Characterisation of the Chemokine Receptor CXCR3 and its Atypical Variants in Human T Lymphocytes

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Characterisation of the Chemokine Receptor CXCR3 and its Atypical Variants in Human T Lymphocytes

A thesis submitted by

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To My Parents

Moim Rodzicom

Acknowledgements

'No man is an island entire of itself; every man is a piece of the continent, a part of the main.'

John Donne

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Abstract

The chemokine receptor CXCR3 and its agonists CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC are involved in a variety of inflammatory disorders including multiple sclerosis, rheumatoid arthritis, psoriasis and sarcoidosis. CXCL11 has also been reported to bind to an additional receptor, namely CXCR7, which also interacts with CXCL12. Two alternatively spliced variants of the human CXCR3 receptor have been described, namely CXCR3-B and CXCR3-alt. The human CXCR3-B has been found to bind CXCL9, CXCL10, CXCL11 as well as an additional agonist CXCL4/PF4. In contrast, CXCR3-alt only binds CXCL11. This work demonstrates that CXCL4 like the original CXCR3 agonists is capable of inducing biochemical signalling, namely intra-cellular calcium elevation, and activation of p44/p42 MAPK and PI3K/Akt pathways in activated human T lymphocytes. Phosphorylation of p44/p42 MAPK and Akt was inhibited by pertussis toxin, suggesting coupling to $G\alpha_i$ protein. In contrast CXCR3 antagonists blocked CXCR3 agonists but not CXCL4-mediated responses. Surprisingly, stimulation of T cells with CXCL4 failed to elicit migratory responses of these cells and did not lead to loss of surface CXCR3 expression. Collectively our evidence shows that although CXCL4 is coupled to downstream biochemical machinery, its function in T cells is distinct from the function of CXCR3 agonists. The work presented in this thesis also indicates that despite considerably lower surface expression in comparison to the full length CXCR3, CXCR3-B and CXCR3-alt transduce biochemical signals in response to CXCL11 in transfected cells.

According to previous reports the role of CXCR7 in signalling and chemotaxis in T cells could not be detected. In T cells and transfected cells system CXCR7 was localised at the plasma membrane and was efficiently internalized in response to CXCL11 and CXCL12.

Studies of the involvement of methylation in T cell chemotaxis suggest that this modification may be required in this process as it was partially inhibited by methylation inhibitor- MTA. Moreover T cell co-stimulation caused increased levels of arginine mono-methylated proteins suggesting the importance of methylation in T lymphocyte signalling.

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Abbreviations

°C	degrees Celsius
μg	microgram
μL	microlitre
-RT	without reverse transcriptase
Ab	antibody
Ag	antigen
ANOVA	analysis of variance
APC	antigen presenting cell
APC	allophycocyanin
BM	bone marrow
bp	basis pair
BSA	bovine serum albumine
Ca^{2+}	calcium ion
CCPs	clathrin-coated pits
CCR	CC chemokine receptor
CCVs	clathrin-coated vesicles
CD	cluster of differentiation
CHO	Chinese hamster ovary
CNS	central nervous system
CO_2	carbon dioxide
CX3CR	CX3C chemokine receptor
CXCR	CXC chemokine receptor
DAG	diacyl-glycerol
DAPI	4, 6-diamidino-2-phenylindole
DARC	Duffy antigen/receptor for chemokines
DC	dendritic cells
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EC	endothelial cell

ECM	extracellular matrix
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
EtOH	ethanol
FACS	fluorescence activates cell sorter
FITC	fluorescein isothiocyanate
FCS	foetal calf serum
g	gram
G418	geneticin
GAG	glycosaminoglycan
GAP	GTPase activating protein
GDP	guanine biphosphate
GEF	GDP exchange factor
GFP	green fluorescent protein
GPCR	G protein couples receptor
GRK	G protein coupled kinase
GTP	guanine triphosphate
h	hour(s)
HEK	human embryonic kidney cells
HRP	horseradish peroxidise
ICAM-1	intracellular adhesion molecule
IFM-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IP3	inositol 1,4,5 triphosphate
IP-10	IFN-γ-induced protein-10
IRES	internal ribosomal entry site
I-TAC	IFN-γ-inducible T cell α chemoattractant
JAK	Janus-family tyrosine kinase
kb	kilobase pair
kDa	kiloDalton

kg	kilogram
KO	knock out
L	litre
LEC	lymphatic endothelial cell
LN	lymph node
LPS	lipopolysaccharide
M	molar
MAPK	mitogen activated protein kinase
MβCD	methyl-β-cyclodextrin
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
Mig	monokine-induced by IFN-γ
min	minute(s)
mL	millilitre
mM	milimolar
mRNA	messenger RNA
MS	multiple sclerosis
MTA	Methylthioadenosine
μM	micro molar
neo	neomycin resistance gene
NFAT	nuclear factor of activated T-cells
nm	nanometre
nM	nanomolar
NK	natural killer
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
pg	picogram

PH	pleckstrin homology
PHA	phytohemagglutinin
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-biphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	12-O-tetradecanoylphorbol-13-acetate
PRMT	protein arginine methyltransferase
PTEN	phosphate and tensin homologue deleted on chromosome ten
PTX	pertussis toxin
RA	rheumatoid arthritis
RBC	red blood cell
rcf	gravitational force
RDC1	receptor dog cDNA
RGS	regulator of G-protein signalling
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RT	reverse transcriptase
RT-PCR	reverse-transcription PCR
SDF-1	stromal cell-derived factor
SDS	sodium dodecyl sulphate
SEB	Staphylococcal Enterotoxin B
sec	second(s)
STAT	signal transducer and activator of transcription
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	tetramethylethylenediamine

Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	IL-17 producing T helper cell
Treg	regulatory T cell
TM	transmembrane
TNF-α	tumor necrosis factor-α
Tween-20	polyoxyethylene-sorbitan monolaurate
VCAM-1	vascular cellular adhesion molecule-1
v/v	volume per unit volume
WT	wild type
w/v	weight per unit volume

Chapter One

Introduction

1.1 Human immune system

Infectious microorganisms are able to reproduce and evolve more rapidly than their human host. In order to protect against these organisms, the human body has developed a variety of complicated mechanisms and cells with highly specified functions in defence, which form the immune system. There are two types of responses to the invasion of the foreign organism; innate immune response (innate immunity) and adaptive immune response (adaptive immunity, Figure 1.1). The first type of immune response is always present and can be triggered rapidly in response to infection. When the innate immune response is not enough to eliminate infection, the more powerful forces of the adaptive immunity are required (Parham, 2000). The adaptive immune response in contrast to innate immunity is highly specific and improves with each successive encounter with the same pathogen. The adaptive immune system 'remembers' the infectious agents and this provides long – lived protection (Roitt et al., 2001).

1.1.1 Innate immunity

The innate immune response provides the first line of defence and its main aim is to limit the infection while the slower, adaptive response is activated. There are several mechanisms of innate immunity: the physical barriers which prevent the entry of a pathogen, such as skin and mucosal surfaces; physiological barriers such as temperature or pH which cause denaturation of pathogen; and chemical barriers such as interferons and complement proteins, responsible for inhibition and destruction of foreign organisms. Cells of the innate immune system are phagocytes which belong to the two major lineages – monocytes / macrophages and polymorphonuclear granulocytes, whose role is to remove particulate antigens. The innate immune system utilizes germline-encoded receptors for recognition of infectious microorganisms, known as pattern recognition receptors

(PRRs). These receptors bind and recognize targets containing particular patterns called PAMPs for pathogen-associated molecular patterns.

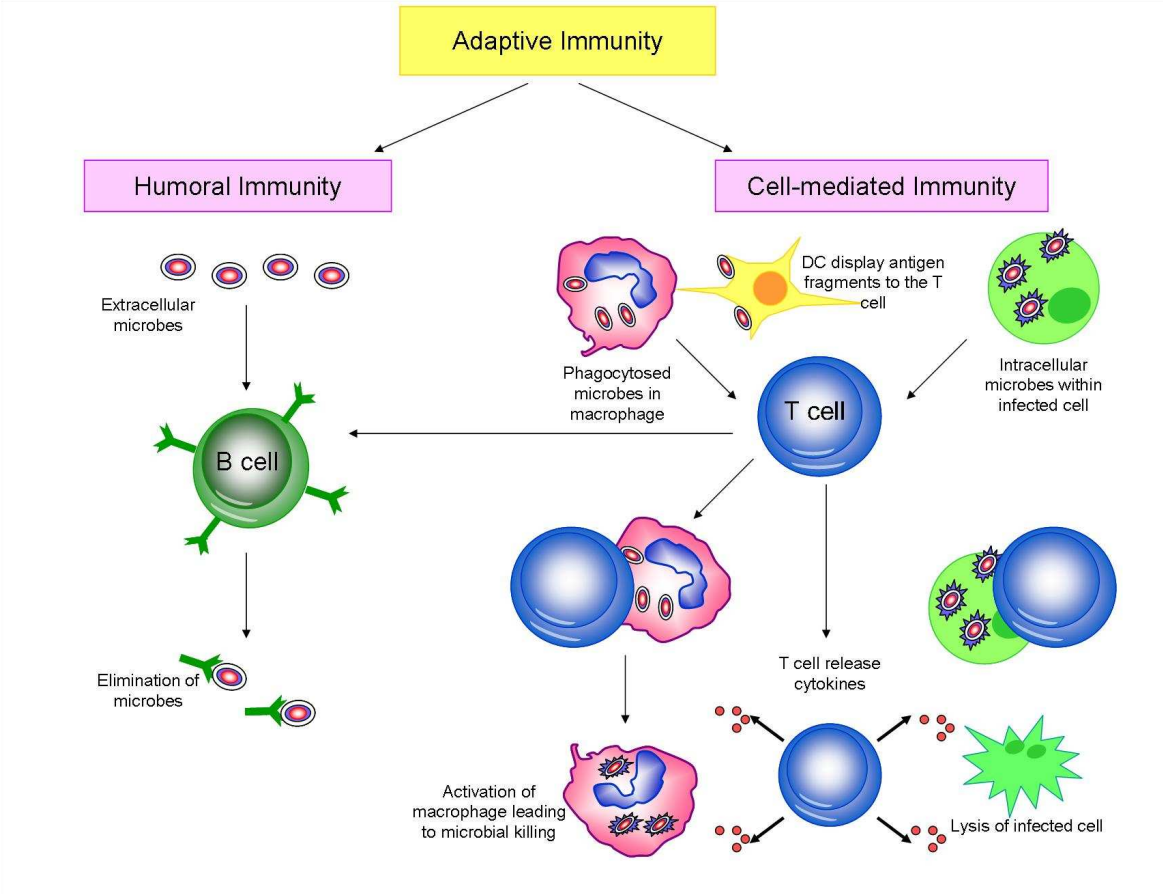


Figure 1.1 The components of adaptive (acquired) immunity. There are two types of adaptive immune defense: humoral, mediated by antibodies secreted by B lymphocytes, and cell-mediated, controlled by T lymphocytes and dendritic cells (involved in antigen presentation). Humoral immunity provides defense against extracellular pathogens, whilst cell-mediated immunity protects body from intracellular pathogens and involves activation of macrophages, antigen-specific cytotoxic T cells and releasing of variety of cytokines which alter the milieu around them. T helper cells also provide a support to B cells during humoral response (Rabb, 2002).

For example the general structure for lipopolysaccharide (LPS) is shared for all gram-negative bacteria and receptors such as Toll-like receptors (TLRs) that recognize conserved sequences of LPS will be able to detect any gram-negative bacterium (Medzhitov and Janeway, 1997; Schulz et al., 2005). The family of mammalian TLRs consists of 12 membrane proteins that induce innate responses through nuclear factor-κB (NF-κB)-dependent and interferon (IFN)-regulatory factor (IRF)-dependent signalling. These receptors recognize bacteria, viruses, fungi and protozoa. In addition to TLR family, which are the important players in microbial

sensing, there are other PRRs which participate in this process (Trinchieri and Sher, 2007). Bacterial and viral components that reach the inside of the cell are recognized by the cytosolic receptors, inducing cytokine production and cell activation. These receptors are categorized into two main families, namely the nucleotide-binding oligomerization domain (NOD)-like receptor family (NLR family), members of which are either NOD receptors or NALPS (NACHT-, LRR- and pyrin-domain-containing proteins); and a family of receptors that have an RNA-helicase domain joined to two caspase-recruitment domains (CARDs), for example retinoic-acid-inducible gene I (RIG-I)' and are named the RIG-I-like receptors (RLR). NLRs recognize bacteria and RLRs detect viral particles (Creagh and O'Neill, 2006; Trinchieri and Sher, 2007). Interplay between these families of receptors provides the efficient functionality of the innate immune system (Figure 1.2) (Creagh and O'Neill, 2006).

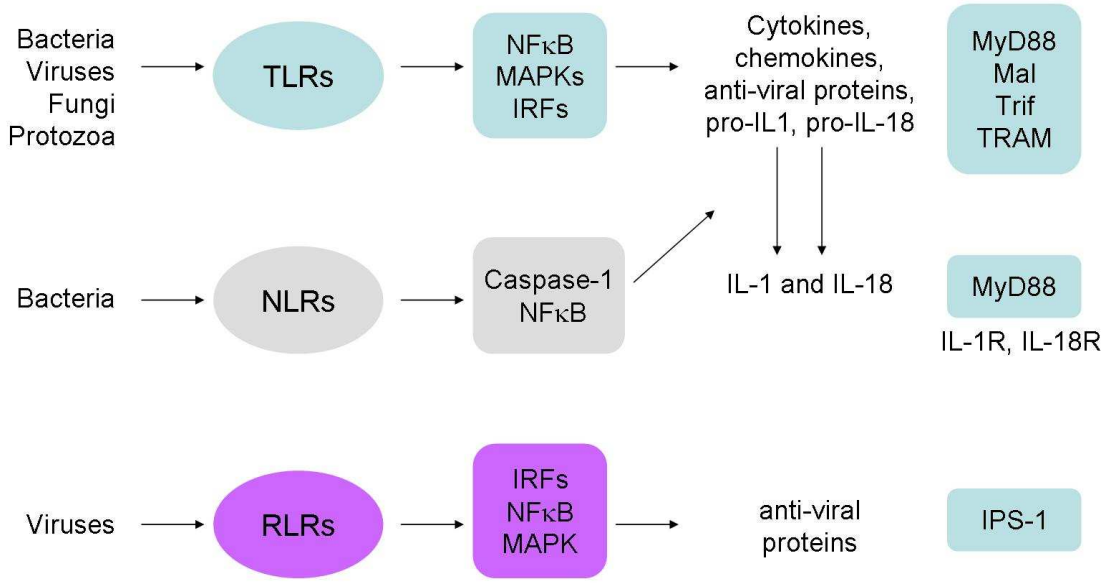


Figure 1.2 Three families of microbes sensors. TLRs recognize multiple pathogens and induce gene expression by the specific recruitment of the adaptors MyD88, Mal, TRIF and TRAM. The downstream activators include NF-kB and mitogen activated protein (MAP) kinases, and, in the case of anti-viral TLRs, IRFs. TLRs induce production of pro-IL-1b and pro-IL-18. NLRs activate NFκB -and caspase-1, which acts on pro-IL-1b and pro-IL-18, resulting in the production of mature bioactive forms whose receptors, in turn, signal through MyD88. RLRs detect viral RNA and recruit the adaptor IPS-1 in order to activate IRFs, NF-kB and MAPKs, resulting in the induction of anti-viral proteins, complimenting the activity of anti-viral TLRs. Adapted from Creagh and O'Neill, 2006.

1.1.2 Acquired (adaptive) immunity

There are two types of adaptive immunity: humoral (mediated by B cells) and cell-mediated (mediated by T cells). Humoral immunity consists of soluble antibodies that are produced in large amounts by plasma cells. These cells (also known as plasmocytes or plasma B cells) are derived from B cells upon stimulation by $CD4^+$ T lymphocytes. The main role of humoral immunity is defence against extracellular pathogens and their toxins due to production of specific antibodies that asist in their elimination.

T lymphocytes are crucial players in cell-mediated immune defense. They can act through the activation of macrophages (to kill phagocytosed pathogens), direct destruction of infected cells or production of cytokines that alter milieu around them. T lymphocyte biology and function will be discussed in further detail later in this chapter.

Another important component of cell-mediated immunity are dendritic cells (DC), which are involved in the process of antigen presentation. Immature DCs bind conserved sequences within pathogens, which in turn initiates their maturation and surface expression of TLRs (Akira et al., 2001) and co-stimulation molecules, such as CD80/CD86 (Schwartz et al., 2001; Vestweber, 2003).

Proper and complex function of the immune system is possible due to the dynamic and effective cooperation between innate and adaptive defenses and is achieved by an array of strictly specialized cells that form this system. Communication between immune cells is controlled by secretion of variety of molecules (cytokines, chemokines) which induce processes like cell differentiation or directed migration, and by regulation of expression of specific receptors on these cells.

1.2 T lymphocytes development in the thymus

Similarly to B lymphocytes, T cells (Thymus-dependent lymphocytes or T lymphocytes) also derive from bone marrow stem cells. They must then undergo gene rearrangements to generate specific antigen receptors (Figure 1.3). The immature T lymphocytes have to leave the bone marrow and enter the thymus

(where they become thymocytes) to mature and rearrange their T cell receptors (TCRs) genes (Parham, 2000). Two lineages of T lymphocytes can develop in the thymus; the majority develop into $\alpha\beta$ T cells and minority (only 5%) into $\gamma\delta$ T cells (Hayday, 2000). These cells develop from a common precursor (Figure 1.3). Despite the fact that only a low number of T cells express $\gamma\delta$ TCR, this cell type shows a distinct receptor profile and has been suggested to play a role in immune defense against infection particularly within the gut (Glatzel et al., 2002). Immature cells entering the thymus do not express any of the T cell receptor complex glycoproteins or the T cell co-receptors CD4 and CD8. Due to lack of expression of both CD4 and CD8, they are called 'double negative' thymocytes. During maturation within the thymus, 'double negative' lymphocytes undergo expansion and differentiation to CD4 and CD8 double positive cells. This process is promoted by IL-7. Only a small subpopulation of double positive thymocytes (1-2% of total) have receptors that can interact with one of the MHC molecules expressed by an individual APC and will be able to respond to antigens presented by these molecules on the surface of APCs. Thymocytes that can recognize self-MHC molecules on the epithelial cells (in the cortex of the thymus) are positively selected. Positive selection is also instrumental in determining whether double positive thymocytes will become CD4 or CD8 expressing T cell ('single positive'), which is related to further effector function of T lymphocytes. An additional mechanism which takes place within the thymus is called 'negative selection' and serves to deplete developing T cells which bind too strongly to self-peptides presented on MHC molecules by dendritic cells or macrophages in the thymus. These potentially autoreactive cells are signalled to undergo apoptosis (Parham, 2000). During processes of positive and negative selection during T cell development within the thymus, large number of cells die and a comparatively low number of cells form a circulating pool of T lymphocytes.

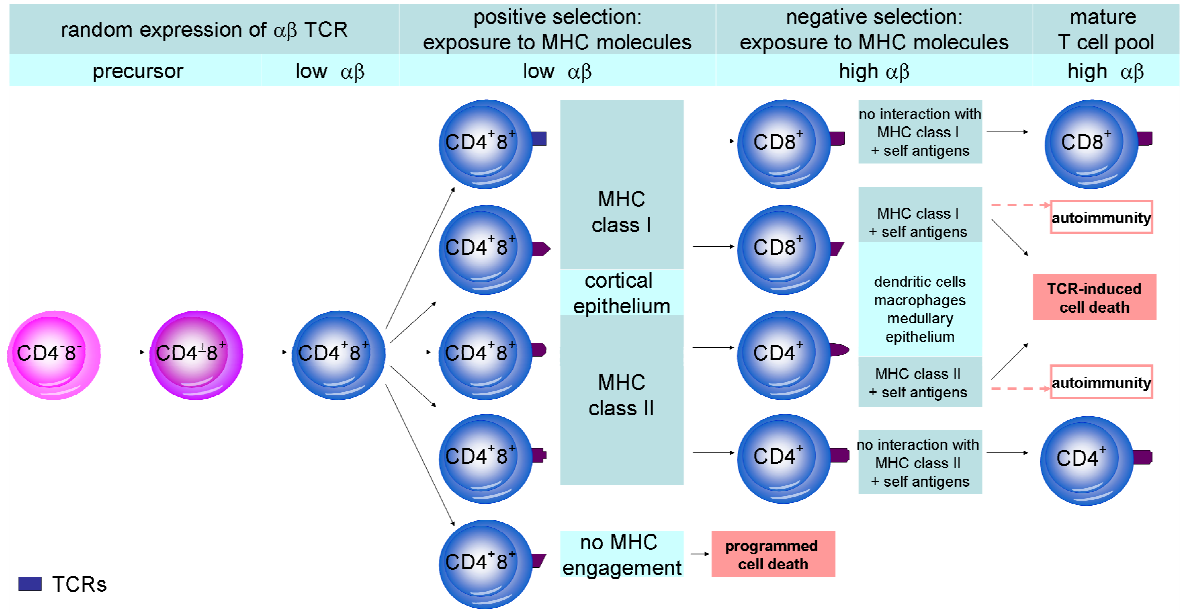


Figure 1.3 Pathways of thymocytes development. Common precursors develop into 'double negative' cells with low levels of TCR αβ expression. These lymphocytes undergo expansion and differentiation to CD4 and CD8 double positive cells. The small number of thymocytes that can recognize self-MHC molecules on the epithelial cells (in the cortex of the thymus) are positively selected. This process is followed by the 'negative selection' which serves to deplete developing T cells which bind too strongly to self-peptides presented on MHC molecules by dendritic cells or macrophages in the thymus. These potentially autoreactive cells are signalled to undergo apoptosis (Adapted from Roitt et al 2001).

As demonstrated in detail in Figure1.4, T lymphocyte development within the thymus is characterised by the surface expression of well-defined markers, including the previously discussed CD4 and CD8, as well as CD44 (or CD117) and CD25, together with the status of the T-cell receptor (TCR). Interactions between Notch receptor that is present on the thymocytes and Notch ligands expressed on the thymic stromal cells induce a complex programme of T cell maturation in the thymus, which leads to the generation of self-tolerant $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, which migrate from the thymus to the periphery to form the T cell pool (Zuniga-Pflucker, 2004).

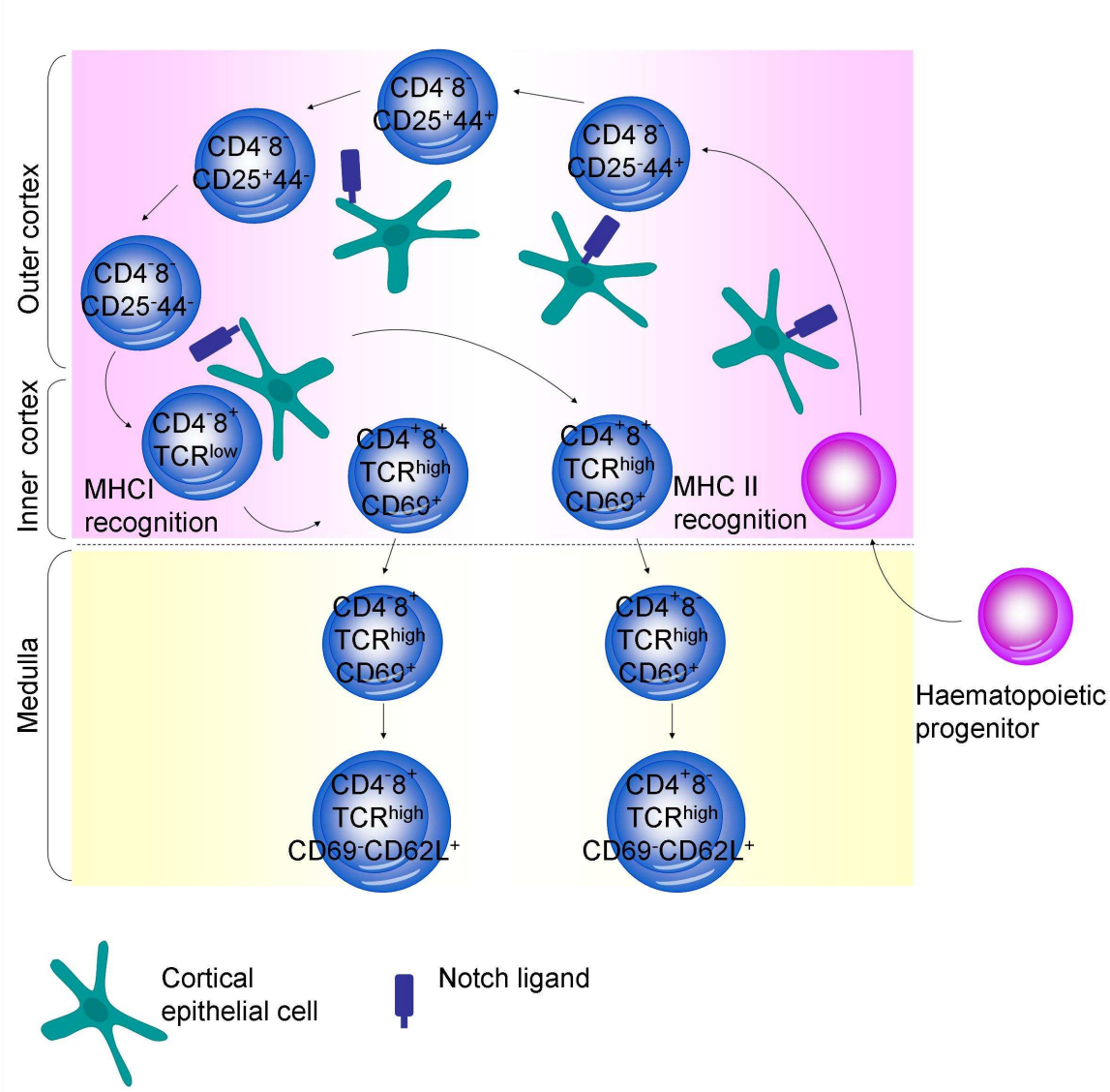


Figure 1.4 T cell development within the thymus. Development of T cells within the thymus can be characterised by the surface expression of well-defined markers, such as CD4, CD8, CD44 (or CD117) and CD25, as well as the status of the TCR. Interactions between Notch receptors and Notch ligands expressed on the thymic stromal cells induce a process of T cell maturation in the thymus, which leads to the generation of a small population of self-tolerant CD4+ helper T cells and CD8+ cytotoxic T cells. These cells migrate from the thymus to form peripheral T cell pool (Zuniga-Pflucker, 2004).

1.3 Antigen presentation and T cell activation

After leaving the thymus, T cells join the population of cells circulating through the lymph - naïve T lymphocytes. To become effective within the immune system, naïve T cells have to achieve an activation stage, clonal expansion by cell division and differentiation into effector cells. Naïve T cells become activated and differentiated into corresponding mature effector cells after encounter with antigen

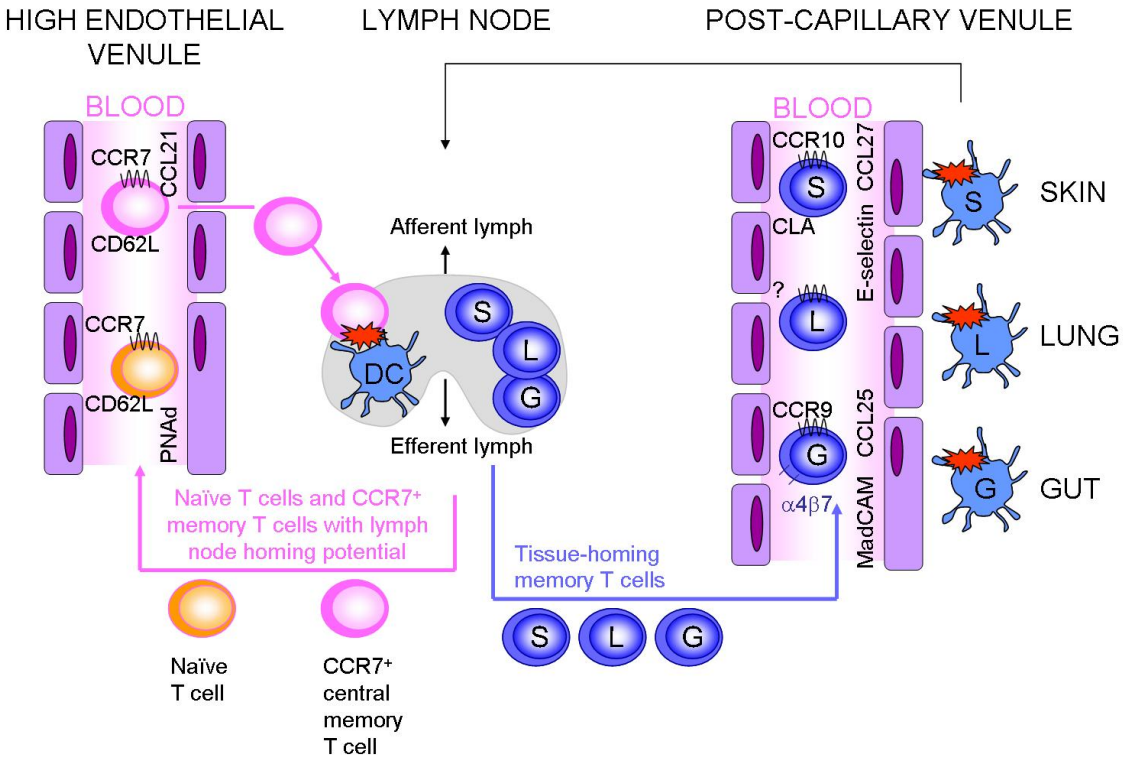
during their recirculation through secondary lymphoid organs. The process of antigen presentation subsequently leading to T cell activation will be discussed below.

1.4 Homing of naïve T cells to secondary lymphoid tissues

T lymphocyte trafficking is a highly regulated and complex process that involves expression of a variety of adhesion molecules and chemokines (Figure 1.5A). These signals allow naïve T lymphocytes to enter the secondary lymphoid tissues such as lymph nodes or Payer's patch through the blood vessels (e.g. HEV - high endothelial venules in lymph nodes), supplying these tissues with oxygen and nutrients (Medoff et al., 2008; von Andrian and Mackay, 2000). This process, also termed extravasation or diapedesis, will be discussed in more detail at a later stage in the chapter.

T lymphocytes passing through the lymphoid tissue are constantly monitoring antigen peptides displayed on MHC molecules expressed on antigen presenting cells using their TCRs (Figure 1.5). Only T cells that encounter a specific antigen stay within the lymph node and start proliferation and differentiation into effector cells. Differentiated, effector T cells leave the lymph node through efferent lymph and then are carried with the blood to the sites of infection, where they perform their specific functions (Figure 1.5B) (Parham, 2000).

A



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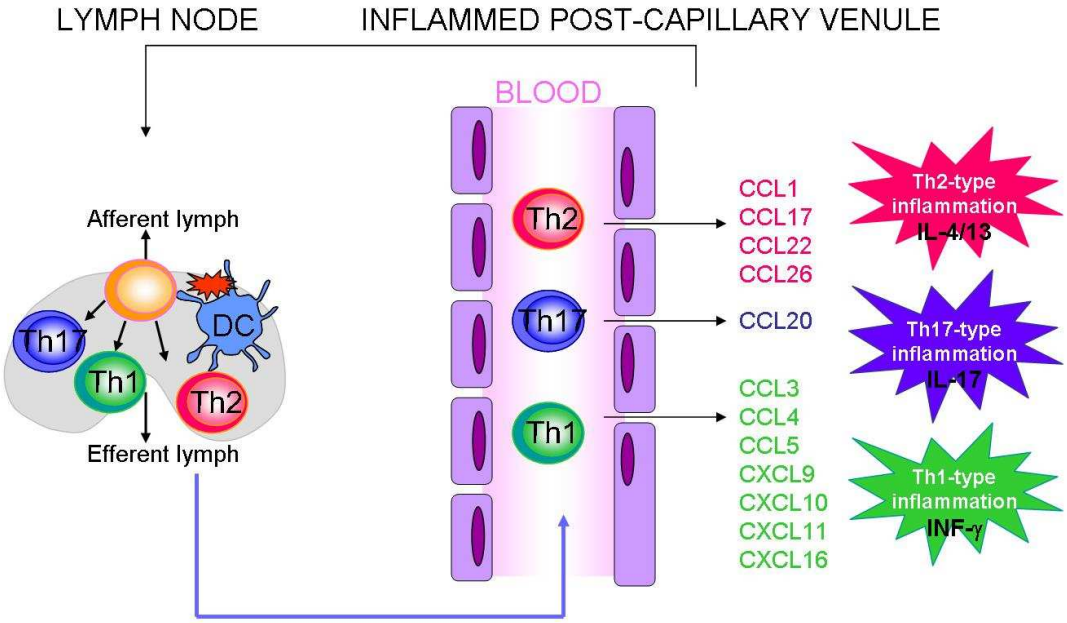


Figure 1.5 Patterns of T cells trafficking. PTO for the figure legend

Figure 1.5 Patterns of T cell trafficking. (A), Homeostatic T cell homing. Specific chemokine receptors such as CCR7 have been associated with T cell homing and migration into lymphoid tissue. "Central" memory CCR7+ T cells have to lose CCR7 expression and gain tissue-specific chemokine receptors in order to home to tissue-specific locations. It has been proposed that CCR7 defines T cells with lymph node–homing potential despite the fact that CCR7+ memory T cells have been found in the tissue. In addition, CCR10 is enriched on CLA+ skin homing T cells (S), whereas CCR9 is enriched on $\alpha4\beta7^{high}$ gut (G) homing T cells. These receptors are proposed to define T cells with either skin- or gut-homing potential. No such chemokine receptor has yet been found for lung-specific T cell homing (L). (B), Inflammation-driven migration of effector T cells. Inflammation driven by CD4 expressing T cells is characterised by the hallmark cytokines produced by each type of infiltrating T cells. Th1 cells mainly secrete IFN-γ, Th2 cells produce IL-4, IL-5 and IL-13 and Th17 cells IL-17. Secretion of these cytokines leads to production of inducible chemokines at the site of inflammation and in turn recruitment of T cells expressing the specific receptors for these chemokines (Medoff et al., 2008).

1.5 The T cell antigen receptor

The antigen receptor on T cells, commonly called T cell receptor (TCR), is composed of two different polypeptide subunits termed TCR α chain (TCRα) and TCR β chain (TCRβ). As a consequence of gene rearrangement during T cell development there is amino acid sequence variability within the N-terminal domains of TCR chains and complementary-determining regions (CDR). There are many millions of different TCRs and each of them define clones of T cells and single antigen-binding specificity (Parham, 2000). The two types of TCR, namely $\alpha\beta$ and $\gamma\delta$, associate with a series of polypeptides that form the CD3 complex. This is required for expression of the TCR complex on the surface. In contrast to TCR, CD3 does not show amino acid variability and cannot generate diversity but is required for signal transduction after antigen binding by TCR heterodimer. The CD3 complex consists of four polypeptides namely, γ, δ,ζ and its alternatively spliced form, η (Figure 1.6). The intracytoplasmic section of the CD3 chain contains specific amino acid sequences known as ITAMs (immuno-receptor tyrosine activation motifs). The ITAM motifs are defined by two tyrosine residues contained within a consensus sequence YxxI/L x(6-12) YxxI/L (where x is any amino acid) (Underhill and Goodridge, 2007). These motifs become phosphorylated by protein kineses during activation of TCR in response to binding of antigen-MHC complexes (Roitt et al., 2001). ITAM motifs are often found in multimers. This multimerisation is believed to amplify signalling due to increasing of the local concentration of signalling molecules. For example TCR complex has

up to ten ITAMs distributed on its CD3 γ, δ, ε and ζ chains (Underhill and Goodridge, 2007).

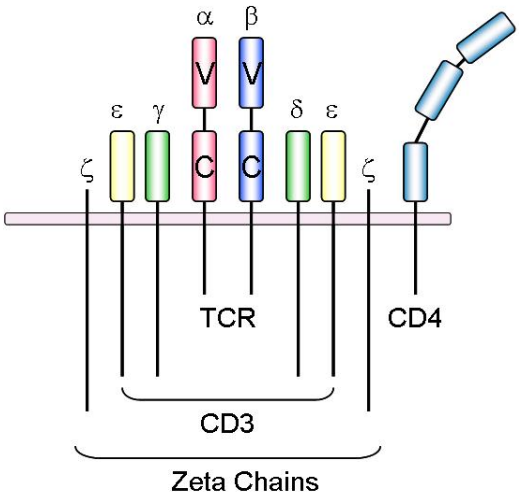


Figure 1.6 The T cell Receptor. Each of the TCR chains (α and β or γ and δ) contain an external V (immunoglobulin (Ig)-variable) and C (Ig-constant) domain, a transmembrane segment and a short cytoplasmic tail. The CD3 γ, δ and ε chains comprise an external immunoglobulin-like C domain, a transmembrane segment and a longer cytoplasmic tail. A dimmer, ζζ (or ζη), is also associated with the complex. Glycoproteins CD4 or CD8 are located near to TCR complexes.(Roitt et al., 2001).

1.6 Antigen presentation

The process of antigen presentation can be subdivided into four different stages: adhesion, antigen-specific activation, costimulation and cytokine signalling. Each stage of this process requires specific interaction between a T cell and an APC and involves multiple cell-surface molecules. The key receptor interactions involved in antigen presentation are described below and summarized in Figure 1.7.

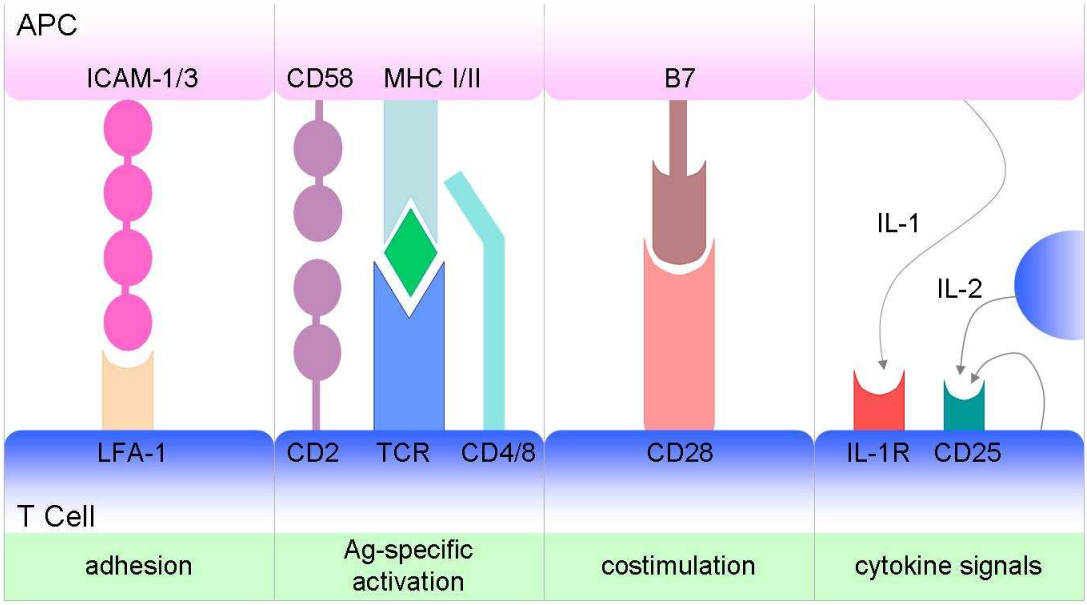


Figure 1.7 The key receptors involved in T cells activation. Initial contact between a T cell and an APC is achieved by interactions through adhesion molecules such as LFA-1 and ICAM. Recognition of antigen peptide bound to MHC molecule by TCR leads to prolonged cell-cell contact. Costimulatory signal is necessary for efficient response of the activated T cell. Activation results in upregulation of cytokines and their receptors which provides additional signals regulating the cell fate (Roitt et al., 2001). Figure adapted from Roit et al., 2001.

1.6.1 Adhesion

Intracellular adhesion molecules (ICAMs), members of the Ig family, are essential in initial contact of T cells with APCs. Transient and non-specific interactions particularly between the integrin, lymphocyte functional antigen-1 (LFA-1 or CD11a/CD18), on the T cell and ICAM-1 (CD54) and ICAM-3 (CD50) on the APC allow the T cell to encounter a large number of different MHC-antigen combinations on different APCs. Encountering the appropriate MHC-antigen, results in conformational changes in LFA-1 leading to tighter cell-cell contact.

1.6.2 Antigen-specific activation

The initial signal required for T lymphocyte activation is generated by the ligation of the TCR with the MHC-antigen complex on the surface of an APC (e.g. dendritic cell) (Davis and van der Merwe, 2006). The signal generated through these interactions subsequently leads to activation of Src family of the kinases such as Lck (associated with CD4 and CD8), ITK and Fyn. Fyn kinase phosphorylates target sequences found in the ζ-chain, ITAMs (previously described in section

1.5), which leads to activation of ζ-chain associate protein kinase (ZAP-70). This initiates recruitment and activation of multiple adaptor proteins such as SH2-containing Leukocyte Protein of 76 kDa and Linker for activation in T cells (LAT) and induces a series of biochemical events by bringing together different intracellular molecules. Activation of signalling molecules such as PLC, PKC and Ras finally leads to activation of transcription factors including activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor κB (NFκB) which, in consequence increases expression of CD25, IL-2 and IL-2 receptor.

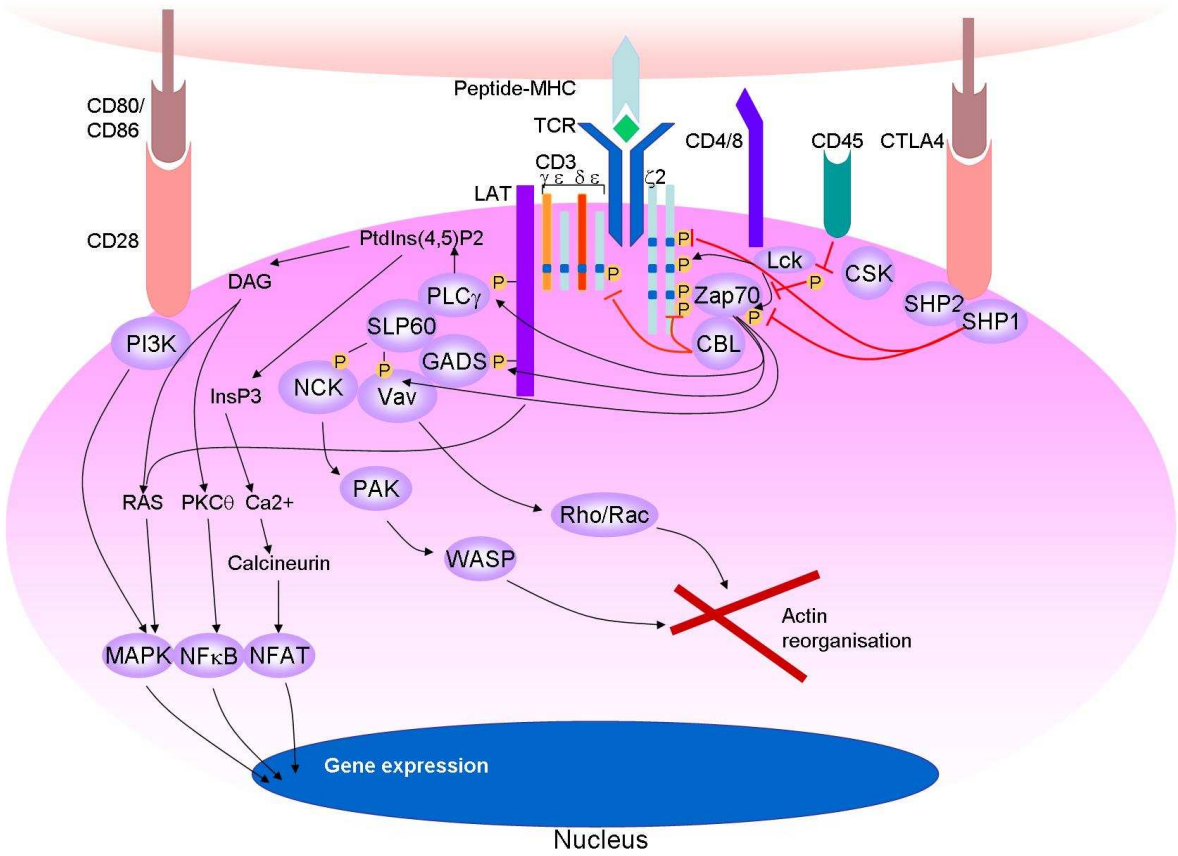


Figure 1.8 Signals involved in TCR-mediated T cell function. Upon T cell activation, SRC protein kinases (PTKs) Lck and Fyn induce phosphorylation of the ζ and ε chains of the TCR at tyrosine residues within ITAM motifs (indicated as the blue bars). Upon phosphorylation, the ITAM motifs in the ζ chain act as docking sites for the ZAP70 (ζ-chain associated protein kinase of 70 kDa), which leads to phosphorylation of following substrates: LAT (linker for activation of T cells), SLP76 (SRC homology 2 (SH2)-domain-containing leukocyte protein of 76 kDa) and PLCγ1 (phospholipase C γ1). These

phosphorylated proteins lead to recruitment of various molecules- Vav, GADS (growth factor receptor bound protein2 (GRB2)-related adaptor protein) and NCK (non-catalytic region of tyrosine kinase). These proteins induce a variety of signalling pathways which leads to activation of Ras, calcium mobilisation, the activation of PKC and polarisation of the actin filaments. Complete activation of T lymphocytes is achieved upon activation of transcription factors NFκB and NFAT, induction of the expression of cytokine genes, secretion of cytokines, T cell proliferation and interaction of various immune cells. CBL, (Casitas B-lineage lymphoma); PTPs, protein tyrosine phosphatases; SHP1 and SHP2, (SH2-domain containing PTP1) (Baniyash, 2004).

1.6.3 Costimulation

MHC/antigen-TCR interactions, although necessary, are not sufficient for full T cell activation. In order to become fully activated the T cell requires a secondary signal, also referred to as costimulation (Bromley et al., 2001). The most potent costimulatory molecules which belong to family of Ig superfamily, are B7 proteins, namely B7-1 (CD80) and B7-2 (CD86). B7 molecules are constitutively expressed on DC but can be upregulated on monocytes and possibly other APCs. B7-1 and B7-2 act as ligands for other members of the Ig superfamily - CD28 and its homologue CTLA-4. Stimulation via CD28 leads to augmented production of IL-2 and expression of IL-2R, entry of the T cell into the cell cycle and enhanced survival. An additional member of the CD28 family, namely inducible costimulatory receptor (ICOS), plays an important role in this process. ICOS is upregulated following T cell activation and functions to maintain T cell-mediated responses (Coyle et al., 2000). CTLA-4 has a higher affinity for B7.1 and B7.2 and is an inhibitory receptor which functions to limit T cell activation, resulting in limitation of IL-2 production (Egen et al., 2002). The discovery of at least two new co-inhibitory molecules, programmed death-1 (PD-1, also known as CD279) and B and T lymphocyte attenuator (BTLA, also known as CD272) (Watanabe et al., 2003), in addition to the unknown ligands that mediate inhibitory actions of B7-H3 (Prasad et al., 2004) and B7-H4 (Suh et al., 2006), has added several layers of complexity to the process of costimulation (Parry et al., 2003).

1.7 Differentiation of effector T cells

In order to perform their specific functions within the immune system, naïve T cells become activated and differentiated into corresponding mature effector cells. CD4 T cells give rise to Th1 or Th2 helper cells whilst mature CD8 T cells are already committed to perform cytotoxic functions (Figure 1.9).

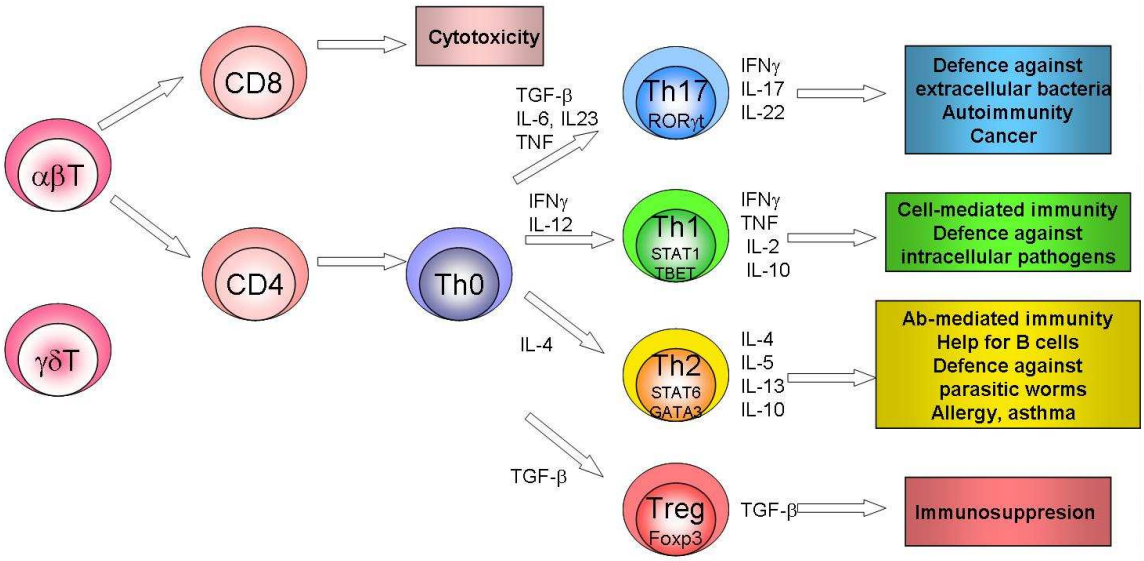


Figure 1.9 T Cell Development and Functions. Immature T cells can display two types of TCR on their surface: first, the commonly expressed αβ and second, present on minority of cells (5%), γδ. Depending on received signals, CD4 T cells give rise to Th1, Th2, Th17 helper cells or Treg cells, whilst mature CD8 T cells are directed to perform cytotoxic functions. Different subsets of CD4 cells are characterised by activation of 'hallmark' transcription factors, secretion of specific cytokines and distinct functions in immune responses.

CD4 T cells become committed to a particular function after the first stimulation by antigen. However most immune responses require a contribution of both Th1 and Th2 T cell types (Hunter, 2005; Hibbert et al., 2003). A decision on which type of CD4 T cell it will differentiate into depends on factors that are not completely understood. One of these is a repertoire of cytokines produced by APC that are already present as a result of an innate system action. It is well known that action of cytokines such as IL-12, IFN-γ, IL-18 (Barbulescu et al., 1998) and IL-23 promote differentiation to Th1 subtype (Oppmann et al., 2000). In addition activation of T-bet transcription factor has been found to be crucial in Th1 regulation. However recent reports also revealed the importance of T-bet

independent pathways (Afkarian et al., 2002; Djuretic et al., 2007; Way and Wilson, 2004).

CD4 T cells give rise to Th2 type upon action of IL-4 which upregulates STAT6, which in turn activates GATA-3, the 'master' transcription factor in Th2 regulation (Lund et al., 2005; Zhu et al., 2001; Ouyang et al., 1998; Ouyang et al., 2000).

The function of effector CD4 T cells (T helper cells) is the secretion of cytokines, which activate and recruit other cell types into sites of immune response. The cytokines secreted by Th1 cells (Figure 1.9) support activation of macrophages which leads to inflammation and a cell-mediated immunity (Figure 1.1). In contrast cytokines produced by Th2 cells (Figure 1.9) are involved in B cell differentiation and antibody production, which forms the humoral immune response (Figure 1.1).

The activation of CD8 T cells to cytotoxic T cells requires stronger costimulatory signals than is needed for CD4 activation. Thus CD8 T cells are activated only when infection is clearly defined (Roitt et al., 2001). Under some circumstances CD4 T cells can help to activate CD8 T cells by secretion of cytokines which upregulate expression of costimulatory molecules on the APCs (Simpson and Gordon, 1977; Bennett et al., 1997; Keene and Forman, 1982). It has also been reported that signalling through CD40 can replace $CD4^+$ T-helper cells in priming of helper-dependent $CD8^+$ CTL responses (Schoenberger et al., 1998). Cytotoxic T cells are involved in cell-mediated immunity and their main role is fighting viral infections (Maggi et al., 1997).

1.8 $CD4^+$ and $CD8^+$ memory T cells

Both CD4 and CD8 expressing T cells are part of immunological memory due to the production of central memory and effector memory T cells which retain immunological memory of particular antigens. After infection has been overcome some of these cells remain available for restimulation, and if the antigen is ever encountered again they provide rapid and specific response. Central memory T cells are maintained within lymphoid circulation in a similar way to naïve T cells. Effector memory T cells can enter peripheral lymphoid tissues and they seem to play a role in the first line of defence upon subsequent exposure to the pathogen. After recognition of the specific antigen, effector memory T cells can rapidly proliferate and respond to this antigen (Sallusto et al., 2004).

Different subsets of T lymphocytes can be also distinguished by the surface expression of surface markers such as hallmark chemokine receptors. The differential expression pattern of chemokine receptors on different types of T cells will be discussed later.

1.9 Th17

IL-17 producing T helper cells have recently been identified as a subset of CD4 expressing cells, derived from the same precursor as Th1 and Th2 cells. Th17 are distinct from Th1 and Th2 subset in their gene expression and biological function. *In vitro* differentiation of Th17 cells is induced by a 'cocktail' of cytokines including IL-6, transforming growth factor-β (TGF-β), TNF, IL-1β and IL-23, together with the neutralisation of the Th1 and Th2 products IFN-γ and IL-4, respectively (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006; Harrington et al., 2005). IL-6 and TGF-β are crucial in differentiation of naïve T cells into Th17 lineage, whereas TNF and IL-1β enhance this pathway and IL-23 stabilizes the Th17 phenotype. RORγt has been found to be the transcription factor important in Th17 development.

Th17 cells are characterised by secretion of proinflammatory cytokine IL-17. Cooperation between the cytokines TGF-β (transforming growth factor-) and IL-6 *in vitro* induces development of Th17 cells in mouse (Dong, 2008; Veldhoen et al., 2006; Weaver et al., 2007; Bettelli et al., 2006; Mangan et al., 2006) and human (O'Garra et al., 2008) systems, whereas IL-23 supports expansion of these cells (Veldhoen et al., 2006; Bettelli et al., 2006; Mangan et al., 2006).

There is evidence to support the involvement of Th17 cells rather then Th1 in autoimmune inflammatory conditions such as experimental autoimmune encephalomyelitis (EAE) or an animal model of multiple sclerosis (Langrish et al., 2005). Moreover, IL-17 has been associated with many other diseases, such as rheumatoid arthritis, asthma, systemic lupus erythematosus (SLE) and allograft rejection (Kolls and Linden, 2004; Moseley et al., 2003).

1.10 Regulatory T cells

Naturally occurring regulatory T cells (Tregs) are additional to effector T cells. Tregs are a subset of $CD4^+$ cells and are derived from the same precursor as Th1, Th2 and Th17. Following their development in the thymus, these cells migrate to periphery, where they control T cell responses to self-antigens (Shevach, 2002; Poitrasson-Riviere et al., 2008). Tregs have been shown to play a role in the suppression of effector T cell responses and prevention of various autoimmune and inflammatory diseases such as inflammatory bowel disease (IBD), gastritis and type I diabetes (Shevach, 2000; Sakaguchi, 2000; Roncarolo and Levings, 2000). 10% of peripheral $CD4^+$ T cells have been found to be Tregs with downregulated expression of CD45RB and upregulated CTLA-4, GITR and LFA-1 expression in comparison to conventional $CD4^+$ T cells (Sakaguchi, 2004; Wing et al., 2008). Their hallmark transcription factor is Foxp3, which is mainly restricted to this subset and is critical for development and function of regulatory T cells.

1.11 Th22

Lately two studies have described a population of previously uncharacterised IL-22- producing human helper T cells (Duhen et al., 2009; Trifari et al., 2009). These cells have been shown to be distinct from Th1 and Th17 helper cells as they secrete IL-22 but neither IFN-γ nor IL-17. Moreover this subset of IL-22 producing cells has been demonstrated to coexpress CCR6 and skin homing chemokine receptors CCR4 and CCR10 (Kupper and Fuhlbrigge, 2004), and has been implicated in skin homeostasis and pathology (Duhen et al., 2009; Trifari et al., 2009).

1.12 Immune cell migration

Immune cell motility and ability to migrate from the circulation to the sites of functional recruitment (a process directed by chemoattractants) are crucial for correct function of the immune system (Figure 1.5 A and B). This is important during immunosurveillance when T cells recirculate from the blood through secondary lymphoid organs (such as lymph nodes and Peyer's patches), and during immune response (e.g. injury or inflammation), when following activation and differentiation, cells migrate to the sites of inflammation. Another important

process which forms part of the innate immune response is leukocyte extravasation - the movement of leukocytes out of the circulatory system, through the blood vessels towards the site of tissue damage or infection. This process is tightly regulated in order to prevent destructive effects of migration of immune cells into healthy tissues. Moreover, the immune cell that is crossing the endothelium must undergo a complex series of steps including tethering and rolling which allow it to initiate interactions with the endothelial cell, firm adhesion and transmigration which will be described in more detail below.

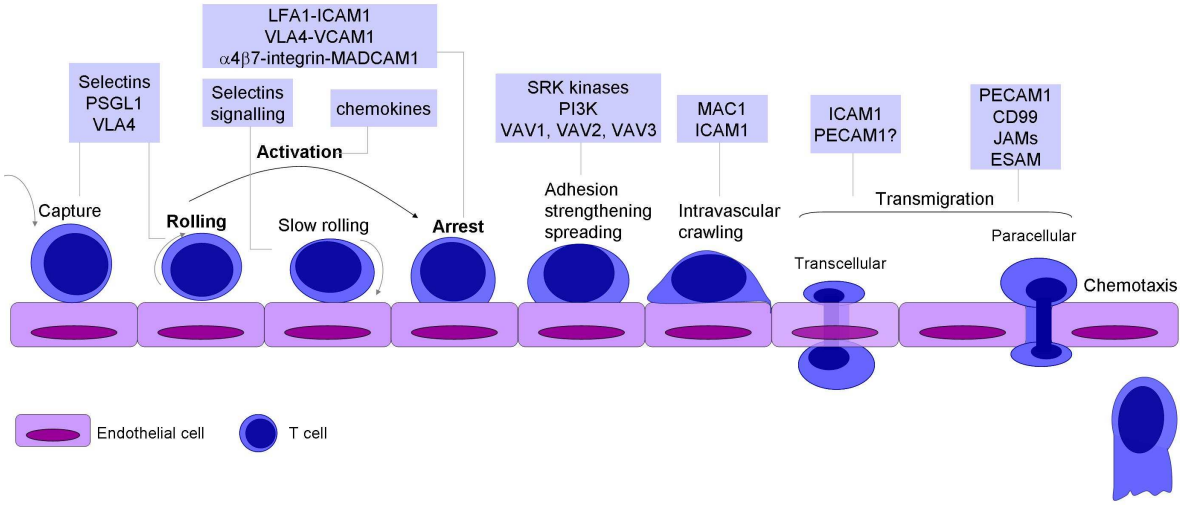


Figure 1.10 Steps of the leukocyte adhesion. Three original steps; rolling, activation and arrest (shown in bold) are mediated by selectins, chemokines and integrins, respectively. Additional steps have been recently defined: capture (tethering), slow rolling, adhesion and strengthening and spreading, intravascular crawling, and paracellular and transcellular tramsmigration. Blue boxes show the key molecules involved in each step. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 (also known as $\alpha_L\beta_2$-integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3□kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known aş $\alpha_4\beta_1$-integrin) (Ley et al., 2007).

1.12.1 Tethering and rolling

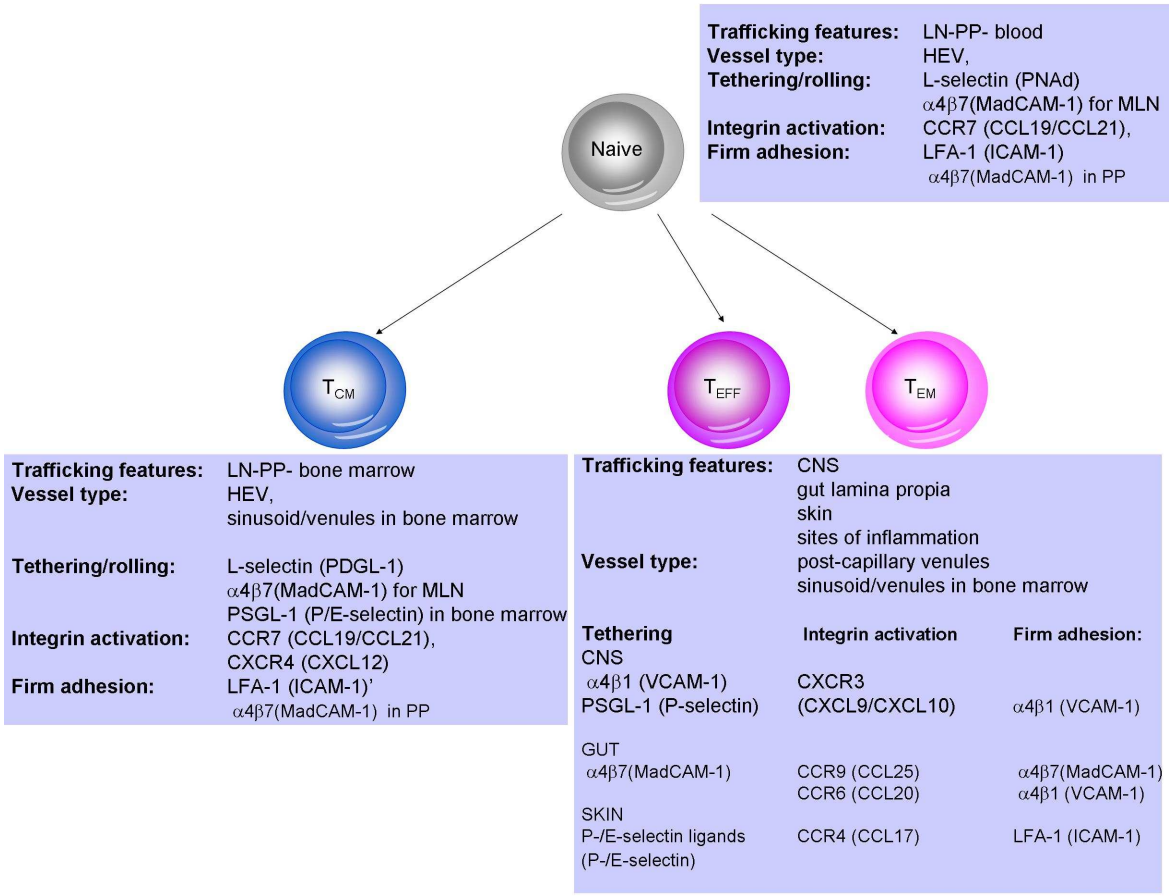


Figure 1.11 Summary of T cell surface molecules involved in the trafficking of T cells. Surface receptors of naïve, memory (T_{CM} and T_{EM}) and T_{EFF} cells are shown, together with their cognate endothelial cell-expressed ligands (indicated in brackets). CNS, central nervous system; HEV, high endothelial venule; LN, lymph nodes; PNAd, peripheral-node addressin; PSGL-1, P-selectin glycoprotein ligand-1 (taken from (Ward and Marelli-Berg, 2009).

Tethering and rolling on the endothelial surface starts the cascade-like process of immune cell extravasation by establishing contact between leukocytes and endothelial cells lining the blood vessel. Under conditions of sheer flow, these interactions are initially transient but eventually allow the cell to 'tether and roll' along the endothelium, leading to the slowing the speed of the passing leukocyte. Molecules that are involved in this process include selectins (e.g. L-selectin), which mediate transient, weak adhesive interactions that result in rolling and integrins sharing α4 subunit (e.g. VLA-4, Very late antigen-4), which mediate lymphocyte rolling mainly in the absence of selectin contribution (Pribila et al., 2004). α4β1 integrin-mediated lymphocyte rolling does not require high affinity

interactions with VCAM-1 (Chen et al., 1999). Another member of the integrin family, LFA-1 (Lymphocyte function-associated antigen-1), despite not being involved in initial tethering of the leukocyte, appears to optimize L-selectin-mediated rolling by reducing leukocyte rolling velocities via its interactions with ICAM-1 (Kadono et al., 2002). During the rolling on the endothelium, receptors on the leukocyte interact with endothelial cell- or tissue-derived chemokines immobilized on the endothelial cell surface. Chemokine binding to GPCRs (Han et al., 2005) expressed by migrating leukocytes trigger conformational changes, leading to increased affinity of leukocyte integrins for their cognate endothelial-cell adhesion molecules, which, in turn, mediate their firm adhesion to the endothelium (Rot and von Andrian, 2004; Ward and Marelli-Berg, 2009). The key surface molecules involved in T cell trafficking events are summarized in Figure 1.11.

1.12.2 Activation and adhesion

Based on weak and transient interactions with endothelium, tethering and rolling of lymphocytes enhances the possibility of sufficient activation and firm adhesion of lymphocyte. Generally, firm adhesion of leukocyte to the endothelium is mediated by ICAM-1 (intracellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule), which bind β2 and β1integrins on leukocytes (Dejana, 2006). In addition, chemokines displayed on the surface of the endothelial cells stimulate lymphocyte rolling via interactions with proteoglycans, causing a rapid, but transient, increase in integrin adhesiveness.

In addition, chemokines modulate integrin functionality by promoting conformational changes of integrins that alter ligand binding affinity and changes in integrin mobility, resulting in integrin clustering and increased avidity of leukocyte adhesion (Pribila et al., 2004).

Most β2 integrins expressed on the surface of unstimulated leukocytes are in their inactive state in which they do not bind ligands and do not signal. Only a small number of integrins present on the cell surface are in an active, high affinity state and these integrins are necessary for spontaneous lymphocyte arrest on the endothelium (Chen et al., 1999; Hynes, 2002). Upon cell activation for example by cytokines, the β2 integrins become activated allowing binding to the receptors.

In this case these are Ig superfamily molecules such as ICAMs present on the endothelial cells.

1.12.3 Transmigration (diapedesis)

To reach the site of infection, following firm adhesion to the endothelium, leukocytes migrate across the vessel wall to infiltrate the underlying tissues, a process known as diapedesis, extravasation and transmigration (Muller, 2003). In contrast to leukocyte rolling, arrest and adhesion, the precise mechanism of diapedesis through endothelium is still poorly defined. The most accepted mechanism of leukocyte transmigration is through interendothelial junctions (paracellular mechanism, Figure 1.6), which open and close rapidly allowing the passage of the cell and maintaining integrity of endothelium, respectively (Muller, 2003; Johnson-Leger et al., 2000). However this model has been challenged by other studies supporting an alternative mechanism of leukocyte diapedesis through individual endothelial cells by penetrating the cell cytoplasm (transcellular pathway). It has also been suggested that these two pathways coexist and are equally possible during leukocyte transmigration (Engelhardt and Wolburg, 2004).

Although the exact mechanisms of extravasation are yet to be fully explored, there are some proteins already identified that are thought to be crucial in this process (Figure 1.10). Studies using antibodies blocking endothelial junctional proteins, revealed the importance of molecules such as JAMs (junctional adhesion molecules) e.g. JAM-1 and PECAM-1 (platelet endothelial adhesion molecule) in leukocyte transmigration (Muller, 2003; Johnson-Leger et al., 2000; (Bird et al., 1993). In addition the process of diapedesis involves interactions of LFA-1 with ICAM-1 (Bird et al., 1993).

1.12.4 Integrin –independent migration

As was discussed above, the role of integrin-mediated adhesion in leukocyte extravasation (two-dimensional migration) is well established. However recent findings by Lämmermann *et al* demonstrate that integrin-mediated adhesion is only necessary to overcome tissue barriers such as the endothelial layer, while interstitial migration is independent from the molecular composition of the extracellular environment. The autonomy from adhesion molecule interaction

allows leukocyte to achieve fast and flexible navigation through any organ without a need to adapt to extracellular ligands (Lammermann et al., 2008).

1.12.5 The role of TCR and co-stimulatory molecules in regulation of T cell trafficking

To recognise tissue-selective integrins and chemokine-chemokine receptor interactions, an additional level of specificity for T cell trafficking into the tissue is provided by specific recognition of antigen displayed by the endothelium (reviewed in Ward and Marelli-Berg, 2009). Within secondary lymphoid organs, antigen-displaying dendritic cells present antigen–peptide–MHC complexes to the TCR on the surface of clone-specific T cells. Co-stimulatory signals delivered to T cells together with TCR engagement are well known to sustain T cell proliferation, differentiation and survival. Moreover, co-stimulators (such as CTLA-4) counteract these effects, thus promoting homoeostatic mechanisms and tolerance induction (Alegre et al., 2001; Parry et al., 2007; Ward and Marelli-Berg, 2009).

1.12.6 Chemotaxis

Immune cells are able to migrate in several different ways. In addition to random motile response (chemokinesis), leukocytes are capable of directional movement either towards a soluble extracellular gradient of chemoattractant (chemotaxis) or when immobilised on tissue structures such as interstitial collagens or a stromal cell network (haptotaxis).

Chemoattractants are substances that are able to induce directed cell migration (or chemotaxis) in motile cells. There are several types of chemoattractants including the most notable large family of chemokines (described in detail within this chapter). Some other molecules namely sphingosine-1-phosphate (S1P) (Sallusto and Mackay, 2004), leukotriene B4 (LTB4) (Funk, 2001; Kamohara et al., 2000) or a member of the complement system C5a (Marder et al., 1985) have also been shown to have chemoattracting properties, attracting T cells out of the thymus, mediation of neutrophils chemotaxis, monocytes and macrophages, respectively.

The process of chemotaxis involves thee distinct features of a migrating cell; establishment of a polarised morphology, formation and extension of protrusions at the leading edge and directional movement. Chemoattractants and the processes required in chemotaxis are described in more detail below.

Extension of pseudopodia

During random migration, cytoplasm protrusions (pseudopodia) form over the whole surface of the cell. Directional migration (e.g. towards chemokine gradient) requires formation of protrusions at the front or leading edge of the cell. This is achieved due the to accumulation of some signalling molecules such as PtdIns(3,4,5)P_3 (phosphatidylinositol (3,4,5)-trisphosphate, or PIP_3). This particular molecule will be discussed in more detail later as this is a vital step in the initiation of chemotaxis.

Polarisation

In order to migrate towards a chemoattractant gradient, the cell must establish polarised morphology, which means formation of the front (leading edge) and back (uropod) (Figure 1.12). Establishment and maintenance of the migrating cell polarity is dependent on molecular events and a set of positive-feedback loops involving PI3K, the Rho family of small GTPases, integrins, microtubules and vesicular transport. The contribution of various signals differs between cell types and depends on specific chemoattractant (Ridley et al., 2003; Ward, 2006). The initial step leading to polarisation and consequently to migration is the activation of surface G-protein coupled receptors (GPCR, e.g. chemokine receptors) by chemoattractants. During polarisation, GPCR are evenly distributed on the cell surface (Servant et al., 1999). Moreover, heterotrimeric G proteins show low preference for the leading edge (Jin et al., 2000). In contrast further downstream effectors exhibit highly asymmetrical distribution. The first detectable molecular event of cell polarisation is accumulation of PI3K lipid products PtdIns(3,4,5)P_3 (PIP_3) at the leading edge of the cell. Studies in neutrophils and dictyostelium provided a body of evidence which indicated that PIP_3 dependent signals were part of a compass mechanism, sensing and responding to extracellular gradients of chemoattractants (Funamoto et al., 2002; Iijima and Devreotes, 2002; Wang et al., 2002; Weiner et al., 2002). Studies using PI3K inhibitors or the genetic loss of

PI3Ks demonstrated a reduction in the chemotactic responses of neutrophils and amoebae in a variety of *in vitro* and *in vivo* migration experiments (Fergus et al., 2007; Funamoto et al., 2002; Hannigan et al., 2002; Hirsch et al., 2000; Iijima and Devreotes, 2002; Weiner et al., 2002). Additional studies using GFP-tagged PH domains selectively reacting with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, and using PtdIns(3,4,5)P_3 –specific antibodies revealed that the levels of PI(3,4,5)P3 become highly polarized in amoebae, neutrophils and neutrophil-like cell lines, with high levels closest to the leading edge (Funamoto et al., 2002; Iijima and Devreotes, 2002; Wang et al., 2002; Weiner et al., 2002). The asymmetric distribution of PIP_3 in migrating leukocytes is thought to be maintained via spatially and temporally regulated positive and negative feedback loops (Barber and Welch, 2006). Positive feedback seems to function through PIP_3, stimulating further production of PIP_3. This mechanism is thought to involve Rho GTPases, most likely Rac (Niggli, 2000; Weiner et al., 2002). In addition to positive regulation, asymmetric localization of PIP_3 is also maintained by excluding it from the rear end of the cell by action of phosphatases PTEN and SHIP which catalyse the conversion of PIP_3 to PtdIns(4,5)P_2 and PtdIns(3,4)P, respectively. The role of PI3K in leukocyte migration will be addressed in greater detail in a later part of the chapter.

