PHD

Chemokines and T cells: Activation requirements for Rantes secretion and CXCR4 signalling in mature T cells

Sotsios, Yannis

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CHEMOKINES AND T CELLS:
ACTIVATION REQUIREMENTS FOR RANTES SECRETION
AND CXCR4 SIGNALLING IN MATURE T CELLS

submitted by

YANNIS SOTSIOS

for the degree of PhD

of the University of Bath

2000

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Abstract.
Chemokines are an important family of molecules involved in various pathophysiological manifestations. The $\beta$-chemokines RANTES, MIP-1$\alpha$ and MIP-1$\beta$ were also implicated to participate in T cell activation. This study demonstrates that freshly purified T cell proliferation and RANTES secretion did not always correlate. CD3/CD28 costimulation or CD2/CD28 costimulation both caused comparable proliferation, but only CD3/CD28 supported modest RANTES secretion. Meanwhile, the phorbol ester PMA caused a robust RANTES secretion without promoting IL-2 production and T cell proliferation. Additions of various $\alpha$- or $\beta$-chemokines, or neutralising anti-chemokine antibodies failed to affect T cell proliferation in response to various costimuli.

The effects of PMA on RANTES secretion appeared to be chemokine-specific as MIP-1$\alpha$ was not equally affected, and depended on protein kinase C (PKC) isoenzyme activation. Signals causing calcium mobilisation caused a reduction in RANTES secretion compared to the PMA response. Ligation of CTLA-4, expression of which depends on sustained calcium elevation, inhibited RANTES secretion induced by CD3/CD28, but had no effect on PMA-stimulated RANTES secretion. The use of inhibitors indicated that PKC-, Ras- and PTK-dependent pathways were crucial for any RANTES secretion, whereas PI3K-, Calcineurin-, mTOR-, ERK1/2 and p38-dependent pathways were only important in CD3/CD28-costimulated RANTES secretion.

Analysis of CXCR4 signalling indicated that calcium mobilisation, unlike $\alpha$-protein-dependent PI3K activation, was not necessary for chemotaxis to SDF-1 and its peptide analogues. CXCR4-induced chemotaxis, D-3 phosphoinositide lipid accumulation, PKB and ERK1/2 activation was sensitive to PI3K or $\alpha$-protein inhibition. In contrast, SDF-1-induced actin polymerisation was partially dependent on PI3K, whereas CXCR4 internalisation was only sensitive to PKC inhibition. Despite the observed PKB activation, SDF-1 did not rescue or promote basal T cell apoptosis, or apoptosis in response to Fas ligation.

Finally, T-tropic HIV-1 gp120s did not cause significant chemotaxis, CXCR4 internalisation or PtdIns(3,4,5)P$_3$ generation and antagonised SDF-1 responses on calcium mobilisation assays.
Acknowledgements

I would like to thank Professor John Westwick and Dr. Steve Ward, my two supervisors, who provided me with this exciting opportunity of research and supported me all along in matter and in spirit. If I could carry away with me people as I can take objects, these two would follow me to my next area of work...

A special mention for all the good advice received from Dr. George Kolios about science and life in general: Μετίωμεν καθ’όδον.

Also, I would like to express my gratitude to Dr. Nicola Jordan for all her patience and practical guidance during this thesis, Mrs. Jane Leithead for moral support and practical help. A big round of applause to all the people who volunteered to donate their precious blood to my experimental altar. If only Dracula had my luck...

Big thanks to Paul, Dr. Paul, for being who he is and being there for me

He would agree that:

Lead heavens
spill
chilled dribble

Bleached souls
landwrecked
forgot how

To cope
without
some chemistry

Finally, I would like to say thank you to Kostas and Flora for many reasons, two of the most important being: a) without them I would not have been born, b) without their multi-faceted support I would not have remained sane.
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein -1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>αCD3</td>
<td>anti-CD3 antibody</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD28RE</td>
<td>CD28-response element</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte antigen</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium-regulated and activated calcium channel</td>
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<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
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<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
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<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic lymphocyte associated antigen</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DEPC-dH20</td>
<td>Diethyl pyrocarbonate-treated distilled water</td>
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<td>DMEM</td>
<td>Dulbecco's modified essential medium</td>
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<td>Deoxyribonucleic acid</td>
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<td>DPP</td>
<td>Dipeptidylpeptidase</td>
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<td>DTH</td>
<td>Delayed type hypersensitivity reaction</td>
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<td>Dithiothreitol</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalopathy</td>
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<td>EDTA</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
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<tr>
<td>F/G-actin</td>
<td>Filamentous / Globular actin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>Fluorescein isothiocyanate</td>
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<td>G-protein-related kinase</td>
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<td>GST-RBD</td>
<td>Glutathione sepharose-Ras-binding domain</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
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<td>Histone 2-B</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’[2-ethanesulphonic acid]</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Human recombinant</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration (50%)</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IgG/M</td>
<td>Immunoglobulin G/M</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D thiogalactoside</td>
</tr>
<tr>
<td>IPs</td>
<td>Immunoprecipitates</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>IP3 receptor</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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</table>
ITAM Immunoreceptor tyrosine-based activation motif
kD/ kDa Kilodalton
Kd Dissociation constant
Luc Luciferase
LFA-1 Leukocyte functional antigen -1
MAPK Mitogen activated protein kinase
mAb Monoclonal antibody
MEK MAPK- kinase
MHC Major histocompatibility complex
mRNA Messenger ribonucleic acid
M-tropic HIV Macrophage tropic HIV
mTOR Mammalian target of Rapamycin
NFATp Nuclear factor of activated T cells (phosphate)
NF-κB Nuclear factor kappa-B
NK Natural killer
NFIL6 Nuclear factor of IL-6
NSI Non syncytium inducing
PBS Phosphate buffered saline
PBMC Peripheral blood mononuclear cells
PBL Peripheral blood lymphocytes
PDGF Platelet derived growth factor
PDK1 Phosphoinositide dependent kinase 1
PHA Phytohaemagglutinin A
PH Pleckstrin homology
PI3K/PI 3-kinase Phosphatidylinositol 3-kinase
PI/PIP/PIP2 Phosphatidylinositol (mono/ bis phosphate
PIP3/PtdIns(3,4,5)P3 Phosphatidylinositol (3,4,5) trisphosphate
PKB /PKC/ PKD Protein kinase B / C / D
PLC / PLD Phospholipase C / D
PMA Phorbol-12-myristate-13-acetate
PRK-2 PKC-related kinase 2
PTK Protein tyrosine kinase
PTX Pertussis toxin
PVPF polyvinylpyrrolidine free
RT Room temperature

XIII
RT-PCR  Reverse transcriptase polymerase chain reaction
SCID   Severe combined immunodeficiency
SEB    Staphylococcal enterotoxin B
SEM    Standard error of means
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH     Src homology
SHIP   SH2-containing inositol 5’ polyphosphatase
SI     Syncytium inducing
STAT   Signal transducer and activator of transcription
TBS    Tris buffered saline
TCR    T cell receptor
TEMED N,N,N',N'-tetramethylethyldiamine
Th     T helper
TLC    Thin layer chromatography
TMR    Transmembrane region
TMS    Transmembrane spanning
TNFRII TNF-receptor II
TNF-α  Tumour necrosis factor –alpha
T-tropic HIV-1 T cell tropic HIV-1
VEA    Very early antigen
VLA    Very late antigen
ZAP    Zeta associated protein
ZVAD-fmk Cbz-Val-Ala-Asp-(Ome)-fluoromethyl ketone

**Single letter amino acid code**

<table>
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<tr>
<td>Meaning</td>
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<td>Cys</td>
<td>Asp</td>
<td>Glu</td>
<td>Phe</td>
<td>Gly</td>
<td>His</td>
<td>Ile</td>
<td>Lys</td>
<td>Leu</td>
<td>Met</td>
<td>Asn</td>
<td>Pro</td>
<td>Gln</td>
<td>Arg</td>
<td>Ser</td>
<td>Thr</td>
<td>Val</td>
<td>Trp</td>
<td>Tyr</td>
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</tbody>
</table>

XIV
INTRODUCTION

1.1 The immune response
The primary function of the immune system is to protect the body from external pathogens and to maintain a healthy responsive state. Effective immunity has to be adaptive, a property characterised by the abilities to recognise and memorise potential pathogens. Therefore cells surviving subsequent exposures of a particular antigenic challenge, elicit responses which are enhanced or adjusted accordingly. Lymphocytes are involved in the recognition of antigens, with certain T-cell populations memorising previous noxious encounters. It is also their responsibility to tolerate the body’s own antigens in order to avoid auto-immunity and, ideally, to identify and deal with defective manifestations of their cellular environment. Apart from their interactive role with B-cells for humoral immunity and specific antibody production, T-cells are crucial for cell mediated effector responses. The type of immune response generated is thought to be determined by the interaction of T-cell subpopulations with themselves and with the other key cells involved in inflammation.

1.2 T cell migration and activation
T-cell activation is thought to require cell-to-cell contact comprising of receptor-ligand type molecular interactions which occur externally between adjacent cell-membranes. Hence, the mechanisms that control cell trafficking, distant recruitment and selective migration of certain populations of lymphocytes to sites of antigenic or auto-immune challenge are cardinal for effective cellular immunity or, on the contrary, responsible for the pathology. Such intercellular communication is achieved with secreted molecules, cytokines like the interleukins, some of which are chemotactic, i.e. directing cellular migration according to their concentration gradient. T cells play a crucial part in providing B cell ‘help’ or cytolytic activity, a commitment achieved by antigenic stimulation of resting T cells. This maturation process takes 3 to 7 days and has been characterised by the coordinated expression of new genes including interleukins, proto-oncogenes and, days later, genes involved in cytolysis and movement through tissues (Ullman 1990, Janeway 1994). Thus, activated, secretory T cells may orchestrate other populations of effector cells like neutrophils, basophils, eosinophils, monocytes and macrophages via chemokines, as well as other T cells.
1.3 Transendothelial migration, adhesion and chemotaxis

Cellular extravasation and localisation to particular extravascular tissues was termed transmigration, a process thought to proceed via several distinct steps with key molecular players (Springer 1994, Mitchison et al. 1996, Lauffenburger et al. 1996) (Figure 1.1). The initial contact of cells with the endothelial cell wall depends heavily on the expression of adhesion molecules called Selectins, that can mediate cell tethering of a flowing cell in the span of a millisecond and, subsequently, rolling (A to B). The second step (B to C) is mediated via chemoattractants or chemokines and their induction of integrin adhesiveness. Integrins bind to receptors that belong to the Ig-superfamily only after rapid and transient conformational changes that allow binding and depend on bivalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)). The transient nature of integrin binding and the fact that some integrin adhesion depends on chemoattractant/chemokine-induced cellular activation, allows for cellular de-adhesion and movement towards higher gradients of chemoattractant/chemokine concentrations (chemotaxis) or integrin adhesiveness (haptotaxis).

Integrins are described by their two subunit composition and lymphocytes are known to express \(\alpha L\beta 2\) (LFA-1, CD11a/CD18) which binds ICAM-1, ICAM-2, ICAM-3, \(\alpha 4\beta 1\) (VLA-4) which binds VCAM-1, fibronectin, and \(\alpha 4\beta 7\) which binds MAdCAM-1, VCAM-1, fibronectin. From the Ig-superfamily integrin receptors, ICAM-1 and ICAM-3 depend on activation whereas ICAM-2 is constitutively expressed. Moreover, all three are expressed also on T lymphocytes and participate in antigenic T cell stimulation (de Fougerolles et al. 1993; Sedwick et al. 1999; also presentation by Dr M. Dustin in the BSI 1999 conference). The constitutive expression of ICAM-2 has been implicated in non-inflammatory traffic such as lymphocyte re-circulation. Furthermore, the fact that carbohydrates bound to MAdCAM-1 can serve as ligands to L-selectin indicates that the integrin system may operate in tethering/rolling, as well as the integrin-mediated strengthening of adhesion, and slow down to arrest. The third step is best described as a transient "arrest" (C). Rolling ceases and adhesion mediated by LFA-1 and VLA-4 becomes much more firm. It was thought that a successive increase in cellular activation via rolling caused by increasing adhesion and chemoattractant/chemokine signalling was the necessary route to step three.
Figure 1.1 Schematic representation of transendothelial migration through activated endothelium.

Legend for Fig. 1.1. Simplified version of trans-endothelial migration and chemotaxis to interstitial sources of chemokine production. The molecules principally involved or mediating the separate phases span the procedure as illustrated by the banners on top. A: Lymphocyte before contact with the endothelium, B: Lymphocyte rolling, C: Lymphocyte “arrest”, D: Extravasation, E: Chemotaxis. See text for details. Selectins are not represented schematically for the sake of clarity.
In the recent BSI 1999 conference, a video presentation by Dr. K Ley showed that signalling from integrin and chemokine/chemoattractant, bound on the endothelium cell surface, indeed integrated together to result in maximal cellular activation (indicated by increases in intracellular calcium ion concentrations), and slowed down the rolling. However, in the slower rolling phase with neutrophils, E-Selectin (not L- or P-) interactions also appeared to be important. Moreover, a second chemokine could display a dramatic effect at any phase of a rolling cell (fast or slow), by causing its arrest a few milliseconds after micro-pipette diffusion.

Cellular arrest is only the initiation of step (D), migration through the endothelial cell wall and subsequent interstitial movement (E) towards the source of the chemoattractant/chemokine. Cellular movement (D and E), is a complex process which entails the overall sequential co-ordination of:

i) cellular polarisation based on cytoskeletal re-arrangements and F-/G- actin re-organisation,

ii) reversible membrane extensions like filopodia and lamellipodia based on a complex regulation of local F-/G- actin re-organisation,

iii) formation and stabilisation of attachments with synthesis and redistribution of activated integrin to the leading edge,

iv) cellular body contraction and adhesive traction involving myosin-based ATP-motors and integrin binding,

v) rear release and retraction including cell surface integrin endocytosis or "ripping" (loss) coupled to cytoskeletal rounding.


1.4 Chemokines

Chemokines were initially distinguished from other chemoattractants because of their specificity for leukocyte subsets. For example, complement fragment C5a attracted neutrophils and monocytes with equal potencies, whereas the chemokine IL-8 attracted neutrophils but not monocytes (Westwick 1989). This distinction supported the proposition that chemokines may be involved in clinical conditions characterised by lesions infiltrated with distinct cell populations (Schall et al. 1994). As chemotactic cytokines, these proteins share some characteristics with the classical cytokines and were initially described as pro-inflammatory. They were thought to be synthesised and secreted by certain cells responding to a stimulus, conveying their effect via high-
affinity receptors on their target cells (Oppenheim et al. 1991). However, during the course of this study, their constitutive involvement in physiology became apparent (Mackay 1996, Rollins 1997). Their classification into families is an area of continuous development as novel ligands and receptors are discovered.

THE CHEMOKINE FAMILY
Chemokines are a rapidly growing superfamily of generally small (8-10 kD) peptides that selectively attract and activate leukocyte populations (Baggiolini et al. 1994; Ward et al. 1998; Baggiolini et al. 1997). Initially, 2 families were defined based on the arrangement of the conserved cysteine (C) residues of the mature proteins (Schall et al. 1994, Mackay 1996). However, now a more complete picture is known, with four families identified so far: the CXC or α-chemokines, CC or β-chemokines, C or γ-chemokines and the CX3C or δ-chemokines (Ward, Westwick, 1998; Ward et al. 1998; Baggiolini et al. 1997) (Figure 1.2). The -x- moiety represents any amino-acid between the N-terminal two cysteines except for the only member- as yet- of the -C- family, lymphotactin, which has only two of the four cysteines. These four families have been located mainly in four separate human chromosomal loci which are 4q12-21 for -CXC, 17q11.2-12 for -CC-, 1q23 for -C- and 16 for the latest -CX3C- family. With new chemokines discovered, several additional positions were found at: 10 for SDF-1α/β, 16q13 for MDC, TARC, and the novel single CX3C member Fractalkine, 2 for MIP-3α, 9 for MIP-3β and 6Ckine, and 7 for Eotaxin-2 (reviewed in Zlotnik et al. 1999).

The ELR motif is present on a subfamily of -CXC- chemokines and is positioned adjacent to the first cysteine and the N-terminal. Apart from a residue-based subdivision of the -CXC- family, the presence or absence of the ELR motif is associated with distinct and opposite functional effects: ELR+ -CXC- chemokines are angiogenic whereas lack of ELR on -CXC- chemokines renders them angiostatic (Schall et al. 1994, Dr. R. Strieter, Gordon Chemokine 1999 conference presentation). The single chemokine member of the -C-X3-C- family, termed fractalkine is a notable exception to the soluble nature of chemokines, since it is found attached to a 200 kD long stalk, heavily substituted with mucin-like carbohydrates and embedded in the cell membrane, not secreted.
This finding supports the view that chemokines although secreted, rapidly bind on heparan sulphate or other glycosaminoglycans (GAGs) on the surface of cells which do not compromise, but instead enhance subsequent signalling (Schall et al. 1994). The property of chemokines to bind cell surface proteoglycans or GAGs can account for gradients fixed on the endothelium, a valid mechanism to mediate extravasation under blood flow conditions. A summary of -CC- and -CXC- chemokines and the various other effects they exert on their target cells apart from chemotaxis is shown in Table 1. (The numerous references for each novel or “traditional” chemokine and their reported effects are comprehensively reviewed in Rollins 1997 and Zlotnik et al. 1999). After the completion of this thesis, chemokine nomenclature was updated in a conference held by the Nomenclature Committee of the International Union of Pharmacology XXII and the various names and acronym designations were replaced with CCL1 to 27 for the -CC- chemokines, and CXCL1 to 14 for the -CXC- chemokines (Murphy et al. 2000). The complete revised list of identified chemokines to date is shown in Appendix I.
### TABLE 1. THE CURRENT STATUS OF CC AND CXC CHEMOKINES

<table>
<thead>
<tr>
<th>CHEMOKINE</th>
<th>RECEPTOR(S)</th>
<th>TARGET CELLS</th>
<th>OTHER EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>-C-X-C- ELR+</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>CXCR-1, CXCR-2</td>
<td>Neut, Bas, T</td>
<td>Degranulation</td>
</tr>
<tr>
<td>GROα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GROβ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GROγ</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ENA-78</td>
<td>CXCR-2</td>
<td>Fibr</td>
<td></td>
</tr>
<tr>
<td>LDGF-PBP</td>
<td></td>
<td>Fibr, Neut</td>
<td></td>
</tr>
<tr>
<td>GCP-2</td>
<td></td>
<td>Neut</td>
<td></td>
</tr>
<tr>
<td><strong>-C-X-C- ELR-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>????</td>
<td>Fibr</td>
<td>Angiostasis</td>
</tr>
<tr>
<td>MIG</td>
<td></td>
<td></td>
<td>Angiostasis, endo anti-proliferative, Anti-tumor</td>
</tr>
<tr>
<td>IP-10</td>
<td>CXCR-3</td>
<td>Act.T (Th1&gt;Th2)</td>
<td></td>
</tr>
<tr>
<td>ITAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDF-1α/β</td>
<td>CXCR-4</td>
<td>Widely expressed and T, DC, BM</td>
<td>Embryonic G.I., CNS, B cell development, t-HIV-1 entry block</td>
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<tr>
<td>BCA-1</td>
<td>CXCR-5</td>
<td>B</td>
<td>B follicle formation</td>
</tr>
<tr>
<td><strong>-C-C-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CCR-1, CCR-5</td>
<td>Mon/Macr, NK, Bas, DC, BM, B, T (Th1&gt;Th2)</td>
<td>NK activation, IgE/IgG4 prod., m-HIV-1 entry block, T costimulation</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>CCR-1, CCR-9</td>
<td>Mon/Macr, NK, Bas, DC, BM, B, T (Th1&gt;Th2)</td>
<td>NK activation, IgE/IgG4 prod., m-HIV-1 entry block, T costimulation</td>
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<tr>
<td>MDC</td>
<td>CCR-4</td>
<td>DC, T (Th2&gt;Th1) IANK, Mon?</td>
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<tr>
<td>TECK</td>
<td>????</td>
<td>Macr, Thy, DC</td>
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<td>TARC</td>
<td>CCR-4</td>
<td>T cell lines</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>CCR-1, CCR-3, CCR-4, CCR-5</td>
<td>Mon/Macr, NK, Bas, DC, memT &gt; T, Bas</td>
<td>T proliferation, m-HIV-1 entry block, IgE/IgG4 prod.</td>
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<td>DC-CK-1 (PARC)</td>
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<td>Anti-proliferative for progenitor BM</td>
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<td>HCC-1</td>
<td>CCR-9</td>
<td>Mon</td>
<td>Lymph node traffic</td>
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<tr>
<td>HCC-4</td>
<td>????</td>
<td>Mon, Lym</td>
<td>NK, T (CD8), Bas degranulation, Mon/ Macr activation</td>
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<td>MIP-3α (LARC)</td>
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<td>MIP-3β (ELC)</td>
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<td>MCP-1</td>
<td>CCR-2, CCR-9</td>
<td>T, Mon, Bas</td>
<td>NK, T (CD8), Bas degranulation, Mon/ Macr activation</td>
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<td>T, Mon, Bas, Eo</td>
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<tr>
<td>MCP-3</td>
<td>CCR-2, CCR-9</td>
<td>T, Mon, Bas, Eo, DC</td>
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<tr>
<td>MCP-4</td>
<td>CCR-2, CCR-3, CCR-9, CXCR-3</td>
<td>T, Mon, Bas, Eo, DC</td>
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<td>Eotaxin</td>
<td>CCR-3, CCR-9, CXCR-3</td>
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<td>Eotaxin-2</td>
<td>CCR-3</td>
<td>Eo, subset Th2, Bas</td>
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<tr>
<td>6C-kine (SLC)</td>
<td>CCR-7, CXCR3</td>
<td>T, B, Mes?</td>
<td>Anti-proliferative for progenitor BM progenitor</td>
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Legend for TABLE 1. Chemokines: IL-8: Interleukin-8, GRO-\(\alpha,\beta,\gamma\): Growth related oncogene-\(\alpha,\beta,\gamma\), ENA-78: Epithelial derived neutrophil attractant-78, LDGF-PBP: Leukocyte derived growth factor-platelet basic protein, GCP-2: Granulocyte chemotactic protein-2, PF4: Platelet factor-4, MIG: Monokine induced by Interferon-gamma(\(\gamma\)), IP-10: Interferon-\(\gamma\) inducible protein-10, ITAC: Interferon-inducible T cell alpha chemoattractant, SDF-1 \(\alpha/\beta\): Stromal-cell derived factor-1 \(\alpha/\beta\), BCA-1: B cell attracting chemokine-1, MIP-1 and-3 \(\alpha/\beta\): Macrophage inflammatory protein-1 and-3 \(\alpha/\beta\), MDC: Monocyte derived chemokine, TECK: Thymus-expressed chemokine, TARC: Thymus and activation-regulated chemokine, RANTES: Regulated on activation normal T cell expressed and secreted, DC-CK-1(PARC): Dendritic cell chemokine-1 (pulmonary and activation-regulated chemokine), HCC-1,2,4: Human CC cytokine-1,2,4, LARC: Liver and activation-regulated chemokine, ELC: EBI1-ligand chemokine (EBI1=CCR7), MIP-3 \(\alpha/\beta\) and MIP-5: see MIP-1 \(\alpha/\beta\) above, MCP-1,2,3,4: Monocyte chemotactic protein-1,2,3,4, MIPF-1: Myeloid progenitor inhibitory factor-1, 6C-kine/SLC: Six-cysteine chemokine/Secondary lymphoid tissue chemokine.


Chemokines in red denote those fully characterised functionally during or after the start of this study (Schall et al. 1994, Mackay 1996, Rollins 1997, Zlotnik et al. 1999). Recent interest in chemokines and their receptors has increased substantially as a result of their emerging role in auto-immune and inflammatory pathologies, in development (hematopoeisis), in physiology (T cell peripheral surveillance and B cell follicle formation), and in infectious diseases (malaria and HIV) (Baggiolini et al. 1994; Moore, 1997; Fauci, 1996; Ward et al. 1998; Baggiolini et al. 1997). This thesis will focus further on two distinct chemokines, namely the –CC- chemokine RANTES and the –CXC- chemokine SDF-1. These are described in more detail below:

RANTES STRUCTURE AND GENE EXPRESSION
Regulated on Activation Normal T cell Expressed and Secreted, forming the acronym RANTES, was rather a long term for a molecule described by Schall et al. 1988 to be specifically associated with T cell activation. Using a subtractive hybridisation method (growth factor-dependent T cell line cDNA minus B-lymphoblastoid cDNA), the genes identified were also dependent on T cell activation. The cDNA encodes for a polypeptide with 91 amino-acids including a 23-residue signal sequence at the aminoterminal, which is cleaved to produce the mature, secreted protein with 68-residues and no potential sites for N-linked glycosylation. The gene encoding for RANTES was found on chromosome 17q11.2-12. Over twenty potential transcription factor binding
Introduction

sites were detected in the immediate upstream region of the RANTES gene, including consensus sites for AP-1, NF-κB, CD28RE and NFIL6. These regulatory sites were originally described in promoters from various cells apart from lymphocytes such as fibroblasts. The presence of these sites indicates multiple transcriptional controls on RANTES expression (Nelson et al. 1993; Nelson et al. 1996; Ortiz et al. 1996; Zlotnik et al. 1999).

Several studies have shown that mRNA for RANTES can be found in numerous tissues apart from T cells. Specifically, RANTES is expressed constitutively in T cells, platelets (Kameyoshi et al. 1992), NK cells (Nelson et al. 1993), lymph nodes with DTH lesions (Devergne et al. 1994), solid tumours, osteoblasts and myeloid precursor cells (Schall et al. 1991). Also, RANTES is expressed after stimulation with anti-CD3 or PHA on T cells (Miller et al. 1989), TNF-α on fibroblasts (Jordan et al. 1996), TNF-α and IL-1β on renal epithelial and mesangial cells (Heeger et al. 1992), and TNF-α on vascular smooth muscle cells (Jordan et al. 1996). However, the production profile and role in T cell physiology where it was originally identified has been under some controversy as it appears expressed constitutively, after stimulation and even found to be down-regulated upon activation in some IL-2-dependent T cell lines (Schall et al. 1988).

SDF-1 STRUCTURE AND GENE EXPRESSION

The genes encoding for Stromal cell-Derived Factor (SDF-1) were originally isolated as orphan clones from bone-marrow stromal cells using a signal sequence trap technique (Tashiro et al. 1993). SDF-1 secreted from mouse bone marrow cells was also identified to be a factor inducing proliferation of B cell progenitors alone and synergistically with IL-7, as well as regulating B cell maturation (Nagasawa et al. 1996). A clone containing cDNA encoding this growth-stimulating factor was isolated by expression cloning, and the nucleotide sequence contained a single substantial open reading frame of 267 nucleotides for an 89-amino-acid polypeptide (Nagasawa et al. 1996). Two isoforms SDF-1α and SDF-1β have been identified that arise from alternative splicing, encoded by 3 exons to give 89 residues or 4 exons to give 93 residues on a single gene, respectively. The mature SDF-1α protein contains 68 amino-acids and is the most abundant isoform in humans (Shirozu et al. 1995). SDF-1α, like Mig, IP-10, ITAC and the recently discovered BLA/BCA-1 lacks the ELR motif and is chemotactic for T lymphocytes rather than neutrophils (Liao et al. 1995).
Although clearly a member of the CXC subfamily, SDF-1α is more closely related phylogenetically to BLA/BCA-1 than the other CXC chemokines (Zlotnik et al. 1999), and is the only CXC chemokine identified to date whose genomic location is not found on the chromosome 4 CXC chemokine cluster, but on chromosome 10 instead (Shirozu et al. 1995). The solution structure and 3-D model of SDF-1 was published by Crump et al. in 1997, and the schematic ribbon-representation of the monomeric SDF-1 molecule is shown in Figure 1.3. The SDF-1 gene is highly conserved since the human and murine SDF-1α differ only at position 18 (valine-human and isoleucine-mouse) (Bleul et al. 1996; Oberlin et al. 1996). Although mRNA for SDF-1 is ubiquitous in all cells except red blood cells, functional expression of SDF-1 remains to be determined. Consistent with the effects of SDF-1 on pre-B cell proliferation, knock-out mice lacking SDF-1α show abnormalities in B cell lymphopoiesis, bone marrow myelopoiesis, cerebellar neuron migration and also have non-fatal ventricular septal defects, but are embryo lethal (Nagasawa et al. 1996). Only very recently in 1999, SDF-1 was reported to be expressed by fibroblasts and mononuclear cells in autoimmune skin lesions (Pablos et al. 1999), glial and neuronal cells (Bajetto et al. 1999), mesothelial cells and biliary duct plate epithelial cells antenatally (Coulomb et al. 1999), and skin dendritic cells (Bouloc et al. 1999). To date lymphocytes have not been reported to produce SDF-1.

**CHEMOKINE RECEPTORS**

So far, all chemokine receptors were found to be members of the seven transmembrane spanning (7-TMS), G-protein coupled receptor family. Similar in structure to the other conventional 7-TMS receptors (Strosberg 1991), they possess an extracellular N-terminus, seven hydrophobic membrane-spanning loops, three extracellular loops, three intracellular loops and a cytoplasmic C-terminus “tail”. Figure 1.4 shows a schematic representation of a generic chemokine receptor. The N-terminus and extracellular loops contain conserved cysteines that form disulphide bridges in an N-terminal-to-second loop and first loop-to-third loop pattern, supporting receptor conformation (Pelchen-Matthews et al. 1999). A most conserved feature of chemokine receptors is the amino-acid sequence DRYLAIV, a structural motif contained in the second intracellular loop domain adjacent to the third transmembrane region (Power et al. 1996, Rollins 1997). A summary of the chemokine receptors identified to date is shown in Table 2, and once again, the red entries denote those discovered during or after this study (Schall et al. 1994, Mackay 1996, Rollins 1997, Zlotnik et al. 1999).
**Legend for Fig. 1.3.** The three dimensional structure of SDF-1 obeys the general features of chemokine structure (outlined by Dr I. Clark-Lewis at the Pasteur institute Conference on Chemokines 1999) which are: The N-terminal domain (shown at the bottom), a disulphide bridge, a loop region (or loop helix), first \( \beta \)-strand, second \( \beta \)-strand, third \( \beta \)-strand, an \( \alpha \)-helix and the C-terminal (shown at the top).

**RANTES RECEPTORS**

RANTES was found to have multiple binding sites on various cell types, as new receptors were identified and tested for RANTES binding. Hence several receptors were found to bind RANTES such as CCR1 (Neote et al. 1993; Proudfoot et al. 1995), CCR4 (Hoogewerf et al. 1996), CCR3 (Combadiere et al. 1995) and CCR5 (Gao et al. 1993; Samson et al. 1996, Raport et al. 1996), which attracted a lot of attention as an HIV-1 fusion cofactor (Alkhatib et al. 1996;). The genes encoding for CCR1, CCR3, CCR4 and CCR5 were all located on chromosome 3 p21.3-p24 (Samson, Soulderue 1996).

**SDF-1 RECEPTOR**

SDF-1\( \alpha \) is the natural ligand for the chemokine receptor CXCR4, another seven transmembrane G-protein coupled receptor (Bleul et al. 1996; Oberlin et al. 1996; Heesen et al. 1997; Loetscher et al. 1994; Feng et al. 1996). CXCR4 is expressed on peripheral blood lymphocytes, monocytes, thymocytes, pre-B cells as well as dendritic...
and endothelial cells (Bleul et al. 1997; Hesselgesser et al. 1998; Forster et al. 1998; Yi et al. 1998; Sallusto et al. 1998). Initially, this receptor termed independently as HUMSTSR (Federspill et al. 1993), LESTR (Loetscher et al. 1994) or FUSIN (Feng et al. 1996), was described as an ‘orphan’ receptor until it was found to be the coreceptor for the binding and entry of T-tropic HIV-strains (Bleul et al. 1996; Oberlin et al. 1996; Moore, 1997; Feng et al. 1996). The cDNA for LESTR encoding 352 amino-acids, was found in a blood monocyte library. The mRNA for what was to be CXCR4, occurred 20 times as frequent as CXCR1 or CXCR2 in monocytes, and in much higher mRNA levels in neutrophils and lymphocytes (Loetscher et al. 1994). The genomic location of HUMSTSR/CXCR4 receptor was reported to be on chromosome 2 (Federspill et al 1993).

**RECEPTOR COUPLING TO G-PROTEINS**

Early examination of seven transmembrane receptors of the chemokine family revealed that they have the capacity to couple to multiple G-protein isoforms (Wu et al. 1993). The conserved DRYLAIV motif in the second intracellular loop has been implicated in G-protein coupling (Damaj et al. 1996), although other intracellular domains are also involved (Strosberg 1991; Damaj et al. 1996), including the cytoplasmic tail (Pelchen-Matthews et al. 1999) (See Figure 1.4). Guanine nucleotide binding proteins were identified as the signal transducers of G-protein-coupled receptor activation, before the sequence of the receptors was known. G-proteins consist of three subunits: $\alpha$-(39-46 kDa) which contains the binding site for GTP, $\beta$-(35-36 kDa) and $\gamma$-(~8 kDa), which form stable non-covalent $\beta\gamma$-complexes. The most diverse species is the $\alpha$-subunit and the various isoforms of the heterotrimeric complexes are classified based on the $\alpha$-subunit identity broadly in 4 classes: Gi (inhibitory for adenyl cyclase), Gs (stimulatory for adenyl cyclase), Gq (stimulatory for phospholipase C) and $G_{12}$ (effects on ion channels and ion exchange pumps). However, there are at least 17 $\alpha$-subunit isoforms, some of which are not members of the 4 classes, such as the poorly characterised $Gz$ (Heldin and Purton 1996). Even more diverse than the G-proteins themselves, is their multiple signal transduction capability; chemokine receptors on the leukocyte population are thought to couple to a limited amount of expressed G-protein isoforms, each one of which can generate several distinct downstream signals (Heldin and Purton 1996). Specifically, IL-8 receptors CXCR1 and CXCR2 were shown to associate with $G\alpha\beta2$ (Damaj et al. 1996).
Introduction

Figure 1.4 Diagram of a generic 7-Trans-Membrane, G-protein-coupled Receptor model, embedded in the cell surface lipid bilayer.

Legend for Figure 1.4.
The extracellular N-terminus and intracellular C-terminus are annotated with “NH₂” and “COOH” respectively. The hydrophobic transmembrane regions (TMRs) are depicted as cylinders embedded between the phospholipid bilayer and numbered with latin numerals (except for ‘IV’ portrayed behind ‘V’). The blue intracellular loop is the first one linking TMR1 to TMR2, green is the second intracellular loop linking TMR3 to TMR4 and red is the third intracellular loop linking TMR5 to TMR6. (Adapted from Strosberg et al. 1991).
### TABLE 2. CHEMOKINE RECEPTORS AND THEIR LIGANDS

<table>
<thead>
<tr>
<th>CHEMOKINE RECEPTOR</th>
<th>TARGET CELL(S)</th>
<th>MIGRATORY</th>
<th>PRINCIPAL CHEMOKINE FAMILY</th>
<th>CHEMOKINE LIGAND(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>-C-X-C- family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR1</td>
<td>Neut- PMN</td>
<td>IL-8, GCP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR2</td>
<td>Neut-PMN</td>
<td>IL-8, GROα, β, γ, NAP-2, ENA-78, GCP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>ActT (Th1&gt;Th2)</td>
<td>IP-10, MIG, ITAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>T, B, Mono, PMN and widespread on other tissues</td>
<td>SDF-1α, β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR5 (BLR-1)</td>
<td>B</td>
<td>BCA-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-C-C- family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR1</td>
<td>Act.T, Mon, Eo, DC</td>
<td>MIP-1α, RANTES, MCP-3, HCC-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR2a and b</td>
<td>Mon, Macr, ActT</td>
<td>MCP-1,-2,-3,-4,5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR3</td>
<td>Eo, Bas, subset of ActTh2</td>
<td>Eotaxin, Eotaxin-2 RANTES, MCP-2,-3,-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>ActT (Th2&gt;Th1), Bas, Plat</td>
<td>TARC, MDC, (RANTES, MIP-1α, MCP-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>ActT (Th1&gt;Th2), Mon, Macr, DC</td>
<td>MIP-1α, MIP-1β, RANTES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>T, B, Mon, bmd-DC</td>
<td>MIP-3α, LARC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR7</td>
<td>T, B</td>
<td>MIP-3β, SLC</td>
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<td></td>
</tr>
<tr>
<td>CCR8</td>
<td>Mon, Macr, T (Th1&gt;Th2)</td>
<td>I-309, TCA-3, TARC, MIP-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR9</td>
<td>BM, T, Mon, non-hematopoietic</td>
<td>Multiple -CC-chemokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-C- family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XCR1</td>
<td>T, NK, ?</td>
<td>Lymphotactin</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-C-X-X-X-C- family</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Neuro, Eno, NK, T(CD8)</td>
<td>Fractalkline / Neurotactin</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Promiscuous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DARC (Duffy Antigen)</td>
<td>postcapillary- Eno, RBC</td>
<td>Multiple CC and ERL+ CXC chemokines</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend for TABLE 2. Chemokines**: See legend for Table 1- also: NAP-2: neutrophil activating protein-2, TCA-3: T cell activation gene-3. Cells: See legend for Table 1-also: PMN: polymorphonuclear cells, Plat: platelets, bmd-DC: bone-marrow-derived dendritic cells, Neuro: neuronal cells, RBC: red blood cells.

CCR1, CCR2A and CCR2B were reported capable of coupling to multiple isoforms such as Gαi, Gαq and Gα16 according to the cell type (Arai, Charo, 1996). In another study, the same receptors i.e. CCR1, CCR2A and CCR2B were all shown to couple to either Gαi causing Gβγ release and PLCβ2 activation, or Gα14, but only CCR2B coupled to Gα16, a hematopoietic cell specific isoform (Kuang et al. 1996). Differential functional G-protein coupling was shown to occur with chemokine receptors using permeabilised IL-2-activated NK cells, treated with neutralising anti-G-protein isoform-
specific antibodies. Hence, anti-Gs, anti-Go and anti-Gz antibodies all blocked migration to MIP-1α, MCP-1 and RANTES, whereas anti-Gi antibodies only blocked migration to RANTES and MCP-1 (Al-Aoukaty et al. 1996). This suggested that more than one G-protein isoforms can mediate chemotaxis, either by differential G-protein coupling to distinct receptors, or multiple G-protein coupling to the same receptors, with chemokine-specific candidate G-protein isoform profiles. Most chemokine responses were found to be sensitive to the ADP-ribosyltransferase action of Pertussis toxin (PTX) on Giα subunits with notable exceptions in IL-8 (Wu et al. 1993), RANTES (Bacon et al. 1995) and MCP-1/CCR2B signalling (Kuang et al. 1996, Turner et al. 1998).

CHEMOKINE RECEPTOR SPECIFICITY AND REGULATION

Upon examination of the -CC- chemokine family of receptors and ligands, receptor promiscuity appeared to be a frequent functional feature. All RANTES receptors were found capable of binding and signalling to a multitude of other ligands, sometimes with equal or greater affinity (Proudfoot et al. 1995, Hoogewerf et al. 1996, Combadiere et al. 1995, Raport et al. 1996). Moreover, the Duffy antigen was described as a chemokine "sink", which bound and inactivated most chemokines by removing them from circulation (Horuk 1994) (see Table 2). In fact, among the very few exceptions to receptor promiscuity was CXCR4, the receptor for SDF-1 (Oberlin et al. 1996). More recently, binding studies for SDF-1 revealed one site for binding and competition with 12G5 antibody, with a Kd = 7.5nM for SDF-1α and 13.7nM for SDF-1β (Hesselgesser et al. 1998). However, several common themes on structural requirements for receptor activation by chemokines started to emerge; truncated analogues of RANTES and MCP-3 were shown to act as promiscuous antagonists in multiple chemokine receptors, defining the NH2-terminal residues as crucial for receptor activation (Gong et al. 1996). The identity of important chemokine N-terminus residues for receptor activation was indicated with studies demonstrating that addition of aminoxypentane on the N-terminal serine of RANTES to give AOP-RANTES generated antagonistic behaviour for CCR5 capable of preventing M-tropic HIV-1 infection, (Simmons et al. 1997). A physiologic way of chemokine regulation has been assigned to dipeptidylpeptidase IV (CD26/DPP-IV), an enzyme widely distributed on the surface of most cells shown to cleave the first two residues from the N-terminal of certain chemokines (Struyf et al. 1999). Hence, CD26/DPP-IV would cleave eotaxin, generating (3-74)-eotaxin, which behaved as an antagonist blocking chemotaxis and HIV-1 entry (Struyf et al. 1999).
The importance of the 3 to 9 N-terminal amino acids in inducing signalling, was later found to exist for non-promiscuous receptors, such as CXCR4; N-terminal peptide fragments up to 9 residues long from SDF-1, were characterised as agonistic and antagonistic for CXCR4 binding, chemotaxis (Loetscher et al. 1998b), and calcium mobilisation (Heveker et al. 1998; Loetscher et al. 1998b). Some of these peptides were used to investigate CXCR4 signalling in this thesis and were: a) the monomer (1-9)-SDF-1: KPVSLSYRC described as a partial agonist, b) the dimer (1-9)-(9-1)-SDF-1: KPVSLSYRC-CRYSLSVPK described also as a partial agonist, c) the substituted dimer (P2G) (1-9)-(9-1)-SDF-1: KGSLSYRC-CRYSLSVGK described as an antagonist. The consensus picture given was a two-site model for receptor selectivity (between CC and CXC chemokines) and subsequent receptor activation. An initial receptor contact, provided by the main body of the chemokine, would define receptor selectivity, as a point mutation in Leu-25 to Tyr-25 in IL-8 for example, transformed the mutant to a CCR1 ligand with monocyte chemoattractant capability (Wells et al. 1996). The structural features that enable receptors to “scan” the chemokine “body” and select for CC or CXC, were assigned to the receptor NH$_2$-terminal 20 amino-acids, since that portion was found to be the least ordered receptor structure in solution (Clark-Lewis et al. 1995). Mutant CCR5 receptors which had the CCR2 N-terminal portion substituted, were shown to switch selectivity towards the CCR2 ligand MCP-1 and not RANTES (Farzan et al. 1997). Importantly, this same region was also shown to mediate HIV-1 binding, since entry of M-tropic HIV-1 which required CCR5 did not occur with N-terminal CCR2/CCR5 mutants (Farzan et al. 1997). A 3-step schematic representation of SDF-1 docking and activating its receptor CXCR4 was published by Crump et al. 1997 and is shown in Figure 1.5.

As a mode of chemokine signalling regulation, chemokine receptors were shown to undergo ligand-induced desensitisation or internalisation. IL-8 was from the first to be described as capable of regulating its receptor expression, and within 10 minutes of IL-8 administration or withdrawal, the receptors were shown to internalise or reappear, respectively (Samanta et al. 1990). Similarly, reciprocal and reversible CCR5 desensitisation was described with RANTES and MIP-1α (Gao et al. 1993). CCR1 endocytosis was shown to occur with both agonist (MIP-1α) and antagonist (NBD-RANTES), however sequestration of the receptor occurred only with agonist treatments (Solari et al. 1997).
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Figure 1.5 Diagram of the proposed 3-step chemokine receptor activation by its cognate ligand (example given of SDF-1 and CXCR4)

1 : approach 2 : recognition 3 : activation

The N-terminal peptide fragments up to 9 residues long from SDF-1 could also cause CXCR4 down-modulation (Heveker et al. 1998). However, up to 1997 and before the advent of specific receptor antibodies, studies using radiolabelled ligands to study internalisation proved liable to dubious interpretation since most chemokines were thought to bind GAGs on the cell surface, later demonstrated by Burns et al. in 1998. Up until 1999 the mechanisms controlling receptor internalisation were still not clarified; only recently the importance of C-terminal cytoplasmic tail phosphorylation at specific residues was addressed.

1.5 Emerging roles for chemokines
Chemotaxis, the initial effect assigned to chemokines, is an ordered migration along a chemical gradient, involving cell polarisation, adhesion molecule upregulation and cell movement with a coordinated formation of lamellipodia and filipodia (Mitchison et al. 1996, Lauffenburger et al. 1996). It is a complex process requiring the integration of several signalling cascades, some of which may account for the non-chemotactic effects chemokines exert on various cell populations (Knall et al. 1996, Rollins 1997). The effects of chemokines on their target cells are however much more diverse and include hematopoietic precursor cell cycling regulation, tumor stasis, megakaryopoiesis, growth factor effects and angiogenesis (Reviewed in Schall et al. 1994; Rollins 1997) (See Table 1). Chemokine involvement in inflammatory pathophysiology has been documented, such as glomerulonephritis, asthma, inflammatory bowel disease,
atheromatous plaques and allogeneic transplant rejection (Reviewed in Schall et al. 1994; Rollins 1997; Homey, Zlotnik 1999). We will consider the biological roles of RANTES and SDF-1 in more detail.

**RANTES ROLES**

The expected effect of RANTES on cells that express one or more of its receptors would be chemotaxis or calcium mobilisation. However, other biological effects and roles in pathophysiology started to accumulate (See Table 1), as RANTES and other -C-C- chemokines were reported to co-stimulate the T-cell proliferative response *in-vitro*, without affecting IL-2 production or IL-2R upregulation (Turner et al. 1995, Taub et al. 1996). Also, incubation of CD56+ cells -but not CD4+ or CD8+ cells- with MIP-1α, MIP-1β and RANTES induced cytolytic activity and proliferation (Maghazachi et al. 1996). MIP-1α, MIP-1β and RANTES but not CXC chemokines were shown to induce NK cell chemotaxis, Ca^{2+} mobilisation and enzyme release (Loetscher et al. 1996). Importantly, the association of RANTES with pathologic lesions led to its designation as an inflammatory chemokine. RANTES was found abundant in inflammatory sites of lymphoma and renal tumour tissues (von Luettichau et al. 1996), as well as inflammatory lesions in Inflammatory Bowel Disease mucosa (Grimm et al. 1996; Berrebi et al. 1997). RANTES was also found in or associated with: asymptomatic renal-allograft rejection (Grimm et al. 1995), bronchial mucosa in atopic asthma (Powell et al. 1996), chronic sinusitis and polypoid disease (Davidsson et al. 1996) by cytokine-stimulated fibroblasts (Meyer et al. 1998), transplant-associated accelerated atherosclerosis (Pattison et al. 1996), plasma at elevated levels in biliary cirrhosis patients (Miyaguchi et al. 1997), polymyalgia rheumatica sufferers (Pulsatelli et al. 1998), psoriatic skin (Fukuoka et al. 1998), blood-vessel endothelium in multiple sclerosis lesions (Simpson et al. 1998) and in experimental EAE (Glabinski et al. 1998).

Most recently RANTES was reported to be produced by lung fibroblasts after Th1 and Th2 cytokine stimulation (Teran et al. 1999), in inflamed joints of rheumatoid arthritis patients causing CCR5+ T cell influx (Suzuki et al. 1999), in advanced breast carcinoma (Luboshits et al. 1999) and in respiratory syncytial viral infection by airway epithelial cells (Becker et al. 1999). The most notorious discovery though, was the finding that RANTES and the related -CC- chemokines MIP-1α, MIP-1β which bind to the same receptors as RANTES produced by CD8+ T cells, blocked HIV-1 infection *in-vitro*.
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(Cocchi et al. 1995), by inhibiting entry of macrophage tropic (mHIV-1), NSI strains of HIV-1, which were found to utilise CCR5 as a cofactor / coreceptor alongside CD4 for intracellular invasion (Drajic et al. 1996; Alkhatib et al. 1996). Moreover, a structural receptor mutation on one RANTES receptor, a Δ32 deletion on CCR5, also prevented HIV-1 binding, affording partial immunity to some fortunate individuals (Paxton et al. 1996, Oberlin et al. 1996). Since then, a lot of effort has been directed towards the elucidation of chemokine receptor expression regulation and subsequently of chemokine responses, especially after CD28-dependent T cell costimulation was shown to downregulate CCR5 and provide additional post-entry anti-viral protection (Levine et al. 1996, Carrol et al. 1997).

SDF-1 ROLES

SDF-1α is widely expressed and is a highly efficacious chemoattractant for monocytes, T lymphocytes and CD34+ human progenitor cells (Bleul et al. 1996; Nagasawa et al. 1996; Aiuti et al. 1997; Sanchez et al. 1997; Shirozu et al. 1995). Studies with knock-out mice showed SDF-1α implicated in B cell lymphopoiesis, bone marrow myelopoiesis, cerebellar neuron migration and ventricular septal formation (Nagasawa et al. 1996). Defects similar to those witnessed by SDF-1−/− mice have been reported in CXCR4−/− mice, which also exhibit defective vascularisation of the gastrointestinal tract and reduced fetal liver (Tachibana et al. 1998; Zou et al. 1998; Ma et al. 1998). In recent studies, SDF-1 and CXCR4 have been reported in autoimmune skin lesions containing fibroblasts and mononuclear cells (Pablos et al. 1999), glial and neuronal cells (Bajetto et al. 1999), mesothelial and biliary duct plate epithelial cells modulating antenatal B cell development (Coulomb et al. 1999) and skin dendritic cells (Bouloc et al. 1999). Moreover, overexpression of CXCR4 was found to correlate with bone-marrow-infiltrates in chronic lymphocytic B cell leukemia (Mohle et al. 1999).

T cell tropic (rHIV-1), SI strains of HIV-1 were found to use LESTR/fusin as a coreceptor for entry alongside CD4. Such rHIV-1 entry was inhibited by the natural ligand SDF-1 of the chemokine receptor LESTR/fusin, later denoted as CXCR4 (Oberlin et al. 1996). Moreover, SDF-1α and its various analogues inhibited CXCR4-mediated T-cell tropic HIV-1 infection in vitro (Bleul et al. 1996; Murukami et al. 1997; Ueda et al. 1997), and such antiviral activity was associated with CXCR4 downmodulation (Heveker et al. 1998). Also, during disease progression, the switch in preference of
chemokine receptors CXCR4 from CCR5 by the transforming virus correlated with the spread of infection to target cells bearing those receptors, and virulence from NSI to SI (Connor et al. 1997).

1.6 Chemokine signalling pathways
Chemokine receptors are usually coupled to $G_{ai}$ proteins with cellular responses to chemokines sensitive to Pertussis toxin treatment: IL-8 on transfected COS-7 cells was portrayed to activate Pertussis-toxin sensitive Gi, release of $G_{i}$ subunits and activation of PI-specific PLC$\beta_2$ (Wu et al. 1993). Generally, elevation of intracellular calcium ion concentration in response to most chemokines is inhibited by PTX. Similarly, inhibition of Adenyl Cyclase resulting in inhibition of cAMP production is also PTX-sensitive (Myers et al. 1995). However, other signal transduction pathways are clearly involved. For example, MCP-1 was shown to induce arachidonic acid release in monocytes (Locati et al. 1994) and IL-8 to cause activation of NADPH oxidase and PLD on neutrophils, via CXCR1 (Jones et al. 1996). In 1995, Jones et al. showed MAPK activation induced by IL-8 to be sensitive to PTX, a signalling cascade associated with receptor tyrosine kinases and cellular growth. MAPK activation by $G_{i}$ subunits was reported to be mediated via a tyrosine phosphorylation event and Ras, resembling receptor-tyrosine-kinase signalling (van Biesen et al. 1995). Indeed, MCP-1 was shown to elicit PTX-sensitive tyrosine phosphorylation and MAPK activation on murine hybridoma cells (Dubois et al. 1996). Moreover, in 1993 Stephens et al. reported PI3K activation by a G-protein mediated pathway, which prompted investigation into the activity of this enzyme in chemoattractant/chemokine responses. Indeed, fMLP-induced activation of MEKK in neutrophils was partially sensitive to PTX but blocked by the PI3K inhibitor Wortmannin (Avdi et al. 1996). However, assigning function to signalling events induced by chemokines has been more elusive as, for example, IL-8 was reported to activate the MEK/MAPK pathway in neutrophils (Knall et al. 96), but neutrophil chemotaxis to IL-8 was insensitive to MEK/MAPK inhibition (Knall et al. 97). A schematic summary of hypothetic and generic chemokine-induced signalling based on evidence up to 1996 is shown in Figure 1.6. Reports which elucidated many aspects of chemokine signalling occurred concomitantly with results from this study and are not included in Figure 1.6. The precise coupling of chemokine receptors to the various signalling cascades and the effects they procure along with chemotaxis, is still subject to intense investigation.
RANTES INDUCED SIGNALLING PATHWAYS

RANTES was reported to inhibit adenyl cyclase in a PTX-sensitive manner (Myers et al. 1995) and, interestingly, high concentrations of RANTES were reported to cause a biphasic Ca\(^{2+}\) ion elevation on T cell clones. The first surge was inhibited by pertussis toxin and the second, delayed and prolonged intracellular calcium rise was PTX-insensitive but sensitive to Herbimycin A (Bacon et al. 1995). Moreover, chemotaxis to RANTES was inhibited by Pertussis toxin, whereas induction of IL-2R expression, IL-2 release and proliferation by high concentrations of RANTES, was inhibited by Herbimycin A (Bacon et al. 1995). A year later in 1996 Bacon et al. reported tyrosine phosphorylation of Focal Adhesion Kinase p125, the focal adhesion protein paxillin and ZAP-70, a molecule involved in T cell receptor signalling. Concomitant with this study, RANTES was reported to activate STAT1 and STAT3 and to induce transcriptional activity of c-fos (Wong et al. 1998).

SDF-1 INDUCED SIGNALLING PATHWAYS

The biological role of SDF-1 was clarified in recent years but before 1997 very little was known about the signalling pathways that may mediate these effects. SDF-1 has been shown to elicit elevation of [Ca\(^{2+}\)]\(_i\) in a number of settings (Bleul et al. 1996; Sanchez et al. 1997) which was susceptible to repeated consecutive ligand administrations implying ligand-induced receptor down-regulation (Oberlin et al. 1996). Also, SDF-1 caused PLC\(\beta\)-induced calcium mobilisation and migration of IL-2 activated natural killer cells (Maghazachi et al. 1997). However, the alpha subunits portrayed to be involved in migration and calcium mobilisation were not sensitive to the same incorporated mAbs; only anti-G(o) and anti-G(q) blocked calcium mobilisation, whereas anti-G(i), anti-G(s) and anti-G(q) were shown to block migration (Maghazachi 1997). Features such as ubiquitous CXCR4 T cell expression, lack of SDF-1 T cell production and the non-promiscuous nature of CXCR4-SDF-1 binding led this study to investigate the ability of SDF-1 to couple to distinct signalling pathways that may mediate cell growth, migration and transcriptional activation on normal T cells where possible, and establish a potential role for SDF-1 in human T cell physiology. Only very recently and concomitant with this study, SDF-1 was reported to enhance tyrosine phosphorylation of focal adhesion complex components (including Pyk-2, paxillin and Crk) and stimulate phosphorylation of both MEK-1 and ERK1/2 in several cell models (Ganju et al. 1998; Davis et al. 1997; Jourdan et al. 1998; Popik et al. 1998).
Figure 1.6 Diagram of potential chemokine-induced signalling cascades from a hypothetical, generic chemokine receptor.
Legend for Figure 1.6. G?α: G-protein α-subunit isoforms such as Gi, or Gq, βγ: G-protein βγ-subunit, P13K?: Phosphatidylnositol 3-kinase isoforms, PLD: Phospholipase D, PLCβ2: Phospholipase C β2 isoform, NADPH oxidase: Nicotinamide Adenine Dinucleotide Phosphate (reduced form) oxidase, PKC: Protein kinase C, RAS: p21 Ras GTP-binding protein, MAPK: Mitogen Activated Protein Kinase, FAKp125: 125kDa Focal Adhesion Kinase, ZAP-70: 70 kDa Zeta (ζ)-chain (T cell receptor) Associated Protein kinase, (?): unidentified kinase or association domain/motif, (P): phosphate group, PC: PhosphatidylCholine, PA: phosphatidic Acid, PIP2: Phosphatidylinositol (4,5) bisphosphate, IP3: Inositol trisphosphate, DAG: Diacylglycerol, AMP: Adenosine monophosphate, cAMP: cyclic AMP. Drugs are depicted in red Italic letters. Curved arrows indicate phosphorylation (eg. (P)), catalysis (cAMP, PA, IP3 + DAG) and dissociation (G-protein to G?α and Gβγ). Straight arrows indicate stimulation (+), inhibition (-), translocation (►), and coupling-activation. The dotted straight arrow from PA to DAG denotes phosphohydrolase activity.

SDF-1 stimulation also increased NFκB activity and induced lipid kinase activity associated with anti-phosphotyrosine immunoprecipitates (Ganju et al. 1998; Davis et al. 1997). These findings are incorporated and discussed alongside results from this study in the Discussion section II.

