Biochemical and Functional Analysis of a Promiscuous β-chemokine receptor, D6

Filipa Lopes de Mendonça
February 2002

This thesis is submitted to the University of Glasgow in accordance with the requirements for the degree of Doctor of Philosophy in the Faculty of Medicine

Beatson Institute for Cancer Research
Garscube Estate
Switchback Road
Glasgow
G61 1BD

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

À minha Mãe

(For my Mum)

Acknowledgement

I would like to thank my supervisor, Dr. Rob Nibbs, for all his help and patience over these last four years. Also thanks goes to my group leader, Prof. Gerry Graham, and my advisor, Prof. Paul Harrison, for all their critical but always encouraging advice. I would also like to thank: Shaeron Wylie that taught me all the Molecular Biology I know, Mary Freshney for her help with tissue culture; Thomas (VGMB), Gloria, Jan, Katrin and Dr Jane for all their technical and intellectual support and more importantly, for always being there when things got tough and when things went well. Iain White and Peter McHardy for their IT support. Deborah and Victoria I would like to thank for .....- where can/should I start?- I guess good friends are always there for each other! Thank you Philip and Gillian for being great sports. Big thank you to Keith and my family for always believing in me regardless of the circumstances. Above all I would like to thank Him that brought me this far.

Table of contents

List of Figures

List of Tables

Abbreviations

α_2-AR	α_2-adrenergic receptor
β_2-AR	β_2-adrenergic receptor
°C	degree centigrade
µg	microgram
µl	microlitre
-/-	knock out
Ag	antigen
AOP	aminooxypentane
APC	antigen presenting cell
apo	apoliprotein
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
CCR	CC chemokine receptor
CFU-GM	colony forming unit granulocyte-macrophage
CHO	Chinese hamster ovary cells
CIA	colagen-induced arthritis
CLA	cutaneous associated antigen
CMV	cytomegalovirus
COS	African green monkey kidney cells
CXCR	CXC chemokine receptor
DAG	diacyl-glycerol
DARC	Duffy antigen/receptor for chemokines
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTH	delayed type hypersensitivity
E. coli	Escherichia coli
EAE	experimental allergic encephalomyelitis
ECL	enhanced chemiluminescence
EST	expressed sequence tag
FCS	fetal calf serum
FDC	follicular dendritic cell
fMLP	formyl-methionyl-leucyl-phenylalanine

FPR	N-formyl peptide receptor
g	gram
GC	germinal centres
GM-CSF	granulocyte-macrophage colony stimulating factor
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
HA	haemagluttinin
HBS	HEPES buffered saline
HEK	human embryonic kidney cells
HEV	high endothelial venules
HHV	human herpes virus
HIV	human immunodeficiency virus
HOS	human osteosarcoma cells
Hr	Hours
HSV	Herpes Simplex Virus
ICAM-1	intracellular adhesion molecule-1
Ig	immunoglobulin
IP_3	phosphatidylinositol 1,4,5-triphosphate
kb	kilobase pairs
kDa	kiloDalton
kg	kilogram
KO	knock out
KSHV	Kaposi's sarcoma-associated herpes virus
l	Litre
LPS	lipopolysaccharide
LT B_4	leukotriene B_4
M	Molar
MadCAM-1	mucosal addressin cellular adhesion molecule-1
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
MCV	Molluscum contagiosum virus
mg	Milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	millimolar

mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NK	natural killer
OD	optical density (light absorbance)
ORF	open reading frame
PAF	platelet-activating factor
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphotidylinositol 3 kinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PKC	phosphokinase C
PLC	phospholipase C
PNAd	peripheral node addressin
PP	Peyer's patches
RA	rheumatoid arthritis
RBC	red blood cell
RNA	ribonucleic acid
rpm	revolutions per minute
sec	second
SLM	special liquid medium
SMC	smooth muscle cells
SV40	simian virus 40
TCR	T cell receptor
TH	T helper cell
TM	Transmembrane
TNF-α	tumour necrosis factor α
UV	ultra violet
v/v	volume per unit volume
vCKBP	viral chemokine binding protein
w/v	weight per unit volume

Single letter amino acid code

Alanine	Ala (A)
Arginine	Arg (R)

Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)
Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

Chemokine Acronyms

6Ckine	chemokine with 6 cysteines
AMAC	alternative macrophage activation-associated CC chemokine
ATAC	activation-induced, chemokines-related molecule exclusively expressed in $CD8^+$ T lymphocytes
BCA-1	B-cell activating chemokine-1
BLC	B lymphocyte chemoattractant
BRAK	breast and kidney chemokine
CC-#	CC chemokine-#
CCCK-#	CC chemokine-#
cκβ#	CC chemokine #
CRG	cytokine responsive gene
CTAP III	connective tissue-activating peptide III
dc/β-ck-1	dendritic cell β-chemokine 1
ELC	Epstein-Barr virus-induced receptor ligand chemokine
ENA-78	epithelial cell-derived neutrophil-activating factor, 78 amino acids

FIC fibroblast-inducible cytokine

GCP-# granulocyte chemoattractant protein **#**

GRO growth-related oncogene

HCC-# hemofiltrate CC chemokine-**#**

IL-8 interleukin-8

γIP-10 γ-interferon-inducible protein-10

I-TAC interferon-inducible T cell α-chemoattractant

LARC liver- and activation-related chemokine

LCC liver CC chemokine

LEC liver-expressed chemokine

Lkn-1 leukotactin-1

LMC lymphocyte and monocyte chemoattractant CC chemokine

LYNAP lymphocyte-derived neutrophil-activating peptide

MARC mast cell activation-related chemokine

MCAFmonocyte chemoattractant an activating factor

MCIF macrophage colony-inhibitor factor

MCP-# monocyte chemoattractant protein-#

MDC monocyte-derived chemokine

MDNCF monocyte-derived neutrophil chemotactic factor

MGSA melanoma growth-stimulatory activity

Mig monokine induced by γ-interferon

MIP-# macrophage inflammatory protein-#

MPIF-# myeloid progenitor inhibitor factor-#

MRP-# MIP-related protein-#

Mtn-1 monotactic-1

NAF neutrophil-activating factor

NAP-# neutrophil-activating protein-#

NCC-# novel CC chemokine-#

PARC pulmonary- and activation-regulated chemokine

PBP platelet basic protein

PBSF pre-B cell-stimulatory factor

PF-4 platelet factor-4

RANTES regulated on activation normal T cell expressed and secreted

SCM-1 single C motif-1

SCY# small cytokine #

SDF-1 stromal cell-derived factor-1

SIS-#	small inducible secreted protein-#
SLC	secondary lymphoid tissue chemokine
STCP-1	stimulated T cell chemoattractant protein-1
TARC	thymus- and activation-related chemokine
TCA-#	T-cell activation protein-#
TECK	thymus-expressed chemokine
TG	thromboglobulin
TLSF	thymic lymphoma cell-stimulating factor
TPAR	TPA-expressed protein

Adapted from (Murphy et al., 2000).

The work presented in this thesis is my own unless otherwise stated.

Abstract

Chemokines were initially defined as host defence proteins involved in cellular migration. They are now known to play a central role in the temporal and spatial positioning of leukocytes required for the successful induction of inflammation and the establishment of immunity. Moreover, chemokines exert many biological responses on other cell types and have been implicated in haemopoiesis, angiogenesis, oncogenesis and development.

In order to carry out their functions, chemokines must bind to and activate seven transmembrane (TM) domain G protein coupled receptors usually expressed on the surface of target cells. The chemokine system is implicated in many diseases such as chronic inflammation, autoimmunity, allergy, AIDS and cancer. This has lead to considerable efforts aimed towards understanding chemokine/receptor interactions with a view to preventing pathological consequences and this interaction.

Human D6 (hD6) is an unusual β chemokine receptor that binds with high affinity to many pro-inflammatory β chemokines, yet is not able to couple to signalling pathways activated by other related chemokine receptors. Moreover, immunocytochemistry has revealed that hD6 is absent from peripheral blood leukocytes and rather is expressed by endothelial cells in a subset of lymphatic vessels in the skin, lung, gut and secondary lymphoid tissue. The function of this receptor on these cells is currently uncertain, but its properties are provocative of a role in leukocyte migration, lymphangiogenesis and possibly metastasis.

In this thesis, chimaeric receptors have been used to understand the atypical biochemistry of hD6. The ultimate aim was to identify domain(s)/residue(s) responsible for the broad ligand binding promiscuity and the high affinity ligand interactions apparent for this receptor and probe the signalling properties of hD6. This work revealed the many problems associated with this approach to biochemical analysis. Chimaeric constructs bearing domains of CC and CXC receptors and large domain swaps between CC chemokine receptors, were shown to be poorly expressed on the surface of transfected cells. Additionally, these studies highlight the importance of the epitope tag, cell lines and the transfection systems used, thus indicating that the design and interpretation of receptor chimaera studies should be carefully considered.

Using small extracellular domain swaps between hD6 and hCCR5 it has been shown that: 1/ the first extracellular, and most highly conserved, loop of hD6 is required for high affinity binding to chemokine; 2/ the second and third extracellular loops appear to weakly influence ligand interaction, although antibody binding studies suggest that this result may be due to the gross structure of the chimaeric receptor being subtly altered; 3/ the N-terminus of hD6 can be replaced with that of hCCR5 with little effect on the binding of most chemokines, although the binding site for RANTES/CCL5 appears to have been altered compared to wild type hD6.

Signalling studies on mutant or chimaeric receptors have revealed that a single amino acid change in hD6 is sufficient to introduce ligand-induced signalling via pertussis toxin sensitive G-proteins into this receptor. Specifically, a single point mutation (E

to A) to convert the DKYLE motif in the second intracellular loop closer to the conserved DRYLA sequence can allow this receptor to induce weak calcium ion fluxes. A reciprocal mutation in hCCR5 blocked signalling through this receptor. Surprisingly, no other chimaeras of hD6 carrying the intracellular domains of hCCR5 were able to induce calcium ion fluxes or enhance the response seen with the E to A mutant. However, these mutants were, unlike the wild type, hD6, able to internalise upon ligand binding. Taken together these data suggest that coupling to calcium ion flux and internalisation are independently regulated events.

The results in this thesis have highlighted the technical difficulties associated with chimaeric receptor work, and the interpretation of these studies. Nonetheless, this work has identified the first extracellular loop as crucial for interaction of hD6 with ligand, derived signalling active mutants of hD6 by a single amino acid change, and generated new ideas on GPCR signalling and receptor internalisation. This work should act as a platform for the more detailed analysis of the biochemistry of hD6.

Chapter 1- Introduction

To guarantee survival, animals must defend themselves against invading pathogens. The immune system has evolved to defend vertebrates against infection. This system is composed of single cells, primarily haemopoeitic, whose function depends on their capacity to traffic, localize within tissues and interact with each other in a precisely coordinated manner. The ultimate aim of these cells is to detect and destroy pathogens and protect against subsequent infection. The body has evolved two different types of immune responses in order to guarantee lifelong immunity against many possible pathogens (immunological memory). When the body is first exposed to the antigen it develops a primary immune (innate) response that appears after a lag period of several days, rises rapidly and exponentially and then more gradually falls again. In the event of the body encountering this antigen again, whether it is within a period of weeks, months or even years, the immune system is capable of eliciting a secondary immune response (adaptive response), based on its immunological memory. This secondary immune response is characterised by a much greater response with a much shorter lag period and a longer duration in comparison to that of a primary response (for review see (Alberts et al., 1989)).

Lymphocytes are cells responsible for immune specificity. They are found in large numbers in the blood, the lymph and in specialized lymphoid organs. Once lymphocytes mature, they migrate from the primary lymphoid organs, the thymus and bone marrow, through the blood stream to the peripheral lymphoid tissues, the lymph nodes, the spleen, and the lymphoid organs associated with mucosa (appendix, Peyer's patches and tonsils). It is in these secondary lymphoid organs that

lymphocyte activation by a foreign antigen first occurs. The lymphatic vessels drain extracellular fluid from the tissues as lymph which passes through the lymph nodes and into the thoracic duct. The thoracic duct subsequently returns the lymph to the blood stream by emptying the lymph into the left subclavian vein. Lymphocytes that circulate in the bloodstream enter the peripheral lymphoid organs, and are eventually carried by lymph to the thoracic duct where they re-enter the bloodstream. Antigens and lymphocytes will eventually encounter each other in the secondary lymphoid organs. Naïve lymphocytes are continually recirculating through these tissues, to which antigen is also carried from all sites of infection and where it is trapped and presented by specialized antigen presenting cells (Alberts et al., 1989; Playfair, 1987). This continuous recirculation of naïve lymphocytes is important during primary/innate responses where the antigen-specific T and B lymphocytes need to increase their possibility of encountering the antigen and each other (Sallusto et al., 2000).

Upon infection/injury, inflammatory mediators such as TNF-α and IL-1 are released and leukocytes adhere to endothelial cells. This L-selectin mediated adhesion of leukocytes to endothelial cells is a loose reversible interaction that allows rolling of leukocytes along the affected segments of the endothelium. Once activated, leukocytes are then capable of adhering strongly to the vascular endothelium and migrating through it into the tissues (Melchers et al., 1999; Rossi & Zlotnik, 2000). The passage of lymphocytes across the endothelium into lymph nodes and Peyer's patches is a multi-step process that involves selectin-mediated rolling, followed by a triggering event and finally firm adhesion (Cyster, 1999). This multi-step process depends on adhesion molecules and chemotactic factors (Melchers et al., 1999; Rossi

& Zlotnik, 2000) and it is driven by chemoattractants. Chemoattractants can not only induce directional migration of leukocytes (Foxman et al., 1997), but also activate leukocytes to release enzymes that can facilitate migration through the extravascular tissue. Chemoattractants identified to this date include C5a, the proteolytic fragment of complement (Didsbury et al., 1992; Gerard & Gerard, 1994) and the bacterial-derived formyl-methionyl-leucyl-phenylalanine (fMLP) (Boulay et al., 1997), shown to attract both neutrophils and mononuclear cells, the lipid mediator platelet-activating factor (PAF) (Wardlaw et al., 1986) and leukotriene B_4 (LT B_4) (Ng et al., 1991), potent chemoattractants for eosinophils and numerous chemokines (Melchers et al., 1999; Schall, 1994a; Rossi & Zlotnik, 2000).

It would be impossible to summarise everything published so far in the chemokine field. In this introduction chapter I intend to introduce the chemokine superfamily, their receptors and illustrate the importance of these molecules in leukocyte trafficking, development, angiogenesis, haemopoiesis, as well as in inflammation and disease by giving examples of chemokine/chemokine receptor pairings shown to be involved in these processes. I will also describe how chemokines interact with their receptors and the outcomes of this interaction. Finally I will concentrate on hD6, a β-chemokine receptor that constitutes the centre of my studies.

1.1. The Chemokine superfamily

In the last decade, chemokines (chemotactic cytokines), previously known as intercrines, Scy (small cytokine) family or SIS (small inducible, secreted) cytokines were identified as heparin binding molecules with chemotactic activity for specific

types of leukocytes (for reviews refer to (Baggiolini, 1998; Baggiolini et al., 1997; Lindhout et al., 1999; Rollins, 1997; Schall, 1994; Wuyts et al., 1999)). Initially, researchers were trying to identify novel genes that were typically expressed in a cell-type-specific (e.g. by T cells or monocytes) or activation-state specific (e.g. after antigen exposure) fashion. The first chemokine to be identified was PF-4/CXCL4 (Walz et al., 1977), because of its presence in blood platelets and because of PF4's ability to bind strongly to heparin. Subsequently other structurally related proteins (e.g. IP-10/CXCL10, mMCP-1/CCL2, Mig/CXCL9, RANTES/CCL5, I-309/CCL1, and MIP-1α/CCL3) were identified on the bases of their cell differentiation and activation properties. IL-8/CXCL8 was the first chemokine to be identified to which a chemoattractant property was demonstrated (Walz et al., 1987; Yoshimura et al., 1987). Chemokines discovered subsequently were purified as chemoattractant activators and then identified through cDNA cloning by signal sequence trapping or by homologous hybridisation. More recently, chemokines have been identified by bioinformatics using expressed sequence tag (EST) databases. Chemokines are particularly easy to find in EST databases because they have relatively small coding sequences that can be captured by a single EST and also because they have conserved sequence motifs (see below) that are easy to recognize (Wells & Peitsch, 1997). Due to the recent 'boom' in the chemokine family, in part facilitated by the use of bioinformatics, many chemokines were codiscovered by different groups of scientists. This has caused some confusion in the chemokine field since the same protein has been give different names for example chemokine ESkine is also known as CTACK, ILC, ALP or skinkine (Murphy et al., 2000). A new systematic nomenclature has been introduced to avoid confusion. In the new nomenclature, the number in the systematic name for each chemokine matches that of the

corresponding human gene name. For example, IL8 which gene alias is SCYB8 is now called CXCL8 where CXC reflects the chemokine family, 8 the number of the gene and L indicates ligand. Throughout this work the most common name of a given chemokine will be used followed by the new systematic name, e.g. ESkine/CCL27.

Chemokines are a growing superfamily of small, inducible, predominantly secreted pro-inflammatory cytokines. The members of this family are structurally related and exhibit from 20% to over 90% identity in their amino acid sequences (Zlotnik & Yoshie, 2000). Structurally, the members of this family share similarities in that they have at least three β- pleated sheets and a C-terminal α-helix (Rollins, 1997). This superfamily is subdivided into four smaller subfamilies (Fig.1.1), which are defined by the position of conserved Cys residues involved in forming disulfide bonds in the tertiary structure of the proteins (Baggiolini et al., 1994; Miller & Krangel, 1992; Schall, 1994). The CC (or β) chemokine subfamily has the first two Cys residues adjacent to each other, whilst CXC (or α) chemokines have one intervening nonconserved amino acid between these two residues. More recently, two other subfamilies have been identified: the C (or γ) subfamily that lacks two (the first and the third) of the Cys residues and the CX_3C (or δ) subfamily, which has three amino acids between the first two Cys residues (Fig. 1.1). To date, many members of the CC (Table 1.1) and CXC (Table 1.2) chemokine subfamilies have been identified, whereas for the C (Table 1.3) and CX_3C (Table 1.4) subfamilies there is only one chemokine identified as yet, namely, lymphotactin and fractalkine, respectively (Bazan et al., 1997; Kelner et al., 1994).

The genes encoding for CXC chemokines are predominantly localized in chromosome 14 although some members of this family have been found to be localized in other chromosomes e.g. SDF-1/CXCL12 in chromosome 10 (Shirozu et al., 1995), and bolekine/CXCL14 found in chromosome 5. These findings suggest that these genes probably arose through gene duplication followed by divergence. Initially, the CXC chemokines were thought to act primarily on neutrophils. However, the recently identified CXC receptors, CXCR3 and CXCR4, were shown to mediate lymphocyte chemotaxis (Bleul et al., 1996; Loetscher et al., 1996; Oberlin et al., 1996). The CXC chemokine subfamily can be further subdivided depending on the presence of a Glu-Leu-Arg (ELR) motif immediately after the first Cys residue localized on the N-terminus of the mature protein (Wuyts et al., 1999) (see more details later).

The CC chemokines, which constitute the largest chemokine family, are known to be attractants and activators of monocytes, lymphocytes, basophils, eosinophils, NK cells and DCs but not generally neutrophils. All CC chemokine genes were thought to be mapped to chromosome 17 but genes encoding for recently identified CC chemokines have been shown to be localized in chromosome 9, for ELC/CCL19 and SLC/CCL21, chromosome 16, for TARC/CCL17 and MDC/CCL22 and chromosome 2, for LARC (reviewed in (Wuyts et al., 1999)).

Lymphotactin/XCL1-2, the only member of the C family, is a chemokine-like molecule specific for lymphocytes chemoattraction (Kelner et al, 1994). Cerdan and colleagues, have shown that lymphotactin is capable of inhibiting $CD4^+$, but co-stimulating $CD8^+$, T cell activation (Cerdan et al., 2000). More recently,

Lymphotactin/XCL1-2 has been reported to enhance TCR-induced apoptosis of $CD4^+$ T cells (Cerdan et al., 2001). In humans, there is evidence for two closely related genes that encode for two isoforms of lymphotactin: ATAC/XCL1 and SCM-1β/XCL2. These two isoforms are thought to have arisen as a result of a very recent gene duplication event and they differ from each other by two amino acids only (Yoshida et al., 1996).

Fractalkine/ CX_3CL1, the sole member of the CX_3C family, is a membrane-anchored chemokine that shares high homology with CC chemokines (Pan et al., 1997). This chemokine, expressed on activated endothelium, can be cleaved to release a soluble form that functions as a chemoattractant for monocytes, NK cell and T lymphocytes (Bazan et al, 1997). Fractalkine/ CX_3CL1 has been shown to be capable of mediating capture, firm adhesion and activation of circulating leukocytes without the involvement of integrins or other adhesion molecules (Fong et al., 1998).

Functionally, chemokines can be broadly divided into two subgroups: homeostatic and inflammatory. The homeostatic chemokines (e.g.: SDF-1/CXCL12, BCA-1/CXC13, LARC/CCL20, ELC/CCL19, SLC/CCL21, TARC/CCL17, TECK/CCL25 and DCCK1/CCL18) are constitutively expressed within lymphoid organs and have a key function in homeostatic leukocyte traffic and cell compartmentalisation within these organs (more details later). On the other hand, inflammatory chemokines (e.g. MCP-1/CCL2, IL-8/CXCL8, GRO-α/CXCL1, GRO-β/CXCL2, GRO-γ/CXCL3, Mig/CXCL9, I-TAC/CXC11, RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, MIP-1γ/CCL15, Eotaxin 1/CCL11 and 2/CCL24, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13 and MCP-5/CCL12) are produced by many different types of cells (such as

leukocytes, endothelial, epithelial and stromal cells) in response to inflammatory stimuli such as LPS, IL-1 and TNF-α, regulate recruitment of leukocytes to inflammatory sites and are mostly expressed during inflammation (Lindhout et al., 1999). Some chemokines such as MDC/CCL22 can have an inflammatory role (Godiska et al., 1997), as well as homeostatic properties (Schaniel et al., 1998; Tang & Cyster, 1999) depending on when and where they are expressed (see more details later).

1.2. Chemokine receptors

Chemokines mediate their activities by binding to cell surface receptors that belong to the 7 transmembrane domain G protein-coupled receptors. In general, chemokine receptors are characterized by having an acidic N-terminal domain, the sequence DRYLAIVHA, or a variation of it, in the second intracellular loop, and a Cys in each of the four extracellular domains. These four conserved Cys residues are thought to be involved in maintaining the tertiary structure of the mature protein, holding the receptors in a cylindrical shape (Fig.1.2) (Murphy et al., 2000).

Chemokine receptors can be classified into four different classes based on the chemokine member(s) they bind. Therefore, they are known as β- or CC-chemokine receptors (CCRs), α- or CXC- chemokine receptors (CXCRs), γ- or C-chemokine receptors (XCRs), δ- or CX_3C-chemokine receptors (CX_3CRs). According to Premack and Schall, (Premack & Schall, 1996), chemokine receptors can also be classified as: specific, shared, promiscuous and viral (Table 1.5).

Specific receptors are defined as those receptors that bind to one ligand only such as CXCR5 and CXCR4 that only bind to BLC/CXCL13 and SDF-1/CXCL12, respectively. Shared receptors bind to more than one receptor within the same family. Examples of shared receptors are CXCR2 that binds to all ELR^+ chemokines (Ahuja et al., 1996; Lee et al., 1992), CXCR3 that binds to the ELR^- chemokines, IP-10/CXCL10, Mig/CXCL9 and I-TAC/CXCL11 (Bao et al., 1999) and CCR5 shown to bind to MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and MCP-2/CCL8. A promiscuous receptor is a receptor that binds to many chemokine ligands of either CXC or CC branches. For example, the Duffy blood group antigen (DARC) which was originally identified as Duffy glycoprotein D (Chauduri et al., 1993) and later as the human erythrocyte chemokine receptor (Horuk et al., 1993, Chaudhuri et al, 1994), binds to many CC and CXC chemokines such as RANTES/CCL5, MCP-1/CCL2, IL-8/CXCL8 and GROα/CXCL1. Moreover, these chemokines have been shown to displace heterologous chemokine binding, suggesting that chemokines of either family compete for a single binding site in DARC (Horuk et al., 1993). Lastly, viral chemokine receptors are shared receptors that have been transduced into the viral genome during evolution (more details later).

Different chemokine receptors have been shown to be expressed on different types of immune cells (Table 1.6), however, in some cases expression of these receptors seems to be restricted to a certain leukocyte subset (Sallusto et al., 1998). For example, Th1 cells, which trigger a cell mediated immunological response, preferentially express CXCR3, and CCR5, whereas Th2 cells (responsible for humoral immunity) express CCR3, CCR4 and CCR8 (Sallusto et al., 1998, Baggiolini, 1998). The ability of leukocytes to express different types of chemokine

receptors allows these cells to respond to many chemoattractant signals presented to leukocytes in a certain spatial/temporal context.

It is worth emphasizing the fact that most chemokines can bind to more than one receptor, that most receptors bind more than one ligand and that under appropriate conditions most cell types can produce chemokines (Tables 1.5 and 1.6). However, this apparent redundancy seems to be important for the robust role of the chemokine system. Knock out mice that lack either a given chemokine or receptor –exception made for SDF-1 or CXCR4 knock out mice that are lethal (more details later)- were shown not to have gross deficiencies in development or survival but rather are found to be different in specialized reactions. For example, natural deletions of CCR5 are known to occur in a certain population of humans that have no obvious phenotype rather than HIV resistance (Samson et al., 1996). In general, blocking of certain chemokines or receptors affects specialized outputs but does not compromise the role of the chemokine system (for review see (Mantovani, 1999)).

1.3. Functions of chemokines and chemokine receptors

1.3.1. Chemokines and leukocyte trafficking

The chemokine-induced migration of cells has been shown to be important not only for immune surveillance, innate and adaptive immune responses, but also of fundamental importance in haemopoiesis, angiogenesis, development and varied forms of pathological inflammation (Foxman et al., 1997; Springer, 1994). Several chemokines and their receptors have been shown to be good candidates for inducing

directional migration of lymphocytes within primary lymphoid organs, from primary to secondary lymphoyd organs, as well as within and between secondary lymphoid organs during humoral immune response (for review see (Campbell et al., 1998b)). Much molecular information has now been gathered on the pathways, the role of adhesion molecules in identification of homing sites and on the chemokines involved in leukocyte trafficking, although it is still not fully clear how this whole system is regulated. A three-step model of leukocyte migration (Fig. 1.3) has been suggested whereby upon activation, leukocytes become firmly adhered and will finally migrate into the tissue space (for review see (Schall, 1994)). Upon infection or injury, chemokines are secreted by resident tissue cells, resident and recruited leukocytes and by cytokine-activated endothelial cells. These secreted chemokines are locally retained on matrix and cell-surface heparan sulphate proteoglycans and therefore establish a chemokine gradient around the inflammatory stimulus as well as on the surface of the overlying endothelium. Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines retained on cell-surface heparan sulfate proteoglycans. Chemokine-induced signalling activates leukocyte integrins leading to firm adherence and finally extravasation (for reviews see (Butcher, 1991; Springer, 1994)).

How the chemotactic gradients are established *in vivo* is not clear. It is known that chemokines are found preferentially immobilized through low affinity binding to proteoglycans on the vascular endothelium and to the extracellular matrix proteins in the tissues where they are presented to leukocytes. The positive charge of chemokines allows these molecules to bind to sulphated proteoglycans found on the cell surface or in the extracellular matrix (Webb et al., 1993). This property intrinsic

to chemokines is of special importance in maintaining chemotactic gradients on the surface of endothelial cells that are constantly exposed to blood flow. The CC chemokine SLC/CCL21, thought to be involved in T cell migration, is expressed by HEVs and is involved in inducing integrin-mediated adhesion of naïve lymphocytes (Gunn et al., 1998b; Tanabe et al, 1997;; Hedrick & Zlotnik, 1997; Hromas et al., 1997; Nagira et al., 1997; Nagira et al., 1998). Treatment of rolling peripheral blood lymphocytes (PBLs) with ELC/CCL19, SLC/CCL21 or SDF-1/CXCL12 in the presence of PNAd (peripheral node addressin- a mixture of glycoproteins expressed on HEV (Berg et al., 1991)) and ICAM-1 (intracellular adhesion molecule-1), induces arrest of PBLs (Campbell et al., 1998b). SLC/CCL21 stimulates a rapid $\alpha 4\beta 7$-integrin-mediated adhesion of lymphocytes to MadCAM-1 (mucosal addressin cellular adhesion molecule-1) expressed on PP and HEV (Pachynski et al., 1998).

In order to attract circulating lymphocytes chemokines must cross the endothelial cells. It was initially thought that chemokines could cross the endothelium by diffusing through intracellular gaps. Work by Middleton *et al* (Middleton et al., 1997) has demonstrated by immuno-electron microscopy that abluminal IL-8/CXCL8 is internalised by venular endothelial cells and subsequently transcytosed to the luminal endothelial cell membrane where it is presented to adherent leukocytes. Binding assays *in situ* have also shown that RANTES/CCL5, MCP-1/CCL2, MCP-3/CCL7 bind to the endothelial cells of venules and small veins but not arteries or capillaries (Hub & Rot, 1998). Additionally, it has been demonstrated by immunocytochemistry that DARC, a promiscuous receptor for α and β chemokines, is expressed on HEV (Hadley et al., 1994). More recently, immunocytochemistry has revealed that D6, a promiscuous β chemokine receptor, is expressed on the lymphatic

endothelium but not on endothelial cells lining blood vessels (Nibbs et al., 2001). Taken together these observations suggest that expression of these receptors on the surface of endothelial cells or lymphatic endothelium might play a role in chemokine transcytosis from the tissue space to the luminal surface of endothelial cells where it is available to attract, interact and activate leukocytes.

The aim of this section is to give examples of chemokine/chemokine receptor pairings shown to be involved in leukocyte trafficking. Firstly, lymphocyte maturation within the primary lymphoid organs, bone marrow and thymus, and the role of chemokines and their receptors for lymphocyte homing to secondary lymphoid organs will be described. Secondly, lymphocyte migration during primary and secondary immune responses will be addressed. Thirdly, the role of chemokines during effector and immune responses will be outlined.

a). Bone marrow

In order to proliferate and differentiate, T and B lymphocytes have to move through different tissue compartments. During maturation, immature lymphocytic precursors either remain in the bone marrow and proceed along the B-lymphoid differentiation pathway or leave the bone marrow and seed into the thymus where they differentiate into mature naïve T cells (see below). While in the bone marrow, immature B cells with high avidity for autoantigens are arrested in their development and die (a process known as negative selection) while the other immature B cells are allowed to leave the bone marrow via the venous sinuses (Melchers et al., 1999).

Bone marrow (BM) stromal cells have been shown to produce the chemokine SDF-1/CXCL12 that is capable of attracting B cell progenitors and to place these

progenitors in contact with the stromal cells (D'Apuzzo et al., 1997). The stromal cells, in turn, release growth and differentiation factors required for B cell maturation. Moreover, SDF-1/CXCL12 has also been shown to induce BM colonization by haemopoietic precursors ($CD34^+$) during embryogenesis via its receptor CXCR4 (Aiuti et al., 1997; Ma et al., 1999). Knock out mice studies have shown that mice lacking CXCR4 or its ligand SDF-1/CXCL12 die perinatally and that mutant embryos have very low numbers of B cell progenitors in both the fetal liver and the bone marrow (Tachibana et al., 1998). These mice were also shown to have other developmental abnormalities that suggest that SDF-1/CXCL12 and CXCR4 are not only important for the haemopoietic system but are also involved in organ development (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998).

MIP-3α/CCL23 has also been shown to be expressed by BM stromal cells (Godfrey & Zlotnik, 1993). Unlike SDF-1/CXCL12, MIP-3α/CCL23, is only expressed in the BM after lipopolysaccharide (LPS) stimulation. MIP-3α/CCL23 is thought to be specifically involved in attracting macrophage precursors into the BM during inflammation (Godfrey & Zlotnik, 1993).

Other chemokines, such as HCC-1/CCL14, MCP-2/CCL8 and MIP-1α/CCL3, have also been shown to be expressed in the bone marrow however their role in the BM environment is still unclear (for review see (Rossi & Zlotnik, 2000)).

b). Thymus

Thymocytes mature in the thymus as they migrate from the cortex to the medulla. In the thymus, thymocytes expressing both CD4 and CD8, encounter processed antigen

on the major histocompatibility complex (MHC) class I or II molecules on specialized antigen presenting cells (APC). As described above for the development of B cells, thymocytes expressing receptors with high avidity for MHC class I or II/autoantigen peptide complexes undergo negative selection. Those thymocytes that survive negative selection will then become either CD8 or CD4 single positive cells, migrate to the medulla and leave the thymus via venous blood to move to arterial blood in the heart.

The role of chemokines present in the thymus is dependent on the maturation status of T cells (Campbell et al., 1999; Kim et al., 1998). T cells found in the cortex and in the medulla of the thymus can respond to TECK/CCL25 (CCR9 ligand), however T cells lose their ability to respond to this chemokine when they reach their late stages of maturation just before they are ready to leave the thymus (Vicari et al., 1997; Zaballos et al., 1999). At the same time, L-selectin expression is upregulated. During the late stages of thymocyte maturation, T cells start to respond to ELC/CCL19 and SLC/CCL21, both ligands for CCR7. Additionally, thymocytes in the process of migrating from the cortex to the medulla, can transiently respond to MDC/CCL22, the CCR4 ligand (Campbell et al., 1999; Imai et al., 1998; Yoshida et al., 1997; Yoshida et al., 1998). These observations point towards a model that supports a role for CCR9 in maintaining cells in the thymus until they are matured, a role for CCR4 in driving the migration of developing cells from the cortex to the medulla and finally, a role for CCR7 and CXCR4 (known to be expressed at all developmental stages (Zou et al., 1998) in migration of mature T cells.

Surprisingly, published work describing knock out studies of CCR4 (Chvatchko et al., 2000) (MDC/CCL22 receptor), SDF-1/CXCL12, CXCR4 (Ma et al., 1998) and or

CCR7 (Forster et al., 1999) (SLC/CCL21 receptor) did not report any abnormal phenotype suggesting that these chemokines might have a redundant effect in thymocyte development.

The observation that different chemokines are expressed at different stages of thymocyte maturation suggests that these molecules may have a role in compartmentalization within lymphoid organs and hence are involved, along with other molecules, in T cell development. Moreover, it is also possible that the absence of phenotype of these knock out mice could reflect functional redundancy.

c). Primary Immune Responses

During a primary immune response T and B lymphocytes need to interact with the antigen presenting cells (APC) and subsequently with each other. These interactions take place within the secondary lymphoid organs: lymph nodes, Peyer's patches (PP) and the spleen. Naïve T and B cells enter the lymph nodes and PP through the high endothelial venules (HEVs) whereas the antigen (Ag) is carried to these secondary lymphoid organs via the afferent lymphatics together with APC or is transcytosed by intestinal M cells into the dome region. In the spleen, the route of entry is the marginal zone sinuses for both lymphocytes and Ag (for review see (Moser & Loetscher, 2001; Sallusto et al., 2000)). Here, the traffic of dendritic cells (DCs) will be described followed by that of T and B cells. Finally, the interactions between these three types of cells will be discussed.

DC traffic

In order to encounter the Ag, DCs and their precursors have to migrate from the blood into the tissue. Once in the tissue these cells are activated, redirected to the lymphatics and then to T cell areas of the secondary lymphoid organs where DCs present the Ag to T cells. Receptors for inflammatory chemokines (e.g. CXCR1, CCR1, CCR2, CCR5 (Godiska et al., 1997; Moser & Loetscher, 2001; Sallusto et al., 2000) and D6 (J. Townson, *pers. comm.*)) that are known to be expressed by DCs are thought to play a role in extravasation of these cells into the site of infection/injury as well as in the local recruitment of resident tissue DCs. On exposure to immune or inflammatory signals DCs undergo maturation which includes downregulation of their endocytic activity, upregulation of MHC, adhesion and costimulatory molecules along with a switch in the chemokine receptor they use (Moser & Loetscher, 2001; Sallusto et al., 2000). Antigen uptake, together with LPS stimulation and/or TNF-α, induces expression of CXCR4 and CCR4 and increases CCR7 expression levels. Simultaneously, the levels of CXCR1, CCR1 and CCR5 are downregulated (Lindhout et al., 1999). These changes in receptor profile enable DCs to migrate from inflammatory sites towards secondary lymphoid organs where they subsequently migrate towards the T cell areas of these organs. DCs' expression of high levels of CCR7 after activation by the Ag regulates migration of these cells towards SLC/CCL21 and ELC/CCL19 (Ngo et al., 1998; Sozzani et al., 1998a; Yoshida et al., 1997), which are produced by lymphatic endothelial cells and interdigitating DCs, respectively. Evidence for the role of CCR7 in regulating DC migration is supported by knock out mice that are deficient in CCR7 (Forster et al., 1999) and by *plt* mice

(Gunn et al., 1999) where maturing DCs cannot migrate from the skin to lymph nodes.

Migration of DCs from the site of Ag capture to the site of Ag presentation can vary depending on anatomical sites (Dieu et al., 1998). For example, in the spleen, immature DCs are found in the marginal zone where blood-borne Ags enter and migrate to the T cell areas after being exposed to maturating stimuli such as LPS. In the tonsils, DCs are found at the site of entry for Ag, the epithelial crypts. These epithelial crypts have been shown to selectively produce LARC/CCL20 (Dieu et al., 1998), a ligand for CCR6 that is expressed (along with other chemokine receptors) by immature DCs present in the skin, Langerhans cells (Yang et al., 1999). Generation of $CCR6^{-/-}$ mice by Cook and *et al* has demonstrated the importance of this receptor in DC migration into the PP (Cook et al., 2000). Once in the PP, the Ag is then transcytosed by the M cells into the dome region where immature DCs are found before they migrate to the T cell areas (for review see (Sallusto et al., 2000)).

A few hours after the DCs stimulation by LPS, MIP-1α/CCL3, MIP-1β/CCL4 and IL-8/CXCL8 are produced. This initial early burst of inflammatory chemokine production by DCs while at the same time downregulating the expression of the cognate receptors is thought to allow DCs to follow different chemokine gradients and at the same time sustain the recruitment of immature DCs as well as other inflammatory cells. At the same time, the late production of lymphoid chemokines (Foti et al., 1999; Sallusto et al., 1999c) such as TARC/CCL17, MDC/CCL22 and DCCK1/CCL18 ensures the correct cell positioning and appropriate cell-cell interaction within the lymph node (for review see (Moser & Loetscher, 2001)).

T and B cell homing to secondary lymphoid organs

In order to enter lymph nodes and PP, T and B lymphocytes need to cross the HEVs. CCR7 is thought to be responsible for lymphocyte arrest on these components of the vasculature. This receptor is widely expressed on naïve T and B lymphocytes (Burgstahler et al., 1995; Campbell et al., 1998a; Sallusto et al., 1998) and one of its ligands, SLC/CCL21, has been shown to be produced by the endothelial cells of the HEV (Gunn et al., 1998b), retained on the surface of these cells and displayed to rolling lymphocytes. Recognition of SLC/CCL21, arrests the lymphocytes on the surface of HEVs under blood flow conditions (Yoshie et al., 1997). Studies with *plt* mice and $CCR7^{-/-}$ support the idea that this chemokine-chemokine receptor paring is important for lymphocyte arrest in the HEVs. Mice lacking SLC/CCL21 (*plt* mice) have been shown to have defective T cell trafficking into lymphoid organs (Gunn et al., 1999; Tangemann et al., 1998) and injection of SLC has been shown to be sufficient to restore T cell trafficking in these mice (Stein et al., 2000). Mice lacking CCR7 have T cells with reduced ability to enter lymph nodes and PP, whereas B cell trafficking is less affected (Forster et al., 1999). These $CCR7^{-/-}$ mice have an abnormal distribution of T and B cells in the secondary lymphoid organs and in fact T and B cells appear in higher numbers in the blood (Forster et al., 1999). Moreover, CCR7 expression is thought to be necessary not only to recognize SLC/CCL21 produced by HEVs to allow lymphocyte entry from blood into secondary lymphoid organs, but also to be required for recognition of SLC/CCL21 produced by stromal cells to allow proper positioning of resting T and B cells into their appropriate areas within the lymphoid organs (Forster et al., 1999).

After crossing the HEV, the T and B lymphocytes follow different routes guided by specific chemokines. The T cell lymphocytes localize to the T cell area where they can scan the surface of DCs in order to detect the specific Ag. Production of DCCK1/CCL18, MDC/CCL22, TARC/CCL17 and ELC/CCL19 by mature DCs probably encourages the interaction of these cells with T cells (Adema et al., 1997). The B cell lymphocytes enter the T cell area and then migrate into the B cell follicle where the Ag is displayed by FDC. These migrating B lymphocytes have been shown to be capable of responding to BCA-1/CXCL13, a CXCR5 ligand shown to be produced by stromal cells (probably FDC) found within the B cell follicles (Gunn et al., 1998a; Legler et al., 1998). Mice lacking CXCR5 have no inguinal lymph nodes and have little or no normal PP. In these CXCR5 null mice, B lymphocytes are not organized into discrete follicles in the remaining lymph nodes but B cell homing into these tissues is not impaired which suggests that CXCR5 is not the only receptor involved in lymphocyte homing (Forster et al., 1996).

Activation of T lymphocytes and T cell-B cell interactions

Segregation of T and B lymphocytes into separate areas within the lymphoid organ allows for separate stimulation by Ag that is displayed to each one of these different types of lymphocytes by the appropriate type of APC. Once B and T cells encounter the Ag they become activated and move towards each other in response to a switch in chemokine receptor usage (for review see (Melchers et al., 1999; Moser & Loetscher, 2001; Sallusto et al., 2000)). Ag stimulated T cells downregulate expression of CCR7 and upregulate expression of CXCR5 and CCR4, by doing so these activated T lymphocytes become sensitive to BLC/CXCL13 and MDC/CCL22 that are produced in the B cell areas (Schaniel et al., 1998; Tang & Cyster, 1999). Simultaneously, Ag

stimulated B cells become responsive to ELC/CCL19, by upregulating expression of CCR7 and therefore are able to migrate towards the T cell area (Ngo et al., 1998).

After B cells interact with specific T cells, some B cells proliferate and differentiate outside the follicle, while others are attracted to the follicular dendritic cells (FDC). The FDC will form a mesh of structures where the B cells will proliferate to form germinal centres (GC) where memory B cells and plasma cells are generated. Memory and plasma B cells migrate into the sites of infection/injury or into the bone marrow through the lymph and blood (for review see (Melchers et al., 1999; Moser & Loetscher, 2001; Sallusto et al., 2000)).

d). Effector and memory responses

After being primed by the Ag, effector T cells acquire new migratory properties that allow them to leave the secondary lymphoid organs and enter the peripheral inflamed tissues. Different types of protective or pathogenic responses are mediated by type 1 (Th1) and type 2 (Th2) polarized T cells that secrete different cytokines, and for this reason interact with and stimulate different types of leukocytic effector cells. Type 1 cells produce IFN-γ and are associated with macrophages and neutrophils in delayed type hypersensitivity (DTH) lesions whereas type 2 cells produce IL-4, IL-5 and IL-13 and are found at sites of allergic inflammation together with eosinophils and basophils (for review see (Sallusto et al., 2000)). Although not only CCR3 (Sallusto et al., 1998), but also CCR4 (Imai et al., 1999) and CCR8 (D'Ambrosio et al., 1998) have been shown to be expressed on Th2 cells it is for CCR3 and its ligand (Eotaxin/CCL11) that the most striking evidence for the role of chemokines and their receptors in polarized T cell response has been gathered. Eotaxin has been shown to be highly produced in mucosal tissues where allergic inflammation is taking place

and CCR3 is found on the surface of eosinophils, basophils and *in vivo* and *in vitro* polarized Th2 cells. It is thought that the sharing of CCR3 by these cells allows for colocalization of these three cell types at the site of inflammation (Gerber et al., 1997).

Unlike Th2 polarized cells, Th1 cells express CCR5, CXCR3 and CCR1 (Sallusto et al., 1998). However, studies have shown that 6 hours after stimulation of the T cell receptor (TCR), the receptors for inflammatory chemokines are transiently downregulated at both the mRNA and the protein level whereas the expression of CCR7, CCR4, CCR8 and CXCR5 is increased (Sallusto et al., 1999a). This change in expression of the chemokine receptors is known to last for a few days and is thought to be involved in relocalizing activated T cells within the tissues where the ligands ELC/CCL19, SLC/CCL21, BCA-1/CXCL13, I-309/CCL1 and TARC/CCL17 are being produced (for review see (Melchers et al., 1999; Moser & Loetscher, 2001; Sallusto et al., 2000)).

From the activated T cell pool generated during a primary immune response, some differentiate to become circulating memory cells. These memory T cells are involved in a much more efficient response upon a secondary challenge.

Interestingly, Sallusto *et al.* have identified two different subsets of memory T cells that are functionally distinct. $CCR7^-$ memory cells express receptors for migration to inflamed tissues and display immediate effector functions. These cells do not migrate through the secondary lymphoid organs but instead are involved in immune surveillance in the peripheral tissues. The $CCR7^+$ memory cells, or central memory cells, expresses lymph-node homing receptors and have no effector function.

However, when these central memory cells come in contact with an Ag they lose their CCR7 expression presumably allowing them to migrate into the site of infection (Sallusto et al., 1999b).

e). Selective homing to skin and gut

Skin homing T cells are identified by their expression of cutaneous lymphocyte associated antigen (CLA) and the chemokine receptor CCR4. TARC/CCL17, the ligand for CCR4, has been shown to be expressed on endothelial cells of inflamed skin (but not gut) and is capable of inducing integrin-dependent adhesion of CLA^+ T cells (Campbell, 1999). Along with its role in extravasation, CCR4 is also involved in directing cell migration within inflamed tissues in response to MDC/CCL22 and TARC/CCL17 that are produced by resident cells in places such as the lung and the liver (Sallusto et al., 2000). More recently, CCR10 has also been shown to be expressed on CLA^+ memory T cells that migrate in response to ESkine/CTACK/CCL27 and MEC/CCL28 (Morales, 1999; Tang & Cyster, 1999).

In contrast, gut homing memory T cells do not express CLA but express the integrin heterodimer $\alpha4\beta7$. $\alpha4\beta7$ is a ligand for the mucosal-addressin-cell adhesion molecule-1 (MAdCAM-1) found on intestinal blood vessels (Melchers et al., 1999; Moser & Loetscher, 2001; Sallusto et al., 2000). The receptor hypothesized to be involved in homing to the gut is CCR9, which binds to TECK/CCL25 a chemokine expressed in the endothelial cells of gut-associated tissues and the thymus (Zabel et al., 1999).

1.3.2. Chemokines and haemopoiesis

Along with the well described effects on mature leukocyte trafficking, chemokines also exert effects on other haemopoietic cell types. MIP-1α/CCL3 (for review refer to (Cook, 1996)), MIP-1β/CCL4, GRO-β/CXCL2 and GRO-γ/CXCL3 have been shown to enhance the formation of CFU-GM (colony -forming unit granulocyte-macrophage) in the presence of M- or GM-CSF (colony stimulating factor) (Broxmeyer et al., 1993). However, chemokines, such as MIP-1α/CCL3, GRO-β/CXCL2, PF-4/CXCL4, IL-8/CXCL8, MCP-1/CCL2, IP-10/CXCL10 and mMIP-1γ/CCL9, have also been shown to be able to act as inhibitors of more immature progenitors (Broxmeyer et al., 1993; Graham et al., 1990; Youn et al., 1995). *In vivo* administration of MIP-1α/CCL3, and pre-treatment of animals with MIP-1α/CCL3 enhances myeloid recovery after treatment with S-phase-active chemotherapeutic agents (Quesniaux et al., 1993). Work by Graham and colleagues reports that MIP-1α/CCL3 does in fact directly inhibit the proliferation of haemopoietic stem cells (Graham et al., 1990). The requirement for MIP-1α/CCL3 for haemopoiesis was investigated using MIP-1α/CCL3$^{-/-}$ mice (Cook et al., 1995). The studies with MIP-1α/CCL3$^{-/-}$ mice did not show an increase in progenitor pool, in fact these knock out mice were shown to have normal numbers of both total nucleated cells and early progenitor cells. From this data, it seems as if MIP-1α/CCL3 is not required to maintain the quiescent state of haemopoietic cells in normal physiology, but one cannot disregard the fact that maybe other factors may compensate for the lack of MIP-1α/CCL3. Indeed, Jacobsen and co-workers demonstrated by analysing the effect of multiple inhibitory chemokines *in vitro* that various inhibitors can synergize with one another to reach levels of inhibition impossible to achieve by a singular

inhibitor (Jacobsen et al., 1994). The generation of mice lacking more than one inhibitor would most certainly provide a means of identifying the role of stem cell inhibitors *in vivo*. Furthermore, the role of MIP-1α/CCL3 in haemopoeisis is thought to be not only restricted to inhibition of early haemopoietic progenitors but has also been shown to be capable of enhancing maturation of mature haemopoietic progenitors (Broxmeyer & Kim, 1999).

