University of Bath

UNIVERSITY OF BATH

PHD

Analysis of CXCR6-mediated signal transduction in T-lymphocytes

Crocker, Jennifer Mary

Award date:
2007

Awarding institution:
University of Bath

Link to publication

Analysis of CXCR6-Mediated Signal

Transduction in T-Lymphocytes

Jennifer Mary Crocker

A thesis submitted for the degree of Doctor of Philosophy

University of Bath
Department of Pharmacy and Pharmacology
September 2006

Accepted 2007

UMI Number: U490220

UMI U490220

Table of Contents

Table of Figures

Acknowledgements

Firstly, I would like to thank Prof. Ward and Dr. Wilson, my supervisors at university and GSK, for the opportunity to carry out this work. However, there are also a number of other people who have helped along the way. To those members of the Ward group whose task it was to teach me lab skills, I was unaware how time consuming it was until my turn came, so thank you!! I would like to thank Jo for her skills as a phlebotomist and Kevin for letting me loose on the FACS machine.

Also to the Ward and Welham groups past and present I would like to thank you for making my time in Bath as enjoyable as possible; I will take away many memories. Finally, but perhaps most importantly I would like to thank my family and friends who have continuously supported me throughout this and other challenges I have faced. I hope you know how much it has been appreciated.

Abstract

Chemokines have a diverse array of effects on the functions of lymphocytes and other immune cells. CXCR6 is a relatively newly discovered chemokine receptor, originally thought only to be expressed on a specific-subset of T-lymphocytes. It has since been identified on a number of cell types, and is not only able to function as a human immunodeficiency virus coreceptor, but has been implicated in the trafficking of T-lymphocytes in certain inflammatory diseases. The ligand for CXCR6, CXCL16, has a novel structure among the members of the CXC chemokine family, it that it possesses a mucin-like stalk which enables it to be both membrane bound or soluble.

There has currently been little work into the signal transduction mechanisms through which CXCR6 and CXCL16 elicit their effects; the aims of this project were to identify signal transduction molecules downstream of CXCR6 and determine whether CXCR6 is coupled to similar pathways utilised by other chemokine receptors. The results indicate that although CXCR6 is implicated in trafficking T-lymphocytes, levels of *in vitro* chemotaxis are very low. However, activation CXCR6 is able to induce polymerisation of actin which is $G_{\alpha i}$-mediated, and dependent on phosphatidylinositol-3 kinase, protein kinase C, Rho, Rac and Cdc42. CXCR6 ligation also has an additive effect on the CD3-mediated production of tumour necrosis factor-α and interferon-γ, through a pathway not utilised by CXCR4. The ability of CXCL16 to induce production of proinflammatory cytokines may indicate that CXCR6, found at high concentrations in areas of inflammation, may not only be involved in trafficking the cells but also in the pathogenesis of the inflammation.

Abreviations

AP1, activator protein-1
aPKC, atypical PKC isoforms (ζ and λ)
Arp2/3, actin-related proteins
ATCC, American Type Culture Collection
ATP, adenosine triphosphate
B-lymphocyte, bone-marrow derived lymphocyte
Btk, Bruton's tyrosine kinase
BSA, bovine serum albumin
CLA, cutaneous lymphocyte antigen
cPKC, classical PKC isoforms(α, βI, βII, γ)
CsK, C-terminal Src kinase
DAG, diacylglycerol
DC, dendritic cell
DGK, diacylglycerol kinase
DMSO, dimethylsulphoxide
EAE, experimental autoimmune encephalomyelitis
ECACC, European Collection of Cell Cultures
eIF4E, eukaryotic initiation factor 4E
ELISA, enzyme linked-immunosorbant assay
ER, endoplasmic reticulum
ERK, extracellular signal-regulated kinase
ESC, erythroid stem cell
FAK, focal adhesion kinase
FBS, foetal bovine serum
FITC, fluorescein
GAPs, GTPase activating proteins
GM, granulocyte-monocyte common precursor
GNEF, guanine nucleotide exchange factor
GPCR, G-protein coupled receptors
Grb2, growth factor receptor bound-2
GRP, guanyl nucleotide releasing factor
GSK3β, glycogen synthase kinase 3β
HIV, human immunodeficiency virus
HRP, horse radish peroxidase
HSC, haemopoietic stem cell
ICAM, intercellular adhesion molecules
ICRF, Imperial Cancer Research Fund
IFN-γ, interferon-γ

IκB, inhibitor of κB
IKK, inhibitor of κB kinase
IL-1, interleukin 1
IL-2, interleukin 2
IL-4, interleukin 4
IL-5, interleukin 5
IL-6, interleukin 6
IL-8, interleukin 8
IL-10, interleukin 10
ILK, integrin-linked kinase
IP$_3$, inositol 1,4,5-trisphosphate
JNK, c-Jun NH_2-terminal kinase
LAT, linker for activated T cells
LPS, lipopolysaccharide
LSC, lymphoid stem cell;
MAPK/MAP-kinase, mitogen activated kinase
Mek, MAP-kinase and ERK kinase
MHC, major histocompatability complex
MK2, MAPK-activated protein kinase 2
MLC, myosin light chain
MLCK, myosin light chain kinases
MSK1, mitogen-and stress-activated protein 1
mTOR, mammalian target of Rapamycin
NEMO, NF-κB essential modulator
NFAT, nuclear factor of activated T cells
NF-κB, nuclear factor-κB
NIK, NF-κB inducible kinase
NK, natural killer cell
nPKC, novel PKC isoforms (ϵ, δ, η, and θ)
p70S6K, S6 kinase of 70kDa
p90RSK, p90 ribosomal S6 kinase
PAK, p21 activating kinase
PBL, peripheral blood derived T-lymphocyte
PBMC, peripheral blood mononuclear cells
PBS, phosphate buffered saline
PDK1, 3-phosphoinsotide-dependent kinase-1
PE, phycoerythrin
PH domain, pleckstrin homology domain

10

PLC, phospholipase C
PI, phosphatidylinositol
PI3K, phosphatidylinositol 3-kinase
PKA, protein kinase A
PKB, protein kinase B
PKC, protein kinase C
PKD, protein kinase D
PMA, phorbol 13-myristate 12-acetate
PMN, polymorphonuclear cell
PTEN, phosphatase and tensin
homologue deleted on chromosome 10
PTX, pertussis toxin
PX, Phox homology domain
PYK2, proline-rich tyrosine kinase 2
RACK, receptors of activated C kinase
ROCK, Rho kinase
SDS-PAGE, sodium dodecyl sulphate-
polyacrylamide gel electrophoresis
SEB, staphylococcus enterotoxin B
SEK1, stress-activated protein kinase-1
SH2 domain, signal relating kinase
homology domain
Shc, Src homology and collagen
SHIP, SH2-containing inositol
polyphosphate 5-phosphatase
SIV, simian immunodeficiency disease

SLP-76, SH2 domain-containing
Leukocyte Protein of 76 kDa
Sos, son of sevenless
STICK, substrates that interact with C
kinase
STRL33, seven-transmembrane
domain receptor L33
SR, sarcoplasmic reticulum
Src, signal relating kinase
TBS, tris-buffered saline
TCR, T cell receptor
T-lymphocyte, thymus derived
lymphocyte
TNFα, tumour necrosis factor α
Tpl-2, tumour progression locus-2
TYMSTR, T-lymphocyte-expressed
seven-transmembrane domain receptor
VASP, vasodilator stimulated protein
VCAM, vascular adhesion molecule
WASP, wiskott-aldrich syndrome
protein
WIP, WASP-interacting protein
ZAP-70, ζ-chain associated protein
70kDa

Chapter 1: Introduction

1.1 The Immune System - an overview

The immune system is similar to other elements of mammalian physiology in being composed of specialised cells with specific functions (figure 1). The granulocyte-monocyte lineage gives rise to a number of cells involved in the innate immune response. Neutrophils, eosinophils and basophils, termed granulocytes because of the presence of cytotoxic granules in the cytoplasm giving a distinct appearance. They circulate in the blood and migrate into tissues when an inflammatory stimulus is detected. The exception to this is the mast cell which bears similar morphology to basophils and is mainly resident in tissues. Granulocytes are involved in different areas of the immune response with neutrophils predominant in the removal and killing of bacteria and fungi and eosinophils predominant in the control of infection by parasites. The exact role of basophils and mast cells is less well defined, but these cells are significant in the pathogenesis of allergic responses. Monocytes have a very short half-life spending approximately 24 hours in the blood before migrating into the tissue; tissue-resident monocytes are termed macrophages. They also contain some cytotoxic granules, are involved in the processing and presentation of antigens and, like neutrophils, are able to ingest and destroy pathogens by phagocytosis. Dendritic cells are bone marrow derived cells which are particularly important in the presentation to, and activation of, naïve T-lymphocytes.

Lymphocytes, the major constituents of the adaptive immune system, are divided into two subtypes, B-lymphocytes and T-lymphocytes. The main functions of B-lymphocytes are the recognition of antigen through surface receptors known as antibodies and the presentation of the antigen to T-lymphocytes. Plasma cells are non-circulating, differentiated B-lymphocytes whose primary function is to produce antibody. T-lymphocytes are pivotal cells in the immune response and are able to influence many aspects of immunity. Their functions include the stimulation of B-lymphocytes providing a signal to mature into plasma cells and release of soluble factors responsible for recruitment, growth, expansion and development of other cells.

Megaka-ryocyte → Platelets

HSC

ESC → Erythrocyte

GM → Monocyte → Macrophage

Eosinophil

Neutrophil → Mast cell

Basophil

STEM CELL

? → Dendritic cell → Follicular Dendritic cell

LSC

B → B-lymphocyte → Plasma cell

NK → Natural Killer cell

T → T-lymphocyte
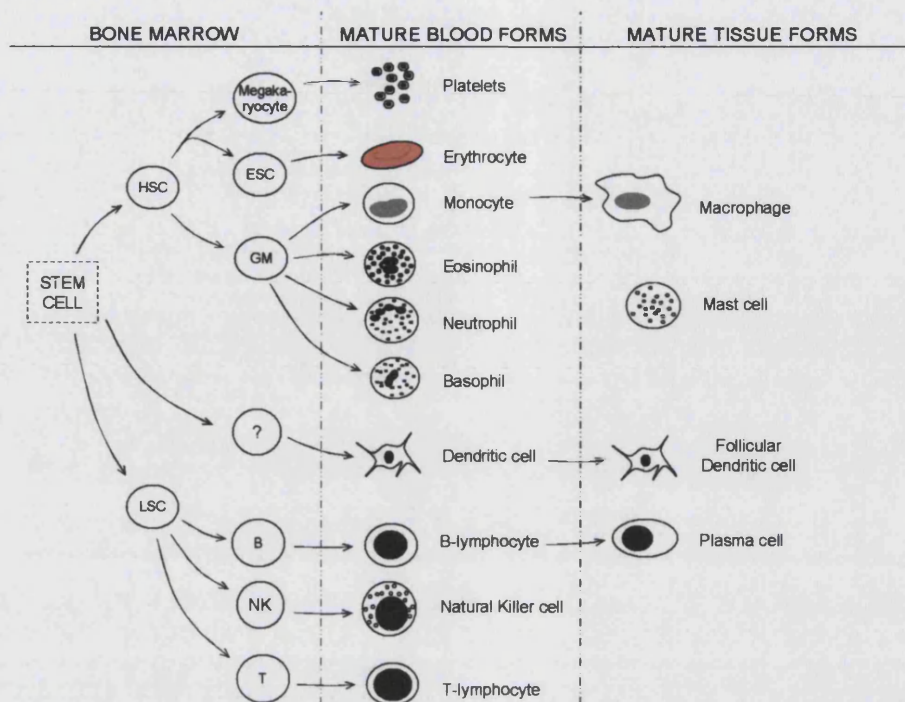
Figure 1. The development of blood cells from pluripotent stem cells to mature circulating and tissue forms.

HSC, haemopoietic stem cell; LSC, lymphoid stem cell; ESC, erythroid stem cell; GM, granulocyte-monocyte common precursor; B, bone-marrow derived lymphocyte; NK, natural killer cell (large granular lymphocyte); T, thymus derived lymphocyte. (based on Peakman & Vergani, 1997)

T-lymphocytes are defined by the presence of several cell surface markers; the receptor for antigen, the TCR (T cell receptor), is present on all T-lymphocytes and forms a complex with CD3, composed of five different transmembrane protein chains, which transduces to signals from the TCR to initiate cell activation. These surface markers are important in T-lymphocyte development (figure 2) (Haks *et al*, 1999). Bone marrow-derived precursor cells (thymocytes) enter the thymus where they generate cytoplasmic forms of the α and β TCR chains. These are then rearranged and are expressed on the surface of the cell; failure to do this will result in cell death. The cell then expresses CD3 as well as CD4 and CD8, accessory proteins with affinity for class II and class I MHC (major histocompatability complex) molecules respectively; these are important in the recognition of antigen and subsequent activation of the T-lymphocyte through the TCR. The double positive

cell is exposed to self-antigen peptides resulting in four possible outcomes: (i) no affinity of the TCR for peptides presented by either class I or II MHC molecules, the cell receives no positive signal and cell death occurs; (ii) the TCR has high affinity for peptide-MHC complex is potentially dangerous if the cell were to encounter this in the periphery, these cells do not continue either; (iii) the TCR has moderate affinity for a peptide presented by class I MHC resulting in positive selection of the cell which loses the CD4 molecule from the cell surface; (iv) the TCR has moderate affinity for a peptide presented by class II MHC resulting in positive selection of the cell which loses the CD8 molecule from the cell surface. These T-lymphocytes then leave the thymus where they may undergo further differentiation.
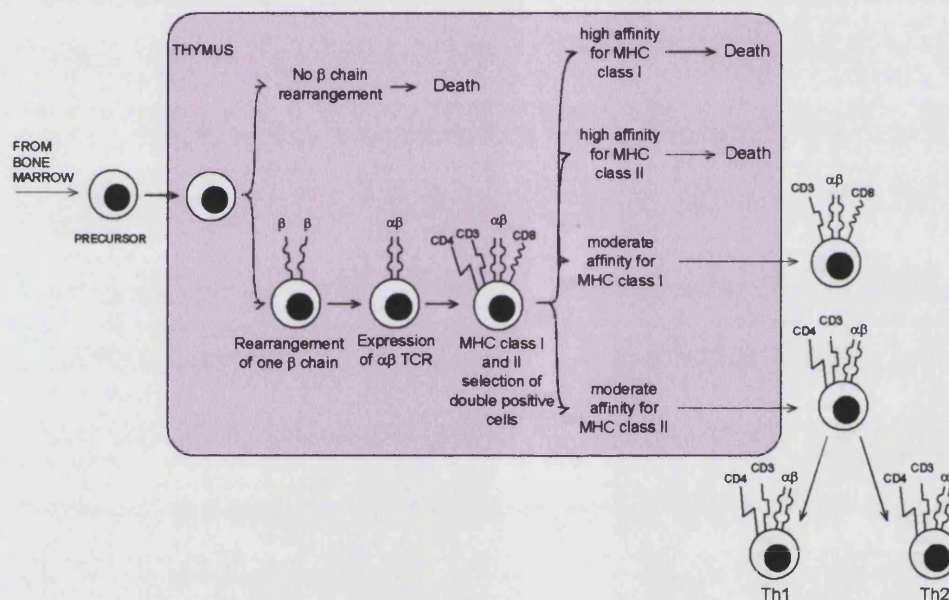
Figure 2. Thymic selection and development of T-lymphocytes.
T-lymphocyte precursors (thymocytes) enter the thymus. Cells expressing a T cell receptor (TCR) which recognise and have moderate affinity for self MHC (major histocompatibility complex) molecules are selected and exit the thymus. These cells can be further developed into effector T-lymphocytes either T helper (Th) CD4+ cells or T cytotoxic (Tc) CD8+ cells. Thymocytes not selected undergo apoptosis. (based on Peakman & Vergani, 1997)

Exposure of CD4$^+$ T-lymphocytes to specific cytokines during their maturation can produce T helper (Th) cells (HayGlass *et al*, 1997; Romagnani, 1996). Th cells are defined by their cytokine profiles; Th1 cells secrete interferon-γ (IFN-γ) and interleukin 2 (IL-2), while Th2 cells produce IL-2, interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6) and interleukin 10 (IL-10). Major functions of these two subsets of T-lymphocytes differ with Th1 cells involved in the activation of macrophages and other cytotoxic cells and Th2 cells important in the activation and maturation of B-lymphocytes.

Natural killer cells are the third member of the lymphocyte family, morphologically similar to T-lymphocytes. Unlike both B- and T-lymphocytes they are able to kill tumour and virally infected cells without prior sensitisation.

The immune response is divided into two parts determined by the speed and specificity of the reaction (reviewed in Parkin & Cohen, 2001; Rollinghoff *et al*, 1997). The innate immune system, present from birth, is the main, first-line defence against invading organisms; it typically involves the activation of neutrophils, monocytes, macrophages and complement. In contrast, the adaptive immune system, based on the development of lymphocytes which respond selectively to antigens, is acquired following exposure to pathogens and has both specificity and memory. While the mechanisms of immunity are highly efficient in protecting the body against antigen, inappropriate or over-activation of the responses are detrimental and can give rise to hypersensitivity and autoimmune disorders, like asthma, rheumatoid arthritis or multiple sclerosis (Vandenbulcke *et al*, 2006). It is thus essential that the system is strictly controlled; central to this is the appropriate recruitment of leukocytes to sites of inflammation. This is a tightly regulated process involving molecules expressed on the vascular endothelium including integrins, selectins, cadherins, connexins and chemoattractants (C5a, formyl peptide, prostaglandins) which direct leukocyte traffic (Worthylake *et al*, 2001). Of particular importance in the trafficking and activation of leukocytes are the members of the chemokine family (Wong & Fish, 2003).

1.2 Chemokines and Chemokine Receptors

1.2.1 Chemokines

Chemokines (chemotactic cytokines) are low molecular weight proteins involved primarily in chemoattraction and regulation of leukocyte trafficking. They are classified into families according to both structural and genetic properties (Zlotnik, *et al*, 2000). All chemokines contain at least 3 β-pleated sheets and an α-helix in the carboxyl-terminus. Most chemokines contain 4 conserved amino-terminal cysteine residues, the positioning of the two nearest the amino-terminal denote the sub-family to which the chemokine belongs (Figure 3) (Rollins, 1997).
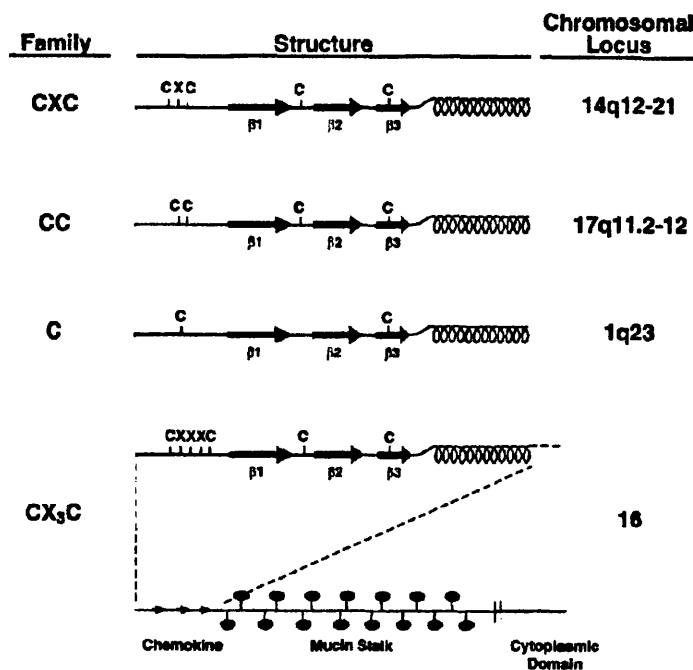
Figure 3. Structural relationship between chemokine families. The genes of the individual chemokine families generally cluster to specific chromosome locations. The basic structure of chemokines consists of 3 β-pleated sheets (β1-3) and an α-helix. The conserved cysteine residues indicated are the basis for the nomenclature of this superfamily (X denotes any amino acid). The CX₃C chemokine has an additional structure not found in other chemokine families (with the exception of CXCL16). The mucin stalk spans the plasma membrane and has a small N-terminal cytoplasmic domain. (Rollins, 1997)

The majority of chemokines are soluble, however the CX₃C chemokine (CX₃CL1/fractalkine), while containing the general features of the other chemokine families, also contains a membrane embedded stalk heavily substituted with mucin-like carbohydrates (Bazan *et al*, 1997). A recently discovered chemokine, CXCL16, has also been found to have this structure; however it can also be cleaved into a soluble form which is also active (Matloubin, *et al*, 2000).

ELR Chemokines

The tripeptide ELR (Glu-Leu-Arg) motif, immediately preceding the first cysteine residue at the amino-terminal, is a common feature of many CXC chemokines which act through CXCR1 and/or CXCR2 allowing further classification of the CXC chemokines. ELR-positive chemokines are potent neutrophil chemoattractants and function as potent angiogenic factors, able to stimulate endothelial cell chemotaxis. ELR-negative chemokines have anti-angiogenic properties; they inhibit endothelial cell chemotaxis induced by ELR-positive chemokines (Strieter *et al*, 1995; Murphy, 1996)

Inducible and Constitutive Chemokines

A more flexible system for categorising chemokines is dependent on a more functional characteristic; the regulation of their production from cells (Mantovani, 1999; Zlotnik, 2000). Chemokines constitutively (constitutive/ homeostatic) expressed in specific organs are believed to have more of a housekeeping role; a specific function involving leukocyte trafficking under normal conditions (figure 4). Inducible (inflammatory) chemokines are strongly upregulated in peripheral tissues following an immune or inflammatory stimulus indicating a role in the development of immune or inflammatory responses (figure 5). The genes which encode inducible chemokines are located in two major clusters on chromosomes 4 and 17 for CXC and CC chemokines respectively; whereas genes for constitutive chemokines are sited alone or in small clusters on chromosomes. It has been suggested that chemokines located on different chromosomes to the major clusters are probably older in evolutionary terms and have been conserved because of their specific functions. They are also less likely to share receptors or have overlapping functions with other chemokines. In contrast, the functional diversity of the chemokines located within the major clusters may be less significant; their main role is likely to be the attraction of neutrophils or monocytes, and probably display some promiscuity to receptors (Zlonik *et al*, 2000).

Chapter 1: Introduction   17

This classification of chemokines does have limitations; some chemokines are constitutively produced, but are also upregulated under inflammatory conditions. For example, CCL19 (EBI(EBV-induced gene)1 ligand chemokine/ ELC) is constitutively expressed on stromal cells where it is involved in trafficking naïve T cells and maturing DC into secondary lymphoid organs, however it can also be upregulated along with CCL21 (secondary lymphoid tissue chemokine/ SLC) at sites of chronic inflammation (Hjelmström *et al*, 2000).
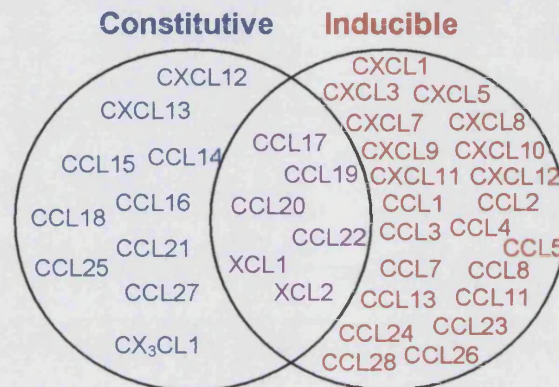
Figure 4. Functional classification of chemokines.

Depending on the regulation of their production, many chemokines can be categorized either as inducible chemokines that are associated with inflammatory and immune responses or constitutive chemokines which regulate normal leukocyte trafficking. Some chemokines, however, play a pivotal role in both homeostasis as well as in inflammatory responses.

1.2.2 Chemokine Receptors

Chemokine receptors are a family of heptahelical G-protein coupled receptors (GPCR); typically 340-370 amino acids in length and contain the conserved amino acid sequence, DRYLAIV, in the second intracellular loop. This motif, exposed following ligation of the receptor by its activating chemokine, is known to be involved in G-protein coupling (Murphy, 2002). The nomenclature of this family of receptors is based on the structure of their activating chemokines; which display some promiscuity between receptors (Table 1). The reason a chemokine can activate a number of receptors is not fully understood; although it may enable the regulation of different leukocyte subpopulations in acute or chronic inflammatory responses.

Receptor	High Affinity Ligands	Functional Expression on Immune Cells
CCR1	CCL3,5,7,14,15,16,23	memory T cells
CCR2	CCL2,7,12,13	monocytes, DC, NK, basophils, PMN
CCR3	CCL5,7,8,13,15,24,26	T cells (Th2>Th1), eosinophils, basophils, mast cells
CCR4	CCL17,22	T cells (Th2>Th1)
CCR5	CCL3,4,5	progenitors, Th1, monocytes, macrophages, DC
CCR6	CCL20	memory T cells, DC
CCR7	CCL19,21	T cells, B cells, DC
CCR8	CCL1,4	Th2 cells
CCR9	CCL25	$\alpha_4\beta_7$+ T cells, DC, macrophages, thymocytes
CCR10	CCL27	CLA+ cells
CCR11	CCL2,8,13	n.a.
CXCR1	CXCL2,3,5,6,7,8	PMN, mast cells, monocytes, macrophages
CXCR2	CXCL1,2,3,5,6,7,8	PMN, mast cells, monocytes, macrophages
CXCR3	CXCL9,10,11	T cells (Th1>Th2), B cells, NK
CXCR4	CXCL12	most progenitor cells, T cells, B cells, PMN, monocytes, macrophages, DC
CXCR5	CXCL13	B cells, memory T cells
CXCR6	CXCL16	T cells
CX_3CR1	CX_3CL1	PMN, monocytes, NK, T cells
XCR1	XCL1,XCL2	T cells

Table 1. Chemokine receptor-ligand pairs and expression profile.
Chemokine receptors are expressed on a wide variety of immune cells. The majority of receptors can be activated by a number of chemokines initiating different functional responses depending on the activating ligand and environment of the cell. NK, natural killer cell; DC, dendritic cell; PMN, polymorphonuclear cell (neutrophil); CLA, cutaneous lymphocyte antigen.

The most widely expressed chemokine receptor is CXCR4, present on T lymphocytes, neutrophils, monocytes, B lymphocytes and blood-derived dendritic cells as well as a number of non-haemopoetic cells. CXCR1 and CXCR2, although expressed on most leukocytes, appear only to be functionally active on neutrophils, monocytes/macrophages and mast cells (Murphy *et al*, 2000; Nilsson *et al*, 1999). CXCR3, CXCR5 and CXCR6 are primarily expressed on lymphocytes although have been identified on other cell types (Forster *et al*, 1994; Qin *et al*, 1998; Unutmaz *et al*, 2000). CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9 and CCR10 are predominantly expressed on lymphocytes, monocytes and

monocyte-derived dendritic cells (Murphy et al, 2000); while CCR3 is found on eosinophils, mast cells, basophils and Th2 lymphocytes (Rubbert et al, 1998; Sallusto et al, 1997).

One receptor that does not fulfil this nomenclature criterion is 'duffy antigen receptor for chemokines' (DARC), a seven-transmembrane domain receptor that does not contain the DRY motif found in other chemokine receptors. DARC is able to bind the majority of members of the CC chemokine family. It is thought that this receptor may act as a buffer enabling concentrations of chemokines to be regulated (Fukama et al, 2003).

Chemokine Receptor Expression on T-lymphocytes

Developing lymphocytes express different chemokine receptor profiles throughout the differentiation process (O'Garra et al, 1998). Naïve T-lymphocytes express receptors which traffic them to secondary lymphoid organs and promote their interaction with dendritic cells, such as CXCR4 and CCR7. Polarisation of naïve T-lymphocytes into effector cells results in selective expression of chemokine receptors at the cell surface, depending on the cytokines present. Priming of T-lymphocytes in the presence of IL-12 promotes production of Th1 cells; these cells preferentially express CCR1, CCR5 and CXCR3. CCR5 and CXCR3 are present on the majority of peripheral blood memory T-lymphocytes; however they are expressed at much higher levels on Th1 cells than Th2 cells (Sallusto et al, 1998). The presence of IL-4 during polarisation drives development of Th2 cells which selectively express CCR3, a receptor originally described on eosinophils and basophils (Gerber et al, 1997). Th2 cells also express CCR4; however this receptor is not a faithful marker of Th2 cells, being expressed on cells which do not produce IL-4, a key feature of Th2 cells (Sallusto et al, 1998). It has become clear that although these profiles are accurate for the majority of cells, there are cells which do not conform to the functional characterisation afforded to them by this chemokine receptor profile.

Chemokines and Disease

The use of chemokine receptor knockout animals has provided insight into the specific roles of these receptors in normal and disease models (Table 2). The role of chemokines and their receptors in the trafficking and activation leukocytes suggests the possibility that they may play a role in a number of inflammatory

diseases. It is clear that the correct functioning of these receptors is maintained in order for the immune system to respond effectively.

Chemokine Receptor	Phenotype of knockout	References
CCR1	Reduced lung eosinophil infiltration in a murine model of asthma Resistance to EAE development	Blease et al, 2000; Khan et al, 2001; Rottman et al, 2000.
CCR2	Reduced macrophage recruitment to inflammatory stimuli Increased resistance to atherosclerosis Attenuation of bronchial hyper-reactivity in a murine asthma model	Blease et al, 2000; Boring et al, 1998; Gaupp et al, 2003; Peters et al, 2001.
CCR3	Absence of eosinophil trafficking to intestinal mucosa Failure of eosinophil migration in a model of skin allergic inflammation	Humbles et al, 2002; Ma et al, 2002.
CCR4	Reduced airway hyper-responsiveness during chronic pulmonary allergic responses to Aspergillus	Schuh et al, 2002; Reiss et al, 2001.
CCR6	Reduction in airway resistance, eosinophil migration and IL-5 production in the lung	Lukacs et al, 2001.
CXCR2	Protection from septic injury	Ness et al, 2003.
CXCR3	Resistance to the development of acute allograft rejection	Hancock et al, 2000
CXCR4	Embryonic lethal Defective development of major organ and immune systems.	Plotkin et al, 2003; Tachibana et al, 1998; Zou et al, 1998.
CX3CR1	Decreased atherosclerosis	Lesnik et al, 2003

Table 2. Phenotype of chemokine receptor knockout models.

This table summarises some of the effects observed in chemokine receptor knockout mice.

Chemokine receptors have been found to be important in the pathogenesis of some diseases. In rheumatoid arthritis, Th1 cells expressing CCR5 and CXCR3 infiltrate into the rheumatoid synovium (Bonecchi et al, 1998); however it is unclear whether these chemokines are involved in the pathogenesis of the disease and what the trigger for the accumulation of these cells is. There are a number of other diseases in which a large number of lymphocytes expressing specific chemokine receptor profiles accumulate at the site of inflammation. In allergic diseases, such as asthma or contact dermatitis, predominantly Th2 cells expressing CCR3 are thought to be involved in the disease pathogenesis (Sallusto et al, 1997; Thomas et al, 2004). CCL25 has been identified as having a role in the pathogenesis of

Crohn's disease by recruiting CCR9+ T-lymphocytes to the small intestine (Konstantinos *et al*, 2001).

In the past decade the involvement of chemokines in pathogenesis of human immunodeficiency virus (HIV) infection has been widely studied. In conjunction with CD4, CXCR4 and CCR5 have been identified as co-receptors for the T-tropic and M-tropic strains respectively (Littman, 1998; Lusso, 2006). The chemokines that bind these receptors are able to inhibit viral entry into the cell; this is thought to occur because either the chemokine directly blocks the viral binding site, or that ligation of the receptor by the chemokine induces a conformational change in the receptor such that the virus is no longer able to bind the receptor (Arenzana-Seisdedos *et al*, 1996).

CXCR6 and its Ligand CXCL16

CXCR6 (BONZO/ STRL33 (seven-transmembrane domain receptor L33)/ TYMSTR (T-lymphocyte-expressed seven-transmembrane domain receptor)) was concurrently discovered by a number of groups in 1997 (Deng *et al*, 1997; Liao *et al*, 1997; Loetsher *et al*, 1997). It has 30% homology with other chemokine receptors and the gene is localised within the major cluster of other CXC chemokine receptors (Liao *et al*, 1997); and was officially classified as a novel chemokine receptor following identification of its ligand, the chemokine CXCL16 (Matloubin *et al*, 2000; Murphy *et al*, 2002; Wilbanks *et al*, 2001). There has been some conflicting evidence about the expression of CXCR6 on cells types, it was suggested that CXCR6 could possibly be a marker of Th1 cells because CXCR6 expression correlates with expression of IFN-γ from cells (Calabresi *et al*, 2002; Heydtmann & Adams, 2002; Kim *et al*, 2001), however it has also been identified on other populations of activated T-lymphocytes (Matloubin *et al*, 2000; Sharron *et al*, 2000; Wilbanks *et al*, 2001), intestinal epithelia cells (Brand, 2002) and aortic smooth muscle cells (Chandrasekar *et al*, 2004). Unlike a number of other chemokine receptors, CXCR6 has only one known ligand, CXCL16; currently thought to exclusively activate CXCR6 (Matloubin *et al*, 2000; Wilbanks *et al*, 2001). CXCL16, also identified as the scavenger receptor SR-PSOX, involved in the uptake of oxidised low density lipoprotein into macrophages (Minami *et al*, 2001). CXCL16 is expressed on a number of cell types, including dendritic cells, macrophages, B cells, vascular endothelial cells (Matloubin *et al*, 2000; Shaskin *et al*, 2003; Wilbanks *et al* 2001). CXCL16 shares similar sequence homology with a number of chemokines with closest homology, among CXC family members, to

CXCL12 (18.8%) (Wilbanks *et al* 2001). CXCL16 exhibits the classic CXC motif in the amino-terminal consistent with other members of this family; however the predicted chemokine domain is followed by a structure predicted to be a mucin-like stalk similar in structure to CX₃CL1 (Wilbanks *et al*, 2001). This allows them to be expressed on the cell surface and be released from cells in a soluble form; both of which are able to elicit a response from the receptor (Nakayama *et al*, 2003). The soluble form of this chemokine has been suggested to act as a chemoattractant, while the membrane-bound form may act as an adhesion molecule (Nakayama *et al*, 2003).

Initially identified as a simian immunodeficiency disease (SIV) co-receptor for specific T-tropic strains of the virus (Deng *et al*, 1997), further studies revealed that it has a similar role in the human form of the virus, HIV (Cilliers *et al*, 2003; Liao *et al*, 1997; Loetsher *et al*, 1997). While the majority of research into the functions of this receptor has been within this field, is has since been implicated in a number of other disease pathologies. CXCR6+ cells are enriched in the synovial fluid of rheumatoid and psoriatic arthritis patients (Kim *et al*, 2001); there are high levels of CXCR6+ T-lymphocytes present in the liver during end-stage hepatitis C disease (Boisvert *et al*, 2003; Wang *et al*, 2004); CXCR6+ T-lymphocytes reactive to myelin basic protein, a feature of multiple sclerosis, accumulate within the inflamed area of the central nervous system (Calabresi *et al*, 2002; Fukumoto *et al*, 2004). The role of CXCL16 and CXCR6 in the development of atherosclerotic plaques is also under investigation (Wågsäter *et al*, 2004; Wuttge *et al*, 2004).

1.3 Signal Transduction Pathways

Initiation of a cellular response following activation of a receptor requires the co-ordinated activity of many intracellular proteins. The GPCR family contains several hundred members which respond to a diverse range of stimuli (neurotransmitters, hormones, light). They consist of seven hydrophobic helices which span the cell membrane and interact directly with G proteins at the cytosolic surface of the cell membrane (Murphy, 2000). G proteins are heterotrimeric proteins containing α, β and γ subunits which transduce the signal initiated by a ligand binding the receptor (Neer *et al*, 1995). There are a number of isoforms of each subunit enabling many potential combinations which can be selectively expressed in a given cell or tissue type. The α subunit contains a binding site for guanine nucleotides, which in an inactive cell is occupied by guanine diphosphate (GDP).

Ligation of the receptor induces a conformational change which enhances the exchange of GDP to guanine triphosphate (GTP), a process which causes the dissociation of the G protein into an α subunit and a βγ subunit both of which are able to modulate a number of effector molecules (al-Aoukaty *et al*, 1996; Neer *et al*, 1995). The hydrolysis of GTP to GDP promotes the dissociation of the Gα subunit from its effectors and allows reassociation with the Gβγ subunit, effectively terminating the response.

The activation of chemokine receptors induces signalling pathways involved in the control of a variety of biological functions, including chemotaxis, proliferation, apoptosis and adhesion. These pathways are complex, often interlinked, and despite considerable advances in our understanding, they remain unclear.

1.3.1 Phosphatidylinositol Lipids

Many signalling pathways rely on the metabolism of plasma membrane phosphatidylinositol (PI) lipids. PI forms the basic structure for the inositol-containing lipids, which consist of a D-myo-inositol-1-phosphate (Ins1P) linked via its phosphate group to diacylglycerol (DAG) (Figure 5). Although the inositol head of PI has five free hydroxyl groups, only those at positions 3, 4, and 5 can be phosphorylated forming lipids collectively known as the phosphatidylinositols or PI lipids. There are currently 8 documented phosphatidylinositols; PI, the PI monophosphates, PI(3)P, PI(4)P and PI(5)P, the PI bis-phosphates, PI(3,4)P$_2$, PI(3,5)P$_2$ and PI(4,5)P$_2$ and PI trisphosphate, PI(3,4,5)P$_3$.

PI lipids reside within membranes and are substrates for kinases, phosphatases and lipases resident in or recruited to the membranes. The production of PI lipids enables the recruitment of signalling proteins to the plasma membrane through the binding of specific lipid binding domains, such as pleckstrin homology (PH), Phox homology (PX) and FYVE domains, contained within the structures of signalling molecules. While FYVE domains tend to selectively bind PI(3)P, PX and PH domains have individual lipid preferences (Simonsen *et al*, 2001) (Table 3).
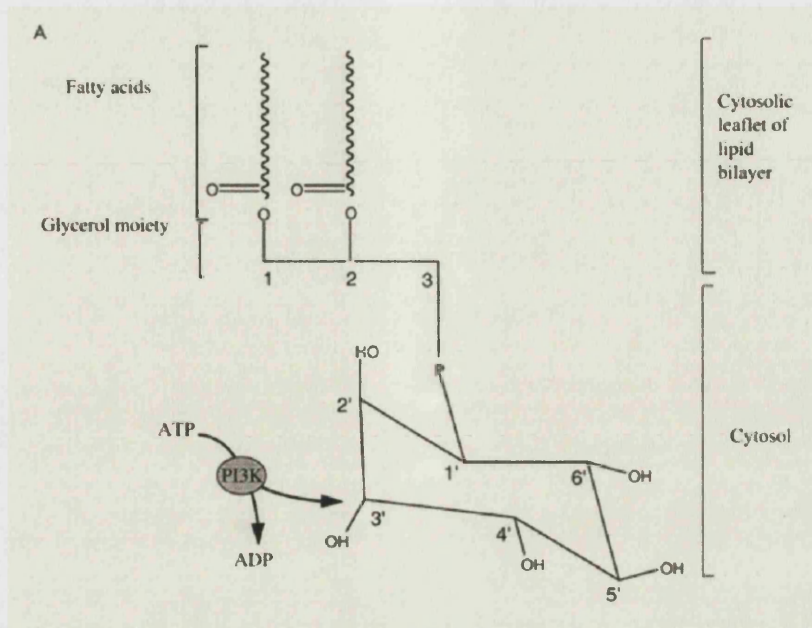
Figure 5. Chemical structure of phosphatidylinositol (PI)

Phosphatidylinositol (PI) forms the basic structure of the inositol-containing lipids located within membranes. The inositol head group of PI has five free hydroxyl groups; positions 3, 4, and 5 can be phosphorylated in different combinations (Sotsios et al, 2000).

Selectivity	Domain	Protein
PI(3)P	PX	Phagocyte NADPH oxidase (phox) complex
	FYVE	Fab1p
	FYVE	YOTB
PI(3,4)P_2	FYVE	Vac1p
	PH	DAPP1
	PH	TAPP-1
	PH	TAPP-2
PI(3,4,5)P_3	PH	BTK
	PH	DAPP1
	PH	Grp1
	PH	PKB

Table 3. Selectivity of lipid binding domain containing proteins.

This table describes the PH, PX and FYVE domain containing proteins that preferentially bind to PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (Maffucci et al, 2001; Lemmon et al, 2003).

1.3.2 Phospholipase C and Calcium

Phospholipase C (PLC) metabolises PI(4,5)P$_2$ into DAG and inositol 1,4,5-trisphosphate (IP$_3$), second messengers which leads to the activation of protein kinase C (PKC) and transient increases of intracellular calcium levels (described below) (Berridge, 2000). The isoform of PLC involved in the response is dependent on the activation mechanism. Of the members of the PLC family (β, γ, δ, ε), only the PLCβ enzymes are regulated by heterotrimeric G proteins (Kelley et al, 2001).

Calcium is a pivotal second messenger implicated in the regulation of a broad range of cellular processes, including gene transcription, proliferation and muscle contraction, through regulation of a number of signalling molecules. Intracellular calcium levels are tightly regulated through channels located at the endoplasmic (ER) and sarcoplasmic reticulum (SR), as well as at the plasma membrane which control the supply of calcium from the stores and extracellular space respectively. IP$_3$ is able to induce the release of intracellular calcium stores through activation of its receptor, IP$_3$R, on the ER (Berridge et al, 1998). The release of calcium regulates the activation of calcium release activated channels (CRACs) allowing entry of extracellular calcium (Lewis, 2001). These channels are critical for correct functioning of lymphocytes; cells deficient of CRAC activity are unable to proliferate or activate transcription factors (Feske et al, 2001; Le Diest et al, 1995). There are also store-independent channels which operate distinct of CRACs activity, however the exact functioning of these has yet to be determined (Owsianik et al, 2006). The subsequent removal of calcium occurs through the combined actions of pumps (Ca^{2+}-ATPase), exchangers (Na$^+$/Ca^{2+} on mitochondria) and buffers (calmodulin) (Berridge et al, 2000). It is currently unclear how cells are able to decode the changes in calcium concentration; however it is thought that the specificity is determined by the amplitude and frequency of the elevation (Dolmetsch et al, 1998).

1.3.3 Diacylglycerol and Protein kinase C

DAG is a lipid mediator generated following the hydrolysis of PI(4,5)P$_2$, it binds to the C1 domain of proteins, traditionally believed to be contained within members of the PKC family, but more recently found in a diverse array of proteins including, diacylglycerol kinases (DGKs), protein kinase D (PKD), chimaerin Rac GAPs (GTPase activating proteins) and RasGRP (Ras guanyl nucleotide releasing factor) (Kazanietz, 2002). DGKs are a family of kinases which may be involved in

the negative regulation of DAG. DGKγ has been shown to phosphorylate DAG resulting in phosphatidic acid, therefore reducing DAG levels (van Blitterswijk & Housa, 2000). The PKD family (PKD/PKCμ, PKD2, PKD3/PKCυ) contains two C2 domains and a PH domain acts downstream of PKC and has been implicated in the activation of the mitogen activated kinase (MAP-kinase) cascade (Brandlin et al, 2002) and nuclear factor-κB (NF-κB) (Storz et al, 2003). Chimaerins, as well as the C1 domain required for activation by DAG, contain a carboxyl-terminal GAP domain which catalyses GTP hydrolysis (inactivation) from Rac1 (Caloca et al, 2003). RasGRPs (RasGRP-1, -2, -3, -4) translocate to the membrane following DAG generation and complexes with, and activates, Ras by promoting loading of GTP (Ebinu et al, 2000).

Although the effects of DAG are not entirely mediated by PKC isoforms, it is safe to say that PKC isoforms are important downstream effectors of DAG. The PKC family is divided into 3 subgroups based on structural similarities and activation requirements. Classical PKC isoforms (cPKC), α, βI, βII, and γ can be activated by calcium, DAG or phorbol esters (natural compounds which bind the C1 domain and mimic DAG signalling); PKC ε, δ, η, and θ are described as novel PKC isoforms (nPKC), these lack a C2 domain conserved in other isoforms and are able to function in a calcium-independent manner. The third PKC subgroup contains the atypical isoforms (aPKC), ζ and λ. This subgroup does not require DAG activity for its activation and is also insensitive to phorbol esters. Some PKC isoforms require phosphorylation by 3-phosphoinsotide-dependent kinase-1 (PDK1) within their catalytic domain to become activated by DAG/calcium binding (Sonnenburg et al, 2001).

Functional responses are isozyme-, cell type- and stimuli-specific allowing selective regulation of signalling cascades. Some of this selectivity is conferred by the activation of distinct PKC-activating protein, such as RACKs (receptors of activated C kinases) and STICKs (substrates that interact with C kinases). PKC is able to directly modulate the MAP-kinase cascade; in general, PKC activates Raf1 which in turn activates other members of the MAP-kinase pathway promoting proliferation, however PKCα activation of the MAP-kinase pathway results in growth arrest of human rhabdomyosarcoma cells (Mauro et al, 2002). The role of PKC isoforms in modulation of transcription factor activity has been widely studied. The main isoform involved in TCR activation of NF-κB and AP1 (activator protein-1) is thought to be PKCθ. PKCθ can phosphorylate and activate IKK2 (IκB (inhibitor of

κB) kinase) upstream of NF-κB (Coudronniere et al, 2000); it was thought that activation of SEK1 (stress-activated protein kinase-1), within the c-Jun NH_2-terminal kinase (JNK) pathway, was responsible for PKC-mediated activation of AP1, however subsequent studies in PKCθ knockout mice revealed only partial inhibition of these factors suggesting involvement of other PKC isoforms (Pfiefhofer et al, 2003). A recent study has shown that PKCα is required for activation of NF-κB by the TCR and has suggested that this isoform is upstream of PKCθ (Trushin et al, 2003). PKC has also been shown to interact with components of the cytoskeleton including actin filaments, MARCKS (Allen et al, 1995), talin and vinculin (Hyatt et al, 1994).