1.7 T cell identities

RESTING AND ACTIVATED T CELL PHENOTYPES

The process stemming from antigenic stimulation could be divided conceptually and temporally into activation, proliferation and differentiation. Activation begins with the APC-T-cell interaction and ends with the secretion of IL-2, an autocrine growth factor for T cells. Proliferation entails DNA replication and cellular division. Differentiation may require several rounds of proliferation with concomitant cytokine signals to result in effector cells. During the first 4 hours of the activation phase, more than 70 genes are newly expressed, initiating several parallel biochemical programs that prepare the cell for division and change them from quiescent (G0) to transformed lymphoblasts (G1) (Ullman et al. 1990; Ward, June 1998). Activated T-cells express molecules on their surface that either lack or exist in much lower densities on resting cells. Such molecules are defined as T cell activation antigens, comprising a heterologous and varied group of molecules. Table 3 displays such activation antigens and other molecules whose expression or function depends on activation.
Table 3. T cell activation markers

<table>
<thead>
<tr>
<th>Name</th>
<th>Brief description</th>
<th>Resting T cell expression</th>
<th>Activated T cell expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25-α-subunit</td>
<td>Part of IL-2 receptor αβ-heterodimer</td>
<td>no</td>
<td>yes; formation of αβ-heterodimer</td>
</tr>
<tr>
<td>LFA-1; CD11a/CD18</td>
<td>β2 Integrin adhesion receptor</td>
<td>yes; inactive conformation</td>
<td>yes; active conformation</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Ig superfamily LFA-1 ligand</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>CD51/CD61</td>
<td>β3 Integrin vitronectin receptor</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>CD29/CD49; VLA-1, VLA-2</td>
<td>β1 Integrin adhesion receptor</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>CD69 ; VEA</td>
<td>C-type selectin adhesion costimulation</td>
<td>no; very low</td>
<td>yes; high</td>
</tr>
</tbody>
</table>

MEMORY AND NAÏVE SUBSETS

T cells found in the periphery are heterogeneous phenotypically as well as functionally. They can be broadly divided into "naïve" and "memory" subsets: naïve are those cells that have not yet encountered their cognate antigen, and memory cells are those that have previously undergone an antigen-driven expansion. After T cell stimulation in vivo CD4+ cells switch expression of membrane CD45 isoforms. The high molecular weight isoform CD45RA is expressed by naïve cells, and the low molecular weight isoform CD45RO is preferentially expressed alongside immunological memory function. Naïve T cells are phenotypically identified as coexpressing CD45RA and low LFA-1, high L-selectin, low CD44 and no activation markers. Memory cells express CD45RO, high LFA-1 low L-selectin, high CD44 and have increased surface expression levels of CD25, CD2, CD28, LFA-3/CD58 and other activation markers (Sanders 1988, Ferrer 1992, Picker et al 1993).

Functionally, memory T cells appear more sensitive to antigenic-TCR triggering and proliferate without requirement for costimulation (Sanders et al. 1988; Ferrer et al. 1992), producing a more robust cytokine response (eg. INF-γ) and a wider variety of cytokines (eg. IL-4) (Ferrer et al. 1992; Austyn, Wood 1993). For some time, the switch from RA to RO was thought to be an irreversible maturational process; however other data indicate that RO cells could revert back to RA expression upon entering a quiescent state in vivo (Tough, Sprent 1994). Memory CD4 T cells are capable of entering non-lymphoid tissue directly from the blood, whereas naïve cells re-circulate to the
secondary lymphoid organs only (Austyn, Wood 1993). During this course of study, the participation of chemokines in naïve and memory physiology emerged as RANTES was shown to be chemotactic for CD45RO⁺ memory T-cells expressing CCR5, whereas SDF-1α was associated with naïve CD45RA⁺ T-cell migration and expression of CXCR4 (Bleul et al. 1997).

**T CELL HELPER SUBSETS (Th1, Th2 and Th0)**

*In-vitro* adapted cells have been used to identify subsets according to function (Mossman, Coffman, 1989), and although some differences have been described between the defined murine and human Th subsets, a consensus model in 1997 presented mature, unprimed or "naïve" CD4⁺ human T cells to produce mainly IL-2 upon antigenic stimulation. These cells, termed T helper cell precursor (Thp) can potentially differentiate to at least 3 functionally distinct subsets which are: a) Th1-secreting IL-2, INF-γ and TNF-α, involved in cell-mediated and delayed-type hypersensitivity (DTH) responses; b) Th2-secreting IL-4, IL-5, and IL-13, regulating the humoral immune response; c) Th0-secreting both Th1 and Th2 cytokines. The development and switching of these types of helper cells is still a matter for debate (Coffman et al. 1997; Borish et al. 1997).

1.8 **T cell activation**

For a productive immune response, T-cells have to achieve full activation, cytokine secretion and clonal expansion. However, apart from recognition of antigen, a second distinct signal, termed costimulatory, emerged as essential for activation. In the absence of such co-stimulus, T cells could die via apoptosis, or enter a state of subsequent unresponsiveness or anergy (Janeway et al. 1994). The major pathway for T cell activation under physiological conditions is the interaction of the antigen-bound-to-MHC with the T cell receptor (TCR)/CD3 complex, together with costimulatory molecule ligation such as CD28. This process can be mimicked in the absence of antigen *in-vitro*, by anti-CD3 plus anti-CD28 antibodies (Lenschow et al. 1996). Moreover, an alternative set of antibodies was described able to induce T cell activation *in vitro*, involving ligation of CD2 together with CD28 (Costello et al. 1993).
**MODEL OF COSTIMULATION**

T-cell activation is a complex procedure which requires the successful delivery of at least two independent signals. Signal 1 is delivered by the TCR/CD3 complex and Signal 2 involves CD28 costimulatory receptor activation. There are several possibilities that explain the requirement for Signal 1 and Signal 2, such as: i) distinct biochemical signals, ii) same signals but varying in onset and duration with non-overlapping kinetics, iii) same signals that are both required to cross critical thresholds for activation. Evidence for distinct signalling was reported by June et al. in 1987 with in-vitro models of T cell activation displaying differential sensitivity to the immunosuppressant Cyclosporin A. Specifically, whereas both CD3/CD28 and PMA/CD28 costimulation conditions can result in T cell proliferation, only CD3 / CD28-induced T cell proliferation is sensitive to Cyclosporin A. In-vivo, CD3/TCR signals occur in association with other molecules like CD4 or CD8 and CD45 which contribute to the formation of the TCR-MHC complex, increase the sensitivity and regulate intracellularly TCR-induced signal transduction. Furthermore adhesion interactions via LFA-1 increase the avidity of the interaction with the APC (Weiss et al 1994; Ward, June 1998). A schematic representation of the key players involved in antigenic costimulation forming the immunological “synapse” is shown in Figure 1.7. In-vitro proliferation of highly purified T cells in the absence of accessory cells that provide the second signal by cell contact or cytokine secretion, requires dual triggering by specific Abs to either CD3 or CD2 in combination with CD28 ligation by either mAb or the natural ligands B7.1/B7.2 (June et al. 1987; Ueda et al. 1995; Costello et al. 1993). In combination with CD28 ligation, such dual CD2 antibody co-ligation has been shown to result in prolonged (>3week) IL2Rα-chain gene transcription and surface expression (Cerdan et al. 1992), as well as long term autocrine IL-2-dependent proliferation (Costello et al. 1993). Alternatively, phorbol esters such as PMA in combination with either calcium ionophores or Abs/natural ligands to CD28 can support T cell proliferation (June et al. 1987; Ueda et al. 1995; Costello et al. 1993).

**INTEGRATION OF TCR, CD3, CD4/8 AND CD45 SIGNALLING**

The T-Cell Receptor/CD3 complex mediates antigen-specific activation as a surface structure containing a duplicate of the disulphide-linked heterodimer TCR α-β, or its alternative form TCR γ-δ, in association with single CD3 polypeptide chains termed CD3 -γ, -δ, two CD3 -ε, the CD3 homodimer ζ-ζ and CD3-η(eta) chains (Figure 1.7).
Structural motifs based on two YXXL/I repeats separated by 6-8 amino-acids termed Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) were identified in the cytoplasmic compartment of the various CD3 chains, with \( \zeta \)-chains containing 3 ITAMs and \( \gamma \)-, \( \delta \)- and \( \epsilon \)- chains each containing 1 ITAM. Hence, the surrogate cytoplasmic domains of the supramolecular complex contain at least 10 ITAMs. The ITAMs are the principal structures which couple the surface receptor to downstream signal transduction (Ward, June 1998). Other molecules can signal to enhance, attenuate or replace \textit{in-vitro} the CD3/TCR-derived Signal 1, such as CD2. The CD2 surface molecule is a 50 kDa surface molecule present on 95% of T cells (Springer et al. 1987; Moigneon et al. 1987). Stimulation \textit{via} CD2 \textit{in-vitro} requires a pair of CD2 antibodies directed against different epitopes (Meuer et al. 1984; Olive et al. 1986), although activation requires the expression of the TCR/CD3 complex (Bockenstedt et al. 1988; Alcover et al. 1988). \textit{In vivo}, CD2 promotes T-cell adhesion and may play a ‘tuning’ role in enhancing the TCR response as well as providing Ag-independent activation \textit{via} ligation with its natural ligand CD58 (LFA3) (Dr. M Dustin, 1999 BSI seminar).

Antigen is presented bound to two types of MHC molecules distinguished by the T-cells with the CD4 and CD8 co-receptors, which bind to invariant regions of the MHC molecules. CD4-coupled TCR/CD3 complexes recognise Ag-MHC-class II and signal for helper activity, producing an array of cytokines which regulate the nature of the adaptive immune response. CD8-coupled TCR/CD3 complexes recognise antigen bound to MHC-class I and signal for cytotoxic activity, e.g. against virally infected cells. CD4 and CD8 molecules provide an essential contribution for TCR/CD3 signal transduction via their associated protein tyrosine kinase (PTK) p56\textsuperscript{ck}. Hence CD4 ligation is required for activation, enhancing CD3 responses and has been recognised as the principal receptor for HIV-1 (Austyn, Wood 1993). CD45 influences MHC/Ag-induced signal transduction, by de-phosphorylating and thus “priming” PTK’s which are coupled to the TCR/CD3 complex and to CD4 /CD8 (Desai et al. 1994). Engagement of the TCR/CD3 complex results in activation of several PTK’s such as p59\textsuperscript{fyn} and p56\textsuperscript{ck} (src family) that phosphorylate key tyrosine residues within the ITAM’s, and recruited ZAP-70, SIp-76, Vav and Itk. Three essential biochemical pathways that convey activation signals follow effective TCR ligation, and they are: a) the calcium pathway, b) the PKC pathways, and c) the Ras pathways. Figure 1.8 is a schematic summary of signal pathways emerging after TCR, CD4 and CD45 co-ligation.
Figure 1.7
Cartoon model of the immunological "synapse" between T cells and APCs

Legend for Figure 1.8: Ag: Antigen, CRAC channel: Calcium-release activated calcium channel, LAT: Linker of Activated T cells a 36 kDa adaptor tyrosine phosphoprotein, Lck, Fyn: protein tyrosine kinases p56 lck and p59 fyn of the src family, ( -I): inactive, ( -A): active, PTPase: protein tyrosine phosphatase, ZAP-70: 70kDa Zeta (ζ)-chain (T cell receptor) associated protein kinase, Pyk-2: Proline-rich tyrosine kinase 2, SHP-1: (src homology 2 (SH2))-containing tyrosine phosphatase 1, Vav: guanine nucleotide exchange factor for the Rho family GTPase Rac1, Slp-76: 76 kDa adaptor tyrosine phosphoprotein, shc: adaptor tyrosine phosphoprotein, Grb-2: Growth factor receptor-bound protein-2 ( adaptor), SOS: mammalian Son of sevenless guanine exchange factor for p21 ras, Ca/CaM: Calcium-Calmodulin, Calcineurin: serine/threonine protein phosphatase 2B, NFAT: Nuclear factor of activated T cells, PLCγ: Phospholipase C γ isoform, PKC: Protein kinase C, RAS: p21 Ras GTP-binding protein, MAPK: Mitogen Activated Protein Kinase, PIP2: Phosphatidylinositol (4,5) bisphosphate, IP3: Inositol trisphosphate, DAG: Diacylglycerol. Drugs are depicted in red italics letters. ITAMs are depicted present on the ζ-chains only for clarity and are not phosphorylated (yellow) or phosphorylated (purple) by src-family proteins, thus recruiting ZAP-70. The stimulation of CRAC channels has not been fully elucidated as calcium ions, IP3 and direct kinase activity from the IP3R have been implicated. Curved arrows indicate de-phosphorylation (PTPase), phosphorylation (Lck-A) and catalysis (IP3 + DAG). Straight arrows indicate stimulation (+), inhibition (-), adaptor association ( ), activation by tyrosine phosphorylation (Fyn-A to Pyk2, Zap-70 to Shp-1, Vav, Slp-76, Shc), translocation/recruitment (PLCγ, NFAT, ZAP-70 to ζ-chain), release/influx (calcium ions) or other activation (e.g. Fyn/Lck - (I) to -(A)).
Figure 1.8 Schematic summary of signal integration from co-ligation of the TCR, CD4 and CD45.
ROLE OF CD28 AND CTLA-4

In the 2 signal model with the T-cell receptor as Signal 1, CD28 is the molecule considered as the main co-stimulatory receptor with a pivotal role in T-cell development. Ligation of CD28 alone has little effect on resting T cell proliferation, but in the presence of limited concentrations of Ag or anti-CD3 antibody, CD28 co-stimulation affords a robust proliferative response. A homodimer composed of two 44 kDa glycosylated chains, CD28 is present on 90% of all CD4+ and 50% of CD8+ T cells and the natural ligands are B7.1 and B7.2 (June et al. 1994; Ward 1996). CD28 has been reported to provide signals distinct from CD3 for T cell activation, since induction of IL-2 mRNA expression, IL-2 receptor surface expression and T-cell proliferation from T-cell costimulation involving CD28 ligation in combination with PMA has been shown to be resistant to Cyclosporin A (June et al. 1987). The CD28 ligands B7.1 and B7.2 combined with CD3 antibody ligation have been reported to be indistinguishable in inducing long term autocrine growth and cytokine secretion, however CD28 antibody ligation appeared less efficient than the natural ligands (Levine et al. 1995). B7 ligands and anti-CD28 antibodies have been found to activate different biochemical events, some of which are summarised in Table 4 (Adapted from Ward 1996).

Curiously, the APC ligands for CD28, CD80 and CD86 (B7.1 and 2), are shared by the CD28 homolog CTLA-4 which provides important signals that negatively regulate T cell activation (Waterhouse et al. 1996; Tivol et al. 1996). The generally accepted paradigm portrays TCR in combination to CD28 ligation affording cellular activation with CTLA-4 subsequently expressed to switch off the response (Ward 1996). TCR engagement, probably in combination with CTLA-4 and/or absence of CD28, can result in anergy manifested in-vivo as a long-lived immunologically unresponsive state of the T-cell. CTLA-4 is also involved in maintaining tolerance to self antigens (Tivol et al. 1996, Dr Carl June personal communication). Moreover, CTLA-4 binding to B7 was thought to limit Th1 expansion, as the effect of B7 on CD3/CD28 costimulation is to promote Th2 differentiation in contrast to CD28-specific antibody ligation (Oosterwegel et al. 1999). Insight into the regulatory activity of CTLA-4 was provided with the use of CTLA-4+ mice, which displayed constitutively active src PTKs (lck, fyn, ZAP-70) and Ras pathways, but did not induce the SH2-containing tyrosine phosphatase SHP-2. Hence, SHP-2 was proposed to be the key CTLA-4-coupled regulatory signalling moiety (Marengere et al. 1996).
Table 4  Biochemical signals generated by CD28 ligation

<table>
<thead>
<tr>
<th>Response</th>
<th>mAb</th>
<th>B7.1(CD80)</th>
<th>B7.2(CD86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine phosphorylation *</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[Ca^{2+}]i elevation</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PI3K activation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ras activation</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sphingomyelinase activation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vav phosphorylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p62 phosphorylation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Raf-1 phosphorylation</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>ERK activation</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JNK activation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Legend for Table 4: (+): stimulation, (-): no effect, ND: not determined. * Tyrosine phosphorylation was reported on a number of unidentified substrates. See also Figure 1.9, Figure 1.10. (Data was obtained from a variety of T cell phenotypes i.e. Jurkat cells or T lymphoblasts or resting T cells reviewed in Ward 1996).

However, much about the mode of action of CTLA-4 is still not understood, as Calvo et al. in 1997 determined the inhibitory site of action of CTLA-4 downstream from the level of tyrosine phosphorylation TCR ζ and ZAP-70, but before the subsequent activation of ERK2. Recently, the role of CD28 in T cell costimulation has been the focus of more attention as CD28 antibody conjugated onto beads resulted in an anti-HIV effect, and when combined with CD3 antibody, it caused long term proliferation with uninfected cells outgrowing infected cells (Levine et al. 1996). Figure 1.9 shows a summary of signalling cascades associated with CD28 that integrate with antigenic T cell costimulation.

ROLE OF CD25

IL-2 and its heteromeric receptor was recognised and described as an autocrine growth factor of T cells, the mechanism by which T cells proliferate after antigenic challenge. Upon T cell stimulation in vitro with supplemented IL-2, CD25 receptor expression has been described as heterogeneous and correlated well with the logarithmic distribution of
individual cell cycle times (Cantrell et al. 1984). Apart from driving activated T cell proliferation, IL-2 is known to be capable of co-stimulating T cells in combination to PMA, cause IL-4 production and induce Natural-Killer-like activity from cytotoxic T cells (Heldin and Purton 1996). IL-2 was also portrayed to regulate -C-C- chemokine receptor expression and chemotactic responsiveness on T-cells (Loetscher et al. 1996), as well as rescuing in-vitro T cell apoptosis from HIV-1-infected patients (Adachi et al. 1996).

**CHEMOKINE PRODUCTION BY T CELLS**

Activation of T lymphocytes with CD2 and CD28 mAbs leads to high-level, autocrine IL-2-dependent CD4+ T cell stimulation (Costello et al. 1993). This stimulation also induces secretion of other cytokines, either T cell-specific such as γ-IFN, or those normally synthesised by accessory cells such as TNF-α, CSF-1 and IL-1 (Cerdan et al. 1992; Cerdan et al. 1991). Similar patterns of proliferation and cytokine production have been reported after TCR-CD3/CD28 costimulation (June et al. 1987, Levine et al. 1995). Alongside established cytokines such as IL-2, new genes were identified to be regulated upon T cell stimulation, such as that encoding for RANTES (Schall et al. 1988). Other chemokines were also reported to be produced upon TCR/CD28-driven T cell activation including MIP-α, MIP-1β, as well as IL-8 (Wechsler et al. 1994; Moriuchi et al. 1997; Herold et al. 1997; Riley et al. 1997). The -C-C- chemokines MIP-α, MIP-1β and RANTES were shown to be associated with a Th1-type immune response and T-cell development (Schrum et al. 1996). Overall, T cells were found to produce IL-8, MIP-α, MIP-1β, IP-10, I309 and RANTES (Rollins 1997). However, understanding of RANTES production and its regulation was deemed very important, given the involvement of this molecule to T cell physiology and the various disease states. Moreover, Ortiz et al. in 1996 reported strong up-regulation of RANTES to occur 3-5 days after activation of resting peripheral blood T cells with either mitogen or Ag. The RANTES promoter activity in T cells involved both early-acting and late-acting transcriptional regulatory events which underlined the importance of tight RANTES production regulation, probably involving multiple pathways in the T cell.
Figure 1.9 Schematic summary of signals associated with CD28 ligation that integrate with and modulate the TCR responses.
1.9 The overlapping nature of chemokine and T cell activation signalling

From the multitude of signalling pathways described for T cell activation and chemokine signalling, several distinct families of enzymes appear to participate in both. For example, PI-3 kinase and protein tyrosine kinase phosphorylation cascades in the T-cell have a major role to play in both TCR/CD28-mediated signal transduction and chemokine receptor signalling. Below is a description of such key enzymes:

**PLC(s) AND CALCIUM MOBILISATION**

Phospholipase C exists in multiple isoforms and catalyses the hydrolysis of Phosphatidylinositol(4,5)P2 to Diacylglycerol (DAG) and Inositol trisphosphate (IP3). 10 mammalian PLC enzymes have been identified that are divided based on size and amino-acid sequence. Table 5 contains a summary of the various isozymes and their activation requirements. Chemokine signalling involves the activation of PLCβ whereas T cell costimulation involves the activation of PLCγ (Heldin, Purton 1996). PLC(s) activation results in IP3 which diffuses to its cognate intracellular receptors (IP3Rs) and causes intracellular release of calcium ions from intracellular stores, a process well described (Heldin and Purton 1996). From the formation of IP3 and intracellular
release / extracellular influx of calcium ions, T cell activation is associated with a sustained calcium rise leading to a pathway with activation of calcium “sensors” and multiple protein kinases.

Table 5

<table>
<thead>
<tr>
<th>PLC isoform</th>
<th>Activators</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLCβ1</td>
<td>Most GPCR; Gqα ++++ / Gβγ +</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>PLCβ2</td>
<td>Most GPCR; Gqα+ / Gβγ ++</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>PLCβ3</td>
<td>Most GPCR; Gqα +++ / Gβγ +++</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>PLCβ4</td>
<td>Most GPCR; Gqα++</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>PLCγ1</td>
<td>PAFR; AngIIIR; GFRs; Y-phosphorylation</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>PLCγ2</td>
<td>TCR; GFRs; Y-phosphorylation</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>PLCδ1, PLCδ2, PLCδ3, PLCδ4</td>
<td>?</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
</tbody>
</table>

Legend for Table 4: GPCR : G-protein coupled receptors, GFRs : polypeptide growth factor receptors, PAFR : PAF receptor, AngIIIR : Angiotensin II receptor, TCR : T cell receptor, Y-phosphorylation : Tyrosine residue phosphorylation, ? : unidentified as yet. Crosses (+) indicate affinity for substrate, so (++++) > (+++) > (++) > (+). (Adapted from Heldin and Purton 1996)

An important calcium “sensor”, termed Calcium-calmodulin kinase, is activated upon binding to calcium ions and exerts multiple functions including activation of the serine/threonine protein phosphatase 2B or Calcineurin. Calcineurin then targets NFATp, a transcription factor for IL-2 gene expression, and by de-phosphorylating NFAT, allows it to cross the nuclear membrane and function (Heldin, Purton 1996). Cyclosporin A, a clinically very useful immunosuppressant, inhibits Calcineurin (see Figure 1.8). The calcium ionophore Ionomycin allows for equimolar influx of extracellular calcium ions, and at 1μM mimics the sustained calcium elevation achieved by the combination of IP3-IP3R-CRAC channel opening. Uncoupled to other pathways like protein kinase C (PKC) activation via DAG or DAG-analogues, such [Ca^{2+}]i rise results in cell death (apoptosis) and in some instances anergy (unresponsiveness) (Ward 1996).
THE PKC(s)

The PKC isozymes comprise a family of serine/threonine kinases that are activated by IP-3 induced \([\text{Ca}^{2+}]_i\) rise, DAG (the other PLC product) and/or D-3 phospholipids such as phosphatidylinositol(3,4,5) trisphosphate (PIP\(_3\)). Table 6 denotes the 11 known PKC isoforms and their activation requirements. PKC isoforms are implicated in many signalling cascades including p21\(^{ras}\) and the MAP kinases ERK and c-Jun N-terminal kinases (JNK 1,2) (Heldin, Purton 1996).

Table 6

<table>
<thead>
<tr>
<th>PKC isoforms:</th>
<th>Activated by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha, \beta I, \beta II, \gamma)</td>
<td>DAG or phorbol esters AND calcium ions</td>
</tr>
<tr>
<td>(\delta, \epsilon, \eta, \theta)</td>
<td>DAG, phorbol esters, PIP(_3) (for (\delta, \epsilon, \eta) only)</td>
</tr>
<tr>
<td>(\zeta, \lambda, \iota)</td>
<td>PIP(_3) (for (\zeta) only), ?</td>
</tr>
<tr>
<td>(\mu) (PKD)</td>
<td>DAG, phorbol esters</td>
</tr>
</tbody>
</table>

The presence of specific PKC isozymes is not well understood, but there is evidence of disparate function between isoforms. In T cell systems, PKC\(\theta\) appears to participate in signals for antigenic stimulation and alongside PKC\(\epsilon\), may regulate transcription factors such as AP-1 and NFAT. Phorbol esters activate many forms of PKC, but there are several PKC isoforms which are not regulated by DAG. Moreover, effects of phorbol esters on T cells that were assigned to PKC activation have been in fact a consequence of Ras activation by the phorbol ester (Ward, June 1998). Other isoforms, such as PKC\(\delta\) and PKC\(\beta 1\), were shown to be important mediators of programmed cell death (apoptosis). Other PKC isozymes known to be present in normal T cells are PKC-\(\alpha, -\beta I, -\beta II, -\delta, -\epsilon, -\eta\) and \(-\zeta\), who would all be subject to activation from both TCR-CD3/CD28 costimulation and chemokine signalling, since IP3 and DAG results from any of the different PLC isoforms that are activated. Moreover, there is evidence that both events also engage the activation of the enzyme responsible for PtdIns(3,4,5)P\(_3\) or PIP\(_3\) production, namely phosphatidylinositol 3-kinases (below).

THE PHOSPHATIDYLINOSITOL 3-KINASE(S)

The prototypical class 1\(_A\) PI 3-kinase (PI3K) consists of an 85 kDa regulatory subunit (responsible for protein-protein interactions via SH2 domain interaction with phosphotyrosine residues, and a catalytic 110 kDa subunit (Vanhaesebroeck et al.
The intracellular domain of CD28 encodes a (p)YXXM binding motif that probably associates to p85, the regulatory subunit of PI3K which catalyses the formation of D-3 phospholipids resulting in the induction of cytokine secretion and expression of the cell survival gene Bcl-xl (Ward 1996). This class 1A PI3K is activated by most receptors coupled to tyrosine kinase, such as CD28. Moreover, the presence of SH3 domains on p85α allow for associations with other proteins, denoting an adaptor role for p85 (Figure 1.9). Table 7 displays the various isoforms of PI3K.

A distinct lipid kinase termed PI 3-kinase-γ (PI3Kγ) is activated by G protein-coupled receptors and is the only characterised member of the class 1B G protein-coupled PI3K family, consisting of a unique 101 kDa regulatory subunit and a distinct 110 kDa catalytic subunit termed p110γ (Stephens et al. 1994; Vanhaesebroeck et al. 1997; Stephens et al. 1997). The catalytic subunit of class II PI3K is somewhat larger at 190 kDa and can only phosphorylate PI and PIP but not PIP2, with the exception of the C2α isoform which, in the presence of phosphatidylinerine may also phosphorylate PIP2. The C2 domain, which characterises class II enzymes, was originally identified as a calcium-sensitive phospholipid binding region in mammalian PKC. Adaptor subunits have not been identified as yet for this class of PI3Ks (Ward 1996). Class III PI3Ks comprising of the yeast Vps34p and Vsp15p subunits and the human homologues PtdIns 3-kinase and p150 can only phosphorylate PI, whose cellular levels remain constant following receptor stimulation. It is known to play a role in vesicular traffic of proteins and not in signal transduction (Ward 1996).

The class I PI3Ks can potentially generate three lipid products namely phosphatidylinositol 3-monophosphate [PtdIns(3)P], phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3], which are collectively known as D-3 phosphoinositide lipids (reviewed in Fruman et al. 1998; Toker, Cantley, 1997). Figure 1.10 displays the structure of Phosphatidylinositol and the cycle of generation of D-3-phospholipids. Nevertheless, there is some evidence that G protein-coupled receptors such as fMLP receptors, are also able to activate the p85/p110 PI3K (Stephens et al. 1993; Kurosu et al. 1996). In this respect, the p85/p110 heterodimer has been demonstrated to be synergistically activated by the βγ subunits of G proteins and by phosphotyrosyl peptides (Kurosu et al. 1996). fMLP-induced D-3 phosphoinositide accumulation in
U937 cells, as well as in-vitro lipid kinase activity from 4G10-phosphotyrosine immunoprecipitates (IPs) was shown to be Pertussis-toxin sensitive (Stephens et al. 1993). Also, both the p85/p110 heterodimer and PI3Kγ exhibit dual specificity as both a lipid kinase and a serine protein kinase (Stoyanov et al. 1995; Dhand et al. 1994).

Table 7

<table>
<thead>
<tr>
<th>CLASS</th>
<th>Catalytic Subunit</th>
<th>Substrate specificity</th>
<th>Adaptor / Binding subunit</th>
<th>Structure of the catalytic subunit</th>
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<td>IA</td>
<td>p110α, p110β, p110δ</td>
<td>PI, PIP, PIP₂</td>
<td>p85α, p85β, p55γ/p55PK</td>
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<tr>
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<td>PI, PIP, PIP₂</td>
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<td>PI</td>
<td>Vps15p / p150</td>
<td><img src="structure_III.png" alt="Structure" /></td>
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</table>

**Substrate lipids:**

PI: Phosphatidylinositol (PtdIns), PIP: Phosphatidylinositol(4)monophosphate (PtdIns(4)P), PIP₂: Phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂). At present, both PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ can be regarded as signalling molecules, whereas PtdIns(3)P is thought to regulate membrane trafficking (Fruman et al. 1998; Toker, Cantley, 1997).

In-vitro PI3K activity and function in various cell models has been investigated mainly with the use of pharmacological inhibitors, various molecular approaches or the use of synthetic analogues of D-3 phosphoinositide lipids which mimic (Derman et al. 1997) or interfere with normal PI3K signal propagation. Synthetic analogues were not utilised in
Introduction

this study. However, the other two approaches for PI3K activity investigation were applied in this study and are detailed below:

a) PI3K activity and D-3 phospholipid formation can be inhibited by the drug Wortmannin as well as the unrelated drug LY 294002. These compounds are relatively specific for lipid-kinases and have been used extensively as the only pharmacological tools to dissect PI3K function. Wortmannin is a cell permeable fungal product which inhibits both serine protein and lipid kinase activities by covalent binding to the p110 catalytic subunit with an IC50 of 5nM. However, some limitations regarding the use of Wortmannin include inhibition of Phospholipase A2 with an IC50 of 2nM, PI 4-kinase with an IC50 of 5nM and at high concentrations eg. 500nM other enzymes such as PLC and PKC (Reviewed in Ward 1996). The synthetic and structurally unrelated inhibitor LY294002, is a reversible ATP-binding site competitor which inhibits PI3K with an IC50 of 1.4μM without affecting PI 4-kinase, src PTKs, MAP kinase, S6 kinase, PKA or PKC (Vlahos et al. 1994). Still, LY 294002 and Wortmannin were reported to inhibit the mammalian target of Rapamycin (mTOR) which regulates p70S6K, one PI3K effector (Brunn et al. 1996). Wortmannin has been reported to affect various responses at PI3K selective concentrations, such as neutrophil respiratory burst in response to fMLP, IL-2 production and T cell proliferation from CD28 costimulation, fibroblast actin rearrangements in response to PDGF, β1 integrin upregulation in T cells after CD2 ligation, leukotriene and histamine secretion following FceR1 stimulation, Fcy-induced phagocytosis, and inhibition of iNOS (Reviewed in Ward 1996). Nevertheless, different PI3K isoforms range in sensitivities to Wortmannin. The human class III is most sensitive (IC50: 2.5 nM), followed by class IA (IC50: 5-10 nM), then class IB (IC50: 40nM) and last the class II C2 domain-containing family which is not inhibited by neither Wortmannin nor LY294002 (Ward 1996).

b) Molecular approaches involve transfections, either with plasmids of constitutively active chimeric constructs (catalytic p110 subunits of class IA fused to the extracellular and transmembrane region of rat CD2 (Reif et al.1996)), or dominant negative mutated forms of p85 which lack the p110 binding domain but still compete for phosphotyrosine sites of class IA recruitment (Hara et al. 1994).
Figure 1.11 Diagram of PhosphatidylInositol and the cycle of phosphoinositide generation.

a) Structure of PhosphatidylInositol
Fatty acyl side chains

Inositol head

Legend for Figure 1.10. a) The red positions 3, 4 and 5 on the inositol ring are the positions which receive a phosphate group, but only position 3 phosphorylation occurs via PI 3-kinase(s) by transfer of the terminal phosphate group from ATP. b) The cycle regulating D-3 phosphoinositide synthesis by PI 3-kinase(s) also showing other related enzymes which contribute as indicated.
PI3Ks are now regarded as an important intracellular signal that is upstream of a variety of responses including insulin-stimulated glucose uptake (Shepherd et al. 1998), membrane ruffling (Wennstrom et al. 1994), superoxide production (Arcaro, Wymann, 1993). Moreover, activation of number of downstream signaling proteins is known to be regulated by PI3K and its lipid products including protein kinase B (PKB), p70S6 kinase and Rac (Chung et al. 1994; Hawkins et al. 1995; Burgering, Coffer, 1995). Hence, PI3Ks have been implicated in events ranging from cell survival, DNA synthesis to chemotaxis. Given the functional role of SDF-1 in chemotaxis (Bleul et al. 1996; Nagasawa et al. 1996; Aiuti et al. 1997; Sanchez et al. 1997; Shirozu et al. 1995) it is interesting to note that in T cells, migration to RANTES was abrogated by Wortmannin and was shown to involve tyrosine kinase-coupled p85/p110 PI3K (Turner et al. 1995).

1.9.1 UPSTREAM / PARRALLEL SIGNALLING TO PI3Ks

**Protein Tyrosine Kinases/ Ras**

The Ras genes and proteins were first associated to transformation, tumours and human malignancies. Ras is a small 21 kDa, membrane-bound, GTP-binding protein that acts as a molecular switch linking receptor tyrosine kinase activation to downstream signalling effects. The p21ras is engaged to the TCR signal via PTK activity, the action of adaptor molecules such as Sos, Grb-2, Shc, p36, Crk, Cbl and may also be independently regulated by PKC. The activity of Ras is directly regulated by the opposing activities of guanine nucleotide exchange factors (GEFs) which activate Ras by exchanging GDP for GTP, and GTPase activating proteins (GAPs) which inhibit Ras by accelerating Ras-intrinsic GTPase activity (p120-GAP and NF1) (Vojtek, Der 1998). Non-receptor PTKs such as the src family of enzymes or ZAP-70, that feature in T cell activation signalling, have been also implicated in chemokine signalling and are responsible for recruiting GEFs to Ras by an interplay of tyrosine phosphorylation and domain-specific association events (Heldin, Purton 1996; Vojtek, Der 1998). Although, molecular approaches have been widely used to study constitutively active or dominant negative isoforms of Ras, two types of pharmacological agents have also been utilised and feature in this study. Herbimycin A was reported to be a selective tyrosine kinase inhibitor with an IC50 for c-src of 900nM, which prevented accumulation of active Ras-GTP following IL-3 and GM-CSF treatment on hematopoietic cell lines (Satoh et al. 1992). Herbimycin A has also been reported to block tyrosine kinase mediated events...
such as PDGF-mediated PLD activation (Heldin, Purton 1996). Manumycin A is another agent used, which inhibits the post-translational modification of farnesylation i.e. the attachment of a 15 carbon (farnesyl) isoprenoid on the C-terminal of Ras (Hara et al. 1993). This process is necessary for functional Ras but may not be limited to Ras; other Ras-related proteins not characterised yet may also depend on farnesylation for activity.

**Small GTPases**

There are five families of small GTPases one of which is Ras; the other four are the Rho-, ARF-, Rab- and Ran-families which together have more than 50 members. The Rho GTPase family consists of seven distinct proteins which are: Rho-A, -B, -C, Rac-1, Rac-2, Cdc42Hs, Cdc42G25K, RhoD, -G, -E and TC10. Like Ras-GTP, these small GTPases cycle between inactive GDP- and active GTP-bound states, and are influenced by a wide variety of stimulatory GEFs and inhibitory GAPs (Reviewed recently in Mackay, Hall 1998). The relevant members from the large Ras superfamily group of enzymes which are implicated in chemotaxis and T cell activation are: Rho –A, and –B regulate stress fibre formation and microfilament networks, and have a role in cell transformation; in particular RhoB plays a role in the G1 to S and/or the S phase of the cell cycle. Cdc42 is thought to be a fundamental component of cytoskeletal rearrangements in response to growth factors, morphogenesis and cycle progression in eukaryotic cells. Rac-1 and -2 regulate lamellipodia formation, membrane ruffling, cytoskeletal actin polymerisation, activation of NADPH-oxidase and of the JNK1/2 cascade. ARFs have been implicated in intracellular vesicular traffic, and (apart from Ral1) PLD activation (Reviewed in Moss, Vaughan 1998).

**Phospholipase D**

Phospholipase D has been implicated to be activated following both G-protein-PLC and PTK activation, and there is evidence for PKC-dependent and PKC-independent activation. Small GTPases are also thought to participate in PLD activation with the Rho-family regulating activity of the membrane-associated PLD, and the ARF-family regulating a cytosolic isoform of PLD. However, presence of PtdIns(4,5)P$_2$ was reported to be essential for stimulation of either PLD isoform (Heldin, Purton 1996). In this study, Brefeldin A which is a specific inhibitor of protein translocation from the ER to the Golgi apparatus, was used to inhibit post-translational modification of small GTPases and PLD (Bacon et al. 1998).
Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPKs) are a group of serine/threonine protein kinases which are proline directed and activated by a wide spectrum of extracellular stimuli. They comprise of many components, essentially of 3 types of regulatory kinase cascades, which serve as information relays, connecting and amplifying cell surface receptor or other type of extracellular signals to specific transcription factors that stimulate expression of specific genes and/or other regulatory proteins that influence key signalling events. The complexity in the number of key enzymes and the “cross-talk” communication between the 3 main cascades has been described extensively (Su et al. 1996, Garrington et al. 1999), and an overview of the signalling MAPK pathway structure, is best illustrated with a diagram in Figure 1.11. Selective pharmacological inhibition used in this study for the ERK1/2 pathway was achieved with the drug PD 98059, whereas the p38/stress pathway was inhibited by the drug SB 203580 (Gould et al. 1995). The SAPK pathway targets c-Jun, ATF2 and Elk1 and no selective inhibitor has been identified as yet (Ward 1996, Su et al. 1996). Convergence of PI3K/Ras signalling and PKC signalling for MAPK activation at the level of MEK has been proposed, since fMLP-induced activation of Ras/Raf-1/MEKK cascade was more potently inhibited by Wortmannin than downstream activation of MEK/MAPK, whereas PKC inhibition did not affect Ras/Raf-1/MEKK and partially inhibited MEK/MAPK activation (Avdi et al. 1996). Wortmannin also blocked IL-8-induced Raf-1/MAPK activation in neutrophils (Knall et al. 1996).

Protein Kinase B

Protein kinase B is serine/threonine protein kinase with a special role in cell survival and a prime effector for PI3K(s). A 60 kDa protein, it contains a PH domain and is activated by growth factor receptor stimulation, whereas it is inhibited with dominant negative p85 mutants and Wortmannin (Coffer et al. 1998). The activation of PKB is thought to entail a two step phosphorylation: firstly a PtdIns(3,4)P2 and PtdIns(3,4,5)P3-mediated co-localisation of PKB with a kinase (via their PH domains), which then phosphorylates PKB on Threonine308 within the catalytic domain; this event ‘unfolds’ PKB rendering it susceptible to a second Serine 473 phosphorylation in the C-terminal regulatory domain, necessary for full activation of PKB. The first kinase has been cloned and termed PtdIns(3,4,5)P3-dependent kinase 1 (PDK1), whereas the second-PDK2- was
provisionally termed so, as it was not cloned or purified (Allessi et al. 1997). Recently however, it is believed that PDK2 is a modified version of PDK1 forming a complex with the PDK1-Interacting –Fragment (PIF), which is either proteolytically cleaved from or still attached to PRK2 (Vanhaesebroeck, Allessi 2000). Alternative pathways also exist to activate PKB, as it is responsive to osmotic stress and heat shock, stimuli which activate the p38 MAPK cascade (Coffer et al. 1998). After dual phosphorylation and full activation at the plasma membrane, PKB translocates to the cytosol and phosphorylates a number of substrates, such as: a) in Insulin signal transduction i.e glycogen synthase kinase 3 (GSK-3), phosphodiesterase 3B (PDE-3B), mammalian target of Rapamycin (mTOR), Insulin-receptor substrate-1 (IRS-1), b) in anti-apoptotic signalling i.e. inhibition of Caspase-9, inhibition of BAD (member of the Bcl-2 family which inactivates the cell survival factors Bcl-xL and Bcl-2), inhibition of Forkhead transcription factors (responsible for Fas ligand expression) and stimulation of IκB kinase and subsequent NF-κB activation, c) in antagonism of MAPK activation by inhibition of Raf-1, d) in tumour development i.e. activation of endothelial Nitric oxide synthase (eNOS) and phosphorylation of the Breast-Cancer-Susceptibility-Gene-1 product (BRCA1) (Coffer et al. 1998; Vanhaesebroeck, Allessi 2000). It should be stressed however, that the physiological mode of PKB activation, its full downstream target repertoire and its role in cellular pathophysiology is still a contentious issue.

p70 S6 Kinase
This kinase is a ubiquitous, mitogen activated serine/threonine kinase which is phosphorylated on multiple sites upon stimulation and in turn phosphorylates the S6 protein on the 40S ribosomal subunit, enhancing mRNA translation. p70 S6 kinase is necessary for G1 to S phase cycle progression and its activity can be inhibited by the immunosuppressant Rapamycin which blocks its phosphorylation. The upstream regulatory component of p70S6 kinase has been termed mammalian target of Rapamycin (mTOR) or FK-506-binding-protein Rapamycin Associated Protein (FRAP), whose interaction with Rapamycin inhibits subsequent activation of p70S6 kinase (Heldin, Purton 1996). The mechanism of p70 S6K activation is not well defined to date, however it is understood that p70S6 kinase activation does depend in part on PtdIns(3,4,5)P3 (reviewed in Pullen and Thomas 1997), as active PKB was subsequently reported to directly phosphorylate mTOR (Nave et al. 1999), whereas PDK1 was reported to phosphorylate p70 S6K involving intermediate PI3K-dependent signalling complexes with PKC ζ (Romanelli et al. 1999).
Figure 1.11 A schematic diagram of the three main MAPK cascades.
Legend for Figure 1.12 MKKKKs: germinal centre kinase (GCK), GCK-like kinase (GLK), mammalian Ste-20-like protein kinase (MST)1. MKKKs: MAPK/ERK kinase kinase (MEKK), tumor progression locus (Tpl)2, dual leucine-zipper bearing kinase (DLK), apoptosis signal-regulating kinase (ASK)1, TGFβ-activated protein kinase (TAK)1, mixed lineage kinase (MLK)3, p21-activated kinase (PAK). MKKs: MAPK/ERK kinase (MEK), MAPKs: extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), stress-activated MAPK (p38). Transcription factors phosphorylated by the MAPKs: ATF-2, Chop, c-Jun, c-Myc, DPC4, Elk-1, Max, MEF2C, NFAT4, Sap 1a, STATS, Tal, p53. Regulatory proteins phosphorylated by the MAPKs: ribosomal p90rsk S6 kinase (p90rsk S6K), Epidermal growth factor (EGF) receptor, SOS (the Ras-GEF), phospholipase A2 (PLA2), MAPK-activated protein kinase (MAPKAPK). The sites of inhibition by the two drugs SB203580 and PD 98059 are indicated by the inhibitory arrows. Single-headed arrows indicate activation/phosphorylation. (?) indicate unidentified signalling components or unknown associations. The diagram encircling PKC, PI 3-kinase and PLC has multiple spikes and a question-mark to indicate that although certain links have been demonstrated (eg. between PKC and Ras, PI 3-kinase and Rac1), the overall integration of proximal signalling events to the possible downstream targets is not fully elucidated to date. Adapted from Su et al. 1996; Garrington et al. 1999.

1.10 Aim of study

β–CHEMOKINE SECRETION IN RESTING AND ACTIVATED T CELLS

The aim is to analyse RANTES secretion from purified human T cells and the effect of distinct T cell activating conditions which provide pharmacologically distinct biochemical signals for T cell proliferation and IL-2 production. Subsequently, to determine the sensitivity of stimulated RANTES secretion to pharmacological inhibition or CTLA-4 ligation and to determine if the requirements for RANTES secretion are common or disparate to those required for T cell proliferation, as well as other related chemokine secretion e.g. MIP-1α.

CXCR4-DERIVED SIGNALS ON T CELLS

The aim is to characterise signal generation following CXCR4 ligation, such as intracellular calcium mobilisation, PI3K activation and protein phosphorylation. Furthermore, to assess the involvement of different PI3K isoforms in CXCR4 signalling and the activation of downstream PI3K effectors such as PKB and MAPK. Finally, to examine functional responses to CXCR4 activation such as receptor internalisation, actin polymerisation, chemotaxis, transcription factor activation and programmed T cell death, and to assign with pharmacological or molecular approaches the signalling pathways that mediate the various CXCR4-ligation responses.
CHEMOKINE ROLE IN T CELL ACTIVATION AND PROLIFERATION

The aim is to establish submaximal responses with distinct T cell activating conditions for proliferation, so that both augmentation and inhibition of the response can be monitored. Subsequently, to examine the effect of exogenous chemokine, or neutralising chemokine antibody addition on submaximal proliferation of purified human T cells. Chemokine receptors have been implicated in T cell phenotypes and responses. Also the aim is to identify purified, resting T cell chemokine receptor expression, in the level of mRNA by PCR analysis and on the cell surface by antibody staining and FACS analysis, where possible. Moreover, to relate chemokine receptor expression with resting or activated T cell phenotypes, and examine the effect of activating signals required for proliferation or RANTES secretion on receptor expression.
### MATERIALS and METHODS

#### 2.1 Materials

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### Materials & Methods

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<td>ATP stock 100mM aq. (−20°C), dissolved in 100mM Tris pH 7.4)</td>
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<tr>
<td>BSA tissue culture grade</td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td></td>
</tr>
<tr>
<td>β–glycerophosphate</td>
<td></td>
</tr>
<tr>
<td>Calcium-free HBSS</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Cell Titer 96 &lt;sup&gt;Aqueous&lt;/sup&gt; one solution reagent</td>
<td>Promega, WI, USA</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Fisons, Loughborough, UK</td>
</tr>
<tr>
<td>Coomassie blue</td>
<td>Sigma, Poole, UK (to dissolve &gt;4h mix in 40% MeOH / 7% gAA / 53% dH₂O)</td>
</tr>
<tr>
<td>Deoxynucleoside triphosphates : dATP, dCTP, dGTP and dTTP</td>
<td>Boehringer Mannheim, Germany</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate (DEPC)</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Digitonin</td>
<td>BDH, Poole, UK</td>
</tr>
<tr>
<td>DMSO</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Dulbeccos’ modified essential medium (DMEM)</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Enhanced Chemiluminescence Reagent</td>
<td>Amersham, Little Chalfont, UK</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>EtOH</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Ethyl formate</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Flo-scint scintillation liquid</td>
<td>Canberra Packard, UK</td>
</tr>
<tr>
<td>MEDIA AND REAGENTS – contd.</td>
<td>Origin/notes</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Foetal Calf Serum Heat Inactivated (FCS)</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Folsch Lipids from Bovine Brain</td>
<td>Sigma, Poole, UK / stored at -20°C</td>
</tr>
<tr>
<td>Fura-2 acetoxyethyl ester (Fura-2AM)</td>
<td>Molecular Probes, Eugene, OR, USA</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Sigma, Poole, UK / Boiled to dissolve in PBS</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>HEPES 1 M buffer solution sterile</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Histone 2B</td>
<td>Boehringer Mannheim, Germany / Stock 1mg/ml in Tris pH 7.5 stored at -20°C</td>
</tr>
<tr>
<td>HCl</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt; 30% v/v aqueous</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Iodine</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>IPTG</td>
<td>Sigma, Poole, UK / Stock 1M in H&lt;sub&gt;2&lt;/sub&gt;O stored at -20°C</td>
</tr>
<tr>
<td>Ladder 15 blunt end fragments 100 base pair (100-1500bp scale)</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Lauryl sulphate (SDS)</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>Leupeptin</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>LiCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Sigma, Poole, Uk / Dissolved in 100mM Tris, 1mM EDTA pH 7.4</td>
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<tr>
<td>L-phosphatidylinositol</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>L-phosphatidylserine</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>Lymphoprep™ (density 1.077)</td>
<td>Nycomed, Birmingham, UK</td>
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<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Anachem, Luton, UK</td>
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<td>2-Mercaptoethanol</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>MeOH</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Methylamine 25% w/v aqueous</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Microscint-O™ scintillation liquid</td>
<td>Canberra Packard, UK</td>
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<td>N-butanol</td>
<td>Fisher Scientific, UK</td>
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<tr>
<td>NaF</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>NP-40 1%</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>1,2 O-Phenylenediamine dihydrochloride</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Orthophosphoric acid</td>
<td>Fisons, Loughborough, UK</td>
</tr>
<tr>
<td>MEDIA AND REAGENTS-contd.</td>
<td>Origin/notes</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------</td>
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<tr>
<td>Paraformaldehyde</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Petroleum ether 40-60%</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Phalloidin-FITC</td>
<td>Sigma, Poole, UK / Stock 0.3mM in PBS stored at -20°C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Sigma, Poole, UK / Stock 100mM in acetone stored at -20°C</td>
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<tr>
<td>Phosphate Buffered Saline (PBS) without Ca²⁺ and Mg²⁺ ions</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Potassium oxalate</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Pd(N)_6 random nucleotide hexamers</td>
<td>Boehringer Mannheim, Germany</td>
</tr>
<tr>
<td>Propan-1-ol</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Protogel acrylamide/ bis acrylamide (30:0.8)</td>
<td>Flowgen, Kent, UK</td>
</tr>
<tr>
<td>PKI-protein kinase inhibitor</td>
<td>Sigma, Poole, UK / Stock 5 μM in PBS 0.05 % BSA stored at -20°C</td>
</tr>
<tr>
<td>RNAsin -a non-competitive Ribonuclease Inhibitor 49.8Kd recombinant E.coli</td>
<td>Promega, WI, USA</td>
</tr>
<tr>
<td>RPMI 1640 culture medium</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Sodium bicarbonate 7.5% aqueous sterile</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>Sodium Orthovanadate</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>Sodium sulphite</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>Streptavidin peroxidase</td>
<td>Sigma, Poole, UK</td>
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<td>Tetrabutyl ammoniumhydrogen sulphate</td>
<td>Sigma, Poole, UK</td>
</tr>
<tr>
<td>TEMED</td>
<td>Sigma, Poole, UK</td>
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<td>Triton X-100</td>
<td>Fisher Scientific, UK</td>
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<td>Trypan Blue</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>Tween 20</td>
<td>Sigma, Poole, UK</td>
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<tr>
<td>Versene</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>Sigma, Poole, UK</td>
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### Materials & Methods

#### RADIOCHEMICALS AND PLASMIDS

<table>
<thead>
<tr>
<th>Origin/notes</th>
<th>Material/Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEN DuPont, Stevenage, UK / Aliquots stored at -20°C</td>
<td>[$\gamma$-³²P] labelled ATP (5 µCi/ml, 3000 Ci/mmol, in aqueous solution)</td>
</tr>
<tr>
<td>NEN DuPont, Stevenage, UK / Room temperature</td>
<td>[³²P] labelled orthophosphate (³²Pi) (5 mCi/ml, 8500-9120 Ci/mmol)</td>
</tr>
<tr>
<td>Amersham, Little Chalfont, UK</td>
<td>[³H]-Thymidine (1 mCi/ml, 2 Ci/mmol)</td>
</tr>
<tr>
<td>Kind gifts from Dr. G. Boulougouris, Bath Institute for Rheumatic Diseases, Bath / Stock 1mg/ml in H₂O at 4°C</td>
<td></td>
</tr>
<tr>
<td>Kind gift from Dr. D. Cantrell, ICRF, London, UK / pEF-BOS vector</td>
<td></td>
</tr>
<tr>
<td>Kind gift from Dr. D. Cantrell, ICRF, London, UK / point mutation in kinase domain R1130P, pEF-BOS vector</td>
<td></td>
</tr>
<tr>
<td>Kind gift from Dr. B. Burgering, Utrecht The Netherlands / pEF-BOS vector</td>
<td></td>
</tr>
</tbody>
</table>

#### CHEMOKINES, CYTOKINES, DRUGS

<table>
<thead>
<tr>
<th>Origin/notes</th>
<th>Material/Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIBCO BRL, Paisley, UK</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>Calbiochem-Novabiochem, UK / Stock 4mg/ml in MeOH stored at -20°C</td>
<td>Brefeldin-A</td>
</tr>
<tr>
<td>GIBCO BRL, Paisley, UK</td>
<td>Geneticin G418</td>
</tr>
<tr>
<td>Calbiochem-Novabiochem, UK / Stock 2mg/ml in DMSO stored at -70°C</td>
<td>Herbimycin-A</td>
</tr>
<tr>
<td>GIBCO BRL, Paisley, UK</td>
<td>Hygromycin B</td>
</tr>
<tr>
<td>Stock 5mg/ml in PBS stored at 4°C</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>Stock 5mg/ml in EtOH stored at -20°C</td>
<td>Fragment peptide monomer (1-9) NH₂-SDF-1</td>
</tr>
<tr>
<td>Kind gift from Dr. Ian C. Lewis, Biomedical Research Centre, Vancouver, Canada / Stock at 4mM with 0.1% BSA stored at 4°C</td>
<td>Fragment peptide dimer (1-9)-(9-1) NH₂-SDF-1</td>
</tr>
<tr>
<td>Fragment peptide dimer (1-9)-(9-1) (P2G) NH₂-SDF-1</td>
<td>Fragment peptide dimer (1-9)-(9-1) (P2G) NH₂-SDF-1</td>
</tr>
<tr>
<td>Fragment peptide dimer (1-9)-(9-1) (P2G) NH₂-SDF-1</td>
<td>gp120 coat from HIV-1 T-tropic IIIB</td>
</tr>
<tr>
<td>Donated from the MRC AIDS reagent project, NIIB, UK / Stock at 1mg/ml with 0.1% BSA stored at -70°C</td>
<td>gp120 coat from HIV-1 T-tropic MN</td>
</tr>
<tr>
<td>gp120 coat from HIV-1 T-tropic SF-2</td>
<td>gp120 coat from HIV-1 T-tropic SF-2</td>
</tr>
<tr>
<td>Heparin, 5000 U/ml Monoparin</td>
<td>Heparin, 5000 U/ml Monoparin</td>
</tr>
<tr>
<td>CP Pharmaceuticals Ltd, Wrexham, UK</td>
<td>hrIL-8</td>
</tr>
<tr>
<td>Sandoz, Basel, Switzerland / Diluted in presence of 0.1% BSA stored at -20°C</td>
<td>hrIL-2</td>
</tr>
<tr>
<td>Sandoz, Vienna, Austria / Stock 20µg/ml in 0.1 % BSA stored at -70°C</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>Calbiochem-Novabiochem, UK</td>
<td>hrIP-10</td>
</tr>
<tr>
<td>Peprotech, Rocky Hill, NJ, USA / Diluted in presence of 0.1% BSA stored at -20°C</td>
<td>LY 294003</td>
</tr>
<tr>
<td>Calbiochem-Novabiochem, UK / Stock 30mM in DMSO stored at -70°C</td>
<td></td>
</tr>
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</table>
### CHEMOKINES, CYTOKINES, DRUGS - contd.  Origin/notes

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Origin/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manumycin-A</td>
<td>Calbiochem-Novabiochem, UK / Stock 10 mM in DMSO stored at -20°C</td>
</tr>
<tr>
<td>hrMCP-1</td>
<td>Peprotech, Rocky Hill, NJ, USA / Diluted in presence of 0.1% BSA stored at -20°C</td>
</tr>
<tr>
<td>hrMIG</td>
<td></td>
</tr>
<tr>
<td>hrMIP-1α</td>
<td></td>
</tr>
<tr>
<td>PD 098059</td>
<td>gift from Parke-Davies, Hants, UK / Stock 10 mM in DMSO stored at -20°C</td>
</tr>
<tr>
<td>Penicillin and Streptomycin</td>
<td>GIBCO BRL, Paisley, UK</td>
</tr>
<tr>
<td>Pertussis Toxin</td>
<td>Sigma, Poole, UK / Stock 100 μg/ml in PBS stored at 4°C</td>
</tr>
<tr>
<td>Phorbol 13-Myristate12-Acetate</td>
<td>Calbiochem-Novabiochem, UK / Stock 5 mM in DMSO stored at -20°C</td>
</tr>
<tr>
<td>Phytohaemagglutinin-A</td>
<td>Fluka, Dorset, UK / Stock 5 mg/ml in 0.1% BSA stored at -20°C</td>
</tr>
<tr>
<td>4α-Phorbol ester</td>
<td>Calbiochem-Novabiochem, UK / Stock 5 mM in DMSO stored at -20°C</td>
</tr>
<tr>
<td>hrRANTES</td>
<td>Peprotech, Rocky Hill, NJ, USA / Diluted in presence of 0.1% BSA stored at -20°C</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Boehringer Mannheim, Sussex, UK / Stock 1 mg/ml in EtOH stored at -20°C</td>
</tr>
<tr>
<td>RO320432</td>
<td>Calbiochem-Novabiochem, UK / Stock 10 mM in DMSO stored at 4°C</td>
</tr>
<tr>
<td>RO 318220</td>
<td>Calbiochem-Novabiochem, UK / Stock 10 mM in DMSO stored at 4°C</td>
</tr>
<tr>
<td>SB 203580</td>
<td>gift from SmithKline Beecham, UK / Stock 10 mM in DMSO stored at -20°C</td>
</tr>
<tr>
<td>hrSDF-1α</td>
<td>Gryphon, Palo Alto, U.S.A / Dilute in presence of 0.1% BSA stored at -20°C</td>
</tr>
<tr>
<td>Staphylococcal Enterotoxin B</td>
<td>Fluka, Dorset, UK / Stock 1 mg/ml in 0.1% BSA stored at -20°C</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Sigma, Poole, UK / Stock 10 mM in DMSO stored at -70°C</td>
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</tbody>
</table>

### ANTIBODIES, BEADS AND CONJUGATES  Origin/notes

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Origin/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-hCD3 (clone UCHT-1)</td>
<td>Gift from Dr. Doreen Cantrell, ICRF, London, UK</td>
</tr>
<tr>
<td>Mouse anti-hCD28 (clone 9.3)</td>
<td>Gift from Dr. Carl June, NMRI, Bethesda USA</td>
</tr>
<tr>
<td>Mouse anti-hCD2 x 2 (clones 6F103 and 39Ci5)</td>
<td>Gift from Daniel Olive, INSRM, Marseille, France / Used cross-linked in equimolar combination</td>
</tr>
<tr>
<td>Mouse anti-hCTLA-4 (3D6)</td>
<td>Gift from Dr. Carl June, NMRI, Bethesda USA</td>
</tr>
<tr>
<td>Goat anti-mouse IgG, Fab’ fragment specific</td>
<td>Sigma Immunochemicals, Poole, UK / For cross-linking soluble αCD3, αCD2, αCD28 and αCTLA-4 abs for T-cell stimulation</td>
</tr>
<tr>
<td>ANTIBODIES, BEADS AND CONJUGATES-contd.</td>
<td>Origin/notes</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tosylactivated magnetic beads M-450, 4.5µm diameter</td>
<td>DYNAL AS, Oslo, Norway / Coated with combinations of αCD3, αCD2, αCD28 and αCTLA-4 abs for T-cell stimulation</td>
</tr>
<tr>
<td>Mouse anti-hCD19 (clone HD 37)</td>
<td>DAKO AS, Denmark / Used in T-cell purification with L243 ab and anti IgG coated M450-beads</td>
</tr>
<tr>
<td>Mouse anti-hCD14 (clone UCHM1)</td>
<td>Purified from murine hybridomas, and kindly donated by the Bath Institute for Rheumatic Diseases, Bath</td>
</tr>
<tr>
<td>Mouse anti-hMHC-II D-R (L243)</td>
<td>DYNAL Merseyside, UK / Used in T-cell purification-negative selection step</td>
</tr>
<tr>
<td>Mouse anti-hCD25 α-chain FITC conjugated (clone ACT-1)</td>
<td>DAKO AS, Denmark / Used for FACS analysis of IL-2 receptor expression</td>
</tr>
<tr>
<td>Goat anti-mouse IgG -FITC conjugated</td>
<td>DAKO AS, Denmark / Used as secondary in FACS analysis</td>
</tr>
<tr>
<td>Mouse anti-hCD69</td>
<td>Kindly donated by the Bath Institute for Rheumatic Diseases, Bath / for FACS</td>
</tr>
<tr>
<td>Mouse anti-rat CD2 (clone OX-34)</td>
<td>DAKO AS, Denmark / Used as primary antibodies in FACS analysis. IgG1 and IgG2a as negative controls for FACS and other assays</td>
</tr>
<tr>
<td>Mouse anti-humanCD4 (clone MT-310)</td>
<td>R&amp;D, Abingdon, UK / Used in chemokine neutralisation assays for proliferation assays. Anti-hRANTES and anti-hMIP-1α also used as coating abs in ELISA assays</td>
</tr>
<tr>
<td>Mouse anti-humanCD8 (clone DK-25)</td>
<td>R&amp;D, Abingdon, UK / Used as detecting antibody in ELISA assays</td>
</tr>
<tr>
<td>Mouse IgG1 isotype</td>
<td>Upstate biotechnology, TCS Biologicals, Bucks, UK / Apoptosis inducer</td>
</tr>
<tr>
<td>Mouse IgG2a isotype</td>
<td>DAKO AS, Denmark / Used as negative control in apoptosis assays</td>
</tr>
<tr>
<td>Mouse anti-hCXCR4 (clone 12G5)</td>
<td>R&amp;D, Abingdon, UK / Used as detecting antibody in ELISA assays</td>
</tr>
<tr>
<td>Mouse anti-hCCR5 (clone 2D7)</td>
<td>R&amp;D, Abingdon, UK / Used as detecting antibody in ELISA assays</td>
</tr>
<tr>
<td>Mouse anti-hCCR3 (7B11)</td>
<td>Upstate biotechnology, TCS Biologicals, Bucks, UK / Apoptosis inducer</td>
</tr>
<tr>
<td>Mouse anti-hIL-8</td>
<td>DAKO AS, Denmark / Used as negative control in apoptosis assays</td>
</tr>
<tr>
<td>Mouse anti-hMCP-1</td>
<td>Upstate biotechnology, NY, USA / Used for immunoprecipitation and as primary antibodies in Western blotting</td>
</tr>
<tr>
<td>Mouse anti-hMIP-1α</td>
<td>Santa Cruz Biotechnologies, USA</td>
</tr>
<tr>
<td>Mouse anti-FAS IgM (clone CH11)</td>
<td>Gift from Dr D. Cantrell ICRF, London UK / Used for immunoprecipitation</td>
</tr>
<tr>
<td>Mouse IgM Isotype control</td>
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</table>
### Materials & Methods

**ANTIBODIES, BEADS AND CONJUGATES-contd.** Origin/notes

<table>
<thead>
<tr>
<th>Antibodies, Beads and Conjugates</th>
<th>Origin/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione-sepharose/Ras-binding-domain from Raf-1 fusion protein (GST-RBD)</td>
<td>Gift from Dr D. Cantrell ICRF, London UK / Used for immunoprecipitation of active Ras</td>
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<tr>
<td>Anti-Myc</td>
<td>Purified and kindly donated by Dr M. J. Welham, Bath, UK / Used as primary for Western blotting</td>
</tr>
<tr>
<td>Mouse anti-Ras (Ab-4)</td>
<td>Calbiochem, Oncogene research products, USA / Used as primary for Western blotting</td>
</tr>
<tr>
<td>Rabbit anti-goat IgG-HRP</td>
<td>DAKO AS, Denmark / Used as secondaries with ECL for Western blot visualisation</td>
</tr>
<tr>
<td>Goat anti-mouse IgG-HRP</td>
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</tr>
<tr>
<td>Rabbit anti-sheep IgG-HRP</td>
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<tr>
<td>Goat anti-rabbit IgG-HRP</td>
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</tr>
<tr>
<td>Protein A sepharose CL4B</td>
<td>Pharmacia, UK / Prepared according to manufacturers instruction and stored sterile at 4°C</td>
</tr>
<tr>
<td>Protein G sepharose beads</td>
<td>Sigma, Poole, UK</td>
</tr>
</tbody>
</table>

**KIT ASSAYS** Origin/notes

<table>
<thead>
<tr>
<th>Kit Assays</th>
<th>Origin/Notes</th>
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<tbody>
<tr>
<td>MACS Pan T-cell™ isolation kit</td>
<td>Miltenyl Biotech, UK</td>
</tr>
<tr>
<td>QuickPrep™ Micro mRNA Purification Kit</td>
<td>Pharmacia Biotech, Herts, UK</td>
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<tr>
<td>Expand™ High Fidelity PCR system</td>
<td>Boehringer Mannheim, Germany</td>
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<tr>
<td>PhosphoPlus™ (Ser473) Akt / PKB antibody detection kit</td>
<td>New England Biolabs, USA</td>
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<tr>
<td>PhosphoPlus™ p44/42 MAP Kinase (Thr202/Tyr204) antibody kit</td>
<td>New England Biolabs, USA</td>
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<tr>
<td>Annexin-V Fluos™ apoptosis detection kit</td>
<td>Boehringer Mannheim, Germany</td>
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<tr>
<td>LucLite™ Bioluminescent Reagent System for Reporter Gene Assays</td>
<td>Canberra Packard, UK</td>
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### 2.2 Methods

#### 2.2.1 CELL CULTURES

All cells were cultured at a constant 37°C in a humidified atmosphere of air supplemented with 5% CO₂. For long term storage, cell lines were frozen under liquid nitrogen. Cells were pelleted (400g, 10min), resuspended at 10⁷ cells/ml in 20% FCS / 10% DMSO and aliquoted into freezing vials. Vials were then lowered into liquid nitrogen, over a number of hours, using a Handi-Freeze freezing tray (Taylor-Warton).
When necessary, cells were thawed rapidly in a water bath (37°C), washed twice in fresh medium and returned to culture. Cell lines and T-lymphoblasts were passaged appropriately and maintained between 0.5-1.5 x10^6 / ml. Cell cultures took place in volumes of 50ml medium for 80cm^2 flasks and 100-150ml medium for 175cm^2 flasks at all times.