To date, studies using the SDF-1/CXCL12 (Nagasawa et al., 1996) or the CXCR4 (Tachibana et al., 1998; Zou et al., 1998) knock out mice provide the strongest evidence supporting the role of chemokines in haemopoiesis. As mentioned before, apart from their developmental defects, these null mice exhibit profound haemopoietic defects such as absence of B lymphopoiesis and of bone marrow myelopoiesis. Chemokines such as ELC/CCL19 and SLC/CCL21 have also been shown to induce chemotaxis in haemopoietic stem and progenitor cells. Broxmeyer and colleagues demonstrated that these two chemokines chemoattract CFU-GM cells through their shared receptor CCR7 (Broxmeyer & Kim, 1999). More recently, IP-10/CXCL10 and Mig/CXCL9 have also shown to be capable of inducing chemotaxis in GM-CSF-stimulated $CD34^+$ cells via their shared receptor CXCR3 (Jinquan et al., 2000). The *in vivo* importance of these observations awaits clarification.

1.3.3. Chemokines and disease

Despite being capable of supporting host defence, chemokines and their receptors can act as amplifiers of inappropriate inflammation in non-infectious inflammatory disorders and therefore cause organ disfunction and increased mortality (Strieter et al., 1996). Chemokines and their receptors have been shown to be involved in many

diseases such as asthma, gastrointestinal allergy, multiple sclerosis, pneumonia, arthritis, atherosclorosis, some types of cancer and allograft rejection (Rollins, 1997). The role of proinflammatory chemokines in these diseases has been demonstrated by detecting chemokine production in the inflamed site as well by the presence of cells known to respond to the chemokine. Most of the studies that generated information on the role of chemokines in disease processes were based on antibody blocking experiments to a given chemokine or chemokine receptor as well as by knock out mice that lack a certain chemokine or chemokine receptor that have subsequently been challenged with an Ag. The following examples demonstrate the wide variety of disease states in which the chemokine system is implicated.

Asthma. Asthma is a chronic inflammatory disease characterized by an exaggerated airway responsiveness that leads to airway inflammation and obstruction (Bertrand, 2000; Gerard & Rollins, 2001; Lukacs et al., 1999; Strieter et al., 1996; Teran, 2000). The recruitment and activation of many cell types such as eosinophils, mast cells, Th2 lymphocytes and neutrophils contribute to the pathogenesis of asthma (Foresi & al, 1997; Kon & Kay, 1999, Lamblin, 1998; Rossi & Oliveira, 1997). IL-8/CXCL8, MDC/CCL22, RANTES/CCL5, MIP-1α/CCL3, MCP-3/CCL7, Eotaxin/CCL11, MCP-5/CCL12, MCP-4/CCL13, HCC-1/CCL14 and Eotaxin-2/CCL24 are examples of chemoattractants and activators of eosinophils (for review see (Teran, 2000; Zlotnik et al., 1999)) that have been isolated from nasal secretions and bronchoalveolar lavage fluid of patients with active asthma (Alam et al., 1996). It seems likely that these chemokines play a role in the pathology of the disease, but the large number of chemokines found is testimony to the complexity and apparent redundancy in the system.

Lukacs and colleagues (Lukacs et al., 1999; Lukacs et al., 1996; Lukacs et al., 1997) have used a murine model of allergic airway inflammation *in vivo* to demonstrate the role of MIP-1α/CCL3 and RANTES/CCL5 in eosinophil chemoattraction. Upon intra tracheal challenge of presensitized mice with parasite egg antigen, an increase in MIP-1α/CCL3, RANTES/CCL5, IL-4 and TNF production was registered (Lukacs et al., 1996). *In vitro* neutralization of MIP-1α/CCL3 or RANTES/CCL5 reduced eosinophils recruitment to the lung tissue and airways during the allergic response, whereas neutralization of MCP-1/CCL2 significantly diminished leukocyte migration in total (Lukacs et al., 1997). Airway hyper-reactivity was seen to diminish when MCP-1/CCL2 is depleted but depletion of RANTES/CCL5 and MIP-1α/CCL3 had no affect on airway hyper-reactivity (Lukacs et al., 1997).

Although many cell types have been demonstrated to be involved in asthma, I will here concentrate on eosinophils to illustrate how complex the interactions within the system can be. Three chemokine receptors were initially isolated from eosinophils: CCR1, CCR3 and CXCR2 (Sabroe et al., 1999). From these, CCR3 was thought to be the major eosinophils chemokine receptor (Heath et al., 1997). Eotaxin/CCL11, the ligand for CCR3, is reported to be released *in vitro* by epithelial cells after stimulation with the cytokines TNF-α, IL-1β and IFN-γ (Lilly et al., 1997). High levels of Eotaxin/CCL11 are produced in the airway epithelium in human asthma; this localized production attracts eosinophils to the epithelium where the release of epithelium-damaging proteins occurs (Lukacs et al., 1999). Eotaxin/CCL11 can also induce basophil degranulation, causing tissue damage characteristic of asthma (Teran, 2000). Moreover, Eotaxin/CCL11 attracts Th2 lymphocytes that promote and maintain the allergic response by ensuring the presence of antigen-specific Th2 cells

(Gutierrez-Ramos et al., 1999). Targeted disruption of the Eotaxin/CCL11 gene in mice demonstrated that this CC chemokine is involved in regulating the constitutive number of eosinophils in the peripheral blood and that it is also involved in enhancing the early recruitment of eosinophils in models of asthma and stromal keratitits (Rothenberg et al., 1997). A recent study has demonstrated that CCR4, and to a lesser extent, CCR8 are also expressed in the majority of T cells infiltrating the bronchial biopsies of allergen-challenged asthmatics (Panina-Bordignon et al., 2001). More recently, Luckacs and co-workers have demonstrated the importance of CCR6 and MIP-3α/CCL20 in allergic pulmonary responses (Luckacs et al., 2001). In this study, the authors challenged CCR6 deficient mice with cockroach antigen and they noticed that these mice had reduced airway resistance, fewer eosinophils around the airway, decreased levels of IL-5 in the lung and low serum levels of IgE.

Rheumatoid arthritis. Infiltration of monocytes and neutrophils into the synovial tissues and synovial fluid of the joints is characteristic of rheumatoid arthrititis (RA) (Strieter et al., 1996). MCP-1/CCL2 and MIP-1α/CCL3 were found to be elevated in synovial fluid from patients suffering from RA (Kunkel et al., 1996). Experiments with CIA (collagen-induced arthritis) mice injected with antibodies against MIP-1α/CCL3 and GROα/CXCL1 demonstrated a delay in the onset, and a reduction on the severity, of arthritis (Kasama et al., 1995). *In situ* hybridisation has detected RANTES/CCL5 mRNA on synovial lining cells of patients with rheumatoid arthritits and MIP-1β/CCL4 has been found in association with synovial fibroblasts and macrophages (Kasama et al., 1995). Additionally, CXC chemokines have also been shown to be involved in the pathogenicity of human rheumatoid arthritis (Kunkel et al., 1996). For example, IL-8/CXCL8 and ENA-78/CXCL5 were shown to be

expressed by both synovial fibroblasts and macrophages (Kunkel et al., 1996). Taken together these studies demonstrate that both CC and CXC chemokines are present in RA, however their involvement in the pathogenesis of arthritis is still unclear. Nevertheless, it is hypothesized that these chemokines are overexpressed at inflammatory focci and are therefore thought to be responsible inducing strong adhesive interactions between rolling leukocytes and the endothelium.

Atherosclerosis. Atherosclerosis, is thought to result from an inflammatory response to arterial damage caused by hypertension, shear stress or disordered blood flow (Ross, 1999). Animal models demonstrated a role for IL-8/CXCL8, SDF-1/CXCL12, Mig/CXCL9, I-TAC/CXCL11, IP-10/CXCL10, I-309/CCL1 and CXCR2 in atherosclerosis (Abi-Younes et al., 2000; Boisvert et al., 2000; Haque et al., 2000; Mach et al., 1999). CXCR2 is expressed on macrophages in atherosclerotic lesions, and CXCR2 deficiency strongly reduces the progression of advanced atherosclerosis in mice (Boisvert et al., 2000). SDF-1/CXCL12 induces platelet aggregation and is expressed in smooth muscle cells, endothelial cells and macrophages in atherosclerotic plaques but not in normal vessels (Abi-Younes et al., 2000). Immunofluorescence staining demonstrated differential expression of IP-10/CXCL10, I-TAC/CXCL11, Mig/CXCL9 and INF-γ in cells composing artherosclerotic plaques (Mach et al., 1999). CX_3CR1 heterozygosity has been shown to be related with a reduced risk of coronary atherosclerosis (Moatti et al., 2001). RANTES/CCL5, Eotaxin/CCL11 and MIP-1α/CCL3 have also been isolated from plaques in atherosclerotic patients (Gerard & Rollins, 2001). Knockout mice of CCR2, the receptor for MCP-1/CCL2, crossed with apoliprotein (apo) E-null mice, which develop severe atherosclerosis, show decreased lesion formation (Boring et al.,

1998). All together these studies indicate that certain chemokines and their receptors paly an important role in modulating the functions of leukocytes and smooth muscle cells involved in the development and progression of atherosclerosis.

Multiple sclerosis. Multiple sclerosis (MS) is an inflammatory, demyelinating disorder of the human central nervous system (Trapp, 1998). Sorensen *et al* found elevated levels of IP-10/CXCL10, Mig/CXCL9 and RANTES/CCL5 in cerebrospinal fluid during MS attacks (Sorensen et al., 1999). CXCR3 was shown to be expressed on lymphocytic cells in virtually every active MS lesion and CCR5 was detected on lymphocytic cells, macrophages, microglia in actively demyelinating MS brain lesions (Sorensen et al., 1999; Balashov, 1999). Several studies have reported that RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL10 and MCP-1/CCL2 mRNA and protein are associated with inflammatory lesions (Gerard & Rollins, 2001). However, MIP-1α/CCL3 and CCR5 deficient mice were shown to contract experimental allergic encephalitis (EAE), the best animal model for MS, whereas $CCR1^{-/-}$ mice has a decreased incidence and less severe clinical score (Rottman et al., 2000; Tran et al., 2000). Mice deficient in MCP-1/CCL2 were shown to be resistant to EAE (Gu et al., 2000), and $CCR2^{-/-}$ mice are completely resistant to the disease (Izikson et al., 2000). It is thought that MCP-1/CCL2 is important in triggering cells expressing CCR2, for example macrophages, involved in producing the characteristic manifestation of this disease (Gerard & Rollins, 2001).

Allograft rejection. Animal models have shown that chemokines also play a role in allograft rejection (Gerard & Rollins, 2001). It is thought that after transplantation of the allograft, the early release of chemokines, triggered by damage to the organs,

attracts leukocytes expressing CCR2, CXCR3 and CCR5. Host natural killer (NK) cells after surveying MHC mismatches at the vascular endothelium respond by producing IFN-γ. IP-10/CXCL10, Mig/CXCL9 and I-TAC/CXCL11 are produced locally and attract CXCR3 expressing T cells as well as DCs. The host cells invade the graft and cause acute and chronic rejection probably independently driven by CXCR3 and MCP-1/CCL2 (Gerard & Rollins, 2001). $CCR1^{-/-}$ (Gao et al., 2000), $CCR2^{-/-}$, $CCR4^{-/-}$, $CCR5^{-/-}$, IP-10/$CXCL10^{-/-}$ (Hancock et al., 2000a), and $CXCR3^{-/-}$ (Hancock et al., 2000b) mice have a much higher allograft survival than the wild-type mice when treated with subtherapeutic doses of immunosuppressants.

It is clear that chemokines play a fundamental role in the development of pathological inflammation and disadvantageous immune responses. Recent research is beginning, particularly through the use of neutralising antibodies and receptor knock-out mice, to elude the function of individual receptor-ligand pairs. Such research is likely to be of considerable use in defining new targets on the treatment of these pathologies. Indeed, small molecule antagonists of several chemokine receptors have been reported to prevent or amelliorate some of these problems in animal disease models.

1.3.4.Chemokines and cancer:

The overall role of chemokines in tumour biology is not clear. The aim of this section is to give examples of studies that have demonstrated a role for chemokines and their receptors regarding metastasis and angiogenesis.

Work by Muller *et al* (Muller et al., 2001) strongly suggests that chemokines and their receptors have a critical role in determining the metastatic destination of tumour cells. In this study, expression of CXCR4 and CCR7 was reported to be elevated in human breast cancer cells, malignant breast tumours and metastases. Additionally, expression of SDF-1/CXCL12, ELC/CCL19 and SLC/CCL21 was found in the most common metastatic sites for breast cancer (Muller et al., 2001). This study also reported that, SDF-1/CXCL12 and SLC/CCL21 stimulated tumour cells were capable of inducing actin polymerisation, a process necessary for cell motility and migration. Moreover, treatment *in vivo* with anti-human CXCR4 monoclonal antibody leads to a significant decrease in lung metastasis (Muller et al., 2001).

Introducing chemokines into tumour cells in animal models has also been shown to modulate tumourgenicity. For example, Botazzi and colleagues showed that transduction of the MCP-1/CCL2 gene in melanoma cells caused growth retardation when injected in mice, but an increase in tumorigenicity and lung metastases is reported when lower numbers of tumour cells are injected into the same animal model (Bottazi et al., 1992). MCP-1/CCL2 was also shown to increase lung metastasis by increasing neovascularization in melanoma cells transfected with this chemokine (Nakashima et al., 1995). Except for one report by Manome *et al* (Manome et al., 1995), MCP-1/CCL2 gene transfer does not seem to confer immunity against parental tumour cells. In fact, Salcedo and co-workers have indeed demonstrated by means of neutralizing antibodies to MCP-1/CCL2, that this chemokine is indeed a direct mediator of angiogenesis (Salcedo et al., 2000). It is therefore thought that MCP-1 can have direct effects in promoting angiogenesis and for this reason therapy employing antagonists of MCP-1 together with other

inhibitors of angiogenesis might prove effective in achieving tumour growth inhibition.

Transduction of IP-10/CXCL10, RANTES/CCL5, I-309/CCL1 and MIP-1α/CCL3 into tumour cells, consistently reduces tumorigenicity and also stimulate hosts immune response (Laning et al., 1994; Luster & Leder, 1993; Mule et al., 1996; Nakashima et al., 1996). Injection of Lymphotactin/XCL1-2 together with the T-cell expanding and activating cytokine IL-2 increased T lymphocyte infiltration in myeloma and gave rise to a synergistic anti-tumour response (Dilloo et al., 1996). More recently, transduction of a colon carcinoma cell line with mouse SLC/CCL21 cDNA was shown to have reduced tumourigenicity (Vicari et al., 2000). In this study the tumours were heavily inlfitrated with leukocytes, including granulocytes, dendritic cells and $CD8^+$ T cells which suggests that the antitumour effects caused by SLC/CCL21 are due to induction of both angiostatic, $CD8^+$ T cell-mediated and possibly NK-mediated tumour resistance mechanisms.

As mentioned before, the CXC chemokines can be subdivided into ELR^+ or ELR^- depending on the presence or absence of the ELR motif. The ELR^+ CXC chemokines (Table 1.2.), functioning through two shared receptors CXCR1 and CXCR2, have been shown not only to be potent neutrophil attractants but also to be powerful promoters of angiogenesis (Belperio et al., 2000), whereas some of the ELR^- chemokines have angiostatic properties (see below). For example, the ELR^+ CXC chemokine IL-8/CXCL8, has been found to be an essential autocrine growth factor for some melanoma cell lines and experiments with neutralized antibodies as well as with IL-8/CXCL8 antisense oligonucleotides has been shown to inhibit growth of

two human malignant cell lines *in vitro* (Schadendorf et al., 1993). Additionally, this chemokine has been reported to be involved in mediating human ovarian carcinoma angiogenesis and tumourigenesis (Yoneda et al., 1998), and to contribute to the overall angiogenic activity of non-small cell lung cancer (Smith et al., 1994). Generation of a mutant of IL-8/CXCL8 lacking the ELR motif showed that this mutant no longer exhibited its normal angiogenic effects and in fact had acquired angiostatic properties (Strieter et al., 1995). Furthermore, when a mutant of Mig/CXCL9 was made where the ELR motif was cloned in after the first Cys residue this new protein was shown to be now capable of angiogenesis (Strieter et al., 1995). These observations suggest that regulation of CXC chemokines expression is important for angiogenesis control.

In contrast to the ELR^+ angiogenic properties the expression of ELR^- CXC chemokines, IP-10/CXCL10 and Mig/CXCL9 were found to be elevated in tumours that regress spontaneously (Sgadari et al., 1996). Intra-tumour injection of IP-10/CXCL10 or Mig/CXCL9 in nude mice previously injected with a lymphoma cell line leads to reduction of tumour-associated angiogenesis (Sgadari et al., 1997; Teruya-Feldstein et al., 1997).

In summary, the chemokine system is capable of regulating tumour growth by exercising their angiostatic and/or angiogenic properties, by initiating tumour immunity or by regulating the tumour metastatic potential. Although it was initially thought that inoculation of tumour cells transfected with various chemokines would result in tumour infiltration by host immune cells that would migrate *in vitro* in response to the transfected chemokine it is now evident that divergent data can be

obtained depending on the animal model and on the chemokine species used. Overall it is believed that, many factors can regulate angiogenesis but the balance between ELR^+ and ELR^- chemokines produced by a given tumour is thought to determine the degree of angiogenesis and invasiveness of the tumour (Strieter et al., 1995; Wang, 1998).

1.4. Host-virus interactions within the chemokine system

Viruses depend on living cells for replication, so in order to escape detection by the immune system, viruses have developed many ways of circumventing the host's immune defences. For example, some viruses have evolved to encode versions of chemokines (viral chemokine homologs) that act as chemokine antagonists and agonists, or chemokine binding proteins or viral chemokine receptor homologs that have been proposed to function as extracellular chemokine scavengers (Alcami & Koszinowski, 2000). The study of viruses that are known to encode genes which are involved in blocking an immune response attracted many researchers to this area as these studies will not only prove helpful in finding how to prevent these viruses from invading the immune system but also provides researchers with a unique model system to study the immune system.

a). Viral chemokines:

Viral chemokines can bind to many chemokine receptors and work as antagonists and thereby block leukocyte migration (Lalani & McFadden, 1999). Alternatively, viral chemokines can act as leukocyte attractants in order to favour viral tropism and

propagation of the virus. Below are some examples of viruses capable of producing viral chemokines that illustrate the widely divergent roles of these molecules in the viral life cycle.

KSHV. The Kaposi's Sarcoma-associated Herpes virus (KSHV)/ Human Herpesvirus 8 (HHV-8), has been shown to encode a number of molecules that mimic cytokine genes, including three proteins homologous to chemokines, named vMIP-I, vMIP-II and vMIP-III (Dittmer & Kedes, 1998). These virally encoded chemokines have 25-40% amino acid similarity to MIP-1α/CCL3 (Lalani & McFadden, 1999) and were initially thought to be involved in dampening the immune response to KSHV infection by inhibiting chemokine signalling by antagonistic binding to receptors (Kledal et al., 1997). vMIP-II has been shown to function as a broad spectrum competitive antagonist since it can bind to CCR1, CCR2, CCR3, CCR5, and CXCR4 but is unable to elicit cellular signalling transduction (Kledal et al., 1997). Moreover, this protein has been shown to block chemoattraction by CC and CX_3C chemokines *in vitro* (Boshoff et al., 1997). In contrast, vMIP-I and vMIP-III are chemokine agonists. vMIP-I binds to and induce calcium mediated signalling through CCR8 selectively expressed in human T cells (Dairaghi et al., 1999), whereas vMIP-III acts selectively on CCR4. It is now known that Th1 polarized cells, which preferentially express CCR5 and CXCR3, usually mediate a host response to a viral infection (Spprigs, 1996). By attracting Th2 cells, which preferentially express CCR3, CCR4 and CCR8 (D'Ambrosio et al., 1998; Sozzani et al., 1998b), these proteins produced by the virus skew the immune response from a Th1 to a Th2 microenvironment within the KS lesion (Stine et al., 2000). In addition to deviating the immune response, these viral peptides have been

reported to be involved in angiogenesis (Boshoff et al., 1997; Stine, 2000). Given that KS lesions are characterized by extensive neovascularization (Hayward, 1999) of the affected tissue it is possible that the angiogenic properties of these viral gene products may contribute to the generation of new blood vessels in KS which in turn may support tumour development.

HCMV. The Human Cytomegalovirus (HCMV) is responsible for severe opportunistic infections and encodes two chemokine antagonists, vCXC-1 and vCXC-2 (MacDonald et al., 1999), and a viral chemokine receptor (see below). Although the biological properties of vCXC-2 are still to be characterized, vCXC-1 is known be biologically similar to IL-8/CXCL8 and GROα/CXCL1 despite the fact that these peptides share very little sequence similarity. Penfold and colleagues showed that vCXC-1 is capable of inducing calcium mobilization, chemotaxis and neutrophil degranulation via high affinity binding to CXCR2, just like IL-8/CXCL8 (Penfold et al., 1999). vCXC-1 has been proposed to be important for disease dissemination by recruiting neutrophils during cytomegalovirus infection, although a definitive role requires further experimentation.

MCV. The human poxvirus, Molluscum contagiosum virus (MCV) causes proliferative lesions on the skin of immunocompromised patients and is characterized by poor infiltration of leukocytes and low inflammation markers. Senkevich and colleagues isolated several genes thought to be potentially involved in preventing an inflammatory response. Amongst these genes, they identified a chemokine homologue, vMCC-1, a gene product of ORF MC148R (Senkevich et al., 1996). vMCC-1 inhibits chemotaxis of multiple leukocytes subsets induced by CC and CXC

chemokines (Damon et al., 1998). Chemotaxis assays, indicated that vMCC-1 is likely to bind to CCR1 and/or CCR5, CCR2, CCR8, CXCR1 and/or CXCR2 and CXCR4 (Damon et al., 1998). For vMCC-1, its antagonistic properties are thought to be attributed to the fact that vMCC-1 lacks a receptor activation domain which is usually found in the N-terminus of cellular chemokines (Damon et al., 1998; Lalani, 1999).

HIV. The HIV transcription factor Tat is a potent chemoattractant for monocytes that can function as a chemokine mimic (Murphy, 2001). Tat is capable of sharing receptors with MCP-1/CCL2, MCP-3/CCL7 and Eotaxin/CCL11. Receptor binding studies demonstrated that Tat can bind to CCR2 and CCR3 but not CCR1, CCR4 and CCR5 (Albini et al., 1998). Moreover, Tat can induce expression of CXCR4 in monocytes/macrophages and lymphocytes, of CCR5 and CCR3 in monocytes/macrophages but not in lymphocytes (de Paulis et al., 2000; Huang et al., 1998). By triggering cell surface receptors, Tat induces leukocyte chemotaxis and possibly allows more efficient virus spread by attracting monocytes towards HIV-infected cells.

b). Chemokine-binding proteins

Viruses can also secrete chemokine-binding proteins (vCKBPs) proposed to protect viruses from host immune and inflammatory responses (reviewed in (Lalani et al., 2000; Lalani & McFadden, 1999; Murphy, 2001; Zlotnik et al., 1999)) e.g. the myxoma virus M-T7 gene product, vCkBP-I, and the poxvirus M-T1 gene product, vCkBP-II (Lalani & McFadden, 1997). vCkBP-I can promiscuously bind to CXC, C and CC chemokines (Lalani et al., 1997) and function as an inhibitor of IFN-γ (Upton

et al., 1992). Studies have shown that vCkBP-I inhibits the formation of a chemokine solid phase gradient on endothelial surfaces by interacting with the C-terminal GAG domain of chemokines known to be involved in binding to glycosaminoglycans (Clark-Lewis et al., 1995; Witt & Lander, 1994). vCkBP-II binds with high affinity to multiple CC chemokines and with low affinity to the CXC chemokine, IL-8 (Smith et al., 1997a). By blocking chemokine interaction with cellular receptors and potentially inhibiting Ca^{2+} mobilization as well as chemokine-mediated chemotaxis, vCkBP-II is believed to inhibit recruitment of inflammatory cells into viral infected tissues (Alcami et al., 1998; Lalani et al., 1998; Smith et al., 1997a)

c). Virally encoded chemokine receptors

The viral receptor US28, is the most characterized of the four human cytomegalovirus (HCMV) GPCR homologs identified to this date. This molecule shares 30% identity with CCR1, and binds to MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MCP-1/CCL2, MCP-3/CCL7 and fractalkine/CX_3CL1 (Kledal et al., 1998; Gao, 1994; Bodaghi, 1998; Neote, 1993 ; Vieira, 1998). The exact role of US28 is not known but work by Bodaghi and colleagues has suggested that US28 might a play a role in chemokine sequestration and thereby block inflammatory events during HCMV infection (Bodaghi et al., 1998). However, a report by Streblow *et al* demonstrates that deletion of US28 decreases smooth muscle cell (SMC) migration in comparison to wild type HCMV (Streblow et al., 1999). This study provides evidence that migration of SMCs upon infection with HCMV depends on the expression of US28 and therefore points towards a disease model whereby HCMV mediates vascular SMC migration through US28.

The murine cytomegalovirus (CMV) M78 GPCR-like protein (Rawlison et al., 1996) appears to be quite different. Interestingly, Oliveira *et al* have shown that M78 is delivered to cells as a constituent of the virion and that this molecule is involved in facilitating the accumulation of immediate-early mRNA (Oliveira & Shenk, 2001). It is thought that M78 is involved in either transcription stimulation or acts posttranscriptionally to influence mRNA stability; it is also possible that M78 initiates a signal that modulates transcriptional regulatory systems whereby it represses some genes and activates others, in order to guarantee survival of the virus.

The KSHV G protein-coupled receptor is a product of KS-associated herpesvirus or HHV8 that is thought to be responsible in part for KS pathogenesis (Bais et al., 1998). Ligands for this viral receptor include both CC and CXC chemokines (Arvanitakis et al., 1997). The G protein coupled receptor encoded by KSHV is constitutively active and is thought to be a viral oncogene since it is capable of driving proliferation of cells transfected with this receptor (Arvanitakis et al., 1997) by activating the protein kinases, JNK/SAPK and p38/MAPK, which activate angiogenesis and are mitogenic for Kaposi's sarcoma and B cells (Bais et al., 1998). Moreover, it has been demonstrated that signalling through this receptor is pertussis toxin resistant and can be altered either negatively or positively by chemokine ligands. In fact, some chemokines such as Mig/CXCL9, bind but do not appear to modulate signalling (Arvanitakis et al., 1997). How these complex interactions conspire to favour the virus survival and replication is by no means clear.

U12 and U51 are also examples of virally encoded GPCRs homologs. These receptors encoded by HHV-6 are capable of eliciting calcium mobilization upon binding to RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4 and MCP-1/CCL2

(Isegawa et al., 1998). The equine herpesvirus 2 can only encode for a viral chemokine receptor, E1, which shares sequence similarities with CC chemokine receptors (Telford et al., 1995) and was shown to mediate signalling transduction in the presence of eotaxin (Camarda et al., 1999). Examples of virally encoded chemokine receptors are the herpes saimiri virus that encodes a receptor-like molecule (ECRF3/ORF74) similar to CXCR1 and CXCR2. This receptor binds to GROα/CXCL1, IL-8/CXCL8 and NAP-2/CXCL7 (Ahuja & Murphy, 1993) and is capable of calcium mobilization.

It is not clearly known why viruses encode chemokine receptors. What it is known is that they are encoded and that bearing in mind the limitations of viral genome size they must be important. Moreover, the fact that these virally-encoded chemokine receptors are signalling competent suggests that this signalling function must be of importance for the virus. At a first glance it is hard to envisage a situation where chemotaxis would benefit the survival of the virus. Alternatively, it is possible that viruses might possibly use these receptors to control cell cycle progression or even by inhibiting apoptosis of the host cell. On the other hand, it is also possible that if the chemokine receptor is present in the virion it may play a role in directing the virus to specific cells with specific chemokines tethered to their surfaces.

1.4.1. Chemokine receptors and pathogen entry

Recently, attention has been paid to the role of chemokine receptors in infectious dieseases. It is now known that chemokine receptors participate in several disease states, either by overexpressing receptors or by facilitating viral entry into host cells. Examples of agents that can invade host cells by manipulating the chemokine

receptor system (for reviews refer to (Murdoch & Finn, 2000; Murphy, 1996; Premack & Schall, 1996)) are described below.

HIV. HIV-1 has been shown to use chemokine receptors together with CD4 to gain access to cells (for review refer to (Murdoch & Finn, 2000; Murphy, 1996; Premack & Schall, 1996)). Feng and colleagues have identified CXCR4 as an entry co-factor for T-cell-tropic HIV isolates (Feng et al., 1996). The CC chemokine receptors, CCR2, CCR3 and CCR5 have also been shown to serve as co-factors along with CD4 to allow entry of macrophage-tropic and dual-tropic strains of HIV-1 (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996). More recently, CCR8 has also been identified as an entry co-factor for T-cell tropic or for macrophage-tropic HIV-1 strains (Horuk et al., 1998). Although many chemokine receptors have been shown to act as co-factors for HIV entry into the host cells, their role *in vivo* has only been well documented for CXCR4 and CCR5 (for review see (Murphy et al., 2000)).

It is thought that the gp120 subunit of HIV recognizes the host-encoded CD4 receptor and undergoes a conformational change that allows this subunit to bind to chemokine receptors (Trkola et al., 1996; Wu et al., 1996). The binding of gp120 to the chemokine receptor induces another conformation change in the Env that leads to activation of the other subunit, gp41. Activation of gp41 allows penetration of the host cell membrane and fusion of the viral and host cell membranes (Berger et al., 1999). The discovery of a group of individuals resistant to HIV-1 infection lead to the identification of a 32bp deletion in the CCR5 gene (CCR5 Δ32). This deletion in the CCR5 gene results in a truncated protein that is not expressed on the cell surface (Samson et al., 1996) and this lack of surface expression is thought to be the reason

why these individuals (with no other apparent phenotype) are more resistant to HIV-1 than others that have a normal CCR5. More recently, a polymorphism in CCR2 has also been identified which is thought to be related to delayed progression to AIDS (Smith et al., 1997b).

Plasmodium. *Plasmodium knowlesi* and *P. vivax*, the parasites responsible for malaria, also uses a chemokine receptor to gain entry into host cells (Chauduri et al., 1989; Hadley & Peiper, 1997; Horuk et al., 1993). Malarial blood infections occur when the parasite invade red blood cells (RBCs) which express the Duffy antigen/receptor for chemokines (DARC). Studies have shown that although malaria is endemic in Africa, 95% of the African population is resistant to the parasites *P.vivax* and *P. knowlesi*. Work by Miller and colleagues showed that the erythrocytes of individuals that are resistant to *P.vivax* do not express the DARC chemokine receptor on their surface and that an anti-DARC antibody could block parasite invasion into normal erythrocytes (DARC-positive erythrocytes) (Miller et al., 1975).

Poxvirus. During evolution, poxviruses have acquired many genes encoding proteins whose function is to counteract the host response to infection (for review see (Alcami & Smith, 1995)). Poxviruses have evolved to modulate the host immune response by producing viral chemokines and chemokine homologs as well as secreting virus-encoded chemokine-binding proteins. Like in the case of HIV (see above), poxviruses can use chemokine receptors, such as CCR5 and CXCR4, to infect cell subtypes (Lalani et al., 1999). Experiments using 3T3 cells have demonstrated that Myxoma virus infection requires the presence of either CCR1, CCR5 or CXCR4 (Lalani et al., 1999). In this study it was also demonstrated that infection of 3T3 cells

transfected with CCR5 by Myxoma virus can be blocked by RANTES/CCL5 or by an anti-hCCR5 polyclonal antibody.

In conclusion, the role of the chemokine system goes beyond simple immune modulators. In order to exploit the therapeutic potential of the chemokine system it is essential to identify specific ligands and receptors that are important in a given disease setting. In the last couple of years, attention has been turned to chemokine receptors that compose attractive targets because they are GPCRs, a class of proteins for which there is a well documented history of developing small, non-peptidyl antagonists. It is therefore of extreme importance to understand the nature of chemokine/ chemokine receptor interactions in order to develop therapeutic targets as well as in developing inhibitors of chemokine/ chemokine receptor interactions.

1.5. Chemokine and chemokine receptor interactions

Many studies have attempted to dissect the molecular requirements for chemokine/chemokine receptor interactions. Initially, in order to determine functionally relevant domains, researchers directly compared primary sequences of chemokines and used mutagenesis to investigate its role in receptor interactions. This approach was soon abandoned not only because it was rather inefficient but also because it was impossible to distinguish between residues that made direct contact with the receptor and residues that are structurally important. Nowadays, structure-function analyses usually include the construction of chimaeric and mutant molecules the biochemical properties of which are then assessed by radioligand binding assays and signalling studies. Although this most recent approach has been successfully

used to study chemokines as well as their receptors, it is possible that the chimaeric protein might have its overall structure disturbed and therefore will not be particularly informative. In order to minimise disturbances of the overall structure, researchers have tried to make chimaeric proteins between more related proteins. Although this approach is thought to generate potentially stable proteins, it might overlook functional contributions attributed to shared determinants. So, together with the generation of chimaeric constructs, Alanine scanning mutagenesis, to target individual residues, and NMR, to detect any structural disturbances, approaches have been used.

Many studies have shown that the N-terminal domain of α and β chemokines is important for chemokine function. Early studies centred on CXCR1 and CXCR2 and their ligands, on MCP-1/CCL2 and its receptor, CCR2. Studies by Clark-Lewis and colleagues demonstrated that the ELR domain found in N-terminal domain of IL-8/CXCL8 is essential for binding to its receptors (Clark-Lewis et al., 1991). Specifically, Alanine scanning mutagenesis of IL-8/CXCL8 identified the Arg residue at position 6, i.e. within the ELR motif, as being critical for ligand binding (Williams et al., 1996) and work carried out by Clark-Lewis reports that deletion the first part of the N-terminus including the ELR motif generates a mutant that retains binding but is no longer biologically active (Clark-Lewis et al., 1991). Although cloning of the ELR domain into PF-4/CXCL4, a non-ELR chemokine that does not bind to CXCR1 or CXCR2 allows binding of the mutant to the IL-8/CXCL8 receptor (Clark-Lewis et al., 1993), the cloning of the same motif into IP-10/CXCL10 generates a mutant that does not bind to the IL-8/CXCL8 receptors (Wuyts et al., 1999). These studies suggest that other parts of the molecules, other than just the

ELR motif are also involved in receptor binding. In fact, the C-terminal α-helix of IL-8/CXCL8 has been reported to be required for binding to CXCR2 but it is not necessary for high affinity binding to CXCR1. Moreover, residues Tyr 13 and Lys15 have been shown to be important for interaction with CXCR1. Similarly, experiments carried out with IL-8/GROα (CXCL8/CXCL1) chimaeric chemokines have also shown that it is not just the ELR motif but also other regions that are necessary and indispensable for high affinity binding (for review see (Wuyts et al., 1999)). Thus, it is quite apparent that multiple domains are required for interaction with receptors and that these domains are different when different receptors are studied. Additional studies have shown that an antibody against CXCR1 can block IL-8/CXCL8-induced activity without affecting binding (Wuyts et al., 1999), suggesting that there must be two distinct sites in the chemokine: one important for binding and another important for receptor activation.

The idea that the initial receptor contact is provided by the main body of the chemokine and activation of the receptor is provided by the N-terminus (two-site model) has been demonstrated for several chemokines such as SDF-1/CXCL12 and MCP-1/CCL2. Alanine scanning of MCP-1/CCL2 allowed identification of residues in this protein involved in signalling transduction and binding (Hemmerich et al., 1999). It is thought that in the case of MCP-1/CCL2, its receptor lies along the hydrophobic groove and that the N-terminus, especially residue Y13, interacts with the receptor with high affinity (Hemmerich et al., 1999). In contrast with the data collected for IL-8/CXCL8, when as little as two amino acid deletions are made in the N-terminus of MCP-1/CCL2 this chemokine lowers its activity and becomes an antagonist (Zhang et al., 1994). Crump and colleagues demonstrated that the N loop

region (region after the CXC motif) of SDF-1/CXCL12 is important for initial docking of the chemokine with CXCR4 and that the N-terminus is also an important binding site where mutations in residues 1 and 2 cause loss of receptor activity (Crump et al., 1997). Alanine-scanning mutagenesis on the N-terminal and the N-loop regions (region after the CC motif) of eotaxin showed that in contrast with MCP-1/CCL2, SDF-1/CXCL12 and IL-8/CXCL8, the residues involved in receptor binding affinity and triggering are scattered throughout the N-terminal and N-loop regions (Mayer & Stone, 2001).

Extensions of the N-terminus have also been shown to affect the function of CC (Proudfoot et al., 1996) and CXC chemokines (Malkowski et al., 1995). Production of RANTES in *E. coli* produced a functionally inactive protein (Met-RANTES) where the initiating methionine was not removed from RANTES/CCL5 endogenous amino peptidases (Proudfoot et al., 1996). This variant of RANTES/CCL5, Met-RANTES was found to be fully folded but was completely inactive in assays measuring calcium mobilization and chemotaxis despite maintaining its ability to bind its receptors (Proudfoot et al., 1996). The finding that N-terminus extensions of RANTES/CCL5 could serve as receptor antagonists lead to the generation of a RANTES/CCL5 analogue, AOP-RANTES where the first eight amino acids of RANTES/CCL5 have been replaced by an aminooxypentane (AOP) group (Simmons et al., 1997). This modified version of RANTES/CCL5 proved to be capable of inducing hCCR5 internalization and therefore acts as a very potent inhibitor of HIV infection (Simmons et al., 1997; Townson, 2000) with slightly altered receptor specificities (Proudfoot et al., 1999). More recently, another antagonist (Met-Ckβ7) has been described that is reported to have a much potent antagonist effect in CCR3

and therefore of potential importance for the treatment of asthma (Nibbs et al., 2000). Met-Ckβ7 is a modified form of MIP-4/CCL18, where the extreme N-terminal residue has been replaced by a methionine (Nibbs et al., 2000). This modified version of MIP-4/CCL18 is reported to be a potent and specific antagonist CCR3 that prevents signalling through this receptor and that at very low concentrations can block eosinophil chemotaxis induced by CCR3 ligands.

Interestingly, naturally truncated forms of chemokines have been identified *in vivo.* It seems as if the immune system exploits the fact that the activity of chemokines can be affected by slight alteration on their N-terminal domain in order to regulate immune responses. It is now known that chemokines can serve as substrates for a number of peptidases. For example, CD26, a dipeptidyl peptidase, has been shown to have a substrate specificity of NH_2-X-Pro that cleaves chemokines such as MCP-1/CCL2, Eotaxin/CCL11, IP-10/CXCL10 and RANTES/CCL5 (Oravecz et al., 1997). Work by Oravecz *et al* demonstrates that the truncated version of RANTES/CCL5 lacking the first two N-terminal residues was incapable of eliciting calcium mobilization in human monocytes but retained this activity in human macrophages stimulated with colony stimulating factor. In this study the authors report that this observation is due to the fact that the truncated version of RANTES/CCL5 has reduced activity, relative to that of RANTES/CCL5, in cells expressing CCR1 but retained the capacity of stimulating CCR5 and inhibiting HIV entry (Oravecz et al., 1997). Another example of proteins that can cleave chemokine is the family of matrix metalloproteinases (MMP). MMP-2, which is naturally secreted by stromal cells, can cleave the first four N-terminal extreme amino acids of MCP-3/CCL7 (McQuibban et al., 2000). This cleaved MCP-3/CCL7 binds to CCR1,

CCR2 and CCR3 but is not capable of inducing calcium mobilization or chemotaxis and therefore acts as an antagonist potentially involved in dampening the immune response (McQuibban et al., 2000). However, these MMP can also serve as positive regulators of inflammation. MMP9 can cleave the first 6 N-terminal amino acid residues of IL-8/CXCL8 and generate an even more potent form of IL-8/CXCL8 thought to be involved in enhancing the inflammation process perhaps by increasing signalling through CXCR1 (Van Den Steen et al., 2000). Proteolytic cleavage is also associated with the generation of a PF-4/CXCL4 variant whose first four amino acids have been cleaved to generate an inhibitor of endothelial cells (Gupta et al., 1995) and with the generation of thrombocidins, microbicidal proteins form human blood platelets have been reported to be C-terminal deletion products of CXC chemokines such as NAP-2/CXCL7 (Krijgsveld et al., 2000).

The ligand binding site on chemokine receptors is also highly complex. It is thought to be composed of multiple non-contiguous domains and at least two distinct sub-sites: one for docking and another for triggering (Ahuja et al., 1996; Murphy et al., 2000). The first evidence for this model regarding chemokine receptors came from studies with CCR1/CCR2 chimaeric receptors (Monteclaro & Charo, 1996). In this study the authors mapped the MCP-1/CCL2 binding site to the N-terminal domain of CCR2 and that other regions were necessary for efficient signal transduction (Monteclaro & Charo, 1996). More evidence supporting this model came from studies with CCR2/CD8 fusion proteins and a truncated form of CCR2 reported by the same authors (Monteclaro & Charo, 1997). This construct was obtained by fusing the N-terminal residues of CCR2 to the transmembrane domain and cytoplasmic tail of CD8 and was shown to bind MCP-1/CCL2 with an affinity similar to that of the

wild type CCR2 (Monteclaro & Charo, 1997). Furthermore, this study also shows that when the truncated mutant of CCR2 that lacks the high affinity binding sites to MCP-1/CCL2 is co-transfected with the CCR2/CD8 fusion protein, calcium mobilization is reported demonstrating that two distinct domains of MCP-1/CCL2 interact with CCR2: one for high affinity ligand binding, the N-terminus, and one for interacting with the distal regions of the receptor to induce activation (Monteclaro & Charo, 1997).

In contrast with the CCR2 data, chimaeric constructs of CCR5 and CCR2b initially reported that the N-terminus of CCR5 is not involved in ligand selectivity, but rather, that the second extracellular loop of CCR5 is the main determinant of ligand specificity (Blanpain et al., 1999). However, N-terminal truncations of CCR5 abrogated high affinity chemokine binding and functional response to MIP-1β/CCL4 and RANTES/CCL5 (Blanpain et al., 1999; Samson, 1997) suggesting that indeed the N-terminal domain of CCR5 was important for ligand binding. Alanine-scanning mutagenesis generated a panel of CCR5 mutants that were used by Blanplain *et al* to investigate the role of specific residues in the N-terminus of CCR5 which are involved in binding to, and signalling in response to, chemokines (Blanpain et al., 1999). In agreement with data published before, this study showed that mutations in the N-terminal domain of CCR5 lead to the generation of mutants with less binding affinity and responsiveness to the ligands (Blanpain et al., 1999). This work shows that not only the first 13 N-terminal amino acids of CCR5 are important for chemokine binding and receptor triggering, but also that other receptors must share structural determinants with CCR5 since swapping the N-terminus of CCR5 with the corresponding domain of other receptors (e.g. CCR2b, CXCR2, CXCR4 and CCR1)

was shown to have little or no effect on chemokine binding and receptor activation. Studies with monoclonal antibodies raised against CCR5, have further confirmed that in fact the domain involved in ligand specificity is localized in the second extracellular loop of this receptor (Wu et al., 1997). Nevertheless, the negatively charged and aromatic residues in N-terminal region of hCCR5 are also reported to be important in binding to the positively charged chemokines (Bannert et al., 2001).

Studies with CXCR4, the SDF-1/CXCL12 receptor, have also shown that this receptor binds to SDF-1/CXCL12 in a way consistent with the two-site model of chemokine-receptor interaction (Brelot et al., 2000). In this study, the authors identified the N-terminus as the site involved in chemokine binding but not signalling and the second extracellular loop as being the site involved in chemokine binding and receptor activation. Moreover, deletions in the N-terminus domain of CXCR4 were shown to abolish signalling but not binding, suggesting that the N-terminus comprises different functional regions (Brelot et al., 2000). Replacement of the extracellular N-terminus of CXCR2 with the corresponding region of DARC showed that the N-terminal domain of DARC is important for the promiscuous binding profile of DARC (Hadley & Peiper, 1997).

In the case of CXCR1, the Cys residues in the extracellular regions of this protein have been shown to be important for binding and mediated signalling in response to IL-8/CXCL8 (Wuyts et al., 1999). Replacement of the N-terminal part of CXCR1 by the corresponding domain of CXCR2 and vice-versa has shown that the N-terminal region of these receptors is responsible for the differences in binding specificity between CXCR1 and CXCR2 (Gayle et al, 1993). Again, as demonstrated for other

chemokine receptors, binding to and signalling through CXCR1 takes place in two distinct steps involving specific regions of the receptor (Clark-Lewis et al., 1995).

The overall conclusion that emerges from these studies is that the interaction between chemokines and their receptors is a two-step process where binding and activation is processed via two domains that operate independently from each other. It is this property of separate domains for activation and binding that allows the generation/design of potent antagonist with potential therapeutic properties.

1.6. Signal transduction

The binding of a chemokine to its receptor results in the activation of G proteins. Activated G proteins are then capable of mobilizing intracellular secondary messengers, which are ultimately involved in coordinating cytoskeleton reorganization and in focal adhesion formation that in turn will be responsible for appropriate cell migration in response to the chemoattractant molecule (for reviews refer to (Bokoch, 1995; Murdoch & Finn, 2000; Murphy, 1996; Premack & Schall, 1996). The complex pathways involved in regulating chemotaxis and other chemokine effects are outwith the scope of this introduction. Instead, I will focus primarily on early receptor-mediated events and on the fate and regulation of the receptor.

G proteins are inactive when GDP is bound to the G-protein subunit and active when GDP is exchanged for GTP. Binding of the ligand to the receptor selects for a receptor conformation state that facilitates the exchange of GDP to GTP on the G protein α-subunit. Once in the active state, G-proteins dissociate into Gα and G$\beta\gamma$

subunits. The Gβγ subunit is capable of activating phospsholipase C (PLC) that cleaves phosphatidylinositol 4,5-biphosphate (PIP_2) to give rise to the secondary messengers, phosphatidylinositol 1,4,5-triphosphate (IP_3) and diacyl-glycerol (DAG). IP_3 has been shown to be involved in calcium mobilization from intracellular stores and DAG, together with calcium ions, activates various forms of protein kinase C, PKC (reviewed in (Bokoch, 1995; Murdoch & Finn, 2000; Murphy, 1996; Premack & Schall, 1996)). Additionally to PKC and calcium-dependent mechanisms, other signalling mechanisms have been found to be involved in leukocyte activation such as activation of phospholipase D (PLD), mitogen activated protein kinase (MAPK), Janus kinase (JAK), phosphatidylinositol 3 kinase (PI3K), tyrosine kinases and phosphatases as well as serine/threonine kinases and phosphatases. Ultimately, these signalling mechanisms lead to cell motility, degranulation, release of superoxide anions and modification of integrins with the response being dictated by the cellular background (reviewed in (Bokoch, 1995; Murdoch & Finn, 2000; Murphy, 1996; Premack & Schall, 1996) (see Fig.1.3). Additionally, it has also been proposed that chemokines through the appropriate GPCR can activate the expression of genes by the phosphorylation and nuclear translocation of signal transducer and activator of transcription (STAT) (Wong et al., 1997; Mellado, 2001). It is now thought that binding of the ligand to the receptor causes a conformation change that results in exposure of the DRY motif to the cell cytoplasm that allows the binding of members of the JANUS family of kinases to bind to the activated receptor (Mellado et al., 1998).