1.3.4 Phosphatidylinositol 3-kinase (PI3K)

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases which phosphorylate the 3-position of the inositol ring of PI lipids PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$. The PI3K family is divided into 4 main classes based on structural similarities and substrate selectivity (figures 6 and 7)(reviewed in Deane & Fruman, 2004). The class I PI3Ks, divided into Class I_A and Class I_B isoforms according to their different activation processes, use PI, PI(4)P and $PI(4,5)P_2$ as substrates. Class I_A PI3Ks consist of a p110 catalytic unit (p110α, β, δ) and an associated regulatory subunit (p85); there are 5 different regulatory units associated with class I_A PI3K derived from different genes (p85β and p55γ) or splice variants (p85a, p55α and p50α). p85 mediates protein-protein interactions via its Src (signal relating kinase) homology (SH2) domain, with phosphotyrosine residues of other proteins. The Class I_B PI3K (p110γ) lacks a p85 binding domain but has a unique regulatory unit, p101. p110γ can be activated by the direct interaction with the dissociated Gβγ subunit following GCPR ligation (Brock et al, 2003).

Currently, less is known about class II and III PI3Ks. In mammals, there are three different isoforms of class II PI3Ks, α, β and γ which do not constitutively interact with a regulatory subunit. These contain a carboxyl-terminal C2 domain; they selectively use PI, and possibly PI(4)P, as substrates in vitro. The single class III PI3K (Vps34p) is evolutionarily conserved among species and uses PI exclusively as its substrate. It has a unique regulatory unit (Vps15p) which in yeast is a serine/threonine kinase. It is thought to be involved in protein and vesicle trafficking (Stevens, 2002).
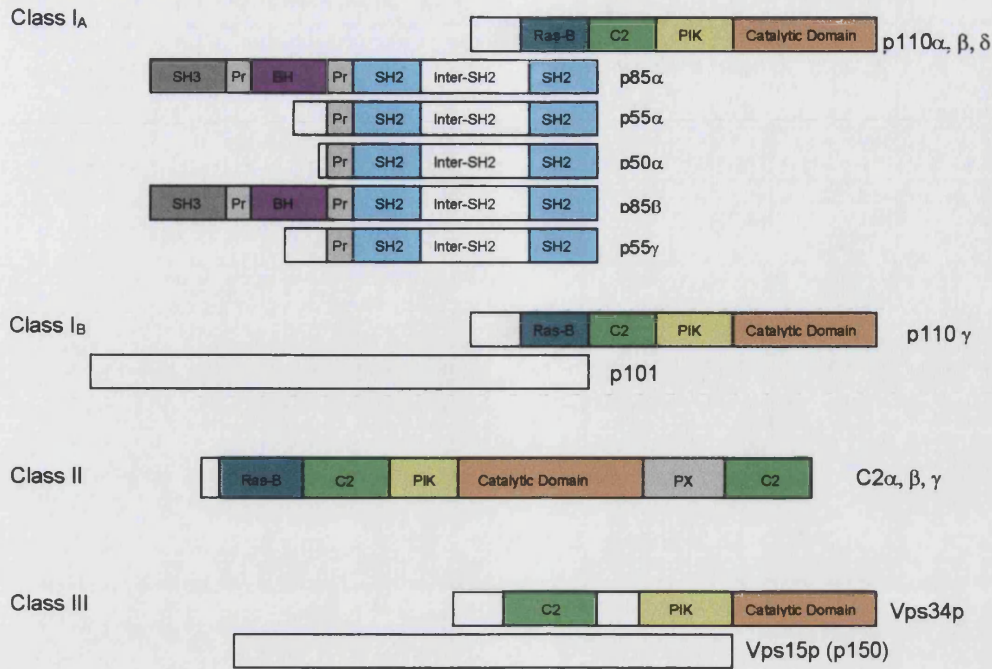
Figure 6. Schematic diagram of mammalian PI3K classes. Each PI3K contains a C2 domain and a catalytic domain connected by the PIK region (a helical section present in lipid, but not protein, kinases). C2 domains are responsible for binding phospholipids in a calcium dependent or independent manner. PX domains can bind PI(3)P and PI(3,4)P and may be involved in recruiting Class II PI3Ks to the membrane. BH, BCR homology domain; Pr, proline rich regions; PX, phox homology domain; Ras-B, Ras binding domain.

Metabolism of PI3K products is strictly controlled; primarily via $PI(3,4,5)P_3$ degradation by the lipid phosphatases SH2-containing inositol polyphosphate 5-phosphatase (SHIP) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Vanhaesebroeck *et al*, 2000; Weber *et al*, 2001; Xiu *et al*, 2002). PTEN hydrolyses the 3-phosphate position converting $PI(3,4,5)P_3$ to $PI(4,5)P_2$, whereas SHIP dephosphorylates the 5-phosphate converting $PI(3,4,5)P_3$ to $PI(3,4)P_2$. $PI(4,5)P_2$ can be further metabolised by PLC into DAG and IP_3. Failure to initiate these regulatory mechanisms results in immune defects, which range from hyper-proliferation (aberrant signalling is seen in many cancers), to debilitating autoimmunity (D'Ambrosio *et al*, 1996).
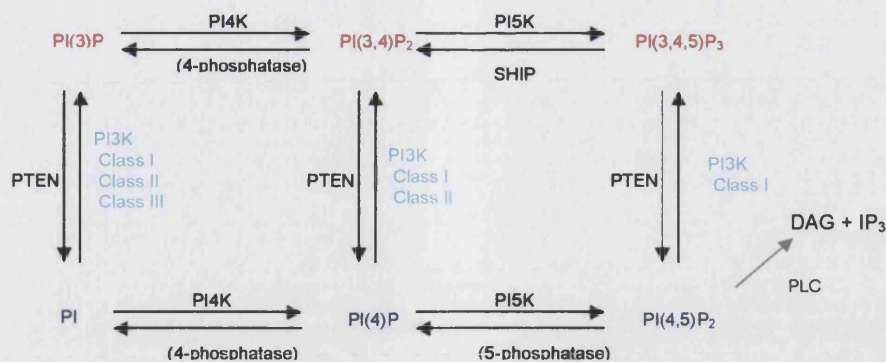
Figure 7. Synthesis and metabolism of phsophoinositides.
PI3K classes have different specificities for phosphatidylinositols. The products of PI3K are involved in multiple signalling pathways, and are therefore tightly regulated by phosphatases (PTEN and SHIP). PI(4,5)P_2 can be cleaved by PLC into two other second messengers, DAG and IP$_3$. DAG, diacylglycerol; IP$_3$, inositol(1,4,5)trisphosphate; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, SH2-containing inositol polyphosphate 5-phosphatase

There is much evidence of chemokine receptors stimulating PI(3,4,5)P_3 accumulation through PI3Kγ. The $G_{\alpha i}$ specific inhibitor, pertussis toxin (PTX) prevents accumulation of PI(3,4,5)P_3 mediated by CXCL12 and CCL2 (Ward *et al*, 1998a; Ward *et al*, 1998b). PI3K$\gamma^{-/-}$ leukocytes derived from genetically engineered mice also fail to produce PI(3,4,5)P_3 in response to CXCL1 (Hirsch *et al*, 2000). PI3Kγ does not appear to be the only isoform activated by chemokine receptors. CXCL12 and CCL5 have been shown to activate the p85/p110 heterodimer in T cells (Sotsios *et al* 1999; Turner *et al*, 1995) and CCL2/MCP-1 in THP-1 cells (Turner *et al*, 1998). This study also reports the activation of PI3K-C2α in CCL2 stimulated THP-1 cells (Turner *et al*, 1998).

1.3.5 Downstream Effectors of PI3K

Numerous studies using chemical PI3K inhibitors, overexpression of mutated forms of PI3K and gene knockout experiments have revealed that PI3K is involved in a wide range of cellular processes mediated by multiple effectors (figure 8).
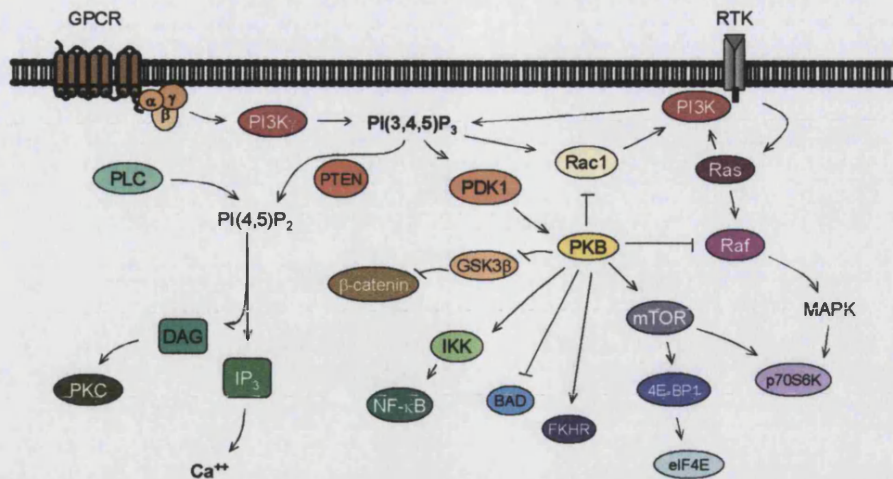
Figure 8. Simplified overview of some PI3K signalling pathways.

PI3K can be activated following ligation of GPCRs and receptor tyrosine kinases resulting in the generation of PI(3,4,5)P_3. This localises PH domain containing proteins to the plasma membrane. PTEN hydroyses PI(3,4,5)P_3 to PI(4,5)P_2 which in turn is hydrolysed by PLC to IP$_3$ and DAG resulting and increase of intracellular calcium and the activation of PKC. PDK-1 phosphorylates PKB resulting in its activation. PKB phosphorylates a number of substrates resulting in either activation or inhibition (described in detail below). Rac1 associates with, and can be activated downstream of, class IA and IB isoforms of PI3K however in a feedback mechanism, Rac1 can increase the activity of the p85 regulatory subunit of class IA PI3Ks. Following ligation of RTKs, Ras can activate PI3K and Raf, which activates the MAP-kinase pathway; also involved in the activation of p70S6K.

DAG, diacylglycerol; eIF4E, eukaryotic initiation factor 4E; FKHR, forkhead transcription factor; GSK3β, glycogen synthase kinase 3β; IKK, inhibitor of κB kinase; IP$_3$, inositol(1,4,5)trisphosphate; MAPK, mitogen activated protein kinase; mTOR, mammalian target of Rapamycin; p70S6K, S6 kinase of 70kDa; PDK1, phosphatidylinositol-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

PKB

PKB is a key mediator of PI3K functions. PKB is a member of the AGC (cAMP-dependent protein kinase A/ protein kinase G/ protein kinase C) superfamily of protein kinases which share sequence homology within their catalytic domain. All three PKB isoforms, PKBα (AKT1), PKBβ (AKT2) and PKBγ (AKT3), have a similar structure consisting of a carboxyl-terminal regulatory domain, a catalytic kinase domain and an amino-terminal PH domain. The PH domain of PKB binds the PI3K

products PI(3,4,5)P$_3$ and PI(3,4)P$_2$ with equal affinity (Frech *et al*, 1997); this allows localisation of the protein to the membrane and induces conformational changes thought to expose the two main sites of phosphorylation, Thr308 and Ser473 (Cantrell, 2002; Scheid & Woodgett, 2003). In recent years there has been much discussion about how PKB is activated. Currently, it is thought that PDK1 phosphorylates Thr308, located within the activation loop of the kinase domain, instigates partial activation of the enzyme (Anderson *et al*, 1997; Stephens *et al*, 1998). PDK1 is another AGC kinase containing a PH domain that binds the 3-phosphoinositol lipids. In order to become fully activated, PKB needs to be subsequently phosphorylated on ser473 within the regulatory domain however, the pathway leading to this remains controversial. Ser473 phosphorylation always parallels the activation of PI3K and full activation of PKB however the mechanism by which this occurs is not completely understood. Initially it was assumed that PDK1 was responsible for phosphorylation of both residues because phosphorylation of ser 473 is dependent on both PI3K activation and phosphorylation of Thr308 (Andjelkovic *et al*, 1999). However, phosphorylation of ser473, but not Thr308, occurs in PDK1 knockout cells (Williams *et al*, 2000). It has been reported that under certain conditions, PKB is able to autophosphorylate ser473 (Toker *et al*, 2000). Integrin-linked kinase (ILK) has been shown to be involved in the phosphorylation of ser473 (Persad *et al*, 2000); alternatively, ser473 may be phosphorylated by an as yet unidentified kinase. Transient regulation of PKB activation by proteins which interact with it has been reported. Both actin and keratin are able to sequester PKB within the cytoskeleton (Cenni *et al*, 2003; Paramio *et al*, 2001).

PKB is involved in many cellular processes via a number of effector molecules (figure 8). One of the main functions of PKB is the control of cell survival by regulation of pro- and anti-apoptotic mediators and transcription factors. Bad is a pro-apoptotic member of the Bcl-2 family of proteins, which binds Bcl-X or Bcl-2 inhibiting their anti-apoptotic activity. Phosphorylation of Bad by PKB releases it from the complex with Bcl-2/ Bcl-X inactivating its pro-apoptotic ability (Datta *et al*, 1997). The Forkhead (FoxO/ FH) family of transcription factors can all be directly phosphorylated by PKB leading to activation of their target genes, which include both pro-and anti-apoptotic proteins (Burgering *et al*, 2003). Another important family of transcription factors is the NF-κB family; dysregulation of these proteins has been found in a number of cancers and autoimmune diseases (Li *et al*, 2000).

Most NF-κB proteins are activated following phosphorylation of IκB, an inhibitor of NF-κB, by an IKK complex, leading to the degradation of IκB, and allowing NF-κB to translocate to the nucleus and activate its target genes (this is discussed in detail in 1.3.8). PKB has been reported to be involved in the activation of the IKK complex (Kane et al, 1999).

PKB has implicated in the regulation of protein synthesis via pathways which include mammalian target of Rapamycin (mTOR/ FRAP/ RAFT) (Scott et al, 1998). mTOR is a serine/ threonine kinase involved in regulating the availability of cellular levels of adenosine triphosphate (ATP) and nutrients required for protein synthesis. PKB can directly phosphorylate mTOR at ser2448 resulting in the inactivation of 4E-BP1, releasing eIF4E (eukaryotic initiation factor 4E) which complexes with other proteins and activates translation; mTOR also phosphorylates p70S6K (S6K1). p70S6K activation is regulated by multi-site phosphorylation; including the phosphorylation at Ser411, Thr421 and Ser424 which is thought to activate p70S6K by relief of an auto-inhibitory mechanism (Pullen & Thomas, 1997). p70S6K phosphorylates the 40S ribosomal subunit protein, S6-ribosomal protein at multiple sites, which include Ser235, Ser236, Ser240 and Ser244 (Ferrari, S. et al, 1991). This phosphorylation is thought to initiate the translational activity induced by S6-ribosomal protein.

PKB is able to regulate cell metabolism through phosphorylation and inhibition of GSK3β, which results in increased storage of glucose as glycogen (Cross et al, 1995). GSK3β is also connected to other signalling pathways; it has been reported to phosphorylate the adaptor protein and transcription factor, β-catenin leading to its subsequent degradation and inhibition of transcription (Yost et al, 1996).

Rho and Rac family of Small GTPases

Of particular importance in PI3K signalling are the small G proteins of the Rac and Rho families. PI3K can activate the small GTPases, Rho, Cdc42 and Rac to initiate several cascades which result in a number of processes including the reorganisation of the actin cytoskeleton and therefore cell polarisation and chemotaxis (Ma et al, 1998; Narumiya et al, 1997). In an active form, these proteins are GTP bound and they alternate between this and an inactive GDP-bound form. This process is highly regulated by guanine nucleotide exchange factors (GNEFs) and GAPs which catalyse activation or inactivation of the protein respectively.

There are numerous studies, using pharmacological inhibitors and knockout models, which indicate these proteins are activated downstream of PI3K in mediating changes in actin organisation (Han et al, 1998; Reif et al, 1996)

Interactions between PI3K and Rac1 are complicated; following TCR ligation p85 has been shown to associate with Rac1 as well as the intracellular signalling domains of the TCR (Kane et al, 2003). Rac1 and Rho have both been shown to be activated downstream of class 1A and 1B PI3K via the GEF Vav (Han et al, 1998; Wennstrom et al, 1994). There is evidence that both GTP-Rac and GTP-Cdc42 can increase the activity of p85 (Weiner et al, 2002), but the exact mechanism for this is currently unknown. However, since $PI(3,4,5)P_3$ can also induce dissociation of GDP from Rac1 and preferentially binds and stabilises the nucleotide free form of the G protein, there is a possible negative regulatory mechanism (Missy, 1998). There is also evidence that Rac1 can act upstream of Class I PI3K (Genot et al, 2000; Keely, 1997). The role of these proteins in chemotaxis is discussed in detail later.

1.3.6 Mitogen Activated Protein Kinase

The MAP-kinase cascade is another of the major signalling pathways involved in signal transduction that is conserved within many organisms. MAP-kinase pathways are a family of serine/threonine protein kinases involved in a number of physiological processes such as cell survival, growth, differentiation, development and death. While the individual pathways differ in their upstream activation sequences and downstream substrate specificities, they consist of a series of kinases in which a MAPKK-kinase phosphorylates and activates a MAPK-kinase, which in turn activates MAP-kinase (figure 9). There are several MAP-kinase which include the extracellular signal-regulated kinase (ERK1/2), JNK and p38 (p38-α, -β, -γ, -δ).
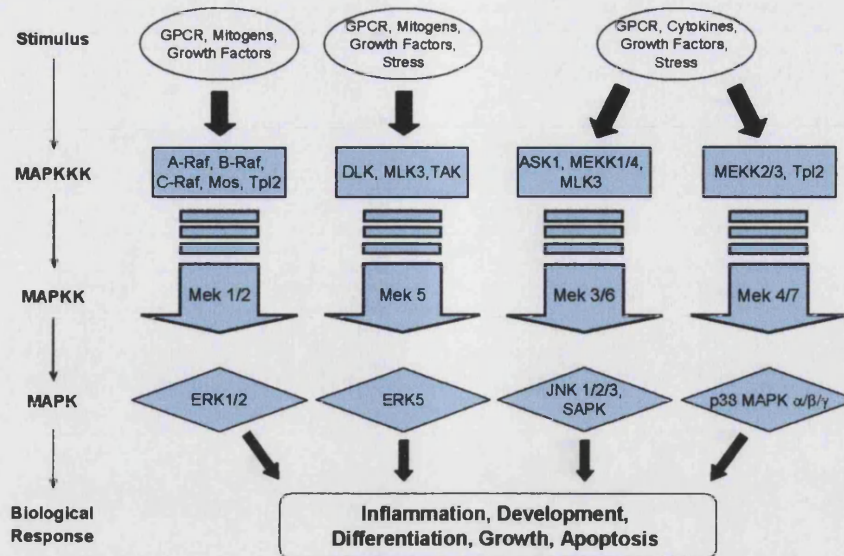
Figure 9. Mammalian MAP-kinase pathway.

MAP-kinase pathways are organized in a three-tier kinase cascade consisting of a MAPKK-kinase that phosphorylates and activates a MAPK-kinase, which in turn activates MAP-kinase, and ultimately leads to activation of transcription factors.

Activation of GPCRs initiates a complex series of signalling events leading to the activation of MAP-kinase cascades (figure 10). $G_{\alpha i}$-linked receptors can activate MAP-kinases through the $\beta\gamma$ subunit in a PKC independent, but Ras dependent manner (Crespo *et al*, 1994). Ras is a membrane bound guanine nucleotide-binding protein (GTPase) regulated by GAPs (p120Ras) and adaptor proteins (SOCS3) (Cacalano *et al*, 2001). Tyrosine phosphorylation has been proposed to play a key role in mediating MAP-kinase signalling. The tyrosine kinase Src can be activated through a direct interaction with either $G_{\alpha i}$- or $G_{\alpha q}$-coupled receptors (Ma *et al*, 2000); $\beta\gamma$-induced phosphorylation of Shc (Src homology and collagen) is mediated by Src (Luttrell *et al*, 1996). Tyrosine phosphorylation of Shc leads to the subsequent formation of Shc, Grb2 (growth factor receptor bound-2), Sos (son of sevenless) complexes which can promotes GTP loading of Ras (van Biesen *et al*, 1995). Non-receptor tyrosine kinases, which include C-terminal Src kinase (Csk), Lyn, Bruton's tyrosine kinase (Btk) and proline-rich tyrosine kinase 2 (PYK2), have been proposed to activate the Ras following ligation of $G_{\alpha i}$- and $G_{\alpha q}$-coupled receptors in a variety of cell types. PI3Kγ, activated by $\beta\gamma$ subunits can activate Src and therefore the Shc-Grb2-Sos mediated activation of Ras; another

PI3K isoform, PI3Kβ, can increase Ras activation of Raf through Cdc42- and PAK (Sun et al, 2000). $G_{\alpha s}$ and $G_{\alpha i}$ which can activate, or inhibit, adenylyl cyclase can mediate MAP-kinase signalling through production of cyclic adenosine monophosphate and activation of PKA, which can activate Rap. Rap is able to activate MAP-kinase through Raf-B. $G_{\alpha q}$ can also stimulate MAP-kinase via a Ras-independent, PKC-dependent pathway through direct phosphorylation of c-Raf; however this phosphorylation may not be enough to induce full activation (Macdonald et al, 1993). The exact mechanism of Ras activation of Raf is currently unclear, however it is thought to be necessary for localisation plasma membrane (Avruch et al, 1994). Activated Raf, a serine/threonine kinase, can phosphorylate and activate Mek (MAP-kinase and ERK kinase). Following LPS activation of TLR4, tumour progression locus-2 (TPL-2), a MAPKKK, present in a cytosolic complex with NF-κB1 p105, is released and can phosphorylate and activate Mek-1 and -2. Mek, a dual specificity kinase, phosphorylates ERK1/2 on both tyrosine and threonine residues within the activation loop; phosphorylation of these residues results in activation of ERK1/2. ERK1/2 translocates to the nucleus where it can mediate activation of expression of transcription factors. ERK1/2 has been shown to phosphorylate a number of residues on p90RSK (p90 ribosomal S6 kinase), thought to be important in the activation of this kinase; however full activation requires phosphorylation by PDK-1. Known substrates of p90RSK include S6 ribosomal protein, GSK3β, and it has been linked to NF-κB activation. ERK1/2 has also been shown to phosphorylate autoinhibitory sites on p70S6K inducing a conformational change crucial for allowing phosphorylation of the catalytic domain inducing activation.

JNK and p38 are MAP-kinases primarily activated in response to environmental stress and inflammatory cytokines. $G_{\alpha 12}$ and $G_{\alpha 13}$ and βγ subunits have been shown to activate JNK in a Rac1 and Cdc42 dependent manner (Gudermann et al, 2000); while $G_{\alpha q}$ as well as βγ have been linked to activation of p38 (Yamauchi et al, 1997).
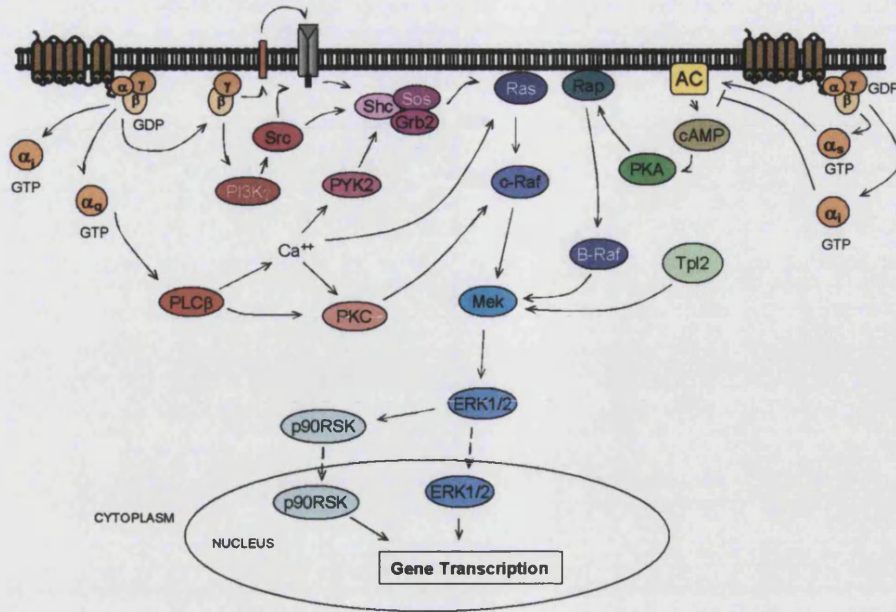
Figure 10. Activation of MAP-kinase pathways by GPCRs.

βγ-subunits can activate Ras via receptor and non-receptor tyrosine kinases, which results in the recruitment of the GEF Sos to the membrane and the exchange of GDP for GTP bound to Ras. $G\alpha_q$ activates protein kinase C (PKC) which can directly stimulate Raf1 or activate Ras via RasGRF and tyrosine kinases acting on Sos. $G\alpha_i$, $G\alpha_o$ and $G\alpha_s$ activate MAP-kinase through pathways which regulate Rap and B-Raf. Mek can also be activated by Tpl2. Activated MAP-kinase translocates to the nucleus and phosphorylates nuclear proteins, including transcription factors, thereby regulating gene expression.

AC, adenylyl cyclase; cAMP, cyclic adenosine triphosphoate; ERK1/2, extracellular-related kinase; MEK, MAPK kinase; PI3K phosphatidylinositol 3-kinase; p90RSK, p90 ribosomal S6 kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PYK2, proline-rich tyrosine kinase 2; RTK, receptor tyrosine kinase; Sos, son of sevenless; Tpl2, tumour progression locus-2.

1.3.7 T Cell Polarisation and Directed Cell Migration

Chemokines and their receptors are important regulators of the trafficking and activation leukocytes in both a normal environment and in response to an inflammatory stimulus. This is a complex process involving the co-ordinated activation of a number of molecules. Inflammatory stimuli initiate the trafficking of leukocytes from the microvasculature to the site of inflammation by activating the expression of selectins, integrin ligands and chemokines on the endothelium. Selectins, involved in the initial tethering of the leukocyte to the endothelium,

provides the leukocyte to 'roll' along the endothelial surface. Firm adhesion is under the control of molecules presented on the surface of the endothelium, such as chemokines, lipid and other pro-inflammatory mediators. These activate adhesion molecules, an important example being the integrin ligands (ICAM-1, VCAM-1), which lead to firm adhesion. The leukocytes are then able to migrate across the endothelium into the tissue, known as diapedesis. This is a complex process in which the leukocyte elongates and extends a pseudopod through the endothelium; dependent on the disassembly and subsequent reassembly of its cytoskeleton. Diapedesis also requires an increase in intracellular free calcium within the endothelial cells between which the cells are passing (Huang *et al*, 1993; Su *et al*, 2000). This rise in intracellular calcium is able to induce retraction of the endothelial cells through activation of myosin light chain kinases (MLCK). (Hixenbaugh *et al*, 1997; Saito *et al*, 1998). Following extravasation, leukocytes are susceptible to chemokine gradients in tissues which allow directed migration directs to a specific site, a process known as chemotaxis.

Chemotaxis is the result of the convergence of multiple signal transduction pathways which cells detect the direction and concentration of, and move toward, an extracellular chemoattractant gradient, some of which have been summarised in figure 11. When cells detect a chemoattractant gradient, they dramatically change their shape, polarizing in the direction of the gradient with the formation of a leading edge and a tail (uropod) (Lauffenburger *et al*, 1996; Sanchez-Madrid *et al*, 1999). At the leading edge of cells, there is an accumulation of chemokine receptors, F-actin, actin-binding proteins and adhesion receptors. Whereas the uropod contains molecules such as myosin II, golgi apparatus and adhesion molecules, such as intercellular adhesion molecules (ICAM)-1,2 and 3, CD43 and CD44. Ezrin, radixin and moesin (ERM family) are linker proteins between the plasma membrane and actin; also found to be localised in the uropod with the adhesion molecules following polarisation of the cell (Serrador *et al*, 2002). This localisation of cell components is partially due to the reorganisation of the actin skeleton which pushes the leading edge forwards and consequently allows actin filaments to move backwards.

The actin cytoskeleton is a flexible system which can be manipulated in order to initiate cell shape change. This involves actin polymerisation on one side of the cell, and depolymerisation on the other. Actin filaments are built up from adenosine 5'-triphosphate (ATP)- actin monomers which bind profilin, an ATP-exchange factor for actin, allowing them to associate with the WASP (wiskott-aldrich syndrome protein) /Arp2/3 (actin-related proteins) complex and form actin

filaments (F actin). Depolymerisation of actin filaments is also important in reorganisation of the actin skeleton, and is mediated mainly by cofilin/actin depolymerising factor proteins. These proteins are activated by dephosphorylation and bind to ADP-actin within the actin filaments, causing them to depolymerise. The cofilin associated-ADP actin is then recycled to ATP-actin by profilin. In this constant cycle, it is therefore possible for cells to constantly change shape.
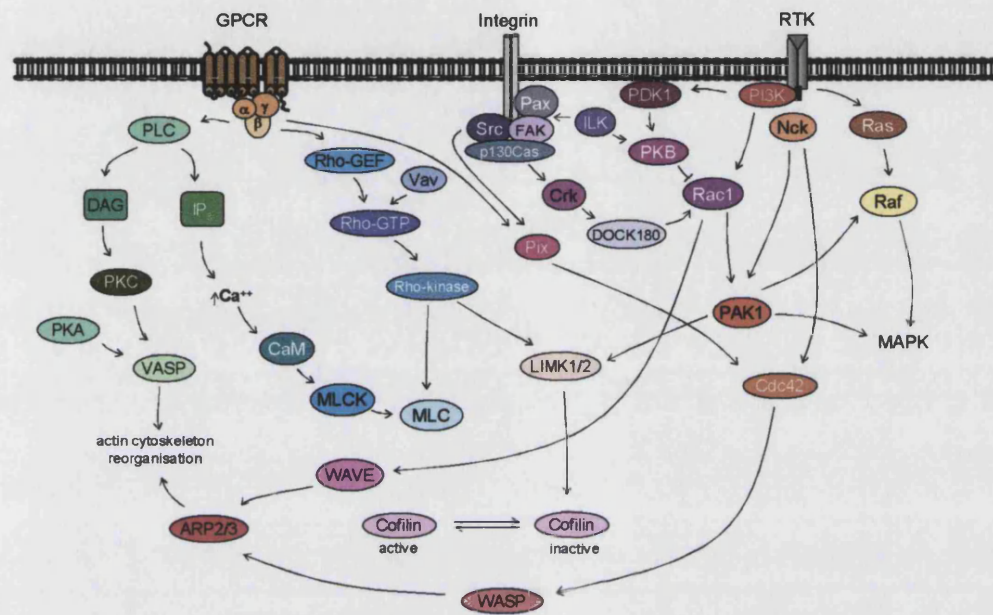
Figure 11. Overview of some signalling pathways involved in the chemotactic response.

Directed cell migration is the culmination of activation of a number of signalling molecules activated downstream of surface receptors.

CaM, calmodulin; DAG, diacylglycerol; FAK, focal adhesion kinse; IP_3, inositol 1,4,5-trisphosphate; LAT, linker for activated T cells; MAPK, mitogen activated kinase; MLC, myosin light chain; MLCK, myosin light chain kinase; PAK, p21 activating kinase; Pax, paxillin; PDK1, phosphatidylinositol kinase-1; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; Src, signal relating kinase; WASP, wiskott-aldrich syndrome protein.

It has become clear over the past few years that PI3K is one of the major players in chemokine mediated chemotaxis. Studies involving PI3K-knockout mutants indicate PI3K-γ is important in neutrophil chemotaxis (Li *et al*, 2000) which is supported by experiments using the PI3K inhibitors LY294002 and wortmannin (Curnock *et al*, 2003; Turner *et al*, 1995; Sotslos *et al*, 1999). Further studies indicate that class 1A PI3Kβ and PI3Kδ are also involved in directional sensing. Following exposure to a chemoattractant, PI3K is localised at the leading edge of cells causing an increase of PI(4,5,6)P$_3$; this localises proteins containing PH-domains to this area (Merlot & Firtel, 2003). Specific PI3K effectors have been identified as being important in aspects of cell migration. PKB is localised at the leading edge of cells (Meili *et al*, 1999) and cells lacking PKB display reduced chemotaxis. While PKB is found at the leading edge of chemotaxing cells, by an unknown mechanism, it can activate PAK1 which is localised at the rear of the cell (Chung *et al*, 2001). The lipid phosphatase, PTEN is also localised to the side and rear of a polarised cell (Lacalle *et al*, 2004) further outlining the requirement for the PI lipids in this process.

The Rho-GTPases Rho, Rac and Cdc42 have been shown to be critical for directed cell migration. Cdc42 is important in determining correct cell polarity with respect to the external signal (Etienne-Manneville, 2004), while Rac is important in formation of the extracellular laminin and orientation of the cell (Sun *et al*, 2004). Both Rac and Cdc42 are thought to be involved in the formation of lamellapodia and the contact these protrusions have with neighbouring cells. Inhibition of Rho disrupts formation of the junctions which are involved in this cell-cell contact, possibly by preventing assembly of the actin filaments required to stabilise them (Takaishi *et al*, 1997). Some of the known Rac-GEFs are PI3K dependent, for example Vav and Sos, and it is thought that binding of PI(4,5,6)P$_3$ to the PH domain of these molecules causes release of an intramolecular inhibition allowing activation (Han *et al*, 1998). DOCK180, while not a conventional Rac-GEF, is known to be an upstream regulator of Rac (Cote & Vuori, 2002); nucleotide-free Rac can bind to DOCK180 causing increased GTP-loading in the presence of GTP; however there is a requirement of an additional enzyme to remove GDP before this can occur (Kobayashi *et al*, 2001). DOCK180 is a member of a class of Rho-GEFs, called CDMs (Ced15/Dock/Myoblasticity), which also includes DOCK2 and ZIZIMIN1. They contain domains which indicate that they may be directly regulated by PI(4,5,6)P$_3$; indicating that PI(4,5,6)P$_3$ is involved in the regulation of this family of

GEFs at the leading edge of chemotaxing cells. p70S6k has also been implicated in the activation of these GTPases; it was recently shown to associate with Rac1 and Cdc42 (Berven, *et al*, 2005).

However, activation of PI3K is not necessarily required for chemotaxis, in fact, recent evidence suggests that in vivo cell migration to the chemokines CXCL12 or CCL19 is unaffected by PI3K inhibitors (Cinamon *et al*, 2001). While ultimately important in most chemotactic responses, it is possible to bypass PI3K to activate the GTPases, in fact the $G\beta\gamma$ subunit could directly activate Rho-GEFs leading to activation of Cdc42, Rac and Rho (Rickert *et al*, 2000). DOCK2 has been found to be important in T lymphocyte migration and more specifically in activation of Rac during chemokine mediated chemotaxis, possibly in a mechanism independent of PI3K (Fukui *et al*, 2001).

Myosin II is localised in the rear of the cell and is thought to be one of the contractile forces in cell movement. Myosin II is a double-headed molecule which can self assemble into filaments which can produce ATP-dependent movement by pulling actin filaments against each other. In non-muscle cells, myosin II has an important role in controlling actomyosin contractility; it can promote tension in the interactions between cell surface integrins and the actin cytoskeleton or their extracellular ligands causing them to break (Arthur, 2002). Activation of myosin II by phosphorylation is regulated by a balance of between MLC phosphatase and MLC kinase in a calcium dependent manner. There are other known molecules which may be involved in activation of myosin, such as p160ROCK. ROCKs (Rho kinases) can phosphorylate a number of substrates, such as myosin light chain (MLC) phosphatase therefore negatively regulating myosin contractility, and the ERM proteins. Other molecules such as LIM kinases (LIMK1, LIMK2), serine/threonine kinases, are also involved in regulating cytoskeletal interactions via phosphorylation of cofilin which inhibits its ability to break up actin filaments. LIMK can not only be activated by ROCKs, but also by Cdc42 and PAK (Yang *et al*, 1998).

Tyrosine phosphorylation of FAK (focal adhesion kinase), a non-receptor tyrosine kinase, following integrin activation creates a binding site for Src family kinases (Schaller *et al*, 1994). FAK can also recruit DOCK180 and the adaptor protein Crk leading to the activation of Rac (Hsia *et al*, 2003). Both FAK and Src can phosphorylate p130Cas, a linker protein involved in mediating a number signals from FAK (Polte *et al*, 1995). FAK, Src and p130Cas are able to complex with

paxillin, an adaptor protein containing a several binding domains enabling interaction of a number of molecules. The F-actin binding protein, vinculin, ILK (integrin linked kinase) and PAK can all directly bind the LIM domains in paxillin (Brown *et al*, 1996; Hasimoto *et al*, 2001; Nikolopolous & Turner, 2001); other proteins are able to link paxillin to PIX, a RAC/Cdc42 GEF (West *et al*, 2001). Paxillin has also been found to localise with LIMK further underlining its importance in regulation in motility (Foletta *et al*, 2004). ILK is also important in the formation of protein complexes which act upstream to affect actin dynamics and has also been implicated in the regulation of PKB and GSK3β (Delcommenne *et al*, 1998).

Further evidence of the complexity of this process is the involvement of protein kinase A (PKA) which is able to interact with a number of molecules which regulate actin and cell migration. PKA phosphorylation of VASP (vasodilator stimulated protein) disrupts its interaction with Abl therefore indirectly regulating WAVE-Abl complex formation (Stuart *et al*, 2006). Abl, an oncoprotein is able to bind actin via binding sites which preferentially bind either G- or F- actin (Van Etten *et al*, 1994). VASP is also able to bind directly to profilin and promote actin polymerisation (Reinhard *et al*, 1995; Walders-Harbeck *et al*, 2002). Through its regulation of PLC and IP$_3$ receptors, PKA can also regulate the calcium dependent activation of MLCK activity (Garcia *et al*, 1997). PKC isoforms are also found localised within the actin structure following activation. PKCβII is activated following F-actin binding (Blobe *et al*, 1996); while other isoforms, α, ε and ζ appear to have a requirement for F-actin binding to elicit certain effects (Gomez *et al*, 1995; Prekeris *et al*, 1995; Schmalz *et al*, 1996).

Activation of the T cell receptor causes activation and recruitment of ZAP-70 (ζ-chain associated protein 70kDa), to the plasma membrane. This induces phosphorylation of LAT (linker for activated T cells) and SLP-76 (SH2 domain-containing Leukocyte Protein of 76 kDa), and links the actin skeleton to TCR activation through Vav1 and the WASP/Arp2/3 complex (Badour *et al*, 2004). Vav is a Rho family GEF and can catalyse activation of Rho, Rac1 and Cdc42; it has recently been shown to be important for CXCR4-mediated chemotaxis in lymphocytes (Vicente-Mazanares *et al*, 2005). It is the direct interaction of Vav with Cdc42 that causes WASP to activate the Arp2/3 complex (Zeng *et al*, 2003). Ras is a GTPase, upstream of the Rho-GTPases, which has been shown to be involved in regulation of cofilin activity through both PI3K- and Mek-dependent pathways (Nebl

et al, 2004). This study suggests that Ras-dependent dephosphorylation of cofilin is partially accountable for the actin destabilising abilities of Ras.

1.3.8 Nuclear Factor-κB (NF-κB)

There are a number of inducible transcription factors responsible for a cell's adaptive responses to environmental changes. NF-κB is an evolutionarily conserved protein important in the control of the immune and inflammatory responses. Following receptor ligation by stimuli including lipopolysaccharide (LPS), interleukin 1 (IL-1), tumour necrosis factor α (TNFα) and γ-irradiation, NF-κB translocates to the nucleus where it binds to the activation site on the target gene. NF-κB is involved in the regulation of the genes of pro-inflammatory cytokines (eg. TNFα, IL-1 and IL-6), chemokines (MIP1α and RANTES) and adhesion molecules required for recruitment of cells to sites of inflammation. However, NF-κB can also regulate a number of genes not involved in the immune response and can therefore affect normal cellular functions. The NF-κB family consists of several proteins including p65 (RelA), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (figure 12); usually present in resting cells as either a homo- or hetero-dimers. Both NF-κB1 and NF-κB2 are synthesised as precursors of 105 and 100 kDa respectively and are proteolysed by the proteasome forming the active NF-κB1 p50 and NF-κB2 p52 subunits. This processing requires the glycine-rich region in the central portion of the protein (Orian *et al*, 1999). The NF-κB family contain a conserved Rel homology domain where the dimerisation, nuclear localisation and DNA binding domains are located. The Rel proteins (p65, RelB and c-Rel) also contain a c-teminal transactivation domain which can strongly activate transcription when bound to the NF-κB binding site on the target genes. RelB can only activate transcription when complexed with NF-κB1 p50 or NF-κB2 p52; RelB/p65 hetrodimers cannot bind DNA and are therefore inhibitory (Marienfeld *et al*, 2003). Since NF-κB1 p50 or NF-κB2 p52 lack the transactivation domain, they can only activate transcription when complexed with one of the Rel proteins. In resting cells, NF-κB is normally sequestered in the cytoplasm as an inactive dimer by a member of the inhibitor of NF-κB (IκB) family (IκBα, IκBβ, IκBε) (figure 12).
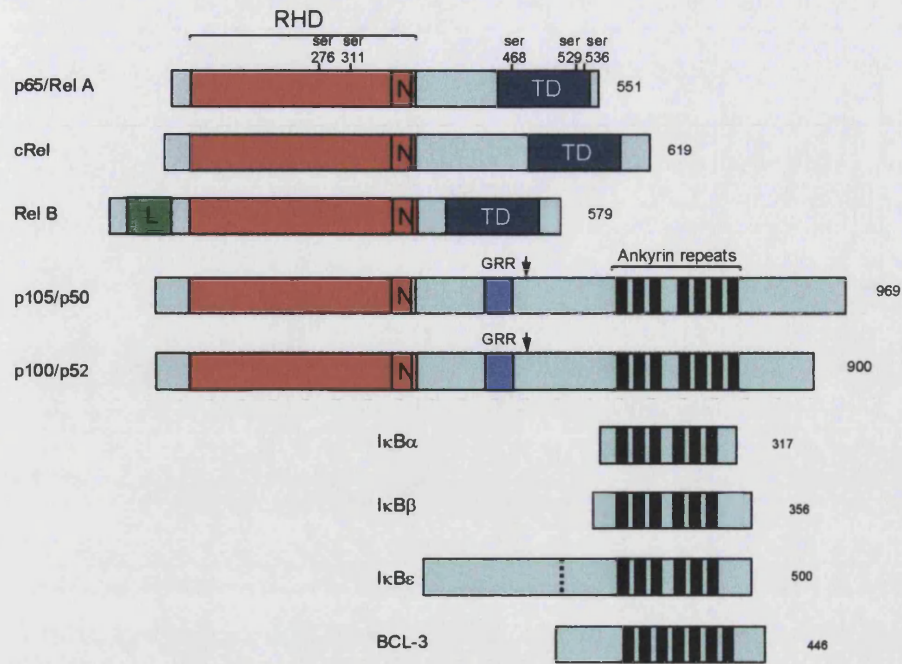
Figure 12. Human NF-κB and IκB family members. The five members of the mammalian NF-κB family, p65 (Rel A), c-Rel, Rel B, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) contain a structurally conserved amino-terminal Rel homology domain (RHD). The RHD is comprised of the dimerisation, nuclear localisation (N) and DNA-binding domains. p65, c-Rel and Rel B contain a transactivation domain (TD) and RelB also contains a leucine-zipper (LZ) motif. p65 contains several residues which can be phosphorylated and may be important for activation. Proteolytic processing of p105 and p100 (indicated by arrows), results in the p50 and p52 NF-κB family members. The glycine rich region (GRR) is required for processing p105 and p100. The inhibitor of NF-κB (IκB) family consists of IκBα, IκBβ, IkBε (which has 2 transcripts shown by the dotted line) and BCL-3. They can be identified by the presence of several ankyrin repeats. p105 and p100 also contain carboxy-terminus ankyrin repeats. The number of residues in each protein is indicated on the right.

IκBs are characterised by the presence of multiple ankyrin repeats which interact with the nuclear localisation sequence in the Rel homology domain of the NF-κB protein to inhibit nuclear translocation. The c- terminal section of NF-κB1 and NF-κB2 also contain multiple ankyrin repeats and can therefore function as an IκB molecule. Messenger RNA encoding only the c-terminal of NF-κB1 has been reported in murine cells and has been termed IκBγ (Moorthy & Ghosh, 2003). Phosphorylation of the N-terminal of IκB induces degradation of the molecule by the proteasome.