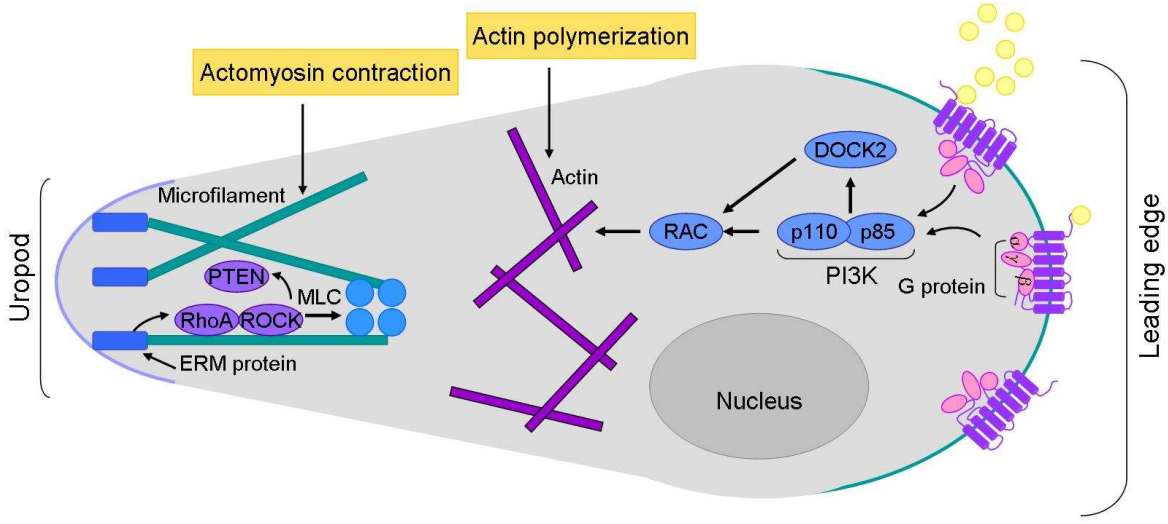


Figure 1.12 Signalling events leading to leukocyte polarisation during chemotaxis. Activation of G-protein coupled chemokine receptors by chemokines leads to stimulation of a dedicator of cytokinesis 2 (DOCK2)-Rac pathway (localized at the leading edge), and actin reorganisation. PI3K is localized mainly at the front of the cell while PTEN phosphatase is distributed to the rear edge of migrating cell. Activated ERM (Ezrin, Radixin and moesin) proteins are recruited to the rear edge, where they stimulate cell

contraction by regulation of myosin light chain (MLC) phosphorylation, propably through the Ras-homologue-gene-family-member-A – Rho associated-coiled-coil-containing-protein-kinase (RhoA-ROCK) cascade (Viola and Gupta, 2007)

Another downstream signal of activated GPCR is the DOCK (dedicator of cytokinesis 2)-Rac-dependent pathway which initiates actin rearrangements within the cell (Viola and Gupta, 2007). Actin plays a fundamental role in motile cells by being continually polymerised and depolymerised allowing for dynamic movement. Actin exists as a globular protein known as G-actin, molecules of which bind together in an ATP-dependent manner, generating filamentous actin (F-actin). Extension of the actin filament (polymerization) occurs at the one end known as the 'barbed end' while depolymerisation takes place at the opposite or 'pointed end' (Figure 1.13). During chemotaxis the generation of new filaments occurs mostly at the leading edge of the cell and pushes the plasma membrane forward, resulting in the protrusion of the cell. Growth of F-actin continues until halted by the binding of capping protein on the barbed end. The role of capping proteins is to maintain and stabilize the filament or to induce its depolymerisation. Capping proteins can be located at the pointed end, where they inhibit dephosphorylation of the filament or at the barbed end preventing addition of further G-actin subunits. During the process of treadmilling the rates of polymerization and depolymerisation are equal thus the length of filament remains constant (Revenu et al., 2004; Vicente-Manzanares et al., 2005).

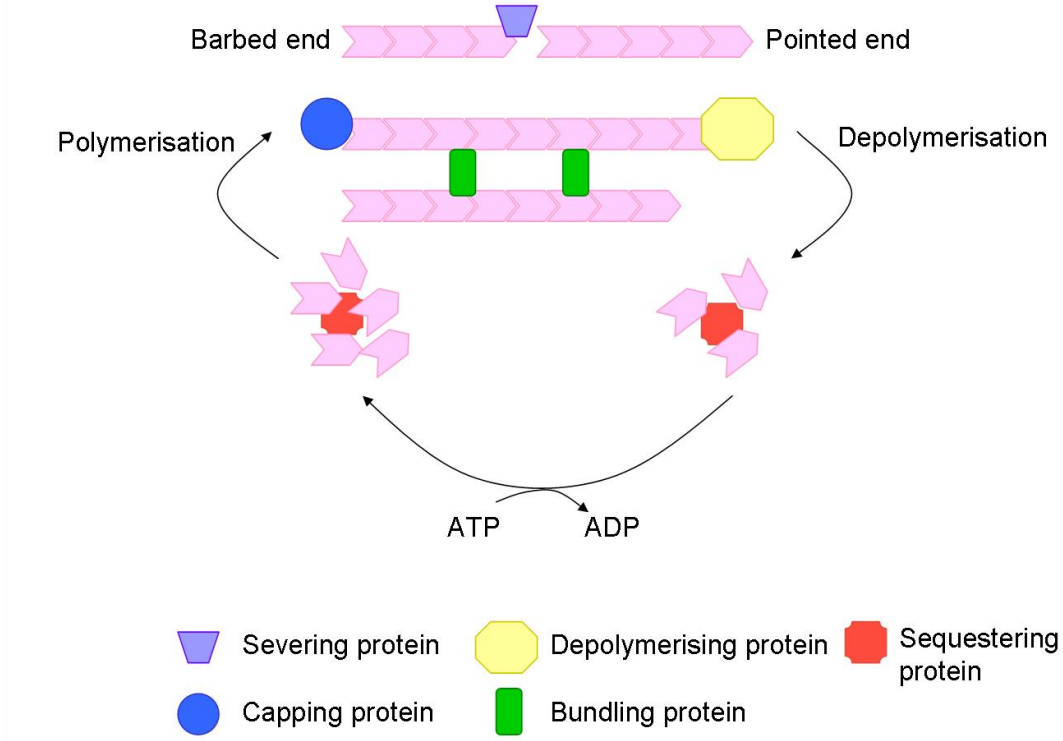


Figure 1.13 Structure and dynamics of actin filaments. Actin exists as a monomeric, globular protein known as G-actin, and as a filamentous actin (F-actin). G-actin subunits bind together upon action of ATP in a process of actin polymerization. Extension of the actin filament occurs at the 'barbed end' while depolymerisation takes place at the opposite 'pointed end'. During the process of treadmilling the rates of the polymerization and depolymerisation are equal, and the length of filament remains unchanged. Capping proteins can bind to the barbed end and prevent addition of further actin monomers. Several other proteins have been shown to bind actin filaments. Some of them act as crosslinking proteins, which stabilize and allow actin filaments to bind together.

1.13 Chemokines and chemokine receptors

1.13.1 Nomenclature and structural characteristics of chemokines

Chemokines are members of a large family of small 8-10 kDa proteins and, to date, at least 50 chemokines and 19 functional receptors have been described. Chemokines show a relatively low level of sequence identity. However their three-dimensional structures exhibit high homology in monomeric fold (Proudfoot, 2002; Schwarz and Wells, 1999). Chemokines are subdivided into four sub-families based on the number and position of highly conserved cysteine residues in the amino terminal end of their primary amino acid sequence, namely C, CC, CXC and CX3C (Rollins, 1997; Sallusto et al., 1998; Zlotnik and Yoshie, 2000). The division

of the chemokines according to their structure represents a new nomenclature (the new versus old nomenclature of chemokines is shown in Figure 7.6 in Appendix). CXC chemokines (or α chemokines) and CC (or β chemokines) are two major families of chemokines. The CXC chemokines contain a single non-conserved amino acid between the first two cysteines of this motif while CC chemokines have these residues juxtaposed. Two other minor chemokine families include CX3C chemokines, which contain three amino acids between the first two cysteins and C chemokines, in which one of these residues is lacking. The CXC chemokines are further subclassified according to the presence or absence of an ELR motif in the N-terminal: Glu-Leu-Arg $(ELR)^+$ and Glu-Leu-Arg $(ELR)^-$. ELR^+ group of chemokines include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 and CXCL15 and this group has been shown to have angiogenic activity and attract mainly neutrophils and polymorphonuclear (PMN) leukocytes to the sites of inflammation. ELR^- chemokines, to which CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16 belong have mainly angiostatic properties and attract lymphocytes and monocytes with poor chemotactic ability for neutrophils (Laing and Secombes, 2004). Chemokines are also divided into 'homeostatic' and 'inflammatory' subfamilies depending whether they are constitutively expressed or whether their expression is upregulated upon inflammatory signals (Figure 1.14).

Many chemokines are clustered in a certain chromosomal location (Figure 7.6 in Appendix). Two main clusters have been recognized. Many human CXC chemokines that mainly act on neutrophils are clustered at chromosome 4q12–13, while many CC chemokines that mainly act on monocytes are located in another cluster at 17q11.2. The CXC chemokines in the 4q21.21 mini-cluster act specifically as T cell chemoattractants (Murphy et al., 2000). Chemokines encoded by the more isolated genes tend to be constitutively produced and have homeostatic roles (Zlotnik and Yoshie, 2000).

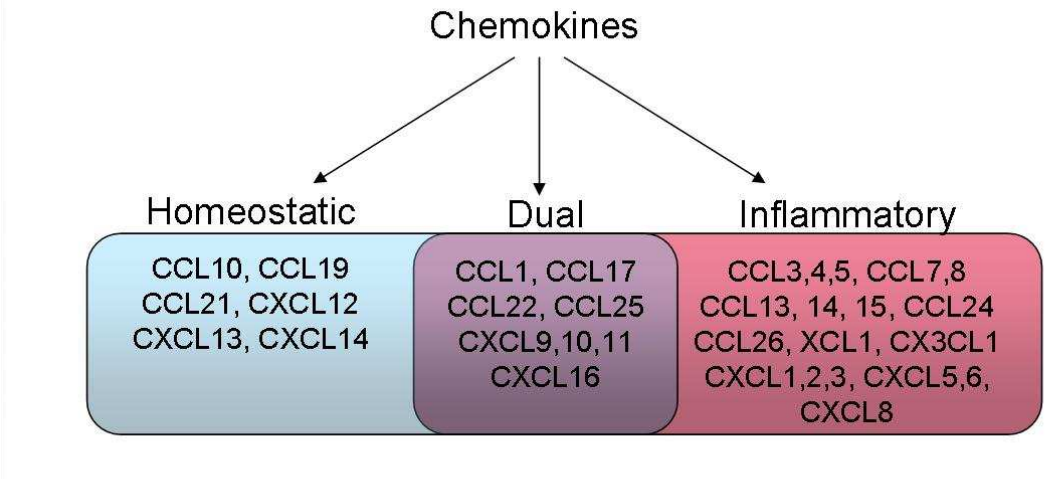


Figure 1.14 Functional classifications of human chemokines. Chemokines can be categorized according to the function they play in immune cells trafficking. Homeostatic (constitutive) chemokines are responsible for trafficking and homing of leukocytes during immunosurveillance. These chemokines are constitutively expressed. Inflammatory (inducible) chemokines are crucial in the guiding of leukocyte migration during injury or disease and their expression is induced e.g. by cytokines.

1.13.2 G-proteins coupled receptors

G-protein coupled receptors or GPCRs are a large and diverse family of receptors and are capable of binding not only chemokines, but also hormones and lipid mediators. Surface GPCRs couple to heterotrimeric intracellular G proteins which mediate activation of downstream effectors including adenyl cyclase, phospholipases, protein kinases and ion channels (Armbruster and Roth, 2005). Figure 1.15A shows characteristic and unique structure of GPCRs: a hydrophobic core of seven transmembrane spanning domains creating three intracellular loops, an extracellular amino terminus and intracellular carboxyl terminus. Binding of the ligand to N-terminal end of the monomeric receptor leads to conformational changes within the receptor that are transduced through 7TM domains, and in consequence its activation. To convert agonist-induced conformational changes of receptor into a functional signal inside the cell, the receptor must undergo three stages of activation (Figure 1.15B). This is achieved by coupling to a heterotrimeric guanine-nucleotide binding protein (G protein) comprising of one each of α, β and γ subunits. Activation by a ligand binding receptor initiates the exchange of guanosine diphosphate (GDP) for guanosine triphosphate on the α subunit, leading to reduced affinity of Gα-GTP for G$\beta\gamma$, and resulting in dissociation of both Gα-GTP and G$\beta\gamma$ from the receptor (Lambright et al., 1994).

Uncoupled G protein subunits are now able to act on their downstream effectors leading to their activation or inhibition. Termination of these actions occurs rapidly via hydrolysis of GTP through activity of intrinsic GTPases. G proteins are switched on and off by the GTPase activating proteins (GAPs) which stimulate their GTPase activity, leading to signal termination. This can be reversed by guanine nucleotide exchange factors (GEFs). Most GAP proteins which act on Gα subunits belong to family of multifunctional Regulators of G protein Signaling (RGSs) (Tesmer et al., 1997).

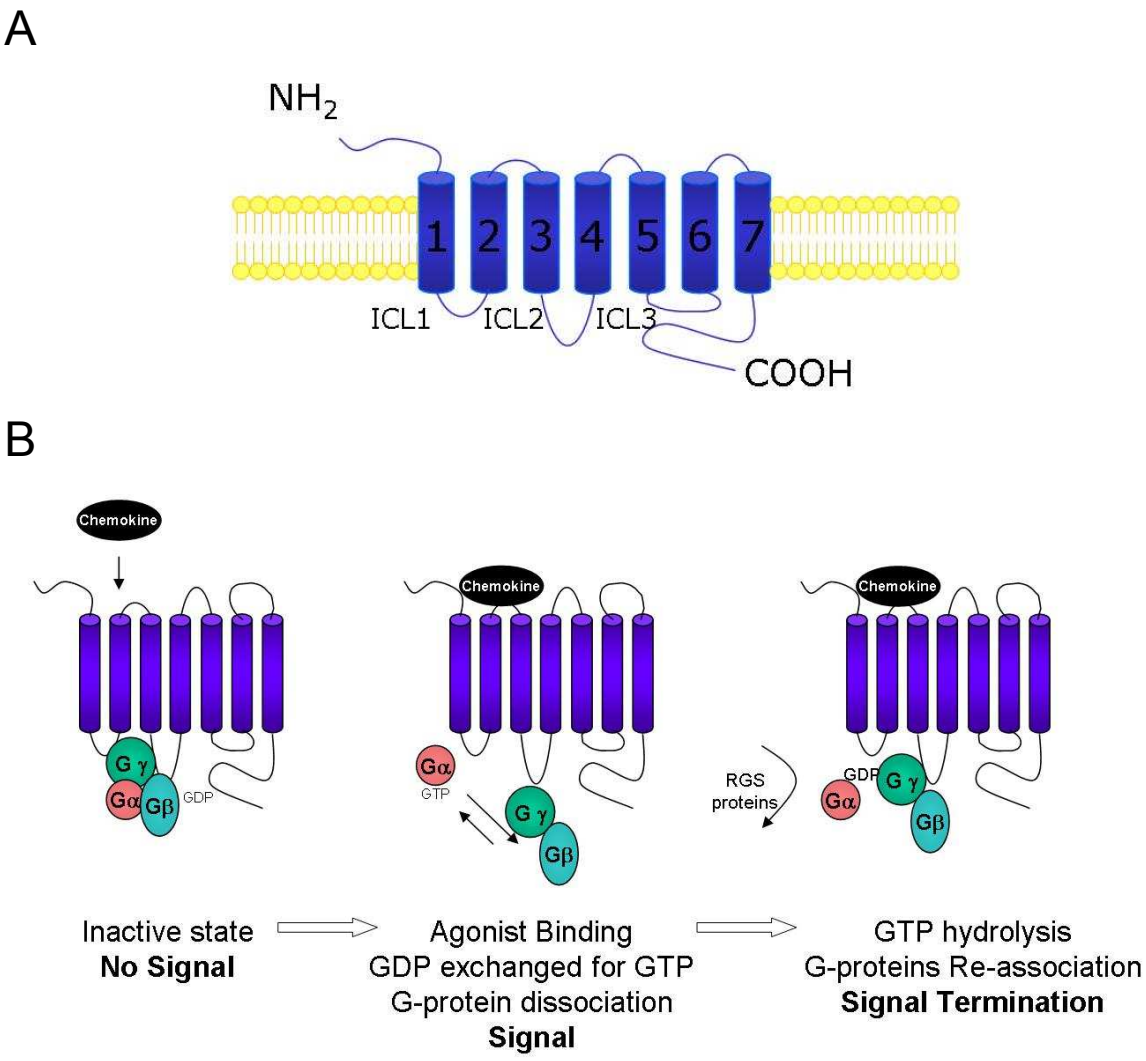


Figure 1.15 A schematic illustration of GPCR and its interactions with chemokine ligand. (A) A 2D representation of an unfolded 7 transmembrane GPCR within the cell membrane with labelled extracellular N- and intracellular C-termini as well as the intracellular (ICLs) loops. As discussed later ICL2 is essential for the activation of GPCRs. (B) Illustration of GPCR activation by its ligand. (B, left panel) inactive state of receptor with associated heterotrimeric G proteins (Gα stays GDP bound); (B, middle panel) receptor in its active state after ligand binding, dissociation of G proteins due to exchange of GDP to GTP, resulting in signal transduction; (B, right panel) signal termination due to hydrolysis of GTP, re-association of G proteins. RGS Regulators of G protein signaling.

1.13.3 Dimerization of GPCRs

It has recently become apparent that interactions between ligand and its receptor are more complex, with these receptors forming homodimers and also heterodimers rather than involving monomeric receptor (Breitwieser, 2004).
For example chemokine receptor CCR5, expressed on T lymphocytes has been reported to form homodimers, and this process was dependent on two residues present in transmembrane domain 1 and 4. Mutations of these residues prevented dimerization of CCR5 and blocked signalling through this receptor (Hernanz-Falcon et al., 2004).

Heterodimerization between CCR2 and CCR5 has been reported to occur and to have functional consequences as their ligands, CCL2/MCP-1 (monocyte chemotactic protein-1) and CCL5/RANTES (regulated upon activation, normal T cell-expressed and Secreted), cooperate to induce PTX-resistant calcium mobilization at concentrations 1- to 100-fold lower than the threshold for each chemokine alone. Heterodomerization, in addition to recruiting each receptor-associated signalling complex, also has unique features such as promoting the specific recruitment of $G_{q/11,}$ leading to PTX insensitive responses and different kinetics of PI3K activation. Therefore it is thought to promote cell adhesion rather than migration (Mellado et al., 2001b).

Another example of chemokine receptor dimerization comes from studies on CXCR4 and CXCR7 which have been demonstrated to exist as homodimers in constitutive association with each other (Babcock et al., 2003; Lagane et al., 2008; Sierro et al., 2007; Toth et al., 2004; Wang et al., 2006; Vila-Coro et al., 1999). Furthermore, using a new firefly luciferase PCA to image dimeric complexes of chemokine receptors in intact cells and living mice, Luker *et al* validated previous findings of CXCR4/CXCR7 homo- and heterodimerization and demonstrated that CXCR7 also exists as preformed dimers (Luker et al., 2009).

These results may have important consequences for T cell physiology. For example during leukocyte extravasation, chemokines are presented to rolling leukocytes by immobilization on heparin bearing proteoglycans on the vascular endothelium. Therefore due to the low concentration of chemokines,

heterodimerization of receptor could stop rolling and trigger cell adhesion and diapedesis through the vessel wall, and finally migration into the surrounding tissues (Rodriguez-Frade et al., 2001).

Although heterologous expression studies suggest that most, possibly even all, GPCRs can form homo- and/or hetero-oligomers, these findings will require further individual case investigation in vivo at physiological level of GPCR expression (Breitwieser, 2004) and cannot be generalized to all chemokine receptors. During inflammation where a variety of soluble mediators are present, the possibility that different chemokine pairs could induce heteromerization of chemokine receptors may add to the complexity of chemokine system physiology (Rodriguez-Frade et al., 2001).

1.13.4 Interactions of Gα and Gβγ with down-stream effectors

Activation of a large number of different downstream effectors is achieved by the existence of variable forms of G proteins. They are categorized into four families based on differences between their α subunits; G_s, $G_{i/o}$, G_q, $G_{12/13}$.

Chemokine binding to their serpentine receptor cause dissociation of Gαi (the type of Gαi most commonly associated with chemokine receptors) and Gβγ subunits of heterotrimeric G proteins, leading to intracellular calcium flux, and activation of a variety of signalling pathways, such as PLC, phosphatidylinositol 3-kinase (PI3K) and the small Rho GTPases (Mellado et al., 2001a).

1.13.5 Regulation of GPCR signalling

Expression of GPCR on the surface and its availability is a tightly controlled process and is a balance between the rate of endocytosis and the rate of replacement (recycling or synthesis of new protein) (Mueller et al 2002, CCR5). Newly synthesized receptors in the endoplasmic reticulum become glycosylated, packaged and transported to the surface where are they ready to interact with their agonists. Activation of GPCR by binding of its agonist turns on mechanisms to limit its own responses. Two processes have been shown to play a role in this phenomenon, namely receptor desensitization and receptor internalization. Desensitization is a very rapid response and leads a cell to be transiently

unresponsive to a subsequent stimulation via that receptor (Sauty et al., 2001; Chuang et al., 1996). This occurs due to phosphorylation of Ser and Thr residues in the intracellular loops and carboxyl-terminus of the receptor by G protein-coupled receptor kinases (GRKs), which results in the uncoupling of G protein subunits from the receptor. Moreover, phosphorylation of these residues is important for the recruitment of adaptor proteins, such as adaptin 2 (AP-2) and β-arrestin which play an important role in receptor internalization (Neel et al., 2005). Internalization is a process in which activated receptors are removed from the surface for degradation or recycling and this results in more prolonged cellular unresponsiveness to these receptor agonists (Signoret et al., 1998; Solari et al., 1997; Aragay et al., 1998; Sauty et al., 2001). The process of chemokine receptor internalization will be presented in more detail later.

1.13.6 Chemokine receptors

Chemokines mediate their effects by binding to 7TM spanning GPCRs which are typical 7TM spanning receptors but with some characteristic features of chemokine receptors features (Smit et al., 2007) such as a D/ERYLAIVHA motif within the second intracellular loop domain (Figure 1.16). Mutation of the first residue of the E/DRY motif in numerous receptors leads to agonist-independent activation (i.e., constitutive activity) (Rasmussen et al., 1999; Scheer et al., 1997) whereas mutation of the R residue cause general impairment of G protein-dependent signaling (Amara et al., 2003; Ballesteros et al., 1998; Chung et al., 2002; Scheer et al., 2000). The interaction between R of the DRY motif, with its adjacent D/E residue at position 3.49 and an additional D/E at position 6.30 near the cytoplasmic end of TM6 is known as the ionic lock (Ballesteros et al., 2001). Charge-neutralizing mutation of D/E6.30 in TM6 results in increased constitutive activity (Ballesteros et al., 2001; Montanelli et al., 2004). Another characteristic feature of chemokine receptors is a highly conserved NPXXY motif found within TM7 in which the N residue acts as an on/of switch by adopting two active and inactive conformational stages (Govaerts et al., 2001; Urizar et al., 2005). Sequences of conserved NPXXY motifs of chemokine receptors are summarized in Figure 7.7 in Appendix.

There are about 50 human chemokines identified to date, with considerably fewer number of chemokine receptors described. Sequence identity for chemokine receptors varies from 36-77% for CXC chemokine receptors and 46±89% for CC chemokine receptors (Baggiolini et al., 1997). The chemokine - chemokine receptor system presents a high level of redundancy and versatility which is achieved by the promiscuous nature of chemokines, as they can bind to several receptors or each receptor may be activated by several different chemokines (Ward and Westwick, 1998). Chemokines binds their receptor with nanomolar affinity and this binding is class restricted, which means that CC receptors are only activated by CC chemokines and CXC receptors by CXC chemokines (Pease and Williams, 2006). The exception to this rule is the Duffy antigen receptor for chemokines (DARC) which binds both CC and CXC chemokines (Lu et al., 1995; Pease and Williams, 2006a).

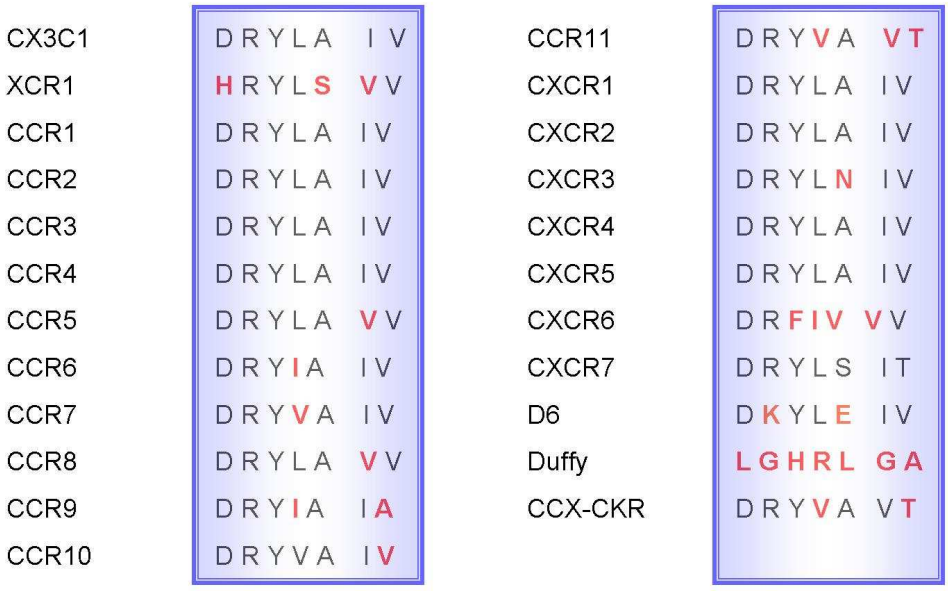


Figure 1.16 Sequences of conservative *DRYLAIV* motif of human chemokine receptors present in the N-terminus of the second intracellular loop of the protein. (Adapted from Thelen, 2008).

As mentioned previously chemokines and chemokine receptors are classified into homeostatic and inflammatory subfamilies. This division however does not seem to be fully categorical. For example CCR10 is thought to be constitutively expressed but is involved in some inflammatory skin conditions (Reiss et al.,

2001), whereas its ligand CCL27 is upregulated by pro-inflammatory cytokines (Homey et al., 2002). Figure 1.17 summarises the network of interactions between chemokines and their receptors.

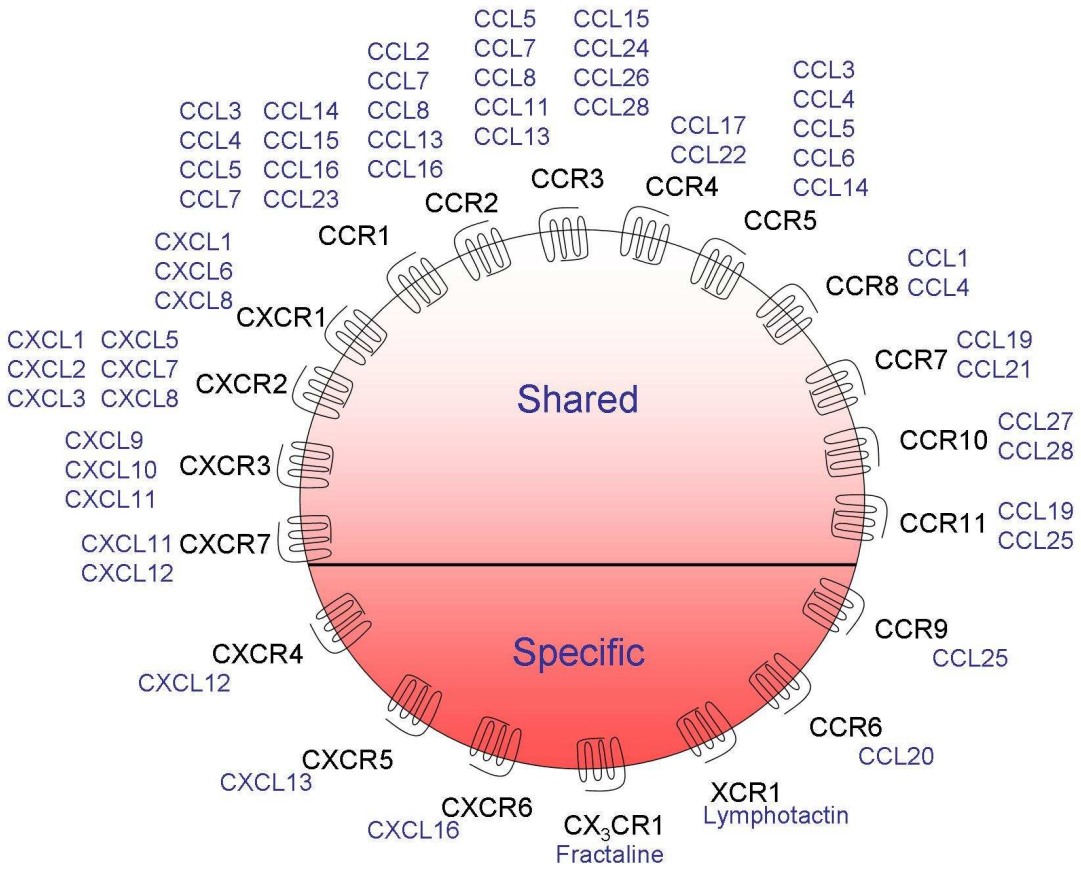


Figure 1.17 Chemokine and chemokine receptors. There are about 50 human chemokines identified in human until now, and 20 chemokine receptors described. The chemokine-chemokine receptor system presents a high level of redundancy and versatility which is achieved by the promiscuous nature of chemokines. Chemokine receptors can be divided into two classes; specific, which bind only one ligand; and shared which interact with more then one chemokine.

1.13.7 CXCR3

CXCR3 is a chemokine receptor and contains structural motifs typical for GPCRs, such as conservative DRY (Figure 1.16) and NPXXY (Figure 7.7, Appendix) motifs at the cytoplasmic ends of transmembrane domains 3 and 7 respectively.

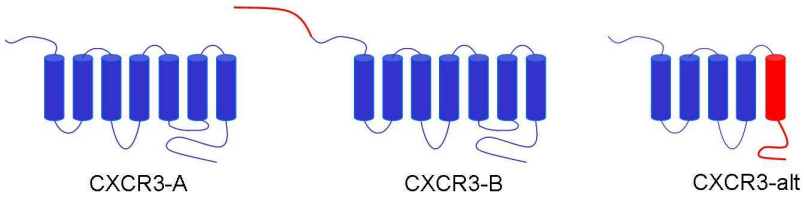
The chemokine receptor CXCR3 is expressed on a wide variety of cells including activated T lymphocytes, NK cells, malignant B lymphocytes, endothelial cells, and thymocytes (Loetscher et al., 1996; Loetscher et al., 1998; Qin et al., 1998; Romagnani et al., 2001; Trentin et al., 1999; Van Der Meer et al., 2001). Three major CXCR3 ligands, CXCL9, CXCL10, and CXCL11, have been identified, all of which are induced by IFN-γ and are therefore thought to promote Th1 immune responses (Cole et al., 1998; Farber, 1990; Luster et al., 1985). Recent studies have shown that different CXCR3 ligands exhibit unique temporal and spatial expression patterns, suggesting that they have non-redundant functions *in vivo*. Moreover, the CXCR3 ligands share low sequence homology (around 40% amino acid identity) and exhibit differences in their potencies and efficacies at CXCR3, with CXCL11 being the dominant ligand in several assays (Cole et al., 1998; Xanthou et al., 2003).

CXCR3 and its agonists have been implicated in the induction and perpetuation of several human inflammatory disorders (Qin et al., 1998) including atherosclerosis (Mach et al., 1999), autoimmune diseases (Sorensen et al., 1999), transplant rejection (Hancock et al., 2000; Hancock et al., 2001) and viral infections (Liu et al., 2000). These findings have made CXCR3 a popular target for the development of new potential anti-inflammatory strategies.

In recent years, two main variants of CXCR3 receptor have been identified, namely CXCR3-B (Lasagni et al., 2003) and CXCR3-alt (Ehlert et al., 2004) (Figure 1.18 A and B). Both variants are generated via alternative splicing of mRNA encoding the original CXCR3 receptor (henceforth referred to as CXCR3-A). In the case of CXCR3-B, alternative splicing resulted in extension of NH3 terminus by 52 amino acids and this form of receptor has been shown to bind Platelet Factor 4 (PF4/CXCL4) in addition to the three original CXCR3 agonists. In contrast CXCR3-alt is a truncated version of CXCR3 (lacking 101 amino acids)

which consequently exhibits a dramatically altered C terminus and with a predicted 4-5 transmembrane domain structure. Despite this drastically modified structure, CXCR3-alt has been shown to bind and respond to CXCL11 (Ehlert et al., 2004).

A



B

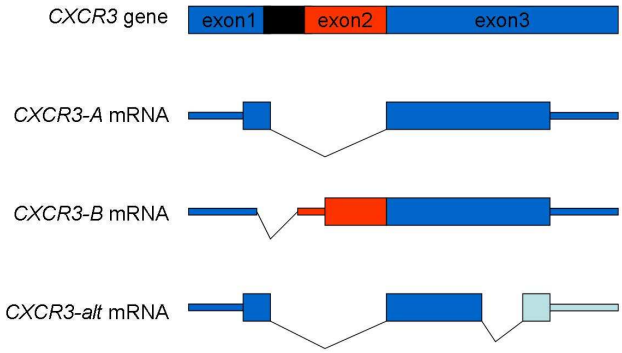


Figure 1.18 Alternatively spliced variants of CXCR3 receptor. (A), Structure of CXCR3 protein and (B), structure of *CXCR3* gene.

1.13.8 CXCR7

CXCR7 has been recently identified as a new member of chemokine receptor family. Despite intensive studies, its function as a typical chemokine receptor (defined by characteristic chemokine receptor signalling) and mechanisms of action are not fully understood and are still to be elucidated. CXCR7 formerly known as RDC1 as its gene was originally cloned from dog cDNA library (*R*eceptor *D*og *c*DNA) (Libert et al., 1990) has been recently described as the second receptor for chemokine CXCL12 (SDF-1) and CXCL11 (I-TAC) (Balabanian et al., 2005; Burns et al., 2006). A connection between RDC1 and chemokine receptors was suggested due to its sequence similarity (43%) and its identity (32%) with CXCR2 and the close location of its gene to *cxcr4, cxc2r*, *cxcr1* on mouse chromosome 1 (Heesen et al., 1998; Moepps et al., 2006). The definition of RDC1 as a CXCR chemokine receptor 7 was based on its high affinity to CXCL12 and CXCL11. Balabanian *et al* first demonstrated that RDC1 is

expressed in T lymphocytes and that anti-RDC1 monoclonal antibody inhibits CXCL12-mediated migration of T cells. The same researchers have also shown CXCL12 -induced internalization and migratory responses in CXCR4-negative and CXCR7 expressing cells. In contrast Burns *et al* reported that CXCR7 binds with high affinity another chemokine, CXCL11, but these receptor-ligand interactions did not lead to Ca^{2+} mobilisation or cell migration. Moreover, the characteristic chemokine receptor coupling to PTX-sensitive G_i-proteins- could not be demonstrated for CXCR7 (Thelen, 2001). As described previously in section 1.6.6, most chemokine receptors contain conserved DRYLAIV motif at the N-terminus of the second intracellular loop that is thought to be necessary but not sufficient for coupling to G_i-proteins. The sequence of CXCR7 receptor is altered in two positions (A/S and V/T alterations, Figure 1.16). It is worth noting that these modifications are present in other receptors (lymphotactin receptor and CXCR6) which are shown to be coupled to G_i-protein (Chandrasekar et al., 2004; Yoshida et al., 1998).

Results from other groups demonstrate that CXCR7 acts as a scavenger receptor during Zebrafish primordial germ cell (PMG) migration, were it is involved in forming a CXCL12 gradient by local scavenging of the chemokine. Initial studies also showed that regulated expression of CXCR4 and CXCR7 on migrating primodial cells, distributed on front and trailing cells, respectively (Dambly-Chaudiere et al., 2007; Valentin et al., 2007). Recent studies also support decoy activities of CXCR7 but its expression was detected on stromal rather than germ cells (Boldajipour et al., 2008).

A distinct role for CXCR7 was highlighted by Sierro *et al* who demonstrated expression of Cxcr7 transcripts on a restricted subset of leukocytes, some T cell subsets, NK cells, and particulary high levels of *Cxcr7* in different subsets of B cells. Despite high expression of CXCR7 in B cells, *Cxcr7* -deficient mice had normal B cell composition. However, *Cxcr7* deficiency in these mice led to death at birth due to ventricular septal defects and semilunar heart valve malformation (Sierro et al., 2007). This study points towards a developmental role of evolutionary conserved CXCR7.

The potential role of CXCR7 in cancer was also proposed as the expression of CXCR7 has been shown to provide cells with growth and survival signals, and increased adhesion properties. *In vivo* tumor growth in animal models was abolished by high affinity CXCR7 antagonists, making this receptor a potential target for a novel cancer therapeutics (Burns et al., 2006).

Although CXCR7 has not been fully accepted as a typical chemokine receptor and its role in T cells is still unclear, the inter-relationship between this receptor and CXCR3 and CXCR4 and their shared chemokines CXCL11 and CXCL12 is intriguing and may provide a new molecular link between inflammation and cancer. Further studies of interactions of CXCR7 with CXCL11 and CXCL12 would be desirable in order to understand the role it plays in T cells.

1.13.9 Atypical chemokine receptors

As well as conventional, signalling chemokine receptors controlling leukocyte recruitment during inflammation, there is a significant body of evidence suggesting that the chemokine receptor system includes 'silent' receptors possessing distinct specificity and tissue distribution. These 'atypical' receptors are unable to sustain signalling activities observed for typical chemokine receptors, such as calcium fluxes and chemotaxis, and are therefore referred to as 'silent' (Mantovani et al., 2001). These modified GPCRs have also been termed 'chemokine receptors' for internalizing receptors. The atypical chemokine receptors display an alteration in the DRYLAIVHA motif that is critical for signalling (Figure 1.16). Two of the best characterised silent chemokine receptors include DARC (Duffy antigen receptor for chemokines) (Horuk et al., 1993) and D6 (Bonini et al., 1997; Horuk et al., 1993; Nibbs et al., 1997) and these receptors will be discussed here together with another atypical chemokine receptor, CCX-CKR (Chemocentryx chemokine receptor) (Gosling et al., 2000). CXCR7 receptor should also be mentioned here as no signalling was detected through this receptor.

D6

The D6 molecule is typical of a chemokine receptor but, like DARC, it lacks the DRY motif (Figure 1.16) and TXP motif (in the second transmembrane domain). D6 binds a wide range of chemokines including inflammatory CC chemokines

(agonists of CCR1-CCR5) (Borroni et al., 2006). D6 is expressed at low levels by circulating leukocytes (Borroni et al., 2006), but high levels of D6 were found on endothelial cells of lymphatic afferent vessels in the skin, gut and lungs (Nibbs et al., 2001), and in the placenta (Bonini et al., 1997). D6 cannot couple with signalling pathways used by chemokines, but instead it posseses unusual intracellular trafficking properties to mediate repeated rounds of chemokine internalization (Fra et al., 2003; Weber et al., 2004; Bonecchi et al., 2004). D6 undergoes rapid constitutive ligand-independent trafficking to and from the plasma membrane, utilizing a β-arrestin- and clathrin-dependent route of internalization. This feature is unique among mammalian chemokine receptors (Weber et al., 2004). Chemokines which bind to D6 are also rapidly internalized, followed by dissociation from the receptor, remaining trapped within the cell for degradation. Meanwhile D6 recycles back to the plasma membrane for further ligand sequestration (Hansell et al., 2006). *In vivo* studies revealed that deletion of D6 can result in increased susceptibility to skin cancer and an inflammatory condition similar to psoriasis (Nibbs et al., 1997). Anti-inflammatory tumour suppressor properties of D6 were also supported by *in vivo* studies using null mice (Bonecchi et al., 2004; Jamieson et al., 2005; Marquez de la Torre et al., 2005).

DARC

A growing body of evidence indicates that another 'silent' receptor DARC also uses chemokine sequestration to control the CC and CXC chemokines to which it binds (Nibbs et al., 2003; Rot, 2005; Du et al., 2002). This limits chemokine availability and regulates blood chemokine levels (Fukuma et al., 2003; Jilma-Stohlawetz et al., 2001). Furthermore, it has been suggested that DARC promotes chemokine transcytosis across blood vessel endothelial cells. While chemokines internalized by D6 are degraded, the fate of chemokines internalized by DARC may be cell context-dependent and it is possible that they can maintain their biological activities. It has been assumed that DARC expressed on erythrocytes and vascular endothelial cells may act as either a biological sink or a transporter for both CC and CXC chemokines. This suggests that, in contrast to chemokine decoy/scavenger D6, DARC plays a more complex role in chemokine homeostasis (Pruenster and Rot, 2006). The modified DRYLAIVHA motif of DARC is shown in

Figure 1.16. Recent data also points towards a role of DARC in transporting of chemokines as well as potentiation of promigratory activities juxtrapolated leukocytes (Pruenster et al., 2009).

Chemokine (C-C motif) receptor-like 1(CCRL1)

Recent work by Comerford *et al* suggests that along with D6 and DARC, CCRL1 (other names CCX-CKR and CCR11) also shows biochemical properties of a chemokine-sequestrating atypical chemokine receptor (Comerford et al., 2006). In contrast to previously described 'atypical' receptors, CCX-CKR binds homeostatic chemokines, namely CCL19, CCL21 and CCL25, and also exhibits weak interactions with human CXCL13 (Gosling et al., 2000). Similarly to DARC and D6, CCX-CKR does not couple with typical signalling pathways and displays alterations within DRYLAIVHA motif (Figure 1.16). CCX-CKR has been shown to internalize and degrade CCL19 *in vitro* with high efficiency and in a β-arrestin independent manner, but in contrast to most chemokine receptors, this process was abolished through caveolin-1 manipulations.

1.13.10 Chemokine receptor expression

As already stated in this thesis, T lymphocytes can be subdivided according to expression of glycoprotein CD4 or CD8 and cytokine profile. They can also be categorized based on expression of characteristic chemokine receptors.

Characterisation of expression profiles of chemokine receptors has been instrumental in defining subsets of human memory T cells with distinct migratory capacity and effector functions. For example, CCR7 expression discriminates between lymph node-homing central memory T cells and tissue-homing effector memory T cells (Sallusto et al., 1999). Equally CXCR3, CXCR6 and CCR5 (Figure 1.19) are preferentially expressed on Th1 cells (Qin et al., 1998) while CCR3, CCR4 and CCR8 (along with the PGD2 receptor CRTH2) are expressed on Th2 cells (Nagata et al., 1999; Sallusto et al., 1998). More recently, CCR2, CCR6 and CCR9 have been reported to be expressed on Th17 cells (Acosta-Rodriguez et al., 2007; Nagata et al., 1999; Sato et al., 2007; Singh et al., 2008; Webb et al., 2008).

Naïve T cells express a limited number of chemokine receptors. CCR7 and CXCR4 are chemokine receptors mostly present on the surface of naïve T lymphocytes. These receptors are involved, for example in naïve T cell recirculation between lymph nodes and blood (Sallusto et al., 1999). The number of chemokine receptors increases upon T cell activation, allowing them to migrate towards a chemokine gradient. These upregulated receptors for example CXCR3, are mostly involved in inflammatory responses.

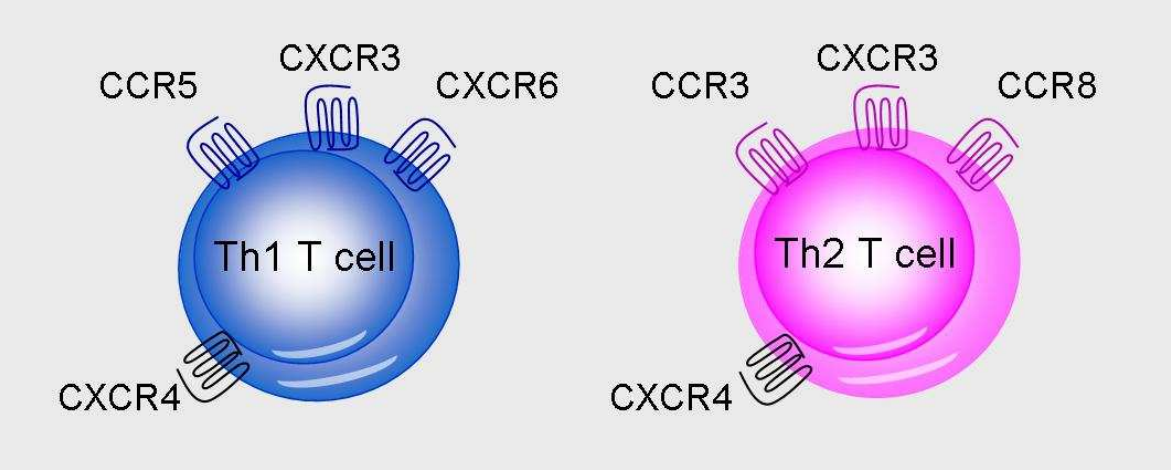


Figure 1.19 Examples of chemokine receptors expressed on Th1 and Th2 lymphocytes.

1.14 Chemokines and chemokine receptors as a potential therapeutic targets

GPCRs, the family of receptors which includes chemokine receptors, are the most attractive and tractable class of receptors for drug design, and are direct targets of ~ 30% of clinically prescribed small-molecule medicines (Milligan and Smith, 2007). Despite the fact that GPCRs are a popular drug targets, no antagonist targeting any chemokine receptor reached the market except for HIV entry inhibitors (Wijtmans et al., 2008; Este and Telenti, 2007). Potential small-molecule chemokine receptor antagonists have been tested on human chemokine receptors, with some promising candidate compounds discovered. However these are often of low affinity (Pease and Williams, 2006b). Applicability of such antagonists has been widely discussed because of the redundancy of chemokine systems (Wijtmans et al., 2008). The fact that most chemokines bind to several chemokine receptors and most chemokine receptors bind more than one chemokine, makes the prediction of therapeutic effect of chemokine receptor

antagonists very complicated. It is of prime importance to establish the specific roles of various chemokine receptors in disease models (Power, 2003). Examples of diseases in which chemokine receptors are involved are shown in Figure 1.20. The detailed role of chemokines and their receptors in a variety of pathologies has been discussed in many reviews (Medina-Tato et al., 2006; Proudfoot, 2002; Ruffini et al., 2007). In view of the character of the presented work, this overview will focus on CXCR3 receptor and its agonists.

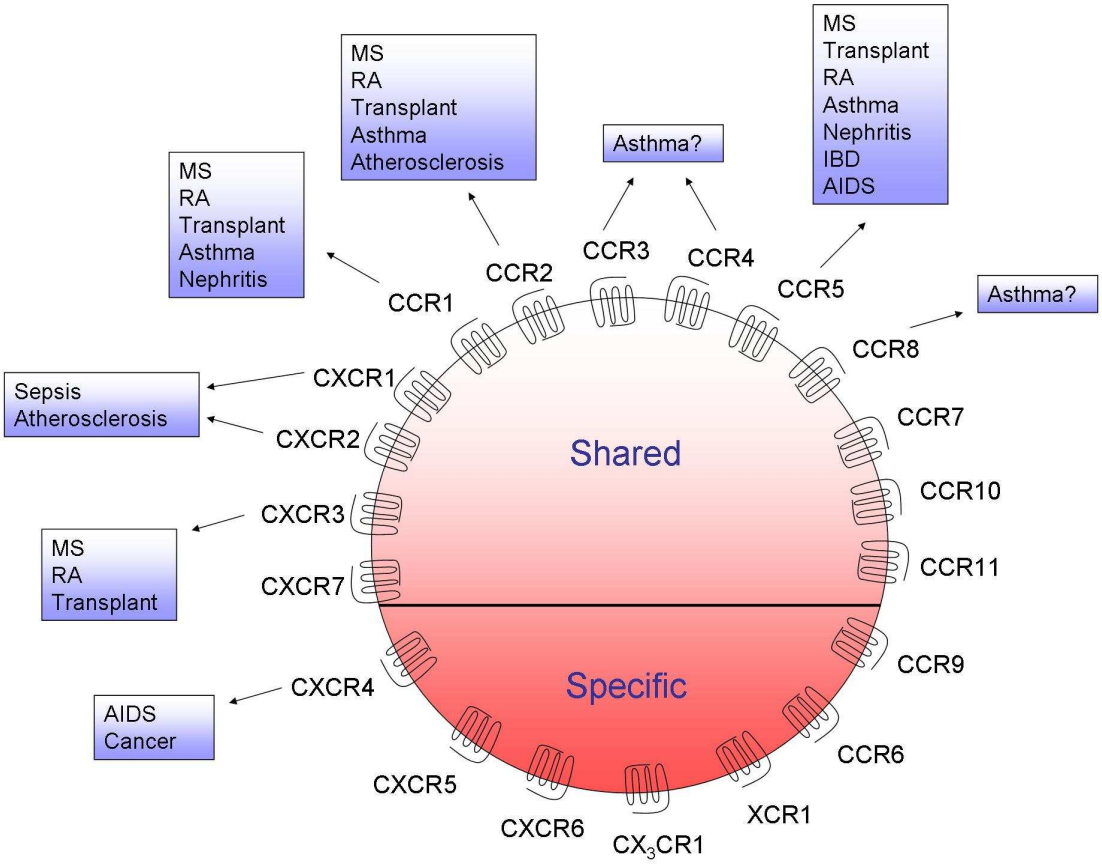


Figure 1.20 Role of chemokine receptors in human pathophysiology. Receptors classified as constitutive do not play a major role in inflammatory diseases. The exception is CCR8, which is constitutively present in thymus but can be upregulated on Th2 cells and is thought to be involved in asthma. CXCR4 is the only constituvely expressed chemokine receptor that has been described to be involved in disease to date. CXCR4 is one of the main HIV co-receptors and plays a role in cancer metastasis (Proudfoot, 2002). IBD, inflammatory bowel disease; MS, multiple sclerosis; RA, rheumatoid arthritis.

CXCR3 and the chemokines it binds CXCL9, CXCL10 and CXCL11, based on their upregulated expression, have been implicated in many inflammatory diseases (summarized in Table 1.1) (Wijtmans et al., 2008). Various pre-clinical approaches (reviewed by Wijtmans et al., 2008), including CXCR3 knockout (KO)

mice, targeting CXCR3 and its agonists by antibodies and targeting of CXCR3 by protein-based or small molecules antagonists, have been applied to confirm its therapeutic potential (Wijtmans et al., 2008). The following are some examples of findings obtained using these approaches.

Studies using CXCR3-KO ($CXCR3^{-/-}$) have shown delayed acute or chronic rejection of cardiac or pancreatic allograft in murine models of transplant rejection. In some cases, especially in combination with immuno-suppressive therapy, $CXCR3^{-/-}$ mice were able to chronically maintained allograft. In similar models, treatment with an antibody directed against either CXCR3 or CXCL10 significantly prolonged allograft survival. In addition, anti-CXCL10 antibodies inhibited chronic experimental colitis, and a Phase II clinical trial has been launched to further study the effect of the one of these antibodies, MDX1100, in the treatment of ulcerative colitis. The same antibody will also be tested in a Phase II trial for rheumatoid arthritis.

Disease	References
Multiple sclerosis	(Sorensen et al., 1999)
Rheumatoid arthritis	(Qin et al., 1998)
Atherosclerosis	(Mach et al., 1999)
Chronic obstructive pulmonary disease	(Saetta et al., 2002)
Inflammatory bowel disease	(Yuan et al., 2001)
Psoriasis	(Rottman et al., 2001)
Hepatitis C	(Shields et al., 1999)
Sarcoidosis	(Agostini et al., 1998)
SARS	(Glass et al., 2004; Danesh et al., 2008)
Transplant rejection	(Hancock et al., 2000; Hancock et al., 2001; Inston et al., 2007)
Metastasis of melanoma and colon cancer cells to the lymph nodes	(Kawada et al., 2004; Kawada et al., 2007)
Metastasis of breast cancer cells to the lungs	(Walser et al., 2006)
HIV	(Hatse et al., 2007)

Table 1.1 Involvement of CXCR3 receptor in human disease. (Reviewed by Wijtmans et al., 2008).

The use of protein-based CXCR3 antagonists also confirmed that blocking of this receptor helped reduce skin inflammation (Proudfoot and Kosco-Vilbois, 2003) and neuroinflammation in mouse model (Vergote et al., 2006).

Small molecule CXCR3 antagonists

A lot of potential small-molecule CXCR3 antagonists have been published and patented over recent years (Johnson et al., 2007; Medina et al., 2005). T487 compound (AMG487), developed by companies Chemocentryx and Tularik (later acquired by Amgen) (Schall et al., 2001; Medina et al., 2002) is the most studied member of its class (Wijtmans et al., 2008). The T487 and its more active analogue NBI74330 (Medina et al., 2002) have been extensively studied in order to characterise their preclinical properties. Both T487 (Verzijl et al., 2008) and NBI-74330 (Heise et al., 2005; Jopling et al., 2007; Verzijl et al., 2008) exhibit noncompetitive antagonism. T487 inhibited CXCR3 mediated chemotaxis (IC_{50}=15 nM, CXCL11) and Ca^{2+} mobilisation (IC_{50}=5 nM, CXCL11) (Johnson et al., 2007). The more potent derivative, NBI-74330, blocked CXCL11 mediated responses in a [^{35}S]-GTPγ-S binding assay (IC_{50}=10.8 nM), chemotaxis (IC_{50}= 3.9 nM) and Ca^{2+} mobilisation (IC_{50}=7 nM) (Heise et al., 2005). Figure 1.21 presents chemical structures of T487, NBI-74330 and its generated *in vivo* N-oxide metabolite, which efficiently binds CXCR3 (Johnson, 2006) and has also been patented (Collins et al., 2004).

Figure 1.21 Chemical structures of CXCR3 antagonists.T487 belongs to the azaquinazolinone class of compounds. NBI-74330 is its more active 4-F, 3-CF_3 analogue. Both ligands represent the best-characterised members of CXCR3 antagonists derived from the azaquinazolinone class. Metabolism of NBI-74330 *in vivo* leads to formation of N-oxide metabolite.

Studies on T487 and NBI-74330 using animal models have shown their great potential in terms of CXCR3 antagonism in many diseases (Wijtmans et al., 2008). The studies of T487 led to clinical studies in two inflammatory disorders: psoriasis

and rheumatoid arthritis. Unfortunately, this compound showed a lack of clinical efficacy in psoriasis and the Phase I trial was attenuated. The current status of the trials on patients with RA which was initiated in Phase II in 2004 is unknown. Work on further modifications of T487 antagonist towards increased efficacy has continued (Wijtmans et al., 2008).

The pool of different classes of existing CXCR3 ligands exhibits high variability. Described here are examples of the most notable CXCR3 antagonists, which are also used as a research tool within the presented work. Despite the positive preclinical results which increased expectations for CXCR3 antagonism therapeutic targeting of CXCR3 in humans still remains an elusive goal.

1.15 Chemokine receptor internalization and intracellular trafficking

The expression of chemokine receptor on the cell surface is a balance between the rate of internalization and the rate of replacement (recycling or synthesis of new protein) (Mueller et al., 2002). Chemokine receptors undergo a basal level of internalization and degradation or recycling in the absence of agonist. Agonist binding can enhance the internalization and trafficking of these receptors, and can increase the rate of receptor sensitization versus desensitization, and recycling versus degradation (Neel et al., 2005). There are two major routes of receptor trafficking: clathrin mediated endocytosis and lipid raft/caveolae dependent internalization. Some receptors can internalise via both pathways while others may utilise one preferred pathway. The decision on which route (or routes) will be chosen by a particular receptor partially depends on the cell type and may be due to the different expression of specific adaptor proteins, membrane lipid composition in the proximity of the receptor, or other unknown factors (Neel et al., 2005).

1.15.1 Pathways for Internalization of Chemokine Receptors

The internalization of receptor and its ligand by clathrin-coated pits is the best characterised endocytic process (Mukherjee et al., 1997). Binding of chemokine ligand to the receptor causes a phosphorylation of Ser and Thr residues in the intracellular loops and C-terminus of the receptor due to action of GRKs (G protein-coupled receptor kinases, which are activated by the kinase activity of second messengers such as PKC), leading to the uncoupling of the G protein from

the receptor and, in some cases, receptor desensitisation. Receptor phosphorylation and/or presence of a di-leucine motif in the C-terminus of chemokine receptor is crucial for the binding of adaptor proteins that link the receptor to a lattice of clathrin that facilitates receptor internalization (Neel et al., 2005). Adaptin-1 and β-arrestin have been found to play an important role in chemokine receptor internalization. β-arrestin binding to receptor occurs through phosphorylated residues in the C-terminus but can also occur through the intracellular loops. It has been reported that β-arrestin binds to both carboxyl-terminus and the third intracellular loop of CXCR4 (Cheng et al., 2000). AP-2 binds to some chemokine receptors, including CXCR2 and CXCR4, via highly conserved Leu-Leu, Ile-Leu and Leu-Ile motifs within the C-terminus (Heilker et al., 1996; Fan et al., 2001). Association of the adaptor proteins is required for the recruitment of clathrin, and through the action of dynamin, formation of clathrin-coated vesicles (Mousavi et al., 2004). Following receptor internalization, clathrin-coated vesicles are uncoated and receptor-ligand complexes are directed to the early endosomal compartment. The chemokine receptor can then be directed to the perinuclear recycling compartment and be recycled back to the plasma membrane, or can enter the late endosomal compartment and be directed to the lysosomal compartment for degradation. Schematic illustration of endocytic pathways is shown in Figure 1.22.

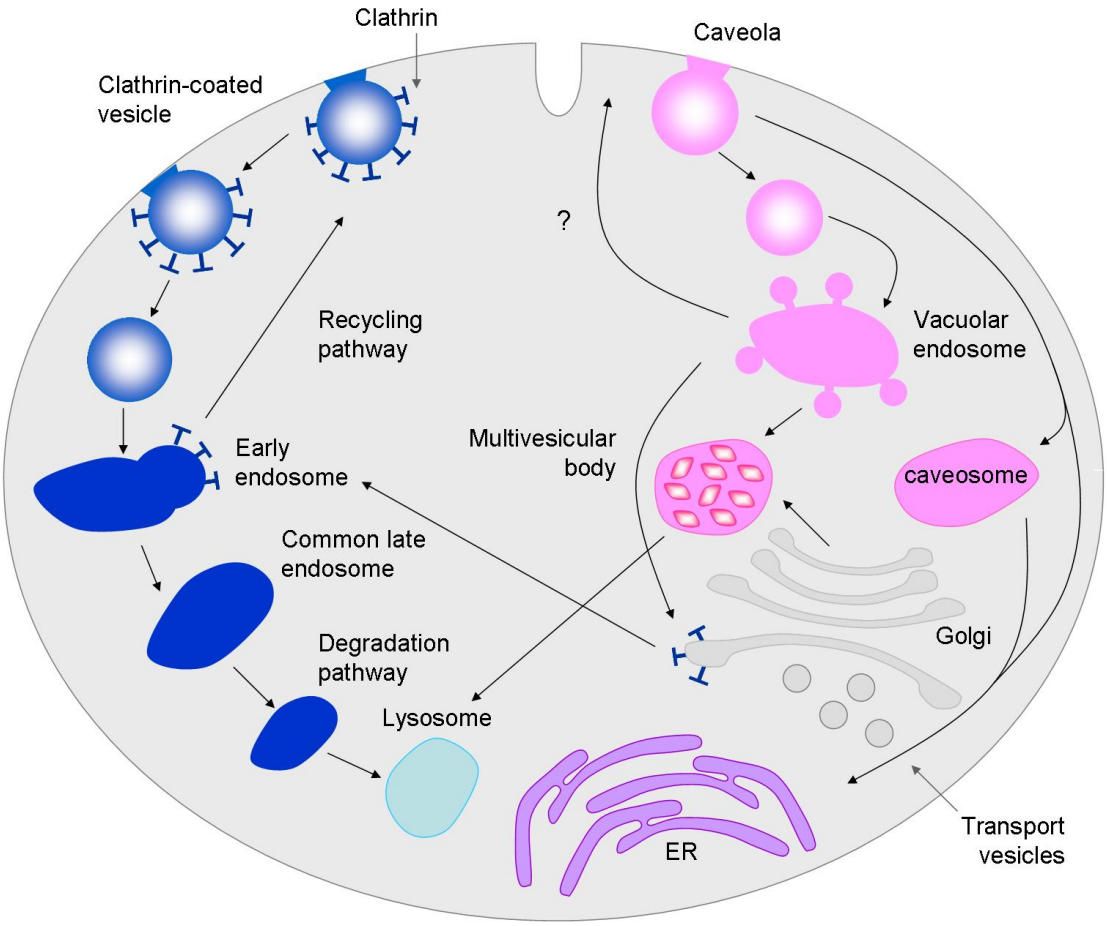


Figure 1.22 Schematic of endocytosis. Two pathways of endocytosis, namely clathrin-dependent and caveola-dependent pathway are shown.

An alternative pathway for internalization of chemokine receptor may occur via lipid rafts or through cholesterol-enriched structures called caveolae (Parton and Simons, 2007; Palade, 1953), Figure 1.22. Caveolae are stabilised by caveolin-1 (CAV-1) and CAV-2 cholesterol binding proteins and form a characteristic flask-shaped structure with no obvious coat (Stan, 2005). Once internalized, some receptors can enter the compartment known as caveosome and fuse with early endosomes also used in clathrin-dependent pathways (Sharma et al., 2003). Despite some reports that chemokine receptors, like CCR5 and CXCR4 (Manes et al., 1999; Manes et al., 2000; Nguyen and Taub, 2002) have been found to some degree in lipid rafts, coveolae/lipid raft dependent internalization does not appear to be a common feature of the chemokine receptors.

1.15.2 Regulation of chemokine receptor trafficking by Rab GTPases

Rabs are small GTPases that are involved in regulation of variety of cellular trafficking events. The exchange of GDP for GTP, GTP hydrolysis, and GDP displacement are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs), respectively.

Rab5 mediates early endocytic responses and is required for the fusion of early endosomes (Rybin et al., 1996). Rab5 interacts with class I PI3K and induces production of phosphatidylinositol 3-phosphate. Rab5 and PIP_3 recruit EEA-1 (early endosomal antigen) and other proteins involved in fusion with early endosomes. Internalization of CXCR2, CXCR4 and CCR5 requires activity of Rab5 and is significantly blocked by expression of Rab5 dominant negative mutant (Fan et al., 2003; Venkatesan et al., 2003). There are two types of endocytic recycling- rapid and slow pathway to which Rab4 and Rab11a contribute, respectively (Ullrich et al., 1996; Sheff et al., 1999; Sonnichsen et al., 2000). The slow recycling pathway, which involves the perinuclear compartment-localised protein Rab11a, seems to be important in intracellular trafficking of chemokine receptors. This was proven for CXCR2, which upon agonist induced internalization localizes to the Rab11a-containing compartment (Fan et al., 2003). Rapid recycling pathway through Rab4 positive endosomes by-passes the perinuclear endosomes and occurs in PI3K-dependent manner (Hunyady et al., 2002). It is not known what mechanisms regulate these different recycling pathways.

Rab7 is involved in directing late endosomes to the lysosomal compartment. Prolonged exposure of chemokine receptors to ligand may result in their degradation in lysosomes, and Rab7 is thought to be important in the process of lysosomal sorting. Involvement of Rab7 was shown for CXCR2 receptor, where blocking of Rab7 activity resulted in decreased localisation of this receptor to the lysosomal compartment and its accumulation in early and recycling endosomes (Fan et al., 2003).

1.15.3 Regulation and functional consequences of internalization

Receptor internalization following ligand binding is the most likely reason for downregulation of most chemokine receptors. The rate of this process can be

dependent on multiple factors that involve the C-terminus of receptor, the type of ligand, cell type or phosphorylation status.

Phosphorylation of Ser and Thr residues and the presence of a di-leucine motif in the carboxyl-terminus were shown to be required for internalization of some chemokine receptors. This has been exemplified in the CXCR4 receptor. In contrast, internalization of CXCR3 by any of its ligands is not affected by the mutation in the LLLRL motif located in the C-terminus. However mutational change of C-terminal Ser and Thr residues inhibited CXCL9 and CXCL10–induced internalization with no effect on CXCL11, the action of which was dependent on the third intracellular loop of CXCR3 in 300-19 cells (Colvin et al., 2004). The rate of internalization also depends on the type of ligand. As previously noted, most chemokine receptors bind with high affinity to more than one chemokine, and these chemokines may differentially induce internalization of the receptor. This situation was observed for CXCR2 which binds two ligands, CXCL6 and CXCL8, with high affinity. CXCL8 is, however, more efficacious in inducing internalization of CXCR2 (Feniger-Barish et al., 2000). Similar findings were reported for the CXCR3 receptor, which became internalized in response to CXCL9, CXCL10 and CXCL11, with CXCL11 being the principal chemokine responsible for CXCR3 internalization (Sauty et al., 2001). Cell context may also affect the rate of internalization and the pathway through which a receptor becomes internalized. This may be due to the availability of different endocytic compartments like caveolae and the expression of various proteins such as β-arrestin and other adaptor proteins (reviewed by Neel et al., 2005). To illustrate, CXCL11 reduced the surface expression of endogenous CXCR3 in T cells to 20%, while in transfected L1.2 cells only 50% reduction of basal expression of surface CXCR3 was detected. Internalization of some receptors like CCR5 have been reported to occur via both clathrin and caveolae-dependent pathways. In some cell types such as Chinese Hamster Ovary (CHO), CCR5 internalises mainly by clathrin-dependent endocytosis (Signoret et al., 2005), while in other cell types, namely Human Embryonic Kidney cells (HEK293), the dominant pathway for CCR5 internalization is via caveolae (Venkatesan et al., 2003).

Little is known regarding the factors which mediate the fate of internalized receptor. These factors possibly include duration and concentration of ligand stimulation and different sorting motifs present within intracellular domains of the receptor (reviewed by Neel et al., 2005). As is the case for CCR5 receptor, short stimulation with the ligand leads it in the direction of the recycling endosomes, while longer stimulation causes its localization to the late endosomal compartment (Signoret et al., 2000). Similarly, CXCR2 at the early time of enters recycling endosomes at early times during stimulation with CXCL8, while the extended stimulation time leads to its direction to the lysosomes (Fan et al., 2003).

Intracellular trafficking is an important aspect of chemokine receptor function and has been studied for multiple receptors. Some findings have suggested a role for internalization of chemokine receptors in transendothelial migration (Sauty et al., 2001). Whether internalization is required in chemotaxis and signalling remain controversial and these issues still need to be addressed.

1.16 Signalling via chemokine receptors

Binding of agonist to its serpentine receptor results in cellular responses such as integrin activation, actin reorganization and directional migration. In T lymphocytes, stimulation of CXCR3 by its agonists leads to elevation of intracellular calcium (Rabin et al., 1999), and activation of phosphoinositol-3-kinase (PI3K) and Akt-dependent signalling, as well as the p42/44 mitogen-activated protein kinase (MAPK) pathways (Smit et al., 2003). CXCR3 activation has also been shown to induce rapid tyrosine phosphorylation of several proteins including Zeta-associated protein of 70 000 MW (ZAP-70), linker for the activation of T cells (LAT) and phospholipase-C-γ1 (PLCγ1) (Dar and Knechtle, 2007). Illustrated below are some of the crucial signalling pathways triggered by chemokine receptors.

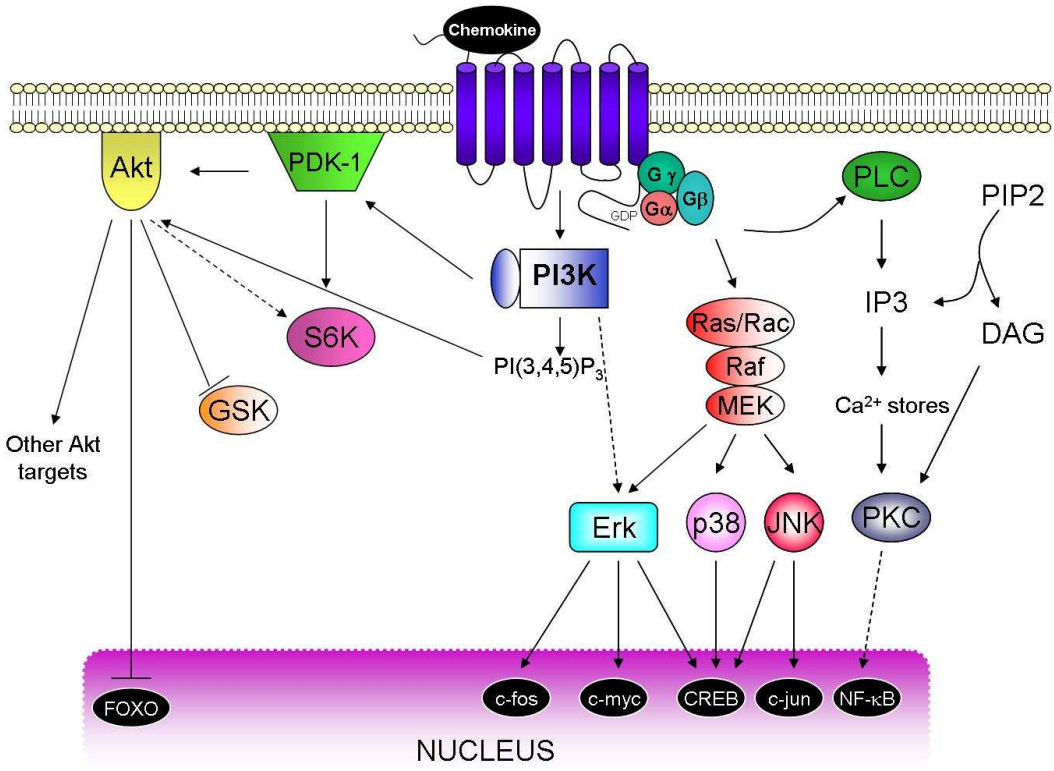


Figure 1.23 Schematic illustration of some signalling events triggered by activation of chemokine receptors.