It is not clear how a single receptor can sort different signalling transduction pathways in response to the ligand. Functional evidence has been accumulated that

shows that chemokine signals can be sorted depending on the cell type, concentration and type of ligand (Murphy, 1996). For example, RANTES/CCL5 has been shown to elicit chemotaxis and degranulation in eosinophils whereas in basophils this chemokine induces chemotaxis but not degranulation (Baggiolini & Dahinden, 1994). MCP-1/CCL2, on the other hand, is capable of inducing basophil degranulation without chemotaxis but is capable of inducing chemotaxis of monocytes and lymphocytes (Baggiolini & Dahinden, 1994). Moreover, different concentrations of RANTES/CCL5 have been shown to elicit biochemically distinct signalling pathways in T cells (Murphy, 1996). Distinct cell type specific signalling pathways have also been demonstrated for IL-8/CXCL8, GROα/CXCL1 and NAP-2/CXCL7. Although these CXC chemokines are all chemoattractants for neutrophils, only IL-8/CXCL8 is capable of inducing PLD activation in these cells (L'Heureux et al., 1995).

The idea of 'one chemokine-one receptor' has been recently challenged by the demonstration of receptor dimerization. Receptor dimerization has been shown to be important for transphosphorylation and activation of JAKs for a variety of GPCRs. By using tagged receptors, monoclonal antibodies and mutant receptors, CCR2 dimerization has been demonstrated (Rodriguez-Frade et al., 1999). Moreover, evidence has now been collected that demonstrates that receptors can not only dimerize with themselves (homodimerization), but also with others (heterodimerization). This ability to dimerize is believed to generate considerable functional interactions. For example, studies with HEK-293 cells co-expressing CCR2 and CCR5 have revealed that calcium mobilization, upon a simultaneous stimulus with MCP-1/CCL2 and RANTES/CCL5, was triggered at a much lower

concentration than that required by other chemokines alone (Mellado et al., 2001). It is now accepted that these two receptors form heterodimers that are more efficient at inducing biological responses due to synergistic interaction of several signalling complexes recruited by each individual receptor. Moreover, the authors report that an additional signalling pathway is triggered and that activation of PI3K is delayed (Mellado et al., 2001). As a consequence, a PTX-resistant calcium flux is elicited and cell adhesion rather than chemotaxis is triggered (Mellado et al., 2001; Rodriguez-Frade, 2001).

As mentioned above, signalling transduction via chemokine receptors has been shown to be dependent on coupling to pertussis toxin-sensitive G-proteins usually those of the G_i-type (Bokoch, 1995; Murphy, 2001). Although most of the chemokine receptors can couple to G_i proteins to activate PLC, Kuang and co-workers have demonstrated that different chemokine receptors have different specificities in coupling to the Gα subunits of the Gq classes (Kuang et al., 1996). Additionally it has been reported that CC chemokine receptors can couple to Gαq proteins whereas CXC chemokine receptors cannot (Arai & Charo, 1996). Furthermore, Arai and colleagues have demonstrated that not only chemokine receptors couple to multiple G-proteins but also that the receptor-G protein pairing is highly cell type-specific (Arai & Charo, 1996).

After activation, receptors can become desensitised to repeated stimulation with the same or other agonists. This is thought to depend on a combination of different mechanisms that include, receptor phosphorylation of Ser and Thr residues by GRKs, internalisation of cell surface receptors, and downregulation of the receptor by

changes in transcription, translation or degradation of pre-existing receptors (Ferguson, 2001; Murphy, 1996; Murdoch, 2000). Studies have shown that a receptor can be desensitised directly by a given ligand or indirectly when a ligand first encountered by the receptor desensitises a second stimulus by a different ligand. For example, work by Sozzani and colleagues demonstrates that MCP-1/CCL2 desensitises monocytes to a second response with MCP-1/CCL2, RANTES/CCL5 or MIP-1α/CCL3. However, when the same cells are treated with RANTES/CCL5 and MIP-1α/CCL3 first, they are capable of eliciting a second calcium flux in response to the second challenge with MCP-1/CCL2 (Sozzani, 1991; Sozzani, 1993). These studies show that MCP-1/CCL2 is capable of cross-desensitising these cells to all other chemokines tested possibly by interfering with the receptors in such a fashion so that they are no longer responsive to MIP-1α/CCL3 or RANTES/CCL5.

Once receptors have bound to the ligand activated G proteins, they become phosphorylated. Phosphorylation of the C-terminal domain or of the third intracellular loop by G protein-coupled receptor kinases promotes β-arrestin binding which in turn will not only promote uncoupling of the G proteins from the receptor but also mediates internalisation of the G protein-coupled receptor. Endocytosis of the receptor is mediated by clathrin that directly interacts with β-arrestins. Once internalised the receptors can either be degraded in lysosomes or recycled back to the surface after they become dissociated from β-arrestins (Ferguson & Garon, 1998).

How it is decided whether to degrade an internalised receptor or to recycle it to the surface is not yet known. Chimaeric studies have suggested that receptors have specific determinants in the C-terminus that determine whether GPCRs are either

recycled back to the surface or are retained within the intracellular compartment of the cell and/or targeted to lysosomes (Anborgh et al., 2000). For example, truncation of the CXCR4 C-terminus is reported to lead to higher receptor mediated activities in inositol phosphate formation and in the induction of a more sustained calcium elevation in the presence of the ligand, SDF-1/CXCL12 (Haribabu et al., 1997). Experiments performed by Cheng *et al* have demonstrated that β-arrestin can regulate internalisation and signalling of CXCR4 and that these functions are mediated through the C-terminus and the third intracellular loop of CXCR4 (Cheng et al., 2000). More recently, it was demonstrated that CCR5 can be internalised through pathways involving clathrin coated pits or caveolae upon stimulation with MIP-1α/CCL3 (Mueller et al., 2002). Following internalization, CCR5 was shown to be transported to early endosomes where dephosphorylation and resensitization occurs before being recycled back to the surface (Mueller et al., 2002).

Another level of receptor trafficking is determined by palmitoylation, a post-translational modification of Cys residues by thioesterification. This type of modification is a dynamic phenomenon shown to affect a broad range of biological activities such as G-protein coupling efficiency and control of receptors phosphorylation and desensitisation (Morello & Bouvier, 1996). Recent studies have demonstrated that a cluster of Cys residues in the C-terminus of CCR5 can be palmitoylated (Blanplain et al., 2001; Percherancier et al., 2001). Moreover, these studies demonstrate that palmitoylation is indeed required for efficient trafficking of CCR5 to the cell surface and for triggering intracellular signal transduction pathways.

Mutagenesis approaches to investigating chemokine receptor signalling

Mutagenesis studies have shown that there are some important motifs within chemokine receptors that are important for G protein coupling and proper signalling transduction (Oliveira et al., 1994; Brelot, 2000; Gosling, 1997; Schraufstatter, 1998). For example, mutation of the highly conserved aspartic acid residue found in the second TM domain of virtually all GPCRs, leads to loss of certain intracellular effects (e.g. calcium flux is no longer detectable) without affecting ligand binding (Farzan et al., 1997). Another important motif is the DRYLAIVHA sequence found at the interface between the third TM and the cytoplasm of most GPCRs. Experiments have demonstrated that mutations in this 'DRY' motif can abolish G protein coupling (Bennet et al., 2000) and unable recruitment and triggering of JAK phosphorylation and association to the receptor (Mellado et al., 1998). Studies carried out by Burger and colleagues have shown that mutation of the 'DRY' motif in CXCR2 to 'VRY' causes constitutive signalling of this receptor and leads to transforming activity (Burger et al., 1999).

Conserved Pro residues in the 5^{th}, 6^{th}, and 7^{th} TM domains of chemokine receptors have been demonstrated to be important for the formation of proline kinks (PK) (Ji et al., 1998; Barlow, 1988; Woolfson, 1990) which are thought to be involved in conferring backbone flexibility required for the conformation change associated with ligand binding induced activation step. Mutations of the conserved Pro residue in TM six was shown to generate impaired surface expression of the new mutant receptor (Kolakowski et al., 1995), reduced functional coupling (Nakayama & Khorana, 1991) and in some cases constitutive signalling (Tonacchera et al., 1998) whereas mutations of this Pro residue in the conserved NPXXY motif in the 7^{th} TM were shown to generate a mutant receptor with impaired activity (Wess et al., 1993; Vichi, 1999;

Barak, 1995). More recently, by analysing the aligned sequences of chemokine receptors, Govaerts *et al* identified another sequence motif (TXP motif) in the 2nd TM helix of these receptors. This TXP motif has been shown to play a key structural role in chemokine receptors where the PK is the main element and the Thr residue works as an accessory element for PK. Mutations of the TXP motif in hCCR5 demonstrated that this newly identified motif is mainly involved in receptor activation and contributes very little for ligand binding (Govaerts et al., 2001).

Additionally, the carboxy-terminus portion of chemokine receptors has been shown to be involved in signalling and receptor internalisation. Truncations and Ala scanning mutagenesis of CCR5's C-terminus have shown that this portion of the receptor is important for high affinity association with β-arrestin, in controlling calcium responses and granular enzyme release in response to the ligand (Kraft et al., 2001). Moreover, C-terminal Ser residues and a dileucine motif have been identified in CXCR4, CXCR2 and CCR5, and have been shown to be involved in differently regulating internalisation of these receptors (Vila-Coro et al., 1999; Aramori, 1997 ; Oppermann, 1999; Fan, 2001; Kraft, 2001). For example, the dileucine motif in CXCR2 was found to be critical for receptor-mediate chemotaxis in response to IL-8/CXCL8 (Ben-Baruch et al., 1995) whereas mutation of the same motif in CCR5 does not impair cellular migration (Kraft et al., 2001).

1.7. hD6, an atypical β-chemokine receptor

In an attempt to further understand the mode of action of MIP-1α/CCL3, murine members of the beta-chemokine receptor family were isolated (Nibbs et al., 1997b).

Fragments obtained by degenerative oligonucleotide-primed PCR from genomic DNA were used as probes to isolate full length cDNAs (Nibbs et al., 1997b). This led to the identification of a novel gene, murine (m) D6, which encodes a protein capable of binding to MIP-1α/CCL3 with high affinity when expressed in CHO cells (Nibbs et al., 1997b). Subsequently, fragments of human D6 used to identify the full length cDNA were generated by PCR using degenerative primers against mD6 from regions which have been shown to be conserved across species in other chemokine receptors (Nibbs et al., 1997a).

The human D6 (hD6) gene encodes a protein of 384 amino acid in length that, like other G protein-coupled receptors, comprises seven predicted transmembrane-spanning domains and four conserved Cys residues involved in maintaining receptor structure. Human D6 shares 71% identity and 86% similarity to the murine counterpart, and like the murine homologue it has an alteration in the highly conserved DRYLAIVHA motif (Nibbs et al., 1997a; Nibbs et al., 1997b). In hD6, a DKYLEIVHA motif is found in place of this highly conserved motif (Nibbs et al., 1997a). Also, the conserved aspartic acid residue found in the second TM domain of other GPCRs is changed to an asparagine. The loss of this aspartic acid residue is not found in any other chemokine receptors apart from DARC and some virally encoded proteins found to be signalling competent (Nibbs et al., 1997a). In common with other chemokine receptors cloned, hD6 has been shown to possesses a single putative N-linked glycosylation site at the N-terminus, a C-terminus rich in Ser and Thr residues that constitute potential phosphorylation sites and a highly acidic N-terminus. Although the highly acidic nature of the N-terminus is characteristic of chemokine receptors, the first thirteen amino acids found in the N-terminus of hD6

are predicted to form a hydrophobic domain that is not seen in other chemokine receptors (Nibbs et al., 1997a).

D6 has been shown to be most closely related to CCR4 and two murine IL-8RL genes. D6 has a lower sequence homology (40% identity and 50% similarity) to CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7 and CCR8 than these receptors have to one another and therefore it is seen as the most divergent member of the β-chemokine receptor family (Nibbs et al., 1997a). See Figures 1.5 and 1.6 for dendogram and sequence alignment of hD6 and other β-chemokine receptors.

Receptor binding studies have demonstrated that hD6 binds with high affinity to MIP-1αP (a hMIP-1α/CCL3 isoform, see below), mMIP-1α/CCL3, RANTES/CCL5, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, MCP-5/CCL12, and weaker affinity to MCP-1/CCL2, eotaxin/CCL11 and HCC-1/CCL14 (Nibbs et al., 1997a; Nibbs et al., 1999). hD6 and mD6 were initially reported to have different binding specificities to hMIP-1α/CCL3. Both D6 homologues and CCR5 bind with high affinity to mMIP-1α/CCL3 whereas binding to hMIP-1α/CCL3 was always seen to be weak (Nibbs et al., 1997a; Boring, 1996; Nibbs et al., 1997b). The basis for this discrepancy was clarified when two distinct but highly related human MIP-1α/CCL3 genes, namely LD78α and LD78β, were isolated (Nibbs et al., 1999). These two isoforms of human MIP-1α/CCL3 bear two reciprocal serine/glycine swaps in the region between Cys three and four and also have different amino acid residues at position 2. LD78α (or MIP-1αS) has a serine residue at position 2 whereas LD78β (or MIP-1αP) has a proline residue at that position. hD6 was shown to bind weakly to the commercially available form of hMIP-1α/CCL3, LD78α or MIP-1αS, but to bind

with high affinity to MIP-1αP. Similarly, CCR5 was shown to bind with high affinity to MIP-1αP and with low affinity to mMIP-1α/CCL3. It is thought that the proline residue at position 2 is important for high affinity binding to hD6. Interestingly, the CXC chemokine, SDF-1/CXCL12 has a proline residue at position 2 yet it does not bind to D6, implying that proline-2 must be presented in the context of a β-chemokine to allow for high affinity binding to D6 (Nibbs et al., 1999).

Surprisingly, in the standard signalling assays for chemokine receptors, such as calcium flux detection, chemotaxis and microphysiometry, hD6, like DARC (Neote et al., 1994) and CCR11 (R. Nibbs, *pers. comm.*), has not been shown to signal (Nibbs et al., 1997a). Bearing in mind the sequence alterations in the DRY motif and in the highly conserved aspartic acid residue, these findings do not necessarily imply that this receptor is signalling incompetent. It is possible that hD6 couples to alternative G proteins, or interacts with other membrane components, which could be expressed in a cell type specific fashion to mediate D6 functional responses. Thus, it was of fundamental importance to identify the cell types that express this receptor.

Northern blot analysis of hD6 expression in tissues revealed that hD6 is expressed in the placenta at high levels, in the liver at lower levels and weakly expressed in the lung and thyroid. Weak expression is detected, after longer exposure times, in a range of tissues including the small intestine and the colon. No hD6 expression was detected in PBLs (Nibbs et al., 1997a).

Binding studies *in situ,* where pieces of human skin were used to determine the binding characteristics of several β-chemokines, revealed that chemokines such as RANTES/CCL5, MCP-1/CCL2, MCP-3/CCL7 but not MIP-1αS or IL-8/CXCL8

bound to lymphatic endothelial cells (Hub & Rot, 1998). However, ^{125}I-MIP-1αP did show binding to these cells (Nibbs et al., 2001) indicating that the receptor expressed in these endothelial cells displays the same binding fingerprint as hD6. Immunocytochemistry in sections of human tissue with an anti-hD6 monoclonal antibody revealed that hD6 is in fact the receptor expressed in the lymphatic endothelium (Nibbs et al., 2001). These detailed studies showed that hD6 is expressed in the lymphatic endothelium of the mucosa and other layers of the gut, in afferent vessels entering lymph nodes and in lymphatic sinuses within secondary lymphoid tissue (Nibbs et al., 2001). hD6 expression was not found in any of the endothelial cells lining blood vessels or in sections of heart, kidney, liver, skeletal muscle, brain, cerebellum, pancreas, prostate or thyroid (Nibbs et al., 2001). Expression of this receptor was also found in a subset of vascular tumours (Nibbs et al., 2001).

The functional role of D6 is not clear, however given its promiscuous binding profile and its expression on the lymphatic it is hypothesised that D6 might play a role in leukocyte trafficking. As suggested for DARC (Horuk et al., 1993), D6 might function as a sink whereby D6's ligands bind to the receptor and are subsequently neutralized. By doing so hD6 would be preventing leukocyte firm adhesion to the lymphatic ECs thus allowing the lymph to passively transport leukocytes from the lymphatics into the lymph node. By doing so hD6 would help dampening leukocyte extravasation from blood vessels so other chemokines such as SLC/CCL21,which is not a ligand for hD6, that has been reported to be expressed by lymphatic ECs and thought to be involved in constitutive lymphocyte and DC traffic (Gunn et al., 1998b), would remain free to interact with their target cells.

Another possibility is that D6 functions as a presenting molecule involved in binding chemokines, which remain fully functional, and then presenting them to the leukocytes. As proposed for DARC (Middleton et al., 1997), it is possible that hD6 might have a role in immune and inflammatory processes by binding chemokines derived from the tissues and therefore inducing leukocyte migration into the lymph node. Conversely, it is possible that by retaining pro-inflammatory β-chemokines in the tissue, D6 might in fact prevent migration of its ligands into the lymphatic vessels, thus ensuring that cells that no longer respond to D6 ligands, for example mature DCs, would be capable of entering the lymph nodes.

Also, hD6 may be involved in transporting chemokines across the endothelium. Like DARC (Middleton et al., 1997), D6 may play a role in transcytosis of tissue derived chemokines across the endothelium into the luminal space.

Given the well documented functions of chemokines in chemotaxis, EC migration and regulation of angiogenesis it is also fair to hypothesize that D6 might also be involved in lymphangiogenesis during development and/or tissue regeneration. Further studies involving *in vivo* and *in vitro* experiments will be necessary to identify the function of D6 on the lymphatic endothelial cells.

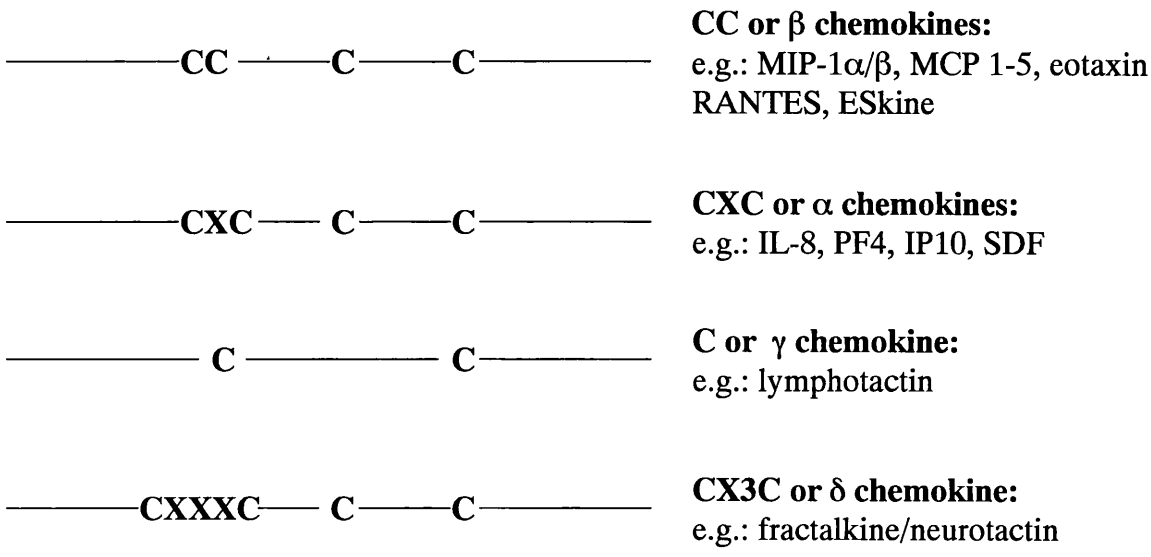


Figure 1.1. The different classes of chemokines. The chemokine superfamily can be divided into 4 subclasses defined by the arrangement of conserved cysteine (C) residues. X indicates an amino acid other than cysteine and unbroken lines other amino acids. Spacing between cysteines is similar in all four classes. N and C terminus can vary in length.

Table 1.1 The CC chemokines

Systematic Name	Common Names	Receptor(s)
CCL1	I-309, TCA-3 (mouse), SIS-f (mouse)	CCR8
CCL2	MCP-1, MCAF, HC11, JE (mouse)	CCR2, DARC, **D6**
CCL3	MIP-1α, MIP-1αS/P, SCI	CCR1, CCR5, CCR3 (mouse), **D6**
CCL4	MIP-1β	CCR5, **D6**
CCL5	RANTES	CCR3, CCR5, DARC, **D6**
CCL6	C10 (mouse); no human homologue known	Unknown
CCL7	MCP-3	CCR2, CCR3, DARC, **D6**
CCL8	MCP-2	CCR2, CCR3, CCR5, **D6**
CCL9	MRP-2, MIP-1γ, CCF18 (no human homologue known)	Unknown
CCL10		Unknown
CCL11	Eotaxin	CCR3, **D6**
CCL12	MCP-5 (mouse); no human homologue known	CCR2, **D6**
CCL13	MCP-4, Cκβ10, NCC-1; no mouse homologue known	CCR1, CCR2, CCR3, DARC, **D6**
CCL14	HCC-1, HCC-3, NCC-2, Cκβ1, CCCK-1/CCCK-3, MCF-1 (no mouse homologue)	CCR1, **D6**
CCL15	HCC-2, NCC-3, MIP-5, MIP-1δ, Lkn-1, CC-2	CCR1, CCR3
CCL16	HCC-4, LEC, HCC-4, LCC-1, Cκβ12, Mtn-1	CCR1
CCL17	TARC, ABCD-2, STCP-1	CCR4
CCL18	DCCK1, PARC, MIP-4, AMAC-1, Cκβ7; (no mouse homologue)	CCR3
CCL19	ELC, MIP-3β, exodus-3, Cκβ11	CCR7, CCR11
CCL20	MIP-3α, LARC, exodus-1; Cκβ1 (mouse)	CCR6
CCL21	6Ckine, SLC, exodus-2, TCA4, cκβ9	CCR7, CCR11
CCL22	MDC, dc/β- cκ (mouse), abcd-1, STCP-1	CCR4
CCL23	MPIF-1, MIP-3, Cκβ8-1 (no mouse homologue)	CCR1
CCL24	MPIF-2, eotaxin-2, Cκβ6	CCR3
CCL25	TECK, Cκβ15	CCR9, CCR11
CCL26	eotaxin-3, MIP-4α (no mouse homologue)	CCR3
CCL27	Eskine, CTACK, ILC (mouse) ALP, skinkine	CCR10
CCL28	MEC	CCR10

Table 1.1. The CC (or β) chemokine family. Adapted from (Murphy et al., 2000). D6's ligands are shown in bold.

Table 1.2. The CXC chemokines

Systematic Name	Common Names	Subclass	Receptor(s)
CXCL1	GROα, MGSA-α, NAP-3, MIP-2 (mouse), N(51)/KC (mouse)	ELR^+	CXCR2, DARC
CXCL2	GROβ, MIP-12α, MGSA-β	ELR^+	CXCR2
CXCL3	GROγ, MGSA-γ, MIP-2β	ELR^+	CXCR2
CXCL4	PF-4	ELR^-	Unknown
CXCL5	ENA-78, LIX	ELR^+	CXCR2
CXCL6	GCP-2, CKA-3	ELR^+	CXCR1, CXCR2
CXCL7	PBP, CTAPIII, β-TG, NAP-2, LA-PF4 (no mouse homologue)	ELR^+	CXCR2
CXCL8	IL-8, NAP-1, GCP-1 (no mouse homologue)	ELR^+	CXCR1, CXCR2, DARC
CXCL9	Mig	ELR^-	CXCR3
CXCL10	IP-10, CRG-2 (mouse)	ELR^-	CXCR3
CXCL11	I-TAC, IP9,β-R1, H174 (no mouse homologue)	ELR^-	CXCR3
CXCL12	SDF-1 (2 human isoforms, α and β), PBSF	ELR^-	CXCR4
CXCL13	BCA-1, BLC	ELR^-	CXCR5
CXCL14	BRAK, bolekine	ELR^-	Unknown
CXCL15	WECHE, lungkine	ELR^+	Unknown
CXCL16	CXCL16	ELR^-	CXCR6

Table 1.2. The CXC (or α) chemokine family. Adapted from (Murphy et al., 2000).

Table 1.3. The C chemokines

Systematic Name	Common Names	Receptor(s)
XCL1	Lymphotactin α, SCM-1α, ATAC	XCR1
XCL2	Lymphotactin β, SCM-1β, ATAC	XCR1

Table 1.3. The C (or γ) chemokine family. Adapted from (Murphy et al., 2000)

Table 1.4. The CX_3C chemokines

Systematic Name	Common Names	Receptor(s)
CX_3CL1	Fractalkine, neurotactin (mouse)	CX_3CR1

Table 1.4. The CX_3C (or δ) chemokine family. Adapted from (Murphy et al., 2000).

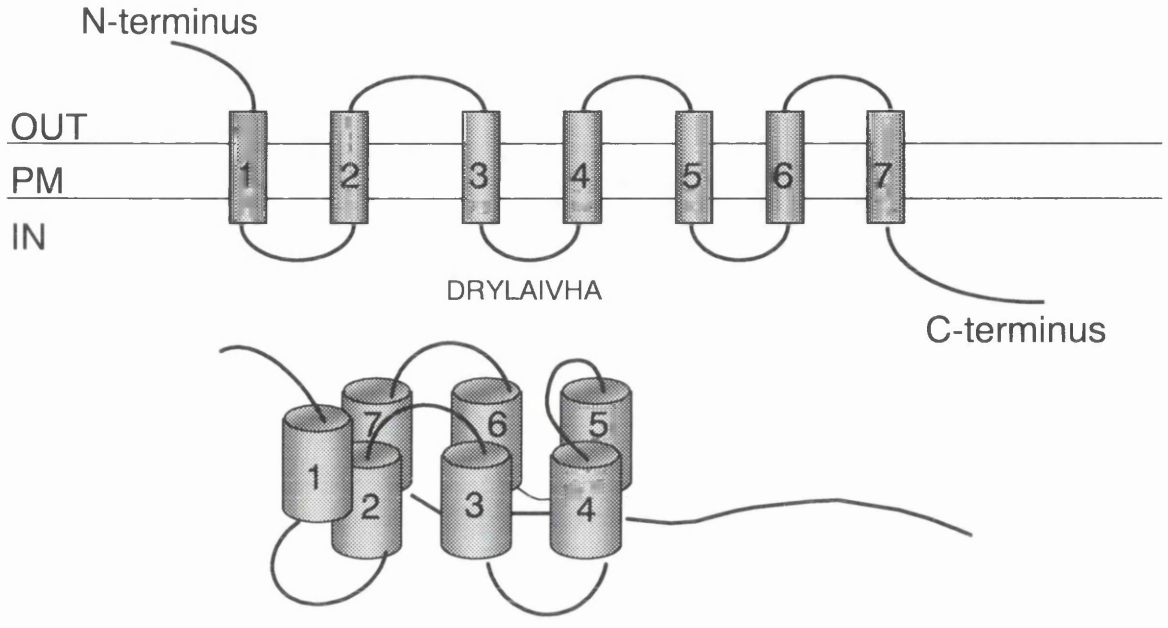


Figure 1.2. Structure of chemokine receptors. In general chemokine receptors are composed of an extracellular N-terminus, an intracellular N-terminus and seven transmembrane domains (grey cylinders numbered 1 to 7) connected by three extracellular loop and three extracellular loops. This structure is held together in a cylindrical shape by two disulphide bonds: one between cysteine residues found in the first and in the second extracellular loops and another between cysteine residues found in the N-terminus and in the third extracellular loop. The highly conserved DRYLAIVHA motif thought to be involved in coupling to G-proteins is shown.

Table 1.5. Chemokine receptors

Group	Receptor(s)	Class	Ligand(s)
Shared	CXCR1	CXC	IL-8, GCP-2
	CXCR2		all ELR^+ chemokines
	CXCR3		IP-10, Mig, I-TAC
	CCR1	CC	MIP-1α, RANTES, MCP-3, MPIF-1
	CCR2		MCP-1, MCP-2, MCP-3, MCP-4
	CCR3		MCP-2, MCP-3, MCP-4, eotaxin-1, eotaxin-2, RANTES
	CCR4		TARC, MDC
	CCR5		RANTES, MIP-1α, MIP-1β, MCP-2
	CCR6		MIP-3α
	CCR7		SLC, ELC
	CCR8		I-309
	CCR9		TECK
	CCR10		ESkine, MEC
	CCR11		TECK, SLC, ELC
	D6		MIP-1α, MIP-1β, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, HCC-1, eotaxin, RANTES
Specific	CXCR4	CXC	SDF-1
	CXCR5		BLC
	CXCR6		CXCL16
	XCR1	XC	Lymphotactin
	CX_3CR1	CX_3C	fractalkine/neurotactin
Viral	CMV US28		MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, fractalkine
	UL12		RANTES, MIP-1α, MIP-1β, MCP-1
	KSHV ORF74		many CC and CXC chemokines
	E1		eotaxin
	ECRF-3		GR0-α, IL-8, NAP-2
Promiscuous	DARC		many CC and CXC chemokines

Table 1.5 The different groups of chemokine receptors. Adapted from (Kunkel, 1999). Chemokine receptors can be classified into four different groups according to their binding profiles, these receptors can also be classified into different classes depending to which members of a certain chemokine family they bind to.

Table 1.6. Haemopoietic cell distribution of chemokine receptors.

Receptor(s)	Haemopoietic cell distribution
CXCR1	N, M, T, NK, Bs, Ms, En
CXCR2	N, M, T, NK, MS, As, Ms, En
CXCR3	Activated T
CXCR4	Myeloid, T, B, Ep, En, DC
CXCR5	B, T
CXCR6	T, NK
CCR1	N, M, T, NK, B, Ms, As
CCR2	M, T, B, Bs
CCR3	Eo, Bs, T
CCR4	T, P
CCR5	T, M, MØ, DC
CCR6	T, B, DC
CCR7	T, B, DC
CCR8	M, Thymus
CCR9	T, Thymus
CCR10	Skin homing T cells (CLA^+)
CCR11	
XCR1	T, B, NK
CX_3CR1	NK, M, T
DARC	En, RBC, T
D6	Dc (Townson, *pers. comm.*)

Table 1.6. Haemopoietic cell distribution of chemokine receptors. N, neutrophil; M, monocyte/macrophage; T, T-lymphocyte; B, B-lymphocyte; NK, natural killer cell; Eo, eosinophils; Bs, basophil; Ms, mast cell; As, astrocyte; P, platelet; En, endothelial cell; Ep, epithelial cell; Hp, hepatocyte; Dc, dendritic cell, MØ, macrophage; RBC, red blood cell. Adapted from (Murdoch & Finn, 2000).

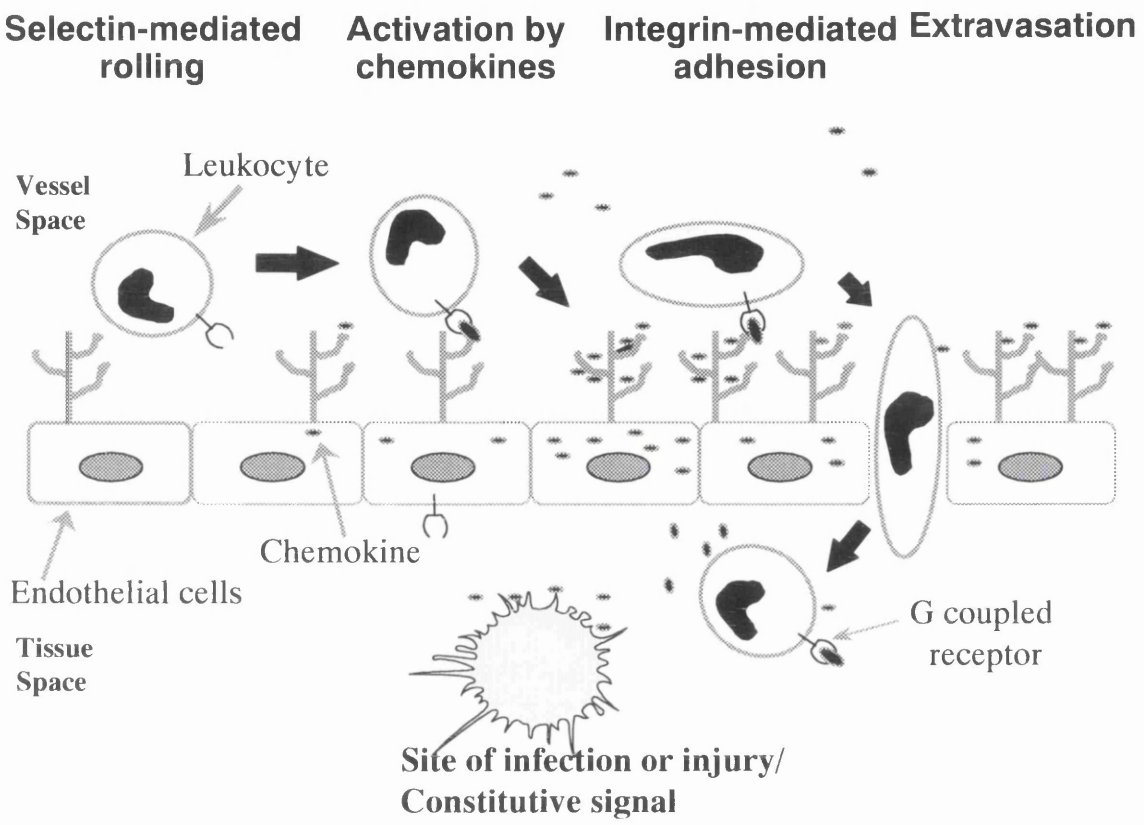
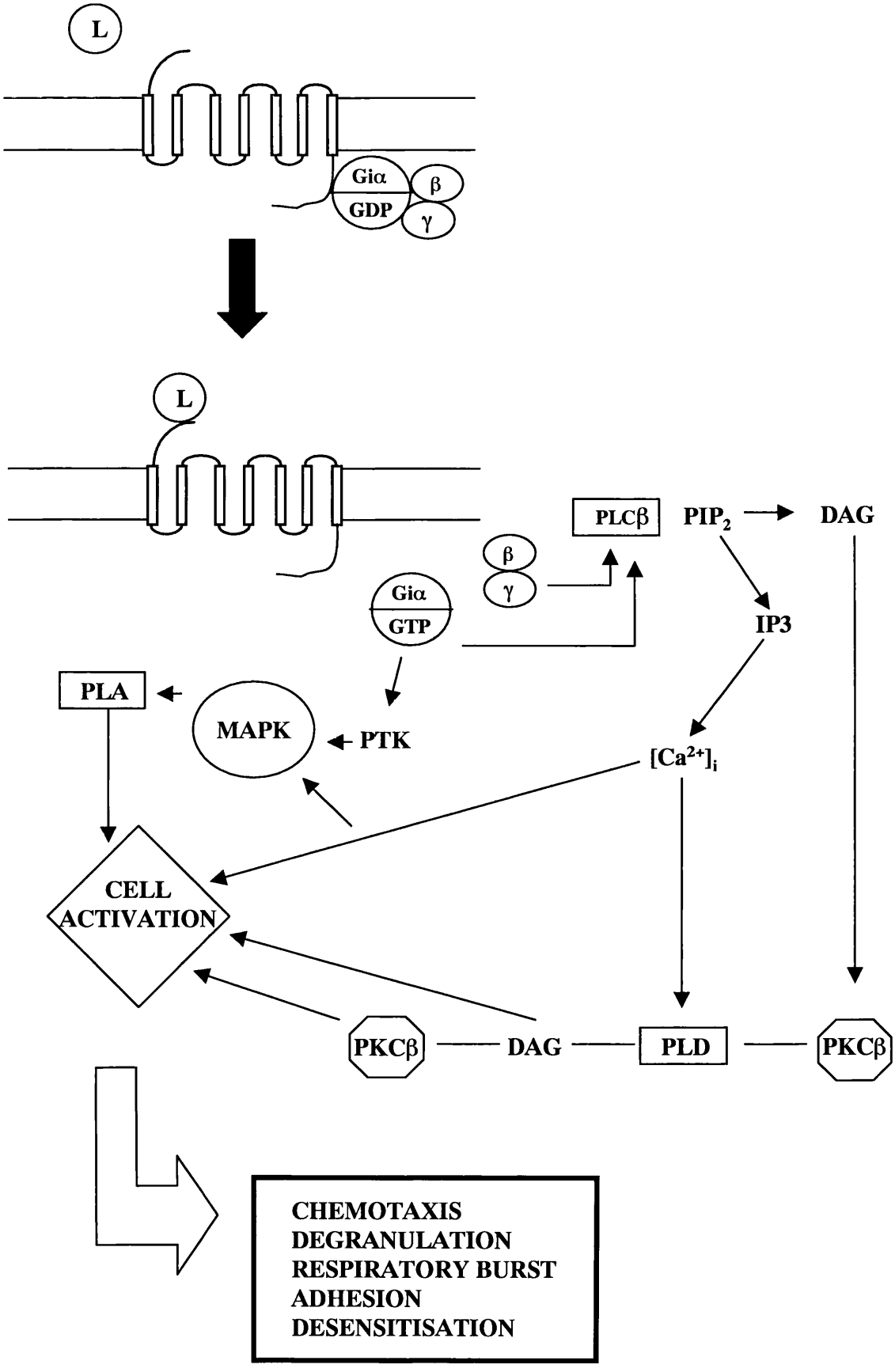


Figure 1.3. Schematic representation of the multi step model of leukocyte extravasation. Production of cytokines in response to a specific stimulus induces changes in the endothelium which include upregulation of adhesion and production of chemokines. Chemokines presented on endothelial cells trigger integrin activation and arrest of those leukocytes that carry the corresponding chemokine receptors. Activated leukocytes become firmly adhered cells which migrate along the endothelial cell surface and eventually into the tissue space. (adapted from (Schall, 1994)).

Figure 1.4. Chemokine receptor signal transduction mechanisms. Ligand (L) binding to the 7 TM domain G-protein coupled chemokine receptor causes guanosine triphosphate displacement (GTP) in the Gαi subunit, which allows dissociation of GαI from Gβγ. Gβ activates phospholipase C (PLCβ), which cleaves PIP_2 into the second messengers DAG and IP_3. DAG activates PKCβ and IP_3 causes the release of calcium from the intracellular stores. The rapid rise in intracellular calcium activates PLD. At the same time Gαi directly activates PTK (protein tyrosine kinase) which in turn activates MAP kinases and phosphorylate serine and threonine residues in the C-terminus of the receptor causing receptor inactivation. Phospholipase A (PLA) is activated by MAP kinases. PLA as well as DAG, intracellular calcium and PKC all interact with specific cell mechanisms leading to actin polymerisation, cell adhesion and motility, degranulation, cytoskeletal rearrengement, chemotaxis and receptor desensitisation. Figure adapted from (Murdoch & Finn, 2000)


L
Giα
GDP
β
γ
L
PLCβ
PIP2
DAG
β
γ
Giα
GTP
IP3
PLA
MAPK
PTK
[Ca2+]i
CELL ACTIVATION
PKCβ
DAG
PLD
PKCβ
CHEMOTAXIS
DEGRANULATION
RESPIRATORY BURST
ADHESION
DESENSITISATION

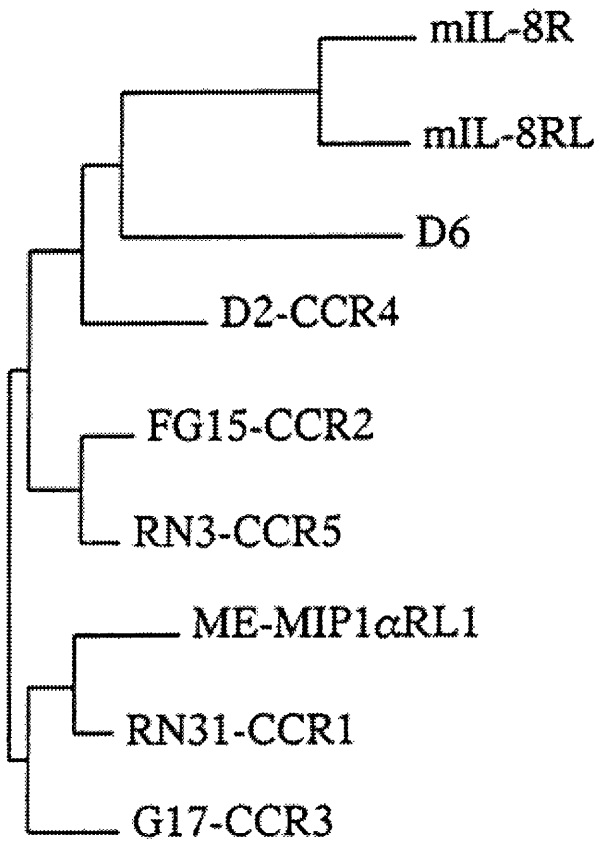
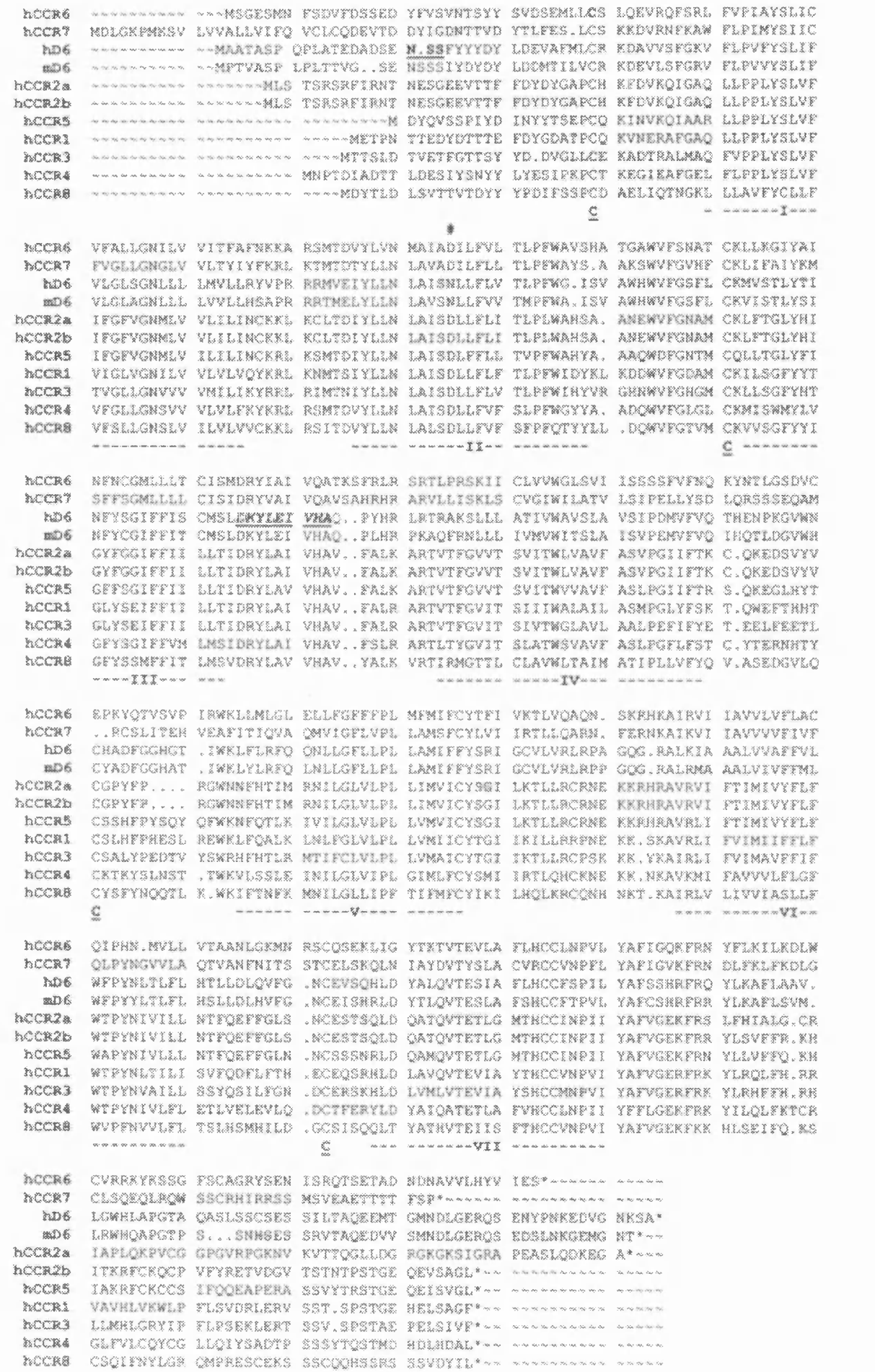


Figure 1.5. Phylogenic relationship of chemokine receptors. The diagram above was obtained by comparing the chemokine receptors' amino acid sequences known at the time this project was designed. The amino acid sequences were compared using the GCG software Pileup and Distances (Kimura method) programs, and then displayed graphically with Growtree (Neighbor-joining program). The distances between each receptor indicate the number of amino acid changes between each receptor. This figure was adapted from (Nibbs et al., 1997b).

Figure 1.8. Protein sequence of hD6, mD6 and other β-chemokine receptors. Dashed lines indicate putative transmembrane domains. The conserved Cys residues are indicated by an underlined C underneath each line up. The DKYLEIV motif characteristic of D6 is shown in bold. A putative N-linked glycosylation site (NSS) is shown in bold and underlined. The # symbol, denotes the aspartic residue conserved amongst G-protein coupled receptors, that is changed to an asparaginine in D6 sequence. This line up was adapted from (Nibbs et al., 1997a).



Chapter 2- Materials and Methods

2.1. Materials

Antibodies

Covance

FITC Labelled Mouse Monoclonal Antibody, HA.11 (clone 16B12)

Boehringer Mannheim

Anti-HA High Affinity rat monoclonal antibody (clone 3F10)

Kodak

Anti-FLAG M2, purified murine IgG1 monoclonal antibody that binds to FLAG fusion proteins

Anti-FLAG M5, purified murine IgG1 monoclonal antibody that binds to Met-FLAG fusion proteins

LeukoSite,Inc (Millenium Pharmaceuticals)

1D4, mouse anti-human D6 antibody. This antibody was made in collaboration with LeukoSite Inc. and optimised using hD6 transfected cell lines as described by Nibbs and colleagues. (Nibbs et al., 2001)

Pharmingen

CCR5 R-FITC- conjugated mouse anti-human monoclonal antibody (clone 2D7/CCR5)

Santa Cruz Biotechnology, Inc

Y11, anti-HA TAG antibody

Sigma

Alkaline Phosphatase conjugated Anti-Mouse IgG (Fc specific)

Anti-Mouse IgG (Fab specific) FITC conjugate

Anti-mouse IgG horseradish peroxidase linked whole antibody (raised in sheep)

Anti-Rat IgG (Whole molecule) FITC conjugate

Bacteriology

Beatson Institute Central Services

LB (Luria Bertani) liquid medium

Glycerol

Becton Dickinson Labware
Falcon 1059 polypropylene tubes
Falcon 2059 polypropylene tubes
Falcon 2501 (miniprep tubes)

Beta Laboratories
Yeast extract

Bibby Sterilin Ltd
90mm bacteriological petri dishes

Difco Laboratories
Bacto-Agar
Bactotryptone

Gibco BRL Europe Life Technologies Ltd
E.coli DH5α competent cells

Nunc
1ml screw cap tubes

Sigma Chemical Co. Ltd
Ampicillin
Chloramphenicol

Cell lines

CHO – Chinese Hamster Ovary cells
COS-7 – African green monkey kidney cells
HEK-293 – Human Embryonic Kidney cells
HOS – Human OsteoSarcoma cells

Chemicals and Reagents

BDH Analar Laboratory Supplies
D- glucose

Fisons Scientific Equipment
Ammonium acetate
Butan-2-ol
Chloroform
Dimethyl sulfoxide (DMSO)
Ethylene diamine tetra acetate (EDTA) disodium salt

EGTA
Methanol
Propan-2-ol
Sodium acetate
Sodium dodecyl sulphate (SDS)

Gibco BRL Europe Life Technologies Ltd
Agarose (ultrapure electrophoresis grade)
Tris base

Severn Biotech Ltd.
30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide
40% (w/v) acrylamide: 2.1% (w/v) bis-acrylamide

Sigma Chemical Co. Ltd
Bovine Serum Albumen (BSA)
Bromophenol Blue
Coomassie Brilliant Blue R
Ethidium Bromide
Goat Serum
HEPES
Ponceau S solution
SIGMA FAST™ pNPP (p- Nitrophenyl Phosphate) substrate tablet set
TEMED (N,N,N',N' – tetraethylenediamine)
Tween-20 (Polyoxyethylene sorbitan nonolaureate)

Chemokines

Beatson Institute
PM2 (non- aggregating murine MIP-1α mutant); a gift from Dr. G. Graham(Graham et al., 1994)

Peprotech
Recombinant Human Eotaxin (carrier free)
Recombinant Human MCAF (Human MCP-1) (carrier free)
Recombinant Human MCP-2 (carrier free)
Recombinant Human RANTES (carrier free)

Enzymes and Kits

ABgene
1.1 x Pre-Aliqouted ReddyMix™ PCR Master Mix in Thermo-Tubes

Gibco BRL Europe Life Technologies Ltd
All restriciton enzymes and reaction buffers were obtained from Gibco BRL unless otherwise stated.