There are three recognised signalling cascades leading to NF-κB activation: the canonical pathway, the alternative (non-canonical) pathway and the p105 pathway (figures 13 & 14). The most characterised is the canonical NF-κB pathway in which the classical IKK complex, composed of the two catalytic proteins IKK1 (IKKα) and IKK2 (IKKβ) and a regulatory subunit, NEMO (NF-κB essential modulator; IKKγ), is recruited to the plasma membrane. It is subsequently activated by phosphorylation by an upstream kinase such as NF-κB Inducible kinase (NIK), MEKK1 or PKB (Factor et al, 2001; Luftig et al, 2004; Zhou et al, 2003). The activated classical IKK complex then phosphorylates IκBα on ser 32 and ser36 resulting in its polyubiquitination and degradation via the proteasome pathway. The released NF-κB hetrodimer, typically p65/p50 or c-Rel/p50, translocates to the nucleus where it binds to specific consensus sequences on the target genes to promote transcription. The IKK complex can also phosphorylate IκBβ and IκBε resulting in ubiquitination and degradation (Heilker et al, 1999). Post-translation modifications of p65 have been shown to affect its transcriptional activity; acetylation of p65 can facilitate retention of the NF-κB complex in the nucleus (Ashburner et al, 2001). Phosphorylation of p65 is important for transactivation as translocation alone is not enough to affect transcription. It has however been suggested that phosphorylation of p65 is required for optimum transcriptional activity. There are several sites for phosphorylation (figure 11) and their individual effects on transcription are currently not fully understood. Phosphorylation of ser 276 in the Rel homology domain by PKA (Zhong et al, 1997) or mitogen-and stress-activated protein 1 (MSK1) (Vermeulen et al, 2003) has been reported to enhance transcriptional activity. Ser 468 can be phosphorylated following TCR activation however the result of this on transcription is unknown (Mattioli et al, 2004). GSK3β is also able to phosphorylate 4 sites within the amino acids sequence 354-551 in the transactivation domain (Buss, H. et al, 2004). There are currently a number of candidates for the kinase that phosphorylates ser536 within the transactivation domain: IKK1 (O'Mahony et al, 1999), IKK2 (Jeong et al, 2005), NIK (Jiang et al, 2003) and PKB (Mattioli et al, 2004) have all been shown to be capable and there has been much debate about whether phosphorylation of this residue is necessary for full transcriptional activity.
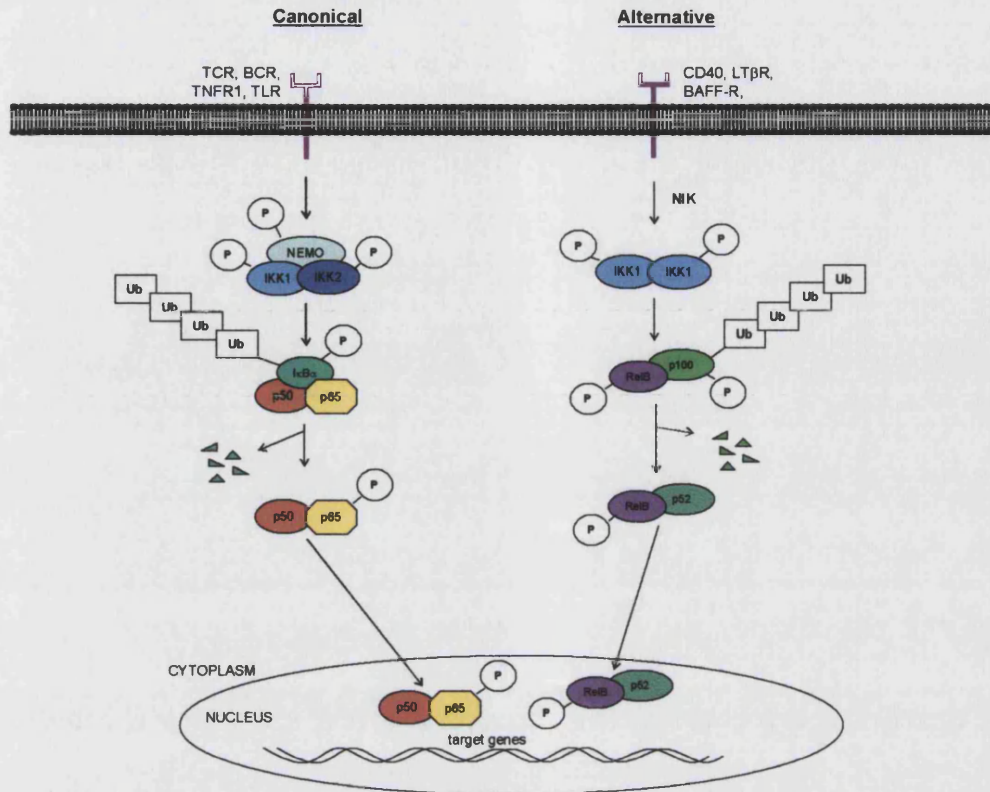
Figure 13. Schematic diagram of the canonical and alternative NF-kB pathways.

The canonical nuclear factor-κB (NF-κB) pathway involves activation of the classical IKK complex, composed of the two catalytic proteins IKK1, IKK2 and a regulatory subunit, NEMO, is recruited to the plasma membrane. The activated classical IKK complex phosphorylates IκBα resulting in its polyubiquitination and degradation via the proteasome pathway. The released NF-κB hetrodimer then translocates to the nucleus. In the alternative NF-κB pathway activated IKK complex phosphorylates the NF-κB2 dimer, typically heterodimers of NF-κB2 p100 and RelB; NF-κB2 p100 is then ubiquitinated and degraded into NF-κB2 p52. The NF-κB2 p52/RelB dimer then translocates to the nucleus to influence transcription.

IκBα, inhibitor of κBα; IKK1, inhibitor of κB kinase 1; IKK2, inhibitor of κB kinase 2; NEMO, NF-κB essential modulator; Ub, ubiquitin.

There is less known about the alternative pathway wherein partial processing of NF-κB2 p100 releases the p52 protein. The IKK complex containing an IKK1 homodimer, independent of both IKK2 and NEMO, is activated by NIK (Ling *et al*, 1998); it is currently unknown whether there are additional regulatory proteins in the complex. The activated IKK complex then phosphorylates the NF-

κB2 dimer, typically heterodimers of NF-κB2 p100 and RelB. NF-κB2 p100, once phosphorylated, is then ubiquitinated and targeted for degradation by the 26S proteasome (Xiao et al, 2004). The NF-κB2 p52/RelB dimer then translocates to the nucleus to influence transcription. It has been suggested that the NF-κB2 p52 protein may be substituted for the NF-κB1 p50 protein following prior to translocation to the nucleus however the implications of this are not clear.

The kinetics of the NF-κB pathways differ depending on the activating stimuli; signal induced degradation of IκBα through the TCR takes about 45 minutes, where as ligation of the TNFR1 by TNFα results in IκBα degradation after about 10 minutes (Heilker et al, 1999). Processing of p100 is much slower than this taking several hours. Among the genes upregulated by NF-κB by TNFα via the canonical pathway are both NF-κB2 p100 and RelB, suggesting that the pathways are linked and have the ability for advanced regulation of NF-κB signalling. There is also possibly some overlap of genes regulated by the canonical and alternative pathways suggesting additional control of gene transcription by NF-κB.

In the third NF-κB pathway, NF-κB1 p105 is partially processed, in a similar way to NF-κB2 p100, resulting in the release of the NF-κB1 p50 protein (figure 14). Following receptor ligation, the c-terminus of p105 is phosphorylated by the classical IKK complex at ser927 and ser932 (Lang et al, 2003; Salmeron et al, 2001). Upstream kinases responsible for the activation of the IKK complex in this pathway have so far not been identified. NF-κB1 p105 is then ubiquitinated and proteolysed releasing NF-κB1 p50 which can then translocate to the nucleus. Since p50 lacks a transactivation domain, modifications, such as phosphorylation can alter its ability to bind DNA. Phosphorylation of p50 at ser337, within the Rel homology domain, by PKA can enhance p50 DNA-binding (Hou et al, 2003). NF-κB1 p105 is constitutively phosphorylated by GSK3β on ser903 and ser907 inducing a stabilised conformation (Demarchi et al, 2003). NF-κB1 p105 also forms a complex with Tpl-2, a MAPKKK in macrophages and following LPS activation of TLR4, the released Tpl-2 can phosphorylate and activate Mek which subsequently phosphorylates ERK1/2 (Bienke et al, 2004). ERK1/2 can translocate to the nucleus where it can activate transcription of genes such as COX-2 and TNFα (Hoffmeyer et al, 1999).
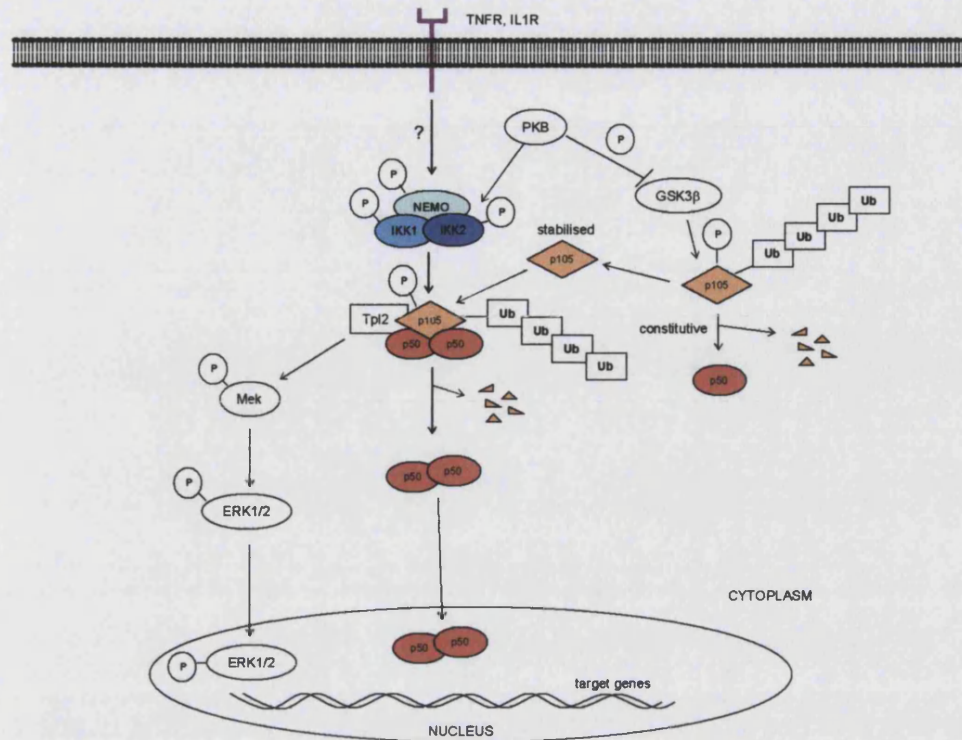
Figure 14. The NF-κB1 p105 pathway.

Following receptor ligation, the c-terminus of p105 is phosphorylated by the classical IKK complex, containing IKK1, IKK2 and NEMO. NF-κB1 p105 is then ubiquitinated and proteolysed releasing NF-κB1 p50 which can then translocate to the nucleus. NF-κB1 p105 can be stabilised by the constitutive phosphorylation by GSK3β. NF-κB1 p105 is found complexed with TPL-2; which can phosphorylate and activate Mek which subsequently phosphorylates ERK1/2. ERK1/2 can also translocate to the nucleus where it can activate gene transcription.

ERK1/2, extracellular related kinase 1/2; GSK3β, glycogen synthase kinase 3β; IKK1, inhibitor of κB kinase 1; IKK2, inhibitor of κB kinase 2; NEMO, **NF-κB essential modulator**, NF-κB, nuclear factor-κB; PKB, protein kinase B; TPL2, tumour progression locus-2; Ub, ubiquitin.

1.3.9 Activation of the TCR

It is not only chemokine receptors which utilise these pathways. It has been recognised that full activation of the TCR requires additional co-stimulatory molecules, one of the most important of which is CD28, activated by the B7 family members on antigen presenting cells. Binding of the TCR by antigen peptide-MHC complex activates a series of complex signalling cascades within the cell (figure

15). Activation of the TCR leads to members of the Src family of protein tyrosine kinases, such as Lck, being recruited to the TCR along with ZAP-70, which is then phosphorylated by the Src-kinases (Howe & Weiss, 1995). Tyrosine phosphorylation is a key mechanism of signal propagation in early TCR signalling. ZAP-70 then phosphorylates the adaptor protein LAT (Zhang *et al*, 1998) which forms a complex with the adaptor SLP-76 and Vav1 (Zhang *et al*, 1999). This complex is capable of activating a number of proteins, such as a complex containing Cdc42, WASP and Arp2/3, or Rac1 responsible for cytoskeletal changes or the MAP-kinase cascade (Salojin *et al*,1999). PI3K is one of the major downstream signalling mediators involved in many of the functional effects elicited by TCR activation. As previously described, PI3K generates the lipid second messenger $PI(3,4,5)P_3$ which is recognised by the PH domain of many proteins, allowing recruitment and activation of proteins like PKB. PKB activates many proteins such as $GSK3\beta$ and the transcription factors Bad and forkhead transcription factors. Activation of PLC results in the metabolism of $PI(4,5)P_2$ to DAG and IP_3 which activates PKC and drives calcium flux resulting in regulation of $NF-\kappa B$ and nuclear factor of activated T cells (NFAT), transcription factors controlling gene regulation. TCR signalling is also mediated by inhibitory signals for example co-stimulation by CD28 represses the inhibitory signals initiated by Cbl-b allowing activation of further signalling mechanisms, like Vav1 or PI3K (Hemdon *et al*, 2005; Li *et al*, 2004).
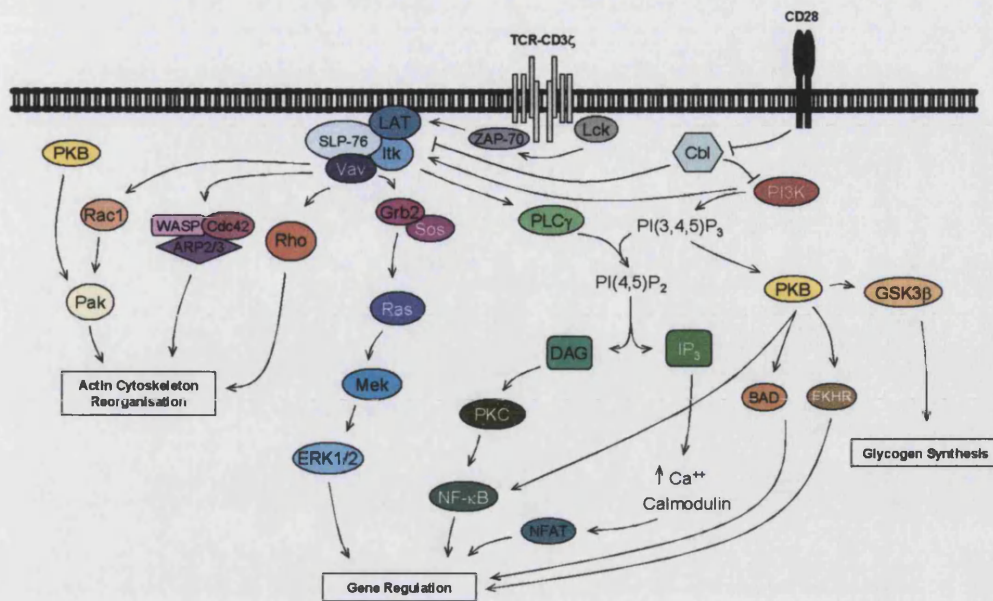
Figure 15. Simplified overview of signalling through the T cell receptor (TCR).

Activation of TCR by antigen results in recruitment and tyrosine phosphorylation of the adaptor proteins Lck and ZAP-70. This leads to activation of a protein complex containing LAT, SLP-76 and Vav. Subsequently, further signalling cascades are activated resulting in reorganisation of the cytoskeleton and regulation of gene transcription. Costimulation of CD28 releases the inhibitory signals of Cbl allowing activation of PI3K and its downstream effectors.

DAG, diacylglycerol; ERK1/2, extracellular-related kinase; FKHR, forkhead transcription factor; GSK3β, glycogen synthase 3β; IP$_3$, inositol 1,4,5-trisphosphate; LAT, linker for activated T cells; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; PAK, p21 activating kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; WASP, wiskott-aldrich syndrome protein; ZAP-70, ζ-chain associated protein 70kDa.

1.4 Analysis of Signal Transduction Pathways

The activation of the pathways described here can be identified by a number of techniques. The generation of transgenic and knockout animals has enabled the identification of the role of specific molecules in embryonic development and normal functioning of cells, organs and animals; as well as their roles in disease progression of animal models. However, a more routine way of determining the interactions and roles of these molecules has been through use of selective inhibitors. There are many commonly available inhibitors reported to target specific molecules. Pertussis toxin is an exotoxin produced by the bacterium *Bordetella pertussis*. It is able to catalyze ADP-ribosylation of the α subunits of G proteins which then remain in their GDP-bound, inactive state (Kaslow & Burns, 1992). This toxin selectivly acts on members of the Gαi family and has been widely used in the invesigation of signalling events downstream of most GPCRs.

There are several inhibitors which target PI3K, the most commonly used ones include LY294002, a synthetic compound, and wortmannin, a fungal metabolite obtained from *Penicillium fumiculosum*. LY294002 is a cell permeable compound that acts on the ATP-binding site of PI3K and blocks the catalytic activity of the enzyme with an IC_{50} of 1.4μM (Vlahos *et al*, 1994). At this concentration it does not affect other known kinases including PKC, PKA, MAPK, S6 kinase or PI4K; however at about 100 fold higher concentrations this compound has been reported to inhibit MLCK, PLC and PI4K it has been shown to inhibit CK2 at similar concentrations used in inhibition on PI3K (Davies et al, 2000). In neutrophils, a concentration of 50μM LY294002 completely inhibits all PI3K activity (Vlahos *et al*, 1994; Vlahos *et al*, 1995). Wortmannin has an IC_{50} of ~ 5nM in CCR2 activated THP-1 cells (Turner *et al*, 1998). Wortmannin has also been shown to inhibit neutrophil MAPK and MLCK activity at 200-300nM (Ferby *et al*, 1994; Cross *et al*, 1995) as well as PI4K and phospholipase D activity (Bonser *et al*, 1991; Nakanishi *et al*, 1992; Nakamura *et al*, 1995). IC87114 is reported to be a selective inhibitor of p110δ with an IC_{50} of 0.5 μM for p110δ inhibition compared to the IC_{50} values for p110α, p110β and p110γ which were >100, 75, and 29 μM, respectively (Sadhu *et al*, 2003). IC87114 was also demonstrated to have no significant effect on other kinases including p38 MAPK, PKB, PKCα or PKCβII (Sadhu *et al*, 2003).

There are a number of PKC inhibitors available with reported specificities to different isoforms. Ro320432 a cell permeable inhibitor of PKC, reported to inhibit PKCα and PKCβI with IC_{50} values of 9nM and 28nM in rat brain. This compound

has also been shown to inhibit other PKC isoforms at higher concentrations (Wilkinson *et al*, 1993). Gö6796 is another widely used PKC inhibitor which targets PKCα and PKCβ1 with IC_{50} of 2.3nM and 6.2nM in rat brain (Gschwendt et al, 1996) and PKCμ with an IC_{50} of 20nM in neutrophils (Wenzel-Seifert *et al*, 1994). It has no reported activity for PKCδ, ε or ζ isoforms. Although rottlerin is reportedly a selective PKCδ inhibitor with an IC_{50} of 3-6μM for purified human PKCδ and PKCθ (Gschwendt *et al*, 1994). It is also able to inhibit PKCα, PKCβ, and PKCγ isoforms at IC_{50} values of 30-42μM, and 80-100 μM for PKCε, PKCη, and PKCζ. Further studies of this inhibitor have revealed that it is also a potent inhibitor of MAPKAPK2, CaM kinase III, p38 MAPK, PKA, and GSK3β (Davies *et al*, 2000) indicating that its status as a selective inhibitor may not be wholly accurate.

Toxins are widely used pharmacological tools in the determination of signalling pathways. Inhibition of members of the small G-protein families by lethal toxin B, from *Clostridium difficile* IP82, preferentially inhibits RhoA over Cdc42 and Rac, and Lethal toxin 82, from *Clostridium sordellii*, preferentially inhibits Rac1 over Cdc42 and Rho. The proposed mechanism of action of these toxins is through the glucosylation or isoprenylation of the target proteins (Just *et al*, 1995). Y27632 is a reported inhibitor of Rho kinase (ROCK) with an IC_{50} of 140nM; it also inhibits ROCKII, PRK2, MSK1 and MAPKAPK1β with IC_{50} values of 800nM, 600nM, 8.3mM and 19mM respectively in purified human kinases (Davies *et al*, 2000; Ishizaki *et al*, 2000).

Aims

As described, chemokine signalling within cells is highly complex and is involved in initiating and regulation of multiple effects. Since CXCR6 and CXCXL16 were discovered 9 years ago, little has been reported regarding the nature of signalling through this chemokine receptor and ligand. The presence of the receptor on activated T lymphocytes suggests an involvement in immune responses and since the receptor has been shown to be up-regulated in a number of disease states, it is important to understand the reason for this. Investigation of signal transduction events following activation of CXCR6 may help to identify the possible importance of this receptor on T lymphocytes. Since this chemokine has a unique structure within its class, and has been identified as a scavenger receptor, we were unsure if it would mediate the same pathways utilised by other chemokines and there receptors. The project aims were therefore two-fold:

1. To identify biochemical targets activated by CXCR6 in T lymphocytes and determine whether it utilises the same signal transduction pathways as other chemokine receptors.

2. To characterise the role of these molecules in functional responses. Is CXCL16/CXCR6 an inflammatory chemokine, can it mediate chemotaxis or gene transcription?

Chapter 2: Materials and Methods

Methods

2.1 Cell Types

The following cell lines were used in this study (descriptions from ECACC or ATCC websites):

- CEM - human Caucasian acute T lymphoblastoid leukaemia cell (purchased from ATCC)
- HUT-78 - Leukaemic T lymphoblast cell (purchased from ATCC)
- Jurkat J6 - Leukaemic T lymphoblast cell (obtained from ICRF)

The following cells were generated from peripheral blood mononuclear cells (PBMCs):

- previously activated human peripheral blood derived T-lymphocytes (PBLs)
  - super antigen-activated T cells or CD3-CD28 activated T cells
- Th1 lymphocytes - T helper 1-like cells

2.2 Culture of Suspension Cell Lines

Suspension cell lines (CEM, HUT-78, Jurkat) were cultured in humidified incubators in 5% CO_2, at 37°C in complete media (RPMI 1640 media supplemented with 10% foetal bovine serum (FBS) (v/v), penicillin (100U/ml) and streptomycin (100μg/ml)). Cells were maintained at a confluency of 1-2x10^6 cells/ml.

2.3 Freezing and Thawing of Cell Lines

Cell lines were stored frozen in liquid nitrogen tanks to provide a continuous stock of cells of a particular age and to minimise transformation of cell lines. Cells at low confluency were resuspended at 5x10^{6}cells/ml in 'freeze mix' (90% (v/v) FBS, 10% (v/v) dimethylsulphoxide (DMSO)) and aliquoted into cryovials at 1ml/vial. The vials were frozen at ‾80°C overnight and then transferred to the liquid nitrogen tanks for storage. Cells were thawed by placing the cryovial in a 37°C water bath to rapidly defrost the cell suspension. The cells were quickly mixed with pre-warmed complete media and centrifuged (5 minutes, 400g). Cells were then resuspended in complete media, seeded into an 80cm^2 tissue culture flask and maintained as described above.

2.4 Isolation of Peripheral Blood Mononuclear Cells

100ml venous blood was taken from a healthy human volunteer into a heparinised syringe (2U/ml). The blood was diluted 1:1 v/v with RPMI 1640 media and 35mls of the mixture was layered over 15mls Lymphoprep in clear 50ml falcon tubes. The tubes were centrifuged at 400g, 20°C for 30minutes (no brake). The milky interface containing the PBMCs (figure 16) was removed into clean tubes and the cells were subsequently washed 3 times in RPMI 1640 medium by centrifuging at 400g for 5 minutes, removing the supernatant and resuspending the cells in RPMI medium. Cells were resuspended in 100mls complete RPMI 1640 medium (supplemented with 10% FBS, 50µg/ml penicillin and 50µg/ml streptomycin). PBMCs were then used to create activated T-lymphocytes.
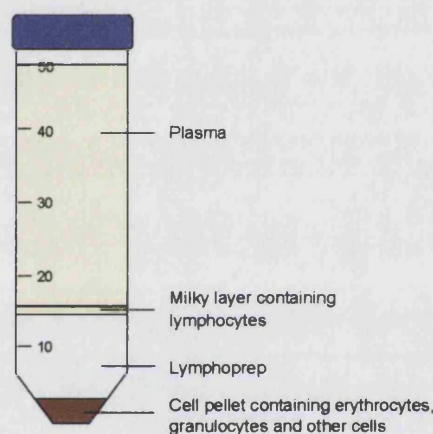
Figure 16. Isolation of mononuclear cells from blood.

Whole blood, diluted with RPMI 1640 media, was layered over 15ml Lymphoprep and centrifuged at 400g for 20 minutes (without brake). The milky interface, containing mononuclear cells, between the plasma and Lymphoprep was removed and cells were cultured.

2.5 Expansion of previously activated human peripheral blood derived T-lymphocytes

Following isolation from blood, PBMCs were then expanded in culture by one of two methods. Either the PBMCs were transferred to a 175cm^2 tissue culture flask with 1µg/ml staphylococcus enterotoxin B (SEB) for 72hrs at 37°C, 5% CO_2. The cells were then washed 3 times in RPMI 1640 (400g, 5 minutes) and resuspended in 100mls complete RPMI 1640 medium with IL-2 (50U/ml) and

transferred to a clean 175cm^2 tissue culture flask. Cells were maintained at a confluency of 1-2x10^6 cells/ml and IL-2 (50U/ml) added every 2 days for up to 14 days. These cells will be referred to as SEB-activated PBLs. Alternatively, PBMCs were transferred to a 175cm^2 tissue culture flask for 3 hours where most adherent cells would be removed from the cell mixture. Suspension cells were removed and seeded at 1x10^6 cells/ml in complete RPMI media with IL-2 (50U/ml) and CD3-CD28 microbeads (1 bead/cell) in an 80cm^2 tissue culture flask. Cells were maintained at a confluency of 1-2x10^6 cells/ml and IL-2 (50U/ml) added every 2 days for up to 20 days. These cells will be referred to as CD3-CD28-activated PBLs. Prior to use in experiments, SEB-activated PBLs were washed free of IL-2, and for CD3-CD28-activated PBLs the CD3-CD28 microbeads were removed, and cells maintained in complete media overnight.

2.6 In Vitro Generation of Human T Helper 1 (Th1) Cells

Preparation of Discontinuous Percoll Gradients

NaCl (9.00g) was dissolved in 100ml MilliQ water to make a 10x isotonic solution. Isotonic Percoll was prepared by diluting mixing Percoll with the 10x isotonic saline solution (9:1). This stock solution ("100%" Percoll) was used to prepare dilutions of 55%, 70% and 81%. Each gradient consisted of 5ml 81% Percoll, 3ml 70% Percoll and 2.5ml 81% Percoll. 1 discontinuous Percoll gradient was prepared for each 20ml whole blood.

Isolation of CD4+/CD45RA+ Cells

100ml venous blood was taken from a healthy human volunteer into a heparinised syringe (2U/ml). The blood was mixed with an equal volume of Hespan plasma expander and left to stand for 30 minutes. The supernatant was removed and centrifuged (400g, 5 minutes). The supernatant was removed and the leukocyte pellet was resuspended in 55% Percoll. The cell suspension was layered over the discontinuous Percoll gradients and centrifuged (1600g, 30 minutes, room temperature). The lymphocytes were removed from the 55% Percoll phase and the 55%/70% Percoll interface (figure 17). Cells were washed twice in phosphate buffered saline (PBS) and CD4/CD45RA+ cells were isolated using a MACS CD4+ T cell isolation kit II and MACS CD45RO microbeads (Miltenyi Biotec, Auburn, CA, USA) in accordance with manufacturer's instructions. Cells were resuspended at 2.5x10^8 cells/ml in buffer (PBS pH 7.2, 0.5% bovine serum albumin (BSA), 2mM EDTA). 10μl/1x10^7 cells Biotin-Antibody Cocktail was added and the cells were

incubated for 10 minutes at 4°C. $30\mu l/1 \times 10^7$ cells buffer and $20\mu l/1 \times 10^7$ cells Anti Biotin Microbeads were added and the cells were incubated for 15 minutes at 4°C. The cells were washed and resuspended in buffer at 2×10^8 cells/ml. Cells were separated using the 'deplete' programme of an autoMACS™ Separator (Miltenyl Biotec, Auburn, CA, USA). The negative fraction eluted contained the CD4⁺ T cells. Cells were resuspended in buffer at $80\mu l/1 \times 10^7$ cells and $5\mu l/1 \times 10^7$ cells MACS CD45RO microbeads was added. Cells were incubated for 15 minutes at 4°C. The cells were washed and resuspended in buffer at 2×10^8 cells/ml. Cells were separated using the 'deplete' programme of an autoMACS™ Separator (Miltenyl Biotec, Auburn, CA, USA). The negative fraction eluted contained the CD4⁺CD45RO⁻ T cells.
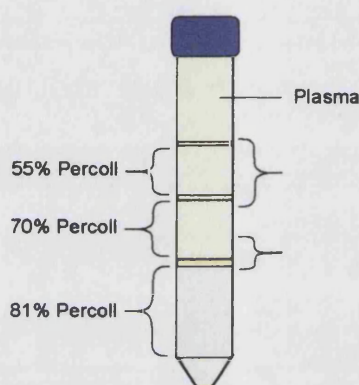
Figure 17. Isolation of lymphocytes from whole blood.
Isotonic Percoll was prepared by diluting mixing Percoll with 10x saline solution. This stock solution ("100%" Percoll) was used to prepare dilutions of 55%, 70% and 81% and discontinuous Percoll gradients were prepared. Whole blood was mixed with an equal volume of Hespan plasma expander and left to stand for 30 minutes. The supernatant was removed and centrifuged (400g, 5 minutes). The leukocyte pellet was resuspended in 55% Percoll, layered over the Percoll gradients and centrifuged (1600g, 30 minutes, room temperature). Lymphocytes were removed from the 55% Percoll phase and the 55%/70% Percoll interface.

Generation of Th1 cells

6-well plates were incubated overnight (37°C, 5% CO_2) with 1.5ml PBS containing 1mM $MgCl_2$, 1mM $CaCl_2$, anti-CD3 (5μg/ml) and anti CD28 (5μg/ml). The wells were washed with PBS prior to addition of cells. CD4⁺CD45RO⁻ T cells were resuspended at 1×10^6 cells/ml in growth media (see Appendix 1) with IL-2

(10ng/ml), IL-12 (5ng/ml) and anti-IL-4 (5ng/ml). 3mls cell suspension was added to each well and incubated for 4 days ($37^{\circ}C$, 5% CO_2). Cells were combined and washed in resting medium (see Appendix 1) and resuspended at 1×10^6 cells/ml in resting medium containing IL-2 (10ng/ml) and IL-7 (1ng/ml). Cells were incubated for 4-7 days ($37^{\circ}C$, 5% CO_2). Cells were resuspended at 5×10^5 cells/ml in growth media with IL-2 (10ng/ml), IL-12 (5ng/ml), anti-IL-4 (5ng/ml) and anti-CD95L (1μg/ml) and incubated for a further 4 days ($37^{\circ}C$, 5% CO_2). IFN-γ production, as a Th1 marker, was measured prior to use.

2.7 Cell Counting and Viability

Cells were resuspended in 50ml media and 100μl was transferred to a 0.5ml centrifuge tube containing 100μl 0.4% trypan blue (a negatively charged chromophore which is able to bind cellular proteins if the membrane is damaged; therefore staining non-viable cells blue). The tube was gently mixed and 10μl was transferred to a haemocytometer. The number of viable cells was counted using a light microscope.

2.8 Cell Stimulation and Lysis

Preparation of Whole Cell Lysates

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended in 1.5ml centrifuge tubes at 5×10^6 cells/500μl in RPMI 1640 medium and allowed to rest for 1 hour at $37^{\circ}C$. Where used, inhibitors were added at the appropriate concentration after 30 minutes. Agonists were added for appropriate times and the stimulations terminated by aspiration of the supernatant and addition of 500μl lysis buffer (see Appendix 1 for recipe). Cells were then lysed by rotating at $4^{\circ}C$ for 10 minutes and centrifuged (400g, 10 minutes $4^{\circ}C$). 400μl of each lysate was transferred to a clean tube containing 100μl 5x reducing sample buffer (see Appendix 1 for recipe) and boiled for 10 minutes. Samples were stored at $20^{\circ}C$ until used for SDS-PAGE and western blotting (described below).

Preparation of Samples for Flow Cytometric Analysis of Phosphorylation and Actin Polymerisation

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended in 1.5ml centrifuge tubes at

5×10^6 cells/500µl in RPMI 1640 medium and allowed to rest for 1 hour at 37°C. Where used, inhibitors were added at the appropriate concentration after 30 minutes. Agonists were added for appropriate times and the stimulations terminated by fixing the cells in 3.7% formaldehyde. Samples were incubated at room temperature for 10 minutes and washed twice in PBS. Cells were permeabilised in PBS containing 0.1% (w/v) triton and 0.1% (w/v) BSA on ice for 5 minutes. Samples were washed twice and resusupended in cold PBS.

Preparation of Samples for Measurement of TNFα and IFNγ

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended in 24-well tissue culture plates at 2×10^6 cells/500µl in RPMI 1640 medium/0.1%BSA and allowed to rest for 1 hour at 37°C. Agonists were added for appropriate times and the stimulations terminated by centrifugation (13000g, 10 minutes, 4°C). The supernatant was transferred to a clean tube and stored at ⁻80°C until used.

Preparation of Nuclear and Cytosolic Fractions

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended in 1.5ml centrifuge tubes at 1×10^7 cells/500µl in RPMI 1640 medium and allowed to rest for 1 hour at 37°C. Where used, inhibitors were added at the appropriate concentration after 30minutes. Agonists were added for appropriate times and the stimulations terminated by aspiration of the supernatant, addition of 400µl buffer 1 (see Appendix 1 for recipe) and incubation on ice for 15 minutes. 15µl 10% NP40 was added and the tubes were mixed and then centrifuged (13000g, 5 minutes, 4°C). The supernatants (containing cytosolic proteins) were transferred to clean tubes and the pellets (containing nuclear proteins) were washed in buffer 1, and then resuspended in 50µl buffer 2 (see Appendix 1 for recipe). The tubes were rotated for 30 minutes at 4°C and centrifuged (13000g, 5 minutes, 4°C). The supernatants (containing nuclear proteins) were transferred to clean tubes. Samples were then analysed to determine protein concentration (described below). 320µl of each cytosolic fraction was transferred to a clean tube containing 80µl 5x sample buffer and 40µl of each nuclear fraction was transferred to a clean tube containing 10µl 5x sample buffer. Samples were boiled for 10 minutes and stored at ⁻20°C until used for immunoblotting (described below).

2.9 Bradford Assay for Protein Concentration

To measure the amount of protein in each sample the Bio-Rad protein assay was used. For each sample to be analysed, 5μl lysate/supernatant was added to a 1.5ml centrifuge tube containing 995μl 20% Bradford solution and vortexed. 100μl was transferred to a 96 well plate in duplicate. A standard curve was also constructed using BSA and the plate was read at 595nm (figure 18) (Versamax, Molecular Devices Corporation, CA, USA). The amount of protein was then calculated from the standard curve by linear regression.
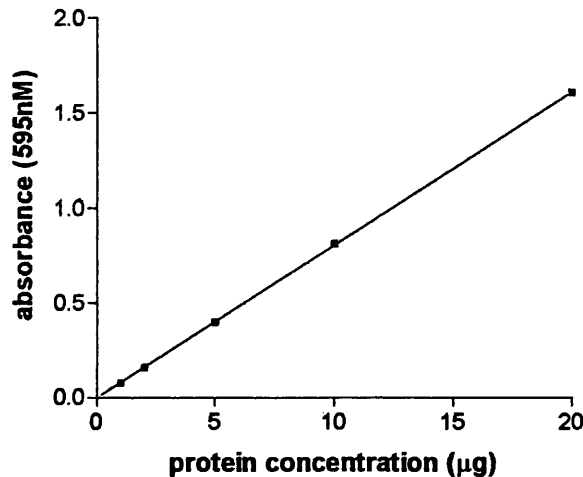
Figure 18. Standard curve for determining protein concentration.
A standard curve of bovine serum albumin (BSA) was constructed to calculate protein concentration in samples. Appropriate concentrations of BSA were added to 20% Bradford solution. 100μl was transferred to a 96 well plate in duplicate and the plate was read at 595nm (Versamax, Molecular Devices Corporation, CA, USA).

2.10 SDS-PAGE & Western Blotting

Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins from whole cell lysates can be identified and measured. The process of boiling samples in the presence of SDS and a reducing agent (2-mercaptoethanol) denatures the proteins into their polypeptide subunits. This allows separation, according to their molecular weight, of individual proteins on a gel when voltage is applied.

SDS-PAGE

SDS-PAGE mini gels were made using BioRad Mini Protean III equipment in accordance with manufacturer's instructions (Bio-Rad Laboratories, Hemel Hempsted, UK). Stacking gels were always 5% bis-acrylamide and resolving gels varied between 7.5% and 12% bis-acrylamide (see appendix 1 for recipes) in accordance with the size of the protein of interest. Glass plates were cleaned before use and 4.5ml resolving buffer was poured in between the plates. MilliQ water was carefully overlaid to ensure a smooth flat gel. The buffer was left to polymerise for 30 minutes and the MilliQ was completely removed. Stacking buffer was poured on top of the resolving gel and a comb was inserted. Following polymerisation of the stacking gel (~ 15 minutes) the comb was removed and the wells were washed with MilliQ water and filled with Running Buffer (see appendix 1 for recipe). Samples were loaded into the appropriate well and a molecular weight marker was also included to aid protein band identification. Gels were electrophoresed at 80V until the samples had passed through the stacking gel and the voltage was then increased to 180V until the samples reached the bottom of the gel. The apparatus was then disassembled and the gel removed from the glass plates and washed in semi-dry transfer buffer (see appendix 1 for recipes). A stack containing 4 pieces of 3MM filter paper and nitrocellulose membrane pre-soaked in semi-dry transfer buffer was assembled on the anode plate of the transfer equipment (Pharmacia-Biotech Multiphor II) and the gel was placed on the top of this. A further 4 pieces of 3MM filter paper pre-soaked in semi-dry transfer buffer were added on top and air bubbles were gently removed. The cathode was placed on top of the stack and the proteins were then transferred from the gel onto the nitrocellulose membrane for 1 hour at $0.8mA/cm^2$ of gel.

Western Blotting (Immunoblotting)

Following transfer of the proteins onto nitrocellulose membrane, the membranes were washed in tris-buffered saline (TBS) (see appendix 1 for recipes) and stained with Ponceau S solution. This enabled visualisation of the protein bands to ensure equal transfer had occurred. The Ponceau S solution was removed by washing the membranes in TBS and they were then blocked for 1 hour in 5% (w/v) non-fat milk powder in TBS. Membranes were washed briefly in TBS and incubated on a rocking platform in the primary antibody overnight. The membranes were washed 4 times (for 5 minutes) in TBS/0.01%Tween (TBST) and incubated with the appropriate horse radish peroxidase (HRP) conjugated secondary antibody

diluted in TBS for 2 hours. Membranes were washed 4 times in TBST and once in TBS before visualisation of the protein bands using Amersham Enhanced Chemiluminescence (ECL) reagent, in accordance with the manufacturer's instructions. Membranes were incubated in the reagent for 1 minute and wrapped in cling film and exposed to autoradiography film.

Membrane Stripping and Reprobing

To verify that the samples were equally loaded on the membranes, membranes were incubated in stripping buffer for 30 minutes at 60°C to remove the antibodies. Membranes were then washed in TBST before being blocked and reprobed in appropriate primary and secondary antibodies as previously described.

2.11 Flow cytometry

A flow cytometer uses the deflection of light to analyse the physical properties of a single cell. Cells in suspension flow through the instrument in a single stream which allows each cell to be analysed. Light, from a laser source, is focused through a lens into a beam of approximately 50µm, perpendicular to the cell stream. As the light crosses the stream of cells, light is deflected and focused onto photodetectors, either in front of or at right angles to the source of light, which convert the light signal into an electrical impulse proportional to the intensity of the light which reaches the detector. Each photodetector is preceded by a filter; the filter ensures that the detector is only exposed to light of a specific colour. One of the detectors to the side of the illuming beam will be fitted with a filter of the same colour as the laser (usually blue); because it is at the side, light will only reach the detector if it has been deflected from the cell. The rougher the cell surface or more granular the cell, the more light will scatter and therefore reach the detector, this signal is known as the side scatter. Other detectors to the side of the laser beam will be preceded by coloured filters which enable detection of cells stained with fluorescent tags (usually fluorescein (FITC) (green) or phycoerythrin (PE) (red/orange)). The flow cytometer also has a detector directly in front of the light source; this is fitted with a metal bar in front of it. Light can only reach the detector if it is bent passing through a particle, this forward scatter is indicative of the size of the particle; a larger cell can refract more light and the signal reaching the detector will be more intense. Some flow cytometers, able to sort cells according to these physical properties, are known as fluorescence activated cell sorters (FACS).
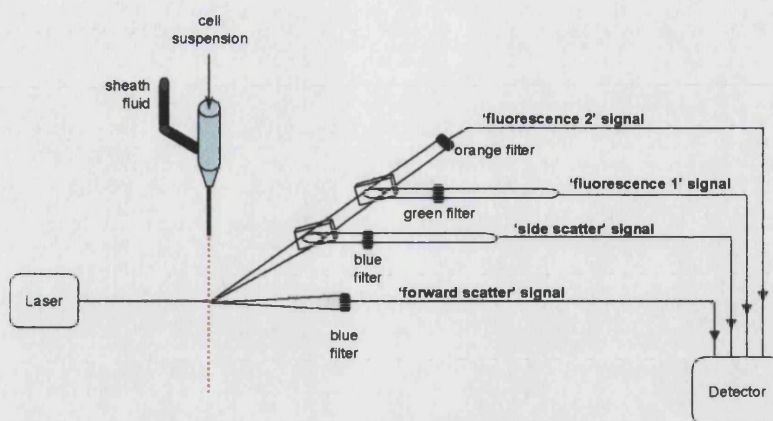
Figure 19. Simplified schematic diagram of a flow cytometer.

Light from the laser deflects from the stream of cells, passes through coloured filters onto photodetectors. The greater the light intensity, the larger the electrical impulse it is converted into. The forward scatter indicates the size of the cell, while the side scatter indicates the granularity. Orange and green filters allow the detection of fluorescent tags on cells.

Analysis of Receptor Expression

Cells were washed twice in PBS and aliquoted into polypropylene FACS tubes at 1×10^6 cells/tube in 80µl PBS/20% FCS. For measurement of CD3, CD28, CXCR4 and CXCR6, 20µl of PE-conjugated mAb or corresponding PE-conjugated isotype control was added to the appropriate tubes. For CD4 and CD8 measurement, 20µl of FITC-conjugated mAb or corresponding FITC-conjugated isotype control was added to the appropriate tubes. A negative control was also included which had 20µl of PBS/20% FCS added in place of antibody to give a reading for the cell's autofluorescence. Cells were incubated for 30 minutes on ice, in the dark with regular mixing. Cells were washed twice in PBS and resuspended in 500µl PBS and immediately analysed by a FACS Vantage (BD Biosciences, San Jose, CA).

Analysis of IL-4 and IFN-γ Production (Th1)

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended in polypropylene FACS tubes at 1×10^6 cells/500µl in RPMI 1640 medium and allowed to rest for 1 hour at $37^{\circ}C$.

Cells were stimulated with PMA (Phorbol 13-myristate 12-acetate) (50ng/ml) and ionomycin (500ng/ml) for 2 hours at 37°C. One aliquot of the cells was left inactivated as a control. Following stimulation, 10μg/ml Brefeldin A was added to all cells and they were incubated for a further 2 hours at 37°C. The cells were washed in 3ml FACS staining buffer (PBS containing 1% BSA) and resusupended at $5x10^5$ cells/ml. Cells were fixed in 3.7% formaldehyde. Samples were incubated at room temperature for 10 minutes and in FACS staining buffer. Cells were permeabilised in PBS containing 0.1% (w/v) triton on ice for 5 minutes. Samples were washed twice in FACS staining buffer and resusupended in 80μl FACS staining buffer. 20μl Alexa Fluor® 488-conjugated anti-IFNγ or Alexa Fluor® 488-conjugated anti-IL-4 was added and the cells incubated for 30 minutes at room temperature in the dark. Samples were washed twice in FACS staining buffer and resusupended in 500μl cold PBS before analysis by a FACS Vantage (BD Biosciences, San Jose, CA).

Analysis of Protein Phosphorylation

Measurement of protein phosphorylation by flow cytometry has recently become more widely used. It enables analysis of the phosphorylation status of individual cells; the use of different coloured fluorescent tags could potentially allow measurement of phosphorylation of multiple molecules in the same cell at a given time point. It also requires fewer cells and is therefore useful when cell numbers are limited.

Following fixing and permeabilisation, cells were incubated in 0.2μg phospho-specific antibody or 0.2μg isotype control for 30 minutes at room temperature. Samples were washed twice in PBS and incubated in FITC-conjugated secondary antibody (1:250 final dilution) for 30 minutes at room temperature. Cells were washed twice in PBS and resuspended in 500μl PBS, transferred to polypropylene FACS tubes and immediately analysed by a FACS Vantage (BD Biosciences, San Jose, CA).

Analysis of Actin Polymerisation

Actin polymerisation occurs in response to a number of cell stimuli; actin monomers, through interaction with a number of proteins, are joined together forming actin filaments (F-actin). Phalloidin is a fungal toxin which binds F-actin monomers in a 1:1 ratio; fluorescently-labelled phalloidin can therefore be used to

measure the concentration of actin in the cytoskeleton because the intensity of the signal will be proportional to the concentration of actin.

Following fixing and permeabilisation, cells were incubated in 0.6μM FITC-conjugated phalloidin for 30 minutes in the dark. Cells were washed twice in PBS, resuspended in 200μl PBS and transferred to polypropylene FACS tubes. To measure autofluorescence of the cells, one sample was fixed and permeabilised but left without phalloidin. Samples were immediately analysed by FACS Vantage (BD Biosciences, San Jose, CA).

2.12 Chemotaxis Assays

There are two methods for analysing cell migration described below. The Neuroprobe disposable Chemotx plates required fewer cells and lower volumes of the chemoattractants, however the individual plates are expensive and it was not possible to use them in all assays. The Neuroprobe reusable chemotaxis chambers were therefore used to measure chemotaxis of cell types in which numbers were not limited.

