and mucosal surfaces (Iwasaki and Kelsall, 2000; Schutyser et al., 2003). Thus, CCL20 produced by Th17 cells not only directs epithelial immunity via antimicrobial activity but also contributes to infiltration of inflammatory cells and IL-23 mediated autoimmune diseases. It is interesting to note that CCR6 has also been reported to be expressed in IFNγ-producing $CXCR3^+$ Th1 cells following exposure to Toll-like receptor ligands, indicating that homing capacity of T helper cells is programmed by the nature of innate immune activation via pattern recognition receptor activation. The relationship between CCR6 expression and the IFNγ-producing cells detected under Th17 polarising conditions is unclear, but certainly CXCR3 is detected in the Th17 polarised population, albeit at lower levels than in the non-polarised cells.

The expression of CCR2 on polarised Th17 cells also has implications for inflammatory disease. It has previously been shown to be expressed on monocytes, activated memory T cells, B cells, and basophils in humans, and also in peritoneal macrophages in mice and its increased expression has been associated with several inflammatory conditions including Experimental Autoimmune Encephalomyelitis (EAE) (Izikson et al., 2000). Interestingly, IL-17 has been shown to be crucial in the development of EAE. Indeed, an anti-IL-17A vaccine protected mice against the induction of EAE (Uyttenhove and Van, 2006). Previously, CCL2 (a major ligand for CCR2) has been implicated in the control of Th2 polarisation (Gu et al., 2000), although it is possible that other CCR2 ligands such as CCL7 and CCL12 may play distinct and more prominent roles in Th17 regulation owing to differences in expression profiles.

Interactions between CCL25 and CCR9 are involved in thymic development and the generation of gut-specific immunological memory (Kunkel et al., 2000; Zabel et al., 1999). In the gut, CCL25 is highly expressed by epithelial cells lining the small intestinal villi but is less prominently expressed in the colon (Kunkel et al., 2000; Zabel et al., 1999). Some T cell subsets are significantly reduced within the intestinal epithelium of CCR9-deficient mice, indicating that CCR9 is necessary for steady state intestinal T cell development and/or migration (Uehara et al., 2002; Wurbel et al., 2001). In addition, adoptive transfer experiments reveal that $CCR9^{+/+}$ lymphocytes are more efficient than $CCR9^{-/-}$ lymphocytes at homing to the small intestine (Johansson-Lindbom et al., 2003). The demonstration of up-regulated CCR9 expression under Th17 polarising conditions is interesting as IL-17 levels are elevated in inflammatory bowel disease (IBD) (Fujino et al., 2003) while the IL-23R gene is a susceptibility factor for IBD (Duerr et al., 2006). Indeed, IL-23 is essential for T cell mediated colitis and promotes inflammation via IL-17 and IL-6 in experimental models (Yen et al., 2006). Paradoxically however, addition of anti-IL-17 antibody worsened intestinal inflammation in a mouse model of colitis (Ogawa et al., 2004). A study of $CCR9^{+}$ T cells isolated from mesenteric lymph nodes (MLN) draining from the small bowel of patients with Crohn's disease showed they produced more IL-17 in response to anti-CD3 or IL-12/IL-18

stimulation, than similar cells from healthy patients, (Saruta et al., 2007) indicating that these $CCR9^+$ T cells are pro-inflammatory.

4.10.2.1 What was the source of the CXCR2?

Analysis of surface chemokine receptor expression revealed low levels of CXCR2 expression. Studies have shown that T lymphocytes do not express CXCR2 (Kershaw et al., 2002), which asks the question; what cells were expressing this receptor? CXCR2 has been shown to play an important role in many diverse signalling pathways such as wound healing, angiogenesis, inflammation and leukocyte chemotaxis (Baugher and Richmond, 2008). Ligands for CXCR2 are ELR+ angiogenic chemokines. This means that they mediate, through CXCR2, the migration and proliferation of endothelial cells, thereby promoting angiogenesis (Raman et al., 2007). CXCR2 is also the main chemokine receptor on neutrophils and mediates neutrophil recruitment (Zarbock et al., 2008). Both endothelial cells and neutrophils are found in the spleen, and although cells were sorted to ensure they were $CD4^+$ T lymphocytes prior to culture, this process is not 100% efficient. However, endothelial cells are adherent cells, meaning that the stick to culture flasks (Liu et al., 2007). In normal culture conditions, the cells are treated with trypsin-EDTA to remove them from the surface of the flask. During routine culture of Th17 cells, this did not occur, ensuring that very few endothelial cells were within the population tested. Primary neutrophils do not survive for very long within culture, coupled with the sorting for CD4 also means that very few would have been within the population tested. While these populations are the likely cells that made up the $CXCR2^+$ population, this explains why expression was so low.