Invitrogen
TOPO TA cloning

Kramel Biotech
Alkaline Phosphatase

New England BioLabs Inc.
Xma I
Xmn I

Roche
Rapid DNA Ligation Kit

Promega
Set of dATP, dCTP, dGTP, dTTP

Qiagen Ltd
QIAprep Spin Miniprep Kit
QIAGEN Plasmid Maxi Kit
QIAquick Gel Extraction Kit
QIAquick PCR Purification Kit

STRATAGENE
QuickChange ™ Site-Directed Mutagenesis Kit
Cloned Pfu DNA polymerase

Miscellaneous

Amersham International plc
Hybond-C^{extra}
ECL Western Blotting Reagents

Alpha Laboratories Ltd
Microcentrifuge tubes

Decon Laboratories Ltd
Decon 75

James Burrough Ltd
Ethanol

Kodak Scientific imaging Systems Ltd.
X-OMAT AR X-Ray film

Gelman Sciences Ltd
Sterile 0.2μm acrodisc filters

Pierce
Iodo-Gen® iodination reagent

Premier Beverages
Marvel (dried skimmed milk)

Vector Labs
VectaShield

Sigma chemical Co. Ltd
Kodak X-ray film

STRATAGENE
Pfu DNA Polymerase

Whatman international Ltd
Whatman 3MM filter paper

DNA and protein markers

Amersham International plc
Rainbow™ coloured protein molecular weight markers

Gibco BRL Europe life Technologies Ltd
1 Kb DNA Ladder
Low DNA mass Ladder

Plasmids

pcDNA.3-mIL8RL-1: plasmid encoding mouse Interleukin 8 receptor-like1 (mIL8RL-1) cloned into pcDNA.3 as a HindIII/NotI insert. This plasmid was constructed by Dr. R. Nibbs (Beatson Institute).

pcDNA.3-hD6: plasmid encoding human D6 cloned into pcDNA.3 as a HindIII/NotI insert. This plasmid was constructed by Dr. R. Nibbs (Beatson Institute).

pcDNA.3-hCCR5: plasmid encoding human CCR5, cloned into pcDNA.3 as a HindIII/NotI insert. This plasmid was constructed by Dr. R. Nibbs (Beatson Institute).

pKSII: plasmid commercially available from STRATAGENE

pcDNA.3: mammalian expression vector commercially available from Invitrogen

pMACS K^k: plasmid encoding for the truncated mouse MHC class I molecules of H-2 K^k haplotype; this plasmid is commercially available from Miltenyi Biotec

Radiochemicals

NEN™ Life Science Product, INC

Iodine-125, carrier free radionuclide (Specific activity, 643.8GBq/mg)

[^{125}I]- MCP-4 (human, recombinant); Specific activity 9.36 MBq/μg

[^{125}I]- RANTES (human recombinant); Specific activity 10.2 MBq/μg

Tissue Culture

BDH

BES (NN-Bis (2-hydroxyethyl)-2- aminoethane sulphonic acid)

Beatson Institute Central Services

Sterile distilled water

Sterile glycerol

Sterile phosphate buffered saline (PBS)

Sterile phosphate buffered saline + EDTA (PE)

Becton Dickinson Labware

90mm tissue culture dishes

100ml plastic pipettes

50ml plastic pipettes

Bibby Sterilin Ltd

Sterile plastic bijoux and universal containers

Gibco BRL Europe Life Technologies Ltd

Special Liquid Medium (SLM)

10x Dulbecco's Modified Eagles Medium

2.5% Trypsin

200mM L-glutamine

7.5% sodium bicarbonate

100mM sodium pyruvate

Geneticin (G418)

Globepharm
Foetal calf serum

Harlan Sera-Lab Ltd
Foetal calf serum

QIAGEN
Effectene™ Transfection Reagent
SuperFect Transfection Reagent

Nunc
Cryotubes
T25, T75, T125 cm^2 tissue culture flasks

Miltenyi Biotec
MACSelect K^k Microbeads
OctoMACS (magnetic cell separator for eight simultaneous separations)
MACS Separation Columns type MS^+

Promega
Transfectam® Reagent for the Transfection of Eukaryotic Cells

Sigma Chemical Co. Ltd
Pertussis toxin
Saponin

2.2. Methods

2.2.1. Tissue culture

All cell culture work was performed using strict aseptic techniques inside a laminar flow hood (Class II Microbiological Safety Cabinets, Medical Air technology Ltd., Manchester, UK). Cells were incubated at 37°C in a dry atmosphere containing 5% (v/v) CO_2 (Heraeus, Essex, UK).

2.2.1.1. Chinese Hamster Ovary (CHO)-K1 Cells

CHO-K1 cells were grown in Special Liquid Medium (SLM) containing 10% (v/v) of FCS and 5mM L-glutamine.

2.2.1.1.1. Stable transfection of CHO cells

CHO cells were transfected using the modified calcium phosphate mediated method especially suitable for stable transfection of CHO cells. 5E5 cells were plated onto 10mm diameter petri dishes containing 10ml of SLM supplemented with 10% of FCS and 5mM of glutamine were incubated overnight in a 'cake box' at 37°C, 5% CO_2. The next day 20μg of DNA were diluted in 0.5ml of 0.25M $CaCl_2$ in a bijoux bottle. 0.5ml of 2xBES Buffered Saline (BBS). (BBS contains 50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4). This mixture was incubated at room temperature for 20 minutes and then added dropwise to the plated cells. Plates were placed back into the 'cake box' and gassed with 3% CO_2. The box was sealed and incubated at 35°C overnight. 18 hours later the medium was aspirated and plates washed twice with warm SLM containing 10% FCS and glutamine. Plates were re-fed with 10ml of

medium and incubated overnight at 37°C, 5%CO_2. 48 hours after transfection the cells were harvested and seeded onto 100mm (diameter) plates at 1E3 cells and 1E4 cells/plate. Plates were again incubated overnight at 37°C, 5% CO_2. 72 hours after transfection 1.6mg/ml of G418 was added for selection of transfected cells. Colonies appeared, in general, around day 10. Once single colonies were visible, clones were picked and transferred into a 24 well plate.

2.2.1.1.2. Transient transfection of CHO cells

1.5E6 cells/100mm diameter plate were seeded the night before transfection and incubated at 37°C, 5% CO_2. The next day 2μg of DNA were transfected into cells using Effectene transfection reagent following manufacturer's instructions. Cells were incubated O/N at 37°C, 5% CO_2. 18 hours later cells were washed once in PBS, re-fed with complete medium, and incubated O/N at 37°C, 5% CO_2. Cells were harvested 24 hours later.

2.2.1.2. Human Embryonic Kidney Cells (HEK.293)

HEK.293 cells were grown in DMEM medium supplemented with 5mM glutamine, 100nM NaPyruvate, 10% FCS, 0.3% Na_2HCO_3, 5mM HCl.

2.2.1.2.1. Stable Transfection of HEK.293

Cells were seeded at 2E6 cells/100mm dish and incubated at 37°C, 5%CO_2 for 2 days. Cells were then gently washed with serum-free DMEM and 1.25ml of serum-

free DMEM added to each plate. 7μg of DNA were mixed with 1.25ml of serum-free DMEM in a bijou bottle (solution A) and 15μl TRANSFECTAM® with 1.25ml of serum-free DMEM in a separate bijou bottle (solution B). Solutions A and B were then mixed together and added dropwise to cells. Cells were incubated for 4 hours at 37°C, 5%CO_2, in a 'cake box'. After 4 hours, 13ml of complete medium was added to each plate and cells were left to incubate for another 2 days. Selection was initiated at day 6 by feeding the cells with complete DMEM supplemented with 800μg/ml of G418.

2.2.1.2.2. Pertussis toxin treatment of stably transfected HEK.293 cells

Stably transfected HEK.293 were grown to reach confluence in a 175 cm^2 flask. Cells were incubated overnight with 100μg/ml of Pertussis toxin. The next day cells were tested for their ability to flux calcium as described in Section 2.2.3.8.

2.2.1.3. COS-7 cells

COS-7 cells were grown in Special Liquid Medium (SLM) containing 10% (v/v) of FCS and 5mM L-glutamine.

2.2.1.3.1. Stable transfection of COS-7 cells

The day before transfection, 5E5 cells were seeded in a 100mm diameter dish in 15ml of growth medium. Cells were incubated O/N at 37°C and 5% CO_2. The next day 10μg of DNA was diluted in serum-free growth medium to a total volume of 300μl. To this DNA solution 40μl of SuperFect Transfection Reagent were added. Mixing was ensured by pipetting up and down 5 times or by vortexing for 10

seconds. Samples were incubated for 5-10 minutes at room temperature to allow complex formation. While complex formation took place, growth medium from the dish was aspirated and the cells washed once with 5ml of PBS. After the incubation period, 3ml of complete growth medium was added to the reaction tube containing the transfection complex. After mixing by pipetting up and down twice, the total volume of transfection complex was added to the cells drop wise.

Selection was started on day 6 by feeding the cells with complete DMEM supplemented with 200μg/ml of G418.

2.2.1.3.2. Transient transfection of COS-7 cells

Transient transfection of COS-7 cells was carried out following the same protocol used for stable transfection of CHO cells (see section 2.2.1.1.2.)

2.2.1.4. Human OsteoSarcoma (HOS) cells:

HOS cells were grown in DMEM medium supplemented with 5mM glutamine, 100nM NaPyruvate, 10% FCS, 0.3% Na_2HCO_3, 5mM HCl.

2.2.1.4.1. Stable transfection of HOS cells

Stable transfection of HOS cells was carried out following the same protocol used for transient transfection of HEK.293 cells (see Section 2.2.1.2.1.)

2.2.2. Molecular Biology

2.2.2.1. Oligonucleotide Synthesis

Oligonucleotides were synthesized by an in-house facility on an Applied Biosystems Model 381A DNA synthesiser using the manufacturers protocols and Cruachem reagents. The oligonucleotides were synthesized without trityl group protection, obtained as a pellet, and resuspended in 600µl sterile distilled water. The final concentration of oligonucleotide was determined by absorbance measurement at 260nm and 280nm using a Beckman DU 650 spectophotometer. An OD_{260} reading of 1 corresponds approximately to 33µg/ml single stranded DNA or 50µg/ml double stranded DNA. The DNA sample purity was estimated from the OD_{260}/ OD_{280} ratio.

2.2.2.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using horizontal gel cast apparatus. Generally, 1% agarose gels were used. Ultrapure electrophoretic grade agarose was dissolved in 1xTAE (40mM Tris-acetate, 1mM EDTA) by boiling the solution. After the solution was cooled down, 0.5µg/ml of Ethidium Bromide were added. The gel was poured into the gel cast and left to set.

To each DNA sample, DNA loading buffer (6x solution: 30% glycerol and bromophenol blue to colour) was added and samples as well as size markers were loaded into the appropriate wells of the gel. The DNA was usually separated by running the gel in 1xTAE buffer at 70-100 constant voltage. DNA bands were visualised using a UV transilluminator and photographed.

2.2.2.3. Restriction Enzyme Digests

DNA was digested in a final volume of 20-50μl using the appropriate enzymes and reaction buffers according to the manufacturer's instructions. In general, 5-10 units of restriction enzyme/μg of plasmid DNA was used. The reactions were carried out at the appropriate temperature (according to manufacturer's instructions) for 60-90 minutes. Digested vectors to be used in ligation reactions (see Section 2.2.2.6.) were treated with 1 unit of Alkaline Phosphatase and further incubated in a water bath at 37°C for 30 minutes to prevent vector religation.

2.2.2.4. DNA purification (gel extraction)

DNA of interest was excised from a gel using a scalpel and purified using a QIAGEN QIAquick Gel Extraction Kit according to manufacturer's instructions. The DNA was eluted from the columns in 30-50 μl of distilled water and the yield determined by running a 5μl aliquot on an agarose gel in the presence of a 1Kb DNA ladder and a DNA mass ladder.

2.2.2.5. DNA Ligation

After the vector and insert were appropriately digested, purified and quantitated, they were ligated by using the Rapid DNA Ligation Kit, according to the manufacturer's instructions. The amount of vector and insert to be used in these reactions was calculated using the following formula:

(50ng of vector x Kb size of insert/ Kb size of vector) x (ratio insert:vector)= ng of insert

The ratio of insert to vector used was 4:1 and $1/10^{th}$ of the above reaction was used to transform *E.coli* DH5α competent cells (see below)

2.2.2.6. Transformation of Competent Bacterial Cells

E.coli DH5α competent cells were used for the propagation of plasmid DNA. A 20μl aliquot of the competent cells were thawed on ice and then transferred to a chilled polypropylene tube (Falcon 2059). The appropriate amount of DNA was added to each tube and left to incubate on ice for 30 minutes. Cells were then heat shocked for 45 seconds in a water bath at 42°C. 80μl of SOC medium (2% bactotryptone, 0.55% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to each sample and each tube was incubated at 37°C for 1 hour with vigorous shaking. The whole of the transformation reaction was then plated on to L-agar plates containing 50μg/ml of ampicillin. Plates were inverted and incubated O/N at 37°C to allow colony formation.

2.2.2.7. Preparation of Plasmid DNA (Miniprep/ Maxiprep)

Single bacterial colonies were picked and incubated overnight in 6ml of LB-medium containing 50μg/ml of ampicillin with vigorous shaking at 37°C. 1.5ml of the overnight culture was centrifuged for 10 minutes at 14,000 r.p.m in a bench top centrifuge. DNA was purified using the QIAprep Spin Miniprep Kit according to manufacturer's instructions. DNA from larger volumes of overnight cultures (100ml) was purified using the QIAGEN Maxi kit following the manufacturer's instructions.

2.2.2.7.1. Plasmid preparation of pMACS K^k

As $pMACSK^K$ is a low copy plasmid an amplification step was carried out in order to achieve high yields of plasmid. DH5α cells were transformed as described in Section 2.2.2.6 with 1μl of commercially available pMACS K^k. The next day 30ml of LB-broth supplemented with 50μg/ml of ampicillin was inoculated with a single bacterial colony. This culture was incubated overnight in a shaker at 37°C and left to reach late-log phase ($O.D._{600}$ ~0.6). 500ml of LB-broth with ampicillin was subsequently inoculated with 25ml of the late log phase culture. This culture was incubated for approximately 2.5 hours at 37°C with vigorous shaking (300 cycles/min on a rotary shaker) until the $O.D._{600}$ reached ~ 0.4. At this stage 2.5ml of chloramphenicol (34mg/ml in ethanol) was added and the culture was left to grow overnight at 37°C in the shaker. The next day DNA from the overnight culture was prepared as described in Section 2.2.2.7.

2.2.2.8. Polymerase Chain Reaction (PCR)

2.2.2.8.1. Pfu method:

For each PCR reaction 10μl of the 10x Pfu buffer, 10μl of DMSO, 10μl of 50% glycerol, 4ul 10mM dNTPs and 0.1μg of each primer per base where mixed in a PCR tube to a final volume of 98μl (made up with dH_2O). 1μl of plasmid template (0.1μg/μl) and 1μl Pfu enzyme (2.5μg/μl) were finally added to each tube. The reactions were overlaid with 100μl of paraffin and heated up to 94°C for one minute. PCR products were obtained after 30 cycles of a one minute denaturing step at 94°C, one minute annealing step at 55°C and a one minute extension step at 72°C. After the

30 cycles, the reactions were left at 72°C for 10 minutes. The whole 100µl of the PCR reaction were analysed by agarose gel electrophoresis and the PCR product was purified using the technique described in section 2.2.2.5

2.2.2.8.2. Pre-Aliquoted ReddyMix™ Master Mix:

To each pre-aliquoted ReddyMix™ PCR Master Mix, 1µl (10µM) of each primer and 50ng of plasmid template were added to a final volume of 50µl. PCR products were obtained after 30 cycles of a one minute denaturing step at 94°C, one minute annealing step at 55°C and a one minute extension step at 72°C. After the 30 cycles, the reactions were left at 72°C for 10 minutes. All of the PCR reaction was analysed by agarose gel electrophoresis and the PCR product was purified using the technique described in section 2.2.2.5.

2.2.2.9. Overlap extension PCR

This technique was used to join two sequences from different receptors when no appropriate restriction enzymes were present. The first round of PCR was set up using the protocol described above. Primers were designed so that the products of the two primary PCR reactions contained at least 20bp overlapping complementary ends; primers used in the secondary reaction were designed to anneal at the non complementary ends and to include restriction sites that allowed cloning of the PCR product into the appropriate backbone. A second PCR reaction was carried out using the products (1.5µl) of the first round as template and 10µM of each primer as before (Section 2.2.2.9.2.). This resulted in the primary products priming on each other and extending to yield a hybrid product. This hybrid product was then analysed by

agarose gel electrophoresis, purified and digested with the appropriate enzymes and then cloned back into the appropriate vector (Fig. 2.1).

2.2.2.10. Site Directed Mutagenesis

Site directed mutagenesis was used to generate single point mutations in either CCR5HACCR5V.pKSII or D6HAD6V.pKSII. The QuickChange™ Site-Directed Mutagenesis Kit was used according to the manufacturer's instructions.

2.2.11. Cloning of DNA fragments into pCR®2.1-TOPO®

Taq polymerase-amplified PCR products were cloned into pCR®2.1-TOPO® using the TOPO TA Cloning® Kit following the manufacturer's instructions. In brief, 4μl of PCR product, 1μl of salt solution and 1μl of linearized pCR®2.1-TOPO® were mixed together and incubated at room temperature for 5 minutes. Reaction mixture was stored on ice until used for transformation.

For each transformation reaction, 2μl of the TOPO® Cloning reaction were transferred into a vial of One Shot® Chemically competent *E. coli*, and mixed gently. Transformation reaction was incubated on ice for 5 minutes and subsequently heat-shocked for 30 seconds at 42°C without shaking. Tubes were immediately transferred to ice and 250μl of room temperature SOC medium were added. After incubating the cells in a shaker (200rpm) for 1 hour at 37°C, 50μl of each transformation were spread in an agar plate containing selective media (L-agar plates supplemented with 50μg/ml of ampicillin). Plates were inverted and incubated overnight in a 37°C

incubator. Colonies were isolated the following day and analysed further to check for successful cloning.

2.2.12. DNA sequencing

An Applied Biosystems 373A automated sequencer was used to verify the correct sequence of all constructs generated. First, PCR amplification of the region to be sequenced was carried out with primers complimentary to domains upstream and downstream from the region to be sequenced. In general PCR reactions contained: 0.5μg plasmid DNA, 3.2pmmoles of primer and 4μl of Big Dye Terminator Reaction premix in a final volume of 10μl (made up with distilled water). 250μl thin walled eppendorf tubes were used for all sequencing PCR reactions. Samples were heated to 95°C for 30 sec, 50 for 30 sec and 60°C for 4 min. This cycle was repeated 25 times. To each reaction 2μl of 3M Sodium Acetate (pH4.5) and 50μl of Ethanol were added. Reactions were incubated at room temperature for 15 minutes and then centrifuged for 10 minutes at top speed in a bench top centrifuge. After discarding of the supernatant, pellets were washed twice with 100μl of ice cold 70% ethanol and again the supernatant was removed. Pellets were left to air-dry to allow evaporation of any residual ethanol. Members of the Beatson Institute technical services staff performed the sequencing gel electrophoresis. Sequencing data was analysed using the CHEVAL computer program.

2.2.2.13. Vector construction:

2.2.2.13.1. hD6/mIL8RL-1 chimaeric constructs

To generate Flag tagged constructs of wild type mIL8RL-1.pKSII and hD6.pKSII a Flag Tag was inserted by PCR after the start codon. Primer sequences are shown in Table2.1 with the new sequence in bold.

hD6.pKSII was amplified by D6Flag and hD6D5. The PCR product generated was then digested with HindIII and BglII and subsequently cloned into hD6.pKSII previously digested with the same enzymes. The new construct obtained was named D6FD6V.pKSII (Fig.2.2)

The IL8Flag and 831 (Table 2.1) primers were used in conjunction with mIL8RL-1.pKSII to yield a PCR product that was digested with HindIII and AccI. To create construct IL8FIL8V.pKSII (Fig.2.2), the HindIII/AccI fragment was cloned into mIL8RL-1.pKSII which was previously digested with the same enzymes.

Primers D6AccI and D6Flag (Table 2.1) were used to change the BglII site of hD6.pKSII into an AccI site. This fragment was then digested with HindIII and AccI and cloned into mIL8RL-1.pKSII, which was previously digested with the same enzymes. This hybrid construct was named D6FIL8V.pKSII (Fig.2.2).

Primers IL8Flag and IL8BglII (Table 2.1), were used to mutate the AccI site of mIL8RL-1.pKSII into a BglII site. This PCR fragment was digested with HindIII and BglII and then cloned into hD6.pKSII to yield the construct named IL8FD6V.pKSII (Fig.2.2).

A HA Tag was cloned after the start codon of hD6.pKSII and mIL8RL-1.pKSII. To clone the HA tag into hD6.pKSII the primers D6HA and hD6d5 (Table 2.1) were used; primer IL8HA together with primer 831 (Table 2.1) were used to clone the HA tag into mIL8RL-1.pKSII. The HA tag was cloned into hD6.pKSII as a HindIII/BglII insert and into mIL8RL-1.pKSII as a HindIII/AccI piece. The new constructs were named D6HAD6V.pKSII and IL8HAIL8V.pKSII (Fig.2.2), respectively. The HindIII/BglII fragment was cloned into HindIII/BglII-cut D6FIL8V.pKSII to generate construct D6HAIL8V.pKSII (Fig.2.2). IL8HAD6V.pKSII (Fig.2.2) was obtained by cloning the HindIII/AccI piece into HindIII/AccI cut IL8FD6V.pKSII.

Once the fidelity of these constructs was checked by restriction enzyme digests and DNA sequencing, the plasmids were digested with HindIII and NotI and subsequently cloned into the corresponding sites of the mammalian expression vector pcDNA.3.

2.2.2.13.2 hCCR5/hD6 chimaeric constructs- large swaps

To add HA Tag sequence on the N-terminus of hCCR5 the primers CCR5HA and CCR5.D1 (Table 2.2) were used in PCR. This fragment was digested with HindIII and MscI and then cloned into HindIII/MscI-cut hCCR5.pKSII to generate CCR5HACCR5V.pKSII (Fig.2.3).

Construct CCR5HAD6V.pKSII, was obtained by cloning the 288bp HindIII/MscI PCR fragment mentioned above into D6HAD6V.pKSII also digested with HindIII/MscI (Fig. 2.3).

Digestion of D6HAD6V.pKSII with HindIII and MscI yield a fragment between nucleotides 1 and 265 of the open reading frame that corresponds to the N-terminus, and a a portion of the adjacent transmembrane I of the translated protein. This fragment was inserted into the digested CCR5HACCR5V.pKSII to generate the chimaeric construct D6HACCR5V.pKSII (Fig. 2.3).

D6HAD6V.pKSII, CCR5HACCR5V.pKSII, CCR5HAD6V.pKSII and D6HACCR5V.pKSII were then subcloned into pcDNA.3.

2.2.2.13.3 hD6/hCCR5 chimaeric constructs- extracellular domain swaps

To create the chimaera 5nt6bd.pKSII, D6HAD6V.pKSII was amplified by primers 5nttop and hD6D4 (Reaction 1) and by primers 5ntbot and CCR5HA (Reaction 2)- for primer sequence see Table 2.3. The PCR products generated by reaction 1 and 2 were further amplified together using the primers CCR5HA and hD6D4 to give a final PCR product. The PCR product was cloned using HindIII/PstI into HindIII/PstI-cut D6HAD6V.pKSII to generate the hybrid construct 5nt6bd.pKSII (Fig. 2.4). This construct encodes the HA tag sequence right after the start codon and the first 19 amino acids of hCCR5's N-terminus joined on to amino acid 36 of hD6's mature protein.

To make the chimaeric construct with the first extracellular loop exchanged (Fig. 2.4), the plasmid D6HAD6V.pKSII was digested with BglII and SmaI which deleted a fragment between nucleotides 322 and 379 of the open reading frame. This fragment corresponds to the first extracellular loop of the translated protein. A similar fragment was obtained from hCCR5 by PCR. D6HAD6V.pKSII was

amplified with primers (Reaction 1) Eloop5Abot and hD6U5 (Table 2.3) and with primers (Reaction 2) Eloop5Atop and hD6D2 (Table 2.3). In a second round of PCR, the PCR products obtained from Reaction 1 and 2 were amplified with the primers hD6U5 and hD6D2 to yield a hybrid fragment. This new fragment comprises the first extracellular loop of hCCR5 (nucleotides 491 to 558 of the open reading frame) flanked at either side by the nucleotides 207 to 322 and nucleotides 379 to 569 of hD6's open reading frame. This fragment was then cloned into the TOPO TA Cloning Kit (Section 2.2.11). After digestion with BglII and SmaI an insert was obtained. This inserted was then cloned into the digested D6HAD6V.pKSII.

Amplification of D6HAD6V.pKSII with primers (Table 2.3) Eloop5Btop in conjunction with hD6D1 and with primers Eloop5Bbot and hD6U1 (Table 2.3) produced two separate PCR products. In a second round of PCR, these 2 PCR products were further amplified with primers hD6U1 and hD6D1 to generate a hybrid fragment. This hybrid fragment corresponds to the second extracellular loop of hCCR5 flanked by portions of hD6 at either side of the hCCR5 loop. Digestion of this fragment with XmaI and XbaI allowed cloning of this fragment into D6HAD6V.pKSII previously digested with the same enzymes. This new chimaeric constructs was named EloopB.pKSII (Fig. 2.4)

Cloning of the third extracellular loop of hCCR5 into the corresponding portion of hD6 was achieved by amplifying D6HAD6V.pKSII with primers stated below (Table 2.3 for full primer sequencing). Two separate reactions were carried out with primers Eloop5Ctop and hD6.3NotI (Reaction 1) and primers Eloop5Bbot and hD6U1 (Reaction 2). The PCR products from reactions 1 and 2 were then used as templates

in a second round of PCR in conjunction with the hD6 internal primers hD6U1 and hD6D1. The resulting PCR product was cloned into TOPO (Section 2.2.11) and then digested with XmaI and NotI. Digestion of D6HAD6V.pKSII with XmaI and NotI allowed cloning of the digested PCR product to generate construct EloopC.pKSII (Fig. 2.4).

Restriction enzyme digests and DNA sequencing verified fidelity of the constructs. All of the above constructs were then cloned into pcDNA.3 as a HindIII/NotI fragments.

2.2.2.13.4. hD6/hCCR5 chimaeric constructs- intracellular mutants

iLoop1.pKSII (Fig.2.5) has the 1st intracellular loop of hD6 swapped for the corresponding region of hCCR5. This construct was obtained by amplification of D6HAD6V.pKSII with the primers iLoop1A in conjunction with D6HA and iLoop1B (Table 2.5) with conjunction with hD6D4 (Table 2.3) in 2 independent PCR reactions. In a second round of PCR the products from the two independent reactions were subsequently amplified with the primers D6HA and hD6D4. This final PCR product was then digested with BamHI and MscI. Digestion of D6HAD6V.pKSII with BamHI and MscI excises the first intracellular loop of hD6's mature protein thus allowing insertion of the digested PCR product. Diagnostic digests and DNA sequencing checked for successful cloning. The insert iLoop1 was digested from pKSII and subcloned into pcDNA.3 as a HindIII/NotI fragment.

D6HAD6V.pKSII was digested with MscI and XbaI to delete a fragment that corresponds to the 2nd intracellular loop of hD6's mature protein. A PCR fragment

was generated by amplifying the products of two primary PCR reactions where the primers iLoop2A/hD6U5 and iLoop2B/hD6D7 (Table 2.4) independently amplified D6HAD6V.pKSII. The final PCR product was digested with MscI and XbaI to allow cloning of this digested fragment into the previously digested vector. Again, the hybrid construct obtained, named iLoop2.pKSII (Fig. 2.5), was analysed by diagnostic digests and DNA sequencing. iLoop2.pKSII was subsequently subcloned into pcDNA.3 as a HindIII/NotI fragment.

The 5' iLoop3B and 3' hD6D7 primers were used in conjunction with the 5' hD6U3 and 3' iLoop3A (see Table 2.4 for primer sequences) to generate a hybrid fragment comprising nucleotides corresponding to the 3rd intracellular loop of hCCR5 flanked by portions of hD6's mature protein. The PCR fragment obtained was digested with XmaI and XbaI and then cloned into D6HAD6V.pKSII (previously digested with the same enzymes), to create construct iLoop3.pKSII (Fig. 2.5). HindIII/Not I digestion of iLoop3.pKSII excises the insert from pKSII to allow subcloning into pcDNA.3. Constructs were analysed by diagnostic digests and DNA sequencing.

C-terminus swaps between hCCR5 and hD6 were obtained by using the primers listed in Table 4. The construct 5ct6bd.pKSII (Fig. 2.6.) bears the C-terminus of hCCR5 cloned into position 946bp of hD6 (straight after the 7th TM domain).

Construct 5ct6bd.pKSII was created following the same procedure described above. 5ctbot and CCR53BnotI (Table 2.4) were used to amplify CCR5HACCR5V.pKSII (Reaction 1); 5cttop in conjunction with hD6U3 (Table 2.4) amplified D6HAD6V.pKSII (Reaction 2). The PCR products from these 2 reactions were further amplified with the primers (hD6U3 and CCR53BNotI) used in the primary

reactions to yield a hybrid PCR fragment containing the C-terminus of hCCR5 and flanked at the 5' end by the 7th TM domain of hD6. The C-terminus of hD6 was excised from D6HAD6V.pKSII as a XbaI/NotI fragment to allow for insertion of the PCR product also digested with XbaI and NotI. Diagnostic digests and DNA sequencing were performed to confirm identity of the new construct (named 5ct6bd.pKSII). 5ct6bd.pKSII was digested with HindIII and NotI and the insert obtained was subcloned into pcDNA.3.

D6HAD6V.pKSII was amplified with primers hD6E-A5' and hD6E-A3' (Table 2.5 for primer sequences) to generate construct hD6E-A.pKSII (Fig. 2.6). Construct hD6K-R.pKSII (Fig. 2.6) was obtained by amplification of D6HAD6V.pKSII with primers hD6K-R5' and hD6K-R3' (Table 2.5 for primer sequences).

CCR5HACCR5V.pKSII was amplified with primers 5Ketop and 5Kebottom (Table 2.5 for primer sequences) to create construct hCCR5DKYLEIV.pKSII (Fig. 2.6).

Fidelity of the constructs hD6E-A.pKSII, hD6K-R.pKSII and hCCR5DKYLEIV.pKSII was checked by DNA sequencing and subsequently subcloned into pcDNA.3 as a HindIII/NotI fragment

2.2.2.13.5. hCCR5/hD6 chimaeric constructs- second generation

To study the cooperative effects of hCCR5 receptor intracellular domains a second generation of chimaeric mutants was generated. These mutants bear a combination of two or more intracellular domains of hCCR5 cloned into the corresponding region of hD6 (Fig. 2.7).

Construct iLoop1+3.pKSII has the first and the third loops of hD6 replaced by the corresponding region of hCCR5. This construct was obtained by cutting the first intracellular loop of iLoop1.pKSII with BamHI and MscI. The digested fragment was subsequently cloned into iLoop3.pKSII previously digested with the same enzymes.

Digestion of hD6DRY.5.pKSII with MscI/XmaI yields a fragment containing the DKYLEIV region of hD6 mutated to DRYLAIV. Constructs iLoop1.pKSII, iLoop3.pKSII, iLoop1+3.pKSII and 5ct6bd.pKSII were separately digested with MscI/XmaI to allow cloning of the DRYLAIV fragment into the corresponding region of these vectors. The new constructs were named iLoop1+DRY.pKSII, iLoop3+DRY.pKSII, iLoop1+DRY+iLoop3.pKSII and DRY+Ct.pKSII.

Digestion of 5ct6bd.pKSII with XbaI and NotI generates a fragment containing the 7TM and the C-terminus of this construct. This fragment was cloned into iLoop1+DRY+iLoop3.pKSII previously digested with the same enzymes to obtain construct iLoop1+DRY+iLoop3+Ct.pKSII.

All cDNAs were subcloned into the mammalian expression vector pcDNA.3 as a HindIII/NotI fragment and diagnostic digests checked for the fidelity of each construct. Each one of these new constructs was stably transfected into HEK.293 cells as described in Section 2.2.1.2.1.

2.2.3. Protein Detection

2.2.3.1. SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

10% SDS polyacrylamide gel was used to resolve protein samples. The resolving gel was made by adding the appropriate volume of 30% (w/v) acrylamide: 0.8% (w/v) bys-acrylamide to 0.45M Tris, 0.1% SDS (final concentration). 0.08% TEMED and 0.1% (w/v) APS were added at the very end to catalyse polymerisation. This solution was poured between two glass plates, the top of the gel was covered with saturated butanol and the gel was left to set for 30 minutes. Once the gel was set the saturated butanol was poured off, washed with water and blotted dry. 10ml of a 5% stacking gel buffer (0.125M Tris, pH6.8, 0.1% SDS, 1.7ml 30% (w/v) acrylamide: 0.8% (w/v) bys-acrylamide, 0.2% APS, 0.3% TEMED; 4x stacking gel buffer: 0.5M TRIS (pH6.8), 0.4% SDS) was poured in top of the resolving buffer, a comb was inserted and the gel left to set.

Cells were trypsinised as usual and washed once with PBS. 1ml of lysis buffer (1x lysis buffer: 50mM Tris (pH6.8), 25mM DTT, 2% SDS) was used to resuspend the harvested cells and samples were boiled for 5 minutes. 60µl of each sample was mixed with an equal volume of 2x SDS loading buffer (2x SDS loading buffer: 100mM Tris-HCL (pH6.8), 2% (v/v) β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for a further 5 minutes. 5µl of Rainbow™ markers was loaded into the first well; a 5µl aliquot of each sample was loaded into separate wells. The gel was left to run at approximately 50mA in 1x SDS-PAGE running buffer (25mM Tris, 250mM glycine, 0.1% (w/v) SDS) until bromophenol blue band reached the bottom of the gel.

2.2.3.2 Transfer of proteins from SDS-PAGE gel to nitrocellulose

Once the proteins were separated by SDS-PAGE, the glass plates were removed and the stacking gel was cut off and discarded. 12 pieces of 3MM filter paper and 1 piece of nitrocellulose membrane (Hybond C^{extra}) were cut to size of the gel. 6 pieces of 3MM paper were soaked in 1x Dry Blot Buffer (48mM Tris, 39mM glycine, 1.3mM SDS and 20% (v/v) methanol) and placed onto graphite plate of blotter. The nitrocellulose membrane was pre-wetted in 1x Dry Blot Buffer and placed carefully onto filter paper. The gel was briefly rinsed with Blot Buffer and placed carefully in top of the nitrocellulose membrane. Another 6 pieces of 3MM filter paper were pre-wet in 1x Dry Blot Buffer and placed over the gel. Any air bubbles were removed by rolling a plastic pipette over the stack of membrane and filter paper, and the transfer was carried out at 180mA for 1 hour. Staining the nitrocellulose with Ponceau S Solution checked the efficiency of the transfer.

2.2.3.3 Western Blotting

Once gel was blotted onto a nitrocellulose membrane, the membrane was incubated overnight in blocking buffer (5% (w/v) dried milk in PBS-T (0.1% (v/v) Tween in PBS)) at 4°C with shaking. The nitrocellulose filter was washed 3x 10 min in PBS-T and then incubated in 10ml of a 1:500 dilution of anti-FLAG M5 or M2 antibody in blocking buffer for 1hr at room temperature with shaking. The primary antibody solution was removed and the membrane was washed 3x 10 min in PBS-T. After this, the membrane was incubated in 25ml of a 1: 5000 dilution of anti-mouse IgG HRP linked antibody in blocking buffer. The secondary antibody solution was removed and the membrane was washed twice for 10 minutes with multiple changes

with PBS-Tween; another 2x 10 min washes were carried out with PBS-Tween. Excess surface liquid was removed from the membrane and the western blot developed by enhanced chemiluminescence (ECL) according to manufacturer's instructions.

2.2.4. Radio-iodination of PM2

PM2 was radiolabeled using Iodogen. 5μg of PM2 (0.1mg/ml) in PBS was incubated with 10μg of Iodogen and 6μl of ^{125}I in an eppendorf tube for 15 minutes on ice. Following this incubation on ice, unincorporated iodine was separated from the labelled protein by applying the reaction mixture to a disposable desalt column and eluting with PBS. 500μl fractions were collected and measured in a gamma counter to detect the peak of protein associated radioactivity, and the active fractions were pooled together to give 1.5ml of a 300nM ^{125}I-PM2 solution.

2.2.5. Functional Assays

2.2.5.1. Enzyme Linked ImmunoSorbent Assay (ELISA)

All constructs were examined in triplicate. 2E5 cells/well were seeded in a 24-well plate one day before the assay. Cells were fixed in 4% paraformaldehyde (in PBS) for 10 minutes on ice, washed twice in PBS and incubated with anti-FLAG antibody (1/1000 in PBS) for 1 hour at room temperature. Cells were then rinsed once with PBS and incubated for 30 minutes with anti-mouse IgG alkaline Phosphatase conjugated (1/10000 dilution in PBS). Cells were washed three times with PBS and developing solution (pNPP substrate tablet set) was added according to the

manufacturer's instructions. Colour development was read at 450nm using a microtiter plate reader.

2.2.5.2. Flow cytometry Analysis

Approximately 5E5 cells were washed with FACS buffer (0.5% Bovine Serum Albumin, 1% sucrose in PBS. This buffer is kept on ice at all times.) and incubated for 30 minutes on ice with 1/100 dilution of HA.11 antibody or 1D4 antibody. Cells were then washed once on ice cold FACS buffer and subsequently resuspended in 400ul of FACS buffer. Cells stained with 1D4 were further incubated for 30 minutes on ice with 1/32 dilution of anti-mouse FITC coupled antibody. At the end of this incubation step cells were washed once with ice cold FACS buffer and then resuspended in 400ul of FACS buffer. All samples were analysed in a FACS (Becton Dickinson).

2.2.5.3. Immunocytochemistry

Cells were seeded at 1E5/well in an 8 well permanox chamber slide and left to grow overnight at 37°C, 5% CO_2. The next day the cells were washed with FACS buffer and incubated for 1 hour with 1/100 High Affinity anti-HA antibody in FACS buffer (slides were kept on a tray of ice at all times). Cells were washed for 1 hour in FACS buffer with multiple changes. After this washing step cells were incubated with 1/32 dilution of the secondary antibody (anti-rabbit FITC coupled antibody) in FACS buffer. Slides washed for a further 30 minutes in FACS buffer, with multiple changes, were subsequently mounted in 2 drops of VectaShield and sealed with nail varnish to prevent the slides from drying out. Cells were visualised in a Biorad MRC

600 confocal attached to a Nikon Diaphot microscope with an oil immersion lense (x10 and x60 magnification). Pictures were viewed on the Confocal Assistant computer program.

2.2.5.4. Internalisation Assay

Cells were incubated with or without 100nM of PM2 in complete medium for 45 minutes at 37°C, 5% CO_2. After the incubation period 1.5ml of ice cold Buffer 1 (Buffer 1: 1% BSA, 0.25% sodium azide, in PBS) was added to each tube. Cells were centrifuged at 1000 r.p.m. for 5 minutes at 4°C and subsequently washed twice with 1.5ml of Buffer 1. Pelleted cells were resuspended in Buffer 1 and incubated for 30 minutes on ice with either 1/10 dilution of anti-hCCR5 FITC-conjugated antibody or with 1/100 dilution of 1D4 antibody. Cells were washed twice in Buffer 1 and either stained with a 1/32 dilution of an anti-mouse FITC coupled antibody (for cells stained with 1D4); or resuspended in 400ul of Buffer 1 and fixed by adding 50ul of 4% PFA. Excess 2^{ry} antibody was washed off twice with Buffer 1, and the cells were finally resuspended in a final volume of 400µl of the same buffer. Again, these cells were fixed by adding 50µl of 4% PFA to each tube.

Relative fluorescence of each sample was determined by the following formula:

$$\frac{\text{fluorescence of sample} - \text{fluorescence of negative control}}{\text{fluorescence of no-chemokine control} - \text{fluorescence of negative control}} \times 100$$

(This method was adapted from (Mack et al., 1998))

2.2.5.5. Receptor Binding Studies

2.2.5.5.1. Adherent Cells

CHO cells were plated at 1E5 / well in a 6 well tissue culture plate and left to grow overnight at 37°C, 5% CO_2. The next day the cells were washed twice with 2ml of warm PBS and incubated with 200µl /well of binding buffer (binding buffer: 0.5% NaAzide, 25mM HEPES in complete SLM; pH 7.4). Various concentrations of unlabeled competitor chemokine, or equivalent volumes of PBS, were added to each well followed by ^{125}I- labelled chemokine. After incubating the cells for 90 minutes at room temperature the wells were washed 3 times with ice cold PBS and then lysed with 0.5ml of 1% SDS. Lysates were transferred to counting vials and each counted for 1 minute in a Beckman Gamma S500B counter. Each point was done in triplicate, the average taken and converted into a percentage of binding in the absence of unlabelled competitor chemokine. On average, the total cpm used was between 17 000 and 20 000 and the specific activity bound was between 3.2 and 4%.

2.2.5.5.2. Suspension assay

1E6 cells were resuspended in 35µl of binding buffer (Section 2.2.3.5.) and incubated with or without 60nM of unlabelled chemokine. To each tube 6nM of I^{125}- labelled PM2 was added and cells were left to incubate for 90 minutes at room temperature. Non specific chemokine binding was removed by adding 50µl of wash buffer (wash buffer: 4% NaCl in complete media) to each tube. Cells were pelleted by centrifugation at 10k r.p.m. for 5 minutes. Pellets were washed once with ice cold and supernatant was pipetted off. Tubes were then cut in half with a hot scalpel and

transferred to counting vials. Samples were counted and data analysed as in Section 2.2.3.6. On average, the total cpm used was between 17 000 and 20 000 and the specific activity bound was between 3.2 and 4%.

For I^{125}-RANTES, and I^{125}-MCP-4 the amounts of unlabelled and labelled chemokine used in the assay were 500nM and 2nM respectively. On average, the total cpm used was between 5 000 and 7 000 and the specific activity bound was between 2.9 and 4%.

2.2.5.5.3. Displacement curves

CHO cells stably expressing HA or Flag Tagged constructs were seeded as in Section 2.2.3.5. G418 resistant pools of HEK.293 expressing HA Tag constructs and transiently transfected COS-7 cells were prepared as described in Section 2.2.3.6.

A full binding curve for each construct was obtained by incubating the cells with I^{125}-PM2 at a constant concentration of 75nM and a varying concentration, 0 to 2uM, of unlabeled PM2. Each point was done in triplicate, the average taken, and converted into a percentage of radioactivity bound in the absence of unlabeled competitor chemokine. IC_{50} was determined using EXCEL. On average, the total cpm used was between 17 000 and 20 000 and the specific activity bound was between 3.2 and 4%.

2.2.5.6. Signalling Assays

Untransfected and stably transfected HEK.293 were assayed for their ability to flux calcium upon ligand binding. Cells were resuspended in 26ml of warm SR buffer (SR buffer: 136mM NaCl, 4.8mM KCl, 5mM Glucose, 20mM HEPES, 1mM $CaCl_2$,

0.05% BSA; pH 7.4), per confluent 175cm^2 flask. Cells were pelleted by centrifugation at 1000 r.p.m. for 5 minutes and then resuspended in 6ml of SR buffer. To each cell suspension 12µl of FURA-2-AM (4µg/µl) was added and cells were incubated for 30-40 minutes in the dark at 37°C, 5% CO_2. After this incubation period cells were washed twice with 20ml of SR buffer and finally resuspended in 6ml of SR buffer. The fluorescence of these samples was measured by a Perkin-Elmer LS50 Spectrometer at an excitation wavelength of 340nm and an emission wavelength of 540nm. Measurements were carried out every 100ms under continuous stirring at 37°C. Once the cells were loaded into the stirred cuvette they were left to equilibrate for 2 minutes and then basal fluorescence was measured, cells were diluted down to achieve a basal fluorescence of between 600-700 units intensity. Cells were then stimulated with 50nM PM2 (final concentration) and the intensity of fluorescence measured for up to 300sec.

2.2.6. The MACSelect- Transfection Cell Selection System

This system was used for the isolation of transiently transfected CHO cells. CHO cells were transiently co-transfected with 1ug of pMACS K^k and 1ug of DNA of interest following the protocol described in Section 2.2.1.1.2.

48 hours after transfection cells were washed once with PBS and 500µl of a weak trypsin solution (90ml PE, 5ml PBS, and 5ml trypsin) were added per 60mm (diameter) plate. Dishes were incubated at 37°C until cells became dissociated from the culture dish and from each other. Trypsinization was stopped by adding 100µl of 100% FCS. To each dish 40µl of MACSelect K^k Microbeads was added and rocking of the dish assured evenly distribution of the beads. The Microbeads were left to

incubate on the plate for 15 minutes at room temperature. After incubation with the Microbeads degassed PBE (PBS supplemented with 0.5% bovine serum albumin and 5mM EDTA) was added to a final volume of 2ml. Cells were resuspended completely to ensure cell suspension. The selection column was placed in the magnetic field and washed with 500µl of PBE. Cell suspension was applied to the column in 500µl aliquots making sure that each aliquot had been resuspended appropriately to avoid blocking of the column with cell clumps. Once all the negative cells (flow-through) had passed through the column was then washed with 500µl of PBE four times and subsequently removed from the separator. The column was placed on a suitable collection universal tube. 1ml of complete media was pipetted into the column. Using the plunger supplied with the column, transfected cells were flushed out of the column into the universal.

Staining the flow through and the sorted cells with anti-Kk antibody checked effectiveness of this system. In general, 5E5 cells were resuspended in 100µl of PBE and 10µl of anti-K^k antibody was added to each tube. Tubes were left to incubate for 5-10 minutes on ice in the dark. Cells were washed by adding 1.5ml of PBE and finally resuspended in 400µl of PBE. Samples were then examined by a FACS.

Primer	Nucleotide Sequence
D6Flag	
	5'gagaga**gtcgacaagcttggatcctccaacatggattacaaggatgacgatgataag**gccgccactgcctctccgca 3'
	HindIII BamHI M D Y K D D D D K A A T A S P Q
hD6D5	5' aggttggagatggccagattcagc 3'
IL8Flag	
	5'gagaga**aagcttggatcctccaacatggattacaaggatgacgatgataag**gccgaggctgaatatttcatctg3'
	HindIII BamHI M D Y K D D D D K A E A E Y T I W
831	5' tcgcctgtataagataaccagca 3'
D6 AccI	5' gagaga**gtcgac**ctcaaccatccgcctgcg 3'
	AccI
IL8 BglII	5' gagag**agatct**ccatgacggatcgggtcc 3'
	BglII
D6HA	
	5'**gagagagtcgacaagcttggatcctccaacatgtacccctacgacgtgcccgactacgccgggccgggggccgccact**
	AccI HindIII BamHI M Y P Y D V P D Y A G P G A A T
	gcctctccg 3'
	A S P
IL8HA	5'**gagagaaagcttggatcctccaacatgtacccctacgacgtgcccgactacgccgggccc**
	HindIII BamHI M Y P Y D V P D Y A G P
	ggggccgaggctgaatatttcatctg 3'
	G A E A E Y F I W

Table 2.1. Primers used to generate hD6/mIL8RL-1 chimaeric constructs. Sequence in bold indicates new sequence.