**Ex-vivo T-cells**

Purified CD3^+ T-lymphocytes and *in-vitro* activated T-lymphoblasts derived from PBMCs of healthy donors were stimulated or cultured in complete medium: RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 µg/ml Streptomycin and 500 ng/ml Amphotericin-B.

**Jurkat cells**

J6CD2^+ , JCam and TCR-deficient J6 (JTcR') subclones of the leukaemic T cell line Jurkat were a kind gift from Dr. A. Alcover, INSERM, Paris and were grown in RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 µg/ml Streptomycin and 500 ng/ml Amphotericin-B. JCam cells were lck deficient, whereas JTcR' cells were deficient in T cell receptor expression.

**U937 cells**

U937 cells were kindly donated by ECACC, UK and were cultured in RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 µg/ml Streptomycin and 500 ng/ml Amphotericin-B.

**U937 cells transfected with ∆p85**

U937 cells transfected with Myc-tagged, ∆p85 (a mutated p85 adaptor subunit of type 1A PI3K, that lacked the p110 binding region) were obtained from Dr. Phil Hawkins, Babraham Institute, UK and were cultured in RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 µg/ml Streptomycin and 500 ng/ml Amphotericin-B. The media were supplemented every 2-3 days with 0.6 mg/ml G418 and 0.1 mg/ml Hygromycin B, so that transfected / resistant populations remained pure in culture. Expression of the dominant negative Myc-∆p85, was induced after overnight incubation with 5nM PMA, 100µM ZnCl_2 and 15 mM IPTG. Verification of expression was obtained with Western blots probing for Myc (See Figure 4.22A).
Chinese Hamster Ovary (CHO) cells

CHO cells (ECACC, UK) were transfected with the cDNA for CD80 (CHO-B7.1) as previously described (Sansom et al. 1993), and kindly donated by Dr. D. Sansom, Bath Institute for Rheumatic Diseases, Bath. Providing a sustainable and replenishable supply of CD28 ligand, CHO-CD80⁺ cells as well as parental untransfected CHO cells were grown in DMEM with 10% v/v FCS, 50 U/ml Penicillin/50 μg/ml Streptomycin, 500 ng/ml Amphotericin-B, 25 mM HEPES, 0.375% (w/v) NaHCO₃, and the nucleosides adenosine, cytidine, uridine, thymidine and guanosine all at 20 μM. When confluent, these cells were passaged by trypsinisation. Briefly, medium was removed from the flasks and adherent cells washed twice in 10ml calcium-free PBS, pH 7.3. Cells were incubated at 37°C for five minutes in the presence of trypsin-EDTA. Trypsinisation was terminated by the addition 20 ml of fresh culture medium. Cells were pelleted at 400g for 5 min and passaged routinely 1:5. Where appropriate CHO / CHO-CD80⁺ cells were fixed after trypsinisation and washing in calcium free PBS with glutaraldehyde. Cells were re-suspended in 2ml calcium free PBS and very gently shaken with another 2ml of 0.05 % w/v glutaraldehyde in calcium free PBS for exactly 2 minutes. After three washes to remove excess glutaraldehyde the cells were fixed and ready for use.

2.2.2 PBMC SEPARATION AND T-CELL PURIFICATION

PBMC isolation

Blood from healthy donors was taken aseptically in 50ml syringes containing heparin 10 U/ml of blood, via 19-gauge ‘butterfly’ needles. The blood was diluted 1:1 with RPMI 1640 culture medium and 35ml aliquots of the mix were carefully layered on to 15ml Lymphoprep™ in 50ml transparent centrifuge tubes and centrifuged brake-free for 30min at 400g. The monocyte/lymphocyte band was carefully removed, washed three times in RPMI (350g, 10min, 20 °C), and counted with a x200 microscope and a Neubauer haemocytometer.

T-cell purification with DYNAL beads

Purified T cells were obtained by negative selection as described previously (Sansom et al. 1993), with some modifications. For T- purification using DYNAL beads and magnet separator, the PBMCs obtained were re-suspended at 1x10⁶ cells/ml in RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 μg/ml Streptomycin and 500 ng/ml
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Amphotericin-B. Adherent cells were removed by plastic adherence on tissue culture petri dishes or 175cm² flasks incubated for 1 hour. The non-adherent cells were pelleted and re-suspended in 15ml centrifuge tubes with 1ml RPMI-1640 for PBMCs derived from 100ml blood, and rotated at 4°C for 45min with the following murine anti-human antibodies at 1 µg/ml: monoclonal αCD19- (B-lymphocyte marker), αCD14- (monocyte and tissue macrophage marker), and αMHC-II D-R- clone L243 (antigen presenting cell marker). The tagged cells were then washed twice and re-suspended in 2ml RPMI-1640 with 2-4x10⁶ /ml magnetic DYNAL beads M-450, 4.5µm diameter, coated with sheep anti-mouse IgG antibodies. After 45min rotation at 4°C, the cells were diluted with 12ml RPMI and passed over a magnetic DYNAL bed three times for 1 minute. Cells were counted and viability was assessed with Trypan Blue exclusion. T-lymphocyte purity was assessed regularly by FACS analysis (See Figure 3.1).

T-cell purification with the MACS system

Later experiments used MACS system to purify cells, since this method was quicker and more efficient. MACS columns, a MACS magnet separator and the MACS pan T-cell isolation kit containing a cocktail of hapten-conjugated anti CD11b, CD16, CD19, CD36 and CD56 antibodies and MACS Microbeads coupled to anti-hapten monoclonal antibodies were used according to manufacturer’s specifications. Briefly, after separation from the Lymphoprep™, the PBMCs were washed three times at 350g, 10min, 20 °C in calcium-free PBS with 2mM EDTA at 6-12°C, and resuspended in 80 µl of PBS with 2mM EDTA and 1% w/v BSA for 1 x 10⁷ viable cells, always at 6-12°C. PBS with 2mM EDTA and 1% BSA constituted the “buffer”. 20 µl of Hapten antibody cocktail per 10⁷ total cells was added, cells were mixed well and incubated for 10 minutes at 6-12°C. Cells were washed two times with 10-20x the labelling volume of buffer and resuspended carefully in 80 µl of buffer per 10⁷ total cells. 20 µl of MACS anti-hapten Microbeads per 10⁷ total cells were added, cells were mixed well and incubated for 15 minutes at 6-12°C with rotation. Cells were then washed with 10-20x the labelling volume of buffer, centrifuged and the supernatant removed completely. The pellet was re-suspended in 500 µl of buffer per 10⁸ total cells (500 µl for less cells). The MACS CS column (for up to 2x10⁸ magnetically labelled cells) was placed in the magnetic field of an appropriate MACS magnet separator and prepared according to manufacturer’s specifications. The cell suspension was pipetted onto the column,
allowing the unlabeled cells to pass through and effluent was collected sterile as the negative enriched T-cell fraction. The column was rinsed with 4x3 ml of buffer and all the effluent collected as before. Cells were counted and viability was assessed with Trypan Blue exclusion. T-lymphocyte purity was assessed regularly by FACS analysis (See Figure 3.1).

2.2.3 MITOGENIC ACTIVATION

T lymphoblast preparation
PBMCs obtained from centrifugation with Lymphoprep™ as described above, were washed three times with RPMI-1640 and re-suspended at 1 x 10^6 cells /ml in RPMI-1640 with 10% v/v FCS, 50 U/ml Penicillin/50 μg/ml Streptomycin and 500 ng/ml Amphotericin-B. They were then incubated for 72 h with one of the following mitogenic stimuli: 1μg/ml Staphylococcal Enterotoxin B, or 5μg/ml PHA, or 1μg/ml Ionomycin with 5ng/ml PMA. After 72 h and every 48h for 10-15 days, the T-lymphoblasts were supplemented with 20ng/ml (0.1 nM) hrIL-2. T-blasts were maintained at a 0.5-1.5 x 10^6 /ml density for up to 12 days. Prior to use, the cells were washed of IL-2 and deprived for 18 hours to allow accumulation in the G0-G1 cell cycle so that they represented a more homogeneous population with respect to IL-2 receptor expression and signalling potential (Ward, Cantrell, 1989), unless otherwise indicated. Alternatively, T-lymphoblasts were created by treating purified T-cells with combinations of anti-CD3/CD28 or anti-CD2/CD28 antibodies conjugated onto beads, as previously described (Levine et al. 1995, Levine et al. 1997). αCD3 antibody or the two αCD2 antibodies in combination to αCD28 antibody all at 1μg/ml each, were used to coat tosylactivated M-450 DYNAL beads, prepared according to the manufacturer’s specifications. Coated beads were used at a 1:1 ratio to the T-cells. Cell counting and viability was assessed with Trypan Blue exclusion.

T cell activation
Purified T cells were stimulated either via ligation of surface receptors, or pharmacologically. Surface receptors were stimulated with specific antibodies i.e. mouse anti-CD3, anti-CD28 mAbs, anti-CTLA-4 Ab, or the 2 distinct anti-CD2 Abs given together. Antibodies were administered soluble alone or at various combinations
(1μg/ml each or the concentrations indicated), always in the presence of 1μg/ml anti-mouse IgG Fab fragment cross-linking antibodies.

Otherwise, αCD3 antibody or the two αCD2 antibodies alone or in combination to αCD28 antibody at 1μg/ml each, with or without 10 μg/ml αCTLA-4 antibody were used to coat tosylactivated M-450 DYNAL beads, prepared according to the manufacturer's specifications. Bead-conjugated antibodies were used at a 1 bead to 1 T-cell ratio, as previously described (Levine et al. 1996; Blair et al. 1998). Alternatively CD28 alone or together with CD2 was ligated with CHO cells expressing surface CD80 or co-expressing surface CD80 and LFA-3, used fixed at a 1:3 T-cells ratio, unless otherwise indicated.

Pharmacologically, T cells were stimulated with PMA or 4α-phorbol ester alone (at 5ng/ml unless otherwise indicated), or in combination to 1μg/ml Ionomycin or CD28 co-ligation. Moreover, various cell types were stimulated with 1 to 100 nM of CC or CXC chemokines, 40 μg/ml of N-terminal modified SDF-1 peptides, 2-20 ng/ml IL-2, 10 μg/ml mouse anti-CXCR4 Ab, 0.001 to 1 μg/ml mouse anti-Fas Ab, and 2 to 200 ng/ml MN, SF2 and IIIB HIV-1 gp120 envelopes.

2.2.4 FACS ANALYSIS
5-10 x10⁴ cells were suspended in 50μl PBS in FACS polypropylene tubes and shaken at 4°C for 30min with 50μl primary antibody or isotype control and 25μl FCS. Following this, they were washed once with calcium free PBS (400g, 5min, 4°C) and re-suspended again in 50μl calcium free PBS with 50μl goat anti-mouse polyvalent (anti-IgM, IgG, IgA) secondary antibody conjugated to FITC and 25μl FCS. After 30min shaking at 4°C, the cells were washed with calcium-free PBS and analysed immediately, or stored in 2% paraformaldehyde at 4°C. All antibodies were used at 1μg/ml final concentration. T-cell purity was assessed with αCD3, αCD2, αCD4, αCD14, αCD16 and αCD19. Cells were also monitored where appropriate for expression of CD28, CD25, CD69, CD80, CXCR4, CCR3, CCR5 and ratCD2 (e.g. to check expression of transfected mutant CD2-p110 plasmid constructs). Propidium Iodide 10μg/ml was used as an indirect indicator of dead cells since this nuclear stain only enters cells with disrupted cell membrane integrity. Sodium azide was included at 0.01% in the ice cold PBS in chemokine receptor down-regulation studies to ensure that cells would not alter surface
expression due to staining antibody ligation or handling. All FACS analyses were performed on a Becton Dickinson FACS Vantage using a 200 mW 488nm argon laser with light being channelled by an FL-1 filter (520nm ± 20) and an FL-2 filter (580nm ± 20). Cell Quest software was used for subsequent analysis and WinMDI software for presentation.

2.2.5 PROLIFERATION ASSAYS
Purified T-cells were re-suspended in complete medium, aliquoted and treated with inhibitors if appropriate, and plated at 150μl /per 6x10^4 cells/per well, in 96-well tissue culture plates. T-cells were stimulated with combinations of anti-CD3, anti-CD2, anti-CTLA-4, anti-CD28 mouse mAb, or CHO-CD80+ cells, PMA, 4α-phorbol and Ionomycin. The various treatments were performed in quintuplicate to minimise internal variation, and all wells adjusted to contain 200μl volumes. The plates were incubated at 37°C, 95% humidity and 5% CO_2, for 44-48 hours and then pulsed with 20μl /1μCi /well ³H-thymidine. 72 h after stimulation the cells were harvested using an automated cell harvester on 96-well filter plates (Unifilter™) and ³H-thymidine incorporated into DNA was measured with a β-scintillation counter (TopCount™) according to the manufacturer’s specifications (Packard Instruments, Groningen, NL).

2.2.6 ELISA ASSAYS
Purified T-cells or T-lymphoblasts were re-suspended in complete medium and aliquoted at 350μl /per 5x10^5 cells/per well, in 24-well tissue culture plates, treated with inhibitors or vehicle when appropriate and stimulated as described above for the proliferation assays. The various treatments were performed in duplicate and all wells adjusted to contain 500μl volumes. Following incubation over a time course, or after 72 hours, the cells were pelleted and the supernatants aliquoted and stored at -70°C.

RANTES- double ligand sandwich Enzyme Linked Immunosorbent Assay
96-well immunosorbent plates were coated with 50μl/well of mouse anti-human RANTES monoclonal antibody at 1μg/ml in coating buffer (25mM Na_2CO_3, 30mM NaHCO_3 in distilled water, pH 9.6) overnight at 4°C. The plates were washed three times with wash buffer (PBS, 0.05 % Tween 20, pH 7.5) to remove excess antibody and known concentrations of RANTES for calibration, along with samples were diluted in sample buffer (wash buffer with 2% FCS). All points were loaded (50μl/well) in
duplicate with a standard curve of: 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 and 0.025 ng/ml of RANTES, and the plates were incubated at 37° C for 2 hours. The plates were then washed three times with wash buffer, and 50μl/well of goat polyclonal anti-human RANTES (R&D) biotinylated antibody was added in sample buffer at 1μg/ml, and plates were incubated for 1 hour at 37° C. The plates were washed three times in wash buffer and incubated another 30 min at 37° C, with streptavidin peroxidase added, 50 μl/well at 0.5 μg/ml in sample buffer. The final three washes were followed with the addition of 100μl/well of 0.2mg/ml 1,2 o-phenylenediamine dihydrochloride, in substrate buffer (3mM citric acid monohydrate, 6mM Na₂HPO₄, pH 5.0) with 0.02 % H₂O₂. Plates were incubated in dark, room temperature, 15-45min or until adequate oxidisation occurred to allow measurement of the coloured end product. The reaction was quenched with 150μl/well of 1 M H₂SO₄. The colour intensity was calculated using a Dynatech MR 5000 plate reader at 490nm wavelength. The average and net Optical Densities with the corresponding RANTES concentrations were derived using the manufacturer’s software. A typical standard curve used to calculate RANTES concentrations is shown in Figure 2.1a.

**MIP-1α double ligand sandwich Enzyme Linked ImmunoSorbent Assay**

96-well immunosorbent plates were coated with 50μl/well of mouse anti-human MIP-1α monoclonal antibody at 4μg/ml in coating buffer (PBS) overnight at 4°C. The plates were washed three times with wash buffer (PBS, 0.05 % Tween 20, pH 7.4) to remove excess antibody and blocked with 300μl/well blocking solution (PBS, 1 % BSA, 5 % Sucrose, 0.05 % Sodium Azide) for 1 hour at room temperature. After another three washes with wash buffer, known concentrations of MIP-1α as standards along with samples were diluted in sample buffer (TBS pH 7.3 : 20 mM Trizma base, 150 mM NaCl, 0.1 % BSA ). All points were loaded (50μl/well) in duplicate with a standard curve of: 2, 1.5, 1, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 ng/ml of MIP-1α. The plates were incubated at room temperature for 2 hours. The plates were washed three times with wash buffer, and 50μl/well of goat polyclonal anti-human MIP-1α (R&D) biotinylated antibody was added in sample buffer at 40 ng/ml, and plates were incubated for 2 hours at RT. Meanwhile, the following buffers were prepared: Gallati buffer (8.4g citric acid in 160ml water, pH 3.95 with 4M KOH) and TMB solution (120mg tetramethylbenzidine in 2.5ml DMSO and 2.5ml 100% EtOH). The plates were washed three times in wash buffer and incubated another 20 min at 37° C, with streptavidin
peroxidase added, 50 μl/well at 0.5 μg/ml in sample buffer. The final three washes were followed with the addition of 50 μl/well of 0.2 mg/ml 1,2 o-phenylenediamine dihydrochloride, in freshly prepared substrate buffer (10 ml Gallati buffer + 2μl of 30% v/v H₂O₂ + 100μl TMB solution). Plates were incubated in dark, room temperature, 15-45min or until adequate oxidation occurred to allow measurement of the coloured end product. The reaction was quenched with 50μl/well of 0.5 M H₂SO₄. The colour intensity was calculated using a Dynatech MR 5000 plate reader at 450nm wavelength with wavelength correction at 550nm. The average and net Optical Densities with the corresponding MIP-1α concentrations were derived using the manufacturer’s software. A typical standard curve used to calculate RANTES concentrations is shown in Figure 2.1b.

2.2.7 CHEMOKINE RECEPTOR RT-PCR ASSAYS
The polymerase chain reaction, a technique applied to amplify virtually any DNA segment which lies between two regions of known sequence, was first described by Kleppe and colleagues in 1979. PCR was applied to amplify DNA encoding for chemokine receptors. The cDNA was generated by reverse transcription from mRNA isolated from T-cells, and RT-PCR enabled visualisation of DNA segment bands in U.V illuminated ethidium bromide gels. With this qualitative process, the extent of constitutive transcription of chemokine receptor mRNA was assessed, as well as responses to stimuli and inhibitors.

Sample mRNA extraction
Sample mRNA was extracted from purified resting T-lymphocytes, stimulated T-lymphocytes, T-lymphoblasts or Jurkat cells at various time points. The commercially available kit QuickPrep™ Micro mRNA Purification Kit by Pharmacia Biotech, Herts, UK, was used and the protocol specified by the manufacturers was followed. 1-5 x10⁶ cells per point were homogenised in Extraction buffer containing guanidinium thiocyanate and N-lauroyl sarcosine, diluted in elution buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and centrifuged briefly at 13000g. The free RNA was then bound to oligo-(dT) cellulose, washed extensively with wash buffers (10mM Tris-HCl, 1mM EDTA, pH 7.5, 0.5 and 0.1 M NaCl) and eluted through a MicroSpin™ column. The mRNA recovered thus was stored in 95% EtOH at -70°C or used immediately.
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Figure 2.1 Representative standard curves for chemokine production assessment by ELISA.

a) RANTES ELISA

![Graph of RANTES ELISA]

b) MIP-1α ELISA

![Graph of MIP-1α ELISA]

Representative Standard curves used for the calculation of a) RANTES or b) MIP-1α, secreted in the supernatant by resting or stimulated purified T cells and T lymphoblasts. See text for details.
**Reverse Transcription (RT) step**

The mRNA was dried from 95% EtOH, dissolved in DEPC-treated sterile water and quantitated, using a deuterium lamp spectrophotometer and absorbance at 260nm, Gene Quant II RNA/DNA calculator, Pharmacia, UK. Protein contamination was assessed by a 260 / 280 nm absorbance ratio of below 1.7. 0.2-1 µg of mRNA from each sample was dissolved in 12.5 µl DEPC- water and denatured for 3 min at 75°C, then cooled on ice. The reverse transcription mixtures were made up in 0.2 ml thin wall PCR tubes, in 7.5 µl reverse transcription buffer (50mM Tris HCl pH 8.3, 75mM KCl, 3 mM MgCl2, 10 mM DTT, supplied by Promega, Madison, WI, USA) and together with the 12.5 µl sample mRNA gave a final volume of 20 µl per PCR tube. The final concentration-in 20 µl-of the constituents was: 1 mM Pd(N)6 random nucleotide hexamers, 0.5mM from each of the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 10 U/µl M-MLV Rtase (Moloney -Murine Leukemia Virus Reverse Transcriptase), and 1 U/µl RNAsin, a non-competitive ribonuclease inhibitor (49.8 kDa recombinant from E.coli). The tubes were placed in a PerkinElmer GeneAmp 2400 thermocycler (Warrington, UK) and followed a reverse transcription program of: 42°C for 60 min, 94°C for 2 min and 4°C thereafter, which was established as optimal in house by Dr. N. Jordan, Bath. The RT products were either used immediately, or briefly stored at 4°C.

**PCR step**

A forward and a reverse primer was obtained for every chemokine receptor available, using Primer 3 Design software available on the Internet. The chemokine receptor and control primers used were developed by Dr N. Jordan, Bath, UK and distributed by PerkinElmer. Each RT template from an experimental sample was tested for five C-C receptors, four C-X-C receptors, a positive control, the house-keeping gene of β-actin and a negative control of original RNA before the RT step, to check for DNA contamination. Each PCR reaction was carried out in 0.2 ml thin wall PCR tubes with 2.5 µl containing ~ 0.02 µg of RT template, 13.85 µl DEPC-dH20, and the following final concentrations of constituents (in appropriate volumes to give in total 25 µl final volume per tube): 200 µM from each of the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 1 µM of forward primer, 1 µM of reverse primer, 1 Unit of Expand™ High Fidelity enzyme mix which comprised of the 2 DNA polymerase enzymes Taq and Pwo, 1x the Expand™ PCR buffer without Mg2+. The polymerase enzymes and PCR buffers were used according to ‘Expand™ High Fidelity PCR system’
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manufacturer's specifications, and the thermocycler was given the following cycle: 15 sec / 95°C, 15 sec 56°C, 15 sec / 72°C repeated for 30 cycles and two holds, one at the beginning, 60 sec / 95°C and one at the end, 6 min / 72°C, prior to cooling to 4°C thereafter. The temperatures set were optimal for the phases of the PCR cycle, 95°C for denaturation of the template, 56°C for annealing of the chemokine receptor oligonucleotide primers to the open DNA strand, 72°C for optimum DNA synthesis by the heat stable polymerase enzymes. Cycle composition and the number of cycles, was optimised for PCR amplification of chemokine receptor primers in house by Dr. N. Jordan, Bath, UK. Mg²⁺ was added to 2 mM final concentration for each tube, 30 sec before the cycling started, during the initial 60 sec / 95°C hold. This ensured all of the enzyme was activated simultaneously and operated uniformly on its substrate. All consumables were purchased from Anachem, Luton, UK.

Detection of PCR products
A 2% agarose gel was made in 0.5x TBE (10 mM Tris base, 10 mM Boric acid, 2 mM EDTA, pH 8.0), boiled and cooled with the addition of 1 µg/ml Ethidium Bromide.

5 µl from each tube containing the PCR end products were coloured with 5 µl of Blue Juice (15% Ficoll 400, 0.25% bromo-phenol blue in water). A 100 base pair ladder comprising of 15 blunt end fragments (100-1500 base pair scale) was also coloured with blue juice. Samples and ladder were loaded onto the agarose-ethidium bromide gel and run by gel electrophoresis with a 100 V/0.1 mA current (BioRad Instruments). The resulting bands were visualised with a U.V illuminator and photographed with a polaroid camera and film.

2.2.8 CALCIUM FLUORIMETRY
Lipophilic compounds such as fura-2 acetoxy methyl ester (fura-2AM) readily penetrate the cell membrane, but are trapped in the intracellular space as fura-2, after cytosolic esterases cleave the ester groups. Changes in free intracellular calcium ion concentrations ([Ca²⁺]i), can be measured using the spectral shift fura-2 undergoes upon chelation of calcium ions. T-lymphoblasts, Jurkat cells, purified T-cells or U937 cells were washed three times (350 g, 5 min, 20°C) in “buffer”: calcium free HBSS with 0.1% BSA, 0.75% w/v sodium bicarbonate, pH 7.4 and re-suspended in a 50 ml centrifuge tube at 10⁷ cells/ml in RPMI with 10% FCS and incubated in the dark with 2.5 µM Fura-2AM for 30 min at 37°C, as described previously (Ward et al. 1995). The loaded cells
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were washed three times in "buffer" and re-suspended at 2 x 10^6 cells per ml. 2 ml aliquots were used in quartz or disposable cuvettes and external calcium and magnesium ions were adjusted to 1mM with addition of CaCl₂ and MgCl₂. The [Ca²⁺]ᵢ was determined with a fluorimeter (Photon Technology International, USA) recording the fluorescence signals at 37°C with dual excitation wavelengths 340 and 380nm and a single emission wavelength 510nm, to account for loading variability between samples and allow comparisons. Cells were stimulated with αCD3, PHA or the chemokines after they were allowed to equilibrate for 2min in a stirred cuvette at 37°C. Quartz cuvettes were washed with distilled water thoroughly prior to use. For calibration an aliquot of cells was lysed with 125μM digitonin which gave the maximum fluorescence signal \( R_{\text{max}} \). Chelation of all free calcium ions, with 10mM EGTA at pH 8.5 adjusted with NaOH, produced the minimum fluorescence signal \( R_{\text{min}} \) equivalent to less than 10nM [Ca²⁺]ᵢ. Sample [Ca²⁺]ᵢ was determined using PTI software based on the formula:

\[
[\text{Ca}^{2+}]_i = \frac{(R - R_{\text{min}})(R_{\text{max}} - R)}{K_d}
\]

where \( R \) is the measured sample fluorescence ratio and \( K_d \) the dissociation constant for the Fura- Ca⁺⁺ complex (224nM).

2.2.9 ³²P METABOLIC LABELLING AND PHOSPHOLIPID HPLC ANALYSIS

³²P metabolic labelling and phospholipid extraction

Jurkat cells, T-lymphoblasts or U937 cells were removed from culture and depleted of intracellular phosphate by three 10 min incubations followed by washing (350g, 10 min, RT) in phosphate-free DMEM supplemented with 20mM HEPES, 0.375% w/v sodium bicarbonate, pH 7.2 at 37°C. The cells were then incubated in 10 ml phosphate free DMEM supplemented with 10% saline dialysed-FCS, 20mM HEPES and 100 μCi/ml ³²P-labelled orthophosphoric acid, at 37°C for 3 to 4 hours, essentially as previously described (Jackson et al. 1992). (FCS was previously dialysed against saline for 18 hours and stored in aliquots at −20°C. Dialysis tubing was prepared by boiling twice for 5 minutes in 5mM EDTA / 200mM Na₂CO₃).

Following labelling, cells were washed three times in phosphate free DMEM, resuspended in RPMI 1640 /20mM HEPES and aliquoted into 1.5 ml screw capped Starsted tubes at 2x10⁷/ 120μl. The cells were stimulated as appropriate at 37°C and the reactions quenched by the addition of 0.7 ml ice cold chloroform/ methanol/ water (32.6% /65.3% / 2.1% v/v/v) (Jackson et al. 1992), vortexing and incubation on ice for
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ten minutes. Phases were separated by the addition of 200 µl freshly prepared chloroform containing 2 µg Folch lipids (1mg/ 100 ml), and 200 µl of 5 mM tetrabutylammonium hydrogen sulphate in 2.4 M HCl. The samples were vortexed and centrifuged at 14000g for five minutes. The lower chloroform layer was carefully removed to a fresh tube containing 400 µl of 5 mM EDTA / 1 M HCl before further vortexing and centrifugation. The lower phases were removed to fresh tubes and dried in vacuo. The samples were then solubilised in water by deacylation with 1 ml 25% w/v aqueous methylamine/ methanol/N-butanol (44.44% / 44.44% / 11.11% v/v/v) per tube. Samples were vortexed, incubated for 40 minutes at 53°C and cooled on ice for one minute before drying in vacuo overnight.

The deacylation generated glycerophosphatidylinositol (GroPtdIns) derivatives that were water soluble compared to the parental phosphatidylinositols (PtdIns) contained in chloroform extracted cell lipid samples or 3H-labeled standards. 0.5 ml sterile water was added to the dried samples followed by 0.6 ml N-butanol/40-60% petroleum ether/ethyl formate (80% / 16% / 4% v/v/v). After vortexing and centrifugation at 14000g for 30 seconds, the upper phase was discarded and the lower phase dried in vacuo.

HPLC analysis

Deacylated lipid samples, prepared as above, were resuspended in 120 µl sterile water in a sonicating water bath before being applied automatically with a Jasco Intelligent sampler 851-AS to an anion exchange Partisphere -SAX column (Whatman) at a flow rate of 1 ml/ min (Jackson et al. 1992). Lipids were eluted from the column using a gradient based on buffer A : dd H₂O, and buffer B : 1.25 M (NH₄)₂ HPO₄ pH 3.8, which were filtered and degassed with bubbling helium gas prior to use. The automated mobile phase gradient fed into the column by a Jasco Intelligent HPLC pump PU-980 connected to a Ternary gradient unit LG-980-02 was : 0 min, 0 % B ; 5 min, 0% B ; 45 min, 12% B ; 60 min, 30% B ; 61 min, 100% B ; 65 min, 100% B ; 66 min, 0% B ; 90 min, 0% A and B (Ward et al 1992). The eluate was fed into a Canberra Packard A-500 Flo-One in-line beta-radio detector, where it was mixed with three volumes of Flo-Scint IV scintillation cocktail (Packard) and the results recorded and analysed using Flo-One data software (Radiomatic, USA). The identity of the various peaks were routinely compared with ³H PtdIns(4)P and ³H PtdIns(4,5)P₂ standards, as well as known elution times for D-3 phosphoinositides described elsewhere (Ward et al. 1993). Retention
times have been previously identified by Stephens et al. 1991 and were subject to slight variation depending on room temperature and age of the column.

2.2.10 TRANSIENT TRANSFECTIONS OF PLASMID DNA CONSTRUCTS
Cells to be transfected were removed from culture, washed three times with RPMI-1640 at 350g for 5 minutes and aliquoted at 1.5x10^7/ 500 µl RPMI 1640. Samples were placed in an electroporation cuvette with 10 µg of desired plasmid DNA prior to electroporation at 310V, 960 µF in a BioRad Gene Pulser. Cell samples were pooled, resuspended in RPMI 1640 supplemented with 10% FCS and cultured over night. The following day the cells were washed twice at 350g for 10 minutes and viable cells were determined with trypan blue exclusion.

2.2.11 CELL LYSIS AND SAMPLE PREPARATION FOR SDS-PAGE
Cell lysis
Cell lysis was performed following the procedure described by Ward et al. (1992). Cells were harvested, washed three times in RPMI-1640 and resuspended at the desired concentration in 0.5 ml RPMI-1640/ 20mM HEPES. After appropriate treatments and stimulations, reactions were terminated by rapid-pulse cell pelleting and supernatant aspiration, followed by lysis with the addition of 0.5 ml/point of freshly prepared ice cold lysis buffer (50mM NaCl, 10mM Tris pH 7.4, 5mM NaF, 5mM Iodoacetamide, 0.5% Nonidet-P40, 1mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1mM phenyl methyl sulphonyl fluoride [PMSF]). The samples were rotated at 4°C for ten minutes before removal of the nuclear lysate/debris by centrifugation for 15 minutes at 13000g / 4°C in a microfuge. Supernatants were removed to a clean tube and used immediately or stored at −20°C.

Total protein preparation and immunoprecipitation
Total protein in the cellular lysates was precipitated by adding 0.7 ml ice-cold acetone to the 0.5ml lysates, followed by 15 minute incubation at 4°C, and centrifugation at 13000g, 4°C in a Beckman F2402 rotor. The supernatants were removed, the pellets dried in-vacuo for 30 minutes, resuspended and boiled in SDS-PAGE buffer (see below). Alternatively, specific proteins of interest were immunoprecipitated from the 0.5 ml cell lysates after pre-clearing by rotation for 30 minutes at 4°C with 20µl of protein-A or -G bead slurry (50% in PBS). Choice of protein-A or -G sepharose beads for pre-clearing
was done according to the specific antibody isotypes to be used. 20 µl of pre-cleared lysate was removed to a fresh tube and kept at 4°C to represent a total lysate control, and the remaining pre-cleared lysates were incubated for 60-90 minutes with 1-10 µg of the specific uncoupled antibody. Then 20 µl of a 50% slurry of the corresponding protein-A or -G sepharose beads was added for another 30-60 minutes rotation at 4°C. With previously coupled antibodies or the fusion protein Ras-binding-domain-glutathione sepharose (GST-RBD), 20 µl of coupled protein-A or -G sepharose bead slurry was applied to the cell lysate for 120 minutes rotation at 4°C. After completing the incubation, the immunoprecipitates (IPs) were washed five times; beads were pelleted by centrifugation and 90% of the supernatant was removed by aspiration. Beads were then resuspended with 1 ml lysis buffer and the wash was repeated three times. For the last wash, the remaining 10% of supernatant was removed completely with the aid of a Hamilton microsyringe. The IPs were used immediately in SDS-PAGE.

Protein samples were resolved essentially as described by Laemmli (1970), according to protocols previously established in our laboratory. Proteins to be analysed either from specific IPs or from acetone precipitated total cell lysates, were solubilised by boiling for 8 minutes in SDS-PAGE sample buffer consisting of 5% SDS, 50% glycerol, 200 mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol in dH2O and coloured appropriately with bromophenol blue.

2.2.12 SDS-PAGE AND WESTERN BLOTS

Sample protein preparation and Gel casting

Where minigels were required, BioRad Mini Protean II equipment was used (Bio-Rad laboratories, Hemel Hempstead, UK) and 8 % or 12 % homogeneous gels were run, prepared as described below. Where large gels were required 15 cm, 7 %– 17 % acrylamide gradient gels were prepared using vertical slab gel units model SE400 (Hoefer Scientific Instruments, San Francisco) and a 2 chamber gel mixer. Once cast, the gels were layered over with water-saturated isobutanol for smoothing and left to polymerise. The isobutanol was then washed off with water and stacking gels poured with appropriate combs in place. APS and TEMED were added immediately prior to casting the gels. For both gel systems running buffer consisted of 192 mM glycine, 25 mM Tris base and 0.1% SDS. Minigels were electrophoresed at 75V for two hours.
whereas larger 15cm gradient gels were run at 75V overnight. The acrylamide gels were prepared as presented in the table below:

<table>
<thead>
<tr>
<th>Component</th>
<th>7% gel</th>
<th>17%</th>
<th>8%</th>
<th>12%</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.2% Acrylamide / 0.8% N,N-methylene-bis-acrylamide (ml)</td>
<td>3.5</td>
<td>8.5</td>
<td>4</td>
<td>6</td>
<td>1.7</td>
</tr>
<tr>
<td>1M Tris pH 8.8 (ml)</td>
<td>5.6</td>
<td>5.6</td>
<td>3.8</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>5.8</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>6.8</td>
</tr>
<tr>
<td>1M Tris pH 6.8 (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.25</td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>-</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.075</td>
<td>0.075</td>
<td>0.15</td>
<td>0.15</td>
<td>0.1</td>
</tr>
<tr>
<td>10% APS (ml)</td>
<td>0.035</td>
<td>0.035</td>
<td>0.15</td>
<td>0.15</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.009</td>
<td>0.006</td>
<td>0.01</td>
</tr>
<tr>
<td>Final volumes (ml)</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

**Gel transfer**

15 cm 7%-17% gradient gels were transferred to “Polyscreen” PVDF membrane (pre-activated for 20 seconds in MeOH, 5 minutes in water and soaked in transfer buffer) in a BioRad Transblot cell at 200 mA / 50 V overnight in transfer buffer consisting 192mM glycine and 25 mM Tris base.

Mini gels were transferred onto nitrocellulose membranes as described previously (Turner et al. 1998), under semi-dry conditions in transfer buffer consisting of 39 mM glycine, 48 mM Tris base, 0.0375% SDS and 20% methanol. The proteins were transferred at 0.8 mA/cm^2^ of gel for 90 minutes.

**Western blots**

Non-specific protein binding to the membranes was blocked by prior incubation of the membranes in filtered (0.25μM nitrocellulose filters) 5% Marvel milk powder in PBS for two hours at room temperature. The membranes were rinsed at least three times in 0.05% Tween 20 (v/v) diluted in PBS (PBS-T), before incubation with the primary antibody. All antibodies were diluted into a sterile filtered solution of 0.05% Marvel in PBS. The membranes were incubated with the primary antibody for at least two hours with gentle agitation, followed by recovery of the primary antibody. The membranes were rinsed as before, prior to incubation with the secondary antibody for at least 45
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minutes. Antibody dilutions ranged from 1:1000 to 1:2000 for primary antibodies and 1:2000 to 1:5000 for secondary antibodies according to efficacy of the current batches. The membranes were then washed with copious changes of PBS-T or, for more stringent washing with PBS-T / 0.5M NaCl, before visualisation with Amersham ECL western blotting reagents according to the manufacturers’ instructions.

Stripping blots
Western blots to be reprobed with a second primary antibody to verify equal loading were submerged in membrane stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 60°C for one hour with occasional agitation. The blots were washed in 200 ml PBS-Tween at room temperature at least three times, before reprobing with a different primary Ab, as above in Western Blots.

2.2.13 IN-VITRO LIPID KINASE ASSAYS
Cells were aliquoted at 2x10^7/ point and lysed as described above in Cell Lysis. The cell lysates were pre-cleared, and IPs were prepared as described above in Total protein preparation and immunoprecipitation. After completing the IP incubation, the beads were pulse-pelleted, the supernatant removed and resuspended in 1ml lysis buffer. The IP-beads were washed sequentially, three times in lysis buffer, once in PBS, twice in 0.5 M lithium chloride (in 100 mM Tris, 1mM EDTA pH 7.6), once in water and finally once with lipid kinase (5mM MgCl2, 0.25 mM EDTA, 20mM HEPES, pH 7.4.) After the last pelleting, the supernatant was aspirated completely with the aid of a Hamilton microsyringe. Lipid kinase activity was determined by the modified method of Whitman et al, also described previously (Turner et al. 1998).

The washed precipitates were resuspended in 30 μl/tube of lipid kinase buffer before the addition of 50 μl/tube of a mixture of 0.5 mg/ml PtdIns and 0.5 mg/ml phosphatidyl serine, dispersed by sonication in 1 mM EDTA, 25 mM HEPES pH 7.4. The reactions were initiated by the addition of 3 μCi of [γ-32P] ATP and 100 μM ATP per tube. After 15 minutes at 30°C, the reactions were terminated by the addition of 100 μl 1M HCl and 200 μl chloroform : methanol (1:1). After vortexing and centrifugation to separate the phases, the lower organic layer was removed to a fresh tube and dried in vacuo with a Savant speedvac. The extracted phospholipids were then resuspended in 50 μl chloroform and separated by thin layer chromatography (TLC) (Turner et al. 1998).
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Laned silica gel 60 (Whatman) plates were pre-treated by spraying with 1% sodium oxalate in water and allowed to dry. The extracted phospholipid samples were loaded onto the plates in 10 μl aliquots and placed in a pre-equilibrated solvent tank containing propan-1-ol : glacial acetic acid (2N) : water (65% : 2% : 33% v/v/v). The samples were resolved overnight and visualised by exposure to iodine vapour prior to autoradiography. Phospholipids were identified by comparison with non-labelled standards, and were previously identified as PtdIns(3)P by extraction and HPLC analysis by Dr S Ward, Bath, UK. A schematic summary of the in-vitro lipid kinase reaction is shown in Figure 2.2a. Below is a diagram of a typical TLC resolution with non-labelled standards (Dr. Ward personal communication).

2.2.14 IN-VITRO HA-PKB ASSAYS

Jurkat cells were co-transfected, as described above in Transient Transfections, with plasmids encoding for hemaglutinin(HA)-tagged-PKB alone, with HA-PKB and ratCD2/p110catalytic domain active chimera, or with HA-PKB and rat CD2/ p110 kinase dead chimera (See Materials : Plasmids and Radiochemicals) (Assay as described in Kulik, Weber, 1998). Cells were washed, aliquoted at 10^7/ point and equilibrated at 37°C prior to appropriate stimulation. Cell lysates were pre-cleared and immunoprecipitated with 2μg anti-HA (12CA5) antibody and 20 μl of a 50% suspension of protein G- sepharose beads per point, as described above in Total protein preparation and immunoprecipitation. Immunoprecipitates were then washed sequentially with 1ml/tube, twice in lysis buffer, twice in 500 mM LiCl2 (in 100 mM Tris, 1 mM EDTA  pH7.5, and finally once in protein kinase B assay buffer (50 mM Tris, 10 mM MgCl2, 1 mM DTT). Once in assay buffer, the beads were removed to screw capped tubes, pulsed in a benchtop microfuge and supernatants aspirated.
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completely using a Hamilton syringe and kinase reactions initiated by addition of 15 µl / tube kinase reaction mixture containing: 0.5 µM protein kinase inhibitor, 2.5 µg histone 2B(H2B) substrate, 50 µM ATP, 3 µCi $^{32}$P-γ-ATP in PKB assay buffer. After 30 minutes at room temperature the reactions were quenched by the addition of 30 µl SDS-PAGE sample buffer, boiling for eight minutes and SDS-PAGE overnight, using the larger 15 cm, 7% to 17% gradient gel apparatus described above in Sample protein preparation and gel casting. Following electrophoresis, the gel was cut horizontally at 40 kDa.

The upper half was transferred on to PDVF and immunoblotted with anti-phospho Ser473 PKB antibody, stripped and reprobed with anti-PKB antibody to demonstrate equal loading, as described above in Western blots & Stripping blots. The lower half of the gel containing the phosphorylated H2B substrate was immersed for 2 hours with gentle agitation in Coomassie blue stain (0.1% w/v Coomassie brilliant blue R). The Coomassie stain was then decanted, and the gel was de-stained with multiple changes of destaining solution (30% methanol/10% acetic acid/60% dH2O v/v/v) for four hours, and fixed by incubation in fixing solution (propan-2-ol: water: glacial acetic acid, 25%: 65%: 10% v/v/v) for two hours. The gels were then placed on filter paper and dried on a BioRad gel dryer (model 583) for two hours. Radiolabelled H2B substrate proteins were visualised by autoradiography. A schematic summary of the in-vitro kinase reaction by PKB is shown in Figure 2.2b.

Figure 2.2 Schematic representation of in-vitro kinase reactions measured by the amount of substrate phosphorylation incorporating $^{32}$P- from ATP labelled at the γ-position with $^{32}$P.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) <em>In-Vitro</em> lipid kinase</td>
<td>PtdIns</td>
<td>PtdIns(3)-$^{32}$P</td>
</tr>
<tr>
<td>B) <em>In-Vitro</em> protein kinase B</td>
<td>Histone 2B</td>
<td>Histone 2B-$^{32}$P</td>
</tr>
</tbody>
</table>

![Diagram of in-vitro kinase reactions](image-url)
2.2.15 CHEMOTAXIS ASSAYS

Chemotaxis was examined using a 96-well chemotaxis chamber (Neuroprobe™, Cabin John, MD, USA) (Figure 2.3). The wells of the 96 well Optiplate I opaque plate were filled with 380 μl of chemoattractant diluted in RPMI 1640 containing 0.1% BSA or vehicle, encased within the chemotaxis chamber and very carefully covered with an adhesive polyvinylpyrrolidine-free (PVPF) polycarbonate membrane (8 μM pore size). After the lid was firmly in place, the chamber was left to equilibrate for 5 minutes at 37°C. The cells were washed and treated with inhibitors if appropriate, and 2 x 10^5 cells were added to each upper well on the lid, in a volume of 200 μl RPMI 1640 / 0.1% BSA in quintuplicate per treatment. The chamber was incubated at 37°C for 2 hours. The cell suspension was subsequently aspirated off and 200 μl of Versene was added to each well. After 20 minutes incubation at 4°C the 96-well plate and membrane were removed from the chamber and centrifuged at 400g for 10 minutes at 4°C. The supernatant was removed and the cells resuspended in 100 μl of RPMI / 0.1% BSA. Cell migration was assessed by adding 20 μl of Cell Titer 96 AQueous solution (Promega, UK) to each well.

After 1-3 hour incubation at 37°C, according to the metabolic rate of the cells, together with plates containing a titration of cells serving as a standard curve. The curve was typically: 500000, 250000, 125000, 62000, 31000, 16000, 8000, 4000, 2000 cells / well in duplicate. The plates were read at λ 490nm, subtracting the readings at a reference λ 650nm to reduce the background contributed by non-specific absorbance, using a Dynatech MR 5000 plate reader. Basal and stimulated migrations were transformed to cell numbers from the standard curve and results were expressed as Chemotactic Index (C.I.): the ratio of stimulated over basal migration. Inclusion of chemokine in both upper and lower compartments served routinely to verify chemotaxis as opposed to chemokinesis. A typical standard curve used for the calculation of migratory cells is shown in Figure 2.4.
Figure. 2.3 Components and assembly of chemotaxis chamber.

Legend for Fig. 2.3 A cross-section view of the apparatus used to measure cell migration.
A : Chemotaxis chamber lid with 96 bores of 200μl capacity when sealed, B : gasket for watertight seal formation, C : Framed adhesive PVDF-free polycarbonate membrane (8 μM pore size), D : 96-well opaque Optiplate, E : Bottom part of chemotaxis chamber. Green arrow indicates direction for apparatus assembly and direction of cell movement.

Below, a cross-section representation of the assembled chamber and the events occurring during the chemotaxis assay: 1) Assembled apparatus. 2) Cells are placed on top of the lid and start to migrate towards the diffusing molecules of chemokine. 3) Cells that crossed through the micropore membrane are stuck on the adhesive membrane underside, to be liberated only after Versene treatment. 4) Centrifugation of the plate with the membrane in situ sediments migrated and detached cells so that resuspension and quantification can occur without handling losses.
2.2.16 ACTIN POLYMERISATION ASSAYS

Cells were washed and aliquoted at 2 x 10⁶ /0.5 ml /point in RPMI-1640 and incubated at 37°C in 24-well plates for the appropriate times and treatments. After stimulation with various concentrations of SDF-1 for the appropriate times, the cells were transferred into FACS polypropylene tubes and fixed using 0.5 ml of 7.4% para-formaldehyde in PBS at RT. After 10 minutes, cells were washed with PBS (350g, 5 minutes) resuspended in 100µl / tube of 1% para-formaldehyde/1% FCS in PBS, and permeabilised with the addition of another 100µl / tube of 0.1% Triton-X solution with 0.3 µM phalloidin-FITC in PBS. The cells were left to stain at 4°C for 30 minutes. The cells were then washed twice in PBS, resuspended in 500 µl of 1% para-formaldehyde/1% FCS solution and analysed immediately or stored at 4°C in dark. Data was analysed on a Becton-Dickinson FACS Vantage, excitation λ 488 nm, emission λ 530 nm (see also FACS Analysis).
2.2.17 CXCR4 DOWN-MODULATION ASSAYS
Various types of cells were washed twice and aliquoted at 1 x 10^6 cells / ml / point in FACS polypropylene tubes. After incubation at 37°C with inhibitors, if appropriate, cells were stimulated with SDF-1, PMA, 4a-phorbol ester or vehicle (0.01% DMSO) for 30 minutes at 37°C. Following treatment, cells were cooled to 4°C with ice and the addition of 4 ml ice-cold PBS with 0.01% sodium azide, and pelleted at 350g for 5 minutes at 4°C. Samples were then stained for CXCR4 expression with 12G5 anti-CXCR4 antibody or IgG2a isotype control and FITC-conjugated secondary antibody as described above in FACS ANALYSIS.

2.2.18 ANNEXIN-V APOPTOSIS ASSAYS
The Apoptosis Annexin-V-FLUOS® staining kit (Boehringer and Mannheim) uses FITC-conjugated annexin-V to detect phosphatidyl serine (PtdS) which is rapidly externalised during apoptosis (Martin et al., 1995). Jurkat cells, purified T-cells or T-lymphoblasts were washed twice and aliquoted into 24-well plates at 1 x 10^6 cells / ml / per point in complete media, and treated with various concentrations of anti-Fas (CH11) antibody or IgM isotype control. Cells were incubated at 37°C either for 8 hours or for 20 hours. The cells were then washed and resuspended in 100 µl HEPES-binding buffer containing propidium iodide and annexin-V conjugated to FITC from a Annexin-V-FLUOS® staining kit as specified by the manufacturers. After 15 minutes incubation at RT, the cells were analysed by FACS. Annexin binding was visualised as an increase in the FL-1 channel for FITC. Membrane degeneration was visualised as an increase on the FL-2 channel for propidium iodide on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm). See also FACS ANALYSIS.

2.2.19 LUCIFERASE CONSTRUCT REPORTER ASSAYS
The effect of SDF-1 on transcription factor activation was examined using Luciferase plasmid constructs transiently transfected in Jurkat cells. The artificial promoter constructs used for determining transcription factor activation were the human (h)NFAT-Luciferase (Luc), hAP-1-Luc and the hIL-2 NF-kB-Luc. Jurkat cells were transiently transfected with 10µg of each plasmid DNA via electroporation as described in Transient Transfections of Plasmid DNA constructs. After recovery overnight, viable cells were washed three times (350g for 10 minutes) in freshly prepared buffer comprising of phenol-red-free RPMI-1640 (GIBCO BRL, Paisley, UK) supplemented to
Materials & Methods

ImM in Ca\(^{2+}\) and Mg\(^{2+}\) ions. Cells were counted, re-suspended with the prepared buffer with 10 % FCS at 1x10\(^6\)/ml and plated in quintuplicate at 100\(\mu\)l / 1x10\(^5\) cells / well in white opaque 96-well plates (Packard Instruments, NL). Cells were treated with appropriate stimulating antibodies and/or chemokines for 8 hours incubated at 37\(^\circ\)C. For assaying luciferase activity, the Luc-Lite\(^\text{TM}\) reagent was prepared according to the manufacturers' specifications. Hence, following the 8-hour incubation, 100\(\mu\)l/well of reconstituted LucLite substrate was added, plates were sealed with TopSeal\(^\text{TM}-\text{A}\) adhesive sealing film and after 15 minutes dark equilibration, the plates were analysed at the single photon emission mode with a TopCount\(^\text{TM}\) spectrophotometer (Packard Instruments, NL).

2.2.20 STATISTICAL ANALYSIS

Statistical analysis was performed on the raw data from (n= number of experiments) using 2-way analysis of variance (ANOVA) and two-way Student’s t-test with p < 0.05 *, p < 0.01 ** and p < 0.001 *** accordingly.
RESULTS I: Chemokines in T cell activation.