4.10.2.2 Why were levels of CXCR4 so low?

CXCR4 is widely expressed on $CD4^+$ T lymphocytes (Bleul et al., 1996; Loetscher et al., 1994) and is involved in both homeostatic and inflammatory trafficking. It is also one of several chemokine receptors that HIV uses to enter cells. Interestingly, it has been reported to also be expressed on Th17 cells. Studies using Th17 cells isolated from secondary lymphoid tissues in the tonsils

revealed that 50% of Th17 cells expressed CXCR4, markedly more than was seen in this study. However, 20% of the Th17 cells from the tonsils were also IFNγ^+, different from the ones used in this study (Lim et al., 2008). Since their discovery, several differences between human and murine Th17 cells have been revealed (McGeachy and Cua, 2008). Expression of CXCR4 appears to be another.

4.10.3 Are the chemokine receptors functional?

Having identified several chemokine receptors that are upregulated upon culture under Th17 polarising conditions, the ability of their ligands to induce migration, receptor internalisation and Akt phosphorylation and activation were assessed. All cells cultured under Th17 polarising conditions migrated to ligands for CCR2, CCR6 and CCR9, although CCL25 less cells migrated to CCR9, consistent with the lower expression of CCR9 on Th17 cells.

Chemokine receptors undergo a basal level of internalisation, followed by degradation or recycling, in the absence of ligand. Upon ligand binding, the rate of internalisation is greatly increased (Neel et al., 2005b). This helps to modify the response of the cell, helping to determine the level of migration. In addition, studies have shown that internalisation is vital for efficient chemotaxis (Richardson et al., 1998). While CCR2 (Ogilvie et al., 2001) and CCR6 (Badr et al., 2005) have previously been shown to undergo internalisation, CCR9 has never been shown to internalise. Further study would be required before it could be determined whether this is a unique function in Th17-polarised cells.

4.10.4 Ligands for CCR2 and CCR6 induce Th17 migration through a PI3K / Akt dependent mechanism.

Ligands for CCR2 and CCR6, although interestingly not CCR9 were able to induce the migration of Th17 cells, and did so preferentially to other cells in the culture. As discussed above, this may help to explain the recruitment of Th17 cells to inflammatory areas, as both IL-17 and CCR2 or CCR6 have been implicated in inflammatory and autoimmune diseases. Using LY294002, an inhibitor of all PI3K isoforms, revealed that PI3K is essential for migration of

Th17 cells. PI3K is activated by most chemokine receptors expressed on T cells, yet paradoxically it is now clear that activation of PI3K by chemokines can be a dispensable signal for directional migration of some T cells particularly after *in vitro* activation and polarisation toward Th2 (Cronshaw et al., 2004) . Studies of the specificity of LY294002 have revealed that it also inhibits some non-lipid kinases such as CK2 and mTOR. In order to prove that this was a PI3K dependent mechanism, another inhibitor Akti-1/2, shown to be very specific for the Akt1 and Akt2 isoforms of Akt, was utilised (Zhao et al., 2005a). This inhibitor also abrogated chemotaxis, at a very similar concentration to LY294002, indicating the PI3K/Akt pathway is essential for murine Th17 cell migration.

Much interest has arisen recently in the role of PI3K and inflammatory diseases, especially as two isoforms, p110δ and p110γ appear to be primarily expressed in leukocytes (Sujobert et al., 2005). Treatment of mice with inhibitors of p110γ suppressed the progression of joint inflammation in mouse models of rheumatoid arthritis (Camps et al., 2005), as well as prolonging survival in mouse models of systemic lupus erythematosus (SLE) (Barber et al., 2005). Both diseases have been shown to have elevated levels of IL-17 (Ziolkowska et al., 2000; Wong et al., 2000). The different dependency of Th17 cells vs. Th2 cell migration on PI3K-dependent signalling offers exciting therapeutic opportunities whereby PI3K isoform-selective inhibitors might be used to suppress Th17-driven inflammatory/autoimmune responses, while leaving other arms of the immune response unaffected.