Primer	Nucleotide Sequence
CCR5HA	
5'	**gagagaaagcttggatcctccaacatgtacccctacgacgtgcccgactacgccgggccgggggattatcaa**
	HindIII BamHI **M Y P Y D V P D Y A G P G** D Y Q
	gtgtcaagtccaatc3'
	V S S P I
CCR5.D1	5'aacaggtcagagatggccag 3'

Table 2.2. Primers used to insert a HA Tag on the N-terminus of hCCR5.pKsII

Primer	Nucleotide Sequence
5nttop	5' **attatacatcggagcc**ctgcag**gaaggatgcagtg** 3'
	PstI
5ntbot	5' accactgcatccttcctgcag**ggctccgatgtataataattg**3'
	PstI
hD6D4	5' gtagggctgagcatgaacg 3'
EloopA5top	5'**tgggactttggaaatacaatgtgtcaactcttgacaggg**ctttatactattaacttttacagtggcatc3'
EloopA5bot	5'**acattgtatttccaaagtcccactgggcggcagcatagt**ggccccagaagggcagtgtcac3'
hD6U5	5' tccttcttctcatggtcttgctccg 3'
hD6D2	5' gacacaagcccatactatggtagc 3'
Eloop5Btop	5'**ctgcagctctcattttccatacagtcagtatcaattctggaagaatttccagacattaaag**cagaac ctcctagggtttctccttccac 3'
Eloop5Bbot	5' **atactgactgtatggaaaatgagagctgcaggtgtaatgaagaccttctttttgaga**tgtctgtac aaagaccatatcaggg 3'
hD6U1	5' cgttcatgctcagccctac3'
hD6D1	5' ctggagtgcgtagtctagatgc 3'
EloopCtop	5' **tttggcctgaataattgcagtagctctaacaggttgcaccaa**gcactccaggtaacagagagcat cgccttc 3'
EloopCbot	5'**actgcaattattcaggccaaagaattcctggaaggtgtt**cagaaacaaggtgagattgtatgg 3'
hD6U1	5' tccttcttctcatggtcttgctccg 3'
hD6.3NotI	5' gagagagagcggccgctcaggctgatttattccccacatcc 3'
	NotI *

Table 2.3. Primers used to generate hD6/hCCR5 chimaeric constructs. Sequence in bold indicates hCCR5 nucleotides. Underlined sequence indicates primer overlap. Box indicates restriction site. Dashed box indicates Stop codon (*).

Primer	Nucleotide Sequence
iLoop1A	5'**gtcatgctcttcagccttttgcagtttatcaggatgag**gagaagaaggaggttcccgct 3'
iLoop1B	5' **atcctgataaactgcaaaaggctgaagagcatgac**atctatctgctgaatctggccatc 3'
hD6D7	5' aggtgccaggtgccatcc 3'
iLoop2A	5'**cctggcttttaaagcaaacacagcatggacgacagccaggtacct**ctgcaggctcatgcagcaaat3'
iLoop2B	5'**gtcgtccatgctgtgtttgctttaaaagccaggacggtcaggttt**agcctgctccttgctaccatag 3'
iLoop3A	5'**gtgcctcttcttctcatttcgacaccgaagcagagtttttaggattcc**ggagtagaagaagatcatggcaa g 3'
iLoop3B	5'**actctgcttcggtgtcgaaatgagaagaagaggcacagggctgtgagg**atagcagcagccttggt ggtgg 3'
hD6U3	5'aagtattcgggaactgtgaggt 3'
CC5.U3	5' ctggtcctgccgctgcttc 3'
5ctbottom	5' cttttcccccatcctg**tatgcctttgtcggggagaag** 3'
5cttop	5' **cttctccccgacaaaggcata**caggatgggggaaaag 3'
CC53BNotI	5'gagagaga[ggcggccgc]tca**caagcccacagatatttcctg** 3'

NotI *

Table 2.4. Primers used to generate construct iLoop1.pKSII. Bold denotes hCCR5 sequence. Underlined sequence shows primer overlap. Box indicates restriction site. Dashed box indicates Stop codon (*).

Primer	Nucleotide Sequence
hD6E-A5'	5' ggaca**a**gtacctggcgatcgttcatgct3'
hD6E-A3'	5' cctgt**t**catggaccgctagcaagtacga3'
hD6K-R5'	5' tgcatg**a**gcctggacaggtacctggagatcg 3'
hD6K-R3'	5' cgatc**t**ccaggtacctgtccaggctcatgca3'
5KEtop	5' tcctgacaatcgat**aa**gtacctgg**ag**gtcgtccatgctgtg 3'
5KEbottom	5' cacagcatggacgac**ct**ccaggtac**tt**atcgattgtcagga 3'

Table 2.5. Primers used to generate point mutations in D6HAD6V.pKSII and CCR5HACCR5V.pKSII. Bold indicates mutated sequences

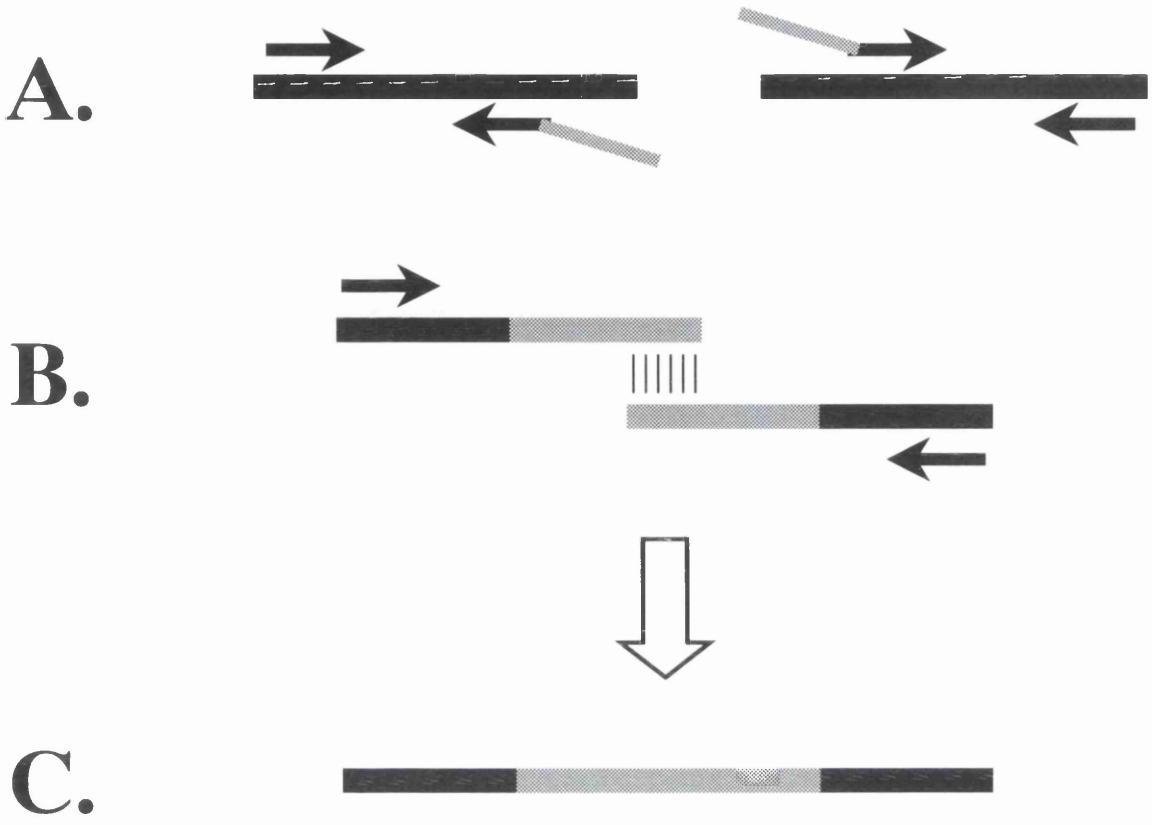


Figure 2.1. PCR strategy for generating chimaeric receptors. Black arrows represent internal primers to the host receptor. Black and grey arrows represent chimaeric primers that prime to the host receptor and have the other receptor's sequence on their 5'end. In step A fragments of the host receptor were amplified to generate chimaeric fragments that overlap. In step B the same internal primers used in step A were used to generate a chimaeric fragment, shown in C) of a certain receptor.

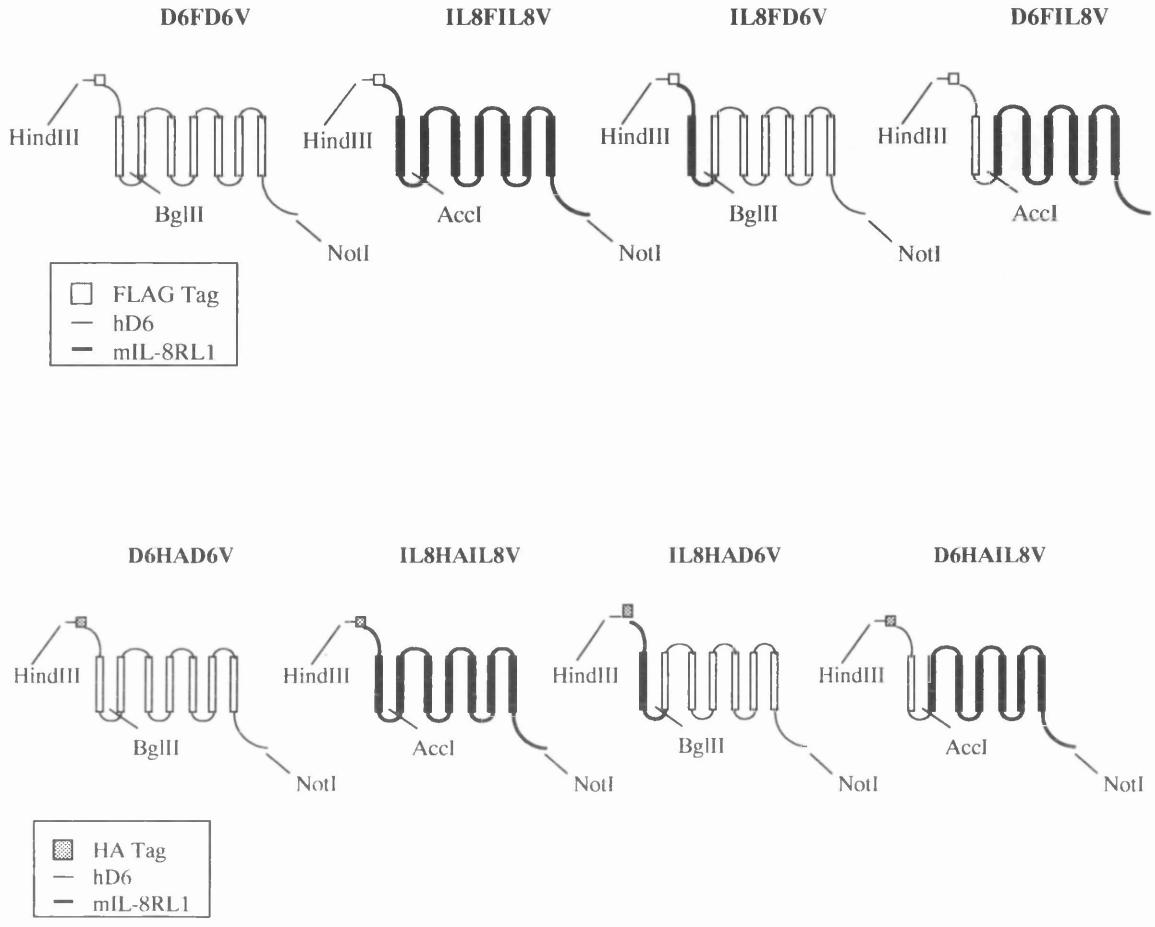


Figure 2.2. hD6/mIL-8RL1 constructs. The white square represents the FLAG Tag and the grey one represents the HA tag epitope. Thin black lines and white rectangles indicate hD6 sequence, thick black lines and filled rectangles indicate mIL8-RL1.

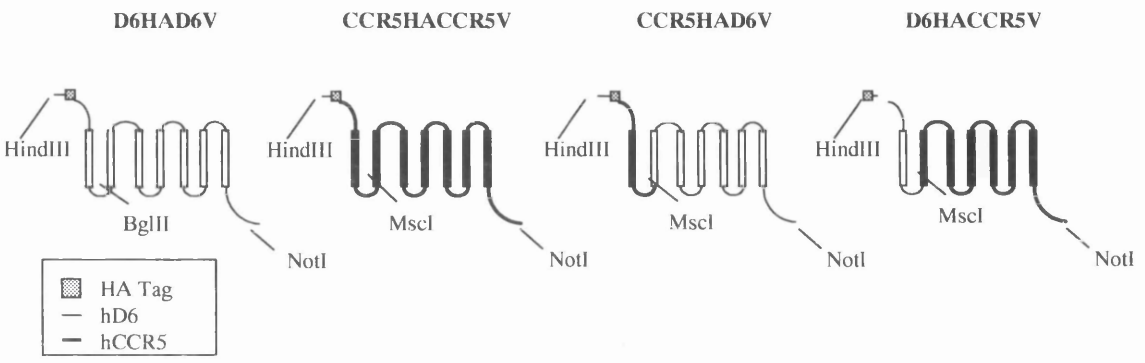


Figure 2.3. hCCR5/hD6 chimaeric constructs. hD6 is represented by thin black lines and white rectangles; thick black lines and black rectangles indicate hCCR5. Grey square represents HA tag.

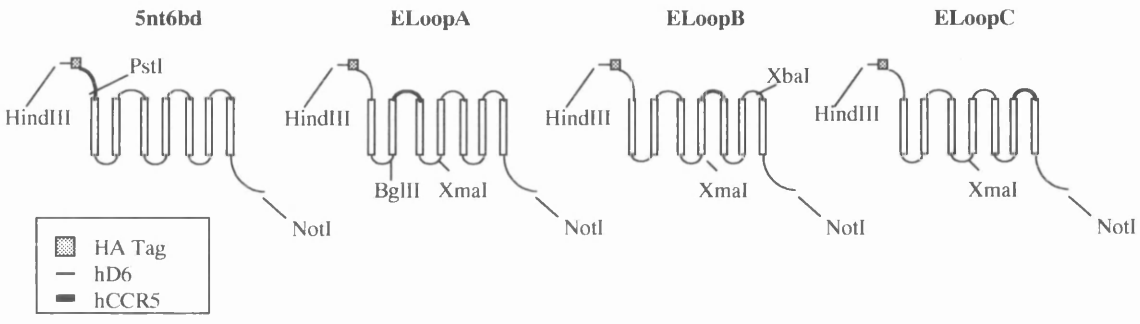


Figure 2.4. Extracellular domain chimaeric constructs. hD6 is represented by thin black lines and white rectangles; thick black lines and black rectangles indicate hCCR5. Grey square represents HA tag.

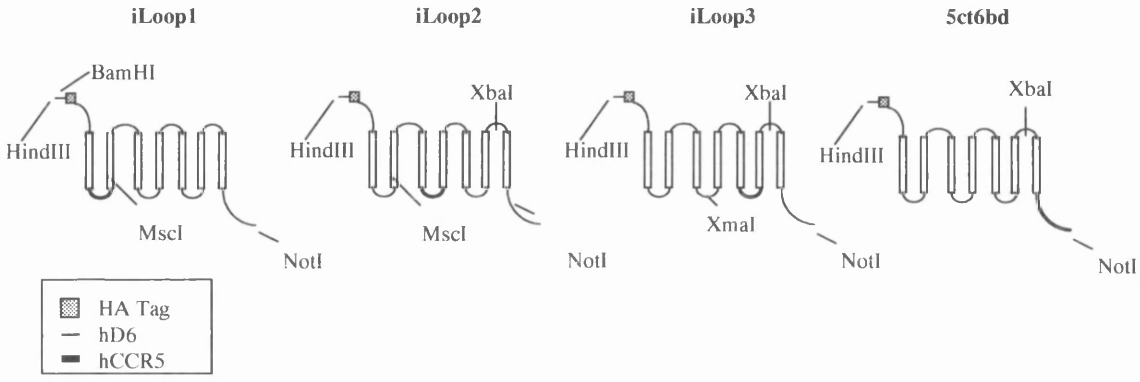


Figure 2.5. Intracellular mutants. hD6 is represented by thin black lines and white rectangles; thick black lines and black rectangles indicate hCCR5. Grey square represents HA tag.

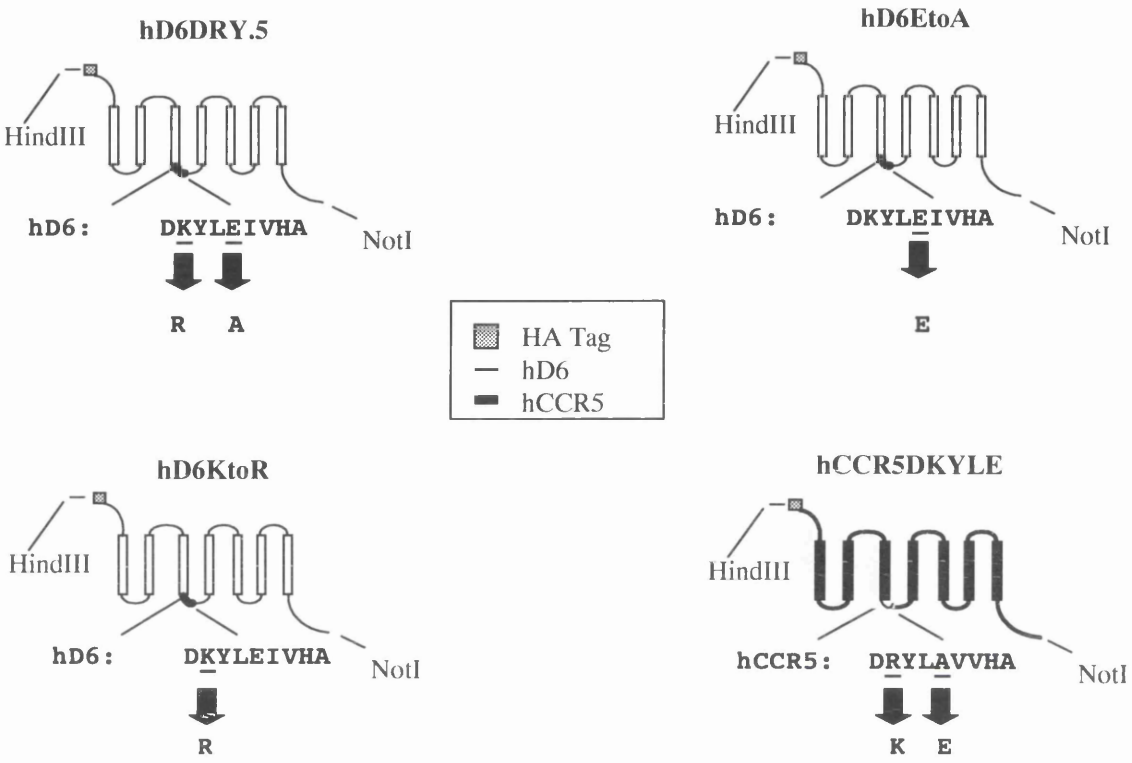


Figure 2.6. 'DRY' mutants. hD6 is represented by thin black lines and white rectangles; thick black lines and black rectangles indicate hCCR5. Grey square represents HA tag.

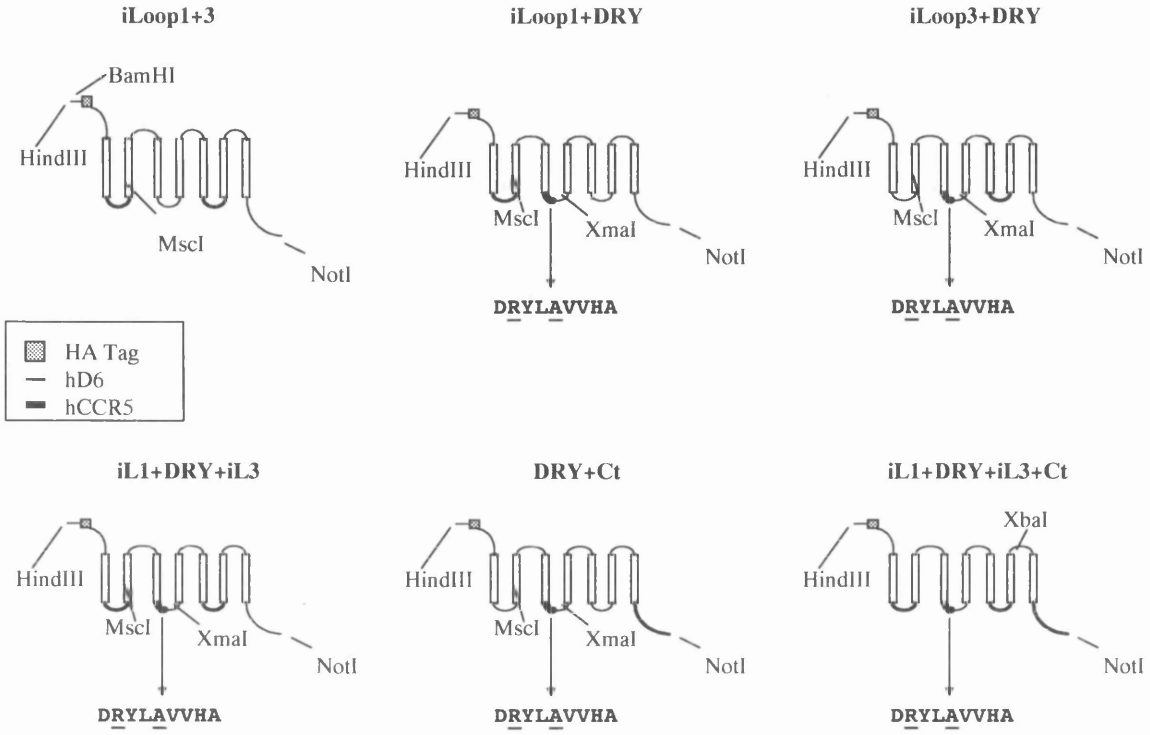


Figure 2.7. Second generation of intracellular mutants. hD6 is represented by thin black lines and white rectangles; thick black lines and black rectangles indicate hCCR5. Underlined residues represent point mutations Grey square represents HA tag.

Chapter 3- Results:

3.1. Identification of hD6's binding domain(s)

Kobilka's pioneering work (Kobilka et al., 1988) in protein engineering set the trend for construction of chimaeric receptors to study the relationship between structure and function in 7TM receptors. Chimaeric work has been facilitated by the degree of identity between members of the superfamily of G protein-coupled 7 TM domain receptors, with the greatest similarity occurring in the transmembrane domains, and the greatest divergence in the N-terminal extension and the three extracellular loops (Schwartz, 1994). Thus, it is generally believed that swapping domains of related receptors permits an analysis of the effect of primary sequence determinants on function whilst maintaining structure.

As discussed in Section 1.5 of the Introduction, in general, binding of a chemokine to a chemokine receptor is a two-step process: in the first step the N-loop region of the chemokine interacts with the N-terminal region of the receptor and in the second step the N-terminus of the chemokine binds to a second site on the receptor, presumably located between or close to the TM helices and extracellular loops, inducing a conformational change of the receptor and consequent signalling.

The aim of this section of the thesis was to identify specific regions and individual residues within hD6 that are responsible for the high surface expression, broad ligand binding promiscuity, and high affinity ligand binding apparent for this receptor. These data will contribute towards an understanding of the biochemistry of hD6 and

will also allow comparisons to be made to other chemokine/receptor pairs, to and may provide useful information in the design of effective blocking reagents.

3.1.1. Generation of mIL-8RL1/hD6 chimaeric constructs

At the outset of this project only CCR1, CCR2, CCR3, CCR4, CCR5, D6, DARC, CXCR1 and CXCR2 had been well characterised and assigned cognate ligands. Phylogenic analysis suggested that this receptor lies between the C-C and C-X-C receptor subfamilies, being most closely related to CCR4 and two murine IL-8/CXCL8 receptor-like genes, mIL-8R and mIL-8RL1 (the likely murine counterparts of CXCR1 and CXCR2, respectively) (Nibbs et al., 1997b).

Although, hD6 and mIL-8RL1 share a fairly high degree of similarity at the amino acid level they are unlikely to show any overlap in ligand specificity as they belong to two separate and distinct chemokine receptor families, the CC and the CXC chemokine receptor families, respectively. hD6 binds to multiple CC chemokines (Nibbs et al., 1997a). mIL-8RL1 was isolated in 1997 (R. Nibbs, *pers. comm.*) and ligands to this receptor are yet to be identified. At the onset of this project it was concerning that the generation of CC/CXC chimaeric receptor constructs could generate constructs with defects in folding and/or expression that would be less likely if the chimaeras were obtained by replacing domains of a CC receptor with the corresponding domains of another CC receptor. Nevertheless, chimaeric constructs of CXCR2 and CCR1 have been successfully generated to identify domains of CXCR2 involved in high affinity binding and receptor activation (Ahuja et al., 1996). Given the promiscuous ligand binding profile of hD6 it was thought that chimaeras obtained by replacing domains of hD6 by the corresponding regions of a CXC receptor would

generate information about the domain(s) of hD6 that are involved both in ligand promiscuity and high affinity ligand binding to hD6. Thus, initially the receptor mIL-8RL1 was chosen for the chimaeric studies with hD6 because it is the CXC receptor shown to be most closely related to hD6.

Since the N-terminus of chemokine receptors has been identified as an important ligand binding domain involved in specificity and activity for receptors (see Section 1.5), it was decided to first investigate the importance of this domain in hD6.

3.1.1.1. FLAG tagged chimaeras

A PCR based cloning strategy (Section 2.2.2.14.1) was used to replace the N-terminal extension along with the first TM domain and part of the first intracellular loop of hD6 by the corresponding region of mIL8RL-1. The reciprocal substitution of hD6 in mIL8RL-1 was also generated (Fig.3.1). A FLAG epitope tag (DYKDDDD) was inserted between the first two amino acids of each construct to allow determination of surface expression of these hybrid receptors. These cDNAs were cloned into the pcDNA.3 vector to allow strong CMV promoter driven expression of the chimaeric constructs in mammalian cells.

Constructs were stably transfected into CHO cells. This cell line has been previously shown to be capable of high surface expression of hD6 protein and many other chemokine receptors (Nibbs et al., 1997a; Nibbs et al., 1997b). Resistant cells were selected using 1.6mg/ml G418 and single cell clones were isolated, expanded and subsequently tested for their ability to show displaceable binding to PM2 (Section 2.2.4.4.1). PM2 is a variant of murine MIP-1α/CCL3 that does not self-aggregate

(Graham et al., 1994), labels well with radioactive iodine, and binds to hD6 with high affinity (Nibbs et al., 1997a; Nibbs et al., 1997b).

Fig 3.2 shows the equilibrium binding assays carried out with CHO cells expressing wild type hD6 (hD6.1), D6FD6V (FLAG tagged wild type hD6) clone C, and the two chimaeras D6FIL8V clone 4 and IL8FD6V clone 1. Many other clones of each construct were tested (data not shown) but they all behaved the same way as shown in Fig. 3.2. D6FD6V was shown to bind five-fold less ^{125}I-PM2 when compared to the wild type protein, hD6.1 and none of the chimaeras was shown to be capable of binding to PM2. The lack of binding to PM2 by the chimaeras D6FIL8V and IL8FD6V could either indicate that these constructs were not being expressed on the surface of the cells or that the N-terminus is essential but not sufficient to promote binding of ligands to hD6. In order to distinguish between these two possibilities detection of surface expression (Section 2.2.4.1) of these receptors was attempted by flow cytometry (Fig.3.3), and by ELISA (not shown). Both approaches proved ineffective in detecting the FLAG epitope in FLAG tagged hD6 (D6FD6V clones A, B, C and D in Fig. 3.3.) and in any of the chimaeric constructs (data not shown) despite using several commercially available antibodies. This was peculiar bearing in mind that D6FD6V expressing cells were capable of binding to ^{125}I-PM2 which therefore indicates that the protein must be expressed on the surface for the CHO cells. Western blot analysis (Fig. 3.4) showed detection of the FLAG tag protein by the same antibodies used in the FACS and in the ELISAs (M2 and M5 anti-FLAG antibodies). In Fig.3.4a the anti-FLAG antibody M2 was used to detect the FLAG tag epitope in extracts from untransfected cells (CHO), wild type untagged hD6 (hD6.1) and two clones of D6FD6V (clones B and C, D6FD6V.B and D6FD6V.C in figure).

Four different sets of bands can be seen. Band 2 has the predicted size of the hD6 tagged protein, band 3 is thought to be a degradation product of this protein and band 4 is a nonspecific band seen in all four lanes. Band 1 is peculiar as it is larger that the expected size of hD6 and the identity of this product remains uncertain although it is possibly weakly present in the untransfected cells. In Fig.3.4b the anti-FLAG M5 antibody was used to detect the FLAG epitope in extracts from the same cell lines as used in panel A. Panel B shows three sets of bands. Band 1 is the predicted size of the tagged protein and band 2 is presumed to be a degradation product of the protein. Band 3 is thought to be a nonspecific band since it is detected in all cell lines. The fact that degradation products are detected by Western blotting might suggest that the FLAG tagged proteins are somehow being degraded or not being expressed appropriately. However, it is also possible that the surface FLAG epitope is somehow masked when the mature protein is expressed on the surface of transfected cells. It should be noted that the M2 antibody binds to FLAG fusion proteins so this antibody can recognise the FLAG epitope anywhere in a given protein whereas the M5 anti-FLAG antibody only recognises Met-FLAG fusion proteins. These antibodies therefore recognise different epitopes and this seems to be the likely explanation for the different patterns of bands seen in the two Western blots shown.

In order to compare binding of tagged and untagged hD6 to PM2, full displacement binding curves were carried out for hD6.1, D6FD6V.A, D6FD6V.B, D6FD6V.C and D6FD6V.D (Fig. 3.5). These data show that cells expressing FLAG tagged hD6 (D6FD6V.A, D6FD6V.B, D6FD6V.C and D6FD6V.D in Fig. 3.5) consistently had a higher binding affinity than untagged wild type hD6 (hD6.1 in figure) The IC_{50} for clones A, B, C and D was estimated to be approximately 4nM, 2nM, 1.2nM and

1nM, respectively, whereas the IC_{50} for hD6.1 was estimated to be approximately 6nM. These results suggest that the FLAG increases the affinity of PM2 for hD6. This was a surprising result as the same tag has been used on other receptors with no affect on binding or signalling (Wong, 1997; Monteclaro, 1996). Perez and colleagues have however shown (Perez et al., 1993) that the very hydrophilic FLAG peptide sequence interferes with ligand binding to the formyl peptide receptor. Consequently, it seems that the hydrophilic eight amino acid stretch in the FLAG tag may either alter the positioning of D6's domains involved in binding, or directly enhance interaction of hD6 with PM2 in such a fashion that a higher affinity to PM2 is achieved. The highly acidic nature of the tag and the N-terminus may suggest that the latter in this case. For this reason along with the lack of detectable surface FLAG epitope, it was concluded that the FLAG tag was not a suitable epitope to tag receptors in the heterologous CHO cell line.

Finally, to eliminate the chance that these observations were the result of a cell-type specific phenomenon, the above constructs were stably transfected into COS-7 and HOS cell lines (Section 2.2.1.3.1 and 2.2.1.4.1). These cell lines were also previously shown to be capable of successfully expressing hD6 on their surface (R. Nibbs, *pers. comm.*). Unfortunately, the FLAG epitope was not detected by flow cytometry or by ELISA in either of the transfected cell lines (data not shown).

3.1.1.2. HA tagged receptors

To determine whether the increasing affinity to PM2 by FLAG tagged wild type hD6 was due to either a particular characteristic of the FLAG epitope or simply due to its hydrophilic nature, HA (haemagglutinin) tagged constructs were generated. This HA

tag (YPYDVPDYAGPG) has been widely used for protein tagging, in particularly with receptors (Rucker et al., 1996).

Initially, all the FLAG epitope of D6FD6V was replaced by an HA tag epitope (Section 2.2.2.13.1.) to investigate not only the ability of this epitope to be detected on the surface of transfected cells, but also to determine whether this new epitope affected binding to PM2. The new construct was stably transfected into CHO cells and pools of clones as well as single cell clones were isolated. A full displacement curve (Fig. 3.6) showed that the HA tag does not affect binding since wild type hD6 and tagged hD6 have the same IC_{50} (approximately 5.5nM). Immunocytochemistry (Fig.3.7a) and flow cytometry (Fig.3.7b) were carried out on a pool of transfectants stably expressing HA tagged wild type hD6 (D6HAD6V). Surface expression of D6HAD6V was now successfully detected by both of these methods. The HA tag was therefore proven to be an appropriate epitope to be used to successfully tag hD6 in CHO cells. So, the FLAG tag in IL8FIL8V, D6FIL8V and IL8FD6V was replaced for a HA tag epitope (Fig.3.8) using a PCR based strategy as before. Pools of transfected CHO cells and single cell clones were derived for each construct and the presence of surface receptor was tested by immunocytochemistry (data not shown) and flow cytometry (Fig. 3.9). The cells were stained with the high affinity anti-HA antibody and their fluorescence compared to the background fluorescence of untransfected CHO cells. As it can be seen in Fig. 3.9, surface expression of all of the HA tagged constructs was now detectable, although surface expression of IL8HAIL8.2, IL8HAD6V.1 and especially of D6HAIL8V.2 was not particularly good. An equilibrium binding assay (Fig. 3.10) was then performed to detect binding of the HA tagged constructs to PM2. Binding to PM2 was not detected for pools of

transfectants expressing IL8HAIL8V, D6HAIL8V or for IL8HAD6V. D6HAD6V.pool bound well to PM2, although it was reduced by 40% when compared to the stable cell line expressing wild type untagged hD6 (hD6.1 in Fig.3.10).

In conclusion, the HA tag is a much better epitope than the FLAG tag for these proteins since it does not affect ligand binding to the wild type tagged receptor and allows detection of this epitope in stable cell lines expressing HA tagged constructs. However, surface expression of IL8HAIL8V, D6HAIL8V and IL8HAD6V was shown to be poor in CHO cells and moreover the continuous passaging of these transfected cell lines was shown to lead to loss of receptor expression. In fact, cells analysed by flow cytometry over an eight-day interval show a totally different fluorescent pattern after staining with the anti-HA antibody, Y11 (Fig. 3.11). These data suggest that surface expression is lost over time. This might be for a number of reasons such as cell survival or promoter silencing, but highlights a problem of the system used.

To determine whether the low detection of chimaeras on the surface was specific to CHO cells, the chimaeric constructs were subsequently stably transfected into HEK-293, COS-7 and HOS cells but detection of the HA epitope on the chimaeras was not observed in any of the cell lines, although surface expression of the wild type tagged receptor (D6HAD6V) was always detected (data not shown).

The poor surface expression of the chimaeric constructs was a disappointment and may be due to a number of reasons, such as incorrect folding. It was concerning that the time in culture from transfection, through the generation of stable lines, was

selecting for cells that lacked good surface expression. The data with CHO cells expressing D6HAD6V indicates that this may be a legitimate concern. Thus, as a final attempt to get good surface expression of the chimaeras, a transient transfection system was employed.

3.1.1.3. Transient transfections:

The HA tagged constructs D6HAD6V, IL8HAIL8V, D6HAIL8V and IL8HAD6V were independently transiently co-transfected into CHO cells with the commercially available Kk plasmid to allow selection of transfected cells. This plasmid encodes for the truncated mouse MHC class I molecule of H-2K^k haplotype. Transfected cells were incubated with the anti-Kk antibody coupled to microbeads. After incubation, cells were applied to a column attached to a magnetic field. Once the magnetic field was removed, cells bound to the column were washed off to generate a sorted population (Section 2.2.4). Cell sorting was carried out 48 hours after transfection along with determination of surface expression (Section 2.2.5.2) and binding (Section 2.2.4.4.1). Fig.3.12 shows a representative flow cytometry profile of the sorted cells where it can be seen that the majority (67%) of purified cells are now Kk positive. This approach proved very effective in generating an enriched cell population, however, the 20-40% recovery of the transfected cells made it technically difficult to produce large number of cells for full displacement binding curves.

Flow cytometry after immunofluorescent staining with anti-HA antibody showed that only the D6HAD6V is expressed well on the surface of these transiently transfected CHO cells (Fig. 3.13a). PM2 was shown to bind to cells transiently expressing D6HAD6V at levels similar to those registered for D6HAD6V stably transfected into

CHO cells (Fig. 3.13b). In accordance with their lack of surface expression, constructs IL8HAIL8V, D6HAIL6V and IL8HAD6V did not bind to PM2 (Fig. 3.13b). Kk plasmid expressing cells did not bind to radiolabelled PM2 at levels higher than untransfected cells (data not shown).

Taken together these results suggest that the HA tagged constructs IL8HAIL8V, D6HAIL8V and IL8HAD6V are poorly assembled on the cell surface. These chimaeras might be unstable and only be expressed at low levels in these cell lines, which could only be determined by Western blotting (not performed), but either way these constructs were not suitable to investigate hD6's properties that form the aim of this project. The poor surface expression of the chimaeric constructs, D6HAIL8V and IL8HAD6V, might be due to the fact that these constructs were obtained by fusing domains from chemokine receptors that belong to different families. However, surface expression of tagged mIL-8RL1 could also not be demonstrated by flow cytometry, immunocytochemistry or by ELISA, showing that it was not expressed on the surface of these heterologous cell lines despite its probable ability of being naturally expressed on the surface of other cells. It has been well documented that there is huge variability in the surface expression of different chemokine receptors in transfected cell lines. hD6 is known to be highly expressed on the surface of transfected cells to an extent that clones isolated in this laboratory were such high expressors that it was difficult to do displacement binding curves with these clones (R. Nibbs, *pers. comm.*). In contrast, for example, the chemokine receptor CCR3 is known to be difficult to express on the surface of heterologous cell lines (Dairaghi et al., 1997); mIL-8RL1 seems to fall into the same category as CCR3.

Whilst the first series of constructs gave little information about hD6 binding domains they did improve many technical aspects of the project. First, they revealed the unsuitability of FLAG tagged constructs and presented the HA sequence as a better epitope. Second, they suggested that chimaeras between receptors for CC and CXC chemokine receptors are unlikely to be of use. Third, a transient transfection protocol was developed for rapid analysis of chimaeras. Thus, chimaeras between hD6 and another chemokine receptor were constructed.

3.1.2. Epitope tagged chimaeras of hCCR5 and hD6

hCCR5 is a CC chemokine receptor that shares 30-37% identity with hD6. This CC chemokine receptor binds to MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 with high affinity [Combadiere et al, 1996; Raport et al, 1996) and weakly to MCP-2/CCL8 (Nibbs et al., 1997b). In addition, hCCR5 has been reported to bind to MCP-3/CCL7, MCP-4/CCL13, MCP-1/CCL2 and Eotaxin/CCL11 (Blanpain et al, 1999), however in this research group binding has only been demonstrated for MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and MCP-2/CCL8 (R. Nibbs, *pers.comm.*). The activity of these other ligands on hCCR5 remains controversial.

hD6 also binds to MIP-1α/CCL3, RANTES/CCL5, MIP-1β/CCL4 and MCP-2/CCL8 although with much higher affinity than hCCR5 (Nibbs et al., 1997a). In addition to these ligands, hD6 also binds to MCP-1/CCL2, MCP-3/CCL7, MCP-4/CCL13, MCP-5 with high affinity, and to Eotaxin/CCL11 and HCC-1/CCL14 with low affinity (Nibbs et al., 1997a). All hD6 ligands have a proline residue at position 2 and this, at least in the context of MIP-1α/CCL3, seems important for binding although not all β-chemokines with a proline residue at this position bind to hCCR5.

Similarly, again in the context of MIP-1α/CCL3 hCCR5 has been shown to be able to bind to MIP-1αP/CCL3 (hMIP-1α isoform that bears a Pro residue at position two) with higher affinity than it binds to MIP-1αS/CCL3 (commercially available hMIP-1α/CCL3, an isoform of MIP-1α/CCL3 that has a Ser residue at position 2) (Nibbs et al., 1999). These data suggest that there may be some similar ligand requirements by hCCR5 and hD6. So, given that hCCR5 is not only well expressed on the surface of heterologous cell lines and shares sequence identity with hD6, plus the fact that some of the hD6 ligands do not bind to hCCR5, and that those that bind to both of these receptors have a higher affinity for hD6, it was decided to construct chimaeras of hD6 and hCCR5. These new constructs were expected to be expressed at higher levels on the surface of transfected heterologous cell lines and to generate information on the binding promiscuity and high affinity ligand binding of hD6.

hCCR5/hD6 chimaeric constructs (Fig. 3.14) were generated by overlap extension PCR (Section 2.2.2.15.) and they bear an HA tag at the N-terminus. Construct CCR5HACCR5V encodes for HA tagged wild type hCCR5. CCR5HAD6V represents the N-terminal portion (including the first TM domain and part of the first intracellular loop) of HA tagged hCCR5 cloned into the corresponding region of hD6. Conversely, D6HACCR5V is the reciprocal chimaera of CCR5HAD6V. These constructs were transiently co-transfected with the Kk plasmid into CHO cells. Once sorted, the cells were tested for surface receptor expression and for their capacity to bind PM2 (Fig. 3.15).

D6HAD6V and CCR5HACCR5V were expressed at similar levels on the surface of sorted CHO cells, whereas sorted cells expressing CCR5HAD6V or D6HACCR5V

showed much lower levels of detectable surface protein (Fig. 3.15a). Displacement of ^{125}I-PM2 by PM2 (Fig. 3.15b) indicates that wild type HA tagged hD6 (D6HAD6V) is capable of PM2 binding. No displaceable binding was registered for CCR5HACCR5V, CCR5HAD6V or D6HACCR5V. This data was not only disappointing but also surprising since binding of PM2 to HA tagged wild type hCCR5 was not detected. hCCR5 has lower affinity for PM2 when expressed in CHO cells (Nibbs et al., 1999) and these results suggest that despite abundant surface expression of hCCR5, the assay is not sufficiently sensitive to detect robust binding by this receptor. Since the surface expression of the chimaeric constructs is even lower than the surface expression of hCCR5, to which no displaceable binding is observed, the absence of binding to PM2 to these constructs cannot be confidently attributed to the changes made.

In an attempt to increase the sensitivity of this assay the same experiment was carried out but this time the binding assay was performed with MIP-1αP/CCL3 which has higher affinity than murine MIP-1α/CCL3 (and to PM2) for hCCR5. Constructs were again transiently transfected into CHO cells and subsequently a binding assay in equilibrium and flow cytometry were performed this time using unsorted populations. In Fig. 3.16a it can be seen that surface expression is detected even without sorting and that in this case all constructs were expressed roughly at the same levels. After staining with the anti-HA antibody 33% of D6HAD6V, 24% of CCR5HACCR5V, 21.5% of D6HACCR5V and 25% of CCR5HAD6V expressing cells were positively stained for the HA epitope. The binding assay shown in Fig. 3.16b shows that cells transfected with D6HAD6V show good displaceable binding of MIP-1αP/CCL3. CCR5HACCR5V shows a much lower amount of binding to ^{125}I-

MIP-1αP but this is above the background seen with untransfected CHO cells and can be competed off with excess unlabeled MIP-1αP. The two chimaeric constructs showed little if any detectable binding. Although the surface expression levels of the two chimaeras is lower than D6HAD6V, this set of data allows to more confidently conclude that the region replaced by hCCR5 sequences in CCR5HAD6V is important for high affinity ligand binding of hD6 to MIP-1αP/CCL3. This domain does not appear sufficient to impart hD6-like binding properties to hCCR5 in the D6HACCR5V chimaera. However, it proved extremely difficult to repeat these experiments to confirm these conclusions as consistently lower levels of chimaeric receptor expression compared to D6HAD6V were achieved, both in the transient CHO cell expression system and in stably transfected CHO and HOS cells (not shown).

The inability to demonstrate significant binding to the ligands could be explained by the fact that the chimaeric constructs tested comprise not only the extracellular N-terminus portion but also the whole of the first TM domain and half of the first intracellular loop of each receptor (refer to Fig.3.14 for cartoon diagram of constructs) which will potentially interfere with the overall structure of the receptor and may alter positioning of the highly conserved Cys residues that are known to be involved in stabilizing the conformation of these G protein-coupled receptors. This may explain why the chimaeras are consistently expressed at such low levels in these experiments, and also why they do not bind ligand. In short, one cannot be certain that the binding differences observed are a direct consequence of a primary determinant of hD6. Overall, the experiments to this point led to conclusion that large changes incorporated into the chimaera not only jeopardise adequate surface

expression but also make it difficult to distinguish the effects of primary sequence on ligand binding, from gross conformation change brought about by the domains changed. It was therefore decided that more subtle changes should be introduced.

3.1.3. Small domain swaps

The first new construct to be generated was 5nt6bd. This construct was obtained by replacing the N-terminal portion (up to the first cysteine residue) of D6HAD6V by the corresponding portion of hCCR5 (Fig. 3.17a). This construct was transiently transfected into CHO cells (Section 2.2.1.1.2); its surface expression and its ability to bind to PM2 was determined 48 hours after transfection. Flow cytometry after immunofluorescence staining with the anti-HA HA.11 antibody showed that D6HAD6V and CCR5HACCR5V have roughly the same levels of surface expression (37% and 30% positively stained cells, respectively) and 5nt6bd is expressed at slightly lower levels with 27.3% of the transfected cells staining positive for the HA tag. Fig. 3.17b shows that, as usual, cells transiently transfected with D6HAD6V bind to ^{125}I-PM2 virtually as well as hD6.1 cells. CCR5HACCR5V binding to ^{125}I-PM2 in the absence of unlabeled ligand is above background, however in the presence of unlabelled competitor not all the labelled ligand was competed off on this occasion (Fig. 3.17b). Nevertheless, Fig. 3.17b clearly shows that 5nt6bd binds well to ^{125}I-PM2, and at significantly higher levels than CCR5HACCR5V implying that 5nt6bd has a higher affinity for this ligand than CCR5HACCR5V. Also, binding of 5nt6bd to PM2 is significantly less than that observed with D6HAD6V, although the chimaera was expressed at lower levels than both HA tagged wild type receptors. It can therefore be concluded that the N-terminus of hD6 is not essential for binding to

PM2, and suggests that replacing it with the hCCR5 N-terminus may reduce the affinity of the receptor for this ligand. Moreover, the fact that the levels of surface expression of this new chimaera were consistently high supports the hypothesis that the large domain swaps previously used might affect folding of the mature protein and prevent PM2 binding to these chimaeras by affecting the overall structure of the receptor.

These were the first results that consistently showed good surface expression and binding by a chimaeric receptor of hCCR5 and hD6. It was therefore decided to embark on more detailed analysis of the role of hD6 extracellular domains by generating smaller domain swaps of hD6 by the corresponding domains of hCCR5. To this end, three other hD6/hCCR5 chimaeric constructs: ExtLoopA, ExtLoopB and ExtLoopC were generated. In these constructs the first, second and third extracellular loops, respectively, of hD6 were independently replaced by the corresponding regions of hCCR5 (Fig. 3.18). As before, these constructs were generated by overlap extension PCR (Section 2.2.2.10 and 2.2.2.15).

However, at this stage another significant technical problem was encountered with the transient transfection of CHO cells. Specifically, it was found that there was highly variable transfection efficiency of these cells that made experimentation difficult. Thus, attempts were made to improve this situation by selecting an alternative cell line. Transient transfection of constructs into COS-7 cells proved to have higher transfection efficiency and to be much more reproducible than the CHO system. For this reason, constructs were transiently transfected into COS-7 cells as described in Section 2.2.1.3.7 and cell surface expression for each tagged construct

was assessed after 48 hours without sorting. Surface expression of wild type and mutant receptors tagged with HA at their N-termini was detected by flow cytometry after immunofluorescence staining with HA.11 (Fig. 3.19). The graph in Fig. 3.19 shows that mutants ExtLoopC and 5nt6bd have consistently higher surface expression than D6HAD6V. Expression of CCR5HACCR5V, ExtLoopA and ExtLoopB is not significantly different from expression of D6HAD6V on the surface of transiently transfected COS-7 cells. The fact that these constructs were expressed at roughly the same levels on the surface of COS-7 cells would facilitate the comparison of ligand binding profiles for each of these receptors. Without full displacement curves being carried out one cannot calculate binding affinities, however, if the receptors are expressed at similar levels one can get an indication of affinity relative to the wild type receptors.