3.1 RANTES and MIP-1α secretion from purified T cell activation

T CELL PURITY VERIFICATION
To verify the purity of T lymphocytes obtained from peripheral-blood derived mononuclear cells, it was important to routinely characterise the identity of these cells according to surface receptor expression. Immediately after Lymphoprep™ separation, the resultant population of peripheral-blood derived mononuclear cells stained 10% positive for the B-cell marker CD19, 9% for the monocyte cell marker CD14, 20% for the Fcγ-IIIa receptor CD16 found on natural killer/macrophage cells, and only 53% for CD2 and 39% for CD3 which denote T-lymphocytes (Fig. 3.1A). However, after a typical T-cell purification using negative selection with the MACS system, the resultant cell population was typically 99% CD2⁺, 97% CD3⁺, but devoid of any specific staining for CD19, CD14 or CD16 (Fig. 3.1B). Moreover, freshly purified T cells typically expressed 95% CD28⁺, 12% CD25⁺, 65% CD4⁺, and 33% CD8⁺ (Fig. 3.2). Similar receptor expression results were obtained with the DYNAL magnetic separation procedure but the MACS system was preferred due to the higher yields and much less handling times involved.

JURKAT AND CHO-CD80⁺ CELL MARKERS
It was important to monitor surface receptor expression on the various cell populations used, since their presence defined their capacity to function in particular assays. The T-cell lymphoma cell line Jurkat was routinely checked for expression of surface receptors and found to be 98% CD2⁺, 98% CD3⁺, and 99% CD28⁺ (Fig. 3.3). CHO cells stably transfected to express human CD80 were routinely monitored for surface expression of CD80 prior to use in CD28 stimulation studies, with typical expression levels of 99% CD80⁺ (Fig. 3.3).

EXAMINATION OF T CELL PROLIFERATION AND RANTES SECRETION
The initial experiments sought to identify the activating stimuli that could elicit RANTES production and secretion, since RANTES was initially characterised as a chemokine regulated upon activation of normal T cells.
Figure 3.1 Effect of T-cell purification by negative selection on surface expression of PBM cell markers

A) Before purification

B) After purification

Figure 3.1 Effect of magnetic purification of T-cells on the surface expression of PBM cell markers. 1 x 10^5 freshly isolated PBM cells / point were analysed for surface expression of CD2, CD3, CD19, CD14 and CD16 using appropriate antibodies before MACS system magnetic purification of resting T-cells (A). The cells were analysed with a FITC-conjugated goat anti-mouse IgG on a Becton Dickinson FACS vantage, as described in Materials and Methods. Expression profiles shown are from one experiment representative of all purified T-cells used elsewhere.
Figure 3.2 Surface expression of CD28, CD25, CD4, and CD8 on purified resting T-lymphocytes.

1 x 10^5 freshly purified resting T-cells per point were analysed for surface expression of CD2, CD3, CD28, CD25, CD4 and CD8 with mouse anti-human antibodies (red histograms), or isotype matched control antibodies (black histograms). The cells were analysed with a FITC-conjugated goat anti-mouse IgG on a Becton Dickinson FACS vantage as described in Materials and Methods. Expression profiles shown are from one experiment representative of all purified T-cells used elsewhere.
Figure 3.3  Surface expression of CD2, CD3 and CD28 on Jurkat cells and CD80 on CHO cells

1x10^5 Jurkat cells / point were analysed for surface expression of CD2, CD3 and CD28, whereas 1x10^5 CHO cells / point transfected with CD80 were verified to express B7.1 with mouse anti-human antibodies (red histograms), or isotype matched control antibodies (black histograms). The cells were analysed with a FITC-conjugated goat anti-mouse IgG on a Becton Dickinson FACS vantage as described in Materials and Methods. Expression profiles shown are from one experiment representative of all Jurkat cells and CD80^+ CHO cells used elsewhere.
Effective T cell activation resulting in proliferation in-vitro, was assessed by the amount of radio-labelled thymidine incorporated after 72 hours of culturing purified T-lymphocytes with appropriate combinations of stimuli (reflecting de novo nucleic acid synthesis necessary for mitosis). As T cell proliferation depends on IL-2 production and secretion, it was necessary to establish if the signals required for these events are also able to support RANTES production and secretion. The concomitant examination of RANTES secretion and proliferation would enable a preliminary assessment of the alleged participation of RANTES in certain modes of T cell activation. Ex-vivo T-cells in culture displayed a basal amount of thymidine incorporation of around 1500 cpm, that was not affected by various stimuli administered alone, with the exception of CD3 ligation by antibodies (Fig. 3.4) or administration of the phorbol ester PMA (Fig. 3.6A), which resulted in similar small but consistent increases of about 5000 cpm.

In vitro RANTES production by T-lymphocytes was assessed with an ELISA assay developed to detect protein secreted (and therefore bioavailable), in the supernatant at set time points during a time course of culture, or after 72 hours, the period that corresponded to proliferation measured amidst such RANTES levels. After 72 hours, unstimulated T-cells in culture released a basal amount of RANTES of around 1.5 ng/ml in their supernatant. Single stimuli administered alone were ineffective in RANTES secretion above basal levels, with the notable exception of 5 ng/ml PMA which resulted in a dramatic RANTES response of 40 ng/ml (Fig 3.6B).

EFFECT OF COSTIMULATION PRESENTATION ON PROLIFERATION

Effective T-cell proliferation was achieved with certain combinations of co-stimulatory stimuli. However, T-cell proliferation was optimised when soluble antibodies were immobilised onto beads. Improvement in proliferative responses appeared to depend on presentations of the stimuli, aimed to mimic the 3-D nature of T-cell – APC / accessory cell interactions by using either CHO cells or beads to serve as “dummy” APCs. Hence, whereas soluble antibodies binding to CD3 and CD28 at 1μg/ml afforded a proliferation of 12000-14000 cpm, the combination of CHO cells expressing the CD28 ligand CD80 in a 1:3 T-cell ratio, together with antibodies against CD3 raised the proliferation levels to 18000-20000 cpm. Conjugation of anti CD3 and anti CD28 antibodies to beads also increased proliferation levels to 20000-22000 cpm. Similarly, an improvement of responses was apparent when a combination of 2 separate anti-CD2 antibodies given together with anti CD28 antibodies were conjugated onto beads, or anti-CD2 antibodies
Results I

together with CHO-CD80⁺ cells were used. Although the improvements in proliferation with the various modes of presentation of CD3/CD28 ligation or CD2/CD28 ligation were significant, CD3/CD28 ligation compared to CD2/CD28 ligation when presented in the same way (i.e. soluble antibodies, or beads, or alongside CHO cells), resulted in proliferation levels that did not differ significantly (Fig. 3.4).

However, PMA at 5 ng/ml together with 1 µM of Ionomycin resulted in proliferation levels comparable to CD3/CD28 or CD2/CD28 co-stimulations with soluble cross-linked antibodies. PMA in combination with such CD28 ligation enhanced proliferation to 31000 cpm and substitution of anti-CD28 antibodies with CHO-CD80⁺ cells increased proliferation even further to 37000 cpm. Thus signal presentation for PMA costimulations appeared important only for proliferation involving CD28. On the contrary, the non-PKC activating 4α-phorbol ester failed to contribute above basal cpm in costimulation studies (Fig.3.6A).

EFFECT OF COSTIMULATION PRESENTATION ON RANTES SECRETION

Unlike proliferation, presentation models of T-cell-costimulation did not improve RANTES secretion equally well. Importantly, whereas soluble cross-linked antibodies binding to CD3 and CD28 resulted in 10 ng/ml RANTES, antibodies to CD2 and CD28 did not affect basal RANTES release. The effect of combining CHO-CD80⁺ cells with anti-CD3 antibody did not differ significantly from the effect of soluble antibody ligation to CD28 and CD3, however conjugation of the latter set of antibodies to beads increased RANTES levels to 18 ng/ml. Conversely, CD2 and CD28 ligation was never effective, not with antibody-coated beads, or even with CHO cells co-expressing LFA3 and CD80 (Fig. 3.5). The robust RANTES secretion response to 5ng/ml PMA could be enhanced only with the combination of soluble antibody against CD28 which gave 68 ng/ml. In marked contrast to their effect on proliferation, substitution of anti-CD28 antibodies with CHO-CD80⁺ cells decreased RANTES secretion to 40 ng/ml, the level of RANTES secreted with PMA alone. Importantly, Ionomycin with PMA resulted in a further reduction down to levels observed from CD3 and CD28 ligation on beads (Fig. 3.6B). Again, the 4α-phorbol ester failed to contribute in RANTES secretion above basal, alone or in costimulation studies (Fig. 3.6B).
Figure 3.4 T-cell proliferation after 72 hours

6x10⁴ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates. After 72h, cells were harvested and ³H-thymidine incorporation was assessed using a β-scintillation counter, as described in Materials and Methods. Cells were stimulated with soluble antibodies, antibody-coated beads or CHO-CD80⁺ cells as described in Materials and Methods. Results are expressed as counts per minute (x1000 C.P.M.) and data represent the means of 3 independent experiments with sem. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
Figure 3.5  RANTES secretion by T-cells after 72 hours

1.5x10^6 cells/well purified T-lymphocytes treated in duplicate as indicated in 24-well plates, were harvested after 72h, pelleted and assayed for RANTES in the supernatant with a double-sandwich ELISA as described in Materials and Methods. Cells were stimulated with soluble antibodies, antibody-coated beads, CHO-CD80^+ cells or CHO-CD80^+/LFA3^+ cells as described in Materials and Methods. Results are expressed as ng/ml of RANTES per well and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.6 Effect of 5ng/ml PMA costimulation on T cell proliferation and RANTES secretion after 72 hours.

A) Proliferation

- 4a-phorbol/Ionom
- PMA / Ionom
- PMA /CHO-CD80
- PMA /anti-CD28
- Ionomycin
- 4a-phorbol
- PMA
- Basal

<table>
<thead>
<tr>
<th>3H-thymidine incorporation (x1000 C.P.M.)</th>
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<tbody>
<tr>
<td>0  5  10  15  20  25  30  35  40  45</td>
</tr>
</tbody>
</table>

B) RANTES secretion

- 4a-phorbol / Ionom
- PMA / Ionom
- PMA / CHO-CD80
- PMA / anti-CD28
- Ionomycin
- 4a-phorbol
- PMA
- Basal

<table>
<thead>
<tr>
<th>RANTES (ng/ml)</th>
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</thead>
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<tr>
<td>0  10  20  30  40  50  60  70  80</td>
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</table>

Figure 3.6 Effect of PMA costimulation on T cells. A) 6x10⁴ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72h, and ³H-thymidine incorporation was assessed using a β-scintillation counter, as described in Materials and Methods. B) 1.5x10⁶ cells/well purified T-lymphocytes were treated in duplicate as indicated in 24-well plates, harvested after 72h, and the supernatant was assayed for RANTES with a double-sandwich ELISA as described in Materials and Methods. Cells were stimulated with PMA, Ionomycin, 4α-phorbol ester, soluble anti-CD28 Ab or CHO-CD80⁺ cells as described in Materials and Methods. Results are expressed as A) : counts per minute (x1000 C.P.M.) , B) : ng/ml of RANTES per well, and all data represent means of 3 independent experiments with sem. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
TITRATION OF RESPONSES WITH CD3 OR PMA COSTIMULATION

**Proliferation with CD3 ligation**
The contribution of CD3 to proliferation was examined using the CHO-CD80\(^+\) cell model by a titration of anti-CD3 antibody concentrations and CHO-CD80\(^+\) cell to T-cell ratios. The contribution of CD3 ligation by antibody was concentration related and proliferation could be augmented further with the presence of CHO-CD80\(^+\) cells. We therefore chose to perform subsequent experiments with the ratio of 1:3 (CHO-CD80\(^+\) cells : T-cells) for submaximal co-stimulations, as it supported concentration-dependent increments to proliferation, with respect to anti-CD3 antibody, that were all above basal levels and below the maximum proliferation observed (Fig. 3.7a).

**RANTES Secretion with CD3 ligation**
Using the 1:3 CHO-CD80\(^+\) cells to T-cells ratio a bell-shaped contribution by anti-CD3 antibody was revealed, with an optimal concentration of 1\(\mu\)g/ml anti-CD3 antibody (10 ng/ml RANTES) (Fig. 3.7b). Unlike proliferation, the presence of increasing CHO-CD80\(^+\) cell : T-cell ratios enhanced RANTES secretion consistently only with 1\(\mu\)g/ml anti-CD3 antibody. The higher 1:1 CHO-CD80\(^+\) cell : T-cell ratio together with 1\(\mu\)g/ml anti-CD3 antibody resulted in the optimal RANTES secretion of 12 ng/ml (Fig. 3.7b).

**Proliferation with PMA costimulation**
To establish optimal and maximal PMA concentrations, T cells were stimulated with a titration of PMA. Anti-CD28 antibody at 1\(\mu\)g/ml appeared more effective than 1 \(\mu\)M Ionomycin with any single PMA concentration, resulting in a maximal proliferative response of 38000 cpm with 50 ng/ml PMA (Fig. 3.8A). Similar maximum proliferation levels were observed to result from 10 \(\mu\)g/ml anti-CD3 antibody with a 1:1 APC : T-cell ratio (Fig. 3.7a), suggesting an upper limit of proliferative responses after 72 hours limited by the initial number of cells aliquoted in the wells.

**RANTES Secretion with PMA costimulation**
PMA alone exhibited bell shaped characteristics with respect to RANTES secretion, with an optimal 40 ng/ml RANTES from 5 ng/ml PMA. However, together with other costimuli, the levels of RANTES secretion witnessed with 50ng/ml PMA were comparable to those observed in the presence of 5 ng/ml PMA. Importantly, in the presence of the two higher PMA concentrations, anti-CD28 antibody ligation enhanced, whereas Ionomycin reduced RANTES secretion compared to PMA alone. Moreover, the CHO-CD80\(^+\) cell : T-cell ratio of 1:3 did not affect the RANTES response to 5ng/ml PMA but was able to enhance RANTES secretion from 50 ng/ml PMA (Fig. 3.8B).
Figure 3.7 Titration of T-cell proliferation and RANTES secretion with anti-CD3 antibody and CHO-CD80+ cells.

a. proliferation

\[( \times 1000 \text{ C.P.M.})\]

![Graph showing proliferation results](image)

b. RANTES

\[(\text{ng/ml})\]

![Graph showing RANTES results](image)

Figure 3.7 Titration of T-cell proliferation and RANTES secretion with anti-CD3 antibody and CHO-CD80+ cells. Purified T-lymphocytes, at: a) 6x10^6 cells/well treated in quintuplicate in 96-well plates were harvested after 72h and \[^3\text{H}-\text{thymidine} \] incorporation was assayed with a \(\beta\)-scintillation counter, b) 1.5x10^6 cells/well treated in duplicate in 24-well plates, were harvested after 72h, pelleted and the supernatants were assayed for RANTES with a double-sandwich ELISA as described in Materials and Methods. Anti-CD3 mAb and CHO-CD80+ cells were administered at the concentrations and ratios shown, as described in Materials and Methods. Results are expressed as a) counts per minute (\(\times 1000 \text{ C.P.M.}\)) b) ng/ml of RANTES per well and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.8 Titration of T-cell proliferation and RANTES secretion with PMA costimulation. Purified T-lymphocytes at: A) 6x10⁴ cells/well were treated in quintuplicate in 96-well plates, harvested after 72h, and ³H-thymidine incorporation was assessed using a β-scintillation counter, B) 1.5x10⁶ cells/well were treated in duplicate in 24-well plates, harvested after 72h, and the supernatant was assayed for RANTES with a double-sandwich ELISA, as described in Materials and Methods. PMA was given at the concentrations shown whereas anti-CD28 mAb, CHO-CD80⁺ cells and Ionomycin were given as described in Materials and Methods. Results are expressed as A): counts per minute (x1000 C.P.M.), B): ng/ml of RANTES per well, and all data represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
TIME COURSE OF RANTES SECRETION WITH T CELL COSTIMULATION

Examination of the time course of RANTES production by T-cells revealed a profile with delayed kinetics compared to IL-2 production (Cantrell et al. 1984), from every stimulation used. At all times examined, with soluble antibodies, or CHO-CD80+ cells, or antibody-coated beads, only costimulations involving CD3 ligation but not CD2 ligation resulted in significant RANTES production. After 48 hours post stimulation, all combinations involving CD3 afforded a modest but significant rise in RANTES released to about double the basal levels. At 72 hours, a steep increase in RANTES production was evident with a further smaller increase at 96 hours. Hence, between 72 and 96 hours, CD3/CD28 ligation with antibody-coated beads was most effective resulting in 18 and 20 ng/ml, soluble cross-linked antibody CD3/CD28 ligation resulted in 11 and 16 ng/ml, and CD3 ligation with CHO-CD80+ cells resulted in 10 and 13 ng/ml respectively (Fig 3.9A).

In the presence of PMA, only costimulation with Ionomycin resembled closely the kinetics of RANTES production by CD3 and CD28 ligation (e.g. 18 ng/ml at 72 hours). In a similar fashion, major increases in RANTES secretion by T cells stimulated with PMA/CHO-CD80+ cells occurred after 72 hours (40 ng/ml as opposed to 12 ng/ml at 48 hours), and subsequently RANTES levels reached a plateau. PMA alone, and even more PMA in combination with anti-CD28 antibody ligation, gave the most dramatic RANTES secretion (69 ng/ml at 72 hours) compared to any other stimulation (Fig. 3.9B).

EXAMINATION OF MIP-1α PRODUCTION BY T CELLS

To examine if the extraordinary effect of PMA on RANTES production was unique to this chemokine or a general feature in other -CC- chemokine production, a comparative assessment of the same costimulations on RANTES and MIP-1α production was carried out. In vitro MIP-1α production by T-lymphocytes was assessed with an ELISA assay developed to detect protein present in the supernatant at set time points during a time course of culture, or after 72 hours, as previously described for RANTES. After 72 hours, unstimulated T-cells in culture released a basal amount of MIP-1α in their supernatant of around 12 ng/ml. Whereas CD3 ligation alone did not affect MIP-1α, soluble anti-CD3 and CD28 antibodies as well as PMA with Ionomycin doubled the MIP-1α production to around 22 ng/ml.
Figure 3.9 Time course of RANTES production by T-cells.

1.5 x10^6 cells/well purified T-lymphocytes treated in duplicate as indicated in 24-well plates, were harvested over a time course after stimulation, and the supernatants were assayed for RANTES with a double-sandwich ELISA as described in Materials and Methods. Cells were stimulated as indicated and as described in Materials and Methods. Results are expressed as ng/ml of RANTES per well and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.10 A) RANTES and MIP-1α secretion at 72 hours, B) Time course of MIP-1α secretion by co-stimulated T-cells.

A)
Chemokine (ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>anti CD3</th>
<th>PMA</th>
<th>PMA anti CD28</th>
<th>PMA Ionom.</th>
<th>anti CD3</th>
</tr>
</thead>
<tbody>
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<td>RANTES</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MIP-1α</td>
<td></td>
<td></td>
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B)
MIP-1α (ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
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<tr>
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<td>96</td>
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</tbody>
</table>

Results are expressed as ng/ml of chemokine per well and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
The combination of PMA with anti-CD28 antibodies elicited even more MIP-1α secretion, maximising it to 90 ng/ml. However, unlike with RANTES, PMA alone failed to induce MIP-1α secretion greater than basal controls (Fig. 3.10A). Examination of the time course of MIP-1α secretion revealed profile similar to that observed for RANTES. Hence, a modest but significant rise in MIP-1α above basal levels was not evident until 48 hours post stimulation. At 72 hours, MIP-1α secretion was optimal at around 25 ng/ml, after which time point, MIP-1α levels reached a plateau. It should be noted that basal levels also increased 3-fold after 72 hours in culture (Fig. 3.10B).

3.2 RANTES secretion from Activated T cells (T-Lymphoblasts).

T LYMPHOBLAST PHENOTYPES
Activated T-lymphocytes were obtained either by incubation of the whole PBMC fraction with a mitogen or by stimulation with the phorbol ester PMA in combination to the calcium ionophore Ionomycin for 72 hours. Alternatively, purified T-cells stimulated with anti-CD3 in combination with anti-CD28 antibodies, or anti-CD2 together with anti-CD28 antibodies also resulted in T-lymphoblast production. When PHA or SEB were used, non-adherent accessory cells became obsolete after 72 hours and the washed T-lymphoblasts were routinely found to be 95-98% CD2+ , 99% CD3+ , 95-97 % CD28+ (Fig. 3.11). T-lymphoblasts derived from costimulation with PMA and Ionomycin stained brightly for CD2, but CD3 and CD28 expression was slightly reduced, possibly because PMA can down-regulate surface receptors (Fig. 3.11). The different stimuli for lymphoblastic transformation appeared to exert differing effects on the CD4+ / CD8+ composition of T-lymphoblasts. After 72 hours with PMA and Ionomycin treatment a tendency towards more CD4+ T-blasts, ~75%, was evident. Conversely, there were less CD4+ T-blasts, about 45% after SEB treatment (Fig. 3.12). The majority of PHA-derived T-blasts were CD4+ (60%), but that composition deviated the least from purified resting T-lymphocyte CD4 (and CD8) expression profiles (Figs. 3.2 and 3.12). With all types of T-lymphoblasts, expression of CD25 was notably up-regulated (Fig. 3.12), compared to the typical 10-12% levels observed with freshly purified resting T cells (Fig. 3.2). Unfortunately, analysis of CD2, CD3 and CD28 surface expression on T-lymphoblasts derived from purified T-cell costimulation with antibodies to CD2 or CD3 and CD28 was hampered by the fact that stimulating antibodies could not be removed
and interfered with the staining process, producing high background fluorescence and artificial peaks.

REGULATION OF RANTES PRODUCTION BY T-LYMPHOBLASTS
Since optimal RANTES secretion was observed after 72 hours T cell activation, it was important to assess RANTES secretion from T-lymphoblasts in long term culture i.e. > 3 days. PBMCs were activated with PHA and maintained in culture for ~2 weeks with hrIL-2 supplementation as described in Materials and Methods. To examine the contribution of IL-2 in the production and secretion of RANTES over a time course, day 7 T-blasts were either maintained in IL-2 or deprived of IL-2 for a period of 18 hours, IL-2 was then added back to the two sets of cells and RANTES secretion was measured during the next four days in culture. Depriving T-blasts of IL-2 appeared to affect RANTES secretion both basaly and in response to subsequent IL-2 addition. In response to additional exogenous IL-2 after day 2 onwards, cells previously maintained in IL-2 displayed significant increases in RANTES secretion compared to RANTES levels from previously deprived cells. (Table 3.1). This lack of effect of IL-2 on deprived blasts was probably due to loss of surface IL-2 receptor expression, an event which has been described to occur upon withdrawal of IL-2 from the culture and can be restored with T cell costimulation (Cantrell et al. 1984)

| TABLE 3.1 |
|---|---|---|---|---|
| DAY | IL-2 DEPRIVED | IL-2 MAINTAINED |
| | 2 ng/ml IL-2 | 20 ng/ml IL-2 | 2 ng/ml IL-2 | 20 ng/ml IL-2 |
| 0 | 2.3 ± 0.4 | 2.5 ± 0.3 | 3.8 ± 0.24 | 3 ± 0.28 |
| 1 | 2.1 ± 0.2 | 4 ± 0.2 ** | 4 ± 0.4 | 5.6 ± 0.45 * |
| 2 | 2.5 ± 0.3 | 3.8 ± 0.3 * | 7 ± 0.3 *** | 8.3 ± 1.8 ** |
| 3 | 3.4 ± 0.4 * | 4 ± 0.18 ** | 8 ± 0.14 *** | 9.6 ± 2.7 |
| 4 | 3.3 ± 0.35 * | 4.1 ± 0.4 ** | 8.3 ± 2 * | 12.3 ± 2.4 *** |

Results are expressed as ng/ml of RANTES per well and represent means of 3 independent experiments with sem. * : p < 0.05, ** : p < 0.01, *** : p < 0.001.
Figure 3.11 Surface expression of CD2, CD3 and CD28 on SEB, PHA or PMA with Ionomycin-transformed T-lymphoblasts after 72 hours.

1 x 10^5 / point T-lymphoblasts derived from PBMC fractions treated in-vitro for 72 hours with SEB, PHA or with PMA and Ionomycin for 72 hours were analysed for surface expression of CD2, CD3 and CD28, with mouse anti-human antibodies (red histograms), or isotype matched control antibodies (black histograms). The cells were analysed with a FITC-conjugated goat anti-mouse IgG on a Becton Dickinson FACS vantage as described in Materials and Methods. Expression profiles shown are from one experiment representative of all such T-lymphoblasts used elsewhere.
Figure 3.12 Surface expression of CD4 and CD25 on SEB, PHA or PMA with Ionomycin -transformed T-lymphoblasts after 72 hours. $1 \times 10^5$ / point T-lymphoblasts derived from SEB-, PHA- or with PMA and Ionomycin- treated PBMC fractions were analysed for surface expression of CD4 and CD25, with mouse anti-human antibodies (red histograms), or isotype matched control antibodies (black histograms). The cells were analysed with a FITC-conjugated goat anti-mouse IgG on a Becton Dickinson FACS vantage as described in Materials and Methods. Expression profiles shown are from one experiment representative of all such T-lymphoblasts used elsewhere.
Figure 3.13 Effect of costimulation and IL-2 on RANTES secretion by T-lymphoblasts after 72h. T-blasts were generated with 5 µg/ml PHA and washed / deprived of IL-2, 18 hours prior to treatment. 1.5x10^6 cells/well treated in duplicate as indicated in 24-well plates, harvested after 72h, and the supernatant was assayed for RANTES with a double-sandwich ELISA as described in Materials and Methods. Antibodies were all administered as described in Materials and Methods. IL-2 was added concomittant to the other treatments. Results are expressed as ng/ml of RANTES per well and data represent means of 3 independent experiments shown with sem. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
Using IL-2 deprived blasts, the effect of CD3 and CD28 ligation alone or in combination, in the presence or absence of concomitant IL-2 administration, was examined after 72 hours in culture. In the absence of exogenous IL-2, CD3 or CD28 ligation alone had no noticeable effect on RANTES secretion whereas CD3/CD28 costimulation afforded a modest but significant increase in RANTES secretion. The addition of IL-2, increased RANTES levels in response to CD3 or CD28 ligation alone but not CD3/CD28 costimulation, which was able to support a modest RANTES secretion alone not significantly different from any combinations of IL-2 with either CD3 or CD2 ligation separately. Although the addition of the higher IL-2 concentration appeared to potentiate RANTES production in response to CD3 and CD28 costimulation, this effect was not significant (Fig. 3.13).

SUMMARY

1. T cell purification resulted in a reproducible, high titer of CD2⁺/CD3⁺ cells with very low expression of the IL-2Rα chain, CD25 (e.g. <12% ).

2. In-vitro lymphoblastic transformation with various mitogens after 72 hours resulted in activated T cells which stained invariably bright for CD2, CD3 and CD28 expression.

3. T lymphoblast populations from the various mitogenic stimuli were heterogeneous in CD4 expression, with apparent trends in CD4⁺ cell phenotypes associated with each mitogen.

4. Expression levels of CD25 on T lymphoblasts were variable but consistently greater than those observed in unstimulated purified T cells.

5. T cell proliferation required the combination of more than one stimuli and could be effectively elicited with CD28 and either CD3, or CD2 costimulation models, as well as PMA and Ionomycin at comparable levels.
6. T cell costimulated RANTES secretion did not correlate with the combined administration of various stimuli since PMA alone caused a robust RANTES secretion, CD3 co-stimulation a modest suboptimal secretion and CD2 costimulation no RANTES secretion above unstimulated levels just like all other stimuli alone.

7. Proliferation levels increased with increments in concentrations of both CD3 and CD28 ligands, whereas RANTES secretion levels showed a positive correlation only with the degree of CD28 ligation. In contrast, there was an optimal amount of CD3 ligand, above or below which RANTES secretion was reduced.

8. Proliferation with PMA was promoted further to an equal degree with either CD28 co-ligation or Ionomycin treatment, and proliferation levels increased with increments in PMA concentration.

9. The notable RANTES secretion with PMA alone was elevated further only with CD28 and markedly reduced with the combination of Ionomycin. RANTES secretion with PMA alone observed a bell shape concentration response with an optimum concentration and the addition of CHO-CD80+ cells together with the optimal PMA concentration did not influence RANTES secretion.

10. The profile of RANTES secretion over a time course was uniform with all stimulation combinations despite the differing amounts produced. A biphasic response was observed, with small to moderate RANTES levels up to 72 hours post-stimulation and a substantial increase in secretion thereafter.

11. The effect of PMA alone was specific to RANTES but not to MIP-1α secretion, however costimulation of T cells with CD28 ligation and either PMA or CD3 revealed MIP-1α secretion with a similar profile to RANTES over time.

12. RANTES production from T lymphoblasts was elevated with increases in IL-2 supplementation but depended on pre-existing and continuous presence of IL-2. With T lymphoblasts previously deprived from IL-2, CD28 ligation could synergise with fresh IL-2 additions in modestly increasing RANTES secretion to levels procured with CD28/CD3 costimulation independently of exogenous IL-2.
3.3 Regulation of T lymphocyte proliferation and RANTES secretion

EFFECT OF CTLA-4 LIGATION
The susceptibility of T-cell proliferation to inhibition, induced by the various activating stimuli, was assessed in order to correlate effects on T-cell proliferation in parallel with RANTES secretion obtained from identical treatments. Ligation of CTLA-4 expressed on recently activated T-cells, is thought to represent an important naturally occurring negative regulation of T-cell activation. T-cells were subjected to the effects of CTLA-4 signalling with the use of three different antibodies, namely the clones 3D6, CT29 and CT36, all provided by Dr. Carl June. From these three clones only 3D6 induced inhibitory signalling (Blair et al. 1998, and Dr. Carl June personal communication). The other two Abs (CT29 and CT36), were tested as negative controls, to account for possible stearic hindrance/obstructive effects caused by CTLA-4 antibody ligation, on the interactions of CD28 with the common ligand CD80. All the anti-CTLA-4 antibodies used, were applied conjugated onto Dynalbeads together with anti-CD28 mAb and either anti-CD3 or CD2 Abs, in approximately a 10-fold greater concentration, as this was shown to be the optimum effective inhibition with clone 3D6 (Blair et al. 1998, and Dr. Carl June personal communication), whereas inclusion of CT29 or CT36 anti-CTLA-4 Abs indeed exerted no effect (Not shown).

Proliferation
With 3D6, when beads were not used (e.g. vehicle- or PMA- treated cells in Fig. 3.14A), the equivalent concentration of 10μg/ml anti-CTLA-4 Ab was used soluble, and such treatment did not affect proliferation. The effect of 3D6 CTLA-4 mAb ligation was most apparent with CD3 and CD28 costimulation where it afforded ~80% inhibition of proliferation. Proliferation with CD2 and CD28 costimulation appeared more resilient, and the modest inhibition observed was not significant. Similarly, proliferation with PMA and anti-CD28 mAb, appeared marginally reduced by 3D6 anti-CTLA-4 ligation, but that effect was not significant (Fig. 3.14A).

RANTES secretion
The effect of CTLA-4 3D6 mAb ligation, previously reported to negatively regulate IL-2 expression and secretion (Blair et al. 1998), was examined on RANTES secretion by T-lymphocytes costimulated for 72 hours. RANTES secretion with CD3 and CD28 costimulation was inhibited by the inclusion of anti-CTLA-4 antibodies by 40%, whereas there was no effect on the non-RANTES inducing CD2 and CD28 costimulation. PMA-stimulated RANTES secretion was not significantly attenuated and
although the enhanced RANTES secretion from PMA / CD28 costimulation was marginally reduced, this effect was not significant (Fig. 3.14B).

**EFFECT OF WORTMANNIN ON T CELL COSTIMULATION**

Class 1A PI3K activation has been implicated in TCR / CD3 signalling (Ward et al. 1992), recognised as an integral and independent part of CD28 signalling (Ward et al. 1993) and the PI3K inhibitor Wortmannin has been shown to block T cell costimulated activation (Ward et al. 1995). As T cell proliferation and RANTES secretion following anti-CD3 Ab ligation occurs only in the presence of CD28 ligation (Figs. 3.4 and 3.5), PI3K-mediated signalling appeared an important candidate enzyme to mediate CD28-costimulation, and Wortmannin was used in order to assess any possible PI3K role.

**Proliferation**

Wortmannin affected T-cell proliferation in a concentration dependent manner and the maximum inhibition observed was with 100nM Wortmannin. The most affected proliferation was induced by CD3 and CD28 co-stimulation, which was inhibited by 60%. Proliferation with CD2 and CD28 costimulation was inhibited by 40%, and with PMA and CD28 costimulation significant inhibition was observed by 20% (Fig. 3.15A).

**RANTES secretion**

Use of the PI3K inhibitor Wortmannin, revealed that RANTES secretion from PMA alone or in combination to CD28 ligands would not be reduced further than 40 ng/ml. Moreover, as RANTES secretion from PMA together with CHO-CD80⁺ cell costimulation did not differ from the RANTES secretion observed with PMA alone, any concentration of Wortmannin tested was not effective with these two stimulating conditions. Interestingly, the enhanced RANTES secretion by PMA and anti-CD28 antibody costimulation was reduced in a concentration-dependent fashion to ~40 ng/ml, the RANTES levels obtained with PMA alone or in combination with CHO- CD80⁺ cells (Fig. 3.15B).

Conversely, the modest RANTES levels secreted by CD3 and CD28 co-ligation, as well as PMA and Ionomycin co-stimulation was inhibited by Wortmannin in a concentration dependent manner. RANTES secretion with 100nM Wortmannin was inhibited by 50% from anti CD3 and CHO- CD80⁺ cell stimulation, by 60% from soluble anti CD3 and CD28 antibody treatment, and by 65% from PMA and Ionomycin stimulation (Fig. 3.15C).
Figure 3.14 Effect of CTLA-4 ligation on T cell co-stimulation

A) 

\(^{3}\text{H}-\text{Thymidine incorporation}

(x1000 cpm)

\[ \begin{array}{cccc}
\text{Vehicle} & \text{Bead CD3} & \text{Bead CD2} & \text{PMA} \\
\text{CD28} & \text{CD28} & \text{CD28} & \text{anti CD28}
\end{array} \]

RANTES secretion (ng/ml)

B) & C)

RANTES secretion (ng/ml)

Vehicle Bead CD3 Bead CD2 CD28 PMA PMA anti CD28

Figure 3.16 Effect of CTLA-4 ligation on T-cell co-stimulation. Purified T-lymphocytes at (A): 6x10^4 cells/well treated in quintuplicate, (B,C): 1.5x10^6 cells/well treated in duplicate, as indicated, were harvested after 72h. (A): \(^{3}\text{H}-\text{thymidine incorporation was assessed using a } \beta\text{-scintillation counter, or (B,C): RANTES secreted was detected by ELISA, as described in Materials and Methods. Cells were stimulated with soluble or bead-conjugated antibodies as indicated and described in Materials and Methods. Vehicle treatment was 0.01% DMSO. Results are expressed as (A): x1000cpm, or (B,C): ng/ml of RANTES per well, and represent means of 3 independent experiments with sem.*: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.15 Effect of Wortmannin on T-cell co-stimulation proliferation.

Purified T-lymphocytes at (A) 6x10^4 cells/well treated in quintuplicate, (B,C): 1.5x10^6 cells/well treated in duplicate, as indicated, were harvested after 72h. (A): ³H-thymidine incorporation was assessed using a β-scintillation counter, or (B,C): RANTES secreted was detected by ELISA, as described in Materials and Methods. T-cells were incubated with Vehicle: 0.001% DMSO or Wortmannin for 10min prior to stimulation, and stimulated as described in Materials and Methods. Results are expressed as (A): x1000 cpm, or (B,C): ng/ml of RANTES per well, and represent means of 3 independent experiments with sem. Unstimulated T cells displayed a basal ~1400 cpm and ~1.2 ng/ml RANTES. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
EFFECT OF CYCLOSPORIN A AND RAPAMYCIN ON T CELL COSTIMULATION

Cyclosporin A was an important agent used for the identification of independent signalling by CD28 during T cell costimulation, since its calcium-dependent target calcineurin, appeared important in proliferation from CD3/CD28 co-ligation, but redundant in PMA/CD28 costimulation. Conversely, the requirement for functional p70S6 kinase as a downstream CD28 effector for proliferation is present in both CD3 or PMA and CD28 costimulations. Hence, Rapamycin is known to inhibit proliferation from both these CD28 costimulations. In order to further assess if stimulation of RANTES secretion shares the differential requirements for functional immunophilins as witnessed in CD28-costimulated proliferations, the effect of CsA and Rapamycin was tested in all RANTES-inducing stimulations.

Proliferation

The established immuno-suppressant Cyclosporin A inhibited in a concentration dependent manner T-cell proliferations stimulated by bead-conjugated anti-CD3/CD28 mAbs and bead-conjugated anti-CD2/CD28 mAbs. Although PMA / Ionomycin-induced proliferation was also significantly inhibited by the higher CsA concentration, PMA and anti-CD28 co-stimulation was the notable exception not inhibited by CsA. Basal and minimal proliferation induced by PMA alone were also not affected. The higher 3μM Cyclosporin A concentration inhibited CD3 and CD28 co-stimulation by ~65%, and both CD2 and CD28 or PMA with Ionomycin costimulation by ~60% (Fig. 3.16).

RANTES secretion

Cyclosporin A affected, in a concentration dependent manner, RANTES secretion from CD3/CD28 costimulation reaching 60% inhibition with 3 μM CsA (Fig. 3.19). PMA and Ionomycin costimulated RANTES secretion was inhibited similarly, with the same higher CsA concentration of 3 μM resulting in 50% inhibition. In contrast, the PMA stimulated RANTES response was not affected with any CsA treatment and PMA/CD28 costimulation appeared marginally attenuated but this was not significant (Fig. 3.17). Rapamycin inhibited in a concentration dependent manner RANTES secretion from CD3 ligation and CHO- CD80+ cells to a maximum inhibition of 70%. Rapamycin also appeared to inhibit RANTES secretion following PMA or PMA / Ionomycin treatment, but in both cases, inhibition did not exceed ~25% even as the concentration of Rapamycin increased, and the modest level of inhibition was not significant until Rapamycin was used at concentrations above 10 ng/ml (Fig. 3.17).
Figure 3.16 Effect of Cyclosporin A on co-stimulated T-cell proliferation.

6x10^4 cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates and after 72 hours were assayed for \(^{3}\)H-thymidine incorporation with a \(\beta\)-scintillation counter as described in Materials and Methods. T-cells were incubated with Vehicle: 0.1% ethanol or CsA for 30min prior to stimulation, and stimulated as described in Materials and Methods. Results are expressed as x1000 CPM and represent means of 3 independent experiments with sem. Unstimulated T cells displayed a basal 1400cpm. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.17 Effect of Cyclosporin A and Rapamycin on RANTES production by T-cell co-stimulation after 72 hours.

PERCENT INHIBITION %

Cyclosporin A

Vehicle 0.1 0.3 1 3 CsA [μM]

Rapamycin

vehicle 0.1 1 10 50 Rapamycin [ng/ml]

Figure 3.17 Effect of Cyclosporin A and Rapamycin on RANTES secretion by T-cell co-stimulation after 72 hours. 1.5x10⁶ cells/well purified T-lymphocytes treated in duplicate as indicated, were assayed for RANTES in the supernatant with a double-sandwich ELISA after 72h, as described in Materials and Methods. T-cells were incubated with Vehicle: 0.1% ethanol or CsA for 30 min or Rapamycin for 60 min prior to stimulation. T cells were stimulated as described in Materials and Methods. RANTES levels in the presence of vehicle only were: Bead CD3 CD28 : (19 ± 2.3 ng/ml), PMA : (43 ± 7.3 ng/ml), PMA antiCD28 : (70 ± 6.7 ng/ml), PMA /Ionom. : (17 ± 3.9 ng/ml), anti-CD3/ CHO- CD80 : (10 ± 0.4 ng/ml). Results are expressed as percent % inhibition of the vehicle response to various stimuli which procured the values shown in brackets and represent means of 3 independent experiments with sem. Unstimulated T cells displayed a basal ~1.2 ng/ml RANTES. * : p < 0.05, ** : p < 0.01, *** : p < 0.001.
EFFECT OF RO 320432 ON T CELL COSTIMULATION

Since the PKC-activating phorbol ester PMA, unlike the non-PKC-activating phorbol ester 4α-phorbol, induced a substantial RANTES secretion this implied that PKC isozymes were principally involved in mediating this effect. However, as PMA does not induce the substantial proliferation in response to CD3/CD28 costimulation, it is possible that the modest RANTES secretion from CD3/CD28 costimulation is regulated by pathways that are alternative to, or regulate PKC isozymes. To examine the involvement of PKC isozymes pharmacologically, the relatively specific PKC inhibitor RO 320432 was assessed in T cell proliferation and RANTES secretion with CD3 or PMA and CD28 costimulations.

RO 320432 affected proliferation dramatically only at 10 μM, the highest concentration used. Proliferation with CD3 and CD28 costimulation was significantly inhibited only with 10 μM by 60-65%, but not abrogated (Fig. 3.18). Proliferation with PMA and CD28 co-stimulation was inhibited significantly with 1 μM by 20% and abrogated entirely with 10 μM RO320432. The minimal proliferation levels with PMA alone were also significantly abolished with all inhibitor concentrations (Fig. 3.18). In contrast to proliferation, 10 μM RO 320432 universally abrogated the RANTES response to basal levels from CD3 and CD28 costimulation, PMA stimulation or PMA/CD28 costimulation (Fig. 3.21).

EFFECT OF "SLEDGEHAMMER" INHIBITORS ON RANTES SECRETION

Manumycin A was described as a farnesyl-transferase inhibitor which led to a disruption of Ras signalling and Ras-dependent proliferation. Herbimycin A was reported as a specific inhibitor of tyrosine kinases which was able to disrupt Ras recruitment or any signalling event dependent on tyrosine phosphorylation. These drugs are arbitrarily termed “sledgehammer” inhibitors, implying broad inhibition of all downstream and parallel coupling, hence affecting multiple downstream effectors. This denomination is justified by the fact that i): both inhibitors may and probably do target more than one family of molecules, and ii): the identified targets of both drugs (i.e. PTKs and Ras) are central in coupling surface receptor activation with downstream signalling components and intracellular cross-talk.
Figure 3.18 Effect of RO 320432 on co-stimulated T-cell proliferation.

$^{3}$H-Thymidine incorporation (x1000 cpm)

Figure 3.20 Effect of RO 320432 on co-stimulated T-cell proliferation. 6x10$^4$ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72h and assayed for $^{3}$H-thymidine incorporation with a β-scintillation counter, as described in Materials and Methods. T-cells were incubated with Vehicle: 0.1 % DMSO or RO 320432 for 30min prior to stimulation. PMA: PMA at 5 ng/ml, PMA anti CD28: PMA at 5ng/ml with 1µg/ml soluble anti-CD28 mAb and 1µg/ml goat anti-mouse IgG Fab' fragment cross-linking Ab, Bead CD3 CD28: 1µg/ml of anti-CD3 and anti-CD28 mAbs conjugated onto Dynalbeads used at a 1 bead : 3 T-cell ratio. Results are expressed as x1000 CPM and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
However, although these inhibitors cannot address which downstream pathway(s) are important in mediating the PTK or Ras effect, they can be used to qualitatively identify the involvement of tyrosine phosphorylation and functional Ras-GTP in the culmination of a response such as RANTES secretion. They were also used to further characterise the robust RANTES response from PMA treatment alone, because Ras activation and not PKCs have been implicated in conveying the effect of phorbol esters in some settings (Ward, June 1998).

Accordingly, complete inhibition of RANTES secretion was achieved which did not vary according to the co-stimulation treatments. Specifically, 2µg/ml of Herbimycin A, or 10 µM of Manumycin A abrogated RANTES production to 1-2 ng/ml irrespective of the stimulation conditions. PMA alone, PMA and CD28 ligation or CD3 and CD28 ligation, although resulting in considerably different amounts of RANTES production, were not able to resist abrogation by any one of the aforementioned inhibitors (Fig. 3.19).

**EFFECT OF PD 98059 AND SB 203580 ON RANTES SECRETION**

These two relatively specific differential MAP Kinase inhibitors have been extensively studied in the past and were not re-evaluated in the proliferation studies (Gould et al. 1995). The “sledgehammer” inhibitors implicated tyrosine kinase cascades and Ras signalling alongside PKC-dependent signalling as important in RANTES secretion. MAP kinase cascades are known to be important signalling components downstream of PTKs, Ras and PKCs. Therefore, PD 98059 and SB 203580 were used in order to dissect the contribution of MEK-MAPK and p38/MAPK respectively, in mediating RANTES secretion from PMA stimulations or CD3/CD28 costimulation.

**PD 98059**

Using the natural ligand for CD28 with CHO- CD80+ cells, the sensitivity of RANTES secretion to the MEK inhibitor PD 98059 (Dudley et al. 1995), with respect to CD3/CD28 costimulation, or the robust PMA response, was examined. Costimulation with anti-CD3 Ab / CHO- CD80+ cells was most affected, with discrete concentration dependent inhibition reaching a maximum of 50% with 10µM PD 98059. Stimulation with PMA was much less susceptible to inhibition, and only PD 98059 concentrations of 3µM and above resulted in a significant but modest inhibition (e.g. 25% ) (Fig. 3.20A).
Figure 3.19 Effect of RO 320432, Manumycin A and Herbimycin A on RANTES secretion by T-cell co-stimulation after 72 hours. 1.5x10^6 cells/well purified T-lymphocytes treated in duplicate as indicated, were assayed for RANTES in the supernatant with a double-sandwich ELISA after 72h, as described in Materials and Methods. T-cells were incubated with vehicle: 0.01% DMSO, with RO 320432 for 30 min, with Manumycin A for 60 min and with Herbimycin A for 18 hours prior to stimulation, as described in Materials and Methods. Results are expressed as ng/ml RANTES and represent means of 3 independent experiments with sem. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 3.20 Effect of PD 98059 and SB 203580 on RANTES secretion by T-cell co-stimulation after 72 hours.

A) PD 98059

Percent (%) Inhibition

anti-CD3 / CHO CD80

PMA

vehicle 0.1 0.3 1 3 10 PD 98059 [µM]

Figure 3.22 Effect of PD 98059 or SB203580 on RANTES secretion by T-cell co-stimulation after 72 hours. 1.5x10^6 cells/well purified T-lymphocytes treated in duplicate as indicated, were assayed for RANTES in the supernatant with a double-sandwich ELISA after 72h, as described in Materials and Methods. T-cells were incubated with vehicle: 0.01% DMSO, PD 98059 or SB 203580 for 60 min, prior to stimulation as described in Materials and Methods. RANTES levels in the presence of vehicle only were: anti-CD3/ CHO CD80: (11 ng/ml), PMA: (43 ng/ml), PMA Ionom.: (17 ng/ml). Results are expressed as percent % inhibition of the vehicle response to various stimuli which procured the values shown in brackets and represent means of 3 independent experiments with sem. Unstimulated T cells displayed a basal ~1.2 ng/ml RANTES. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
SB 203580
The p38/MAPK inhibitor SB 203580 revealed that, again, anti-CD3 Ab / CHO- CD80+ cell costimulated RANTES secretion was reduced in a concentration dependent way to a maximum 75 % inhibition with 10μM SB 203580. However, with PMA, or PMA / Ionomycin the modest 20% inhibition of RANTES secretion with SB203580, became significant only at 10μM (Fig. 3.20B).

SUMMARY
1. CTLA-4 ligation by inhibitory antibody significantly inhibited proliferation and RANTES secretion from CD3/CD28 costimulated T cells. T cell proliferation from CD2 or PMA and CD28 costimulations, as well as RANTES secretion from PMA alone or PMA and CD28 costimulation were not affected significantly by CTLA-4 inhibitory antibody.

2. Wortmannin significantly inhibited RANTES secretion from co-stimulations which incorporated CD28, CD3, or Ionomycin stimulations but not PMA. Proliferation from co-stimulations which incorporated CD3 or CD2 stimulations was most affected by Wortmannin, and only 100nM was capable of a modest inhibition on PMA/CD28 costimulated proliferation.

3. Cyclosporin A significantly inhibited proliferation from co-stimulations which incorporated CD3, CD2, or Ionomycin stimulations. RANTES secretion from CD3/CD28 costimulation was resistant to Cyclosporin A and weakly sensitive to Rapamycin. RANTES secretion from PMA/Ionomycin treatment was less sensitive to Cyclosporin A and resistant to Rapamycin.

4. RO 320432 abrogated RANTES secretion from all types of costimulation. RO 320432 abolished proliferation to costimulations which incorporated PMA but only partially inhibited proliferation from CD3/ CD28 costimulation. Manumycin A and Herbimycin A abrogated RANTES secretion from all types of costimulation.

5. PD 98059 and SB 203580 treatment potently inhibited RANTES secretion from CD3 costimulation. Instead, only higher concentrations slightly attenuated RANTES secretion with PMA-costimulations.
3.4 Chemokine participation in costimulated T lymphocyte proliferation

CHEMOKINES AND T CELL PROLIFERATION
As induction of T-cell proliferation was compared between the various stimuli applied alone and the various models of co-stimulation, it was evident that effective proliferative responses resulted from combinations of delivered signals. Chemokines have been implicated in the past as having a role to play in T cell costimulated proliferation (Turner et al. 1995, Maghazachi et al. 1996, Murphy et al. 1996, Taub et al 1996). The involvement of autocrine or paracrine chemokines in participating alongside established mitogenic stimuli was assessed with addition of exogenous peptide(s) or, where possible, addition of neutralising anti-chemokine antibodies. Firstly, several chemokines were examined for their capacity to substitute a costimulatory signal and provide significant proliferation either alone, or in combination with one established stimulus. RANTES, MIP-1α, MCP-1, IL-8, SDF-1 and IP-10 were administered at their optimal physiologic concentration of 10 nM, alone, or in combination to antibodies against CD3, CD2, or CD28, as well as in combination to CHO-CD80+ cells. In every case, the chemokines failed to display a stimulatory or costimulatory effect on proliferation (Fig. 3.21).

Using the natural ligand for CD28 presented on fixed CHO cells in combination with CD3 antibody ligation, submaximal levels of T-cell proliferation were defined as T cell proliferation clearly below the maximum proliferative capacity possible (from 60000 T cells per well), when stimulated optimally. Such optimal stimulation would be e.g. 10 μg/ml anti-CD3 antibody and a ratio of 1 CHO-CD80+ cell for 1 T cell. The influence of RANTES, MIP-1α, MCP-1 and IL-8 was assessed on less vigorous costimulation, to allow for potential enhancement. Again, over a range of 5 physiologically relevant concentrations, namely 1, 3, 10, 30 and 100nM, none of these chemokines tested appeared capable of affecting the established submaximal T-cell proliferation induced by CD3 with CHO-CD80+ cells (Table 3.2). Moreover, using the same costimulation conditions to examine autocrine chemokine proliferative effects, neutralising antibodies against RANTES, MIP-1α, MCP-1 and IL-8 were administered together with the anti-CD3 Abs plus CHO-CD80+ cells. The neutralising antibodies were tested routinely for efficacy in functional assays such as calcium mobilisation and chemotaxis. Over a range of 4 concentrations, the neutralising antibodies failed to affect submaximal proliferation (Table 3.2).
Figure 3.21 Effect of various chemokines on T-cell proliferation with single stimuli at 72h.

3H-Thymidine incorporation (x1000 cpm)

Vehicle RANTES MIP-1α MCP-1 IL-8 SDF-1 IP-10

Chemokine Treatment [10 nM]

Figure 3.21 Effect of various chemokines on T-cell proliferation with single stimuli at 72h. 6x10^4 cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72 hours and were assayed for 3H-thymidine incorporation with a β-scintillation counter as described in Materials and Methods. T-cells were incubated with Vehicle : 0.1% BSA or chemokine, concomitant to the various stimulations as described in Materials and Methods. Results are expressed as x1000 CPM and represent means of 3 independent experiments with sem. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
Table 3.2  Effect of various chemokines and chemokine-neutralising antibodies on submaximal anti-CD3/ CHO-CD80 co-stimulated T-cell proliferation.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Basal</th>
<th>1 μg/ml anti CD3 mAb + CHO-B7.1(3:1 T cell)</th>
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<tr>
<td>RANTES</td>
<td></td>
<td>19000 ± 2100</td>
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<tr>
<td></td>
<td></td>
<td>18000 ± 2200</td>
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<td></td>
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<td>20000 ± 2300</td>
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<tr>
<td></td>
<td></td>
<td>19000 ± 2000</td>
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<tr>
<td>MCP-1</td>
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<td>19000 ± 2100</td>
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<tr>
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<td>19000 ± 2050</td>
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<td></td>
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<td>19000 ± 2000</td>
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<td>anti MCP-1 Ab</td>
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<td>anti MIP-1α Ab</td>
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<tr>
<td>Mouse IgG</td>
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<tr>
<td>Chemokines in [nM]</td>
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<td>Vehicle</td>
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<tr>
<td>Antibodies in [μg/ml]</td>
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</table>

Legend for Table 3.2
6x10⁴ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72h and were assayed for ³H-thymidine incorporation with a β-scintillation counter as described in Materials and Methods. T-cells were stimulated as described in Materials and Methods. Chemokines, chemokine-specific neutralising antibodies, isotype-matched control mouse IgG, or vehicle: 0.1% BSA, were given together with the stimulations. Results are expressed as CPM ± sem and represent means of 3 independent experiments.

The lack of effect of chemokines and neutralising chemokine Abs may have reflected changes in chemokine receptor surface expression and availability during the course of the assay. Therefore, again using the natural ligand for CD28 presented on CHO cells as APCs, submaximal T-cell proliferation with CD3 ligation and CHO-CD80⁺ cells, 10 nM RANTES or 10μg/ml anti-RANTES neutralising Ab were added at different times to the CD3/CD80 stimulated cell cultures. The time of administration ranged from 15 minutes prior co-stimulation, to 24 hours after co-stimulation. Again, no effect on
proliferation was observed at any time point with either RANTES or neutralising anti-RANTES antibody (Table 3.3).

The effect of RANTES or anti-RANTES neutralising antibody was examined on T-cell proliferative responses with PMA alone, Ionomycin alone, and PMA in combination with CD28 stimuli. The addition of 10nM RANTES or 10μg/ml anti-RANTES antibody failed to influence T-cell proliferations induced by PMA with anti-CD28 antibody, or PMA with CHO-CD80+ cells, or PMA alone, or the basal cpm resulting from Ionomycin alone (Fig. 3.22).

Table 3.3 Effect of RANTES and neutralising anti-RANTES antibody administered at various times on submaximal anti-CD3/CHO-CD80 co-stimulated T-cell proliferation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of RANTES or anti-RANTES antibody addition (t)</th>
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<tbody>
<tr>
<td></td>
<td>-15 min</td>
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<tr>
<td>None</td>
<td></td>
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<tr>
<td></td>
<td>1400 ± 450 cpm (t = 0)</td>
</tr>
<tr>
<td>Anti-CD3 / B7.1</td>
<td></td>
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<tr>
<td></td>
<td>19500 ± 2300 cpm (t = 0)</td>
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<tr>
<td>+ RANTES 10nM</td>
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<tr>
<td></td>
<td>21000 ± 2200 cpm</td>
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<tr>
<td>+ anti-RANTES Ab 10μg/ml</td>
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<tr>
<td></td>
<td>21000 ± 2000 cpm</td>
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</tbody>
</table>

Legend for Table 3.3

Effect of RANTES and neutralising anti-RANTES antibody administered at various times on submaximal anti-CD3/CHO-CD80 co-stimulated T-cell proliferation. 6x10⁴ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72h and were assayed for ³H-thymidine incorporation with a β-scintillation counter as described in Materials and Methods. T-cells were stimulated at time t = 0, as described in Materials and Methods. RANTES or neutralising anti-RANTES Ab was administered at t = -15 min to t = + 24 h, as indicated. Results are expressed as CPM ± sem and represent means of 3 independent experiments.
Figure 3.22 Effect of RANTES and neutralising anti-RANTES antibody on PMA co-stimulated T-cell proliferation.

In order to assess chemokine signalling on the various cell populations it was important to establish if the effects or lack of effects observed, could be correlated to suitable chemokine receptor expression. Expression on the surface could only be assessed with soluble antibodies specific for chemokine receptors, which were of limited availability at the time of experimentation. Unfortunately the only available antibodies for surface expression assessment were against CXCR4, CCR5 and CCR3. Due to antibody availability against chemokine receptors, only CCR3, CCR5 and CXCR4 were assessed on resting T-cells. After purification only CCR5 but not CCR3 could be detected on a small percentage (~14%) of resting T-cells (Fig. 3.23). Another approach to chemokine receptor expression was to examine the presence of mRNA levels of particular receptors with RT-PCR. This approach involved PCR amplification and is a powerful qualitative way of checking the presence or absence of mRNA for receptors to which forward and reverse primers were available. Hence mRNA RT-PCR was performed for CCR1, CCR2, CCR3, CCR4, CCR5, CXCR1, CXCR2, CXCR3, CXCR4 and a house-keeping gene for β-actin.
Figure 3.23 CCR3 and CCR5 surface expression on (A) peripheral T lymphocytes and (B) Jurkat cells.

Figure 3.23 CCR5 and CCR3 surface expression on various cell types. 1 x 10^5 cells / tube purified resting T-cells (A) or Jurkat cells (B) were stained with 7B11 anti-CCR3 antibody or 2D7 anti-CCR5 antibody (red histograms) or isotype matched mouse IgGs (black histograms) and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm), as described in Materials and Methods. Expression levels for A) : 3 % isotype controls, 3 % CCR3, 14 % CCR5, expression levels for B) : 5 % isotype controls, 5 % CCR3, 5 % CCR5. Results from one experiment representative of three other independent experiments.
Resting purified T-cells consistently had mRNA for CXCR4 and with isolation and in-vitro exposure after 3 days there was consistent mRNA upregulation for CCR4 and CXCR3 (Fig. 3.24). In addition to CCR4 and CXCR3, mRNA for CCR1 was detected after 3 day CD28 ligation, CD2/CD28 co-stimulation and PMA/Ionomycin treatment (Fig. 3.24). Moreover, CD3/CD28 costimulated T-cells expressed mRNA for CCR5 whereas CXCR4 mRNA could be always detected in treated or untreated cells (Fig. 3.24). However the presence of mRNA for a particular receptor did not guarantee surface expression available for ligation. Indeed when CXCR4 surface expression was monitored days after isolation in conjunction with the activation marker CD69, an inverse correlation became evident. Resting T-cells immediately after isolation expressed 2% (basal) CD69 and ~80% CXCR4, however after 72 hours in-vitro incubation without any other treatment, purified T-cells expressed 16% CD69 whereas CXCR4 expression declined to 60%. Transformation of the T-cells into IL-2 maintained T-blasts at day 5 reduced CXCR4 expression to ~10%, but CD69 stained to ~20% (Fig. 3.25). Hence, surface CXCR4 was clearly detected on purified T-cells but expression declined significantly on T-lymphoblasts, whereas those changes in surface expression were not reflected with changes in CXCR4 for mRNA expression.