Neuroprobe Reusable MB Series 96 Well Chemotaxis Chambers (Cell lines and activated PBLs) (figure 20)

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium. Cells were resuspended at 1x10^6 cells/ml in RPMI 1640 medium/0.1% BSA and where used, inhibitors were added at the appropriate concentration. The 96-well reusable chemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA) was prepared in accordance with the manufacturers instructions. The appropriate agonists were placed in the lower 96 well plate (disposable) within the lower chamber and a 5μm polyvinylpyrrolidine-free polycarbonate membrane was carefully fitted on top. The chamber lid was gently closed and 200μl of the cell suspension was added to each well of the upper chamber. The chamber was left for 3 hours at 37°C (5% CO$_2$). At the end of the incubation period, cells remaining in the upper chamber were aspirated off and 200μl versene was added to each well. The chamber was incubated at 4°C for 20 minutes and the versene was aspirated off. The chamber was dismantled and the 96-well plate in the lower chamber was centrifuged (400g, 10 minutes). The membrane was detached and the media was the aspirated off and the cells were resuspended in 100μl RPMI/0.1% BSA. Cell numbers were determined from

standard curves of cells from the same experiment using 20μl/well Cell Titer 96 AQ$_{ueous}$ reagent. Plates were incubated at 37°C until the colour developed. Absorbances were read on a plate reader at 490nm (Versamax, Molecular Devices Corporation, CA, USA).
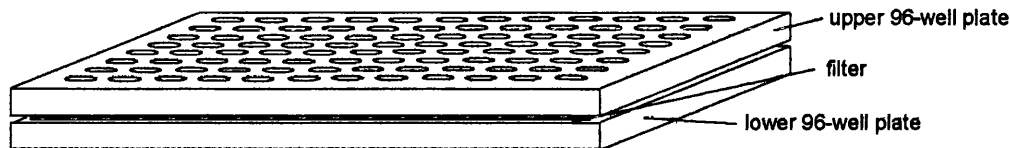
Figure 20. Neuroprobe reusable chemotaxis chambers.

Chemoattractants were added to the 96 wells of the lower plate and a membrane (filter) was fitted over the top. The lid (forming the upper 96-well plate) was closed ensuring air bubbles did not form and that the upper and lower wells were aligned. Cells were added to the upper wells and the chamber was left for 3 hours at 37°C (5% CO$_2$). Cell numbers were determined using Cell Titer 96 AQ$_{ueous}$ reagent.

Neuroprobe Disposable Chemotx System (Th1) (figure 21)

Cells were harvested by centrifugation (400g, 5 minutes) and washed 3 times in RPMI 1640 medium (without phenol red). Cells were resuspended at 1x10^7 cells/ml in RPMI 1640 medium (without phenol red)/0.1% BSA. Cells were labelled with 5mg/ml calcein-AM (Molecular Probes, Leiden, Netherlands) for 30 minutes in the dark at 37°C. Cells were then washed twice and resuspended at 4x10^6 cells/ml in RPMI 1640 medium (without phenol red)/0.1% BSA. The 96-well disposable chemotaxis plates (Neuroprobe, Gaithersburg, MD, USA) was prepared in accordance with the manufacturers instructions. 29μl of the appropriate agonists were placed in the 96 well plate and a 5μm filter was carefully placed on top. 25μl of the cell suspension was then placed on top of the filter over each well and the plate was incubated at 37°C (5% CO$_2$) for 1.5 hours. At the end of the incubation time cells remaining on the top of the filter were gently washed off with PBS and the filter top was scraped with 3MM chromatography paper. The plate was read using a fluorescent plate reader (Fluoroskan Ascent, Thermo Labsystems, Finland) at 485nm (excitation) and 538nm (emission). Cell numbers were determined from standard curves of cells from the same experiment.

cell suspension

filter
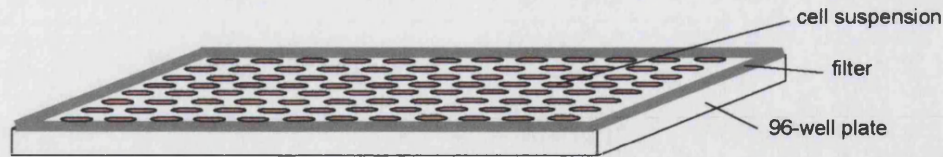
96-well plate

Figure 21. Neuroprobe Chemotx plates.

Chemoattractants were added to the 96-well plate and a membrane (filter) was fitted over the top. Calcien-AM labelled cells were placed in the filter, over each well and were incubated for 1.5 hours at $37^{\circ}C$ (5% CO_2). Cells were washed off the filter and the fluorescence was read using a plate reader.

2.13 Measurement of IFNγ and TNFα by ELISA

ELISAs (enzyme linked-immunosorbant assay) can be used for the quantitative detection of a variety of small biological assays. The principle of the assay is to capture the 'antigen' with immobilised antibody and then add a secondary antibody coupled to an enzyme. Unbound antibodies are removed and the activity of the enzyme can be measured.

Samples were thawed on ice and levels of cytokines were measured by ELISA in accordance with the manufacturer's instructions. A standard curve was constructed from recombinant human TNFα or IFNγ. 50μl of sample diluent was added to each well of the 96-well plate precoated with anti-TNFα antibody (for measurement of IFNγ this was not required). 50μl sample or standard was added to the wells in duplicate; the plate was covered and incubated for either 1 or 2 hours at room temperature for or TNFα or IFNγ resepctively. Following incubation, the plate was washed 3 times in wash buffer. 100μl biotinylated antibody reagent was added to each well and incubated for an hour at room temperature. The plate was washed 3 times and 100μl strepavidin-HRP solution was added to each well. The plate was covered and incubated for a further 30 minutes at room temperature. Following incubation, the plate was again washed 3 times in wash buffer. 100μl TMB substrate solution was added to each well and plate was incubated in the dark for 30 minutes at room temperature to allow colour change to occur. 100μl stop solution was added to each well and the plate was read on a plate reader at 450nm (Versamax, Molecular Devices Corporation, CA, USA) and TNFα concentrations were calculated by linear regression from the standard curves (figure 22).
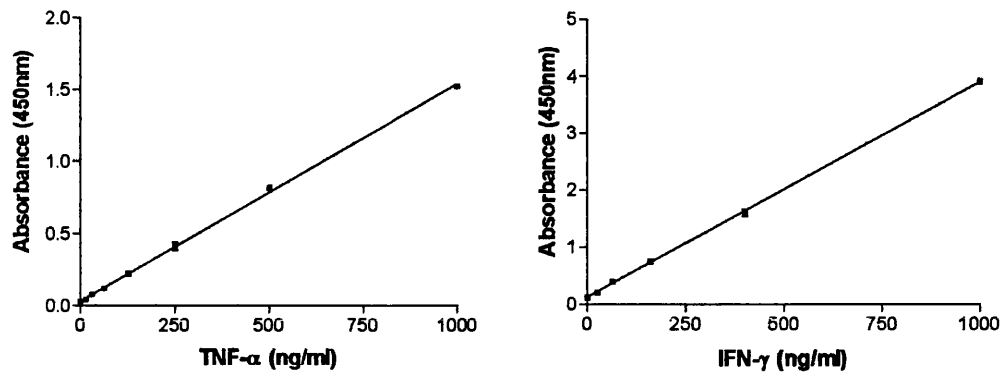
Figure 22. Examples of ELISA standard curves.

2.14 Statistical analysis

Statistical analysis was performed using ANOVA and Student's t test, with Bonferroni correction where necessary.

Materials

Antibodies:

Antibody	Species	Monoclonal/ Polyclonal	Source
Anti-AKT1	Goat	Polyclonal	Santa Cruz, CA (USA)
Anti-CD3 (clone UCHT1) (for stimulations)	Mouse	Monoclonal	Doreen Cantrell, School of Life Sciences, Dundee (UK)
Anti-CD3 (Phycoerythrin conjugated) (for flow cytometry)	Mouse IgG_1	Monoclonal	Pharmingen, San Diego, CA (USA)
Anti-CD3 (for Th1 polarisation)	Mouse	Monoclonal	Pharmingen, San Diego, CA (USA)
Anti-CD4 (Phycoerythrin conjugated) (for flow cytometry)	Mouse IgG_1	Monoclonal	Pharmingen, San Diego, CA (USA)
Anti-CD8 (Carboxyfluorescein conjugated)	Mouse IgG_{2B}	Monoclonal	R & D Systems, Abingdon (UK)
Anti-CD28 (for Th1 polarisation)	Mouse	Monoclonal	Pharmingen, San Diego, CA (USA)
Anti-CD28 (clone 9.3) (for stimulations)	Mouse	Monoclonal	Carl June, Uni. Pennsylvania (USA)
Anti-CD95L	Mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-CXCR4 (Phycoerythrin conjugated)	Mouse IgG_{2B}	Monoclonal	R & D Systems, Abingdon (UK)
Anti-CXCR6	Mouse	Monoclonal	R & D Systems, Abingdon (UK)
Anti-ERK1	Rabbit	Polyclonal	Santa Cruz, CA (USA)
Anti-goat immunoglobulin (HRP conjugated)	Rabbit	Polyclonal	Dako, Glostrup (Denmark)
Anti-IFNγ (Alexa Fluor® 488 conjugated)	mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-IL-4 (Alexa Fluor® 488 conjugated)	mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-Lamin A/C (636)	Mouse	Monoclonal	Santa Cruz, CA (USA)
Anti-mouse IgG_1 (Alexa Fluor® 488 conjugated)	mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-mouse IgG_1 (FITC conjugated)	Mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-mouse IgG_1 (phycoerythrin conjugated)	Mouse	Monoclonal	BD Pharmingen, San Diego, CA (USA)
Anti-mouse IgG_{2B} (phycoerythrin conjugated)	Mouse	Monoclonal	R & D Systems, Abingdon (UK)

Anti-mouse immunoglobulin (HRP conjugated)	Rabbit	Polyclonal	Dako, Glostrup (Denmark)
Anti-NF-κB p105/p50	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-p70S6 kinase	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho ERK1/2$^{Thr202/Tyr204}$	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho Mek1/2$^{ser217/221}$	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho NF-κB p65^{ser276}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho NF-κB p65^{ser536}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho NF-κB2 p100^{ser864}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho p105 NF-κB2^{ser933}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho p70S6 kinase $^{thr421/ser424}$	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho PKB^{Thr308}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho PKB^{Ser473}	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-phospho S6 ribosomal protein$^{ser 235/236}$	Rabbit	Polyclonal	Cell Signalling Technologies (UK)
Anti-rabbit immunoglobulin (HRP conjugated)	Goat	Polyclonal	Dako, Glostrup (Denmark)
Anti-Rabbit IgG (FITC conjugate)	Goat	Polyclonal	Sigma Aldrich, Gillingham (UK)

Cell culture materials:

Material	Source
6-well tissue culture plates	Costar
CD4$^+$ T cell isolation kit	Miltenyi Biotec, Auburn, CA (USA)
CD45RO Microbeads	Miltenyi Biotec, Auburn, CA (USA)
Cryotubes	Fisher Scientific, Loughborough (UK)
Dynabeads® CD3/CD28 T Cell expander	Dynal Biotech (UK)
Foetal bovine serum	Gibco BRL, Paisley (UK)
Foetal calf serum	Gibco BRL, Paisley (UK)
Hepes	Gibco BRL, Paisley (UK)
Hespan plasma expander	Geistlich Pharm,
Interleukin-2 (IL2) (for T-lymphoblasts) (for Th1-like cells)	Chemicon, Hampshire (UK) R & D Systems, Abingdon (UK)
Interleukin-7 (IL7)	R & D Systems, Abingdon (UK)
Interleukin-12 (IL12)	R & D Systems, Abingdon (UK)

Lymphoprep (Ficoll-paque 1.077g/ml density)	Axis-Shield, Oslo (Norway)
Media	Gibco BRL, Paisley (UK)
Mercaptoethanol	Sigma Aldrich, Gillingham (UK)
Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}	Gibco BRL, Paisley (UK)
Penicillin/Streptomycin	Gibco BRL, Paisley (UK)
Percoll	Sigma Aldrich, Gillingham (UK)
Plastics	Nunc (UK)
RPMI 1640 media	Gibco BRL, Paisley (UK)
Sodium heparin	Sigma Aldrich, Gillingham (UK)
Sodium pyruvate	Gibco BRL, Paisley (UK)
Staphylococcal enterotoxin B	Sigma Aldrich, Gillingham (UK)
Trypan Blue	Gibco BRL, Paisley (UK)
Trypsin/EDTA	Gibco BRL, Paisley (UK)

Other:

Material	Source
2-mercaptoethanol	Sigma Aldrich, Gillingham (UK)
96-well plates for chemotaxis	Greiner-bioone (Germany)
Acrylamide/bis acrylamide	Bio-rad (UK)
Ammonium persulphate	Sigma Aldrich, Gillingham (UK)
Aprotinin	Sigma Aldrich, Gillingham (UK)
Bovine serum albumin (BSA)	Sigma Aldrich, Gillingham (UK)
Brefaldin A	Sigma Aldrich, Gillingham (UK)
Bromophenol blue	BDH, Poole (UK)
Cell Titer 96® Aqueous One Solution Cell Proliferation Assay	Promega UK, Southampton (UK)
DMSO	Sigma Aldrich, Gillingham (UK)
EDTA	Sigma Aldrich, Gillingham (UK)
Enhanced chemiluminescence detection kit for Western blotting (ECL)	Amersham International (UK
Ethanol	Fisher Scientific, Loughborough (UK)
Filter paper	Whatman, Maidstone (UK)
Formaldehyde	Sigma Aldrich, Gillingham (UK)
Glycerol	Sigma Aldrich, Gillingham (UK)
Glycine	Sigma Aldrich, Gillingham (UK)
Gö6976	Calbiochem, Nottingham (UK)
HEPES	Sigma Aldrich, Gillingham (UK)
Human IFNγ ELISA Kit	Pierce Endogen, Rockford IL (USA)
Human TNFα ELISA Kit	Pierce Endogen, Rockford IL (USA)
Hydrochloric acid	BDH, Poole (UK)
IC87114	Tocris Cookson, Avonmouth (UK)
Ionomycin	Sigma Aldrich, Gillingham (UK)
Lethal toxin 82	
Leupeptin	Sigma Aldrich, Gillingham (UK)
LY294002	Tocris Cookson, Avonmouth (UK)
Marvel (fat-free dry milk)	Supermarket
Methanol	Fisher Scientific, Loughborough (UK)
Molecular weight protein markers	Bio-rad (UK)

Nitrocellulose	BDH, Poole (UK)
Nonidet P-40	Sigma Aldrich, Gillingham (UK)
Pan T Cell Isolation Kit II (human)	Miltenyi Boitec, CA (USA)
PD98059	Calbiochem, Nottingham (UK)
Pertussis toxin	Sigma Aldrich, Gillingham (UK)
Plastics	Greiner-bioone (Germany)
Phalloidin - FITC conjugated	Sigma Aldrich, Gillingham (UK)
Phorbol 13-myristate 12-acetate (PMA)	Calbiochem, Nottingham (UK)
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich, Gillingham (UK)
Ponceau S	Sigma Aldrich, Gillingham (UK)
Rapamycin	Calbiochem, Nottingham (UK)
Recombinant human CXCL16 (rhCXCL16)	R & D Systems, Abingdon (UK)
Recombinant human CXCL10 (rhCXCL10)	R & D Systems, Abingdon (UK)
Recombinant human CXCL12 (rhCXCL12)	R & D Systems, Abingdon (UK)
RO-32-0432	Calbiochem, Nottingham (UK)
Rottlerin	Calbiochem, Nottingham (UK)
Sodium azide	Sigma Aldrich, Gillingham (UK)
Sodium chloride	Sigma Aldrich, Gillingham (UK)
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, Gillingham (UK)
Sodium fluoride	Sigma Aldrich, Gillingham (UK)
Sodium molybdate	Sigma Aldrich, Gillingham (UK)
Sodium orthovanadate	Sigma Aldrich, Gillingham (UK)
TEMED	Sigma Aldrich, Gillingham (UK)
Toxin B	
Tris (Trizma base)	Sigma Aldrich, Gillingham (UK)
Tween-20	Sigma Aldrich, Gillingham (UK)
Versene	Gibco BRL (UK)
Wortmannin	Calbiochem, Nottingham (UK)
X-OMAT film	Amersham International (UK)
XTT	Sigma Aldrich, Gillingham (UK)
Y27632	Calbiochem, Nottingham (UK)

Chapter 3 - Results

Identification of a T cell model for investigation into signalling through CXCR6

CXCR6 messenger RNA was first identified in T-lymphocytes (Loescher *et al*, 1997; Deng *et al*, 1997) and has since been reported to be expressed on a number of other primary cell types (Chandrasekar *et al*, 2004; Deng *et al*, 1997; Sharron, M. *et al*, 2000). Despite this the majority of research into CXCR6 has remained focused on primary T-lymphocytes and T cell lines. In order to investigate signalling mechanisms following activation of CXCR6 it was necessary to develop a working T cell model which expresses a functional CXCR6 receptor on the cell surface.

Characterisation of functional CXCR6 on T cell lines

3.1 Expression of CXCR6 on T cell lines

Several leukaemic T cell lines were analysed for expression of CXCR6 in order to develop a working model for further experiments (Figure 23). The leukaemic T cell lines, Jurkat, HUT-78 and Cem cells, are widely used within the chemokine research field as a model T cell. Initial analysis of Jurkat cells showed expression of CXCR6 on a small population of cells (figure 23A), as a T cell line, it would usually be expected that cells would be uniform in their phenotype, so the reason only a small population of cells express the receptor is therefore unclear. Unfortunately, CXCR6 was unable to be detected in future experiments (figure 23B). Both of these samples originated from the same source however, they represent aliquots thawed at different times and CXCR6 was only detected in the first. Although some work into CXCR6 function has been carried out in Jurkats, it used cells transfected with CXCR6 in order to obtain a consistently high expression (Matloubin *et al*, 2000).

The lack of consistency of CXCR6 expression in Jurkat cells lead to the use of alternative T cell lines. HUT-78 cells are another commonly used T cell line, which have previously been reported not to express CXCR6 RNA (Deng *et al*, 1997). However, analysis of these cells by flow cytometry indicated that CXCR6 was expressed on the cell surface (figure 23C). Deng *et al* were also unable to

---

detect CXCR6 RNA in Cem cells, figure 23D however shows that they also display a high level of expression over the entire population.
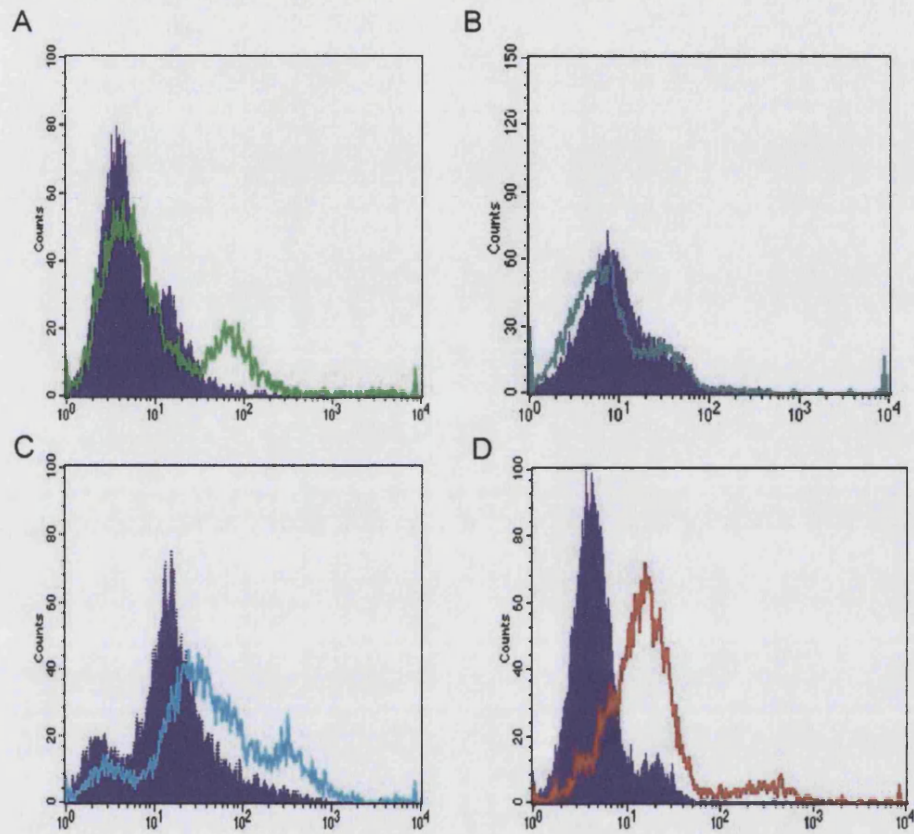
Figure 23. Flow cytometric analysis of CXCR6 expression on cell lines. **A** Initial Jurkat sample showing a sub-population of cells expressing CXCR6 (green line). **B** Second Jurkat sample showing no CXCR6 expression (green line). **C** Expression of CXCR6 on the entire Hut 78 cell population (blue line). **D** High expression of CXCR6 on Cem. Blue shaded area represents isotype control (mouse IgG_{2B}) Cells were incubated with either 2.5µg/ml phycoerythrin-conjugated anti-CXCR6 antibody or isotype control for 30minutes. Samples were then analysed by flow cytometry. (n=5)

3.2 Analysis of signalling pathways coupled to CXCR6 in T cell lines

Although these cell lines express CXCR6 at the cell surface, it was important to verify that the receptor was coupled to signalling pathways. As has been previously described, ligation of CXCR6 by CXCL16 activates the MAPK pathway (Matloubin *et al*, 2000). Phosphorylation of ERK1/2 on Tyr204 and Thr202 within the activation loop results in activation of ERK1/2. Figure 24a shows that in these Jurkat cells, CXCL16 initiates both a concentration-dependent and time dependent phosphorylation of ERK1/2, at Thr202 and Tyr204, which reaches a maximum at 5nM and 2 minutes respectively. CXCR6 expression on Jurkats was only detectable in the initial aliquot thawed; as would be expected, lack of detectable receptor expression correlated with loss of CXCL16-induced phosphorylation of ERK1/2. PI3K and PKB are components of another important signalling pathway whose activation following TCR engagement and chemokine receptor ligation has been widely reported. Although there has been much discussion about the exact requirement for phosphorylation of serine 473 for full activation of PKB, it has been shown to correlate with activation of PI3K. However, due to a lack of PTEN and SHIP in Jurkats (Astoul *et al*, 2001; Shan *et al*, 2000), these cells have high basal levels of phosphorylated PKB, and in these initial experiments in Jurkats, PKB phosphorylation was not measured.

The HUT-78 express CXCR6 over a larger percentage of the population than seen in the Jurkat sample, however signalling experiments in this cell line were disappointing. There was high levels of basal ERK1/2 phosphorylation which decreased with increasing concentrations of CXCL16 (figure 24b). Basal ERK1/2 phosphorylation decreased at 30 seconds and reappeared at 1 and 2 minutes and again after 30 minutes. Due to the complex signalling pathways leading to phosphorylation of ERK, the later activation of ERK1/2 is not surprising, however the high basal levels make analysis of stimuli-induced phosphorylation difficult to decipher.

Cem cells express high levels of CXCR6 however there was no detectable CXCL16 induced phosphorylation of ERK1/2 or PKB. CXCL12 did increase phosphorylation of these proteins suggesting that in these cells, CXCR6 is not functionally coupled to downstream effectors (figure 24c).
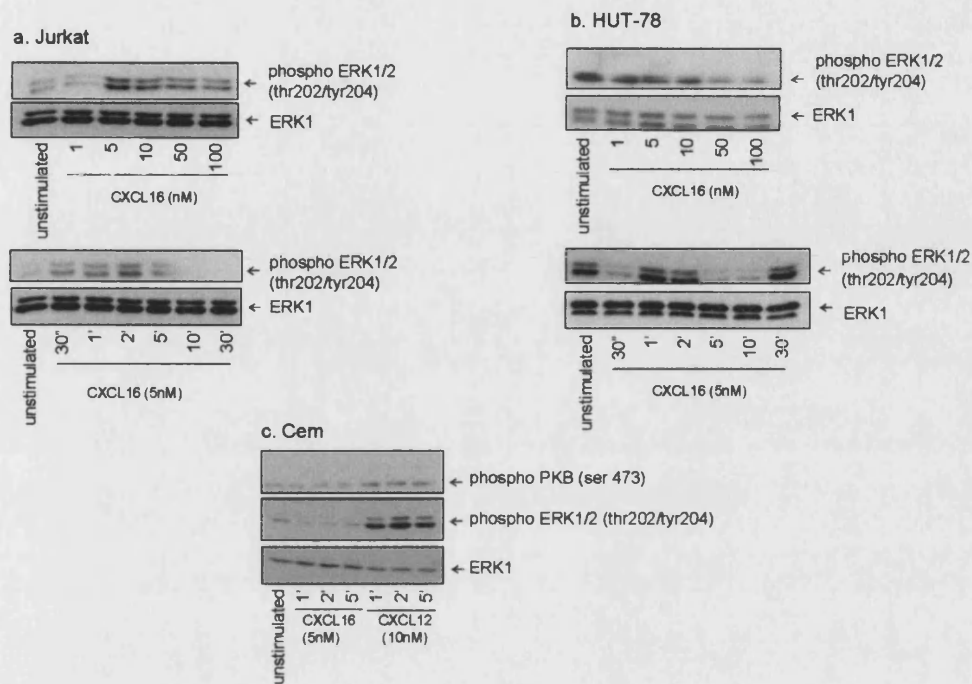
Figure 24. CXCR6 is coupled to signal transduction pathways in some T cell lines.

a. concentration-dependent phosphorylation of ERK1/2 by CXCL16 (1nM-100nM) at 2 minutes in Jurkats and time-dependent phosphorylation of ERK1/2 by CXCL16 (5nM) in Jurkats. **b**. concentration-dependent phosphorylation of ERK1/2 by CXCL16 (1nM-100nM) at 2 minutes in HUT-78 cells and time-dependent phosphorylation of ERK1/2 by CXCL16 (5nM) in HUT-78 cells. **c** time-dependent phosphorylation of ERK1/2 and PKB by CXCL16 (10nM) and CXCL12 (10nM) in cem cells. Following stimulation ($5x10^6$ cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho ERK1/2 (thr42/tyr44) antibody. Equal loading of gels was verified using anti-ERK1 antibody. (n=3).

Characterisation of functional receptor expression on primary T-lymphocytes

Th1 cells

3.3 Expression of CXCR6 on Th1 cells

Following its discovery on T-lymphocytes, CXCR6 was described as a Th1-specific chemokine receptor because expression of the receptor correlates with production of IFN-γ from cells (Calabresi et al, 2002; Heydtmann & Adams, 2002; Kim et al, 2001). While CXCR6 is no longer thought to be exclusively expressed on Th1 cells, they were thought to be a more reliable cell type for the investigation into signal transduction through CXCR6. Polarisation of naïve T-lymphocytes (CD4$^+$/CD45RO$^-$) into a Th1 phenotype using cytokine stimulation produced a population of cells which express CXCR6 (Figure 25 a & b). The percentage of cells which expressed the receptor was highly donor dependent and ranged from 5 - 35%. These cells were characterised as Th1-like by their ability to produce IFN-γ (figure 25c) and lack of IL-4 production (figure 25d); although there was some variation between donors, In all experiments, the percentage of cells producing IFN-γ was above 80% and below 5% for cells producing IL-4 .

3.4 CXCL16 Induces chemotaxis of Th1 cells

Following an inflammatory stimuli, chemokines are able to induce directed cell migration towards the site of inflammation. This is an important phenomenon in the immune response; although chemotaxis of cells is also important in the homeostatic roles of chemokines. Initiation of a chemotactic response involves the polarisation of the cell in which they become elongated and develop a wide lamallipod at the leading edge and a tail-like projection (uropod) at the trailing end. The cells generate filamentous actin at the leading edge and the uropod contracts driving the cell forwards.

CXCR6 and its ligand CXCL16 have been implicated in the trafficking of activated T-lymphocytes to areas of chronic inflammation (Boisvert et al, 2003; Kim et al, 2001; Wang et al, 2004). CXCR6 has been shown to induce directed migration towards CXCL16 in primary T-lymphocytes and Jurkat cells transfected with CXCR6 in vitro. Initial experiments in the polarised Th1 cells show that CXCL16 significantly increases cell migration in a concentration dependent manner which peaks at ~10nM (figure 26).
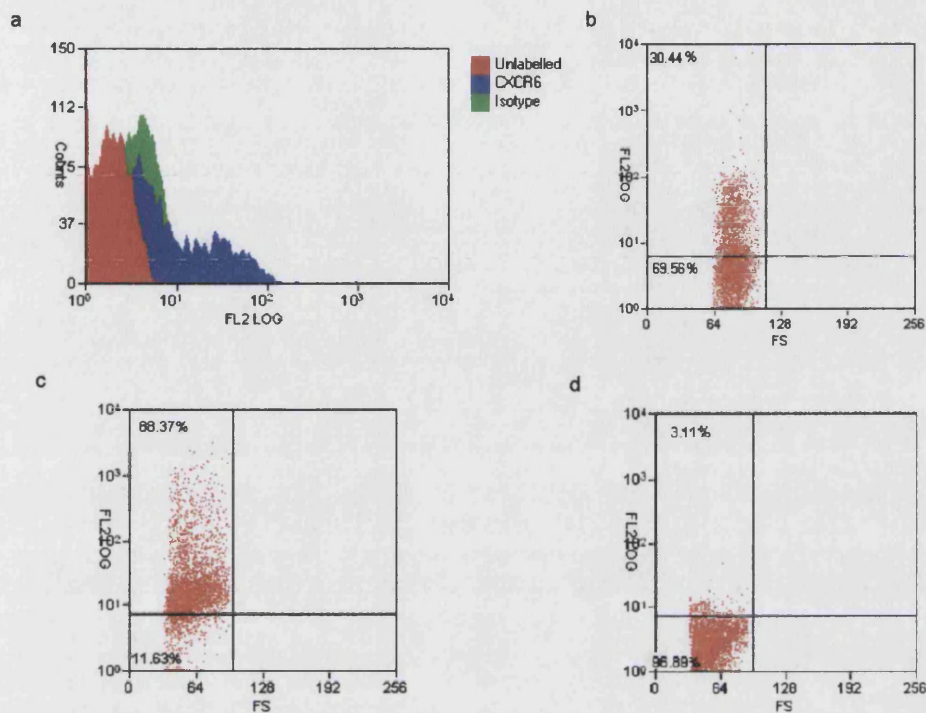
Figure 25. Flow cytometric analysis of CXCR6 expression on Th1 cells.

a, b. A representative population of Th1-like cells express CXCR6. c, d. production of IFN-γ and IL-4 from cells indicating the Th1-like phenotype. For CXCR6 measurement, cells were incubated with either 2.5μg/ml phycoerythrin-conjugated anti-CXCR6 antibody or isotype control for 30minutes. For analysis of IFN-γ and IL-4 production, cells were activated with PMA (Phorbol 13-myristate 12-acetate) (50ng/ml) and ionomycin (500ng/ml) and incubated with 10μg/ml Brefeldin A. Cells were fixed in 3.7% formaldehyde, permeabilised in 0.1% (w/v) triton and incubated with Alexa Fluor® 488-conjugated anti-IFNγ or Alexa Fluor® 488-conjugated anti-IL-4 antibody. Samples were then analysed by flow cytometry. Data corresponds to one donor (n=15)
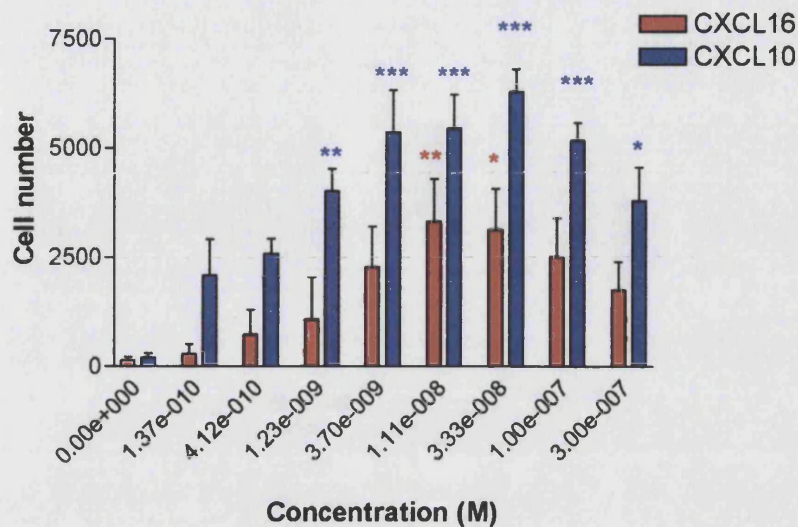
Figure 26. Chemotaxis of Th1 cells to CXCL16 and CXCL10. T cells (CD4⁺/CD45R0⁻) were purified from whole blood and incubated with cytokines to induce a Th1-like phenotype. Cells were labelled with 5mg/ml calcein-AM and chemotaxis to CXCL16 and CXCL10 was measured using disposable chemotaxis plates in accordance with the manufacturer's instructions (Neuroprobe, Gaithersburg, MD, USA). Cell numbers were calculated from a standard curve. Data represents the mean +/- SD from 5 donors. * = p< 0.05, ** = p< 0.01, *** = p< 0.001 compared to basal chemotaxis.

Th1 cells also express the receptor for the chemokine CXCL10, CXCR3; as a positive control for this experiment, chemotaxis towards CXCL10 was also measured. This produced a concentration dependent response which was highly significant. Although the response was statistically significant, the overall numbers of cells which migrated was low compared to the total number of cells used at the start of the assay, with a maximum of ~4% and ~7% of cells migrating towards CXCL16 and CXCL10 respectively. This does correlate to a previous study in which ~5% of cells migrate towards CXCL16 (Wilbanks *et al*, 2001).

3.5 Analysis of signalling pathways coupled to CXCR6 in Th1 cells

Isolation and polarisation of these cells is expensive, time consuming and does not result in the numbers required to carry out extensive investigations using SDS-PAGE western blotting techniques routinely used in determining the activation of signalling pathways. In the past few years the phospho-specific antibodies to a number of proteins used for western blotting have been validated for use in flow cytometry. This process uses fewer cells and would ultimately be useful in determining simultaneous phosphorylation of multiple proteins in a single cell. The antibodies used in this study were raised to recognise Mek1 phosphorylated at ser217/221, p70S6K phosphorylated at thr421/ser424 and PKB phosphoriated at ser473. These antibodies were all obtained from New England Biolabs, UK, and have been validated by this company to demonstrate their specificity to the particular target, through use of specific blocking peptides. Recent experiments by the company and others, to validate these antibodies for flow cytometry have also been reported for Mek1/2 (Chow *et al*, 2001; Furuno *et al*, 2001), p70S6K (Perez *et al*, 2004) and PKB (Krutzik *et al*, 2004). Methodolgy for this technique has also been published (Krutzik & Nolan, 2003; Irish et al, 2004; Perez *et al*, 2004)

A method was developed in order to minimise the number of polarised Th1 cells required; following stimulation with the relevant ligand, cells were fixed with formaldehyde and permeabilised with triton and incubated with a phospho-specific antibody. 10000 cells were analysed by flow cytometry and the median fluorescence was calculated. Traditional SDS-PAGE western blotting was also carried out using the same antibodies in order to detect whether these antiboies recognised other proteins.

Activation of the MAPK pathway is well characterised in T lymphocytes following activation of GPCRs and the TCR. Mek1 is a dual specificity MAPKK activated following phosphorylation by an upstream kinase. Ligation of CXCR6 by CXCL16 increases phosphorylation of Mek, however the extent of phosphorylation and kinetics were very variable (figure 27a). In general, CXCL16 induced an initial phosphorylation of Mek at between 1 and 1.5 minutes which then quickly diminished. PMA has been used in the validation of this phospho-Mek antibody for flow cytometry; is a potent activator of some PKC isoforms and has been shown to induce phosphorylation of Mek and ERK1/2 in several cell types. PMA also increased phosphorylation of Mek in a time dependent manner, but again the

magnitude of the response was variable (figure 27b). Concurrent western blot experiments also confirmed this result (figure 28).
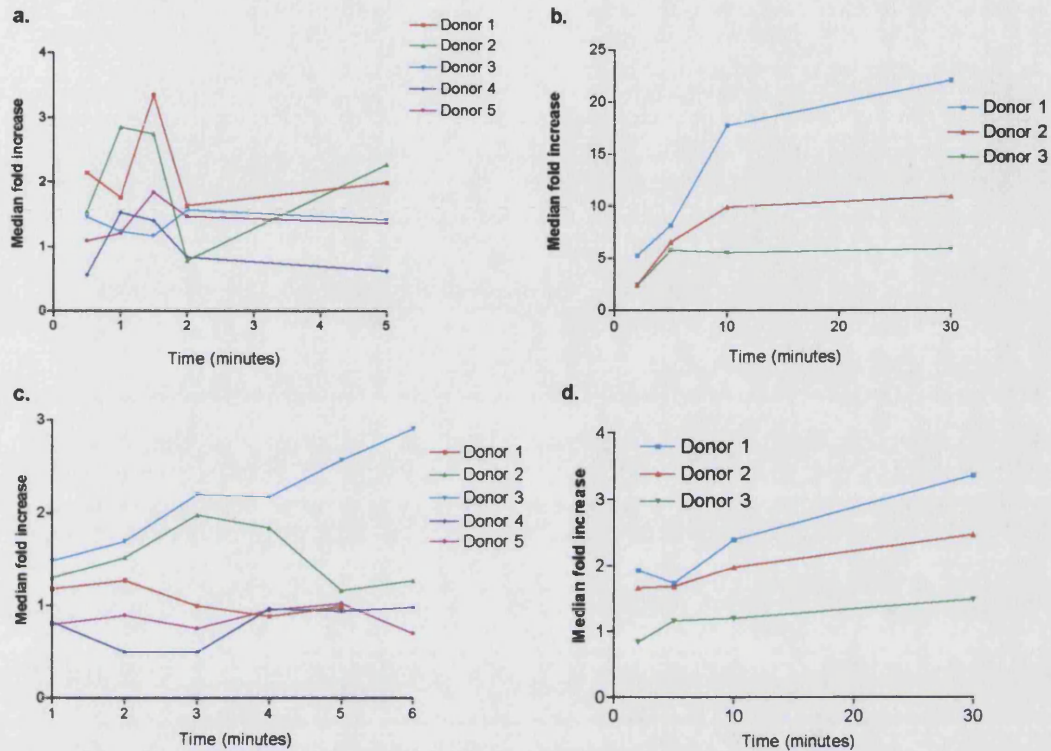
Figure 27. Flow cytometric analysis of MAPK signalling in Th1 cells.

a CXCL16 (10nM) -induced Mek (MAP-kinase and ERK kinase) phosphorylation (ser217/221). b PMA (Phorbol 13-myristate 12-acetate) (10nM) -induced Mek phosphorylation (ser217/221). c CXCL16 (10nM) -induced p70S6K phosphorylation (thr421/ser424). d PMA (10nM) mediated p70S6K phosphorylation (thr421/ser424). Donors exhibited different sensitivities to activation by CXCL16 and PMA which did not correlate to the level of CXCR6 expression. Cells were activated for the indicated times. Stimulations were terminated by fixing the cells in 3.7% formaldehyde and cells were permeabilised in 0.1% (w/v) triton. Cells were incubated in 0.2μg phospho-specific antibody or 0.2μg isotype control and then in FITC-conjugated secondary antibody. Samples were analysed by flow cytometry. The median fluorescence was then used to calculate the increase above basal levels.

Figure 28. Western blot analysis of MAPK signalling in Th1 cells

Concentration-dependent phosphorylation of Mek (MAP-kinase and ERK kinase) by CXCL16 at 2 minutes (top panel). Time-dependent phosphorylation of Mek and p70S6K by CXCL16 (10nM) or PMA (Phorbol 13-myristate 12-acetate) (10nM) (lower panel).

Following stimulation ($5x10^6$ cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-Mek (Ser217/221), or -p70S6K (thr241/424) antibody and visualised by chemoluminescence. Equal loading of gels was verified using anti-ERK1 or anti-p70S6K antibodies. (Data shown is representative of one donor; n=3).

CXCL16 phosphorylates Mek in a concentration-dependent manner which peaked at 10nM. Unlike the responses shown by flow cytometry, the time-dependent phosphorylation of Mek was later more sustained appearing at 1.5 minutes and disappearing by 5 minutes; the response elicited by PMA was much greater than for CXCL16, as was seen with the flow cytometry.

One of the downstream effectors of the MAPK is p70S6K which it activated following by phosphorylation of a number of residues. Phosphorylation of threonine 421 by ERK1/2 is required for the subsequent phosphorylation of the activation loop. An antibody specific for this phosphorylation site has been validated for use in flow cytometry; however unlike the phosphorylation of Mek, neither CXCL16 nor PMA produced any consistent trends in the phosphorylation of p70S6K between donors making analysis difficult (figure 27 c & d). Western blot analysis of p70S6K phosphorylation using the same antibody showed that CXCL16 can induce a time-dependent response which appears at 2 minutes and lasts until 5 minutes after stimulation (figure 28); although the strength of the response did vary between donors. PMA consistently activated a strong phosphorylation of p70S6k at Thr421 in western blot experiments. This antibody also recognises a p70S6K homologue, p85S6K which displays a similar phosphorylation profile following cell stimulation by CXCL16 although it has higher basal activity.

PKB is another signalling molecule whose activation following TCR engagement and chemokine receptor ligation has been widely reported. Phosphorylation of PKB has been reported in T cells following CXCL16 ligation of CXCR6 (Wilbanks *et al*, 2001). Phosphorylation of ser473 is thought to be required for full activation of PKB and can be used as an indirect marker of this. Flow cytometric analysis of CXCL16-induced phosphorylation of PKB at ser473 showed a general trend of a maximum at 2 minutes in 3 out of the 5 donors tested, but the magnitude of the response varied (figure 29). Western blot analysis showed a concentration dependent increase of PKB phosphorylation in which phosphorylation was induced at 3nM and was maintained to 100nM, the maximum concentration used (figure 30). Jurkat cells have high basal levels of phosphorylated PKB, therefore unstimulated Jurkat cells were used as a positive control to verify the results of the western blot. A time dependent response was also seen in some donors that peaked at 2 minutes which correlates with the results seen from the flow cytometry. In all of these experiments, CXCR6 expression was measured but levels of receptor expression did not correlate with the magnitude of the response.
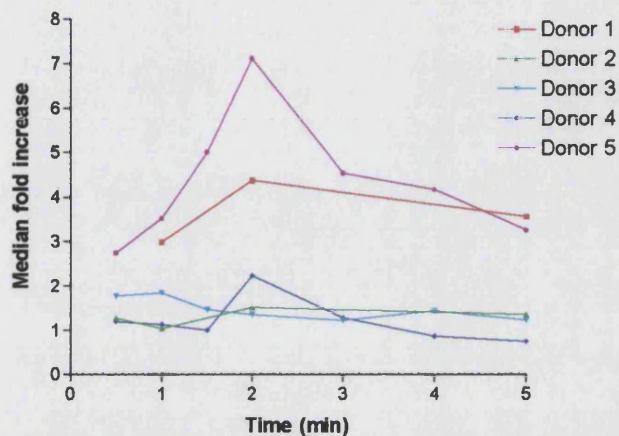
Figure 29. Flow cytometric analysis of PKB signalling in Th1 cells

CXCL16 (10nM) -induced protein kinase B (PKB) (ser473) phosphorylation. Cells were activated for the indicated times. Donor variation was observed with donors inducing different magnitudes of CXCL16 -induced PKB phosphorylation which did not correlate to the level of CXCR6 expression. Stimulations were terminated by fixing the cells in 3.7% formaldehyde and cells were permeabilised in 0.1% (w/v) triton. Cells were incubated in 0.2µg phospho-PKB (ser473) antibody or 0.2µg isotype control and then in FITC-conjugated secondary antibody. Samples were analysed by flow cytometry. The median fluorescence was then used to calculate the increase above basal levels.
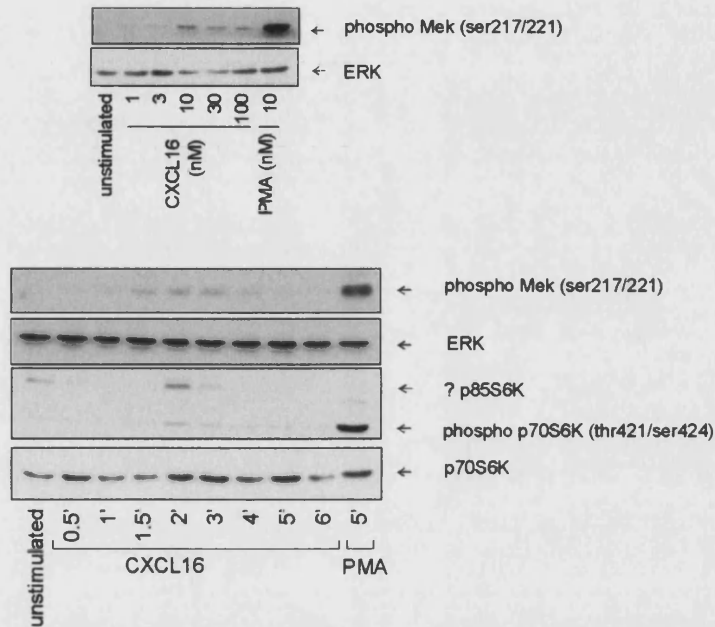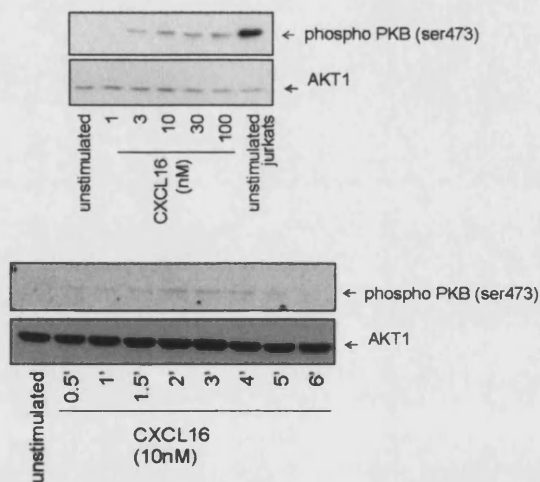
Figure 30. Western blot analysis of PKB signalling in Th1 cells

Concentration-dependent phosphorylation of protein kinase B (PKB) (ser473) at 2 minutes (top panel). Time-dependent phosphorylation of PKB by CXCL16 (10nM) (lower panel). Following stimulation (5×10^6 cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho PKB (ser 473) antibody and visualised by chemoluminescence. Equal loading of gels was verified using anti-AKT1 antibody. (Data representative of 1 donor; n=3).