Using the reproducible COS-7 cells expression system, the HA tag, and the small domain changes it was now possible to perform a detailed analysis of binding of the chimaeras to a selected spectrum of hD6 ligands.

3.1.4. N-terminal domain of hD6

CCR5HACCR5V, D6HAD6V and 5nt6bd were independently transiently transfected into COS-7 cells (Section 2.2.1.1.2). Surface expression of each receptor was assessed after 48 hours by flow cytometry after immunofluorescence staining with the anti-HA antibody HA.11. From the flow cytometry profiles shown in Fig. 3.20a it can be seen that all constructs were expressed at similar levels on the surface of the transiently transfected COS-7 cells. These HA tagged receptor expressing cells were subsequently tested for their ability to show displaceable binding to ^{125}I-PM2 using

an array of hD6 ligands (Fig. 3.20b). D6HAD6V, as expected, binds to ^{125}I-PM2 and this binding can be effectively competed away in the presence of an excess of unlabelled PM2, MCP-1/CCL2, Eotaxin/CCL11, MCP-2/CCL8 or RANTES/CCL5. Again, no binding to ^{125}I-PM2 was observed for CCR5HACCR5V although this construct was expressed at the same levels of D6HAD6V suggesting that the reduced affinity this receptor has for PM2 puts it below the level of detection of this assay. As before, construct 5nt6bd was shown to be capable of binding to PM2 and this could be displaced by the presence of an excess unlabeled PM2, MCP-1/CCL2, Eotaxin/CCL11 and MCP-2/CCL8. It is likely that 5nt6bd has a higher affinity to PM2 than CCR5HACCR5V since they are both expressed at the same levels in these cells but only binding of 5nt6bd to PM2 is detected. This displacement data shows that 5nt6bd still retains the promiscuous binding profile characteristic of hD6 but intriguingly RANTES/CCL5 seems no longer able to displace ^{125}I-PM2 from this receptor. To further investigate this chimera, alternative labelled chemokines were employed. A binding assay in equilibrium was performed where 2nM of ^{125}I-MCP-4/CCL13 were displaced by 0.5μM of unlabeled MCP-4/CCL13. MCP-4/CCL13 chemokine was chosen because it does not bind to hCCR5. Fig. 3.21 shows that 5nt6bd binds to MCP-4/CCL13 at levels similar to that of D6HAD6V. More importantly, in an attempt to determine whether RANTES/CCL5 bound to 5nt6bd, another binding assay was carried out using ^{125}I-RANTES/CCL5 (2nM) (Fig. 3.22). As it can be observed in the graph, replacement of the N-terminus of hD6 by the corresponding region of hCCR5 leads to a 40% reduction in binding to RANTES/CCL5 when compared to 100% of D6HAD6V but it is notable that even at low nanomolar concentrations of labelled RANTES/CCL5, binding to 5nt6bd is

easily detected. CCR5HACCR5V binding to RANTES/CCL5 was not detected in this assay.

Taken together these data suggest that substitution of the N-terminus of hD6 with the corresponding domain of hCCR5 creates a receptor that has an affinity for PM2 like wild type hD6, and that can still bind to all ligands recognised by hD6. Thus, other domains of hD6 must be responsible for binding promiscuity. However, the inability of RANTES/CCL5 to displace radiolabelled PM2 suggests that RANTES/CCL5 and PM2 bind different domains of the 5nt6bd receptor, in contrast to the wild type receptor where these sites overlap. Binding of two different chemokines to different sites in the same receptor is not novel and has been reported before for IL-8/CXCL8 and GROα/CXCL1 on CXCR1/CXCR2 chimaeric receptors (Ahuja et al., 1996) and for MCP-1/CCL2 and MIP-1α/CCL3 on chimaeric receptors (Monteclaro et al, 1996). It is possible that inclusion of this N-terminus of hCCR5 (a receptor able to bind RANTES/CCL5) in this chimaera preferentially attracts RANTES/CCL5 to bind to this domain away from the site it usually binds to in wild type hD6 that overlaps with the PM2 binding site. In this way, 5nt6bd could accommodate a molecule of RANTES/CCL5 and another of PM2 on its surface without competition.

3.1.5. The first extracellular loop of hD6 plays a critical role in PM2 binding

The chimaeric construct ExtLoopA comprises wild type hD6 where the first extracellular loop (the extracellular domain of hD6 most strongly conserved between species), has been replaced by the corresponding region of hCCR5 (Fig.3.23a). This

construct was obtained by overlap extension PCR (Sections 2.2.2.10 and 2.2.2.15), and includes an HA tag at the N-terminus.

The ExtLoopA HA tagged construct along with D6HAD6V and CCR5HACCR5V were transiently transfected into COS-7 cells and after 48 hours flow cytometry was performed in these cells. The flow cytometry profile (Fig. 3.23a) indicates that ExtLoopA has the same surface expression as D6HAD6V. Binding assays were performed on the transfected cells and ExtLoopA showed no detectable PM2 binding above background (Fig. 3.23b). Not surprisingly, no displacement of ^{125}I-PM2 was registered in the presence of excess unlabeled MCP-1/CCL2. These data show that when the first extracellular loop of hD6 is replaced by the corresponding domain of hCCR5, the mutant created can longer behave like hD6. However, the fact that binding of PM2 to CCR5HACCR5V was not detected by this assay could imply that binding of PM2 to ExtLoopA is not completely lost but is below the detection levels of this assay.

To examine if other ligands were similarly affected by alterations in the first extracellular loop, ^{125}I -MCP-4/CCL13 and ^{125}I-RANTES/CCL5 were used. As for PM2, binding of ^{125}I-MCP-4 to ExtLoopA was below the detection levels of the assay (Fig.3.24). Similarly, binding to ^{125}I-RANTES/CCL5 by ExtLoopA was not observed (Fig. 3.25). It seems, therefore that the first extracellular loop of hD6 is important for high affinity binding to RANTES/CCL5 and MCP-4/CCL13. One cannot rule out the possibility that construct ExtLoopA, like construct CCR5HACCR5V, has a low affinity to RANTES/CCL5 and MCP-4/CCL13 that is

below the detection levels of this assay. Nonetheless, these data show that the first extracellular loop of hD6 is important for ligand binding.

3.1.6. The second extracellular loop

ExtLoopB is an HA tagged chimaeric receptor that comprises the second extracellular loop of hCCR5 cloned into the corresponding region of hD6 (Fig.3.26a). This construct was generated by overlap extension PCR and subsequently transiently transfected into COS-7 cells. Surface expression of this construct was assessed by flow cytometry after immunofluorescence staining with the HA.11 antibody. Fig. 3.26a shows that ExtLoopB is expressed on the surface of COS-7 cells at the same level as D6HAD6V. Despite the same levels of surface expression ExtLoopB only exhibits 50% of the binding to ^{125}I-PM2 seen with D6HAD6V. Binding of ^{125}I-PM2 to ExtLoopB could be displaced (Fig. 3.26b) by addition of an excess (0.5μM) of unlabelled PM2, MCP-1/CCL2, MCP-2/CCL8 or RANTES/CCL5, but not by Eotaxin/CCL11.

Binding of ExtLoopB to radiolabelled MCP-4/CCL13 (Fig. 3.27) and to radiolabelled RANTES/CCL5 (Fig. 3.28) was also shown to be reduced by over 60% when compared to D6HAD6V binding.

Taken together these data suggest that the second extracellular loop of hD6 is required for high affinity binding to PM2, MCP-4/CCL13 and RANTES/CCL5 as binding to these labelled ligands is reduced in the chimaera. Eotaxin/CCL11 did not significantly displace PM2 binding. This may be because PM2 and Eotaxin/CCL11 do not compete for the same binding site in this mutant. An alternative and probably

more likely interpretation is that it could simply indicate that PM2 displacement by Eotaxin/CCL11 is not detected because binding to PM2 is reduced in the first place. More detailed analysis would be required to distinguish between these possibilities.

3.1.7. The third extracellular loop of hD6

ExtLoopC is an HA tagged chimaeric construct that bears the third extracellular loop of hCCR5 in place of the corresponding loop of hD6 (Fig. 3.29a). This construct was again generated by overlap extension PCR and its ability to be expressed on the surface of transiently transfected COS-7 cells was assayed by flow cytometry using the HA.11 antibody as before. As it can be seen in Fig. 3.29a, the construct ExtLoopC is expressed on the surface of COS-7 cells at roughly the same level of D6HAD6V. Binding data (Fig. 3.29b) shows that this new chimaeric mutant still retains its ability to bind to ^{125}I-PM2 although at a lesser extent (approximately 70% less) than D6HAD6V. Data from equilibrium binding assay presented in Fig.3.29b shows that ^{125}I-PM2 binding can only be displaced by an excess of unlabeled PM2 or MCP-2/CCL8. None of the other chemokines tested displaced binding of ^{125}I-PM2 to ExtLoopC. The lack of detectable displacement could be due to the fact that the ability of PM2 to bind to this chimaeric receptor is reduced to an extent that small changes in displacement might not be detected as by this assay.

To test the ability of RANTES/CCL5 and MCP-4/CCL13 to bind to ExtLoopC two separate binding assays in equilibrium were performed. (Fig.3.30 and Fig.3.31). This chimaeric construct was shown to still be capable of binding to MCP-4/CCL13 and RANTES/CCL5 albeit at lower levels than D6HAD6V.

As with ExtLoopB, it appears as though alteration of the third extracellular loop reduced the affinity of hD6 for ligands, but the promiscuity appears likely to be retained. Thus, in summary, the data suggests that the first extracellular loop is the main determinant of ligand binding in the hD6 molecule, with the second and third extracellular loops also contributing to ligand recognition. This will be further discussed in Chapter 4.

FLAG tagged constructs

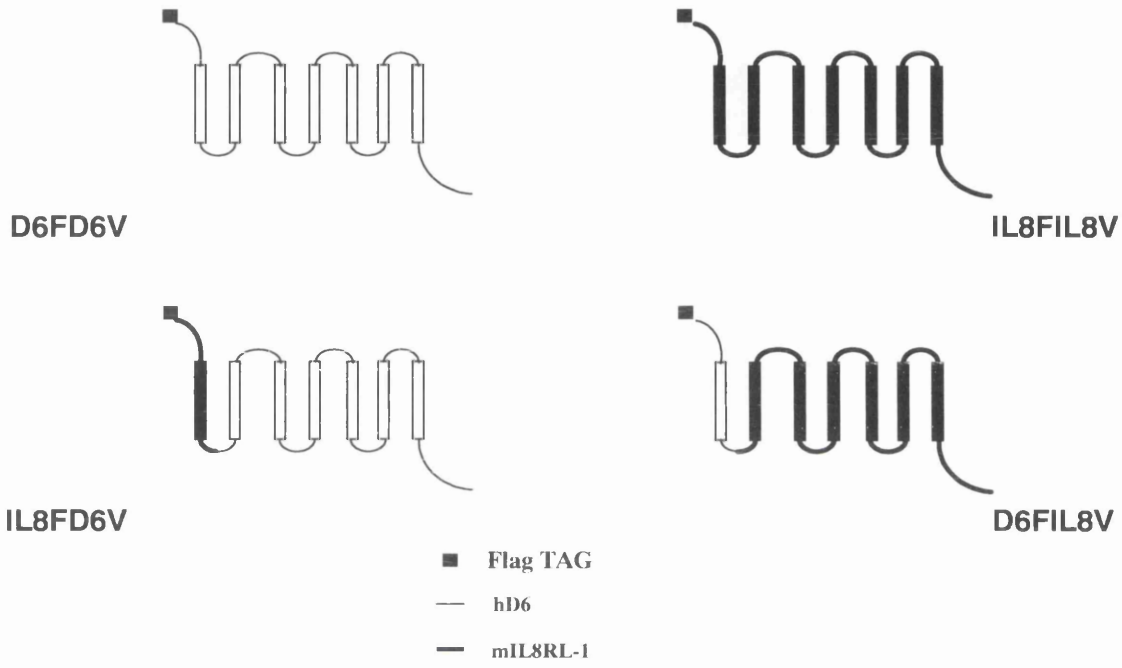


Figure 3.1. Schematic diagram of FLAG tagged wild-type and chimaeric receptors. White rectangles and thin black lines indicate hD6 sequence. Black rectangles and thick black lines represent mIL-8RL1. The filled black square at the N-terminus of each construct represents the FLAG tag epitope. The transmembrane domains are represented by rectangles, and the N-terminus, the intracellular and extracellular loops and cytoplasmic tail are shown as solid lines. The chimaeric receptors were generated by PCR as described in Section 2.2.2.14.1.

Binding of ^{125}I-PM2 to FLAG tagged chimaeras

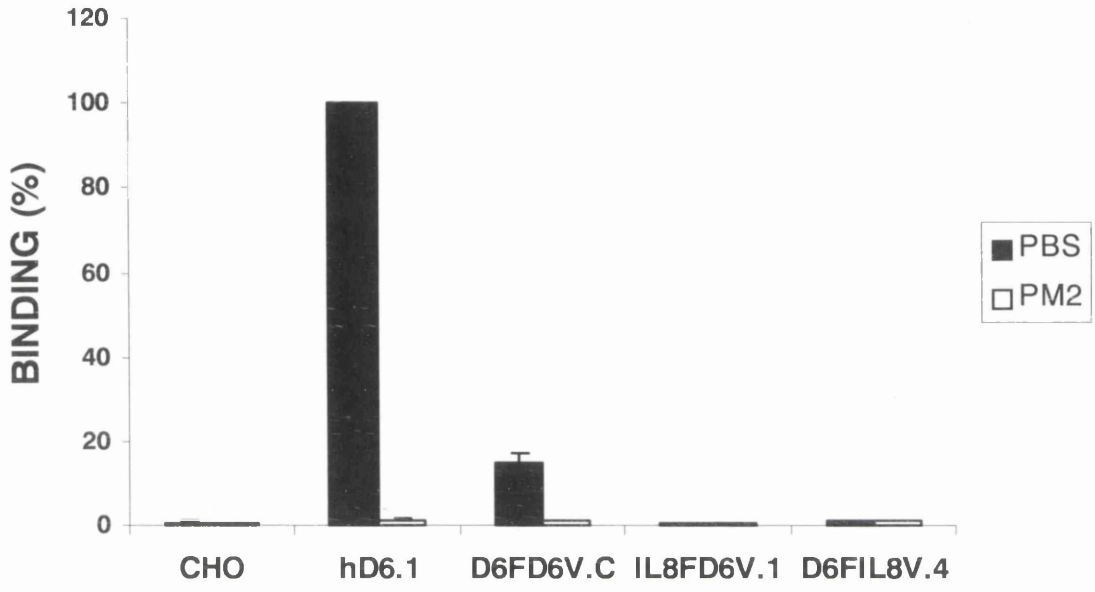
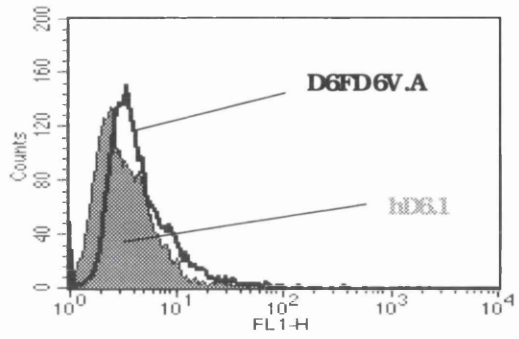
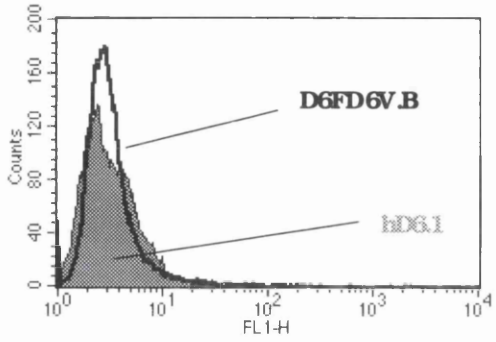


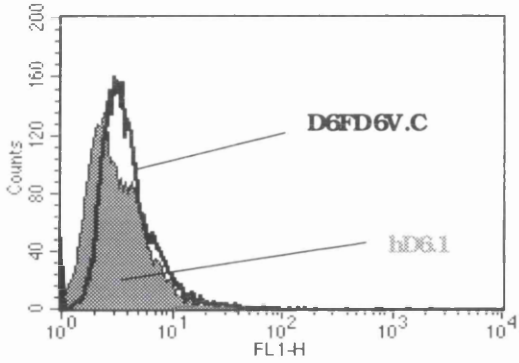
Figure 3.2. Binding of ^{125}I-PM2 to wild type and chimaeric receptors. Displacement of ^{125}I-PM2 binding in the presence of unlabeled PM2 by untransfected CHO cells (CHO) and by CHO cells expressing the wild type hD6 receptor (hD6.1), the FLAG tagged wild type hD6 (D6HAD6V.C) as well as the chimaeras (IL8FD6V.1 and D6FIL8V.4) is shown. 1E5 cells were incubated at room temperature in 0.6nM ^{125}I-PM2 and 120nM unlabeled PM2 or equivalent volumes of PBS for 90 minutes. Cells were then washed three times with ice cold PBS and subsequently lysed in 0.1% SDS. The remaining ^{125}I-PM2 in the lysate was counted for one minute in a gamma counter as before. Each point was carried out in triplicate, the average taken and then converted into a percentage of binding relative to hD6.1 binding to ^{125}I-PM2 in the absence of competitor. Graph shows the average of three experiments carried out in triplicate. Error bars represent standard deviation.

Figure 3.3. Expression of wild type FLAG tagged hD6 in CHO cells. Stably transfected CHO cells were examined for expression of the FLAG tag epitope by flow cytometry following incubation with the FLAG monoclonal antibody M2 and a FITC-conjugated secondary antibody. Untagged wild type hD6 is denoted in grey; A, B, C and D are clones of D6FD6V and are represented by a thick black line. Approximately 5E5 cells were harvested, incubated on ice for 30 min. with 1/100 dilution of the M2-anti FLAG primary antibody. Cells were subsequently washed with FACS buffer and further incubated with a 1/32 dilution of anti-mouse FITC-coupled antibody for 30 min. on ice. Cells were then washed with FACS buffer and resuspended in a final volume of 400μl of the same buffer. Fluorescence of each sample was determined by a FACS and compared to the background fluorescence of untagged cells (hD6.1). Profiles shown are representative of seven different experiments carried out in triplicate. The appropriate isotype controls were carried out were appropriate.

Flow cytometry profiles of FLAG tagged constructs







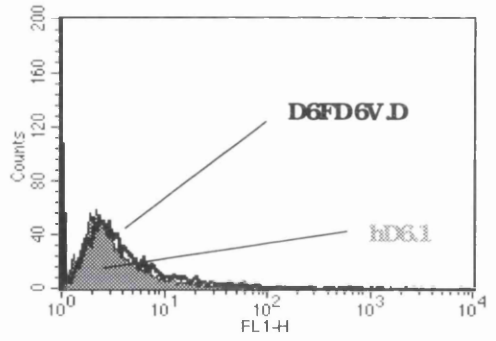
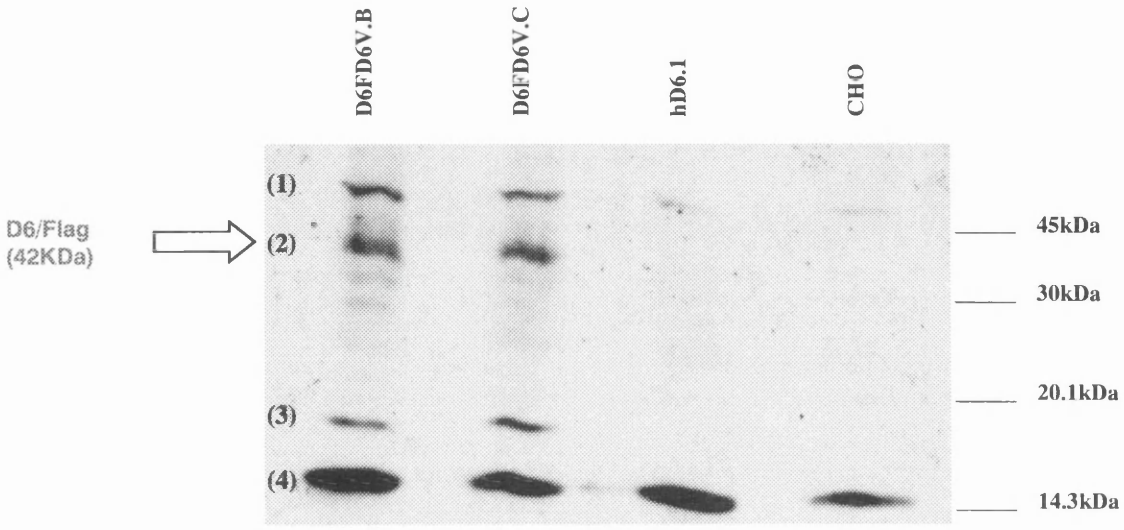


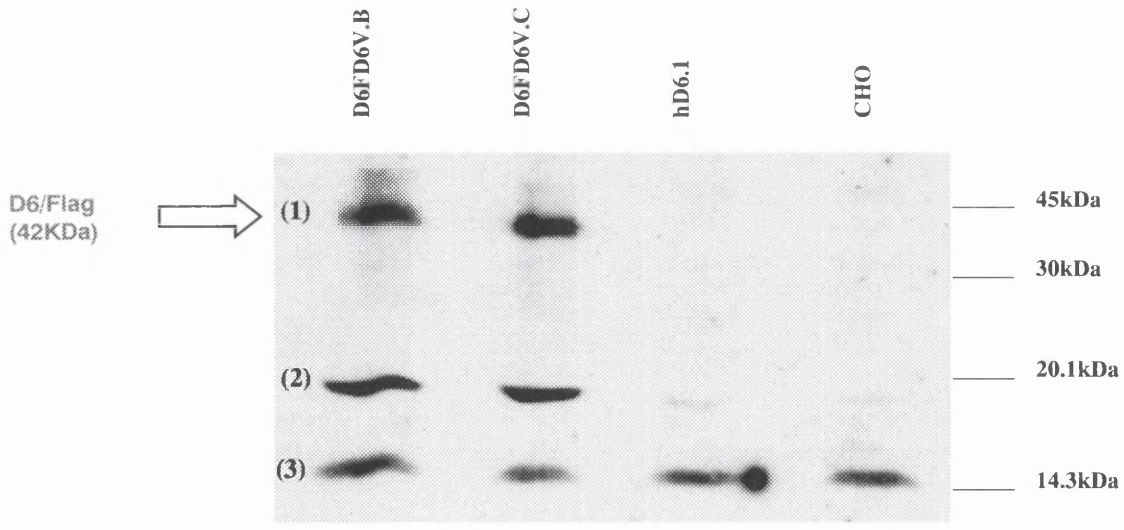
Figure 3.4. Detection of the FLAG tag epitope by Western blotting analysis. Whole cell extracts of untransfected CHO cells (CHO) as well as stably transfected CHO cells expressing FLAG tagged (D6FD6V.B and D6FD6V.C) and untagged wild type hD6 (hD6.1) were isolated from 1E6 cells, separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with the M2 anti-FLAG antibody (panel A) or the M5 anti-FLAG antibody (panel B). Arrow indicates predicted size of hD6 tagged protein. See text for discussion of detected bands.

Western Blot analysis of CHO cells stably expressing FLAG tagged hD6

A.



B.



FLAG tagged hD6 has higher affinity to PM2 than wild type hD6

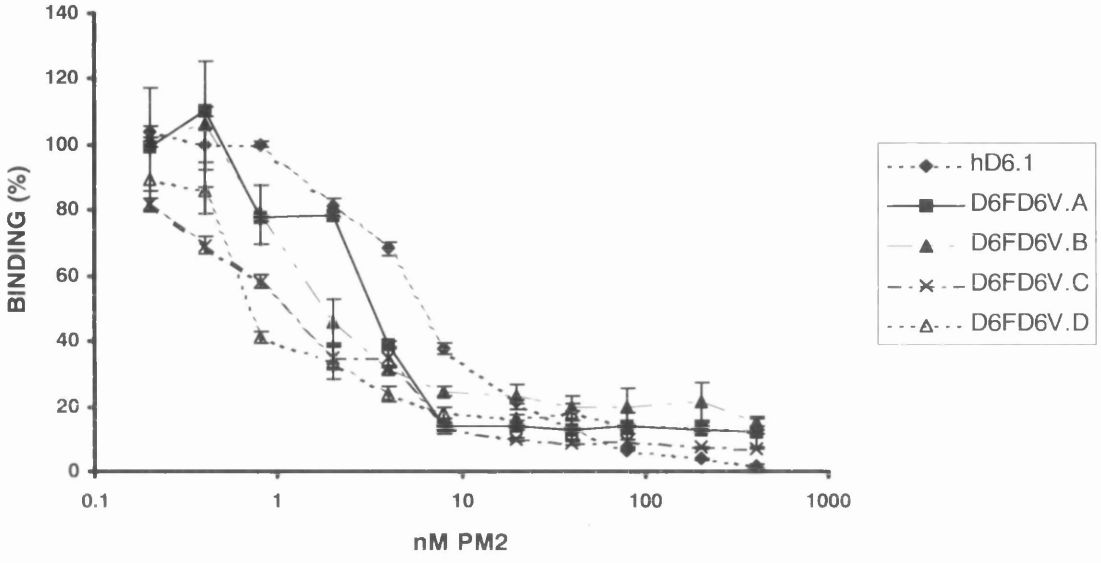


Figure 3.5. FLAG tag constructs have higher affinity to ^{125}I-PM2 than wild-type untagged hD6. Displacement of ^{125}I-PM2 by increasing amounts of unlabeled PM2 from CHO cells expressing wild type hD6 (hD6.1) as well as different clones of FLAG tagged hD6: D6FD6V. A, D6FD6V.B, D6FD6V.C, D6FD6V.D. 1E5 cells were seeded overnight and then tested for their ability to displace ^{125}I-PM2 (0.6nM) with increasing amounts of unlabeled PM2. After a 90 min. incubation, cells were lysed with 0.1% SDS and the remaining ^{125}I-PM2 in the lysate counted for 1 min. Each point was done in triplicate, the average taken and converted into a percentage of the binding observed in the absence of unlabelled competitor chemokine. The estimated IC_{50} for the FLAG tagged clones is 4nM, 2nM, 1.2nM and 1nM, for clones A, B, C and D, respectively. hD6.1 IC_{50} is estimated to be approximately 6nM.

The HA tag epitope does not affect PM2 binding

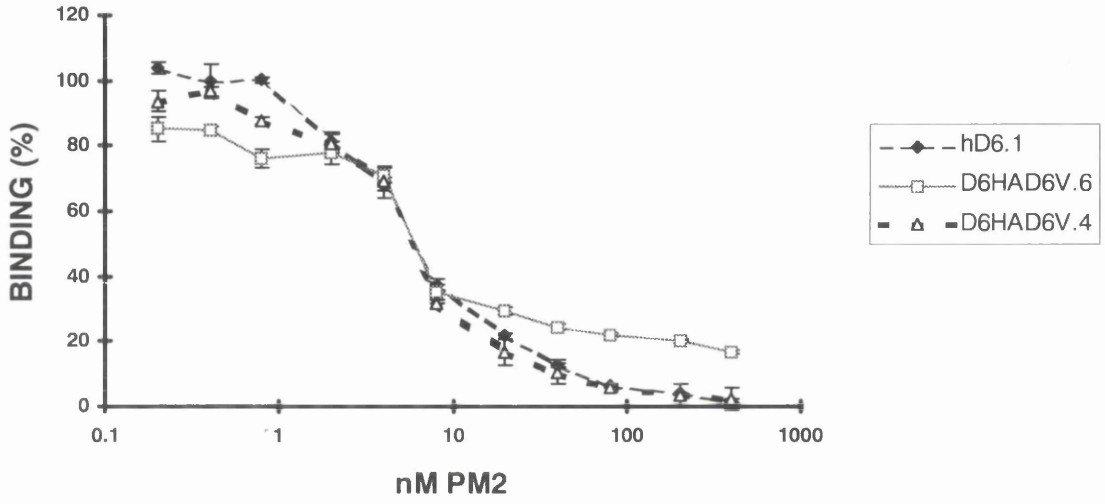
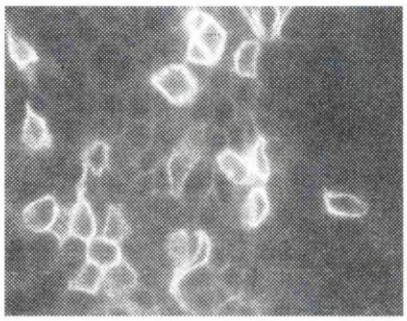


Figure 3.6. HA tagged constructs have the same binding affinity to ^{125}I-PM2 as wild type hD6. Stably transfected CHO cells expressing HA tagged wild type hD6 (D6HAD6V.6 and D6HAD6V.4) have the same binding affinity to ^{125}I-PM2 as CHO cells stably expressing wild type hD6 (hD6.1). 1E5 cells were seeded overnight and then tested for their ability to displace ^{125}I-PM2 (0.6nM) in the presence of increasing amounts of unlabeled PM2. After a 90 min. incubation at room temperature, cells were lysed with 0.1% SDS and the remaining ^{125}I-PM2 in the lysate counted for 1 min. Each point was done in triplicate, the average taken and converted into a percentage of binding observed in the absence of unlabelled competitor chemokine. The estimated IC_{50} for all cell lines is approximately 7nM.

Figure 3.7. Surface expression of HA tagged wild type hD6. A. Pools of CHO cells stably expressing HA tagged wild type hD6 (D6HAD6V.pool) were stained with high affinity anti-HA antibody and subsequently with anti-mouse FITC secondary antibody. Untransfected CHO cells (CHO) showed little cross-reaction with this antibody. Cells were stained while adherent to permanox chamber slides and after immunostaining were visualized in a Biorad MRC 600 confocal microscope. Data shown is representative of three experiments carried out independently from each other. **B.** Approximately 5E5 cells were harvested and subsequently immunostained with the high affinity anti-HA and the FITC-coupled anti-mouse secondary antibody. Fluorescence of each sample was analysed by a FACS and compared to the background fluorescence of the untransfected cells (CHO, in grey). Incubation with the secondary antibody alone or with the appropriate isotype control showed no increase in fluorescence of the samples in comparison to untransfected cells. FACS profile shown is representative of four experiments independently performed.

HA tag detection

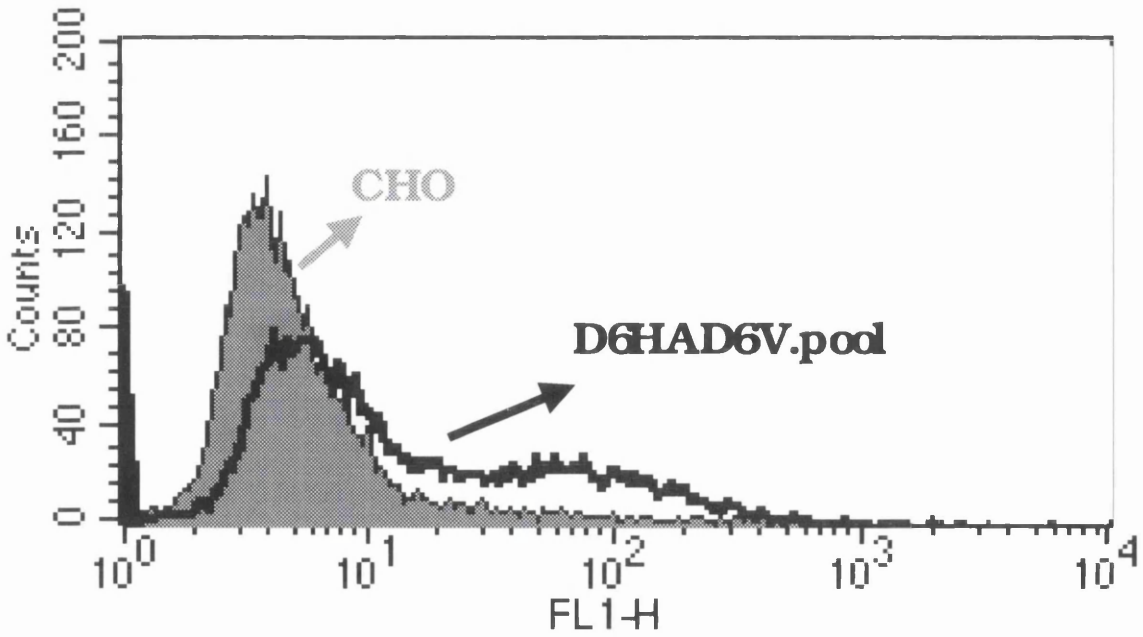
A.



D6HAD6V.pool

CHO

B.



HA tagged IL-8RL1/D6 chimaeric constructs

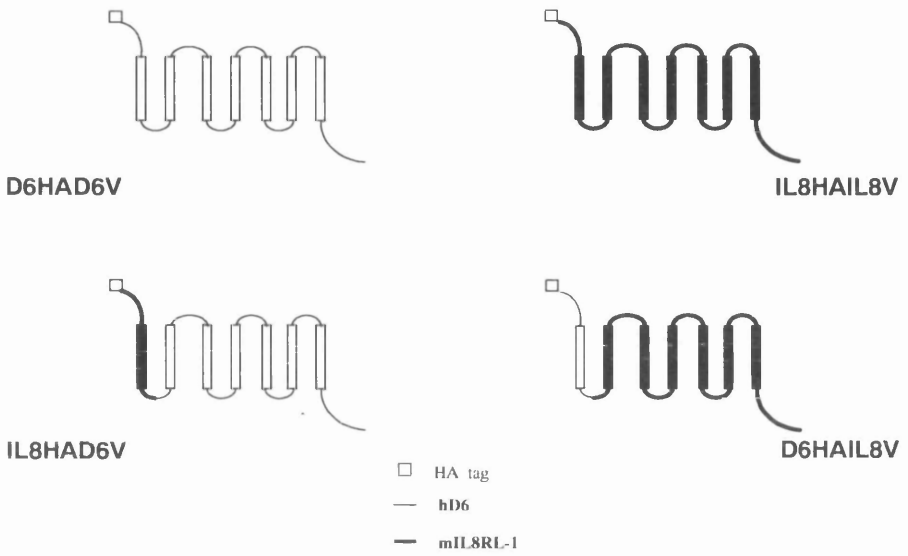


Figure 3.8. Schematic diagram of HA tagged wild-type and chimaeric receptors. White rectangles and thin black lines indicate hD6 receptor. The mIL8Rl-1 is represented by black rectangles and thick black lines. The white square indicate the HA tag epitope cloned at the N-terminus of each construct. The transmembrane domains are represented by rectangles, the N-terminus, the intracellular and extracellular loops as well as the cytoplasmic tail are shown as solid lines. Chimaeric receptors were generated by overlap extension PCR as described in Section 2.2.2.9.

Flow cytometry profiles of D6/IL-8RL1 HA tagged constructs

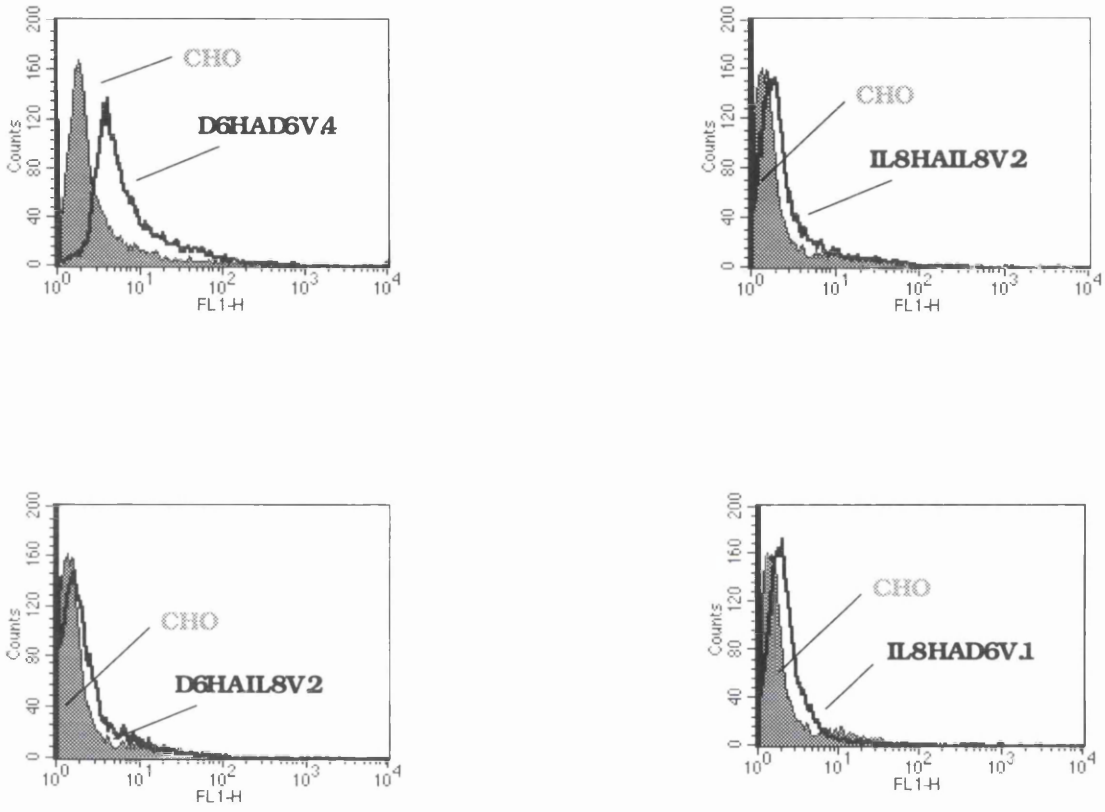


Figure 3.9. HA tag detection by flow cytometry. Surface expression of HA tagged wild type hD6 (D6HAD6V.4), HA tagged wild type mIL-8RL1 (IL8HAIL8V) as well as of the HA tagged constructs D6HAIL8V and IL8HAD6V was determined by flow cytometry after immunofluorescence staining with the anti-HA high affinity primary antibody and the anti-mouse FITC coupled secondary antibody. Fluorescence of each transfected cell line (black thick lines) was compared to the background fluorescence of untransfected cells, CHO cells (in grey). Isotype control carried out where appropriate. FACS profiles are representative of three experiments carried out separately.

IL-8RL1/D6 HA tagged chimaeras do not bind ^{125}I-PM2

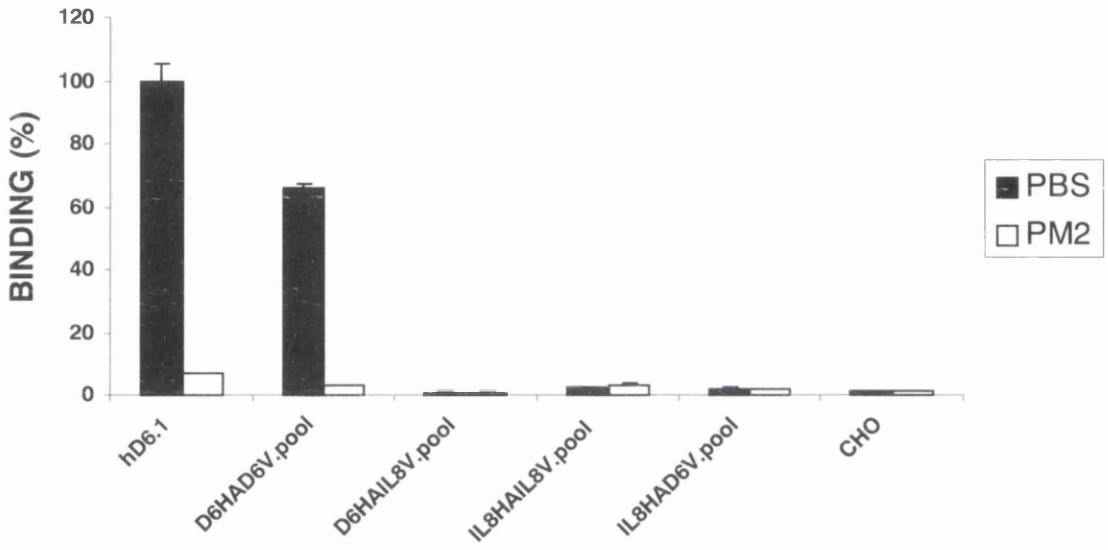


Figure 3.10. Binding assay in equilibrium. Stably transfected pools of CHO cells expressing wild type hD6 (hD6.1), HA tagged wild type hD6 (D6HAD6V.pool), HA tagged wild type mIL8RL-1 (IL8HAIL8V.pool) or the HA tagged chimaeras IL8HAD6V.pool and D6HAIL8V.pool were tested for their ability to displace ^{125}I-PM2 binding in the presence of unlabeled PM2. 1E5 cells were incubated at room temperature with 0.6nM ^{125}I-PM2 and 120nM of unlabeled PM2 or equivalent volumes of PBS. Each point was done in triplicate, the average taken and then converted in a percentage relative to hD6.1 binding to ^{125}I-PM2 in the absence of unlabeled PM2 (set arbitrarily at 100%). Data shown is the average of three experiments carried out in triplicate.

HA tagged hD6 expressing stable cell lines lose expression over time

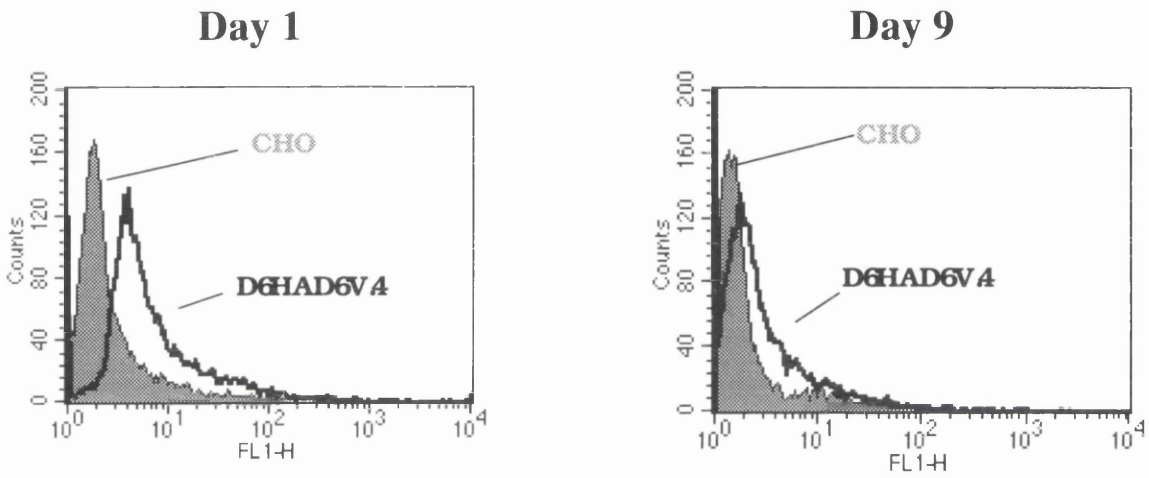


Figure 3.11 HA tagged hD6 stable cell lines lose expression through time. A clonal population of CHO cells stably expressing HA tagged wild type hD6 (D6HAD6V.4) were assessed for their surface expression by flow cytometry. Untransfected CHO cells (CHO, in grey) and D6HAD6V.4 (thick black line) expressing CHO cells were stained with the anti-HA Y11 antibody (2μg/ml) on Day 1 and Day 9. Fluorescence of D6HAD6V.4 expressing cells stained with anti-HA antibody decreases with time in culture. The appropriate isotype controls were carried out as appropriate.

Enrichment of transfected cells using the MACS system

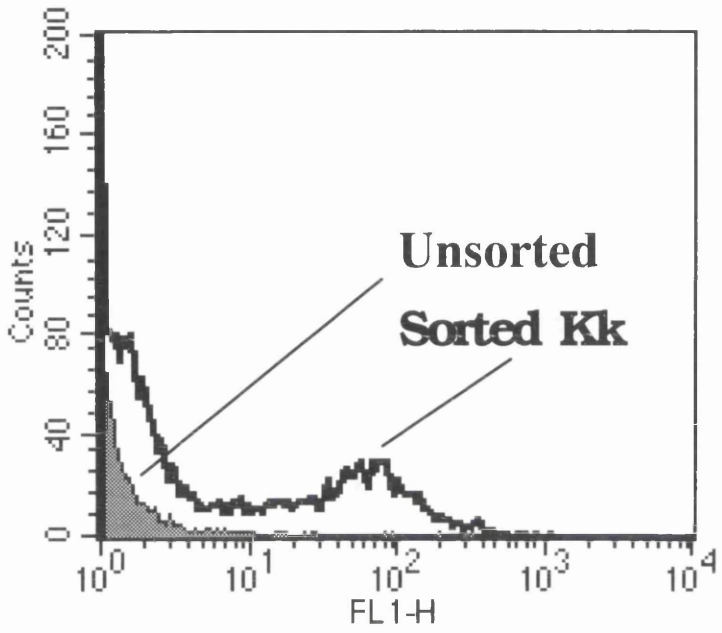
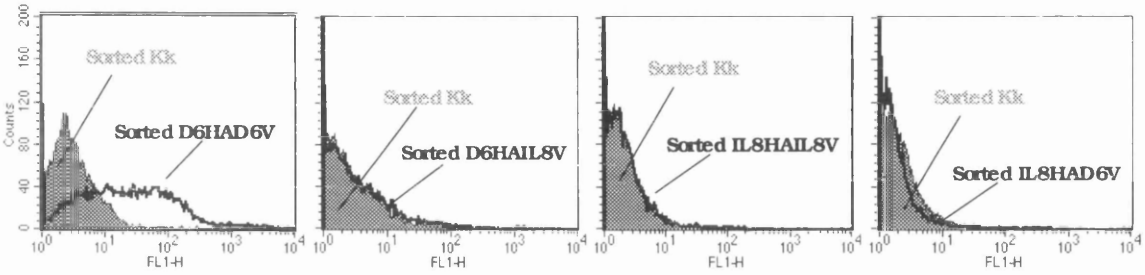


Figure 3.12. Detection of a Kk sorted population by flow cytometry. CHO cells transiently transfected with the Kk cDNA were incubated with the anti-Kk antibody coupled to magnetic microbeads and were subsequently sorted down a magnetic column (Sorted Kk). 5E5 sorted Kk and 5E5 unsorted cells (these are cells transiently transfected with the Kk cDNA that have not been sorted) were both stained with the FITC-coupled anti-Kk antibody. Fluorescence of CHO cells transiently expressing the Kk protein was compared to the fluorescence of unsorted CHO cells transiently transfected with the Kk cDNA. This graph shows a representative flow cytometry profile of the sorted cells. The same number of cells was analysed by flow cytometry for both samples, however due to sensitivity settings on the flow cytometer, less cells are seen for the unsorted population. Data shown are representative of five independent experiments.