4.10.4.1 Why didn't CCR9 mediate migration of Th17 cells?

Surface expression of CCR9 was increased in response to Th17 polarising conditions, while CCL25 induced migration of cells cultured under Th17-polarising conditions, as well as internalisation of CCR9 and Akt activation, indicating that the receptor was functional. However, when migrated cells were analysed, no significant migration of Th17 cells had occurred in response to CCL25. One possible explanation for this is that the increased surface receptor expression of CCR9 was not on Th17 cells. However analysis of receptor

expression on Th17 cells using flow cytometry revealed that a similar proportion of Th17 cells expressed CCR9 as CCR6. Therefore the reasons for this result are currently unclear, but may reflect differences in the functional efficacy of CCL25 verses CCL2 and CCL20. In this regard, CCL25 was much less potent that CCL2 or CCL20 in inducing Akt phosphorylation. It could also result from a difference between cell types, whereby CCR9 on Th17 cells is non-functional, while CCR9 expressed on other cells within the Th17-polarised cell culture is functional. Studies on melanoma cells revealed a population of cells that express CCR9, but where the CCR9 was not functional. In this case, a dysfunctional signaling pathway was suggested as a possible explanation (Letsch et al., 2004).

4.10.5 A role for p110δ in Th17 cell polarisation?

Differentiation of T lymphocytes occurs upon engagement of the T cell receptor (TCR) with APCs, as well as the presence of varying cytokines. Once this has occurred, a signalling cascade is initiated involving several tyrosine kinases. The transmembrane adapter protein, linker for activation of T cells (LAT), nucleates this complex and controls recruitment and activation of phospholipase Cγ, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol trisphosphate and diacylglycerol (Zhang et al., 1998). Inositol trisphosphate triggers the cytoplasmic release of Ca^{2+}, which induces the translocation of NF-AT transcription factors to the nucleus, while diacylglycerol activates PKC and Ras guanyl-releasing protein, which in turn activate the Ras-ERK1/2 pathway and AP-1 nuclear translocation (Genot and Cantrell, 2000).

Other proteins can influence this system by altering the level of PIP2. One such protein is PI3K, which is thought to couple to the TCR through LAT (Ward and Cantrell, 2001). Previous work by Okkenhaug *et al* (Okkenhaug et al., 2006) revealed a role for the p110δ isoform of PI3K in the polarisation of Th1 and Th2 cells. Use of mice lacking a functional p110δ isoform, or IC87114, a p110δ-specific inhibitor, revealed that loss of p110δ did inhibit Th17 cell polarisation, although not to the same extent as in Th1 and Th2 cells.

Akt has also been shown to be essential for T helper cell differentiation (Arimura et al., 2004), as well as a number of PIP3-dependent kinases such as Itk (Fowell et al., 1999), although to-date, Itk has only been reported to have a role in Th2 cell polarisation. It is likely that p110δ signals through these proteins during T helper cell differentiation. Other proteins have been implicated in differentiation of T lymphocytes following TCR engagement including the p110α isoform of PI3K (Sauer et al., 2008) and PKCθ (Villalba et al., 2000). Further work needs to be completed before it can be determined what role these proteins may have in Th17 cell differentiation, and whether they account for the 50% of Th17 cells that differentiated in the absence of P110δ.

4.11 Conclusions

In this section of the study, I have shown that surface expression of CCR2, CCR6 and CCR9 is increased on the surface of $CD4^+$ splenocytes cultured under Th17 polarising conditions. I have also shown that surface expression of CXCR3 is downregulated on these cells, in line with the reduced numbers of $IFN\gamma^+$ cells in the culture population. Ligands for CCR2, CCR6 and CCR9 were able to induce the migration of cells, as well as receptor internalisation, indicating that the receptors are functional. It has also been shown that ligand binding of these receptors induces Akt phosphorylation and activation. CCR2, CCR6 and CCR9 have been implicated in a number a number of inflammatory diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease, all diseases that IL-17 has been implicated in. Therefore this work describes a model whereby IL-17 producing cells localise to this area through their expression of these chemokine receptors.

Analysis of the cells by flow cytometry post-migration to ligands of CCR2, CCR6 and CCR9 revealed that CCR2 and CCR6 mediated the migration of Th17 cells, but that CCR9 did not. Further studies would need to be performed to determine exactly why, but other studies have also identified the expression of non-functional CCR9. The migration of Th17 cells was shown to be via the PI3K / Akt pathway, and may help to explain why treatment with p110γ (an

isoform of PI3K) inhibitors suppressed joint inflammation in mouse models of rheumatoid arthritis, a disease mediated by IL-17.