The effect of CD3 or CD2 activation of T-cells as opposed to plain in-vitro exposure on CXCR4 surface expression was examined with great difficulty due to antibody cross-reactivity. Unfortunately, analysis of CXCR4 surface expression on T-lymphoblasts derived from purified T-cell CD3/CD28 or CD2/CD28 antibody costimulation was hampered by the fact that the stimulating antibodies could not be removed and were stained with the secondary FITC-conjugated antibody used to detect CXCR4-bound antibody. The resulting high background fluorescence and artificial peaks impeded routine analysis (Fig. 3.26). Nevertheless, several chemokines were tested for their ability to influence submaximal CD3- or CD2- and CD28-costimulated proliferation. Soluble antibody ligation of CD3 with CD28 and CD2 with CD28 provided comparable submaximal proliferation (Fig 3.4), but not equal RANTES production (Fig 3.5), which could allow for differential effects of chemokines present during costimulation. The effect of three chemokine concentrations added with the respective costimuli was examined. With 1 – 100 nM of RANTES (Fig. 3.27a), of IP-10 (Fig. 3.27b), of SDF-1 (Fig. 3.27c), or of all these three chemokines together (Fig. 3.27d), there was no observable effect on CD3/CD28 or CD2/CD28 proliferation levels.
Figure 3.24 Chemokine receptor mRNA regulation in purified T-lymphocytes.

RT-PCR was performed on mRNA extracted from 5x10⁶ freshly purified resting or stimulated T cells per point, as indicated and described in Materials and Methods. D0 denotes freshly purified T-lymphocytes, whereas D3 denotes 72 hours in culture. Results are from single experiments representative of 3 other replicate experiments.
Figure 3.25   Effect of *In-Vitro* exposure on CXCR4 and CD69 surface expression of purified T-lymphocytes and T-lymphoblasts.

**DAY 0 Resting T-lymphocytes**

- **CD69**: 4%
- **CXCR4**: 80%

**DAY 3 *In-vitro* "resting" T-lymphocytes**

- **CD69**: 16%
- **CXCR4**: 60%

**DAY 5 IL-2 maintained T-lymphoblasts**

Figure 3.25. Effect of *in-vitro* exposure on CXCR4 and CD69 surface expression of purified T-lymphocytes and T-lymphoblasts. 1 x10^6 freshly purified T-lymphocytes, purified T-lymphocytes after 72 hour *in-vitro* incubation or 1 x10^6 day 5 IL-2 maintained T-lymphoblasts (from PHA) were stained with anti-CXCR4 or anti-CD69 antibody (red histograms), or isotype matched control antibodies (black histograms) and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm), as described in Materials and Methods. Expression levels shown are from one experiment representative of all purified T-cells and PHA transformed T-lymphoblasts used elsewhere. SEB transformed T-lymphoblasts displayed similar levels of CXCR4 expression after culture with IL-2.
Figure 3.26 Effect CD3/CD28 and CD2/CD28 costimulation of purified T-lymphocytes on CXCR4 surface expression after 72 hours.

CD3/CD28 activated T-cells

CD2/CD28 activated T-cells

Figure 3.26. Effect CD3/CD28 and CD2/CD28 costimulation of purified T-lymphocytes on CXCR4 surface expression after 72 hours. Purified T-lymphocytes were co-stimulated with Dynalbead-conjugated antibody combinations as shown and described in Materials and methods. Following incubation, 1 x10^6 cells / point from each co-stimulation were stained with 12G5 anti-CXCR4 antibody or isotype matched mouse IgG2a and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm ) as described in Materials and Methods. Expression levels shown are from one experiment representative of all CD3 or CD2 and CD28 co-ligation-activated T-cells used elsewhere.
Figure 3.27 a, b, c and d. Effect of various chemokines alone or in combination, on submaximal CD3 or CD2 co-stimulated T-cell proliferation.

Results

Figure 3.27 a, b, c and d Effect of various chemokines alone and in combination, on submaximal CD3 or CD2 co-stimulated T-cell proliferation. $6 \times 10^4$ cells/well purified T-lymphocytes were treated in quintuplicate as indicated in 96-well plates, harvested after 72 hours and were assayed for $^3$H-thymidine incorporation with a $\beta$-scintillation counter as described in Materials and Methods. T cells were stimulated as described in Materials and Methods. Results are expressed as cpm ± sem and represent means of 3 independent experiments.
Figure 3.28 Chemokine receptor mRNA regulation in SEB transformed T-lymphoblasts.

RT-PCR was performed on mRNA extracted from 5x10⁶ SEB-derived T lymphoblasts per point, as described in Materials and Methods. D3-D8 denotes the days following SEB administration, arrows indicate origin of cells stimulated with the treatments in brackets. Anti-CD28 antibody was administered as described in Materials and Methods. Results are from single experiments representative of 3 other replicate experiments.
Figure 3.29 Chemokine receptor mRNA regulation in PHA transformed T-lymphoblasts.

RT-PCR was performed on mRNA extracted from 5x10⁶ PHA-derived T-lymphoblasts per point, as described in Materials and Methods. D3-D8 denotes the days following PHA administration, arrows indicate origin of cells stimulated with the treatments in brackets. Anti-CD28 antibody was administered as described in Materials and Methods. Results are from single experiments representative of 3 other replicate experiments.
3.5 Effect of IL-2 and CD28 ligation on T cell chemokine receptor mRNA expression

Following the 72 hour transformation, SEB T-lymphoblasts expressed mRNA for CCR4, CXCR3, CXCR4 and faint CCR1; exposure to 20 ng/ml IL-2 for 2 days upregulated mRNA for all receptors tested at day 5 (Fig. 3.28). The effect of IL-2 was reversible since washing and deprivation of IL-2 for 2 days caused a loss in all receptor mRNA at day 7, except for the cardinal activated profile of CCR4, CXCR3 and faint CXCR4 (Fig. 3.28). Re-introduction of IL-2 for 24 hours upregulated mRNA for CCR5 and faint CCR1 and CCR2 at day 8, however, mRNA upregulation for CCR5 and CCR1 was inhibited by concomitant 24 hour CD28 ligation (Fig. 3.28). Similarly, PHA T-lymphoblasts expressed mRNA for CCR4, CXCR3, CXCR4 and faint CCR1 at day 3; exposure to 20 ng/ml IL-2 for 2 days upregulated mRNA for all receptors tested at day 5 apart from CCR3 and CXCR2 (Fig. 3.29). Again, the IL-2 effect was abrogated after washing and deprivation of IL-2 for 2 days; at day 7 only mRNA for CCR4, CXCR3 and faint CXCR4 was detected (Fig. 3.29). Re-introduction of IL-2 for 24 hours upregulated mRNA only for CCR1 at day 8 which was again inhibited by concomitant 24 hour CD28 ligation (Fig. 3.29).

SUMMARY

1. No chemokine tested at any concentration was capable of providing efficient costimulation in combination to single CD3, CD2 or CD28 ligation and also could not maximise submaximal CD3 or CD2 and CD28 costimulated proliferations.


3. Time of administration of RANTES or anti-RANTES antibody did not affect the lack of noticeable effect of those treatments on submaximal proliferations by CD3/CD28.

4. RANTES or anti-RANTES antibody did not affect proliferation induced by costimulations involving PMA.
5. With the available antibodies surface CCR5 could only be detected on a subpopulation of resting T cells but surface CCR3 could not be detected.

6. Expression levels of surface CXCR4 inversely correlated with CD69 expression levels. Activation of resting purified T cells with CD3/CD28, CD2/CD28 as well as T lymphoblast transformation resulted in markedly lower surface CXCR4 levels.

7. Resting T cells contained mRNA for CXCR4 only, which could also be detected in activated T cells and T-lymphoblasts. In-vitro exposure in the absence of other stimulation, as well as any type of mitogenic costimulation consistently up-regulated mRNA for CXCR3 and CCR4.

8. mRNA for most other CC and CXC chemokine receptors was reversibly up-regulated on T lymphoblasts with the addition of IL-2, whereas CD28 ligation down-regulated CCR1 and CCR5 mRNA expression in the presence of IL-2.
DISCUSSION I

T CELL COSTIMULATION AND RANTES PRODUCTION

For this study, the established main T cell activation pathways were used, namely the antigen receptor dependent pathway involving signals provided by CD3/TCR complex antibody ligation together with CD28 (Lenschow et al. 1996), an antigen receptor independent pathway which required ligation of CD2 with a pair of CD2 antibodies directed against different epitopes (Meuer et al. 1984; Olive et al. 1986) together with ligation of CD28 (Costello et al. 1993), or the pharmacological approach with phorbol esters such as PMA in combination with either calcium ionophores or Abs/natural ligands to CD28 (June et al. 1987; Ueda et al. 1995; Costello et al. 1993). Proliferation of T cells via CD3/CD28 or CD2/CD28 costimulation models with anti-CD28 antibodies conjugated onto beads or CHO-CD80+ cells was enhanced in comparison to proliferation from stimulations with soluble cross-linked antibodies. This improvement in proliferative responses was probably due to optimised signal delivery from presentation of costimulatory ligands in a physiological 3-D context. CD3/CD28 and CD2/CD28 costimulations were affected to a similar degree, in accordance with the notion that both types of costimulation are thought to involve similar intracellular signalling, such as activation of MEK1 and NFAT (Hutchcroft et al. 1998).

CD80 was documented to be advantageous as a costimulatory ligand, as CD80 was reported more potent for PLCγ activation than CD86 or anti-CD28 antibody, whereas only CD80, and to less extent anti-CD28 antibody, was able to cause CD28 cytoplasmic tail phosphorylation (Slavik et al. 1999). However, both CD80 and CD86 costimulated NFAT activation, cytokine production and multiple protein tyrosine phosphorylation alike (Slavik et al. 1999), as well resulting in identical levels of PI3K activation (Ueda et al. 1995). In this study, proliferation was enhanced by the substitution of soluble anti-CD28 antibodies with CHO cells expressing CD80, but when antibody-coated beads were utilised, anti-CD28 antibodies tended to drive proliferation to an equal, or even greater degree than CD80 on CHO cells, combined with either CD3 or CD2 costimulation. This may suggest an intrinsic negative regulatory activity from the natural CD28-ligand CD80, absent when anti-CD28 antibody treatment is used. This property of CD80 could either be due to more efficient induction of CTLA-4 expression, or more efficient negative regulatory signalling by CD28 via enhanced Cbl, Itk or SHIP.
recruitment (Liao et al. 1997; Bachmaier et al. 2000; Chiang et al. 2000). Certainly, enhancement of T cell proliferation from bead-conjugated compared to soluble antibody stimulation, corroborates reports of efficient long term T cell proliferation promotion only when CD28 antibody was conjugated onto beads, and not when soluble CD3/CD28 antibodies, or combinations of CD3 antibody ligation/IL-2, PHA/IL-2 or PMA and IL-2 were used for T cell costimulation (Levine et al. 1997).

However RANTES, whose name suggests a dependence on T cell activation, proved to be regulated by pathways not necessarily identical to those required for proliferation. Upon examination of the stimuli required for secretion of RANTES from T lymphocytes the phorbol ester PMA, which is unable to support T cell proliferation, was found to be able to elicit strong secretion of RANTES. Conversely, stimuli such as CD2 and CD28 ligation which were able to support T cell proliferation were unable to elicit RANTES secretion, whereas T cell proliferation by CD3 and CD28 co-ligation was driven to a similar degree as CD2 and CD28 co-ligation, yet also supported modest RANTES secretion. CD3 can support RANTES secretion from highly purified T cells in the presence of CD28 stimulation. However, it appears that CD2 is unable to provide a key signal(s) that can complement those provided by CD28 in order to support RANTES production, although CD2 and CD28 causes T cell activation and induces production of cytokines (Costello et al. 1993; Cerdan et al. 1992; Cerdan et al. 1991).

It is surprising that CD2 ligation is unable to support RANTES secretion in the presence of CD28 stimulation since previous observations are consistent with CD2 generating similar intracellular biochemical signals to TCR/CD3 complex. For instance, they both stimulate Ras (Graves et al. 1991), activate phospholipase C and elevate 

$[Ca^{2+}]_i$

(Pantaleo et al. 1987; Weiss et al. 1984), activate PKC (Friedrich et al. 1989), stimulate PI3K and the accumulation of D-3 phosphoinositide lipids (Ward et al. 1992) as well as stimulate tyrosine phosphorylation of CD3ζ (Monostori et al. 1990) and Lck (Danielian et al. 1989). However, whilst it has been demonstrated that CD3 and CD2 stimulate the tyrosine phosphorylation of a similar pattern of polypeptides (Ley et al. 1991), they may not be identical. Indeed, CD2 ligation unlike CD3, is unable to induce tyrosine phosphorylation of HS1, a signalling protein of unknown function (Hutchcroft et al. 1998).
The time course profile of RANTES secretion observed from the various costimulations follows a similar profile to that of distinct transcription factors for the RANTES gene. Hence, in activated T cells, there are distinct early and late-acting responsive elements within the RANTES promoter that bind distinct transcription factors. For instance, the "early" site interacts with NFIL6 within 24 hours and the "late" site interacts with the novel R(C)-FLAT and 'E'-region FLAT after 3 to 5 days post stimulation (Ortiz et al. 1996). The late (3-5 day) regulatory region of the RANTES promoter was found to be critical for RANTES production by T cells (Nelson et al. 1996). Although the investigations on RANTES transcription factor activation were performed with lectin-transformed T-lymphoblasts, with all stimulations causing RANTES release in this study, maximum levels were not detected prior to 72 hours. This result does not support the notion of RANTES being produced and stored in vesicles in T cells for any length of time prior to its secretion, unlike RANTES de-granulation from platelets (Schall TJ 1991). Hence, measurements of secretion at 72 hours could be considered to correlate with 'fresh' RANTES production. The quantities of RANTES and MIP-1α secreted by CD3/CD28 costimulated human T cells after 72 hours found in this study, were similar to those reported elsewhere as 19 ng/ml and 137 ng/ml, respectively (Riley et al. 1997).

The data indicates a strong up-regulation of RANTES secretion by PMA alone that involves activation of PKC isoenzymes, since RANTES secretion was unaffected by the non-PKC activating 4α-phorbol ester. This effect of PMA appears to be chemokine-specific since PMA was unable to increase levels of the related CC chemokine MIP-1α. Moreover, the relatively specific PKC inhibitor RO-32-0432, abolished PMA-stimulated or any other costimulated RANTES secretion. The PKC inhibitors used in this study, RO 328220 and the most recent RO 320432, although based on bisindolemaleimide analogues of staurosporine, have improved selectivity for PKC isoforms over other kinases. Both these compounds specifically block PKCα, PKCε, PKCγ, PKCβI and PKCβII (Beltman et al. 1996; Wilkinson et al. 1993). However, RO 328220 has been reported to have effects on other enzymes such as inhibition of Mitogen-Activated-Protein-Kinase-Phosphatase, activation of JNK and induction of c-Jun expression (Beltman et al. 1996). It is also possible that other elements within the RANTES promoter may respond to PMA targets other than PKCs, and such targets could also lack from the 4α-phorbol ester spectrum of activity. Other studies showed that phorbol esters can activate the 9E3/cCAF chemokine gene via multiple signal transduction pathways.
that were characterised predominantly PKC-independent (as assessed by resistance to Calphostin C) and which converged on MEK1/ERK2 and activated the Elk1 transcription factor (Li et al. 1999). In this respect, activation of MAPK by PMA was portrayed to require only functional Raf-1 and not PKC (Della Roca et al. 1997), supporting the notion that PMA may have additional targets distinct from PKC (eg. Raf-1), that may also support the RANTES secretion observed. PKC can regulate a number of T cell activation genes via control of transcription factors and the RANTES promoter region contains PKC responsive elements such as sites for NFkB, AP-1 and NF-AT (Moriuchi et al. 1997). Mutation of these sites has been found to disrupt PMA-ionomycin stimulated RANTES promoter activity (Moriuchi et al. 1997). Interestingly, CD3 and CD28 have both been reported to activate PKC (Ward 1996, Friedrich et al. 1989), although ligation of either receptor alone is unable to support RANTES production. This may suggest that PKC activation has to occur at critical thresholds of stimulation to enable RANTES secretion. Hence, whilst stimulation of PKC by PMA is sufficient to cross this threshold, either CD3 or CD28 alone provide insufficient activation of PKC to support RANTES secretion.

The role of IL-2 in the regulation of chemokine production has been addressed with CD3/CD28 costimulated murine T cells, where antibody blockade of CD80 and CD86 interaction with CD28 disrupted IL-2 production and IL-2-driven T cell proliferation. However, only MIP-1α but not RANTES secretion inhibited, implicating CD28 and not IL-2 signalling for MIP-1α production (Herold et al. 1997). The contribution of IL-2 in RANTES secretion by T lymphoblasts appeared to be equal and superimposable to costimulation signals that can induce IL-2 production. CD3/CD28 or CD2/CD28 costimulated IL-2 production (and proliferation) is likely to be an event distinct from RANTES production as the latter occurred only with CD3/CD28 costimulation (Figure I. a). Moreover, absence of IL-2 production with PMA or PMA/CD28 T cell costimulation does not correlate with the highest levels of concomitant RANTES secretion observed, although PMA/CD28 may stimulate signalling pathways that are shared with IL-2R activation. PMA-stimulated RANTES secretion was further enhanced by CD28 antibody ligation. There are several potential explanations for this observation. Firstly, an AP1/CRE like element at about -215 to -192 has been identified in the RANTES promoter (Peter Nelson, personal communication) and CD28 has been demonstrated to synergistically activate cAMP-responsive-element binding protein in T lymphocytes
(Hsueh et al. 1997). This mechanism may underly the enhanced RANTES secretion observed in response to CD28/PMA. Secondly, CD28 has been demonstrated to enhance PMA-stimulation of NFκB as well as AP-1 (Boulougouris et al. 1999) and one of the NFκB binding sites serves as a CD28RE (Ortiz et al. 1996; Moriuchi et al. 1997). In this respect, bronchial epithelial cell RANTES production induced by respiratory syncytial virus was reported dependent on NFκB nuclear binding (Thomas et al. 1998). Since CD28 elicits little if any calcium mobilisation (Ward et al. 1992), this suggests that further biochemical signals can contribute to and exacerbate RANTES promoter activity along with those signals provided by CD3. A major biochemical event elicited by CD28 is activation of the PI3K-dependent signalling cascade (Parry et al. 1997). Downstream effectors of this pathway include PKB (Parry et al. 1997), which has been shown to be involved in activation of NFκB in several systems including T cells (Romashkova, Makarov, 1999; Ozes et al. 1999; Kane et al. 1999). It is therefore tempting to speculate that the enhancement of PMA-stimulated RANTES secretion due to CD28 ligation is mediated by PI3K-dependent signalling. Studies with PI3K inhibitors revealed that a functional PI3K-dependent signalling mechanism is indeed required for the CD28-mediated enhancement of PMA-stimulated RANTES secretion (see below Regulation of T cell Activation and RANTES production).

However, whilst CD28-stimulated AP-1 generation is inhibited by PI3K inhibitors, it should be noted that CD28-mediated induction of NFκB is resistant to PI3K inhibitors in a T lymphoblast cell model (Edmead et al. 1996), and the contribution of other pathways cannot therefore be discounted. Figure 1b displays a diagram of the RANTES promoter with the putative key players arising from surface receptor ligation and PMA. Moreover, no enhancement of RANTES secretion was observed when CHO-CD80+ cells were combined to concentrations of PMA (5 ng/ml) which alone gave optimal RANTES release, suggesting an intrinsic regulatory activity from CD80 compared to anti-CD28 antibody more prominent with RANTES secretion than with proliferation. The disparity between anti-CD28 antibody and CD80 could involve: i) different signalling pathways or, ii) different thresholds of activation for RANTES secretion, so that in order to match the anti-CD28 antibody enhancement of RANTES release, either more CD80 ligand or more PMA would be required. Interestingly, the increase in RANTES secretion observed with the combination of CHO-CD80+ cells and the higher concentration of PMA (50 ng/ml), does not exceed the optimal levels of RANTES secreted by the lower PMA concentration (5ng/ml) alone.
Figure 1. A Schematic summary of the divergence between signals required for IL-2 production/proliferation and RANTES production.

Red denotes CD3 and CD3-derived signalling, black denotes CD28 and CD28-signalling, yellow denotes CD2 and CD2-derived signalling and purple represents signalling events required for IL-2 production and proliferation. The question mark inside the octagon represents unknown signalling entities which, like HS1, are activated only following CD3 stimulation. The question mark in the circle denotes the unknown nature of integration (potentiation, synergy, antagonism, etc.) between CD3-specific and CD3/CD28 signals required for RANTES production.
Figure I. b  Schematic diagram of the RANTES promoter and the upstream identified requirements for transcription activation.

Identified transcription factors and their relative binding sites on the RANTES promoter. Solid black arrows represent transcription factors / binding as a result of activation of the upstream receptors / signalling molecules indicated. Arrows with plus sign (+) represent CD28-mediated synergistic activation-enhancement of established activation pathways mediated by PKC/PMA. Dotted arrows indicate unidentified interactions with (?): unidentified transcription factors / regulators. The graph on the top right corner shows the time profile of three transcription factors after T cell stimulation, red : NFIL6, blue : R(C)FLAT, green : “E”-region FLAT. Interestingly, the maximal increase in RANTES secretion occurred at day 3, coinciding with the time point where all these three transcription factors are expressed at roughly equal levels. See also text for details. Adapted from Ortiz et al. 1996.
This may suggest a concentration-dependent shift in the intracellular target repertoire for PMA, some of which regulate and others which promote RANTES secretion. At high PMA concentrations the regulatory targets are possibly more prominent but may be antagonised more effectively by CD28 signalling.

REGULATION OF T CELL ACTIVATION AND RANTES PRODUCTION

CTLA-4 SIGNALLING
From the above data, it appears that under conditions where one or more stimuli (e.g. CD2, CD3 or ionomycin) can elicit increases in \( [\text{Ca}^{2+}]_i \), RANTES secretion can either be severely compromised or completely inhibited compared to the optimal amounts seen in response to the phorbol ester PMA. One possible interpretation of the data is that PKC-dependent pathways are sufficient for optimal RANTES secretion, whilst an as yet unidentified calcium-dependent factor is activated, which negatively regulates RANTES gene transcription/secretion. Certainly, a negative regulatory region of the RANTES promoter has been described (Moriuchi et al. 1997). Whilst there may well be an intracellular calcium-dependent factor(s) that can modulate RANTES gene/transcription, an alternative explanation for this consistent observation revolves around the fact that optimal mRNA induction and surface expression of CTLA-4 is dependent on a sustained calcium signal (Linsley et al. 1996; Freeman et al. 1992). Repeatedly activated T cells are known to upregulate expression of firstly B7.2 followed by B7.1 (Greenfield et al. 1997; Azuma et al. 1993) and the B7.2 on T cells has been suggested to preferentially bind CTLA-4 (Greenfield et al. 1997).

Whilst B7.1 and B7.2 up-regulation has been reported to occur much later (e.g. 10 days) than the time courses examined in this study, it is possible that these molecules are expressed at low undetectable levels. Given the very high affinity of CTLA-4 for its B7 ligands (Ward 1996), this low expression of B7.1 and/or B7.2 may be sufficient to exert functional effects via CTLA-4. Certainly, inhibitory anti-CTLA-4 Abs abrogated CD3/CD28-driven RANTES secretion in this study. Hence, the sub-optimal amounts of RANTES secretion observed in response to CD3/CD28 ligation may indeed reflect an inhibitory signal that is initiated following CTLA-4 binding B7 which is expressed on activated T cells. This explanation does not correlate too well with the observation that CD3 plus CD28 ligation is able to initiate robust T cell proliferation. However, T cell proliferation is driven by IL-2 production which is initiated within 6 hours of stimulation.
(June et al. 1987), whilst optimal RANTES gene up-regulation occurs much later at 72 hours (Ortiz et al. 1996). It is certainly possible therefore, that the mechanisms for IL-2 and RANTES gene transcription, mRNA stability, translation and/or secretory processes have different thresholds for regulation by CTLA-4. That is to say, inhibition of IL-2 secretion may occur at very robust levels of CTLA-4 activation, whilst inhibition of RANTES secretion may occur at quantitatively lower levels of CTLA-4 activation. Hence, the modest amounts of RANTES secretion observed in response to CD3/CD28 ligation may reflect a selective regulation of RANTES secretion by CTLA-4, robustly expressed, binding limited amounts of B7 expressed on activated T cells. Moreover, the lack of enhancement in RANTES secretion with PMA/CD80 at certain concentrations of PMA, may be due to ligation of a limited basal amount of CTLA-4 expressed, which can dominate over CD28 in contributing negative signalling for RANTES.

Integration of CTLA-4 in CD28 and TCR costimulation has been documented to provide regulation in proliferative responses, CD69 and IL-2Rα chain expression and cytokine secretion (Chambers, Allison, 1999), and CTLA-4*7 * deficient mice exhibit a severe lymphoproliferative disorder and multi-organ tissue destruction (Tivol et al. 1997). CTLA-4 antibody ligation was found to inhibit IL-2 secretion and proliferation of CD3/CD28 costimulated cells, but allowed Bcl-  expression and cell survival (Blair et al. 1998), corroborating the notion that especially in the absence of CD28 engagement, CTLA-4 can exert signals in part complimentary to CD28. Moreover, apart from CTLA-4, there is evidence that negative signalling via CD28 is also very important in regulating T cell activation, as T cells from Itk* or Cbl * deficient mice exhibit hyper-responsiveness to CD3/CD28 resulting in marked hyper-proliferation (Liao et al. 1997; Bachmaier et al. 2000; Chiang et al. 2000). RANTES secretion from anti-CTLA-4/anti-CD28/PMA treatments appeared slightly elevated compared to secretion from CD80/PMA. This may indicate: i) a greater efficiency for CD80 rather than anti-CTLA-4 antibody in inducing inhibitory signalling via CTLA-4, ii) a greater efficiency for CD80 rather than anti-CD28 antibody in inducing inhibitory signalling via CD28, iii) marginally higher levels of surface CTLA-4 expression induced by CD28 signalling via ligation with CD80 rather than anti-CD28 antibody. The specificity of the anti-CTLA-4 Ab inhibitory effect was confirmed by the fact that it inhibited CD3/CD28-stimulated RANTES secretion but had no effect on RANTES secretion in response to PMA. This correlates with the fact that PMA has previously been reported to be unable to induce detectable CTLA-4 expression in this cell model (Boulougouris et al. 1999).
Apart from simply reflecting a lack of CTLA-4 expression, the lack of effect of anti-CTLA-4 Abs on PMA-stimulated RANTES secretion could be attributed to a distal site of action for PMA, bypassing the inhibitory targets of CTLA-4. However, the former explanation is more likely as maximal CTLA-4 expression requires a sustained calcium signal (Linsley et al. 1996; Freeman et al. 1992), which is lacking when cells are stimulated with PMA alone.

**PI3K inhibition**

There has been some controversy over the specificity of widely used inhibitors which calls for caution over the interpretation of results. The structurally unrelated PI3K inhibitors Wortmannin and LY 294002 were reported to directly block mTOR autokinase activity at low concentrations (Brunn et al. 1996), and their inhibitory activity on insulin signalling was indistinguishable from that of the translation inhibitor Rapamycin (Srivastava, 1998). Although non-specific effects of PI3K inhibitors could be responsible for this observation, the mechanism of activation of p70S6K, one PI3K effector, might explain a convergence of the sites of action of the two types of inhibitors, on a common pathway. Catalytic activation of p70S6K is thought to occur following phosphorylation by the PI3K-dependent PDK-1 (Wortmannin sensitive), as well as interaction with mTOR/FRAP (Rapamycin sensitive). Even so, the potential for Wortmannin and LY294002 to affect lipid kinase-like domains on other high molecular weight PI3K homologues awaits further confirmation and should not be discounted (Brunn et al. 1996). Proliferation, and even more so, RANTES secretion from stimulations incorporating either TCR/CD3 ligation or Ionomycin were more sensitive and were inhibited partially by Wortmannin, in contrast to PMA-costimulated proliferation and RANTES secretion which were more resistant to inhibition by Wortmannin. The results suggest that the modest RANTES secretion from costimulations involving CD3 results from a combination of PI3K-dependent and PI3K independent signalling pathways. Certainly, costimulations incorporating CD28 ligation and CD3 were inhibited with the greatest potency by Wortmannin in either proliferation or RANTES secretion assays. Moreover, an interesting facet of CD28-enhancement of PMA-stimulated RANTES secretion was that, unlike NF-κB induction (Edmead et al. 1996), the CD28 effect appeared to be sensitive to PI3K inhibition since it was abolished by Wortmannin, suggesting PI3Ks as a key CD28 downstream effector promoting RANTES secretion.
Cyclosporin A and Rapamycin

A pattern of partial inhibition of RANTES secretion from costimulatory treatments known to cause robust calcium mobilisation (CD3, Ionomycin), but no inhibition in RANTES secretion from treatments with PMA, was also observed with Cyclosporin A. A pathway dependent on calcineurin is selectively affected by CsA which can block certain types of T cell activation proving a clinically valuable immunosuppressant. T cell proliferation stimulated by CD3/CD28 was inhibited by CsA. However, T cell proliferation stimulated by PMA and anti-CD28 ligation was unaffected, an effect observed previously (June et al. 1987). Although CsA inhibited RANTES secretion from costimulations involving either TCR/CD3 ligation or Ionomycin, again, PMA-driven secretion was unaffected by CsA. Unlike PMA/CD28-stimulated proliferation, Cyclosporin-A displayed a tendency to attenuate the levels of RANTES detected from PMA/CD28 treatment (~70ng/ml) close to the levels seen with PMA alone (~40 ng/ml).

There are several reasons for the surprising effect of CsA on PMA/CD28-stimulated RANTES secretion: (i) It is possible that the DAG-sensitive PKC isoforms stimulated by PMA are different from PKC isoforms recruited in costimulations with CD28 (eg. PKC ζ), and as CsA has been shown to inhibit PKC isoform activity in T cells (Szamel et al. 1993), such a CsA-sensitive PKC may coincide with the PKC involved in the CD28-induced enhancement of RANTES secretion by PMA. Importantly, the inhibitory effect of CsA on PKC appeared to be indirect, affecting targets downstream of PKC, like the activation of nuclear Activator of DNA Replication (ADR), rather than direct PKC inhibition (Kimball et al. 1993). Regulation of NF-κB in T cells was shown to involve a convergence of calcium-dependent, CsA-sensitive pathways with PKC-dependent and additional PKC-independent pathways, at the level of IκBα phosphorylation and degradation (Steffan et al. 1995). (ii) There is evidence that extracellular calcium influx in different cell types can synergise or antagonise NF-κB activation by PMA (Aoki et al. 1997). Hence, it is possible that calcium influx may activate multiple signalling pathways, some that limit, together with others that are essential for CD3/CD28-stimulated RANTES secretion. In the presence of PMA, a modest rise in intracellular calcium levels occurring with cross-linked CD28 antibody ligation, may not be sufficient to counteract the activation of additional PI3K-dependent PKC isoforms (Derman et al. 1997). The net effect is an increase in RANTES production, already robust in response to PMA. CsA has been shown to potentiate calcium influx in platelets in response to
thrombin (Haller et al. 1994), so it may be that such CsA-dependent augmentation of CD28-induced calcium influx can effectively counteract the increment in PMA-induced RANTES secretion in response to CD28. An inhibitory profile similar to CsA was evident with Rapamycin potently affecting RANTES secretion from CD3/CD28 costimulation, but not inhibiting greatly PMA-stimulated RANTES secretion. Activation of p70S6 kinase is a process which has been documented to rely on upstream PI3K-dependent signalling, involving effectors such as PKB, PDK1 and PKCζ (Nave et al. 1999; Romanelli et al. 1999). The engagement of such signalling moieties from CD3/CD28 costimulation is to be expected given the involvement of CD28. However, RANTES secretion by PMA appears to bypass the requirement for mTOR activation. This could result because: (i) PMA affects PKC(s) / PKC-independent targets which contribute to p70S6K activation downstream of mTOR, (ii) PMA stimulates PKC-independent targets that mediate RANTES secretion only partly relying or independent of p70S6K activation. In contrast to CsA, PMA/Ionomycin-stimulated RANTES secretion was not inhibited by Rapamycin, presumably because the calcium influx afforded by Ionomycin does not affect the activation of p70S6K more than it compromises the robust RANTES secretion from PMA stimulation alone.

**Ras and Protein Tyrosine Kinase inhibition**

Established signalling events associated with CD3/CD28 costimulation involve transient activation of Ras and concomitant engagement of non-receptor tyrosine kinases (Heldin, Purton 1996; Vojtek, Der 1998). As molecular approaches or specific inhibitors were not available, the involvement of Ras and PTKs in supporting RANTES secretion were examined with the use of Manumycin A, a farnesyl-transferase inhibitor capable of blocking Ras-mediated signalling (Hara et al. 1993) and Herbimycin A, a competitive, reversible inhibitor of protein tyrosine kinase(s) (Satoh et al. 1992). The inhibitory effects of Manumycin A and Herbimycin A on RANTES secretion, similar in severity to PKC inhibition, may be due to inhibition of closely associated pathways. Herbimycin A was reported to have no effect on PKC or cAMP-dependent protein kinase, but could disrupt the pathway for Ras-mediated proliferation by blocking non-receptor tyrosine kinase(s) coupling to GAP/GEF regulators of Ras-GTP (Satoh et al. 1992). A similar effect with these inhibitors was reported for PMA-stimulated TNF-α production from U937 cells (Callaghan et al. 1996). There are several examples of PKC / Ras / PTK signalling integration, with PKC proposed to lie: (i) upstream of tyrosine kinase
cascades in angiotensin-II- and TGF-β-induced signal pathways (Motojima et al. 1999; Watanabe et al. 1998), (ii) downstream of tyrosine kinase cascades in hyperkapnia (CO₂)-induced cerebral vasodilation (Rama et al. 1997). Moreover, Ras-GTP-Raf complexes were found to be required for Raf-1 activation by PKC (Marais et al. 1998), whereas the DAG-insensitive PKC-ζ associated with Ras was shown to critically mediate Ang-II stimulation of ERK-1/2 (Liao et al. 1997).

**ERK and p38/MAPK inhibition**

Since the effect of Manumycin A on RANTES secretion was so dramatic and the MAPK pathway is one established downstream effector pathway for Ras, the effect of MEK inhibitor PD 98059 was examined on RANTES secretion. Moreover, as CD3/CD28-costimulated T-cell proliferation was shown to involve the related stress/p38/MAPK pathway (Ward et al. 1997), and CD3/CD28 co-ligation was reported to couple to the p38 MAPK pathway via ZAP-70 / Rac-1 / PAK-1 (Salojin et al. 1999) (sensitive to Herbimycin A), the effect of SB 203580 on RANTES secretion was examined. The inhibitory actions of PD 98059 and SB 203580 on the MEK-1/MAPK pathway and p38/MAPK pathway respectively have been elucidated in the past (Gould et al. 1995). PD 98059 affected RANTES secretion only slightly (20% inhibition) with PMA stimulation, but did achieve considerable inhibition of RANTES secretion from CD3/CD28 costimulation. SB 203580 affected RANTES secretion from PMA or PMA/Ionomycin stimulation to a similar degree as PD98059 (20% inhibition), but inhibited more robustly CD3/CD28-costimulated RANTES secretion. However, inhibition of the MAPK pathway either by PD 98059 (Dr. R.V. Parry personal communication), or genetically with dominant negative MEK-1 transgene expression (Alberola-Ila et al. 1995) was reported not to affect CD3/CD28 costimulated T cell proliferation. This is in contrast to the correlation shown between p38/MAPK inhibition by SB203580 and inhibition of T cell proliferation (Ward et al. 1997). Hence, although the p38/MAPK pathway, but not the MAPK pathway, is considered indispensable for T cell proliferation, both these two pathways appeared to partially contribute to CD3/CD28-stimulated RANTES secretion. PMA-induced RANTES secretion appeared capable of by-passing their requirement, although the slight attenuation witnessed with the higher concentrations of either inhibitor, implied a modest degree of PMA-driven stimulation of MAPK and p38/MAPK activation that is redundant for the majority of RANTES detected. Figure Ic depicts the various sites of pharmacological inhibition.
and the hypothetical integration of the pathways affected, responsible for RANTES production/secretion by the T cell.

**THE ROLE OF CHEMOKINES IN T CELL COSTIMULATION**

Initially, β-chemokines like RANTES, MIP-1α and MIP-1β were reported to exert costimulatory signals with concomitant CD3 ligation (but not with PMA), capable of inducing CD25 expression, IL-2 secretion and T cell proliferation (Taub et al. 1996). An endogenous autocrine/paracrine role in T-cell activation was proposed for β-chemokines, distinct from their ability to order migration exemplified by the T cell infiltration of sites subcutaneously injected with MIP-1β on SCID mice (Murphy et al. 1996). In this study, chemokines failed to exert a role in T cell proliferation; the model of sub-optimal T-cell costimulation was chosen so that a window for detecting enhancement or suppression of responses was available and the lack of effect could not be discounted as beyond the limits of detection. Moreover, if there was a case for maximal stimulation of the cells, chemokines were still not capable of enhancing proliferation in combination with single stimuli like CD3 or CD28 ligation alone. Possible reasons for the disparity between these results and data published elsewhere may include: a) indirect facilitation of TCR/CD28 engagement via increased, chemokine-induced cellular motility to fixed, planar sites of costimulation (antibody-coated plates), b) increased chemokine-induced bystander-cell recruitment resulting from increased cellular polarisation and adhesion, c) different receptor expression and biochemical requirements for cellular activation between primary (normal) T cells, immortalised T-cell-lines, and *in-vitro* generated transfected cells or clones. If the supposed costimulatory effects of RANTES were mediated via CCR5 signalling, it may also be possible that the ~10% CCR5⁺ T cell subpopulation contained in freshly purified T cells from one donor would respond to RANTES-induced augmentation of CD3/CD28-costimulated proliferation, but this may be below the threshold of detection, thus being "drowned" by less proliferation of many more CCR5⁻ T cells.

Although RANTES and MIP-1α were reported to activate STAT-1:1 and STAT-1:3, cause tyrosine phosphorylation of multiple proteins and induce c-fos expression in Jurkat cells (Wong, Fish, 1998), curiously, the costimulatory activation of Jurkat cells by RANTES correlated with, and depended upon, CD3/TCR surface expression (Dairaghi et al. 1998).
Figure I. Schematic integration of the various pathways important for RANTES production / secretion from CD3/CD28 T cell costimulation.

Moreover, RANTES treatment of Th0 T cell clones was found to stimulate homotypic aggregation and phosphorylation of p125FAK, paxillin and ZAP-70 (Bacon et al. 1996). As similar signalling has been reported to occur on NK cells, where α4β1 or α5β1 integrin binding to fibronectin resulted in p125FAK, Fyn, Lyn and ZAP-70 phosphorylation (Rabinowich et al. 1996), it may be possible that signalling reported to occur with RANTES ligation could be a result of RANTES-upregulated adhesion molecule engagement. SDF-1 on B cells was shown to cause redistribution of CXCR4 to the leading edge and ICAM-3 to the posterior uropod, with bystander cellular recruitment reported to occur via LFA-1/ICAM-1 interactions (Vicente-Manzanares et al. 1998). Chemokine-induced activation and up-regulation of adhesion molecules is an established event required for cellular transmigration. Hence, in the absence of direct costimulation, an indirect facilitation of T cell costimulation by chemokines mediated by adhesion molecules in an in-vivo setting (eg. in the presence of activated endothelium), cannot be discounted. Moreover, the only chemokine receptor detected on the surface and at the mRNA level on freshly purified T cells was CXCR4. This may account for the lack of costimulatory effects of CC-chemokines but not of SDF-1. Surface expression of CXCR4 appeared very sensitive to in-vitro handling and was inversely proportionate to the activation state of the cells as assessed by CD69 expression. Moreover, in a recent BSI 1999 seminar, Dr. M. Dustin presented data which portrayed ligands for CCR7 and CXCR3 as the only ones capable of causing migration, disrupting immuno-synapse formation/co-stimulated activation involving TCR / CD28 / LFA-1 interactions. The model used a planar lipid layer onto which chemokines and adhesion molecules were embedded. SDF-1 or CCR5 ligands although chemotactic alone, were subservient to the TCR and could not disrupt synapse formation when initiated by the ICAMs. A potential
facilitation of synapse formation by SDF-1, was reported to be exerted via adhesion molecule upregulation only with murine TCR transgenic T cells. However, no facilitation was evident with normal T cells with a level of constitutive adhesion molecule expression and a normal potential for adhesive interactions.

CHEMOKINE RECEPTOR REGULATION

Regulation by mitogenic stimulation

In this study, mitogenic stimuli either by accessory cells and antigenic lectin, or CD3/CD28 costimulation certainly appeared to reduce surface expression of CXCR4, but invariably induced mRNA expression for CCR4 and CXCR3 and did not abrogate CXCR4 mRNA. The effect of transition from resting to activated T cell via CD3/CD28 costimulation on receptor expression has been studied elsewhere and TCR stimulation was reported to cause rapid and transient switch of surface chemokine receptor expression with CXCR4 least affected (Sallusto et al. 1999). Moreover, PHA stimulation was reported to upregulate CXCR4 and decrease CCR5 surface expression (Bleul et al. 1997).

Regulation by Cytokines

In the T lymphoblasts used in this study, IL-2 appeared to regulate the expression of mRNA for most receptors in a reversible fashion, except for CCR4, CXCR3 and CXCR4. However CD28 ligation could selectively inhibit the mRNA expression of CCR5 in the presence of IL-2. This effect of CD28 coupled to CD3 co-ligation, has important applications in HIV-1 ex-vivo adoptive therapy (Levine et al. 1996; Carroll et al. 1997). Elsewhere, CD3/CD28 costimulation was shown to dominate over the presence of IL-2 on Day 12 T lymphoblasts and cause a loss in CC receptor expression and migration to the respective CC chemokines (Loetscher et al. 1996). IL-2 has been reported to regulate chemokine receptor expression both in the level of mRNA and upregulation on the surface; in particular, IL-2 was shown to cause increases in mRNA for CCR2A and CCR2B in NK cells (Polentarutti et al. 1997), upregulation of surface CCR5 and CXCR4 in T cells (Bleul et al. 1997), multiple functional CC receptor expression which correlated with T cell ability to migrate to RANTES and MCP-1 but not IL-8 (Loetscher et al. 1996). The effect of IL-2 was shown to be reversible and depend on the continuous presence of the cytokine, since upon withdrawal/wash of IL-2 from the cultures, CC receptor expression and responsiveness to CC chemokines was lost (Loetscher et al. 1996).
However recently, the related cytokine IL-15 was reported to be more efficacious than IL-2 after 12 hours in T cell culture, in inducing expression of mRNA for CC receptors, but not CCR3 and CXC receptors like CXCR4, as well as mRNA for CC chemokines, IP-10 and IL-8 (Perera et al. 1999). IL-15 was shown to signal via the IL-2Rβ chain and recently was presented to be a survival factor for memory T cells (Perera et al. 1999). Moreover, IL-4 which like IL-15 also uses part of the IL-2 receptor, was reported to reverse CXCR4 downregulation following CD3, CD2 or CD28 costimulation, by re-inducing CXCR4 T cell expression (Jourdan et al. 1998). Hence, identification of the effects of cytokines on chemokine receptor expression is by no means complete as yet.

**CHEMOKINE RECEPTOR EXPRESSION AND T CELL PHENOTYPES**

A great interest in allocating T cell chemokine receptor expression to phenotype has occurred recently because chemokines were initially defined as preferably attracting specific subsets of T cells. MIP-1α, MIP-1β and RANTES were found to be selectively produced by Th1-T-cells, in a study using either Th1 clones or PBMCs transformed with Th1 versus Th2 models, such as the nematode *A. Suum* and the intracellular parasite *Y. Enterocolitica*, respectively (Schrum et al. 1996). Th1 cells were reported to respond more efficiently to RANTES, MIP-1α and MIP-1β, whereas SDF-1 was equally efficacious for both Th1 and Th2 cells (Siveke, Hamann, 1998). Moreover, Bonecchi et al. in 1998 reported CCR2, CXCR4 and CCR1 to be found on both Th1 and Th2 cells, whereas other receptors to be more phenotype-specific such as CCR3, CCR4 for Th2 and CCR5, CXCR3 for Th1. Also, (Loetscher et al. 1998a) assigned CCR5 to T cells of the Th1 phenotype, and selective Th1 cell diapedesis was demonstrated upon expression of endothelial RANTES, attributable to CCR5 T cell expression (Kawai et al. 1999).

With the rapid discovery of new chemokines and their receptors several groups presented models linking T cell phenotypes with receptor expression and migrational capacities. Chemokine receptors served to further divide memory T cells to effector or resting, which correlated their expression and tissue preference for trafficking (Mackay, 1999). Hence, memory T cells (CD45RA-) were divided into CCR7+ and CCR7-, but naïve T cells (CD45RA+) were all CCR7+, a recently described receptor which allows recirculation to the lymph nodes (Sallusto et al. 1999). Effector T cells were defined as CCR7- and other receptors could assign their migration to specific tissues, CCR4+ and CLA+ for skin homing, as opposed to CCR5+ and α4β7 integrin expression for gastric mucosa homing (Sallusto et al. 1999). The model proposed for chemokine receptor
allocation on T cell subsets had: i) CCR7 and CXCR4 expression on naïve T cells allowing their recirculation to secondary lymphoid organs, ii) upon priming with antigen T cells became memory cells with Th1 expressing CCR1, CCR2, CCR5, CXCR3 or Th2 expressing CCR2, CCR3, and CCR4, allowing them to play a role in peripheral inflammation, DTH and allergy, iii) exposure to further antigen alters chemokine receptor expression so that Th1 activated cells express CCR7 and CXCR5 to recirculate to the lymph nodes whereas Th2 activated cells express CCR7, CXCR5, CCR8 and CCR4, as candidate mediators of chronic inflammation (Sallusto et al. 1999).

The mRNA data from this study for chemokine receptors expressed after lymphoblastic transformation and CD3/CD28 costimulation indicate, in the first instance, both Th1 (CCR4) and Th2 (CXCR3) phenotype potential. Subsequent IL-2 promotion of chemokine receptor mRNA was not restricted to CC chemokines, and with no apparent Th1/Th2 trends. Furthermore, it was the balance of local IL-4 and INF-γ reported to be crucial in Th1-Th2 switching and the development of Th1 or Th2 effector phenotypes (Nakamura et al. 1997). Therefore, switching of T cell responses to chemokines has been proposed to critically direct lymphocytes to localised cytokine/cellular millieu which mediate Th1/Th2 differentiation, therefore chemokine receptors expressed could be considered as part of Th1/Th2 differentiation and used as alternative Th markers (Campbell et al. 1999). Figure I.d is a diagram of the integration between chemokines and cytokines as they are thought to occur in T cell effector differentiation.
The uncommitted, CXCR4-expressing naïve T cell has an equal opportunity to migrate to areas of either Th1- or Th2-type costimulation based on the ubiquitous expression of SDF-1. Upon contact/priming with antigen in the designated Th1 (faint green) or Th2 (faint red) areas/lymphoid organs, Th-specific phenotype evolves with expression of certain chemokine receptors, ensuring that migration will occur to peripheral Th1- (green) or Th2-(red) sites secreting Th1- or Th2-associated chemokines. Thus Th1 and Th2 type T cells can contribute to both the secondary lymphoid organ cytokine milieu responsible for inducing Th1 or Th2 differentiation, and to the secretion of chemokines form the sites of challenge responsible for distinguishing and attracting Th1- or Th2-type T cells.
CONCLUSIONS I

RANTES AND T CELL ACTIVATION

RANTES secretion can occur independently of T cell proliferation and is thus driven by biochemical signals distinct from those that are required for IL-2 production and T cell proliferation. RANTES secretion like IL-2 production and T cell proliferation is also subject to inhibitory modulation by CTLA-4, although this can be by-passed if cells are activated under conditions in which CTLA-4 expression has not occurred. One important goal of future studies will be to obtain a greater understanding of the biochemical signals required for RANTES gene expression and secretion, as these may offer potential routes for manipulation of RANTES secretion:

➢ Identification of the PKC pathways that are important in mediating RANTES secretion by molecular approaches. The aim would lie in identifying selective inhibition of the PKC and/or other pathways for RANTES production as this may offer novel and selective targets for therapeutic intervention in a number of disease settings where it may be beneficial to regulate chemokine secretion without necessarily altering overall immune capabilities. For example, a co-ordination of MCP-1, RANTES and Eotaxin activity in the lung was shown to regulate the development of a model for asthma which could be inhibited with anti-chemokine antibodies or receptor antagonists (Gonzalo et al. 1998).

➢ Selective induction of RANTES secretion in the absence of T cell proliferation may be required in the threat of an HIV-1 proliferative spread. For instance, targeted modest up-regulation of RANTES secretion from T lymphocytes may be beneficial during the early stages of M-tropic HIV infection, where it may be possible to compete out the M-tropic HIV binding to CCR5 by the up-regulation of the endogenous chemokine ligand.
RESULTS II: Chemokine signalling in T cells

4.1 Calcium Mobilisation

Changes in intracellular free calcium ions are a frequent outcome of receptor ligation which may result from second messenger-stimulated release of Ca\(^{2+}\) ions from intracellular stores and/or influx of extracellular Ca\(^{2+}\) ions through the activation of a calcium channel. Lipophilic compounds such as fura-2 acetoxymethyl ester (Fura-2AM) after penetrating the cell membrane undergo catalysis by cytosolic esterases which cleave the ester groups. The resulting non-lipophilic Fura is trapped in the intracellular space and will chelate free Ca\(^{2+}\) ions with high affinity. Changes in free Ca\(^{2+}\) ion concentrations can be measured using the spectral shift Fura undergoes upon chelation of calcium ions.

COMPARISON OF CHEMOKINE AND ANTI-CD3 ANTIBODY RESPONSES

Established mitogenic stimuli often result in a sustained Ca\(^{2+}\) ion elevation. Ligation of CD3 is known to be a strong stimulus for mobilisation of calcium, whilst in comparison CD28 elicits little if any elevation of [Ca\(^{2+}\)]\(_i\) (Ward 1996). With all the T-cell types tested, anti-CD3 antibody caused a substantial rise with an onset of response between 10 and 30 seconds on resting T-cells (Fig. 4.1a), activated T-lymphoblasts (Figs 4.3a & 4.4a) and Jurkat cells (Fig. 4.2a). The increase in Ca\(^{2+}\) ion concentration was greatest with Jurkat cells from 50 nM basal to an initial peak of 500 nM and a sustained level of 300 nM over 3 minutes (Fig. 4.2a). A set of atypical PHA derived IL-2-T-lymphoblasts from a unique donor presented a similar profile with 40 nM basal [Ca\(^{2+}\)]\(_i\) rising initially to 275 nM and over 3 minutes remaining at 200 nM (Fig. 4.3a). More representative responses from typical PHA-derived IL-2-T-lymphoblasts (Fig. 4.4a) as well as the profile from resting T-cells (Fig. 4.1a) involved a basal 30-40 nM [Ca\(^{2+}\)]\(_i\) rising to 250 nM or 120 nM respectively, without as pronounced an initial peak as that observed with Jurkat cells. The response of T-cells to CD3 ligation was subsequently used as a positive control for calcium mobilisation in experiments investigating chemokine receptor mediated responses. The antibody against CXCR4, a monoclonal mouse anti-CXCR4 antibody 12G5 (Endres et al. 1996), was readily available and hence, CXCR4 was the only receptor whose surface expression was routinely assessed. All the chemokine responses observed had a common profile of a transient [Ca\(^{2+}\)]\(_i\) rise with a rapid onset,
irrespective of the chemokine. Resting T-cells responded in such a way to 100 nM SDF-1 with a maximum rise of ~40 nM $[Ca^{2+}]_i$ and a return to basal levels in 60-70 seconds (Fig. 4.1b) which correlated with the bright CXCR4 surface expression observed (Fig 4.5), and the only chemokine receptor mRNA detected (Fig 3.27). 100 nM RANTES as well as IP-10 did not appear to affect resting T-cell intracellular $[Ca^{2+}]$ (Fig 4.1c and d). With Jurkat cells, 100 nM RANTES or IP-10 also had no effect (Fig. 4.2 c and d), but surprisingly neither did 100 nM SDF-1 (Fig. 4.2b). The responsiveness of Jurkat cells was verified with CD3 ligation which initiated the expected rise in $[Ca^{2+}]_i$ in the SDF-1 treated cell aliquot (Fig. 4.2b). However, Jurkat cells expressed CXCR4 brightly on their surface (Fig 4.5) and also contained mRNA for CXCR4 as well as CCR4, CXCR2 and CXCR3 (Fig. 4.7). With the addition of 100 nM SDF-1, every set of PHA- or SEB-derived T-lymphoblasts, unlike Jurkat cells, responded rapidly with a rise of 50 nM $[Ca^{2+}]_i$ although from different basal levels (Figs. 4.3b and 4.4b). Interestingly, surface CXCR4 expression was markedly reduced to levels ~10%, and this profile was consistent with either PHA or SEB transformed cells (Fig 4.5), whereas CXCR4 mRNA was always detected (Fig. 4.6). Moreover, the response with the atypical T-lymphoblasts appeared more protracted, over 2 minutes (Fig. 4.3b), in comparison to the ~80 seconds duration with the normal T-lymphoblasts (Fig. 4.4b).

The atypical T-lymphoblasts also responded to 100 nM RANTES or IP-10, again in a rapid and transient manner with maximum rises of 30 nM $[Ca^{2+}]_i$ (Fig. 4.3c and d). However, normal T-lymphoblasts resembled resting T-cells in that 100 nM RANTES or IP-10 did not exert a noticeable effect (Fig. 4.4c and d). Although it was not possible to assess the surface expression of the receptors for IP-10 and RANTES, it is interesting to note that mRNA for CXCR3, CCR5, CCR4, CCR2 and CCR1 could always be detected from PHA- or SEB-derived T-lymphoblasts as well as from the unique PHA-derived atypical T-lymphoblasts (Fig 4.6).
Figure 4.1 Calcium mobilisation in purified resting T-cells.

a) CD3 ligation

b) SDF-1

c) RANTES

d) IP-10

Figure 4.1. Calcium mobilisation in purified resting T-cells. Purified resting T-cells were loaded with Fura-2AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, 2 x 10⁶ T-cells per cuvette were stimulated with: a) 10 µg/ml anti-CD3 antibody, b) 100 nM SDF-1, c) 100 nM RANTES, d) 100 nM IP-10. Traces are in M [Ca²⁺]i from one experiment representative of 3 other independent experiments.
Figure 4.2 Calcium mobilisation in Jurkat cells.

Jurkat cells were loaded with Fura-2AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the black arrows, 2 x 10^6 Jurkat cells per cuvette were stimulated with: a) 10 μg/ml anti-CD3 antibody, b) 100 nM SDF-1 (red arrow was positive response to anti-CD3), c) 100 nM RANTES, d) 100 nM IP-10. Traces are in M [Ca^{2+}]_i from one experiment representative of 3 other independent experiments.
Figure 4.3 Calcium mobilisation in atypical T-lymphoblasts.

Atypical Day 10 IL-2-T-lymphoblasts from PHA treated PBMCs were loaded with Fura-2AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, $2 \times 10^6$ atypical T-lymphoblasts per cuvette were stimulated with: a) 10 µg/ml anti-CD3 antibody, b) 100 nM SDF-1, c) 100 nM RANTES, d) 100 nM IP-10. Traces are in M $[Ca^{2+}]_i$ of one experiment out of two, with cells derived from one unique donor not representative of other donors.
Figure 4.4 Calcium mobilisation in normal T-lymphoblasts.

Day 10 IL-2-T-lymphoblasts from PHA treated PBMCs were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, 2 x 10^6 T-lymphoblasts per cuvette were stimulated with: a) 10 μg/ml anti-CD3 antibody, b) 100 nM SDF-1, c) 100 nM RANTES, d) 100 nM IP-10. Traces are in M [Ca^{2+}]_i from one experiment representative of 5 other experiments. Similar calcium mobilisation profiles were obtained later with SEB-derived IL-2-T-lymphoblasts.
Figure 4.5 CXCR4 surface expression on various cell types.

A) RESTING T-CELLS

B) Day 10 T-LYMPHOBLASTS (IL-2)

C) JURKAT CELLS

D) U937 CELLS

1 x 10^5 cells / tube purified resting T-cells (A), Day 10 IL-2 maintained T-lymphoblasts (B), Jurkat cells (C), or U937 cells (D) were stained with 12G5 anti-CXCR4 antibody (red histograms) or isotype matched mouse IgG2a (black histograms) and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm), as described in Materials and Methods. CXCR4 expressions shown are representative for all corresponding cell types subsequently used in other assays. SEB-derived IL-2 T-lymphoblasts expressed identical levels of surface CXCR4.
Figure 4.6 Chemokine receptor mRNA expression in Day 10 IL-2 maintained T-lymphoblasts. Mitogenic stimulation as indicated in the brackets, was given for 3 days, followed by IL-2 supplementation for 7 days, and RT-PCR was performed on mRNA extracted from $5 \times 10^7$ T lymphoblasts per point, as described in Materials and Methods. T-lymphoblasts were derived from PBMCs with all mitogens, except for CD3/CD28 stimulation with bead-conjugated antibodies on purified T-cells, as described in Materials and Methods. “Atypical PHA” denotes PHA-derived T-lymphoblasts from a unique donor. Results are from single experiments representative of 3 other replicate experiments except for the unique atypical PHA T-lymphoblasts.
Figure 4.7 Chemokine receptor mRNA expression in Jurkat cells.