1.16.1 Phospholipase C/protein kinase C pathway

The Phospholipase C/Protein Kinase C (PLC/PKC) signalling cascade has been demonstrated to be activated downstream of chemokine receptors and TCRs (Figure 1.23 and 1.24). PLC function involves the modulation of phosphoinositides at the plasma membrane. The PLC family comprises of six isoforms, namely β, γ, δ and recently identified ε, ζ and η (Harden and Sondek, 2006; Katan, 1998). Activation of chemokine receptor by ligand binding leads via activation of several protein kinases to the activation of PLCβ. The resulting hydrolysis of PtdIns(3,4)P_2 into two second messengers; Diacyloglycerol (DAG) and inositol triphosphate (IP_3), leads to binding of IP_3 to the IP_3 receptor (IP_3 R), which is itself a calcium-permeable ion channel, and the release of Ca^{2+} from intracellular endoplasmic reticulum (ER) Ca^{2+} stores (Feske, 2007). Ca^{2+} released from the cellular stores causes only a moderate and transient increase in intracellular Ca^{2+} levels due to the small size of the ER in lymphocytes. The reduction of the Ca^{2+} concentration in the ER induces activation of a SOCE (store-operated Ca^{2+} entry) pathway, resulting in the opening of store-operated Ca^{2+} channels in the plasma membrane (Feske, 2007). In lymphocytes, SOCE through calcium-release-activated calcium

(CRAC) channels is the main mechanism to increase intracellular Ca^{2+} concentration, and is crucial for the activation of transcription and cytokine gene expression (Lewis, 2001; Parekh and Putney, 2005; Prakriya and Lewis, 2003). Two molecules are reported to be involved in this process; stromal interaction molecule 1 (STIM1), located within the ER calcium sensor and the ORAI1 (also known as CRACM1 or TMEM142A), a functional component of the CRAC channel (Feske et al., 2006; Prakriya et al., 2006; Luik et al., 2006). The Ca^{2+} binding EF hand motif of STIM1 is localized within the ER, facing the lumen, and therefore it is postulated that STIM1 proteins act as sensors of Ca^{2+} concentration in the ER. Depletion of Ca^{2+} from intracellular stores triggers redistribution of STIM1 from diffuse into discrete 'puncta' in close proximity to the plasma membrane, leading to the interaction with CRAC channels and refilling of the intracellular stores (Zhang et al., 2005; Liou et al., 2005). DAG is highly hydrophilic therefore remains associated within the membrane recruiting cytoplasmic PKC and activating both conventional PKC (cPKC) and novel PKC (nPKC).

PLCβ, the most abundantly expressed within the immune system has been implicated in a variety of cellular functions in neutrophils (Rhee and Bae, 1997). However no role in chemotaxis was demonstrated. In contrast PLC has been found to be involved in migration of T cells (Li et al., 2000; Cronshaw et al., 2006). The prominent role of PLCβ was demonstrated to be specifically dependent on the production of IP_3 and the subsequent increase in intracellular calcium, and not the activation of PKC (Bach et al., 2007).

The family of PKC serine/threonine kinases is composed of 9 members categorized according to their structure and function. Members of the conventional PKC family α, β and γ, exhibit DAG sensitive calcium responsiveness. Novel PKC isoforms δ, ε, η and θ, are DAG sensitive and calcium insensitive. Atypical PKCs ζ and λ contain altered C1 domains and are both DAG and calcium insensitive. *In vitro* PKC can be activated independently of plasma membrane receptors with the use of phorbol esters such as PMA, which does not distinguish between isoforms and results in a strong, robust activation of the signalling cascade (Kazanietz et al., 2000).

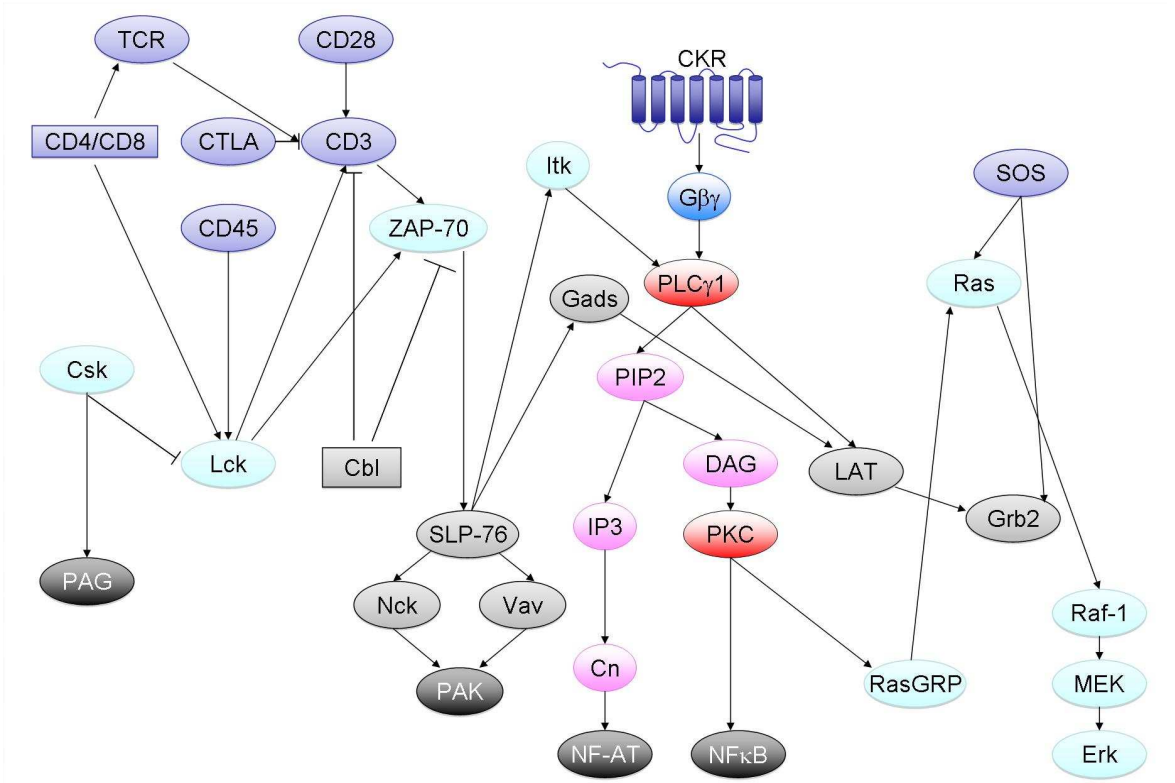


Figure 1.24 PLC/PKC pathway. Following T cell receptor (TCR) stimulation, a member of the Src family of protein tyrosine kinases, Lck phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) located within the cytoplasmic domains of the CD3 complex. Subsequently, ZAP-70 (a member of the Syk family kinases) is recruited by its Src homology-2 (SH2) domains binding to the phosphorylated ITAMs sites. Activated ZAP-70 transduce signal through the phosphorylation of downstream effectors such as adaptors LAT and SLP-76, which in turn leads to the activation of PLCγ1. Following chemokine receptor stimulation, dissociated Gβγ subunits also stimulate PLCγ1. Activation of PLC results in cleavage of phosphatidylinositol 4,5-biphosphate [PI(4,5)P_2] to inositol 1,4,5-triphosphate (IP_3) diacylglycerol (DAG). IP_3 induces calcium mobilization, which activates NF-AT (nuclear factors of activated T cells). DAG leads to activation of RasGRP and PKCθ, which in turn stimulate the Ras-MAPK (Ras-mitogen-activated protein kinase) and NF-κB (nuclear factor of kappa-B) pathways, respectively.

PKCδ has been implicated in cell motility and migratory responses of many non-immune cells (Iwabu et al., 2004). Pharmacological interventions revealed the role of DAG-dependent PKC isoforms, in particular PKC δ, in the CCL4 migratory response. It is not well understood how PKC isoforms regulate cell motility/migration, although it is thought that PKC isoforms can have an effect on actin reorganization/polymerization (Hartwig et al., 1992; Sasahara et al., 2002).

1.16.2 Phosphoinositide 3-kinase

Phosphoinositide 3-kinases (PI3Ks) are a family of enzymes that catalyse the phosphorylation of the 3-OH position of inositol ring groups of phophoinositide (PI)

lipids, namely phosphatidylinositol (PtdIns), phosphatidylinositol(4)phosphate [PtdIns(4)P], and phosphatidylinositol(4,5)phosphate [PtdIns(4,5)P_2]. This results in the formation of 3'-phosphoinositide lipids: PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively (Figure 1.25) (Curnock et al., 2002; Vanhaesebroeck et al., 2001). PtdIns(3)P is the precursor to all phophoinositides and is constitutively present in cells. Its level remains unaltered upon cell stimulation and it is thought to be involved in the regulation of membrane trafficking (Vanhaesebroeck et al., 2001). PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are not present in resting cells but their intracellular concentration increases rapidly upon cell stimulation via a variety of receptors, suggesting a likely function as second messengers (Sotsios and Ward, 2000).

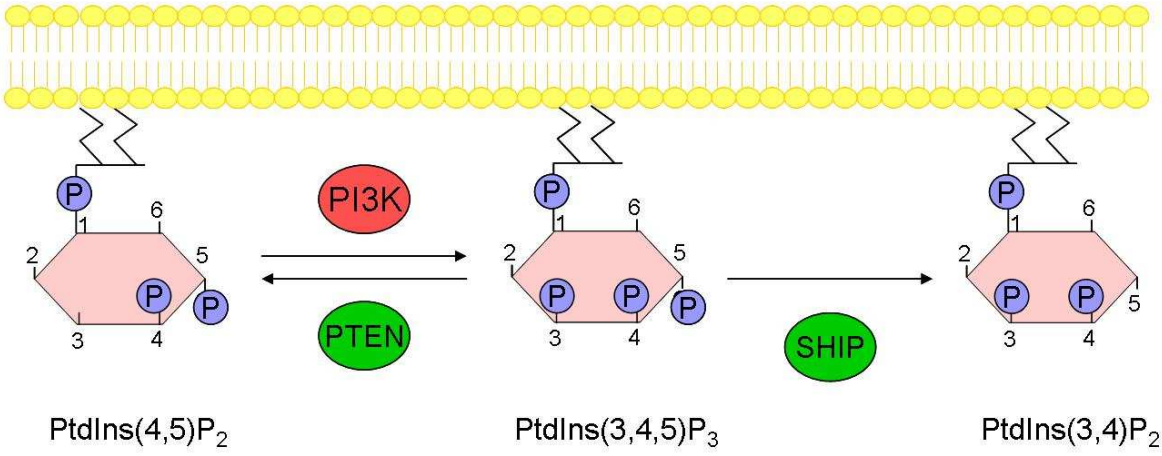


Figure 1.25 Generation of PtdIns(3,4,5)P_3 by catalytic action of Phosphoinositide 3-kinase. PI3K phosphorylates the inositol ring of its substrate PtdIns(4,5)P_2 converting it to PtdIns(3,4,5)P_3. Action of PI3K is regulated by two phosphatases, namely PTEN and SHIP which convert PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 and PtdIns(3,4)P_2, respectively.

PI3Ks can be subdivided into three main classes according to their *in vitro* lipid substrate specificity, structure and regulation (Figure 1.26). It was shown that *in vitro* class I PI3Ks can phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, however *in vivo*, PtdIns(4,5)P_2 is thought to be their preferred substrate (Vanhaesebroeck and Waterfield, 1999). The members of this class interact with Ras and form heterodimeric complexes with adaptor proteins which link them to a variety of upstream signals. This class is further subdivided into two subfamilies, class IA and IB. The class IA PI3Ks are activated by Tyr kinases heterodimers, consisting of; p85 (85 kDa molecular weight); a regulatory/adaptor protein (p85α/β,

p55α/γ or p50α), responsible for protein-protein interactions via SH2 (Src homology) domain with phosphotyrosine residues of other proteins; and catalytic subunit p110 (110 kDa), which is encoded by 3 genes p110α, p110β and p110δ. The single member of class IB PI3K, PI3Kγ, associates with a unique adaptor protein, namely p101 and it is activated by GPCRs. In contrast to class IA PI3Ks, class IB do not interact with the SH2-containing adaptors. (Stephens et al., 1994; Stephens et al., 1997; Sotsios and Ward, 2000).

The class II PI3Ks (e.g. PI3K-C2α) contain a characteristic C2 domain on their carboxy terminus (Figure 1.22), and their preferential substrates *in vitro* are PtdIns and PtdIns(4)P. They can also utilize PtdIns(4,5)P_2, when presented with phosphatidylserine. In contrast to members of other classes of PI3K, PI3K-C2α is resistant to treatment from PI3K inhibitors, Wortmannin and LY294002 (Domin et al., 1997).

The class III PI3Ks consists of only one member, the human homologue of the yeast vesicular sorting protein, Vps34 (vacuolar protein sorting 34). This enzyme catalyzes only one substrate, PtdIns, both *in vitro* and *in vivo*, and it has been suggested to have a role in intracellular trafficking (Yan and Backer, 2007).

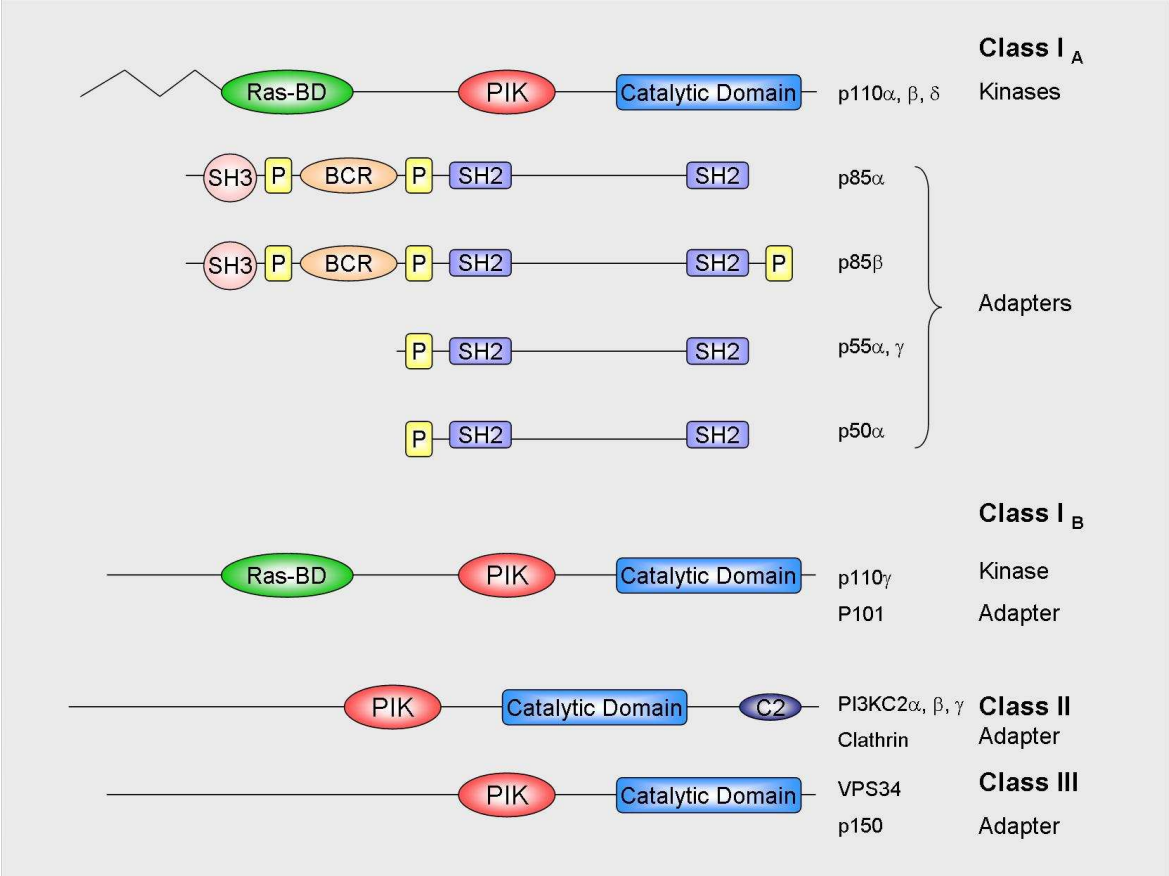


Figure 1.26 Classification of phosphoinositide 3-kinase family members. PI3Ks have been subdivided into three classes based on substrate specificity, structure and regulatory mechanisms. Class I is further subdivided into class I A and class I B according to association with specific regulatory/adapter subunit. The class IA and IB catalytic subunits consist of a Ras binding domain (Ras-BD) that mediates activation by the small GTPase Ras, and a Phosphatidylinositol kinase homology domain (PIK). Class II is similar to class Ie but its members lack Ras-BD and contain C2 domain at their C-termini. The catalytic subunit of the single member of class III Vps34 also lacks Ras-BD. The protein domains within adapter subunits are as follows, BCR, breakpoint-cluster region; P, proline-rich motif; SH2, src-homology domain 2; SH3, src-homology domain 3 (Adapted from Curnock et. al 2002).

Regulation of PI3K by SHIP and PTEN

Catalytic activity of PI3K is tightly regulated by the lipid phosphatases SHIP (SH_2-containing inositol phosphates) and tumour suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10) (Rohrschneider et al., 2000; Astoul et al., 2001; Cantley and Neel, 1999). Both enzymes act on PI3K activity by modulating its products via dephosphorylation. SHIP is a 5-phophoinositide phosphatase converting PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2, while PTEN is a 3-phophoinositide phosphatase and catalyse dephosphorylation of PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. These regulatory components are crucial as their loss causes dysregulation of phosphoinositide 3-kinase-dependent signaling and function in

leukocytes, and has been implicated in many inflammatory and autoimmune diseases (Harris et al., 2008). The importance of SHIP and PTEN phosphatases is also supported by the fact that their function is commonly lost in many leukemias and leukemic cell lines (Cully et al., 2006). As will be discussed in more details below, PI3K has been involved in migratory responses of certain cell types. Its localization in resting cells is mainly cytoplasmic with minimal catalytic activity, while PTEN is located at the plasma membrane. Following chemokine stimulation, the cell became polarized and PI3K is translocated to the leading edge while PTEN is moved from this area to the sides and rear of the cell. This promotes generation and maintenance of lipid products on the front, dephosphorylation of PI3K products trailing edge, and further maintaining of polarized shape of the migrating cell (Figure 1.12).

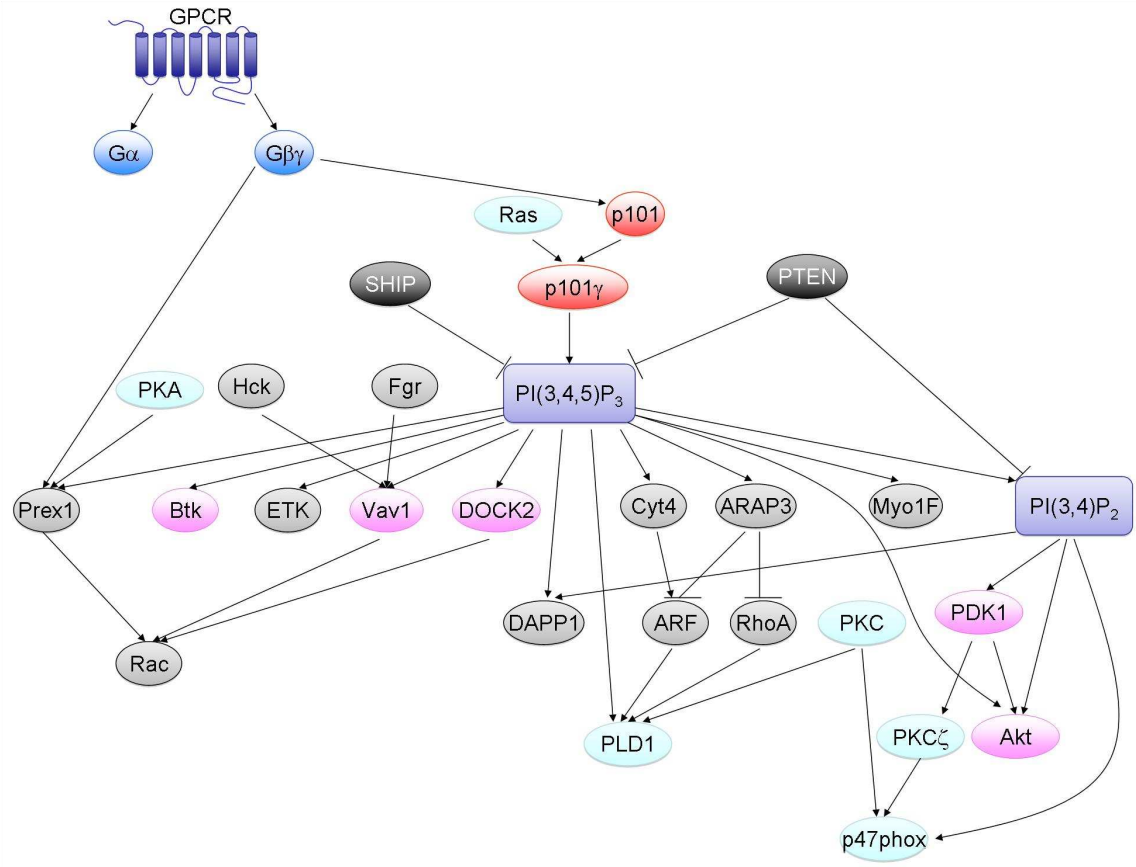


Figure 1.27 Signalling events following formation of phosphoinositide lipids by PI3K. Agonist stimulation of chemokine receptor activated class IB PI3K via action of Gβγ subunits and the Ras. This leads to accumulation of PI3K products PtdIns(3,4,5)P_3, and indirectly PtdIns(3,4)P_2, in the membrane and interaction with variety of effector proteins by binding to the PH domain.

PI3K in cell polarisation and migration

Chemokines have been shown to stimulate accumulation of PtdIns(3,4,5)P_3. PI3Kγ is a key isoform activated downstream of chemokine receptors. Several chemokine receptors however can activate other PI3K isoforms (Sotsios and Ward, 2000; Ward, 2004).

The role of PI3K in chemotactic response has been established primarily in neutrophils (Fergus et al., 2007; Hannigan et al., 2002; Hirsch et al., 2000; Ridley et al., 2003; Wang et al., 2002). Contribution of PI3Kγ in directional migration has been also shown in murine T cells, (Nombela-Arrieta et al., 2004; Reif et al., 2004; Webb et al., 2005), although its role in primary human T lymphocytes has been harder to verify.

For many years it was thought that PI3K was important for the directional migration of immune cells including T lymphocytes. Evidence from different groups have demonstrated PI3K-dependent CCL20, CCL2, CXCL8 and CXCL12-induced chemotaxis of eosinophils, THP-1 monocytic cell line, neutrophils and T lymphocytes, respectively (Knall et al., 1997; Sotsios et al., 1999; Sullivan et al., 1999; Turner et al., 1998). The involvement of PI3K in directional migration towards chemokine gradient was also demonstrated for CCL5/RANTES (Regulated on Activation, Normal T cell Expressed and Secreted)-induced polarisation and chemotaxis of T lymphocytes using inhibitors such as LY294002 and Wortmanin (Turner et al., 1995).

In contrast, in experiments that more closely mimic the physiological conditions encountered by lymphocytes undergoing transendothelial migration, there is now a growing body of evidence suggesting that PI3K activation is a dispensable signal and is not crucial for chemokine-stimulated T cell chemotaxis. PI3K inhibition have minimal effect on *in vivo* T lymphocyte arrest, on adhesion to high endothelial venules in exteriorised Peyer's patches (Sotsios et al., 1999), and on transendothelial migration in laminar flow chambers (Cinamon et al., 2001), in response to either CXCR4 and/or CCR7 ligation. In addition CCR4-agonist-stimulated migration of human Th2 differentiated cells (Cronshaw et al., 2004),

and CXCR3 ligand-stimulated migration of PBLs (Smit et al., 2003) in transwell assays is also insensitive to PI3K inhibitors.

Furthermore, several recent studies examining the effects of either the genetic loss of PI3Ks, or of selective PI3K inhibitors on the chemotactic efficiency of both neutrophils and *Dictyostelium* amoebae revealed no specific deficiencies (Andrew and Insall, 2007; Hoeller and Kay, 2007; Weiner et al., 2002). The genetic loss of PI3Kγ or selective PI3K inhibitors led to the reductions in the chemokinetic responses which could explain some of the apparent reductions in chemotactic migration reported previously (Fergus et al., 2007). .

1.16.3 Downstream effectors of PI3K

As already established, the stimulation of a chemokine receptor by ligand leads to the activation of PI3K. This activation triggers a range of T lymphocyte functions including growth, proliferation, cytokine secretion and survival (summarized in Figure 1.27). Multiple cellular proteins have been identified as downstream effectors of PI3K and some of them will be discussed below. Monitoring the phosphorylation of the downstream effectors such as Akt/PKB, Erk 1/2 MAPK or S6 can be an indirect readout of PI3K activity.

Protein kinase B PKB/Akt

Protein kinase B (PKB), also termed Akt, is a serine/threonine kinase that is the best characterised downstream effector of PI3Kγ and p85/p110. PKB is a key mediator in many cellular processes (summarized in Figure 1.28), including growth factor-induced cell survival and protection against c-myc induced cell death (Dudek et al., 1997; KauffmanZeh et al., 1997; Kulik et al., 1997). Modulation of PKB is dependent on recruitment to the plasma membrane via its PH domain by PtdIns(3,4,5)P_3. Membrane bound PKB is then thought to be phophorylated in phosphoinositide-dependent kinase-1 (PDK-1)-mediated manner at Thr308, while the rictor-mTOR complex directly phosphorylates Ser473 in the hydrophobic region of PKB. PKB requires the phosphorylation of both of these residues to become fully activated. Upon activation PKB localises to the site of action within the cytoplasm and its activity is controlled by the dephosphorylation of

PtdIns(3,4,5)P_3 by SHIP. A decrease in PtdIns(3,4,5)P_3 levels at the membrane leads to the localisation of PKB in cytosol in an inactive state.

Several chemokine receptors have been shown to activate PKB in a PI3K-dependent manner. IL-8 is not able to activate PKB in PI3Kγ-deficient neutrophils. However in mammalian cells several cellular events such as cytoskeletal reorganization and lamellipodium formation have been demonstrated to be PI3K- but not PKB-dependent. Furthermore, CXCR3-induced chemotaxis of T lymphocytes has been shown to occur in PKB independent manner (Smit et al., 2003). A number of phosphorylation targets for PKB have been described (Figure 1.28). These include proteins involved in the regulation of glucose metabolism and cell survival such as GSK-3 and several transcription factors. It has become apparent that PKB may be able to regulate activation of NF-κB but the exact mechanism remains controversial

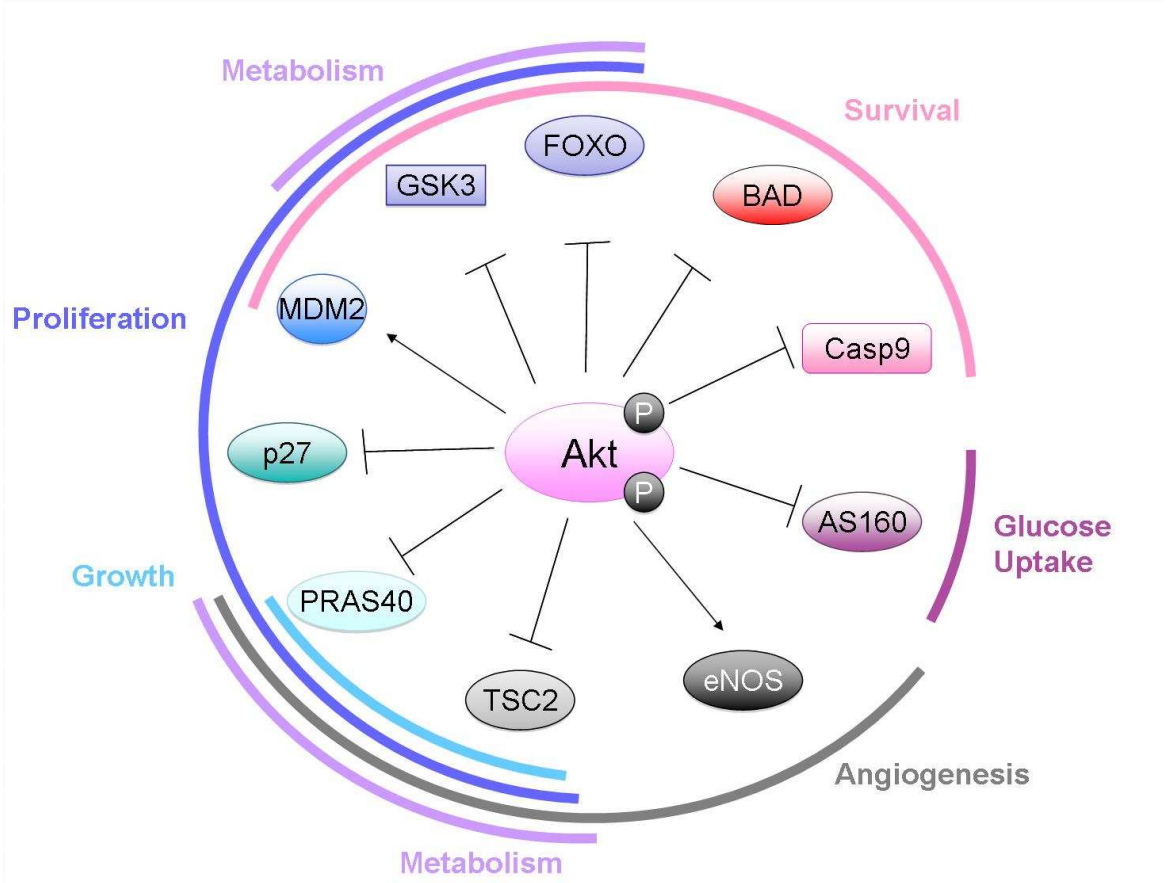


Figure 1.28 The role of PKB/Akt and proposed mechanism of activation of its downstream effectors.

S6 Ribosomal protein

PI3K has been found to mediate activation of S6 kinase, which is a component of the PI3K/mTOR pathway (Martin et al., 2001). S6 kinase in turn induces the phosphorylation of the ribosomal protein. S6 kinases are tightly controlled by the mTOR (mammalian target of Rapamycin). There are several lines of evidence supporting the fact that S6 plays the a role in chemotaxis (Richardson et al., 2004). Notably GM-CSF (granulocyte-macrophage colony-stimulating factor)–induced chemotaxis and chemokinesis of neutrophils is attenuated after pre-treatment with immunosuppressant rapamycin, which blocks mTOR (Gomez-Cambronero, 2003). Despite the clear role mTOR plays in neutrophil migration and chemokinesis, its role in T cells is still to be elucidated.

1.16.4 Tyrosine kinases

JAK/STAT pathway

It has been demostrated that in addition to activating G-protein coupled signalling pathway, chemokines can also activate the Jak/STAT pathway (Janus kinase / signalling transducer and activator of transcription) (Mueller and Strange, 2004). The JAK family of non-receptor tyrosine kinases is comprised of four members, JAK1, JAK2, JAK3 and Tyk2 (Tyrosine kinase 2) (Schindler, 1999). These kinases together with STAT proteins form the so called JAK/STAT pathway. STAT monomeric proteins are present in the cytosol and become recruited to the plasma membrane. There they become activated and act as a substrates for JAK family members. Binding of the chemokine to its receptor promotes binding of specific Jak members which become rapidly activated and promote tyrosine phosphorylation of the receptor (Schindler, 1999). SH2-domain (Src homology domain)-containing STAT proteins are then recruited to the plasma membrane, where they are activated then released, homodimerized, and translocated to the nucleus where they can regulate gene expression.

Several chemokines have been reported to signal via the Jak/STAT pathway, including CCL2, CCL5, CCL3 and CXCL12, (Wong et al., 2001; Zhang et al., 2001; Mueller and Strange, 2004; Vila-Coro et al., 1999). Contrasting data suggests that CXCL12 signalling via CXCR4 is Jak independent (Muehlinghaus et

al., 2005). This, in turn, implies that the role of the Jak family members is still not as well established as previously thought.

Tec Kinases

TCR stimulation leads to the activation of a series of biochemical events resulting in the induction of gene expression in the activated cell. The initiating signals involve protein tyrosine kinases, such as members of the Src family, Syk/Zap-70 family, and Tec family. The Tec kinase family are non-receptor tyrosine kinases widely expressed in T cells and essential in T cell signalling. Five members have been described within the Tec family; Tec, Btk, Itk, Rlk and Bmx, of which only Tec, Itk and Rlk are expressed in T cells (Mano et al., 1993; Miyazato et al., 1996; Yang et al., 1999).

Over the last few years interest in Tec kinases has risen due to their role in T cell development, effector function and other processes such as regulation of actin adhesion (Berg et al., 2005). Tec kinases have also been observed to be activated downstream of chemokine receptors. Recruitment of Itk to the plasma membrane was demonstrated upon stimulation with CXCL12 (Fischer et al., 2004). These authors also showed impaired chemotaxis towards CXCL12 in Ikt deficient T cells.

1.16.5 Ras superfamily of small GTPases

Guanosine triphosphatases belong to the Ras superfamily of small GTPases and are downstream effectors of PI3K. Upon activation by a variety of extra-cellular stimuli, the members of this superfamily engage a wide range of multiple downstream effectors which control cellular processes such as re-arrangements of cytoskeleton and membrane involved in cell migration (Hawkins et al., 2006; Suire et al., 2006). The Ras superfamily is divided into 5 smaller families based on their sequence, similarity and functionality: namely Ras, Rho, Rab, Ran and Arf. These molecules are present in many signalling cascades within the cell and are known as cell switches.

1.16.6 Ras family and mitogen-activated protein kinases

The Ras Sarcoma oncoprotein family consists of four members; Rap, Ras, Ral and Rheb. They can be activated by GEFs (guanine-nucleotide-exchange factors),

which are divided into 3 main classes, namely SOS (Son of Sevenless), Ras-GRF (Ras guanyl releasing factor) and Ras-GRP (Ras guanyl releasing protein). The most studied and the best charcterised pathway within this family is the Ras GTPase pathway. Activation of this pathway leads to the subsequent activation of the MAPK signalling cascade.

The Mitogen-activated protein kinase (MAP kinase) family of serine/threonine kinases comprises of multiple members among which the best characterised are ERK (extracellular signal-regulated kinase), JNK (c-jun-NH_3-terminal kinase) and p38 MAPK. The activation of MAPK occurs via subsequent phosphorylation of the upstream kinases MAPK kinase kinase and MAPK kinase (Figure 1.29). There is a wide range of protein kinases involved in each step of this activation and each of them is differentially regulated by G-proteins, scaffold, adaptor, substrates and regulatory proteins. In resting cells MAPKs are localized in the cytoplasm and following activation they trigger phosphorylation of nuclear substrates including other protein kinases, regulators of cell cycle and transcription factors. Not surprisingly, MAP kinases are involved in multiple cellular processes such as proliferation and differentiation, development, inflammatory response and apoptosis (Weston and Davis, 2007).

ERK

Erk is the best characterised member of the MAPK family, activated downstream of receptor tyrosine kinases, GPCR, cytokine receptor and integrins. This signal initiates recruitment of the small GTPase Ras which, in turn, activates the serine/threonine kinase Raf (MAPKKK), followed by the phosphorylation of Erk1/2 by its MAPKK, MEK1 or MEK2. Phosphorylation of Erk triggers its homodimerization and translocation to the nucleus where further activation of Erk downstream effectors occur.

Various CC and CXC chemokines have been demonstrated to activate the Erk1/2 pathway (Boehme et al., 1999; Knall et al., 1997; Lopez-Ilasaca et al., 1998; Sotsios et al., 1999; Turner et al., 1998). Inhibition of Erk activation by blocking MEK activity abrogated CXCL12, CCL20 and eotaxin – induced actin polymerization and/or cell migration of T lymphocytes and eosinophils, respectively (Boehme et al., 1999; Sotsios et al., 1999; Turner et al., 1998). In

contrast, Erk1/2 activation appears not to be crucial for chemotaxis induced via all chemokine receptors, as it has been reported that CXCR3-mediated directional migration of T cells is insensitive to inhibition of MAPK pathway (Smit et al., 2003).

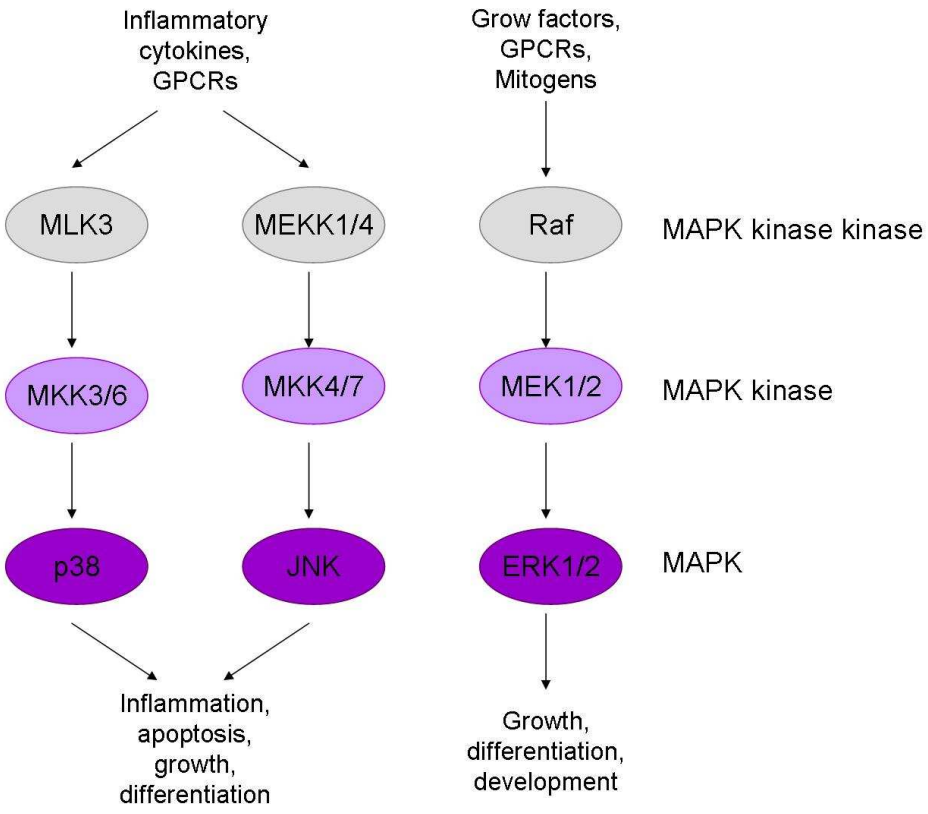


Figure 1.29 Activation of MAPK members by their upstream kinases.

1.17 Protein arginine methylation: a potential modification of signalling molecules

The main transmethylation pathway involves methyltransferase-induced donation of methyl a group by *S*-adenosylmethionine and its conversion to *S*-adenosyl-L-homocysteine (SAH), a potent feed-back inhibitor of methyltransferases (Yuan et al., 1999). This reaction is regulated by *S*-adenosyl-L-homocysteine hydrolase (SAHase), an enzyme that catalyzes the hydrolysis of SAH into adenosine and homocysteine.

Protein arginine methylation is a post-translational modification resulting in the transfer of a methyl group from S-adenosylmethionine by protein arginine methyltransferases (PRMTs). This can lead to modifications in which arginine residues are either mono- or dimethylated. The dimethyl arginines can occur either symmetrically (sDMAs) or asymmetrically (aDMAs) (Figure 1.30). (Aleta et al.,

1998; McBride and Silver, 2001). Two types of protein arginine methyltransferases have been described based on their final reaction product. Type I (PRMT1, PRMT3, CARM [PRMT4], PRMT6) and type II (PRMT5, PRMT7, PRMT 9). Both generate the MMA (monomethyl-arginine) as the intermediate product, but aDMAs are catalyzed specifically by type I PRMTs while type II PRMT activity leads to formation of sDMAs (Cook et al., 2006; Frankel et al., 2002; Mowen et al., 2004). Methylated arginine was detected on abundant proteins like histones, nuclealin, fibrillarin and heterogenous nuclear ribonucleoproteines (hnRNP) (Blanchet et al., 2005). It has been shown that protein arginine methylation regulates subcellular localization and modulates protein-protein interactions and is involved in many cellular processes such as RNA processing, transcriptional regulation, signal transduction and DNA repair (Bedford and Richard, 2005; Cheng et al., 2005; Shen et al., 1998). Recent evidence points towards a role for protein arginine methylation in T cell antigen signaling and cytokine gene activation (Blanchet et al., 2005; Mowen et al., 2004).

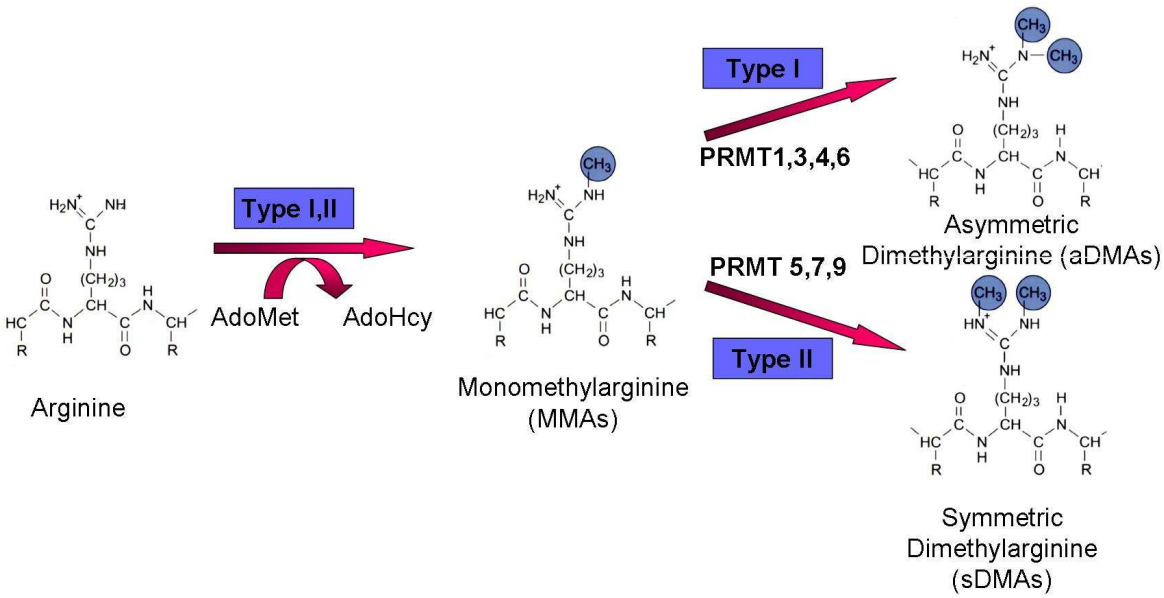


Figure 1.30 Schematic of PRMTs activity products

1.17.1 Protein arginine methylation in lymphocyte signaling

Two recent investigations present evidence of involvement of protein arginine methylation in T-cell activation, showing that TCR and CD28 signaling exploit this pathway (Blanchet et al., 2005; Mowen et al., 2004).

TCR signaling activates a cascade of post-translational events resulting in cytokine gene expression. Phosphorylation and ubiquitylation of specific proteins are known to be involved in this process. Results presented by Mowen *et al* provide evidence that arginine methylation is also involved in signalling via TCR and cytokine secretion. It has been shown that the NFAT co-activator NIP45 (NFAT Interactin Protein 45 kDa) is arginine methylated by PRMT, and this modification is required for NFAT/NIP45 interaction (Mowen et al., 2004). It was also demonstrated previously that methylation of STAT proteins regulates interaction with its inhibitor Protein Inhibitor of Activated STAT (PIAS1) thus plays role in cytokine signaling (Mowen et al., 2004).

Blanchet *et al* presented evidence in 2005 that CD28 'second signal' increases PRMT activity and arginine methylation of several proteins including two key effectors of CD28 signaling: interleukin-2 tyrosine kinase (Itk) and GDP-GTP exchange factor (GEF) for Rho-family GTPases, Vav-1. Vav-1 plays a critical role in T cell development and activation, and is required in TCR-induced events like calcium flux, activation of the Erk MAP kinase pathway and activation of NF-κB transcription factors. The importance of Vav-1 methylation remains unclear but the fact that methylated Vav-1 protein was found in the nucleus may be significant for its putative function in the nucleus (Tybulewicz, 2005).

It is well established that methylation of *Escherichia coli* chemotaxis receptors is crucial in allowing the organism to migrate towards chemoattractant gradients (Silverman and Simon, 1977; Parkinson et al., 2005). The role of protein methylation in mammalian lymphocyte migration is unexplored but there are some data which might suggest that this modification could be involved in chemotaxis of human T cells. The embryonic/perinatal lethality of PRMT1 and PRMT4 deficiency in mice may suggest that arginine methylation orchestrates directional cell migration during developmental processes (Pawlak et al., 2000; Yadav et al., 2003). Further evidence suggests that increases in prenylcysteine carboxyl methylation of Ras-related proteins correlate with their activation in neutrophils following chemoattractant treatment whilst inhibitors of prenylcysteine methylation abrogate chemotaxis of endotoxin-activated macrophages (Philips et al., 1993; Volker et al., 1991). It has also been demonstrated that methylation of transducin γ

subunit correlates with enhanced PLC and PI3K activation (Volker et al., 1991). In addition, selective antibodies specific for sDMA and aDMA recognise several cytoskeletal proteins (Boisvert et al., 2003).

1.18 Aims of the Study

Chemokine-directed migration of immune cells plays a significant role in immune surveillance and in disease. T lymphocytes are crucial players in cell-mediated adaptive immune defence. The chemokine receptor CXCR3 has been identified infiltrating effector T cells in a variety of inflammatory conditions including arteriosclerosis, rheumatoid arthritis, multiple sclerosis and psoriasis. Similarly CXCR3-binding ligands are highly expressed and secreted by cells within the same lesions. Therefore CXCR3 is a potential target for anti-inflammatory agents. There is a growing body of evidence for the existence of modified CXCR3 receptors, which may present a major limitation for developing CXCR3-specific inhibitors. Until now two alternatively spliced variants of the human CXCR3 receptor have been described, namely CXCR3-B and CXCR3-alt. These atypical CXCR3 receptors have not been completely investigated in terms of expression, ligand-induced signalling heterogeneity or functionality. Commercially available antibodies do not distinguish between CXCR3 and its variants and, as such, expression of CXCR3-B and CXCR3-alt at protein level, their internalization in response to chemokines, or signalling is still not fully characterised.

CXCR7 has been demonstrated to share ligands both with CXCR3 and the homeostatic receptor CXCR4. However, despite binding CXCL12 and CXCL11 with high affinity, its role in typical chemokine receptor signalling and functional responses is controversial, with a number of conflicting reports published.

The aims of the first part of this project were as follows:

- To characterise agonist-induced responses mediated via CXCR3 and its spliced variants CXCR3-B and CXCR3-alt in T lymphocytes and in transfected cell lines.
- To investigate the role of CXCR7 in T cell signalling and examine agonist-induced responses in CXCR7-expressing transfectants.

Hence, the following hypotheses were proposed:

- CXCL4, similarly to CXCR3, is involved in T lymphocyte signalling and functional responses such as chemotactic activity. Moreover CXCR3-B and CXCR3-alt localize to the plasma membrane and are responsive to CXCL11, and, in case of CXCR3-B, CXCL4.
- CXCR7 is involved in T lymphocyte signalling and chemotaxis. CXCL11 and CXCL12 induce down-regulation and biochemical signalling in CXCR7 transfectants.

The following objectives were established in order to test these hypotheses:

- Examine the expression, chemotaxis and signalling mediated by CXCR3 and CXCR3-B in human T lymphocytes.
- Investigate the mechanisms of CXCL11-induced down-regulation of surface CXCR3.
- Utilize small-molecule CXCR3 antagonists to explore the role of CXCR3 in human T cells.
- Analyze CXCL4-mediated responses in T cells to address the role of CXCR3-B.
- Construct hCXCR3-A (full length), CXCR3-B and CXCR3-alt receptors tagged on their C terminus with the EGFP protein (Enhanced Green Fluorescent Protein) in a mammalian expression vector. Expression of these DNA constructs in mammalian cells to compare internalization and biochemical signalling of CXCR3-A versus CXCR3-B and CXCR3-alt after ligand binding.
- Verify if currently available anti-human CXCR3 antibodies are able to bind to spliced variants of the CXCR3 receptor.
- Investigate the expression of endogenous CXCR7 in human T lymphocytes.
- Examine the involvement of CXCR7 on CXCL11- and CXCL12-mediated signalling and chemotaxis.
- Analyze expression and agonists-induced internalization of surface CXCR7 in transfected cells.
- Examine signalling via CXCR7 in transfected cells.

As discussed in this introduction, there is a growing body of evidence that methylation is involved in lymphocyte function. Hence, the aim of the second part of the study was to characterise the role of protein arginine methylation in T cell signalling and migration. In order to test the hypothesis that protein arginine methylation is up-regulated following stimulation of T lymphocytes, and that this is important in chemokine-induced directional migration of T cells, the following objectives were proposed:

- Examine the effect of T cell stimulation on levels of protein arginine methylation.
- Investigate the effect of methyltransferase inhibitors on *in vitro* migration of activated T lymphocytes.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Antibodies and secondary reagents

Flow cytometry

1. Fluorescein Isothiocyanate (FITC)-conjugated mouse anti-human CD3 antibody (isotype IgG1κ, clone WT31) (BD Biosciences, Oxford, UK).
2. FITC-conjugated mouse anti-human CD4 (isotype IgG1κ, clone 11830) (BD Biosciences, Oxford, UK).
3. FITC-conjugated mouse anti-human CD8 (isotype IgG2B, clone 37006) (R&D Systems, Abingdon, UK).
4. Phycoerythrin (PE) / Allophycocyanin (APC) / FITC-conjugated mouse monoclonal anti-human CXCR3 (isotype IgG1, clone 49801) all at concentration of 25 μg/mL (R&D Systems, Abingdon, UK).
5. PE-conjugated mouse monoclonal anti-human RDC1/CXCR7 antibody (clone 358426) (R&D Systems, Abingdon, UK).
6. PE / APC / FITC-conjugated mouse IgG1 (R&D Systems, Abingdon, UK).
7. PE-conjugated mouse IgG2A (R&D Systems, Abingdon, UK).
8. FITC-conjugated mouse IgG2B (R&D Systems, Abingdon, UK).
9. FITC-conjugated mouse IgG1κ isotype control (BD Biosciences, Oxford, UK).
10. Rabbit IgG control (Sigma-Aldrich, Poole, UK).
11. FITC conjugated anti-Rabbit IgG (whole molecule) antibody produced in sheep (Sigma-Aldrich, Poole, UK).

Immunoblotting

1. Polyclonal anti- phospho-S6 Ribosomal Protein (Ser235/236) Antibody (cat. no. 2211) produced in rabbit (Cell Signaling Technology, (NEB), Hitchin, UK).
2. Polyclonal anti- phospho-p44/42 Map Kinase (Thr202/Tyr204) Antibody produced in rabbit (Cell Signaling Technology, (NEB), Hitchin, UK).

3. Polyclonal anti- phospho-Akt (Ser473) Antibody produced in rabbit (Cell Signaling Technology, (NEB), Hitchin, UK).
4. Polyclonal anti- phospho-GSK3β (Ser9) Antibody produced in rabbit (Cell Signaling Technology, (NEB), Hitchin, UK).
5. Akt1 (C-20) antibody (Santa Cruz, (Insight Biotechnology), Wembley, UK).
6. Erk1 (C-16) antibody (Santa Cruz, (Insight Biotechnology), Wembley, UK).
7. Mouse monoclonal anti-human CXCR3 antibody clone 49801 (R&D Systems, Abingdon, UK).
8. Polyclonal goat anti-rabbit immunoglobulins (Dako, UK Ltd, Cambridge).
9. Polyclonal rabbit anti-goat immunoglobulins (Dako, UK Ltd, Cambridge).
10. Polyclonal rabbit anti-mouse immunoglobulins (Dako, UK Ltd, Cambridge).
11. Monoclonal mouse anti-mono- and dimethyl arginine antibody (clone 7E6), (Abcam, Cambridge, UK).
12. Monoclonal mouse anti-monomethyl arginine antibody (clone 5D1), (Abcam, Cambridge, UK).
13. Polyclonal rabbit anti-dimethyl-Arginine, asymmetric (Asym 24) antibody, (Upstate (Milipore), Watford, UK).
14. Polyclonal rabbit Anti-dimethyl-Arginine, symmetric (SYM11) antibody, (Upstate (Milipore), Watford, UK).

2.1.2 Bacteriology

1. 90mm bacteriological petri dishes (Sterilin, Caerphilly, UK)
2. Ampicillin (Sigma-Aldrich, Poole, UK)
3. *Escherichia coli* DH5α competent cells (Invitrogen, Paisley, UK)
4. One Shot® TOP10 competent *Escherichia coli* (Invitrogen, Paisley, UK)
5. Kanamycin (Sigma-Aldrich, Poole, UK)
6. Difco LB (Luria Bertani) Broth Miller medium and Difco LB (Luria Bertani) Agar Miller medium (BD Biosciences, Oxford, UK)
7. S.O.C. medium (Invitrogen, Paisley, UK)

2.1.3 Buffers and Solutions

1. *Etotal buffer* (147 mM NaCl, 2 mM KCl, 10 mM HEPES, 12 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.3 with NaOH, MQ water)

2. *FACS buffer* (5% of FBS in PBS, optional 0.1% of sodium azide)
3. *MACS buffer* (0.5% FBS, 2 mM EDTA pH 7.2 in PBS)
4. *SDS-PAGE 4 X resolving buffer* (1.5 M Trizma base pH 8.8, 0.4% (w/v) SDS, MQ water)
5. *SDS-PAGE running buffer* (25mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS)
6. *SDS-PAGE 5 X sample buffer* (10% SDS, 50% glycerol, 200mM Tris HCL pH 6.8, Bromophenol blue, 5% 2-Mercaptoethanol)
7. *SDS-PAGE 4 X stacking buffer* (0.5 M Trizma base pH 6.8, 0.4% (w/v) SDS, MQ water)
8. *Semi-dry transfer buffer* (39mM glycine, 48nM Tris-HCl, 0.0375% SDS, 20% (v/v) methanol)
9. *Solubilization buffer* (1% (v/v Nonidet P-40, 150 nM NaCl, 50 mM Tris pH 7.5, 5mM EDTA, 10 mM sodium fluoride*, 1 mM sodium molybdate*, 1 mM sodium orthovanadate*, 1 mM phenylmethylsufonyl fluoride*, 10 μg/mL leupeptin*, 10 μg/mL aprotinin,1 μg/mL soybean trypsin inhibitor*, 1 μg/mL pepstatin A*, MQ water)
10. *Stripping buffer 5x concentrate* (100mM 2-Mercaptoethanol, 2% SDS, 62.5nM Tris-HCl pH 6.7)
11. *TAE Buffer (Tris-Acetate-EDTA) 50x concentrate* (242g Tris base, 57.1ml Acetic acid, 100ml 0.5M EDTA, dH_2O to 1 litre, pH 8.5)
12. *TBS buffer (Tris-buffered saline) 10x concentrate* (20mM Tris-HCl, 150mM NaCl, pH 7.5)

* *denotes added on the day of use*

2.1.4 Cell culture and T cells isolation and expansion

Cell lines:

Chinese Hamster Ovary Cells (CHOK1)

Human Embryonic Kidney Cells (HEK-293)

Jurkat T Cell line

1. 10 mL plastic pipettes (Greiner Bio-One, UK)

2. 25 mL plastic pipettes (Greiner Bio-One, UK)
3. 3 mL sterile Pasteur-Plast pipettes (Fisher Scientific, UK).
4. 6 well tissue culture dishes (Nunc, UK)
5. 75 and 175 cm^3 tissue culture flasks (Nunc TM, UK)
6. 50 mL transparent polypropylene centrifuge tubes (Greiner Bio One, UK)
7. Cryotubes (Nunc, UK)
8. Sterile plastic bijoux and universal containers (Greiner Bio One, UK)
9. Accutase (Innovative Cell Technologies)
10. 0.25% Trypsin-EDTA (Gibco®/Invitrogen, Paisley, UK)
11. 200mM L-glutamine (Gibco®/Invitrogen, Paisley, UK)
12. Lipofectamine™ 2000 (Invitrogen, Paisley, UK)
13. Lymphoprep (Ficoll-paque1.077 g/mL density) (Axis-Shield, Cambridgeshire, UK)
14. *Trans*IT®-LT1 Transfection Reagent (Mirus Bio, UK)
15. Mowiol (Calbiochem, Merk Chemicals, (VWR), Leicestershire, UK, UK)
16. Non-Essential Amino Acids Solution 10 mM (100X) (Gibco®/Invitrogen, Paisley, UK)
17. Phosphate buffer saline (PBS) (Gibco®/Invitrogen, UK)
18. Poly-L-lysine 0.01%, mol wt 70,000-150,000, sterile-filtered (Sigma-Aldrich, Poole, UK)
19. RPMI-1640 and DMEM tissue culture medium (Gibco®/Invitrogen, Paisley, UK)
20. Heat inactivated Foetal Bovine Serum (FBS) (Gibco®/Invitrogen, Paisley, UK)
21. Streptomycin (50 mg/mL) and Penicillin (50 U/mL) solution (Gibco®/Invitrogen, Paisley, UK)
22. Heparin (500 U/mL in H_2O) (Sigma-Aldrich, Gillingham, UK)
23. Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, Gillingham, UK)
24. Trypan Blue (Gibco®/Invitrogen, Paisley, UK)
25. Phytohemagglutinin (PHA) (Sigma-Aldrich, Gilingham, UK)
26. Dynabeads® CD3/CD28 T cell expander (Invitrogen, Dynal AS, Oslo, Norway)
27. Interleukin -2 (IL-2) (Peprotech, London, UK)
28. Human T lymphocyte isolation kits: pan-T cell isolation kit, $CD8^+$ T cell isolation kit II, $CD4^+$ T cell isolation kit II (Miltenyi Biotec GmbH)
29. 75 and 175 cm^3 tissue culture flasks (Nunc TM, UK)

30. 50 mL transparent polypropylene centrifuge tubes (Greiner Bio One, UK)
31. MACS magnetic cell separator or Dynal magnetic particle concentrator

2.1.5 Chemicals

1. 30% Acrylamide/Bis solution, 37.5:1 (Bio-Rad, Hemel Hempsted, UK)
2. Agarose (electrophoresis grade) (Sigma-Aldrich, Poole, UK)
3. Ammonium persulfate for electrophoresis >=98% (Sigma-Aldrich, Poole, UK)
4. Aprotinin (Sigma-Aldrich, Poole, UK)
5. Bromophenol blue (Sigma-Aldrich, Poole, UK)
6. Chloroform (Fisher Scientific, UK)
7. ECL (Amersham Bioscience, Little Chalfont, UK)
8. Enhanced Chemiluminescent reagent (ECL) (Amersham Biosciences, UK)
9. Ethanol (Fisher Scientific, UK)
10. Ethidium bromide (BioRad, Hemel Hempsted , UK)
11. Glycine for electrophoresis ≥99% (Sigma-Aldrich, Poole, UK)
12. Glycerol (Sigma-Aldrich, Poole, UK)
13. Leupeptin (Sigma-Aldrich, Poole, UK)
14. 2-Mercaptoethanol, >=99% (Sigma-Aldrich, Poole, UK)
15. Methanol (Fisher Scientific, UK)
16. Methylthioadenosine (MTA), (Sigma-Aldrich, Poole, UK)
17. Nonidet P40 (BDH, (VWR), Leicestershire, UK)
18. Pepstatin (Sigma-Aldrich, Poole, UK)
19. Pertussis toxin (Calbiochem, Merk Chemicals, (VWR), Leicestershire, UK)
20. Phenylmethylsulphonyl fluoride, (Sigma-Aldrich, Poole, UK)
21. Propan-2-ol (isopropyl alcohol) (Fisher Scientific, UK)
22. Protein Assay Dye reagent concentrate (Bio-Rad, Hemel Hempsted, UK)
23. Soybe trypsin inhibitor (Sigma-Aldrich, Poole, UK)
24. Sodium azide (BDH, (VWR), Leicestershire UK)
25. Sodium chloride, ACS reagent, >=99.0% (Sigma-Aldrich, Poole, UK)
26. Sodium fluoride (BDH, (VWR), Leicestershire, UK)
27. Sodium molybdate (BDH, (VWR), Leicestershire, UK)
28. Sodium vanadate (BDH, (VWR), Leicestershire, UK)
29. TEMED for electrophoresis, approx. 99% (Sigma-Aldrich, Poole, UK)
30. Tris-base (Sigma-Aldrich, Poole, UK)

31. Triton X (Sigma-Aldrich, Poole, UK)
32. TRIzol® reagent (Invitrogen, Paisley, UK)
33. Tween 20 (Sigma-Aldrich, Poole, UK)

2.1.6 Chemokines

1. Human recombinant chemokines CXCL4, CXCL9, CXCL10, CXCL11 and CXCL12 (Peprotech, London, UK)
2. Biotinylated chemokines CXCL10 and CXCL11 were chemically synthesised and then refolded (Almac, Elphinstone, UK)

2.1.7 Assay systems, kits and molecular biology reagents

1. DNA 1kb Ladder (New England BioLabs, UK; Promega, Southampton, UK, UK)
2. Easy-A High Fidelity PCR Master Mix (2x, 0.1 U/μl) (Stratagene, Cheshire, UK)
3. 6 x gel loading buffer (Sigma-Aldrich, Poole, UK)
4. TransIT-LT1 –lipid based transfection reagent (Mirus, (Cambridge Bioscience), UK)
5. Oligo (dT) (Promega, Southampton, UK)
6. Oligonucleotides designed to binds specifically to the gene of interest (synthesized by Sigma-Aldrich, Poole, UK or Invitrogen, Paisley, UK)
7. Omniscript RT kit containing 10 x buffers, 5 mM dNTP mix, Omniscript RT (Qiagen, Crawley, UK)
8. Plasmid purification Mini and Maxi Prep kits (Qiagen, Crawley, UK)
9. Pre-Aliquoted ReddyMix PCR Master Mix (ABgene, Epsom, UK)
10. QIAQuick Gel Extraction Kit and QIAQuick PCR purification Kit (Qiagen, Crawley, UK)
11. Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Hemel Hempsted, UK)
12. Rapid DNA Ligation Kit (Roche, Hertfordshire, UK)
13. RNAse inhibitor, RNAsin Plus (Promega, Southampton, UK)
14. Restriction enzymes: HindIII, KpnI, NotI, AgeI/PinAI (New England BioLabs, UK)
15. Strips of 8 Thermo Tubes (ABgene, Epsom, UK)
16. 0.5 mL Thermo Tubes (Sigma-Aldrich, Poole, UK)

17. Topo TA Cloning System (Invitrogen, Paisley, UK)

2.1.8 Plasmids used for Molecular Cloning

pCR®2.1-TOPO® 3.7kb (Invitrogen, Paisley, UK)

pEGFP 3.4 kb vector encoding EGFP (Clontech, UK)

pcDNA 3 5.4 kb expression vector (Invitrogen, Paisley, UK)

pIRES neo 5.2 kb exression vector (Clontech, UK)

2.1.9 Primers

With the exception of the T7 and PS6 oligonucleotides (purchased from Promega, Southampton, UK), all primers were supplied by Sigma.

NO	OLIGO NAME	SEQUENCE 5' TO 3'
1.	CXCR3-A /CXCR3-alt (*hCXCR3 PRI2-S*)	CCAAGTGCTAAATGACGCCG
2.	CXCR3-A (*hCXCR3 PRI3-A*)	CAAAGGCCACCACGACCACCACCA
3.	CXCR3-alt * (*hCXCR3 PRI4-A*)	CTCCCGGAACTTGACCCCTGTG
4.	CXCR3-B	ATGGAGTTGAGGAAGTACGGCCCTGGAAG
5.	CXCR3-A/B	AAGTTGATGTTGAAGAGGGCACCT GCCAC
6.	hCXCR3-A-alt H3+Kozak	TAGTAGAAGCTTCCGCCACCATGGTCCTTGAG GTGAGTGACCACC
7.	hCXCR3-B H3+Kozak	TAGTAGAAGCTTCCGCCACCATGGAGTTGAG GAAGTACGGCCCTGGAAG
8.	hCXCR3-A–B stop Kpn1	TAGTAGGGTACCGCCAAGCCCGAGTAGGAGG CCTCTGAGGTC
9.	hCXCR3-alt stop Kpn1	TAGTAGGGTACCGCGACTGTGGGCGAAAGGG GAGCCCGGATTC
10.	hCXCR3-A/B stop Not1	TAGTAGGCGGCCGCTCACAAGCCCGAGTAGG AGGCCTCTGAGGTC
11.	hCXCR3-alt stop Not1	TAGTAGGCGGCCGCTCAGACTGTGGGCGAAA GGGGAGCCCGGATTC
12.	hCXCR7 -S	CCAGGGAACTTCTCGGACATCAGC
13.	hCXCR7 -A	TGTGCTCGGGGTAGAAGGACCGGC
14.	M13R	CAGGAAACAGCTATGAC
15.	T7 promoter primer	TAATACGACTCACTATAGGG
16	BGH	TAGAAGGCACAGTCGAGG
17.	PS6	ATTTAGGTGACACTATAG

18.	EGFP-seq1	CACTTTATGCTTCCGGCTCGTATG
19.	EGFP-seq2	TGCAGCGGCAGGTCGAGCTGGTCC
20.	EGFP-seq3	CAGCTTGCCGTAGGTGGCATCGCC

Table 2.1 Sequences of oligonucleotides used in the study.

*primer bind within positions 692-695 and 1039-1049.

2.2 Methods

2.2.1 Cell culture and cell culture conditions

Cell culture was performed using aseptic techniques in a Medical Air Technology LTD Class II Microbiological Cabinet laminar flow hood. All cell types used in the present study were cultured in the humidified, 37°C, 5% (v/v) CO_2 incubator.

2.2.2 Chinese Hamster Ovary Cells

Chinese Hamster Ovary (CHO) cells were maintained in 175cm^2 tissue culture flasks in DMEM medium supplemented with 10% foetal bovine serum (FBS), 1% NeAA (Non essential Amino Acids), 1% L-glutamine and 50 U/mL of penicillin and 50 μg/mL of streptomycin. Cultures were monitored for density, and maintained until achieving confluence up to about 95%. At that point used culture medium wash removed and cells were washed once in 30 mL of PBS, followed by trypsinisation for 3-5 mins at 37°C with 5 mL of Trypsin:EDTA or 5mins 37°C with 5 mL of Accutase. Cells were then vigorously dislodged from flask, and reaction was stopped by addition 25 mL of growth DMEM medium, ensuring complete mixing with detached cells. Fresh flasks were seeded with about 3 mLs of stock cells and 40 mL of growth medium. CHO cells were maintained until 35 passages. For seeding a day before transfection, detached cells were counted, diluted to 6x 10^5 per mL in growth medium and 2 mL of cell suspension was placed per well in 6-well plate.

2.2.3 Human Embryonic Kidney Cells

Human Embryonic Kidney (HEK293) cells were maintained in 175cm^2 tissue culture flasks in RPMI-1640 medium supplemented with 10% of FBS, and 50 U/mL

of penicillin and 50 μg/mL of streptomycin. Cultures were monitored for density, and maintained until achieving confluence up to about 95%. Cell were then harvested and seeded as described previously in 2.2.2.

2.2.4 Freshly Isolated and Activated T Lymphocytes

Routinely in the present study PBMCs were isolated from human blood and activated *in vitro* using bacterial superantigens or lectins, and cultured up to 12 days. Alternatively freshly isolated pan T cells were obtained from PBMCs, used on the day of isolation or cultured *in vitro* in the presence of antibodies. Activated T lymphocytes were utilized in experimental procedures between 9-12 days post-isolation and activation.

2.2.5 Isolation of PBMCs by gradient centrifugation

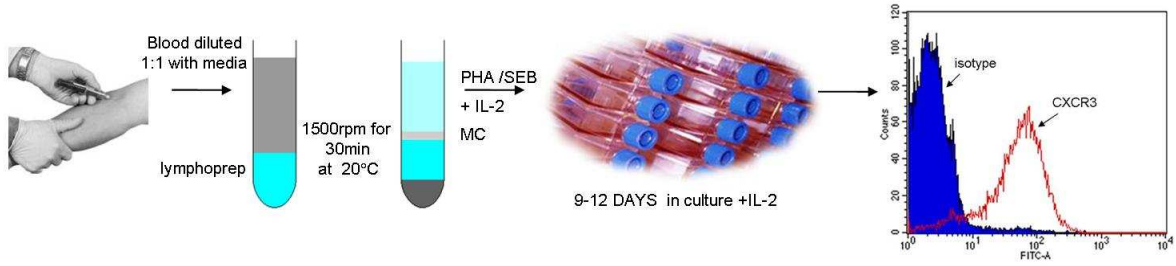
T cell isolation from freshly donated human peripheral blood and their *ex vivo* expansion provides a useful protocol for studying biochemical and functional events in T lymphocytes. After separation from peripheral blood, the mononuclear cells (a mixure of monocytes and lymphocytes) are activated and kept in culture up to 12 days under conditions which promote T lymphocyte proliferation, activation and up-regulation of CXCR3.

Whole blood donated by healthy human volunteers* was collected in heparinized syringe (500 U per 50ml of blood), diluted 1:1 in a sterile175 cm^3 tissue culture flask with RPMI- 1640 medium and mixed gently. 35ml of blood/medium mix was carefully overlaid on 15mL of Lymphoprep in 50mL transparent conical centrifuge tubes (e.g. Falcon tubes) and centrifuged at 400g at 20^oC with the brake off for 30 minutes. Following centrifugation, the PBMCs fraction containing lymphocytes and monocytes, seen as a 'milky' layer on a top of higher density Lymphoprep (Figure 2.1) was carefully removed and transfer to fresh 50mL tube.

Removed cells (representing PBMCs) ware washed three times in 50 mL of RPMI-1640 medium and resuspended in a volume of RPMI-1640 containing 10% FBS and 50 U/mL of penicillin and 50 μg/mL of streptomycin equivalent to the volume of blood from which they were isolated.

* Procedures for the use of human blood was carried out under University and Departmental safety and ethical guidelines for the use of human tissue.

A



B

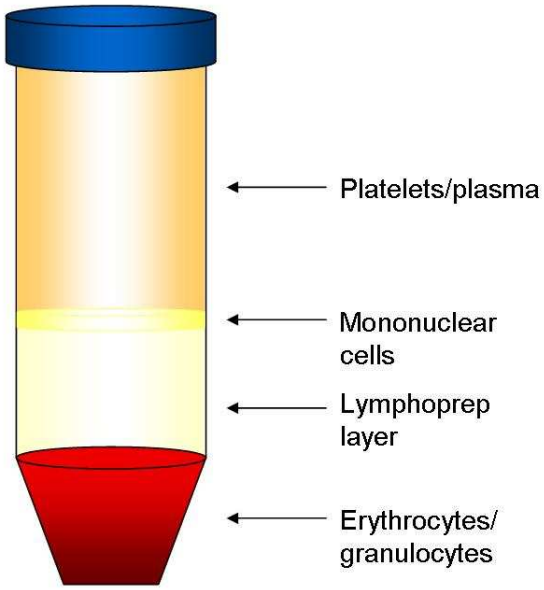


Figure 2.1 Isolation and in vitro expansion of PBMC. Steps of isolation and culture of peripheral blood-derived human T lymphocytes, (A). Schematic illustration of PBMC separation with Lymphoprep after centrifugatio, (B). The diagram is representative of the separated cell layers observed after centrifugation with Lymphoprep.