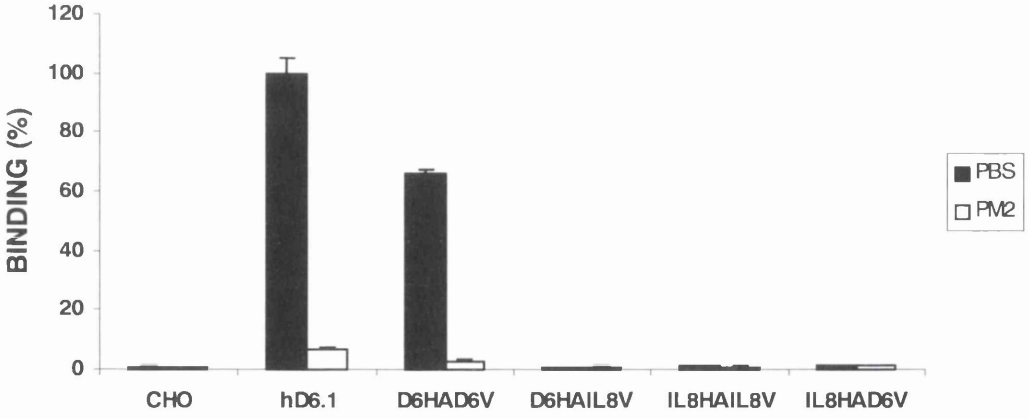
3.13. Surface expression and binding profiles for the sorted cell lines. A. Transiently transfected CHO cells expressing the Kk molecule or co-expressing Kk and the HA tagged constructs D6HAD6V, IL8HAIL8V, D6HAIL8V and IL8HAD6V, were incubated with the anti-Kk microbead-coupled antibody for 15 min. at room temperature. After incubation, cells were sorted down a magnetic column and Kk positive cells were subsequently stained with the anti-HA, HA.11, antibody. Flow cytometry compared fluorescence of each sorted population (black line) to the background fluorescence given by sorted CHO cells transiently expressing the Kk protein (Sorted Kk, in grey), that does not cross-react with HA.11. Profiles shown are representative of three independent experiments. **B.** Panel B shows a binding assay in equilibrium where the transiently transfected CHO cells co-expressing the Kk plasmid and the HA tagged wild type hD6 (D6HAD6V), HA tagged wild type mIL-8RL1 (IL8HAIL8V) as well as the chimaeras D6HAIL8V and IL8HAD6V were tested for their ability to displace ^{125}I-PM2 with unlabeled PM2. In brief, 1E5 cells were incubated with 0.6nM ^{125}I-PM2 and 120nM unlabeled PM2 or equivalent volumes of PBS, for 90 minutes at room temperature. Cells were subsequently washed with ice cold PBS and the remaining activity of ^{125}I-PM2 was counted for 1 minute. Each point was done in triplicate, the average taken and then converted into a percentage relative to hD6.1 binding in the absence of competitor (set arbitrarily at 100%). Data shown as the average of three separate experiments carried out in triplicate. hD6.1 is a stable cell line expressing untagged wild type hD6. Untransfected CHO cells (CHO) showed little background binding. Error bars represent standard deviation.

Surface expression and binding analysis of MACS sorted mIL-8RL-1/hD6 chimaeric constructs

A.



B.



hD6/hCCR5 HA tagged chimaeric constructs

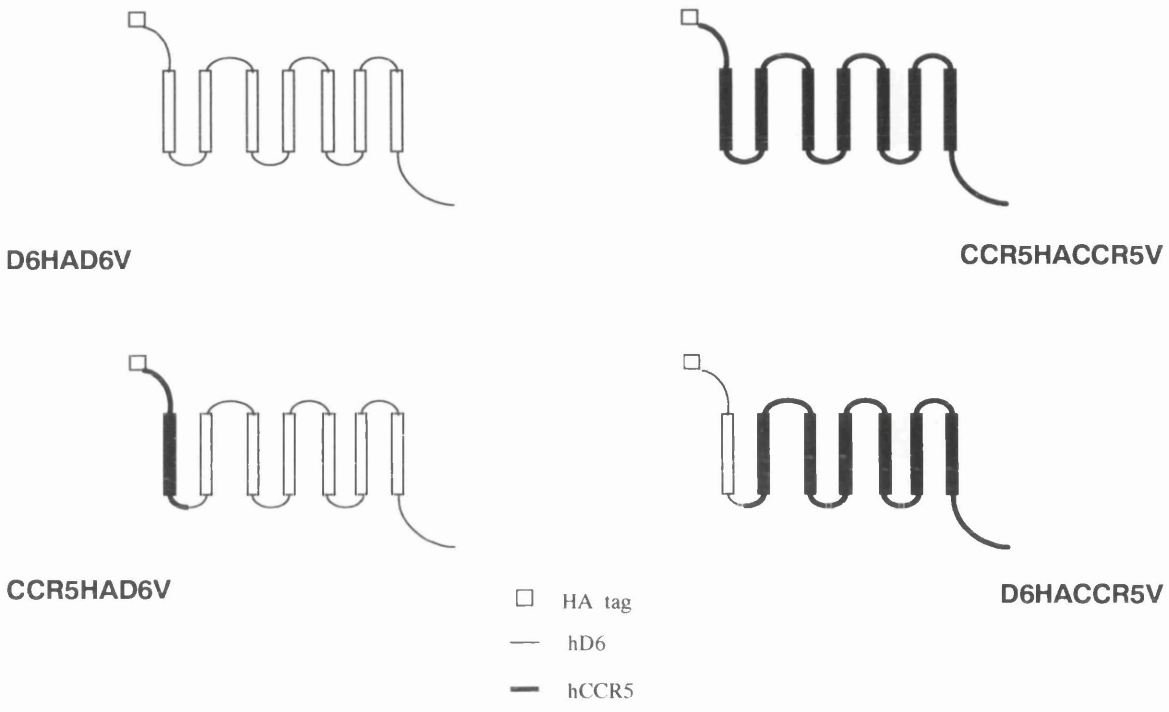
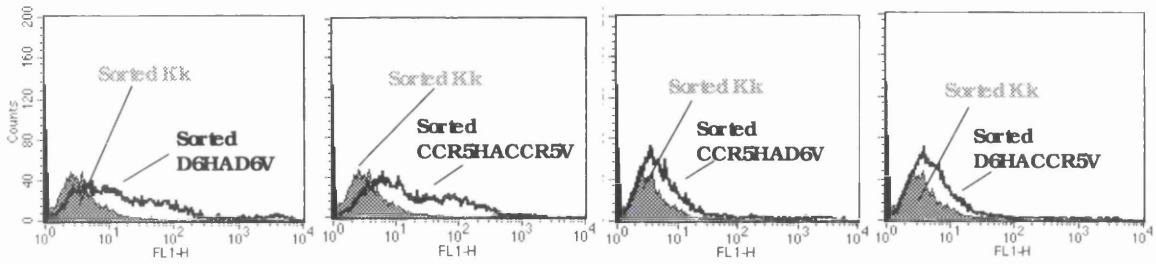


Figure 3.14. Schematic diagram of hD6/hCCR5 HA tagged chimaeric constructs. White rectangles and thin black lines indicate hD6 receptor. hCCR5 is represented by black rectangles and thick black lines. The white square represents the HA tag sequence cloned at the N-terminus of each construct. The transmembrane domains are represented by rectangles, and the N-terminus, the intracellular and extracellular loops and cytoplasmic tail are shown as solid lines. The chimaeric receptors were generated by PCR as described in Section 2.2.2.14.1.

Figure 3.15. Flow cytometry and binding analysis of MACS sorted hD6/hCCR5 HA tagged constructs. Transiently transfected CHO cells were sorted using the MACS system and subsequently tested for their surface expression (graph A) and for their ability to displace ^{125}I-PM2 (graph B), 48 hours after transfection. **A.** Transiently transfected CHO cells expressing the Kk protein or co-transfected with the Kk and the HA tagged receptors cDNA were incubated with the anti-Kk antibody coupled to magnetic beads. After incubation cells were sorted down a magnetic column and Kk positive cells were purified. The sorted populations were subsequently stained with the anti-HA antibody, HA.11 and surface expression was analysed by a FACS. Fluorescence of each transfected cell line (black line) was compared to the background fluorescence of CHO cells transfected with the Kk plasmid only (Sorted Kk, in grey). Incubation of samples with the appropriate isotype controls showed no increase in fluorescence in comparison to untransfected cells. **B.** Displacement of 0.6nM ^{125}I-PM2 by 120nM unlabeled PM2 by untransfected CHO cells (CHO) and by CHO cells expressing the wild type hD6 (hD6.1), the HA tagged wild type hD6 (D6HAD6V), wild type hCCR5 (CCR5HACCR5V) as well as the chimaeras (D6HACCR5V and CCR5HAD6V). 1E5 cells incubated at room temperature in the presence of 0.5% azide for 90 minutes before washing with ice cold PBS. Cells were lysed in 0.1% SDS and the remaining ^{125}I-PM2 in the lysate counted. Experiment was performed once in triplicate. Average of values is presented as a percentage of binding calculated in relation to hD6.1 in the absence of unlabelled competitor (set arbitrarily at 100%). Error bars represent standard deviation of a population.

Flow cytometry and binding analysis of MACS sorted HA tagged constructs

A.



B.

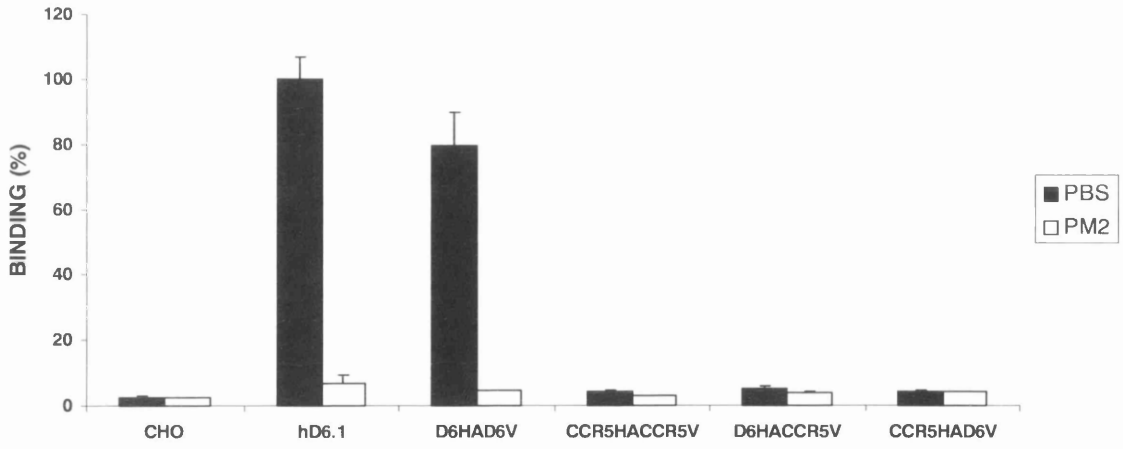
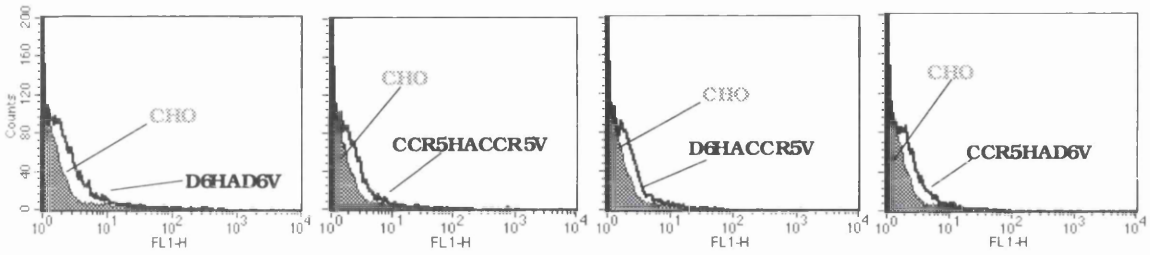


Figure 3.16. Displacement of ^{125}I-MIP-1αP by HA tagged constructs. Transiently transfected CHO cells were tested for their ability to express HA tagged proteins surface expression (graph A) and for their ability to displace ^{125}I-MIP-1αP binding (graph B) 48 hours after transfection. **A.** CHO cells transiently expressing HA tagged constructs were stained with the anti-HA antibody, HA.11 and surface expression of each receptor was analysed by a FACS. Fluorescence of each transfected cell line was compared to the background fluorescence of untransfected cells, CHO (in grey). Isotype controls were carried out where appropriate and showed no specific staining. **B.** Displacement of ^{125}I-MIP-1αP (30nM) with unlabeled MIP-1αP (1μM) by untransfected CHO cells (CHO) and by CHO cells expressing the wild type hD6 (hD6.1), the HA tagged wild type hD6 (D6HAD6V), wild type hCCR5 (CCR5HACCR5V) as well as the chimaeras (D6HACCR5V and CCR5HAD6V) was assayed by a binding assay in equilibrium. 1E5 cells incubated at room temperature in the presence of 0.5% azide for 90 minutes and then washed three times with ice cold PBS. Cells were subsequently lysed in 0.1% SDS and the remaining ^{125}I-PM2 in the lysate counted. Experiment was performed in triplicate, the counts averaged and then expressed as a percentage of binding to hD6.1 binding in the absence of unlabelled competitor (set arbitrarily at 100%). Error bars represent standard deviation of a population.

Chimaeric constructs of hD6/hCCR5 do not bind to ^{125}I-MIP-1αP

A.



B.

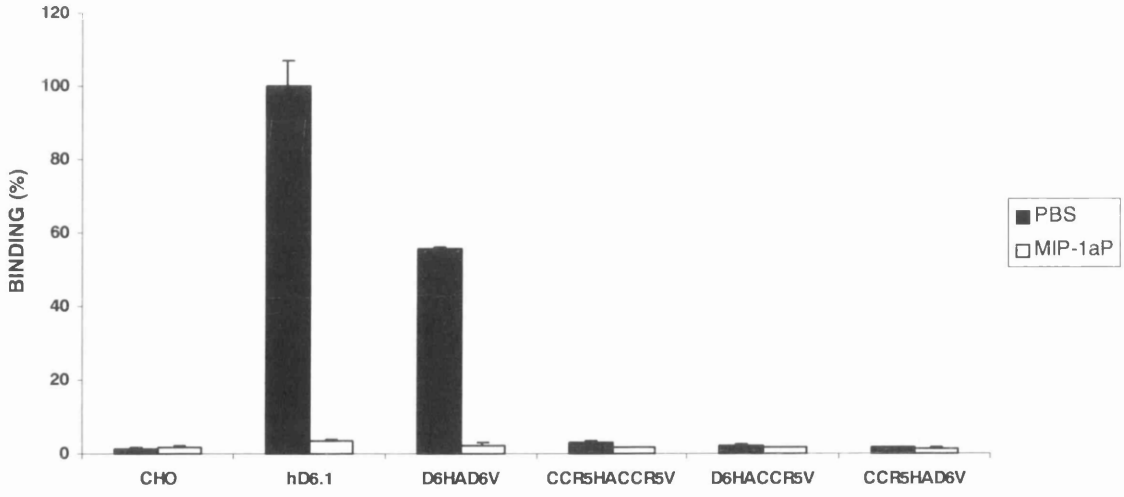
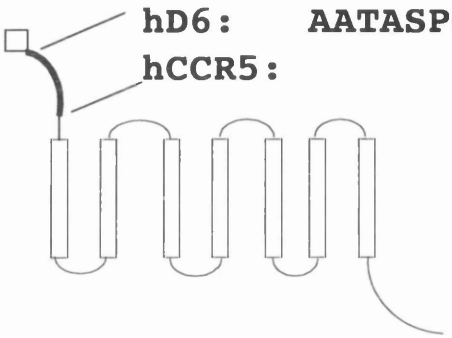


Figure 3.17. Displacement of ^{125}I-PM2 by 5nt6bd. A. Construct 5nt6bd is a HA tagged chimaeric construct of hD6 and hCCR5 where the extracellular domain of hD6's N-terminus up to the first Cys residue has been replaced by the corresponding region of hCCR5. The white square represents the N-terminal HA tag, hD6 is represented by thin black lines and white rectangles whereas hCCR5 is represented by a thick black line. DNA sequence of hCCR5 and hD6 is shown beside the replaced domain. **B.** 2μg of D6HAD6V, CCR5HACCR5V and 5nt6bd cDNA were separately transiently transfected into CHO cells. 48 hours after transfection cells were harvested and immunostained with the anti-HA antibody, HA.11. 37% of D6HAD6V, 30% of CCR5HACCR5V and 27% of 5nt6bd expressing cells stained positively for the HA antibody. Binding to PM2 by the transiently transfected cell lines expressing either D6HAD6V, 5nt6bd or CCR5HACCR5V, by the stable cell line expressing wild type untagged hD6, (hD6.1) and untransfected CHO cells (CHO) is shown. These cell lines were tested for their ability to displace 30nM of ^{125}I-PM2 with 1μM of unlabeled PM2. Untransfected cells are also shown (CHO). Binding assays were done twice in triplicate. Error bars represent standard deviation.

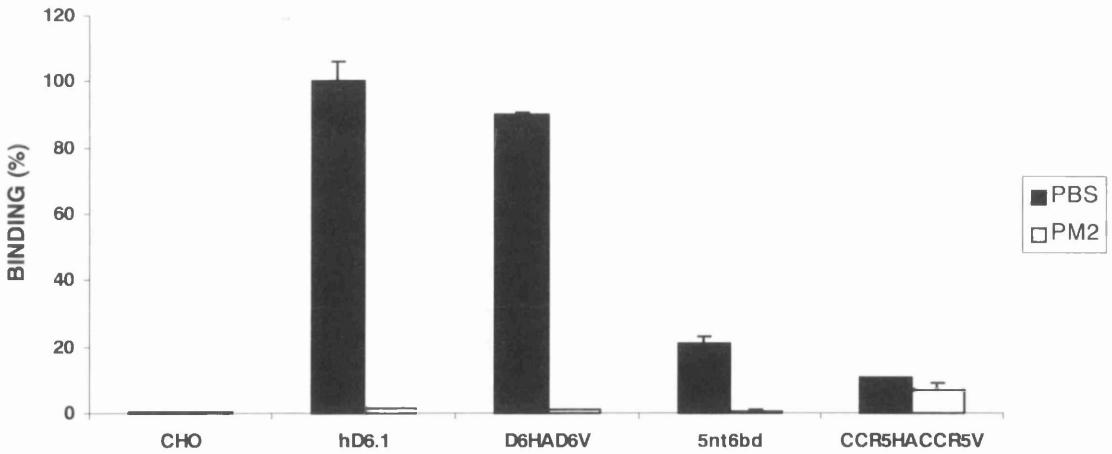
Chimaeric construct 5nt6bd binds to PM2

A.



B.

Surface expression: 37% 27% 30%



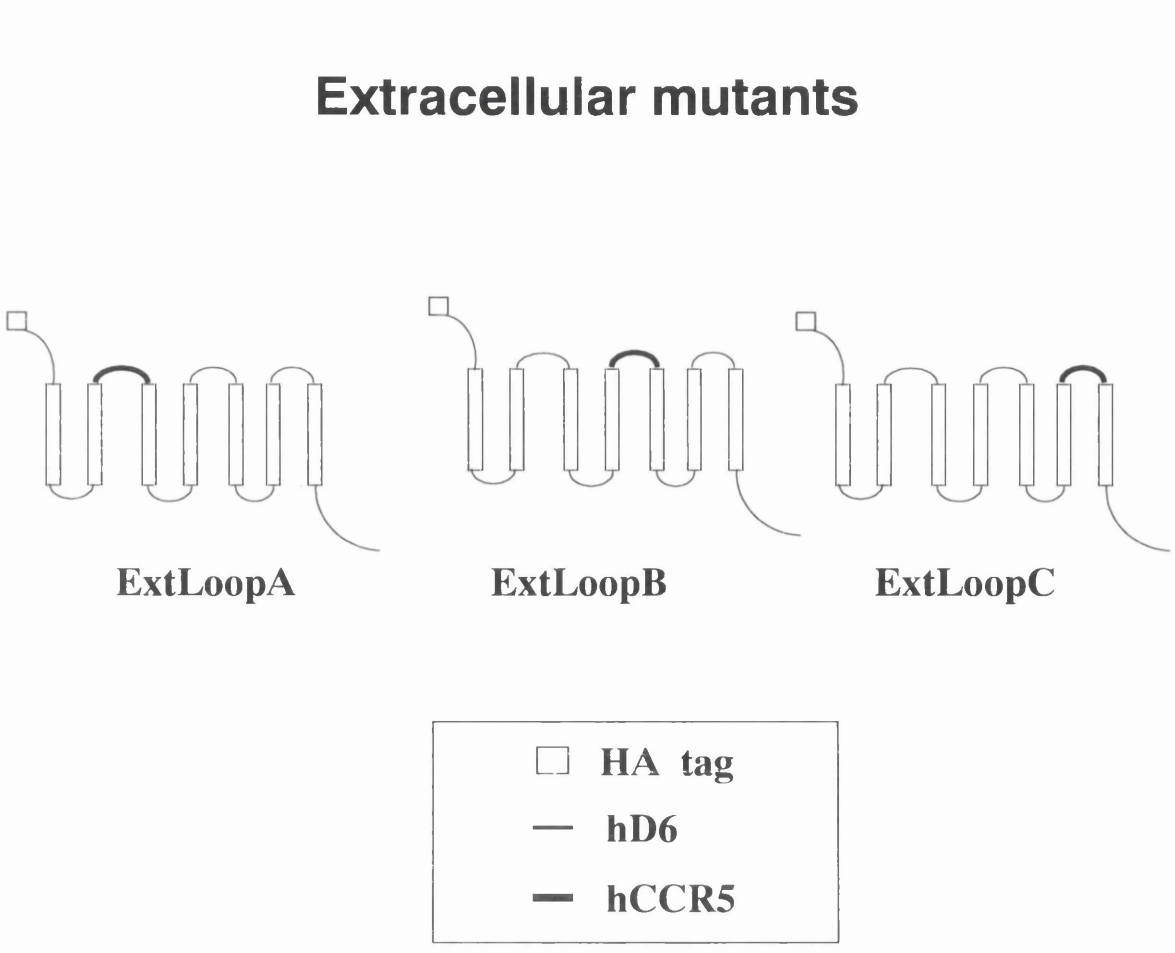


Figure 3.18. Schematic cartoon diagram of the extracellular domain mutants. The extracellular loops of hD6 were replaced with the corresponding domains of hCCR5 by overlap extension PCR. ExtLoopA represents a chimaeric construct where the first extracellular loop of hD6 has been replaced by the corresponding loop of hCCR5. ExtLoopB, bears the second extracellular loop of hCCR5 cloned into the corresponding domain of hD6. ExtLoopC has the third extracellular loop of hD6 replaced by the corresponding domain of hCCR5. All constructs were HA tagged at the N-terminus to facilitate detection of surface expression. HA tagged is represented by the white square, thin black lines and white rectangles represent hD6 whereas thick black lines indicate hCCR5 domains.

Surface expression of extracellular mutants

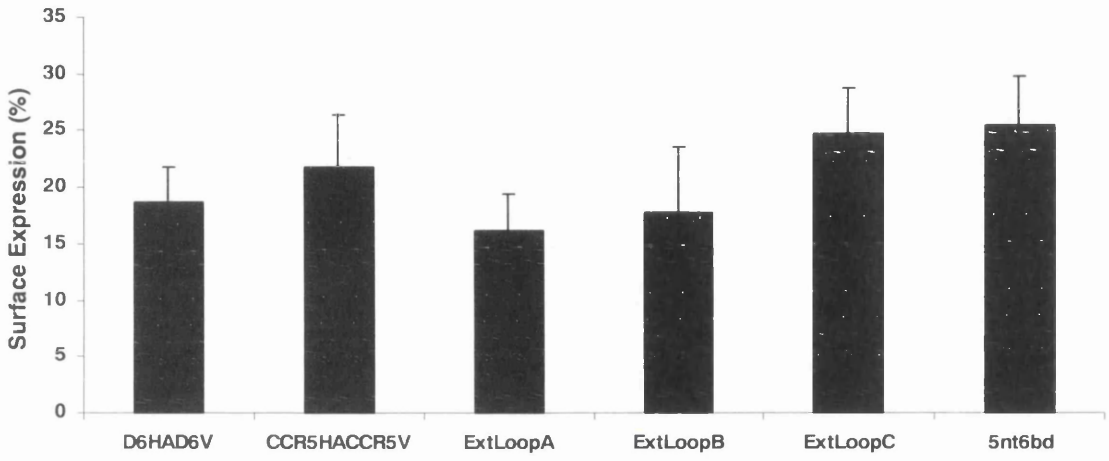
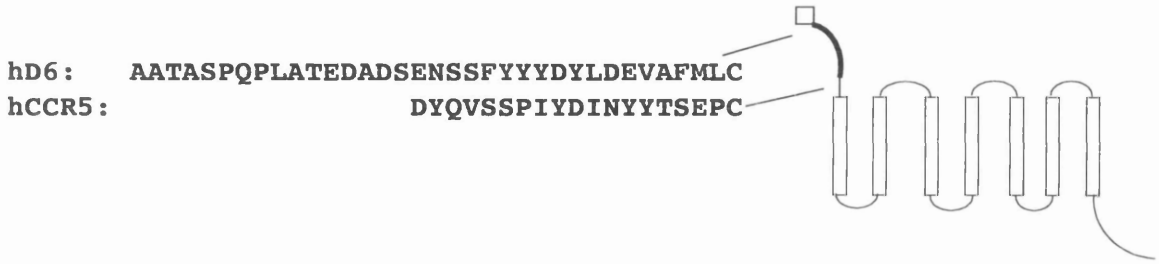


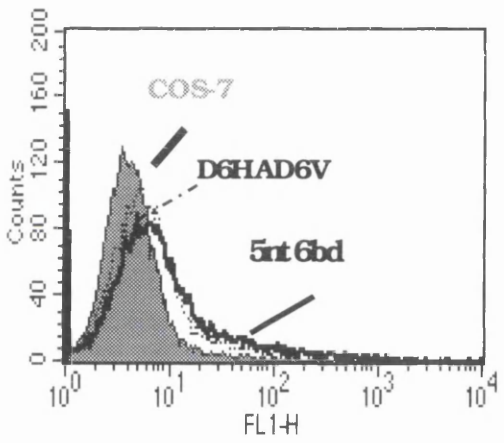
Figure 3.19. Surface expression of extracellular mutants. cDNAs encoding for the extracellular mutants, ExtLoopA, ExtLoopB, ExtLoopC or 5nt6bd as well as cDNA encoding for HA tagged wild type hD6 (D6HAD6V) and hCCR5 (CCR5HACCR5V) were separately transiently transfected into COS-7 cells. Cells were harvested and immunostained with the anti-HA antibody 48 hours after transfection. Percentage of positive cells was determined by comparison with untransfected cells. D6HAD6V, CCR5HACCR5V, ExtLoopA and ExtLoopB are expressed at the same levels on the surface of COS-7 cells. ExtLoopC and 5nt6bd are consistently expressed at higher levels than D6HAD6V ($P<0.05$). Results shown are the average of nine separate experiments. Error bars represent standard deviation.

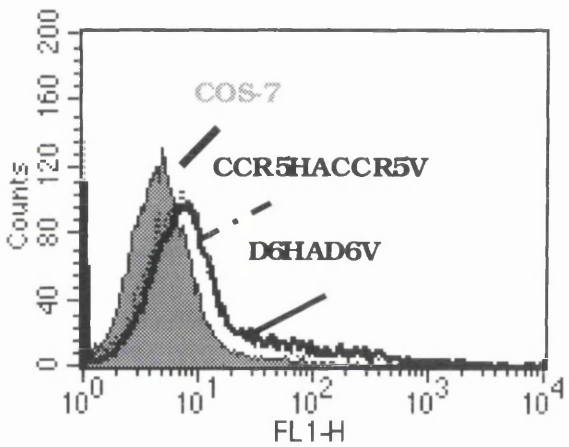
Figure 3.20. Binding of ^{125}I-PM2 to 5nt6bd is displaced by MCP-1, eotaxin, MCP-2 but not by RANTES. Schematic diagram of 5nt6bd is shown. The extracellular N-terminus domain (up to the first Cys residue) of hCCR5 was cloned into the corresponding region of hD6. Grey square indicates HA tag epitope cloned at the N-terminus of this mutant receptor. Thin black lines and white rectangles indicate hD6 domains whereas hCCR5 sequence is represented by a thick black line. **A.** Untransfected (COS-7, in grey) and transiently transfected COS-7 cells expressing either D6HAD6V (thick black line), CCR5HACCR5V (dotted line) or 5nt6bd (dotted line), were stained with the FITC coupled anti-HA antibody, HA.11, 48 hours after transfection. Fluorescence of each cell line was compared to fluorescence of untransfected cells by a FACS. HA tagged constructs 5nt6bd and CCR5HACCR5V were expressed on the surface of COS-7 cells at the same level of D6HAD6V. Incubation of samples with the isotype control showed no unspecific staining. Profiles shown are representative of two separate experiments. **B.** On the same day transiently transfected and untransfected COS-7 cells were tested for their ability to bind ^{125}I-PM2 and whether this binding could be displaced by the presence of an excess unlabeled chemokine. In brief, 1E6 cells were incubated with 120nM ^{125}I-PM2 and 1μM unlabeled chemokine or equivalent volumes of PBS. After 90 minutes of incubation at room temperature, cells were washed with wash buffer and with ice cold PBS. The remaining ^{125}I-PM2 activity was counted for one minute in a gamma counter as before. The results shown are the average of two experiments carried out in triplicate. Percentage of binding is calculated in relation to hD6.1 binding of ^{125}I-PM2 in the absence of competitor (set arbitrarily at 100%).

Binding of ^{125}I-PM2 to 5nt6bd can be displaced with other β-chemokines

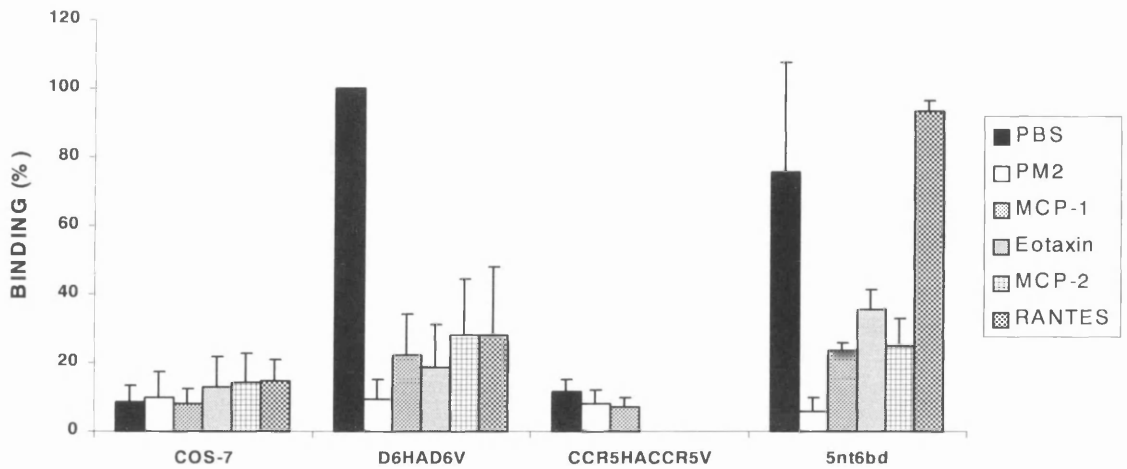
A.







B.



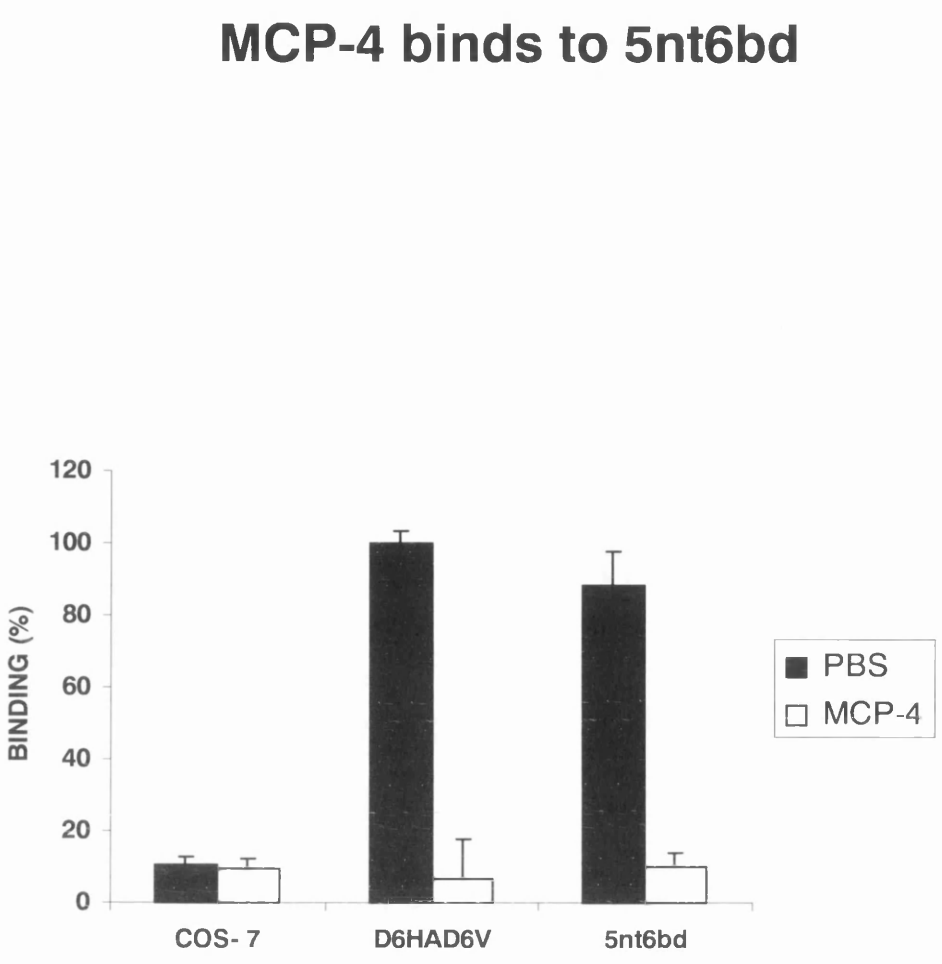


Figure 3.21. 5nt6bd binds to MCP-4. Untransfected (COS-7) and transiently transfected COS-7 cells expressing either D6HAD6V or 5nt6bd were tested for their ability to bind to 2nM ^{125}I-MCP-4 and to displace it in the presence or absence of 0.5μM of MCP-4, 48 hours after transfection. 5nt6bd binds to MCP-4 at levels comparable to those registered for HA tagged wild type hD6 (D6HAD6V). The results shown are the average of two experiments carried out in triplicate. Percentage of binding is calculated in relation to binding of ^{125}I-PM2 to D6HAD6V expressing cells in the absence of competitor (set arbitrarily at 100%).

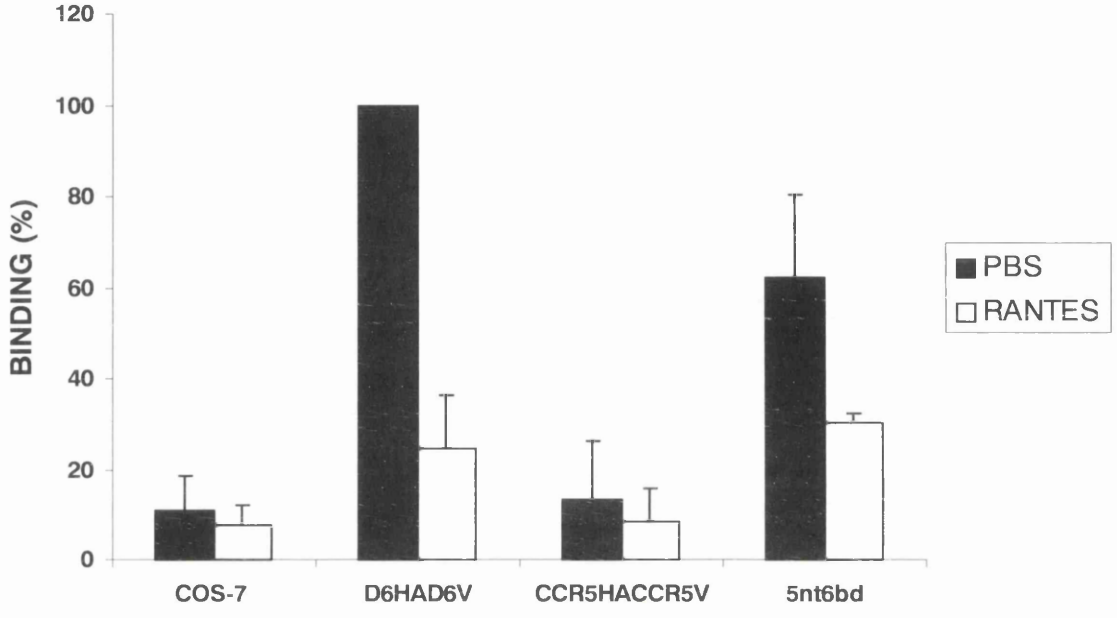
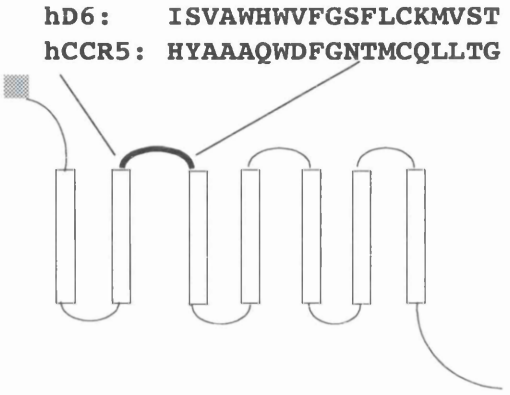


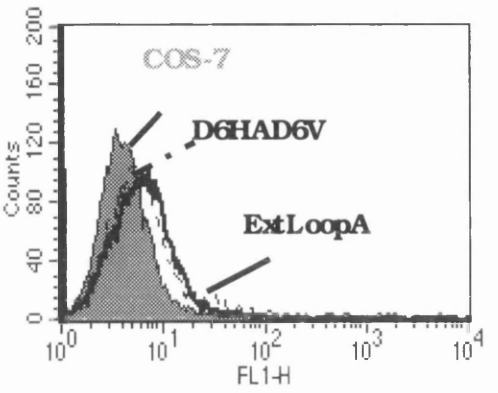
Figure 3.22. RANTES binds to 5nt6bd. Untransfected and transiently transfected COS-7 cells with D6HAD6V, CCR5HACCR5V or 5nt6bd cDNAs were tested for their capacity to bind to ^{125}I-RANTES (2nM) in the presence or absence of 0.5μM of unlabeled RANTES, 48 hours after transfection. Binding of CCR5HACCR5V to RANTES is below the detection levels of this assay. 5nt6bd binds to RANTES. The results shown are the average of two experiments carried out in triplicate. Percentage of binding is calculated in relation to binding of ^{125}I-PM2 to D6HAD6V expressing cells in the absence of competitor (set arbitrarily at 100%).

Figure 3.23. Binding of ^{125}I-PM2 to cells expressing ExtLoopA is not detected, demonstrating the importance of the first extracellular loop of wild type hD6. A. ExtLoopA bears the first extracellular loop of hCCR5 (thick black line) cloned into the corresponding region of hD6 (thin black lines and white rectangles) with a HA tag (grey square) cloned at the N-terminus. Alignment of the two sequences that encode for the two extracellular loops is shown. COS-7 cells were transiently transfected with ExtLoopA or D6HAD6V cDNAs. 48 hours after transfection, transfected (black and dotted lines) and untransfected (COS-7, in grey) cells were assayed for surface expression of the HA epitope by flow cytometry. Surface expression of each constructed was determined by a FACS and compared to the background fluorescence of untransfected cells (COS-7, in grey), as shown. Isotype controls were carried out as appropriate. **B.** Binding assay. In brief, 1E6 cells were incubated with 120nM ^{125}I-PM2 and 1μM unlabeled cold competitor or equivalent volumes of PBS. After 90 min. incubation at room temperature, cells were washed with ice cold PBS and the remaining ^{125}I-PM2 activity counted. Error bars represent standard deviation. Experiments were done twice in triplicate. Percentage of binding is calculated in comparison to D6HAD6V binding (set arbitrarily at 100% in the absence of competitor).

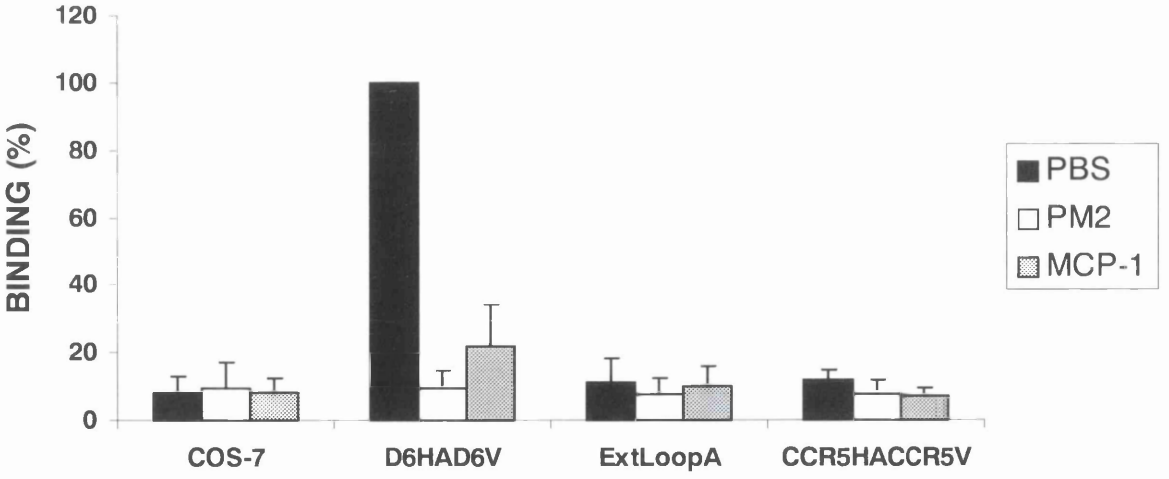
The first extracellular loop of hD6 is important for ^{125}I-PM2 binding

A.





B.



The first extracellular loop of hD6 is important for ^{125}I-MCP-4 binding

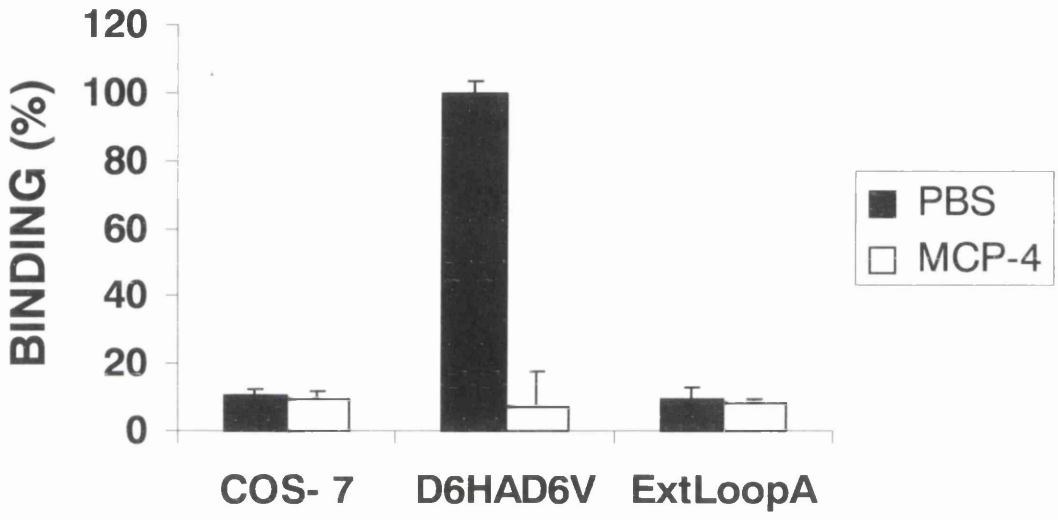


Figure 3.24. The first extracellular loop of hD6 is necessary for binding to MCP-4. 1E6 untransfected (COS-7) and transiently transfected COS-7 cells expressing D6HAD6V or ExtLoopA were incubated with 2nM ^{125}I-MCP-4 in the absence or presence of unlabelled MCP-4 (0.5μM). After 90 min. incubation at room temperature cells were washed and the remaining activity counted. Results are presented as the average of two experiments carried out in triplicate. Percentage of binding was calculated in relation to ^{125}I-MCP-4 binding to D6HAD6V expressing COS-7 cells in the absence of competitor (set arbitrarily at 100%). Error bars indicate standard deviation.

The first extracellular loop of hD6 is important for RANTES binding

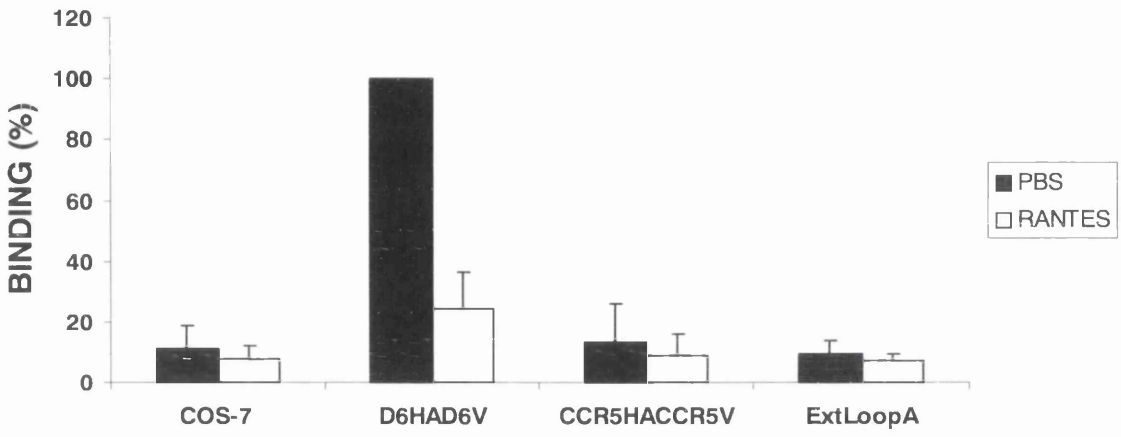
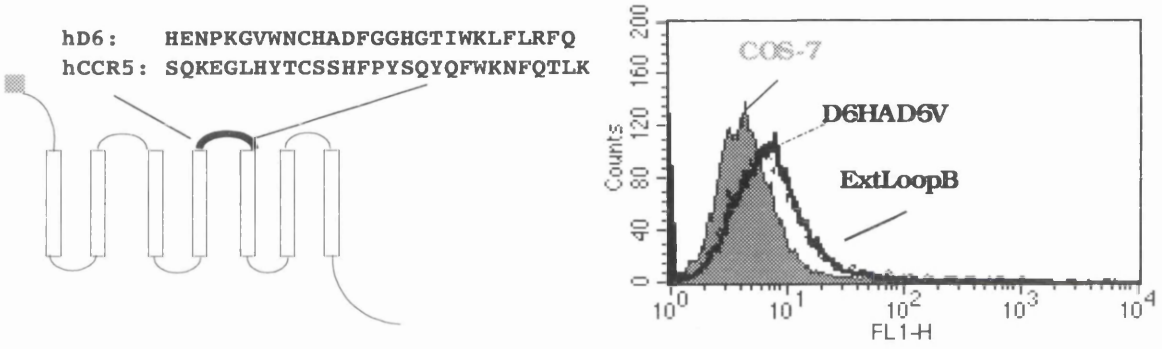


Figure 3.25. The first extracellular loop of hD6 is important for binding to RANTES. 1E6 transiently transfected COS-7 cells expressing D6HAD6V, CCR5HACCR5V or ExtLoopA and untransfected cells (COS-7) were assayed for their ability to bind to ^{125}I-RANTES. Cells were incubated with 2nM ^{125}I-RANTES and either 0.5μM unlabeled RANTES or an equivalent volume of PBS. Graph shows the average of two experiments done in triplicate. Percentage of binding is calculated in relation to ^{125}I-RANTES binding to D6HAD6V in the absence of cold competitor (set arbitrarily at 100%). Error bars represent standard deviation.