Characterisation of CXCR4 Mediated Calcium Mobilisation

Dose-Responses

The highest concentration tested, 100 nM SDF-1, on T-lymphoblasts resulted in a 50 nM [Ca$^{2+}$]$_i$ rise, whereas responses from 1-30nM SDF-1, all resulted in 30 nM [Ca$^{2+}$]$_i$ rises (Fig. 4.8a). The same quantum-like concentration responses were also evident with the U937 cell line which was found to stain brightly for CXCR4 surface expression (Fig.4.5). Although 100nM SDF-1 resulted in a robust rise of 120 nM [Ca$^{2+}$]$_i$, 10 nM SDF-1 and 1 nM SDF-1 both produced 30 nM [Ca$^{2+}$]$_i$ rises (Fig. 4.8b).

Extracellular Calcium Requirement

Attempting to identify the origin of the SDF-1 induced rise in [Ca$^{2+}$]$_i$, experiments were performed without the addition of 1mM CaCl$_2$ to the calcium free media and in the presence of calcium chelators such as EGTA. Although such treatment resulted generally in lowered basal intracellular calcium levels and more fragile cell preparations, the response of normal T-lymphoblasts to 100nM SDF-1 was not altered, with maximum transient rises of 50nM [Ca$^{2+}$]$_i$ ( Fig. 4.9a ). U937 cells in the absence of calcium and with EGTA present, also responded to 100 nM SDF-1 with unaltered kinetics, albeit a modest ~30 nM [Ca$^{2+}$]$_i$ rise instead of the robust rise observed previously (Fig. 4.9b).
Effect of PMA, Pertussis toxin or repeated SDF-1 administration.

CXCR4 mediated calcium responses to SDF-1 on T-lymphoblasts were sensitive to a variety of treatments; pre-treatment of T-lymphoblasts to 50 ng/ml PMA for 15 minutes abrogated the response to 100nM SDF-1 but did not affect the CD3 mediated $[Ca^{2+}]_i$ rise (Fig. 4.10a). Ligand-induced down-modulation was demonstrated with repeated administrations of $2 \times 100$ nM SDF-1, one given in the wake of the previous response and without effect (Fig. 4.10 b). Pertussis toxin pre-treatment also abolished the SDF-1 response on T-lymphoblasts, indicating Gia-protein coupling to CXCR4-mediated calcium mobilisation (Fig. 4.9c). Cell responsiveness was not impaired since, once again, a receptor not coupled to Gi α-proteins was able to initiate calcium rises, such as CD3 ligation with antibodies (Fig. 4.10c).

Structural requirements of CXCR4-ligands for calcium mobilisation.

Peptide analogues corresponding to the N-terminal sequence of SDF-1 have been described which act as either partial agonists or antagonist in chemotaxis and calcium signalling assays (Loetscher et al. 1998). The ligand requirements of CXCR4-mediated calcium mobilisation were examined with such various peptide fragments of SDF-1 and the anti-CXCR4 antibody 12G5 on U937 cells. Peptide fragments, 9 amino-acid long from the NH$_2$-terminal of native SDF-1 behaved differently, depending on their conformation in solution and amino-acid composition. Hence, at 40 μM, a concentration previously demonstrated to elicit a biochemical event such as elevation of $[Ca^{2+}]_i$ (Loetscher et al. 1998), the dimeric (1-9)-(9-1) fragment from NH$_2$-SDF-1 behaved as a full agonist resulting in a robust and transient rise of ~140 nM $[Ca^{2+}]_i$ whereas the same fragment, in solution as a monomer (1-9) at 40 μM, exerted only a weak agonistic effect of a transient 50 nM $[Ca^{2+}]_i$ rise (Fig. 4.11). Conversely, 40 μM of the substituted proline for glycine at position 2 (P2G), dimeric (1-9)-(9-1) NH$_2$-SDF-1 fragment, as well as 10 μg/ml of 12G5 did not exert a rise in calcium levels when administered alone (Fig. 4.11). However, when the (P2G) dimer fragment at 40 μM was co-administered with 10nM SDF-1, it drastically reduced the SDF-1 response from a 90 nM $[Ca^{2+}]_i$ rise to ~20 nM $[Ca^{2+}]_i$ rise with much shorter kinetics (Fig. 4.12a). Similarly, 10 μg/ml of 12G5 antibody pre-treatment, also reduced the response to 10 nM SDF-1 from a 40 nM $[Ca^{2+}]_i$ rise to a ~10 nM$[Ca^{2+}]_i$ rise, whereas pre-treatment of the cells with 10 μg/ml IgG2a did not affect the 10nM SDF-1 response significantly (Fig. 4.12b).
Figure 4.8 a and b. SDF-1 induced calcium mobilisation.

a) SDF-1 dose response on T-lymphoblasts

Free $[Ca^{2+}]$ i. M

![Graph showing calcium concentration over time for different SDF-1 concentrations](image)

b) SDF-1 dose response on U937 cells

Free $[Ca^{2+}]$ i. M

![Graph showing calcium concentration over time for different SDF-1 concentrations](image)

Figure 4.8. SDF-1 induced calcium mobilisation.
a) T-lymphoblasts from PHA treated PBMCs or b) U937 cells were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, $2 \times 10^6$ cells per cuvette were stimulated with SDF-1 at the concentrations indicated. Traces are in M $[Ca^{2+}]$; from one experiment representative of 3 other independent experiments.
Figure 4.9 Calcium mobilisation by chemokines in the absence of extracellular calcium.

Normal T-lymphoblasts from PHA treated PBMCs (a) or U937 cells (b) were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, $2 \times 10^6$ cells per cuvette were stimulated as shown. Traces are in $M \ [Ca^{2+}]$, from one experiment representative of 3 other independent experiments.
Figure 4.10 a, b and c. Effect of PMA, tandem SDF-1 and Pertussis Toxin on CXCR4- calcium mobilisation in T-lymphoblasts.

- **a)** Effect of 50 ng/ml PMA pre-treatment for 15 minutes.

- **b)** SDF-1 induced CXCR4 down-regulation.

- **c)** Effect of 100ng/ml Pertussis toxin pre-treatment for 8 hours.

Figure 4.10. Effect of PMA, tandem SDF-1 and Pertussis toxin on T-lymphoblast calcium mobilisation. T-lymphoblasts from PHA transformed PBMCs were pre-treated as indicated and then loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. 2 x 10^6 cells per cuvette were stimulated as indicated by the arrows. Traces are in M [Ca^{2+}], from one experiment representative of 3 other independent experiments.
Figure 4.11 Calcium mobilisation with other CXCR4 ligands on U937 cells. U937 cells were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, 2 x 10^6 cells per cuvette were stimulated as indicated. Traces are in M [Ca^{2+}]_i from one experiment representative of 3 other independent experiments.
Figure 4.12 Effect of other CXCR4 agonists on SDF-1 induced calcium mobilisation.

a) Effect of the (P2G) dimer fragment on the SDF-1 induced response

b) Effect of 12G5 pre-treatment on the SDF-1 response

Figure 4.12 Effect of other CXCR4 ligands on SDF-1 induced calcium mobilisation. U937 cells were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. At the times indicated by the arrows, 2 x 10^6 cells per cuvette were stimulated with 10 nM SDF-1. Cells were pretreated with the antibodies as indicated at the green arrow. Traces are in M [Ca^{2+}], from one experiment representative of 3 other independent experiments.
Figure 4.13 Effect of PI3K inhibitors on SDF-1-induced calcium mobilisation.

Effect of LY 294002 on T-lymphoblasts

Figure 4.14. Effect of PI3K inhibitors on SDF-1 induced calcium mobilisation. T-lymphoblasts from PHA treated PBMCs were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. 2 x 10^6 cells per cuvette were pre-treated for 30 minutes with LY 294002 at the concentrations indicated, and stimulated at the times indicated by the arrows. Traces are in M [Ca^{2+}]_i from one experiment representative of 3 other independent experiments.
**Effect of PI3K Inhibition on CXCR4-calcium mobilisation.**

In other systems optimal calcium mobilisation has implicated recruitment of PLCγ via its tandem SH2 domains and/or its PH domain and the D-3 products of PI3K(s). The involvement of PI3Ks in CXCR4 mediated calcium mobilisation, was assessed with the use of the two unrelated pharmacological inhibitors, Wortmannin (Wymann et al. 1996) and LY294002 (Vlahhos et al. 1994), but did not appear significant. The reversible competitive PI3K inhibitor LY 294002, with all concentrations tested on T- lymphoblasts, did not affect the response to 100 nM SDF-1, yet manifested concentration dependent increments in basal [Ca2+]i levels of a maximum 20 nM [Ca2+], resembling the Wortmannin effect (Fig. 4.13 and not shown).

**SUMMARY**

1. Surface chemokine receptor expression, mRNA chemokine receptor expression and ability to mobilise calcium did not correlate. Jurkat cells were unable to mobilise calcium to SDF-1 although mRNA for CXCR4 and surface CXCR4 expression was readily detected.

2. SDF-1 was the only chemokine consistently able to cause calcium mobilisation in resting T cells, T lymphoblasts and U937 cells in a concentration-dependent manner that did not appear to depend on extracellular calcium ion influx.

3. CXCR4-induced calcium mobilisation was abrogated by pre-treatment with Pertussis toxin, PMA and repeated SDF-1 exposure.

4. The anti-CXCR4 antibody 12G5 and the peptide fragment dimer from the N-terminal of SDF-1 with proline for glycine substituted at position 2, behaved as antagonists.

5. PI3K appeared to have no role to play in CXCR4-mediated calcium mobilisation.
4.2 Involvement of PI 3K(s) in CXCR4 signalling

PHOSPHATIDYL-INOSITOL(3,4,5)P₃ ACCUMULATION IN INTACT CELLS UPON SDF-1 LIGATION

Analysis of the deacylated phospholipids extracted from cell lysates of Jurkat cells metabolically labelled with [³²P], resulted in a highly reproducible trace which enabled detection and quantification of changes in glycerophosphoryl sample derivatives (GroPtdIns lipids). For reasons of clarity these will be referred to as PtdIns(3,4,5)P₃. The appropriate eluate peaks were identified from their retention times with the aid of [³H]-labelled phosphoinositide lipid standards (Fig. 4.14). Treatment of labelled Jurkat cells with SDF-1 produced a rapid and transient increase in PtdIns(3,4,5)P₃ levels which was dependent on the SDF-1 concentration in a bell-shaped manner. After 1 minute SDF-1 ligation, equivalent optimum responses were obtained with concentrations between 1-100 nM SDF-1, raising the basal cpm of about 1100 to 4400 cpm (Fig. 4.15a).

Ligands for established PI3K-coupled receptors, such as CD80 for CD28 on Jurkat cells, applied for 5 minutes elevated total PtdIns(3,4,5)P₃ levels to around 9000 cpm. This treatment served as a positive control for [³²P] loading and a positive standard for assessing SDF-1 induced PtdIns(3,4,5)P₃ formation (Fig. 4.15a). PtdIns(3,4,5)P₃ elevations were consistent with respect to each experiments’ basal, unstimulated PtdIns(3,4,5)P₃ levels but differed according to [³²P] loading efficiencies, as well as different cell types. Hence, for comparative reasons between experiments and cell types, results were expressed as fold increases from basal. 100 nM SDF-1 induced PtdIns(3,4,5)P₃ elevations in Jurkat cells, U937 cells as well as IL-2 maintained T-lymphoblasts which had comparable kinetics, the maximal responses obtained at 1 minute with Jurkat cells and U937 cells and 30 seconds with T-lymphoblasts. Overall, the maximum increases ranged from 3 to 7-fold the basal PtdIns(3,4,5)P₃ levels (Fig. 4.15 b and c). In the first attempts to identify the PI3K(s) responsible for the total PtdIns(3,4,5)P₃ elevation, the inhibitor Wortmannin was able to abrogate the SDF-1 responses in T-lymphoblasts (Fig. 4.16a) as well as in Jurkat cells, where the Gi(α)-protein inhibitor Pertussis Toxin also abolished SDF-1 induced responses (Fig. 4.16b).
Figure 4.14  HPLC separation and analysis of GroPInsP₃ generation from SDF-1 stimulated Jurkat cells

Figure 4.14 HPLC separation and analysis of phosphatidyl-inositol(3,4,5)P₃ (PtdInsP₃) generation in Jurkat cells. Jurkat cells at 1x10⁷ cells / point were metabolically labelled with [³²P], stimulated with vehicle, or 100 nM SDF-1 for 1 minute, and phospholipids were extracted as described in Materials and Methods. The resulting glycerophosphoryl sample derivatives (GroPIns lipids) were separated by anion exchange HPLC and analysed with an on-line Flo-One radiomatic program, as described in Materials and Methods. Retention times of eluate peaks were verified for each experiment against ³H-labeled phosphoinositol lipid standards. Main trace shows SDF-1 stimulated full run, zoom regions show basal and stimulated GroPInsP₃ eluate peaks (rt:62-64 min). Peaks are expressed in cpm, time scale in 1/10 of a minute (min x 10⁻¹). This trace is from one experiment representative of all lipid extraction experiments that provided data.
Figure 4.15. CXCR4-mediated Phosphatidyl-Inositol (3,4,5)P₃ generation in various cell types.

4.15a

**PtdIns(3,4,5)P₃**

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
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<tr>
<td>cpm</td>
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SDF-1 [nM] for 1 minute

4.15b

Fold increase from basal PtdIns(3,4,5)P₃

4.15c

Fold increase from basal PtdIns(3,4,5)P₃

Figure 4.15. CXCR4-mediated PtdIns(3,4,5)P₃ generation in various cell types. 1x10⁷ cells / point of Jurkat (a) and (b), T-lymphoblasts or U937 cells (c) were metabolically labelled with [³²P], stimulated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Cells were stimulated:

(a) for 1 min with 0.1% BSA, 0.1-1000 nM SDF-1, or for 5 min with CHO-CD80+ cells at a 1:3 T-cell ratio, (b) and (c) with 0.1% BSA, or 100 nM SDF-1 for the times indicated. Results are expressed as (a) cpm of ³²P-labelled PtdIns(3,4,5)P₃, (b) and (c) fold increases in levels of PtdIns(3,4,5)P₃ from unstimulated cells and represent means ± sem from 3 independent experiments. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 4.16 Effect of Wortmannin and Pertussis toxin on CXCR4-mediated PtdIns(3,4,5)P$_3$ generation by 100 nM SDF-1.

a) T Lymphoblasts

Fold increase from basal PtdIns(3,4,5)P$_3$

![Graph showing fold increase from basal PtdIns(3,4,5)P$_3$ for T lymphoblasts.]

b) Jurkat cells

Fold increase from basal PtdIns(3,4,5)P$_3$

![Graph showing fold increase from basal PtdIns(3,4,5)P$_3$ for Jurkat cells.]

Time course

Figure 4.16 Effect of Wortmannin and Pertussis toxin on CXCR4 mediated PtdIns(3,4,5)P$_3$ generation by 100 nM SDF-1. (a) T-lymphoblasts, or (b) Jurkat cells 1x10$^7$ cells / point were metabolically labelled with $[^{32}$P], stimulated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Cells were pre-treated for 10 minutes with 0.001% DMSO or 100nM Wortmannin (a) and (b), or for 16 hours with 100 ng/ml Pertussis Toxin, and then stimulated with 0.1% BSA, or 100 nM SDF-1 as indicated. Results are expressed as fold increases in levels of PtdIns(3,4,5)P$_3$ from unstimulated cells and represent means ± sem from 3 independent experiments. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
Results II

PtdIns 3,4,5P3 ACCUMULATION IN RESPONSE TO OTHER CXCR4 LIGANDS

CXCR4 mediated PtdIns(3,4,5)P3 formation was not an SDF-1 exclusive effect, although other ligands capable of generating PtdIns(3,4,5)P3 above basal levels could also interfere with SDF-1 signalling. The use of peptide fragments, 9 amino-acids long, (1-9) from the NH2-terminal of the native SDF-1, defined certain requirements for CXCR4 receptor mediated PtdIns(3,4,5)P3 responses. On Jurkat cells, the NH2-(1-9) SDF-1 fragments that existed in solution as monomers and, slightly more efficacious, the covalently bound dimers (1-9)-(9-1), behaved as classic partial agonists. Albeit at 1000-fold higher µM concentrations, the peptide fragments alone produced about ½ to ¾ of the SDF-1 PtdIns(3,4,5)P3 response, and when given together with SDF-1, they compromised the SDF-1 response down to their own levels (Fig. 4.17). Substitution of the proline for glycine at position 2 with the (P2G) fragment NH2-(1-9) SDF-1 dimer, produced an antagonist, resulting in negligible PtdIns(3,4,5)P3 accumulation alone but blocking the SDF-1 induced response down to basal levels (Fig. 4.17). The antibody used widely for CXCR4 FACS analysis was also examined on Jurkat PtdIns(3,4,5)P3 accumulation. Jurkat cells treated with 10 µg/ml of the 12G5 antibody did not result in significant PtdIns(3,4,5)P3 generation alone but after 10 minute pre-treatment, it blocked the 10 nM SDF-1 response considerably- by about 60%. This effect was specific to CXCR4 binding since an isotype matched IgG did not affect the SDF-1 response significantly (Fig. 4.17).

4.3 Identification and role of PI3K isozymes in CXCR4 signalling

IN-VITRO LIPID KINASE ACTIVITY WITH p110 γ-IMMUNOPRECIPITATES FOLLOWING CXCR4 LIGATION TO SDF-1.

HPLC analysis of total phospholipid production from cells stimulated with CXCR4 ligands portrayed the total PtdIns(3,4,5)P3 produced by all the PI3K(s) coupled to this chemokine receptor. To examine specific PI3K isozyme coupling and contribution to the total D-3-phospholipid-messenger produce, in-vitro lipid kinase assays were performed with PI3K isozyme -specific immunoprecipitates from cells stimulated with CXCR4 ligands. From the candidate PI3K isozymes that are sensitive to wortmannin, one is thought to be activated directly by G-protein activation. (type 1B-p110γ) and consequently expected to be sensitive to pertussis toxin.
Figure 4.17 Jurkat cell PtdIns(3,4,5)P3 generation after 1 minute with other CXCR4 ligands alone or in combination to SDF-1.

Fold increase from basal PtdIns(3,4,5)P3

Figure 4.17 Jurkat cell PtdIns(3,4,5)P3 generation after 1 minute with other CXCR4 ligands alone or in combination to SDF-1. 1x10^7 cells / point Jurkat cells were metabolically labelled with [32P], stimulated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Cells were stimulated for 1 minute with 10nM SDF-1 (red columns) or Vehicle: 0.1% BSA (white columns) together with 0.1% BSA or peptide (1-9) fragments from the NH2-terminal of SDF-1 as described in the text and Materials and Methods. Antibodies were given 10 minutes prior to SDF-1. Results are expressed as fold increases in levels of PtdIns(3,4,5)P3 from unstimulated cells and represent means ± sem from 3 independent experiments. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
With an antibody suitable for immunoprecipitation made available for p110 \( \gamma \), the coupling of this PI3K type 1B could also be investigated \textit{in-vitro}. The kinetics of p110 \( \gamma \) recruitment to CXCR4 upon SDF-1 ligation was investigated in SDF-1 stimulated IL-2 maintained T-lymphoblasts, as well as Jurkat cells. With T-lymphoblasts, SDF-1 caused a transient and rapid increase in p110 \( \gamma \) precipitated lipid kinase activity, with an onset of 30 seconds and still detectable after 10 minutes of SDF-1 stimulation (Fig. 4.18a). p110-\( \gamma \)-precipitated lipid kinase activity from SDF-1 treated Jurkat cells was detected as soon as 15 seconds after stimulation but also subsided faster, returning to basal levels after 5 minutes (Fig. 4.18b).

\textbf{IN-VITRO LIPID KINASE ACTIVITY WITH p85-IMMUNOPRECIPITATES FOLLOWING CXCR4 LIGATION TO SDF-1.}

An antibody specific for the p85 adaptor unit of the p85/p110 heterodimer PI3K (type 1A) was also available and therefore used for \textit{in-vitro} kinase assays. Indeed, purified resting T-cells in response to SDF-1 displayed p85-associated lipid kinase activity which appeared to peak at 30 seconds and swiftly subsided by 1 minute disappearing after 5 minutes. Wortmannin treatment at 100 nM abolished basal or stimulated lipid phosphorylation (Fig. 4.19A). Stimulation of p85-co-precipitated lipid kinase activity was also demonstrated in SDF-1 treated U937 cells (4.19B), where both the kinetics of PI3K activation and the effect of 100 nM Wortmannin closely resembled those seen with the Jurkat T cell line (Fig. 4.20). When Jurkat T-cells were stimulated over a time course with 100 nM SDF-1, co-precipitated lipid kinase activity of p85 IPs displayed a transient increase which, resembling the kinetic profile of PtdIns(3,4,5)P3 production examined by HPLC, peaked at 1 minute and subsided 5 minutes after SDF-1 stimulation (Fig. 4.20). The PI3K(s) inhibitor Wortmannin at 100 nM abrogated basal and SDF-1 stimulated p85-associated lipid kinase activity, whereas 100 ng/ml pertussis toxin treatment did not (Fig. 4.20). The p85-associated lipid kinase activity from pertussis toxin treated Jurkat cells in response to SDF-1 was more prolonged and with a faster onset, appearing at 30 seconds and still maintained at 5 minutes after stimulation (Fig. 4.20).
Figure 4.18 Effect of SDF-1 on p110γ-Immunoprecipitated in-vitro Lipid Kinase activity from Day 10 IL-2 maintained T-lymphoblasts and Jurkat cells.

a) T-lymphoblasts

b) Jurkat cells

Figure 4.18 Effect of SDF-1 on p110γ-Immunoprecipitated in-vitro Lipid Kinase activity from day 10 T-lymphoblasts and Jurkat cells. 1 x 10^7, a) IL-2 maintained, SEB-T-lymphoblasts or b) Jurkat cells per point were stimulated at 37°C with vehicle (0.1% BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 1 μg/ml anti-p110γ Ab and the IPs subjected to in-vitro lipid kinase assays as described in Materials and Methods. 32P-labelled PtdIns(3)P was visualised by autoradiography of the TLC plates run overnight. Results shown from 1 experiment representative of another 3 independent experiments.
Figure 4.19 A and B. Effect of SDF-1 on p85-Immunoprecipitated *in-vitro* Lipid Kinase activity from resting purified T-lymphocytes or U937 cells.

A: Resting T cells

100 nM SDF-1

vehicle

30 sec 1 min 5 min

PtdIns(3)P

+ Vehicle

B: U937 cells

100 nM SDF-1

vehicle

30 sec 1 min 5 min

PtdIns(3)P

+ Vehicle

Figure 4.19 Effect of SDF-1 on p85-Immunoprecipitated *in-vitro* Lipid Kinase activity from resting T-cells or U937 cells. 1 x 10⁷ (A) purified, resting T-cells or (B) U937 cells per point were pre-treated at 37⁰ C for 10 minutes with vehicle (0.01 % DMSO) or with 100 nM wortmannin and then stimulated with vehicle (0.1 % BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 1 µg/ml anti-p85 mAb and the IPs subjected to in-vitro lipid kinase assays as described in Materials and Methods. ³²P-labelled PtdIns(3)P was visualised by autoradiography of the TLC plates run overnight. Results shown from 1 experiment representative of another 3 independent experiments.
Figure 4.20 Effect of SDF-1 on p85-Immunoprecipitated *in-vitro* Lipid Kinase activity from Jurkat T-cells.

100 nM SDF-1

- **Vehicle**
  - 30 sec
  - 1 min
  - 2 min
  - 5 min

- **+ Vehicle**

- **PtdIns(3)P**

- **+ 100 nM wortmannin**

- **+ 100 ng/ml Pertussis Toxin**

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Figure 4.20 Effect of SDF-1 on p85-Immunoprecipitated *in-vitro* Lipid Kinase activity from Jurkat T-cells. 1 x 10^7 Jurkat T-cells per point were pre-treated at 37°C for 10 minutes with vehicle (0.01 % DMSO) or with 100 nM wortmannin, or for 12 hours with 100 ng/ml pertussis toxin and then stimulated with vehicle (0.1 % BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 1 μg/ml anti-p85 mAb and the IPs subjected to in-vitro lipid kinase assays as described in Materials and Methods. 32P-labelled PtdIns(3)P was visualised by autoradiography of the TLC plates run overnight. Results shown from 1 experiment representative of another 3 independent experiments.
RELATIVE LIPID KINASE ACTIVITY CONTRIBUTION OF RECRUITED PI3K ISOSYME WITH CXCR4 LIGATION

Having established that upon SDF-1 ligation p85 is recruited, protein tyrosine kinase phosphorylation events are thought to be a prerequisite for interaction of the receptor/adaptor molecules with the SH2-domains of p85. Therefore, it was important to establish if any possible catalytic contribution in PtdIns(3,4,5)P3 generation from p85 recruitment and class 1A p110 subunits depended on PTK activation. Hence, the role of PTK activation in recruiting PI3K(s) involved in SDF-1-induced PtdIns(3,4,5)P3 responses in Jurkat cells was examined pharmacologically. Surprisingly, Herbimycin A appeared to affect the SDF-1 response very modestly by decreasing it from 4.5-fold to 3.8-fold, but this effect was not statistically significant (Fig. 4.21). However, results should be considered with caution, since such inhibitors probably have multiple targets within the cellular signalling compartments, some of which may still be unidentified. PI3K type 1A, is usually associated with receptors coupled to protein tyrosine kinases, such as CD28 (Ward, 1995), although there is some evidence that G-protein activation may synergise alongside activated PTKs in recruiting type 1A (Okada et al. 1996).

Therefore, the involvement PI3K type 1A was dissected out with a molecular approach, with a model of U937 cells transfected with an inducible, Myc-tagged, dominant negative mutant of the regulatory p85 subunit. Expression of the Δp85 mutant lacking the binding site required for coupling to the catalytic p110 subunit, was induced overnight in the presence of 5nM PMA, 15 mM IPTG and 100 μM ZnCl2. Δp85 expression was routinely checked with Western blot analysis for the presence of the co-expressed Myc moiety in the cell lysate (Fig. 4.22A). Moreover, as PMA is known to down-regulate surface receptor expression, the effect of the induction treatment on CXCR4 surface expression was routinely checked and found to be minimal; both uninduced and induced U937 cells stained consistently for CXCR4 ≥ 50% above basal levels (Fig. 4.22B). Importantly, when expression of Δp85 in U937 cells was induced and [32P]-labelled phospholipids were extracted, deacylated and measured, such disruption of p85/p110 coupling did not affect the kinetics of the SDF-1 induced PtdIns(3,4,5)P3 response. Even the modest attenuation of the 30 second PtdIns(3,4,5)P3 rise, from 6-fold to 4-fold was not significant (Fig. 4.23). It should be noted however, that basal cpm levels were reduced by half in the Δp85 expressing U937 cells, although it is not clear if this reflected an effect on [32P] loading, or denotes a role for type 1A
PI3K in basal PtdIns(3,4,5)P$_3$ maintenance. PI3K type 1A- p85/p110 may be recruited to G-protein receptors via synergistic G-protein and PTK activity (Okada et al. 1996), although it was usually associated with receptors coupled to protein tyrosine kinases (Lu et al. 1998; Suzuki et al. 1999). Hence, with 4G10, an antibody specific for tyrosine-phosphorylated protein substrates, one could possibly immunoprecipitate only recruited i.e. activated PI3K type 1A for in-vitro lipid kinase activity examination. PI3K type 1B- p110$\gamma$ is thought to be activated directly by G-protein activation (Vanhaesebroeck et al. 1997), and consequently would not depend on phosphotyrosyl-mediated recruitment. This hypothesis was examined by dissecting out catalytic PI3K type 1A coupling in U937 cells induced or not induced to express $\Delta$p85, and subsequent investigation of SDF-1-stimulated in-vitro lipid kinase activity with either p110$\gamma$ or 4G10 immunoprecipitates. Expression of $\Delta$p85 in U937 cells did not affect the SDF-1 stimulated increase in p110$\gamma$ associated lipid kinase activity (Fig. 4.24A). However, the more delayed 4G10-associated lipid kinase increase in activity after SDF-1 treatment was blocked in $\Delta$p85 expressing U937 cells (Fig. 4.24B). Following stimulation with 100 nM SDF-1 over a time-course, Jurkat cells were lysed and immunoprecipitated with either p110$\gamma$ or with 4G10 as well. Interestingly, although lipid kinase activity increased with both immunoprecipitates, the onset and duration of activation did not overlap; p110$\gamma$ precipitated lipid kinase activity was transient peaking at 1 minute and returning to basal levels by 5 minutes as witnessed before, however, phosphotyrosyl co-precipitated lipid kinase activity appeared late in onset at 2 minutes, peaking after 5 minutes following SDF-1 stimulation (Fig. 4.25).

PROTEIN TYROSINE PHOSPHORYLATION WITH CXCR4 LIGATION
Recruitment and activity of PI3K type 1A-p85/p110 has been shown to require tyrosine phosphorylation of appropriate amino acid residues, therefore tyrosine phosphorylation in response to CXCR4 ligation was examined. Day 10, IL-2-maintained T-lymphoblasts were treated with 100 nM SDF-1 over a time-course and cell lysates were immunoblotted with the anti-phosphotyrosine antibody 4G10. Indeed, SDF-1 caused tyrosine phosphorylation of multiple substrates, the most consistent of which were: a ~ 40 kDa, a ~ 80 kDa, a ~100 kDa and a ~130 kDa protein. Maximum phosphorylation occurred at 1 minute for the 40 and 100 kDa species, 30 seconds for the 80 kDa protein and the 130 kDa substrate appeared phosphorylated only at 1 min (Fig. 4.26).
Figure 4.21 The effect of Manumycin A and Herbimycin A on PtdIns(3,4,5)P$_3$ generation in intact Jurkat cells with 100nM SDF-1 for 1 minute.

Figure 4.21 Manumycin A and Herbimycin A on PtdInsP$_3$ generation in Jurkat cells with 100nM SDF-1 for 1 minute. $1 \times 10^7$ $[^{32}P]$-labelled Jurkat cells / point were pre-treated and stimulated as indicated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Cells were pre-treated with vehicle (0.1% DMSO), or Manumycin A for 60 minutes, or with Herbimycin A for 18 Hours and stimulated with vehicle (0.1% BSA)(white histobars), or 100nM SDF-1 (red histobars) for 1 min. Results are expressed as fold increases in levels of PtdIns(3,4,5)P$_3$ from unstimulated cells and represent means ± sem from 3 independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. 
Figure 4.22. Inducible expression of Δp85 in U937: Δp85 cells and effect of Δp85 induction on CXCR4 expression. U937: Δp85 cells were induced to express Δp85 as described in the Materials and Methods. A) 5 x 10⁶ induced or non-induced cells were lysed, total protein precipitated with acetone, and samples were immunoblotted with 1 μg/ml anti-Myc, or anti-p85 antibody after electrophoresis in 10 % acrylamide gels and semi-dry transfer as described in Materials and Methods.

B) 1 x 10⁵ induced (2) or non-induced (1) U937: Δp85 cells were suspended with 12G5 anti-CXCR4 antibody or isotype matched mouse IgG2a and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm) as described in Materials and Methods. Results shown are from one induction experiment representative of all U937: Δp85 experimental data.
Figure 4.23 SDF-1 induced PtdIns(3,4,5)P$_3$ generation in U937:Δp85 cells.

U937:Δp85 cells were either Induced or Uninduced to express Δp85 and 1x10$^7$ $[^{32}]$P]-labelled cells per point were stimulated with either 0.1% BSA for 1 minute, or 100 nM SDF-1 for the times indicated. Cells were metabolically labelled with $[^{32}]$P], stimulated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Induction did not affect surface CXCR4 expression (Fig 4.22B), whereas Δp85 expression was verified with anti-Myc Western blots (Fig. 4.22A). Results are expressed as fold increases in levels of PtdIns(3,4,5)P$_3$ lipids from unstimulated cells and represent means ± sem from 3 independent experiments. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
Figure 4.24 Effect of SDF-1 on the in-vitro Lipid Kinase activities of p110γ or 4G10 - Immunoprecipitates from induced or uninduced Δp85 U937 cells.

A) p110γ IPs

Uninduced Δp85 U937 cells

Induced Δp85 U937 cells

B) 4G10 IPs

Uninduced Δp85 U937 cells

Induced Δp85 U937 cells

Figure 4.24 Effect of SDF-1 on the in-vitro Lipid Kinase activities of p110γ or 4G10 - Immunoprecipitates from induced or uninduced U937: Δp85 cells. U937 : Δp85 were either induced, or not induced over 12 hours with 5nM PMA, 100μM ZnCl₂, and 15 mM IPTG. The following day 1 x 10⁷ cells per point from either treatment were stimulated at 37°C with vehicle (0.1 % BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 1 μg/ml anti-p110γ Ab (A) or anti-phosphotyrosine 4G10 Ab (B) and the IPs subjected to in-vitro lipid kinase assays as described in Materials and Methods. ³²P-labelled PtdIns(3)P was visualised by autoradiography of the TLC plates run overnight. Results shown from single experiments representative of another 3 independent experiments.
Figure 4.25 Effect of SDF-1 on the \textit{in-vitro} Lipid Kinase activities of p110$\gamma$ or 4G10 - Immunoprecipitates from Jurkat T-cells.

\textbf{Results II}
PYK2 PHOSPHORYLATION

One tyrosine kinase which is itself tyrosine phosphorylated upon activation, is the proline-rich protein tyrosine kinase (Pyk-2), also known as Focal Adhesion kinase-related PTK (Sieg et al. 1998; Lev et al. 1995; Qian et al. 1997). Pyk-2 activation has been implicated in signal transduction from a wide range of stimuli, including G-protein coupled receptors (Davis et al. 1997; Blaukat et al. 1999; Della Roca et al. 1997). Jurkat cells were treated with 100 nM SDF-1 over a time-course, cell lysates were immunoprecipitated with the anti-phosphotyrosine antibody 4G10 and immunoblotted with an antibody specific for Pyk-2. In response to SDF-1, Pyk-2 was rapidly tyrosine phosphorylated by 60 seconds but returned to basal levels by 5 minutes. To verify equal loading and protein transfer, cell lysates were immunoprecipitated and immunoblotted with Pyk-2 (Fig. 4.27).

RAS ACTIVATION

Ras is an important signalling molecule implicated in chemokine responses (Voice et al. 1999), that may link PI3K type 1A-p85/p110 and type 1B-p110 γ with each other and downstream effector activation (Giglione, Parmeggiani, 1998; Hedin et al. 1999). Moreover, Manumycin A, clearly blocked the SDF-1-induced PtdIns(3,4,5)P3 response in intact cells, partially lowering it from 4.5-fold to 2-fold (Fig 4.21). To examine directly if ligation of CXCR4 resulted in activated Ras, a protein construct comprising of the Ras-GTP binding portion of Raf-1 conjugated to glutathione sepharose beads was used to co-precipitate the active GTP-Ras but not the inactive GDP-Ras. When Jurkat cells were treated with 100 nM SDF-1 over a time-course and cell lysates were co-precipitated with the GTP-Ras-binding protein construct, a clear recruitment of active Ras by 30 seconds which diminished to basal levels by 5 minutes was evident (Fig. 4.28A). Both Ras and PTK appear to be activated in response to CXCR4 ligation. PI3K (p85/p110) type 1A is thought to require tyrosine phosphorylation of sites close to the substrate membrane that enable p85 recruitment and subsequent p110-mediated lipid kinase activity. Indeed, active Ras recruitment by CXCR4 ligation after 1 minute with 100 nM SDF-1 was abolished after pre-treatment with 10 μM of the farnesyltransferase-Ras inhibitor Manumycin A and also abrogated after pre-treatment with 2 μg/ml of the general tyrosine kinase inhibitor Herbimycin A (Fig. 4.28B).
Figure 4.26  SDF-1 stimulates tyrosine phosphorylation of multiple substrates in day 10 IL-2 maintained T-lymphoblasts.

100 nM SDF-1

Vehicle

15 sec 30 sec 1 min 5 min

m. w. markers

200 kDa

98 kDa

67 kDa

44 kDa

29 kDa

Figure 4.26  SDF-1 stimulates tyrosine phosphorylation of multiple substrates in mid-term IL-2 maintained T-lymphoblasts. 1 x10⁷ mid-term, IL-2 maintained, T-lymphoblasts per point were stimulated at 37° C with vehicle (0.1 % BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, total protein precipitated with acetone, and samples were immunoblotted with 1µg/ml anti-phosphotyrosine (4G10) antibody after 10 % acrylamide gel electrophoresis and semi-dry transfer, as described in Materials and Methods. Results shown from 1 experiment representative of another 3 independent experiments.
Figure 4.27 SDF-1 stimulates tyrosine phosphorylation of Pyk-2 in Jurkat T-cells.

1 x 10^7 Jurkat T-cells per point were stimulated at 37°C with vehicle (0.1% BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 1 μg/ml anti-phosphotyrosine (4G10) mAb, and samples were immunoblotted with 1 μg/ml anti-Pyk-2 antibody after 10% acrylamide gel electrophoresis and semi-dry transfer, as described in Materials and Methods. To verify equal loading, cell lysates were immunoprecipitated and immunoblotted with Pyk-2 (lower panel). Results shown from 1 experiment representative of another 3 independent experiments.
Figure 4.28 SDF-1 activates Ras in Jurkat T-cells

1 x 10^7 Jurkat T-cells per point were: (A) stimulated at 37°C with vehicle (0.1% BSA) or 100 nM SDF-1 for the times indicated, (B) pre-treated at 37°C for 1 hour with vehicle (0.01% DMSO) or Manumycin A, and for 18 hours with Herbimycin A and then stimulated with vehicle (0.1% BSA) or SDF-1 for 1 minute as indicated. Cells were then lysed, immunoprecipitated with 1 μg/ml RBD-GST fusion protein, and samples were immunoblotted with 1μg/ml anti-RAS antibody after electrophoresis through 10% acrylamide gels and semi-dry transfer, as described in Materials and Methods. Results shown from 1 experiment representative of another 3 independent experiments.
4.4 Activation of PI3K downstream effectors via CXCR4 ligation

PROTEIN KINASE B
An established effector of PI3Ks is protein kinase B (PKB) (Burgering, Coffer, 1995), thought to be recruited by and depend on phospholipid products of PI3K for subsequent phosphorylation and activation. After witnessing SDF-1 mediated, G-protein dependent PtdInsP$_3$ formation, which lies upstream of PKB, the effect of SDF-1 on PKB activation was examined. Jurkat cells were stimulated with 100 nM SDF-1 and the cell lysates immunoblotted with a phosphospecific anti-PKB antibody which recognised the active 473 phosphoserine-PKB. SDF-1 caused an increase in PKB phosphorylation which was evident 30 seconds after ligation and still evident after 1 minute ligation (Fig. 4.29). PKB activation by SDF-1 in Jurkat cells was assessed in the presence of 100 nM Wortmannin or 100 ng/ml Pertussis toxin. Both these inhibitors abolished PKB activation confirming the involvement of G-protein and PI3Ks as upstream signalling components (Fig. 4.29). Alternative, PI3K-independent pathways are also thought to activate PKB, in response to osmotic stress and heat shock, stimuli which activate the p38 MAPK cascade (Coffer et al. 1998). Hence, the Ras inhibitor Manumycin A at 10 µM as well as the MEK kinase inhibitor PD 98059 at 10 µM could be considered to inhibit SDF-1 induced PKB phosphorylation, implicating Ras and the MAPK pathway as mediators of PKB activation by SDF-1 (Fig. 4.29). However, the levels of non-stimulated, active endogenous PKB were high in the immortalised Jurkat cell line and treatment with most inhibitors appeared to stimulate an increase in basal endogenous PKB phosphorylation (Fig. 4.29). Consequently, the extent of SDF-1-stimulated increase in PKB phosphorylation, or lack of effect, did not appear ideally clear cut.

Moreover, fully active PKB has been shown to be dually phosphorylated on threonine 308 as well as serine 473 (Pullen et al. 1998; Allesi et al. 1997), an aspect of PKB activation not addressed with the 473-phosphospecific antibody available. An alternative approach which allowed assessment of PKB activation in functional terms, utilised Jurkat cells transiently transfected with HA-PKB or co-transfected with p110 - the catalytic domain from type 1A PI3K. Analysis of PKB activation using HA-PKB-transfected Jurkat cells was performed as described previously (Parry et al. 1997). The p110 domains were part of chimeric constructs which also contained the extracellular region of rat CD2.
Figure 4.29 SDF-1 stimulates activation of PKB in Jurkat T-cells. $1 \times 10^7$ Jurkat T-cells per point were pre-treated at 37°C either for 1 hour with vehicle (0.01% DMSO), Manumycin A, or PD 98059, or for 10 minutes with Wortmannin, or for 12 hours with Pertussis Toxin, and then stimulated with vehicle (0.1 % BSA) or SDF-1 as indicated. Cells were lysed, and acetone-precipitated protein samples were immunoblotted with 1µg/ml phospho-specific at Ser 473 anti-PKB antibody (upper panels) after electrophoresis through 12% acrylamide gels and semi-dry transfer. Blots were stripped and reprobed with anti-PKB antibody to verify equal loading and efficiency of protein transfer (lower panels), as described in Materials and Methods. Results shown from 1 experiment representative of another 3 independent experiments.
This was important in order to verify expression by FACS and to localise p110 to the membrane, rendering it constitutively active and a positive control-ratCD2/p110(a). Negative controls were obtained by a kinase dead version of the construct with a point mutation in the p110 catalytic domain (Arg to Pro 1130), resulting in rat CD2/p110(i) which also localised to the surface but with catalytically inactive p110. After immunoprecipitation with anti-HA antibody, activated HA-PKB due to SDF-1 treatment or constitutive p110 activity (positive control) was examined in-vitro, with a kinase assay using histone H2B as substrate. The degree of HA-PKB phosphorylation as well as the phosphorylated \(^{32}\text{P}\)-labeled H2B was then visualised (Fig.4.30B). Indeed, both 100 nM SDF-1 and the active p110 positive control caused an increase in active PKB as visualised by immunoblotting with the anti-phosphoserine-473-PKB. This activation was also reflected in the degree of H2B phosphorylation from the respective treatments. Maximum PKB phosphorylation was seen after 1 minute SDF-1 ligation, whereas the maximum levels of H2B phosphorylation were evident after 5 minutes (Fig. 4.30B). In order to check for adverse effects from the transfection process, the surface expression of CXCR4 as well as rat CD2 was routinely checked by FACS (Fig. 4.30A).

CXCR4-INDUCED MAPK (p42/p44, ERK -1/2) ACTIVATION
Since both Pyk-2 and Ras are implicated in downstream culmination of MAPK activation and were shown to be engaged in CXCR4 signal propagation, the effect of SDF-1 on ERK-1/2 activation was examined. Jurkat T-cells were stimulated with 100 nM SDF-1, lysed and immunoblotted with anti-phospho ERK-1/2 antibody which detected the active form of ERK-1/2. SDF-1 caused rapid phosphorylation of ERK-1/2 by 30 seconds which decreased gradually over 5 minutes to disappear after 10 minutes (Fig. 4.31A). This ERK-1/2 phosphorylation in response to SDF-1 was strongly inhibited by pre-treatment with 100 nM Wortmannin (Fig. 4.31B) and abrogated after 100 ng/ml Pertussis toxin pre-treatment, which suggested that PI3Ks recruited by G-protein coupled receptors had a cardinal role in mediating MAPK activation (Fig. 4.31C). The effect of Wortmannin on SDF-1 induced ERK-1/2 activation was examined over a range of concentrations in Jurkat cells and significant inhibition was evident only with inhibitor concentrations above 10 nM, namely 100 and 1000 nM (Fig. 4.32A). A report demonstrated that PI3K activation resulted in PKC\(\delta\) and PKC\(\zeta\) phosphorylation and activation by PDK1 (Le Good et al. 1998). To investigate another possible input to ERK-1/2 activation by SDF-1, the relatively selective PKC(s) inhibitor RO 320432 was also tested over a range of concentrations.
Figure 4.30A  CXCR4 and rat CD2 surface expression on Jurkat cells co-transfected with HA-tagged PKB and a rat CD2/p110α chimeric construct. Following overnight recovery from transfection, 1 x 10^5 Jurkat cells / tube were stained with either 12G5 anti-CXCR4 antibody or OX-34 anti-rat CD2 antibody or isotype matched mouse IgG and FITC-conjugated goat anti-mouse IgG antibody. The cells were analysed on a Becton Dickinson FACS vantage (excitation wavelength 488nm - emission wavelength 530nm ) as described in Materials and Methods. Similar rat CD2 expression levels were observed on cells transfected with catalytically inactive (i) rat CD2/p110α constructs. Results from one experiment representative of five other independent experiments.
Figure 4.30B  SDF-1 stimulates increased *in vitro* kinase activity of transfected HA- PKB in Jurkat T-cells. Jurkat T-cells were transiently transfected with HA-PKB or co-transfected with ratCD2/p110(a)-active or ratCD2/p110(i)-inactive. 1 x10⁷ Jurkat T-cells per point were stimulated at 37°C with vehicle (0.1 % BSA) or 100 nM SDF-1 for the times indicated. Cells were then lysed, immunoprecipitated with 2.5 μg/ml anti-HA Ab and the IPs subjected to in-vitro PKB kinase assays, followed by SDS-PAGE 7-17 % gradient electrophoresis as described in Materials and Methods. The 60kDa HA-PKB-containing upper part of the PDVF membranes was probed with 1 μg/ml phospho-specific at Ser⁴⁷³ anti-PKB Ab ( upper panel). Blots were stripped and reprobed with anti-PKB Ab (middle panel). The lower part of the gels were dried and the 15 kDa phospho-histone H2B substrate was detected by autoradiography (lower panel). Results shown from 1 experiment representative of another 3 independent experiments.
Figure 4.31 SDF-1 stimulates phosphorylation of ERK-1/2 in Jurkat T-cells

Jurkat T-cells per point were pretreated at 37°C (A) for 1 hour with vehicle (0.01% DMSO), (B) for 10 minutes with Wortmannin, or (C) for 12 hours with Pertussis Toxin and then stimulated with vehicle (0.1% BSA) or SDF-1 as indicated. Cells were then lysed, and acetone-precipitated protein samples were immunoblotted with 1μg/ml phospho-specific anti-ERK 1/2 antibody (upper panels) after electrophoresis through 10% acrylamide gels and semi-dry transfer. Blots were stripped and reprobed with anti-ERK 1/2 antibody to verify equal loading and efficiency of protein transfer (lower panels), as described in Materials and Methods. Results shown from single experiments representative of another 3 independent experiments.
Figure 4.32 Effect of PI3K and PKC isozyme inhibitors on SDF-1 mediated phosphorylation of ERK-1/2 in Jurkat T-cells. 1 x 10^7 Jurkat T-cells per point were pre-treated at 37°C (A) for 10 minutes with vehicle (0.1 % DMSO) or Wortmannin, or (B) for 1 hour with vehicle or RO 320432 and then stimulated with vehicle (0.1 % BSA) or SDF-1 for 1 minute, as indicated. Cellular protein samples were immunoblotted with 1µg/ml phospho-specific anti-ERK 1/2 antibody (upper panels) after 10 % acrylamide gel electrophoresis and semi-dry transfer. Blots were stripped and reprobed with anti-ERK 1/2 antibody to verify equal loading and efficiency of protein transfer (lower panels), as described in Materials and Methods. Results shown from 1 experiment representative of another 3 independent experiments.
Unlike Wortmannin, RO 320432 attenuated SDF-1 induced ERK-1/2 phosphorylation with the lowest concentration of 0.5 μM. This effect appeared concentration dependent since inhibition of ERK-1/2 phosphorylation increased with 10 μM and was total with 30 μM RO 320432 (Fig. 4.32B).

SUMMARY

1. CXCR4 ligation caused a rapid and transient elevation of PtdIns(3,4,5)P$_3$ levels in intact Jurkat, U937 cells or T lymphoblasts which was abrogated by pre-treatment with Pertussis toxin or Wortmannin.

2. Ligands other than SDF-1 which were characterised as partial agonists or antagonists in calcium mobilisation exerted similar effects on PtdIns(3,4,5)P$_3$ generation

3. CXCR4 ligation caused protein tyrosine kinase activation, Ras activation and tyrosine phosphorylation of multiple protein substrates including Pyk2. CXCR4-induced elevation of PtdIns(3,4,5)P$_3$ levels in intact cells was also partially blocked by Manumycin A.

4. CXCR4 ligation induced activation of both p85/p110 type 1A and p110$\gamma$ type 1B PI3K. However, loss of p85 coupling to p110 type 1A with expression of a dominant negative mutant Δp85 or tyrosine kinase inhibition with Herbimycin A did not compromise significantly total PtdIns(3,4,5)P$_3$ generation.

5. Lipid kinase activity recruited by phosphotyrosine residues appears with a late onset and is lost with Δp85 expression, whereas p110$\gamma$-associated lipid kinase activity is unaffected by dominant negative mutant Δp85 expression.

6. CXCR4 ligation caused phosphorylation and activation of PKB that peaked after 1 minute and appeared sensitive to Gic$\alpha$-protein, PI3K, Ras and MEK inhibition.

7. CXCR4 ligation caused rapid and transient phosphorylation and activation of ERK-1/2 that appeared sensitive to Gic$\alpha$-protein, PI3Ks and PKC(s) inhibition.
5.1 CXCR4 functional characterisation: Actin Polymerisation

CXCR4 INDUCED ACTIN POLYMERISATION MEASURED BY PHALLOIDIN BINDING

Rearrangement of the actin cytoskeleton is an early cellular response during chemotactic responses (Howard, Meyer, 1984). A motile response such as ordered migration to SDF-1 clearly implicates cytoskeletal re-arrangement, which in turn involves continuous turnover of structured filamentous actin (F-actin) and soluble globular actin (G-actin). As F-actin dissipates to G-actin in one cellular compartment, the inverse re-assembly occurs elsewhere in the cytosol, in the direction of movement. A way of detecting changes in the F-actin to G-actin ratio is provided by the high affinity of phalloidin binding to polymerised F-actin but not to “resting” G-actin. Thus, provided cells have been fixed and permeabilised, phalloidin conjugated with a fluorophore moiety such as FITC can enter the intracellular space, bind and be detected with a FACS apparatus. This principle was useful with purified resting T-lymphocytes where SDF-1 induced a reproducible and measurable shift in FITC fluorescence (Fig. 5.1), confirming previous observations (Bleul et al. 1996). However, with activated T-cells such as T-lymphoblasts, and more so with cell lines such as Jurkat cells and U937 cells, basal phalloidin-related fluorescence from untreated cells was already at very high levels, constituting any SDF-1 related effect immeasurable (Fig. 5.1). Since SDF-1 clearly signalled in these cells at all the other assays performed and actin assembly is an integral part of any type of cellular motility, this high basal phalloidin fluorescence may point out to a highly active but probably non ordered motility profile of these cells.

Purified, resting T-lymphocytes responded to SDF-1 in a concentration dependent manner which procured a maximum increase of about 30% from basal fluorescence levels, with concentrations of 10 nM SDF and greater (Fig. 5.2 a). The effect of SDF-1 on actin polymerisation could only be detected transiently; with a rapid onset, maximum increase in phalloidin binding occurred by 30 seconds and was concluded in 30 minutes (Fig. 5.2 b). The signal mediators required for actin polymerisation were assessed pharmacologically, since primary resting T cells were not suitable for molecular approaches, with the use of inhibitors as specific as possible.
Figure 5.1 Effect of SDF-1 on actin polymerisation in various cell types assessed with phalloidin binding. Purified T-cells (a), T-lymphoblasts (b), Jurkat cells (c) or U937 cells (d) at 2x10⁶ cells /point were treated for 1 minute with 100nM SDF-1 (red histograms) or vehicle (black histograms), fixed and treated with FITC-phalloidin and sample fluorescence was analysed on a Becton-Dickinson FACS Vantage (excitation wavelength 488nm - emission wavelength 530 nm ) as described in Materials and Methods. Histograms shown are from one experiment representative of three.
Figure 5.2 a and b. SDF-1 mediated actin polymerisation in resting T-lymphocytes.

2x10^6 purified T-cells /point were incubated (a) for 1 minute with either 0.1% BSA, or SDF-1 at the concentrations indicated, or (b) with 100 nM SDF-1 for the times indicated. Following treatment, the purified T-cells were fixed and actin polymerisation was assessed as described in Materials and Methods. Results are expressed as percentages of phalloidin binding fluorescence above basal levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p< 0.01, *** : p< 0.001.
The requirement for functional coupling of CXCR4 to Gi(α)-protein was demonstrated with the abrogation of the SDF-1 induced response at all time points following treatment with Pertussis toxin (Fig. 5.3a). The effect of Wortmannin and LY294002 on the 100 nM SDF-1 response was at all times partial, with LY294002 appearing more efficacious at the 30 second SDF-1 response. 50% of the SDF-1 response after 1 minute was the highest inhibition level for both PI3K inhibitors (Fig. 5.3a). Conversely, the reputed Ras inhibitor Manumycin A did affect the 1 minute 100 nM SDF-1 actin polymerisation greatly, abolishing any rise in fluorescence, as well as appearing to slightly increase basal phalloidin-binding induced fluorescence levels (Fig. 5.3b). However, the inhibitor of one established signalling pathway downstream of Ras, namely PD98059 the MEK inhibitor, displayed a very modest effect on the same SDF-1 response. The stimulated fluorescence levels by SDF-1 which were around 45% above basal fluorescence, in the presence of the inhibitor reached 35% above unstimulated levels (Fig. 5.3b).

SUMMARY

1. CXCR4 ligation caused a detectable and rapid increase in filamentous actin in resting T cells that peaked in 30 seconds and subsided after 30 minutes.

2. CXCR4–induced actin polymerisation was abrogated with Pertussis toxin and Manumycin A, partially blocked by PI3K inhibitors and very modestly attenuated by MEK inhibition.
Figure 5.3 a. Effect of PI3K inhibitors and Pertussis toxin on the actin polymerisation time course induced by 100nM SDF-1.

% actin polymerised

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<tr>
<th>Time (min)</th>
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Time course for 100 nM SDF-1

* : p < 0.05, ** : p < 0.01, *** : p < 0.001.
Figure 5.3b. Effect of Manumycin A and PD 98059 on the actin polymerisation induced by 100nM SDF-1 for 1 minute.

2x10^6 cells purified T-cells/point were incubated for 1 minute with 0.1% BSA (white histobars), or 100nM SDF-1 (black histobars) after pre-treatment for 60 minutes with vehicle: 0.1% DMSO, PD 98059, or Manumycin A, as indicated. Following treatment the purified T-cells were fixed and actin polymerisation was assessed as described in Materials and Methods. Results are expressed as percentages of phalloidin binding fluorescence above basal levels and represent means ± sem from 3 independent experiments. *: p < 0.05, **: p < 0.01, ***: p < 0.001.
5.2 CXCR4 functional characterisation: Internalisation

SDF-1 AND PMA INDUCED CXCR4 DOWN-REGULATION

An important characteristic of chemokine receptor function is the ability to internalise transiently in response to ligand activation. This represents one method to conclude chemotaxis at areas with high agonist concentrations, where further chemotactic signalling ceases due to diminished receptor availability. PMA, SDF-1 and the anti-CXCR4 antibody 12G5 were found to cause PTX-insensitive CXCR4 internalisation and recycling (Signoret et al. 1997; Forster et al. 1998). Although chemokine receptors have been shown to continually recycle between the surface and internal cytoplasmic stores, with ligand-induced receptor internalisation there is notably less receptor expressed on the cell surface than the cytoplasm, since for a limited period the dynamic equilibrium is shifted to favour inward movement. It is important to note however, that while CXCR4 expression assessed with flow cytometry represents a snapshot of such an equilibrium at a specified time, any effects on the rate of receptor recycling cannot be addressed. Hence, CXCR4 was found to be strongly expressed in unstimulated purified resting T-cells and Jurkat cells, but following 100 nM SDF-1 applied for 30 minutes, CXCR4 expression was markedly affected on both cell types (Fig. 5.4). Similar effect on CXCR4 expression was also observed with 50 ng/ml PMA for 30 minutes, again with both resting T-cells and Jurkat cells (Fig. 5.5 a and c). The effect of PMA appeared to be a result of PKC(s) activation since the non-PKC activating 4α-phorbol ester failed to affect CXCR4 receptor expression (Fig. 5.5 b).

EFFECT OF PKC INHIBITORS ON SDF-1 AND PMA INDUCED CXCR4 DOWN-REGULATION

The involvement of PKC(s) as a mediator of CXCR4 internalisation in both PMA- and SDF-1- induced responses was investigated with the use of pharmacological inhibitors. While older and not very selective inhibitors, such as staurosporine or bisindolylmaleimide were reported not to affect SDF-1-induced CXCR4 down-regulation (Haribabu et al. 1997; Signoret et al. 1997) and indeed did not affect it (result not shown), the relatively more selective PKC(s) inhibitor RO 318220, blocked both types of internalisation on Jurkat cells. PMA-induced responses were most sensitive, with an abrogation of internalisation at 500 nM RO 318220, the lowest concentration used (Fig. 5.6). SDF-1-induced internalisation was inhibited in a concentration dependent manner,
with complete inhibition evident only at 50 μM RO-318220 (Fig. 5.6). The PKC(s)-
sensitive nature of the SDF-1 response was further investigated in resting T-cells and
Jurkat cells with the advent of a compound with improved PKC(s) selectivity, RO
320432. On purified T-cells, the SDF-1 induced internalisation was reversed in a step-
wise fashion with increasing RO-320432 concentrations starting from 1 μM.
Interestingly, at 30μM of the inhibitor, CXCR4 receptor expression on SDF-1 treated
cells was equal to that on unstimulated cells, with a 25% cell fraction expressing more
CXCR4 than basal levels (Fig. 5.7). Jurkat cells appeared equally sensitive to the same
inhibitor as treatments of 30μM RO 320432 restored CXCR4 expression on SDF-1
treated cells back to basal unstimulated levels, again with a fraction of cells (~15%)
over-expressing CXCR4 above basal unstimulated levels (Fig. 5.8).