2.2.6 Isolation and purification of Pan T cells

An alternative model of the activated T cell system was the utilization of purified, freshly isolated T cells. These cells were obtained from PBMCs fraction using Pan T cell Isolation Kit. This is an indirect magnetic labelling system, in which unbound cells pass through the magnetic column (negative selection) and can be utilized within experimental procedures. The protocol was followed as per manufacturer instructions. Briefly, PBMCs were isolated as described in section 2.2.5, washed three times in MACS buffer, resuspended in 40 μL of buffer with 10 μL of specific

Biotin-Antibody Cocktail (biotin-conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A) per 10^7 total cells and incubated at 4°C for 10 minutes. 30 μL of buffer and 20 μL of Anti-Biotin-Micro Beads per 10^7 total cells were added and incubated for a further 15 minutes. Cell were then washed once in 10 mL of MACS buffer and resuspended in 500 μL of the same buffer. Cell suspension was then applied to the LS MACS Separator column and column was washed three times with 3 mL of buffer. The eluted, enriched T cell fraction was collected, washed in RPMI-1640 medium and resuspended in FACS buffer for use in surface receptor expression study or resuspended in complete RPMI-1640 and activated as described below.

2.2.7 Ex-vivo activation and clonal expansion of T lymphocytes

Ex-vivo activation of T cells and subsequent expansion mimics processes of *in vivo* activation of T cells in response to antigen presentations. Described here are the methods of *in vitro* activation used within presented study.

One of the most commonly used activators of human T cells is PHA, a lectin isolated from plants that acts as a mitogen which induces activation of T cells by cross linking to glycoproteins on the cell surface. PHA will yield activated T lymphocytes that are predominantly $CD8^+$. Alternatively, SEB is one of the best known superantigens, which function by binding to MHC class II molecules expressed on the surface of professional antigen presenting cells (APC) present within the PBMC population. SEB acts then as cross linker subsequently binding to α chain of T cell receptor (TCR), stimulating robust activation of T lymphocytes. SEB will yield activated T lymphocytes that are predominantly $CD4^+$.

Isolated PBMCs were stimulated with either 5 μg/mL of PHA with addition of 20 ng/mL of IL-2 or 1 μg/mL SEB. Three days post isolation, the non-adherent cells were removed, washed once in RPMI-1640 media (50 mL) and re-suspended in complete RPMI-1640 complete medium (containing 10% FBS, 1% Penicillin-Streptomycin solution) and supplemented with IL-2 (20 ng/mL). T lymphocytes were then kept in culture up to 12 days, fresh medium was added and cells were supplemented with 20 ng/mL of IL-2 every 2-3 days.

For the rapid expansion of freshly isolated T cells anti-CD3/CD28 mAb-coated Dynabeads were used (approximately 1 bead per T cell). This method of

stimulation more accurately mimics the *in vivo* presentation of antigen to T cells, but avoids engagement/activation of the co-inhibitory receptors such as CTLA-4 (34). Furthermore this method does not require large numbers of autologous/ MHC matched APC and antigen or allogenic mononuclear cells and mitogen. The T lymphocyte population should first be purified by negative selection using pan-T cell isolation kits (as described in section 2.2.6). T lymphocytes are not contaminated with monocyte/macrophages and the antibody-coated beads are simply removed using a magnet. T lymphocytes can be sustained and expanded in culture for several weeks.

2.2.8 Cell count

To prepare an appropriate concentrations of cell suspension, cells were counted using haemocytometer. Briefly, 20 μL of well mixed cell suspension was transferred in to the eppendorf tube and mixed with 20 μL of trypan blue. 10 μL of the mixture was then carefully transferred to one side of a haemocytometer and the number of cells in two opposite corner squares was counted under light microscope. Cell which appeared as non-viable (stained blue with trypan) were excluded. The cell concentration was calculated using the following formula:

$$\text{Cells/mL} = (\text{Average cell count per square}) \times \text{dilution factor} \times 10^4$$

2.2.9 Freezing/thawing of cells

For storage, 5-10 x 10^6 cells/mL in exponential growth were resuspended in freeze medium containing 10% dimethylsulphoxide (DMSO), and 90% of foetal bovine serum. The cell suspension was transferred to cryotubes (1mL/tube) and placed into freezing down container filled with 250 mL of propan-2-ol. The container was placed into -80°C over night and cells were cooled down at 1°C / minute before being transferred to liquid nitrogen for long-term storage. For resuscitation of cells of cells from liquid nitrogen, cells were rapidly defrosted for one minute in a 37°C water bath, washed once in RPMI or other appropriate medium and resuspended in 20 mLs of complete medium and cultured as stated previously.

2.2.10 Transfection of CHOK1 cells

24 hours before transfection cells were seeded into CHOK1 culture medium (see 2.2.2 for details) at $6x10^5$ per mL. 2 mL of cell suspension loaded into each well in 6-well plate. On the day of transfection the cell confluency was >95%. 4 μg of DNA was diluted into 250 μL of serum free DMEM medium in polycarbonate tube. 12.5 μL of Lipofectamine 2000 was diluted into 250 μL of DMEM as before. Both dilutions were combined immediately and incubated for 20 minutes at room temperature to establish DNA-Lipofectamine complexes. During incubation time, cells were washed once in DMEM medium which was aspirated off before adding complexes. 0.5 mL of DMEM was added to 0.5 mL of DNA-Lipofectamine solutions and cells were carefully overlaid with 1 mL per well of mixture. Complexes were aspirated off after 5 hours incubation at 37°C and replaced with 3 mL of CHO growth medium. Cells were analysed 48 hours post-transfection.

2.2.11 Transfection of HEK293 cells

24 hours before transfection HEK293 cells were seeded into RPMI-1640 culture medium (see 2.2.3 for details) at $6x10^5$ per mL. 2 mL of cell suspension loaded into each well in 6-well plate for confluency of >95% on the day of transfection. 2 μg of DNA was diluted into 250 μL of RPMI-1640 medium followed by addition of 4 μL of *Trans*IT®-LT1 transfection reagent. Obtained mixture was then incubated for 20 minutes at room temperature to establish complexes between DNA and lipid-based transfection reagent. After incubation, 250 μL solutions were added drop wise to each well containing cells prepared day before kept in culture medium. Cells were analysed 48 hours post-transfection.

2.2.12 Molecular biology

2.2.12.1 Nucleic acid preparation

RNA extraction

The commercially available anti-CXCR3 antibodies are unable to distinguish between CXCR3-A, CXCR3-B or CXCR3-alt, while reported CXCR3-B antibodies

are either not widely available or have limited specificity. In addition, there are no reported antibodies to CXCR3-alt. So, the most accurate way of assessing expression of CXCR3 isoforms in human T lymphocytes is to monitor the mRNA expression for the individual forms of CXCR3 as described below.

RNA extraction using TRIzol reagent was performed according to manufacturer instructions, briefly. Approximately 9 days post-isolation and initial activation, human T lymphocytes (5-10x10^6 cells) were removed from culture, pelleted and lysed in 1ml of TRIzol reagent and incubated for 5 minutes at room temperature. This step allowed to complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform is added per 1 mL of TRIzol used and samples are agitated by hand for 15 seconds and incubated for 2-3 minutes at room temperature followed by centrifugation for 15 minutes at 4°C and at no more than 12,000g. After centrifugation, the mixture separated into different phases: lower red phenol-chloroform phase, an interphase and upper aqueous phase containing RNA. The aqueous phase was carefully collected from each sample and transferred to a fresh eppendorf tube and RNA was precipitated by mixing with 0.5 mL of isopropyl alcohol. Samples were then incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 4°C at no more than 12,000g. Precipitated RNA was seen as gel-like pellet on the side and the bottom of the tube. Supernatants were carefully discarded and RNA was washed in at least 1 mL of 75% ethanol. Samples were mixed by vortexing and centrifuged for 5 minutes at 4°C at no more than 7,500g. Washed RNA pellets were briefly dried by air or vacuum dry for approximately 10 minutes and dissolved in RNase free water of 0.5% SDS solution by passing a few times through a 1 mL pipette tip and incubation for 10 minutes at 55-60°C. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in spectrophotometer. RNA dilutions (e.g. 1:50) were prepared in RNAse free water. The same water in which the RNA was diluted was used to calibrate the spectrophotometer. Purity of RNA was estimated by the ratio of the readings at 260 nm and 280 nm (A260/A280). Partially dissolved RNA solutions have an A260/280<1.6, which exclude contamination with contaminants such as protein and phenol.

Plasmid DNA preparation (Mini/Maxipreps)

Single bacterial colonies were carefully picked from agar Petri dish and incubated over night with shaking at 37°C in 5 mL of LB medium containing 50 μg/mL of ampicillin or 50 μg/mL of kanamycin. Next day cultures were pelleted by centrifugation (10 min, 6000g) and DNA was purified using QIAGEN Mini Kit according to the manufacturer's instructions. DNA from larger volumes of over night cultures (100-200 mL) was purified using Qiaquick QIAGEN Maxiprep Kit following manufacturer protocol.

Synthesis of cDNA from RNA

The cDNA was prepared by reverse transcription of cellular RNA using Oligo (dT) as the primers (binding to poly (A) tail at the 3' end of messenger RNA) and Omniscript RT kit, according to manufacturer protocol.

Briefly, approximately 1μg of each RNA sample was mixed with 0.5 μL of Oligo(dT) and 0.5 μL RNAsin Plus and incubated for 5 minutes at 65°C followed by incubation on ice ~ 1 min. This step helps opening secondary structures of RNA and binding primers. The rest of reagents including 1 μL of Reverse Transcriptase (RT) enzyme, 2 μL of 10x buffer and 2 μL of 5 mM dNTP mix was added and incubated for 60' at 40°C and 10' at 70°C.

The RT-PCR Reaction mix was as follow: 10x buffer (2 μL), 5 mM dNTP mix (2μl), oligo (dT) (0.5 μL), RNAsin Plus (0.5μL), Omniscript RT (1 μL, 4 units per reaction), 50 ng-2 μg RNA template (1 μg), dH_2O (up to 20 μL volume).

2.2.12.2 Polymerase chair reaction (PCR)

PCR for most applications was performed using Easy-A High Fidelity PCR Master Mix (Stratagene, Cheshire, UK). Alternatively for performing PCR using bacterial colonies as a template, Pre-Aliquoted ReddyMix PCR Master Mix (ABgene) was used. Reaction mix for PCR was as follows: 2x Easy-A High Fidelity PCR Master Mix (12.5 μL), 10μM 5' primer (2 μL), 10μM 3' primer (2 μL), approximately 1 μg cDNA and distilled water (up to 25 μL volume) or alternatively Pre-Aliquoted ReddyMix PCR Master Mix (45 μL), 5' and 3' primers (2 μL each), single bacterial

colony collected from Petri dish. All reactions were mixed well by pipetting before being placed in a thermocycler.
Appropriate thermocycler programs were used according to the application and are detailed below:

Amplification of cDNA and expression constructs: Initial denaturation: 94°C 5 mins, (denaturation: 94°C 1 min, annealing: 60°C 30 sec, extension: 72°C 1 min) x 35, final extension: 72°C 10 min, final hold: 4°C;

Amplification of full length CXCR3: 94°C 5 mins, (94°C 1 min, 60°C 30 sec, 72°C 2 min) x 35, 72°C 10 min, 4°C;

Colony PCR: 94°C 10 mins, (94°C 1 min, 50°C 30 sec, 72°C 1 min) x 35, 72°C 10 mins, 4°C.

PCR products were then analysed by agarose gel electrophoresis as described below. If required, PCR products were purified as stated in 2.2.1ed2.5.

2.2.12.3 Agarose gel electrophoresis

1.2% of agarose was prepared in 100 ml of 1xTAE buffer and melted by heating in a microwave for 2 minutes, followed by gentle mixing until completely dissolved. 2 μL of ethidium bromide (10 mg/mL) was added to 100 mL of cooled agarose solution, mixed and slowly poured in an appropriate tank. Required combs were placed into the gel and any air bubbles were removed. Gel was left to set for 30-60 minutes. 1x TAE buffer (running buffer) was added to an appropriate gel running tank. The gel was then placed in the tank and the comb removed. PCR products were mixed with 6 x gel loading buffer (not if Pre-Aliquoted ReddyMix PCR Master Mix was used with loading buffer already included) and loaded on to the gel alongside with DNA ladder. Gel was run at 90V in the TAE running buffer until bromophenol blue (from the loading buffer) has run 3/4 the length of the gel. After that point gel (preferably within its holder) was carefully transferred to visualise and photograph PCR products on the UV transluminator with photo camera build.

2.2.12.4 Restriction enzyme digest

Unless otherwise stated, restriction enzyme digest was performed in 30-50 μL volume in reaction mix which included appropriate to the enzyme 10 X buffer; enzyme, between 5-10 units per 1 μg of DNA (volume of enzyme was no more than 10% of final volume of reaction mix); dH_2O; DNA; 1% of BSA (if required). Reactions were carried out for 3 hours at 37°C. Digested DNA was then purified as described below, and analysed on agarose gel before being used in ligation reactions.

2.2.12.5 Gel purification of DNA fragments

Appropriate DNA bands were excised from agarose gel using a scalpel and purified using QIAGEN QIAquick Gel Extraction Kit according to manufacturer's protocol. DNA bound to the column was eluted using 30-50 μL of elution buffer (included in the kit) and concentration was determined by spectrophotometry. In order to confirm the purity and size of nucleic acid, 5 μL of DNA was run on an agarose gel alongside with DNA ladder marker. Purified DNA of known concentration was then used in ligation reactions as stated below.

2.2.12.6 DNA ligation

Ligation reactions were performed using Rapid DNA Ligation Kit according to manufacturer's protocol. Briefly, vector DNA and insert DNA were dissolved in 1 x DNA Dilution Buffer to the final volume 10 μL followed by addition of 10 μL of T4 DNA Ligation Buffer and 1 μL of T4 DNA Ligase. Ligation mix was then incubated at the room temperature for 5 minutes before being used to transform *E. Coli* DH5α or TOP 10 competent bacteria. The amount of vector and insert used in ligations was calculated using formula:

((ng of vector) x (kb size of insert) / (kb size of vector)) x (molar ratio of (insert/vector)) = (ng insert)

Generally, the ratio of insert to vector used was 4:1.

2.2.12.7 Transformation of competent bacteria

The tubes of 50 μL of *E. Coli* DH5α or TOP 10 competent bacteria was thawed on ice. 5μL of ligation mix was added to each sample and cells were mixed gently and incubated for 30 min on ice. Competent cells were then transferred in to the water bath set up for 42°C and incubated for 30 sec (heat shock) and immediately transfer on ice. After 2 min of incubation on ice, 250 μL of RT SOC medium was added to each sample and cells were placed into the 37°C incubator with vigorous shaking (250 rpm) for 1h. 100 μL of bacterial suspension was then placed on LB-agar plates containing 50 μg/mL of required selection agent, ampicillin or kanamycin. Plates were inverted and incubated at 37°C over night in order to allow colony growth.

2.2.12.8 Analysis of transformants

The presence of an insert and its size was determined by growing bacteria from each colony in liquid culture (using LB broth medium) at 37°C with shaking (200 rpm) over night. Plasmid DNA was then purified using Mini Prep Kit as stated in 2.2.12.1 and analysed by restriction digest using enzymes that excise the insert (see 2.2.12.4) followed by separation on agarose gel electrophoresis. Alternatively, in order to quickly screen for plasmid inserts directly from *E. coli,* colony, PCR protocol was used. PCR reaction mix was prepared as stated in 2.2.12.2. The small amount of each colony picked up gently from appropriate plate using yellow pipette tip was added to each PCR reaction. Samples were mixed by pipetting and placed in thermocycler using program detailed in 2.2.12.2. PCR products were then analysed by agarose gel electrophoresis for the presence of insert.

2.2.12.9 DNA sequencing

DNA sequencing was performed using dideoxy method (Sanger method). Reaction requires appropriate primer, polymerase enzyme, normal nucleotide mixture and DNA template. Critical role in this method play dideoxy nucleotides that lack the -OH at the 3′ carbon atom. These nucleotides are also added to the growing DNA and cause the chain elongation to stop. Therefore products of this reaction are different length DNA fragments, which are then separated from

longest to shortest. Each of the four dideoxynucleotides is labelled with a different 'tag' and fluoresces in different colours. Fragments are then separated by length from longest to shortest. A difference of one nucleotide is enough to separate one strand from another. Labelled bands appear at each location where the dideoxynucleotide was added and terminated elongation reaction.

Briefly, region of DNA to be sequenced was amplified by PCR using primers binding to sequence upstream or downstream of the region of interest (primers are listed in Table 2.1 in section 2.1.9). Reaction mix was as follow: buffer (2 μL), primer (1μL, 3.2 pmoles), enzyme mix (1 μL), DNA (1.5 μL, 0.5 μg), H_2O up to 7.5 μL. Reaction was performed using thermocycler program detailed below:

96°C 30 sec, (96°C 10 sec, 50°C 5sec, 60°C 4 mins) x 24, 4°C.

In some instances DNA was sequenced by Gene Service (Oxford).

2.2.12.10 Cloning of PCR products for sequencing

PCR products were prepared as described in 2.2.12.2 using cDNA obtained from Human Immune System MTC™ Panel as a template and oligonucleotides specific to bind to each variant of CXCR3 receptor (CXCR3-A, -B and –alt, detailed in Table 2.1). Products were then gel purified and cloned into pCR®2.1-TOPO® vector using Topo TA cloning system according to manufacturer protocol. Cloned fragments were verified by DNA sequencing using M13R oligonucleotide (see Table 2.1 for sequence).

2.2.12.11 Generation of constructs encoding EGFP-tagged hCXCR3-A, hCXCR3-B and hCXCR3-alt receptors

Full length hCXCR3-A, CXCR3-B and CXCR3-alt were amplified by PCR using cDNA obtained from Human Immune System MTC™ Panel as a template (cDNA from spleens and leukocytes) and following oligonucleotides: 'hCXCR3-A-alt H3+Kozak' and 'hCXCR3-A–B stop KpnI' to amplified CXCR3-A; 'hCXCR3-B H3+Kozak' and 'hCXCR3-A–B stop KpnI' to amplified CXCR3-B and 'hCXCR3-A-alt H3+Kozak' and 'hCXCR3-alt stop KpnI' to amplified CXCR3-alt. Thermocycler conditions: 'Amplification of full length CXCR3' were as detailed in section 2.2.12.2. Fragments ~1kb, were then purified from agarose gel, digested using

HindIII and KpnI enzymes and subcloned into pEGFP vector previously digested using the same enzymes. Plasmid DNA was then purified using Quantum Plasmid Miniprep Kit and the presence of insert was confirmed by HindIII and AgeI/PinAI digest. DNA from each miniprep containing insert was then again resolved and extracted from agarose gel and digested using HindIII and NotI enzymes which allowed cutting fragments encoding CXCR3 with the EGFP tag on its C terminus. Fragments were then cloned into pcDNA3.1 expression vector which has been cut with the same enzymes. Presence of the insert was verified by PCR using bacterial colonies as a template and primers complementary to upstream (T7 promoter primer, see Table 2.1 in section 2.1.9) and downstream (BGH primer, see Table 2.1) of cloned fragment. To confirm that C-terminal EGFP tag was correctly inserted, DNA sequencing was performed using following oligonucleotides: T7, EGFP-seq1, EGFP-seq2 and EGFP-seq3.

2.2.12.12 Generation of constructs encoding hCXCR3-A, hCXCR3-B and hCXCR3-alt receptors

Full length hCXCR3-A, CXCR3-B and CXCR3-alt were amplified by PCR using pcDNA3.1-hCXCR3-A, pcDNA3.1-hCXCR3-B and pcDNA3.1-hCXCR3-alt, as the template, respectively. Follow oligonucleotides were used: 'hCXCR3-A-alt H3+Kozak' and 'hCXCR3-A–B stop NotI' to amplified CXCR3-A; 'hCXCR3-B H3+Kozak' and 'hCXCR3-A–B stop NotI' to amplified CXCR3-B and 'hCXCR3-A-alt H3+Kozak' and 'hCXCR3-alt stop NotI' to amplified CXCR3-alt. Thermocycler conditions used were as above. Amplified fragments were prepared and cloned into pcDNA3.1 expression vector as detailed in 2.2.12.11. To verify presence of correctly inserted fragment, colony PCR and sequencing was performed.

2.2.13 Immunoblotting

Immunoblotting also known as Western blotting provides useful tool in the study of expression and/or posttranslational modifications of protein of interest. Within presented work, Western blotting technique was mainly utilized in order to study changes in protein phosphorylation occurring due to cell stimulation with agonist. This was obtained by using anti-phospho-protein specific antibodies as primary the antibodies within immunoblotting procedures as described below.

2.2.13.1 Cell stimulation and preparation of cell lysates

Cells were washed three times by centrifugation at 300 g in RPMI 1640 medium, re-suspended to concentration $1x10^6$ / 500μL and placed in eppendorf tubes. Cells were then incubated for 1 hour in a 37°C water bath. During that time inhibitors were added (if required). Cells were stimulated for indicated periods of time with appropriate concentration of agonist diluted in RPMI 1640 medium. Stimulation was terminated by quick centrifugation for 15-30 seconds at no more than 6,000g at room temperature, aspiration of supernatant and addition of 100 μL of solubilisation buffer (see Materials 2.1.3.). To the control sample RPMI 1640 was added instead of a stimulant. The samples were mixed and rotated at 4°C for 20 minutes followed by centrifugation at 10,000 g for 10 minutes. The protein-containing supernatants were transferred to the fresh tubes and samples were diluted with 2x SDS containing sample buffer (see 2.1.3.) and boiled for 5 minutes at 95°C before being loaded in to the gel or stored at -20°C.

2.2.13.2 Protein determination by Bradford assay

In order to determine the concentration of proteins, a colorimetric protein assay (a Bradford assay) was performed. The assay is based on an absorbance change in the dye coomassie when it stabilizes into coomassie blue from the previously red form after binding to proteins. Bradford reagent was diluted 1 in 5 in volume of PBS and transferred to the fresh eppendorf tubes (1 mL per tube). 5 μL of the protein sample of the unknown concentration was added to the 1 mL of Bradford reagent, mixed thoroughly and left for 5 minutes. In the meantime protein standards were prepared by addition 0, 1, 2, 4, 8 or 16 μg of BSA to 1 mL of the protein reagent. The absorbance of the standards and the samples was measured using the spectrophotometer at 595 nm wave length.

2.2.13.3 SDS-PAGE and Western blotting

Protein lysates were resolved in one dimensional 10% SDS-polyacrylamide gel (SDS-PAGE) using the Bio-Rad Mini Protein II System (Bio-Rad Labs, UK). Appropriate concentrations of resolving and stacking polyacrylamide gels were prepared, poured between glass plates and left to polymerize. 10% APS and TEMED were used to catalyze polymerization reaction. 20 μL of each sample was

loaded into the wells in stacking gel along side with protein standards and run at 80V current in running buffer (see 2.1.3.). Upon reaching the resolving gel, the current was increased to 180V. Proteins were electro-transferred for 60 minutes at 40 mA per gel onto the 0.45 μm nitrocellulose membrane soaked in semi-dry buffer. After being transferred proteins were dyed red by putting the membranes into the Ponceau S in order to confirm successful transfer and washed once for 5 minutes in 1x TBST buffer. Membranes were then incubated for 60 minutes in blocking buffer containing 5% non-fat milk in TBST with slight agilation and rinsed once for 5 minutes in TBST. Membranes were incubated overnight in 10 mL of specified primary antibody solution (diluted 1:1,000 in TBST, supplemented with 0.1% sodium azide and 5% BSA) at 4°C with gentle agitation. The next day membranes were washed three times in TBST and incubated with secondary antibody conjugated to horse radish peroxidase (HRP) diluted 1:10,000 in TBST supplemented with 0.1% of milk for 1-2 hours at room temperature and washed three times for 5 minutes in TBST. Visualization of the protein bands was due to incubation of the membranes in 4 mL of Enhanced Chemiluminescent reagent (ECL) for 1 minute and exposure to X-ray film.

2.2.13.4 Membrane stripping and reprobing

In order to verify protein transfer and equal loading into the gel, pan antibodies which recognize all posttranslational forms of particular protein were used. Membranes were first rehydrated in 1x TBST and then incubated at 60°C for 20 minutes in 20 mL of stripping buffer to remove bound antibody. After extensive washing, 3 times for 5 minutes in TBST, membranes were incubated in blocking buffer for one hour at room temperature. After incubation time blots were rinsed once in TBST and primary antibody was added for overnight incubation. Next day the immunoblotting procedures were carried out as described above.

2.2.14 Flow cytometry

Flow cytometry technology measures and analyzes multiple physical features of single cells, as they flow in a fluid stream through a laser. These measured characteristics include a relative cell size, granularity or internal complexity, and fluorescence intensity. Flow cytometry has found a multiple implications in cell

biology study. In present work this technique was utilized in several different ways, to determine purity of isolated cell populations and expression of surface markers and receptors of interest, to monitor expression of fluorescent reporters such as GFP, and finally to determine the numbers of migrated cells following a chemotaxis assays.

2.2.14.1 Setting up the flow cytometer

To adjust the instrument 'negative sample' representing unstained cells was used. Cells were gated using forward scatter (FSC) and side scatter (SSC) (Figure 2.2A, left and middle panel). Gated events were considered as viable cells and were used for subsequent analysis. The instrument was set up that >95% of negative population of the cells (M1) was between 10^0 and 10^1 on the FL-1 (for FITC or EGFP) or FL-2 (for PE) channels (Figure 2.2A, right panel). For double staining two additional, single stained controls are required to set up compensation. These samples represented FITC and PE positive controls (Figure 2.2B). Each sample, including negative control, was analysed with at least 10,000 gated events counted per sample.

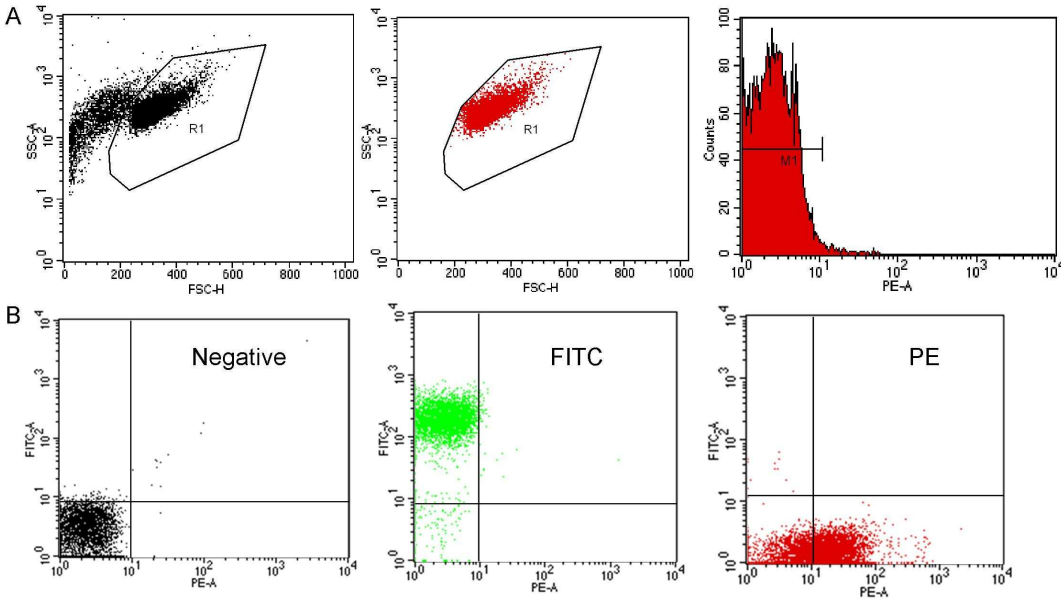


Figure 2.2 Setting up the Flow Cytometer for Single and Two Colour Analyses. (A) Events within region R1 (left and middle panels) are considered viable cells and only these cells are used for analysis. Instrument is adjusted the way so that >95% of gated, negative control cell fall within M1 region between fluorescence intensity 10^0 and 10^1 (right panel). (B) Controls required for setting up compensation for two colour analysis. That include unstained, negative cells (bottom left quartile on the left panel), and single stained cells representing positive controls labelled with FITC (top left quartile on the middle panel), and PE (bottom right quartile on the right panel).

2.2.14.2 Analysis of cell surface receptors expression

Freshly isolated or activated T cells (alternatively other cell types if required) were washed twice in RPMI 1640 medium and resuspended at $1x10^6$ cells per sample in 90 μL of ice cold FACS buffer (see 2.1.3.). 10 μL of required fluorescently-labelled anti-human antibody against the receptor of interest or appropriate isotype-matched immunoglobulin controls (typical concentration 2.5-5 μg/mL) was added to cell suspensions and samples were incubated for 30 minutes at 4°C in the darkness. Cells were washed once in 1 mL of pre-chilled FACS buffer and resuspended in 300 μL of the same buffer for flow cytometry analysis using Becton Dickinson FACSCanto system. The same protocol was used to determine purity of T cell populations by analysing expression of surface markers (e.g. CD3). In order to characterize expression of a receptor present on only the sub-population of interest, a double staining with antibodies conjugated to two different fluorophores was used. Obtained data was analyzed using FACSDiva or CellQuest software.

2.2.14.3 Internalization assay

In order to perform receptor internalization study, cells were prepared and stimulated as described in section 2.2.13.1. Stimulations were terminated by addition of 90 μL of ice cold FACS buffer. 10 μL of required fluorescently-labelled anti-human antibody against the receptor of interest or appropriate isotype-matched immunoglobulin controls (typical concentration 2.5-5 μ/mL) was added to cell suspensions and samples were incubated for 30 minutes at 4°C in the darkness. Following incubation time, cells were washed and analysed using FACSCanto flow cytometer as above. Obtained data was analyzed using FACSDiva or CellQuest software. Mean fluorescence intensity (MFI) values were obtained by subtracting the MFI of the isotype control from the MFI of the positively stained sample. Decrease in CXCR3 surface expression was expressed as a percent of baseline expression. Following formula was used: MFI of stimulated cells/MFI of untreated cells *100.

2.2.14.4 Actin polymerisation assay

In order to perform actin polymerisation study, cells were prepared and stimulated as described in section 2.2.13.1. Stimulations were terminated by adding 500 μL of

fixing solution (methanol free 4% para-formaldehyde in PBS) at RT. After 10 minutes incubation, samples were washed with PBS (350g, 5 minutes), resuspended in 100 μL / tube of 1% para-formaldehyde / 1% FCS in PBS and permeabilised with addition of another 100 μL / tube of 0.1% Triton-X solution with 0.3 μM TRITC phalloidin in PBS. Cells were left to stain at 4°C for 30 minutes. After incubation, cells were washed twice in PBS, resuspended in 300 μL of 1% para-formaldehyde / 1% FCS solution and analyzed immediately or stored at 4°C in dark. Analysis was performed using FACSCanto flow cytometer and FACSDiva or CellQuest software.

2.2.14.5 Analysis of transfection efficiency using EGFP reporter

Within presented work, flow cytometry analysis was also used to evaluate expression of EGFP-tagged CXCR3 receptor in transfected HEK293 cells. 48 hours post-transfection HEK293 cells were harvested, washed and resuspended in pre-chilled FACS buffer. Expression of EGFP reporter was analysed using FACSCanto system.

2.2.15 Fluorescent/confocal microscopy

2.2.15.1 Suspension cells

Cells were stimulated, fixed and stained with TRITC-conjugated phalloidin as detailed in sections 2.2.13.1 and 2.2.14.4, respectively. After two washes (in cooled PBS), cells were resuspended in 200 μL of PBS. Microscope slides mounted with the paper pad and the cytospin cuvette in the metal holder. Cell suspensions were loaded in to each cuvette and centrifuged using cytospin on to the cover slides at 500 rpm for 10 minutes at room temperature. Slides, paper and cuvette were then carefully extracted and cover slice was gently placed on the drop (around 15 μL) of Mowiol containing 10 μg/mL of 4, 6-diamidino-2-phenylindole (DAPI) aliquoted on the microscope slide. Slides were dried in the dark and stored at 4°C until analysed.

2.2.15.2 Adherent cells

22-25 mm diameter glass cover slips were placed in to each well of a 6 well tissue culture plate. 1 mL of sterile 0.01% poly-L-lysine was added to each well and plate was incubated at 37°C overnight. The poly-L-lysine was then aspirated off and wells were rinsed once with sterile MilliQ water. $2x10^5$ cells in 2 mL of complete RPMI-1640 medium was added into each well and cultured for 24 hours. Cells were then transfected as detailed in 2.2.11 and kept in culture for additional 48 hours. Following protocol, cell were rinsed gently in PBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were again washed in PBS and incubated with 1 mL of conjugated antibody solution (10 μg/mL of antibody in PBS containing 5% FBS) at 4°C for 30 minutes in the darkness with gentle agitation. After incubation cells were rinsed twice with PBS and cover slides were carefully removed from the wells, air-dried and mounted on to glass microscope slides in Mowiol containing 10 μg/mL of DAPI. Slides were dried in the dark and stored at 4°C until analysed.

2.2.16 Neuroprobe In vitro migration assay

To study directional migration of T lymphocytes in vitro in the presented study, we used the Neuroprobe ChemoTx® System 96-well chemotaxis chamber which permits rapid, sensitive and consistent measurement of T lymphocyte migrational responses to chemoattractants. Before performing cell migration assays, T lymphocytes were washed twice in RPMI-1640 and re-suspend at 3.2 x 10^6 cells/mL in RPMI-1640 medium containing 0.1% BSA. Cells were then left to rest for 30 minutes in water bath set up at 37°C and during that time inhibitors were added (if required). Appropriate concentrations of chemokines were prepared in RPMI-1640 medium containing 0.1% BSA. Each well of the plate was filled with 29 μL of chemokine dilutions or media aolone. Each point was prepared in triplicates. Plate was the carefully overlaid with filter. 25 μL of previously prepared cell suspension was loaded on the top of the filter, as explained in Figure 2.3.

Chemotaxis plate was then placed in the humidified incubator set up at 37°C, 5 % CO_2 for 3 hours. After incubation non-migrated cells were removed from the top of the filter by carefully wiping surface twice with a piece of Whatmann paper and plate was centrifuged for 10 minutes at 400 g with brake off.

Filter was then carefully removed and cells that had migrated into wells (bottom chamber) were transferred to polystyrene tubes containing 300 μL of ice cold PBS. Migrated cells were then counted using FACS Canto system for 30 seconds.

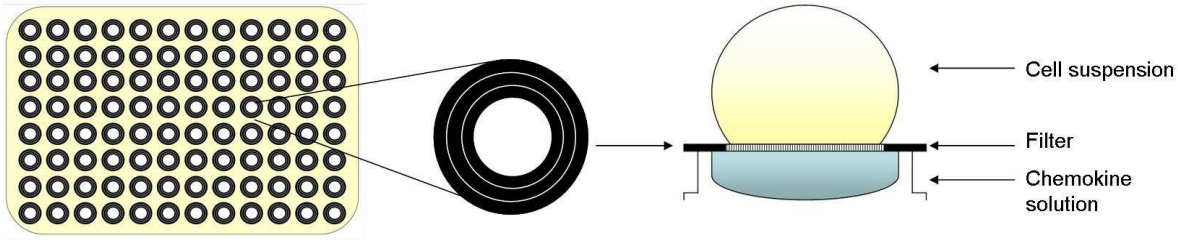


Figure 2.3 Schematic illustration of Neuroprobe ChemoTx® System 96-well chemotaxis plate. (A), 5 μm pore filter with hydrophobic surface surrounding area above each well. (B), Schematic illustration of single well loaded with chemokine solution, overlaid with filter and cell suspension loaded on the top.

2.2.17 Calcium mobilisation assay

Previously activated T cells were washed twice in RPMI-1640 medium and resuspended in Etotal buffer at the concentration $1x10^6$ cells/mL. HEK293 cells were washed twice, harvested and resuspended as above. Cells were loaded with Flou4 – calcium binding fluorescent dye (excited at 485) at the final concentration 5 μM, and incubated at the room temperature for 30 min in the dark. Following incubation, cells were washed twice resuspended at $1x10^6$ cells/mL and transferred to 96-well plate. Appropriate concentration of chemokines was added and real-time fluorescence was recorded at 520 nm every 3-10 s using a multimode platereader (Fluostar Optima, BMG Labtech, UK).

2.2.18 Biotinylated ligand uptake assay

Previously activated T cells were washed twice in RPMI-1640 medium and resuspended at the concentration $1x10^6$ cells/mL and incubated at 37°C for 30 minutes. Biotinylated-CXCL11 (or –CXCL10), was mixed with 5μL of streptavidin-conjugated to appropriated flourophore (for example FITC) in a final volume of 10μL (made up in PBS), and incubated at room temperature for an hour to generate biotinylated-chemokine/FITC complexes. T cells were stimulated with

FITC labelled chemokine for 30-45 minutes at 37°C (control cells were stimulated with PBS containing only FITC streptavidine), washed in PBS, fixed in 4%PFA/PBS and washed again. T cells were then resuspended in 200 μL of PBS and microscope slides were prepared as described in 2.2.15.1.

2.2.19 Data Analysis

All statistical analyses were performed using GraphPad Prism version 4 software by ANOVA test with Dunnett's corrections. If not applicable, data sets were analysed with Student *t*-test. Results with p values<0.05 were considered to be statistically significant.

Chapter Three

Results & Discussion

3. Characterisation of the CXCR3 receptor in human T lymphocytes and CXCR3 transfected cells

3.1 Characterisation of CXCR3 receptor expression and its atypical variants in human T lymphocytes

The transition from bone marrow-resident hematopoietic stem cells, to development as T cell precursors in the thymus, migration into secondary lymphoid organs for immune response initiation and maturation into circulating memory and effector T cells, involves sequentially co-ordinated changes in the profiles of chemokine receptor expression to guide cells into the appropriate microenvironment. Characterisation of expression profiles of chemokine receptors has been instrumental in defining subsets of human memory T cells with distinct migratory capacity and effector functions. For example, CCR7 expression discriminates between lymph node-homing central memory T cells and tissue-homing effector memory T cells. In addition, CXCR3, CXCR6 and CCR5 are preferentially expressed on Th1 cells, while CCR3, CCR4 and CCR8 (along with the PGD2 receptor CRTH2) are expressed on Th2 cells (Sallusto et al., 1998). More recently, CCR2, CCR6 and CCR9 have been reported to be expressed on Th17 cells (Sato et al., 2007; Singh et al., 2008).

Chemokines and their receptors have been divided into two classes depending on whether their expression is constitutive or inducible. Naïve T cells express a limited number of chemokine receptors. Following T cell activation, the number of chemokine receptors present on the surface increases, allowing the cells to respond to chemokine gradients. For example, CXCR3 receptors become up regulated on the surface of activated Th1 cells. CXCR3 has been linked to many inflammatory disorders including atherosclerosis (Mach et al., 1999), autoimmune diseases (Sorensen et al., 1999), transplant rejection (Hancock et al., 2000; Hancock et al., 2001), and viral infections (Liu et al., 2000). These findings have made CXCR3 and its agonists CXCL9, CXCL10 and CXCL11 a popular target for the development of new potential anti-inflammatory strategies. In recent years

however, two main variants of the CXCR3 receptor have been identified, namely CXCR3-B (Lasagni et al., 2003) and CXCR3-alt (Ehlert et al., 2004). Both variants are generated via alternative splicing of mRNA encoding the original CXCR3 receptor (henceforth referred to as CXCR3-A). In the case of CXCR3-B, alternative splicing resulted in extension of the NH_3 terminus by 52 amino acids and this form of receptor has been shown to bind Platelet Factor 4 (PF4/CXCL4) in addition to the three classical CXCR3 agonists (Lasagni et al., 2003). In contrast, CXCR3-alt is a truncated form of CXCR3 (lacking 101 amino acids) with a drastically changed COOH terminus and 4-5 transmembrane domain protein structure (Ehlert et al., 2004).

Expression of the CXCR3 receptor has been mainly associated with T lymphocytes, preferentially with Th1 phenotype. Freshly isolated T cells have been shown to have low levels of CXCR3 expression on the surface, and this expression is highly up-regulated during T cell activation. In the present study, we evaluate surface expression of the CXCR3 receptor on human T lymphocytes by flow cytometry, using fluorescently labelled anti-human CXCR3 antibodies (clone 49801). This antibody, similarly to other commonly used anti-human CXCR3 antibodies, does not distinguish between existing spliced variants of CXCR3. Therefore, we investigated the presence of RNA transcripts of each of the known isoforms of CXCR3 in human T lymphocytes by reverse transcription (RT) PCR. RT PCR analysis was also used to determine expression of CXCR3 and its atypical isoforms in other human blood cells and tissues.

3.1.1 Determination of surface expression of CXCR3 in freshly isolated and activated human T lymphocytes

As shown in Figure 3.1A, freshly isolated pan T cells, separated using the MACS system, represent a pure population of CD3 positive cells. These cells exhibit moderate levels of CXCR3 on the surface, which was significantly increased following activation with CD3/CD28 antibody-coated micro beads and culture in the presence of IL-2 (Figure 3.1C). High expression of surface CXCR3 was also detected on day 9 PBMCs, activated with SEB and IL-2 (Figure 3.1B). In addition, the presence of CXCR3 was detected on $CD3^+$ cells and subsequently on both $CD4^+$ and $CD8^+$ populations (Figure 3.1D).

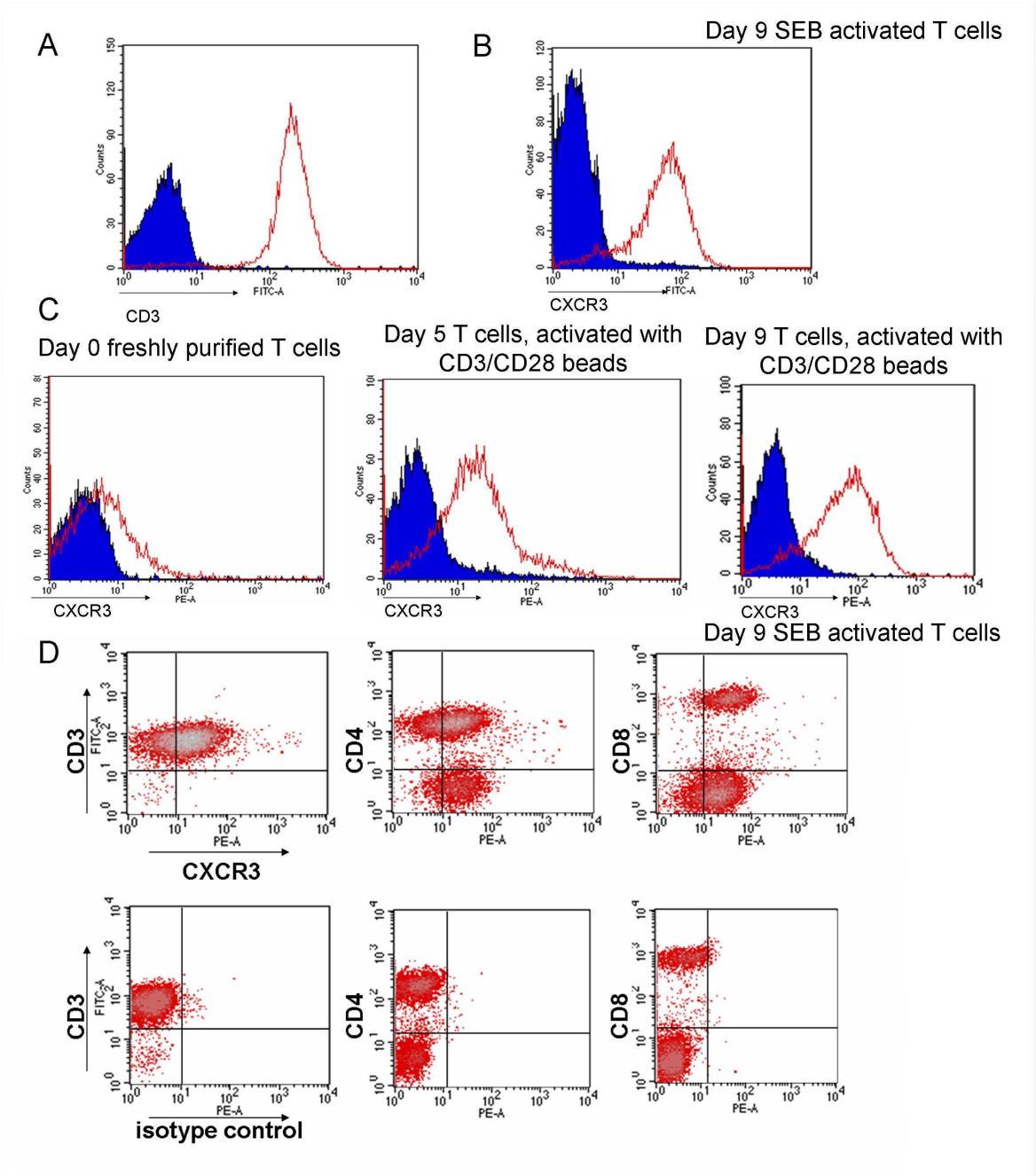


Figure 3.1 Surface expression of the CXCR3 receptor on freshly isolated and activated human T lymphocytes. (A) T cells purified on the MACS column using the Pan T cells isolation kit, represent a pure population of $CD3^+$ cells. (B) Surface expression of CXCR3 on day 9, SEB/IL-2 activated PBMCs. (C) Surface expression of CXCR3 on freshly isolated (left histogram) and CD3/CD28 and IL-2 activated, day 5 and 10 T cells (middle and right histogram, respectively). (D) Representative staining for CXCR3 on $CD3^+$, $CD4^+$ and $CD8^+$ cells activated with SEB and IL-2 (day 9) is shown in the upper panel, staining with suitable isotype control is below. Staining with anti-CD3 or anti CXCR3 antibodies is shown as open histograms (red line), staining with isotype controls is presented as filled histograms (blue). Presented data are representative for at least four independent experiments using blood from different donors.

3.1.2 Determination of expression of different variants of CXCR3 in T lymphocytes at mRNA level

This data shows that at the mRNA level, three spliced variants of CXCR3, namely CXCR3-A, CXCR3-B and CXCR3-alt are expressed on SEB/IL-2 activated T lymphocytes. Primers designed to specifically bind to CXCR3-A and CXCR3-B gave 770 and 545bp products, respectively (Figure 3.2). We were also able to detect the presence of an additional variant of CXCR3, namely CXCR3-alt. This is a truncated version of CXCR3, generated by alternative splicing via exon skipping, with an altered C terminus and predicted to have only 4-5 transmembrane domains (Ehlert et al., 2004).

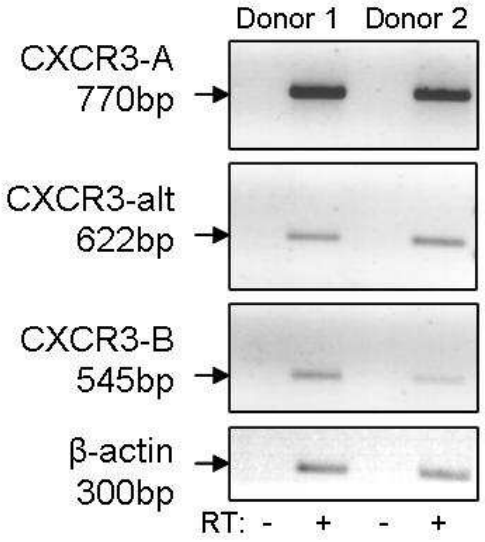


Figure 3.2 Expression of CXCR3 and its atypical variants at mRNA level in human activated T lymphocytes. PCR analysis was performed using cDNA from day 9-12 SEB/IL-2 stimulated T cells as template and specific primers sets. Arrows point out bands corresponding to mRNA expression of each of CXCR3 variants, CXCR3-A, CXCR3-alt, CXCR3-B and β-actin. RT +/- indicates presence or absence of Reverse Transcriptase. Presented data are representative for at least 3 independent experiment using blood from different donors.

3.1.3 Determination of the expression of different variants of CXCR3 in other blood cells and human tissues

In order to determine expression of CXCR3-A and its two splice variants, CXCR3-B and CXCR3-alt in other blood cells and tissues, PCR analysis using first strand cDNA preparations from RNA from various tissues/cells (MTC Panels, Clontech) as templates and primers specific to CXCR3-A, CXCR3-B and CXCR3-alt genes (the same primers set which was used in section 3.1.2) was performed. G3PDH PCR primers (Clontech) were used as a control.

According to PCR results, mRNA encoding CXCR3 and its variants was present in different types of human blood cells. That includes both resting and activated $CD4^+$ and $CD8^+$ T cells, B cells and monocytes (Figure 3.3 upper case). PCR analysis revealed expression of CXCR3-A mRNA in samples obtained from human spleen, lymph node, thymus leukocytes and at low levels in samples from tonsil, bone marrow and foetal liver. CXCR3-B was found in all samples apart from bone marrow and foetal liver. These results should be carefully interpreted because a contamination of templates with genomic DNA cannot be excluded. The commercial sources of cDNA used lacked the appropriate RT- controls which would be required in order to confirm the presence of mRNA for CXCR3 variants and absence of genomic DNA in the sample. To overcome this problem, primers should be designed to bind within different exons as was done for CXCR3-alt. PCR using primers specific for CXCR3-alt, apart from band predicted for this variant (detected in all samples apart from bone marrow and foetal liver), gave an additional product of 1000bp (pointed out by the large arrow). Presence of this band suggests that samples may be contaminated, as it is the correct size to be a product amplified from genomic DNA.

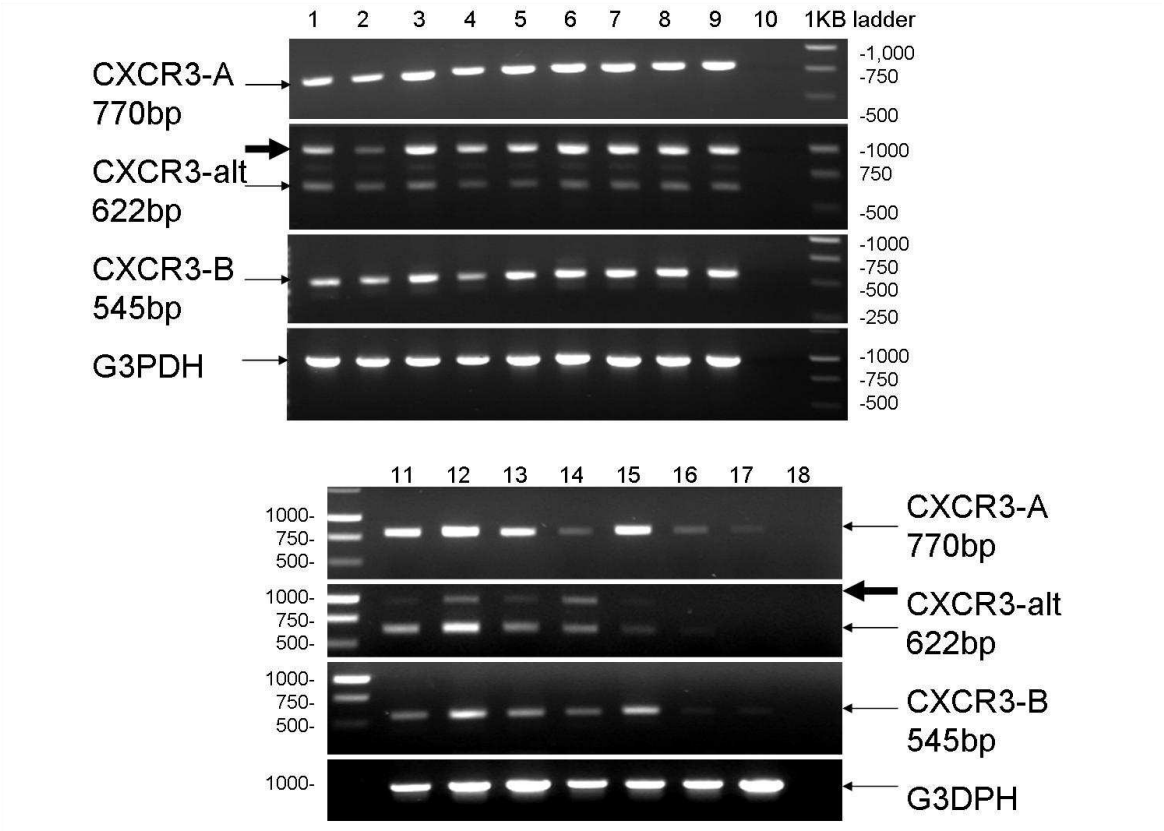


Figure 3.3 PCR analysis of CXCR3 expression and its atypical variants in human blood cells and the human immune system. PCR analysis was performed using cDNA preparations from the Human Blood Fractions MTC™ Panel (upper case) or Human Immune System MTC™ Panel (lower case) (MTC Panels, Clontech) as template and specific primers sets. Arrows indicate bands corresponding to mRNA expression of each of the CXCR3 variants; CXCR3-A, CXCR3-alt, CXCR3-B and G3PDH. Large arrows indicate an additional band (size around 1000bp) appearing after using primers binding to CXCR3-alt cDNAHuman Blood Fractions MTC™ Panel

1. mononuclear cells (B & T-lymphocytes and monocytes)
2. resting $CD8^+$ cells (T-supressor/cytotoxic)
3. resting $CD4^+$ cells
4. resting $CD14^+$ cells (monocytes)
5. resting $CD19^+$ cells
6. activated $CD19^+$ cells
7. activated mononuclear cells
8. activated $CD4^+$ cells
9. activated $CD8^+$ cells
10. control (dH_20)

Human Immune System MTC™ Panel

11. spleen
12. lymph node
13. thymus
14. tonsil
15. leukocyte, peripheral blood
16. bone marrow
17. foetal liver
18. control (dH_20)

3.2 Agonist-induced down-regulation of CXCR3 surface expression in human T lymphocytes

Prolonged agonist stimulation leads to receptor desensitisation, which in many cases leads to receptor internalization. This provides a regulatory mechanism for intracellular responses by reducing the number of surface-expressed receptors. Following ligand binding, there are two major routes whereby GPCRs are internalized into cells. The first and most well-defined route involves the binding of arrestin to the phosphorylated receptor, which leads to clathrin binding. The receptor-arrestin complex is then sequestered in clathrin-coated pits. This pathway is often considered a default system for degradation and recycling of receptors (Pelchen-Matthews et al., 1999; Shenoy and Lefkowitz, 2003). The second pathway involves invaginations of the cell membrane known as caveolae and functions independently of clathrin-coated pits (Mueller et al., 2002; Orlandi and Fishman, 1998).

Within this study, down regulation of CXCR3 receptor surface expression upon exposure to its agonists CXCL9, CXCL10, CXCL11 and CXCL4 in human SEB/IL-2 activated T cells was investigated. The effect of small noncompetitive CXCR3 antagonists on CXCL11 induced internalization was also examined. Furthermore, mechanisms of CXCL11-mediated CXCR3 internalization using a variety of inhibitors blocking distinct endocytosis pathways and cell signaling pathways including PI3K, PLC and PKC were re-evaluated (Sauty et al., 2001).

Internalized receptors can face two potential fates. Firstly, the receptor can be recycled back to plasma membrane though rapid or slow recycling pathways (Neel et al., 2005). Another potential fate is that receptors pass through the endosomal system followed by direction to lysosomes for degradation. This may lead to down-regulation of receptor expression (Neel et al., 2005).

3.2.1 Concentration and time dependent internalization of CXCR3 in response to CXCL9, CXCL10, CXCL11 and CXCL4

To examine the effect of agonist stimulation on down regulation of CXCR3 surface expression, we performed a concentration-dependence study. Day 9-12 SEB/IL-2 activated T lymphocytes were exposed to various concentrations of each CXCR3

agonist, CXCL9, CXCL10, CXCL11 over 30 minutes (Figure 3.4A) and then analysed by FACS for CXCR3 surface expression. Incubation with the highest (300 nM) concentrations of CXCL9 and CXCL10 led to a 50-60% reduction in surface expression of CXCR3 respectively. Similar to published data, results have shown that CXCL11 is the most potent and efficient inducer of CXCR3 internalization. Maximum loss of CXCR3 from the surface in response to CXCL11 (85% reduction) was observed after stimulation with 300 nM of the agonist. In contrast, no loss of surface CXCR3 was observed in response to CXCL4. In a kinetic analysis of loss of surface receptor expression it was observed that in the case of all three agonists: (CXCL9, 10 and 11), maximum receptor internalization occurred within the first 5 minutes of stimulation with 100 nM of agonist (Figure 3.4B). This detected loss of CXCR3 surface expression was a ~75% reduction with CXCL9, ~60% with CXCL10 and ~20% with CXCL11. Again it was not possible to observe any effect with CXCL4. In order to examine any reappearance of CXCR3 to the surface in the presence of agonist, T cells were exposed to CXCL9, CXCL10 and CXCL11 for up to 3 hours and CXCR3 expression was analysed as before (Figure 3.4C). Results showed that at longer time points in the presence of agonist, there was a sustained slow down-regulation of CXCR3 with little or no recovery of surface expression.

In order to visualize the internalized agonist, biotin-labelled recombinant CXCL11 was incubated with FITC-streptavidin to form complexes, and used to stimulate T cells. A biotinylated agonist uptake assay was performed as described in Materials and Methods. Analysis using fluorescent microscopy revealed the presence of fluorescent puncta and aggregates in the cell cytoplasm indicating a possible uptake of labelled CXCL11 (Figure 3.4D). Stimulated cells exhibited different levels of chemokine uptake and in some cases uptake was difficult to detect. Therefore in order to compare the abilities of biotinylated and native CXCL11 to induce down-regulation of CXCR3, T cells were stimulated in the presence of each agonist (100 nM) and flow cytometry analysis was performed as before. Obtained results indicated an impaired ability of biotinylated CXCL11 to internalize surface CXCR3 suggesting that the addition of biotin affects its interaction with CXCR3 (Figure 3.4E).

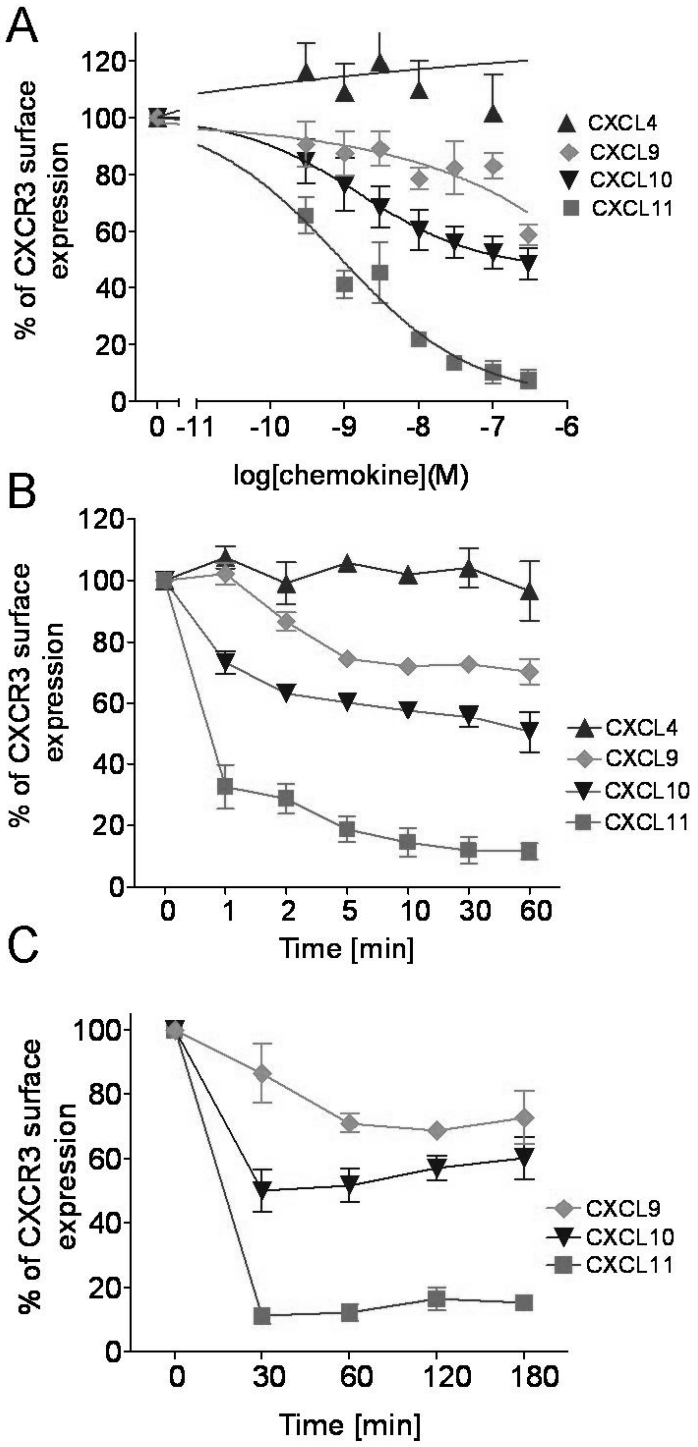


Figure 3.4 Concentration and time dependent agonist-induced down-regulation of surface expression of CXCR3 receptors on human activated T lymphocytes. PTO for figure legend.

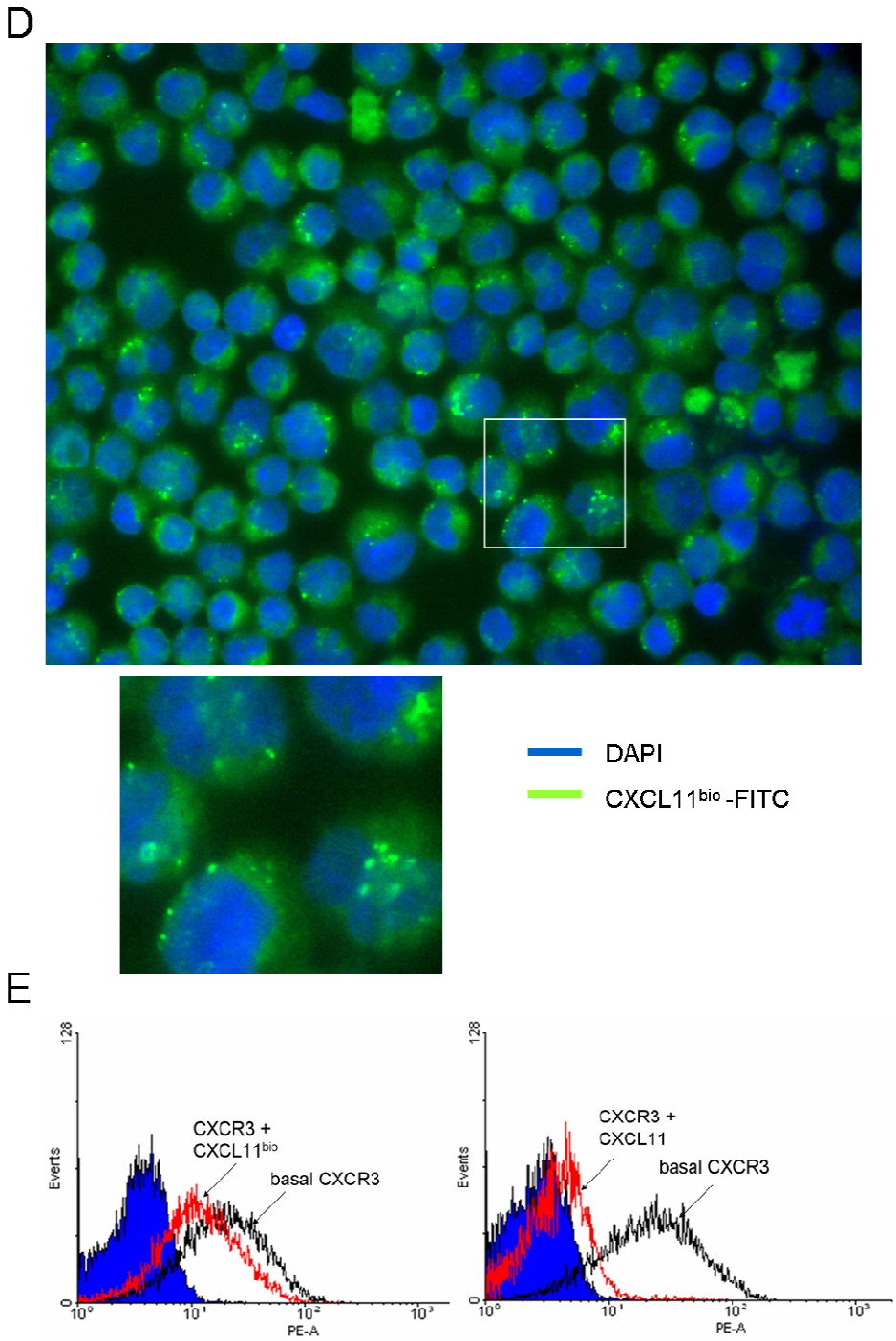


Figure 3.4 Concentration and time dependent agonist-induced down-regulation of surface expression of CXCR3 receptors on human activated T lymphocytes. (A), Day 9-12, SEB/IL-2 activated T cells were stimulated with increasing concentrations of CXCL11, CXCL10, CXCL9 or CXCL4 over 30 minutes or (B) with 100 nM of each agonist over various periods of time, up to 60 minutes or 3 hours (C). All incubations were done at 37°C. Agonists were then washed off and cells were incubated with an anti-CXCR3 antibody or isotype control at 4°C followed by FACS analysis as described in *Materials and Methods*. Decrease in CXCR3 surface expression is expressed as a percentage of baseline surface expression using following formula: MFI (Mean Fluorescence Intensity) of stimulated cells/MFI of untreated cells *100. Shown data represents an average +/- SEM of at least 3 independent experiments using blood from different donors.(D), Uptake of biotin-streptavidin-FITC labelled CXCL11. Day 9-12, SEB/IL-2 activated T cells were incubated for 30 minutes in the presence of 100 nM biotin-streptavidin-FITC labelled

CXCL11. Cells were then washed, incubated with DAPI at a concentration of 10μg/ml (blue), centrifuged and mounted onto microscope cover slips using non-fluorescent mounting solution. Samples were then left O/N and analyzed by fluorescence microscopy. (E), Comparison of abilities of native, recombinant CXCL11 versus biotinylated CXCL11 to induce CXCR3 down-regulation.

3.2.5 3.2.2 Effect of CXCR3 antagonists on CXCR3 agonist-induced internalization of CXCR3

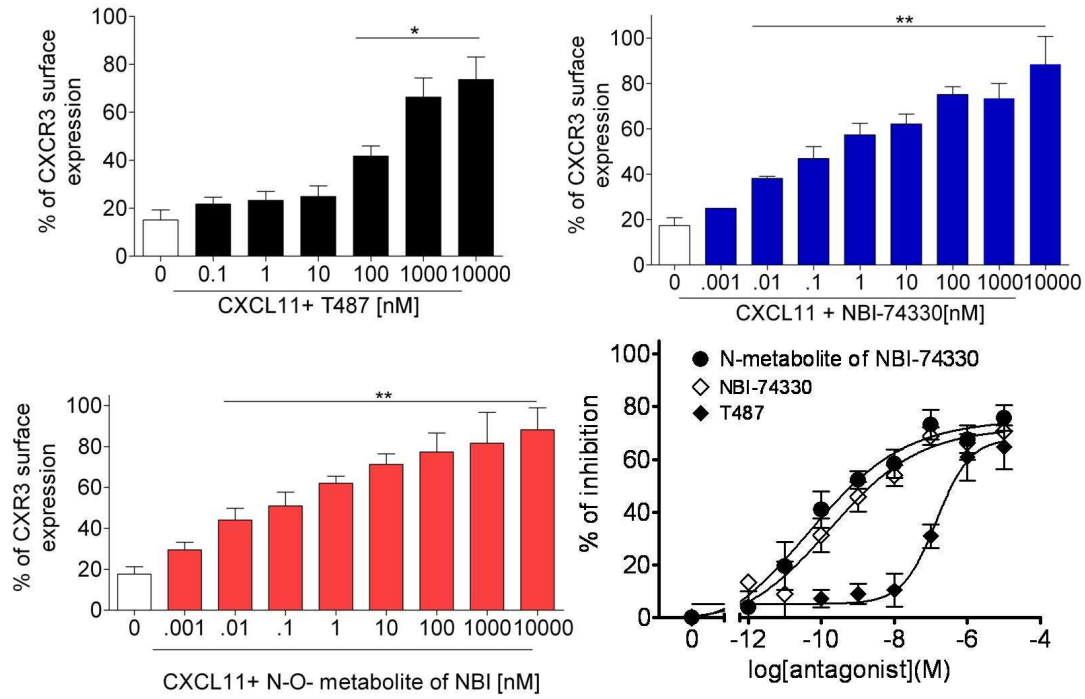
For further analysis of agonist-mediated down-regulation of CXCR3 surface expression on T cells, we used three small non-competitive CXCR3 antagonists namely, T487, its derivative, NBI-74330 and the N-oxide metabolite of NBI-74330 (Jopling et al., 2007; Medina et al., 2002; Medina et al., 2005; Schall et al., 2001; Wijtmans et al., 2008; Johnson et al., 2007). Within presented work an effect of increasing concentrations of CXCR3 antagonists against CXCL11 mediated responses was addressed. We choose this chemokine because it was found to be the most potent and efficient inducer of CXCR3 internalization in our previous experiments.

Pre-incubation of T cells with various concentrations of each antagonist (0.001 nM -10 μM) for 30 minutes followed by stimulation with agonist led to a concentration dependent inhibition of CXCL11-induced internalization of CXCR3 (Figure 3.5A). T487 was less potent than both NBI-74330 and N-oxide-NBI-74330 which presented similar potency (IC50s can be found in Chapter Seven, Section 7.5). 100 nM of T487 decreased the CXCL11-induced effect on CXCR3 surface expression significantly, whereas NBI-74330 and its oxidised metabolite showed a similar effect at 0.1 nM. At the highest concentration (10 μM) all three antagonists have shown comparable potency, however maximal inhibition was achieved with 100 nM of NBI-74330. Total inhibition of CXCL11 mediated internalization was not achieved after treatment with the compounds. The maximum effect was observed after using 100 nM - 10 μM concentration of antagonists and led to an 80% inhibition of CXCL11 induced internalization.

After examination of the effect of CXCR3 antagonists on CXCR3 surface down-regulation mediated by CXCL11, the ability of NBI-74330 to inhibit CXCL9 and CXCL10-induced down-regulation of CXCR3 was investigated (Figure 3.5B). In this experiment, T cells were pre-treated with a single, 100 nM concentration of the

compound followed by stimulation with 100 nM of each agonist for 30 min. Higher concentrations of CXCL9 and CXCL10 (in comparison to CXCL11) were chosen due to their lower efficiency and potency in previous internalization assays. Upon treatment with CXCL9 and CXCL10, expression of CXCR3 was reduced by 20 and 45%, respectively. NBI-74330 had an effect on this loss of surface expression of CXCR3 however it was not able to block it completely (Figure 3.5B). Similar to our previous results using CXCL11, some level of agonist-induced CXCR3 internalization seems to occur despite the presence of antagonist.

A.



B.

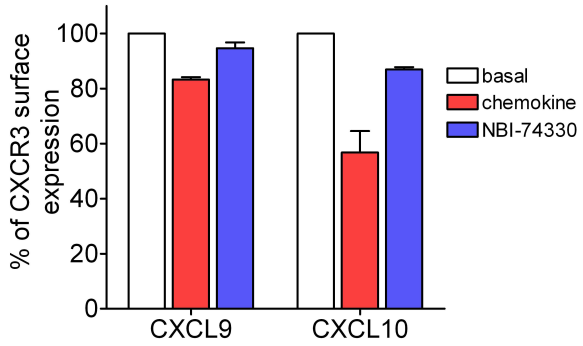


Figure 3.5 receptors in T lymphocytes. (A), Effect of CXCR3 antagonists on CXCL11-induced internalization of CXCR3. Day 9-12, SEB/IL-2 activated T lymphocytes were incubated in the presence or absence (DMSO only, open bars) of various concentrations of one of the compounds: T487 (0.1 nM-10 μM) (top left panel), NBI-74330 (0.001 nM-10 μM) (top right panel) or N-oxide-metabolite, followed by stimulation with 30 nM CXCL11 for 5 mins. The percentage inhibition by all three CXCR3 antagonists is depicted in bottom right panel. White bars indicate cells stimulated by CXCL11, without antagonists treatment. (B), Effect NBI-74330 on CXCL9 and CXCL10 induced internalization of CXCR3 in T lymphocytes. Day 9-12, SEB/IL-2 activated T cells were incubated in the presence or absence of of NBI-74330 (100 nM) compound for 30 min before being stimulated for 30 mins with CXCL9 or CXCL10 (100 nM). All incubations were done at 37°C. Agonists were then washed off and cells were incubated with anti-CXCR3 antibody or isotype control at 4°C followed by analysis on FACS as described in *Materials and Methods*. Decrease in CXCR3 surface expression was expressed as a percentage of baseline surface expression. Shown data represent average +/- SEM of at least 3 independent experiments using blood from different donors. Data was analysed using One-way Anova with repeated measures followed by Dunnett's correction.* $p<0.05$, **$p<0.01$ compared with CXCL11 treatment alone.