SEB-activated PBLs

3.6 Expression of CXCR6 on SEB-activated PBLs

The availability and donor variation limited the use of the Th1 cells so a different type of T cell derived from PBMCs was cultured. These are PBLs activated using SEB and expanded in culture. Consistent with the reported expression on only activated T cells, at early stages in the culture, these cells have very low expression of CXCR6, but expression increases after 10 days to a maximum at day 12 before levels decrease and disappear by day 14 (figure 31). As was seen with the Th1 cells, expression of CXCR6 did vary between donors but was typically between 15-30% on days 11 and 12.
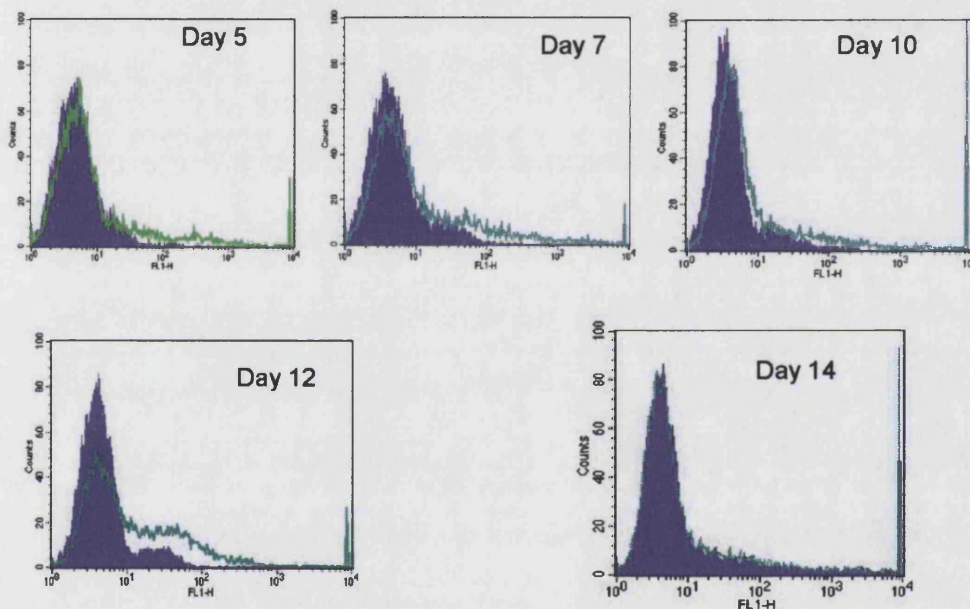
Figure 31. Flow cytometric analysis of CXCR6 expression on SEB-activated PBLs.
CXCR6 expression on the surface of SEB-activated PBLs. Blue shaded area represents the isotype control (mouse IgG_{2B}) the green line represents CXCR6 expression. Cells were incubated with either 2.5μg/ml phycoerythrin-conjugated anti-CXCR6 antibody or isotype control for 30 minutes. Samples were then analysed by flow cytometry. (Representative of 2 experiments)

3.7 Analysis of signalling pathways coupled to CXCR6 in SEB-activated PBLs

As has been shown in other cells, in these cells CXCR6 is coupled to the MAPK pathway (figure 32). CXCL16 induces a time dependent increase in phosphorylation of Mek1 which peaks at 1 minute and subsequently decreases. Activated Mek is known to phosphorylate ERK1/2 at Thr202 and Tyr204 and following CXCR6 ligation, ERK1/2 is also phosphorylated. This peaks at 1 minute and disappears by 4 minutes. CXCL12, the ligand for CXCR4, elicits a stronger and more sustained phosphorylation of both of these proteins than CXCL16, however typically ~60% of these cells express CXCR4. Marginal phosphorylation of PKB at serine 473 is also observed following activation of CXCR6. This increases up to 2 minutes and then decreases, again CXCL12 elicits a stronger response. Experiments to determine signal transduction were carried out on either day 11 or 12 after isolation from blood because the cells expressed the highest levels of CXCR6 at this time point.
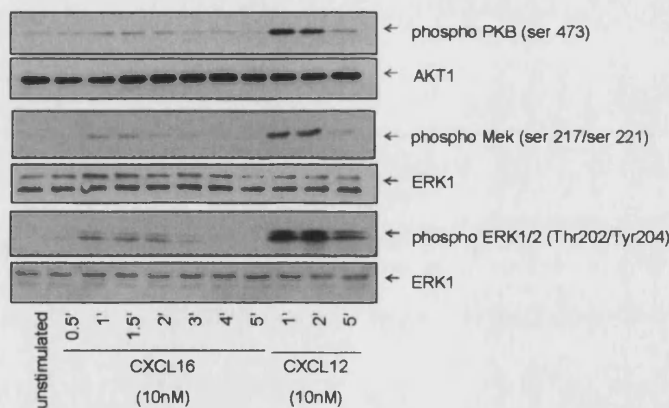
Figure 32. CXCR6 is coupled to signal transduction pathways in SEB-activated PBLs. Time-dependent phosphorylation of protein kinase B (PKB) (top panel), Mek (MAP-kinase and ERK kinase) (middle panel) and ERK (extracellular signal-regulated kinase) (lower panel) by CXCL16 (10nM). Following stimulation (5×10^6 cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-PKB (Ser473), anti-phospho-Mek (Ser217/221) or -ERK1/2 (thr42/tyr44) antibody and visualised by chemoluminescence. Equal loading of gels was verified using anti-ERK1 or anti-AKT1 antibodies. (Data shown is representative of one donor; n=3).

CD3-CD28-activated PBLs

3.8 Expression of CXCR6 on CD3-CD28-activated PBLs

Although SEB T-lymphoblasts express functional CXCR6, they are only able to be used for a limited time, because the receptor expression decreases. CD3-CD28-activated PBLs have a more sustained expression of CXCR6 (figure 33). Typical expression was ~20% at day 7 and increased and was maintained up to day 15, when expression was at ~35-40%. Over 95% of the cells expressed CD3 and ~70% expressed CXCR4 (figure 33).

Chemotaxis of CD3-CD28-activated PBLs

3.9 CXCL16 induces Chemotaxis of CD3-CD28-activated PBLs

Since CXCR6 and its ligand CXCL16 have been implicated in the trafficking of activated T-lymphocytes, it was logical to investigate whether CD3-CD28-activated PBLs chemotax towards CXCL16. During the measurement of chemotaxis in CD3-CD28-activated PBLs, CXCL12 was used as a positive response because ~70% of the cells expressed its receptor, CXCR4, and CXCL12 is known to mediate chemotaxis of T-lymphocytes. In CD3-CD28-activated PBLs, CXCL12 exhibits a highly significant concentration dependent response which peaks at 10nM with a chemotactic index of ~ 6 (the chemotactic index is calculated by the division of the raw data by the basal level of chemotaxis, therefore a chemotactic index of 1 represents no chemotaxis) (figure 34). CXCL16 only induces minimal chemotaxis which is statistically significant at 10nM; however the response is only weakly concentration-dependent and the maximum chemotactic index observed is under 2. The difference between CXCL12 and CXCL16 in their chemotactic responses is not solely due to the percentage of cells expressing the relevant receptor because the magnitude of the difference does not correlate, although this would be a contributing factor.
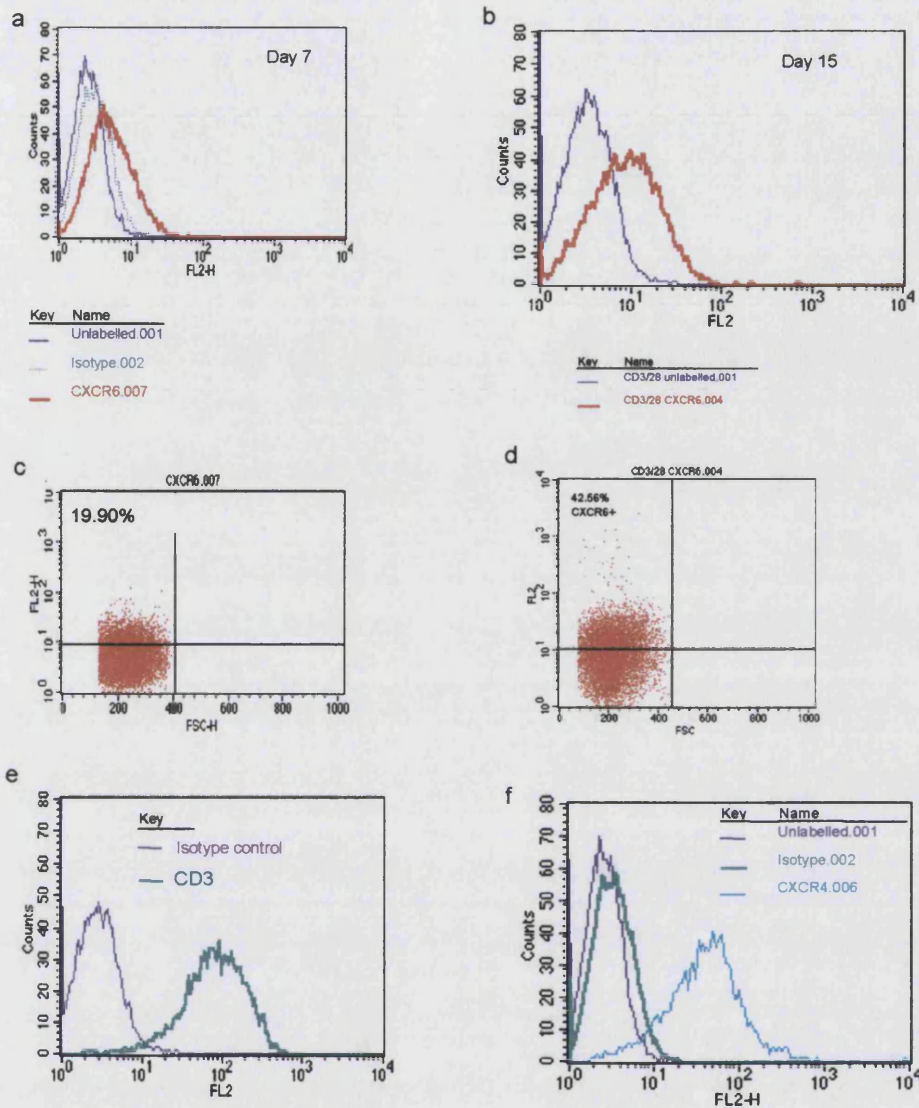
Figure 33. Expression of surface receptors on CD3-CD28- activated PBLs.

a, c. Expression of CXCR6 on CD3-CD28 activated PBLs on day 7 after isolation. Data represented as a histogram (panel a) and on dot plot (panel c). b, d. Expression of CXCR6 on CD3-CD28 activated PBLs on day 15 after isolation. e, Expression of CD3 on CD3-CD28 activated PBLs on day 7 after isolation. f, Expression of CXCR4 on CD3-CD28 activated PBLs on day 7 after isolation. Levels of CD3 and CXCR4 were maintained through out the culture period. Data represented as a histogram (panel b) and on dot plot (panel d). Cells were incubated with either 2.5µg/ml phycoerythrin-conjugated anti-CXCR6 antibody, 2.5µg/ml phycoerythrin-conjugated anti-CD3 antibody or 2.5µg/ml phycoerythrin-conjugated anti-CXCR4 antibody or isotype control for 30minutes. Samples were then analysed by flow cytometry. (Data shown is representative of one donor; n=5)
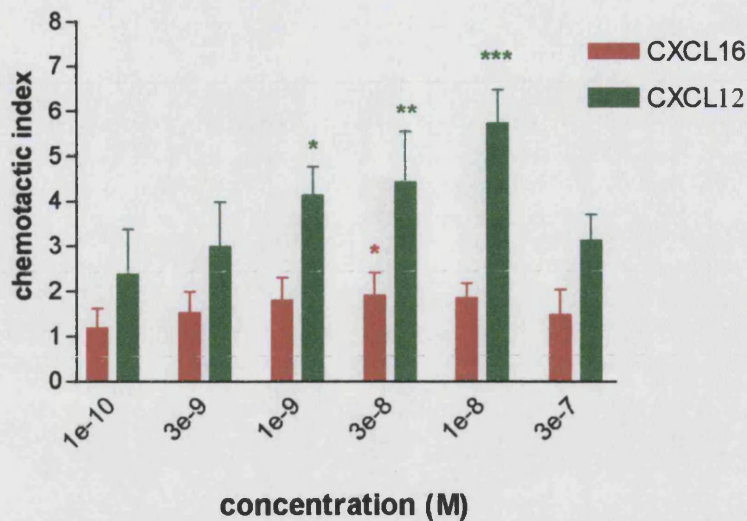
Figure 34. Chemotaxis of CD3-CD28-activated PBLs to CXCL16 and CXCL12.
PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Chemotaxis to CXCL16 and CXCL12 was measured using 96-well reusable chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Cell numbers were determined using Cell Titer 96 AQ$_{ueous}$ reagent and the chemotactic index was calculated from basal levels of chemotaxis. Data represents the mean +/- SD from 3 individual experiments. * = p< 0.05, ** = p< 0.01, *** = p< 0.001 compared to basal chemotaxis.

3.10 Inhibition of chemotaxis by PI3K inhibitors

PI3K has been widely demonstrated to be important in several aspects of cell migration; studies involving PI3K-knockout cells indicate PI3K-γ is important in neutrophil chemotaxis (Li *et al*, 2000) this data is supported by experiments using the PI3K inhibitors LY294002 and wortmannin (Curnock *et al*, 2003; Turner *et al*, 1995; Sotsios *et al*, 1999). Other studies have indicated that class 1A PI3Kβ and PI3Kδ are also involved in directional sensing. CXCR6 mediated chemotaxis is completely inhibited by the PI3K inhibitor, LY294002 (10μM) (figure 35). This inhibition is only significant at a CXCL16 concentration of 10nM. CXCL12 induced chemotaxis is only partially abrogated by LY294002, inhibiting chemotaxis to an index of ~ 2 and all concentrations of CXCL12 used. LY294002 preferentially inhibits class I PI3K isoforms, however at the concentration used, most isoforms would be inhibited; the results in CD3-CD28-activated PBLs suggest that CXCL12

utilises pathways not inhibited by LY294002, which combine with PI3K to induce chemotaxis. The PI3Kδ selective inhibitor, IC87114, does not significantly inhibit chemotaxis induced by either CXCL16 or CXCL12 (figure 35), indicating that this isoform may not be important in mediating chemotaxis of these cells to CXCL16 or CXCL12.
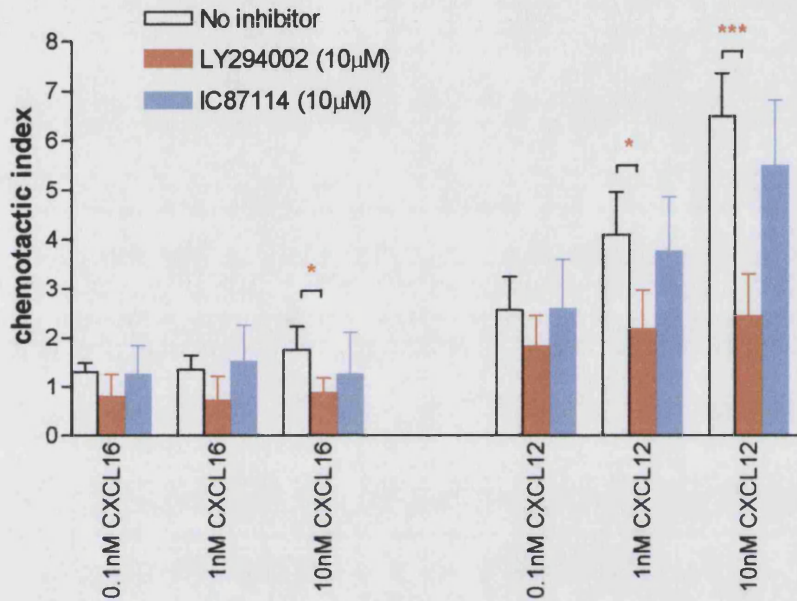
Figure 35. Inhibition of chemotaxis of CD3-CD28-activated PBLs to CXCL16 and CXCL12 by PI3K inibitors.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with 10μM LY294002 or 10μM IC87114 for 30 minutes. Chemotaxis to CXCL16 and CXCL12 was measured using 96-well reusable chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Cell numbers were determined using Cell Titer 96 AQ$_{ueous}$ reagent and the chemotactic index was calculated from basal levels of chemotaxis. Data represents the mean +/- SD from 3 individual experiments. * = p< 0.05, *** = p< 0.001 compared to basal chemotaxis.

Measurement of Actin Polymerisation - an indicator of chemotaxis?

Since migration of cells towards CXCL16 is difficult to assess using the available techniques, an alternative assay quantifying the amount of filamentous actin in a cell was used as a potential measure of chemotaxis. Phalloidin is a fungal toxin which binds to F-actin monomers in a 1:1 ratio. Fluorescent labelling of phalloidin allows levels of F-actin in individual cells to be measured using flow cytometry. Actin polymerisation occurs at the leading edge of chemotaxing cells, allowing the extension of lamellipodia and subsequent motility of the cell.

3.11 CXCR6-induced actin polymerisation

CXCL16 elicits both a time- and concentration dependent increase in actin polymerisation (figure 36). The response was almost instant with the maximum occurring at 15 seconds after stimulation of the cell with a concentration of 10nM CXCL16 and continually decreasing over 30 minutes. 10nM CXCL12 elicited a similar response which was maximal at 30 seconds and subsequently decreasing. The vehicle did not significantly induce actin polymerisation indicating that the responses observed following stimulation of the cells with CXC16 and CXCL12 were due to receptor activation (figure 36).

3.12 Activation of heterotrimeric G-proteins

There are four main classes of G protein α subunit, $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 12}$ which are coupled to downstream signalling cascades. Pertussis toxin (PTX) selectively inhibits activation of the $G_{\alpha i}$ isoform and can therefore determine whether GPCRs are coupled to it.

F-actin polymerisation entirely mediated by CXCR6 and CXCR4 is mediated via $G_{\alpha i}$ coupled receptors (figure 37a). The generation of F-actin is completely abolished following incubation of the cells with (pertussis toxin) PTX (100ng/ml) for 16 hours prior to stimulation. Incubation of these cells with the PTX vehicle did not affect the chemokine generated response (figure 37b). PTX slightly decreased the basal level of F-actin, however this was not significant.
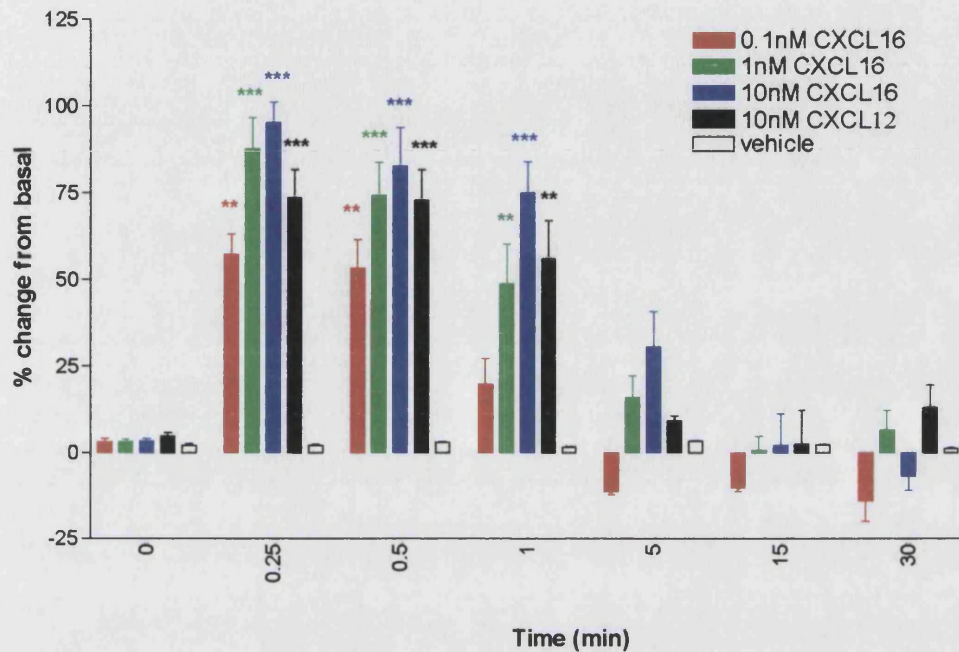
Figure 36. CXCL16 and CXCL12 increase actin polymerisation.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were activated by CXCL16 or CXCL12 for appropriate times and fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % change from basal fluorescence calculated from the median fluorescence +/- SD from 3 separate experiments. * = p< 0.05, ** = p< 0.01, *** = p< 0.001 compared to basal fluorescence.
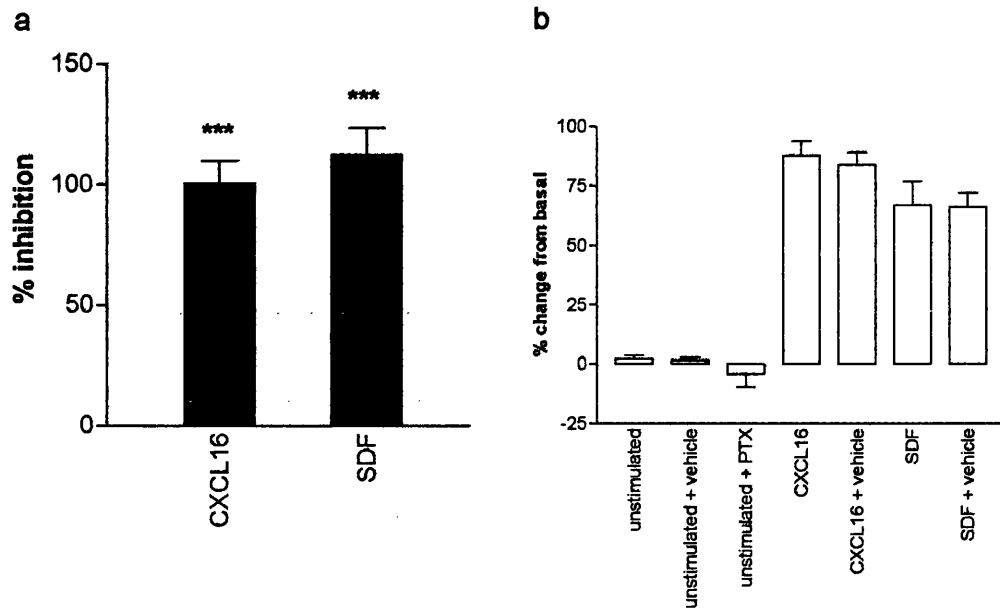
Figure 37. Pertussis toxin (PTX) inhibits CXCL16 and CXCL12 mediated actin polymerisation.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were incubated with PTX (100ng/ml) or vehicle (0.025% glycerol) for 16 hours. Cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for 30 seconds and fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 or CXCL12 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). *** = p< 0.001 compared to CXCL16 or CXCL12 response. (n=3)

3.13 Actin polymerisation is abrogated by PI3K inhibitors

PI3K isoforms are localised at the leading edge of polarised cells, and have been linked to F-actin assembly in *Dictyoostelium* and mammalian fibroblasts through activation of Rac. In CD3-CD28-activated PBLs, LY294002 is able to completely abolish CXCL16 induced actin polymerisation with an IC_{50} of 1.25μM (figure 38a). At the concentration shown to inhibit CXCL16-mediated chemotaxis in these cells, actin polymerisation is inhibited by ~85% of the maximum. The vehicle controls do not significantly affect either the basal levels of F-actin or the CXCL16 mediated increase (figure 38b).

Figure 38. LY294002 inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with LY294002 for 30 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). ** = p< 0.01 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

Wortmannin, another non-selective PI3K inhibitor, inhibits actin polymerisation in a concentration dependent response with an IC_{50} of 10.1nM (figure 39a). It reaches maximum inhibition at about 85% of the total actin polymerisation induced by CXCL16. The vehicle controls do not affect either the basal levels of F-actin or the CXCL16 mediated increase (figure 39b).

Figure 39. Wortmannin inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with wortmannin for 10 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). * = p< 0.05 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

IC87114, the selective PI3Kδ inhibitor had no significant effect on the chemotactic response to CXCL16 (figure 40), however it is able to inhibit actin polymerisation induced by CXCL16 to a maximum of ~70% with an IC_{50} of 2.3 μM (figure 40a). This suggests that PI3Kδ is possibly involved in cell polarisation, but does not have a significant role in chemotaxis. There is no significant effect of the vehicle controls on unstimulated or stimulated actin polymerisation (figure 40b).

Figure 40. IC87114 inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with IC87114 for 30 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). * = p< 0.05 compared to CXCL16 stimulated response, ** = p< 0.01 compared to CXCL16 stimulated response. (n=3)

3.14 mTOR is involved in actin polymerisation

Rapamycin inhibits mTOR, a protein phosphorylated by a number of proteins including PKB; phosphorylation of mTOR results its inactivation. Experiments in smooth muscle cells and fibroblasts have found that rapamycin is able to inhibit cell migration (Poon *et al*, 1996) and stress fibre formation (Crouch *et al*, 1997). Rapamycin partially inhibits F-actin formation in CXCL16 stimulated CD3-CD28-activated PBLs (figure 41a); it also slightly increased basal F-actin, but this was not significant (figure 41b).

Figure 41. Rapamycin inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with rapamycin for 60 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). * = p< 0.05 compared to CXCL16 stimulated response. (n=3)

3.15 Actin polymerisation is inhibited by PKC inhibitors

PKC isoforms have been found to be localised with various cytoskeletal proteins, including F-actin. These associations are isoform-, stimuli- and cell type specific allowing complex regulation of the responses. PKC activity has been identified in the cytoskeleton of both resting and activated cells. There are a number of pharmacological inhibitors of protein kinase C isoforms with varying specificities. RO320432 inhibits cPKC and nPKC isoforms, whereas Gö6976 and rottlerin are described as selective inhibitors of cPKC and PKCδ, respectively, although there are reports suggesting that rottlerin is also able to inhibit PKCθ. In CD3-CD28-activated PBLs, Ro320432 significantly inhibits CXCL16 mediated actin polymerisation in a concentration dependent manner with a maximum inhibition of ~50% of the stimulated response (figure 42a).
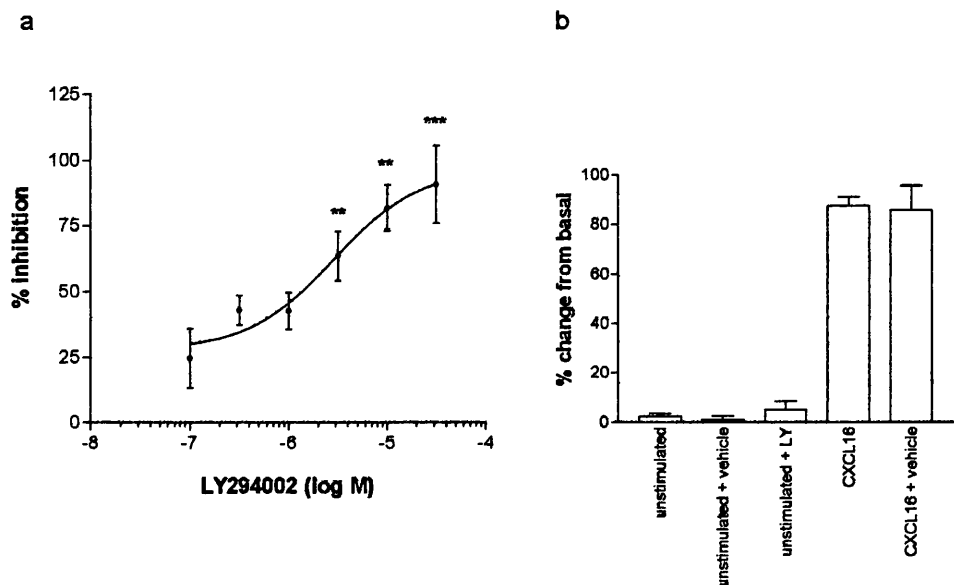

Figure 42. RO320432 inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with RO320432 for 30 minutes and were activated by CXCL16 for appropriate times. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). * = p< 0.05 compared to CXCL16 stimulated response, ** = p< 0.01 compared to CXCL16 stimulated response. (n=3)

At 3nM, Gö6976 inhibits actin polymerisation by ~40%, but at 30nM this decreases slightly (figure 43a) suggesting a role for cPKC isoforms in actin polymerisation by CXCL16. Rottlerin, at 1-10μM, inhibits F-actin polymerisation by ~70% indicating that nPKCs are possibly more potent at inducing actin polymerisation. The vehicle controls do not significantly affect either unstimulated or stimulated actin polymerisation (figures 42b & 43b).
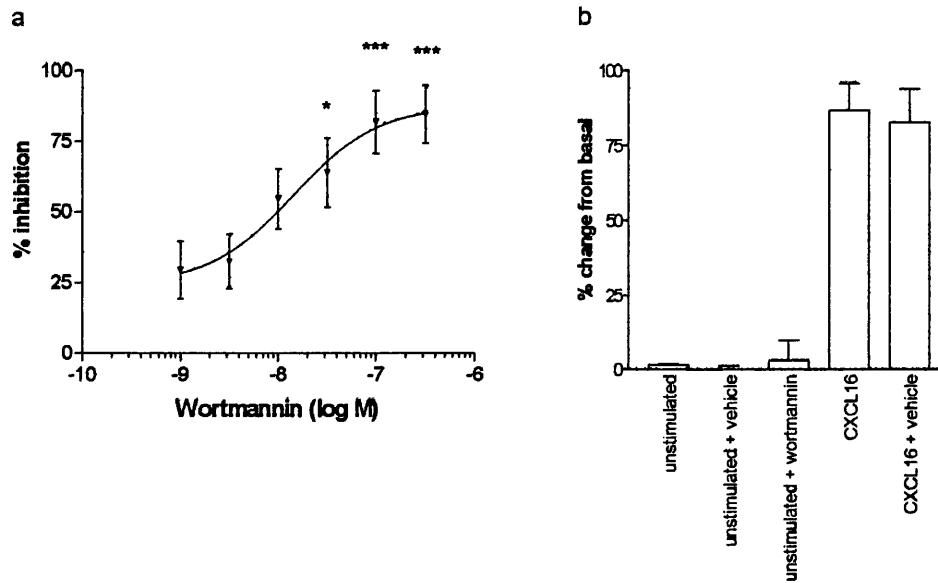

Figure 43. PKC Inhibitors reduce actin polymerisation in CD3-CD28-activated PBLs.
PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with either Gö6976 (Go) or rottlerin (Rott) for 30 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). * = p< 0.05 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

3.16 MAPK pathway is linked to actin polymerisation

The MAPK pathway can be activated downstream of numerous, complex signalling cascades; some of which are involved in actin polymerisation, for example PKC can activate Raf1, a MAPKKK, which activates Mek and ERK1/2.

Inhibition of Mek, by PD98059, induces a highly significant concentration dependent inhibition of CXCL16 mediated actin polymerisation with an IC_{50} of 3.8µM (figure 44a). PD98059 slightly inhibits basal polymerisation of actin, but this was not significant (figure 44b). The inhibitor vehicle did not affect either stimulated or unstimulated actin polymerisation.
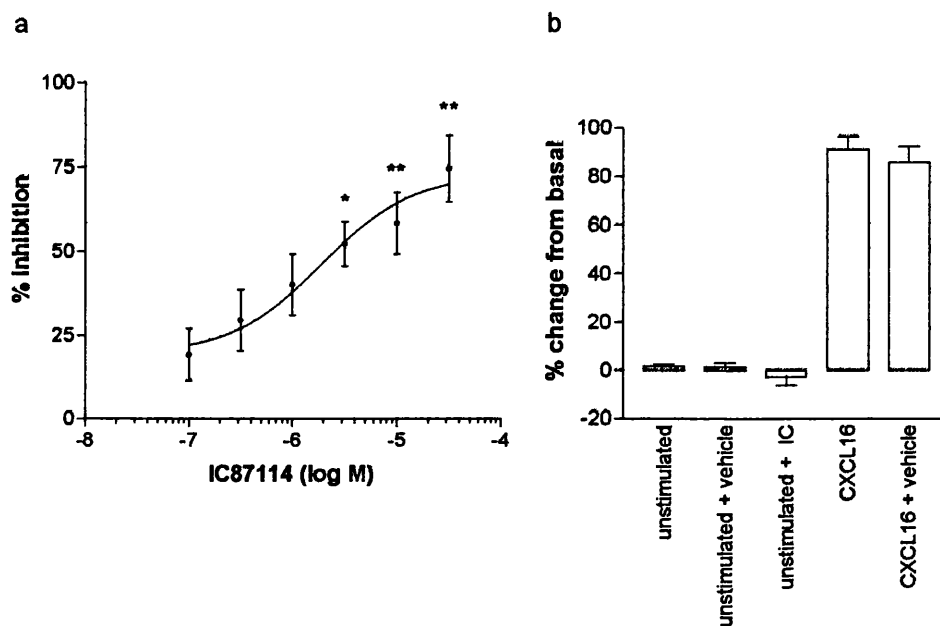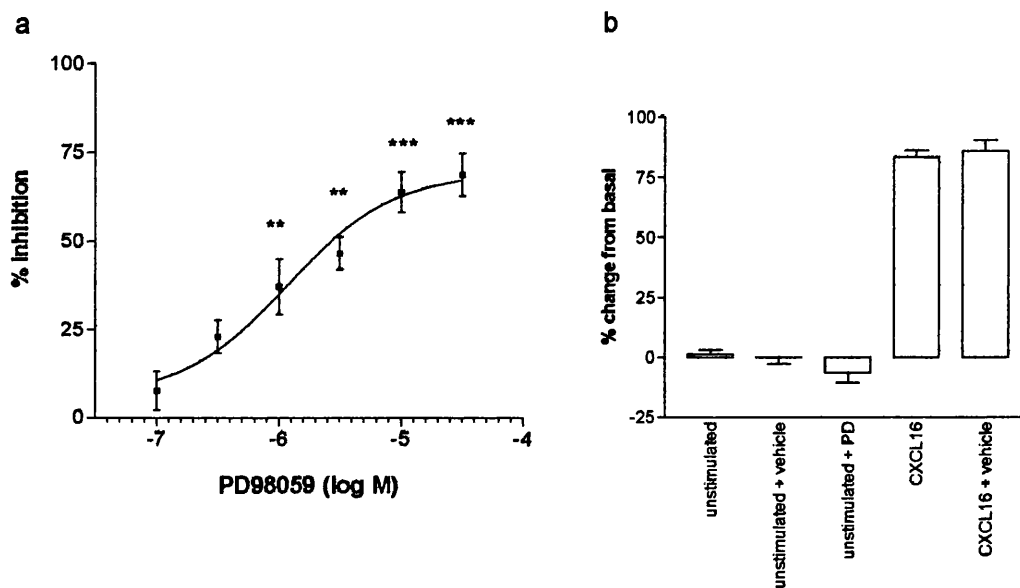

Figure 44. PD98059 inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with PD98059 for 60 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). ** = p< 0.01 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

3.17 Rho, Rac and Cdc42 are involved in actin polymerisation

Small GTPases of the Rho family (including Rho, Rac and Cdc42) are critical many aspects of cell behaviour, such as lamellipod formation, gradient sensing and gene transcription. In the pseudopod, PI3K and PI(3,4,5)P$_3$ colocalise with the small GTPase Rac1 (Huang *et al*, 2003; Gardiner *et al* 2002). Rac1 and Cdc42, and their downstream effectors, PAK, WASP and WAVE stimulate a complex containing Arp2/3 which induces actin polymerisation. While Rac1 is important in polymerisation, Cdc42 is important in determining correct cell polarity with respect to the external signal. Rho is also critical in cytoskeletal reorganisation, via its downstream effectors, ROCK and mDia.

It is therefore not surprising that, inhibition of ROCK by Y27632 decreases actin polymerisation mediated by CXCR6 (figure 45a). The maximum CXCL16 mediated actin polymerisation is inhibited by Y27632 at an IC$_{50}$ of 389nM, however this inhibition of ROCK only elicits a maximal inhibition of ~60%, indicating the requirement of the other proteins in initiating actin polymerisation.

Lethal toxin B (LT-B) from *Clostridium difficile* preferentially inhibits RhoA over Cdc42 and Rac. LT-B inhibits CXCL16-activated actin polymerisation in a concentration dependent manner, which produces ~75% inhibition of the stimulated response at 10ng/ml, the hightest concentration of toxin used (figure 46a). Lethal toxin 82 (LT-82) from *Clostridium sordellii* preferentially inhibits Rac1 over Cdc42 and Rho. At 10ng/ml, LT-82 completely inhibits actin polymerisation induced by CXCL16 (figure 47a). The IC$_{50}$ values for Tox B and LT-82 are 0.12ng/ml and 0.11ng/ml respectively. The vehicle did not significantly affect actin polymerisation however both of the toxins reduced basal levels of F-actin (figure 46b & 47b).
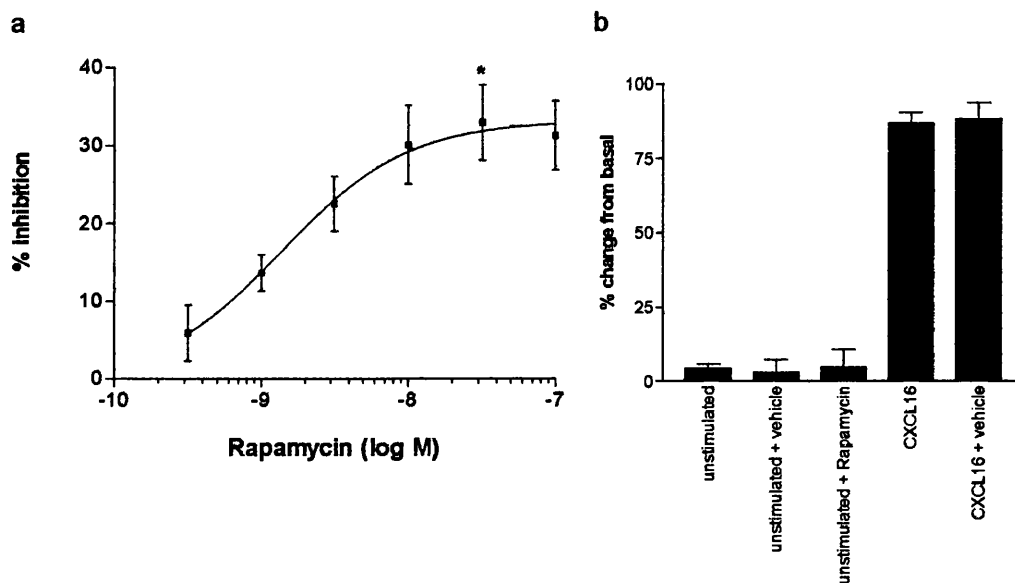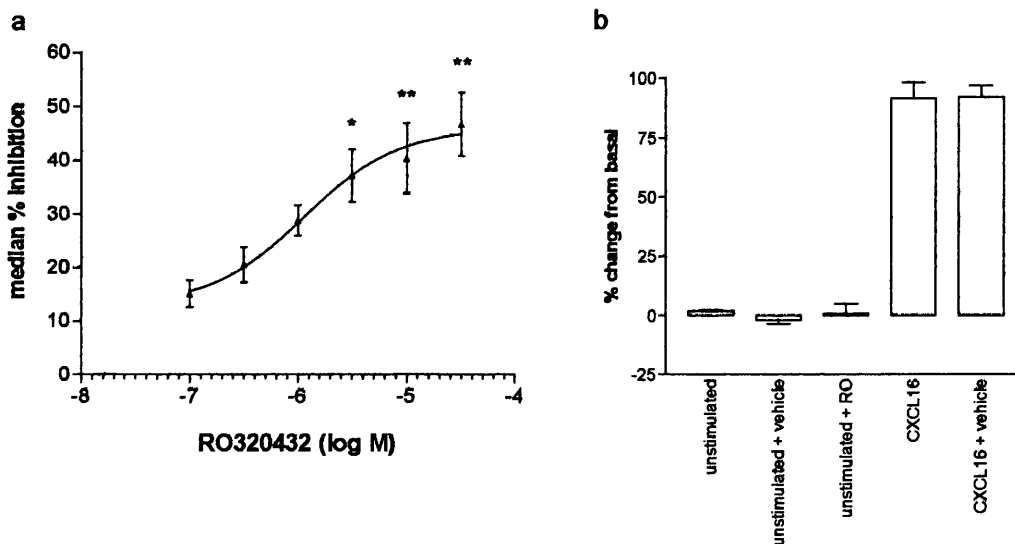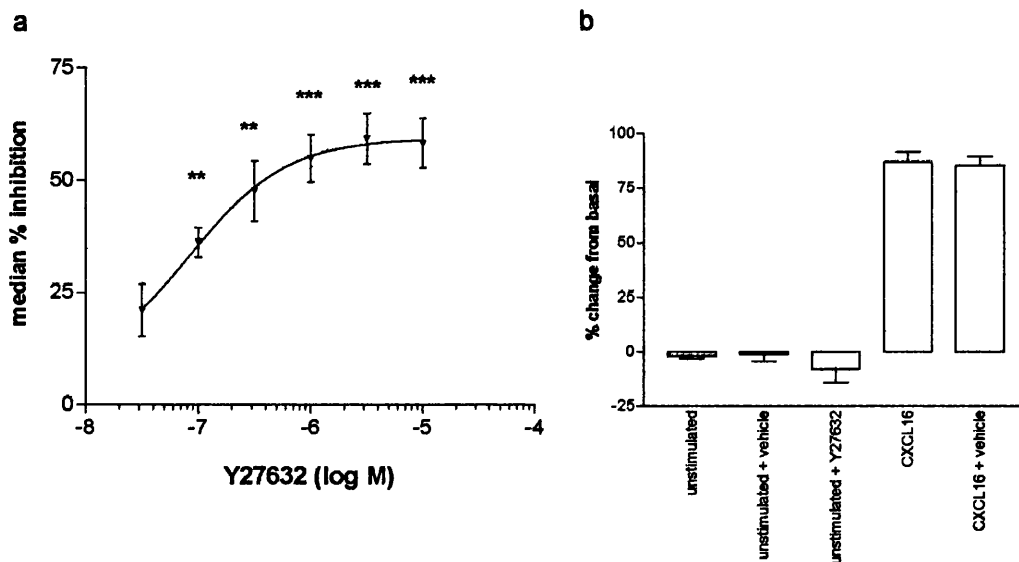
Figure 45. Y27632 inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with Y27632 for 60 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). ** = p< 0.01 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)
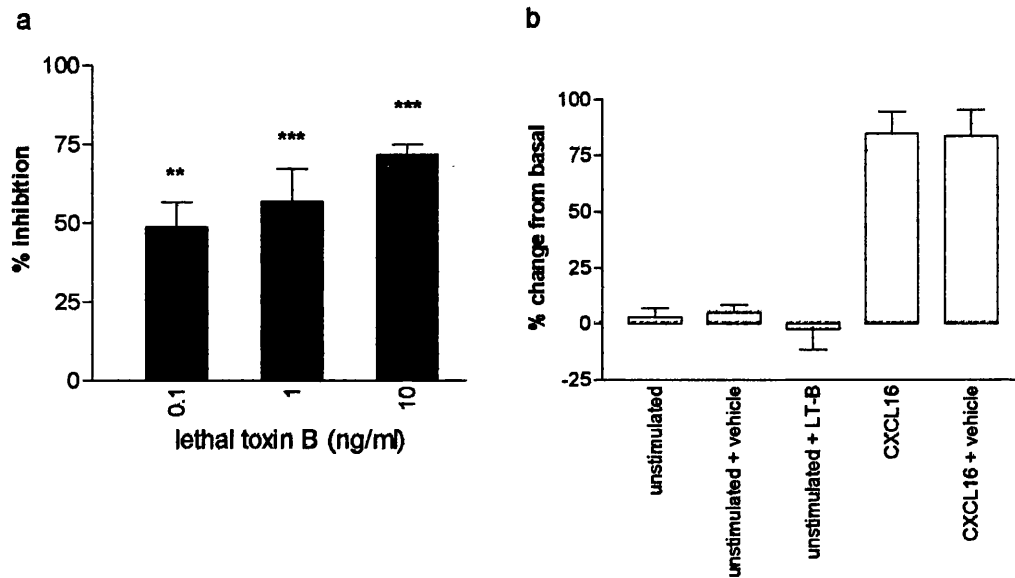
Figure 46. Lethal toxin-B inhibits actin polymerisation in CD3-CD28-activated PBLs.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with lethal toxin-B (LT-B) for 60 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). ** = p< 0.01 compared to CXCL16 stimulated response, *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

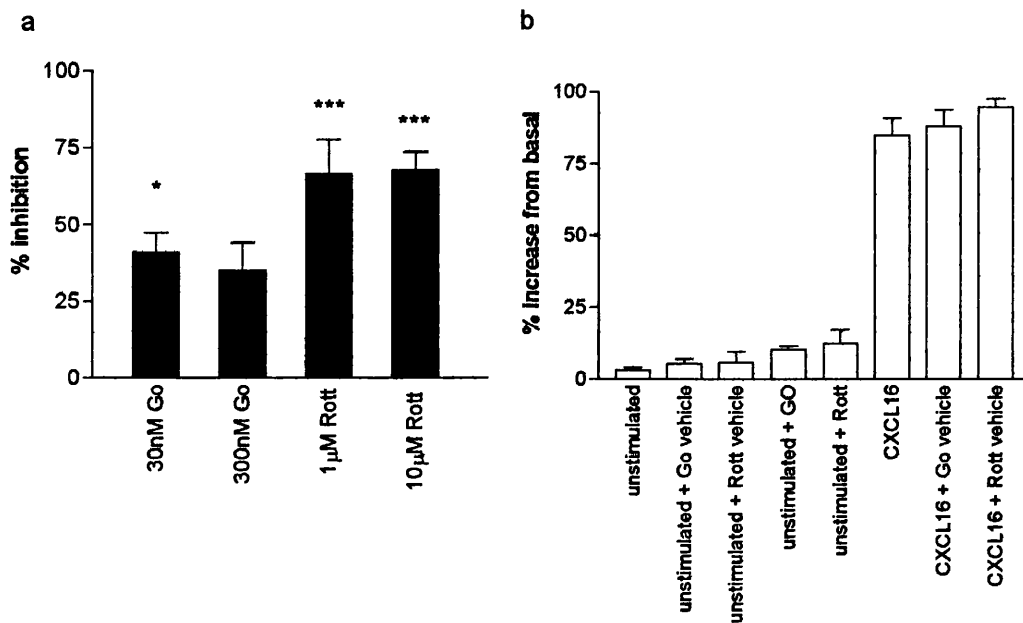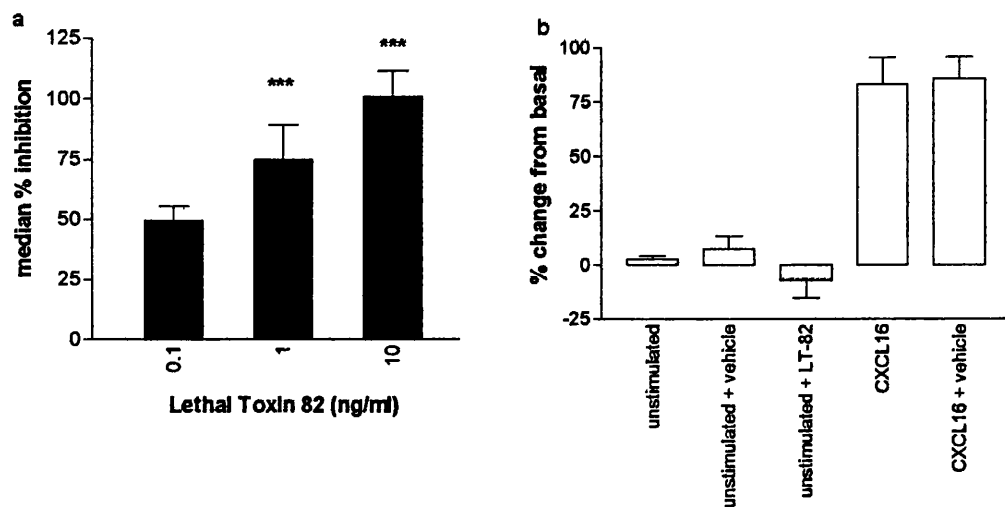**Figure 47. Lethal toxin-82 inhibits actin polymerisation in CD3-CD28-activated PBLs.**
PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. Cells were pretreated with lethal toxin-82 (LT-82) for 60 minutes and were activated by CXCL16 for 30 seconds. Cells were fixed with 3.7% formaldehyde and permeabilised using 0.1% (w/v) triton. Actin polymerisation was measured using FITC-conjugated phalloidin. Data represented as the % inhibition of the CXCL16 response calculated from the median fluorescence +/- SD (a) or the % change from basal fluorescence calculated from the median fluorescence +/- SD (b). *** = p< 0.001 compared to CXCL16 stimulated response. (n=3)

Analysis of signalling pathways coupled to CXCR6 in CD3-CD28-activated PBLs

CD3-CD28-activated PBLs express CXCR6 which is coupled to signal transduction mechanisms that can regulate actin polymerisation. Further investigation to elucidate the interactions between these pathways indicated that CXCR6 is coupled to pathways utilised by other receptors, however it also revealed that CXCR6 may be coupled to pathways not currently reported for other chemokine receptors in these cells.