EFFECT OF OTHER INHIBITORS ON SDF-1 INDUCED CXCR4 DOWN-
REGULATION
Conversely, a range of inhibitors that were shown to exert an effect on other CXCR4
signalling assessments, did not appear to affect SDF-1 induced CXCR4 down-
modulation. At their highest concentrations, PI3Ks inhibitors Wortmannin (100 nM) and
LY 294002 (10 μM), the MEK inhibitor PD 98059 (10 μM), as well as the Gia-protein
inhibitor Pertussis Toxin (100 ng/ml), failed to affect ligand-induced CXCR4
internalisation on both Jurkat cells (Fig. 5.9) and resting T-cells (Fig. 5.10). However,
whereas 10 μM of the p38/MAPK inhibitor SB 203580 did not influence receptor
responses on Jurkat cells (Fig. 5.9), the reputed Ras inhibitor Manumycin A on resting
T-cells afforded a partial inhibition of SDF-1-induced CXCR4 internalisation (Fig.
5.10). The effect of Manumycin A appeared to be concentration dependent, with the
highest 10 μM concentration restoring SDF-1 treated CXCR4 expression to about two
thirds of basal unstimulated expression levels (Fig. 5.10).
Figure 5.4 Effect of SDF-1 on CXCR4 expression on a) Jurkat cells and b) resting T-cells.

Jurkat cells (a) or purified resting T-cells (b) at $1 \times 10^6$ cells / point were incubated for 30 minutes with vehicle-0.1 % BSA (red histograms) or 100 nM SDF-1 (purple histograms). Following treatment, cells were cooled to 4°C and stained for CXCR4 expression with 12G5 Ab (purple and red histograms) or isotype matched mouse IgG2a (black histograms) and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 5.5. Effect of phorbol esters on CXCR4 expression on Jurkat cells and resting T-cells. Jurkat cells (a) and (b), or purified resting T-cells (c) at 1 x 10^6 cells / point were incubated 37°C / 30 minutes with vehicle- 0.01 % DMSO (red histograms), 50 ng/ml PMA-(a) and (c), or 50 ng/ml 4α-phorbol ester -(b) ( purple histograms). Following treatment, cells were cooled to 4°C, stained for CXCR4 with 12G5 antibody (purple and red histograms) or isotype matched mouse IgG2a (black histograms) and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 5.6 Effect of RO 318220 on SDF-1 and PMA induced CXCR4 down-regulation on Jurkat cells. 1 x 10^6 Jurkat cells/ point were pre-treated for 1 hour with RO 318220 at the concentrations indicated, and stimulated at 37°C for 30 minutes with 0.1% BSA, 100 nM SDF-1, or 50 ng/ml PMA as indicated in the legend. Following treatment, cells were cooled to 4°C and stained for CXCR4 expression with 12G5 antibody and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results are expressed as the percentage (%) of cells staining for CXCR4, above basal-isotype control staining levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p < 0.01, *** : p < 0.001.
Figure 5.7. Effect of RO 320432 on SDF-1 induced CXCR4 down-regulation on resting T-cells.

**a)**

![Graph showing FITC-fluorescence log scale with different concentrations of RO 320432 and SDF-1.]

**b)**

![Graph showing cell number vs. FL-1 FITC-fluorescence (log) with various concentrations of RO 320432 and SDF-1.]

Figure 5.7 a and b Effect of RO 320432 on SDF-1 induced CXCR4 down-regulation on resting T-cells. 1 x 10^6 / point purified T-cells were (a): treated for 30 minutes with 0.1 % BSA (red histogram) or 100nM SDF-1 (purple histogram) and stained with 12G5 anti-CXCR4 antibody (red and purple histogram) or isotype matched mouse IgG2a (black histogram), or (b): pre-incubated at 37°C for 1 hour with 1-30 μM RO 320432 and then either treated with 0.1 % BSA (black histogram) or 100 nM SDF-1 (all histograms except black and red) as indicated. Following treatment, cells were cooled to 4°C and stained with 12G5 anti-CXCR4 antibody (all histograms except red) or isotype matched mouse IgG2a (red histogram) and analysed on a Becton Dickinson FACS vantage, as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 5.8 Effect of RO 320432 on SDF-1 induced CXCR4 down-regulation on Jurkat cells. 1 x 10^6 cells / point Jurkat cells were (a) incubated for 30 minutes with 100 nM SDF-1 (purple histogram) or 0.1% BSA (red histogram) and stained with 12G5 anti-CXCR4 antibody (purple and red histograms) or isotype matched mouse IgG2a (black histogram), or (b) incubated at 37 °C for 1 hour with RO 320432 and then treated with 100 nM SDF-1 for another 30 minutes, as indicated. Following treatment, cells were cooled to 4°C and stained with 12G5 anti-CXCR4 antibody (all histograms except black) or isotype matched mouse IgG2a (black histogram) and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 5.9 Effect of various inhibitors on SDF-1 induced CXCR4 down-regulation on Jurkat cells. 1 x 10^6 Jurkat cells / point were untreated (0.1% DMSO) or treated with 100 nM Wortmannin for 15 minutes, 10 μM LY294002 for 30 minutes, 100 ng/ml Pertussis toxin for 12 hours, 10 μM PD 98059 for 1 hour, 10 μM SB 203580 for 1 hour, and stimulated at 37°C for 30 minutes with 0.1% BSA, or 100 nM SDF-1 as indicated in the legend. Following treatment, cells were cooled to 4°C, stained with 12G5 anti-CXCR4 antibody and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results are expressed as the percentage (%) of cells staining for CXCR4, above basal-isotype control staining levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p< 0.01, *** : p< 0.001.
Figure 5.10 Effect of various inhibitors on SDF-1 induced CXCR4 down-regulation on resting T-cells. 1 x10^6 purified resting T-cells / point were untreated (0.1% DMSO) or treated with 100 nM Wortmannin for 15 minutes, 10 μM LY294002 for 30 minutes, 100 ng/ml Pertussis toxin for 12 hours, 10 μM PD 98059 for 1 hour, 0.1, 1 or 10 μM Manumycin A for 1 hour, and stimulated at 37°C for 30 minutes with 0.1 % BSA, or 100 nM SDF-1 as indicated in the legend. Following treatment, cells were cooled to 4°C, stained with 12G5 anti-CXCR4 antibody and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results are expressed as the percentage (%) of cells staining for CXCR4, above basal-isotype control staining levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p< 0.01, *** : p< 0.001.
SUMMARY

1. SDF-1 and PMA resulted in a marked downregulation of CXCR4 in Jurkat cells and resting purified T cells.

2. PKC inhibition with RO 328220 and RO 320432 blocked CXCR4 down-regulation induced by PMA and, at higher concentrations, that induced by SDF-1.

3. CXCR4 down-modulation by SDF-1 was insensitive to inhibition of Gia-protein, PI3Ks, MAPK, p38 but was blocked by the Ras inhibitor Manumycin A.

5.3 CXCR4 functional characterisation: Chemotaxis

CHARACTERISATION OF PI3K(s) MEDIATING CHEMOTAXIS TO SDF-1

The term chemokine was coined on a functional basis, with the best description of these molecules being defined as their ability to direct migration up their concentration gradient. Hence, the cardinal effect resulting from CXCR4 ligation and signalling should be ordered migration regardless of the cell type. The ability of SDF-1 to serve as a chemokine was assessed with the use of a 96-well Neuroprobe © chemotaxis chamber. Although this in-vitro system does not take into account physiological trans-endothelial cell layer interactions, nevertheless one can dissect and differentiate chemotactic and chemokinetic effects of molecules in solution; With every new batch, SDF-1 was tested for non-ordered motility effects, by placing the same concentration of chemokine in both the cellular-top and the emigration-bottom chambers. Since chemokine receptor stimulation can have biological effects in the absence of measurable calcium mobilisation (Turner et al. 1995), we investigated whether SDF-1 could stimulate chemotaxis of Jurkat cells, alongside all the other cell types which expressed CXCR4. SDF-1 did not exert chemokinetic effects above untreated or vehicle levels, but did behave as a chemokine with all cells that expressed CXCR4 i.e. Jurkat cells, U937 cells, purified resting T-lymphocytes and T-lymphoblasts (Fig. 5.11). Chemotaxis to SDF-1 observed a bell-shaped, concentration dependent response with all cell types, characteristic of chemokine-induced chemotaxis (Turner et al. 1995).
The optimum concentration of SDF-1 was about 10 nM universally. Greatest migration was evident with the long term cell lines rather than with the *ex-vivo* T-cells and the lowest chemotactic index displayed with T-lymphoblasts, may have been a consequence of diminished CXCR4 expression (see Fig. 4.5)(Fig. 5.11). However, it should be noted that T-lymphoblasts also had a high basal motility which acted as “background noise” when results were converted into ratios of stimulated over unstimulated migrated cells. To examine the CXCR4 signalling components that mediate chemotaxis, naturally occurring CXCR4 expression and coupling was respected with the use of pharmacological inhibitors. Jurkat cell and resting T-lymphocyte chemotaxis to SDF-1 was sensitive to Gi(α)-protein inhibition, since Pertussis toxin abolished the chemotactic response in both cell types. More surprisingly, the same responses in both cell types appeared comparably sensitive to abrogation by Wortmannin, implicating Wortmannin-sensitive PI3K(s) as key mediators of chemotaxis to SDF-1 (Fig. 5.12a and b).

The involvement of PI3K(s) on SDF-1 mediated chemotaxis could only be characterised up to a degree with the use of pharmacological tools, namely that purified T-cell and Jurkat cell chemotaxis was Wortmannin sensitive. To verify this, LY 294002, the other PI3K(s) inhibitor, was applied in a more extensive concentration range to reveal an IC\textsubscript{50} of below 1μM on chemotaxis of both T-cell types to 10 nM SDF-1 (Fig 5.13). At this concentration, LY 294002 is relatively selective for PI3K(s). Having the opportunity of using a molecular approach to address PI3K type 1A coupling to receptor activation, the SDF-1 induced migration of U937 cells transfected with an inducible, Myc-tagged, dominant negative mutant of the regulatory p85 subunit was examined. The expression of Δp85 was verified with anti- Myc Western blot analysis (see Figure 4.22A), and CXCR4 expression was not compromised by the induction process (see Figure 4.22B). Expression of Δp85 in U937 cells did not abolish the bell-shaped migration to SDF-1, however there was a modest reduction in migration towards 1 and 10 nM of SDF-1. Migration of the Δp85-expressing U937 cells was 70% of the uninduced U937 cells, to both these SDF-1 concentrations (Fig. 5.14a). Moreover, migrations to 1 nM SDF-1 with induced and uninduced Δp85-U937 cells were equally sensitive to Wortmannin and Pertussis toxin abrogation (Fig. 5.14b), just as with Jurkat cells and resting T-cells (Fig. 5.12). The profile generated with pharmacological and genetic manipulations indirectly pointed out to PI3K type 1B( p110γ), as the principal lipid kinase mediator of chemotaxis.
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Figure 5.11 Chemotaxis to SDF-1 with various cell types.

Purified T-lymphocytes, Day 10 IL-2-maintained T-lymphoblasts, Jurkat cells, or U937 cells, at 1x10^5 cells/ point were assayed for chemotaxis over 2h to 0.1% BSA, or concentrations of SDF-1 as indicated, in a 96-well Neuroprobe © chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration and represent means ± sem from 3 independent experiments. *: p<0.05, **: p< 0.01, ***: p< 0.001.
Figure 5.12. Effect of Pertussis toxin and Wortmannin on SDF-1 induced chemotaxis of T-lymphocytes and Jurkat cells.

Figure 5.12. Effect of Pertussis toxin and Wortmannin on SDF-1 induced chemotaxis of T-lymphocytes and Jurkat cells. 1x10^5 resting T-lymphocytes ( a) or Jurkat cells (b) per point were pre-treated with inhibitors and assayed for chemotaxis over 2h to 0.1% BSA, or the concentrations of SDF-1 as indicated, in a 96-well Neuroprobe chemotaxis chamber. Migration was determined as described in Materials and Methods. Cells were pre-treated with 0.001% DMSO for 1 hour, 100 nM Wortmannin for 10 min, and 100 ng/ml Pertussis Toxin for 16 hours, as indicated in the legends. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration and represent means + sem from 3 independent experiments. *: p<0.05, **: p< 0.01, *** : p< 0.001.
Figure 5.13 Effect of LY 294002 on Jurkat and purified T-cell chemotaxis to SDF-1.

1x10^6 resting T-lymphocytes or Jurkat cells per point were assayed for chemotaxis to 10 nM SDF-1 over 2h after pre-treatment for 30 min with concentrations of LY 294002 as indicated, in a 96-well Neuroprobe © chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.): the ratio of stimulated over basal migration and represent means ± sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
Figure 5.14a and b. Effect of Ap85 expression on U937 cell chemotaxis to SDF-1.

1x10^3 U937 : Ap85 cells per point were induced or uninduced to express Ap85 as indicated in the legends and assayed for chemotaxis in a 96-well Neuroprobe chemotaxis chamber over 2h to (a) 0.1% BSA, or the concentrations of SDF-1 as indicated, or (b) to 0.1% BSA, or 1nM SDF-1 following pre-treatment with 0.1 % DMSO, 100 nM wortmannin for 10 minutes, or 100 ng/ml Pertussis toxin for 16 hours. Induction of Ap85 expression was verified with anti-Myc Western blots and the process did not affect surface CXCR4 expression-see Figure 4.22. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.): the ratio of stimulated over basal migration and represent means + sem from 3 independent experiments. *: p<0.05, ** : p<0.01, *** : p<0.001.
EFFECT OF VARIOUS INHIBITORS ON CXCR4 MEDIATED CHEMOTAXIS

Although SDF-1-directed chemotaxis of in-vitro adapted Jurkat cells and fresh, ex-vivo T-cells, was equally sensitive to inhibition by pertussis toxin and wortmannin, the effect of other inhibitors was addressed using the more physiologically relevant resting T-cells. PI3Kγ has been demonstrated to mediate Gβγ-dependent regulation of the MAPK signalling pathway in other systems (Takeda et al. 1999; Lopez-Ilasaca et al. 1997). In various other systems multiple signalling molecules have been implicated in chemokine or other G-protein coupled signal transduction pathways: Eotaxin-induced eosinophil MAPK activation, as well as rolling, actin polymerisation and chemotaxis was shown to be inhibited by PD 98059 (Boehme et al. 1999); Neutrophil migration to IL-8 was reported to be blocked by wortmannin and LY 294002 but not PD 58059; however IL-8 induced activation of the Ras/Raf/MAPK cascade and p38, but not JNK (Knall et al. 1997); activation of PLD by RANTES in Jurkat cells was shown to be inhibited by Brefeldin A and bisindolylmaleimide, and required functional RhoA and ARF (Bacon et al. 1998); the PKC inhibitors Calphostin C, GO 6850, GO 6976 and bisindolylmaleimide were investigated in fMLP- and IL-8-induced neutrophil integrin-adhesion and migration, and were found to exert no effect, although myristoylated PKCζ pseudosubstrates were capable of blocking chemokine induced actin assembly and in-vitro PKCζ activity could be stimulated by chemokines but not PMA (Laudanna et al. 1998).

Therefore, the role of PKC(s), Ras signalling, MAPK(ERK-1/2) cascade and PLD-Rho/ARF GTPases, on chemotaxis to CXCR4 ligation was addressed with inhibitors as specific as possible to these events. Hence, resting T-cell migration to SDF-1 was sensitive to Manumycin A, as well as RO 320432 in a concentration dependent manner. Manumycin A appeared more efficient than RO 320432 at 1μM, blocking the response to 25% as opposed to 75% of control migration to SDF-1 respectively, yet both inhibitors abrogated the chemotactic response with the highest concentrations tested, 10 μM (Fig. 5.15). On the other hand, the effect of PD 98059 on SDF-1 induced chemotaxis of resting T-cells was always partial, and although inhibition observed concentration-dependent increases, the highest concentration of 10μM reduced the SDF-1 response to about 35% of the control SDF-1 response (Fig. 5.16). A partial inhibitory profile was also generated with the use of a single concentration of Brefeldin-A.
Figure 5.15 Effect of Manumycin A and RO 320432 on T-lymphocyte chemotaxis induced by 10nM SDF-1.

Figure 5.15 Effect of Manumycin A and RO 320432 on T-lymphocyte chemotaxis induced by 10nM SDF-1. 1x10⁵ resting T-lymphocytes per point were treated with 0.1% DMSO, or Manumycin A for 60 min or RO-320432 for 30 min at the concentrations indicated and assayed for chemotaxis to 10 nM SDF-1 over 2h, in a 96-well Neuroprobe chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.): the ratio of stimulated over basal migration and represent means + sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
Figure 5.16 Effect of PD 98059 on T-lymphocyte chemotaxis induced by 10nM SDF-1. 1x10^5 resting T-lymphocytes per point were and assayed for chemotaxis to 10 nM SDF-1 over 2h after pre-treatment for 60 min with the indicated concentrations of PD 98059, in a 96-well Neuroprobe© chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration. Means of 3 independent experiments with sem and represent means + sem from 3 independent experiments. *: p<0.05, **: p< 0.01, ***: p< 0.001.
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Figure 5.17 Effect of Brefeldin A on T-lymphocyte chemotaxis induced by SDF-1.

1x10^5 resting T-lymphocytes per point were pre-treated as indicated in the legend and assayed for chemotaxis over 2h to medium: 0.1% BSA or concentrations of SDF-1 as indicated, in a 96-well Neuroprobe® chemotaxis chamber. Cells were pre-treated for 3 hours with 0.1% DMSO, or 1.4 μM Brefeldin A. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration and represent means ± sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
At 1.4 μM, Brefeldin-A partially inhibited to about 50% the chemotactic response to 0.1-10 nM SDF-1 (Fig. 5.17). It must be noted however, that although inhibitor concentrations were kept as low as possible for optimising relative target selectivity, results using Brefeldin A, Manumycin A and to a lesser extent, RO320432, PD 98059 and Wortmannin should be considered with caution, since they may convey effects on targets other than those principally assigned, some of which may still be unidentified.

**CHEMOTACTIC EFFECTS OF OTHER CXCR4 LIGANDS**

CXCR4 mediated chemotaxis was examined with ligands other than SDF-1, that were capable of generating CXCR4 specific responses in other assays. Agonists other than SDF-1 that were specific for CXCR4 alone did produce comparable chemotactic responses. The peptide fragments, 9 amino-acids long, (1-9) from the NH₂-terminal of the native SDF-1 were tested on CXCR4 mediated chemotaxis on U937 cells and resting T-cells. The monomer and dimer NH₂-(1-9) SDF-1 soluble fragments at 40 μM elicited comparable T-cell chemotaxis between them, which was of about 75% the 10 nM SDF-1 response. With U937 cell chemotaxis, the dimer appeared a more efficient agonist than the monomer, producing ~80% of the SDF-1 response as opposed to 35%, respectively. The point mutated proline for glycine at position 2(P2G), NH₂-(1-9)-(9-1) SDF-1 covalently bound dimer fragment did not elicit significant chemotaxis, and neither did the anti-CXCR4 antibody 12G5 (Fig. 5.18). However, co-administration of the other ligands together with SDF-1 on resting T-cells exposed their partial agonist or antagonist identities on chemotaxis. The NH₂-(1-9) SDF-1 monomer as well as the dimer, at 40 μM induced chemotaxis in the order of 75% of the 10 nM SDF-1 response, but when given together with SDF-1, they antagonised the SDF-1 response down to 50%, behaving as partial agonists (Fig 5.19). Moreover, 40 μM of the NH₂-(1-9)-(9-1) SDF-1 (P2G) dimer, as well as 10 μg/ml 12G5 antibody, behaved as a true antagonists, not eliciting chemotaxis on their own but blocking the SDF-1 induced response down to basal levels. This effect was specific to CXCR4 binding since incubation with 10 μg/ml isotype-matched IgG2a did not affect the SDF-1 response significantly (Fig 5.19).
Figure 5.18 Effect of other CXCR4 ligands on cellular chemotaxis.  

![Chemotactic Index Graph](image)

Figure 5.18 Effect of other CXCR4 ligands on cellular chemotaxis. 1x10^5 resting T-lymphocytes or U937 cells per point were assayed for chemotaxis over 2h to 0.1% BSA, 10 nM SDF-1, 40 μM of monomeric, dimeric or (P2G)-substituted peptide NH2-(1-9) SDF-1 fragments, or 10μg/ml 12G5 anti-CXCR4 antibody, in a 96-well Neuroprobe © chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration and represent means ± sem from 3 independent experiments. *: p<0.05, ** : p< 0.01, *** : p< 0.001.
Figure 5.19 Chemotaxis of resting T-lymphocytes to other CXCR4 ligands alone or in combination to SDF-1.

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Results II

Figure 5.19 Chemotaxis of resting T-lymphocytes to other CXCR4 ligands alone or in combination to SDF-1. $1 \times 10^5$ resting T-lymphocytes per point were assayed for chemotaxis over 2h to 0.1% BSA, or 10 nM SDF-1, as indicated in the legend, in the presence of vehicle treatment, 40 µM monomeric, dimeric or (P2G)-substituted peptide NH$_2$-(1-9) SDF-1 fragments, 10µg/ml 12G5 anti-CXCR4 or isotype-matched mouse IgG2a as indicated, in a 96-well Neuroprobe chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.) : the ratio of stimulated over basal migration and represent means + sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
SUMMARY

1. Migration to SDF-1 occurred with varying efficiency according to cell type with optimum SDF-1 concentration of around 10 nM. Migration was abrogated by PI3K and Gi-protein inhibition. LY 294002 IC$_{50}$ ~1µM.

2. Migration to SDF-1 was not compromised with the disruption of type 1A PI3K p85 subunit coupling to the catalytic subunit p110 isoforms.

3. Ras and PKC(s) inhibitors afforded concentration-dependent complete inhibition of migration to SDF-1. Conversely, the MEK inhibitor and the PLD/RhoGTPase(s) inhibitor afforded concentration-dependent partial inhibition of migration to SDF-1.

4. All CXCR4 ligands characterised as partial agonists or antagonists in PtdIns(3,4,5)P3 generation and calcium mobilisation, behaved as such also in chemotaxis assays of resting T cells and U937 cells.

5.4 CXCR4 functional characterisation: T cell transcription factor activation

EFFECT OF SDF-1 ON LUCIFERASE CONSTRUCT TRANSCRIPTION ASSAYS
The issue of whether chemokines exert additional effects besides chemotaxis has been documented recently for members of the CXC receptor family, in particular CXCR1 and CXCR2 in endothelial cell settings propagating angiogenesis. T-cell proliferation costimulation studies with SDF-1 represent the culmination of T-cell activation were cytokine and cytokine receptor expression, such as IL-2, play a major role for the ultimate response measured, i.e. cell division. Since SDF-1 alone or in combination with other stimuli did not have a significant effect on T-cell proliferation, yet there was clear evidence of MAPK cascade engagement in response to SDF-1, the possibility that SDF-1 may exert a sub threshold effect on part of the cytokine gene transcription process was examined. Unfortunately, transfection of luciferase promoter constructs was not successful with either resting T-cells or T-lymphoblasts, but only with Jurkat cells which
had the capacity to recover. Therefore, an insight on more physiological transcription activation with normal ex-vivo cells was not available with this system. When Jurkat cells were stimulated with antigen dependent CD3 and CD28 co-stimulation for 8 hours, a positive response above basal luminescence of 3-fold with the NF-AT luciferase construct (Luc), 2.5-fold with AP-1-Luc and 4.5-fold with NF-κB-Luc was produced (Fig. 5.20). The addition of individual antibodies to CD28 did not have a significant effect above basal levels with all three reporter construct assays. CD3 ligation alone on the other hand, did produce a modest increase from basal activity of ~1.8 fold with NF-AT-Luc and 2-fold with NF-κB-Luc (Fig. 5.20a and c). The cell survival percentages after electroporation varied from 60-80% of the original cell number as assessed by trypan-blue exclusion and some normalisation was achieved with adjustment of the viable cell numbers per well for every experiment. However, there was no direct method of assessing how many viable cells were positively transfected, apart from the consistent observation that the basal luminescence from transfected cells versus control non-transfected cells was three-fold greater. Hence, there was adequate sensitivity to differentiate between mitogenic stimuli, non-mitogenic stimuli and basal transfected activity with NF-AT-Luc and NF-κB-Luc transfected cells. However, the addition of SDF-1 either alone or in combination to any of the antibody treatments did not affect in a major way the outcome from all three reporter assays (Fig. 5.20). A modest but significant exception was the ~1.4 fold increase from basal levels as a result of SDF-1 alone in the NF-κB-Luc assay (Fig. 5.20c).

EFFECT OF SDF-1 ON RANTES SECRETION BY T-CELLS

In order to investigate the possibility of “homeostatic” chemokine signals influencing “inflammatory” chemokine production, the effect of SDF-1 on RANTES production following costimulation was examined. CXCR4 is expressed functionally on resting and activated T-cells albeit with different densities (see Fig 4.5). RANTES production by purified T-lymphocytes following in vitro costimulation for 72 hours was examined, in the presence or absence of hrSDF-1. As mentioned previously in 3.1, CD3 ligation or CD28 ligation alone did not procure production of RANTES above basal levels. Co-stimulation with CD3 and CD28 ligation together, resulted in 11ng/ml RANTES production. The addition of 100 nM SDF-1 did not affect basal or co-stimulated levels of RANTES produced with any of the above treatments (Fig 5.21).
Figure 5.20. Effect of SDF-1 on transcription factor activation in Jurkat cells transfected with Luciferase constructs.

- **a** NFAT-Luc
  - Fold increase from basal luminescence
  - Vehicle vs. SDF-1

- **b** AP-1-Luc
  - Fold increase from basal luminescence
  - Vehicle vs. SDF-1

- **c** NF-κB-Luc
  - Fold increase from basal luminescence
  - Vehicle vs. SDF-1

Figure 5.20. Effect of SDF-1 on transcription factor activation in Jurkat cells transfected with Luciferase constructs. 15x10⁶ Jurkat cells per point were transfected with 10µg of hNFAT-Luciferase (a), hAP-1-Luciferase (b) and the hIL-2 NF-κB-Luciferase (c) plasmid DNA via electroporation as described in Materials and Methods. After recovery overnight, 1x10⁵ competent cells / well were treated for 8 hours with media, 1µg/ml of soluble anti-CD3, or anti-CD28, or anti-CD3 and anti-CD28 antibodies, in the presence of 0.1% BSA, or 100 nM SDF-1, as indicated. Luciferase activity was determined as described in Materials and Methods. Results are shown as fold increases of basal luminescence from transfected unstimulated cells, and represent means ± sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
Figure 5.21 Effect of SDF-1 on RANTES secretion by T-cell co-stimulation at 72 hours. 1.5x10^6 cells/well purified T-lymphocytes were treated in duplicate in 24-well plates, harvested after 72h, pelleted and assayed for RANTES in the supernatant, with a double-sandwich ELISA as described in Materials and Methods. All antibodies were administered as described in Materials and Methods in the presence of 0.1% BSA or 100nM SDF-1. Results are expressed as ng/ml of RANTES per well and represent means from 3 independent experiments with sem.
5.5 CXCR4 functional characterisation: T cell apoptosis

EFFECT OF SDF-1 ON T-CELL APOPTOSIS

Apoptosis, or programmed cell death is an integral part of T-cell physiology which can be induced by a variety of stimuli (Green, 1998; Li, Yuan, 1999; Los et al. 1999). CD95 or Fas ligation constitutes a physiological way of dispensing unwanted activated T-cells, since it is up-regulated on the surface of activated T-cells. Fas ligation is also a method of cytotoxic CD8+ T-cell mediated killing. Apoptosis is crucially different from necrosis as a method of cell death, in that the cytoplasmic constituents neatly pack in apoptotic bodies which are disposed of by phagocytosis, thus cell membrane integrity is never breached to expose potentially antigenic intracellular contents and provoke an immune reaction. An early event which can characterise cells committed to undergo apoptosis, is the exposure of phosphatidylserine (PS) residues on the outside of the cell membrane which otherwise always reside on the intracellular side. These cell membrane “flips” can be detected with fluorophore-conjugated Annexin-V which binds with high affinity to PS. Since necrotic cell fragments will also bind Annexin-V, a way to differentiate between the two modes of death is the use of propidium iodide, a nuclear stain that requires a damaged-perforated or absent cell membrane to reach its target. Hence, apoptotic cells with intact cell membranes exposing PS, will stain with Annexin-V but will exclude propidium iodide. Necrotic cells will either appear double positive, or, if they constitute nuclear cell fragments, stain for propidium iodide only. CXCR4 ligation has been reported to induce programmed cell death in several cellular settings either directly (Hesselgesser et al. 1998) or in the presence of accessory cells (Herbein et al. 1998).

The effect of SDF-1 on T-cell apoptosis was examined and compared to established T-cell death induction such as anti-Fas antibody ligation. As expected, purified resting T-cells did not respond to Fas ligation after 8 hours of incubation. Moreover, the presence of 100 nM SDF-1 did not appear to induce apoptosis or necrosis above basal levels when administered either alone or in combination to anti-Fas antibodies (Fig. 5.22). However, with Jurkat cells which represent an immortalised T-cell with an activated phenotype, after 8 hours of incubation with 0.1 µg/ml anti-Fas antibody, 40% of cells stained positive for Annexin-V (Fig. 5.23). On the contrary, 100 nM SDF-1 for 8 hours on its own did not have a significant effect on Jurkat cell apoptosis and, alongside concomitant Fas ligation, was not capable of protecting or augmenting the levels of apoptosis.
witnessed with Fas ligation alone (Fig. 5.23). The effect of anti-Fas antibody was a specific Fas ligation event since control isotype IgM did not alter basal conditions (Figs. 5.22 and 5.23). The possibility that the initial concentration of anti-Fas antibody tested, was too high to allow any detection of possible synergistic or antagonistic effects from CXCR4 ligation, was addressed by a range of anti-Fas antibody concentrations from 1 ng/ml up to 1μg/ml and a prolonged incubation period of 20 hours. With Jurkat cells, a step-wise concentration-dependent increase in Annexin-V binding revealed a basal level of around 5%, which increased to 27% with 1 ng/ml anti-Fas antibody and climaxed at 50% with 1μg/ml anti-Fas antibody (Fig. 5.24a). 100 nM SDF-1 did not appear to exert a significant effect in combination to Fas ligation, and alone, although there was a tendency to increase up to 2-fold the basal Annexin-V binding levels, this effect was not significant (Fig. 5.24a). The same concentrations of anti-Fas antibodies and SDF-1 over a 20 hour incubation period were performed with day 10 IL-2-maintained T-lymphoblasts, which displayed much higher spontaneous apoptosis levels of about 35% (Fig. 5.24b). With T-lymphoblasts, neither Fas ligation nor the presence of SDF-1 had a significant effect on the elevated spontaneous levels of Annexin-V binding (Fig. 5.24b).

SUMMARY

1 SDF-1 did not appear to affect transcription factor NFAT-, AP-1-, or NF-κB-luciferase activation in combination to CD3, CD28 or CD3/CD28 ligation. Alone, SDF-1 caused a very modest elevation in NF-κB-luciferase activity.

2 SDF-1 did not appear capable of costimulating RANTES secretion in the presence of CD3 or CD28 ligation alone, and neither did it affect CD3/CD28-costimulated RANTES secretion.

3 SDF-1 ligation for 8 hours was not capable of increasing apoptosis levels of purified T cells or Jurkat cells.

4 In combination to anti-Fas antibody ligation, SDF-1 ligation did not prevent or potentiate control or Fas-induced levels of apoptosis on T lymphoblasts and Jurkat cells.
Figure 5.22 Effect of SDF-1 and anti-FAS antibody on apoptosis of purified resting T-cells using Annexin-V binding.

Figure 5.22 Effect of SDF-1 and anti-FAS antibody on apoptosis of purified resting T-cells using Annexin-V binding. 1x10⁶ resting T-cells / per point were treated in 24-well plates with 0.1 μg/ml control mouse IgM or CH11 anti-FAS antibody in combination to 0.1% BSA or 100 nM SDF-1 as indicated, and incubated at 37°C for 8 hours. Apoptosis was assessed with Annexin-V binding as described in Materials and Methods. Lower right quadrants indicate annexin-V binding only, Upper right quadrants indicate annexin-V binding with propidium iodide staining and Upper left quadrants show propidium iodide staining only. No significant difference in staining was evident with either treatment. Results from one experiment representative of another three independent experiments.
Figure 5.23 Effect of SDF-1 and anti-FAS antibody on apoptosis of Jurkat cells using Annexin-V binding.

1x10^6 Jurkat cells / per point were treated in 24-well plates with 0.1 μg/ml control mouse IgM or CH11 anti-FAS antibody in combination to 0.1% BSA or 100 nM SDF-1 as indicated, and incubated at 37° C for 8 hours. Apoptosis was assessed with Annexin-V binding as described in Materials and Methods. Lower right quadrants indicate annexin-V binding only, Upper right quadrants indicate annexin-V binding with propidium iodide staining and Upper left quadrants show propidium iodide staining only. Results from one experiment representative of another three independent experiments.
Figure 5.24. Effect of SDF-1 on FAS-induced apoptosis of Jurkat cells and T-lymphoblasts.

**a Jurkat cells**

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**b T-lymphoblasts**

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<td>% Annexin-V binding</td>
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Figure 5.24. Effect of SDF-1 on FAS-induced apoptosis of Jurkat cells and T-lymphoblasts. 1x10^6 Jurkat cells (a) or T-lymphoblasts (b) per point were treated in 24-well plates with 0.1% BSA, or 100 nM SDF-1 as indicated in the legends together with 1 μg/ml mouse IgM Ab, or CH11 anti-FAS Ab at the concentrations indicated, and incubated at 37°C for 20 hours. Apoptosis was assessed with Annexin-V binding as described in Materials and Methods. Results are expressed as percentage (%) of annexin-V binding from lower right quadrants (see Figs. 5.26 and 5.27) from one experiment representative of another three independent experiments, and represent means ± sem.
6.1 Signalling from CXCR4 co-ligands T-tropic HIV-1 gp120 coats

The only exception to CXCR4 specific ligands used in this study, were the T-tropic HIV-1 gp120s, which remain elusive as to whether or not they propagate signalling via CD4 or CXCR4 ligation. gp120 from M-tropic and T-tropic HIV-1 isolates were reported to cause signal transduction including protein tyrosine phosphorylation, calcium mobilisation (Davis et al. 1997; Popik et al. 1998), even chemotaxis (Iyengar et al. 1999). However, whether such gp120 signalling occurs from chemokine receptor ligation, CD4 ligation or both, still remains uncertain (Madani et al. 1998; Su et al. 1999). CD4 ligation has been shown to affect TCR/CD28 costimulation signal propagation (Goedert et al. 1996; Pallier et al. 1999), thus also having the potential of interfering with chemokine signalling. Recently, a soluble ligand for the CD4 receptor was identified as IL-16 and was reported to have chemotactic properties for T-cells (Mashikian et al. 1999). However, classic chemokine signalling from 7-TMRs was also shown to be desensitised with concomitant CD4 ligation and vice-versa (Mashikian et al. 1999). Hence, the effect of T-tropic MN-HIV-1 glycoprotein coat (gp120) as a CXCR4 / CD4 co-ligand was examined on various signalling and functional assays in this study. It should be noted that the gp120s are all ligands for CD4, and CXCR4 is concomitantly occupied.

**Calcium mobilisation**

The effect of rHIV-1 gp120 glycoprotein coats as CXCR4 co-ligands on calcium mobilisation was examined on U937 cells and T-lymphoblasts since these cells both expressed CXCR4 and CD4. All the gp120s tested at various concentrations on calcium mobilisation with all the cell types did not exert a measurable effect alone. Representatively, 20 ng/ml of 3B-gp120 on U937 cells, as well as 20 ng/ml of MN-gp120 on T-lymphoblasts did not alter basal [Ca^{2+}]i levels (Fig. 6.1a and b). However, when T-lymphoblasts were pre-treated for 80 seconds with 20 ng/ml MN-gp120 and then 100 nM SDF-1 was administered, the response was severely impaired (Fig. 6.1c). This HIV-1 coat impairment of the SDF-1 response could not be attributed at this stage to CD4 signalling, CXCR4 down-modulation or simply CXCR4 occupancy-hindrance.
Results II

Generation of PtdIns(3,4,5)P₃

The effect of the T-tropic MN, SF2 and 3B- HIV-1 glycoprotein coats (gp120) as CXCR4 co-ligands was examined on PtdIns(3,4,5)P₃ accumulation on cells of the T-lineage. With T-lymphoblasts, the gp120s at 20 ng/ml were mostly ineffective. With a tendency towards a slight increase of 1.5-fold the basal levels after 1 minute, the 3B-gp120 was singled out as the most efficacious (Fig. 6.2A). On Jurkat cells, a time course study with 20 ng/ml 3B-gp120 revealed a significant effect on PtdIns(3,4,5)P₃ levels with a maximal 2-fold increase after 30 seconds (Fig. 6.2B). Again, with this assay it is not possible to assign any effect or lack of effect to CXCR4 alone.

Actin polymerisation

The effect of the T-tropic 3B HIV-1 glycoprotein coat (3B gp120) on actin polymerisation in T-lymphocytes was perhaps the most robust signalling effect measured compared to any other assay. 20 ng/ml 3B gp120 resulted in an increase of fluorescence which was comparable in magnitude to the SDF-1 response. Reaching a maximum 30% fluorescence above unstimulated levels after 1 minute, the 3B gp120 coat effect declined much faster than SDF-1, to 10% by 5 minutes and returned to basal by 30 minutes (Fig. 6.3).

CXCR4 downregulation

The possibility that T-tropic rHTV-1 gp120 glycoprotein coats as CXCR4 co-ligands may induce CXCR4 internalisation was investigated with two T-tropic strains, MN- and 3B- gp120s. On resting T-cells, CXCR4 expression was reduced by 25% after 30 minutes of 200 ng/ml MN-gp120 but appeared unaffected after 30 minutes of 200 ng/ml 3B-gp120 (Fig. 6.4). The same sensitivity to these gp120 coats was displayed by Jurkat cells, where 2, 20 or 200 ng/ml MN-gp120 caused some degree of down-modulation whereas 200 ng/ml 3B-120 again did not affect CXCR4 expression (Fig. 6.5). The effect of MN-gp120 appeared bell shaped with the middle 20 ng/ml concentration exhibiting an optimal 30% reduction in CXCR4 expression from basal levels (Fig. 6.5). The effect of T-tropic gp120s on CXCR4 receptor expression has been reported to be a result of CD4 ligation mediated via a p56 lck pathway (Su et al. 1999). Moreover, ligation of antigen receptors on B-cells has also been reported to cause PKC-mediated CXCR4 internalisation (Guinamard et al. 1999). To assess whether CD4 or even the T-cell receptor have a role to play in the partial CXCR4 down-modulation observed with T-tropic gp-120s, lck-deficient Jurkat cells termed JCam and T-cell receptor-deficient
Jurkat cells termed $J^{\text{TcR}^-}$ were used in comparison to normal Jurkat cells for assessment of surface CXCR4 expression. 100 nM SDF-1 induced efficient CXCR4 internalisation with all the Jurkat cell types, although JCam cells appeared to internalise more CXCR4 upon SDF-1 ligation (Fig. 6.6). Conversely, 20 ng/ml MN-gp120 caused partial internalisation with Jurkat cells which was abolished when JCam cells were used. Similarly, CXCR4 expression on $J^{\text{TcR}^-}$ cells also appeared resilient to 20 ng/ml MN-gp120 ligation (Fig. 6.6).

Chemotaxis
The MN- HIV-1 gp120s did not precipitate significant chemotaxis with resting T-cells, over a range of 2-200 ng/ml concentrations. With Jurkat cells, MN-gp120 appeared to exert a limited chemotactic effect, where, at 20 ng/ml, nearly twice as many Jurkat cells migrated to the gp-120 containing wells compared to basal migration (Fig. 6.7).

SUMMARY

1. MN-or IIIB- gp120 T-tropic HIV-1 glycoprotein coats behaved as antagonists causing no calcium flux on their own but blocking responses to SDF-1.

2. HIV-1 gp-120 coats caused very modest PtdIns(3,4,5)P$_3$ generation.

3. T-tropic gp120 HIV-1 caused a rapid increase in actin polymerisation comparable in magnitude and onset to SDF-1, but subsided much faster after 5 minutes.

4. T-tropic HIV-1 gp-120 afforded very modest if any chemotaxis on resting T cells and Jurkat cells.

5. T-tropic gp120 HIV-1 coats caused a very modest attenuation of surface CXCR4 expression that was abolished in the absence of functional lck or the TCR signalling complex. SDF-1 induced CXCR4 internalisation was unaffected by the absence of functional lck or the TCR signalling complex.
Figure 6.1 Effect of HIV-1 gp120s on calcium mobilisation.

a) Effect of 20 ng/ml 3B-gp120 on U937 cells

b) Effect of 20 ng/ml MN-gp120 on T-lymphoblasts

c) Effect of MN-gp120 on the SDF-1 response in T-lymphoblasts

Figure 6.1 Effect of HIV-1 gp120s on calcium mobilisation. U937 cells (a) or T-lymphoblasts from PHA treated PBMCs (b) and (c) were loaded with Fura-2 AM and assayed for calcium mobilisation as described in Materials and Methods. 2 x 10^6 cells per cuvette were stimulated at the times indicated by the arrows. Traces are in M [Ca^{2+}]i from one experiment representative of 3 other independent experiments.
Figure 6.2 A and B. Effect of HIV-1 glycoprotein coats (gp120) on PtdIns (3,4,5)P₃ generation.

**Figure 6.2.** A and B. Effect of HIV-1 glycoprotein coats (gp120) on PtdIns(3,4,5)P₃ generation. 1x10⁷ cells / point of T-lymphoblasts (A), or Jurkat cells (B) were metabolically labelled with [³²P], stimulated and phospholipids were extracted, deacylated and analysed by HPLC, as described in Materials and Methods. Cells were stimulated either (A) for 1 min with 0.1% BSA, or 20 ng/ml MN, SF2, or 3B strains of HIV-1 gp120s or (B) with 20 ng/ml 3B gp120 for the times indicated. Results are expressed as fold increases in levels of PtdIns(3,4,5)P₃ from unstimulated cells and represent means ± sem from 3 independent experiments. * : p < 0.05, **: p < 0.01, *** : p < 0.001.
Figure 6.3 Effect of the T-tropic 3B HIV-1 glycoprotein (3B gp120) on actin polymerisation in T-lymphocytes.

Figure 6.3 Effect of the T-tropic 3B HIV-1 glycoprotein (3B gp120) on actin polymerisation in T-lymphocytes. 2x10^6 cells purified T-cells/point were incubated with 20 ng/ml 3B gp120 for the times indicated. Following treatment the purified T-cells were fixed and actin polymerisation was assessed as described in Materials and Methods. Results are expressed as percentages of phalloidin binding fluorescence above basal levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p< 0.01, *** : p< 0.001.
Figure 6.4
Effect of HIV-1 gp120s on CXCR4 expression on resting T-cells.

1 x 10^6 cells / point purified resting T-cells were incubated at 37°C for 30 minutes with 0.1 % BSA (red histograms), or with 200 ng/ml MN-gp120-(a) or 3B-gp120-(b) (purple histograms). Following treatment, cells were cooled to 4°C and stained with 12G5 anti-CXCR4 antibody (purple and red histograms) or isotype matched mouse IgG2a (black histograms) and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 6.5 Effect of HIV-1 gp120s on CXCR4 expression on Jurkat cells.

1 x 10^6 / point Jurkat cells were incubated at 37°C for 30 minutes with 0.1 % BSA (red histograms), or MN-gp120 at 2 ng/ml (a), 20 ng/ml (b), 200 ng/ml (c), or 200 ng/ml 3B-gp120 – (d) / purple histograms. Following treatment, cells were cooled to 4°C and stained with 12G5 anti-CXCR4 antibody (purple and red histograms) or isotype matched mouse IgG2a (black histograms) and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results shown are from one experiment representative of another 4 independent experiments.
Figure 6.6 Effect of SDF-1 and HIV-1 MN-gp120 on CXCR4 expression on Jurkat, JCam and J^{TcR^{-}} cells.

Figure 6.6 Effect of SDF-1 and HIV-1 MN-gp120 on CXCR4 expression on Jurkat, JCam and J^{TcR^{-}} cells. Jurkat, JCam or J^{TcR^{-}} cells were stimulated at 37°C for 30 minutes with 0.1% BSA, 100 nM SDF-1, or 20 ng/ml MN-gp120, as indicated in the legend. Following treatment, cells were cooled to 4°C, stained for CXCR4 expression with 12G5 anti-CXCR4 antibody and analysed on a Becton Dickinson FACS vantage as described in Materials and Methods. Results are expressed as the percentage (%) of cells staining for CXCR4, above basal-isotype control staining levels and represent means ± sem from 3 independent experiments. * : p < 0.05, ** : p< 0.01, *** : p< 0.001.
Figure 6.7 Effect of the T-tropic MN- HIV-1 glycoprotein coats (MNgp120) on cellular chemotaxis.

Chemotactic Index

- Jurkat
- Resting T-cells

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Figure 6.7 Effect of the T-tropic MN- HIV-1 glycoprotein coats (MNgp120) on cellular chemotaxis. 1x10^5 resting T-lymphocytes or Jurkat cells per point were assayed for chemotaxis over 2h to 0.1% BSA, 10 nM SDF-1 or 2, 20 or 200 ng/ml MNgp120 as indicated, in a 96-well Neuroprobe chemotaxis chamber. Migration was determined as described in Materials and Methods. Results are expressed as Chemotactic Index (C.I.): the ratio of stimulated over basal migration and represent means ± sem from 3 independent experiments. *: p<0.05, **: p<0.01, ***: p<0.001.
DISCUSSION II

INVESTIGATION OF CHEMOKINE RECEPTOR SIGNALLING

A significant difference between the currently described chemokines was the degree of promiscuity existing for receptor activation. Chemokines like RANTES were initially described as capable of activating multiple receptors like CCR1, CCR4 and CCR5. However, with the available reagents during this study, surface and mRNA expression of CCR5 could only be detected on a very small subpopulation of normal resting T cells purified by negative selection. Therefore, studies of RANTES-induced signal transduction would have to utilise long-term, in vitro-adapted cell lines, already expressing a RANTES receptor (e.g. J6 Jurkat cells) or, transfected with a suitable receptor. Such deviation from normal T cell physiology was subject to dubious interpretation and questionable universal application. Elusively, multiple binding sites apart from CCR1 and CCR5 have also been reported for RANTES, but not MIP-1α, which mediate a transient increase in RANTES binding on ex-vivo cells from between the first 6 to 15 hours in culture; antibody ligation of CD3 could block this effect (Utsunomiya et al. 1997). No other natural ligand apart from SDF-1 to date has been shown to ligate CXCR4 (Wells et al. 1998, Zlotnik et al. 1999), a receptor found in our hands to be always present at the mRNA level, irrespectively of the activation state of the T cells. CXCR4 surface expression was also always detected, albeit at different densities according to the activation state of the cells. This universal CXCR4 expression on normal resting T cells, T lymphoblasts and cell lines, alongside the fact that SDF-1 is not been shown to be produced by T cells (Rollins 1997), indicated this receptor as most appropriate for signal analysis.

Although calcium mobilisation has been the first parameter used to examine chemokine receptor signalling capacity, a multitude of other signalling pathways have also been reported to be activated in response to chemokines (Power et al. 1996, Ward, Bacon et al. 1998, Lucacs et al. 1999). A possible group of molecules that could mediate multiple signal transduction in response to chemokines are the PI3Ks. As such, p85/p110 PI3K was shown to be activated by RANTES on T cells, in the absence of calcium mobilisation (Turner et al. 1995). Subsequently, it was established that different G-protein-coupled 7-transmembrane receptors activated distinct signalling pathways.
Carbachol-ligation of m2 muscarinic receptors induced activation of JNK, Ras, Rac1, Pak, cdc42 and RhoA. However, Wortmannin or Gβγ inhibition did not affect cdc42 and RhoA activation, partially inhibited Pak activation, and blocked JNK, Ras and Rac1 activation (Lopez-Ilasaca et al. 1998).

CALCIUM MOBILISATION FOLLOWING CXCR4 LIGATION

Expression of a chemokine receptor did not guarantee calcium mobilisation, unlike ligation of CD3. This became apparent when mRNA expression of CCR5, CCR1 and CXCR3 was compared to calcium mobilisation responses with RANTES and IP-10 on T lymphoblasts. Moreover, surface CXCR4 expression did not correlate with calcium mobilisation responses to SDF-1 on Jurkat cells. The interesting observation that SDF-1 was unable to stimulate increases in $[Ca^{2+}]_i$ in Jurkat cells, although these cells still elicited a chemotactic response to SDF-1, further supported the notion that chemokine receptor stimulation can have biological effects in the absence of measurable calcium mobilisation (Turner et al. 1995). Recently, a splice variant receptor for SDF-1 was described that was termed CXCR4-Lo and was co-expressed on PBL; CXCR4-Lo was described as 9 aa residues longer than CXCR4 inserted between residues 16 and 17 from the N-terminal. However, this receptor also fluxed calcium, but less efficiently than CXCR4, with an SDF-1 EC$_{50}$ of 20nM, as opposed to 6nM for the originally described spliced CXCR4 (Gupta, Pillarisetti, 1999). Therefore it is unlikely that absence of calcium mobilisation is attributed to differing receptor expression.

SDF-1 has been shown to prevent T-tropic HIV-1 fusion, cause calcium mobilisation that was PTX-sensitive and cause CXCR4 down-modulation following repeated administration (Hesselgesser et al. 1998). The anti-CXCR4 antibody 12G5, was reported to inhibit calcium mobilisation and migration to SDF-1 (Bleul et al. 1997), as well as responses to the N-terminal peptide fragments (Heveker et al. 1998; Loetscher et al. 1998b). With cells that did flux calcium in response to SDF-1, the CXCR4 response to its other various ligands had all the characteristics that were described previously. SDF-1-induced calcium mobilisation was sensitive to Gi-protein inhibition by PTX and completely dependent on intracellular calcium release, implying activation of PLC isoforms without contributory extracellular calcium ion influx. Moreover, calcium mobilisation by CXCR4 ligation was subject to inhibition by repeated ligand
administration and PKC activation with PMA. The first 3 residues from the N-terminal of SDF-1 were important in defining receptor agonism or antagonism by the N-terminal peptide fragments used in this study and 12G5 antibody could compromise agonist responses. The mode of attenuation of the SDF-1 response could be receptor internalisation, competition for surface binding, and/or uncoupling of the receptor signalling capacity. As elucidated with concomitant studies of CXCR4 internalisation described in more detail later, the mode of inhibition of calcium mobilisation with repeated SDF-1 administration, and PMA or 12G5 pre-treatment was due to receptor loss from the cell surface, whereas PTX disabled G-protein mediated PLCβ activation, an event subsequent to receptor ligation.

Studies with PI3K inhibitors in other systems have demonstrated a requirement for D-3 phosphoinositides in the activation of Phospholipase C-γ and hence for optimal calcium mobilisation in response to ligation of the B cell antigen receptor (Scharenberg et al. 1998; Rhee, Bae, 1997). Such a mechanism is thought to involve direct interaction of D-3 phosphoinositide lipids with the tandem SH2 domains and/or the amino terminal PH domains of phospholipase C-γ. In addition, the D-3 phosphoinositides can interact with the PH domains of the Tec family of protein tyrosine kinases, thereby influencing their membrane targeting and activation, which in turn influences Phospholipase C-γ activation. However, CXCR4 is likely to be coupled to the pertussis toxin-sensitive Phospholipase Cβ in normal T cells, similar to the effect of SDF-1 on IL-2 activated NK cells (Maghazachi et al. 1997), or IL-8 on Cos cells (Wu et al. 1993). In contrast, it is unlikely that Phospholipase Cγ is activated by CXCR4. The G protein-coupled β isoforms of Phospholipase C also contains a PH domain that can potentially interact with the D-3 phosphoinositides formed in response to SDF-1 and hence facilitate optimal calcium mobilisation. However, this seems an unlikely event given that we were unable to detect any inhibitory effect of the PI3K inhibitor Wortmannin on elevation of [Ca^{2+}]_i in T lymphoblasts in response to SDF-1 stimulation. Moreover, Knall et al. in 1997 reported that calcium mobilisation to IL-8 in neutrophils was not inhibited by Wortmannin, LY 294002 or PD 98059.

PtdIns(3,4,5)P3 GENERATION FOLLOWING CXCR4 LIGATION
SDF-1 and its peptide analogues induced a concentration and time-dependent accumulation of PtdIns(3,4,5)P3 in T lymphoblasts, U937 cells and Jurkat cells. This SDF-1-stimulated generation of D-3 phosphoinositide lipids was inhibited by pre-treatment of the cells with either an SDF-1 peptide antagonist, an anti-CXCR4 Ab and PI3K inhibitors as well as the G protein inhibitor pertussis toxin. Moreover the SDF-1 fragment peptides retained their agonistic or antagonistic qualities based on calcium mobilisation. The elevation of PtdIns(3,4,5)P3 observed in response to SDF-1 appeared to be the result of activation of more than one PI3K (e.g. the p85/p110 PI3K and PI3Kγ). However, the accumulation of PtdIns(3,4,5)P3 in Jurkat cells stimulated by SDF-1 could be completely inhibited by pre-treatment with Pertussis toxin, strongly indicating that D-3 phosphoinositide lipid accumulation occurs via a Gi protein-coupled PI3Ks. To date, the only characterised Gi protein-coupled PI3K is the class 1B PI3Kγ (Vanhaesebroeck et al. 1997). Previous studies using different cell models, such as a murine pre-B-cell lymphoma L.1.2 cell line transfected with CXCR4, have reported that SDF-1 stimulation induces a lipid kinase activity to co-associate with anti-phosphotyrosine immunoprecipitates (4G10 IPs), implying the activation of the class 1A p85/p110 heterodimer, although this was not formally demonstrated (Ganju et al. 1998). Other groups have reported an increase in PI3K activity associated with anti-phosphotyrosine immunoprecipitates after activation of G protein-coupled receptors (Stephens et al. 1993; Turner et al. 1995; Turner et al. 1998). Certainly, SDF-1 can induce the protein tyrosine phosphorylation of a number of substrates (Ganju et al. 1998; Davis et al. 1997; Jourdan et al. 1998; Popik et al. 1998), whilst other G-protein-coupled receptors have also been shown to stimulate protein tyrosine kinases after appropriate stimulation with bombesin and vasopressin (Zachary et al. 1991) or MCP-1 (Turner et al. 1998). Since synergistic activation of the p85/p110 PI3K by tyrosine-phosphorylated peptides and βγ subunits of GTP binding proteins has been reported (Okada et al. 1996; Kurosu et al. 1996), it is possible that the p85/p110 heterodimer may contribute to the accumulation of PtdIns(3,4,5)P3 observed after stimulation with SDF-1.

**ACTIVATION OF MULTIPLE PI3K ISOFORMS BY CXCR4 LIGATION**

In our hands, increases in *in-vitro* lipid kinase after SDF-1 treatment could be detected with p85 immunoprecipitates, from normal T cells and cell lines. However with Jurkat cells, this lipid kinase activity was not inhibited by Pertussis toxin, but only
Wortmannin. This indicates that although in-vivo generation of lipid kinase products by CXCR4 ligation appears to require and depends on functional Gi-protein, in-vitro analysis of lipid kinase activity contribution from p85 IPs can be misleading since the dependence on functional Gi-protein is lost. Hence, p85 may have other, non-lipid kinase roles in Gi-protein-coupled receptor activation that are not addressed, such as p85 adaptor functions in tyrosine kinase signalling complexes. Tyrosine kinase activation and substrate phosphorylation is clearly detected following CXCR4 ligation by SDF-1. However, it seems unlikely that tyrosine kinase coupled p85/p110 heterodimeric PI3K makes an important contribution to SDF-1 stimulated PtdIns(3,4,5)P3 accumulation, since this response is completely abrogated by pertussis toxin pretreatment, whilst the protein tyrosine kinase inhibitor Herbimycin A had no effect on the PtdIns(3,4,5)P3 accumulation. The use of U937: Ap85 cells confirmed the pharmacological data, since loss of potential coupling to p110 α/β/δ isoforms by Δp85 expression did not affect the SDF-1 stimulated PtdIns(3,4,5)P3 accumulation. SDF-1-stimulated activation of Gi protein-coupled PI3K plays a pivotal role in chemotaxis given that PI3K inhibitors prevented chemotaxis of Jurkat cells, and peripheral blood-derived T lymphocytes. This correlates well with previous studies which have indicated that PI3K and its metabolic products play an important role in signalling pathways mediating chemotaxis (Thelen et al. 1995; Knall et al. 1997; Turner et al. 1995; Turner et al. 1998; Kundra et al. 1994). In another study, monocyte migration to MCP-1 and MIP-1α was Wortmannin sensitive (Weber et al. 1998). Also, SDF-1-induced polarisation and chemotaxis of whole PBL cell fractions were inhibited by Wortmannin, as well as inhibition of moiesin and ICAM-3 redistribution to the uropod (Vicente-Manzanares et al. 1999).