3.2.3 Mechanisms and regulation of CXCL11–induced internalization of CXCR3 in human T cells

Examination of endocytic pathways involved in agonist-induced surface down-regulation of CXCR3 receptor

To examine which of the endocytic pathways are involved in CXCL11-induced CXCR3 internalization, the inhibitors which have been shown to abolish either clathrin or caveolae-dependent routes of endocytosis were utilized. Hypertonic sucrose and chlorpromazine have been demonstrated to inhibit the assembly of clathrin-coated pits, whereas nystatin and filipin can block internalization via caveolae (Harder et al., 1997; Okamoto et al., 2000). Filipin is a polyene antibiotic with antifungal properties, which binds selectively to cholesterol, forming complexes in the plasma membrane that sequester cholesterol and induce structural disorder (Bolard, 1986; Mcgookey et al., 1983; Ohtani et al., 1989; Robinson and Karnovsky, 1980). methyl-β-cyclodextrin (MβCD) was also used to evaluate the involvement of cholesterol in CXCR3 internalization. MβCD is a membrane impermeable, small cyclic oligosaccharide with a hydrophobic core that selectively and rapidly extracts cholesterol from the plasma membrane (Kilsdonk et al., 1995; Ohtani et al., 1989).

Incubation of T cells with 0.4 M sucrose did not have any effect on CXCR3 internalization, in contrast to chlorpromazine which, at 25 μg/mL, significantly inhibited internalization by 40% (Figure 3.6A). Treatment of cells with 5 μg/mL of filipin or 50 μg/mL of nystatin internalization led to minor inhibition of agonist-induced loss of surface expression of CXCR3, however it did not reach statistical significance (Figure 3.6B). In contrast, cholesterol disruption using 10 mM MβCD resulted in significant inhibition of CXCR3 surface down-regulation by 40% (Figure 3.6C). Similarly, no effect with either clathrin or caveolae-dependent pathway inhibitors was observed in HEK293 cells expressing CXCR3 (Figure 3.6 C and D). In addition, it is important to notice that in transfected HEK293 cells, stimulation with CXCL11 led to down-regulation of surface CXCR3 to a lesser extend than it was observed in T cells (Figure 3.36D).

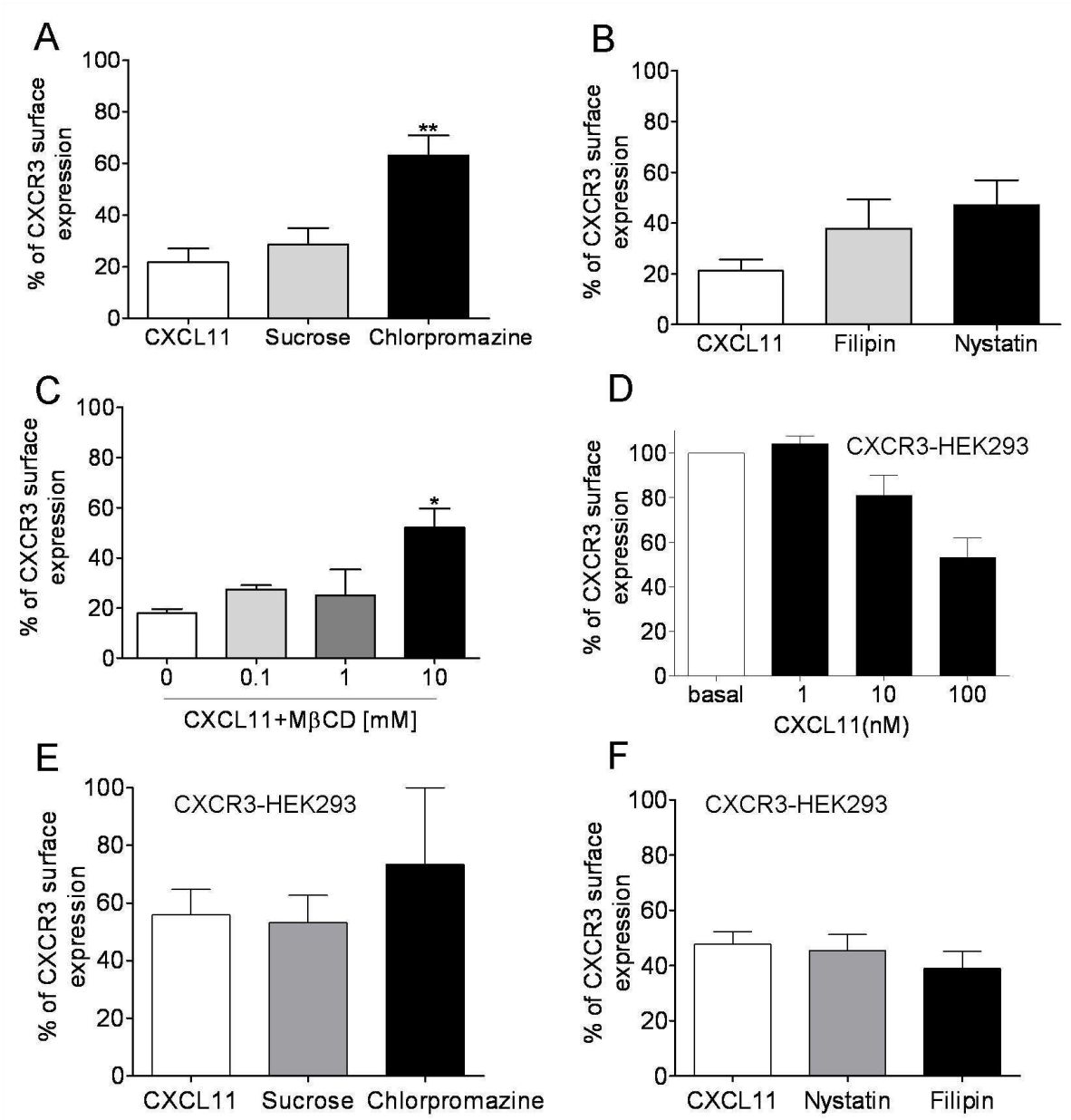


Figure 3.6 Mechanisms of CXCR3 internalization in human activated T cells and transfected HEK293 cells. The level of CXCR3 internalization in T cells in response to stimulation with 30 nM CXCL11 after pretreatment in the presence or absence of 0.4 M sucrose and 25 μg/mL chlorpromazine (A), 5 μg/mL filipin and 50 μg/mL nystatin (B), or up to 10 mM MβCD (C). CXCL11-induced down-regulation of surface CXCR3 in transiently transfected HEK293 cells (D). Effect of sucrose and chlorpromazine (E), nystatin and filipin (F) on CXCL11 (100nM) –induced CXCR3 internalization in CXCR3 expressing HEK293 cells. All incubations were done at 37°C. Cell surface CXCR3 expression was measured using flow cytometry as described in *Materials and Methods*. Decrease in CXCR3 surface expression is expressed as a percentage of baseline surface expression. Data was analysed using One-way Anova with repeated measures followed by Dunnett's correction.* $p<0.05$, **$p<0.01$ compared with CXCL11 treatment alone. Data represent the mean +/- SEM of at least three different experiments for T cells or two different experiments for HEK293 cells.

Despite some negative results obtained using pharmacological interference with endocytic pathways, these data should be carefully interpreted considering the lack of positive controls showing pharmacological activity of compounds. Demonstration of the compounds activities in well known endocytic pathways for example transferrin endocytosis would be adviced in order to draw a definite conclusions from obtained results.

In order to examine other mechanisms responsible for agonist-induced CXCR3 internalization, we investigated the involvement of different intracellular signalling pathways using selective inhibitors and activators. Agonist binding to chemokine receptors leads to activation of variety of intracellular signal transduction pathways, including heterotrimeric G proteins, tyrosine kinases, PI3K and PKC (Neer, 1995; Ward et al., 1998).

Effect of pertussis toxin on CXCL11-induced CXCR3 surface down-regulation

Gi has been described to be crucial for CXCR3-induced chemotaxis and elevation of extracellular calcium (Neer, 1995). To address the role of Gi in CXCR3 internalisation, T cells were pre-treated with 10 ng/mL of PTX for 16h at 37°C prior to stimulation with 30 nM CXCL11 and the expression of CXCR3 analysed as above (Figure 3.7A). PTX, at the concentration that inhibited CXCR3-mediated Erk and Akt phosphorylation in previous experiments (shown in Figure 3.12), did not have any effect on down-regulation of surface CXCR3. This suggests that CXCL11 induced internalisation of CXCR3 in independent of Gαi activation.

Effect of PI3K and PIKfyve inhibitors in CXCL11-induced CXCR3 surface down-regulation

To evaluate the role of PI3K in mediating the CXCR3 internalisation signal, three broad spectrum PI3K inhibitors, namely LY294002, Wortmanin and ZSTK474 (Figure 3.7 B,C,D) were utilized. 60 min incubation with increasing concentrations of LY294002 (3-30 μM), Wortmannin (30-300 nM) and ZSTK474 0.1-10 μM did not affect the loss of CXCR3 surface expression stimulated by CXCL11, suggesting that PI3K is not required for this biological process.

PIKfyve is the mammalian type III PtdInsP kinase that generates PtdIns(3,5)P2 from PtdIns3P substrate (Mcewen et al., 1999). PIKfyve has been shown to be involved in maintaining endomembrane homeostasis in mammalian cells. Overexpression of a mutated form of PIKfyve or knockdown of PIKfyve using short interfering RNA siRNA in COS7 or human embryonic kidney 293 cells causes swelling of an endocytic vacuole-like compartment that resembles late endosomes (Ikonomov et al., 2003; Rutherford et al., 2006). PtdIns(3,5)P2 has also been shown to have a role in the retrieval of cargoes to the trans-Golgi network (TGN; Rutherford et al., 2006) and at the TGN (Ikonomov et al., 2003).

In order to determine the role of PIKfyve in CXCR3 endocytosis, a pyridofuropyrimidine compound, YM201636, with high in vitro inhibitory activity against PIKfyve (IC_{50} of 33 nM) identified during a drug discovery programme directed at PI3K was introduced to the study (Hayakawa et al., 2006). T cells were incubated in the presence of 3, 10 or 30 μM of YM201636 and its effect on CXCL11-induced down-regulation of surface CXCR3 was assayed. We observed that only the highest concentration of YM201636 significantly blocked CXCL11-mediated internalisation of CXCR3 (Figure 3.8A). Because the concentration used was approximately 1000 fold higher then the published half-maximal inhibitory concentration towards PIKfyve, observed inhibition may be explained as an off-target effect. In parallel, an effect of YM201636 on CXCL11-induced phosphorylation of Akt and p44/p42 MAP kinase in T cells was tested and inhibitory effects of the compound using lower 1-10 μM concentrations was observed (Figure 3.8B). 30 minutes incubation with 1 μM YM201636 led to complete attenuation of the phospho- Akt signal and partial inhibition of phospho-p44/p42 MAPK suggesting that unlike CXCR3 internalisation, PIKfyve may be involved in CXCR3- mediated signalling in T cells.

Effect of PKC inhibitors on CXCL11-induced CXCR3 surface down-regulation

It has been previously reported that activation of PKC leads to internalisation of CXCR4 and CCR3 chemokine receptors (Zimmermann et al., 1999; Signoret et al., 1997). Therefore in our study a known PKC activator – phorbol myristate acetate (PMA) was used to determine its ability to induce CXCR3 internalisation

(Figure 3.8C). Treatment of T cells with 1, 10 and 100 nM of PMA led to a concentration dependent down-regulation of CXCR3 surface expression, as assessed by FACS, by 50%. This suggests that global activation of PKC can lead to agonist independent CXCR3 internalisation and may also be important for internalisation in response to agonist binding. Therefore, cells were pre-treated with widely used PKC-delta inhibitors, Rottlerin and RO31-8220 which inhibit conventional (α, β, γ) and novel (δ, ε, η) PKC isoforms at relatively low concentrations (EC_{50}s, approximately 20–100 nM) and the atypical PKC-ζ at higher concentrations (EC_{50}, 1–4 μM) (Standaert et al., 1997). Incubation with both inhibitors at the 30 μM concentration resulted in significant reduction of CXCL11-induced internalisation of CXCR3 (Figure 3.8D, E). Therefore, PKC appears to be important in agonist independent and dependent down-regulation of surface CXCR3.

Effect of PLC inhibition on CXCL11-induced CXCR3 surface down-regulation

The effect of the selective PLC inhibitor U73122 and its inactive close analogue U73343 (Bleasdale et al., 1990) was tested. Incubation of T cells for 30 min with 3 and 10 μM with both compounds did not have any effect on CXCL11-mediated down-regulation of surface CXCR3 (Figure 3.8F). In contrast increasing the concentration of U73122 to 30 μM had a dramatic effect and completely blocked CXCR3 down-regulation. No effect was observed using 30 μM U73343. These results suggest a potential involvement of PLC in agonist-induced down-regulation of CXCR3 in T cells.

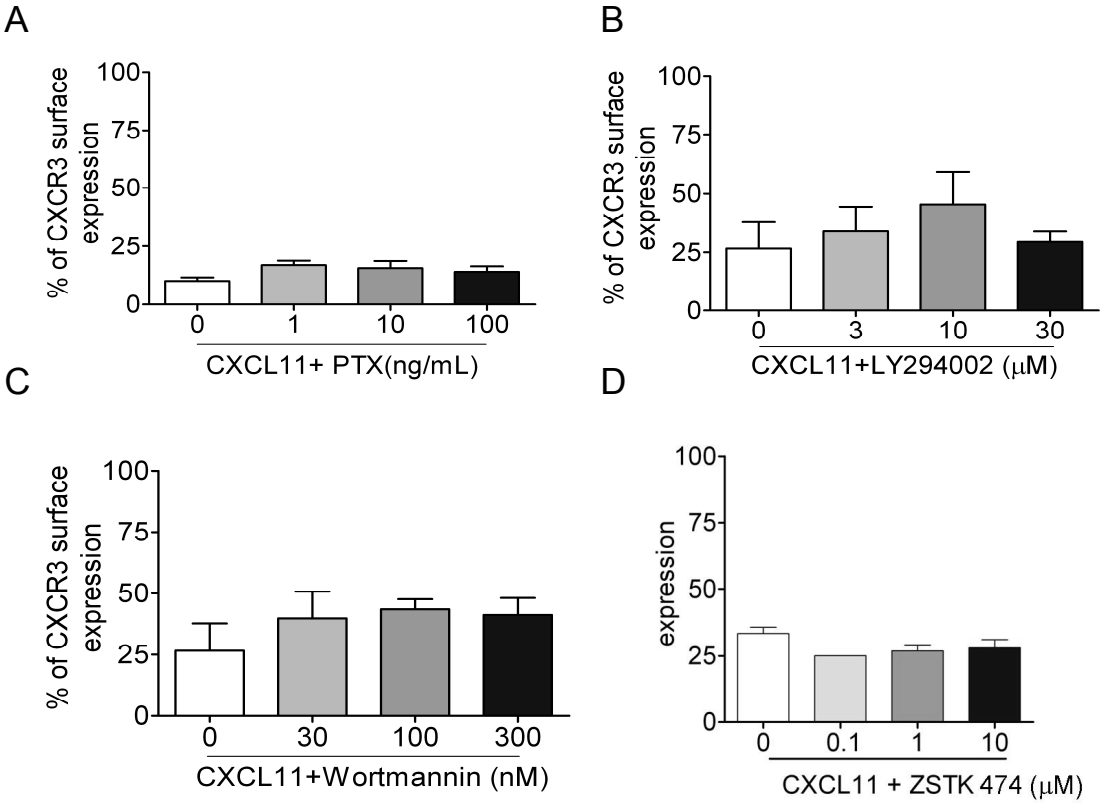


Figure 3.7 Involvement of Gαi and PI3K in CXCL11-induced internalization of CXCR3 in human T cells. Internalization of CXCR3 in response to 30 nM CXCL11 in the presence or absence of PTX (1-100 ng/mL) (A), PI3K inhibitors: LY294002 (3-30 μM) (B), Wortmannin (30-300 nM) (C) and ZSTK474 (0.1-10 μM) (D). Cells were incubated with appropriate concentrations of each inhibitor or vehicle control for 30 mins at 37°C before being stimulated with 30 nM CXCL11 for 5 mins at 37°C. Cell surface CXCR3 expression was measured using flow cytometry as described in *Materials and Methods*. Decrease in CXCR3 surface expression was expressed as a percentage of baseline surface expression. Data was analysed using One-way Anova with repeated measures followed by Dunnett's correction, compared with CXCL11 treatment alone. Data represent the mean +/- SEM of at least three different experiments.

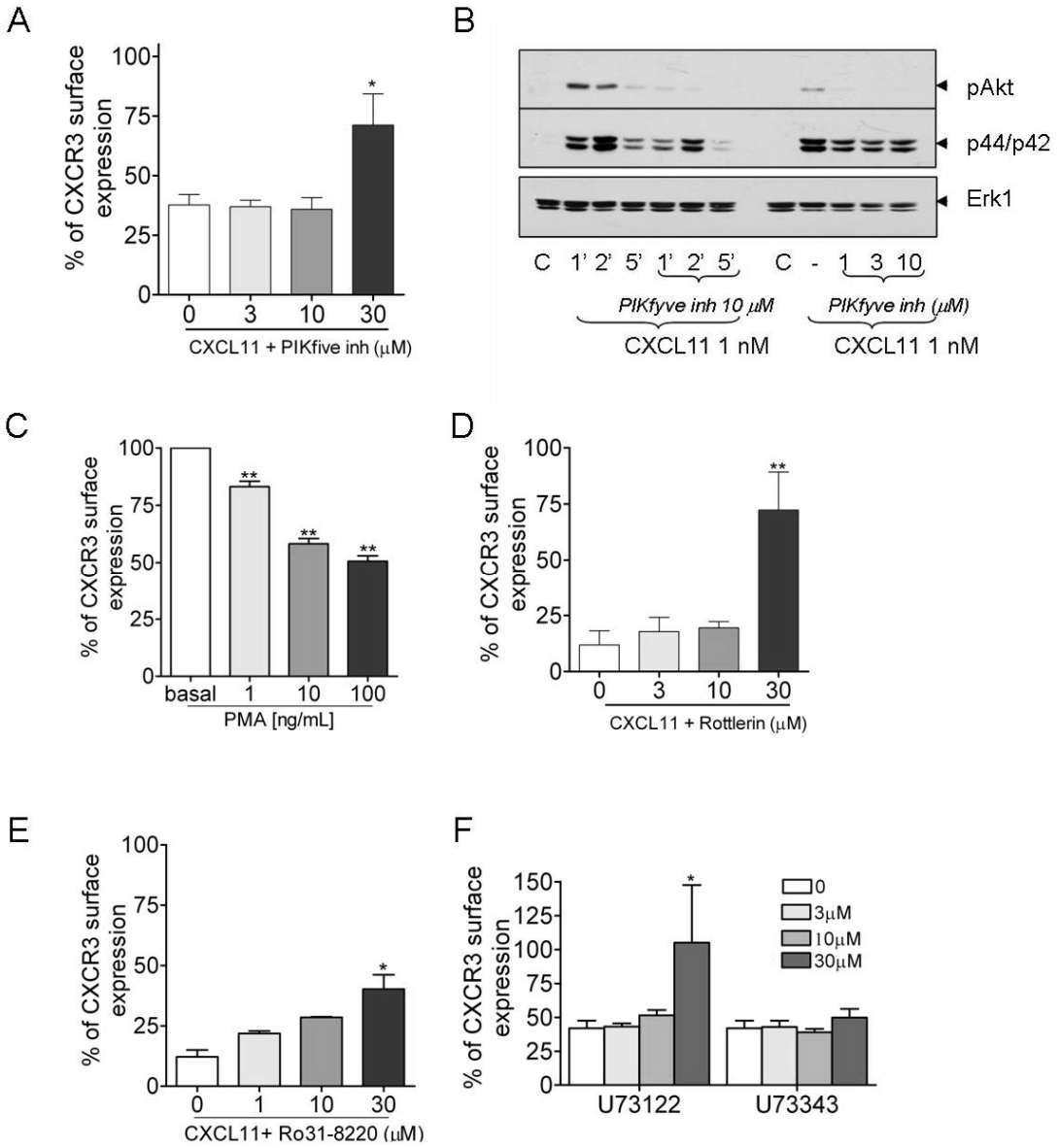


Figure 3.8 Involvement of PIKfyve, PKC and PLC in CXCL11-induced internalization of CXCR3 in human T cells. Effect of PIKfyve inhibititor (3-30 μM) (A) PMA (1-100 ng/mL) (C), Rottlerin (3-30 μM) (D), Ro31-8220 (1-30 μM) (E) and U73122 (3-30 μM) (F) on CXCL11 induced internalization of CXCR3 in T lymphocytes. Day 9-12 SEB/IL-2 activated T cells were incubated with appropriate concentrations of each inhibitor or vehicle control for 30 mins at 37°C before being stimulated with 30 nM CXCL11 for 5 mins at 37°C. Cell surface expression of CXCR3 was measured using flow cytometry as described in *Materials and Methods*. Decrease in CXCR3 surface expression was expressed as a percentage of baseline surface expression. Panel (B) shows the effect of PIKfyve inhibition on CXCL11-induced Akt and Erk phosphorylation in T cells. Previously activated T cells were incubated with appropriate concentrations of each inhibitor or vehicle (DMSO) for 30 mins at 37°C before being stimulated with 1 nM of CXCL11 for the required period of time. Phosphorylation of p44/42 MAPK and Akt/PKB was determined by Western blot analysis using specific anti–phosho-p44/p42 MAP kinase (Thr202/Tyr204) and anti-phospho-Akt (Ser476) antibodies, respectively. To confirm equal loading, membranes were stripped and reprobed using anti-Erk1 antibody. Data were analysed using One-way Anova with repeated measures followed by Dunnett's correction.*, $p<0.05$, **$p<0.01$ compared with CXCL11 treatment alone. Data represent the mean +/- SEM of at least three different experiments.

3.2.4 CXCR3 surface expression recovery following agonist exposure

To determine CXCR3 cell surface replenishment after agonist stimulated internalization, receptor down-regulation was initiated as described above. After incubation with CXCL11 for 5 minutes, cells were washed three times with pre-warmed Hank's Buffered Salt Solution (HBSS) to remove unbound CXCL11. Surface expression of CXCR3 was assessed as described previously, after 30, 60, 120 and 180 minutes incubation in serum free medium (Figure 3.9A). Reappearance of CXCR3 on the cell surface occurred relatively slowly, and only around 80% recovery of basal CXCR3 surface levels was observed within 180 minutes incubation after stimulation with agonist. This slow rate of CXCR3 cell surface recovery may suggest involvement of slow recycling pathway as is the case for class B GPCR such as vasopressin type to receptor (Innamorati et al., 2001; Le Gouill et al., 2002) or its degradation and reappearance to the membrane due to de novo synthesis receptor proteins. To examine if protein synthesis plays role in CXCR3 surface recovery in T cells, we preincubated cells with 10 μg/mL of cycloheximide for 1h prior to induction of internalization with CXCL11 and left them to recover in the presence of cycloheximide. Treatment of T cells with cycloheximide significantly inhibited CXCR3 cell surface replenishment suggesting that CXCR3 surface recovery occurs at least partially due to synthesis of the new receptor molecules. Alternatively involvement of a chaperon protein could be possible. This finding is opposite from these reported on other chemokine receptors such as CXCR6 and CCR5 (Mueller et. al 2002; Le Gouill et. al 2002).

To address the involvement of endocytic recycling in CXCR3 surface recovery, we used the fungal metabolite Brefeldin A (BFA), a known PLD inhibitor, which has been shown to interfere with the function of Golgi apparatus therefore, to inhibit recycling. Internalization of CXCR3 was initiated as before and T cells were incubated for recovery, as described, in the presence or absence of BFA. This should allow normal internalization, and BFA should only affect the recovery phase. Due to inhibitory effect of BFA on surface CXCR3 reappearance (Figure 3.9C), it was concluded that the recovery of the receptor is dependent of the Golgi function.

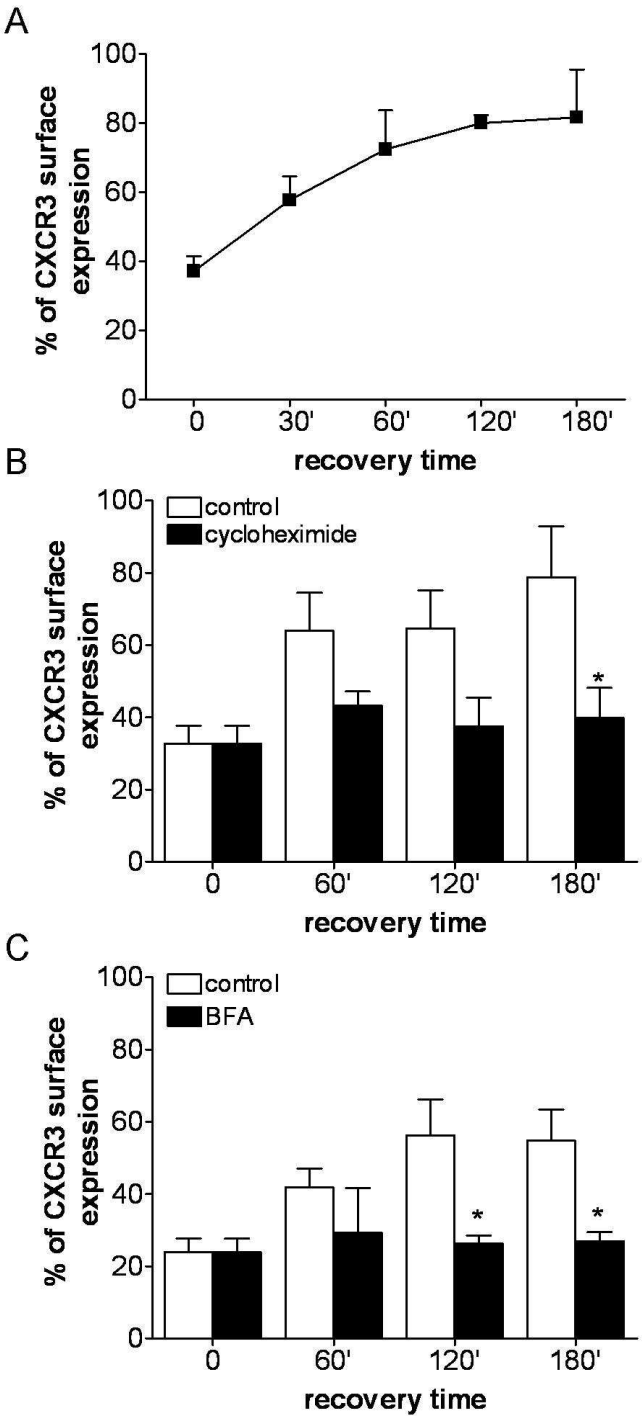


Figure 3.9 Recovery of CXCR3 surface expression on T lymphocytes after agonist stimulation. (A), Reappearance of CXCR3 surface expression after stimulation with agonist. (B), effect of protein synthesis inhibition on the surface CXCR3 recovery. (C), effect of BFA on CXCR3 surface recovery. Day 9-12 SEB/IL-2 activated T lymphocytes were exposed to 30 nM of CXCL11 for 5 min. Alternatively T cells were incubated with 10 μg/mL of cycloheximide for 1 hour before exposure to agonist and also added during recovery period. BFA (10 μg/mL) was added during recovery phase. Samples were then washed 3 times in HBSS buffer prewarmed at 37°C to remove unbound chemokine and resuspended in serum free RPMI-1640 medium. CXCR3 surface expression was determined at times 0, 30, 60, 120 and 180 mins after chemokine removal by washing. Data represent the mean +/- SEM of at least three different experiments. Data was analysed using student t test. *, $p<0.05$ compared with time matched control.

3.3 Analysis of biochemical signals mediated via CXCR3 receptor

Binding of agonist to CXCR3 results in cellular responses such as integrin activation, actin reorganization and directional migration. In T lymphocytes, stimulation of CXCR3 by its agonists leads to elevation of intracellular calcium (Rabin et al., 1999) and activation of phosphoinositol-3-kinase (PI3K) and Akt-dependent signaling as well as the p44/p42 mitogen-activated protein kinase (MAPK) pathways (Smit et al., 2003). CXCR3 activation have also been shown to induce rapid tyrosine phosphorylation of several proteins including Zeta-associated protein of 70 000 MW (ZAP-70), linker for the activation of T cells (LAT) and phospholipase-C-γ1 (PLCγ1) (Dar and Knechtle, 2007). In this study we focused on further examination of several aspects of cellular signalling via CXCR3, including intracellular calcium elevation and phosphorylation of certain downstream effectors including p44/p42 Mitogen Activated Protein Kinase (Erk1/2) and PI3K/Akt dependent pathways.

The aim of this study was examine the role of CXCL4 and CXCR3-B in biochemical responses in previously activated T cells and to re-investigate the role of chemokines CXCL9, CXCL10 and CXCL11 in T cells signalling. To achieve this, the biochemical responses induced by CXCL4 such as calcium elevation and phosphorylation of chemokine receptor downstream effectors such as Akt or MAPK were examined and compared with responses mediated by CXCR3 agonists. In order to further examine CXCL4-induced responses we utilized pertussis toxin to establish coupling of CXCR3-B to Gi protein and introduced to the study small CXCR3 antagonists to re-investigate the connection between CXCL4 and CXCR3. Finally we investigated the effect of CXCR3 and CXCL4 chemokines stimulation on Akt downstream effector GSK3β protein and mTOR pathway compartment S6. Phosphorylation of S6 is also PI3K-dependent and therefore can be used as an indirect measurement of PI3K activity.

Mitogen-activated protein kinases (MAP kinases) family of serine/threonine kinases comprises of multiple members among which the best characterized are ERK, JNK and the p38 MAPK. The activation of MAPK occurs via subsequent phosphorylation of upstream kinases which are differentially regulated by G-proteins, scaffold, adaptor, substrates and regulator proteins. MAP kinases are

involved in multiple cellular processes such as proliferation and differentiation, development, inflammatory response and apoptosis (Weston and Davis, 2007).

Protein kinase B (PKB/Akt), is a serine/threonine kinase that is the best characterized downstream effector of PI3Kγ and p85/p110 and therefore can be used as an indirect method of measuring of PI3K activity. Akt is a key mediator in many cellular processes including growth factor-induced cell survival and protection against c-myc induced cell death (Dudek et al., 1997; KauffmanZeh et al., 1997; Kulik et al., 1997).

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase implicated in cellular processes including determination of cell fate and differentiation and its action usually inhibits the target proteins (as is the case of glycogen synthase or NFAT) (Doble and Woodgett, 2003; Embi et al., 1980; Neal and Clipstone, 2001). GSK3 is inhibited by Akt phosphorylation, therefore acting as an activator of many signalling pathways blocked by GSK3 (such as dephosphorylation of NFAT). In addition GSK3 have been found to be phosphorylated in vitro by p70 S6 kinase which is directly activated by MAP kinases, therefore agonists of this cascade inhibit GSK3 function (Sutherland et al., 1993; Stambolic and Woodgett, 1994).

The ribosomal protein S6 is phosphorylated by S6 kinases which are tightly controlled by the mTOR (mammalian target of Rapamycin). There are several lines of evidences supporting the fact that S6 plays the role in chemotaxis (Richardson et al., 2004) but despite the fact of clear role mTOR plays in neutrophil migration its role in T cell is still to be elucidated.

3.3.1 CXCR3 agonists and CXCL4 stimulate elevation of intracellular calcium

Due to the limitations of existing CXCR3-B antibodies, we utilized CXCL4 as a indirect marker to establish whether CXCR3-B was functionally expressed on T cells, since this has been reported to activate CXCR3-B but not other CXCR3 isoforms. First the ability of CXCL4 to induce elevation of intracellular calcium in comparison with CXCL9, 10 and 11 responses was tested. Previously activated T lymphocytes were therefore stimulated with various concentrations of CXCR3

agonists or CXCL4 and maximal responses for each concentration were plotted to create concentration response curves (Figure 3.10A). High, micromolar concentration of CXCL4 was required to induce response comparable to CXCL9 and CXCL10. In contrast CXCL11 mediated more robust response with the same as CXCL9 and CXCL10 concentrations. In kinetics study we stimulated T cells with 10 nM concentration of each CXCR3 antagonists and 1 μM of CXCL4. Responses induced by CXCL11 were about 2-fold higher than CXCL9, CXCL10 and CXCL4 (Figure 3.10B).

3.3.2 Activation of PI3K/Akt and p44/p42 Mitogen Activated Protein Kinase pathways by CXCR3 agonists and CXCL4

It has been previously shown that CXCR3 agonists activate PI3K/Akt and p44/p42 MAPK however the role of CXCL4 in stimulation of these pathways in T cells is to be elicited (Smit et al., 2003). Therefore to address this issue, further study of biochemical signals activated in response to these chemokines was performed. Previously activated with SEB and expanded in IL-2 day 9-12 T lymphocytes were stimulated with CXCR3 agonists and CXCL4, and effect each chemokine on PI3K/Akt and p44/p42 MAPK (Erk1/2) pathways was assessed by immunoblotting (Figure 3.11). Densitometry was also used to quantify detected responses. The CXCR3 agonists CXCL9, CXCL10 and CXCL11 (0.3-100 nM) as well as CXCL4 (0.3-100 nM) stimulated PI3K/Akt-dependent signalling as measured by phosphorylation of Akt/PKB at Ser473. In addition, all agonists stimulated p44/p42 MAPK phosphorylation at positions Thr 202 and Tyr 204. While the responses to CXCL9, CXCL10 and CXCL11 were concentration-dependent, the responses to CXCL4 were atypical and did not exhibit obvious concentration-dependency (Figure 3.11A). The Akt and p44/p42 MAPK phosphorylation responses to all agonists occurred rapidly and transiently with complete attenuation of responses after 10 minutes stimulation with each agonist. However, CXCL11-stimulated Akt and p44/p42 MAPK phosphorylation was detectable earlier (within 30 seconds) and exhibited more sustained kinetics in comparison to the other agonist responses (Figure 3.11 B). Furthermore, as shown by Figure 3.11C, phosphorylation of Akt induced by CXCL4 as well as CXCL11 (which was used as a positive control) was inhibited by pre-incubation with micromolar concentration of LY294002 (10 μM). Despite the ability of CXCL4 to induce phosphorylation of

p44/42 MAPK and Akt, these responses were generally at lower strength in comparison to CXCR3 agonist (blots required longer exposure time). Moreover there was noticeable variability in the results obtained with CXCL4. Examples of these variations are illustrated in Figure 3.11 (panels showing responses obtained for CXCL4- A, B and B'). These differences were exhibited in the strength of signal, and could result from variations between donors (e.g. differential expression of CXCR3-B). Further reasons for such variability will be highlighted in discussion.

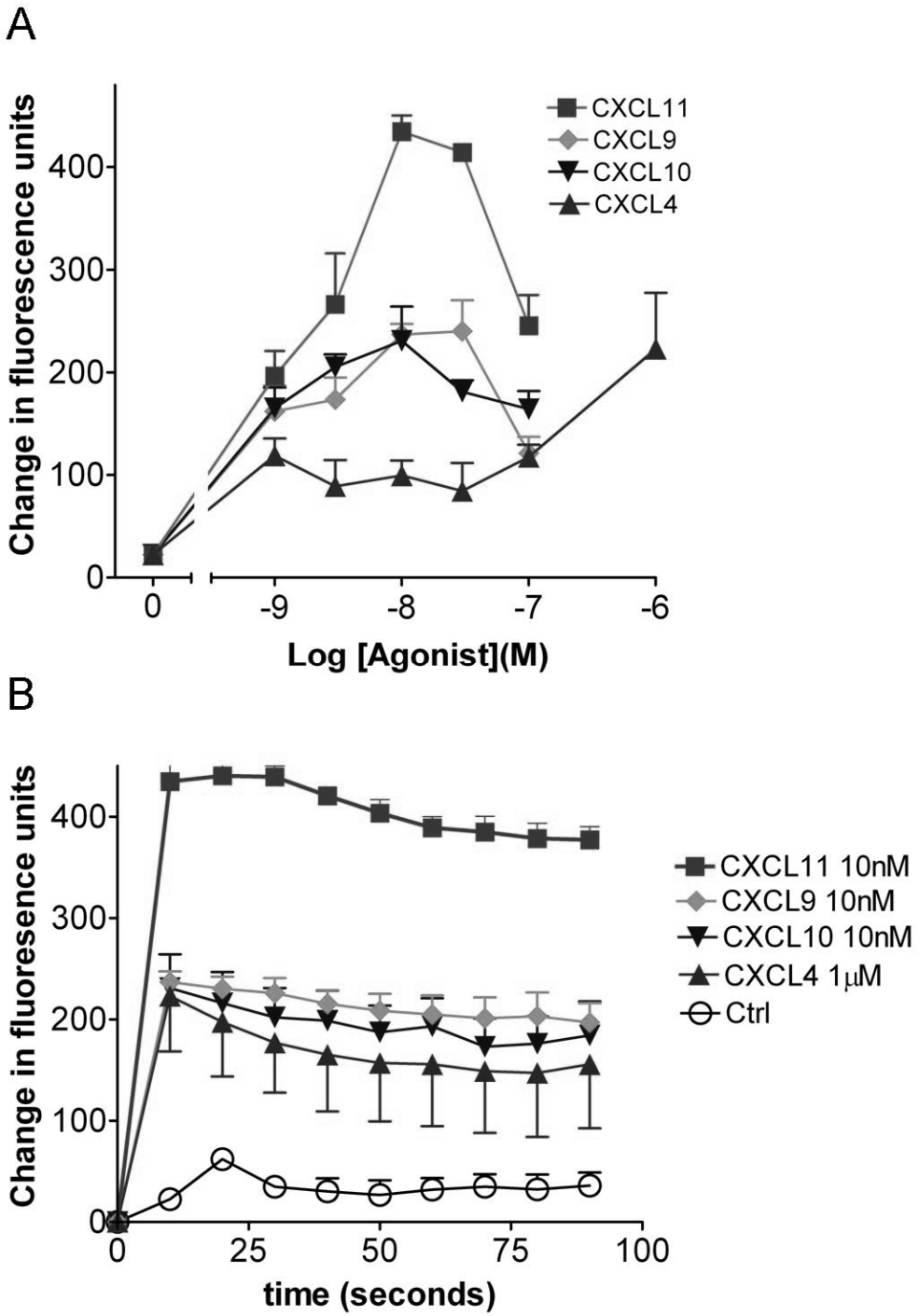


Figure 3.10 Intracellular calcium flux obtained from activated T lymphocytes in response CXCL9, CXCL10, CXCL11 and CXCL4. (A) Concentration response of intracellular calcium flux induced in T cells. Day 9-12 SEB/IL-2 activated T cells were washed, resuspended in buffer containing calcium as described in *Materials and Methods*, and loaded with 5 μM Fluo-4. Cells were stimulated with different concentrations of each agonist (0.3-300 nM or 0.3-1 μM for CXCL4). Changes in fluorescence were measured using fluorescence reader. Peak response from each stimulation was taken to created a concentration-response curve. (B) Chemokine-induced time-dependent mobilisation of intracellular calcium in T cells. Activated T cell were assayed as above, stimulated with single concentration of CXCL4, CXCL9, CXCL10 and CXCL11, and induced responses were observed against time. Presented data are representative for three independent experiments.

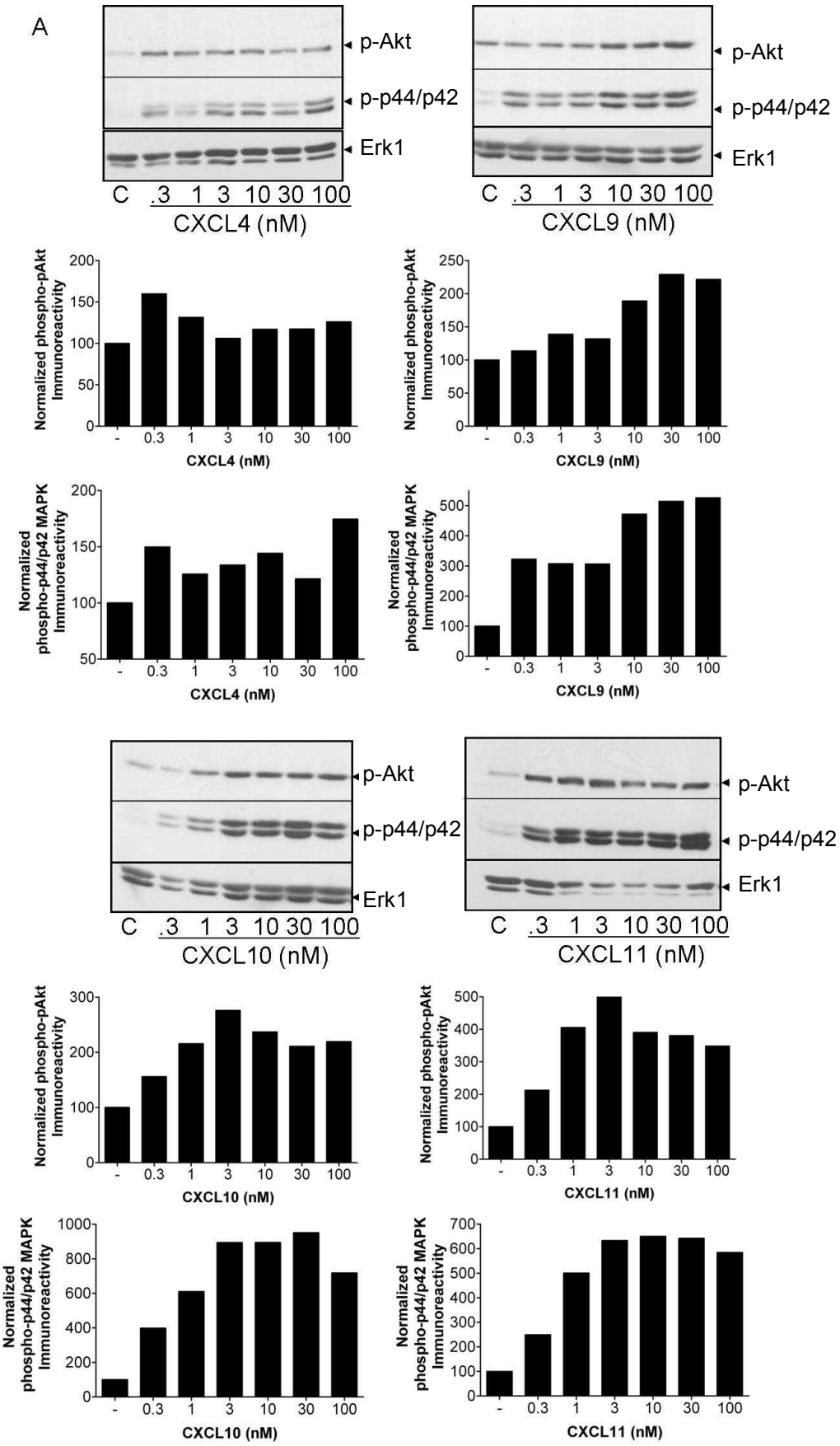


Figure 3.11 CXCR3 agonists and CXCL4-stimulated activation of PI3K/Akt and p44/p42 MAPK in human T cells. PTO for figure legend.

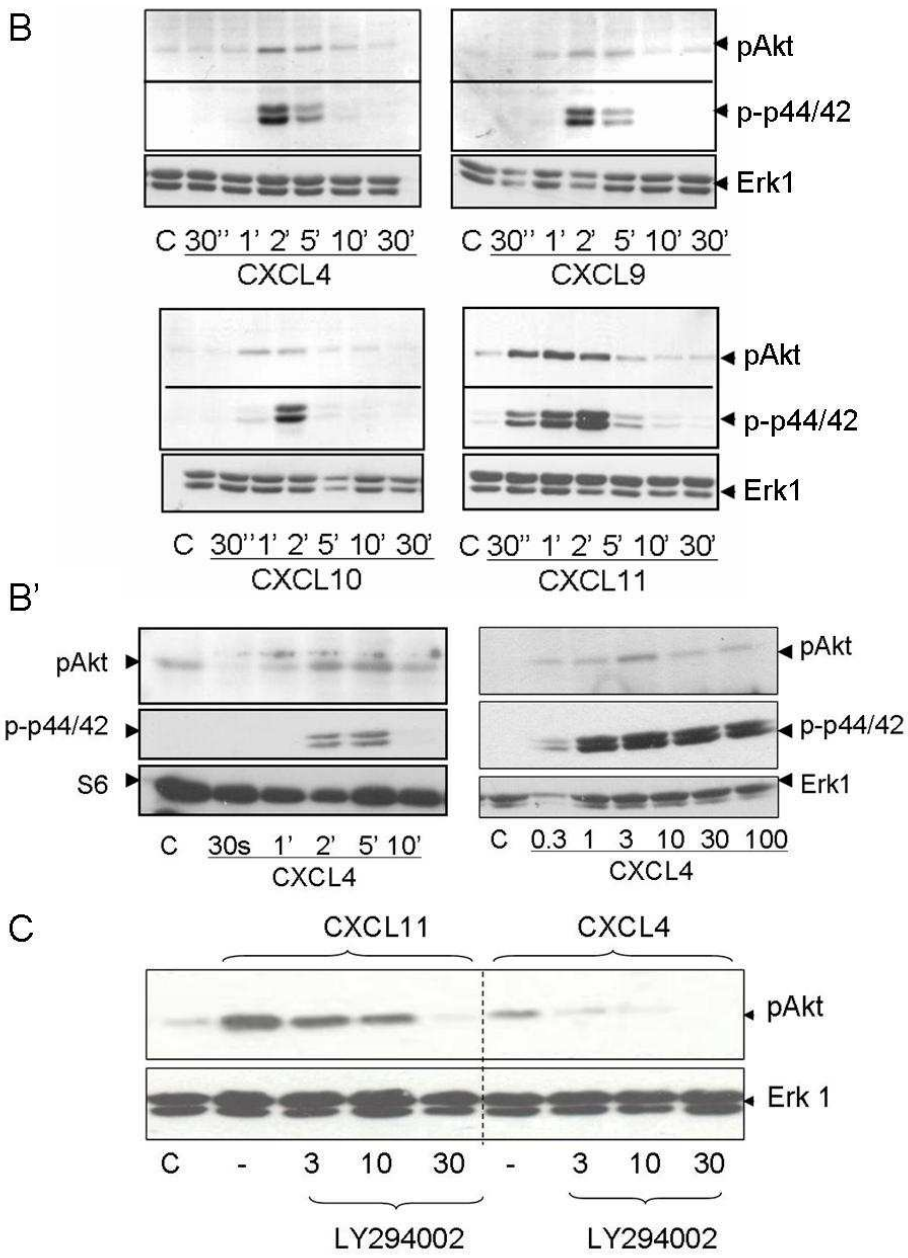


Figure 3.11 CXCR3 agonists and CXCL4-stimulated activation of PI3K/Akt and p44/p42 MAPK in human T cells. (A), Concentration dependent and (B), time dependent phosphorylation of Akt and p44/p42 MAPK. (C), Effect of LY294002 (10 μM) on CXCL11 and CXCL4 –induced Akt and p44/42 MAPK phosphorylation. Day 9-12 SEB/IL-2 activated T cells were washed twice in RPMI-1640 media, resuspended at $2x10^6$ cells/mL and stimulated with 0.3-100 nM concentration of CXCL9, 10, 11 or CXCL4 for 2 min or with 1 nM of each agonist for required period of time. Control samples were stimulated with media. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt ab with the affinity for the active Ser^{473}-phosphorylated form of Akt or p44/p42 MAPK ab with the affinity for the active Thr^{202}/Tyr^{204} form of Erk. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 (or S6) antibody to verify equal loading and efficiency of the protein transfer. In concentration response study p44/p42 MAPK and Akt phosphorylation (from individual experiments) was quantified by chemiluminescence and corrected for total Erk 1 expression on stripped blots. Presented data are representative for at least three independent experiments using blood from different donors.

3.3.3 Effect of PTX on CXCR3-mediated signalling

The CXCR3 receptor has been previously shown to be coupled to pertussis toxin (PTX)-sensitive Gi protein, yet the CXCR3-B variant has been reported to be pertussis toxin-resistant and possibly Gs coupled (Lasagni et al., 2003). Therefore, for further investigation of signaling through CXCR3-A and CXCR3-B, T lymphocytes were pre-treated for 16 h with 10 ng/ml of pertussis toxin and its effect on Akt and Erk1/2 activation was determined. As expected, pre-treatment with pertussis toxin completely inhibited CXCL9-, CXCL10- and CXCL11-induced phosphorylation of both Akt as well as p44/p42 MAP kinases (Figure 3.12). Given the previous reports that CXCL4 mediated effects were via pertussis toxin-resistant CXCR3-B, it was surprising to find that CXCL4-induced phosphorylation of Akt and p44/p42 was inhibited by pertussis toxin in our experimental system.

3.3.4 Inhibition of CXCR3 signalling by small molecule CXCR3 antagonists

In order to further investigate whether the biochemical signalling elicited by CXCL4 was indeed mediated by a CXCR3 receptor, we utilized the small non-peptide CXCR3 antagonists namely, T487 and NBI-74330 compounds. First, we verified that these inhibitors targeted CXCR3 by examining their effect on CXCL11-induced phosphorylation of Akt and p44/p42 MAPK. Effect of oxidized metabolite of NBI-74330 was also tested (Figure 3.13A). 1 μM of T487 compound was required to completely inhibit CXCL11-triggered signals (Figure 3.13 A, left panel). In contrast, NBI-74330 and its metabolite were much more potent and 100 and 1000 times lower concentrations, respectively were sufficient to blocked signal mediated by CXCL11 to basal level (Figure 3.13A, middle and right panels). We then examined the effects of single concentrations of CXCR3 antagonists on Akt and p44/p42 MAPK phosphorylation induced by CXCL9, CXCL10 and CXCL4 as well as CXCR4 agonist – CXCL12 (Figure 3.13 B and C). Both, T487 and NBI-74330 at the respective concentrations 1 μM and 100 nM, inhibited biochemical signal mediated by CXCL9 and CXCL10. In contrast these antagonists had no effect on neither CXCL4 nor CXCL12- induced responses.

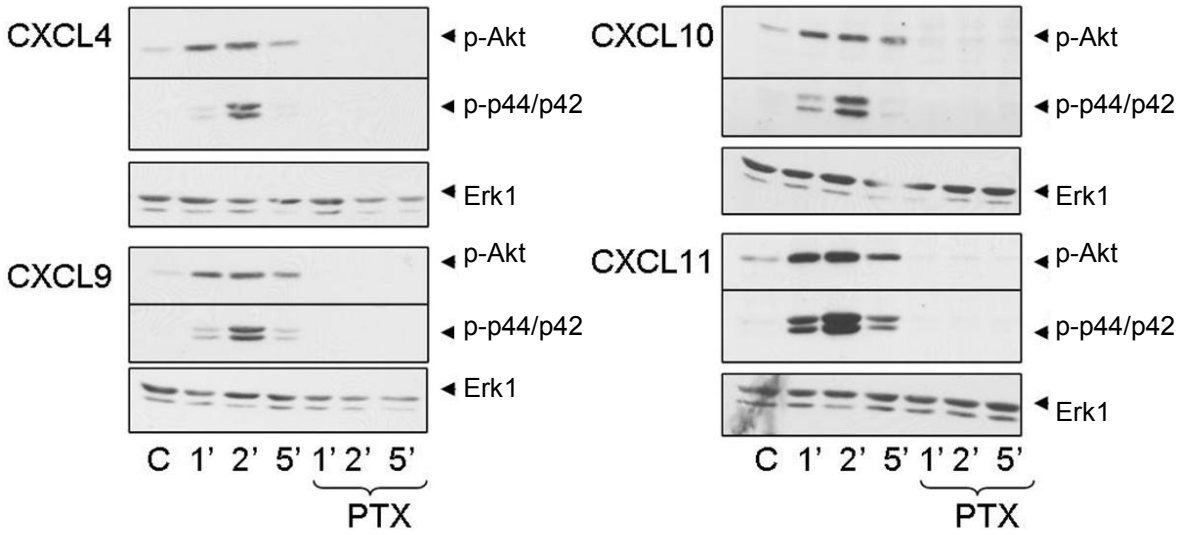


Figure 3.12 Signalling mediated by CXCR3 agonist and CXCL4 is pertussis toxin sensitive. Day 9-12 SEB/IL-2 activated T cells were incubated with 10 ng/mL of PTX for 16 hours or left untreated and stimulated with 1 nM of each agonist for required periods of time. Control samples were stimulated with media. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt ab with the affinity for the active Ser^{473}-phosphorylated form of Akt or Erk1/2 (p44/p42) ab with the affinity for the active Thr^{202}/Tyr^{204} form of Erk. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. Presented data are representative for at least three independent experiments using blood from different donors.

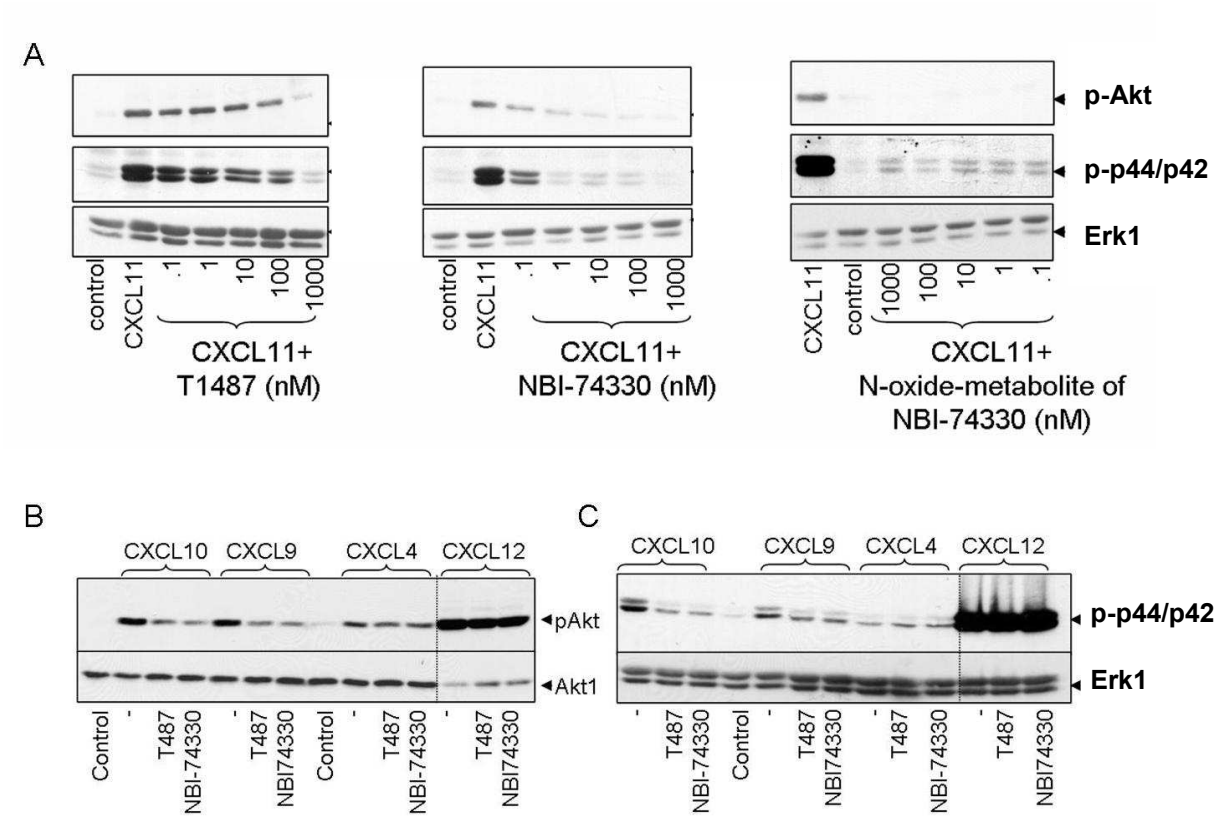


Figure 3.13 Biochemical signalling mediated by CXCR3 agonists but not CXCL4 is sensitive to treatment with small CXCR3 antagonists. Day 9-12 SEB/IL-2 activated T cells were pre-incubated for 30 min with (A), 0.1-1000 μM of T487, NBI-74330 or its N-oxidised metabolite prior to stimulation with 1 nM CXCL11 or (B, C), 1 μM of T487 or 100 nM of NBI-74330 prior to stimulation with 1 nM of CXCL10, -9, -4 or -12 or left untreated (control). Samples were lysed and lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt ab with the affinity for the active Ser^{473}-phosphorylated form of Akt or Erk1/2 (p44/p42) ab with the affinity for the active Thr^{202}/Tyr^{204} form of Erk. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. Presented data are representative for at least three independent experiments using blood from different donors.

3.3.5 Phosphorylation of GSK3β and S6 protein by CXCL4 and CXCR3 chemokines

In this part of the study another read-out of PI3K/Akt signalling pathway was assessed, namely the activation of its down stream effectors glycogen synthase kinase 3β (GSK3β) and ribosomal protein 6 (S6). The relative potencies of CXCL4 and CXCR3 agonists CXCL9, CXCL10 and CXCL11 to phosphorylate GSK3β protein which results in its inhibition were examined. Exposure of previously activated human T cells to increasing concentrations of CXCL4 for 2 minutes led to the minor phosphorylation of endogenous GSK3β with maximum signal observed at 3 nM of the chemokine, as assessed using an antibody recognizing the phosphorylated form of GSK3β (Figure 3.14A). Stimulation with CXCR3 chemokines induced concentration-dependent phosphorylation of GSK3β and the strongest signal was detected at the highest 100 nM concentration of CXCL9 and CXCL11 and 30 nM of CXCL10. Incubation of T cells with 1 nM concentration of each chemokine resulted in time-dependent increase in GSK3β phosphorylation. A maximal transient increase in phosphorylation of GSK3β was observed after 5 minutes exposure with CXCL11 and after 2 minutes exposure with CXCL10 (Figure 3.14B, right panel). The other CXCR3 agonist CXCL9 as well as CXCL4 also induced time dependent increase in phosphorylation of GSK3β, and maximal response occurred after 1 minute of the stimulation (Figure 3.14B, left panel).

The ability of CXCL4 and CXCR3 agonists to induce the phosphorylation of ribosomal protein S6 was also examined. A maximal increase in S6 phosphorylation was similar for all chemokines used in the experiment and was observed after 5 minutes stimulation (Figure 3.14C), suggesting that CXCR3 agonists and CXCL4 exhibit similar abilities to activate this protein and in turn induce increase of translation.

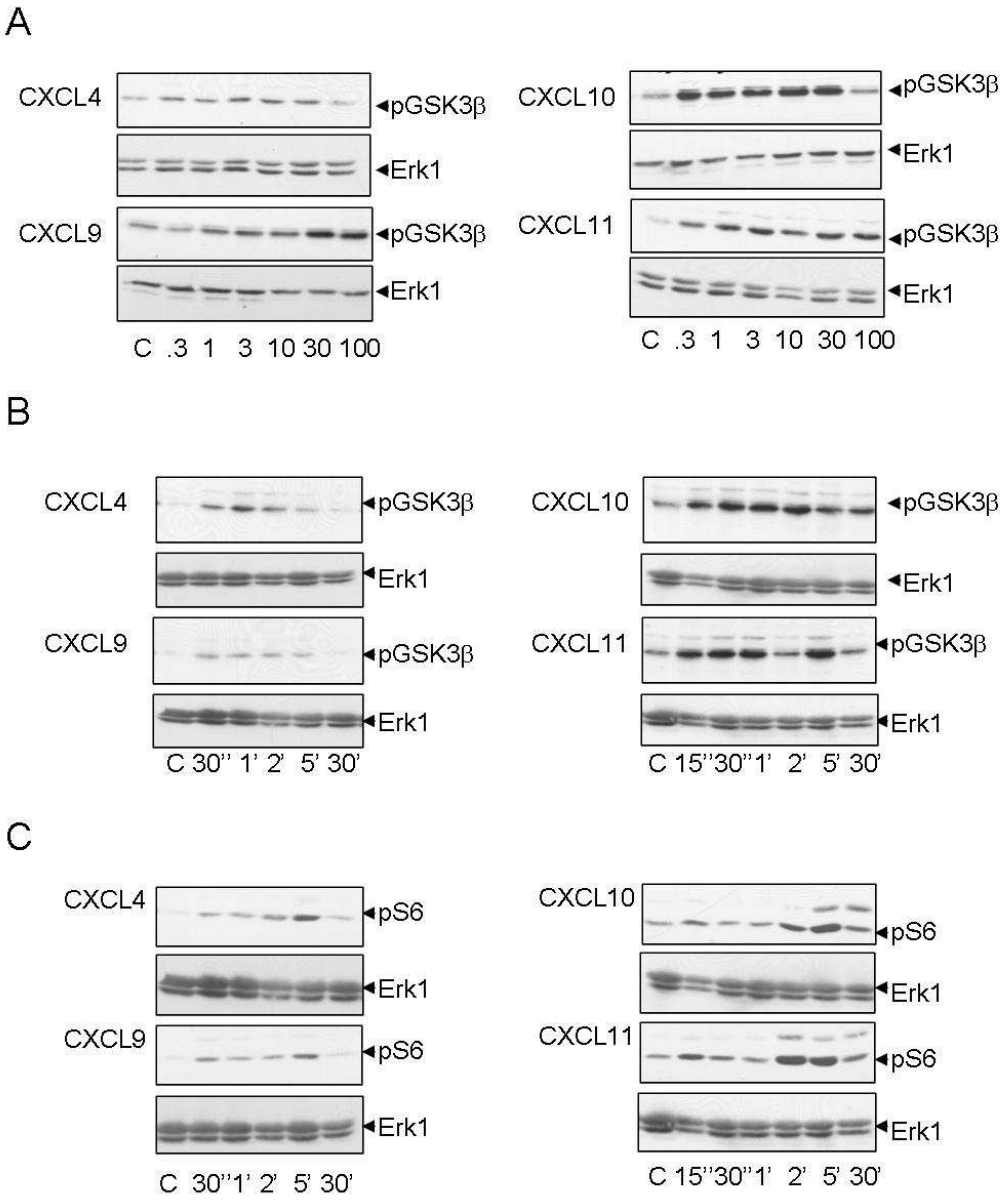


Figure 3.14 CXCR3 agonists and CXCL4-stimulated phosphorylation of GSK3β and S6 in human T cells. Day 9-12 SEB/IL-2 activated T cells were washed twice in RPMI-1640 media, resuspended at $2x10^6$ cells/mL and stimulated (A), with 0.3-100 nM concentration of CXCL9, 10, 11 or CXCL4 for 2 min or (B and C), with 1 nM of each agonist for required periods of time. Control samples were stimulated with media. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific GSK3-β ab with the affinity for the active Ser^9 (A and B) or with a phospho-specific S6 ab with the affinity for the active $Ser^{235/236}$-phosphorylated form of S6 (C). Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. Presented data are representative for three independent experiments using blood from different donors.

3.4 Analysis of CXCR3 – mediated chemotaxis in human T lymphocytes

Chemokines are crucial components of immunosurveillence and the immune response against pathogens, coordinating directional leukocyte migration and trafficking of immune cells under homeostatic and inflammatory conditions. CXCL9, 10 and 11 are an inflammatory chemokines and their expression is up-regulated during a variety of inflammatory disorders possibly due to increased secretion of interferon-γ. Similarly, CXCR3 expressing effector T cells has been localized in inflammatory lesions suggesting the importance of CXCR3 – CXCR3 agonists interactions in the recruitment of effector T cells to the site of inflammation, resulting in T-cell-mediated inflammatory responses (D'Ambrosio et al., 2003; Proudfoot, 2002; Viola and Luster, 2008).

CXCR3-B is an alternatively spliced variant of CXCR3 and it was found to mediate apoptotic but not chemotactic signal to micromolar concentrations of CXCL4 in microvascular endothelial cell (Lasagni et al., 2003). CXCL4, previously identified as a ligand binding to CXCR3-B – stored and released from platelets in micromolar concentrations upon platelets activation (Dawes et al., 1983; Files et al., 1981). Recent findings report the role of platelets and chemokines CCL5 and CXCL4 in stimulation of monocyte arrest upon atherosclerotic endothelium (Schober et al., 2002; von Hundelshausen et al., 2001; von Hundelshausen et al., 2005). Moreover, a clinical study by Pitsilos at al presents evidence for the involvement of CXCL4 in early and symptomatic atherosclerosis (Pitsilos et al., 2003).

Excessive recruitment of leukocytes is a characteristic feature of inflammation. Most anti-inflammatory therapies target leukocytes which have already migrated to the site of inflammation. The example of such successful therapy may be the interferon-β treatment in patients with multiple sclerosis. Prevention of excessive migration of leukocytes by antagonizing chemokine receptors such as CXCR3 might be a more successful therapeutic approach in treatment of inflammatory disorders (Proudfoot, 2002). Moreover the potential specificity of action of chemokine receptors makes this class of molecules particularly attractive as a drug targets. Therefore antagonism of a given chemokine receptor could have a specific action and avoid deleterious side effects (D'Ambrosio et al., 2003). As

stated previously, CXCR3 and its agonists are involved in variety of inflammatory diseases making this receptor an attractive target in designing of novel anti-inflammatory therapeutics. It is important therefore, to study and better understand chemokine mediated migration of immune cells in vitro. Moreover, an in vitro model of lymphocyte migration can be a useful tool in characterization of an effect of chemokine receptor antagonism.

The aim of this part of the project was to re-investigate chemotactic responses induced by CXCR3 agonists; CXCL9, CXCL10 and CXCL11 and clarify the role of CXCL4 in chemotaxis of previously activated T lymphocytes.

3.4.1 CXCR3 agonist and CXCL4 induce actin polymerisation in activated T cells

First, the ability of CXCL4 and CXCR3 agonists to induce F-actin polymerisation of activated T cells was tested. Flow cytometry technique was used to measure increase in TRITC-conjugated phalloidin binding, that stabilizes the filaments against depolymerization, following agonist stimulation. Exposure to 1 nM concentration of CXCL4 resulted in increase of actin polymerisation with maximum effect after 1-2 minute of incubation (Figure 3.15). Similarly rapid and transient effect on actin polymerisation was observed following 30-60 seconds stimulation with CXCR3 agonists. Longer, 5 minutes incubation with each agonist led to decrease of actin polymerisation below basal level.

3.4.2 CXCR3 agonists but not CXCL4 stimulate migratory responses in activated T cells

As we were able to induce F-actin polymerisation in response to CXCL4 in T cells and this induction was comparable to that induced by CXCR3 agonists, we chose to further investigate the role CXCL4 in mediating chemotaxis of T cells. All CXCR3 agonists induced migratory responses from T lymphocytes activated either with SEB/IL-2 or CD3/CD28 antibodies coated beads (Figure 3.16A and B left panels, respectively). CXCL11 appeared to be the most potent and efficacious chemoattractant and induced a typical bell-shaped migratory response with optimal chemotaxis at 1-10 nM. Other CXCR3 agonists, CXCL9 and CXCL10 were less efficacious and had lower potency with optimal chemotactic responses at 10-

100 nM concentration. In contrast we were not able to detect any migratory response following stimulation with CXCL4 in activated T cells (Figure 3.16 A and B right panels) at the concentration range inducing biochemical signalling (1 nM – 1 μ).

Because of the lack of effect of CXCL4 on T cells chemotaxis we then decided to examine how CXCR3 antagonists affects chemotaxis mediated by CXCL9, CXCL10 and CXCL11. Experiments were started by examining the effect of T487, NBI-74330 and N-o-metabolite of NBI-74330 compounds on migratory responses using CXCL11 as a chemoattractant. All three antagonists inhibited directional migration to CXCL11 in a concentration-dependent manner (Figure 3.17A). T487 exhibited the lowest potency with IC50's of 69 nM whereas NBI-74330 and its oxidised metabolite were more potent with following IC50's, 2.3 nM and 0.53 nM- respectively (Table showing IC50 values is placed in Appendix chapter). We then tested the effects of single concentrations of CXCR3 antagonists on CXCL9 and CXCL10 – induced chemotaxis of T cells. Migratory responses to CXCL9 and CXCL10 were inhibited by both T487 and NBI-74330 at concentrations known to elicit close to maximum inhibition of CXCL11 responses (Figure 3.17B). Neither T487 nor NBI-74330 had any effect on responses to the CXCR4 agonist CXCL12 which was used as negative control (Figure 3.17B).

3.4.3 Effect of PIKfyve inhibition of CXCL11 and CXCL12-mediated chemotaxis of T cells

Due to previous observations that treatment with PIKfyve inhibitor had an effect on agonist-induced CXCR3 internalization and signalling, we decided to investigate its effect on CXCR3-mediated chemotaxis of activated T cells. Therefore prior to assaying chemotaxis towards CXCL11, T cells were incubated in the presence of 1 or 10 μM of PIKfyve inhibitor YM203616. Treatment with the higher concentration of the compound only partially inhibited CXCL11- induced directional migration of T cells however this effect did not reach statistical significance (Figure 3.18). Moreover YM203616 had no effect on chemotaxis towards CXCL12. These results indicate that activity of PIKfyve is not involved in T cell directional migration.

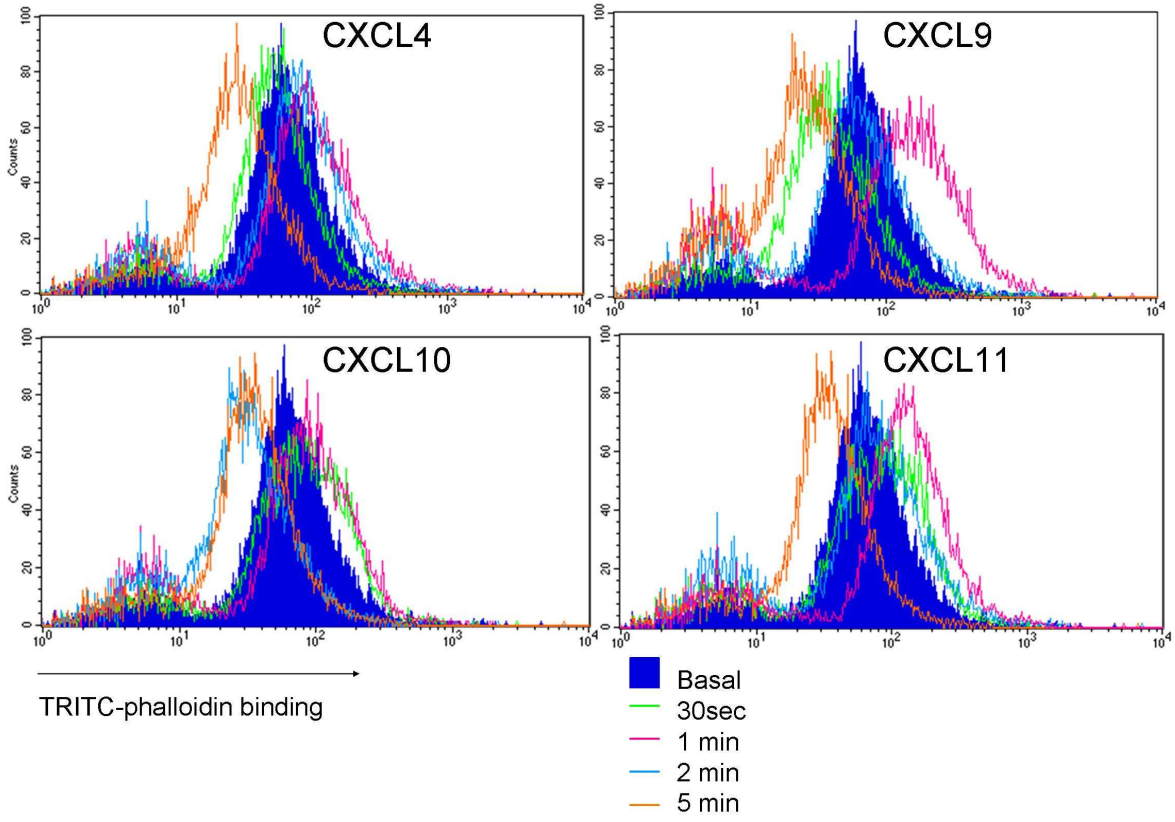


Figure 3.15 Agonist-induced F-actin polymerisation in human T lymphocytes. Day 9-12 SEB/IL-2 activated T cells were stimulated for 30 seconds, 1, 2 or 5 minutes with 10 nM of CXCL4, 9, 10, or 11, fixed in 4% para-formaldehyde, permeabilized and stained with TRITC-labelled phalloidin. F-actin polymerization was monitored by FACS as described in *Materials and Methods* chapter. Data shown is representative for two other experiments using cells isolated from different donors.