In this section of the study, I have also shown that another isoform of PI3K, p110δ, is involved in the polarisation of Th17 cells. Studies using mice expressing a kinase-dead form of p110δ, or a p110δ inhibitor revealed that 50% less cells polarised to a Th17 phenotype. However, further work would need to be performed to determine which protein was responsible for the other 50%.

Chapter 5: Final Conclusions and Future Work

5. Final Conclusions and Future Work

5.1 Final Conclusions

During the course of this study, a crucial role for Akt has been shown in T lymphocytes. Using both primary PBMCs and the T cell line CEM cells, inhibition of Akt using the specific inhibitor Akti-1/2 significantly reduced the number of cells that migrated to either CCL22 or CXCL12. PI3K, commonly thought to be responsible for activation of Akt, was inhibited using three separate inhibitors. The results revealed that although PI3K inhibition reduced cell migration in CEM cells stimulated with CXCL12, it had no effect on CCL22 stimulated CEM cells or CXCL12 stimulated PBMCs. Analysis of Akt activity revealed differences in the effects of PI3K inhibition on Akt activity depending on the cell type and chemokine. Further experiments are required to explain this result, but one possible explanation is that Akt represents a 'pinch point' in the cell signalling cascade so that inhibition of Akt is more effective than inhibition of PI3K for inhibiting migration in certain cell types. Akt also has an important role in the regulation of L-selectin, a cell adhesion molecule important in lymph-node trafficking. Akt has been shown to phosphorylate FOXO, a transcription factor, and thus prevent its entry into the nucleus and the subsequent transcription of L-selectin (Fabre et al., 2008). Thus, Akt has multiple different effects on cell migration, and another explanation of Akt's crucial role in migration is could be that it's vital in many different processes.

The role of other kinases thought to be important in chemokine receptor signalling and cell migration was also investigated. Using CCL22 mediated cell migration of CEM cells as a model; I have shown that inhibition of GSK-3 leads to an increase in cell migration, consistent with Akt's inhibition of GSK-3 under normal cell conditions. In addition, using inhibitors of PKCδ and splenocytes from mice containing a kinase-dead mutant of PKCδ revealed a role for PKCδ in T lymphocyte migration. The signalling pathways through which PKCδ is involved in T lymphocyte migration are still to be determined. However, studies have shown that PKC isoforms are involved in several processes crucial to cell migration. These include regulation of changes in integrin affinity and lateral mobility (Giagulli et al., 2004), effects on actin reorganisation and

polymerisation (Sasahara et al., 2002; Hartwig et al., 1992) and phosphorylation of myosin light chain (MLC) (Zemlickova et al., 2004; Iwabu et al., 2004a). In addition, during lymphocyte function-associated antigen-1-mediated locomotion of activated T cells, PKCßI and PKCδ associate with microtubules in the uropod, the trailing extension of migrating T cells. (Volkov et al., 2001; Fanning et al., 2005a), indicating a role for PKCδ in the migration of T cells.

Taken together, these results suggest a possible model of the signalling pathway in CCL22 mediated migration of CEM cells, and link PI3K / Akt activation to cell polarisation and migration (Fig. *5.1*).