Recently, the biochemical and physiologic role of PI3Ky has been assessed using PI3Ky−/− mice (Hirsch et al. 2000; Li et al. 2000; Sasaki et al. 2000). Hence, IL-8 was unable to stimulate accumulation of PtdIns(3,4,5)P3 in neutrophils from PI3K−/− mice, which correlated with a marked reduction of neutrophil recruitment in response to IL-8. Similarly, marked reductions of chemotaxis of PI3K−/− macrophages were observed in response to SDF-1, RANTES, MDC and to a lesser extent MIP-5 (Hirsch et al. 2000). Interesting anomalies have arisen from work with the PI3K−/− mice, in that genetic knock-out of PI3Ky resulted in varying and incomplete inhibition of chemokine-stimulated cell migration, depending on the individual chemokine analysed (Hirsch et al.
This suggests that alternative mechanisms can still sustain varying amounts of cell migration depending on the chemokine investigated. In this respect, Δp85 expression does not prevent any possible direct interaction of p110 isoforms with CXCR4 or activated G-protein subunits, as was recently reported for p110β (Murga et al. 2000). Moreover, loss of p85 coupling to p110 isoforms did not affect SDF-1 stimulated lipid kinase activity from p110γ IPs but abrogated SDF-1 stimulated lipid kinase activity from 4G10 IPs. SDF-1 stimulated lipid kinase activity from p110γ IPs could be detected with Jurkat cells, T lymphoblasts and U937 cells with a time course that matched SDF-1 stimulated PtdIns(3,4,5)P3 accumulation in intact cells. 4G10 IPs displayed a delayed onset of activation that indicated a different time frame of p85/p110 lipid kinase activity recruitment via SH2 domain affinity to phosphotyrosyl residues, from the observed profile of lipid kinase activity associated with p85 IPs. Unfortunately the adaptor functions of p85 where not addressed with the cell model of Ap85 expression, since mutations left the SH2 domains intact to participate in protein-protein interactions in the absence of recruited p110 isoform catalysis.

Interestingly, the rapid onset of lipid kinase activity from SDF-1-stimulated p110γ IPs coincided with that from p85 IPs, leaving the possibility that upon CXCR4 ligation, p85 is recruited as an adaptor by PTK activation, concomitant to direct Gβγ-mediated activation of p110γ and/or p110β. It possible that in intact cells, p110 isoforms other than p110γ may be activated in a p85-independent way and also contribute in PtdIns(3,4,5)P3 formation, with p85 preferentially engaged in adaptor functions. Consequently, from the activated p110 isoforms by CXCR4 ligation, after cell lysis for the in-vitro kinase assay only the isoforms capable of binding to p85 (i.e. p110 α/β/δ) will co-precipitate with p85. Recent studies using murine bone-marrow cells with dominant negative mutants of SHP-1, the SH2-domain specific tyrosine phosphatase which "switches off" the signals procured from tyrosine kinase recruitment, reported enhancement of migration, actin polymerisation and MAPK activation in response to SDF-1 (Kim et al. 1999). The very modest attenuation of chemotaxis following abrogation of p85/p110 coupling to CXCR4 ligation with the use of U937 : Ap85 cells, supported the notion that p110γ was the key kinase mediator for chemotactic responses to SDF-1. An additional role for PTK-dependent recruitment of adaptor molecules, as well as p85 in mediating chemotaxis would be subject to SHP-1 regulatory activity. Herbimycin A certainly did not affect PtdIns(3,4,5)P3 accumulation following treatment
with SDF-1, which corroborates the notion that signals from PTKs do not contribute towards the established PtdIns(3,4,5)P₃ requirement for chemotaxis, but PTK recruitment to CXCR4 ligation may indeed play a role in accessory signalling for chemotaxis, such as an adaptor-dependent/catalytic-independent role for PI3K. In addition, the observed *in vitro* activation of p85/p110 PI3K may still be physiologically relevant, and may account for the lack of complete inhibition of chemokine-stimulated migration of PI3Kγ⁺/⁻ macrophages (Hirsch et al 2000). For example, Wortmannin inhibits MCP-1 stimulated THP-1 cell chemotaxis, even though Wortmannin has no effect on MCP-1-stimulated PtdIns (3,4,5)P₃ accumulation (Turner et al. 1998). Indeed, overexpression of a membrane-localised constitutively active mutant of p110α induces ICAM-3 redistribution in a T cell line, thus indicating that class 1A PI3K activation is not only necessary but also sufficient to induce membrane receptor polarisation (Vicente-Manzanares et al. 1999). There are two possibilities to explain how the p85/p110 heterodimer may influence cell migration: (i) the p85/p110 may make only a small, undetectable but nevertheless significant and important contribution to the overall pool of 3'-OH lipids formed in response to chemokine stimulation; (ii) the physiological role for p85/p110 may reside in the protein serine kinase activity rather than its lipid kinase activity. In this respect, the leukocyte-specific δ isoform of class 1A PI3K exhibits unique protein serine kinase substrate specificity compared to p110α (Vanhaesebroeck et al. 1999).

The mechanism which G-coupled chemokine receptors use to recruit and activate the various PI3K isoforms still remains not well defined. Even the mode of p110γ activation by Gβγ subunits remains unclear, since p110γ is reported to be constitutively associated to the N-terminal of a regulatory p101 subunit and although recruitment by Gβγ requires bound p110γ to p101, both p101 and p110γ can also bind directly to Gβγ. Hence, Gβγ could directly bind to p110γ or release it indirectly by binding p101 (Krugmann et al. 1999). Even more perplexing, serum has been reported to cause nuclear translocation of p110γ that depends on the N-terminus (Metjian et al. 1999). Alternative pathways for PI3K isoform activation have been described. For example, a pathway for activation of p110γ downstream of receptor tyrosine kinases has been proposed involving Tec-type tyrosine kinases containing SH2- and PH-domains like Btk/Itk; following receptor stimulation, recruited p85/p110 PI 3-kinase and its lipid products would attract Btk/Itk via its PH-domains. Once beside the plasma membrane, Btk/Itk could bind via its SH2-
domains the γ-chain of the Gβγ subunit, which in turn would activate p110γ (Melendez et al. 1998). Reversing the order of activation, a study using a non-hematopoietic cell line demonstrated that following G-protein-coupled receptor activation by LPA, phosphorylation and transactivation of the unrelated receptor for EGRF occurs, which recruited Gab1/p85 and subsequent p85/p110 PI 3-kinase activity in the absence of p110γ (Laffargue et al. 1999). However, cellular permeabilisation followed by neutralising antibodies indicated the involvement of PI3Kγ following stimulation with RANTES, MCP-1, IP-10 and SDF-1 on NK cells, where also an association of p110γ with Gβγ subunits was identified with western blots (Al-Aoukaty et al. 1999). Concomitant to this study, more than one isoforms of PI3K were also shown to be involved in signalling from a chemokine receptor other than CXCR4. Differential sensitivity of \textit{in-vitro} lipid kinase and chemotaxis assays, to either Wortmannin (p85/p110 PI3K), or PTX (PI3K-C2α), indicated their respective involvement in MCP-1 signalling on THP-1 cells (Turner et al. 1998). Cross-linking of FcγR1, resulted in p85/p110 phosphotyrosine-coupled PI3K and then PI3Kγ activation; the authors selectively dissected out the isoforms' contribution with either induction of U937 : Δp85 expression or anti-sense oligonucleotides, respectively (Melendez et al. 1998). Hence, the observed different levels of PtdIns(3,4,5)P3 accumulation stimulated by SDF-1 and CD28 in this study may be simply explained by the fact that their principal-but not exclusive- targets for stimulation are different sub-classes of PI3K, namely PI3Kγ and PI3K-p85/p110 respectively.

\textbf{PTK ADAPTOR MOLECULE RECRUITMENT OTHER THAN p85 FOLLOWING CXCR4 LIGATION}

Stimulation of GPCR other than CXCR4 results in a rapid increase in the tyrosine phosphorylation of docking proteins such as Shc (Ptasznik et al. 1995; Luttrell et al. 1996) and Gab1 (Daub et al. 1997) followed by Grb-2-mediated recruitment of Ras guanine nucleotide exchange factors (GEFs) to the plasma membrane and subsequent Ras activation (Van Biesen et al. 1995). Src family kinases as well as FAK-related tyrosine kinases (Dikic 1996; Clark et al. 1995; Lev et al. 1995) have been suggested to mediate βγ-induced activation of MAP kinase and it is interesting to note that both FAK and Pyk-2 have been reported to be activated by RANTES and SDF-1, respectively.
(Bacon et al. 1996; Davis et al. 1997). CXCR4 ligation in our hands caused transient tyrosine phosphorylation of Pyk2 in Jurkat cells. Pyk2 was reported to be activated following CXCR4 ligation in other cell systems (Davis et al. 1997, Misse et al. 1999). An adaptor/linker role has been assigned to Pyk2 in signal transduction in various systems. G-protein-coupled receptors in response to bradykinin were shown to phosphorylate Pyk2, recruiting Shc / Grb2 /Sos and resulting in MAPK activation, with concomitant calcium-ion elevation and PKC activation (Lev et al. 1995). Moreover, the Grb2/Sos complex was thought to link Pyk2 with MAPK activation, whereas the p130Cas/Crk complex to link Pyk2 to JNK activation (Blaukat et al. 1999). Given that CXCR4 ligation by SDF-1 was shown to cause phosphorylation of Pyk2, Paxillin, as well as association of Crk, but resulted only in MAPK activation, and not JNK activation (Ganju et al. 1998), the former pathway (i.e. Pyk2 to MAPK) appears to be a more relevant model. Certainly the recent report that dominant negative mutants of SHP-1 caused enhancement of migration, actin polymerisation and MAPK activation in response to SDF-1, implicates PTKs in signalling that mediates, at least in part, the chemotactic response to SDF-1 (Kim et al. 1999). Another role for Pyk-2 could be its potential to mediate CXCR4 regulation of the TCR signalling threshold; Pyk-2 was reported to be tyrosine phosphorylated by SDF-1, T-tropic gp120s (Davis et al. 1997), as well as by ligation of the TCR via fyn but not lck (Qian et al. 1997). In this respect, some studies have demonstrated that SDF-1 can exert inhibitory effects on critical components of the TCR signalling cascade such as reduced tyrosine phosphorylation of ZAP-70, SLP-76 and LAT (Peacock, Jirik, 1999).

**RAS ACTIVATION FOLLOWING CXCR4 LIGATION**

There is little direct information concerning the effect of chemokines on Ras activation, although this study demonstrates that SDF-1 can activate Ras in Jurkat cells, whilst IL-8 has been demonstrated to activate Ras/Raf in neutrophils (Knall et al. 1996). The participation of Ras in chemotactic signalling was recently demonstrated in a study using transfected isoforms of human Ras; K-Ras-4B was significantly better in mediating Raf-1 activation and migration than H-Ras, K-Ras-4A and N-Ras (Voice et al. 1999). The mechanisms of Ras activation by chemokines are similarly poorly defined, although CXCR4 ligation caused Ras activation in Jurkat cells that was blocked with Manumycin A and depended on tyrosine kinase activity, since it was also blocked by Herbimycin A. However, only Manumycin A affected SDF-1 stimulated PtdIns(3,4,5)P3 accumulation
with a 60% inhibition. Assigning the effects of Manumycin A exclusively on Ras inhibition may be misleading as there may be a number of small GTPases which require farnesylation and also participate as mediators in CXCR4 signal transduction. Nevertheless, the putative involvement of Ras in coupling CXCR4 ligation to PI3K activation does not implicate dependence on tyrosine kinase activity, otherwise Herbimycin A would have an effect on PtdIns(3,4,5)P3 accumulation. A possible role for Ras is that of a mediator promoting SDF-1-stimulated Gβγ–subunit coupling to its downstream effectors, by binding p85-coupled p110 isoforms and p110γ at their Ras-GTP-binding domain. Inhibition of Ras with Manumycin A resulted in abrogation of the chemotactic response to SDF-1, indicating a central role for Ras alongside PI3K as mediators of chemotaxis. Active GTP-bound Ras can directly interact with the catalytic subunits of PI3K (Rodriguez-Viciana et al. 1994) and PI3Kγ (Rubio et al. 1997) and cells expressing constitutively active Ras have greater levels of PI3K products (Rodriguez-Viciana et al. 1994; Rodriguez-Viciana et al. 1997). In addition, there is evidence that Ras lies downstream of, and is regulated by PI3K (Hu et al. 1995; Giglione et al. 1998). Ras-binding-domains from Raf-1 or p110α, were shown to modulate Ras activity in both aspects of intrinsic GTP/GDP exchange and GEF-stimulated GTP/GDP exchange (Giglione, Parmeggiani, 1998).

Several studies have now indicated that ERK1/2 activation in response to several chemokines can be inhibited by PI3K inhibitors (Sotsios et al. 1999; Knall et al. 1996, Knall et al. 1997). This suggests the involvement of PI3K in regulating ERK activation, although at present it is unclear as to the relative proximal versus distal positioning of Ras and PI3K with respect to chemokine receptor signal transduction. It is interesting to note however, that in COS cells, LPA-induced activation of Ras and MAP kinase is blocked by inhibitors of PI3K or by dominant-negative inhibitors of the p85 subunit of PI3K, suggesting that p110α or p110β isoforms are required for Gi-mediated MAP kinase activation at a point upstream of Ras activation (Hawes et al. 1996). Furthermore, the PI3Kγ isozyme has also been shown to be involved in Gβγ-stimulated activation of MEK and ERK1/2 in COS cells at a point upstream of Ras (Lopez-Ilasaca et al. 1997). The precise mechanism by which these p110α/β and PI3Kγ facilitate Ras activation is not clear, although it has been postulated that recruitment of the PI3K to the plasma membrane results in enhanced recruitment and activity of a tyrosine kinase which is in turn responsible for mediating activation of Shc-Grb-2-Sos-Ras pathway
leading to increased MAPK activity (Hawes et al. 1996; Lopez-Ilasaca et al. 1997). In this respect, Gβγ-stimulated Shc phosphorylation is sensitive to tyrosine kinase inhibitors and Wortmannin (Touhara et al. 1995), whilst the lipid products of PI3K activity PtdIns(3,4,5)P3 has been reported to bind with high affinity to SH2 domains of proteins such as Src (Rameh et al. 1995), such that it may serve as a platform for the recruitment of signalling molecules required for MAPK activation.

**PI3K-MEDIATED, RAS-INDEPENDENT MAPK ACTIVATION**

Studies with α2A-coupled to PTX-sensitive Gi, showed that MAPK activation stimulated by the Gi-receptor required functional Ras/Sos and Raf-1 (Della Roca et al. 1997). However, p110γ was shown to be activated upon Gi-coupled LPA receptor ligation to mediate MEK but not Raf-1 activation, i.e. Ras-independent MAPK activation. In that study, Ras was activated via the LPA receptor but its contribution could be lost (Takeda et al. 1999). Ras is an established upstream signalling component for MAPK activation, yet p110γ has been reported to directly activate MAPK (Lopez-Ilasaca et al. 1997, Bondeva et al. 1998), rendering the requirement for a Ras/Raf-1/MAPK activation pathway redundant. Molecular approaches studying Ras function by Hedin et al. in 1999 implied agonist-induced Ras-dependent and Ras-independent pathways for MAPK activation. With Jurkat cells transfected with PTX-sensitive, G-protein-coupled δ opioid receptor DOR1, MAPK phosphorylation resulted from agonist ligation as well as Gβγ subunit overexpression. However, dominant-negative Ras expression only inhibited Gβγ-induced effects, but not agonist-mediated MAPK activation. Wortmannin also could not block the agonist-mediated effects (Hedin et al. 1999). In this respect it is important to note that in CHO cells transfected with IL-8 receptors, dominant-negative forms of Ras and Raf have no effect on IL-8 stimulated ERK1/2 activation (Shyamala et al. 1998) suggesting that there may be Ras-independent routes for activation of ERK1/2 by IL-8 and perhaps other chemokines.

There is emerging evidence to suggest that Ras-independent ERK1/2 activation by chemokines may potentially involve PI3Kγ activation. Thus, whilst PI3Kγ has been reported to lie upstream of Ras during Ras-dependent activation of ERK1/2 in COS cells (Rubio et al. 1997), it has also been demonstrated to stimulate Ras-independent activation of both MEK and ERK1/2 in CHO cells (Lopez-Ilasaca 1997). The action of
PI3Kγ on ERK1/2 in CHO cells may involve activation of the atypical ζ isoform of PKC (Takeda et al. 1999). PKCζ as well as PKCδ can be phosphorylated in the activation loop sites by PDK-1 in a 3'-phosphoinositide-dependent manner (LeGood et al. 1998; Chou et al. 1998). There is also evidence that PKCζ may be involved in the signalling pathways leading to neutrophil adhesion and chemotaxis in response to IL-8 (Laudanna et al. 1998). One might therefore, predict that the reported Ras-independent ERK1/2 activation stimulated by IL-8, to be Wortmannin-sensitive. However, in CHO cells transfected with the IL-8 receptors, Ras-independent ERK1/2 activation stimulated by IL-8 is not significantly inhibited by PI3K inhibitors (Shyamala et al. 1998). The significance of these observations is not fully understood, although it is interesting to note that IL-8-stimulated MAPK activation is severely abrogated by PI3K inhibitors in human neutrophils (Knall et al. 1996; Knall et al. 1997).

Thus, there is considerable diversity in signalling characteristics of individual chemokine receptors between cell types and there may be a certain degree of redundancy in certain systems. One interesting observation relating to the Wortmannin-sensitivity of IL-8-stimulated ERK1/2 activation in neutrophils, is that Ras activation is unaffected (Knall et al. 1997). However, IL-8-stimulated Raf activation is severely abrogated by PI3K inhibitors (Knall et al. 1997). There is indeed evidence from other systems that PI3K can regulate Raf-1 activation via a mechanism involving p21-activated kinase 3 (PAK) (Sun et al. 2000; Chaudhary et al. 2000). This suggests that PI3Ks and their lipid products may be required for either localisation and/or activation of Raf. The first possibility is that Ras lies upstream of PI3K and utilises PI3K as an intermediate to effect the membrane localisation and activation of Raf to the plasma membrane. Secondly, it is possible that Ras is not necessarily required for ERK activation and rather, Ras activation is involved in other functions. There are indeed multiple Ras effector pathways including RalGEF and Rac. Indeed, Rac has been implicated in regulating chemokine-stimulated cell migration as will be discussed later, although it is unclear whether Ras is required for Rac activation. The effect of chemokine receptor stimulation on other Ras effectors such as Ral-GEF is not known, although a role for Ral actin cytoskeletal rearrangements (a process important in cell migration), has been previously reported (Cantor et al. 1995). Nevertheless, some suggested mechanisms by which PI3Kγ and the p85/p110 heterodimers may be involved in ERK activation by chemokine receptors are summarised in Figure II. a.
Figure II.a. Possible roles for PI3Ks to link MAPK activation following CXCR4 ligation.

ROLE OF MAPK ACTIVATION IN CXCR4-INDUCED CHEMOTAXIS

Ras isoforms were shown to control chemotaxis to PDGF (Anand-Apte et al. 1997), independently of downstream Raf-1 activation (Kundra et al. 1995). In this study, the capacity for CXCR4 ligation to induce MAPK activation was abolished with PTX and drastically reduced with Wortmannin, implicating Gβγ-stimulated PtdIns(3,4,5)P3 generation as a key mediator in Jurkat cells. Moreover, such MAPK activation was sensitive to PKC inhibition, although the precise PKC isoforms involved were not determined. However, if PLCβ-generated DAG, stimulated PKC and resulted in MAPK phosphorylation independently of PI3K, the response to CXCR4 ligation would be expected to be less sensitive to Wortmannin. Indeed, a very faint phosphorylation signal
was detected with some blots after 1 minute CXCR4 ligation in the presence of Wortmannin, implicating some, redundant signal propagation via a G-protein / PLC / PKC / Raf-1 / MAPK pathway. Several other studies have also reported that SDF-1 stimulates phosphorylation of MEK-1 and ERK1/2 in leukemic T cell lines, T cell clones and a pre-B cell lymphoma cell line (Davis et al. 1997; Jourdan et al. 1998; Popik et al. 1998). SDF-1 was shown to cause phosphorylation of NF-κB and MAPK, but not p38/MAPK or JNK (Ganju et al. 1998). Our data indicates that PI3K inhibitors prevent SDF-1-stimulated activation of ERK1/2, implying an upstream requirement for PI3K catalytic activation. These observations correlate well with observations that PI3Kγ has been demonstrated to mediate Gβγ-dependent regulation of both the ERK1/2 MAP kinase and PKB signalling pathway in other systems (Bondeva et al. 1998; Takeda et al. 1999; Lopez-Ilasaca et al. 1997). The study with dominant negative mutants of SHP-1, by Kim et al. in 1999, implied PTKs involvement in MAPK activation from CXCR4 ligation. In other chemokine studies, ligation of CCR6 by MIP-3α was shown to cause MAPK activation, and migration was abrogated by the PI3K inhibitor LY294002 and by the MEK inhibitor PD 58059 (Sullivan et al. 1999). Fractalkline was also reported to activate MAPK kinase (Maciejewski-lenoir et al. 1999).

In our hands, the MEK inhibitor PD098059 partially inhibited SDF-1-stimulated chemotaxis suggesting that ERK1/2 activation may be involved at least in part, as a downstream effector of a PI3K-regulated signalling cascade which culminates in a chemotactic response. This would correlate with previous observations indicating a role for MAP kinases in amoeboid chemotaxis in response to cAMP and fibroblast chemotaxis in response to fibronectin (Anand-Apte et al. 1997; Wang et al. 1998). However, it should be emphasised that chemotaxis of neutrophils in response to the related chemokine IL-8 or fMLP has been reported to be independent of ERK1/2 (Thelen et al. 1995; Knall et al. 1997; Kundra et al. 1994). Hence, our observation that ERK1/2 activation is involved at least partially, in SDF-1-stimulated chemotaxis in T cells, may reflect differences between cell types and/or chemoattractants with respect to the biochemical pathways that facilitate chemotactic responses. The possible pathways for CXCR4 ligation inducing MAPK activation are represented schematically in Figure II. b.
Possible routes for MAPK activation after CXCR4 ligation

Legend for Fig.II.b: Gi: inhibitory G-protein, Gα and Gβγ: G-protein α and βγ subunits, ?PTK: unidentified protein tyrosine kinase, ? : unidentified PTK substrate/adaptor tyrosine-phosphoprotein, PKC\textsubscript{DAG}: Diacylglycerol-sensitive Protein kinase C isoforms, PKC\textsubscript{ζ}: Protein kinase C ζ, PLC\textsubscript{β}: Phospholipase C β, p85: Class1A PI3K adaptor subunit, p110α: Class1A PI3K catalytic subunit, p110γ: Class1B PI3K catalytic subunit, sos: GEF for Ras, Ras: p21-GTP-binding protein, Pyk-2: proline-rich tyrosine kinase 2, Raf: serine/threonine protein kinase, MEK: MAPK/ERK kinase, MAPK: mitogen-activated protein kinase. Adaptor tyrosine phosphoproteins : Gab1, Shc, Grb2. Single headed arrows show activation and/or phosphorylation, double-headed arrows denote association/recruitment.
RHO FAMILY GTPases AND CXCR4-INDUCED ACTIN POLYMERISATION

Reorganisation of the actin cytoskeleton is an important step in cell migration and different chemokines are able to induce the polarisation of lymphocytes with generation of specialised cell compartments. PI3K\(\gamma\) has been shown to play an important role in regulating reorganisation of the actin cytoskeleton (Ma et al. 1998), a process thought to be a prerequisite for cell movement (Howard, Meyer, 1984). However, it is interesting to note that SDF-1 stimulated actin polymerisation is only partially inhibited by PI3K inhibitors. This may indicate that whilst chemotaxis is fully dependent on PI3K activation, actin polymerisation is subject to distinct biochemical regulation involving additional PI3K independent pathways. For example, Wortmannin and LY294002 were reported to block macrophage Ig-mediated phagocytosis, but not cup formation and actin re-assembly (Cox et al. 1999). In this respect, it is interesting to note that SDF-1 induces CXCR4 coupling to both Gi (pertussis toxin-sensitive) (Bleul et al. 1996; Hesselgesser et al. 1998) and Gq (pertussis toxin-insensitive) (Bleul et al. 1996; Hesselgesser et al. 1998; Amara et al. 1997; Forster et al. 1998) family members of G proteins, so it is possible that CXCR4 uses more than one G protein subunit and may initiate signalling pathways that are independent of PI3K(s). However, resting T cell actin polymerisation in response to SDF-1 depended on functional Gi-protein implying G\(\alpha\)- and/or G\(\beta\)\(\gamma\)-dependent signal transduction. Another possibility is that more than one receptor is activated by SDF-1 and these receptors may be differentially coupled to signalling pathways and functional events. Indeed, while SDF\(^{\gamma}\) and CXCR4\(^{\gamma}\) mice have similar phenotypes relating to B cell development, they may not be identical in other respects (Tachibana et al. 1998; Nagasawa et al. 1996; Zou et al. 1998; Ma et al. 1998) and a splice variant of CXCR4 has indeed recently been identified (Gupta, Pillarisetti, 1999).

Interestingly, optimal changes in actin polymerisation were observed 30 secs-1 min after SDF-1 stimulation, but chemotaxis occurred and was measured 2 hours after stimulation. In this respect, although dependence on actin re-organisation for cellular orientation of T cells towards the APCs was described, initial LFA1/ICAM-1 interactions caused polarisation of the actin binding protein Talin, in the absence of actin polarisation (Sedwick et al. 1999). Therefore, a final possibility to explain the different...
sensitivities of SDF-1 stimulated chemotaxis and actin polymerisation to PI3K inhibitors is that whilst the observed changes in polymerised actin may well be a representation of initiation of motile response, these changes may have little to do with the subcellular contractile machinery for sensing chemotactic gradients and facilitating ordered and co-ordinated cell migration.

Different data suggests that Rho GTPases regulate cell polarity during leukocyte migration such as during leukocyte adhesion through integrins activated by chemoattractants as well as in LFA-1/ICAM--dependedent leukocyte aggregation migration (Laudanna et al. 1996; Tominaga et al. 1993). Previously, Cdc42 has been implicated in T cell polarisation toward antigen presenting cells (Stowers et al. 1995). Cdc42 also appears to have a major role in the control of directional migration of leukocytes, since a dominant-negative mutant of Cdc42 displays a much more potent inhibitory effect on leukaemic T cell line chemotaxis towards SDF-1 gradients than dominant-negative mutants of RhoA and Rac (DelPozo et al. 1999). Interestingly, active mutants of Rho, Rac and Cdc42 also inhibit SDF-1 induced migration, although this is likely due to the observed inhibition of uropod formation and also continuous activation of integrins that may impede release of certain adhesion contacts during detachment phase of cell migration. In addition, expression of Cdc42 mutants in monocytic cells demonstrated that rearrangement of the actin cytoskeleton in response to CC chemokines (MCP-1 and MIP-1α) is regulated via Cdc42 (Weber et al. 1998). Rac and Cdc42 have been reported to associate with p85/p110 (Tolias et al. 1995), whilst expression of active mutants of p110α in fibroblasts can induce actin reorganisation in the form of Rac-mediated lamellipodia and focal complexes and Rho-mediated stress fibres and focal adhesions (Reif et al. 1996). Similarly, reorganisation of the actin cytoskeleton and membrane ruffling induced by overexpression of wild type PI3Kγ or expression of an active mutant of PI3Kγ required Rac but not Cdc42 (Ma et al. 1998). Interestingly, MCP-1 and MIP-1α, but not Cdc42-stimulated cytoskeletal reorganisation can be inhibited by Wortmannin indicating the involvement of PI3K upstream of Cdc42 in chemokine-stimulated cell migration (Weber et al. 1998).

The precise mechanism by which PI3K(s) activate Rho family GTPases has not been elucidated. There is evidence however, that the Rac guanine nucelotide exchange factor Vav whose expression is restricted to hematopoietic cells may play a role in coupling
certain chemokine receptor-activated PI3K to Rho GTPases. In this respect, *in vitro* studies imply that Rac is downstream of PI3Kγ because activation of the fMLP receptor results in cytoskeletal rearrangements in a pathway involving Gβγ, PI3Kγ, Rac and Vav (Ma et al. 1998). Studies using murine bone-marrow cells with dominant negative mutants of SHP-1, reported enhancement of actin polymerisation in response to SDF-1 (Kim et al. 1999). This would imply PTKs involvement in actin polymerisation from CXCR4 ligation. The guanine nucleotide exchange activity of Vav is regulated by tyrosine phosphorylation as well as a PH domain that can bind 3'-phosphoinositide lipids (Han et al. 1998). Overexpression of Vav1 in fibroblasts induces a dynamic reorganisation of the actin cytoskeleton and initiates Rac-mediated formation of actin stress fibres and focal adhesion mediated by RhoA GTPase (Olson et al. 1996). Consistent with the regulatory effects of Vav on Rac and Rho, severe defects in the regulation of the actin cytoskeleton occur in Vav-deficient lymphocytes (Holsinger et al. 1998; Fischer et al. 1998). Furthermore, other GEFs for Cdc42 and Rac such as SOS and PIX/Cool are also regulated by PI3K (Nimmual et al. 1998; Yoshi et al. 1999). In our hands, pharmacologic inhibition of Ras with Manumycin A, but not MEK with PD 98059 abrogated actin polymerisation in response to SDF-1, indicating that the inhibition of actin polymerisation by Manumycin A is not related to inhibition of either Ras-dependent or Ras-independent signal propagation downstream to MAPK. Manumycin A, but not PD 98059, also caused an elevation of basal actin polymerisation. It is possible that Ras inhibition feeds back to disrupt signalling complexes containing p85, Rho and cdc42, but a direct inhibitory effect of farnesyltransferase inhibitors on cdc42/Rho/Rac1 cannot be discounted.

Recently, a novel binding site for F-actin but not G-actin was described, contained in the PH domains of Btk and of other Tec family PTK members. PIP2 was portrayed to compete with actin for these PH-actin binding sites, but not for the PH domains in PKC or Gβγ (Yao et al. 1999). The authors postulated a link between RhoGTPases RhoA, Rac1 and cdc42 activation, actin reorganisation, Btk co-localisation and JNK activation (Yao et al. 1999). Hence, a potential mechanism by which PI3K may be coupled to Rho GTPases, involves the Tec family kinase Btk which can be activated by SDF-1 in B cells and links PI3K to the Rho family GTPases (Nore et al. 2000). Btk is translocated to the membrane following a wide range of receptor-mediated stimuli including SDF-1 and probably occurs via interaction of its PH domain with 3’phosphoinositides (Nore et al.
2000; Scharenberg et al. 1998). A gain-of-function mutant of Btk also induced membrane ruffles and lamellipodia which can be blocked by dominant-negative forms of Rac (Nore et al. 2000). Whilst Btk may couple chemokine receptors to the Rho family of GTPases, the involvement of other tyrosine kinases cannot be ruled out.

**PLD ACTIVATION FOLLOWING CXCR4 LIGATION**

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine to generate choline and the fusogenic lipid phosphatidic acid (Roth et al. 1999). It is a vital component of the Golgi membrane and is absolutely required for vesicular transport of proteins. PLD has been reported to be activated by RANTES in T cells (Bacon et al. 1998). Additionally, IL-8 on neutrophils caused the formation of PLD products PA and PtdEtOH (Sallusto et al. 1999), whereas PLD activation was shown to coincide with Raf-1 and MAPK activation by the insulin receptor (Rizzo et al. 1999). There are several possibilities for interactions between PI3K, PLD activation and actin polymerisation induced by chemokines. Firstly, at least 3 distinct cytosolic Ca\(^{2+}\) dependent PLD isozymes may exist with specificity for PtdIns(3,4,5)P\(_3\), indicating that PLD may represent a major pathway for PtdIns(3,4,5)P\(_3\) metabolism (Ching et al. 1999). Secondly, PI3K activation of Ras may lead indirectly to the activation of the Ras effector Ral which has been reported to be involved in PLD activation (Jiang et al. 1995). Thirdly, a link between PI3K and certain members of the ARF family of proteins was recently reported. ARFs (consisting of ARF1-6) cycle between inactive GDP- and active GTP-bound forms and are regulated by ARF-GEFS and ARF-GAPS. ARFs1-5 bind intracellular membranes upon activation where they regulate intracellular vesicular transport (Roth et al. 1999). ARF-GEFs include GRP-1, ARNO and cytohesin-1 which contain a C-terminal PH domain. The use of GFP constructs has demonstrated that GRP1, ARNO and cytohesin-1 can all be recruited to the plasma membrane via a PI3K dependent mechanism (Hmama et al. 1999; Venkateswarlu et al. 1998; Venkateswarlu, Oatey et al. 1998) and PtdIns(3,4,5)P\(_3\) can enhance the ARF exchange activity of GRP1 (Klarlund et al. 1998).

Hence, one mechanism by which chemokines induce chemotaxis may be to regulate actin polymerisation via the PtdIns(3,4,5)P\(_3\)-dependent regulation of ARFs such as ARF6, which in turn leads to PLD activation. This is a particularly interesting
possibility given that PI3Kγ has been shown to play an important role in regulating reorganisation of the actin cytoskeleton (Ma et al. 1998), whilst RANTES-stimulated PLD activation in Jurkat cells is dependent on the GTP-binding proteins ARF-1 and RhoA (Bacon et al. 1998). The partial inhibitory effect of Brefeldin A on T cell chemotaxis to SDF-1, may point out to a possible degree of participation of PLD, assigning a functional role to the reported PLD activation by RANTES in Jurkat cells, which requires RhoA and ARF (Bacon et al. 1998).

**PKC(s) ACTIVATION FOLLOWING CXCR4 LIGATION**

**PKC(s) role in chemotaxis**

Calcium mobilisation caused by CXCR4 ligation did not depend on extracellular calcium and the candidate enzyme PLCβ, apart from hydrolysing calcium-releasing IP3, also generates DAG. Classic PKC isoenzymes are thought to be activated by DAG. Moreover, PtdIns(3,4,5)P3 generation is thought to recruit the atypical PKCζ. Hence, after CXCR4 ligation, the potential for both DAG-sensitive and PtdIns(3,4,5)P3-sensitive PKC isoform activation is present, although pharmacological PKC inhibitors do not distinguish between them. Inhibition of PKC with RO 320432 resulted in abrogation of the chemotactic response to SDF-1, indicating a central role for PKC alongside PI3K as mediators of chemotaxis. PKC isoforms were shown to be involved in the migratory response to MCP-1 (Yen et al. 1997), and were shown to play an essential part in the pathology of metastasis, namely the αβ4-integrin-mediated chemotaxis of carcinoma cells to EGF (Rabinovitz et al. 1999). Moreover, calcium-independent PKC isoforms were shown to mediate PI3K-regulated chemotaxis (Derman et al. 1997). The general PKC inhibitors Calphostin C, GO 6850, GO 6976 and bisindolylmaleimide were investigated in fMLP- and IL-8-induced neutrophil integrin-adhesion and migration, and were found to exert no effect, however, myristoylated PKCζ pseudosubstrates were capable of blocking chemokine induced actin assembly (Laudanna et al. 1998). Moreover, in-vitro PKCζ activity could be stimulated by chemokines but not PMA, yet in-vivo PMA treatment caused the translocation of PKCζ from the plasma to the membrane (Laudanna et al. 1998). This may suggest in-vivo interactions between different PKC isoforms. A novel PKCμ/PKD was recently described as a DAG- or PMA-sensitive protein kinase that could be inhibited in-vivo but not in-vitro by RO 328220, hence was thought to lie downstream of other PKCs (Zugaza et al. 1997). In other studies, PKC-dependent PKD activation was reported to occur in parallel and
downstream of PKCs (Cole et al. 1998), whereas PKD was found to contain a PH domain which had a regulatory role in intrinsic protein kinase activity (Iglesias, Rozengurt, 1998).

**PKC(s) role in CXCR4 internalisation**

Both SDF-1 and PMA caused CXCR4 internalisation in Jurkat cells and resting T cells. The involvement of DAG/PMA sensitive PKC isozymes in mediating receptor internalisation by SDF-1 was demonstrated with the use of 2 relatively selective PKC inhibitors RO 320432 and RO 318220. PMA-induced internalisation was more sensitive to PKC inhibition, although SDF-1 responses were eventually inhibited with the higher concentrations. Using pharmacological methods there has been some discrepancy in assigning PKC dependency in SDF-1-induced CXCR4 internalisation: The broad spectrum PKC inhibitors Staurosporine (Haribabu et al. 1997), as well as Calphostin C blocked PMA but not SDF-1 induced internalisation (Signoret et al. 1997). However, anti-CD3- and PMA-induced CXCR4 internalisation was inhibited by RO 328220 but not Staurosporine, reported to also block T cell chemotaxis (Peacock, Jirik, 1999). T cell CXCR4 down-regulation by treatment with SEA, anti-CD3 and PHA, was blocked by another PKC inhibitor GF109203X (Bermejo et al. 1998). Demonstrations of differences between PMA- and SDF-1- induced internalisation were possible with the advent of molecular approaches: PMA- but not SDF-1-induced CXCR4 internalisation was found to depend on a serine rich motif SSLKILS in the cytoplasmic -COOH region of CXCR4 which was not found on CCR5, and unlike CCR5, occurred via clathrin coated pits (Signoret et al. 1998).

Elsewhere with B cells, PKC activation and CXCR4 COOH-tail phosphorylation mediated by binding to the SSXXXIL motif was shown to be the mechanism of functional CXCR4 down-regulation after BCR ligation, via PLCγ2 activation and DAG formation (Guinamard et al. 1999). The contribution of IP3 / calcium mobilisation was dissected out with the use of IP3-receptor mutants (Guinamard et al. 1999). On the other hand, CXCR4 mutants which lacked 40 residues at the COOH tail including the SSLKIL motif were found to have normal expression, were completely unable to internalise with PMA, but some internalisation, albeit reduced, still occurred with SDF-1 (Haribabu et al. 1997). Using mutants of multiple Threo / Ser sites on the COOH tail, Serine324, Isoleucine328 and Leucine329 were defined as the essential residues for SDF-1-mediated CXCR4 internalisation, an event partially dependent on receptor phosphorylation. SDF-
1-mediated CXCR4 internalisation utilised arrestin-3 to link CXCR4 to clathrin-coated pits and was insensitive to bisindolylmaleimide, unlike PMA-induced internalisation which was inhibited by the general PKC inhibitor (Orsini et al. 1999). Moreover, although expression of dominant negative arrestin-3 and dynamin-1 mutants blocked SDF-1 and PMA-induced CXCR4 internalisation, arrestin-3 co-localisation and subsequent plasma membrane recruitment still occurred with SDF-1-internalised CXCR4 (Orsini et al. 1999).

**ROLE FOR Gi-PROTEIN INDEPENDENT CXCR4 SIGNALLING**

**G-protein coupled receptors and chemotaxis**

The dependence on functional Gi-protein for chemotaxis to SDF-1 corroborated a well characterised observation; inhibition of chemotaxis by PTX was proposed to occur as long as Gβγ release depended on Gαi activation (Arai et al. 1997; Neptune et al. 1999), since Giα-GTP, and effects on cAMP, were considered not to be important (Neptune, Bourne, 1997), whereas release of Gβγ following activation of Gαi was demonstrated as the key mediator of chemotaxis (Arai et al. 1997; Neptune, Bourne, 1997; Neptune et al. 1999). In CXCR2-ligation with IL-8, coupling to various isoforms of G-protein was shown to be possible, but only Gi and not Gs or Gq, was shown to mediate chemotaxis (Neptune, Bourne, 1997).

**CXCR4 internalisation**

In contrast to any other functional readout of CXCR4 in this study, PTX did not affect SDF-1-induced CXCR4 internalisation which could be explained by the fact that GRK-mediated receptor phosphorylation and clathrin-coated pit internalisation is a signalling event independent of receptor coupling to functional Gi-protein. Alternatively, a PTX-insensitive Gq-protein also capable of coupling to CXCR4 activation in response to SDF-1 (Bleul et al. 1996; Hesselgesser et al. 1998; Amara et al. 1997; Forster et al. 1998), could be involved in signalling for internalisation. PI3K inhibitors also did not affect SDF-1 induced CXCR4 internalisation. This is not surprising, since PI3K(s) are thought to be involved in vesicular trafficking, an event separate and subsequent to receptor internalisation. Surface CXCR4 expression examination does not address the post-endocytosis fate of the receptor. The fact that in this study only the higher concentrations of the PKC inhibitors RO 328220 and RO 320432 were capable of
blocking SDF-1-induced internalisation may indicate non-specific inhibition of kinases other than the PKCs that are selectively recruited by SDF-1 and not by PMA. However, differences between cell types have been reported in the trafficking of internalised receptors. One study found PMA and SDF-1 internalised receptors to be localised in endosomes close to the nucleus and recycled to the surface, with internalised and sequestered CXCR4 undisturbed in cycloheximide-treated cells implying re-circulation (Signoret et al. 1997). On the other hand, cell lines were shown to have clathrin-coated pit mediated spontaneous internalisation of CXCR4 and to a lesser extent CCR5, and with SDF-1-internalised CXCR4 some receptors were degraded in lysosomes and CHX treatment irreversibly reduced total CXCR4 available for traffic (Tarasova et al. 1998).

The partial inhibitory effect witnessed with Manumycin A on SDF-1-induced receptor internalisation appeared concentration-dependent and did not correlate with inhibition of PI3K. If this indicates a role for Ras in propagating receptor internalisation after CXCR4 ligation via GRK/arrestin/Clathrin-coated pits, cannot be clarified at present. The effect of Manumycin A could also be attributable to additional secondary, as yet unidentified targets (such as small GTPases), or even feedback effects from Ras-inhibition to upstream/downstream signalling moieties, also involved separately in internalisation pathways. Receptor internalisation as a way of CXCR4 signalling regulation may be the principal method for PMA- and SDF-1- induced desensitisation of the calcium response, but is not the only one. CXCR4-mutants lacking 40 C-terminal tail residues including the SSLKI motif, were expressed normally on the cell surface, and calcium mobilisation responses between wild type and mutant CXCR4 were demonstrated as identical with the first exposure to SDF-1. However, with the second SDF-1 administration, a desensitisation of signalling independent of receptor phosphorylation and internalisation was revealed and attributed to phosphorylation of PLCβ2 (Haribabu et al. 1997).

CXCR4-INDUCED TRANSCRIPTION FACTOR ACTIVATION

Transcription factor activation by engagement of surface receptors on T cells has been demonstrated previously with the use of luciferase reporter assays which allow functional assessment, as opposed to in-vitro binding or phosphorylation. Engagement of the TCR was demonstrated to activate NFAT-Luciferase activity via a Ras/JNK, and a lck/Nck/PAK pathway (Yablonski et al. 1998). In another report, luciferase assays were
used to demonstrate Ras/JNK mediated activation of NFAT-AP-1 in response to CD3/CD28 costimulation (Faris et al. 1996). The SDF-1-induced activation of a Wortmannin-sensitive PI3K appears to be an important signal required for SDF-1 stimulated biochemical events such as ERK1/2 and PKB phosphorylation. Whilst SDF-1 can couple to these distinct signalling pathways that can mediate cell survival, growth, migration and transcriptional activation, it appeared unable to support IL-2 production and T cell proliferation either alone or in combination with anti-CD3 or anti-CD28 Abs. The very modest but significant increase in NF-κB-luciferase basal activity in the presence of SDF-1, may be regarded up to a degree as corroborating the report from Ganju et al. 1998, where CXCR4 is implicated in modulating NF-κB activity. Consideration should be taken for the fact that NF-κB activity was assayed as increases of in-vitro binding and not productive transcription activation, as well as the potential chemokine signalling differences between human T cell and murine pre-B-cell lymphoma lines. Also, as NF-κB has been shown to be a key mediator of RANTES gene transcription, one potential functional result from SDF-1-stimulated NF-κB activity i.e. RANTES secretion, was not observed.

The lack of substantial transcription factor activation by SDF-1 could be explained by the possibility that SDF-1 may have a functional role in regulating the threshold for T cell activation and not in independent mitogenic transcriptional activity. Indeed, some studies have demonstrated that SDF-1 can exert inhibitory effects on critical components of the TCR signaling cascade such as reduced tyrosine phosphorylation of ZAP-70, SLP-76 and LAT (Peacock, Jirik, 1999), whilst PI3K has been proposed to play a negative role in TCR function (Reif et al. 1997). Another possibility is that the same pathways engaged at different frequencies, could differentiate short duration signals from chemokines that regulate cytoskeletal and chemotactic events, or more persistent/sustained co-stimulatory signals which control cellular survival and growth. There is indirect support for a distinction between chemokine and mitogenic signalling pathways, based on quantitative/temporal terms as opposed to qualitative terms: CXCR4-induced signal duration is strictly regulated via rapid internalisation as well as downstream signal desensitisation (Haribabu et al. 1997), in contrast to the TCR-APC "synapse" which engages several adhesion and accessory molecules to enhance the avidity and duration of interaction.
CXCR4 AND T CELL APOPTOSIS

Activation of T lymphoblasts with SEB or CD3/CD28 costimulation was reported to protect against anti-CD95-induced apoptosis up until day 8 (Mcleod et al. 1998), although another study demonstrated that following activation with SEB, T lymphoblasts undergo a discrete number of cell divisions and then become susceptible to an endogenous increase in apoptosis (Renno et al. 1999). The basal apoptosis witnessed with day 10 T lymphoblasts was again unaffected by SDF-1 treatment, suggesting an inability for CXCR4 signalling to counteract mitogenic determination of pro-apoptotic or anti-apoptotic T cell behaviour. PKB is a key mediator of growth factor-induced cell survival and protection agonist for c-Myc-induced cell death (Dudek et al. 1997; Kauffmann-Zeh et al. 1997; Kulik et al. 1997). Interestingly, as activated PKB detaches from the membrane and translocates through the cytosol and then to the nucleus (Datta et al. 1999), recent reports have demonstrated a similar serum-mediated nuclear translocation of PI3Kγ (Metjian et al. 1999). It is now well established that PKB is a downstream effector of PI3Kγ (Bondeva et al. 1998), p110β (Murga et al. 2000), and other p85-regulated p110 isoforms (Vanhaesebroeck et al. 1999, Datta et al. 1999). Supporting the role of a main PI3K effector, Th2 type T cell clones were shown to be resistant to Caspase-8 cleavage and apoptosis with concomitant elevated p85/p110 PI3K activity after CD3 ligation (Varadhachary et al. 1999). Indeed, several G-protein coupled receptors including those activated by the chemokines SDF-1, RANTES and IL-8 have been shown to activate PKB in a PI3K-dependent manner (Sotsios et al. 1999; Tilton et al. 1997; Murga et al. 1998). Furthermore, IL-8 is unable to stimulate PKB in PI3Kγ-deficient neutrophils (Hirsch et al. 2000).

Our observation that SDF-1 activates PKB fits well with the previous demonstration that PKB is a downstream effector of multiple PI3K isoforms (Bondeva et al. 1998; Murga et al. 2000). However, pre-treatment of Jurkat cells with SDF-1 was not sufficient to protect against Fas-induced Jurkat cell death. PKB is thought to require dual phosphorylation for activation. Firstly a Thr308 phosphorylation by PDK-1 occurs, which is followed by a second phosphorylation on Ser473 to fully activate PKB (Pullen et al. 1998). Jurkat cell CXCR4 ligation has the capacity to result in PKB phosphorylation and kinase activation via activation of a PI3K identified principally as PI3Kγ. It is possible
that SDF-1-induced PKB activation does not occur to a degree sufficient to counteract apoptotic Fas-induced signals on Jurkat cells. However, PKB activation via m2 receptor stimulation with carbachol was reported to be mediated via both $G\alpha$ and $G\beta\gamma$ subunits and result in survival against UV-induced apoptosis (Murga et al. 1998). This PKB activation was sensitive to Wortmannin and overexpression of PI3K$\gamma$ mutants (Murga et al. 1998). The inhibitory effects of PTX and Wortmannin on CXCR4-induced PKB activation can be explained with the uncoupling of p110$\gamma$ (and possibly other p110 isoform activated by G-protein) lipid kinase activity. Examination of PKB activation assessed by the degree of phosphorylation with phospho-Ser$^{473}$ specific antibodies proved difficult because of high basal / background, CXCR4-unrelated PKB activation, possibly induced by PI3K-independent, stress signalling (Coffer et al. 1998). Alternatively, a PI3K dependent survival mechanism was reported to involve coupling to PKB / GSK-3 (Pap, Cooper, 1998). Whatever the case, treatment with Manumycin A and PD 98059 appeared to elevate basal phospho-PKB levels so that any additional SDF-1 effect was undetectable. However, the possible contribution of the Ras/Raf-1/MEK/MAPK pathway in regulating or maintaining basal PKB activation levels remains to be clarified. A regulatory role for PTEN/MMAC1 phosphatase in counteracting PI3K / PKB activity was proposed as a mechanism for tumour development inhibition (Wu et al. 1998). The possibility that, after administration of Ras and/or MEK inhibitors, indirect inhibitory effects on such phosphatases could affect the background regulation of PKB activity, cannot be dismissed.

Nevertheless, activation of PKB by SDF-1 is hard to reconcile with evidence implicating SDF-1 and CXCR4 with the promotion of cell death in various systems (Herbein et al. 1998; Hesselgesser et al. 1998; Berndt et al. 1998). IIIB gp120 and SDF-1$\alpha$ were reported to induce CD4-independent apoptosis with an equal or better efficiency than anti-Fas antibody treatment, after 2-5 days incubation with a sensitive human NT neuronal cell line (Hesselgesser et al. 1998). Herbein et al. in 1998 demonstrated an indirect facilitation, i.e. SDF-1 or IIIB gp120 treatment of CD8$^+$ T cells and macrophages was reported to induce CD8$^+$ T cell apoptosis indirectly and only after 18h of cell-to-cell contact, occurring via upregulation of macrophage-bound TNF$\alpha$ and T cell- TNFRII. Moreover, a new type of PBL or Jurkat cell death was described, with either anti-CD4 or anti-CXCR4 cross-linked antibody treatment; this apoptotic pathway was described as distinct from CD95-induced cells death, since it occurred after just 2
hours incubation independently of Caspase-3 and -8 cleavage, poly(ADP-ribose) polymerase or GÎ± activation, did not involve the presence of TNFÎ± or p56lck, and was insensitive to CD95 receptor decoys and the caspase inhibitor ZVAD-fmk (Berndt et al. 1998). Interestingly, SDF-1 was able to block this novel anti-CXCR4 antibody-induced T cell apoptosis (Berndt et al. 1998). SDF-1 treatment in our hands did not induce or potentiate apoptosis in Jurkat cells, T lymphoblasts or resting T cells.

SIGNALLING INDUCED BY T-TROPIC HIV-1 gp120s

Misse et al. in 1999, using CD4⁺ CXCR4⁺ cells, showed IIIb gp120 to induce Pyk2 phosphorylation but not MAPK activation. However, several reports have corroborated the notion that CD4 ligation is the principal mediator of any signalling by T-tropic gp120s: In a recent report HIV-1 gp120s as well as anti-CD4 antibody treatment but not SDF-1, caused Ras-independent activation of lck, Raf-1 and MAPK, as well as activation of NF-κB, AP-1 and NF-IL6(C/EBP) (Popik et al. 1998). Expression of INF-γ, TNF-α, RANTES and MIP-1β genes detected by RT-PCR was greater in response to anti-CD4 antibodies than to gp120s, and the authors concluded that gp120-stimulated signalling could be assigned predominantly to CD4 ligation and not the chemokine receptors (Popik et al. 1998). In our hands, T-tropic gp120s from three different strains did not elicit signalling events comparable to SDF-1 ligation and these assays are discussed in more detail below:

Calcium mobilisation by T-tropic HIV-1 gp120s

T-tropic gp120s as CXCR4/CD4 co-ligands, did not appear capable of mobilising calcium on their own but were able to compromise calcium mobilisation responses to SDF-1. This finding corroborates another study with Xenopus Laevis oocytes transfected with CXCR4 and/or CD4, where the strain IIIb failed to cause calcium mobilisation alone but partially blocked responses to SDF-1 (Madani et al. 1998). The HIV-1 mediated inhibition of SDF-1 responses depended completely on CD4 for adsorption (Madani et al. 1998).
CXCR4 internalisation and chemotaxis induced by T-tropic HIV-1 gp120s

HIV-1 gp120 as CXCR4/CD4 co-ligands at best partially reduced surface CXCR4 expression but did not cause the loss of receptor expression to the degree seen with SDF-1 or PMA treatment. However, the modest gp-120 effect, unlike the robust SDF-1 response was dependent on lck and functional TCR expression. In accordance to this observation, T-tropic gp120s were shown to result in both CXCR4 and p56lck phosphorylation, SDF-1 could only phosphorylate CXCR4, and anti-CD4 antibodies could only phosphorylate p56lck (Su et al. 1999). Although all three treatments caused CXCR4 internalisation, Herbinycin A did not inhibit SDF-1 induced internalisation, therefore gp120-CXCR4 internalisation was thought to be mediated via CD4/p56lck (Su et al. 1999). Hence, steric hindrance as well as a degree of CXCR4 receptor internalisation, were likely to be responsible for the compromised calcium mobilisation responses to SDF-1. In contrast, HIV-1 gp120 was reported to compete with SDF-1 for CXCR4 binding and internalisation (Hesselgesser et al. 1997). Moreover, Misse et al. in 1999, using CD4+ CXCR4+ cells, showed IIIB gp120 to induce CXCR4 internalisation. In this study, the gp120 CXCR4-co-ligands elicited a very modest, if any chemotactic response. This finding is contrasted by a study showing that both heat-denatured and native IIIB gp120 induced chemotaxis of CD4+ and CD8+ cells from PBL or the PM1 T cell line, thus demonstrating CD4-independent gp120 binding and migration (Iyengar et al. 1999). Differences between normal ex-vivo cells, cell lines and cell types may possibly account for the contrasting reports in the literature.

CONCLUSIONS II

CHEMOKINES, T CELL ACTIVATION AND HIV-1 INFECTION

A comparative study of T cells from HIV+ and HIV+ patients, reported a correlation between the T cell activated state (HLA-DR), CCR5 expression and HIV infection, but no link between CXCR4 expression, type of virus i.e. NSI or SI, and T cell activation state (Ostrowski et al. 1998). The outcome of CD3/CD28 costimulation on HIV-infected T cells appears to depend heavily on memory or naïve phenotype and has been the subject of some controversy, since inhibition of HIV-1 production has been reported to occur with naïve T cells (Roederer et al. 1997), and stimulation of HIV replication
has been reported to preferentially occur in memory T cells (Spina et al. 1997). Interestingly, the main growth factor for memory T cells was presented to be IL-15 (1999 BSI meeting), and the upregulation of chemokine receptors by IL-15 was also shown to result in an increase in HIV infectivity (Perera et al. 1999). Multiple possible pathways mediating the CD3/CD28 antiviral effect, also reported by Carroll et al. in 1997, were proposed such as: i) downregulation of CCR5 mRNA / surface expression which impeded M-tropic HIV-1 entry but did not affect CXCR4 expression and T-tropic HIV-1 entry, ii) indirect inhibition of M-tropic HIV-1 entry by production of competing CC chemokines (Riley et al. 1997), iii) reversible inhibition of production of HIV-1 by infected T cells, dependent on constant CD3/CD28-bead exposure (Barker et al. 1998). Hence, medium from CD3/CD28-stimulated T-cells could delay and partially inhibit M-tropic HIV-1 infection of PHA-derived T lymphoblasts, an effect abolished with neutralising anti-CC chemokine antibodies, whereas direct CD3/CD28 activation could prevent and/or abolish infection completely, with neutralising anti-CC chemokine antibodies having no effect (Riley et al. 1997). However, another report described that CC-chemokines actually enhance T-tropic replication and advance the emergence of new viral particles via a Gi-protein-sensitive mechanism (Kinter et al. 1998). Moreover, HIV-1-derived Tat protein may be a candidate mediator of this T-tropic viral exacerbation by signalling-dependent CC-chemokines, as Tat has been shown to be responsible for i) immune hyperactivation causing elevated levels of T cell IL-2 secretion from CD3/CD28 co-stimulation (Ott et al. 1997), ii) upregulation of CXCR4 but not CCR5 surface expression in a CHX-sensitive way (Secchiero et al. 1999) and iii) production of MCP-1 by astrocytes and subsequent monocyte-CNS infiltration (Weiss et al. 1999). In contrast, mutant CCR5 receptors lacking the DRY motif of the second intracellular loop and various mutations on the COOH cytoplasmic tail which disrupt coupling to Goi and PLCβ / calcium mobilisation respectively, were still capable of supporting HIV entry, indicating that functional chemokine receptor signalling does not participate in HIV entry (Gosling et al. 1997).

**MODEL FOR CXCR4 SIGNALLING IN T CELLS**

Figure II.c is a schematic model of the signalling events that occur following CXCR4 activation as suggested by the results in this study and other related work. The activation of PI3K-dependent signalling cascades plays a pivotal role in chemotaxis to SDF-1 of Jurkat cells and peripheral blood-derived T lymphocytes. SDF-1 activates
Discussion II 273

Figure II.d  Schematic model of proposed T cell CXCR4 signalling pathways.

Pertussis toxin-sensitive PI3K(s) that appear necessary for the activation of PKB and ERK in response to SDF-1. Whilst the role of PKB in SDF-1 functional responses is not understood, it seems that PI3K-dependent ERK activation is required for SDF-1-stimulated chemotaxis. Interestingly, other studies have reported that G protein-coupled receptors can activate multiple PI3K effectors such as Rac and PKB (Van Weering et al. 1998; Ma et al. 1998). However, whilst cytoskeletal reorganisation and lamellipodium formation are PI3K-mediated events, they occur independently of PKB (Van Weering et al. 1998; Ma et al. 1998). Use of PI3K inhibitors such as Wortmannin and LY294002 does not distinguish between the lipid or protein kinase activities of PI3Ks. Given that PKB activation is dependent on D-3 phosphoinositide products of PI3Kγ, whilst MAPK activation is mediated by the protein kinase activity of PI3Kγ in other systems (Bondeva et al. 1998), it will be an important aim of future studies to ascertain whether it is the lipid or protein kinase activity of PI3K responsible for mediating functional effects of SDF-1:

- Elucidate further the role and mechanism of PKB and MAPK activation in CXCR4 signalling. Clarify the role and mechanism of Ras recruitment and activation after CXCR4 ligation

- Identify the receptor responsible for actin polymerisation induction by T-tropic HIV-1 gp120s. Investigate the role of such T-tropic HIV-1 gp120-induced actin polymerisation and other signalling events. Determine the contribution of CXCR4 binding to signalling from T-tropic HIV-1.
## APPENDIX I

### THE NEW NOMENCLATURE FOR CHEMOKINES

#### CXC (α) chemokines

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