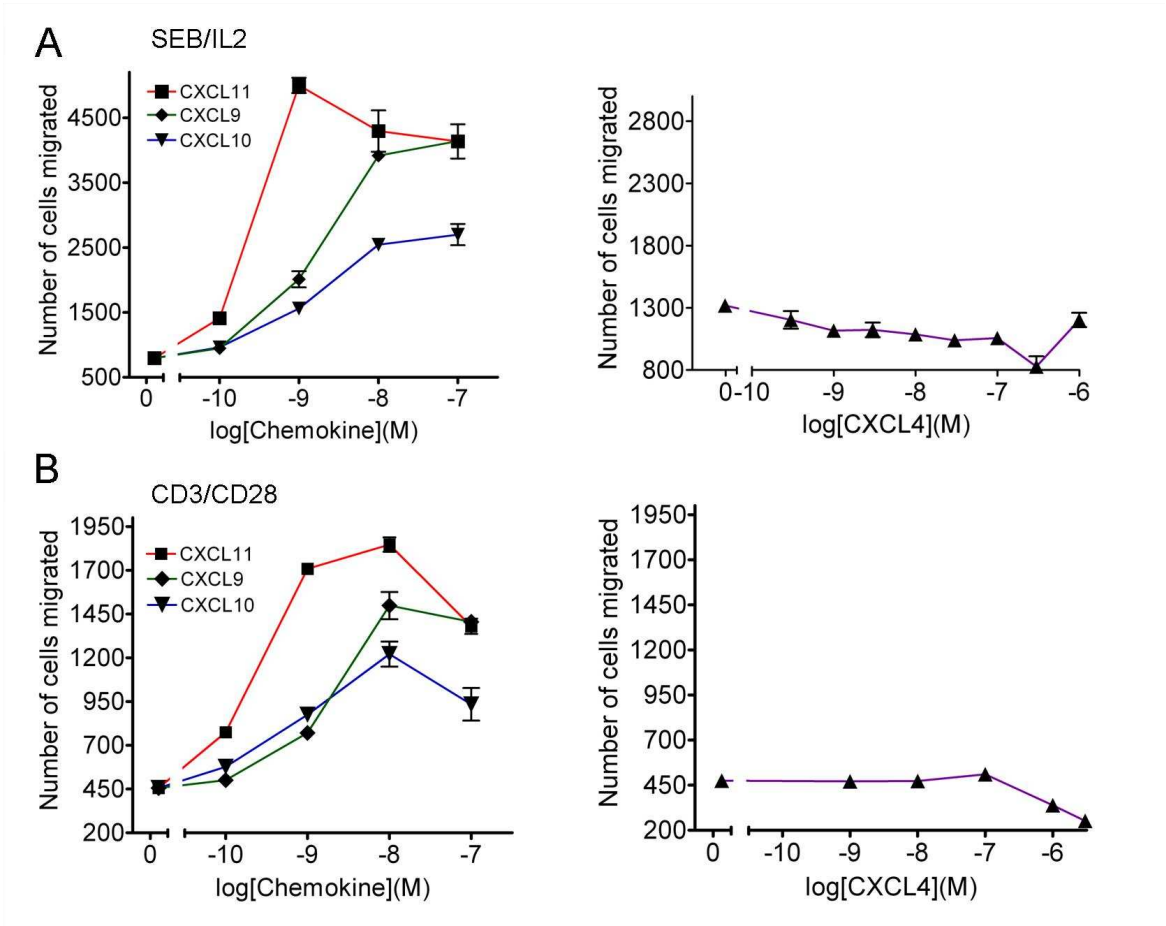


Figure 3.16 Activated human T lymphocytes are migratory responsive to CXCR3 agonists but not to CXCL4. (A), chemotaxis of day 10, SEB/IL-2 activated T cells to increasing concentrations of CXCL9 (♦), CXCL10 (▼) and CXCL11 (■) (left panel) or CXCL4 (▲) (right panel). (B), chemotactic responses of day 10 T cells, activated with CD3/CD28 antibodies-coated beads to CXCL9, 10 and 11 (left panel) or CXCL4 (right panel). Previously activated T cells were washed, resuspended at 3.2 x 10^6 / mL and placed (25μL per well) on the filter membrane above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* T cells migrated to increasing concentrations of chemokines (0.1-100 nM of CXCL9, 10 and 11 or 0.1- 3 μM of CXCL4). Presented data, expressed as number of migrated cell (mean +/- SD)is taken from a single experiment with triplicates and is representative to at least three different experiments using cells isolated from different donors.

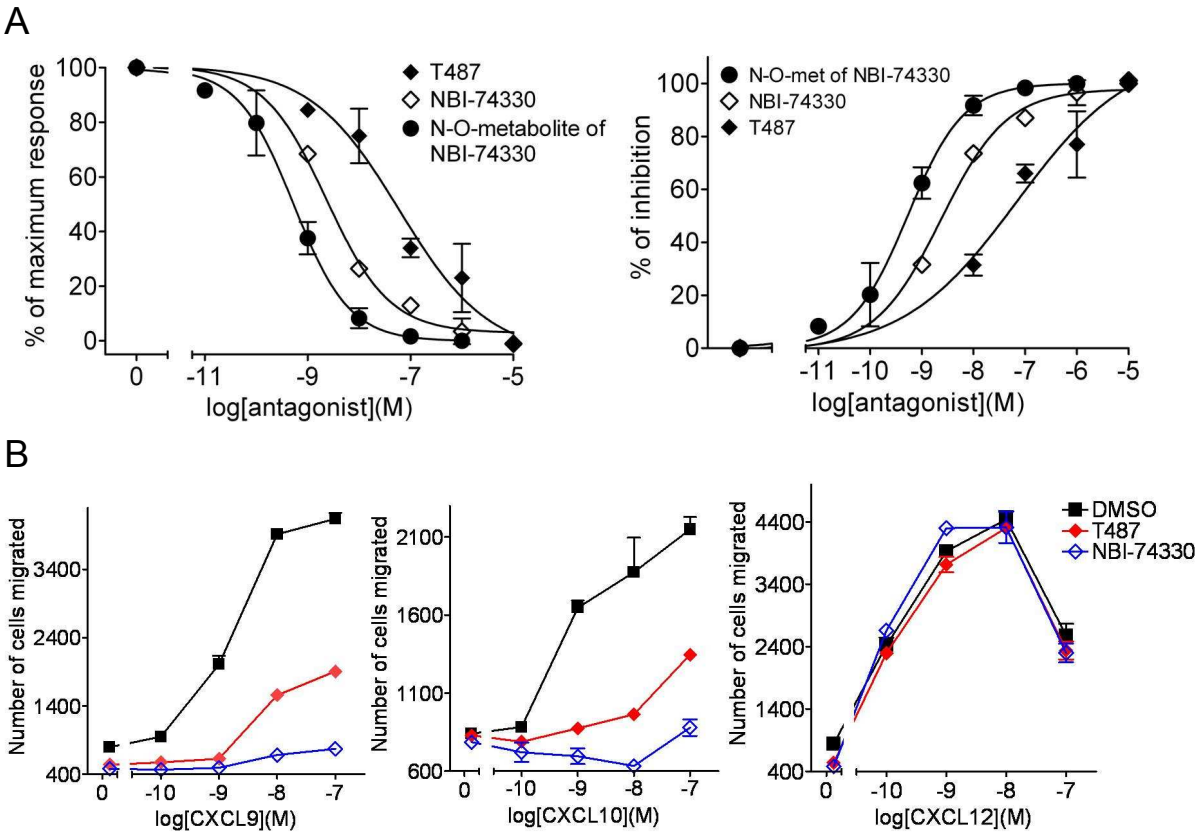


Figure 3.17 CXCR3 antagonists inhibit CXCR3-mediated chemotaxis of activated human T lymphocytes. Previously activated T cells were washed, resuspended at 3.2 x 10^6 / mL and incubated with appropriate concentrations of CXCR3 antagonists. Cells were then placed (25μL per well) on the filter membrane above lower chambers containing 1 nM of CXCL11 (A), indicated chemokine solutions (B) or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* Presented data, expressed as number of migrated cell (mean +/- SD)is taken from a single experiment with triplicates and is representative to at least three different experiments using cells isolated from different donors.

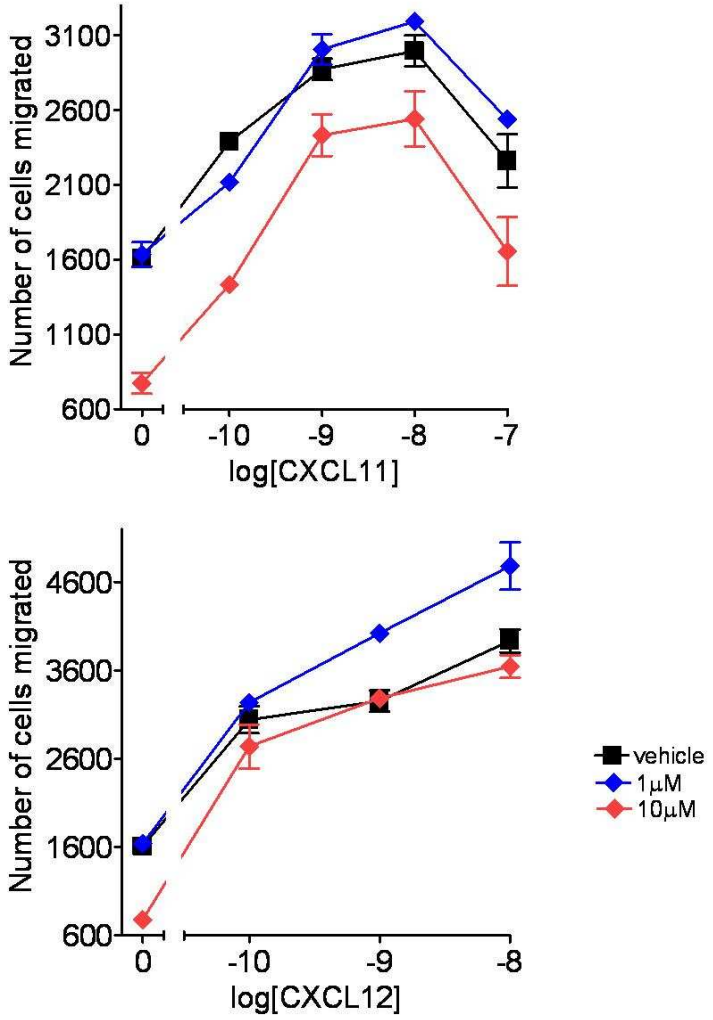


Figure 3.18 Effect of PIKfyve inhibition on CXCL11 and CXCL12 mediated chemotaxis in T cells. Day 9-12 SEB/IL-2 activated T cells were washed, resuspended at 3.2 x 10^6 / mL and incubated with appropriate concentrations of PIKfyve inhibitor – YM203616 or DMSO. Cells were then placed (8 x 10 10^4 / 25μL per well) on the filter membrane above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* Presented data, expressed as number of migrated cell (mean +/- SD)is taken from a single experiment with triplicates and is representative to at least three different experiments using cells isolated from different donors.

3.5 Analysis of human CXCR3-A, CXCR3-B and CXCR3-alt in transfected cell model

As described in the previous parts of presented work, there are three identified alternative CXCR3 protein isoforms encoded by a single human gene and generated via alternative splicing. Expression both of these 'atypical' variants of CXCR3 at mRNA level has been detected in some cell types including T lymphocytes (Lasagni et al., 2003). There are a small number of studies reporting on responses mediated via CXCR3-B (Petrai et al., 2008; Romagnani et al., 2004) and CXCR3-alt (Ehlert et al., 2004), thus functionality and biochemical analysis of these receptors is still to be fully elucidated. This is especially important due to the fact of CXCR3 being a potential drug candidate in targeting of inflammatory diseases, where existence of various isoforms of the target protein might be additional limitation and should be considered.

As a part of this project it was planned to characterize biochemistry of two existing spliced variants of CXCR3 receptor, namely CXCR3-B and CXCR3-alt in comparison to original version of the receptor – renamed as CXCR3-A, using *in vitro* transfected cell system. Thus the aim of this part of presented work was to generate constructs encoding human CXCR3-A, CXCR3-B or CXCR3-alt (with or without EGFP tag); and their expression in cell lines for further analysis. Due to the lack of commercially-available antibodies specific to each variant of CXCR3, the constructs with C-terminal EGFP (Enhanced Green Fluorescent Protein) tag would allow us to monitor their location and detection where these receptors are expressed in the cell (intracellularly or on the cell surface), using fluorescent visualization. A schematic illustration of all generated CXCR3 constructs is presented in Figure 3.19B.

3.5.1 Generation of constructs encoding human CXCR3-A, -B and –alt

Full length fragments encoding CXCR3-A, -B and –alt were amplified by PCR using appropriate primers adding to each product required restriction sites at 5' and 3' end and Kozak sequence and codon STOP at 5' and 3' end, respectively (sequence of primers can be found in Table 2.1).

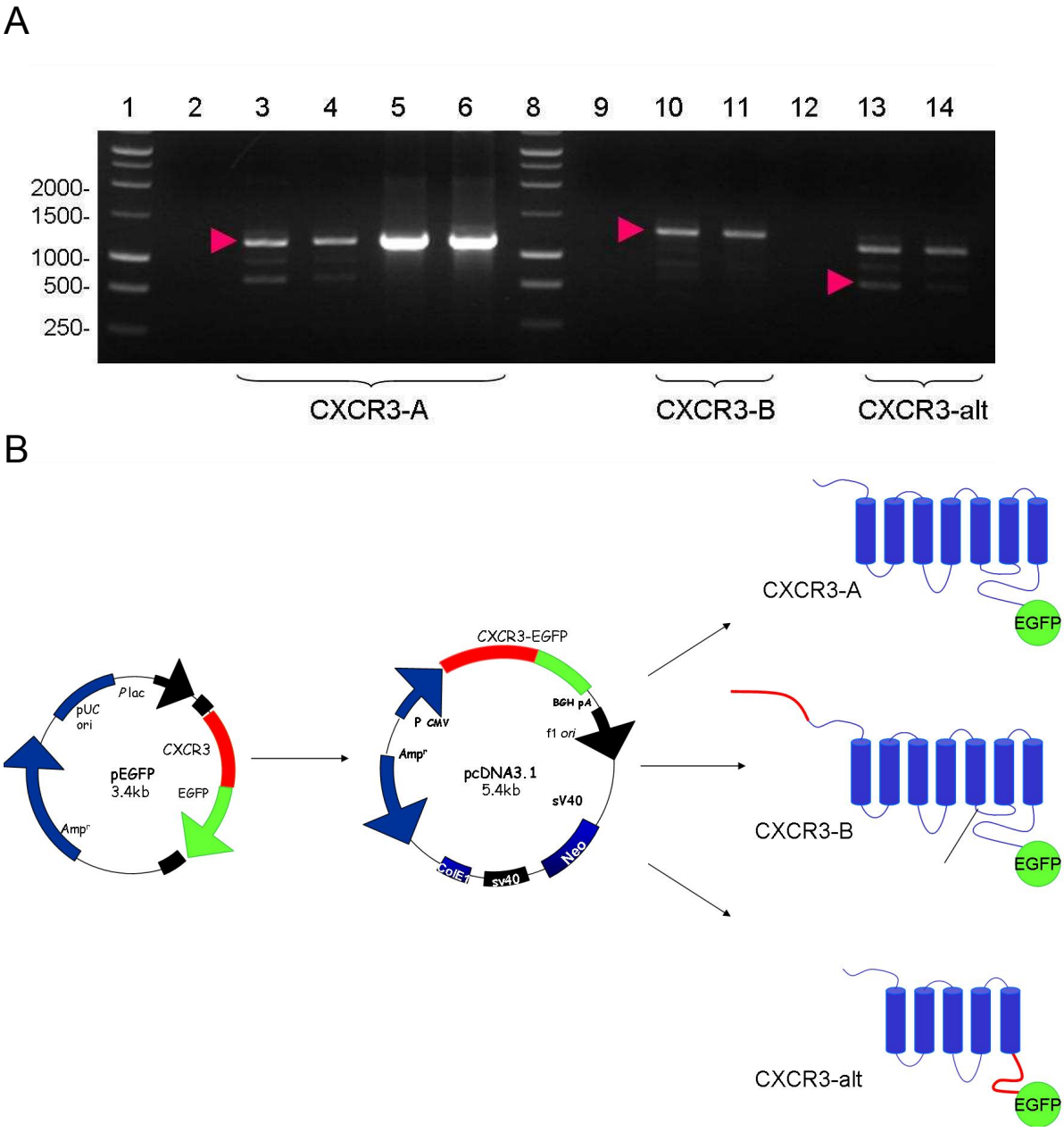


Figure 3.19 Generation of EGFP-tagged variants of CXCR3 receptor. (A), PCR of full length human CXCR3-A, CXCR3-B and CXCR3-alt. PCR was performed using cDNA from spleens or leukocytes (Clontech), or pIRES-Neo plasmid encoding CXCR3 as a templates and primers specific for CXCR3-A, -B and alt. Sense and anti-sense primers contain sequences recognized by HindIII and KpnI restriction enzymes, respectively. Lanes 1 and 8 – 1 kilo base pair molecular marker; lanes 2, 9 and 12 – control (dH_20); lane 3 - CXCR3 product amplified using cDNA from spleen, 4 – from leukocytes, lanes 5 and 6 – from pIRES-Neo vector; lane 10 - CXCR3-B from spleen, lane 11 – from leukocytes; lanes 13 and 14 – CXCR3-alt from spleen. Arrows point out bands corresponding to specific CXCR3 PCR products. (B), Plasmids and tagged receptors used in the study. Schematic illustration of constructs on the left hand side with resulting proteins being expressed on the right hand side. Inserts were subcloned into pEGFP vector follow by cloning into pcDNA3.1 expression vector.

PCR was performed using commercially-available cDNA from spleens or leukocytes (Clontech) (Figure 3.19A). Prepared inserts were then cloned into expression vector pcDNA3.1 (as described in details in sections 2.2.12.11 and 2.2.12.12 of Materials and Methods chapter) and verified by DNA sequencing. In order to generate CXCR3 constructs with C-terminal EGFP tag, fragments were subsequently cloned into pEGFP vector (Figure 3.19B).

3.5.2 Expression of CXCR3-A, -B and –alt in CHOK1 and HEK293 cell lines

Constructs obtained in 3.5.1 were transiently expressed in HEK293 and CHO cells (Figures 3.21 and 3.22). Both of these cell lines do not express any detectable endogenous chemokine receptors and have been shown to be capable to express high levels of chemokine receptors surface expression following transfection with similar constructs (Dagan-Berger et al., 2006; Proost et al., 2007). First expression of EGFP-CXCR3-A, -B and –alt was assessed by flow cytometry by the analysis of EGFP reporter. Transfection of HEK cells was optimised by using variable amounts of DNA and transfection reagent (Figure 3.20) while transfection of CHO cells was performed using previously optimised protocol (as described in Materials and Methods). Figure 3.21A shows the expression of 1 μg DNA encoding EGFP-tagged CXCR3 variants in HEK293 cells. Mean fluorescence intensity in HEK293 cells transfected with all three receptors was much higher compared with control cell, indicating expression of EGFP-tagged CXCR3. HEK-CXCR3-B-EGFP cells were less fluorescent then HEK-CXCR3-A-EGFP and HEK-CXCR3-alt-EGFP and expression of CXCR3-A form of receptor appeared to be at higher level then CXCR3-alt form (summarized in Figure 3.21B). Expression of CXCR3 variants was also assessed using mouse anti-human CXCR3 (clone 49801) to examine the amounts of each receptor present on the surface of the HEK cells (shown in Figure 3.21C and D). Figure 3.21B shown representative dot plot indicating expression of EGFP and anti-CXCR3 antibody staining. CXCR3-A transfectants represent two main populations of cells, one EGFP and PE positive, indicating expression of CXCR3-A-EGFP on the surface and EGFP positive and PE negative population of cells expressing transfected receptor intracellularly.

Representative histogram of anti-CXCR3 staining of cells expressing each CXCR3 variant (without a EGFP tag) is shown in Figure 3.21C and summarized in 3.21D

EGFP-tagged and non-tagged variants of CXCR3 are expressed at the similar levels (left and right panels of Figure 3.21E, respectively) suggesting that reporter tag does not affect the expression of the receptor on the surface. Consistently we observed relatively high surface expression of CXCR3-A, much lower expression of CXCR3-B and even lower expression of CXCR3-alt.

The antibody 49801 used in FACS analysis also reacted in immunoblotting studies and we were able to detect the bands around 70 kDa corresponding to predicted EGFP-tagged CXCR3-A (43 for CXCR3 and 27 for EGFP), higher band for CXCR3-B and slightly lower for truncated CXCR3-alt, (Figure 3.21E). The visible smears above expected protein sizes (about 100 kDa in CXCR3 lines) may be due to post-translational modifications of receptors. Bands of high molecular weight visible on the top of the gel may suggest SDS-stable aggregates of CXCR3 molecules. The lower molecular weight bands (between 37-50 kDa) may be result of degradation of receptor proteins. This is seen especially for CXCR3-alt which because of its dramatically altered structure may be less stable. Migration at different than predicted height in SDS-PAGE demonstrating posttranslational modification and aggregation of molecules was previously reported for D6 receptor (Blackburn et al., 2004).

In order to analyze the distribution of CXCR3 variants within the cell we performed the confocal microscopy analysis. We examined EGFP localization as well as anti-CXCR3 antibody staining in order to analyze surface expression of each CXCR3 variant (Figure 3.21F). CXCR3-A-EGFP was found in intracellular stores as well as at the surface of transfected HEK cells. PE-signals corresponding to anti-CXCR3 antibody staining were also found at the surface. CXCR3-B-EGFP was also expressed intracellularly (as indicated by expression of EGFP reporter) and some signal was also detected on the surface of the cell. However much fewer number of cells with PE staining at the surface was found suggesting lower expression levels or lower immunoreactivity of anti-CXCR3 antibody towards CXCR3-B variant. No reactivity of the same antibody was detected at the surface of CXCR3-alt, despite of clear expression of CXCR3-alt-EGFP (Figure 3.21F lower panel).

Similar analysis was performed when the constructs were expressed in CHO cell line. FACS analysis revealed that in this cell line, each variant of CXCR3 was expressed at similar levels to those found in HEK293 cells (Figure 3.22A). EGFP-tagged CXCR3-A, CXCR3-B and CXCR3-alt we expressed at the comparable levels however surface expression differed between each variant showing pattern found on HEK cells (CXCR3-A>CXCR3-B>CXCR3-alt). Two different antibodies gave comparable results in FACS analysis. Expression is CXCR3 isoforms was also detected by immunoblotting (Figure 3.22B). Analysis revealed presence of bands with predicted as well as higher molecular weight (their appearance was explained above). Bands about 37 kDa may be effect of non-specific imuno-reactivity of the antibody as they are present in mock control. Lower bands (less than 30 kDa), seen especially in CXCR3-A and CXCR3-alt lanes may represent degradation products. Fluorescent microscopy using EGFP reporter and anti-CXCR3 antibody revealed both intracellular and surface expression of CXCR3-A (Figure 3.22C). In contrast no surface expression CXCR3-B and CXCR3-alt was observed (data not shown).

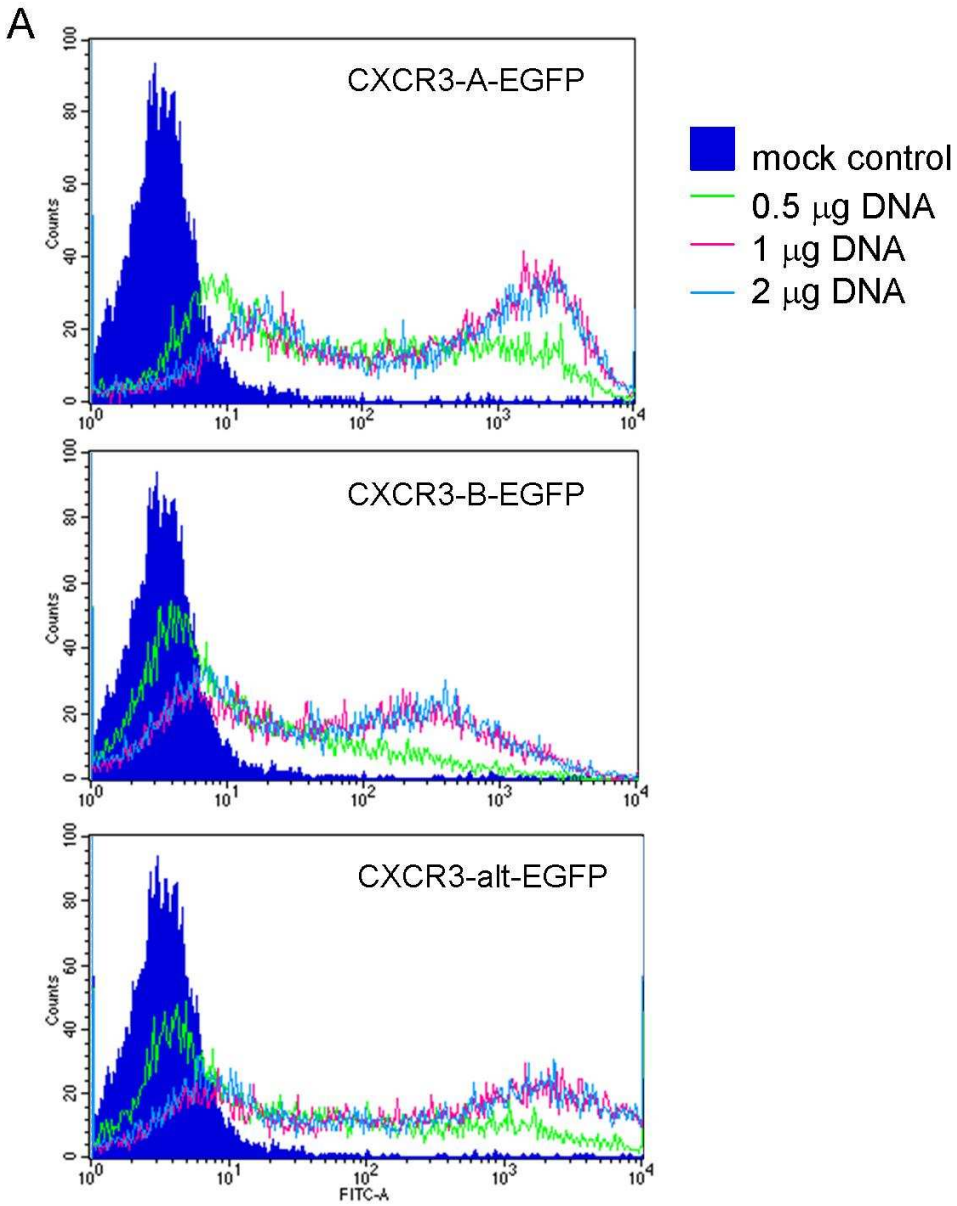


Figure 3.20 Optimisation of transfection efficiency in HEK293 cell line. (A), HEK293 cells were transfected with different amounts of DNA encoding CXCR-A, CXCR-B, CXCR3-alt or with empty vector and 48 hours post-transfection the expression of each receptor was assessed by FACS.

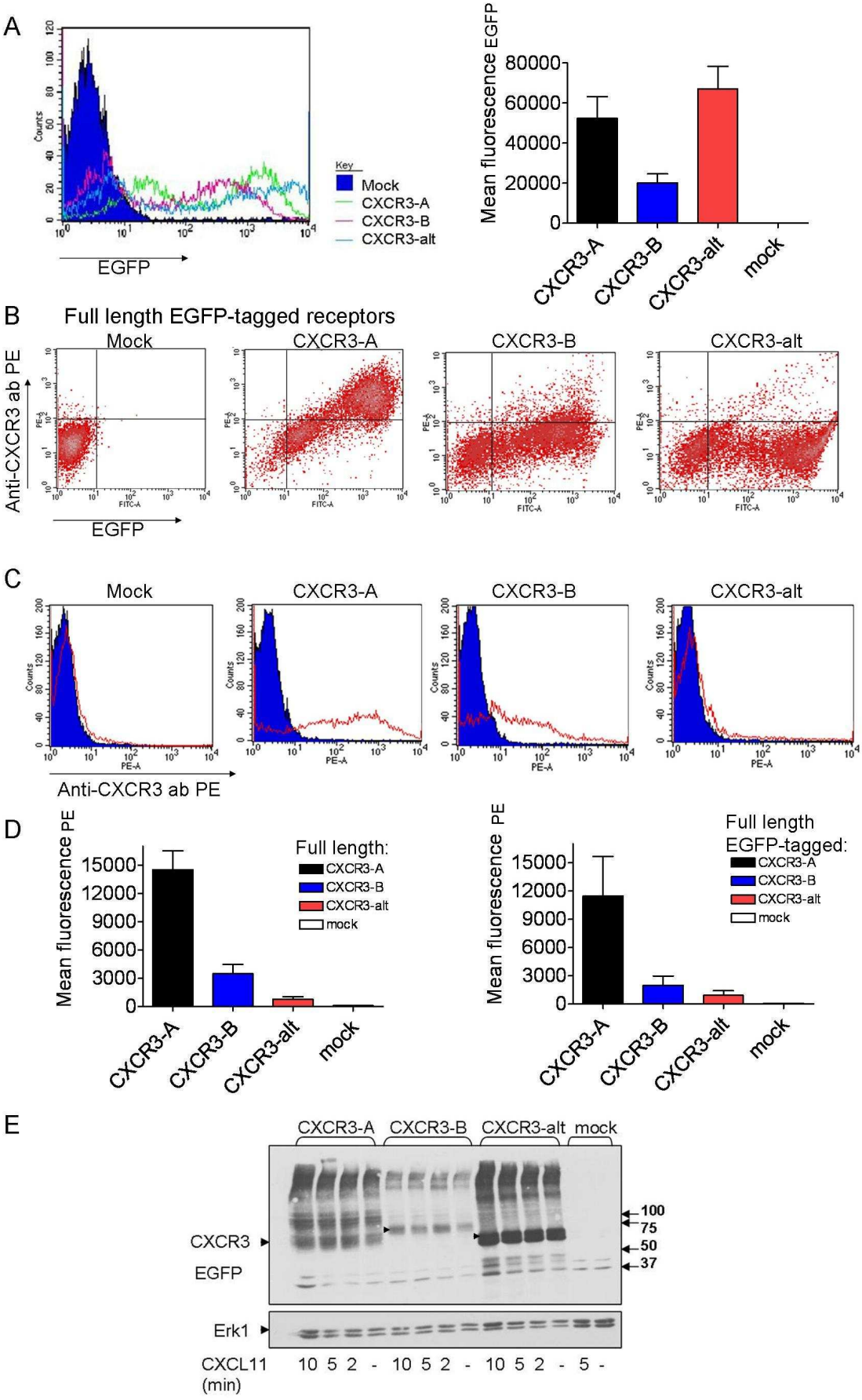


Figure 3.21 Expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected HEK293 cells. PTO for figure continuation and figure legend.

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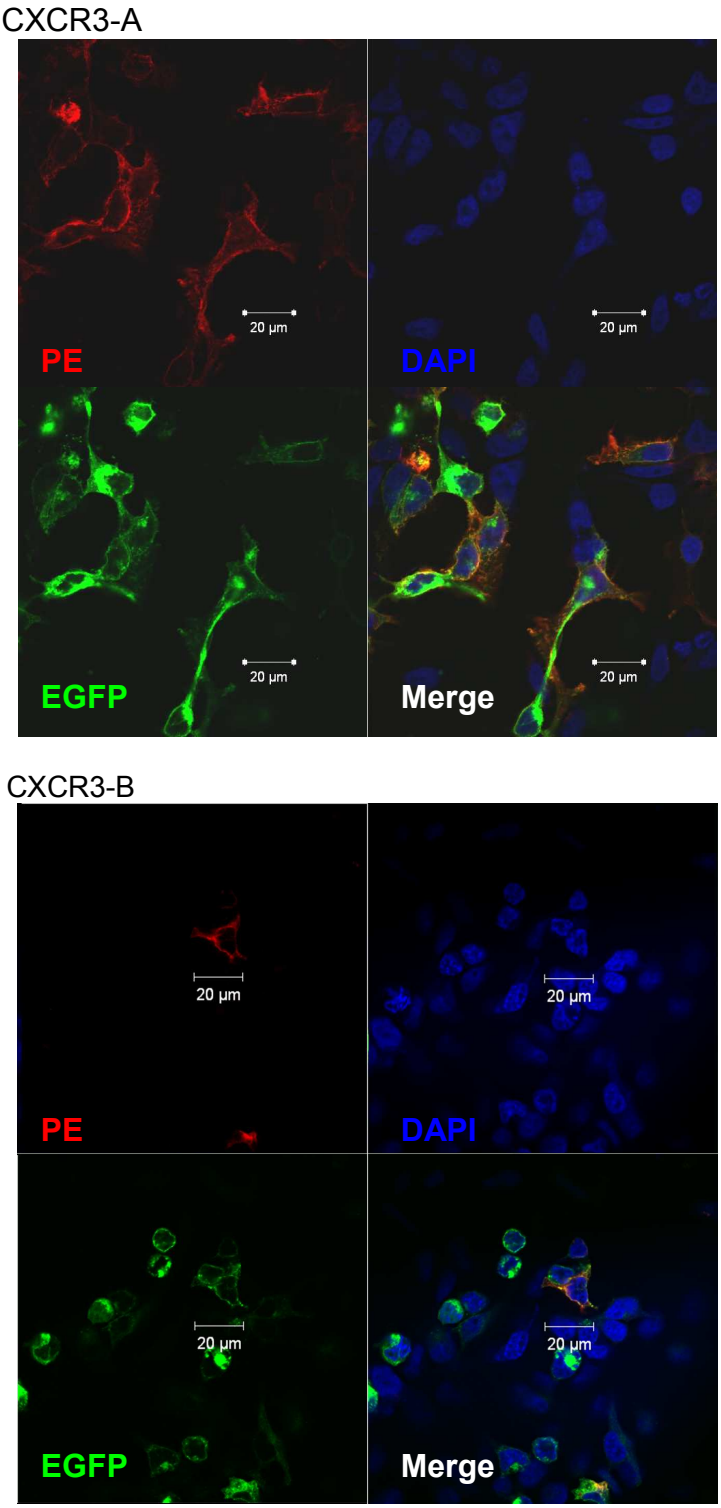


Figure 3.21 Expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected HEK293 cells. PTO for figure legend.

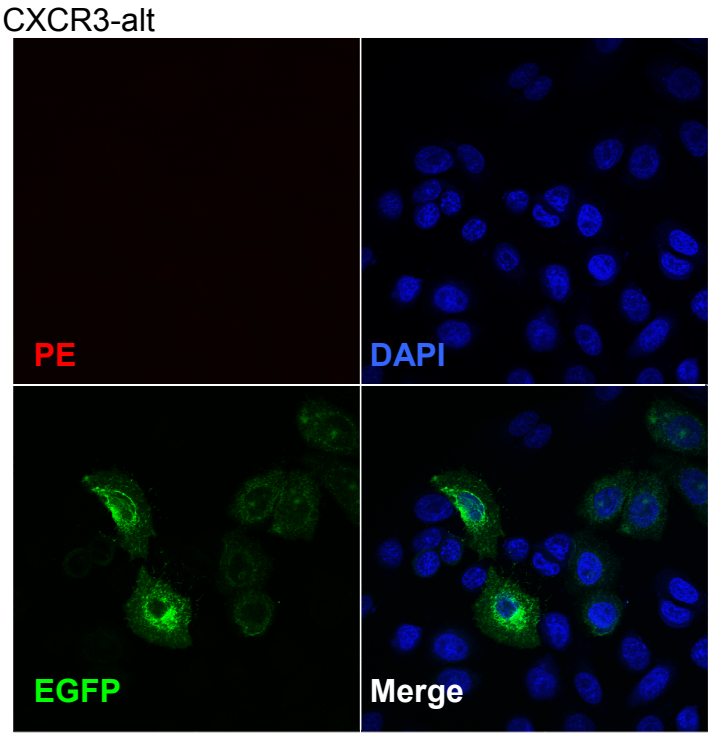
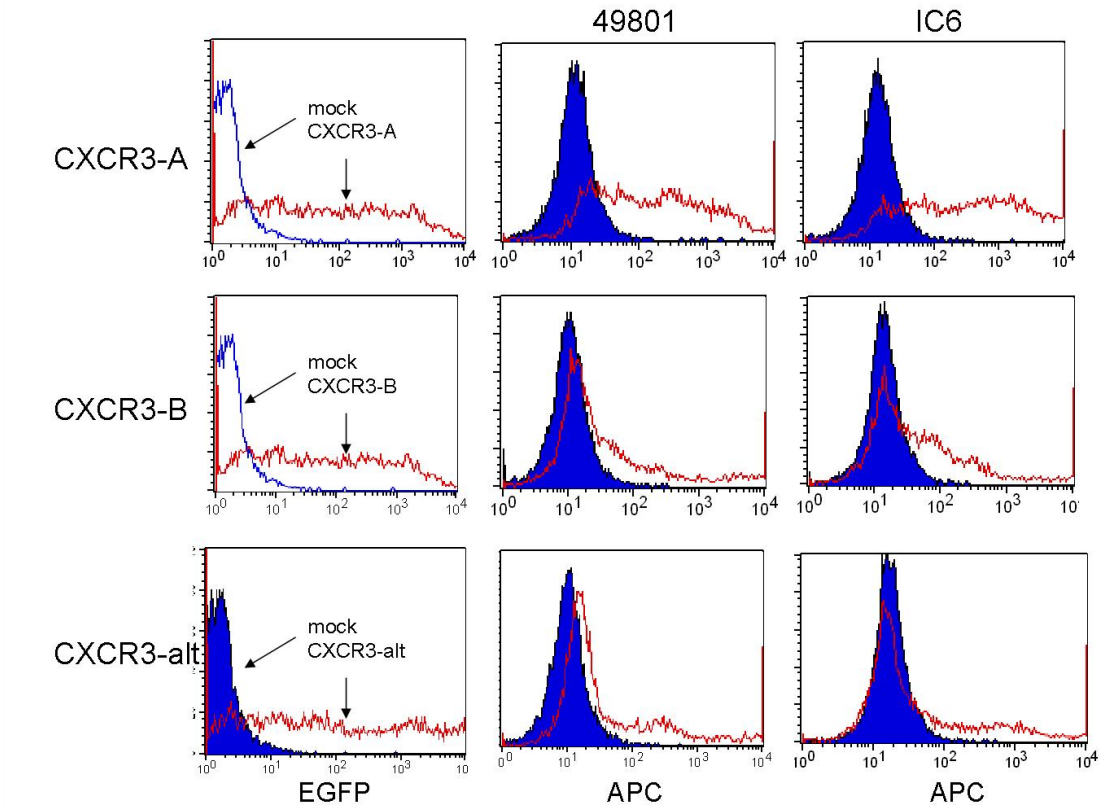


Figure 3.21 Expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected HEK293 cells.HEK293 cells were transiently transfected with 1 μg plasmid encoding appropriate chemokine receptor; CXCR3-A, CXCR3-B or CXCR3-alt with or without EGFP tag as described in Materials and Methods. 48 hours post transfection expression of each of receptor was analyzed. (A), Flow cytometry analysis of expression of EGFP reporter; left panel shows representative histogram and right panel shows average of three independent experiments. (B), Flow cytometry analysis of CXCR3-A, -B and –alt expression by examination of EGFP reporter expression and surface staining with anti-CXCR3 antibody conjugated with PE. Presented data are representative for at least five different experiments. (C), Analysis of surface expression of CXCR3-A, -B and –alt without EGFP tag assessed by FACS analysis using anti-CXCR3 antibody conjugated with PE. Experiment is representative for three other experiments. (D), Surface expression of CXCR3-A, -B and –alt assessed by FACS using anti-CXCR3-PE antibody staining, presented as an average of at least four different experiments; expression of receptors with or without EGFP tag is shown on right and left panel, respectively. (E), Western blot analysis of EGFP-tagged CXCR3-A, -B, and –alt expression. Briefly, lysates from $2x10^6$ cells transfected HEK293 cells were resolved on SDS gel. Membranes were immunoblotted with mouse monoclonal anti-CXCR3 antibody. Arrow heads indicate the position of EFGP-CXCR3 receptors. The membranes were stripped and reprobed with anti-Erk1 antibody (lower panel) to demonstrate loading. Mock – control cells transfected with empty vector and/or transfection reagent only. (F), Confocal images of HEK293 cells expressing CXCR3-A (top panel), CXCR3-B (middle panel) and CXCR3-alt (bottom panel). HEK293 cells grown on poly-L-lysine-coatd coverslips, were transfected with constructs encoding CXCR3-A-EGFP and CXCR3-B-EGFP and 48 hours post-transfection expression of both receptors was analysed by confocal microscopy. Briefly, cells were fixed, stained with anti-CXCR3-PE antibody and coverslips were mounted with Mowoil containing 10 μg/mL of DAPI (blue) for nuclear visualisation. Red represents PE, green- EGFP.

A



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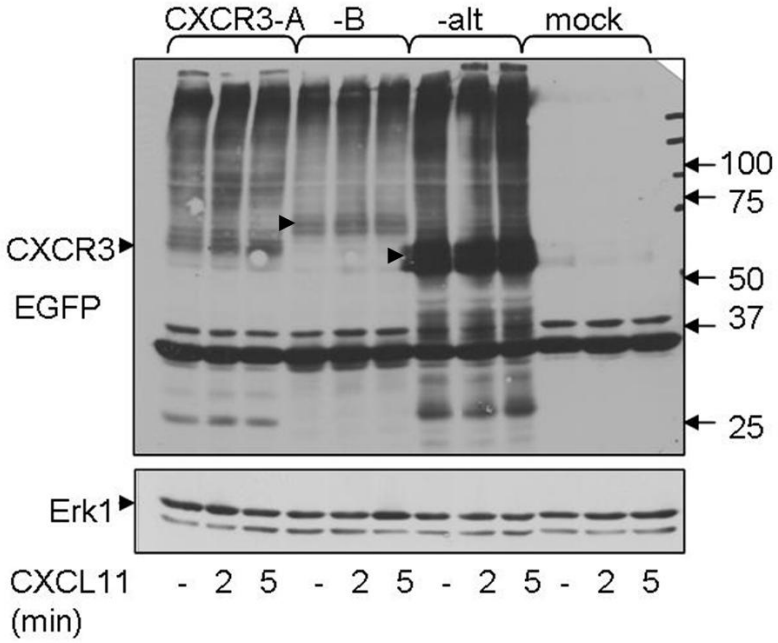

Figure 3.22 Expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected CHO cells. PTO for figure legend.

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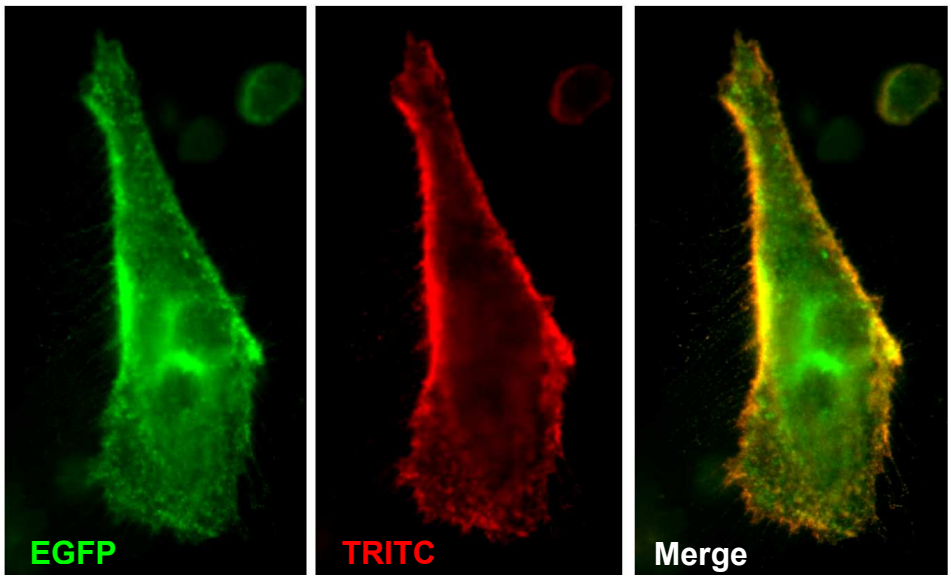

Figure 3.22 Expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected CHO cells. CHO cells were transiently transfected with 1 μg of DNA encoding appropriate chemokine receptor; CXCR3-A, CXCR3-B or CXCR3-alt C-terminally tagged with EGFP reporter as described in Materials and Methods. 48 hours post transfection expression of each of receptor was analyzed. For surface staining cells were incubated at 4°C with APC-conjugated appropriate anti-CXCR3 antibody or isotype control before FACS analysis. (A), Flow cytometry analysis of expression of EGFP reporter (left panels); surface staining with anti-CXCR3 clone 49801 antibody (central panels) or IC6 antibody (right panels). (B), Western blot analysis of EGFP-tagged CXCR3-A, -B, and –alt expression. Briefly, lysates from $2x10^6$ cells transfected CHO cells were resolved on SDS gel. Membranes were immunoblotted with mouse monoclonal anti-CXCR3 antibody. Arrow indicate the position of EFGP-CXCR3 receptors. The membranes were stripped and reprobed with anti-Erk1 antibody (lower panel) to demonstrate loading. Mock – control cells transfected with empty vector and/or transfection reagent only. (C), Images of CHO cells expressing CXCR3-A receptor. Red represents staining with 49801 antibody and TRITC labelled secondary antibody, green- EGFP. Presented data are representative for 3 other experiments.

3.5.3 Agonists-induced down-regulation of CXCR3-A, -B and –alt surface expression

Stimulation of T cells with CXCR3 agonists led to decrease of surface expression of the receptor, in contrast no effect was observed following stimulation with CXCL4. In order to further study this issue, HEK293 transfectants expressing each variant of CXCR3, CXCR3-A, -B or –alt fused at their C termini with EGFP reporter were stimulated first with CXCL11 which was previously showed to be the most efficacious inducer of CXCR3 internalization (Figure 3.23A, left panel). However according to obtained results and previous reports (Lasagni et al., 2003; Mueller et al., 2008), consistently higher levels of expression were obtained for CXCR3-A transfectants compared with CXCR3-B, making direct comparison difficult. Basal surface expression of each receptor was normalised to 100% and any changes following chemokine stimulations were analyzed. Only low percentage (about 25%) of CXCR3-A and CXCR3-B become internalized upon stimulation with 100 nM CXCL11 and levels of CXCR3-alt increased following agonist treatment up to 125%. The possible explanation was that internalization can be impaired due to EGFP fusion at the C terminals of the CXCR3 variants which may prevent interaction with adaptors. Therefore, constructs encoding full length receptors without fusion protein were generated and experiments were repeated as previously (Figure 3.23A right panel). This time about 40% decrease in surface expression of CXCR3-A and CXCR3-B variants was observed following stimulation with 100 nM CXCL11 for 5 and 30 minutes suggesting that adding of EGFP to the C termini of the receptors partially affected their internalization. Moreover these results indicate that CXCL11 interact with both variants of CXCR3 receptor. In contrast stimulation of CXCR3-alt expressing HEK293 cells resulted in increased expression of the receptor for about 20% (Figure 3.23A left panel) and further extension of incubation time to 120 minutes led to nearly 80% rise of CXCR3-alt surface levels in comparison to basal (Figure 3.23B). It was then examined if CXCL4 is able to induce any down-regulation of particular receptor variants. No obvious down-regulation of CXCR3-A and only 20% decrease of CXCR3-B surface was detected after incubation of CXCR3-A-HEK293 and CXCR3-B HEK293 cells, respectively with 100 nM CXCL4 (Figure 3.23C).

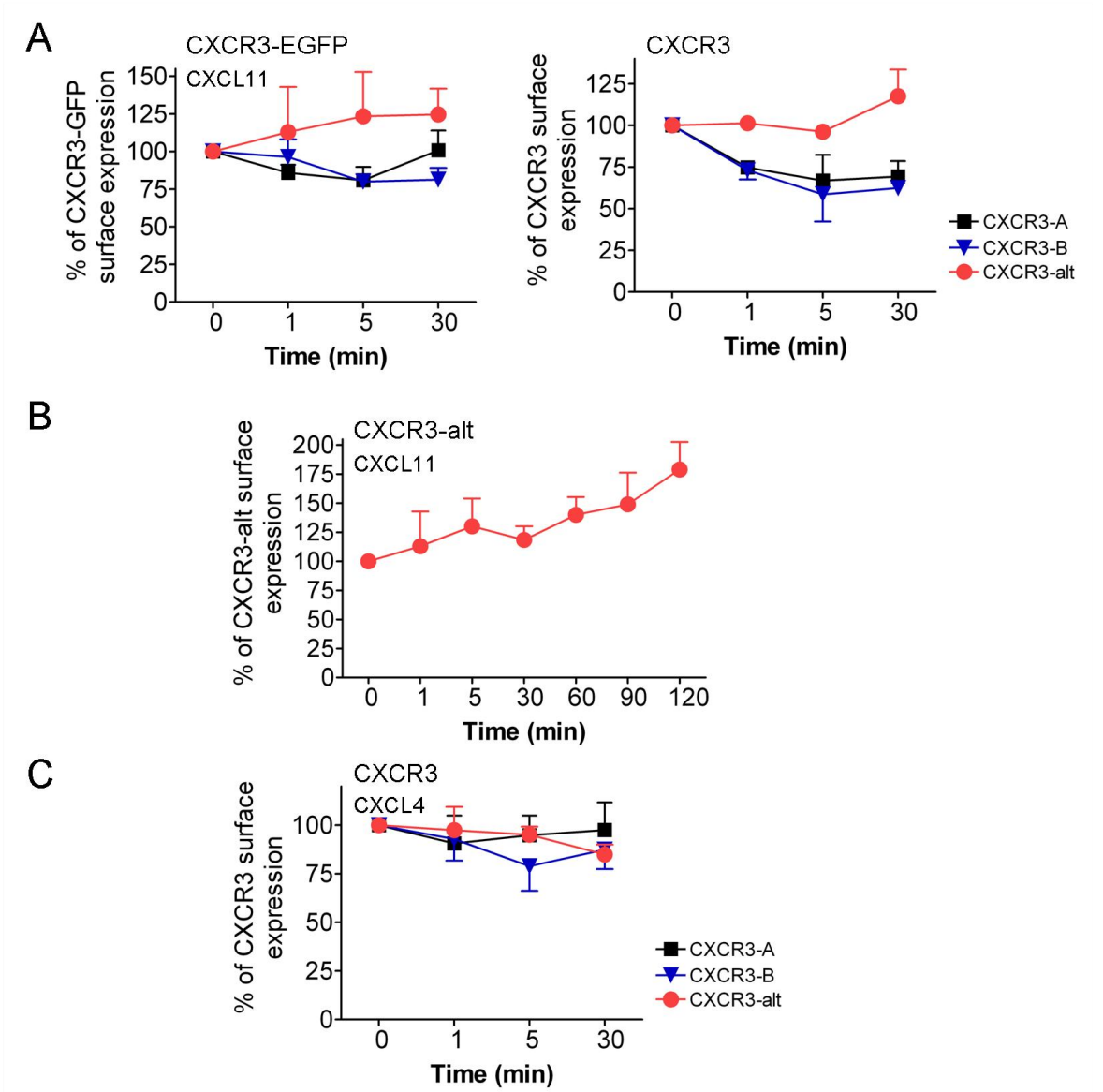

Figure 3.23 Effect of CXCL11 and CXCL4 on down-regulation of surface expression of CXCR3-A, CXCR3-B and CXCR3-alt in transfected HEK293 cells. HEK 293 cells previously transfected with constructs encoding EGFP-tagged CXCR3-A (■), CXCR3-B (▼)and CXCR3-alt (●) were harvested and exposed to 100 nM of CXCL11 for 1, 5 and 30 mins. All incubations were done at 37°C. Agonists were then washed off and cells were incubated with anti-CXCR3 antibody or isotype control at 4°C followed by analysis on FACS as described in *Materials and Methods*. Decrease in CXCR3 surface expression was expressed as a percentage of baseline surface expression using following formula: MFI (Mean Fluorescence Intensity) of stimulated cells/MFI of untreated cells *100. Shown data represent average +/- SEM of at least 3 independent experiments.

Similarly as expected CXCL4 had no major effect on surface CXCR3-alt as this form of CXCR3 has been shown to bind and respond only to CXCL11.

3.5.4 Intracellular calcium mobilisation induced by CXCL11 and CXCL4 in HEK293 cells transiently expressing CXCR3-A, CXCR3-B and CXCR3-alt

The ability of CXCL11 and CXCL4 to induce intracellular calcium mobilisation in HEK293 cells expressing different variants of CXCR3 was then assessed. Transfected HEK293 cell were stimulated with single concentrations of CXCL11 and CXCL4 and response over time was observed. 30 nM concentration of CXCL11 was effective at inducing elevation of intracellular calcium levels in cells expressing each variants of CXCR3, as is shown by Figure 3.24A. CXCR3-A-expressing cells gave the highest response (5 fold increase in fluorescence above basal level) in comparison with CXCR3-B and CXCR3-alt transfectants which responses were approximately 3 and 2 fold increase above basal fluorescence level, respectively. In keeping with the calcium mobilisation data in T cells, high (300 nM) concentration of CXCL4 was required to induce responses in transfected HEK cells (Figure 3.24B). Stimulation with CXCL4 led to approximately 3.5 fold increase in fluorescence levels in HEK293 expressing CXCR3-A similarly to response of CXCR3-B transfectants (3 fold increase). As expected, no response was detected in cells expressing CXCR3-alt.

3.5.5 Activation of PI3K/Akt and p44/p42 MAPK pathways in cells expressing CXCR3-A, CXCR3-B or CXCR3-alt receptors by chemokines

It was demonstrated in Section I of presented work that stimulation of activated T cells with CXCR3 agonists as well as CXCL4, a reported agonists for CXCR3-B variant, resulted in increase of phosphorylation of Akt and p44/p42 MAPK indicating activation of these crucial signalling pathways. To further examine these responses, HEK293 cells transiently expressing one of each CXCR3 variants CXCR3-A, CXCR3-B or CXCR3-alt were first exposed to 10 nM of CXCL11 and changes in phosphorylation of Akt and Erk kinase were analysed by immunoblotting (Figure 3.25A). Stimulation of CXCR3-A-expressing HEK293 cells with CXCL11 resulted in small increase in p44/p42 MAPK phosphorylation as they exhibited high levels of basal phospho-MAPK. Moreover CXCL11 induced clear

phosphorylation of p44/p42 MAPK in cells expressing CXCR3-B and CXCR3-alt without any obvious effect on the cells transfected with the mock. HEK293 cell expressing either variant of CXCR3 as well as control cells exhibited high basal level of phosphorylated Akt and no obvious increase upon stimulation with CXCL11 was detected. CXCL11-triggered phosphorylation of Erk was also observed in CHO cells expressing CXCR3-B and CXCR3-alt but surprisingly again we were struggling to detect a clear signal in CXCR3-A transfected cells following stimulation with CXCL11. CHO cells expressing CXCR3-A and CXCR3-alt following stimulation with CXCL11, exhibited certain decrease in levels of phospho-Akt in comparison to untreated cells. In contrast in CHO cell expressing CXCR3-B stimulation with 10 nM CXCL11 resulted in Akt phosphorylation only at one time point. There were noticeable variations of the responses obtained within these experiments between basal levels of phosphorylation, and repeats of samples stimulated with chemokines (two examples are shown in Figure 3.2A and A'). These variations were possibly caused by differences in surface expression of CXCR3 variants between samples, as a result of transient transfection of the cells in individual wells and subsequent stimulation.

Furthermore the ability of CXCL4 to induce activation of both Akt and 44/42 MAPK was tested. Upon stimulation with 100 nM of CXCL4 no increase in phosphorylation above basal levels of neither Akt nor p44/p42 MAPK could be observed in HEK293 cells expressing different CXCR3 variants in exception of CXCR3-A expressing cells which exhibited low level of Akt phosphorylation (Figure 3.25B).

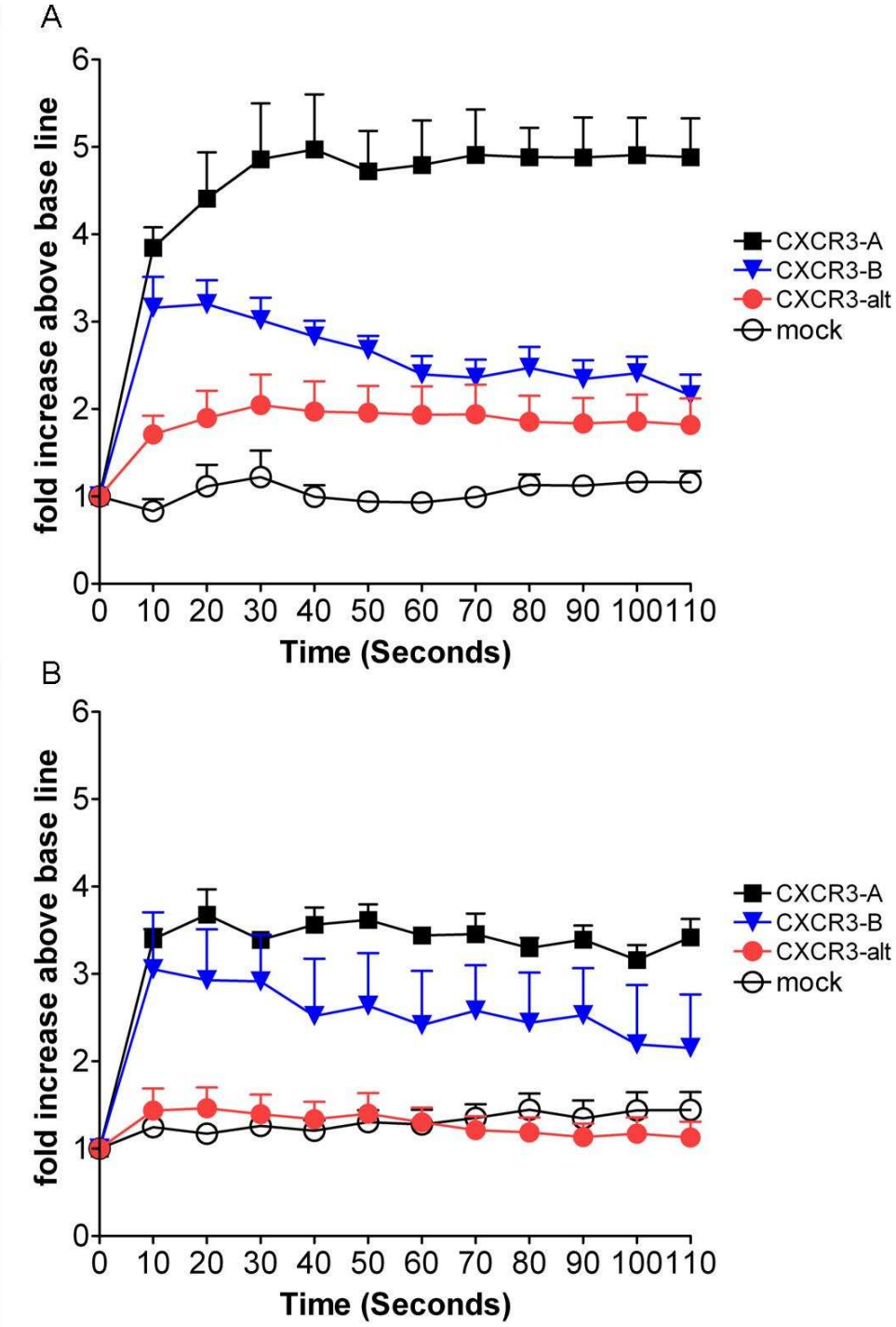

Figure 3.24 Elevation of cytosolic calcium concentration obtained from HEK293 cells expressing full length CXCR3-A, -B and –alt. 48 hours post-transfection, HEK293 cells were harvested, washed, and resuspended in buffer containing calcium as described in *Materials and Methods*, prior by loading with 5 μM Fluo-4. Cells were then stimulated with 30 nM of CXCL11 (A) or 300 nM of CXCL4 (B) and changes in fluorescence were measured over time using fluorescence reader. Data shown are the mean of two separate experiments performed in duplicates.

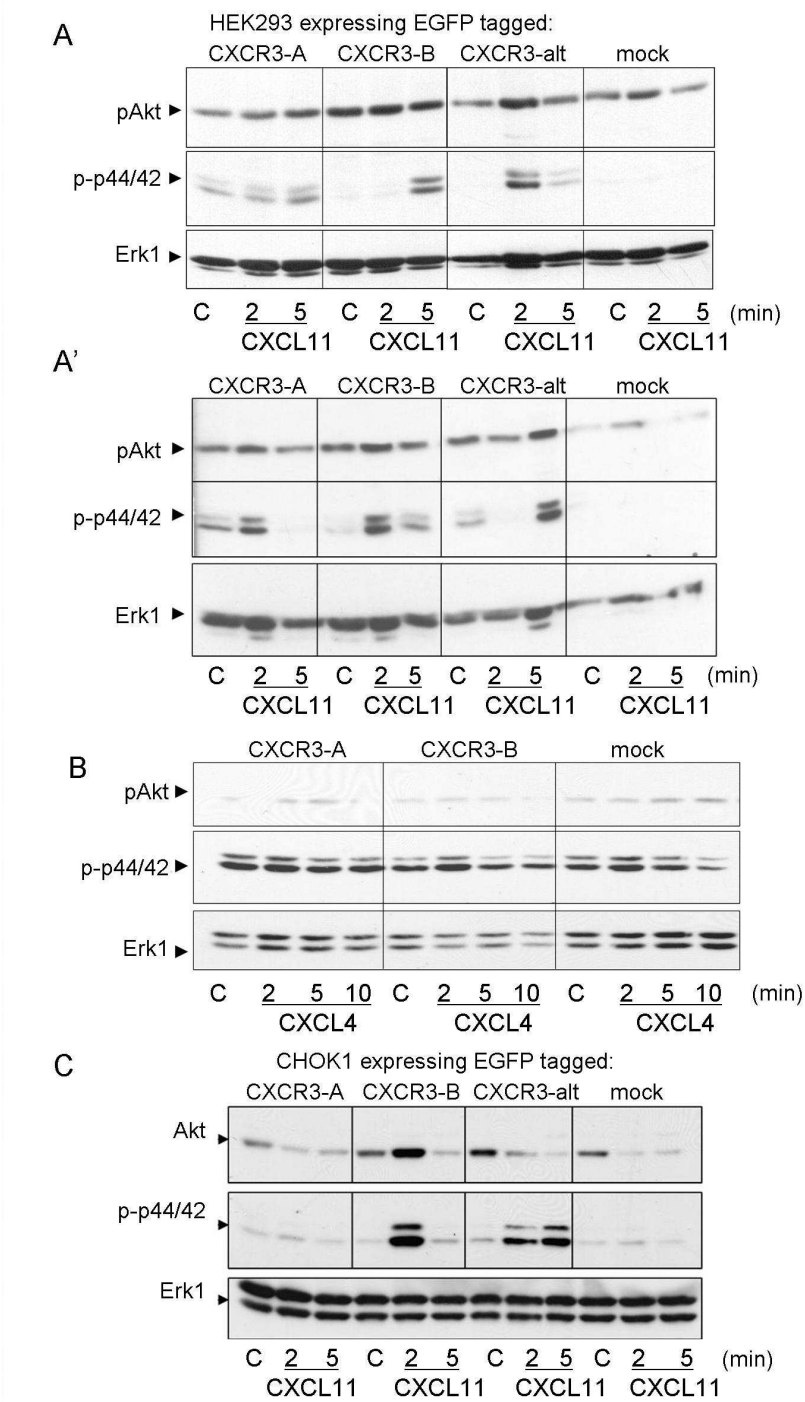

Figure 3.25 Activation of Akt and p44/p42 MAPK in CXCR3-A, CXCR3-B or CXCR3-alt-expressing HEK293 and CHO cells. 48 hours post-transfection, HEK cells (A,A' and B) or CHOK1 cells (C) were harvested, washed, and stimulated with 10 nM of CXCL11 (A,A'), 100 nM of CXCL4 (B) or 10 nM CXCL11 (C) for indicated periods of time. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt ab with the affinity for the active Ser^{473}-phosphorylated form of Akt or p44/p42 MAPK ab with the affinity for the active Thr^{202}/Tyr^{204} form of Erk. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. Presented data are representative for at least two independent experiments.

3.6 Summary of Chapter Three

In this chapter it was aimed to analyze expression and biochemical and functional responses mediated by CXCR3 receptor and its existing spliced variants – CXCR3-B and CXCR3-alt. CXCL4 chemokine which has been reported to bind and respond via CXCR3-B was utilized to study this atypical variant of CXCR3. The following observations were made:

- Freshly isolated T lymphocytes express moderate levels of CXCR3 receptor on the surface and this expression is up-regulated following stimulation with either CD3/CD28 antibodies –coated microbeads or SEB, along with culture in the presence of IL-2. Moreover CXCR3 was present on both CD4 and $CD8^+$ T cells.
- SEB/IL-2 activated T lymphocytes express three known spliced variants of CXCR3 namely, CXCR3-A, CXCR3-B and CXCR3-alt at the mRNA level.
- CXCR3 agonists, CXCL9, CXCL10 and CXCL11 but not CXCL4 induced concentration and time-dependent down-regulation of CXCR3 surface expression. Following functional hierarchy in terms of chemokine-induced receptor internalization was observed, CXCL11>CXCL10>CXCL9.
- Small CXCR3 antagonists inhibited CXCL11-induced internalization of CXCR3 showing following potencies: T487- 380 nM, NBI-74330- 2.44 nM and N-oxidized metabolite of NBI-74330- 0.712 nM.

  Down-regulation of CXCR3 mediated by CXCL9 and CXCL10 was sensitive to NBI-74330 treatment.
- CXCL11-induced CXCR3 internalization was insensitive to hypertonic sucrose treatment however was inhibited by chlorpromazine indicating a potential role of clathrin-dependent pathway of endocytosis. Moreover detected down-regulation of surface CXCR3 was not significantly inhibited by nystatin and filipin suggesting that CXCL11- induced CXCR3 internalization does not utilize lipid rafts/caveolae. In contrast it was partially blocked by MβCD indicating a possible distinct cholesterol-dependent pathway.
- CXCL11- mediated CXCR3 surface down-regulation did not appear to require Gαi and PI3K involvement as it was insensitive to pertussis toxin and PI3K inhibitors treatment, respectively.

- Stimulation of T cells with PKC activator – PMA led to significant down-regulation of surface CXCR3, moreover treatment with PKC inhibitors rottlerin and RO31-8220 and PLC inhibitor U73122 but not its inactive analogue U73343 led to inhibition of agonist (CXCL11) triggered internlisation of CXCR3.
- Pretreatment of T cells with PIKfyve inhibitor - YM201636 led to decrease of agonist-induced CXCR3 surface down-regulation, however due to high concentration used observed inhibition was possible off-target effect.
- Following induction of down-regulation with CXCL11 the recovery of surface CXCR3 levels occurred relatively slowly with around 80-90% recovery of the basal CXCR3 surface levels detected 180 minutes after incubation with the agonist.
- CXCR3-surface replenishment following agonist stimulation in T cells is dependent upon synthesis of a new receptor proteins as well as the Golgi function.
- CXCL9, CXCL10, CXCL11 and CXCL4 induced phosphorylation of Akt and p44/p42 and these biochemical events involved PTX-sensitive Gi signalling.
- Responses induced by CXCL4 were at lower strength in comparison to CXCR3 agonists and exhibited certain level of variability in order to the signal strength.
- Small CXCR3 antagonists inhibited CXCL11-induced signalling showing different inhibitory activities: N-oxide-NBI-74330>NBI-74330>T487. T487 and NBI-74330 also attenuated CXCL9 and CXCL10-mediated signalling but they had no effect on signals induced by neither CXCL4 nor CXCL12.
- CXCL9, CXCL10, CXCL11 and CXCL4 induced phosphorylation of GSK3β and S6 phosphorylation in T cells.
- CXCR3 agonists and CXCL4 induced actin polymerisation in activated T cells.
- CXCR3 agonists stimulated chemotaxis of T cells with CXCL11 being the most potent and efficient chemoattractant. In contrast CXCL4 failed to induce a chemotactic response in activated T cells.
- Small CXCR3 antagonists inhibited CXCL11-induced chemotaxis showing following inhibitory activities: T487, IC50 - 69 nM, NBI-74330, IC50 - 2.3 nM and oxidised metabolite of NBI-74330 - 0.53 nM. T487 and NBI-74330 also

had an inhibitory effects on CXCL9 and CXCL10 but not on CXCL12–induced chemotaxis.

- PIKfyve inhibitor YM201636 partially blocked chemotaxis induced by CXCL11 and had no effect on CXCL12- mediated chemotaxis.

Analysis of variants of human CXCR3 expressed in HEK293 and CHO cell lines led to the following observations:

- In both, HEK293 as well as CHO cells, EFGP-tagged full length CXCR3 receptor was highly localized to the cell surface however its expression was also detected in intracellular stores.
- CXCR3-B-EGFP was expressed at lower levels in both cell lines. Moreover much lower proportion of this variant was found on the cell surface as indicated by pan isoform anti-CXCR3 antibody binding.
- Truncated CXCR3-alt –EGFP was expressed as similar levels to full length CXCR3 however only small proportion of the receptor was present on the surface and recognized by anti-CXCR3 antibody in HEK293 and CHO cells.
- EGFP-tagged variants of CXCR3 were not efficiently down-regulated from the cell surface of HEK293 cells following stimulation with CXCL11. In contrast expression of non-tagged versions of CXCR3-A and CXCR3-B was decreased for about 25% due to exposure to CXCL11 suggesting that EGFP tag affected internalization. Expression of surface CXCR3-alt was increased for about 25% prior stimulation with CXCL11 and extension of stimulation time up to 120 minutes led to further increase for approximately 75%.
- Stimulation with CXCL4 resulted in minor (approximately 25%) down-regulation of CXCR3-B surface expression whereas levels of CXCR3-A and CXCR3-alt remained unaltered.
- CXCL11 stimulated calcium mobilisation in HEK cell expressing all three isoforms of CXCR3 whereas CXCL4 triggered calcium influx in CXCR3-A and CXCR3-B-expressing cells.
- CXCL11 induced phosphorylation of Erk in CXCR3-A, CXCR3-B and CXCR3-alt transfectants without obvious effect on Akt activation in both HEK and CHO cells lines with exception of CXCR3-B positive CHO cell

where we observed increase in phosphorylation of Akt. These results exhibited noticeable variability and would have to be improved.