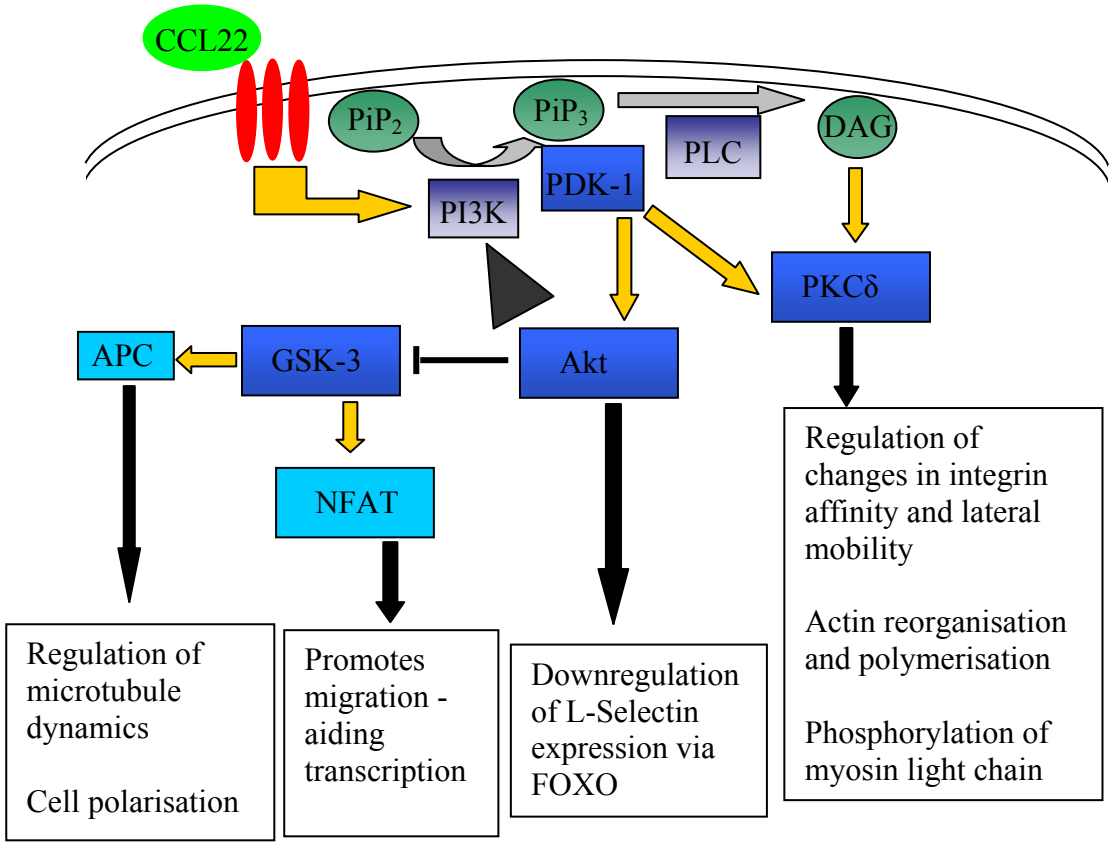


Figure 5.1: Model of a possible intracellular signalling pathway resulting from CCL22 binding its receptor in CEM cells. Upon CCL22 binding to CCR4, PI3K is activated and converts PIP_2 to PIP_3 in the cell membrane. This allows PDK1 and Akt to bind, resulting in the activation of Akt. Akt then phosphorylates GSK-3, inactivating it and preventing GSK-3 from phosphorylating APC and NFAT. NFAT can therefore translocate to the nucleus where it promotes migration through the transcription of other proteins, while APC can associate with microtubules and promote cell polarisation. In addition, PLC converts PIP_3 into IP_3 and DAG. DAG binds to PKCδ, activating it in association with phosphorylation by PDK-1. PKCδ then phosphorylates other proteins to promote cell polarisation and migration. Akt can also directly influence migration. By phosphorylating FOXO and excluding it from the nucleus, Akt prevents transcription of L-selectin. The black triangle denotes the relative quantities of each protein within the cell.

Further studies are required to determine whether this model is applicable to all chemokine receptors, although this study has already shown differences in the susceptibility of cell migration to PI3K inhibition in different cells. This difference in signalling pathways could be a method by which cells that receive multiple stimuli from different chemokine receptors prioritise their response to a

specific signal, i.e. signals from homeostatic chemokines versus inflammatory chemokines, by down regulating specific proteins.

Many cells have been shown to express specific chemokine receptors on their cell surface, targeting them to specific regions of the body or allowing them to participate in specific inflammatory responses. A new type of T helper cell, Th17 cells, was investigated in this study to determine whether they also express a unique chemokine receptor profile. Using murine splenocytes polarised to a Th17 phenotype, this study has shown that murine Th17 cells express the chemokine receptors CCR2 and CCR6. Following on from the previous section, it was also shown that CCR2 and CCR6 mediated migration is PI3K and Akt dependent. Further study is required to determine whether CCR2 and CCR6 are expressed on the same cells, or whether they represent two distinct populations of cells. In humans, CCR2 has been shown to be expressed on cells that are also CCR5- while CCR6 has been shown to be co-expressed with CCR4.