3.18 CXCR6 induces phosphorylation of PKB and MAPK

The MAPK pathway and PKB, as an indirect marker of PI3K activation, are acknowledged as two of the major signal transduction pathways in most cells. Their activation can regulate a number of functional responses conserved within cells. The full activation of a number of these molecules requires the phosphorylation of specific residues; this may induce a conformational change, releases an inhibitory mechanism or exposes further activation sites.

As described for some of the other cell types used so far, in CD3-CD28-activated PBLs CXCR6 can mediate phosphorylation of both the MAPK and PKB signalling pathways (figure 48). CXCL16 phosphorylates ERK1/2 and PKB in both a concentration and time dependent manner detectable at 1 and 2 minutes and then declines; it is maximal at 10nM. This response is initiated by the ligand, indicated by the vehicle control which did not elicit any response.

Further evidence of the activation of these pathways is the phosphorylation of downstream effectors. In CD3-CD28-activated PBLs, CXCL16 elicits a greater and more sustained phosphorylation of PKB than CXCL10 but the response at 10nM is maximal at 1 minute, unlike the response from CXCL12 which peaks at 2 minutes (figure 49). GSK3β can be phosphorylated by PKB at ser9 following ligation of chemokine receptors or the TCR. CXCL16 stimulation increases phosphorylation of GSK3β in a time-dependent manner, a response seen with both CXCL12 and CXCL10 (figure 49). Phosphorylation of GSK3β induced by CXCL16 is not as great as the response from both CXCL12 and CXCL10 however the kinetics display a similar pattern reaching a maximum at 2 minutes.
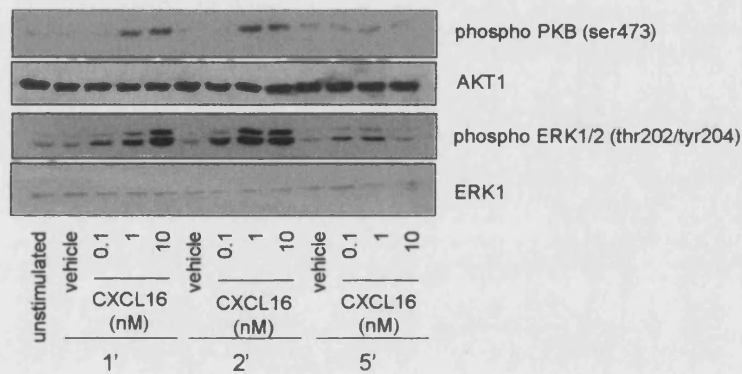
Figure 48. CXCR6 is coupled to signal transduction pathways in CD3-CD28-activated PBLs

Time-dependent phosphorylation of protein kinase B (PKB) (top panel) and ERK (extracellular signal-regulated kinase) (lower panel) by CXCL16 (0.1-10nM). Following stimulation ($5x10^6$ cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-PKB (Ser473) or -ERK1/2 (thr42/tyr44) antibody and visualised by chemoluminescence. Equal loading of gels was verified using anti-ERK1 or anti-AKT1 antibodies. (Data shown is representative of one donor; n=3).
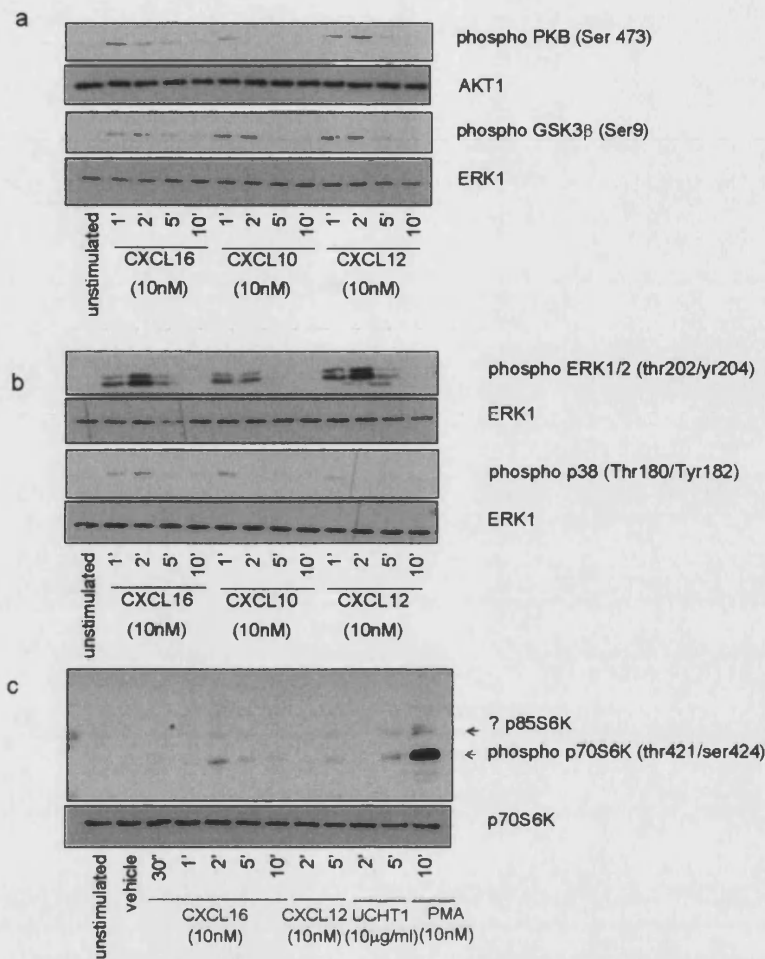
Figure 49. Chemokine receptor activated signal transduction pathways in CD3-CD28-activated PBLs

a, Time-dependent phosphorylation of protein kinase B (PKB) (ser473) and glycogen synthase 3β (GSK3β) (ser9) by CXCL16 (10nM), CXCL10 (10nM) and CXCL12 (10nM). b, Time-dependent phosphorylation of ERK (extracellular signal-regulated kinase) (tyr202/thr204) and p38 MAPK (thr180/tyr182) by CXCL16 (10nM), CXCL10 (10nM) and CXCL12 (10nM). c, Time-dependent phosphorylation of p70S6K (thr421/ser424) by CXCL16 (10nM), CXCL12 (10nM), UCHT-1 (10μg/ml) and PMA (Phorbol 13-myristate 12-acetate) (10nM). This antibody also possibly recognises the p70S6K homologue, p85S6K. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation with the appropriate agonist. Following stimulation (5x10^6 cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-specific antibody to the relevant residues and visualised by chemoluminescence. Equal loading of gels was verified using anti-AKT1, anti-ERK1 or anti-p70S6K antibodies. (Data shown is representative of one donor; n=3).

As discussed, CXCL16 activates the MAPK pathway, shown by the phosphorylation of ERK1/2. In a similar pattern to that seen in the phosphorylation of PKB, the response from CXCL10 less sustained than that of CXCL16 and CXCL12; CXCL12 also elicits a stronger phosphorylation than that of CXCL16 (figure 49). Another member of the MAPK family is p38, a protein involved in activation of gene transcription. CXCL16 induced phosphorylation of this protein is more prolonged than that of CXCL12 and CXCL10 (figure 49).

One of the proteins that can be activated downstream of MAPK and PI3K is p70S6K. As shown in Th1 cells, PMA is a potent activator of this protein and has been used as a positive control along with the UCHT1 antibody which activates CD3 (figure 49). CXCL16 phosphorylation of p70S6K at Thr421 and Ser424 peaks at 2 minutes; these are residues known to be phosphorylated by ERK1/2 and this phosphorylation enables the activation of p70S6K following phosphorylation of other residues. CXCL12, like UCHT1 generates a weaker signal which appears at 5 minutes.

3.19 CXCR6 is coupled to $G_{\alpha i}$

Actin polymerisation induced by CXCL16 and CXCL12 was completely abrogated following incubation of cells with PTX; CXCR6 mediated signal transduction is also dependent on $G_{\alpha i}$. PTX completely abolishes the phosphorylation of ERK1/2 and PKB induced by CXCL16 (figure 50). Cells were incubated with either PTX or the vehicle for 16 hours prior to stimulation; cells incubated with the vehicle slightly increased basal levels of PKB phosphorylation in unstimulated cells. This was not observed with the addition of the CXCL16 vehicle or with cells treated with PTX.
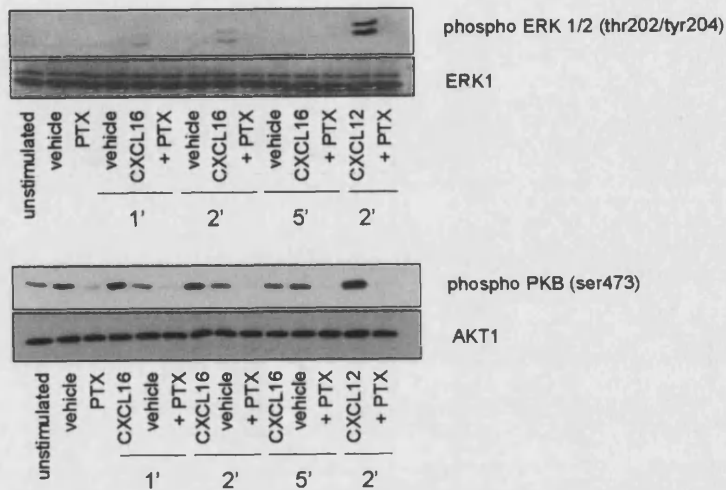
Figure 50. Pertussis toxin (PTX) inhibits CXCL16 and CXCL12 mediated signal transduction.

Pertussis toxin (PTX) inhibits the time-dependent phosphorylation of ERK (extracellular signal-regulated kinase) (tyr202/thr204) and protein kinase B (PKB) (ser473) induced by CXCL16 (10nM) and CXCL12 (10nM). PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were incubated with pertussis toxin (100ng/ml) or vehicle (0.025% glycerol) for 16 hours and cells were activated with CXCL16 (10 nM) or CXCL12 (10 nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.

3.20 CXCL16 induces phosphorylation of down stream effectors of PI3K

There are a number of signalling molecules activated downstream of PI3K; of these, PKB is sometimes used as an indirect measure of PI3K activation. As has been previously reported, CXCL16 induces phosphorylation of PKB in T-lymphocytes. In CD3-CD28-activated PBLs inhibitors of PI3K, LY294002, wortmannin and IC87114, are all able to completely inhibit phosphorylation of PKB at ser473 in a concentration dependent manner (figures 51, 52 & 53). IC87114, a selective PI3Kδ inhibitor is only able to mediate complete inhibition of PKB phosphorylation at the maximum concentration used; the specificity for this compound at this concentration is not known and may therefore indicate that the PI3Kδ isoforms is only partially responsible for phosphorylation of PKB.

These PI3K inhibitors partially inhibit CXCL16-induced ERK phosphorylation in CD3-CD28-activated PBLs (figures 51, 52 & 53). Phosphorylation of ERK1/2 is more sensitive to inhibition by wortmannin, than by LY294002 or IC87224. This contradicts a recent report in which CXCL16-induced activation of ERK in intestinal epithelial cells was independent of PI3K, but dependent on Mek (Brand *et al*, 2002); however this could be due to differences in cell types.

LY294002, wortmannin and IC87114 are able to reduce phosphorylation of GSK3β to basal levels at 10μM, 100nM and 30μM respectively (figures 51, 52 & 53); at this concentration the IC87114 may inhibit other PI3K isoforms. S6-ribosomal protein is a substrate downstream of PKB and ERK1/2, all three inhibitors decrease CXCL16-induced phosphorylation of S6-ribosomal protein in a concentration dependent response although none of them completely inhibit the phosphorylation (figures 51, 52 & 53). However, S6 ribosomal protein can be activated down stream of a number of pathways, not all of which are PI3K-dependent.
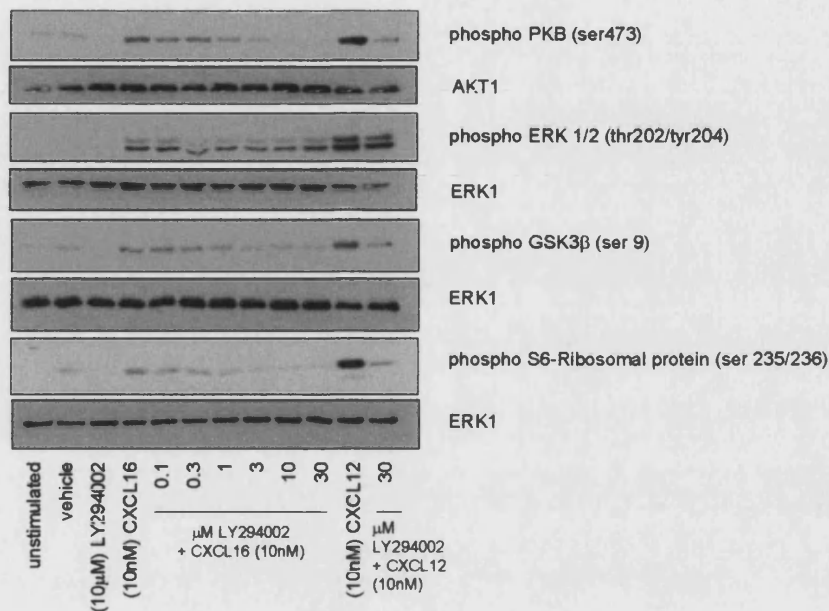
Figure 51. LY294002 inhibits CXCR6 signal transduction CD3-CD28-activated PBLs

The PI3K inhibitor LY294002 (0.1-30µM) attenuates the time-dependent phosphorylation of protein kinase B (PKB) (ser473), glycogen synthase 3β (GSK3β) (ser9), ERK (extracellular signal-regulated kinase) (tyr202/thr204) and S6-ribosomal protein (ser235/236) induced by CXCL16 (10nM) and CXCL12 (10nM). PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs ($5x10^6$ cells/point) were incubated with LY294002 or vehicle for 30 minutes and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.
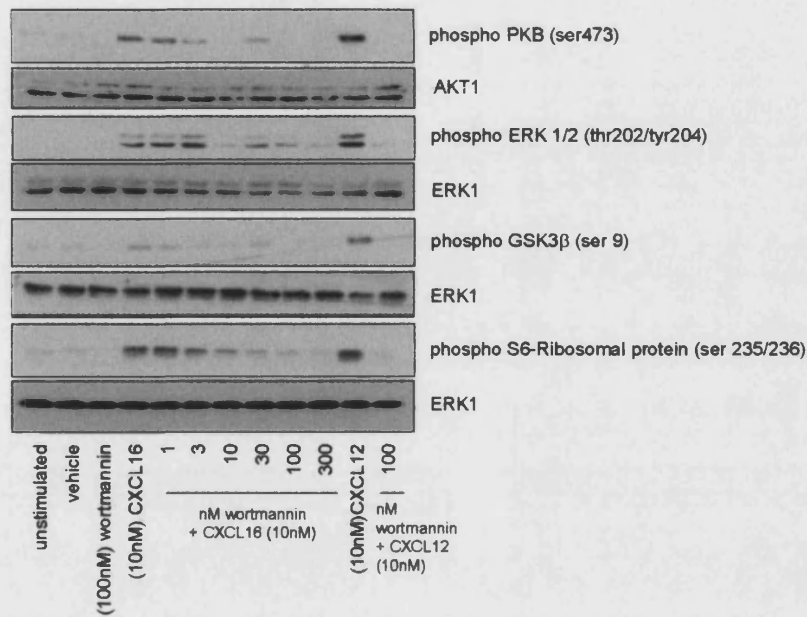
Figure 52. Wortmannin inhibits CXCR6 signal transduction CD3-CD28-activated PBLs
The PI3K inhibitor wortmannin (1-300nM) attenuates the time-dependent phosphorylation of protein kinase B (PKB) (ser473), glycogen synthase 3β (GSK3β) (ser9), ERK (extracellular signal-regulated kinase) (tyr202/thr204) and S6-ribosomal protein (ser235/236) induced by CXCL16 (10nM) and CXCL12 (10nM). PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs (5x10^6 cells/point) were incubated with wortmannin or vehicle for 10 minutes in the dark and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.
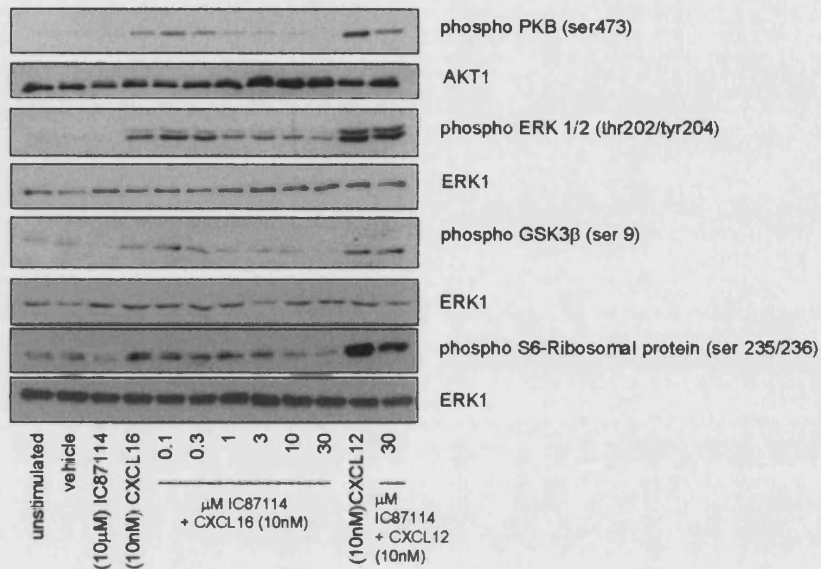
Figure 53. IC87114 inhibits CXCR6 signal transduction CD3-CD28-activated PBLs.
The PI3K inhibitor IC87114 (0.1-30µM) attenuates the time-dependent phosphorylation of protein kinase B (PKB) (ser473), glycogen synthase 3β (GSK3β) (ser9), ERK (extracellular signal-regulated kinase) (tyr202/thr204) and S6-ribosomal protein (ser235/236) induced by CXCL16 (10nM) and CXCL12 (10nM). PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs ($5x10^6$ cells/point) were incubated with IC87114 or vehicle for 30 minutes and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.

3.21 CXCL16 Activates Rapamycin-Sensitive Signalling Pathways

mTOR is a protein which can be phosphorylated by PKB resulting in its activation. mTOR has been reported to phosphorylate Thr389 on p70S6K, required for full activation. Inhibition of mTOR by rapamycin completely abolishes the phosphorylation of p70S6K at thr421 and ser424 induced by CXCL16 in a concentration-dependent manner (figure 54). CXCL12 induced phosphorylation of p70S6K is only partially inhibited by rapamycin, indicating that the chemokines may be linked to this protein through different mechanisms. Further evidence of this comes from S6-ribosomal protein, a downstream target of p70S6K, although it can be phosphorylated by p90RSK downstream of MAPK. The CXCL16 induced phosphorylation of S6-ribosomal protein is inhibited by rapamycin in a concentration dependent manner, however rapamycin has no effect on CXCL12 induced S6-ribosomal protein phosphorylation (figure 54).

3.22 CXCL16 activates cPKC and nPKC dependent signalling pathways

PKC isoforms can be activated by a number of stimuli and can regulate different signalling pathways; downstream effectors of PKC include members of the MAPK family although these can be either activated or inhibited depending on the activating stimuli, cell type and isoform activated. cPKCs can activate Raf which ultimately leads to phosphorylation of ERK, PKCδ has also been shown to activate ERK1/2. CXCL16 induced phosphorylation of ERK1/2 is inhibited by Gö6976 and rottlerin, but not RO320432 (figure 55). Gö6976 inhibits ERK1/2 more strongly at 30nM than 300nM, while rottlerin is able to completely inhibit phosphorylation of ERK1/2. PKC can also phosphorylate p70S6K via either MAPK dependent or independent pathways. All three inhibitors decrease CXCL16 mediated phosphorylation of p70S6K. Again, Gö6976 mediated inhibition of p70S6K is stronger at 30nM than 300nM, while rottlerin and RO320432 are able to completely inhibit phosphorylation. RO320423 inhibition of p70S6K phosphorylation therefore appears to function independent of MAPK signalling. A substrate of p70S6K is S6-ribosomal protein, unlike its inhibition of phosphorylation of ERK1/2 and p70S6K or inhibition of actin polymerisation, Gö6976 inhibits S6-ribosomal protein more strongly at 300nM than at 30nM indicating that one of the isoforms it inhibits is able to activate S6-ribosomal protein independently of MAPK and p70S6K (figure 54). Ro320432 and rottlerin completely inhibit S6-ribosomal protein phosphorylation at 10µM and 30µM respectively.
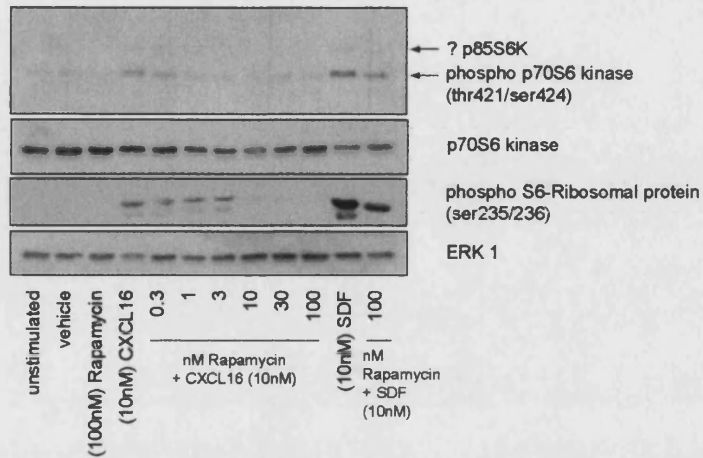
Figure 54. Rapamycin inhibits CXCR6 signal transduction in CD3-CD28-activated PBLs.

Rapamycin (0.3-100nM) attenuates the time-dependent phosphorylation of p70S6K (thr421/ser424) and S6-ribosomal protein (ser235/236) induced by CXCL16 (10nM) and CXCL12 (10nM). The p70S6K antibody also possibly recognises the p70S6K homologue, p85S6K. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs (5×10^6 cells/point) were incubated with rapamycin or vehicle for 60 minutes and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.
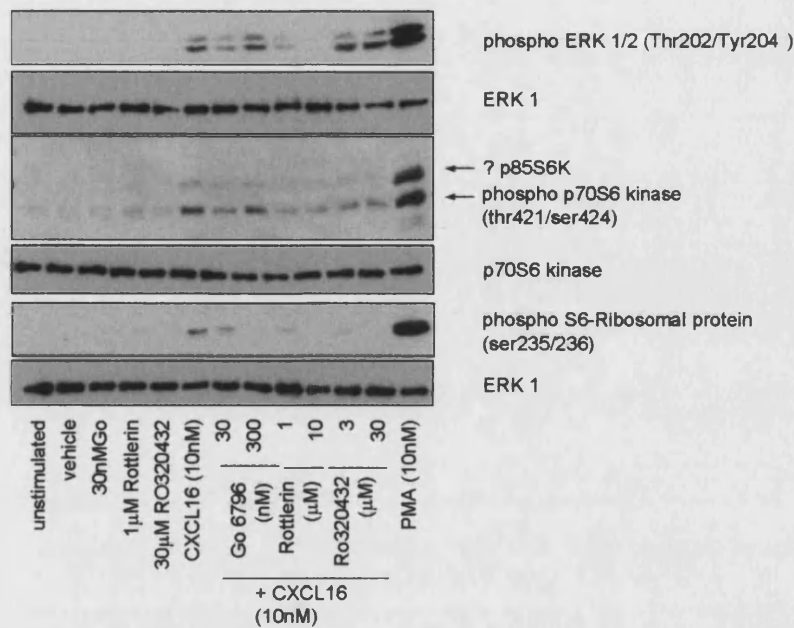
Figure 55. Inhibition of CXCR6 mediated signal transduction by PKC inhibitors in CD3/CD28 lymphoblasts

The PKC inhibitors Gö6976, rottlerin and RO320432 attenuate the time-dependent phosphorylation of ERK (extracellular signal-regulated kinase) (tyr202/thr204), p70S6K (thr421/ser424) and S6-ribosomal protein (ser235/236) induced by CXCL16 (10nM). PMA (Phorbol 13-myristate 12-acetate) (10nM) was used as a positive control to verify phosphoryaltion of the proteins. The p70S6K antibody also possibly recognises the p70S6K homologue, p85S6K. RO320432 inhibits cPKC and nPKC isoforms, whereas Gö6976 and rottlerin are described as selective inhibitors of cPKC isoforms and PKCδ, respectively.

PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs (5×10^6 cells/point) were incubated with Gö6976 (30-300nM), rottlerin (1-10μM), RO320432 (3-30μM) or vehicle for 30 minutes and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.

3.23 CXCL16 Activates MAPK

As has been previously mentioned, CXCL16 can mediate phosphorylation of MAPK pathways. The phosphorylation of ERK1/2 has been shown in other cells to be independent of PI3K, but dependent on Mek (Brand *et al*, 2002). In CD3-CD28-activated PBLs, CXCL16 mediated phosphorylation of ERK1/2 completely abrogated by inhibition of Mek with PD98059 (figure 56). Phosphorylation of GSK3β is also sensitive to inhibition of Mek in CXCL16 or CXCL12 stimulated cells (figure 56).
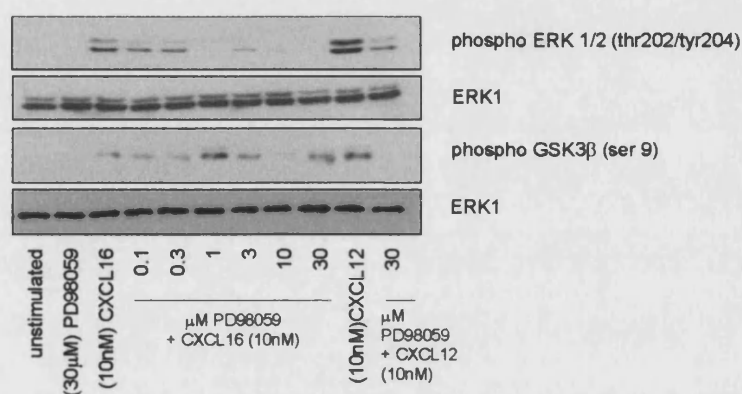
Figure 56. PD98059 inhibits CXCR6 signal transduction in CD3-CD28-activated PBLs

The Mek1/2 inhibitor PD98059 (0.1-30μM) attenuates the time-dependent phosphorylation of ERK (extracellular signal-regulated kinase) (tyr202/thr204) and glycogen synthase 3β (GSK3β) (ser9) induced by CXCL16 (10nM) and CXCL12 (10nM). PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation. CD3-CD28-activated PBLs (5×10^6 cells/point) were incubated with PD98059 or vehicle for 60 minutes and cells were activated with CXCL16 (10nM) or CXCL12 (10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. Data representative of 3 separate experiments.

3.24 CXCR6 can induce NF-κB phosphorylation

A large number of signal transduction pathways ultimately have an effect on transcription factors. CXCL16 activates a number of these pathways (PKB, MAPK, and Cdc42/Rac1) and CXCR6+ T-lymphocytes are upregulated in many chronic inflammatory conditions. It has recently been published that CXCL16 has pro-inflammatory properties and can activate the transcription factor NF-κB and induce production of TNF-α in smooth muscle cells (Chandresakaar, *et al*, 2005). It is therefore possible that CXCR6 may be coupled to transcription factor activation in T-lymphocytes.

The canonical NF-κB pathway involves degradation of the inhibitory molecule IκB which allows phosphorylation of p65 NF-κB at a number of residues. The significance of phosphorylation of these sites in determining transcriptional activity of p65 NF-κB has been widely discussed. *Okazaki et al* suggests that ser 276 is the most important marker of p65 NF-κB activation in murine embryonic fibroblasts; however in T cells it is thought that phosphorylation of ser536 is the most accurate marker of p65 NF-κB activation (Mattioli *et al*, 2004). Although CXCL12 has previously been shown to activate NF-κB in murine astrocytes (Han Y *et al*, 2001), in CD3-CD28-activated PBLs, neither CXCL16 nor CXCL12 induce phosphorylation of p65 NF-κB on ser276 or induce degradation of IκBα (figure 57 a, b, c). Costimulation of CD28, with 9.3 antibody, and PMA, in the same cells, induces both degradation of IκBα and phosphorylation of ser 276 in a time-dependent manner (figure 57 a & b). However, CXCL16 but not CXCL12, induces a time dependent phosphorylation of p65 NF-κB at ser536 which is maximal at 30 minutes (figure 57d). This phosphorylation is not as strong as the response elicited by stimulation of cells with PMA and 9.3 antibody. Phosphorylation of ser536 was measured in cytosolic and nuclear fractions separately (figure 57e). Cytosolic phosphorylation of p65 increased up to 120 minutes following CXCR6 activation; an increase of phosphorylated p65 was also seen in the nucleus after 90 minutes. This activation of p65 indicates that CXCR6 is possibly coupled to the canonical pathway, although IκBα is not degraded, activation of p65 complexed with other members of the IκB may occur following CXCR6 ligation.
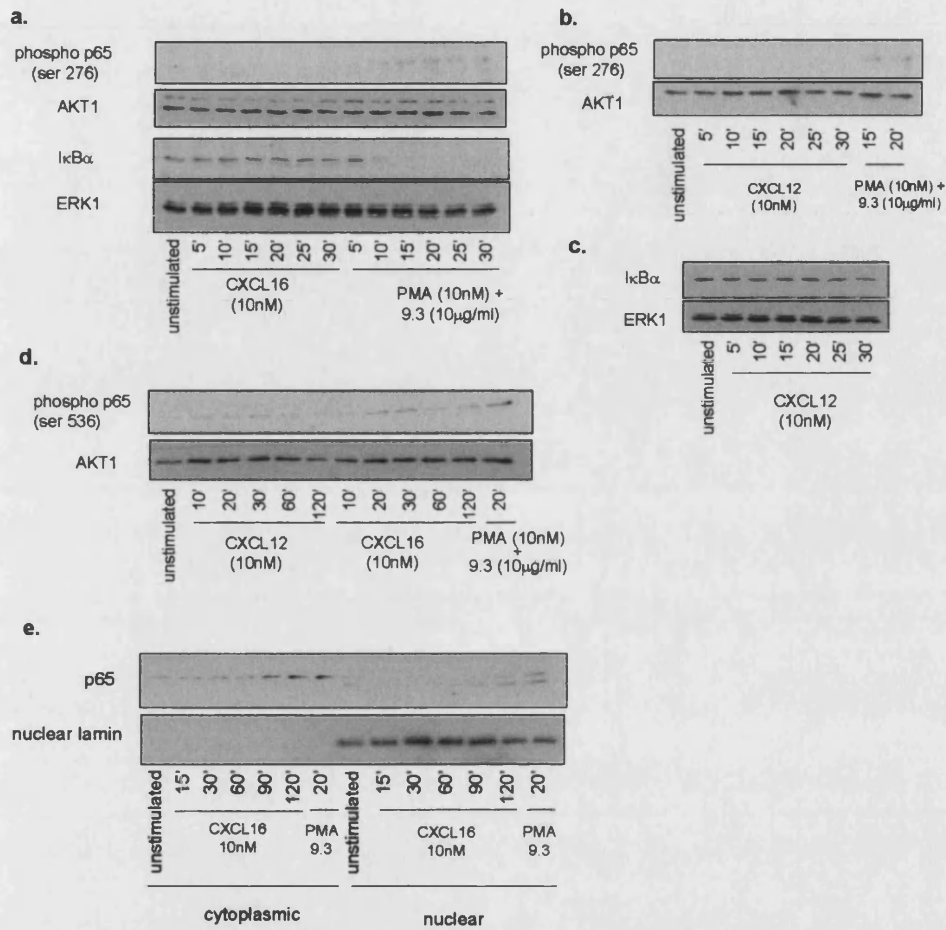
Figure 57. Does CXCL16 activate the canonical NF-κB pathway?

a, PMA (Phorbol 13-myristate 12-acetate) (10nM) and 9.3 antibody (10µg/ml) but not CXCL16 (10nM) induces time-dependent phosphorylation of p65 (ser276) and degradation of IκBα. b, c, CXCL12 (10nM) does not induce phosphorylation of p65 (ser276) (b) or degradation of IκBα (c). d, CXCL16 (10nM), but not CXCL12 (10nM) induces time-dependent phosphorylation of p65 (ser536). e, CXCL16 (10nM) induces translocation of phosphorylated p65 (ser536) from the cytosol to the nucleus. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation with the appropriate agonist. Following stimulation (5×10^6 cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-specific antibody to the relevant residues and visualised by chemoluminescence. Equal loading of gels was verified using antibodies as described (Data shown is representative of one donor; n=3).

The canonical NF-κB pathway is not the only mechanism of activating NF-κB dependent gene transcription. p105 NF-κB1 is also known to be activated in T-lymphocytes. Following agonist stimulation, p105 is phosphorylated and degraded releasing the associated NF-κB proteins (p50, p65 or c-Rel), allowing them to translocate to the nucleus. CXCL16 induces a gradual increase in phosphorylation of p105 which reaches a maximum at 30 minutes and has disappeared by 60 minutes (figure 58a). By contrast, costimulation with PMA and 9.3 antibody induces phosphorylation of p105 after 5 minutes and is sustained for at least 30 minutes. Using an antibody for p105 it was possible to see the degradation of p105 in the cytoplasm following activation of CXCR6 (figure 58b). By 45 minutes there was complete degradation of p105 and levels did not return by 90 minutes. In conjunction with this, there was a concurrent sustained increase of nuclear p50 after 15 minutes which correlates with the phosphorylation and degradation of p105 Levels of p50 in the cytoplasm also decreased slightly after 30 minutes it is not clear whether this has recognised the N-terminal of the p105 protein which is degraded or if it is translocation of p50 proteins to the nucleus. This antibody also recognises a protein of ~75kDa which is possibly the product of p50 cleavage from p105, although it is present in both the cytoplasm and nucleus, which indicates it may be a longer form of the p50 molecule.

3.25 CXCR6 mediates production of proinflammatory cytokines from CD3-CD28-activated PBLs

Dysregulation of NF-κB signalling has been linked to the overproduction of pro-inflammatory cytokines associated which chronic inflammatory conditions in which numbers CXCR6+ T-lymphocytes are also increased. Since CXCL16 may activate these NF-κB pathways it is possible that activation of CXCR6 on T-lymphocytes may contribute to the release of these cytokines. One of the most important pro-inflammatory cytokines regulated by NF-κB is TNFα. There is evidence that CXCL12 is able to induce TNFα production from murine astrocytes (Han Y et al, 2001), and CXCL16 has been reported to induce TNFα production from smooth muscle cells (Chandrasekar et al, 2004); although there are currently no reports of either of these chemokines promoting TNFα release from T-lymphocytes.
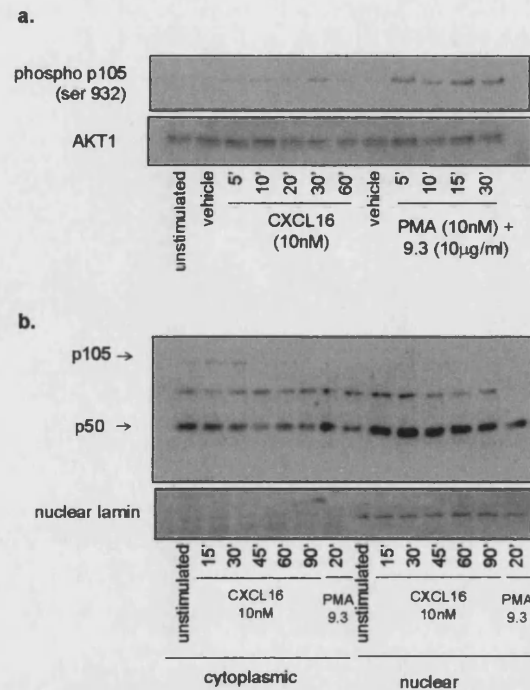
Figure 58. Activation of the p105 NF-κB pathway.

a, CXCL16 (10nM) and PMA (Phorbol 13-myristate 12-acetate) (10nM) and 9.3 antibody induces time-dependent phosphorylation of p105 (ser932). b, CXCL16 increases nuclear concentrations of p50. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were washed and left to rest overnight prior to stimulation with the appropriate agonist. Following stimulation (5×10^6 cells/point), cells were lysed and the proteins separated on a 10% SDS-PAGE gel. Changes in phosphorylation were detected using anti-phospho-specific antibody to the relevant residues and visualised by chemoluminescence. Equal loading of gels was verified using antibodies as described (Data shown is representative of one donor; n=3).

Both CXCL16 and CXCL12 significantly increase production of TNFα from CD3-CD28-activated PBLs (figure 59). At 48 hours, CXCL16 regulated TNFα production increased in a concentration-dependent manner up to 10nM. By 72 hours, this TNFα production had decreased, however there was still a significant increase at 10nM CXCL16. CXCL12 induced production of lower concentrations of TNFα than CXCL16 however it demonstrated a concentration-dependent increase which was statistically significant. At 72 hours, CXCL12 induced production low concentrations of TNFα which was not statistically significant. At both 48 and 72 hours the vehicle slightly increased TNFα production, but this was not significant at either time point.
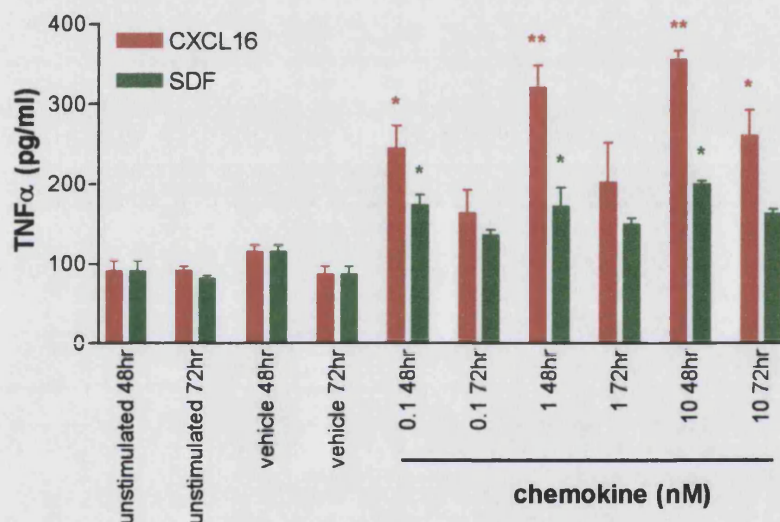
Figure 59. Chemokine induced TNF-α production from CD3-CD28-activated PBLs.
CXCL16 and CXCL12 induce production of tumour necrosis factor-α (TNF-α) from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were activated with CXCL16 (0.1-10 nM) or CXCL12 (10 nM) for indicated times. The supernatants were removed and concentrations of IFN-γ were measured by ELISA in accordance with the manufacturer's instructions. Data represented as mean +/- SD. * = p< 0.05 compared to unstimulated control, ** = p< 0.01 compared to unstimulated control. (n=2)

Activation of the TCR is known to promote TNFα production from T-lymphocytes. Stimulation of CD3 with UCHT1 elicited a concentration dependent increase in TNFα production in CD3-CD28-activated PBLs at both 48 and 72 hours which was approximately double the concentration induced by CXCL16 (figure 60). Stimulation of these cells with both UCHT1 and CXCL16 produced an additive increase over stimulation with UCHT1 alone in the concentration of TNFα. At 1nM CXCL16, this increase was not significant at any time point or concentration of UCHT1. Using 10nM CXCL16, at 48 and 72 hours, with 1μg/ml UCHT1, the additive effect of the costimulation was statistically significant. It is possible that at 10μg/ml, UCHT1 alone elicit the maximal effect on TNFα production and not further response is possible.
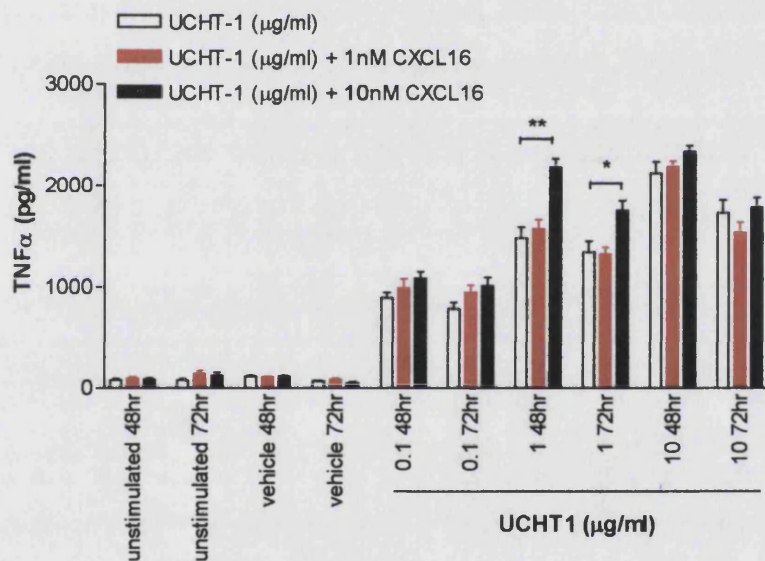
Figure 60. CXCL16 increases CD3-induced TNF-α production from CD3-CD28-activated PBLs.

CD3 alone and co-stimulation of CD3 with CXCL16 induce production of tumour necrosis factor-α (TNF-α) from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were activated with either UCHT1 (0.1-10μg/ml) or UCHT1 (0.1-10μg/ml) and CXCL16 (1-10 nM) for indicated times. Where more than one agonist was used, UCHT-1 was added for 30minutes prior to addition of the chemokine. The supernatants were removed and concentrations of TNF-α were measured by ELISA in accordance with the manufacturer's instructions. Data represented as mean +/- SD. * = $p < 0.05$ compared to UCHT1 alone stimulated response, ** = $p < 0.01$ compared to compared to UCHT1 alone stimulated response. (n=2)

Although CXCL12 also increased TNFα production from these cells, costimulation with UCHT1 and CXCL12 did not induce the same additive effect as was found with CXCL16 and UCHT1 (figure 61).
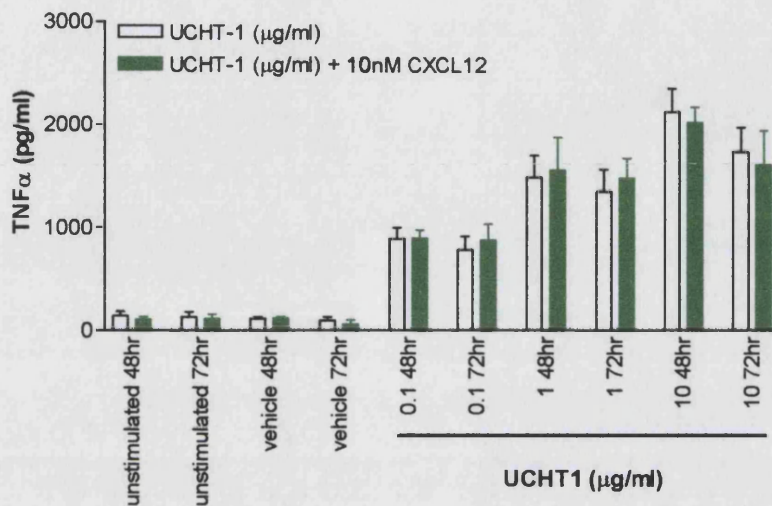
Figure 61. CXCL12 has no effect on CD3-induced TNF-α production from CD3-CD28-activated PBLs.