- Following stimulation with CXCL4 we were not able to detect any increase in levels of neither Erk nor Akt phosphorylation in HEK cell expressing with CXCR3-A and CXCR3-B.

3.7 Discussion

The first part of the presented thesis is focused on characterization of the CXCR3 receptor present on human T cells. The work included examination of the expression of CXCR3 and its spliced variants in activated human T lymphocytes, loss of CXCR3 surface expression following agonist stimulation, signalling induced by CXCR3 agonists, and CXCR3-mediated chemotactic responses. Analysis of CXCR3 and its variants, namely CXCR3-B and CXCR3-alt, was also performed in transfected HEK293 cells.

3.7.1 The culture conditions

T cell isolation from freshly donated human peripheral blood and their *ex vivo* expansion provides a useful protocol for studying biochemical and functional events in T lymphocytes. After separation from peripheral blood, the mononuclear cells (a mixure of monocytes and lymphocytes) are activated and kept in culture up to 12 days under conditions which promote T lymphocyte proliferation, activation and up-regulation of CXCR3. Many factors can influence the number of chemokine receptors expressed on the cell surface. The age and health condition of the donor are contributing factors, similarly to the method of *in vitro* activation and cytokine milieu in cell environment such as the presence of IL-2.

Different methods of T cells activation which were utilised within the presented work are discussed below. PHA is a lectin isolated from plants and acts as a mitogen which induces activation of T cells by cross linking to glycoproteins on the cell surface. PHA will yield activated T lymphocytes that are predominantly $CD8^+$. Alternatively, SEB is one of the best known superantigens, which function by binding to MHC class II molecules expressed on the surface of professional antigen presenting cells (APC) present within the PBMC population. SEB acts then

as cross linker subsequently binding to α chain of the TCR, stimulating robust activation of T lymphocytes. SEB will yield activated T lymphocytes that are predominantly $CD4^+$. This method of T cells activation was preferentially used within presented work.

For the rapid expansion of freshly isolated T cells, stimulation with anti-CD3/CD28 mAb-coated Dynabeads was used. This method of stimulation more accurately mimics the *in vivo* presentation of antigen to T cells, but avoids engagement/activation of the co-inhibitory receptors such as CTLA-4 (Parry et al., 2003). Furthermore this method does not require large numbers of autologous/ MHC matched APC and antigen or allogenic mononuclear cells and mitogen. The T lymphocyte population should first be purified by negative selection using pan-T cell isolation kits. T lymphocytes are not contaminated with monocyte/ macrophages and the antibody-coated beads are simply removed using a magnet. T lymphocytes can be sustained and expanded in culture for several weeks.

3.7.2 CXCR3 expression in freshly isolated and activated T cells

It has been shown that freshly isolated, resting T cells show a certain level of inflammatory chemokine receptor CXCR3 which is dramatically up-regulated after activation (Mueller et al., 2008; Rabin et al., 1999). Data obtained within the study presented here correlates with these findings making activated and *ex vivo* expanded human T cells a good model for studying CXCR3.

3.7.3 Responsiveness of T cells to CXCR3 agonists

Naïve T lymphocytes expressed limited number of chemokine receptors. Following T cells activation, the number of chemokine receptors present on the surface increases, allowing the cell to respond to chemokine gradient. An interesting feature about CXCR3 is that approximately 40% of freshly isolated human T cells express this receptor without ability to respond to CXCR3 agonists (Loetscher et al., 1998). This responsiveness is gain during T cell activation with IL-2 and a mitogen such as PHA or Con A which leads to increasing receptor density (Loetscher et al., 1998).

3.7.4 Expression of CXCR3 variants in T cells

The commercially available anti-CXCR3 antibodies are unable to distinguish between CXCR3-A, CXCR3-B or CXCR3-alt, while reported CXCR3-B antibodies are either not widely available or have limited specificity. In addition, there are currently no reported antibodies to CXCR3-alt. However, expression of individual CXCR3 isoform mRNA in human T lymphocytes can be monitored. Expression of CXCR3-B and CXCR3-alt mRNA in activated human T cells was previously reported (Ehlert et al., 2004; Lasagni et al., 2003). In addition to full length CXCR3 (CXCR3-A), the expression of CXCR3-B and CXCR3-alt at mRNA was also detected within these investigations and it would therefore be tempting to surmise that all functional or signalling responses generated should be attributed to all variants of CXCR3 (in the case of CXCR3-alt that would include only responses induced by CXCL11). It appears that in T cells CXCR3-A is expressed at higher levels in comparison to the other two isoforms. However this was determined by semi quantitative PCR analysis, therefore it would be good practice to utilize several methods to detect the presence of these receptors, such as qPCR and immunoblotting or immunofluorescence using antibodies generated against specific epitopes of each receptor variants. In addition immunoblotting using pan isoform anti-CXCR3 antibody could be helpful in determination of endogenous expression of CXCR3 variants at the protein level which could be distinguish based on differences in weight. This approach was attempted to be used however had major limitations as the antibody resulted in variety of non-specific bands (some possible due to receptor aggregation) making identification of endogenous CXCR3 protein very difficult (data not shown).

3.7.5 CXCR3 agonists CXCL9, CXCL10 and CXCL11 but not CXCL4 induce down-regulation of CXCR3 surface expression in activated T cells

Agonist-induced desensitisation and internalization of chemokine receptors is believed to be a crucial process allowing immune cells to maintain their capacity to respond to small changes in a chemotactic gradient (Murphy, 1994).

Internalized CXCL11 was visualized using a fluorescently labeled (using FITC streptavidine) biotinylated version of the chemokine. Fluorescent microscopy analysis revealed FITC puncta within stimulated and fixed cells suggesting uptake

of labeled agonist. This technique was planned to be used to examine the fate of chemokine-chemokine receptor complexes after internalization. However further experiments revealed that the ability of biotinylated CXCL11 to induce down-regulation of CXCR3 is highly impaired in comparison to unlabelled recombinant CXCL11. Moreover biotinylated CXCL11 induced phosphorylation of p44/p42 MAPK and chemotaxis of SEB/IL-2 activated human T cells to much lesser extent that its 'native' analogue (Figure 7.8). Similar observations were found for biotinylated CXCL10. Possible explanation of the lack of bioactivity of biotinylated chemokines may be fact that they were chemically synthesised and then refolded in contrast to other chemokines used which are recombinant, produced in bacteria.

As a part of the study, chemokine-induced endocytosis of CXCR3 in T lymphocytes was investigated. In agreement with similar receptor internalization studies CXCR3 agonists caused receptor down-regulation in a rapid, concentration- and time-dependent manner (Arai et al., 1997; Jopling et al., 2007; Sauty et al., 2001). CXCL11 appeared to be the most potent and efficacious agonist. Results of the study were similar to the findings by Sauty et al who shown that recombinant CXCL11 was a more potent inducer of CXCR3 internalization than CXCL10 and CXCL9, and that second, CXCL11 present at the surface of activated endothelial cells is responsible for inducing CXCR3 internalization despite higher expression of CXCL10 and CXCL9 (Sauty et al., 2001). A similar functional hierarchy has also been demonstrated for other chemokines such as CCR5, CCR3 and CXCR2 ligands (Mack et al., 1998; Zimmermann et al., 1999; Feniger-Barish et al., 2000). Moreover Colvin et al demonstrated that CXCR3 utilize different intra- and extracellular domains for specific signalling and functional responses. The carboxyl terminus has been demonstrated to be required for CXCL9- and CXCL10- mediated internalization, while CXCL11 appeared to required the third intracellular loop of CXCR3 to induce its internalization (Colvin et al., 2004). This phenomenon may therefore explain the differential effects of CXCL11 in comparison to other chemokines CXCL9 and CXCL10. In addition CXCL9 and CXCL10 has been demonstrated to act as full or partial agonists depending upon the assay system used (Clark-Lewis et al., 2003; Gonsiorek et al., 2003; Heise et al., 2005; Jopling et al., 2007).

In contrast to CXCR3 agonists, stimulation with CXCL4 (even at high nanomolar and micromolar concentrations) had no noticeable effect on CXCR3 expression. However from these experiments it is unclear if CXCR3-B surface expression has been altered because the antibody used in the study is not directed to a specific CXCR3 variant. Moreover it is unknown if the antibody binds to each variant with the same affinity. Therefore experiments to investigate an effect of CXCL4 on CXCR3 (CXCR3-A) and CXCR3-B surface expression were carried out in transfected HEK293 cells which do not express endogenous chemokine receptors and will be discussed further in this chapter.

Agonist-induced CXCR3 internalization assay was previously described to be the useful and informative method of determining agonist potency and antagonist affinity measurements (Jopling et al., 2007).
As a part of the CXCR3 internalization study, three small nonpeptidergic, noncompetitive CXCR3 antagonists developed by Chemocentryx and Tularik (later acquired by Amgen), namely T487 (AMG487) and its analogue NBI-74330 and N-oxide metabolite were examined. An action of T487 and NBI-74330 has been extensively studied in variety of in vitro assays, including [^{35}S]-GTPγS, elevation of intracellular free calcium and chemotaxis (Johnson et al., 2007). NBI-74330 has also been shown to inhibit CXCL11-induced internalization of endogenous CXCR3 in murine splenocytes and its generated in vivo N-oxide metabolite exhibited antagonistic properties (Jopling et al., 2007). In these studies, all three antagonists were able to block, in a concentration dependent manner, internalization of CXCR3 induced by CXCL11. Both, NBI-74330 and its metabolite were more potent then T487 however total inhibition of surface receptor down-regulation was not achieved with either compound.

3.7.6 Mechanisms of CXCL11–induced internalization of CXCR3 in human T cells

Two main pathways have been reported for the internalization of chemokine receptors following ligand binding. The clathrin-mediated pathway of endocytosis is the best described route for receptor surface down-regulation. During this type of endocytosis the activated receptor become phosphorylated through the action of arrestins, and is directed to clathrin-coated pits. Receptor–agonist complexes

are then internalized in vesicles which are then released from the cell surface by dynamin and transported to endosomes. In endosomes receptors are dephosphorylated, resensitised and recycled back to the plasma membrane (Conner and Schmid, 2003). Certain CXC family chemokine receptors such as CXCR1 (Barlic et al., 1999), CXCR2 (Yang et al., 1999) and CXCR4 (Venkatesan et al., 2003) have been reported to utilize clathrin-dependent route for internalization. A second pathway of receptor internalization occurs via lipid rafts, also known as membrane rafts, which are a glycosphingolipid-enriched microdomains, or detergent-resistant microdomains, relatively resistant to solubilization with commonly used detergents such as Triton-X. They are believed to contribute to the structure and function of caveolae- cholesterol-enriched, highly organised membrane structures (Anderson, 1998). Despite being described in macrophages (Kiss and Geuze, 1997), it is still a debate whether T cells also contain caveolae (Fra et al., 1994; Fra et al., 1995). Caveolae-dependent pathway have been demonstrated for endocytosis of CCR4 and CCR5 (Mariani et al., 2004; Mueller et al., 2002). Studies on mechanisms of receptor internalization are usually carried out using a genetic approach (dominant negative constructs such as dynamin or clathrin mutants) or pharmacological compounds to interfere with a particular pathway (Marchese et al., 2003; Neel et al., 2005).

Here, in the study of CXCR3 endocytosis, several inhibitors which have previously been shown to inhibit clathrin and caveolae –dependent pathways were utilised. Hypertonic sucrose and chlorpromazine have been demonstrated to inhibit the assembly of clathrin-coated pits, whereas cholesterol-binding agents such as polyene antimycotics nystatin and filipin can block internalization via caveolae (Harder et al., 1997; Okamoto et al., 2000). Methyl-β-cyclodextrin (MβCD), a specific cholesterol depleting agent, was also used to evaluate the involvement of cholesterol in CXCR3 internalization (Cardaba et al., 2008; Rodal et al., 1999).

In both human SEB/IL-2 activated T cells and CXCR3-expressing transfected HEK 293 cells the use of inhibitors suggested that the pathway mediating CXCL11-induced internalization did not appear to require caveolae. These results are in agreement with studies carried out by Meiser et al using a similar approach and cell model to study CXCR3 (Meiser et al., 2008). Surprisingly treatment of T cells

with the cholesterol depleting agent MβCD had a noticeable effect (around 40% inhibition) on agonist-triggered loss of surface expression of CXCR3. It has been previously reported that filipin and cyclodextrin modify cholesterol-rich microdomains through different modes of action with different consequences on B cell receptor (BCR) signalling (Awasthi-Kalia et al., 2001). This may explain the differential effect they had on CXCR3 internalization observed in this study. Awasthi-Kalia et al concluded that MβCD, through extracting cholesterol without membrane binding, leads to dispersion of a large proportion of lipid rafts and prevents BCR translocation into rafts (Awasthi-Kalia et al., 2001). It is possible that this effect was also apparent for CXCR3, making it less available for agonist binding. Filipin does not cause cholesterol extraction but instead it inserts into the membrane and quenches cholesterol *in situ* (Elias et al., 1978; Mcgookey et al., 1983; Robinson and Karnovsky, 1980). Therefore filipin causes partial dispersal of lipid rafts constituents and its effect may not be pronounced enough to inhibit internalization of CXCR3. In addition filipin has been shown to have a little effect on CXCL11 binding to CXCR3 (Meiser et al., 2008).

In conclusion it is important to notice the existence of significant amount of apparently conflicting reports on the role of lipid rafts in chemokine receptor internalization (Neel et al., 2005). One of the reasons may be derived from similar studies using cholesterol depletion agents which may be too broad and may interfere with aspects of other trafficking pathways. Therefore in order to appropriately assess the importance of alternative endocytic pathways, more specific and targeted approaches need to be used. Moreover according to the results obtained it is unclear whether internalization of CXCR3 in T cell requires clathrin, as no effect of hypertonic sucrose was observed. In contrast, treatment with chlorpromazine caused significant inhibition of CXCL11-triggered internalization of CXCR3. An inhibition was also observed in HEK293 transfectants, however the inhibitory effect of chlorpromazine did not reached statistical significance. It is possible that inhibition of CXCR3 internalization by chlorpromazine was an off-target effect and it requires further investigation. However another explanation may be due to a possible antagonizing effect of chlorpromazine on β-arrestin binding to the CXCR3 receptor, which has been previously reported for the dopamine D2 receptor (Masri et al., 2008). Moreover β-

arrestin has been shown to be partially involved in CXCR3 internalization (Kohout et al., 2001; Meiser et al., 2008). Data shown in this thesis is partly consistent with findings reported by Meiser et al who found that agonist – induced CXCR3 internalization in transfected L1.2 cells (but not in T cells) was blocked by inhibition of clathrin (Meiser et al., 2008).
As a continuation of the study, considering the limitations of the pharmacological approach other more direct strategies, such as overexpression of a dominant-negative mutants of important endocytic components or siRNA technique would need to be conducted.

3.7.7 Signalling pathways involved in CXCL11–induced internalization of CXCR3 in human T cells

Various pharmacological agents targeting well-described signalling cascades were used in this part of the presented work, to investigate the regulation of agonist-induced down-regulation of surface CXCR3.

Like most chemokine receptors, CXCR3 couples to PTX-sensitive G_i and CXCR3-mediated chemotaxis of T cells is completely inhibited by PTX (Smit et al., 2003). In contrast internalization of CXCR3 is unaffected by PTX at the same (and higher) concentration which completely abolished chemokine – induced activation of p44/42 MAPK and PI3K/Akt pathways. These data indicate that CXCR3 internalization is not dependent upon G_i-protein coupling or p44/p42 MAPK and PI3K/Akt activation. Insensitivity of CXCR3 internalization to PTX treatment has been previously reported by Sauty et al (Sauty et al., 2001). Similar findings have also been reported for other chemokine receptors such as CXCR4 (Forster et al., 1998).

Moreover no effect was observed by broad spectrum inhibitors of PI3K such as LY294002, Wortmannin or ZSTK474. Similar results for CXCR3 were previously observed by Sauty at el in human T cells (Sauty et al., 2001).
The role of PIKfyve enzyme in vesicle trafficking has been well established (Rutherford et al., 2006; Jefferies et al., 2008). Here its role in CXCR3 endocytosis was examined using the selective PIKfyve inhibitor YM201636. This agent is reported to inhibit PIKfyve with an IC50 of 33 nM, and 3 μM for p110α (Jefferies et

al., 2008). Treatment of T cells with the PIKfyve inhibitor at micromolar concentrations up to 10 μM had a strong and moderate effect of Akt and p44/42 MAPK phosphorylation, respectively, but no effect on CXCR3 internalization. However increasing the concentration of YM201636 to 30μM significantly inhibited the loss of CXCR3 surface expression suggesting the involvement of p110α. Therefore to confirm that result PI-103 (which has at least 10 fold higher affinity for p110α (IC50 8 nM) over other members of class I of PI3K) was used (Workman et al., 2006). No effect of PI-103 (0.3-30 μM) on CXCR3 internalization was detected supporting the results using broad spectrum PI3K inhibitors (Figure 7.9 in Chapter Seven). Therefore the effect of YM201636 on CXCR3 down-regulation observed at higher concentration is possibly due to a yet unidentified off-target effect.

In the T cell model used in the study, PMA induced internalization of CXCR3 suggesting that intracellular signal transduction pathways that activate PKC may induce agonist-independent down-regulation of surface CXCR3. These results were consistent with previous findings for CXCR3 (Sauty et al., 2001) as well as CXCR4 (Signoret et al., 1997) and CCR3 (Zimmermann et al., 1999) Similarly, the PKC-delta inhibitor Rottlerin, and RO31-8220 (which inhibits conventional (α, β, γ) and novel (δ, ε, η) PKC isoforms at relatively low concentrations (EC_{50}s, approximately 20–100 nM) and the atypical PKC-ζ at higher concentrations (EC_{50}, 1–4 μM) (Standaert et al., 1997) attenuated CXCR3 internalization at high concentration. Therefore PKC appears to be involved in agonist independent and dependent down-regulation of CXCR3 from the surface. In addition, treatment with the PLC-blocking compound U73122 but not with its inactive analogue U73343 had an inhibitory effect on CXCR3 endocytosis. These studies contrast with the results of Sauty et al who observed no effect of PKC inhibition in CXCL11 – mediated CXCR3 internalization using staurosporin.

3.7.8 CXCR3 surface expression recovery following agonist exposure

Consistently with previous findings (Meiser et al., 2008) a slow rate of surface recovery of CXCR3 was observed suggesting slow recycling or degradation of CXCR3 following agonist stimulation. Treatment with cycloheximide confirmed that protein synthesis is important in CXCR3 surface replenishment. Therefore upon

agonist binding CXCR3 is possibly degraded and new receptor molecules are de novo synthesized in order to recover surface expression. CXCR3 surface expression recovery was also inhibited by disrupting of endoplasmic reticulum (ER) function suggesting that newly synthesized receptor needs to be efficiently transported through the ER to the Golgi. Similar results were obtained by Meiser et al who found that recovery of CXCR3 at the cell surface is dependent upon newly synthesized receptor trafficking through functional Golgi apparatus in the cell (Meiser et al., 2008).

3.7.9 CXCR3 agonists and CXCL4 induce biochemical signalling in human activated T lymphocytes

The focus of these studies was to investigate the biochemical signals induced by CXCR3 agonists CXCL9, CXCL10 and CXCL11 as well as CXCL4. First the ability all agonists to induce elevation of intracellular free calcium was assessed in SEB/IL-2 activated T cells. CXCL 11, 10, 9 and 4 induced calcium mobilization, although considerable differences between these chemokines were observed. CXCL11 was more potent and efficacious than other CXCR3 agonists. These results were in agreement with the reported reduced affinities for CXCL9 and 10 compared with 11 for CXCR3, with CXCL11 being unique as it interacts with 2 receptor states of CXCR3 (Cox et al., 2001; Smit et al., 2003). CXCL4 exhibited the lowest potency and micromolar concentrations were required to induce response, however its efficiency was comparable to CXCL9 and CXCL10. Similar findings have been reported for ConA activated T cells where high nanomolar concentration of CXCL4 had to be used in order to obtain intracellular calcium elevation (Mueller et al., 2008).

In addition to calcium responses, CXCR3 agonists and CXCL4 triggered activation of p44/42 MAPK and PI3K/Akt signalling pathways in activated T cells. Again, it was observed that CXCL11 was the most potent in its action (especially at the earliest time points of p44/p42 MAPK and Akt phosphorylation). Signalling induced by stimulation with all four agonists exhibited similar patterns, showing peak response after 2 minutes of incubation followed by rapid attenuation after 5 minutes of exposure. This may be explained by receptor desensitisation and/or internalization and possibly degradation. These data partially differ from results

obtained by Smit et al who detected a sustained Akt activation in response to low (1-10 nM) concentrations of CXCL11. In addition the same research group observed a maximum increase of p44/p42 MAPK and Akt phosphorylation at later time points of agonists stimulation in activated T cells in comparison to this study (Smit et al., 2003).

Sustained activation of MAPK and Akt was previously reported for CXCL12 binding to its receptor CXCR4 (Tilton et al., 2000). However in contrast to CXCR3, the CXCL12–CXCR4 couple is involved in homeostasis rather than inflammation, and both CXCL12 and CXCR4 have been reported to be required for embryogenesis (Tachibana et al., 1998; Zou et al., 1998). Rapid and transient phosphorylation of Akt and MAPK was also demonstrated for other chemokines such as CCL2, CCL5, CCL19 and CXCL10 which act through CCR2 (Charo et al., 1994), CCR5 (Raport et al., 1996; Samson et al., 1996), CCR7 (Yoshida et al., 1998) and CXCR3 (Loetscher et al., 1996; Yoshida et al., 1998), respectively (Tilton et al., 2000; Yoshida et al., 1998).

In comparison to CXCR3-mediated responses, the signal detected due to activation of CXCR3-B by CXCL4 was generally of lower strength, which may be explained by lower expression of CXCR3-B on the cell surface as a consequence of lower expression levels of its transcript, or alternatively the modified amino terminus of CXCR3-B might promote changes in receptor conformation and affect agonist binding and subsequently signalling. Moreover there was a certain level of variability in the results obtained with CXCL4. One possible reason underlying these differences could be variations between donors caused by different levels of CXCR3-B expression between individuals. The levels of CXCR3-B expression may differ with donor age, gender (the gene of CXCR3-B is present on chromosome X) or race. CXCR3-B expression could also depend on general health of the individuals however this possibility seems rather unlikely because human blood was donated by healthy volunteers. This potential correlation was not investigated within presented thesis but would be interesting to study further. Another possible reason of observed inconsistency could be due to variations in batches of recombinant CXCL4 or difference in quality and purity of CXCL4 purchased from different suppliers.

3.7.10 CXCL4 induces signalling in activated T cells in pertussis toxin-sensitive manner

Coupling of CXCR3-B to Gi protein is unclear due to opposite findings previously reported by two groups (Lasagni et al., 2003; Mueller et al., 2008). One study demonstrated PTX insensitivity and the lack of calcium influx in response to ligands and suggests the coupling of CXCR3-B to other types of G proteins in CXCR3-B transfectants (Lasagni et al., 2003). In contrast study by Mueller et al (2008) reported that CXCL4-mediated responses in both activated T cells and in CXCR3-B -expressing L1.2 cells were inhibited by PTX (Mueller et al., 2008). According to the results obtained within this thesis, in activated T cells CXCR3 agonists as well as CXCL4 couple to Gi protein as activation of both p44/42 MAPK and Akt pathways was abolished by PTX, suggesting that coupling to different Gα proteins may depend upon cell type.

3.7.11 Effect of CXCR3 antagonists on CXCR3-mediated responses

CXCL11-induced activation of p44/p42 MAPK and Akt was sensitive to treatment with CXCR3 antagonists. Potencies of these compounds were similar to those demonstrated for CXCL11-mediated CXCR3 surface down-regulation. In contrast using the highest concentrations of inhibitors it was possible to decrease responses nearly to basal levels. The inhibitory effect of a single concentration of T487 and NBI-74330 was also detected in migration and signalling induced by CXCL9 and CXCL10, whilst CXCL12 responses mediated by CXCR4 were not affected. Surprisingly p44/p42 MAPK and Akt phosphorylation stimulated by CXCL4 was not affected by CXCR3 antagonists. This result was opposite from that found by Mueller et al (2008) who observed that CXCL4-mediated chemotaxis was significantly (but not completely) inhibited by the CXCR3 antagonist T487. That may be explained either by CXCL4 interactions with different receptors than the CXCR3-B receptor present on the surface of T lymphocytes or insensitivity of the CXCR3-B isoform to CXCR3 antagonists. It would be of interest to further study this issue in both T cells and CXCR3-A and CXCR3-B expressing transfected cells. For example the effect of the CXCR3 antagonists on other CXCL4-triggered responses such as intracellular calcium elevation in both proposed cell systems could make for an interesting study.

3.7.12 Phosphorylation of GSK3β and S6 protein by CXCL4 and CXCR3 chemokines

As an alternative read-out of PI3K/Akt signalling pathway in addition to Akt activation, namely the phosphorylation of other down stream effectors such as GSK3β and ribosomal protein 6 S6 were assessed. This approach of PI3K pathway studying was previously used by others (Huang et al., 2007; Smith et al., 2007). The abilities of CXCR3 agonists and CXCL4 to stimulate phosphorylation of these proteins were investigated by immunoblotting. In day 9-12 SEB/IL-2 activated T cells CXCL9, CXCL10 and CXCL11 induced PI3K-dependent phosphorylation of both GSK3β and S6. Low levels of phosphorylation were also detected upon stimulation with CXCL4. Taken together with my earlier findings that demonstrate the ability of CXCL4 to induce p44/p42 MAPK and Akt in a PTX-sensitive manner, these results suggest that signalling events initiated by CXCR3 agonists and CXCL4 are similar.

3.7.13 CXCR3 agonist and CXCL4 induce actin polymerisation in activated T cells

Polymerization and a reorganization of the actin cytoskeleton is an early cellular event in the chemotactic response to chemokines. CXCR3-mediated actin rearrangements have been well studied (Dagan-Berger et al., 2006; Smith et al., 2007). According to data presented here, CXCL4 is also capable to induce actin polymerization in activated human T cells.

3.7.14 CXCR3 agonists but not CXCL4 induced chemotaxis in activated T lymphocytes

The effects of CXCL4 on T cells previously demonstrated by different groups suggested a role of CXCL4 in immunomodulation. CXCL4 has been reported to have an inhibitory effect of T cell proliferation via an antigen-specific stimulation (Fleischer et al., 2002) and the ability of CXCL4 to induce CD4+CD25+ regulatory T cell proliferation and to inhibit CD4+CD25- T cell proliferation (Liu et al., 2005) has also been demonstrated. In addition another study has shown a potential ability of CXCL4 to modulate Th1/Th2 polarization via differential regulation of transcription factor T-bet and GATA-3 (Romagnani et al., 2005). Therefore, the

actions of CXCL4 are opposite to several other chemokines, which are reported to promote T cell functions (Bacon et al., 1995; Taub et al., 1996).

Although CXCL4 was effective in inducing biochemical signalling (seen as phosphorylation of p44/p42 MAPK, Akt, GSK3-β and S6) and functional events (actin reorganisation) no chemotactic response of T cells was detected, even at high, micromolar concentrations. Data presented in this thesis opposite from the results described by Mueller et al, showing that CXCL4 induced directional migration of activated human T lymphocytes (Mueller et al., 2008). The possible explanation of this may be due to difference in responsiveness between SEB and ConA activated T cells. No chemotactic activity has been also detected in resting T cells (Fleischer et al., 2002) and previous reports have shown that even high concentrations of CXCL4 lack chemotactic activity for polymorphonuclear neutrophils (Petersen et al., 1996). Hence it would be interesting to further investigate how the method of T cells activation modulate their ability to respond to CXCL4 and determine the existence of possible distinct signalling pathway in differentially activated T lymphocytes.

As was suggested previously (Fleischer et al., 2002), CXCL4 may play a role in maintaining peripheral tolerance and in suppressing of autoreactive T cell responses at the sites of inflammation where high amounts of proinflammatory cytokines are present. Thus, although CXCL4 induces biochemical signalling events similarly to other chemokines, it does not share a typical chemokine features such as an ability to induce chemotactic activity.

3.7.15 Analysis of expression of human CXCR3-A, CXCR3-B and CXCR3-alt in transfected cells

Transfected cells systems are a commonly used model in chemokine receptors studies. Neither cell line used within this study (CHOK1 and HEK293) express any detectable endogenous chemokine receptors and have been shown to be capable of expressing high surface levels of chemokine receptors following transfection with constructs similar to these used here (Dagan-Berger et al., 2006; Proost et al., 2007). Thus in order to analyze three CXCR3 spliced variants the transfectants system was used.

Full length, C-terminal EFGP-tagged CXCR3 (referred to as CXCR3-A to distinguish it from other variants) CXCR3-B and CXCR3-alt were successfully expressed in HEK293 and CHOK1 cell lines. Flow cytometric analysis of the EGFP signal revealed comparable levels of CXCR3-A and CXCR3-alt expression whereas CXCR3-B was found to be expressed at a lower level. In addition analysis of surface expression of each receptor form demonstrated in either CHOK1 or HEK293 cells transiently transfected with either construct exhibited high expression of CXCR3-A (as shown by anti-CXCR3 antibody staining and EGFP expression) whilst CXCR3-B and CXCR3-alt surface expression appeared to be lower. This phenomenon may be a consequence of lower expression of their transcript (which was also observed in T cells). Moreover, due to alternative splicing of CXCR3 mRNA which results in extension of the CXCR3 protein by 52 amino acids at its N terminus, the receptor expression and function may be affected as this region of CXCR3 has been shown to be important for activation (Xanthou et al., 2003). Similarly, low levels of CXCR3-B expression were previously reported in transfected L1.2 cell line (Mueller et al., 2008). In contrast to the extended protein of CXCR3-B, in case of CXCR-alt differentially processed mRNA results in a dramatically altered receptor with only four to five predicted TMD (Ehlert et al., 2004). This impact is more extensive compared with other chemokine receptors which exists in more than one alternatively transcribed variants (Gupta and Pillarisetti, 1999; Lasagni et al., 2003; Wong et al., 1997; Yu et al., 2000). The most similar situation has been reported for the genomic mutant of CCR5, CCR5Δ32, in which case transcription results in protein with predicted structure lacking three TMD (Liu et al., 1996). This altered mRNA was considered not to be translated into protein or if so not to be transported to cell surface leading to lack of biological activity. These properties may be partially true for CXCR3-alt as its surface expression appeared to be dramatically lower in comparison to full length receptor. A similar observation was reported by Ehlert et al who demonstrated a 15-fold lower expression of CXCR3-alt than the full-size receptor in transfected HEK293 cells, however it appeared to traffic as functional receptor to the cell surface (Ehlert et al., 2004).

Difference in expression of spliced variants of CXCR3 (-B and –alt) in comparison to the original receptor may be also due to lower protein stability of these isoforms and faster degradation. It is not known if CXCR3 could form dimers with its other

isoforms but it would be interesting to examine if co-expression with CXCR3-A could improve stability of CXCR3-B and CXCR3-alt due to their aggregation. Finally high expression of some forms of CXCR3 (such as CXCR3-B) could lead to cell death. It is important to notice that cells expressing EGFP-tagged CXCR3-B exhibited different phenotype (round shape, less healthy look) comparing to cells expressing other variants (Figure 3.21F).

Due to lack of specific commercially available antibodies which distinguish between different CXCR3 proteins, in the study of surface expression of CXCR3 receptor and its variants, the mouse monoclonal pan anti-CXCR3 antibody (clone 49801) was used. Another available anti-CXCR3 antibody (clone IC6) directed against the 37 N-terminal amino acid residues of human CXCR3, previously used by Ehlert et al to detect CXCR3-alt, gave the same results. However it needs to be addressed that the affinities of these antibodies towards CXCR3-B and CXCR3-alt may be different to CXCR3-A due to structural changes within the protein. Therefore it would be good practice to develop antibodies directed against epitopes specific for each variant of CXCR3.

3.7.16 Agonists-induced down-regulation of CXCR3-A, -B and –alt surface expression

Although expression of surface CXCR3 is rapidly and dramatically reduced upon stimulation with CXCL11 in T cells, this effect is not that pronounced in CXCR3-expressing transfected cells. However a transfectant system expressing the human ortholog of CXCR3 is well established (Xanthou et al., 2003) and has been previously used in studies of CXCR3 internalization (Meiser et al., 2008). Despite that this model is useful in situation when more than one form of receptor exists.

C-terminal fusion of EGFP to CXCR3 variants resulted in impaired internalization of CXCR3-A and CXCR3-B in response to CXCL11 in comparison to unlabelled receptors, suggesting that EGFP tag affects interaction with adaptors. In contrast for both EGFP-CXCR3-alt and non-tagged CXCR-alt an increase in surface following CXCL11 stimulation was observed. This up-regulation is possibly due to release of previously preformed and intracellularly stored proteins of CXCR3-alt. A similar observation was previously reported for CXCR3 receptor in $CD4^+$ T cells

stimulated with PHA or exposed to the cyclooxygenase substrate arachidonic acid (Gasser et al., 2006). In comparison to the moderate effect CXCL11 of down-regulation of CXCR3-A and CXCR3-B, an effect of CXCL4 was only marginal which is similar to my previously discussed observations in T cells that CXCL4 failed to internalize surface CXCR3.

3.7.17 Intracellular calcium mobilisation induced by CXCL11 and CXCL4 in HEK293 cells transiently expressing CXCR3-A, CXCR3-B and CXCR3-alt

A previous study by Lasagni et al reported that in transfected microvascular endothelial cells CXCR3-B, but not CXCR3-A, was able to bind CXCL4 with high affinity and that CXCR3-B was able to induce neither calcium responses nor chemotaxis (Lasagni et al., 2003). In contrast another study demonstrated that when expressed in the L1.2 cell line both CXCR3-A and -B variants bind and chemotacticly respond to CXCL4 (Mueller et al., 2008). Moreover as previously discussed the same group reported intracellular calcium flux and chemotaxis in T cells in response to CXCL4. As reported in this thesis, both CXCR3-A and CXCR3-B isoforms induce intracellular calcium elevation in transfected HEK293 cells triggered by CXCL11 and CXCL4 suggesting that both receptors interact with CXCL4. In addition CXCR3-alt transfectants were capable of inducing calcium responses upon stimulation with CXCL11 although to a lesser extend. CXCR3-alt has been previously shown to be responsive to CXCL11 in transfected HEK293 cells (Ehlert et al., 2004).

3.7.18 Activation of PI3K/Akt and p44/42 MAPK pathways in cells expressing CXCR3-A, CXCR3-B or CXCR3-alt receptors by chemokines

Petrai et al has previously shown that p38MAPK is a downstream target of CXCR3-B activation. These results were obtained in CXCR3-B expressing HEK 293 cell using CXCL10 or CXCL4 as a stimulus (Petrai et al., 2008). Here the effect of CXCR3-B activation on other members of the MAPK family, namely p44/42MAPK and Akt, was investigated. Surprisingly only CXCL11, but not CXCL4, induced phosphorylation of p44/p42 MAPK CXCR3-A and CXCR3-B transfectents. In contrast no obvious effect of either CXCL11 or CXCL4 on Akt activation in CXCR3-B expressing cells was observed. This results were unexpected as CXCL4 did induce calcium responses in previous, presented here

experiments in transfected HEK293 cells and activated T cells and was capable to induce both p44/p42 MAPK and Akt in T cells. Therefore re-evaluation of these results in other cell lines could prove useful. This study also demonstrates for the first time that CXCR3-alt is able to induce both p44/p42 MAPK and Akt activation in HEK293 and CHOK1 cell lines following stimulation with CXCL11. Despite the ability of CXCL11 to induce phosphorylation (at least of p44/p42 MAPK) in transfected cell lines, there were noticeable variations between control samples and repeats of agonist-stimulated samples. The possible reason underlying this variability may be the transient method of cell transfection, which could result in different levels of surface expression of CXCR3 variants, especially when cells were transfected and then stimulated in individual wells. In order to avoid this error it would be a good practice to develop a stable cell lines expressing CXCR3 variants. That would allow a better reproducibility of results and help to avoid such variability between samples.

Chapter Four

4. Biochemical analysis of human CXCR7

RDC1/CXCR7 is a recently identified new member of chemokine receptors family. Despite intensive studies, its function as a typical chemokine receptor and mechanisms of action are not fully understood and are still to be elicited.

CXCR7 has been previously identified as a novel receptor for CXCL12. Moreover CXCL12 has been found to signal and promote chemotaxis of T cells through CXCR7 (Balabanian et al., 2005). Further studies by Burns et al also revealed additional ligand for CXCR7, namely CXCL11. Considering previous findings next part of presented work will focus on re-evaluation of the involvement of CXCR7 in CXCL12 and CXCL11-mediated chemotaxis and signaling of human T lymphocytes. CXCR7-expressing HEK293 cells will also be utilized to study CXCR7 internalization and biochemical signaling events in response to stimulation with agonist.

4.1 Expression of CXCR7 in human T lymphocytes

Expression of CXCR7 was investigated in human T lymphocytes activated by SEB/IL-2 and CD3/CD28 beads by semi-quantitative PCR and by flow cytometry, respectively. PCR analysis of revealed expression of CXCR7 at mRNA level (Figure 4.1, and Table 2.1 for primers sequences). Moreover, using flow cytometry analysis and 358426 mAb directed against CXCR7 we observed low levels (around 20%) of surface expression of CXCR7 in freshly isolated T cells and this expression was comparable to expression of CXCR3 in these cells (Figure 4.1 B and Figure 3.1C, respectively). Upon stimulation with CD3/CD28 antibodies-coated beads, level of CXCR7 expression decreased slightly and reached 13% on day 10 post-isolation (Figure 4.1B), compared with high expression of surface CXCR3 (Figure 3.1C). Changes in CXCR7 expression shown as mean fluorescence are shown in Figure 4.1C). Freshly isolated T cells contained marginally higher proportion of CXCR7+ cells comparing to activated cells however mean fluorescence showing surface expression of CXCR7 was moderately lower.

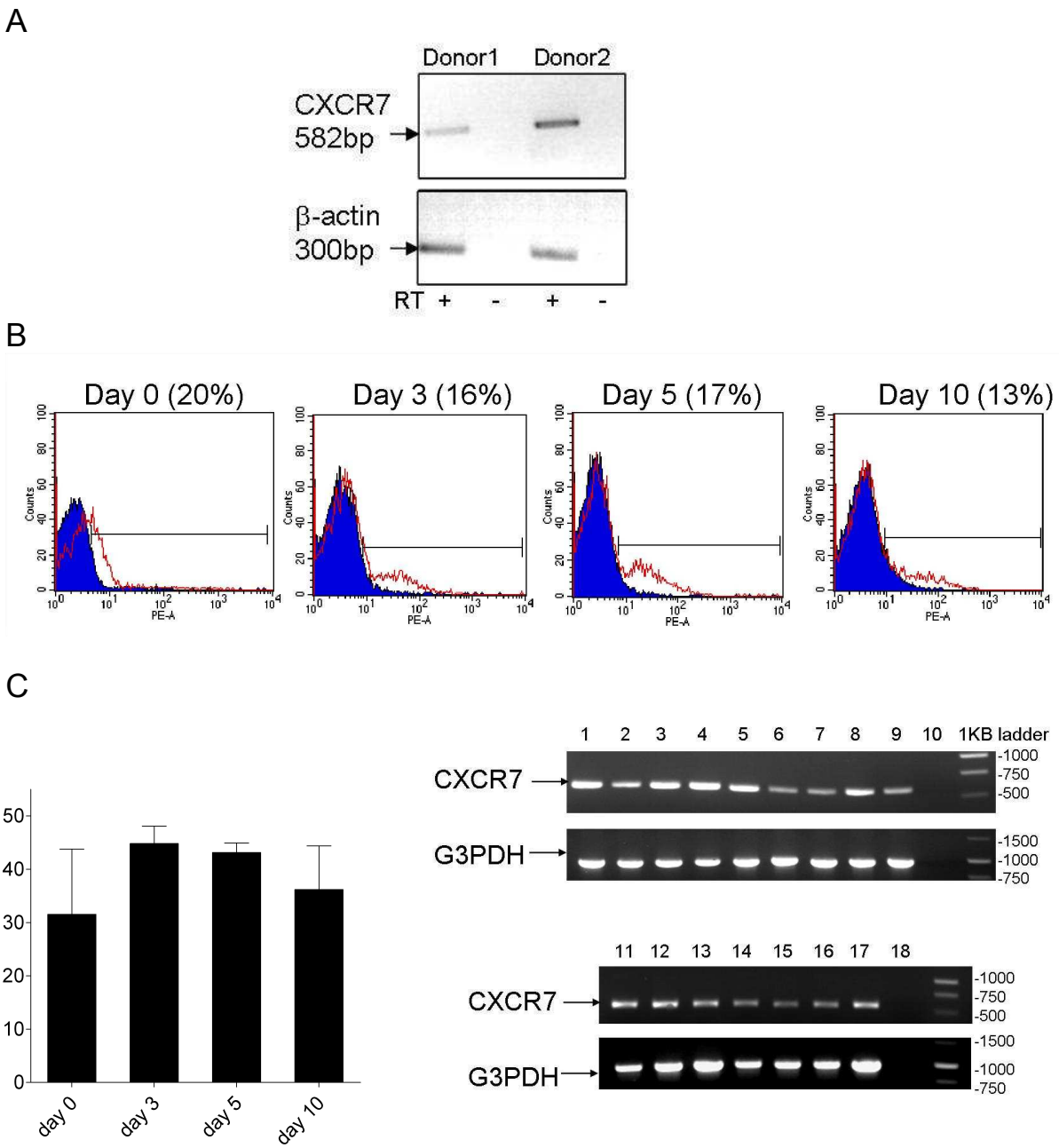

Figure 4.1 Expression of CXCR7 in human T lymphocytes and other cell types. PTO for the figure legend.

Figure 4.1 Expression of CXCR7 in human T lymphocytes and other cell types. (A), expression of CXCR7 RNA in day 9 SEB/IL-2 activated human T cells. Arrows point out bands corresponding to mRNA expression of CXCR7 and β-actin. RT +/- indicates presence or absence of Reverse Transcriptase. (B), expression of surface CXCR7 on freshly isolated (day 0) and CD3/CD28 activated T cells. Staining with anti-CXCR7 antibody is shown as open histograms (red line), staining with isotype controls is presented as filled histograms (blue). (C), Expression of surface CXCR7 on resting and activated T cells presented as MF. Data derived from at least two experiments. (D), Expression of CXCR7 at mRNA Level in Human Blood Cells and Human Immune System. PCR analysis was performed using cDNA preparations from Human Blood Fractions MTC™ Panel (upper case) or Human Immune System MTC™ Panel (lower case) (Clontech) as template and specific primers sets. Arrows point out bands corresponding to mRNA expression of CXCR7 (around 550 bp) and G3PDH (around 1000 bp).

Human Blood Fractions MTC™ Panel

1. mononuclear cells (B & T-lymphocytes and monocytes)
2. resting $CD8^+$ cells (T-supressor/cytotoxic)
3. resting $CD4^+$ cells
4. resting $CD14^+$ cells (monocytes)
5. resting $CD19^+$ cells
6. activated $CD19^+$ cells
7. activated mononuclear cells
8. activated $CD4^+$ cells
9. activated $CD8^+$ cells
10. control (dH_20)

Human Immune System MTC™ Panel

11. spleen
12. lymph node
13. thymus
14. tonsil
15. leukocyte, peripheral blood
16. bone marrow
17. foetal liver
18. control (dH_20)

Similar expression of CXCR7 was detected on the surface of T cells activated with SEB. The expression of CXCR7 mRNA was also assessed in other leukocytes and different tissues, by using PCR analysis. We utilized CXCR7-specific primers (as above), and commercially available cDNA probes derived from different fractions of human blood and tissues from immune system (Figure 4.1D).

Obtained results demonstrated that CXCR7 encoding mRNA was widely present in resting and activated T cells, B cells and monocytes, as well as spleen, lymph nodes, thymus, tonsils, bone marrow and foetal liver. These results however cannot exclude genomic contamination of used templates due to lack of RT-controls. Therefore it would be useful to re-design primers which will bind within different exons as CXCR7 has predicted 4-exons containing gene structure (Broberg et al., 2002; Zhang et al., 2009).

4.2 Effect of anti-CXCR7 antibody on chemotaxis and signalling of human T lymphocytes

Despite the high affinity of CXCR7 to CXCL12 and CXCL11, its role in CXCL12 and CXCL11-dependent chemotaxis is currently controversial issue (Balabanian et al., 2005; Burns et al., 2006). One study suggests that CXCL12 signals through CXCR7 in primary T cells and that CXCR7 cooperate with CXCR4 in lymphocyte motility (Balabanian et al., 2005). In contrast, evidence from other group did not confirmed a contribution of CXCR7 in T cell migration (Burns et al., 2006). To further address this issue, the potential involvement of CXCR7 in directional migration of activated human T cells was investigated. According to obtained results, chemotaxis of T cells induced by CXCL11 and CXCL12 was insensitive to treatment with anti-CXCR7 mAb 358426 (Figure 4.2A). No significant effect was also observed on random T cells motility (Figure 4.2B). The effect of the same antibody was then tested on CXCL11 and CXCL12-triggered activation of the PI3K/Akt and p44/42 MAPK pathways. Treatment with anti-CXCR7 mAb had no noticeable effect on the degree or kinetics of CXCL11 and CXCL12 induced phosphorylation of Akt and p44/42 MAPK (Figure 4.2C). Taken together presented results argue against any role of CXCR7 in CXCL11 or CXCL12 driven or random migration and CXCR3 or CXCR4 mediated signalling events such as Akt and p44/42 MAPK activation in activated T cells.

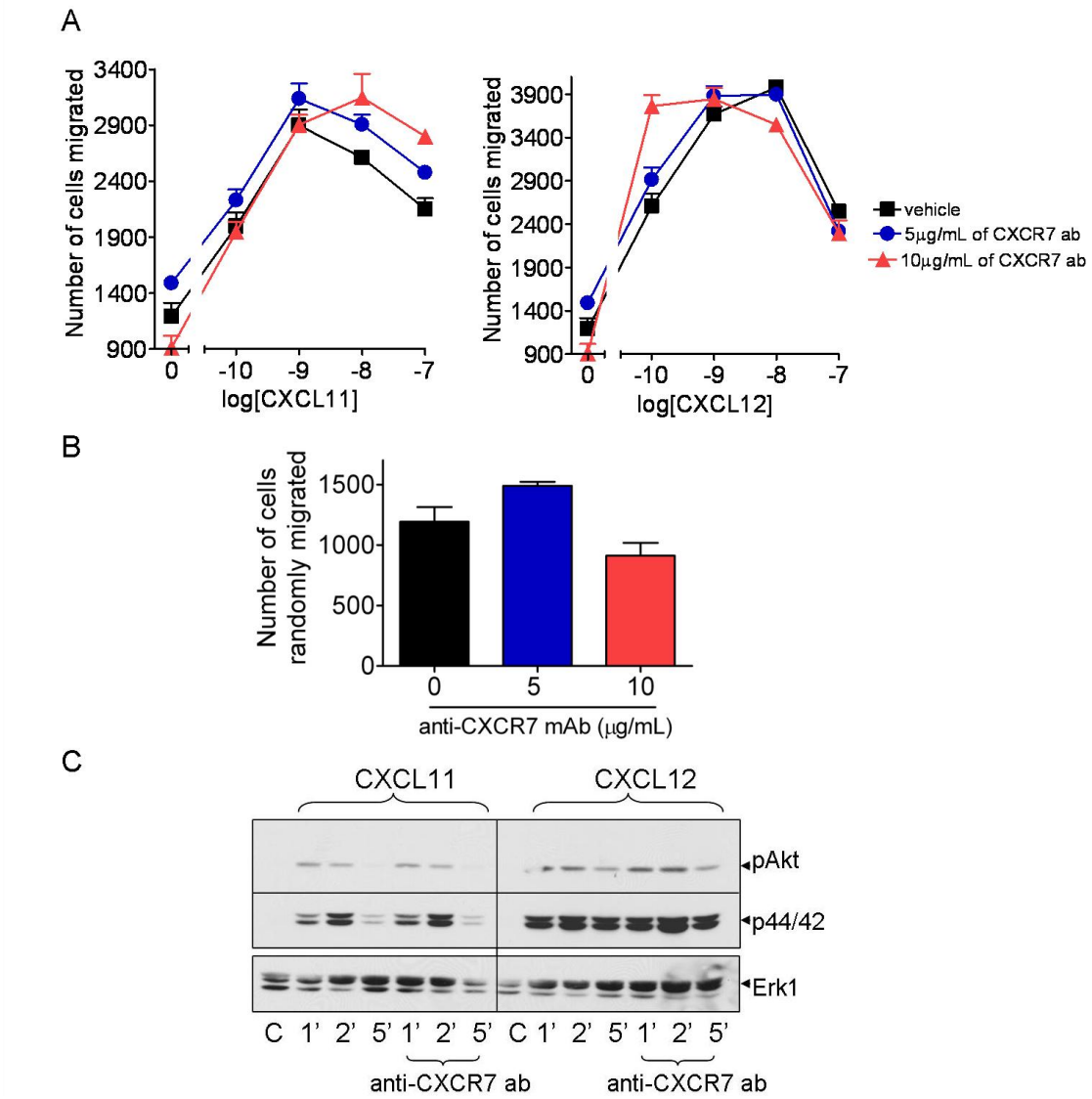

Figure 4.2 CXCL11 and CXCL12 induced chemotaxis and signalling in activated T lymphocytes is insensitive to anti-CXCR7 antibody treatment. (A), chemotaxis of day 9-12 activated T cells to increasing concentrations (0.1-100 nM) of CXCL11 (left panel) and CXCL12 (right panel) in the presence or absence of anti-CXCR7 antibody. Previously activated T cells ($3.2x10^6$) were washed, incubated with 5 or 10 μg/mL of anti-CXCR7 mAb for 30 minutes at 37°C and placed (25μL per well) above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* Presented data, expressed as number of migrated cell (mean +/- SEM) is taken from a single experiment with triplicates and is representative to two different experiments using cells isolated from different donors. (B), Effect of anti-CXCR7 mAb on random migration (C), T cells were incubated with 10 μg/mL of anti-CXCR7 mAb for 30 min at 37°C follow by stimulation with 1 nM of CXCL11 or CXCL12 for 1, 2 or 5 min. Control samples were stimulated with media. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-Ser^{473}specific Akt ab or anti-phospho- Thr^{202}/Tyr^{204} Erk1/2 (p44/42) ab. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. Presented data is representative for two different experiments performed using blood from different donors.

4.3 Expression of CXCR7 in HEK293 cell line

In order to further examine CXCR7 mediated responses a system consisting of HEK293 cells transiently transfected with constructs encoding human CXCR7 receptor with YFP tag on its amino terminus or non-tagged version of the receptor (both were gifts from Marcus Thelen, Switzerland) was used. Efficiency of transfection was determined 48 hours after procedures by flow cytometry (Figure 4.3). In the analysis, YFP reporter detection along side with anti-CXCR7 mAb clone 358426 was utilized in order to examine expression of tagged and non-tagged CXCR7 receptor in HEK293 cells. Both FACS analysis (Figure 4.4A left and central panel) and confocal microscopy (Figure 4.4B) confirmed high expression of CXCR7 on the surface of transfectants as assessed by PE-conjugated anti-CXCR7 mAb. This correlated with YFP expression in cells transfected with YFP-tagged construct, which also revealed robust intracellular expression of CXCR7 (Figure 4.4B).

4.4 CXCL11- and CXCL12- induced down-regulation of CXCR7 surface expression in HEK293 cells

Agonist- induced chemokine receptors internalization is a widely studied cellular process (Thelen, 2001). Internalization of CXCR7 mediated by CXCL12 has been previously shown for pre-B 300.19 cells and dermal fibroblasts expressing hCXCR7 (Balabanian et al., 2005). Therefore having established a good expression system, we investigated the effect of CXCL11 and CXCL12 stimulation on CXCR7 surface expression in the HEK293 cells model. Treatment of HEK293 cells expressing human CXCR7 or YFP-CXCR7 with increasing concentrations of either CXCL11 or CXCL12 induced a concentration-dependent internalization of CXCR7 (Figure 4.5A and B left panels). Maximum decrease in CXCR7 surface expression was observed from 100 to 30-40% after stimulation with 100nM of either CXCL11 or CXCL12 for non-tagged and YFP-tagged receptor. Time-course experiments revealed that CXCR7 surface expression decreased from 100 to around 40% within the first 5 min of incubation and after this time point we observed a small increase in expression, particularly in YFP-CXCR7-expressing cells (Figure 4.5A and B, right panel).

A

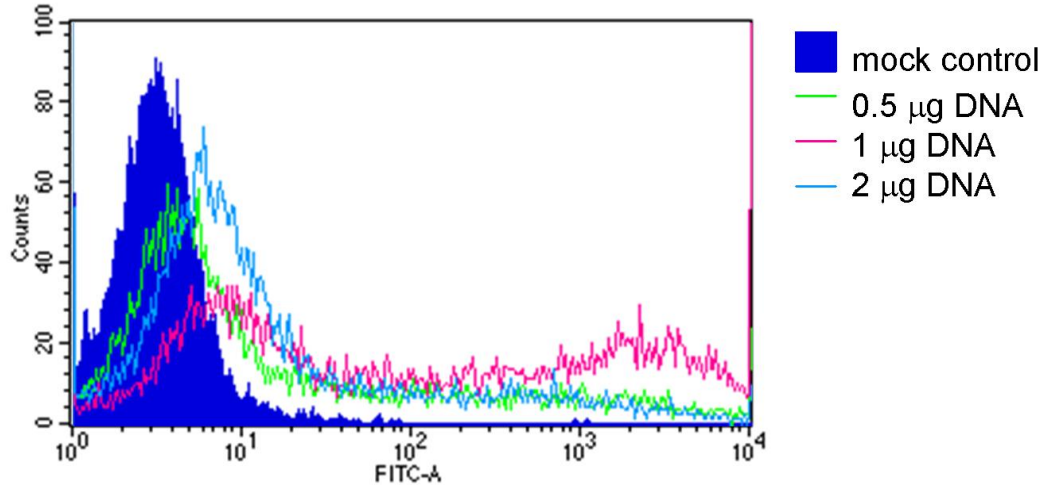

Figure 4.3 Optimisation of CXCR7 expression in HEK293 cell line. (A), HEK293 cells were transfected with different amounts of DNA encoding CXCR7-YFP or with empty vector and 48 hours post-transfection the expression of the receptor was assessed by FACS.

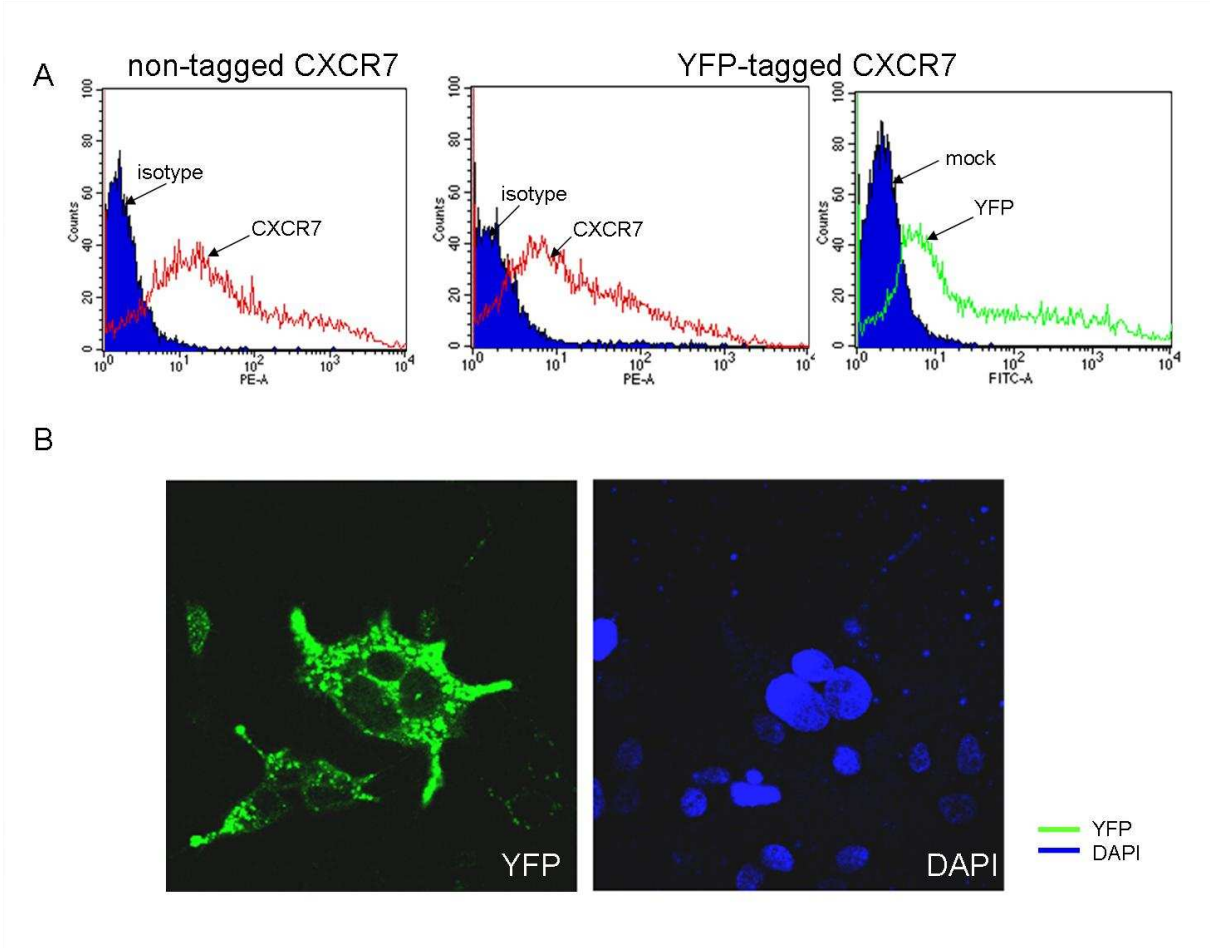

Figure 4.4 Expression of CXCR7 receptor in HEK293 cells. HEK293 cell were transiently transfected with CXCR7 chemokine receptor with or without YFP tag as described in Materials and Methods. 48 hours post transfection expression of CXCR7 was analyzed. (A), Flow cytometry analysis of CXCR7 expression by examination of surface staining with anti-CXCR7 mAb conjugated with PE (left and middle panels) and YFP reporter expression (right panel). (B), Confocal image of HEK293 cells expressing CXCR7 HEK 293 cells grown on poly-L-lysine-coated coverslips, were transfected with constructs encoding YFP-CXCR7 and 48 hours post-transfection expression of the receptor was analysed by confocal microscopy. Briefly, cells were fixed and coverslips were mounted with Mowoil containing DAPI (blue) for nuclear visualisation. Presented data are representative for 3-5 independent experiments.

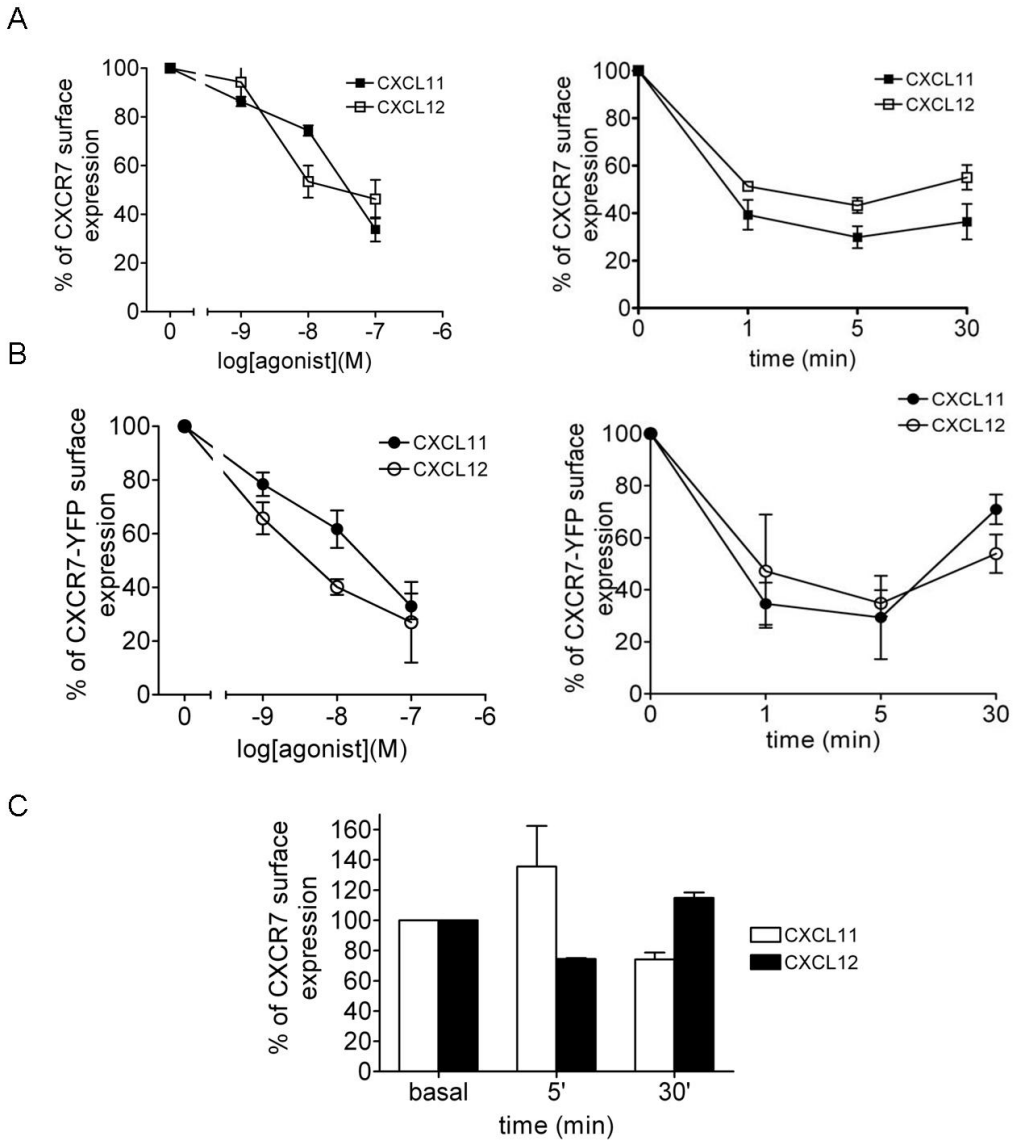

Figure 4.5 CXCR7 is down-regulated in response to CXCL11 and CXCL12. 48 hours post-transfection CXCR7 (A), or YFP-CXCR7 (B)-expressing HEK293 were incubated for 30 min at 37°C with the indicated concentrations of CXCL11 or CXCL12 (A and B left panels) or for the time indicated with 100 nM of each agonist (A and B, right panels) or left untreated. (C), Previously activated T cells were incubated with 100 nM of CXCL11 or CXCL12 for 5 or 30 minutes. Cell were then stained with the anti-CXCR7 mAb or IgG2A isotype control at 4°C and amount of receptors that remains at the cell surface after incubation with the chemokine was assessed by FACS. 100% corresponds to receptor expression at the surface of cell incubated in medium alone. Results represents the mean +/- SE of three independent experiments.

Despite of relatively low surface expression of CXCR7 in T lymphocytes an effect of CXCL11 and CXCL12 (Figure 4.5C) on this expression was also examined. A small decrease in CXCR7 surface expression for about 20% following stimulation with 100 nM of CXCL11 or CXCL12 for 30 or 5 minutes, respectively. In contrast shorter, 5 minutes exposure to CXCL11 and longer, 30 minutes exposure to CXCL12 led to increase of basal level of CXCR7 for about 20-40%.

4.5 Mechanisms of CXCR7 Internalization

Due to CXCR7 sharing agonists with CXCR3 and CXCR4, the effect of NBI-74330 and AMD3100, which inhibit CXCR3 and CXCR4 respectively on CXCL11- and CXC12- driven loss of surface of CXCR7 expression in transfected HEK293 was examined (Figure 4.6A). Neither NBI-74330 nor AMD3100 had any effect of down-regulation of CXCR7 following stimulation with CXCL11 and CXCL12. This suggest that the antagonists are receptor and not agonist selective.

As discussed previously there are two major pathways by which chemokine receptor can be internalized; either via clathrin-coated vesicles and then clathrin-mediated endocytosis or via cholesterol enriched structures-caveolae. To examined which pathway may be involved in agonist- triggered CXCR7 internalization we used chlorpromazine and hypertonic sucrose medium which have been shown to block the assembly of clathrin (Mueller et al., 2002; Okamoto et al., 2000) and filipin and nystatin which can inhibit internalization via caveolae (Harder et al., 1997). We examined the effect of these inhibitors on CXCR7 down-regulation in CXCR7-expressing HEK293 cells (Figure 4.6B). Sucrose and chlorpromazine had only a minimal effect of CXCL11- induced down-regulation of CXCR7 (5-15% of basal expression) suggesting that clathrin- mediated pathway may not play significant role in CXCR7 endocytosis. Similarly, no effect on CXCL12- triggered internalization of CXCR7 was observed. Neither filipin nor nystatin had any inhibitory effect on both CXCL11- and CXCL12- induced CXCR7 down-regulation suggesting that caveolae are not involved in down- regulation of CXCR7 in transfected HEK293 cells. These inhibitors were also addressed in study of CXCR3 down-regulation in transfected HEK293 cells following incubation with 30 nM CXCL11.

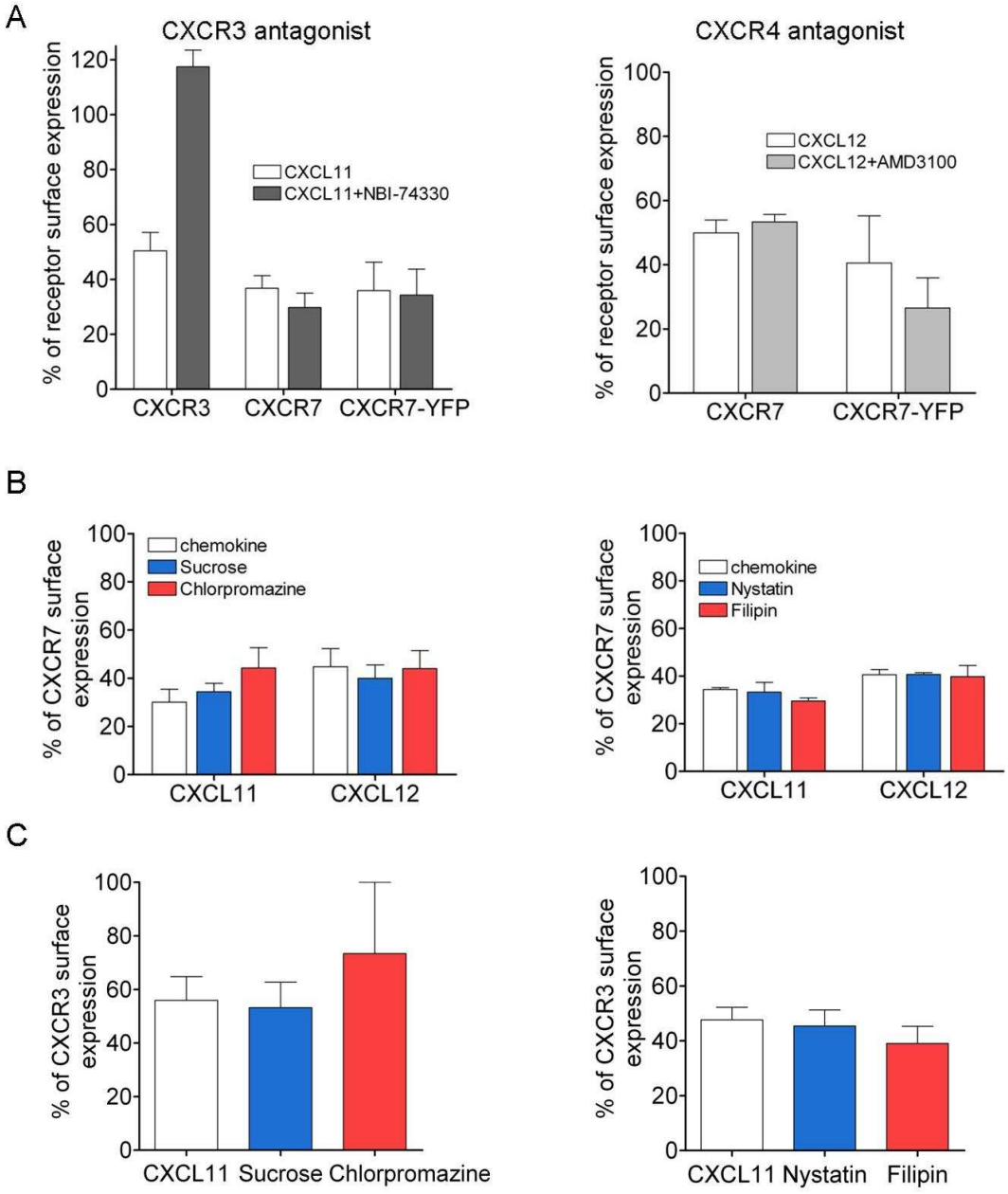

Figure 4.6 Mechanisms of CXCR7 down-regulation in HEK293 cells. (A), 48 hours post-transfection CXCR7 expressing HEK293 were incubated for 30 min at 37°C with 300 nM of NBI-74330 or AMD3100 follow by stimulation with CXCL11 or CXCL12, respectively. (B), CXCR7-HEK293 cells or (C), CXCR3-HEK293 cells were treated with 0.4M sucrose, 10 μg/mL of chlorpromazine (left panels), 50 μg/mL of nystatin or 5 μg/mL of filipin (right panels) follow by incubation with 100 nM of CXCL11 or CXCL12 (B) or 30 nM of CXCL11 (C). Cell were then stained with the anti-CXCR7 mAb or IgG2A isotype control or alternatively anti-CXCR3 mAb and IgG1 control at 4°C and amount of receptors that remains at the cell surface after incubation with the chemokine was assessed by FACS. 100% corresponds to receptor expression at the surface of cell incubated in medium alone. Results represents the mean +/- SE of 3 (or 2 in panel C) independent experiments.

Similarly to CXCL11- induced down-regulation of CXCR7, CXCR3 internalization was partially but not significantly reduced by chlorpromazine but not by sucrose treatment and was insensitive to nystatin and filipin (Figure 4.6C).

4.6 Analysis of biochemical signals mediated via CXCR7 receptor

In this part of the study two signalling pathways, Akt/PI3K and p44/p42 MAPK of CXCL11 and CXCL12 were delineated. Despite the fact that both CXCL11 and CXCL12 appeared to be inducers of CXCR7 surface down-regulation in the previous experiment they were unable to trigger any notable activation of the Akt/PI3K and p44/p42 MAPK signalling pathways in CXCR7-transfected HEK293 cells following stimulation with 100 nM concentration of each agonist (Figure 4.7). These data suggest that CXCR7 is not coupled to PI3K and p44/p42 MAPK signalling, at least in the transfected HEK293 cells model.