However, some caution should be taken in drawing conclusions between human Th17 cells and the murine cells used in this study. Several recent studies have highlighted differences between human and mouse cells, one of the most basic of which is the requirement for TGF-β. While murine Th17 cells require the presence of TGF-β and IL-6 to differentiate, studies have shown that in human Th17 cells, TGF-β is dispensable. A study by Acosta-Rodriguez *et al* has shown that human Th17 cells only require IL-1β and IL-6 (costa-Rodriguez et al., 2007b), while Wilson *et al* showed that Th17 cells derive from naïve precursors in Man in response to either IL-1β or IL-23, with no synergistic effect occurring when both were used (Wilson et al., 2007). However, some recent studies have contradicted these results, suggesting that TGF-β is required and that previous studies were flawed as they used media containing bovine serum, a source of TGF-β. (Volpe et al., 2008; Yang et al., 2008). Murine Th17 cells appear to have the same developmental origin as $FoxP3^+$ Treg cells, with both requiring TGF-β to differentiate. Indeed, $FoxP3^+$ Treg cells have been shown to produce IL-17A in the presence of TGF-β. However human Th17 cells appear to be more closely linked to Th1 cells, with studies showing the existence of large

numbers of cells that produce both IL-17 and IFNγ (Annunziato et al., 2007), as well as human Th17 cells that express T-bet (the Th1 transcription factor) in addition to RoRγt (Annunziato et al., 2007).

This study has identified a role for PI3K in both the migration of Th17 cells and their polarisation. Inhibition of PI3K or Akt greatly reduced the migration of Th17 cells to ligands of both CCR2 and CCR6. Several companies are currently investing in inhibitors of PI3K for use in inflammatory diseases (Medina-Tato et al., 2007) and with Th17's major role in these diseases, this result makes PI3K an even more attractive target. In addition, the p110δ isoform of PI3K has been shown to be involved in the polarisation of Th17 cells. While this could also be an attractive target for anti-inflammatory drugs, it is worth noting that these results have also been shown in other $CD4^+$ cells including Tregs (Patton et al., 2006), preventing specific targeting of Th17 cells. Thus significant side-effects could result from the administration of PI3K-inhibiting compounds.

5.2 Future Work

5.2.1 Confirm the specificity of Akti-1/2

The Akti-1/2 compound had raised some interesting questions regarding Akt and whether it lies solely downstream of PI3K. A lot of these conclusions are based around the assumption that this inhibitor is specific for Akt at the concentrations used. Analysis of the inhibitor's effects on the MAPK family of proteins has revealed no inhibition. However, it would be useful to run a much wider screen against this inhibitor, to confirm that there are no other kinases mediating these effects in T lymphocyte chemotaxis. This could be achieved though, for example, a high-throughput kinase screen that detects binding against a wide range of different kinases. Such screens are commercially available, although expensive.

5.5.2 Explore whether Akt is activated independently of PI3K

One of the most interesting outcomes of this work was the discovery that Akt inhibition can still inhibit PI3K inhibitor resistant migration. There are two

possible explanations for this: i) that Akt is being activated independently of PI3K, ii) that there is a difference in the relative abundances of the two kinases within the cell, allowing Akti-1/2 to have a much more potent effect than the PI3K inhibitors. Therefore, it would be useful to inhibit PI3K using siRNA or a similar system and then repeat the chemotaxis experiments. If PI3K is non-functional but the cells still migrate and can be inhibited by Akti-1/2 then this indicates a PI3K-independent role for Akt. Also, it would be interesting to monitor the activity of PI3K directly, after adding the PI3K inhibitors. If some PI3K is still active, it could indicate a difference in the abundance of the two kinases within the cell. If it turns out that Akt is activated independently of PI3K, the next logical step would be to try and determine what kinases are responsible for activating it. Some evidence has been published that shows that PKC isoforms can phosphorylate Akt after activation through GPCR induced activation of PLC and the subsequent production of DAG. These isoforms would make a good first target for the investigation.

5.5.3 Further investigate the expression of chemokine receptors on the surface of Th17 cells

Several questions remain unanswered regarding the expression of chemokine receptors on the surface of Th17 cells. Limitations with equipment and commercially available antibodies meant that I was unable to determine whether any of the chemokine receptors were co-expressed on the surface of the Th17 cells. This could be of particular importance in identifying a Th17-specific marker. In addition, further work also needs to be carried out to determine why CCR9 does not induce chemotaxis of Th17 cells. The expression of CCR9 on Th17 cells, as well as the intracellular signals generated upon ligand binding, need to be investigated.

5.5.4 Determine what proteins are vital for Th17 cell polarisation

During the course of this study it was determined that Th17 cells, unlike Th1 and Th2 cells are not completely dependent on the p110δ isoform of PI3K to polarise. This could be of particular interest in identifying anti-inflammatory

drug-targets and so it would be useful to determine what proteins are vital for Th17 cell polarisation.

Chapter 6: References

6. References

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