CD3 alone and co-stimulation of CD3 with CXCL12 induce production of tumour necrosis factor-α (TNF-α) from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were activated with either UCHT1 (0.1-10μg/ml) or UCHT1 (0.1-10μg/ml) and CXCL12 (10nM) for indicated times. Where more than one agonist was used, UCHT-1 was added for 30minutes prior to addition of the chemokine. The supernatants were removed and concentrations of TNF-α were measured by ELISA in accordance with the manufacturer's instructions. Data represented as mean +/- SD. (n=2)

The signalling mechanisms behind this additive effect are unclear. There was no augmentation in phosphorylation of PKB or S6 ribosomal protein when CD3-CD28-activated PBLs were co-stimulated with 1μg/ml UCHT1 and 10nM CXCL16 compared to stimulation with either UCHT1 or CXCL16 (figure 62). There is however an increase in ERK1/2 phosphorylation; this experiment would need to be repeated to verify this.

phospho PKB (ser 473)												
AKT1												
phospho ERK1/2 (thr202/tyr204)												
ERK1												
phospho S6 ribosomal protein (ser235/236)												
ERK1												

UCHT1 (1μg/ml) - - + - + - + - + - + - +
CXCL16 (10nM) - - - + + - - + + - - + +
                    2'   5'   10'
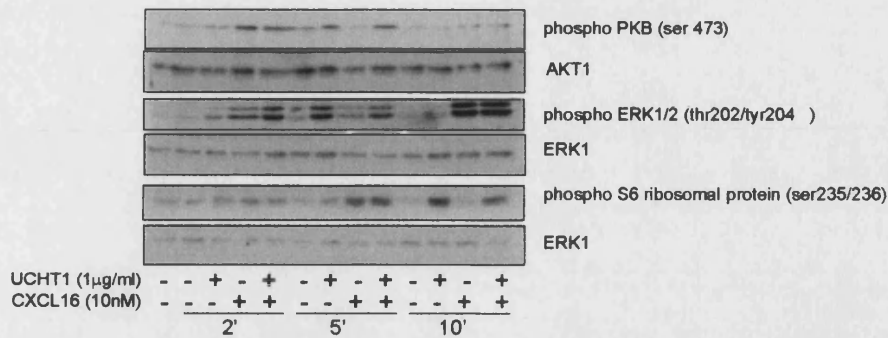
Figure 62. Activation of signal transduction pathways by CD3 activation and CXCL16
CD3 and CXCL16 alone and in combination induce phosphorylation of a number of signalling molecules from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28 T lymphoblasts were activated with either UCHT1 (1μg/ml), CXCL16 (10nM) or UCHT1 (1μg/ml) and CXCL16 (1-10nM) for indicated times. Whole cell lysates were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed using appropriate antibodies. Membranes were stripped and reprobed with indicated antibodies to verify equal loading of samples. (n=1)

Expression of CXCR6 on T-lymphocytes positively correlates with the production of IFNγ, another proinflammatory cytokine, from cells. Ligation of this receptor significantly induces production of IFNγ which increases in a concentration dependent manner at 48 and 72 hours (figure 63). At 48 hours, 10nM (the only concentration used) CXCL12 also induces a significant increase in IFNγ production; as was found in the measurement of TNFα production, the concentration of IFNγ produced was much lower for CXCL12 than CXCL16. Ligation of CD3 by UCHT1 also elicits a concentration dependent production of IFNγ in CD3-CD28-activated PBLs at 48 and 72 hours (figure 64). While costimulation CXCL12 did not significantly alter this response, costimulation with CXCL16 did (figure 64). At 48 hours, 10nM CXCL16 significantly increased the concentration of IFNγ produced by UCHT1 (0.1, 1, 10 μg/ml) above that produced by UCHT1 alone (0.1, 1, 10 μg/ml). At 72 hours there was also an additive effect with costimulation using 1μg/ml UCHT1 and 10nM CXCL16 which was statistically significant.
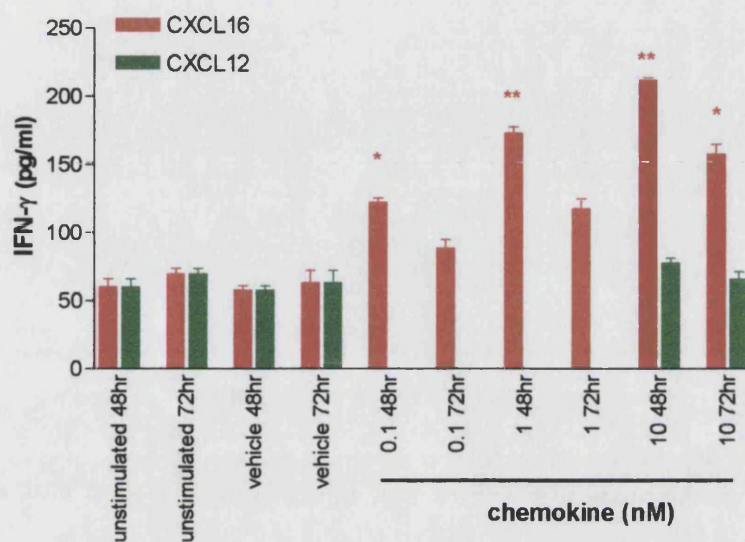
Figure 63. Chemokine-mediated IFN-γ production from CD3-CD28-activated PBLs.

CXCL16 and CXCL12 induce production of interferon-γ (IFN-γ) from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28-activated PBLs were activated with CXCL16 (0.1-10 nM) or CXCL12 (10 nM) for indicated times. The supernatants were removed and concentrations of IFN-γ were measured by ELISA in accordance with the manufacturer's instructions. Data represented as mean +/- SD * = p< 0.05 compared to CXCL16 stimulated response, ** = p< 0.01 compared to unstimulated control. (n=2)
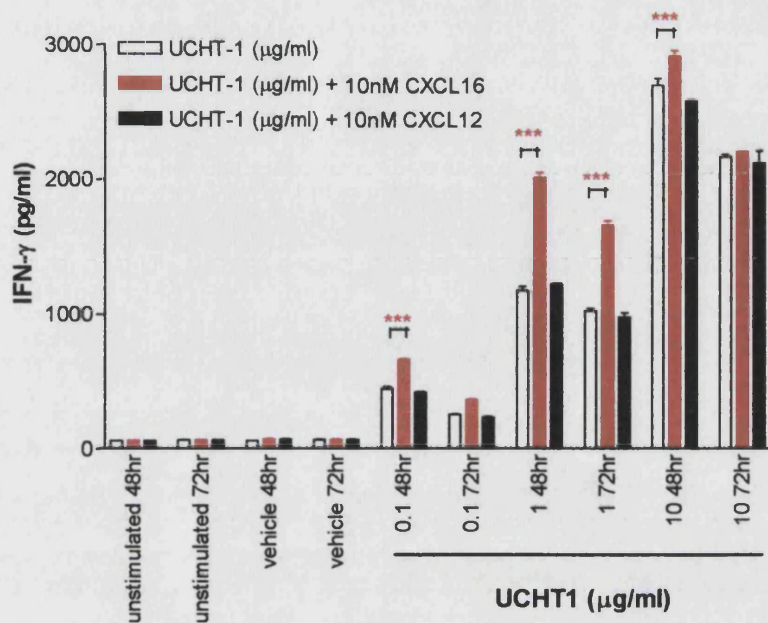
Figure 64. CD3 and Chemokine-mediated IFN-γ production from CD3-CD28-activated PBLs.

CD3 alone and co-stimulation of CD3 with CXCL16 or CXCL12 induce production of interferon-γ (IFN-γ) from CD3-CD28-activated PBLs. PBMCs were isolated from whole blood and activated using CD3-CD28 microbeads. CD3-CD28 T lymphoblasts were activated with either UCHT1 (0.1-10μg/ml) or UCHT1 (0.1-10μg/ml) and CXCL16 (10 nM) or CXCL12 (10 nM) for indicated times. Where more than one agonist was used, UCHT-1 was added for 30minutes prior to addition of the chemokine. The supernatants were removed and concentrations of IFN-γ were measured by ELISA in accordance with the manufacturer's instructions. Data represented as mean +/- SD. * = p< 0.05 compared to CXCL16 stimulated response, *** = p< 0.001 compared to unstimulated control.

Chapter 4: Discussion

Cell Lines vs Primary Cells

Identification of a T-cell model with which to investigate the activation of CXCR6 was a critical step in this project. Previous studies about CXCR6 used either primary T-lymphocytes or Jurkats transfected with CXCR6. There are a number of T cell lines routinely used in the chemokine field which were available for this study. Cell lines have the advantage over primary cells in that they offer a sustainable source of cells and are easy and cheap to culture. Analysis of Jurkat, HUT-78 and Cem cells identified that they all expressed the receptor CXCR6 and they were therefore a logical starting point. Unfortunately none of them provided a suitable model with which to continue; while the initial data from the Jurkat cells was good, further aliquots of cells from the same source, thawed at later time points did not express the receptor. The reasons for this are unclear. HUT-78 and Cem cells continued to express similar levels of the receptor each time receptor expression was measured, however the signalling data obtained from these cells was neither reliable nor consistent. The high basal levels of phosphorylated MAPK observed with HUT-78 cells were unexpected and therefore made these cells unsuitable for analysing further signal transduction pathways. Cem cells, while surface expression of CXCR6 was high, the receptor did not appear to be coupled to signal transduction pathways. The receptor CXCR4 was able to induce phosphorylation of both PKB and ERK1/2 demonstrating that the cells were functional. Ultimately, the cell lines available were not viable for investigating the signal transduction pathways activated by CXCR6.

Primary T-lymphocytes have been used in a number of studies looking at CXCR6, and for a time, CXCR6 expression was thought to be confined to Th1 cells. Although receptor expression has now been reported on a number of cell types, CXCR6 is highly expressed on activated T cells and receptor expression correlates with the production of IFN-γ from cells. Isolation of naïve T-lymphocytes (CD4⁺/CD45RO⁻), and their subsequent activation with several rounds of cytokines produced cells with a Th1-like phenotype, identified by the ability to produce IFN-γ, but not IL-4. Some of these cells expressed CXCR6, however the percentage was not very high and varied widely between donors. This variation in CXCR6 expression did not correlate with the variation in the responses found when measuring phosphorylation of proteins making analysis of the results difficult to

interpret. The inconsistency between donors in the receptor expression and response to CXCR6 ligation, the low numbers and the cost of generating the cells limited their use.

Activated PBLs were a source of primary T-lymphocytes which expand in culture and were easier and cheaper to generate that the Th1 cells. Cells activated with SEB express the receptor after several days in culture and expression increases to a peak at around day 10 or 11 before decreasing. This limited the period in which experiments can be performed. Cells activated with CD3-CD28 microbeads express CXCR6 for longer time periods, with receptor expression on ~ 20% of cells by day 7, a level that was maintained, and sometimes increased for over a week. The appearance of CXCR6 after several days in culture during which cells are activated, is consistent with reports of CXCR6 expression primarily on activated T cells. While there was some variation between donors in levels of receptor expression, the CD3-CD28-activated PBLs produced more consistent data and were therefore used in the majority of experiments.

Measurement of Signal Transduction

Phosphorylation is required by a number of signalling molecules in order for activation to occur, and the phosphorylation of certain residues can be used as a marker of protein activation. In general these phosphorylation events were detected using western blotting techniques. Since the availability of Th1 cells was limited, it was necessary to employ a method of detecting phosphorylation events that did not require as many cells as western blotting. In recent years some of the antibodies used in western blotting have been validated for flow cytometry; this presented a possibility for measuring phosphorylation in the Th1 cells. Following stimulation of cells, they were fixed and permeabilised and incubated with antibodies specific for phosphorylated forms of the proteins. The addition of a fluorescent tag to the antibody allowed the quantitative measurement of changes in phosphorylation by flow cytometry. This would allow the simultaneous analysis of multiple phosphorylation sites/proteins in a single cell, with the use of different coloured tags. The data was analysed by using the light scatter to gate the population of cells, this removes dead or disrupted cells, which often fluoresce brightly, from the analysis. The fluorescence was then plotted on a histogram and the median florescence used in subsequent calculations. The principles of this technique mean that it sensitive enough to detect small changes in the

Chapter 4: Discussion

phosphorylation status of proteins, which may not be detected by western blots, however it is very dependent on the specificity of the antibody. This limitation is demonstrated by the use of the phospho-p70S6K antibody which also recognises p85S6K; the western blot shows up an additional band to represent this protein, but it is not able to be distinguished from the p70S6K signal when using flow cytometry. Another factor to consider with the use of this technique is the cost of the antibodies used, because the volumes required for flow cytometric analysis of phosphorylation is greater than that required for western blotting.

Consistent with the activation of other chemokine receptors, ligation of CXCR6 induces the phosphorylation of a number of signalling molecules, consistent with their activation.

Comparison of CXCR6 mediated responses with those of other chemokine receptors was used to determine whether similar signalling pathways were utilised; CXCL10, the ligand for CXCR3 was used as a control for some of the Th1 and CD3-CD28-activated PBL data. CXCR3 is predominantly expressed on Th1 cells and activated T-lymphocytes; however CXCL12, through its receptor CXCR4, which is expressed on the majority of cells, elicited a greater response in signalling experiments in CD3-CD28-activated PBLs and was therefore also used to compare with CXCL16 responses.

The unique structure of CXCL16, compared to other CXC chemokines, and its presence as a soluble or membrane bound form suggested that it may have functions not performed by other chemokines. This study used a recombinant form of the soluble chemokine in all experiments, it is currently unknown whether this is able to elicit different responses through CXCR6 that the membrane bound form, however chimeric studies using CXCL16 and CXC_3L1 indicate that the membrane bound form of the chemokine is able to activate CXCR6 as well (Heydtmann et al, 2005; Shimoaka et al, 2002)

CXCL16-induced Chemotatic Responses

Chemokine-induced regulation of lymphocyte migration is important for their development and the ability of correct inflammatory responses. CXCR4 is known to be important in the trafficking of T-lymphocytes throughout development and routine immune surveillance and the CD3-CD28-activated PBLs used here migrated towards CXCL12. In these experiments, CXCL16 was not a very strong

Chapter 4: Discussion

chemoattractant and the levels of migrating cells observed were very low, although comparative to those seen in previous studies using the soluble form of the chemokine. It is therefore surprising, based on in vitro experiments, that CXCR6 has been implicated in trafficking of T-lymphocytes. The difference in levels of cells migrating towards CXCL16 compared to CXCL12 is partly explained by the levels of their receptors on the cell surface. However, the variation in response is not solely due to the percentage of cells expressing the relevant receptor because the magnitude of the difference does not correlate; the percentage of cells expressing each receptor does not correlate to the percentage of cells which actually migrate. This may be due to the limitations of the assay used; these in vitro assays may not reflect the in vivo capabilities of a chemokine present in both soluble and membrane bound forms. Cell movement is an extremely complex phenomenon, requiring the protrusion of a leading edge, adhesion of that edge to the extracellular matrix, translocation of the cell and finally the retraction of the trailing edge. Chemokines provide a gradient important in the directional movement of the cell, however it is possible that the cell surface expression of CXCL16 also plays an active role in the adhesion of lymphocytes which would enable more effective movement of the cell (Heydtmann et al, 2005). Since in these assays only the soluble chemokine was used it is not possible to demonstrate whether this occurs, but it cannot be ruled out as a reason why only low levels of cells migrate towards CXCL16 in vitro when in vivo there is an accumulation of CXCR6+ cells at inflammatory sites.

The low levels of chemotaxis observed in CD3-CD28-activated PBLs to CXCL16 mean that the effects of inhibitors were difficult to detect. An alternative to chemotaxis was the measurement of F-actin following cell stimulation. This is not a direct measure of chemotaxis, but it demonstrates a functional mechanism required for chemotaxis. Polymerisation of F-actin occurs at the leading edge of migrating cells and can be quantitatively measured using phalloidin which binds F-actin monomers in a 1:1 ratio. This technique is easier and quicker than chemotaxis assays; the data produced allowed detection of small changes induced by inhibitors and was more repeatable than the chemotaxis assays. In these assays, while CXCL12 did not induce as much actin polymerisation as CXCL16, which is surprising considering the chemotactic responses, the response was highly significant for both chemokines. This difference may just reflect differential signalling mechanisms activated by the different receptors, or an ability of CXCL16 to induce actin polymerisation for a function other than cell migration.

Chapter 4: Discussion

Polymerisation of actin occurs following activation of a cell in order to induce shape change and cell polarity or formation of the immunological synapse as well of a number of other cell processes.

The signalling mechanisms which result in movement of a cell are in the process of being unravelled, however it will be many years before the process is fully understood. The involvement of PI3K isoforms in the chemotactic response has been widely studied, but many questions remain unanswered. PI3K inhibitors have been shown to inhibit leukocyte chemotaxis and imaging of cells has revealed a concentration of $PI(3,4,5)P_3$ at the leading edge of cells (Sadhu et al, 2003). Since chemokine receptors are GPCRs, it is not surprising that p110γ knockout mice display reduced chemokine mediated neutrophil and macrophage chemotaxis; these cells also indicate a requirement for this PI3K isoform in $PI(3,4,5)P_3$ production, PKB and Rac activation and accumulation of F-actin at the leading edge of cells (Li et al, 2000; Sasaki et al, 2000). Class IA isoforms have also been shown to be activated downstream of GPCRs. While the non-selective PI3K inhibitors reduced chemokine mediated chemotaxis, the selective p110d inhibitor had little effect on CXCL16 mediated chemotaxis. In contrast to the results found here, study of neutrophil chemotaxis showed a requirement for p110δ, however actin polymerisation was not affected (Sadhu et al, 2003). This raises questions about the requirement of specific PI3K isoforms in chemotaxis, and how stimuli- and cell-specific these are. The lack of effect of IC87114 may indicate that other class IA isoforms are more important than p110δ in the chemotactic response to CXCL16. What was surprising about the role of p110δ, is the reduction of F-actin polymerisation in response to CXCL16. Downstream of GPCR activation, pRex-1, a Rac-GEF, has been demonstrated to be important in the $PI(3,4,5)P_3$-dependent GTP loading of Rac1 (Weiner et al, 2002; Welch et al, 2002). It has been postulated that p110δ is activated downstream of Rac1 following G$\beta\gamma$ activation of Rac1 through pRex-1 (Deane & Fruman, 2004). This mechanism of p110d activation may allow an additional regulatory pathway, allowing differential activation of the PI3K isoforms in different functional responses. However it is also possible that the chemotaxis assay used in this study was not sensitive enough to detect a reduction of chemotaxis.

133

Chapter 4: Discussion

A number of PKC isoforms have been shown to interact with actin and the cytoskeleton. Of the nPKC isoforms, PKCδ has been shown to bind actin in airway epithelial cells (Liedtke *et al*, 2003). PKCε is involved in the spreading of fibroblasts through its interactions with actin filaments and myosin II (England *et al*, 2002). PKCθ can phosphorylate WIP (WASP-interacting protein) and therefore directly regulate actin reorganisation (Sasahara *et al*, 2002). cPKC isoforms also potentially have a role in the reorganisation of the actin cytoskeleton; PKCβI is involved in cell polarity and has been associated with microtubules (Volchov *et al*, 1998; Volchov *et al*, 2001). Conversely, PKCα can directly bind fascin, a protein involved in bundling actin which localises at the leading edge of cells; inhibition of this interaction attenuates cell migration (Anilkumar *et al*, 2003). Through inhibition of actin polymerisation, using the chemical inhibitors, CXCL16 is able to activate members of cPKC and nPKC families, and these are involved in CXCL16-mediated actin polymerisation. Of the isoforms within these sub-groups, rottlerin-sensitive isoforms appear to have most effect on actin polymerisation and phosphorylation of ERK1/2.

Since CXCR6 mediated phosphorylation of ERK1/2 is mediated via $G_{\alpha i}$, indicated by the complete abrogation of the response by PTX, the upstream activation processes can be speculated from previous studies. $G_{\alpha i}$ can be coupled to ERK phosphorylation via a number of pathways (described in the introduction), and in these cells, CXCL16 mediated phosphorylation of ERK occurs only partially through a PI3K-dependent pathway. Inhibition of ERK phosphorylation appears to be more dependent on PKC, with rottlerin having a much greater effect, implicating PKCδ and possibly PKCθ in the response. PKC has isoform-specific interactions with activation of ERK1/2, and in general, PKC activates Raf which is upstream of other MAPKs. Since PKCα appears to have opposite effects on ERK phosphorylation to other PKC isoforms, it is possible that PKCα in these cells is responsible for the lack of effect of RO320432 and Gö6976, while rottlerin exhibited a stronger attenuation of ERK phosphorylation. It also possibly explains the stronger inhibition of phosphorylation seen at lower concentrations of Gö6976.

The ability of Gö6976 to inhibit phosphorylation of S6-ribosomal protein without inhibiting either ERK phosphorylation or phosphorylation of p70S6K indicates several possibilities: the isoform responsible for attenuating the inhibition of ERK phosphorylation at the higher concentration is not involved in

Chapter 4: Discussion

phosphorylation of S6 ribosomal protein or that this isoform is coupled to an ERK and p70S6K independent pathway.

The role of MAPK in chemotaxis is not well defined. In these cells the Mek1/2 inhibitor was able to inhibit F-actin polymerisation. A recent study has implicated MAPK as critical in the CXCL16-mediated recruitment of cells to rheumatoid arthritis synovial tissue (Ruth *et al*, 2006). Paxillin has an important role in the organisation of the actin cytoskeleton; It has been found to associate with Mek, Raf and ERK1/2 (Ishibe *et al*, 2003). ERK1/2 is able to phosphorylate paxillin, promoting the association with FAK and ultimately the activation of Rac which promotes actin polymerisation (Ishibe *et al*, 2004).

PD98059 was also able to inhibit CXCL16 mediated phosphorylation of GSK3β. This is possibly through the MAPK effector p90RSK which has been shown to be upstream of GSK3β (Ding *et al*, 2005). This interaction may also be involved in the inhibition of actin polymerisation by PD98059, since GSK3β has been shown to interact with Cdc42 (Etienne-Manneville & Hall, 2003).

The MAPK p38 is phosphorylated downstream of CXCL16 in activated-PBLs; while p38 is traditionally believed to be involved in gene transcription following stress-activated signals, it has also been linked to regulation of the actin cytoskeleton. Both ERK1/2 and p38 are able to activate MAPK-activated protein kinase 2 (MK2); MK2 deficient mice show abnormal migration of mouse embryonic fibroblasts and neutrophils (Hannigan *et al*, 2001; Kolyarov *et al*, 2002). p38 has also been shown to activate PAK6, a PAK family member not activated by Cdc42 or Rac and whose functions are so far not determined (Kaur *et al*, 2005).

There are a number of signalling events which indicate that CXCL16 and CXCL12 may be able to regulate different cellular processes. The ability of rapamycin to inhibit CXCL16-induced, but only partially inhibit CXCL12 induced phosphorylation of p70S6K and S6 ribosomal protein, suggesting a potential role for CXCL16 in protein translation or glucose metabolism. CXCL16 induced phosphorylation events also appear to be more sensitive to IC87114 than those of CXCL12, indicating more of a role for p110δ in CXCL16 mediated responses. Since PI3K is potentially involved in most cellular functions, it is not really possible to say how this may affect the cellular responses to the different chemokines.

Chapter 4: Discussion

Does CXCL16 activate NF-κB?

The canonical NF-κB pathway traditionally involves the phosphorylation, ubiquitination and degradation of IκB allowing phosphorylation of the NF-κB p65 subunit which then translocates to the nucleus. CXCL16 does not affect IκBα but induces phosphorylation of p65 at ser536 and translocation to the nucleus. This leads to a number of questions regarding whether the canonical NF-κB pathway is activated by CXCL16. The ability of phosphorylation of ser536 to determine the activation of NF-κB is debatable, with some studies suggesting it is important in p65 activation in T-lymphocytes, while other studies suggest this is not the case. Phosphorylation of p65 at ser536 is not always associated with degradation of IκBα (Doyle *et al*, 2005; Sasaki *et al*, 2005). In macrophages Btk is involved in a pathway in which there is no degradation of IκBα, but p65 is phosphorylated at ser 536 and there is increased transcriptional activity (Doyle *et al*, 2005). In this study, it was suggested that phosphorylation of p65 at ser536 was responsible for the LPS induced increase in transcriptional activity. Previously this group had found a role for Btk in TLR4 signalling to NF-κB (Jefferies *et al*, 2003). Activation of NF-κB by LPS involves a pathway which includes the adaptors MyD88 and Mal which activate IKKs via IRAKs and TRAF6. Since over expression of the adaptor proteins has been shown to promote transactivation (Mansell *et al*, 2004) and Btk has been found in complexes with both of them (Jefferies *et al*, 2003) it is probable that interactions between the adaptor proteins and Btk are responsible for the effects of Btk on NF-κB. T cells do not contain Btk, but it is possible that an alternative member of the Tec family of kinases could be involved in the phosphorylation of p65 by CXCL16.

Redundancy of individual IκB isoforms, shown by isoform deficient mice indicates that although IκBα is traditionally viewed as the main isoform involved in the canonical NF-κB pathway, other IκB molecules are important in NF-κB signalling. Thus, it is possible that the p65 is complexed with IκBβ or IκBε. IκBβ is able to strongly bind p65/p50 heterodimers and masks both of the NLS, functioning to retain NF-κB in the cytoplasm (Malek *et al*, 2001).

In T cells, the proteasome inhibitor MG132 has been shown to reduce translocation of total p65 but not p65 phosphorylated at ser536 (Sasaki *et al*, 2005). This study found that this phospho-p65 was not dimerised with either p50 or associated with IκBα, IκBβ or IκBε. It also describes a possible selective effect for

p65 phosphorylated on ser536 on gene activation, binding only the IL-8, not ICAM-1 promoter region.

This all suggests that CXCL16 may be able to activate the canonical NF-κB pathway in a way which does not involve the degradation of IκBα. It also leads to the possibility that the phosphorylation of p65 on ser536 by CXCL16 is not connected with the activation of the canonical pathway, but that the p65 is dimerised with p105.

Since phosphorylation does not always mean activation, further experiments will be required to verify if CXCL16 can activate NF-κB, or whether the phosphorylation events are independent of activation.

The production of TNFα can result from the activation of a number of transcription factors, one of which is NF-κB. CXCL16 alone was not able to induce significant amounts of TNFα from cells; however the additive effects of TCR and CXCR6 ligation provide an intriguing possibility for costimulation. The additive effect of co-stimulation of the TCR and CXCR6 can be the result of a number of explanations; the activation of differential transcription factors; enhanced transcription factor activation through different signal transduction pathways or the activation of a positive feedback loop.

TNFα, and other pro-inflammatory cytokines, have been shown to increase the expression of CXCR6 on the cell surface (van der Voort et al, 2005). The CD3 mediated TNF-a produced from these cells may therefore upregulate the expression of CXCR6 on the surface of the cell. It may be possible that CXCL16 can induce an autocrine positive feedback loop to enhance the expression of CXCR6 and therefore production of TNF-α; however in these experiments the concentration of TNF-α produced may not be enough to activate this.

Activation of NF-κB induces production of TNF-α, itself a potent activator of NF-κB, creating a positive feedback loop. Since the mechanisms by which CXCR6 may activate NF-κB are currently unknown, NF-κB may be activated by several pathways following costimulation of these cells, which induces an additive effect on the production of TNF-α. For example, CARMA1, activated by PKC downstream of TCR ligation, is required for TCR, but not IL-1- or TNF-α -activation of NF-κB, indicating a potential mechanism by which activation of CD3 can induce transcription (Wang et al, 2002).

Chapter 4: Discussion

Synergism between TCR and IL-1R in the activation of NF-κB has been shown to be dependent on PKC (Li et al, 2005); in this study the NF-κB nuclear localisation was primarily mediated by IL-1R activation, but prolonged by TCR engagement, this was thought to antagonise proteins involved in the normally transient NF-κB nuclear localisation. It is therefore possible that TCR activation of transcription factors can be potentiated by the chemokine receptor which prolongs NF-κB activation through a similar mechanism.

Activation of the MAPK pathway may be a way in which the CD3 signal could be enhanced by CXCL16, since there is possibly an additive phosphorylation of ERK1/2 in cells which were stimulated with UCHT-1 and CXCL16. The activation of Ras isoforms is clearly an important upstream event in ERK activation, and a number of studies have shown the compartmentalisation of Ras within the cell is important for eliciting responses induced by different stimuli (Bivona et al, 2003; Chiu et al, 2002; Rocks et al, 2005). The ability of MAPK phosphatases to dephosphorylate only the tyrosine residue on ERK, creating a pool of primed proteins, leads to a possibility that inhibition of these phosphatases creates a more sustained ERK activation (Tonks et al, 2001). If CXCL16 was able to inhibit these phosphatases, there could be increased gene activation via CD3 activated ERK.

Another mechanism by which the MAPK pathway may affect TNF-α production is through GSK3β. Inhibition of Mek by PD98059 inhibited phosphorylation of GSK3β, a protein recently implicated in the regulation of proinflammatory cytokines (Woodgett & Ohasi, 2005).

The activation of TLRs has recently been shown to induce activation of ERK1/2 through a mechanism dependent on NF-κB1 p105 (Dumitri et al, 2000; Waterfield et al, 2004). The degradation of p105 releases TPL-2 which can activate ERK1/2. The results here indicate that CXCL16 can induce phosphorylation of p105 which is degraded in the cytosol. This may allow the activation of ERK1/2 by TPL-2 which can then activate gene transcription. TCR activation of TNF-α is unaffected in TPL2-/- cells; however LPS-induced upregulation of TNF-α is dramatically reduced in TPL-2-/- macrophages (Dumitru et al, 2000). It is therefore possible that CXCL16 and the TCR are able to induce TNF-α production through different mechanisms.

p38 MAPK is crucial to production of proinflamatory cytokines, including TNF-α (Kyriakis et al, 2001). TNF-α gene regulation in mouse macrophages has been found to be mainly dependent on a promoter region which contains NF-κB

binding motifs (Shakhov *et al*, 1990), however a more recent study has identified another two regions which are able to maximise the LPS induced activation of this gene (Yao *et al*, 1997). These regions are able to bind other transcription factors, some of which are activate by p38 allowing maximal activation of the TNF-α gene through the co-ordinated activation of a number of transcription factors. p38 is able to affect NF-κB -dependent gene expression, possibly through regulation of TATA-binding protein (Carter *et al*, 1999). Another way in which p38 is able to increase TNF-α production is through the stabilisation of its mRNA (Neiniger *et al*, 2002). It is therefore possible that the increase in TNF-α production seen by co-stimulation of CD3 and CXCR6 could be mediated by activation of p38, since CXCL16 was able to induce greater phosphorylation of p38 than CXCL12 and CXCL12 does not induce an additive production of TNF-α as seen with CXCL16.

Another protein through which GPCRs and the TCR may cooperate is in the activation of p70S6K. This protein, shown to be phosphorylated by CXCL16, has been identified as a mechanism by which receptor tyrosine kinases and GPCRs synergise in DNA synthesis (Billington *et al*, 2005). This study suggests that sustained PKB activity (4 hours) correlated with the increased DNA synthesis when cells following GPCR and receptor tyrosine kinase activation.

Since TNF-α production can be regulated through a number of transcription factors, most of which can be activated downstream of the TCR, it is possible that in these cells the TCR and CXCR6 activate different ones and can therefore co-operate to enhance gene activation; however without further investigation this cannot be determined.

Chapter 4: Discussion

Future work

The data obtained from this project has raised a number of questions which can only be explained following further investigation. In order to answer some of these questions, the immediate experiments I would have performed would include the following.

- Measurement of actin polymerisation using combinations of inhibitors, for example, PD98059 with a PI3K inhibitor or combined PKC and PI3K inhibition.

- While CXCL16 does not induce degradation of IκBα, there are other IκB isoforms which may by complexed with NF-κB p65. Therefore measuring IκBβ or IκBϵ degradation may help elucidate the pathway following CXCR6 ligation.

- Phosphorylation of NF-κB p65 does not necessarily confer activation; therefore the use of EMSA experiments would indicate whether CXCL16 can activate NF-κB.

- The use of inhibitors of PI3K, PKC and MAPK may shed light on the upstream effectors responsible for the phosphorylation of NF-κB p65 and NF-κB p105 by CXCL16.

- Co-stimulation of CD3 with CXCR6 induced increased TNF-α production, which is possibly partly regulated by MAPK family members therefore measurement of p38 and also NF-κB phosphorylation following co-stimulation may help elucidate the mechanism.

- Since CXCR6 expression on cells may be upregulated by TNF-α, measurement of levels of CXCR6 expression following CD3 ligation may provide insight into a mechanism of costimulation or possibly an autocrine mechanism by which CXCR6 can increase its own surface expression.

- Since CXCL16 can induce production of TNFα and IFNγ the activation of other genes using micro array would be and interesting area of further study.

Summary & Conclusion

Since its identification on T-lymphocytes in 1997, little has been reported regarding the signal transduction mechanisms coupled to CXCR6 in these cells. The aims of this project were to identify biological targets linked to ligation of this receptor. By comparing some of the responses obtained in response to CXCL16 with responses elicited by other chemokines it has become clear that CXCR6 is similarly coupled to many signalling molecules; however it may also activate pathways not utilised by these receptors.

The experiments carried out through the duration of this project have identified a number of signalling molecules activated downstream of CXCR6 which are involved in the polymerisation of actin and also possibly the induction of gene transcription however, these pathways have not been fully characterised (figure 65).
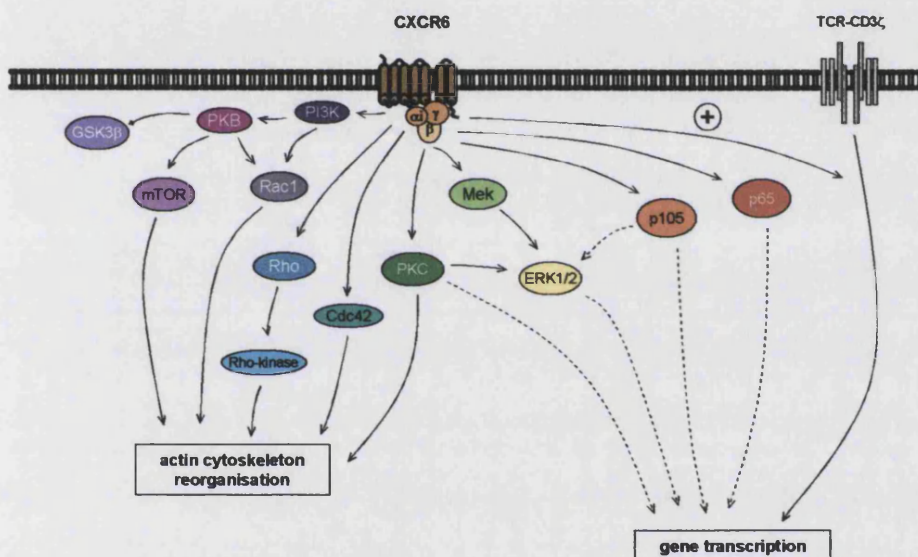
Figure 65. Summary of CXCR6 activated signalling pathways.

CXCR6 induces phosphorylation of key molecules involved in a number of signalling pathways. CXCR6 is a $G\alpha_i$ coupled receptor which is able to activate PI3K and its downstream effectors. This receptor is also coupled to the MAPK pathway and can mediate functional responses through these and other molecules, including PKC, small GTPases and NFκB. The ability of CXCR6 to induce and increase CD3-mediated cytokine production occurs through an, as yet, unidentified pathway. Dashed lines represent possible interactions. ERK1/2, extracellular-related kinase; GSK3β, glycogen synthase 3β; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; PI3K phosphatidylinositol 3-kinase; PKC, protein kinase C.

While CXCR6 is able to induce low levels of T cell migration in vitro, the increased number of T cells expressing CXCR6 found at sites of inflammation indicates that in vivo this receptor may have a more profound effect. As discussed, membrane bound CXCL16 may be involved not only as a gradient for directional purposes, but also have a role in events prior to extravasation, such as adhesion, which would enable greater levels of migration.

The ability of these CXCR6+ cells to produce pro-inflammatory cytokines raises new questions about their actual role in the disease states. In conditions in which an accumulation of CXCR6+ T-lymphocytes are found, there is often a high concentration of TNFα and IFN-γ and suggesting the possibility that CXCR6 may be responsible for more than just trafficking the cells.

Ultimately, CXCR6 and its ligand, CXCL16, are coupled to complex signal transduction pathways which result not only in the reorganisation of the actin cytoskeleton and migration of T cells, but also the production of pro-inflammatory cytokines.

Reference List

Al-Aoukaty A, Schall TJ, Maghazachi AA (1996) Differential coupling of CC chemokine receptors to multiple heterotrimeric G proteins in human interleukin-2-activated natural killer cells. *Blood* **87,** 4255-4260.

Allen LH, Aderem A (1995) A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. *J.Exp.Med.* **182,** 829-840.

Andjelkovic M, Maira SM, Cron P, Parker PJ, Hemmings BA (1999) Domain swapping used to investigate the mechanism of protein kinase B regulation by 3-phosphoinositide-dependent protein kinase 1 and Ser473 kinase. *Mol.Cell Biol.* **19,** 5061-5072.

Anilkumar N, Parsons M, Monk R, Ng T, Adams JC (2003) Interaction of fascin and protein kinase Calpha: a novel intersection in cell adhesion and motility. *EMBO J.* **22,** 5390-5402.

Arenzana-Seisdedos F, Virelizier JL, Rousset D, Clark-Lewis I, Loetscher P, Moser B, Baggiolini M (1996) HIV blocked by chemokine antagonist. *Nature* **383,** 400.

Arthur WT, Noren NK, Burridge K (2002) Regulation of Rho family GTPases by cell-cell and cell-matrix adhesion. *Biol.Res.* **35,** 239-246.

Ashburner BP, Westerheide SD, Baldwin AS, Jr. (2001) The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol.Cell Biol.* **21,** 7065-7077.

Astoul E, Watton S, Cantrell D (1999) The dynamics of protein kinase B regulation during B cell antigen receptor engagement. *J.Cell Biol.* **145,** 1511-1520.

Astoul E, Edmunds C, Cantrell DA, Ward SG (2001) PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol.* **22,** 490-496.

Avruch J, Zhang XF, Kyriakis JM (1994) Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem.Sci.* **19,** 279-283.

References

Badour K, Zhang J, Siminovitch KA (2004) Involvement of the Wiskott-Aldrich syndrome protein and other actin regulatory adaptors in T cell activation. *Semin.Immunol.* **16**, 395-407.

Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ (1997) A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**, 640-644.

Beinke C, van BD, Cordes N (2003) Ionizing radiation modules of the expression and tyrosine phosphorylation of the focal adhesion-associated proteins focal adhesion kinase (FAK) and its substrates p130cas and paxillin in A549 human lung carcinoma cells in vitro. *Int.J.Radiat.Biol.* **79**, 721-731.

Beinke S, Ley SC (2004) Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem.J.* **382**, 393-409.

Berridge MJ, Bootman MD, Lipp P (1998) Calcium—a life and death signal. *Nature* **395**, 645-648.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. *Nat.Rev.Mol.Cell Biol.* **1**, 11-21.

Berven LA, Crouch MF (2000) Cellular function of p70S6K: a role in regulating cell motility. *Immunol.Cell Biol.* **78**, 447-451.

Berven LA, Willard FS, Crouch MF (2004) Role of the p70(S6K) pathway in regulating the actin cytoskeleton and cell migration. *Exp.Cell Res.* **296**, 183-195.

Billington CK, Kong KC, Bhattacharyya R, Wedegaertner PB, Panettieri RA, Jr., Chan TO, Penn RB (2005) Cooperative regulation of p70S6 kinase by receptor tyrosine kinases and G protein-coupled receptors augments airway smooth muscle growth. *Biochemistry* **44**, 14595-14605.

Bivona TG, Perez DC, I, Ahearn IM, Grana TM, Chiu VK, Lockyer PJ, Cullen PJ, Pellicer A, Cox AD, Philips MR (2003) Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. *Nature* **424**, 694-698.

References

Blease K, Mehrad B, Standiford TJ, Lukacs NW, Gosling J, Boring L, Charo IF, Kunkel SL, Hogaboam CM (2000) Enhanced pulmonary allergic responses to Aspergillus in CCR2-/- mice. *J.Immunol.* **165**, 2603-2611.

Blease K, Mehrad B, Standiford TJ, Lukacs NW, Kunkel SL, Chensue SW, Lu B, Gerard CJ, Hogaboam CM (2000) Airway remodeling is absent in CCR1-/- mice during chronic fungal allergic airway disease. *J.Immunol.* **165**, 1564-1572.

Blobe GC, Stribling DS, Fabbro D, Stabel S, Hannun YA (1996) Protein kinase C beta II specifically binds to and is activated by F-actin. *J.Biol.Chem.* **271**, 15823-15830.

Boisvert J, Kunkel EJ, Campbell JJ, Keeffe EB, Butcher EC, Greenberg HB (2003) Liver-infiltrating lymphocytes in end-stage hepatitis C virus: Subsets, activation status, and chemokine receptor phenotypes. *Journal of Hepatology* **38**, 67-75.

Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D, Mantovani A (1998) Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* **92**, 2668-2671.

Bonser RW, Thompson NT, Randall RW, Tateson JE, Spacey GD, Hodson HF, Garland LG (1991) Demethoxyviridin and wortmannin block phospholipase C and D activation in the human neutrophil. *Br.J.Pharmacol.* **103**, 1237-1241.

Boring L, Gosling J, Cleary M, Charo IF (1998) Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894-897.

Brand SA, Sakaguchi T, Gu XB, Reinecker HC (2002) Expression of a novel chemokine ligand-receptor pair in intestinal epithelial cells (IEC): CXCL16 induced signals regulate epithelial restitution. *Gastroenterology* **122**, 621.

Brandlin I, Hubner S, Eiseler T, Martinez-Moya M, Horschinek A, Hausser A, Link G, Rupp S, Storz P, Pfizenmaier K, Johannes FJ (2002) Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways. *J.Biol.Chem.* **277**, 6490-6496.

145

References

Brock C, Schaefer M, Reusch HP, Czupalla C, Michalke M, Spicher K, Schultz G, Nurnberg B (2003) Roles of G beta gamma in membrane recruitment and activation of p110 gamma/p101 phosphoinositide 3-kinase gamma. *J.Cell Biol.* **160**, 89-99.

Brook M, Sully G, Clark AR, Saklatvala J (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS Lett.* **483**, 57-61.

Brown MC, Perrotta JA, Turner CE (1996) Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J.Cell Biol.* **135**, 1109-1123.

Burgering BM, Medema RH (2003) Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. *J.Leukoc.Biol.* **73**, 689-701.

Buss H, Dorrie A, Schmitz ML, Frank R, Livingstone M, Resch K, Kracht M (2004) Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity. *J.Biol.Chem.* **279**, 49571-49574.

Cacalano NA, Sanden D, Johnston JA (2001) Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras. *Nat.Cell Biol.* **3**, 460-465.

Calabresi PA, Yun SH, Allie R, Whartenby KA (2002) Chemokine receptor expression on MBP-reactive T cells: CXCR6 is a marker of IFN-gamma-producing effector cells. *Journal of Neuroimmunology* **127**, 96-105.

Caloca MJ, Wang H, Kazanietz MG (2003) Characterization of the Rac-GAP (Rac-GTPase-activating protein) activity of beta2-chimaerin, a 'non-protein kinase C' phorbol ester receptor. *Biochem.J.* **375**, 313-321.

Cantrell D (2002) Protein kinase B (Akt) regulation and function in T lymphocytes. *Semin.Immunol.* **14**, 19-26.

References

Carter AB, Knudtson KL, Monick MM, Hunninghake GW (1999) The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J.Biol.Chem.* **274**, 30858-30863.

Cenni V, Sirri A, Riccio M, Lattanzi G, Santi S, de PA, Maraldi NM, Marmiroli S (2003) Targeting of the Akt/PKB kinase to the actin skeleton. *Cell Mol.Life Sci.* **60**, 2710-2720.

Chandrasekar B, Bysani S, Mummidi S (2004) CXCL16 signals via Gi, phosphatidylinositol 3-kinase, Akt, I kappa B kinase, and nuclear factor-kappa B and induces cell-cell adhesion and aortic smooth muscle cell proliferation. *J.Biol.Chem.* **279**, 3188-3196.

Chow S, Patel H, Hedley DW (2001) Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. *Cytometry* **46**, 72-78.

Chung CY, Potikyan G, Firtel RA (2001) Control of cell polarity and chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. *Mol.Cell* **7**, 937-947.

Cilliers T, Nhlapo J, Coetzer M, Orlovic D, Ketas T, Olson WC, Moore JP, Trkola A, Morris L (2003) The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C. *Journal of Virology* **77**, 4449-4456.

Cinamon G, Grabovsky V, Winter E, Franitza S, Feigelson S, Shamri R, Dwir O, Alon R (2001) Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. *J.Leukoc.Biol.* **69**, 860-866.

Cinamon G, Shinder V, Alon R (2001) Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat.Immunol.* **2**, 515-522.

Cote JF, Vuori K (2002) Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. *J.Cell Sci.* **115**, 4901-4913.

147

References

Coudronniere N, Villalba M, Englund N, Altman A (2000) NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. *Proc.Natl.Acad.Sci.U.S.A* **97**, 3394-3399.

Crespo P, Xu N, Simonds WF, Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* **369**, 418-420.

Cross DA, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS, Cohen P (1994) The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem.J.* **303** (Pt 1), 21-26.

Crouch MF (1997) Regulation of thrombin-induced stress fibre formation in Swiss 3T3 cells by the 70-kDa S6 kinase. *Biochem.Biophys.Res.Commun.* **233**, 193-199.