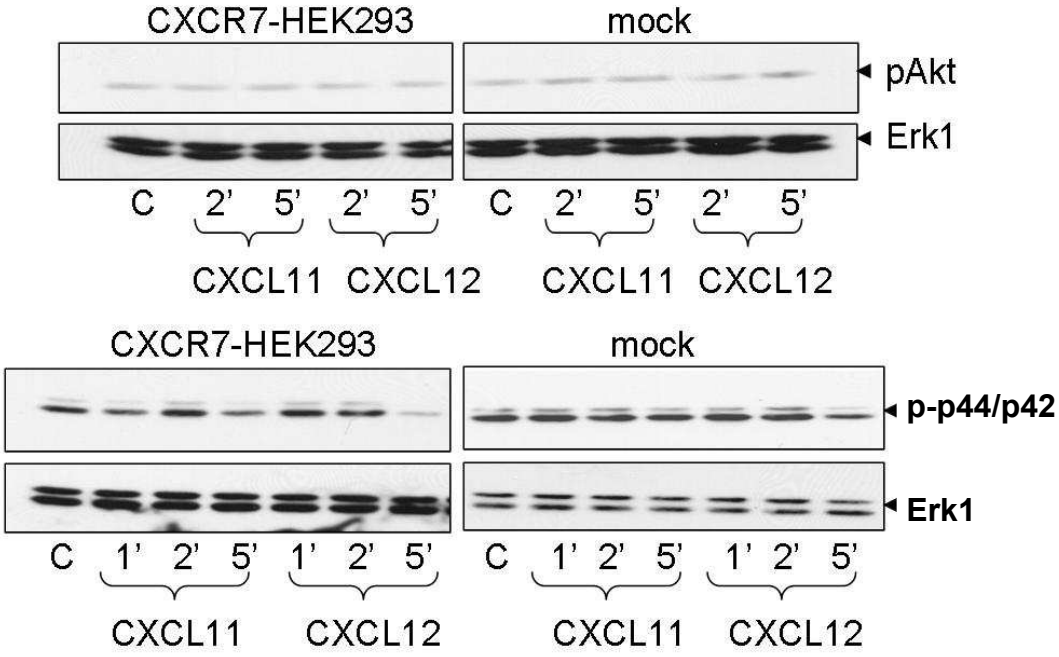

Figure 4.7 Effect of CXCL11 and CXCL12 stimulation on Akt and Erk phosphorylation in CXCR7- expressing HEK293 cells. 48 hours post-transfection HEK cells were stimulated with 100 nM concentration of CXCL 11 or CXCL12 for 1-5 min. Control samples were stimulated with media. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific Akt ab with the affinity for the active Ser^{473}-phosphorylated form of Akt or p44/p42 ab with the affinity for the active Thr^{202}/Tyr^{204} form of Erk. Proteins were visualized by ECL. The blots were stripped and reprobed with anti-Erk1 antibody to verify equal loading and efficiency of the protein transfer. In concentration response study p44/p42 MAPK and Akt phosphorylation was quantified by chemiluminescence and corrected for total Erk 1 expression on stripped blots. Presented data is representative for three independent experiments.

4.7 Summary of Chapter Four

In this part of the study our aim was to analyse the expression of CXCR7 in T cells and its role in T cells chemotaxis and signalling. Moreover we analyzed the CXCR7-mediated responses such as agonist-induced down-regulation and biochemical signalling in CXCR7-expressing HEK293 cells. Our major findings are as follows:

- CXCR7 in expressed in human T lymphocytes at mRNA level and low levels of CXCR7 are expressed on the surface of freshly isolated and activated T cells. Surface expression of CXCR7 was not dependent on the way of activation, similar levels of CXCR7 surface expression were detected on both CD3/CD28 antibodies-coated beads– as well as SEB- stimulated T lymphocytes.
- Treatment with anti-CXCR7 358426 mAb had no effect on neither CXCL11 and CXCL12 – mediated chemotaxis nor Akt and p44/p42 MAP kinase phosphorylation in T cells.
- HEK293 cells transiently transfected with CXCR7 or YFP-CXCR7 – encoding constructs exhibited high levels of CXCR7 expression on the cell surface.
- Surface CXCR7 and YFP-CXCR7 were down-regulated following CXCL11 and CXCL12 stimulation and this down-regulation was insensitive to CXCR3 and CXCR4 antagonists, NBI-74330 and AMD3100, respectively. Moreover endogenous CXCR7 expressed in T cells was partially internalized upon stimulation with both CXCL11 and CXCL12.
- Down-regulation of CXCR7 appeared to occur in clathrin and caveolae-independent manner.
- No Akt phosphorylation and no obvious p44/p42 MAPK phosphorylation could be detected following stimulation with CXCL11 or CXCL12 in CXCR7 transfectants.

4.8 Discussion

4.8.1 Expression of chemokine receptor CXCR7 in Human T cells

Formerly known as RDC1, chemokine receptor CXCR7, exhibits phylogenic relation to the chemokine receptor family and binds inflammatory and homing chemokines CXCL11 and CXCL12 (Balabanian et al., 2005). Despite that it has not yet found general acceptance as typical chemokine receptor as characteristic chemokine signaling could not be demonstrated (Burns et al., 2006).

Study of CXCR7 expression revealed that *CXCR7* gene is expressed in SEB/IL-2 activated and in vitro expanded IL-2 T lymphocytes. mRNA encoding CXCR7 was previously detected in brain, heart, kidney, spleen, and PBLs (Balabanian et al., 2005; Eva and Sprengel, 1993; Libert et al., 1990; Shimizu et al., 2000). However surface CXCR7 was mainly demonstrated on transformed tumor cells (Burns et al., 2006). Here, expression of surface CXCR7 was examined by immunofluorescence using mAb 358426. This antibody detected a relatively small (around 20%) population of CXCR7-expressing cells within freshly isolated $CD3^+$ T cells and this number changed minimally upon T cell activation. In a study by Balabanian et al. surface CXCR7 was also detected on PBL and the A0.01 T-cells using different monoclonal antibodies directed against CXCR7 (clones 9C4 and 12G5) (Balabanian et al., 2005). In contrast Hartmann et al. were not able to show any detectable surface expression of CXCR7 in freshly isolated T cells using three different antibodies (clones 9C4, 11G8 and 358426) and have reported CXCR7 to be present only intracellularly (Hartmann et al., 2008).

4.8.2 CXCL11 and CXCL12 induced chemotaxis and signalling in activated T lymphocytes is insensitive to anti-CXCR7 antibody treatment

Despite its high affinity for CXCL11 and CXCL12, the role of CXCR7 in CXCL11 and CXCL12-mediated chemotaxis and signaling is unclear (Balabanian et al., 2005; Burns et al., 2006). In order to identify the potential involvement of CXCR7 in CXCL11 and CXCL12-mediated responses in T cells, the effect of anti-CXCR7 mAb 358426 on was investigated. The chemotactic effect of neither CXCL11 nor CXCL12 was inhibited in the presence of the antibody (5 and 10 μg/mL). In addition treatment of T cells with the same antibody did not affect phosphorylation of p44/p42 MAPK or Akt. These observations were in disagreement with results

obtained by Balabanian *et al* who, using the anti-CXCR7 antibody 9C4, were able to partially interfere with CXCL12-induced migration of T cells (Balabanian et al., 2005). The lack of effect in these studies may be due to use of different antibodies and as the neutralizing abilities of anti-CXCR7 mAb 358426 has not been confirmed. However in another study, Hartmann *et al* reported that chemotaxis toward different doses of CXCL12 as well as Gi dependent signaling and motility of T lymphocytes and $CD34^+$ cells was also insensitive to CXCR7-blocking mAb (9C4, 11G8) and to the small molecule CXCR7 antagonist CCX733 (Hartmann et al., 2008). Taken together, it appears that T cells do not utilize their CXCR7 to transduce CXCL12- and possibly CXCL11-dependent intracellular and functional responses.

4.8.3 CXCL11- and CXCL12- induced down-regulation of CXCR7 surface expression in HEK293 cells

Because surface-receptor endocytosis is a well known process occurring upon chemokine stimulation, the effect of CXCL11 and CXC12 on CXCR7 internalization was investigated. In these studies CXCR7-expressing transfected HEK293 cells was utilized. Both full length YFP-tagged and non-tagged human CXCR7 receptors were found to be expressed at high levels at the surface of these cell making them an appropriate system to study CXCR7, as previously reported (Hartmann et al., 2008). Treatment of CXCR7-HEK293 cells with CXCL12 induced time and concentration dependent down-regulation of surface CXCR7. Similar results were demonstrated by Balabanian et al in pre-B 300.19 cells expressing human CXCR7, dermal fibroblasts, CHOK1 and A0.01 cells (Balabanian et al., 2005). In addition, transfected HEK293 cells were responsive CXCL11 as stimulation with this chemokine gave the same results. In contrast both CXCL11 and CXCL12 had only a minute effect on surface expression of endogenous CXCR7 in T cells.

4.8.4 Mechanisms of CXCR7 internalization

Despite some reports that CXCR7 becomes internalized following agonist binding (Balabanian et al., 2005), the mechanisms of this process are unknown. In order to establish the pathway of agonist induced endocytosis of CXCR7, previously discussed pharmacological approach were addressed. Unfortunately, the results obtained were unclear as there was no obvious effect of inhibition of either clathrin or caveaolin-dependent pathways on CXCL11 or CXCL12-mediated surface down-regulation of CXCR7 in HEK293 cells. Moreover, lack of controls proving biological activities of used compounds, makes any conclusions difficult. Furthermore, considering the limitation of using the low selectivity pharmacological inhibitors such as filipin, further studies using more directed approaches such as dominant mutant strategy or RNA interference are required.

4.8.5 Effect of CXCL11 and CXCL12 stimulation on Akt and Erk phosphorylation in CXCR7- expressing HEK293 cells

Having established the effect of CXCL11 and CXCL12 on surface CXCR7 in transfected HEK293 cells, their ability to induce biochemical signaling was investigated. No Akt phosphorylation and no obvious p44/p42 MAPK phosphorylation could be detected following stimulation with CXCL11 or CXCL12 in CXCR7 transfectants. As was proposed before in by Hartmann et al, the lack of signaling typical for chemokine receptor may be explained by alteration within its DRY motif similar to other atypical chemokine receptors such as Duffy antigen receptor for chemokines (Horuk et al., 1993), D6 (Bonini et al., 1997; Nibbs et al., 1997), or CCXCKR (Gosling et al., 2000). Results obtained here support previous observations in other cellular systems, indicating that CXCL12 binding to CXCR7 does not trigger calcium mobilization or chemotaxis (Burns et al., 2006; Sierro et al., 2007). Therefore as CXCR7 does not appear to be involved in typical signaling events, motility or chemotaxis, its role may be distinct to other chemokine receptors. One proposed possibility is that CXCR7 serves as an adaptor for a subset of CXCR4 molecules specialized in transducing rapid CXCL12-mediated integrin activation but is not essential for global CXCR4-mediated signaling implicated in cell motility or survival (Hartmann et al., 2008). In contrast a connection between CXCR7 and CXCR3 still remains unexplored and requires more attention. Moreover CXCR7 has been demonstrated to provide a

growth/survival signal and increased adhesiveness of cells and is suggested to be constitutively active in tumor cells, thus a role in tumor development has also been proposed (Burns et al., 2006).

Chapter Five

5. Role of protein arginine methylation in human T cells

It is well established that protein phosphorylation initiates signal transduction that in turn leads to lymphocyte activation. However, it has been demonstrated that other posttranslational modifications such as protein methylation may contribute to this process. There are several studies providing evidence for involvement of protein arginine methylation in T-cell activation (Blanchet et al., 2005; Mowen et al., 2004). NFAT co-activator NIP45 (NFAT Interactin Protein 45 kDa) is arginine methylated by PRMT and this modification is required for NFAT/NIP45 interaction (Mowen et al., 2004). It was also demonstrated previously that methylation of STAT (Signal Transducer Activator Transcription) proteins regulates interaction with its inhibitor PIAS1 (Protein Inhibitor of Activated STAT) thus plays role in cytokine signaling (Mowen et al., 2004). Further evidence came from the study by Blanchet et al who demonstrated that CD28 stimulation increases PRMT activity and arginine methylation of several proteins including two key effectors of CD28 signaling: interleukin-2 tyrosine kinase (Itk) and GDP-GTP exchange factor (GEF) for Rho-family GTPases – Vav-1. The importance of Vav-1 methylation remains unclear. However, the fact that methylated Vav-1 protein was found in the nucleus may be significant for its putative function in nucleus (Tybulewicz, 2005).

5.1 Profiles of arginine methylated proteins in T cells

Protein arginine methylation is a post-translational modification resulting in transfer of a methyl group from S-adenosylmethionine by protein arginine methyltransferases (PRMTs). This can lead to modifications in which arginine residues are either mono- (MMAs) or dimethylated (DMA). The dimethyl arginines can occur either symmetrically (sDMAs) or asymmetrically (aDMAs). In order to re-evaluate the effect on T cell activation of arginine-methylation of cellular proteins commercially available antibodies 5D1, 7E6, Asym 24 and Sym 11 reacting with MMA, MMA and DMA, aDMA and sDMA, respectively were utilized in immunoblotting of lysates derived from unstimulated and stimulated PHA/IL-2 T cells (Figure 5.1 A-D). Anti-monomethyl-arginine antibody recognized small amount of proteins in unstimulated T lymphocyte. Upon stimulation with anti-CD28

antibody (a process which mimics T cell costimulation), the amount of bands detected by antibody gradually increased after 5 to 30 minutes (around 100, 75, 40 and 30 kDa molecular weight Figure 5.1A). In addition, stimulation with CD28 A6 together with anti-CD3 antibody UCHT1 (which mimics TCR stimulation by antigen) for 30 minutes led to further increase of protein methylation. 7E6 antibody which reacts with mono- as well as dimethylarginine-containing proteins detected several bands in unstimulated cells and stimulation with either anti-CD28 or CD3 antibody resulted in increased levels of methylated proteins (Figure 5.1B). It appeared that additional bands (with molecular weights 60 and 25 kDa) were detected after CD28 then CD3 stimulation suggesting that co-stimulation might be more sufficient to trigger methylation of arginine residues. It is important to notice that protein bands detected at around 50 and 27-30 kDa correspond to the heavy and light chains of either CD28 or CD3 mouse antibodies, respectively. We then examined the effect of T cell stimulation on the levels of asymmetric and symmetric dimethyl-arginines-containing proteins. Asym24 and Sym 11 antibodies which react with aDMAs and sDMAs, respectively detected different patterns of protein bands in unstimulated T cells (Figure 5.1C and D). In contrast it was not possible to observe any increase in the levels of proteins containing dimethylated arginines neither asymmetrically or symmetrically methylated substrates following stimulation with anti-CD28 or both anti-CD28 and CD3 antibodies. The effect of the methyltransferases (MTases) inhibitor, methylthioadenosine (MTA) on the basal levels of protein arginine methylation was examined. The results revealed that both asymmetrical and symmetrical dimethylation of arginine decreased slightly after pretreatment with 1 mM of MTA for 30-60 minutes (MTA, Figure 5.2A).

To examine if MTA has an effect on cell viability, T cells were incubated in the presence of 1 mM MTA or DMSO (control cells) at 37°C for 5 hrs in serum free RPMI medium. Cells were then washed, resuspended in FACS buffer and 1μg/mL of propidium iodide (PI) was prior to flow cytometry analysis. PI binds to the DNA of the cell only when disruption of plasma membrane occurred, thus PI binding may be a marker of cell death. No difference of PI binding was found between MTA and DMSO treated samples suggesting that this compound has no obvious effect on cell viability (Figure 5.2B).

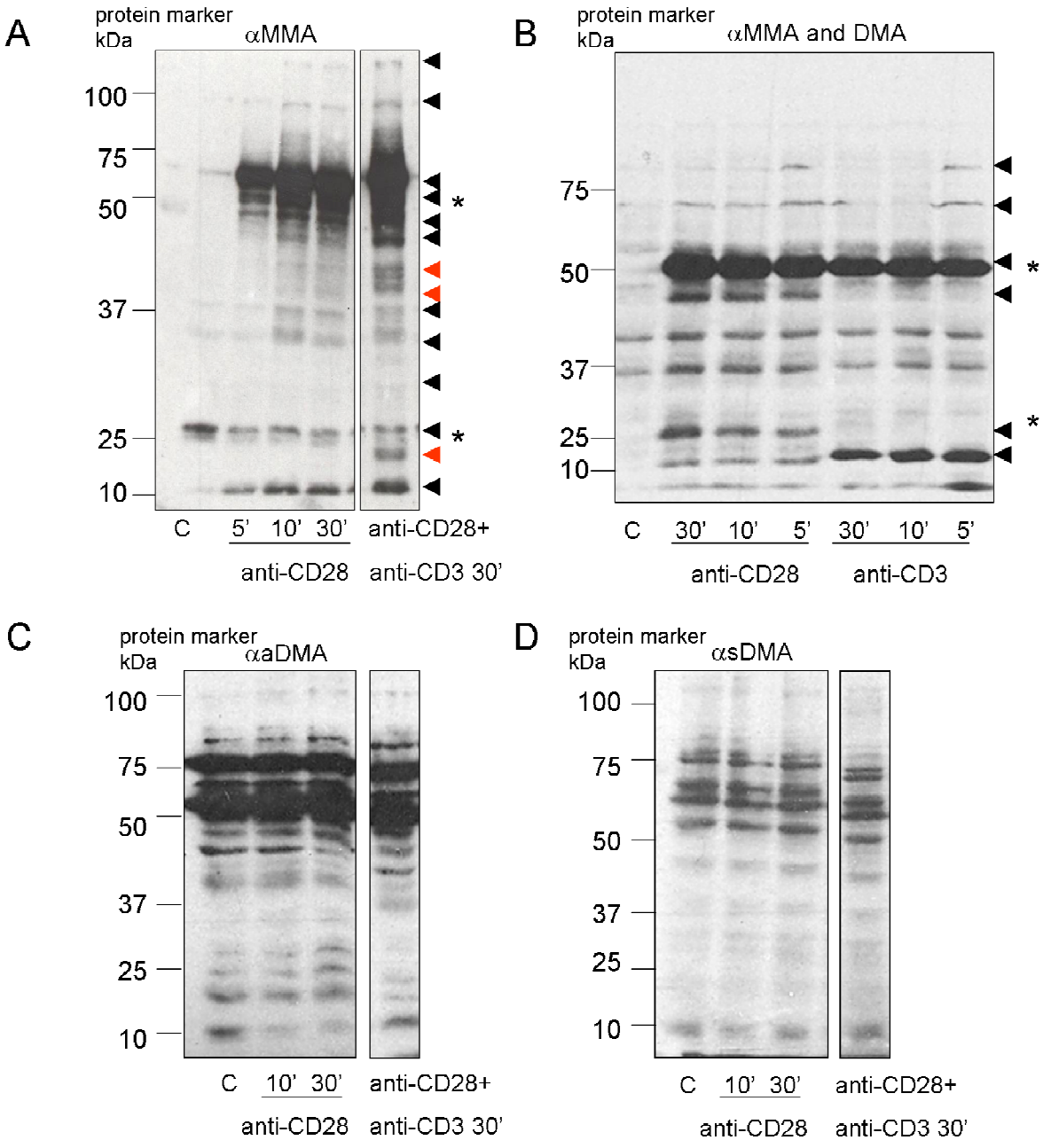

Figure 5.1 Profiles of protein arginine methylation in T cells. Day 9-12 PHA/IL-2 activated T lymphocytes were stimulated with 10μg/mL of anti-CD28 (clone 9.3), anti-CD3 (clone UCHT1) or both antibodies for times indicated. Control samples were stimulated with media. Samples were lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with (A), anti-MMA (clone 5D1) antibody; (B), anti-MMA and DMA (7E6) antibody; (C), anti-aDMA (Asym24); (D), anti sDMA (Sym11) antibody. Black arrow heads indicate bands induced by CD28 stimulation. Red arrow heads indicate bands induced by additional CD3 activation. Asterisks indicate predicted weights of heavy and light chains of antibodies used for stimulation. The data is representative to two to three independent experiments using T cell isolated from different donors.

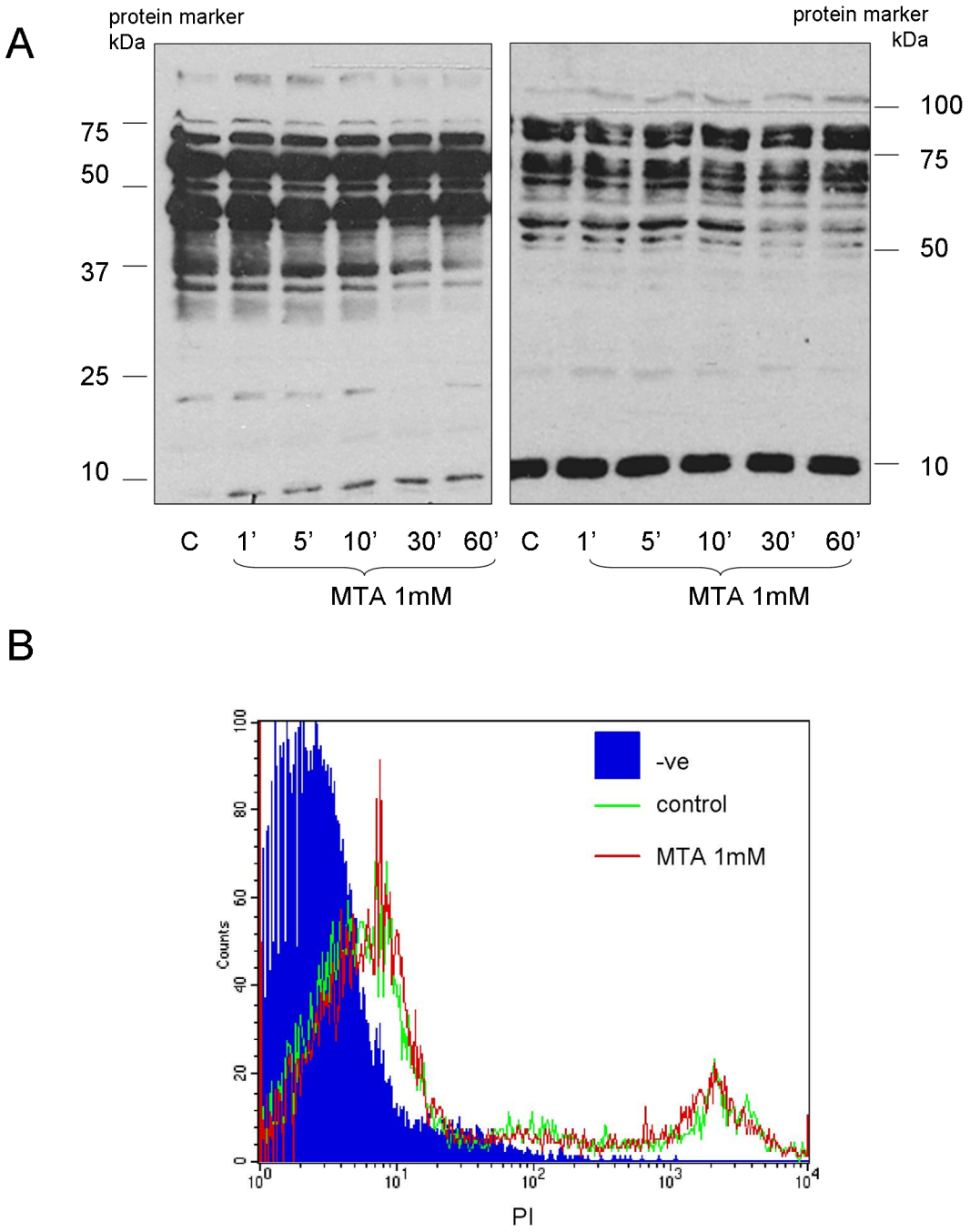

Figure 5.2 Effect of MTA of the basal levels of dimethylarginine-containing proteins in T cells. (A), Previously activated T cells were incubated with 1 mM of MTA for times indicated at 37°C. Samples were then lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies: anti-aDMA (Asym24, left panel) or anti-sDMA (Sym11, right panel). (B), T cells were incubated with 1 mM of MTA or DMSO (control) for 5 hours at 37°C. After incubation samples were washed and 1 μg/mL of PI was added. Samples were the analysed by FACS, fluorescence was collected at FL-2. The data is representative to three independent experiments using T cell isolated from different donors.

5.2 Effect of okadaic acid on the PRMT activity

Protein phosphorylation plays a key role in many cellular processes and is regulated by phosphatase activity. Thus, the balance of enzyme activity between kinases and phosphatases is critical in mediation of cell functional response.

Duong et al showed the first evidence that PRMT1 activity is negatively regulated via direct interaction with serine/threonine phosphatase PP2A (protein phosphatase 2A) (Duong et al., 2005). To investigate its effect on PRMT1 in T cells, the incubation with known PP2A inhibitor okadaic acid (500nM for 30 minutes) was performed follow by stimulation with anti-CD28 antibody. Okadaic acid alone had no effect on basal protein methylation profile (Figure 5.3C). Treatment of T cells with okadaic acid followed by activation with anti-CD28 antibody resulted in shift in migration of certain protein bands in the gel (detected proteins had molecular weight around 75 and 60 kDa) and sDMAs (proteins around 50 kDa) (Figure 5.3A and B). This up-shift in gel migration of highlighted proteins could be wrongly interprete as an increase in the level of methylated protein however is more likely caused by higher level of phosphorylation, the result of phosphatase inhibition. This could also explain a decrease within dimethylated proteins either containing aDMAs (50kDa) and sDMAs (40, 60 and 70 kDa). This proteins are possibly 'stuck' within up-shifted bands discussed above therefore giving impression of increase or decrease in methylation of some proteins. It is also worth notice the effect of okadaic acid had on total Erk1 levels (shown in Figure 5.3A and B). Samples in which cells were treated with okadaic acid migrated further up the gel in comparison to untreated samples presumably due to higher levels of protein phosphorylation in these cells.

In order to confirm the inhibitory effect of okadaic acid on PP2A, we examined its effect on Akt phosphorylation, which is down regulated by this phosphatase (Figure 5.3 E), (Valverde et al., 2000).

The effect of okadaic acid was also examined on arginine monomethylation using the antibody directed against MMAs. Again level of monomethylarginine-containing proteins raised in order to CD28 stimulation. Treatment with PP2A inhibitor had no detectable effect on MMAs-containing proteins level (Figure 5.3C). Antibody used in the experiment also detected a single band which appeared after 10' CD28 stimulation followed by treatment with okadaic acid however significance of that band is unknown.

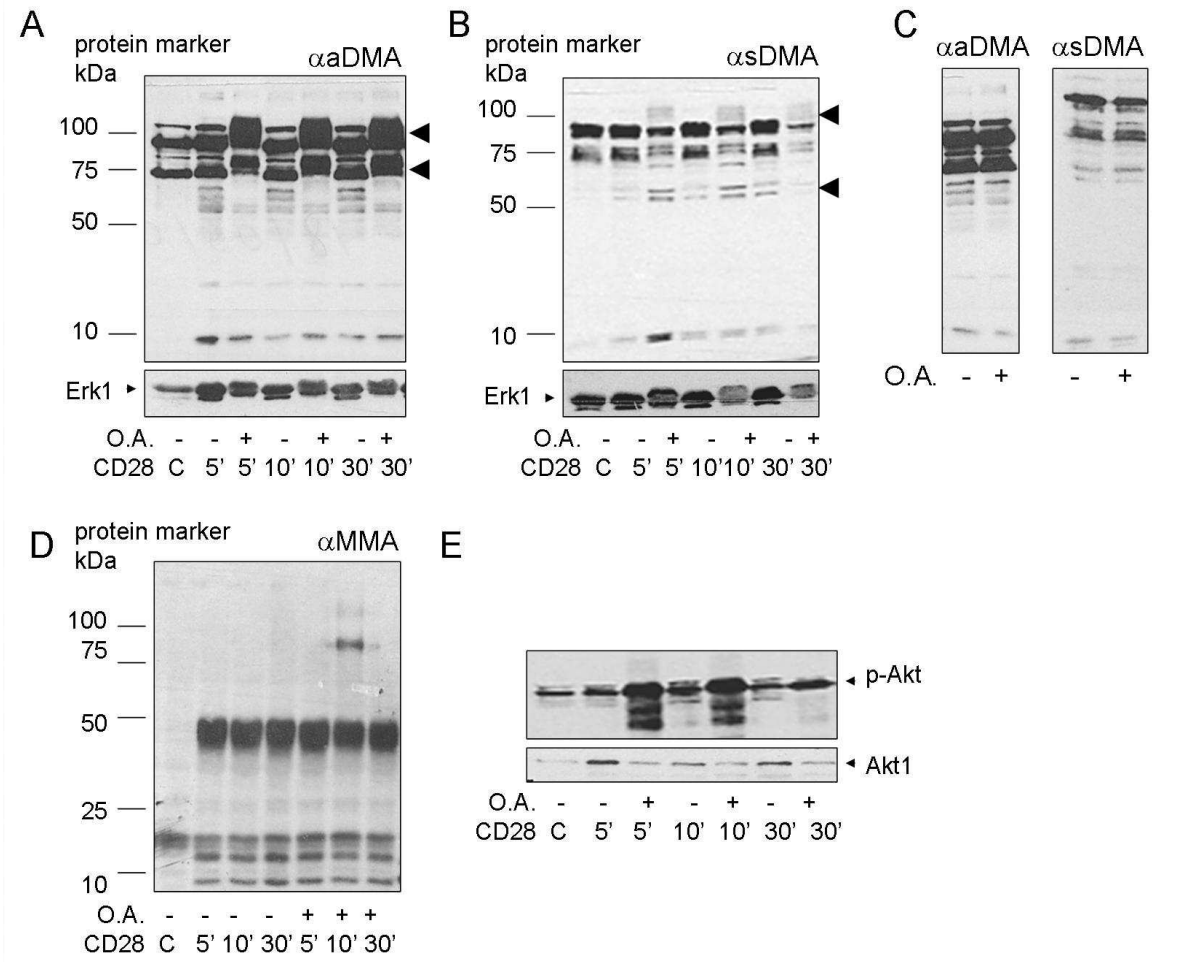

Figure 5.3 Effect of okadaic acid on PRMT1 activity. Previously activated T cells were incubated with 500 nM of okadaic acid for 30 minutes at 37°C followed by stimulation with the anti-CD28 antibody. Samples in panel (C) were not stimulated. Samples were then lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibody: (A, C left panel), anti-aDMA (Asym24); (B, C right panel), anti-sDMA (Sym11); (D), anti-MMA (7E6); (E), anti-phospho-Akt (Ser^{473}). Bands changed after okadaic treatment are indicated by arrow heads.

5.3 The role of protein methylation in T cell migration

Despite the well established role of methylation in chemotaxis of *Escherichia coli* (Silverman and Simon, 1977; Parkinson et al., 2005), the role of this modification in mammalian lymphocyte migration is unexplored. Some of exciting findings suggesting a potential role of protein methylation in migration of T cells are discussed in chapter 1.17 of Introduction.

Considering described findings it was aimed to investigate the effect of inhibition of methylation in vitro migration of activated T lymphocytes. First the effect of methyltransferase inhibitor, methylthioadenosine (MTA) on CXCL12 stimulated actin polymerisation of T cells was examined. MTA (1 mM) pre-treatment abrogated F-actin activation to basal levels as shown by phalloidin binding (Figure 5.4A). In order to determine the role of protein methylation in T cell migration, cells were pre-treated with increasing concentration of MTA (10-3000 μM) prior to performance of chemotaxis assay using 1 nM CXCL11 as a stimulus. Only the highest concentration significantly but not completely inhibited on the CXCL11-trigered directional migration (Figure 5.4B). In contrast, in some cases MTA treatment had a much stronger effect on migration, suggesting that sensitivity to the inhibition of methylation may be donor dependent. As shown in Figure 5.4C directional migration of T cells towards rising concentrations of CXCL11 was completely abolished by 3 mM MTA. We also examined the effect of inhibition of methylation on chemotaxis of freshly isolated pan T lymphocytes. These cells exhibit moderate expression of CXCR3 but high levels of CXCR4 (Mueller, 2008; Sotsios et al., 1999) therefore CXCL12 was chosen to stimulate chemotaxis in the experiments. Pretreatment with 3 mM MTA had a partial effect on directional migration of T cells with the significant effect on peak response (towards 10 nM CXCL12, Figure 5.4D).

In the further study the effect of other known inhibitors of protein methylation on T cells migration was also investigated. First the effect of sinefungin which is a natural nucleoside produced by Streptomyces incarnatus and Streptomyces griseolus was examined (Yebra et al., 1991). It is a structural analog of S-adenosylmethionine (SAM), which is a known inhibitor of transmethylation reactions related to DNA proteins phospholipids and other molecules (Barbes et

al., 1990; Fuller and Nagarajan, 1978; Mccammon and Parks, 1981; Paolantonacci et al., 1985). As is shown in Figure 5.5A, 30 minutes incubation of T cells with 1, 10 and 100 μM of sinefungin did not affect neither chemotaxis towards 1 nM of CXCL11 and 10 nM of CXCL12 nor random migration. We then proceeded to examine an effect AMI-1, small molecule that specifically inhibit protein arginine *N*-methyltransferase (PRMT) activity (Cheng et al., 2004). Treatment of T cells with 1-100 μM concentration of AMI-1 did not affect chemotaxis induced by CXCL11 and CXCL12 or random migration (left and right panels, respectively Figure 5.5B).

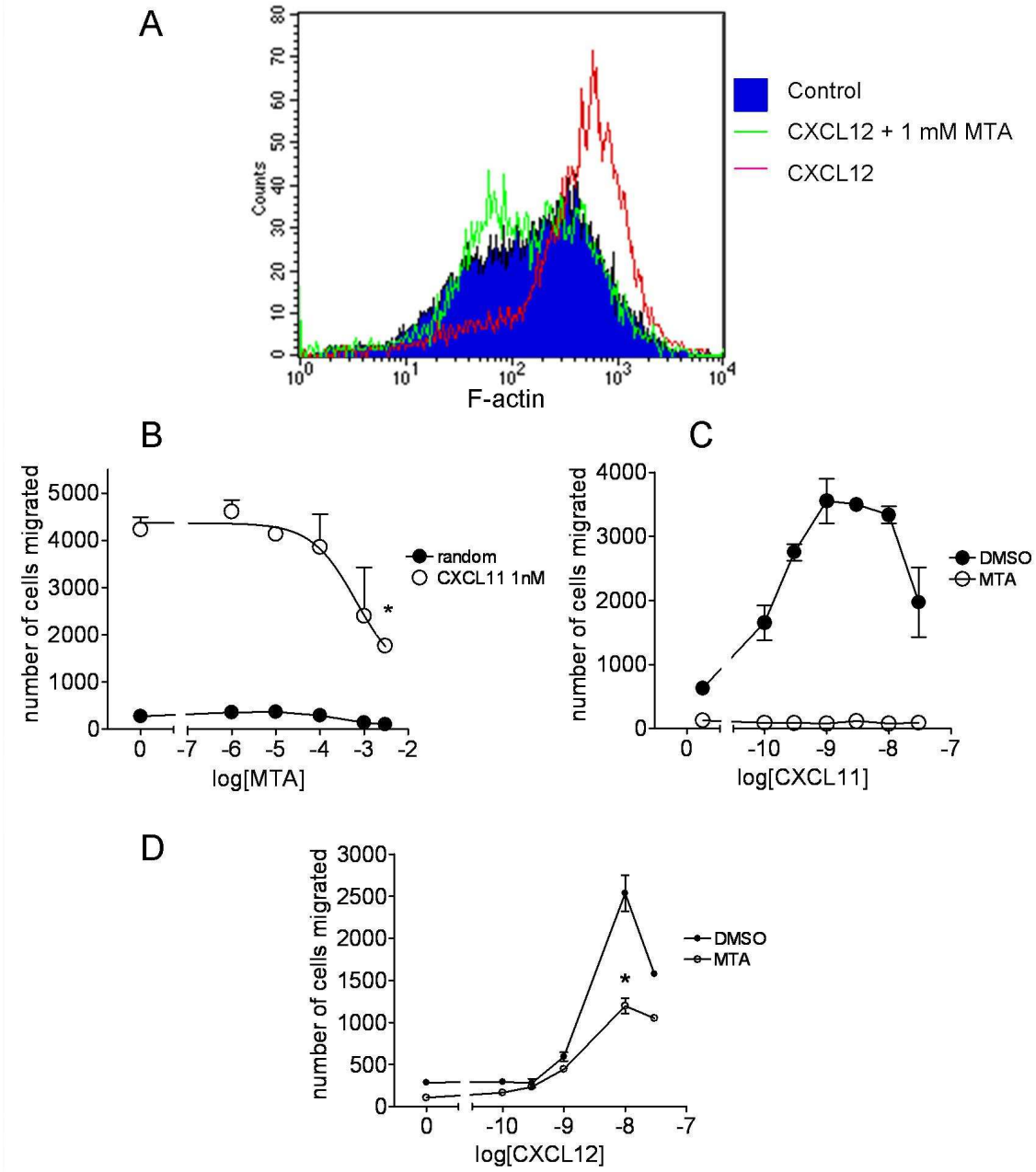

Figure 5.4 Effect of MTA on T lymphocyte migration. PTO for the figure legend.

Figure 5.4 Effect of MTA on T lymphocyte migration. (A), Inhibition of actin polymerisation in T cells. Day 9-12 SEB/IL-2 activated T cells were incubated in the presence of 1 mM MTA and stimulated for 1 minute with 0.3 nM CXCL12. Cells were then fixed in 4% para-formaldehyde, permeabilized and stained with TRITC-labelled phalloidin. F-actin polymerization was monitored by FACS. Data is representative for three other experiments. (B), Inhibition of T cells chemotaxis towards 1 nM CXCL11 by increasing concentration of MTA. (C), Inhibition of T cell chemotaxis to increasing concentration of CXCL11 by 3 mM of MTA. (D), Inhibition of chemotaxis of freshly isolated pan T cells towards increasing concentrations of CXCL12 by 3 mM MTA. T cells were washed, incubated with appropriate concentrations of MTA for 30 minutes at 37°C and placed (25μL per well) above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* Presented data, expressed as number of migrated cell (mean +/- SEM) is taken from a single experiment with triplicates and is representative of two different experiments using cells isolated from different donors.

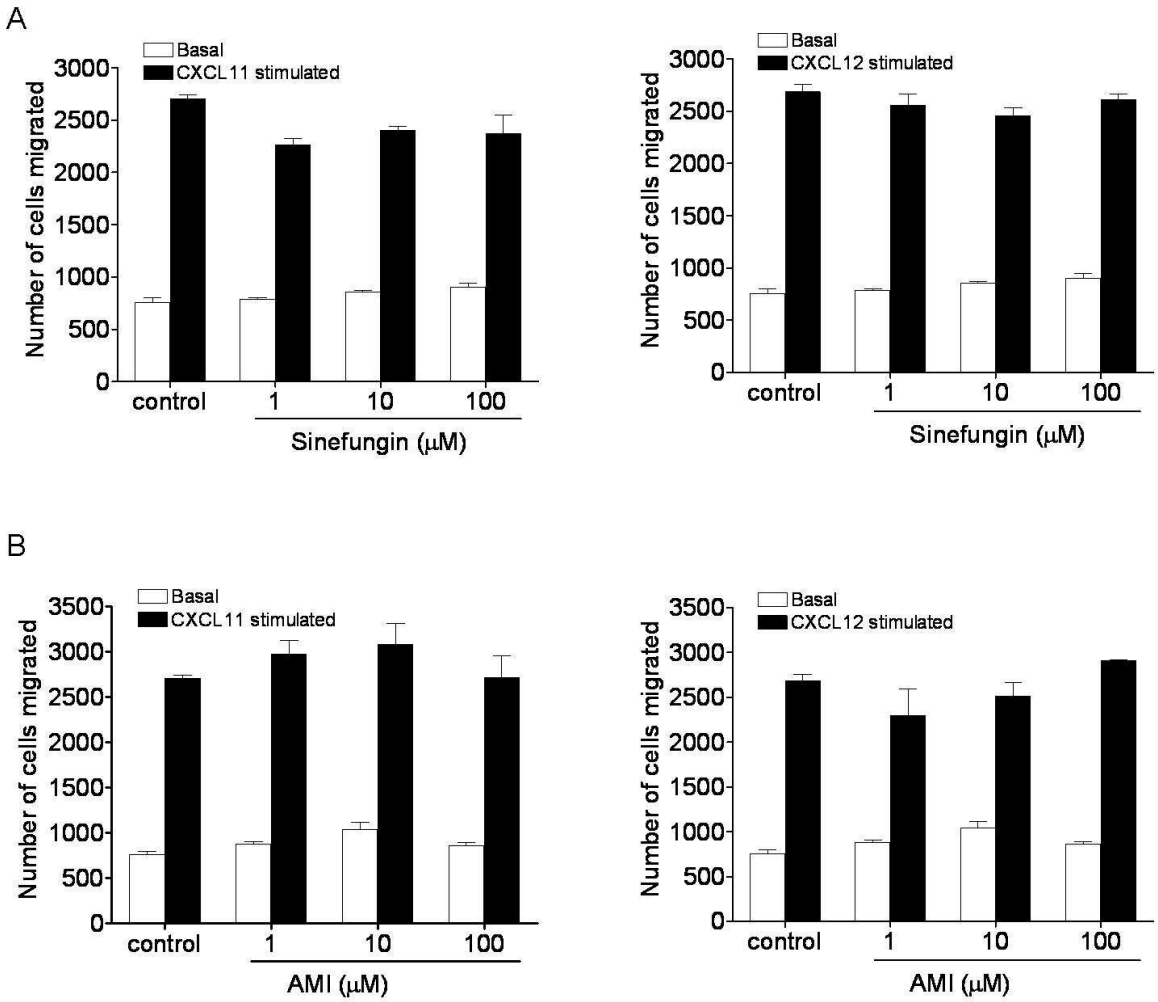

Figure 5.5 Effect of Sinefungin and AMI on T lymphocyte migration. Previously activated T cells were washed, incubated with appropriate concentrations of Sinefungin (A) or AMI-1 (B) for 30 minutes at 37°C and placed (25μL per well) above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.* Presented data, expressed as number of migrated cell (mean +/- SEM) is taken from a single experiment with triplicates and is representative of three different experiments using cells isolated from different donors.

5.4 Summary of Chapter Five

In this section we aimed to re-investigate the effect of T cell stimulation on the levels of protein arginine methylation. Moreover we were interested in the possible effect of protein methylation in chemokine-induced directional migration of T cells. During study the following observation were made:

- Using antibodies directed against MMA, aDMA and sDMA we were able to detect characteristic profiles of mono- and dimethylarginine-containing proteins in T cells, respectively.
- Levels of monomethylarginie methylation increased upon CD28 stimulation and addition of both anti-CD3 along with anti-CD28 antibodies resulted in further increase.
- No effect on dimethylation of arginines was observed following T cells stimulation with anti-CD28 or both-CD28 and CD3 antibodies.
- Inhibition of PP2A with okadaic acid led to increased protein phosphorylation (slower migration in the gel of phosphorylated proteins) but no obvious effect on arginines methylation could be detected.
- Treatment with broad spectrum methylation inhibitor MTA blocked CXCL12-induced actin polymerisation.
- MTA had a significant inhibitory effect on T cells chemotaxis induced to CXCL11.
- Chemotaxis of freshly isolated pan T cells towards CXCL12 was also affected by MTA treatment.
- No inhibition of chemotaxis was detected after treatment with sinefungin and AMI-1.

5.5 Discussion

5.5.1 The role of protein arginine methylation in T cells activation

Posttranslational modifications often determine a final molecular state and activity of the proteins. In addition to extensively studied phosphorylation, protein arginine methylation has recently begun to attract attention as it has been shown to contribute to many cellular processes by modifying proteins as well as nucleic acids and phospholipids (Banerjee, 1980; Chiang et al., 1996; Ueland et al., 1984).

Methylation is particularly crucial in the function of the immune system. For example, interference with SAHase was associated with decreased Ig production and slower growth of lymphoblastoid cells (Tsuchiya et al., 1981) and impaired humoral and cell mediated immune responses (Fu et al., 2006; Wolos et al., 1993a; Wolos et al., 1993b; Wu et al., 2005). Mice deficient in arginine methyltransferase CARM1 exhibited developmental arrest at the early stage of thymocyte progenitor differentiation (Kim et al., 2004). Moreover methylation reactions appear to be required more in lymphocytes than other cell types, particularly after activation (German et al., 1983; Kim et al., 2004). First, the role of this modification has been demonstrated in regulation of cytokine gene transcription in Th lymphocytes. Mowen and colleagues have shown that inhibition of arginine methylation affected the expression of several cytokine genes, including the hallmark T helper cell cytokines, IFN-γ, and IL-4 (Mowen et al., 2004). Moreover the same group also reported that T cell receptor signalling enhanced expression of the protein arginine methyltransferase PRMT1, which in turn methylated the nuclear factor of activated T cells (NFAT) cofactor protein, NIP45 (Mowen et al., 2004). Another study demonstrated that CD28 co-stimulatory signal induced protein arginine methyltransferase activity and methylation on arginine of several proteins, including Vav1 (Blanchet et al., 2005). A different study by Lawson et al. performed in $CD4^+$ T cells also reported that inhibition of transmethylation leads to immunosuppression by reducing phosphorylation of several key proteins involved in TCR signaling, including Akt, Erk1/2, and NF-κB and this effect is correlated with reduced methylation of Vav1.

In this study the effect of T cell activation on protein arginine methylation was examined using anti-CD28 and anti-CD3 antibodies, whose actions mimic the costimulatory signal and TCR activation respectively. In order to detect changes in arginine-methylation of cellular proteins commercially available antibodies 5D1, 7E6, Asym 24 and Sym 11 reacting with MMA, MMA and DMA, aDMA and sDMA, respectively were utilized. Levels of monomethylarginie methylation increased upon CD28 stimulation and addition of both anti-CD3 along with anti-CD28 antibodies resulted in further increase. No effect on dimethylation of arginine was observed following T cell stimulation with anti-CD28 or both-CD28 and CD3 antibodies. This lack of effect may be due to limited reactivity of antibodies which would make detection of gentle changes impossible. Therefore in order to further investigate that issue a more sensitive method would be required. For example immunoprecipitation of methylated arginine-containing proteins followed by mass spectrometry analysis would be advised. This would be more specific than immunoblotting and it could reveal the identity of methylated proteins.

It has been shown by Duong et al that PRMT1 activity is negatively regulated via direct interaction with the serine/threonine phosphatase PP2A (protein phosphatase 2A) (Duong et al., 2005). Here, in a T cell model, activity of PRMT1 appeared to be up-regulated following inhibition of PP2A with okadaic acid as the increase in asymmetric dimethyl-containing proteins was increased. In contrast no increase was detected in symmetric dimethyl-containing proteins suggesting no effect of PP2A inhibition on type two PRMTs. Moreover as observed by up-shifted migration on SDS-PAGE, some of methylated proteins were also phosphorylated. This was observed in both symmetric and asymmetric dimethyl-containing proteins.

5.5.2 Role of protein arginine methylation in cell migration

Several studies point toward a role of methylation in the chemotactic activity of a cell. The first evidence was derived from study of *Escherichia coli* chemotaxis in which methylation was demonstrated to be crucial in allowing the organism to migrate towards chemoattractant gradients (Silverman and Simon, 1977; Parkinson et al., 2005). The role of protein methylation in mammalian lymphocyte migration is unexplored but there are some data which might suggest that this

modification might be involved in chemotaxis of human T cell. The embryonic/perinatal lethality of PRMT1 and PRMT4 deficiency in mice may suggest arginine methylation orchestrating directional cell migration during developmental processes (Pawlak et al., 2000; Yadav et al., 2003). There is also evidence that increases in prenylcysteine carboxyl methylation of Ras-related proteins correlates with their activation in neutrophils following chemoattractant treatment whilst inhibitors of prenylcysteine methylation abrogates chemotaxis of endotoxin-activated macrophages (Philips et al., 1993; Volker et al., 1991). Taking all these findings into consideration, it was aimed to investigate the role of methylation in T lymphocytes. Despite the fact that the broad spectrum methylation inhibitor MTA impaired directional migration of freshly isolated pan T cells and activated T cells, this effect was not consistent and appeared to be donor dependent. Moreover another known inhibitor of transmethylation reactions, namely structural analog of S-adenosylmethionine (SAM) – sinefungin (Yebra et al., 1991), did not interfere with CXCL11 and CXCL12 -induced chemotaxis. In addition AMI-1, small molecule that specifically inhibit protein arginine *N*-methyltransferase (PRMT) activity (Cheng et al., 2004) had no effect on chemotaxis mediated by CXCR3 and CXCR4. These data suggest that methylation of residues other than arginine, such as lysine, may possibly be involved in chemokine driven T cell migration. It is also important to consider that effect of MTA may be an off-target effect due to the high concentration used in the experiments. Therefore to clarify this issue novel specific compounds which target arginine and lysine methylation would be required. Moreover introduction of siRNA in order to knock down the particular PRMT enzymes would allow a further investigation of the role of arginine methylation in T cell migration.

Chapter Six

Summary

6.1 Summary

The presented work characterises the role of chemokine receptor CXCR3 and its spliced variants, namely CXCR3-B and CXCR3-alt, in human T lymphocytes and transfected cell lines. Part of this study also examined the chemokine receptor CXCR7 as it has been reported to share the ligand CXCL11 with CXCR3.

In the investigation of CXCR3 in T cells, responses mediated by CXCL9, CXCL10 and CXCL11 and their ability to induce CXCR3 internalization, intracellular signalling or chemotaxis were re-examined. In comparison with CXCL9 and CXCL10, CXCL11 was a more potent and efficacious agonist in all assays performed. These observations are consistent with previous findings demonstrating full agonism of CXCL11 towards CXCR3 (Gonsiorek et al., 2003; Heise et al., 2005; Sauty et al., 1999). In contrast, CXCL9 and CXCL10 have been demonstrated to behave as full or partial agonists depending on the assay system used (Clark-Lewis et al., 2003; Gonsiorek et al., 2003; Heise et al., 2005). Furthermore CXCL9 and CXCL10 have been reported to interact with the carboxy-terminus of CXCR3 to induce down-regulation of the receptor, whilst CXCL11 has been shown to preferentially activate a distinct, carboxy-terminal independent internalization pathway (Sauty et al., 2001).

CXCL11-mediated surface down-regulation of CXCR3 was further investigated within the study in terms of mechanisms and signalling pathways involved. In conjunction with previous studies by Meiser et al. (Meiser et al., 2008), results obtained here in both human SEB/IL-2 activated T cells and CXCR3-expressing transfected HEK 293 cells suggest that the pathway mediating CXCL11-induced internalization does not appear to require caveolae. Surprisingly, the treatment of T cells with the cholesterol-depleting agent MβCD had a noticeable effect on the agonist-triggered loss of surface expression of CXCR3. This revelation indicates the involvement of another cholesterol-dependent pathway. In addition the role of clathrin in CXCL11-mediated internalization was unclear due to contrasting results using two inhibitors reported to block clathrin-dependent endocytosis. Based on results obtained in this study, showing that neither use of endocytosis blockers nor

CXCR3 antagonists was able to fully attenuate this process, it appears that internalization of CXCR3 in response to agonist binding may be important in the regulation of T cell recruitment in vivo due to prevention of amplification of CXCR3 signalling. Moreover no complete surface recovery of CXCR3 was observed after CXCL11 stimulation suggesting receptor degradation and synthesis of new proteins as treatment with cycloheximide inhibited re-appearance of CXCR3. These observations compared closely to these reported by Meiser et al. who demonstrated constitutive and CXCL11-induced degradation of CXCR3 (Meiser et al., 2008).

Studies on CXCR3 internalization also revealed that this process occurs independently of Gi and PI3K coupling but may require the involvement of PKC and PLC. A possible role of PIKfyve may also be considered, however additional approaches would be desired in order to confirm the effect of pharmacological inhibition.

A recently identified spliced variant of CXCR3, CXCR3-B, in addition to binding of the three original agonists, has been reported to also interact with CXCL4 (Lasagni et al., 2003). This work demonstrates for the first time, that CXCL4 is effective in inducing biochemical signalling (seen as phosphorylation of p44/p42 MAPK, Akt, GSK3-β and S6) and functional events (actin reorganisation) in activated T cells. Similarly to studies conducted by Mueller et al., CXCL4 also induced intracellular calcium mobilisation in both activated T cells and CXCR3-A and CXCR3-B transfectants and exhibited lower potency in comparison to other CXCR3 agonists. The results presented here also indicate that CXCL4-stimulated p44/p42 MAPK and PI3K/Akt activation in T cells occurs via coupling to PTX sensitive Gi. Surprisingly these signals were not affected by treatment with CXCR3 antagonists. A possible explanation may be differential interaction of CXCL4 with CXCR3 / CXCR3-B in comparison to its other ligands as shown by Mueller et al. The same research group also reported the inability of specific CXCR3 antagonist (identical to the compound T487 used here) to displace CXCL4 from either variant of CXCR3 (Mueller, 2008).

Results obtain in this thesis also demonstrate that CXCR3-B expressing transfectants are responsive to CXCL11 as the treatment with this agonist induced

intracellular signalling such as phosphorylation of p44/p42 MAPK, Akt and elevation of intracellular calcium.

Although Mueller and colleagues described a role of CXCL4 signalling chemotaxis of ConA activated T cells, the work presented in this thesis indicates that pathway is not involved in inducing of chemotactic activity of SEB-activated T lymphocytes. In addition CXCL4 did not have any effect on down-regulation of CXCR3 surface expression in activated T cells which may explain the lack of chemotaxis. Notably only a moderate decrease of surface CXCR3-B was detected in transfected HEK293 cells.
Collectively these findings show that CXCL4 does not share functional properties with CXCR3 agonists. An ability to induce intracellular signalling together with a failure to induce T lymphocyte chemotaxis suggests distinct roles in T cell biology.

A study by Ehlert et al., showed evidence of an additional spliced variant of CXCR3, namely CXCR3-alt. This particular variant has been detected in T cells and has been shown to bind and functionally respond to CXCL11 (Ehlert et al., 2004). In contrast the role of this receptor in biochemical signalling and its ability to be internalized in response to agonist binding is unexplored. This thesis demonstrates, for the first time, that, despite low surface expression in transfected HEK293 cells, CXCR3-alt was able to induce an elevation of cytosolic calcium concentration and activate phosphorylation of p44/p42 MAPK and Akt following stimulation with CXCL11. However no internalization of CXCR3-alt was observed in response to CXCL11.

Previously known as orphan receptor RDC-1, the chemokine receptor CXCR7 has been recently demonstrated to bind CXCL12 and CXCL11 (Balabanian et al., 2005; Burns et al., 2006) but its role in typical chemokine receptor signalling is still unclear. Moreover CXCR7 has been highlighted to serve as an adaptor for CXCR4 in transducing CXCL12-mediated integrin activation without being involved in CXCR4-mediated cell motility or survival (Hartmann et al., 2008).
Within this thesis a partial characterisation of CXCR7 receptor was performed. As previously reported (Hartmann et al., 2008) it was not possible to observe any

effect of anti-CXCR7 antibody on CXCL12-mediated chemotaxis and signalling in T cells. In addition the same antibody did not affect responses induced by CXCL11. Moreover CXCL12 as well as CXCL11 induced internalization in both T cells and transfected cells but no biochemical signalling could be detected in CXCR7-expressing HEK293 cells.

Initially in this thesis it was postulated that protein arginine methylation is up-regulated following stimulation of T lymphocytes and that this is important in chemokine-induced directional migration of T cells. Based on the results obtained, levels of monomethylarginie methylation increased upon CD28 and CD3. In contrast no effect on dimethylation of arginine was observed following T cell stimulation with anti-CD28 or both-CD28 and CD3 antibodies. Notably studies of the involvement of methylation in T cell chemotaxis were inconclusive, as MTA, a broad spectrum inhibitor of methylation, partially blocked chemotactic activity but another compound, sinefungin, and the more specific protein-arginine methylation inhibitor AMI-1 gave negative results.

Chapter Seven

Appendix

7.1 The genetic code

	U	C	A	G	
U	UUU ***Phe***	UCU ***Ser***	UAU ***Tyr***	UGU ***Cys***	U
	UUC ***Phe***	UCC ***Ser***	UAC ***Tyr***	UGC ***Cys***	C
	UUA ***Leu***	UCA ***Ser***	UAA **STOP**	UGA **STOP**	A
	UUG ***Leu***	UCG ***Ser***	UAG **STOP**	UGG ***Trp***	G
C	CUU ***Leu***	CCU ***Pro***	CAU ***His***	CGU ***Arg***	U
	CUC ***Leu***	CCC ***Pro***	CAC ***His***	CGC ***Arg***	C
	CUA ***Leu***	CCA ***Pro***	CAA ***Gln***	CGA ***Arg***	A
	CUG ***Leu***	CCG ***Pro***	CAG ***Gln***	CGG ***Arg***	G
A	AUU ***Ile***	ACU ***Thr***	AAU ***Asn***	AGU ***Ser***	U
	AUC ***Ile***	ACC ***Thr***	AAC ***Asn***	AGC ***Ser***	C
	AUA ***Ile***	ACA ***Thr***	AAA ***Lys***	AGA ***Arg***	A
	AUG ***Ile***	ACG ***Thr***	AAG ***Lys***	AGG ***Arg***	G
G	GUU ***Val***	GCU ***Ala***	GAU ***Asp***	GGU ***Gly***	U
	GUC ***Val***	GCC ***Ala***	GAC ***Asp***	GGC ***Gly***	C
	GUA ***Val***	GCA ***Ala***	GAA ***Glu***	GGA ***Gly***	A
	GUG ***Val***	GCG ***Ala***	GAG ***Glu***	GGG ***Gly***	G

Third position (3' end)

Third position (3' end)

U= Uracil, C= Cytosine, A= Adenine, G= Guanine

7.2 The amino acids

Amino Acid	3 letter code	1 letter code	Property
Alanine	Ala	A	Hydrophobic
Arginine	Arg	R	Hydrophilic
Asparagine	Asn	N	Hydrophilic
Aspartic Acid	Asp	D	Hydrophilic
Cysteine	Cys	C	Hydrophilic
Glutamine	Gln	Q	Hydrophilic
Glutamic Acid	Glu	E	Hydrophilic
Glycine	Gly	G	Hydrophilic
Histidine	His	H	Hydrophilic
Isoleucine	Ile	I	Hydrophobic
Leucine	Leu	L	Hydrophobic
Lysine	Lys	K	Hydrophilic
Methionine	Met	M	Hydrophobic
Phenylalanine	Phe	F	Hydrophobic
Proline	Pro	P	Hydrophobic
Serine	Ser	S	Hydrophilic
Threonine	Thr	T	Hydrophilic
Tryptophan	Trp	W	Hydrophobic
Tyrosine	Tyr	Y	Hydrophobic
Valine	Val	V	Hydrophobic

Negatively charged amino acids are highlighted in blue, positively charged amino acids are shown in yellow.

7.3 The Greek alphabet

Α	α	Alpha
Β	β	Beta
Γ	γ	Gamma
Δ	δ	Delta
Ε	ε	Epsilon
Ζ	ζ	Zeta
Η	η	Eta
Θ	θ	Theta
Ι	ι	Iota
Κ	κ	Kappa
Λ	λ	Lambda
Μ	μ	Mu
Ν	ν	Nu
Ξ	ξ	Xi
Ο	ο	Omicron
Π	π	Pi
Ρ	ρ	Rho
Σ	σ	Sigma
Τ	τ	Tau
Υ	υ	Upsilon
Φ	φ	Phi
Χ	χ	Chi
Ψ	ψ	Psi
Ω	ω	Omega

7.4 Recipes for SDS-PAGE gels

	Resolving Gels			Stacking Gel
	7.5%	10%	12%	4 X
4 X Resolving/ Stacking Buffer (mL)	5	5	5	3
MilliQ Water (mL)	9.84	8.17	6.84	6.85
Acrylamide	5	6.67	8	2
APS 10% (μL)	150	150	150	150
TEMED (μL)	15	15	15	15

7.5 Table showing IC_{50} of CXCR3 antagonists

Compound	IC_{50} (nM)		
	CXCL11-induced chemotaxis	CXCL11-induced internalization	CXCL11-induced chemotaxis (literature)
T487 (AMG487)	69	380	3.9 (Heise et at., 2005)
NBI-74330	2.3	2.44	15 (Johnson et al., 2007)
N-oxide-NBI-74330	0.53	0.712	-

7.6 Families of human chemokines and chemokine receptors.

The Human Chemokine/ Chemokine Receptor family

CXC Chemokine/Receptor family

Systematic name	Human Chromosome	Human Ligand	Chemokine receptor(s)
CXCL1	4q12-q13	GROα/MGSA-α	CXCR2 > CXCR1
CXCL2	4q12-q13	GROβ/MGSA-β	CXCR2
CXCL3	4q12-q13	GROγ/MGSA-γ	CXCR2
CXCL4	4q12-q13	PF4	Unknown
CXCL5	4q12-q13	ENA-78	CXCR2
CXCL6	4q12-q13	GCP-2	CXCR1, CXCR2
CXCL7	4q12-q13	NAP-2	CXCR2
CXCL8	4q12-q13	1L-8	CXCR1, CXCR2
CXCL9	4q21.21	Mig	CXCR3
CXCL10	4q21.21	IP-10	CXCR3
CXCL11	4q21.21	I-TAC	CXCR3
CXCL12	10q11.1	SDF-1α/β	CXCR4
CXCL13	4q21	BLC/BCA-1	CXCR5
CXCL14	Unknown	BRAK/bolekine	Unknown
CXCL15	Unknown	Unknown (Lungkine in mouse)	Unknown
CXCL16	17p13	SRPSOX	CXCR6

C Chemokine/Receptor family

Systematic name	Human Chromosome	Human Ligand	Chemokine receptor(s)
XCL1	1q23	Lymphotactin	XCR1
XCL2	1q23	SCM-1b	XCR1

CX3C Chemokine/Receptor family

Systematic name	Human Chromosome	Human Ligand	Chemokine receptor(s)
CX3CL1	16q13	Fractaline	CX3CR1

CC Chemokine/Receptor family

Systematic name	Human Chromosome	Human Ligand	Chemokine receptor(s)
CCL1	17q11.2	I-309	CCR8
CCL2	17q11.2	MCP-1/MCAF	CCR2
CCL3	17q11.2	MIP-1α /LD78α	CCR1, CCR5
CCL4	17q11.2	MIP-1β	CCR5
CCL5	17q11.2	RANTES	CCR1, CCR3, CCR5
(CCL6)		Unknown (mouse only)	Unknown
CCL7	17q11.2	MCP-3	CCR1, CCR2, CCR3
CCL8	17q11.2	MCP-2	CCR3
(CCL9/CCL10)		Unknown (mouse only)	Unknown
CCL11	17q11.2	Eotaxin	CCR3
(CCL12)		Unknown (mouse only)	Unknown
CCL13	17q11.2	MCP-4	CCR2, CCR3
CCL14	17q11.2	HCC-1	CCR1
CCL15	17q11.2	HCC-2/Lkn-1/MIP-1δ	CCR1, CCR3
CCL16	17q11.2	HCC-4/LEC	CCR1
CCL17	16q13	TARC	CCR4
CCL18	17q11.2	DC-CK1/PARC/AMAC-1	Unknown
CCL19	9p13	MIP-3β /ELC/exodus-3	CCR7
CCL20	2q33-q37	MIP-3α /LARC/exodus-1	CCR6
CCL21	9p13	6Ckine/SLC/exodus-2	CCR7
CCL22	16q13	MDC/STCP-1	CCR4
CCL23	17q11.2	MPIF-1	CCR1
CCL24	7q11.23	MPIF-2/Eotaxin-2	CCR3
CCL25	19p13.2	TECK	CCR9
CCL26	7q11.23	Eotaxin-3	CCR3
CCL27	9p13	CTACK/ILC	CCR10
CCL28	5p12	MEC	CCR3, CCR10

7.6 Human chemokine and chemokine receptor families. Many chemokines are clustered in certain chromosomal location. Two main clusters have been recognized. Many human CXC chemokines that mainly act on neutrophils are clustered at chromosome 4q12–13 (shown in red), while many CC chemokines that mainly act on monocytes are located in another cluster at 17q11.2 (purple). The CXC chemokines in the 4q21.21 mini-cluster (blue) act specifically as T cell chemoattractants. Chemokines encoded by the more isolated genes tend to be constitutively produced and have mostly homeostatic roles (shown in black) (Adapted from Murphy et al, 2000).

7.7 Sequences of conservative *NPXXY* motif of human chemokine receptors

CCR1	EVISYTHCCV**NPVIY**AFV**G**ERFRKYLRQL
CCR2	ETLGMTHCCINPIIYAFVGEKFRSLFHIA
CCR3	EVIAYSHCCMNPVIYAFVGERDRKYLRHF
CCR4	ETLAFVHCCLNPIIYFFLGEKFRKYILQL
CCR5	TETLGMTHCCNPIIYAFVGEKFRNYLLYF
CCR6	EVLAFLHCCLNPVLYAFIGQKFRNYFLKI
CCR7	YSLACVRCCVNPFLYAFIGVKFRNDLFKL
CCR8	EITSFTHCCVNPVIYAFVGEKFKKHLSEI
CCR9	QTIAFFHSCLNPVLYVFVGERFRRDLVKT
CCR10	SGLALARCGLNPVLYAFLGLRFRQDLRRL
CCR11	ESIALFHSCLNPLLYAFLDGASFKNYVMKV

CXCR1	EILGFLHSCLNPIIYAFIGQNFRHGFLKI
CXCR2	EILGILHSCLNPLIYAFIGQKFRHGLLKI
CXCR3	SGLGYMHCLLNPLLYAFVGVKFRERMWML
CXCR4	EALAFFHCCLNPILYAFLGAKFKTSAQHA
CXCR5	EFLGIAHOCLNPMLYTFAGVKFRSDLSRL
CXCR6	EAIAYLRACCNPVLYAFVSLKFRKNFWKLVKDIG
CXCR7	QCLSLVHCCVNPVLYSFINRNYRYELMKAFIFKYSAKTG

CX_3CR1	ETVAFSHCCLNPLIYAFAGEKFRRYLYHL
XCR1	RNLAFSHCCFNPVLYVFVGVKFRTHLKHV
D6	ESIAFLHCCFSPILYAFSSHRFRQYLKAFLAAVLG

X/G = Transmembrane X/G = Cytoplasmic but juxta TM domain X/G = end of C-terminus

7.8 Comparison of abilities of biotinylated versus 'native' agonists to induce signaling and chemotaxis of activated human T cells

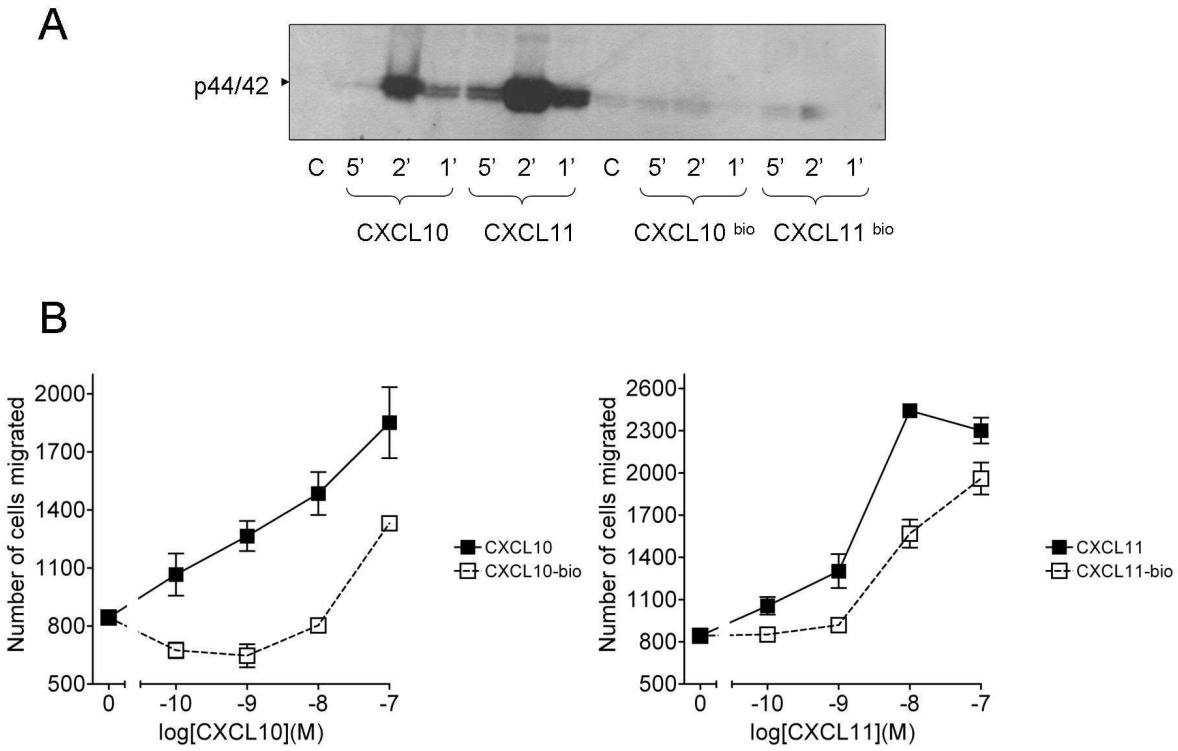

Figure 7.8 Comparison of abilities of biotinylated versus 'native' agonists to induce signaling and chemotaxis of activated T cells. Day 9, SEB/IL-2 treated T cells were stimulated with biotinylated or unlabelled CXCL10 or CXCL11 (100 nM). Control samples were treated with media. Samples were then lysed by centrifugation and addition of solubilisation buffer. Lysates containing 1x sample buffer were separated by electrophoresis in 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the p44/p42 MAPK ab with the affinity for the active Thr^{202}/Tyr^{204} form of MAPK (A). Chemotaxis of day 9, SEB/IL-2 activated T cells to increasing concentrations of either biotinylated or 'native' CXCL10 or CXCL11 (0.1-100 nM). Previously activated T cells were washed, resuspended at 3.2 x 10^6 / mL and placed (8 x 10 10^4 / 25μL per well) on the filter membrane above lower chambers containing chemokine solutions or media. Chemotaxis across 5 μm pore size membrane was determined after a 3 hour incubation at 37°C in 5% CO_2 as described in *Materials and Methods.*

7.9 Effect of PI-103 inhibitor on CXCL11-induced down-regulation of surface CXCR3 in T cells

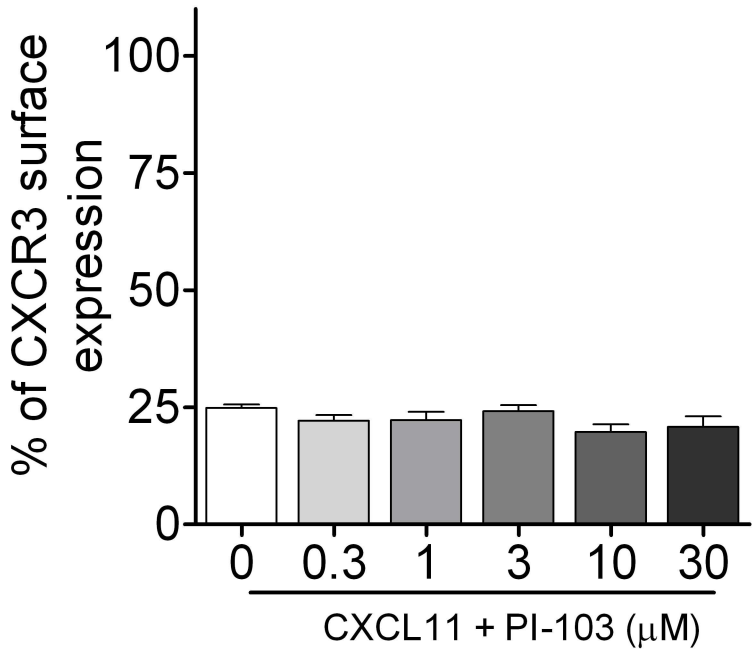

Figure 7.9 Effect of PI-103 inhibititor on CXCL11 induced internalization of CXCR3 in T lymphocytes. Day 9-12 SEB/IL-2 activated T cells were incubated with appropriate concentrations of each inhibitor (0.3-30 μM) or vehicle control (white bar) for 30 mins at 37°C before being stimulated with 30 nM CXCL11 for 5 mins at 37°C. Cell surface expression of CXCR3 was measured using flow cytometry as described in *Materials and Methods*. Decrease in CXCR3 surface expression was expressed as a percentage of baseline surface expression. Data show average of two independent experiments.

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