Curnock AP, Logan MK, Ward SG (2002) Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology* **105**, 125-136.

Curnock AP, Sotsios Y, Wright KL, Ward SG (2003) Optimal chemotactic responses of leukemic T cells to stromal cell-derived factor-1 requires the activation of both class IA and IB phosphoinositide 3-kinases. *J.Immunol.* **170**, 4021-4030.

D'Ambrosio D, Fong DC, Cambier JC (1996) The SHIP phosphatase becomes associated with Fc gammaRIIB1 and is tyrosine phosphorylated during 'negative' signaling. *Immunol.Lett.* **54**, 77-82.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241.

Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem.J.* **351**, 95-105.

Deane JA, Fruman DA (2004) Phosphoinositide 3-kinase: diverse roles in immune cell activation. *Annu.Rev.Immunol.* **22**, 563-598.

References

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S (1998) Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc.Natl.Acad.Sci.U.S.A 95, 11211-11216.

Demarchi F, Bertoli C, Sandy P, Schneider C (2003) Glycogen synthase kinase-3 beta regulates NF-kappa B1/p105 stability. J.Biol.Chem. 278, 39583-39590.

Deng HK, Unutmaz D, KewalRamani VN, Littman DR (1997) Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature 388, 296-300.

Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, Bartholomeusz G, Li Y, Pan Y, Li Z, Bargou RC, Qin J, Lai CC, Tsai FJ, Tsai CH, Hung MC (2005) Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol.Cell 19, 159-170.

Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392, 933-936.

Doyle SL, Jefferies CA, O'Neill LA (2005) Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFkappaB activation by lipopolysaccharide. J.Biol.Chem. 280, 23496-23501.

Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103, 1071-1083.

Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science 280, 1082-1086.

England K, Ashford D, Kidd D, Rumsby M (2002) PKC epsilon is associated with myosin IIA and actin in fibroblasts. Cell Signal. 14, 529-536.

149

References

Etienne-Manneville S (2004) Cdc42--the centre of polarity. *J.Cell Sci.* **117**, 1291-1300.

Factor V, Oliver AL, Panta GR, Thorgeirsson SS, Sonenshein GE, Arsura M (2001) Roles of Akt/PKB and IKK complex in constitutive induction of NF-kappaB in hepatocellular carcinomas of transforming growth factor alpha/c-myc transgenic mice. *Hepatology* **34**, 32-41.

Ferby IM, Waga I, Sakanaka C, Kume K, Shimizu T (1994) Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J.Biol.Chem.* **269**, 30485-30488.

Ferrari S, Bandi HR, Hofsteenge J, Bussian BM, Thomas G (1991) Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. *J.Biol.Chem.* **266**, 22770-22775.

Feske S, Giltnane J, Dolmetsch R, Staudt LM, Rao A (2001) Gene regulation mediated by calcium signals in T lymphocytes. *Nat.Immunol.* **2**, 316-324.

Foletta VC, Lim MA, Soosairajah J, Kelly AP, Stanley EG, Shannon M, He W, Das S, Massague J, Bernard O (2003) Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. *J.Cell Biol.* **162**, 1089-1098.

Foletta VC, Moussi N, Sarmiere PD, Bamburg JR, Bernard O (2004) LIM kinase 1, a key regulator of actin dynamics, is widely expressed in embryonic and adult tissues. *Exp.Cell Res.* **294**, 392-405.

Forster R, Emrich T, Kremmer E, Lipp M (1994) Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* **84**, 830-840.

Frech M, Andjelkovic M, Ingley E, Reddy KK, Falck JR, Hemmings BA (1997) High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J.Biol.Chem.* **272**, 8474-8481.

References

Fukui Y, Hashimoto O, Sanui T, Oono T, Koga H, Abe M, Inayoshi A, Noda M, Oike M, Shirai T, Sasazuki T (2001) Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. *Nature* **412**, 826-831.

Fukuma N, Akimitsu N, Hamamoto H, Kusuhara H, Sugiyama Y, Sekimizu K (2003) A role of the Duffy antigen for the maintenance of plasma chemokine concentrations. *Biochem.Biophys.Res.Commun.* **303**, 137-139.

Furuno T, Hirashima N, Onizawa S, Sagiya N, Nakanishi M (2001) Nuclear shuttling of mitogen-activated protein (MAP) kinase (extracellular signal-regulated kinase (ERK) 2) was dynamically controlled by MAP/ERK kinase after antigen stimulation in RBL-2H3 cells. *J.Immunol.* **166**, 4416-4421.

Garcia LJ, Rosado JA, Gonzalez A, Jensen RT (1997) Cholecystokinin-stimulated tyrosine phosphorylation of p125FAK and paxillin is mediated by phospholipase C-dependent and -independent mechanisms and requires the integrity of the actin cytoskeleton and participation of p21rho. *Biochem.J.* **327 (Pt 2)**, 461-472.

Gardiner EM, Pestonjamasp KN, Bohl BP, Chamberlain C, Hahn KM, Bokoch GM (2002) Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. *Curr.Biol.* **12**, 2029-2034.

Gaupp S, Pitt D, Kuziel WA, Cannella B, Raine CS (2003) Experimental autoimmune encephalomyelitis (EAE) in CCR2(-/-) mice: susceptibility in multiple strains. *Am.J.Pathol.* **162**, 139-150.

Genot E, Cantrell DA (2000) Ras regulation and function in lymphocytes. *Curr.Opin.Immunol.* **12**, 289-294.

Genot EM, Arrieumerlou C, Ku G, Burgering BM, Weiss A, Kramer IM (2000) The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. *Mol.Cell Biol.* **20**, 5469-5478.

151

References

Gerber BO, Zanni MP, Uguccioni M, Loetscher M, Mackay CR, Pichler WJ, Yawalkar N, Baggiolini M, Moser B (1997) Functional expression of the eotaxin receptor CCR3 in T lymphocytes co-localizing with eosinophils. *Curr.Biol.* **7**, 836-843.

Gomez J, Martinez de AA, Bonay P, Pitton C, Garcia A, Silva A, Fresno M, Alvarez F, Rebollo A (1995) Physical association and functional relationship between protein kinase C zeta and the actin cytoskeleton. *Eur.J.Immunol.* **25**, 2673-2678.

Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem.Biophys.Res.Commun.* **199**, 93-98.

Haks MC, Oosterwegel MA, Blom B, Spits HM, Kruisbeek AM (1999) Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. *Semin.Immunol.* **11**, 23-37.

Han J, Luby-Phelps K, Das B, Shu X, Xia Y, Mosteller RD, Krishna UM, Falck JR, White MA, Broek D (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science* **279**, 558-560.

Han Y, He T, Huang DR, Pardo CA, Ransohoff RM (2001) TNF-alpha mediates SDF-1 alpha-induced NF-kappa B activation and cytotoxic effects in primary astrocytes. *J.Clin.Invest* **108**, 425-435.

Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C (2000) Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J.Exp.Med.* **192**, 1515-1520.

Hannigan MO, Zhan L, Ai Y, Kotlyarov A, Gaestel M, Huang CK (2001) Abnormal migration phenotype of mitogen-activated protein kinase-activated protein kinase 2-/- neutrophils in Zigmond chambers containing formyl-methionyl-leucyl-phenylalanine gradients. *J.Immunol.* **167**, 3953-3961.

Hashimoto S, Tsubouchi A, Mazaki Y, Sabe H (2001) Interaction of paxillin with p21-activated Kinase (PAK). Association of paxillin alpha with the kinase-inactive and the Cdc42-activated forms of PAK3. *J.Biol.Chem.* **276**, 6037-6045.

152

References

HayGlass KT, Li Y, Rempel JD, Wang M, Simons FE (1997) Exogenous IL-12 and directed induction of human and murine Th1-associated responses. *Int.Arch.Allergy Immunol.* **113**, 281-283.

Heilker R, Freuler F, Pulfer R, Di PF, Eder J (1999) All three IkappaB isoforms and most Rel family members are stably associated with the IkappaB kinase 1/2 complex. *Eur.J.Biochem.* **259**, 253-261.

Herndon TM, Pirone DM, Tsokos GC, Chen CS (2005) T cell-to-T cell clustering enhances NF-kappaB activity by a PI3K signal mediated by Cbl-b and Rho. *Biochem.Biophys.Res.Commun.* **332**, 1133-1139.

Heydtmann M, Hubscht SG, Briskin M, Adams DH (2001) The chemokine receptor CXCR6 (BONZO) is enriched in the liver of patients with hepatitis C and in normal liver. *Journal of Hepatology* **34**, 209-210.

Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP (2000) Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* **287**, 1049-1053.

Hixenbaugh EA, Goeckeler ZM, Papaiya NN, Wysolmerski RB, Silverstein SC, Huang AJ (1997) Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. *Am.J.Physiol* **273**, H981-H988.

Hjelmstrom P, Fjell J, Nakagawa T, Sacca R, Cuff CA, Ruddle NH (2000) Lymphoid tissue homing chemokines are expressed in chronic inflammation. *Am.J.Pathol.* **156**, 1133-1138.

Hoffmeyer A, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S (1999) Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. *J.Biol.Chem.* **274**, 4319-4327.

Hopkins CR, Miller K, Beardmore JM (1985) Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. *J.Cell Sci.Suppl* **3**, 173-186.

References

Hou S, Guan H, Ricciardi RP (2003) Phosphorylation of serine 337 of NF-kappaB p50 is critical for DNA binding. *J.Biol.Chem.* **278**, 45994-45998.

Howe LR, Weiss A (1995) Multiple kinases mediate T-cell-receptor signaling. *Trends Biochem.Sci.* **20**, 59-64.

Hsia DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, Spencer KS, Cheresh DA, Schlaepfer DD (2003) Differential regulation of cell motility and invasion by FAK. *J.Cell Biol.* **160**, 753-767.

Huang AJ, Manning JE, Bandak TM, Ratau MC, Hanser KR, Silverstein SC (1993) Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J.Cell Biol.* **120**, 1371-1380.

Huang YE, Iijima M, Parent CA, Funamoto S, Firtel RA, Devreotes P (2003) Receptor-mediated regulation of PI3Ks confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. *Mol.Biol.Cell* **14**, 1913-1922.

Humbles AA, Lu B, Friend DS, Okinaga S, Lora J, Al Garawi A, Martin TR, Gerard NP, Gerard C (2002) The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc.Natl.Acad.Sci.U.S.A* **99**, 1479-1484.

Hyatt SL, Liao L, Aderem A, Nairn AC, Jaken S (1994) Correlation between protein kinase C binding proteins and substrates in REF52 cells. *Cell Growth Differ.* **5**, 495-502.

Ishibe S, Joly D, Zhu X, Cantley LG (2003) Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. *Mol.Cell* **12**, 1275-1285.

Ishibe S, Joly D, Liu ZX, Cantley LG (2004) Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis. *Mol.Cell* **16**, 257-267.

References

Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S (2000) Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol.Pharmacol.* **57**, 976-983.

Jefferies CA, Doyle S, Brunner C, Dunne A, Brint E, Wietek C, Walch E, Wirth T, O'Neill LA (2003) Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. *J.Biol.Chem.* **278**, 26258-26264.

Jeong SJ, Pise-Masison CA, Radonovich MF, Park HU, Brady JN (2005) A novel NF-kappaB pathway involving IKKbeta and p65/RelA Ser-536 phosphorylation results in p53 Inhibition in the absence of NF-kappaB transcriptional activity. *J.Biol.Chem.* **280**, 10326-10332.

Jiang X, Takahashi N, Ando K, Otsuka T, Tetsuka T, Okamoto T (2003) NF-kappa B p65 transactivation domain is involved in the NF-kappa B-inducing kinase pathway. *Biochem.Biophys.Res.Commun.* **301**, 583-590.

Jiang X, Takahashi N, Matsui N, Tetsuka T, Okamoto T (2003) The NF-kappa B activation in lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. *J.Biol.Chem.* **278**, 919-926.

Kane LP, Shapiro VS, Stokoe D, Weiss A (1999) Induction of NF-kappaB by the Akt/PKB kinase. *Curr.Biol.* **9**, 601-604.

Kane LP, Weiss A (2003) The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol.Rev.* **192**, 7-20.

Kaslow HR, Burns DL (1992) Pertussis toxin and target eukaryotic cells: binding, entry, and activation. *FASEB J.* **6**, 2684-2690.

Kaur R, Liu X, Gjoerup O, Zhang A, Yuan X, Balk SP, Schneider MC, Lu ML (2005) Activation of p21-activated kinase 6 by MAP kinase kinase 6 and p38 MAP kinase. *J.Biol.Chem.* **280**, 3323-3330.

155

References

Kazanietz MG (2002) Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol.Pharmacol.* **61**, 759-767.

Keely PJ, Westwick JK, Whitehead IP, Der CJ, Parise LV (1997) Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390**, 632-636.

Kelley GG, Reks SE, Ondrako JM, Smrcka AV (2001) Phospholipase C(epsilon): a novel Ras effector. *EMBO J.* **20**, 743-754.

Khan IA, Murphy PM, Casciotti L, Schwartzman JD, Collins J, Gao JL, Yeaman GR (2001) Mice lacking the chemokine receptor CCR1 show increased susceptibility to Toxoplasma gondii infection. *J.Immunol.* **166**, 1930-1937.

Kim CH, Kunkel EJ, Boisvert J, Johnston B, Campbell JJ, Genovese MC, Greenberg HB, Butcher EC (2001) Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. *Journal of Clinical Investigation* **107**, 595-601.

Kim CH, Johnston B, Butcher EC (2002) Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. *Blood* **100**, 11-16.

Kim CH, Nagata K, Butcher EC (2003) Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naive to effector T cell differentiation. *Journal of Immunology* **171**, 152-158.

Kobayashi S, Shirai T, Kiyokawa E, Mochizuki N, Matsuda M, Fukui Y (2001) Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P3. *Biochem.J.* **354**, 73-78.

Konstantinos AP, Sheridan JF (2001) Stress and influenza viral infection: modulation of proinflammatory cytokine responses in the lung. *Respir.Physiol* **128**, 71-77.

References

Krutzik PO, Irish JM, Nolan GP, Perez OD (2004) Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clin.Immunol.* **110**, 206-221.

Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* **81**, 807-869.

Lacalle RA, Gomez-Mouton C, Barber DF, Jimenez-Baranda S, Mira E, Martinez A, Carrera AC, Manes S (2004) PTEN regulates motility but not directionality during leukocyte chemotaxis. *J.Cell Sci.* **117**, 6207-6215.

Lang V, Janzen J, Fischer GZ, Soneji Y, Beinke S, Salmeron A, Allen H, Hay RT, Ben-Neriah Y, Ley SC (2003) betaTrCP-mediated proteolysis of NF-kappaB1 p105 requires phosphorylation of p105 serines 927 and 932. *Mol.Cell Biol.* **23**, 402-413.

Lauffenburger DA, Horwitz AF (1996) Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Lemmon MA (2003) Phosphoinositide recognition domains. *Traffic.* **4**, 201-213.
Ref ID: 1153

Lesnik P, Haskell CA, Charo IF (2003) Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J.Clin.Invest* **111**, 333-340.

Lewis TS, Shapiro PS, Ahn NG (1998) Signal transduction through MAP kinase cascades. *Adv.Cancer Res.* **74**, 49-139.

Li D, Gal I, Vermes C, Alegre ML, Chong AS, Chen L, Shao Q, Adarichev V, Xu X, Koreny T, Mikecz K, Finnegan A, Glant TT, Zhang J (2004) Cutting edge: Cbl-b: one of the key molecules tuning. *J.Immunol.* **173**, 7135-7139.

Li M, Shillinglaw W, Henzel WJ, Beg AA (2001) The Rela(p65) subunit of NF-kappaB is essential for inhibiting double-stranded RNA-induced cytotoxicity. *J.Biol.Chem.* **276**, 1185-1194.

157

References

Li Y, Sedwick CE, Hu J, Altman A (2005) Role for protein kinase Ctheta (PKCtheta) in TCR/CD28-mediated signaling through the canonical but not the non-canonical pathway for NF-kappaB activation. *J.Biol.Chem.* **280**, 1217-1223.

Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D (2000) Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* **287**, 1046-1049.

Liao F, Alkhatib G, Peden KW, Sharma G, Berger EA, Farber JM (1997) STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. *J.Exp.Med.* **185**, 2015-2023.

Liedtke CM, Cole T, Ikebe M (1997) Differential activation of PKC-delta and -zeta by alpha 1-adrenergic stimulation in human airway epithelial cells. *Am.J.Physiol* **273**, C937-C943.

Ling L, Cao Z, Goeddel DV (1998) NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. *Proc.Natl.Acad.Sci.U.S.A* **95**, 3792-3797.

Littman DR (1998) Chemokine receptors: keys to AIDS pathogenesis? *Cell* **93**, 677-680.

Loetscher M, Amara A, Oberlin E, Brass N, Legler D, Loetscher P, D'Apuzzo M, Meese E, Rousset D, Virelizier JL, Baggiolini M, renzana-Seisdedos F, Moser B (1997) TYMSTR, a putative chemokine receptor selectively expressed in activated T cells, exhibits HIV-1 coreceptor function. *Curr.Biol.* **7**, 652-660.

Luftig M, Yasui T, Soni V, Kang MS, Jacobson N, Cahir-McFarland E, Seed B, Kieff E (2004) Epstein-Barr virus latent infection membrane protein 1 TRAF-binding site induces NIK/IKK alpha-dependent noncanonical NF-kappaB activation. *Proc.Natl.Acad.Sci.U.S.A* **101**, 141-146.

Lukacs NW, Prosser DM, Wiekowski M, Lira SA, Cook DN (2001) Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J.Exp.Med.* **194**, 551-555.

158

References

Lusso P (2006) HIV and the chemokine system: 10 years later. *EMBO J.* 25, 447-456.

Luttrell LM, Daaka Y, Lefkowitz RJ (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr.Opin.Cell Biol.* 11, 177-183.

Ma AD, Metjian A, Bagrodia S, Taylor S, Abrams CS (1998) Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. *Mol.Cell Biol.* 18, 4744-4751.

Ma L, Rohatgi R, Kirschner MW (1998) The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. *Proc.Natl.Acad.Sci.U.S.A* 95, 15362-15367.

Ma M, Wei T, Boring L, Charo IF, Ransohoff RM, Jakeman LB (2002) Monocyte recruitment and myelin removal are delayed following spinal cord injury in mice with CCR2 chemokine receptor deletion. *J.Neurosci.Res.* 68, 691-702.

Macdonald SG, Crews CM, Wu L, Driller J, Clark R, Erikson RL, McCormick F (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. *Mol.Cell Biol.* 13, 6615-6620.

Macdonald SG, Crews CM, Wu L, Driller J, Clark R, Erikson RL, McCormick F (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. *Mol.Cell Biol.* 13, 6615-6620.

Maffucci T, Falasca M (2001) Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett.* 506, 173-179.

Malek S, Chen Y, Huxford T, Ghosh G (2001) IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J.Biol.Chem.* 276, 45225-45235.

159

References

Mansell A, Brint E, Gould JA, O'Neill LA, Hertzog PJ (2004) Mal interacts with tumor necrosis factor receptor-associated factor (TRAF)-6 to mediate NF-kappaB activation by toll-like receptor (TLR)-2 and TLR4. *J.Biol.Chem.* **279**, 37227-37230.

Mantovani A (1999) The chemokine system: redundancy for robust outputs. *Immunol.Today* **20**, 254-257.

Marienfeld R, May MJ, Berberich I, Serfling E, Ghosh S, Neumann M (2003) RelB forms transcriptionally inactive complexes with RelA/p65. *J.Biol.Chem.* **278**, 19852-19860.

Matloubian M, David A, Engel S, Ryan JE, Cyster JG (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nature Immunology* **1**, 298-304.
Ref ID: MATLOUBIAN2000
Reprint: Not in File

Mattioli I, Sebald A, Bucher C, Charles RP, Nakano H, Doi T, Kracht M, Schmitz ML (2004) Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. *J.Immunol.* **172**, 6336-6344.

Mauro A, Ciccarelli C, De CP, Scoglio A, Bouche M, Molinaro M, Aquino A, Zani BM (2002) PKCalpha-mediated ERK, JNK and p38 activation regulates the myogenic program in human rhabdomyosarcoma cells. *J.Cell Sci.* **115**, 3587-3599.

Meili R, Ellsworth C, Lee S, Reddy TB, Ma H, Firtel RA (1999) Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. *EMBO J.* **18**, 2092-2105.

Merlot S, Firtel RA (2003) Leading the way: Directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J.Cell Sci.* **116**, 3471-3478.

Minami M, Kume N, Shimaoka T, Kataoka H, Hayashida K, Akiyama Y, Nagata I, Ando K, Nobuyoshi M, Hanyuu M, Komeda M, Yonehara S, Kita T (2001) Expression of SR-PSOX, a novel cell-surface scavenger receptor for phosphatidylserine and oxidized LDL in human atherosclerotic lesions. *Arterioscler.Thromb.Vasc.Biol.* **21**, 1796-1800.

160

References

Missy K, Van P, V, Raynal P, Viala C, Mauco G, Plantavid M, Chap H, Payrastre B (1998) Lipid products of phosphoinositide 3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. *J.Biol.Chem.* **273**, 30279-30286.

Moorthy AK, Savinova OV, Ho JQ, Wang VY, Vu D, Ghosh G (2006) The 20S proteasome processes NF-kappaB1 p105 into p50 in a translation-independent manner. *EMBO J,* **25**, 1945-1956.

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol.Rev.* **52**, 145-176.

Murphy PM (2002) International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacological Reviews* **54**, 227-229.

Murphy WJ, Tian ZG, Asai O, Funakoshi S, Rotter P, Henry M, Strieter RM, Kunkel SL, Longo DL, Taub DD (1996) Chemokines and T lymphocyte activation: II. Facilitation of human T cell trafficking in severe combined immunodeficiency mice. *J.Immunol.* **156**, 2104-2111.

Nakamura I, Takahashi N, Sasaki T, Tanaka S, Udagawa N, Murakami H, Kimura K, Kabuyama Y, Kurokawa T, Suda T, . (1995) Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. *FEBS Lett.* **361**, 79-84.

Nakanishi S, Kakita S, Takahashi I, Kawahara K, Tsukuda E, Sano T, Yamada K, Yoshida M, Kase H, Matsuda Y, . (1992) Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J.Biol.Chem.* **267**, 2157-2163.

Nakayama T, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O (2003) Cutting edge: Profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues. *Journal of Immunology* **170**, 1136-1140.

Narumiya S, Ishizaki T, Watanabe N (1997) Rho effectors and reorganization of actin cytoskeleton. *FEBS Lett.* **410**, 68-72.

161

References

Nebl G, Fischer S, Penzel R, Samstag Y (2004) Dephosphorylation of cofilin is regulated through Ras and requires the combined activities of the Ras-effectors MEK and PI3K. *Cell Signal.* **16**, 235-243.

Neer EJ (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80**, 249-257.

Ness TL, Hogaboam CM, Strieter RM, Kunkel SL (2003) Immunomodulatory role of CXCR2 during experimental septic peritonitis. *J.Immunol.* **171**, 3775-3784.

Nikolopoulos SN, Turner CE (2001) Integrin-linked kinase (ILK) binding to paxillin LD1 motif regulates ILK localization to focal adhesions. *J.Biol.Chem.* **276**, 23499-23505.

Nilsson G, Mikovits JA, Metcalfe DD, Taub DD (1999) Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/Interleukin-8RB. *Blood* **93**, 2791-2797.

O'Garra A, McEvoy LM, Zlotnik A (1998) T-cell subsets: chemokine receptors guide the way. *Curr.Biol.* **8**, R646-R649.

O'Mahony A, Lin X, Geleziunas R, Greene WC (2000) Activation of the heterodimeric IkappaB kinase alpha (IKKalpha)-IKKbeta complex is directional: IKKalpha regulates IKKbeta under both basal and stimulated conditions. *Mol.Cell Biol.* **20**, 1170-1178.

Okabe S, Fukuda S, Kim YJ, Niki M, Pelus LM, Ohyashiki K, Pandolfi PP, Broxmeyer HE (2005) Stromal cell-derived factor-1alpha/CXCL12-induced chemotaxis of T cells involves activation of the RasGAP-associated docking protein p62Dok-1. *Blood* **105**, 474-480.

Okazaki T, Sakon S, Sasazuki T, Sakurai H, Doi T, Yagita H, Okumura K, Nakano H (2003) Phosphorylation of serine 276 is essential for p65 NF-kappaB subunit-dependent cellular responses. *Biochem.Biophys.Res.Commun.* **300**, 807-812.

References

Orian A, Schwartz AL, Israel A, Whiteside S, Kahana C, Ciechanover A (1999) Structural motifs involved in ubiquitin-mediated processing of the NF-kappaB precursor p105: roles of the glycine-rich region and a downstream ubiquitination domain. *Mol.Cell Biol.* **19**, 3664-3673.

Owsianik G, Talavera K, Voets T, Nilius B (2006) Permeation and selectivity of TRP channels. *Annu.Rev.Physiol* **68**, 685-717.

Paramio JM, Segrelles C, Ruiz S, Jorcano JL (2001) Inhibition of protein kinase B (PKB) and PKCzeta mediates keratin K10-induced cell cycle arrest. *Mol.Cell Biol.* **21**, 7449-7459.

Parkin J, Cohen B (2001) An overview of the immune system. *Lancet* **357**, 1777-1789.

Peakman M, Vergani D (1997) 'Basic and Clinical Immunology.' (Churchill Livingstone: New York)

Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S (2001) Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. *J.Biol.Chem.* **276**, 27462-27469.

Peters W, Scott HM, Chambers HF, Flynn JL, Charo IF, Ernst JD (2001) Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc.Natl.Acad.Sci.U.S.A* **98**, 7958-7963.

Plotkin J, Prockop SE, Lepique A, Petrie HT (2003) Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. *J.Immunol.* **171**, 4521-4527.

Polte TR, Hanks SK (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc.Natl.Acad.Sci.U.S.A* **92**, 10678-10682.

Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR (1996) Rapamycin inhibits vascular smooth muscle cell migration. *J.Clin.Invest* **98**, 2277-2283.

163

References

Prekeris R, Mayhew MW, Cooper JB, Terrian DM (1996) Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *J.Cell Biol.* **132**, 77-90.

Pullen N, Thomas G (1997) The modular phosphorylation and activation of p70s6k. *FEBS Lett.* **410**, 78-82.

Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, Thomas G (1998) Phosphorylation and activation of p70s6k by PDK1. *Science* **279**, 707-710.

Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J.Clin.Invest* **101**, 746-754.

Reif K, Nobes CD, Thomas G, Hall A, Cantrell DA (1996) Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr.Biol.* **6**, 1445-1455.

Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch BM, Walter U (1995) The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *EMBO J.* **14**, 1583-1589.

Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC (2001) CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J.Exp.Med.* **194**, 1541-1547.

Rickert P, Weiner OD, Wang F, Bourne HR, Servant G (2000) Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. *Trends Cell Biol.* **10**, 466-473.

Rollinghoff M (1997) Immunity, components of the immune system and immune response. *Biologicals* **25**, 165-168.

Rollins BJ (1997) Chemokines. *Blood* **90**, 909-928.

Romagnani S (1996) Development of Th 1- or Th 2-dominated immune responses: what about the polarizing signals? *Int.J.Clin.Lab Res.* **26**, 83-98.

164

References

Rubbert A, Combadiere C, Ostrowski M, Arthos J, Dybul M, Machado E, Cohn MA, Hoxie JA, Murphy PM, Fauci AS, Weissman D (1998) Dendritic cells express multiple chemokine receptors used as coreceptors for HIV entry. *J.Immunol.* **160,** 3933-3941.

Ruth JH, Haas CS, Park CC, Amin MA, Martinez RJ, Haines GK, III, Shahrara S, Campbell PL, Koch AE (2006) CXCL16-mediated cell recruitment to rheumatoid arthritis synovial tissue and murine lymph nodes is dependent upon the MAPK pathway. *Arthritis Rheum.* **54,** 765-778.

Sadhu C, Dick K, Tino WT, Staunton DE (2003) Selective role of PI3K delta in neutrophil inflammatory responses. *Biochem.Biophys.Res.Commun.* **308,** 764-769.

Sadhu C, Masinovsky B, Dick K, Sowell CG, Staunton DE (2003) Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J.Immunol.* **170,** 2647-2654.

Saito H, Minamiya Y, Kitamura M, Saito S, Enomoto K, Terada K, Ogawa J (1998) Endothelial myosin light chain kinase regulates neutrophil migration across human umbilical vein endothelial cell monolayer. *J.Immunol.* **161,** 1533-1540.

Sallusto F, Mackay CR, Lanzavecchia A (1997) Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* **277,** 2005-2007.

Salmeron A, Janzen J, Soneji Y, Bump N, Kamens J, Allen H, Ley SC (2001) Direct phosphorylation of NF-kappaB1 p105 by the IkappaB kinase complex on serine 927 is essential for signal-induced p105 proteolysis. *J.Biol.Chem.* **276,** 22215-22222.

Salojin KV, Zhang J, Delovitch TL (1999) TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1-/PAK-1/p38 MAPK signaling pathway. *J.Immunol.* **163,** 844-853.

Sanchez-Madrid F, del Pozo MA (1999) Leukocyte polarization in cell migration and immune interactions. *EMBO J.* **18,** 501-511.

References

Sasahara Y, Rachid R, Byrne MJ, de la Fuente MA, Abraham RT, Ramesh N, Geha RS (2002) Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol.Cell* **10**, 1269-1281.

Sasaki CY, Barberi TJ, Ghosh P, Longo DL (2005) Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway. *J.Biol.Chem.* **280**, 34538-34547.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol.Cell Biol.* **14**, 1680-1688.

Scheid MP, Woodgett JR (2003) Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett.* **546**, 108-112.

Schmalz D, Kalkbrenner F, Hucho F, Buchner K (1996) Transport of protein kinase C alpha into the nucleus requires intact cytoskeleton while the transport of a protein containing a canonical nuclear localization signal does not. *J.Cell Sci.* **109** (Pt 9), 2401-2406.

Schuh JM, Power CA, Proudfoot AE, Kunkel SL, Lukacs NW, Hogaboam CM (2002) Airway hyperresponsiveness, but not airway remodeling, is attenuated during chronic pulmonary allergic responses to Aspergillus in CCR4-/- mice. *FASEB J.* **16**, 1313-1315.

Scott PH, Brunn GJ, Kohn AD, Roth RA, Lawrence JC, Jr. (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc.Natl.Acad.Sci.U.S.A* **95**, 7772-7777.

Serrador JM, Vicente-Manzanares M, Calvo J, Barreiro O, Montoya MC, Schwartz-Albiez R, Furthmayr H, Lozano F, Sanchez-Madrid F (2002) A novel serine-rich motif in the intercellular adhesion molecule 3 is critical for its ezrin/radixin/moesin-directed subcellular targeting. *J.Biol.Chem.* **277**, 10400-10409.

References

Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J.Exp.Med.* **171**, 35-47.

Shan X, Czar MJ, Bunnell SC, Liu P, Liu Y, Schwartzberg PL, Wange RL (2000) Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol.Cell Biol.* **20**, 6945-6957.

Sharron M, Pohlmann S, Price K, Lolis E, Tsang M, Kirchhoff F, Doms RW, Lee B (2000) Expression and coreceptor activity of STRL33/Bonzo on primary peripheral blood lymphocytes. *Blood* **96**, 41-49.

Shashkin P, Simpson D, Mishin V, Chesnutt B, Ley F (2003) Expression of CXCL16 in human T cells. *Arteriosclerosis Thrombosis and Vascular Biology* **23**, 148-149.

Shimaoka T, Nakayama T, Fukumoto N, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita T, Ohsumi J, Yoshie O, Yonehara S (2004) Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. *Journal of Leukocyte Biology* **75**, 267-274.

Simonsen A, Wurmser AE, Emr SD, Stenmark H (2001) The role of phosphoinositides in membrane transport. *Curr.Opin.Cell Biol.* **13**, 485-492.

Sonnenburg ED, Gao T, Newton AC (2001) The phosphoinositide-dependent kinase, PDK-1, phosphorylates conventional protein kinase C isozymes by a mechanism that is independent of phosphoinositide 3-kinase. *J.Biol.Chem.* **276**, 45289-45297.

Sotsios Y, Whittaker GC, Westwick J, Ward SG (1999) The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J.Immunol.* **163**, 5954-5963.

Sotsios Y, Ward SG (2000) Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. *Immunol.Rev.* **177**, 217-235.

167

References

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* **279**, 710-714.

Stevens SW, Ryan DE, Ge HY, Moore RE, Young MK, Lee TD, Abelson J (2002) Composition and functional characterization of the yeast spliceosomal penta-snRNP. *Mol.Cell* **9**, 31-44.

Storz P, Toker A (2003) NF-kappaB signaling--an alternate pathway for oxidative stress responses. *Cell Cycle* **2**, 9-10.

Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D, . (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J.Biol.Chem.* **270**, 27348-27357.

Stuart JR, Gonzalez FH, Kawai H, Yuan ZM (2006) C-ABL interacts with the wave2 signaling complex to induce membrane ruffling and cell spreading. *J.Biol.Chem.*

Su B, Karin M (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr.Opin.Immunol.* **8**, 402-411.

Su WH, Chen HI, Huang JP, Jen CJ (2000) Endothelial [Ca(2+)](i) signaling during transmigration of polymorphonuclear leukocytes. *Blood* **96**, 3816-3822.

Sun CX, Downey GP, Zhu F, Koh AL, Thang H, Glogauer M (2004) Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. *Blood* **104**, 3758-3765.

Sun H, King AJ, Diaz HB, Marshall MS (2000) Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. *Curr.Biol.* **10**, 281-284.

References

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 393, 591-594.

Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y (1997) Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J.Biol.Chem.* 272, 23371-23375.

Thomas MS, Kunkel SL, Lukacs NW (2004) Regulation of cockroach antigen-induced allergic airway hyperreactivity by the CXCR3 ligand CXCL9. *J.Immunol.* 173, 615-623.

Toker A, Newton AC (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J.Biol.Chem.* 275, 8271-8274.

Tonks A, Cooper RA, Price AJ, Molan PC, Jones KP (2001) Stimulation of TNF-alpha release in monocytes by honey. *Cytokine* 14, 240-242.

Trushin SA, Pennington KN, Carmona EM, Asin S, Savoy DN, Billadeau DD, Paya CV (2003) Protein kinase Calpha (PKCalpha) acts upstream of PKCtheta to activate IkappaB kinase and NF-kappaB in T lymphocytes. *Mol.Cell Biol.* 23, 7068-7081.

Turner L, Ward SG, Westwick J (1995) RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *J.Immunol.* 155, 2437-2444.

Turner SJ, Domin J, Waterfield MD, Ward SG, Westwick J (1998) The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2alpha. *J.Biol.Chem.* 273, 25987-25995.

Unutmaz D, Xiang W, Sunshine MJ, Campbell J, Butcher E, Littman DR (2000) The primate lentiviral receptor Bonzo/STRL33 is coordinately regulated with CCR5 and its expression pattern is conserved between human and mouse. *J.Immunol.* 165, 3284-3292.

References

van Blitterswijk WJ, Houssa B (2000) Properties and functions of diacylglycerol kinases. *Cell Signal.* **12**, 595-605.

Van Etten RA, Jackson PK, Baltimore D, Sanders MC, Matsudaira PT, Janmey PA (1994) The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. *J.Cell Biol.* **124**, 325-340.

van d, V, van Lieshout AW, Toonen LW, Sloetjes AW, van den Berg WB, Figdor CG, Radstake TR, Adema GJ (2005) Elevated CXCL16 expression by synovial macrophages recruits memory T cells into rheumatoid joints. *Arthritis Rheum.* **52**, 1381-1391.

Vandenbulcke L, Bachert C, Van CP, Claeys S (2006) The innate immune system and its role in allergic disorders. *Int.Arch.Allergy Immunol.* **139**, 159-165.

Vanhaesebroeck B, Alessi DR (2000) The PI3K-PDK1 connection: more than just a road to PKB. *Biochem.J.* **346 Pt 3**, 561-576.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu.Rev.Biochem.* **70**, 535-602.

Vermeulen L, De WG, Van DP, Vanden BW, Haegeman G (2003) Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J.* **22**, 1313-1324.

Vicente-Manzanares M, Cruz-Adalia A, Martin-Cofreces NB, Cabrero JR, Dosil M, varado-Sanchez B, Bustelo XR, Sanchez-Madrid F (2005) Control of lymphocyte shape and the chemotactic response by the GTP exchange factor Vav. *Blood* **105**, 3026-3034.

Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J.Biol.Chem.* **269**, 5241-5248.

References

Vlahos CJ, Matter WF, Brown RF, Traynor-Kaplan AE, Heyworth PG, Prossnitz ER, Ye RD, Marder P, Schelm JA, Rothfuss KJ, . (1995) Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. *J.Immunol.* **154**, 2413-2422.

Wagsater D, Hugander A, Dimberg J (2004) Expression of CXCL16 in human rectal cancer. *International Journal of Molecular Medicine* **14**, 65-69.

Walders-Harbeck B, Khaitlina SY, Hinssen H, Jockusch BM, Illenberger S (2002) The vasodilator-stimulated phosphoprotein promotes actin polymerisation through direct binding to monomeric actin. *FEBS Lett.* **529**, 275-280.

Wang D, You Y, Case SM, lister-Lucas LM, Wang L, DiStefano PS, Nunez G, Bertin J, Lin X (2002) A requirement for CARMA1 in TCR-induced NF-kappa B activation. *Nat.Immunol.* **3**, 830-835.

Wang J, Holmes TH, Cheung R, Greenberg HB, He XS (2004) Expression of chemokine receptors on intrahepatic and peripheral lymphocytes in chronic hepatitis C infection: its relationship to liver inflammation. *J.Infect.Dis.* **190**, 989-997.

Ward SG, Westwick J (1998) Chemokines: understanding their role in T-lymphocyte biology. *Biochem.J.* **333** (Pt 3), 457-470.

Ward SG, Bacon K, Westwick J (1998) Chemokines and T lymphocytes: more than an attraction. *Immunity.* **9**, 1-11.

Waterfield M, Jin W, Reiley W, Zhang M, Sun SC (2004) IkappaB kinase is an essential component of the Tpl2 signaling pathway. *Mol.Cell Biol.* **24**, 6040-6048.

Weiner OD (2002) Rac activation: P-Rex1 - a convergence point for PIP(3) and Gbetagamma? *Curr.Biol.* **12**, R429-R431.

Welch HC, Coadwell WJ, Ellson CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, Tempst P, Hawkins PT, Stephens LR (2002) P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell* **108**, 809-821.

References

Wennstrom S, Hawkins P, Cooke F, Hara K, Yonezawa K, Kasuga M, Jackson T, Claesson-Welsh L, Stephens L (1994) Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr.Biol.* **4**, 385-393.

Wennstrom S, Siegbahn A, Yokote K, Arvidsson AK, Heldin CH, Mori S, Claesson-Welsh L (1994) Membrane ruffling and chemotaxis transduced by the PDGF beta-receptor require the binding site for phosphatidylinositol 3' kinase. *Oncogene* **9**, 651-660.

Wenzel-Seifert K, Schachtele C, Seifert R (1994) N-protein kinase C isoenzymes may be involved in the regulation of various neutrophil functions. *Biochem.Biophys.Res.Commun.* **200**, 1536-1543.

West KA, Zhang H, Brown MC, Nikolopoulos SN, Riedy MC, Horwitz AF, Turner CE (2001) The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL). *J.Cell Biol.* **154**, 161-176.

Wilbanks A, Zondlo SC, Murphy K, Mak S, Soler D, Langdon P, Andrew DP, Wu LJ, Briskin M (2001) Expression cloning of the STRL33/BONZO/TYMSTR ligand reveals elements of CC, CXC, and CX3C chemokines. *Journal of Immunology* **166**, 5145-5154.

Wilkinson SE, Parker PJ, Nixon JS (1993) Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem.J.* **294** (Pt 2), 335-337.

Wong MM, Fish EN (2003) Chemokines: attractive mediators of the immune response. *Semin.Immunol.* **15**, 5-14.

Woodgett JR, Ohashi PS (2005) GSK3: an in-Toll-erant protein kinase? *Nat.Immunol.* **6**, 751-752.

Worthylake RA, Burridge K (2001) Leukocyte transendothelial migration: orchestrating the underlying molecular machinery. *Curr.Opin.Cell Biol.* **13**, 569-577.

172

References

Wuttge DM, Zhou X, Sheikine Y, Wagsater D, Stemme V, Hedin U, Stemme S, Hansson GK, Sirsjo A (2004) CXCL16/SR-PSOX is an interferon-gamma-regulated chemokine and scavenger receptor expressed in atherosclerotic lesions. *Arterioscler.Thromb.Vasc.Biol.* **24,** 750-755.

Xiao G, Fong A, Sun SC (2004) Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. *J.Biol.Chem.* **279,** 30099-30105.

Xiu Y, Nakamura K, Abe M, Li N, Wen XS, Jiang Y, Zhang D, Tsurui H, Matsuoka S, Hamano Y, Fujii H, Ono M, Takai T, Shimokawa T, Ra C, Shirai T, Hirose S (2002) Transcriptional regulation of Fcgr2b gene by polymorphic promoter region and its contribution to humoral immune responses. *J.Immunol.* **169,** 4340-4346.

Yamauchi J, Nagao M, Kaziro Y, Itoh H (1997) Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of Gbetagamma and Galphaq/11 subunits. *J.Biol.Chem.* **272,** 27771-27777.

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393,** 809-812.

Yao J, Mackman N, Edgington TS, Fan ST (1997) Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J.Biol.Chem.* **272,** 17795-17801.

Yost C, Torres M, Miller JR, Huang E, Kimelman D, Moon RT (1996) The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10,** 1443-1454.

Zeng R, Cannon JL, Abraham RT, Way M, Billadeau DD, Bubeck-Wardenberg J, Burkhardt JK (2003) SLP-76 coordinates Nck-dependent Wiskott-Aldrich syndrome protein recruitment with Vav-1/Cdc42-dependent Wiskott-Aldrich syndrome protein activation at the T cell-APC contact site. *J.Immunol.* **171,** 1360-1368.

References

Zhang W, Han SW, McKeel DW, Goate A, Wu JY (1998) Interaction of presenilins with the filamin family of actin-binding proteins. *J.Neurosci.* **18,** 914-922.

Zhang Z, Elly C, Qiu L, Altman A, Liu YC (1999) A direct interaction between the adaptor protein Cbl-b and the kinase zap-70 induces a positive signal in T cells. *Curr.Biol.* **9,** 203-206.

Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, Ghosh S (1997) The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89,** 413-424.

Zhou L, Tan A, Iasvovskaia S, Li J, Lin A, Hershenson MB (2003) Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-kappaB-mediated gene expression in airway epithelial cells. *Am.J.Respir.Cell Mol.Biol.* **28,** 762-769.

Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. *Immunity.* **12,** 121-127.

References

Appendix 1: Recipes for buffers and solutions

1. Media for Th1 polarisation

Growth Media

500ml RPMI 1640 media

5% (v/v) Heat inactivated foetal calf serum

5ml Non essential amino acids

5ml Sodium Pyruvate

10mM Hepes

100U/ml Penicillin

100μg/ml streptomycin

50μM Mercaptoethanol

Resting Media

500ml RPMI 1640 media

10% (v/v) Heat inactivated foetal calf serum

5ml Non essential amino acids

5ml Sodium Pyruvate

10mM Hepes

100U/ml Penicillin

100μg/ml streptomycin

50μM Mercaptoethanol

2. Lysis buffers

2.1 Lysis buffer for whole cell lysates

20mM Tris pH 7.5

137mM NaCl

1% (v/v) Nonidet P-40

10% (v/v) glycerol

1mM EDTA

1mM sodium vanadate *

1mM sodium molybdate *

10mM sodium fluoride *

40μg/ml phenylmethylsulfonyl fluoride *

0.7μg/ml pepstatin A *

10μg/ml aprotinin *

10μg/ml leupeptide *

10μg/ml soyabean trypsin inhibitor *

MilliQ H_2O

* added just before use

2.2 Nuclear extract buffers

Buffer 1	Buffer 2
10mM Hepes	10mM Hepes
10mM KCl	400mM NaCl
100μM EDTA	100μM EDTA
100μM EGTA	100μM EGTA
1mM sodium molybdate *	1mM sodium molybdate *
1mM sodium vanadate *	1mM sodium vanadate *
10mM sodium fluoride *	10mM sodium fluoride *
MilliQ H_2O	MilliQ H_2O

* added just before use

Appendix

3. Solutions and buffers used in immunoblotting

3.1 5x SDS-sample buffer

10% (w/v) SDS

50% (v/v) glycerol

200mM Tris pH 6.8

Bromophenol blue

5% (v/v) 2-mercaptoethanol

MilliQ H_2O

3.2 Resolving gel buffer   Stacking gel buffer

Resolving gel buffer	Stacking gel buffer
1.5M Tris pH8.8	0.5M Tris pH6.8
0.4% SDS	0.4% SDS

To make 4 gels:

	Resolving gel			Stacking gel
	7.5%	10%		5%
MilliQ H_2O	9.84ml	8.17ml	MilliQ H_2O	6.85ml
Resolving gel buffer	5.0ml	5.0ml	Stacking gel buffer	3.0ml
Bis-Acrylamide	5.0ml	6.67ml	Bis-Acrylamide	2.0ml
10% (w/v) ammonium persulphate *	150μl	150μl	10% (w/v) ammonium persulphate *	150μl
TEMED *	15μl	15μl	TEMED *	15μl

* added just before use

3.3 Buffers for immunoblotting

SDS-page running buffer

25mM Tris

192mM glycine

0.1% (w/v) SDS

MilliQ H_2O

Semi-dry transfer buffer

39mM glycine

48mM Tris

0.0375 % (w/v) SDS

20% (v/v) methanol

MilliQ H_2O

Appendix

Tris-buffered saline (TBS)

20mM Tris pH7.5

150mM NaCl

MilliQ H_2O

Tris-buffered saline + tween (TBST)

TBS as above

0.01% (v/v) Tween-20

Stripping buffer

62.5mM Tris pH6.8

2% (w/v) SDS

0.0077% (v/v) 2-mercaptoethanol

MilliQ H_2O

Appendix