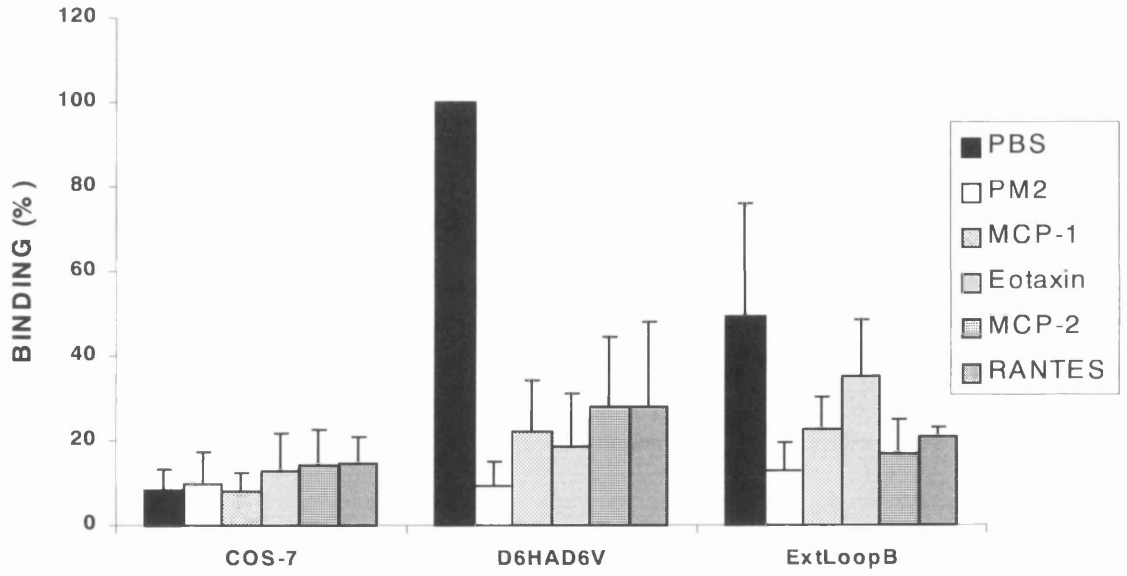
Figure 3.26. ExtLoopB expressing cells displace ^{125}I-PM2 binding in the presence of other chemokines. A. ExtLoopB is a chimaeric construct which bears the second extracellular loop of hCCR5 (thick black line), cloned into the corresponding region of hD6 (thin black lines and white rectangles). A HA tag (grey square) was cloned at the N-terminus. Untransfected (COS-7) and transfected COS-7 cells were transiently expressing D6HAD6V or ExtLoopB receptors were immunostained with the anti-HA antibody and fluorescence of each cell line was determined by a flow cytometry 48 hours after transfection. Fluorescence of each cell line was compared with background fluorescence of untransfected cells (COS-7 cells, in grey). Isotype controls were carried out as appropriate and showed no unspecific binding. Profile shown is representative of two separate experiments. **B.** D6HAD6V, ExtLoopB expressing COS-7 cells and untransfected COS-7 cells were incubated with 120nM ^{125}I-PM2 and 1μM of unlabelled PM2, MCP-1, eotaxin, MCP-2, RANTES or equivalent volumes of PBS. Binding assay was carried out as described before. Graph shows the average results of two experiments done in triplicate. Percentage of binding was calculated in relation to D6HAD6V expressing cells binding to ^{125}I-PM2 in the absence of cold competitor (set arbitrarily at 100%). Error bars represent standard deviation.

Surface expression and ^{125}I-PM2 binding of ExtLoopB expressing COS-7 cells

A.



B.



^{125}I-MCP-4 binding to ExtLoopB expressing cells

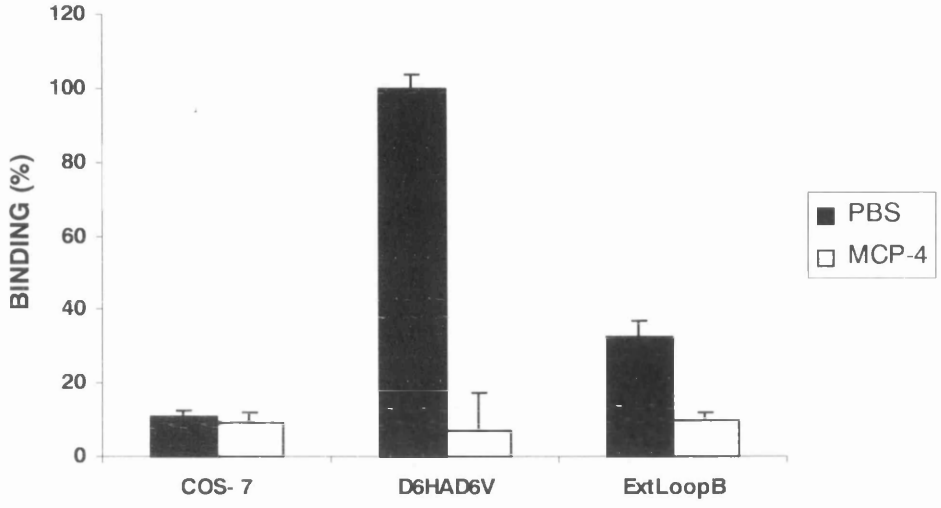


Figure 3.27. Displacement of ^{125}I-MCP-4 binding from ExtLoopB expressing cells. Approximately 1E6 transiently transfected COS-7 cells expressing D6HAD6V or ExtLoopB as well as untransfected cells (COS-7) were incubated with 2nM ^{125}I-MCP-4 and 0.5μM unlabeled MCP-4 or equivalent volumes of PBS. Graph represents data from two experiments done in triplicate. Percentage of binding is calculated in relation to D6HAD6V expressing cells binding to ^{125}I-MCP-4 in the absence of competitor (set arbitrarily at 100%). Untransfected cells showed no binding to ^{125}I-MCP-4. Error bars represent standard deviation.

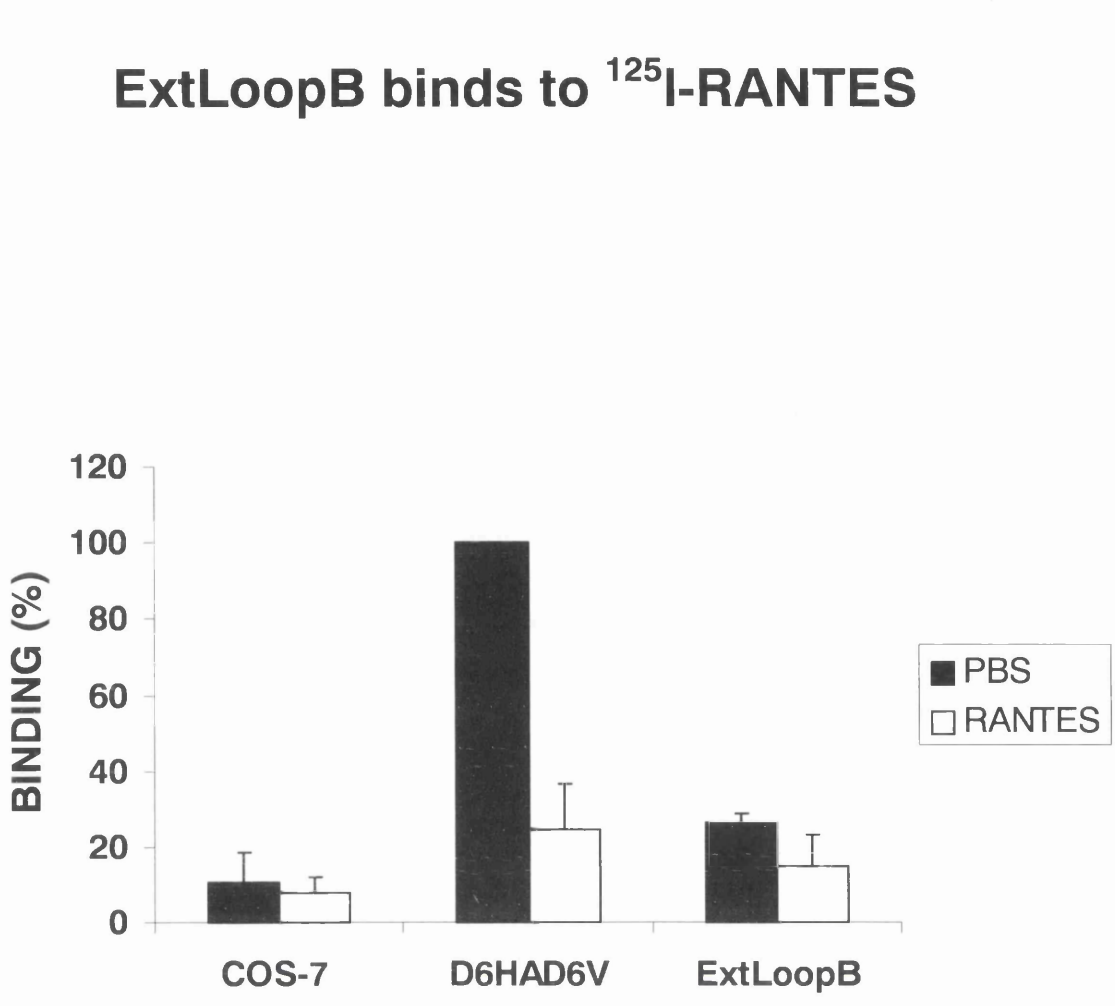
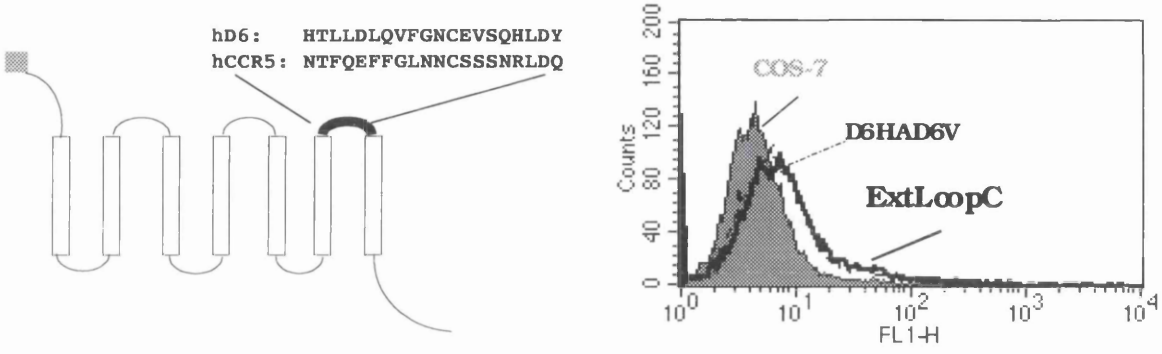


Figure 3.28. Displacement of ^{125}I-RANTES from ExtLoopB expressing cells. Approximately 1E6 transiently transfected cells expressing D6HAD6V or ExtLoopB as well as untransfected cells (COS-7) were incubated with 2nM of ^{125}I-RANTES and 0.5μM of unlabeled RANTES or equivalent volumes of PBS. Data presented in this graph is the average of two experiments done in triplicate. Percentage of binding was calculated in relation to ^{125}I-RANTES binding to D6HAD6V expressing cells in the absence of competitor, set arbitrarily at 100%. Error bars represent standard deviation.

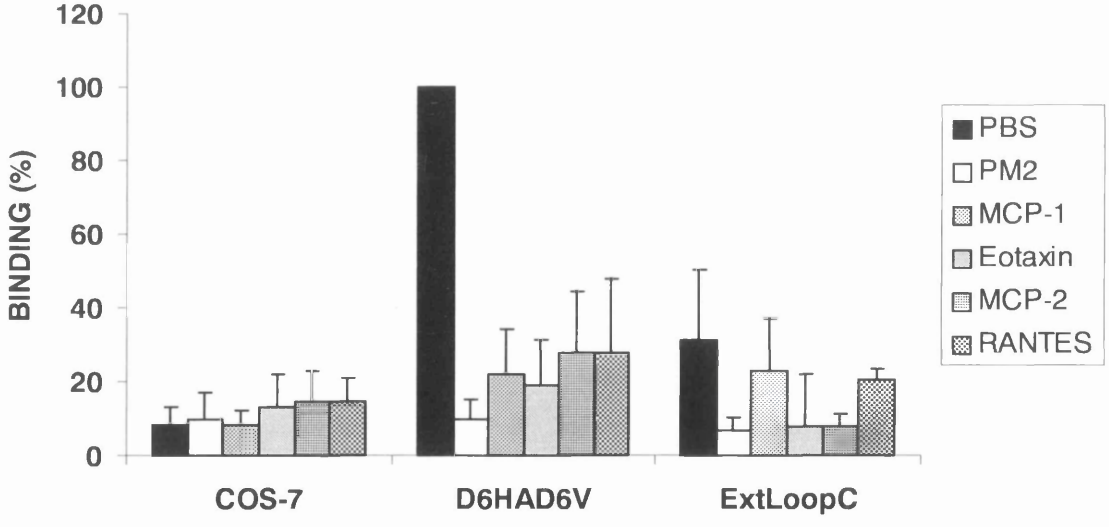
Figure 3.29. Displacement of ^{125}I-PM2 from ExtLoopC expressing COS-7 cells. **A.** Schematic diagram of chimaeric construct ExtLoopC is shown in A. The HA tag is represented by the grey cylinder square, transmembrane domains of hD6 are represented by white rectangles, hD6 sequence is represented by thin black lines, thick black line represents hCCR5. The sequence encoding for the third extracellular loop of hD6 and hCCR5 is shown. Approximately 5E5 COS-7 cells transiently expressing D6HAD6V or ExtLoopC were stained with the anti-HA antibody and subsequently analysed by a flow cytometry 48 hours after transfection. Fluorescence of transfected cells was compared to the background fluorescence of untransfected cells (COS-7). Isotype controls were carried out and showed no unspecific staining. Profiles shown are representative of two separate experiments. **B.** On the same day cells were incubated with 120nM ^{125}I-PM2 and 1μM unlabeled competitor or equivalent volumes of PBS. Percentage of binding is calculated in relation to ^{125}I-PM2 binding for D6HAD6V expressing COS-7 cells in the absence of competitor (set arbitrarily at 100%). Results shown are the average of two experiments done in triplicate. Error bars represent standard deviation.

Extracellular loop C- surface expression and binding to ^{125}I-PM2

A.



B.



^{125}I-MCP-4 binding to the chimaeric receptor ExtLoopC

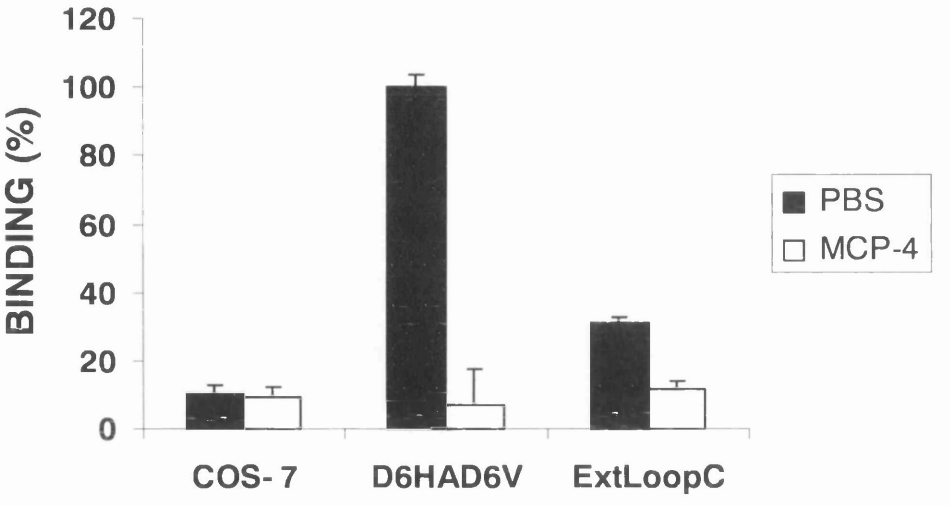


Figure 3.30. Displacement of ^{125}I-MCP-4 binding from COS-7 cells transiently transfected with ExtLoopC cDNA. Approximately 1E6 untransfected (COS-7) or transiently transfected COS-7 cells with D6HAD6V or ExtLoopC cDNAs were incubated with 2nM ^{125}I-MCP-4 and 0.5μM unlabeled MCP-4 or equivalent volumes of PBS. Binding assay in equilibrium was performed as described before. Graph shows the average of two experiments done in triplicate; error bars represent standard deviation of a population; Percentage of binding is calculated in relation to ^{125}I-MCP-4 binding for D6HAD6V expressing COS-7 cells in the absence of competitor (set arbitrarily at 100%). Results shown are the average of two experiments done in triplicate. Error bars represent standard deviation.

^{125}I-RANTES binding to ExtLoopC chimaera

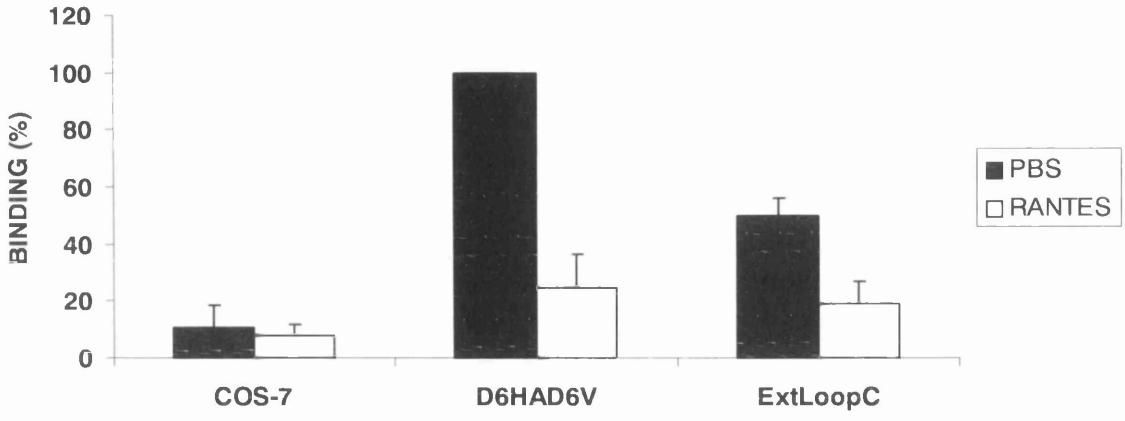


Figure 3.31. Displacement of ^{125}I-RANTES binding from cells expressing ExtLoopC. Approximately 1E6 untransfected (COS-7) and COS-7 cells transfected with D6HAD6V or ExtLoopC cDNAs were tested for their ability to bind to RANTES 48 hours after transfection. Cells were incubated with 2nM ^{125}I-RANTES and 0.5μM unlabeled RANTES or equivalent volumes of PBS. Data shown in graph is an average of two experiments done in triplicate; error bars represent standard deviation of a population; Percentage of binding was calculated in relation to ^{125}I-PM2 binding for D6HAD6V expressing COS-7 cells in the absence of competitor (set arbitrarily at 100%). Results shown are the average of two experiments done in triplicate. Error bars represent standard deviation.

3.2. Recognition of chimaeric receptors by an anti-hD6 monoclonal antibody

During the course of this work, a mouse anti-hD6 monoclonal antibody (1D4) was generated that recognizes hD6 expressed on heterologous cell lines and in tissue sections. This work is described in (Nibbs et al., 2001). However, 1D4 could not successfully recognise hD6 immobilised in nylon filters using conventional Western blotting techniques (R. Nibbs, *pers.comm.*). Thus, 1D4 recognises a conformation-dependent epitope on hD6. It was thought that this antibody would be of use to study ligand binding and at the same time study its ability to interact with the chimaeric receptors. This will determine the position of the 1D4 epitope, and also may provide information concerning the overall structure of the chimaeric receptors.

HEK.293 cells stably expressing HA tagged 5nt6bd, ExtLoopA, ExtLoopB and ExtLoopC were stained with the anti-HA antibody HA.11, and the anti-hD6 antibody 1D4. The data presented in Fig.3.32 has been normalized for HA.11 staining and shows that the N-terminus is the major epitope recognized by 1D4 monoclonal antibody. However, the second and third extracellular loop chimaeras also show reduced interaction with the antibody. These results suggest that the hD6 epitope recognised by 1D4 is within the N-terminal region and that the structure of this epitope is disrupted to some degree in the chimaeric constructs ExtLoopB and ExtLoopC. ExtLoopA binds 1D4 as efficiently as the wild type hD6 protein.

To determine whether this antibody competed with PM2 for binding to hD6, the same cells were stained with the 1D4 with or without PM2 and incubated for 45

minutes. Flow cytometry (Fig. 3.33) showed that addition of PM2 still allows epitope recognition by the 1D4 antibody. Likewise, radiolabelled ligand binding experiments on wild type hD6 transfected cells by Dr. Nibbs have demonstrated that 1D4 does not interfere with PM2 binding. Therefore, PM2 and 1D4 bind to non-overlapping sites on the receptor.

These findings corroborate the binding studies with the chimaeric receptors that suggest that the N-terminus of hD6 is not responsible for ligand binding or promiscuity. Also, it can be hypothesized that the decrease in ligand binding observed with ExtLoopB and ExtLoopC could be due to an abnormal conformation assumed by these chimaeric receptors that does not allow appropriate interaction with the ligands, rather than a direct consequence of the primary sequence interaction with ligand.

In summary, the work described here has demonstrated the difficulties associated with chimaeric receptor studies. By mixing receptors it was hoped that receptor structure would be fairly well conserved so the effect of primary sequence determinants could be assessed. Only by swapping small regions of closely related receptors can it confidently be assumed that the overall gross structure of the resultant chimaeras has not been disrupted. In fact, the studies with the anti-hD6 antibody suggest that even small changes may disrupt distant structure. Nonetheless, these data do implicate the highly conserved first extracellular domain in ligand binding to human D6. Discussion of the technical and scientific aspects of these studies is expanded in Chapter 4.

N-terminus of hD6 is the major epitope recognized by 1D4

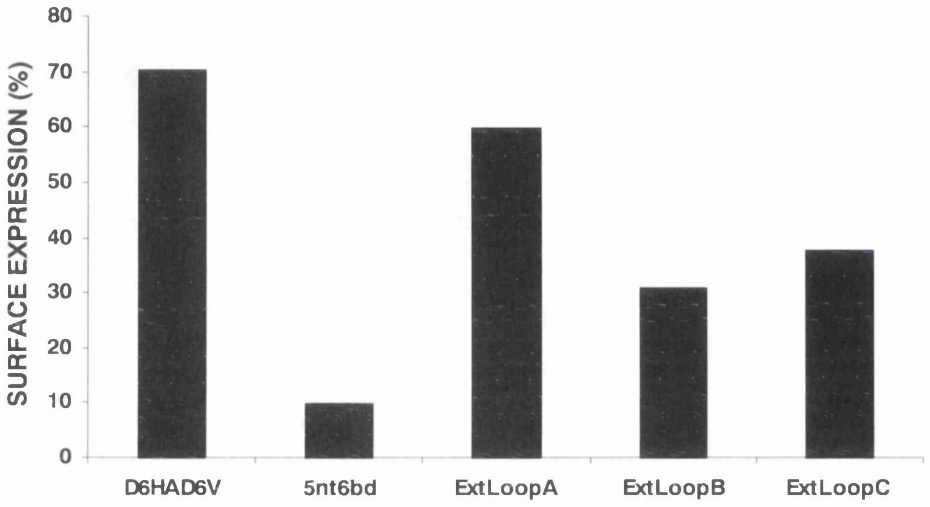


Figure 3.32. Surface expression of stably transfected HEK.293 expressing wild type hD6 and chimaeric constructs. HEK.293 cells were stably transfected with D6HAD6V, 5nt6bd, ExtLoopA, ExtLoopB or ExtLoopC cDNAs and immunostained with the anti-HA antibody HA.11 and the anti-hD6 monoclonal antibody 1D4. Fluorescence of each sample was assessed by flow cytometry. Surface expression detected by 1D4 antibody was calculated as a percentage of HA.11 staining. Appropriate isotype controls were carried out for both antibodies used and showed no unspecific staining. Graph shows the average of three separate experiments.

1D4 antibody does not block PM2 binding

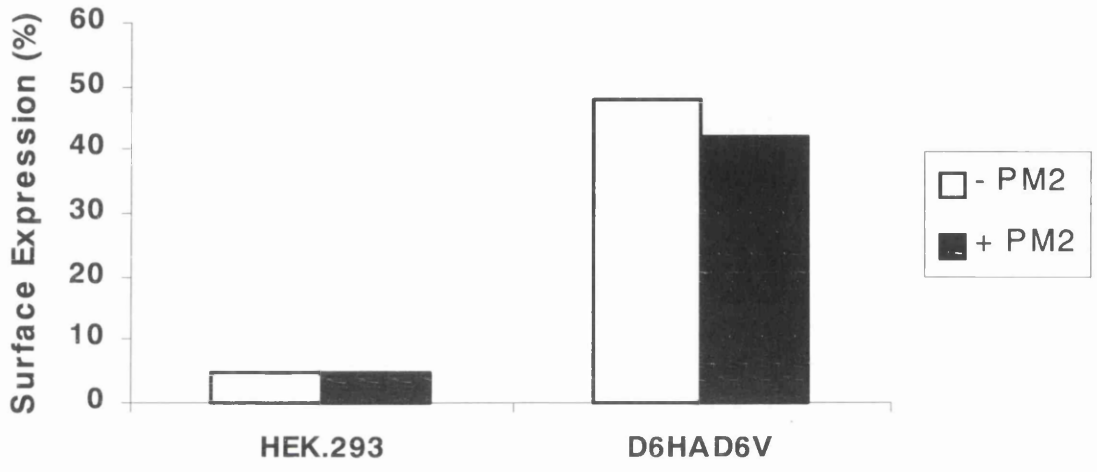


Figure 3.33. Human D6-specific monoclonal antibody does not compete with ligand binding to hD6. HEK.293 cells stably expressing HA tagged wild-type hD6 (D6HAD6V), and untransfected cells (HEK.293) were stained with the anti-hD6 antibody, 1D4, in the presence or absence of PM2. Data shown are the average of three separate experiments.

3.3. Signalling studies

G protein-coupled receptors mediate leukocyte activation by coupling to heterotrimeric G proteins. Binding of the ligand to the receptor activates G proteins resulting in regulation of a wide variety of second messenger systems. Selection of a G protein partners is likely dependent on sequence determinants within the intracellular domains.

In general, seven transmembrane receptors have an extended third intracellular loop that interacts directly with G proteins. Chemokine receptors, however, have an extremely short third intracellular loop so it is hypothesized that other intracellular portions, such as the C-terminus domain, may be critically involved in signal transduction via these GPCRs (Bennet et al, 2000; Oliveira et al, 1994; Scheer et al, 1996). Also, the conserved DRYLAIVHA motif located at the interface between the third TM domain and the cytoplasm and the conserved aspartic acid residue found in the second TM domain, have been shown to be important for G protein activation and coupling (Bennet et al, 2000; Oliveira et al, 1994; Scheer et al, 1996).

Standard signalling assays for chemokine receptors such as calcium flux, chemotaxis, and microphysiometry demonstrated that hD6 cannot elicit signalling upon ligand binding when expressed in a variety of heterologous cells (Nibbs et al., 1997a) (Nibbs, R., *pers. comm.*). Signalling of hCCR5 has been most commonly detected by calcium flux, a very sensitive technique that can register calcium fluxing in the presence of very low concentrations of ligand and at low levels of receptor occupancy. In these assays, stably transfected cells, are loaded with FURA-2AM, a

fluorescent dye that binds to calcium ions. Upon ligand binding Ca^{2+} is released from the intracellular calcium stores and an increase in fluorescence is registered. Fig. 3.34 shows a typical calcium flux profile obtained when cells expressing hCCR5 or hD6 are challenged with PM2. Additionally, in heterologous cells hD6 transfectants do not internalise in the presence of ligand (R. Nibbs, *pers. comm.*) whereas hCCR5 transfected cells have been shown to be internalised upon ligand binding (Alkhatib et al., 1997).

These observations suggest that hD6 most likely does not transduce an intracellular signal in the transfected cells tested, although one cannot rule out the possibility that other secondary messengers, distinct from these tested so far, are activated by ligand binding to exogenous D6. What these results do suggest however is that hCCR5 and hD6 must be structurally distinct as one couples to G proteins in transfected cells whilst the other does not. hD6 might not be capable of undergoing the necessary conformational change to allow coupling, or alternatively, hD6 might contain primary sequence determinants that prevent interactions with the G proteins present in heterologous cell lines. The divergent C-terminus, the altered DRY motif at the end of the third TM domain, or the absence of an aspartic acid residue in the second TM (all known to be important for G protein coupling in other chemokine receptors (Brelot et al., 2000; Gosling et al., 1997; Oliveira et al., 1994; Schraufstatter et al., 1998)) might contribute to this. In fact, at the outset of this work it was already known that introducing an aspartic acid residue onto the second TM of hD6 did not introduce signalling to calcium flux (R. Nibbs, *pers. Comm.*).

Through the generation of chimaeric constructs between hCCR5 and hD6, it was hoped that domains/residues would be identified that could introduce ligand-induced G protein coupling into hD6. This would hopefully yield some information about the ability of wild type hD6 to undergo ligand-induced conformational change, which has implications for the understanding of hD6 biochemistry and function. hD6 also provides a signalling-negative background that would allow the study of hCCR5 G protein coupling domains. Calcium fluxes and receptor internalisation assays were used as a functional read-out, both seen with hCCR5 but not hD6.

3.3.1. Sequence comparison of intracellular loops of hD6 and hCCR5

The intracellular domains of hD6 and the corresponding regions of hCCR5 share virtually no overlap in their primary sequence and are different in their overall charge and length. In the first and second intracellular loop, hD6 has more positively charged residues than hCCR5, whereas in the third intracellular loop hCCR5 has more positively charged residues than hD6. Within the second intracellular loop of hD6 the DKYLEIV motif is variable from the canonical DRYLAIV motif of chemokine receptors, with the notable exclusion, at residue 134, of a negatively charged Glu residue. The remaining eleven amino acids that constitute the second intracellular loop of hD6 are mostly positively charged whereas in hCCR5 they are mostly non polar. It should also be noticed that hD6 has a Pro residue at position 140 that is also found in CCR11, to which signalling cannot be demonstrated in HEK.293 cells (R. Nibbs *pers. comm.*). The C-terminus domain of hD6 is longer and encodes for an extra seventeen amino acids and is found to be overall negatively charged

whereas the same region in hCCR5 is overall positive. hD6 C-terminus has more Ser residues than the C-terminus of hCCR5. Moreover, hCCR5 has three palmitoylated Cys residues recently shown to play a role in intracellular trafficking of this receptor (Blanplain et al., 2001) whereas hD6 only has one putative palmitoylation site. In comparison with the C-terminal region of hCCR5, the corresponding region of hD6 has more Ser and Pro residues which have been shown to be involved in post translational modifications and bends or folds within the mature protein (Barlow & Thornton, 1988; Ji et al., 1998; Woolfson & Williams, 1990).

3.3.2. The second intracellular loop is necessary for expression of hD6 on the surface of HEK.293 cells

Four chimaeric constructs (Fig. 3.35) were initially generated by overlap extension PCR (Sections 2.2.2.10 and 2.2.2.17.1). Constructs iLoop1, iLoop2 and iLoop3 carry, respectively, the first, second and third intracellular loop of hCCR5 cloned into the corresponding region of hD6. 5ct6bd is a construct where the C-terminus domain of hD6 has been replaced by the C-terminus of hCCR5. To facilitate detection of surface expression of each of these chimaeric constructs a HA tag sequence was cloned in the N-terminus of each construct. Each one of these constructs was transfected into HEK.293 cells (Section 2.2.1.2.1) and, to avoid clonal differences, a pooled population was obtained after selection with 800μg/ml of G418.

Surface expression of wild type and chimaeric chemokine receptors was detected by flow cytometry after immunofluorescence staining with the anti-HA mAb, HA.11. These constructs were subsequently tested for their capacity to flux calcium upon ligand binding. This assay was chosen to assess signalling, not only because it is

commonly used to determine the signalling capacity of chemokine receptors, but also because it has been shown to be extremely sensitive in detecting signalling even when there is little receptor occupancy. Nibbs and colleagues have shown that low concentrations of PM2 are still capable of eliciting a detectable calcium flux (Nibbs et al., 1999).

Construct 5ct6bd was expressed at the same levels as wild type HA tagged hD6 (D6HAD6V), whereas iLoop3 and iLoop1 are expressed at lower levels. 45% of cells expressing iLoop1 and 70% of those expressing iLoop3 stained positively for the HA tag epitope (Fig.3.36). Immunofluorescence staining with the same antibody detected the HA epitope in approximately 80% of the cells expressing D6HAD6V and 5ct6bd. CCR5HACCR5V was also seen to be expressed at lower levels than D6HAD6V, with 50% of its cells testing positively for the presence of the HA tag epitope. Construct iLoop2, which bears the second intracellular loop of hCCR5 cloned into the corresponding region of hD6, was notably completely undetectable on the surface of HEK.293 cells (Fig. 3.36). However, when these cells were permeabilized with 0.01% of saponin over 50% of the cells were shown to be positive for HA tag (Fig.3.37). This data indicates that this chimaera does not get appropriately transported to the surface suggesting that its folding and therefore its trafficking to the membrane are likely to be compromised.

It should be noticed that the percentage of cells staining with the anti-HA antibody fluctuated over time in culture therefore prior to each subsequent experiment, cells were stained with the anti-HA antibody to determine receptor surface expression.

To determine whether constructs iLoop1, iLoop3 and 5ct6bd retained their ability to bind to PM2 a binding assay was carried out (Fig. 3.38). All these hD6 mutants exhibited displaceable binding to ^{125}I-PM2. 5ct6bd and iLoop1 bound PM2 at levels comparable to D6HAD6V (Fig. 3.38). iLoop3 was shown to have its ability to bind to PM2 reduced by approximately 50%, even though this chimaera was expressed at much higher levels than the other receptors when this experiment was performed. The fact that iLoop3 was seen to bind the least ^{125}I-PM2 may indicate that the affinity of this chimaeric receptor is reduced relative to wild type hD6.

Despite the abundant surface expression, and the ability to bind PM2, none of the new chimaeras were able to detectably flux calcium upon treatment with PM2 (data not shown). Large calcium fluxes were however detected for the HEK.293 cell line stably expressing hCCR5 that were used as a positive control (Fig. 3.39). It was therefore concluded that simple single loop changes were insufficient to allow coupling of hD6 to calcium ion fluxing.

3.3.3. A single point mutation in the DKYLEIV motif of hD6 is sufficient to elicit signalling

Given that the iLoop2 mutant did not get expressed on the surface of HEK.293 cells, it was thought that by introducing smaller changes in this region one could investigate the effect of changing the DKYLEIV motif of hD6 whilst maintaining surface expression. Site directed mutagenesis was used to introduce changes in the DKYLEIV motif of hD6. Complete cDNA sequencing (Section 2.2.13) was performed and these constructs were stably transfected into HEK.293 cells (Section 2.2.1.2.1). A stable pool of clones was obtained by selection with 800μg/ml of G418.

Mutant hD6K-R has a point mutation changing the hD6 Lys131 residue to an Arg and mutant hD6E-A has a point mutation changing the Glu134 residue to an Ala. Both of these constructs have an HA tag epitope cloned after the start codon. Two point mutations changing the hD6's residues Lys131 to an Arg and the Glu134 to an Ala generated construct hD6DRY. Cartoon diagrams of these constructs are shown in Fig. 3.40. The hD6DRY.5 is a cell line stably expressing the untagged hD6DRY mutant given by Dr. R. Nibbs.

In order to be able to compare surface expression of the HA tagged intracellular mutants of hD6 with the surface expression of the untagged cell line expressing hD6DRY.5, the anti-hD6 mAb, 1D4 was used. Flow cytometry analysis revealed that the mutants hD6E-A and hD6DRY.5 were expressed at roughly the same levels as wild type HA tagged hD6 (D6HAD6V) although the mutant hD6K-R was expressed on the surface of the HEK.293 cells at slightly lower levels than D6HAD6V, hD6E-A and hD6DRY.5 (Fig. 3.41).

To determine the ability of these constructs to bind PM2 an equilibrium binding assay was performed (Fig. 3.41). The hD6E-A mutant was shown to be capable of binding ^{125}I-PM2 at the same levels as the wild type HA tagged receptor. hD6K-R and hD6DRY.5 mutants have their ability to bind to PM2 reduced by 70 and 60%, respectively, when compared to D6HAD6V. These results suggest that the mutations introduced in hD6 to generate the hD6K-R or the hD6DRY.5 constructs have somehow disrupted the conformation of the mature protein in such a fashion that this mutant has its ability to bind PM2 diminished to some extent.

The ability of these constructs to flux calcium upon ligand binding was tested as before. Interestingly, constructs hD6DRY.5 and hD6E-A were shown to be able to elicit an increase in intracellular calcium upon stimulation with PM2 (Fig. 3.42). This calcium flux was much smaller than the one registered for wild type hCCR5 (Fig. 3.42b). Mutant hD6K-R did not detectably flux calcium upon ligand binding (data not shown).

Previous research has demonstrated that chemokine receptors couple to Pertussis toxin-sensitive G-proteins (Zhao et al., 1998). As shown in Fig. 3.43, pre-treatment of cells stably expressing the hD6DRY.5 mutant with Pertussis toxin (Ptx) abolished calcium flux indicating that hD6DRY.5 can couple to Ptx-sensitive G proteins.

Taken together these results show that a single point mutation in hD6 can introduce the ability to generate ligand-induced signalling via pertussis toxin-sensitive G protein. Moreover, it seems fair to suggest that the C-terminal eleven amino acids of the second intracellular loop of hD6 are involved in regulating surface expression, possibly by maintaining correct receptor conformation. These issues will be discussed in greater depth in the discussion section (Chapter 4.2).

3.3.4. hCCR5 signalling mutants

Two point mutations in the highly conserved 'DRY' region of wild type hCCR5 were performed so that the role of the 'DKYLE' motif of hD6 could be studied in an hCCR5 background. hCCR5DKYLE is a mutant of wild type hCCR5 that bears two point mutations changing the amino acid residue Arg115 to a Lys and the residue Ala118 to a Glu. A cartoon diagram of this construct can be seen in Fig. 3.44. This

construct was subcloned into a mammalian expression vector, pcDNA.3 and once the DNA sequence was verified it was stably transfected into HEK.293 cells. A pool of positive clones was selected with 800μg/ml G418.

Flow cytometry after immunofluorescence staining with the anti-hCCR5 antibody 2D7 was performed to determine the surface expression of this construct (Fig. 3.45). hCCR5DKYLE was shown to have a much higher surface expression than CCR5HACCR5V. It is interesting to note that two point mutations in the second intracellular loop of hCCR5 are sufficient to increase the surface expression of hCCR5 by more than 30%.

A binding assay was performed to determine whether the ability to bind to PM2 had been affected. HEK.293 cells stably expressing CCR5HACCR5V were shown to still be able to bind PM2 although hCCR5DKYLE expressing cells bound approximately 20% less PM2 (Fig. 3.45).

A calcium flux assay was performed to test the signalling capacity of these constructs upon ligand binding. CCR5HACCR5V-expressing cells produced robust calcium fluxes as previously shown (Fig. 3.34 and 3.39). Although hCCR5DKYLEIV was shown not only to be expressed at high levels on the surface of HEK.293 cells but also to be capable of PM2 binding, this mutant was not capable of fluxing calcium upon ligand binding (Fig. 3.46). This data shows that, in agreement with previous studies (Gosling et al., 1997), the conserved 'DRY' domain of hCCR5 is essential for G protein activation.

3.3.5. Second generation of chimaeric receptors

To determine whether the weak signalling detected for the hD6DRY.5 and hD6E-A cell lines could be increased by a cooperative effect from other domains of hCCR5, a second generation of intracellular chimaeric constructs was obtained in which two or more intracellular domains of hCCR5 were cloned into the corresponding region of hD6. Five new constructs were generated (Section 2.2.2.18) and cartoon diagrams of these constructs are shown in Fig.3.47. Construct iLoop1+3 bears the first and third intracellular loops of hCCR5 cloned into the corresponding region of hD6. iLoop1+DRY+iLoop3 is the same as iLoop1+3 and has the DKYLEIV motif in the second intracellular loop mutated to DRYLAIV. iLoop1+DRY and iLoop3+DRY are mutants of iLoop1 and iLoop3, respectively, where the DKYLEIV motif has been mutated to DRYLAIV. Cloning of the hCCR5 receptor C-terminus into iLoop1+DRY+iLoop3 generates construct iLoop1+DRY+iLoop3+Ct. These constructs were subcloned into the mammalian expression vector pcDNA.3, stably transfected into HEK.293 cells (Section 2.2.1.2.1) and a pool of positive clones were selected using 800μg/ml G418.

The surface expression of each construct was assessed by flow cytometry after staining with the 1D4 antibody and the HA.11 antibody (Fig. 3.48). D6HAD6V was shown to have the highest surface expression (88% of the cells stained positive) and iL1+DRY, DRY+Ct and CCRHACCR5V have approximately 50% of detectable receptors on the surface of HEK.293 cells. Constructs, iL1+3, iL3+DRY, iL1+DRY+iL3 and iL1+DRY+iL3+Ct have the lowest percentage of stained cells: 21%, 13%, 14% and 30% respectively (Fig. 3.48). Saponin treatment of cells expressing these constructs allowed intracellular staining with the same antibody and

revealed that these constructs were abundantly expressed intracellularly (Fig. 3.49) suggesting that these constructs were not efficiently transported to the cell membrane.

Equilibrium binding assays were performed (Fig. 3.48) that showed that D6HAD6V-expressing cells bound radiolabelled PM2, whilst cells expressing CCR5HACCR5V, or the chimaeric constructs iLoop1+DRY, iLoop1+DRY+iLoop3 and DRY+Ct all showed displaceable binding to PM2 with variable efficiency. No binding was detectable to iLoop1+3 or the mutant in which all the intracellular loops were altered (iLoop1+DRY+ iLoop3+Ct). It seems likely that this lack of detectable binding was due to a combination of low receptor surface expression and a decrease in ligand binding affinity, although this has not been rigorously tested. iLoop3+DRY was peculiar as binding was detected to radiolabelled PM2, but this was not displaced by unlabelled PM2. Currently, there is no explanation for this phenomenon but it is possible that this particular pool of transfected cells has upregulated molecules that enhance non-specific binding to PM2. This observation does however, demonstrate the importance of examining displaceable binding, rather than just radiolabelled ligand binding.

All these cell lines were next tested in the calcium flux assays in comparison to cells transfected with hD6DRY or hCCR5. As expected, hCCR5 transfected cells gave a large robust flux, whereas those expressing hD6DRY gave a small flux. However, none of the new chimaeric receptors were able to elicit detectable calcium fluxes (data not shown).

It can be concluded from these results that the introduction of additional domains from hCCR5 into a background of hD6 already carrying a mutant DRYLA motif, does not bring about a dramatic increase in ability to elicit calcium fluxes. In fact, these multiple mutants are often compromised in their ability to correctly assemble at the surface of the cell. However, if hCCR5-like signalling activity had been introduced into these chimaeras, we would have expected to detect calcium fluxes even with low surface expression because of the sensitivity of the assay. The absence of signalling by those receptors that are abundantly expressed (iLoop1+DRY, DRY+Ct), although detectable signalling is observed with DRY mutants alone, cannot be confidently attributed as a loss of signalling in itself, but rather as a consequence of the small size of the flux combined with the low surface expression.

3.4. Internalization Studies

As an alternative way of assessing signalling by the intracellular domain chimaeras it was decided to examine receptor internalisation. This was thought to perhaps reveal activation of alternative signalling pathways by ligand binding, independent of calcium fluxes.

The surface expression of wild type HA tagged hD6 (D6HAD6V) and its intracellular mutants stably transfected into HEK.293 cells was measured by flow cytometry using the hD6-specific mAb 1D4 (Section 2.2.4.3). Wild type hCCR5 and hCCR5 mutants surface expression was determined by using the anti-hCCR5 mAb, 2D7. As it can be seen in Fig. 3.50, wild type HA tagged hD6 (D6HAD6V) was not internalised after 45 minutes incubation with 100nM of PM2. Wild type hCCR5

(hCCR5 in Fig. 3.50) surface expression in contrast, decreased by 35% after 45 minutes incubation with 100nM of PM2 in agreement with previous studies in the same cell line (Ling et al., 1999). Surprisingly, PM2 treatment for 45 minutes caused approximately 28-56% reduction in cell fluorescence in cells expressing iLoop1, iLoop3, 5ct6bd, hD6E-A, iLoop1+DRY, DRY+Ct, and no reduction in cells expressing hD6K-R (Fig 3.50). The only mutant that showed no reduction, and in fact, possibly an increase in surface expression was mutant hD6K-R.

Fig. 3.51 shows that treatment of HEK.293 stably expressing wild type (hCCR5 in Fig.) and HA tagged hCCR5 (CCR5HACCR5V in Fig.) with PM2 for 45 minutes induced a 50% reduction in cell surface expression in both of these cell lines. However, surface expression of the hCCR5 intracellular mutant, hCCR5DKYLE, was reduced by 23% after treatment with PM2 for 45 minutes. Thus, although changing the DRYLA domain of hCCR5 to DKYLE abolishes the ability of hCCR5 to couple to calcium fluxes, it reduces, but does not completely prevent, internalisation of this mutant receptor.

These data are somewhat surprising as they suggest that all the mutants, except hD6K-R, are able to signal into HEK.293 cells to mediate internalisation in response to PM2. The inability to detect ligand-induced calcium ion fluxes from some of these mutated receptors, in particular iLoop1, iLoop3, 5ct6bd and hCCR5DKYLE where abundant surface receptor is detectable, suggests that receptor internalisation is not dependent on coupling to calcium ion fluxes. An alternative mechanism is more likely. This will be discussed in greater detail in chapter 4.2.

In summary, the results detailed here show that a single point mutation in hD6 can introduce the ability to couple to pertussis toxin-sensitive G proteins in HEK.293 cells and stimulate calcium ion fluxes. This change, E to A neutralises a charged residue in the conserved DRYLAIV motif of hD6. Introducing DKYLE into hCCR5 completely blocks coupling to calcium ion fluxes. These data reinforce the importance of the DRYLAIV motif on G protein partner selection. The receptor internalisation data suggest that many domains of hCCR5 are involved in receptor internalisation, and that this is probably not dependent on coupling to calcium ion fluxes. The implications and interpretation of the results are discussed below.

Ligand induced calcium flux undetectable in hD6 expressing HEK.293 cells

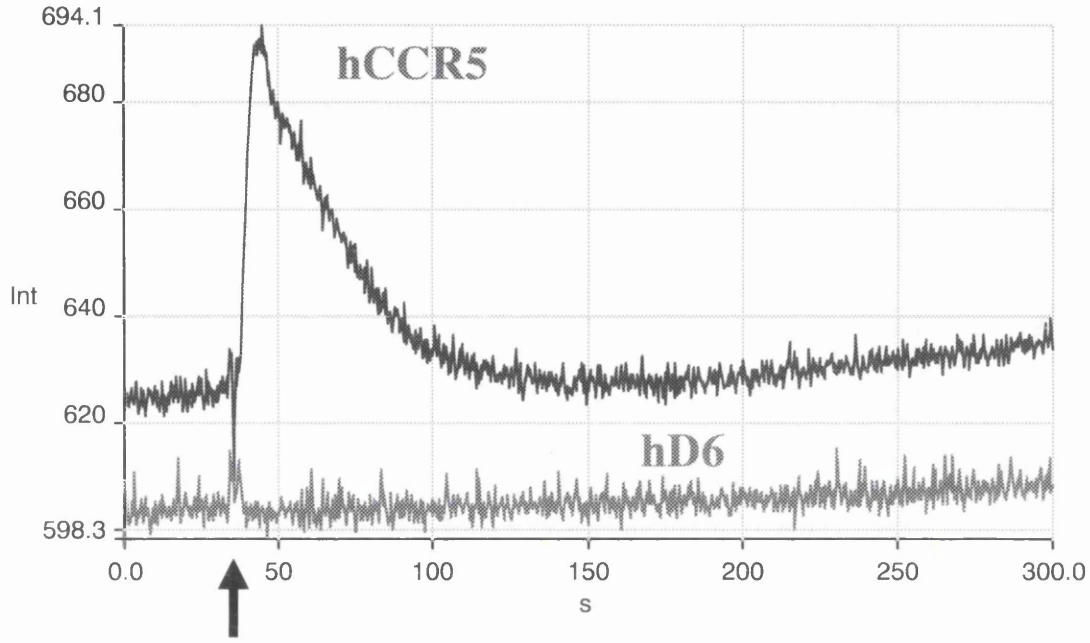


Figure 3.34. Calcium flux detection in HEK.293 cells expressing hCCR5 and hD6 cDNAs. Pools of HEK.293 cells were stably transfected with hCCR5 and hD6 cDNA. Pools of G418-resistant clones were loaded with Fura-2AM and assayed at 37°C in a continuously stirred cuvette in a Perkin-Elmer LS50 Spectrometer (340nm (λ_{ex}); 500nm (λ_{cm})), with fluorescence emission recorded every 100ms for 300s. PM2 was added to a final concentration to a final concentration of 50nM at the time indicated by the black arrow. The figure shows that after addition of PM2 an increase in fluorescence is registered in hCCR5 expressing HEK.293. This increase in fluorescence reflects detection of intracellular calcium No change in basal fluorescence is seen for hD6 expressing cells upon addition of PM2. The graph shown is representative of the calcium flux profile observed in many occasions (n >20). Int, intensity; s, seconds.

Schematic diagram of intracellular chimaeric constructs

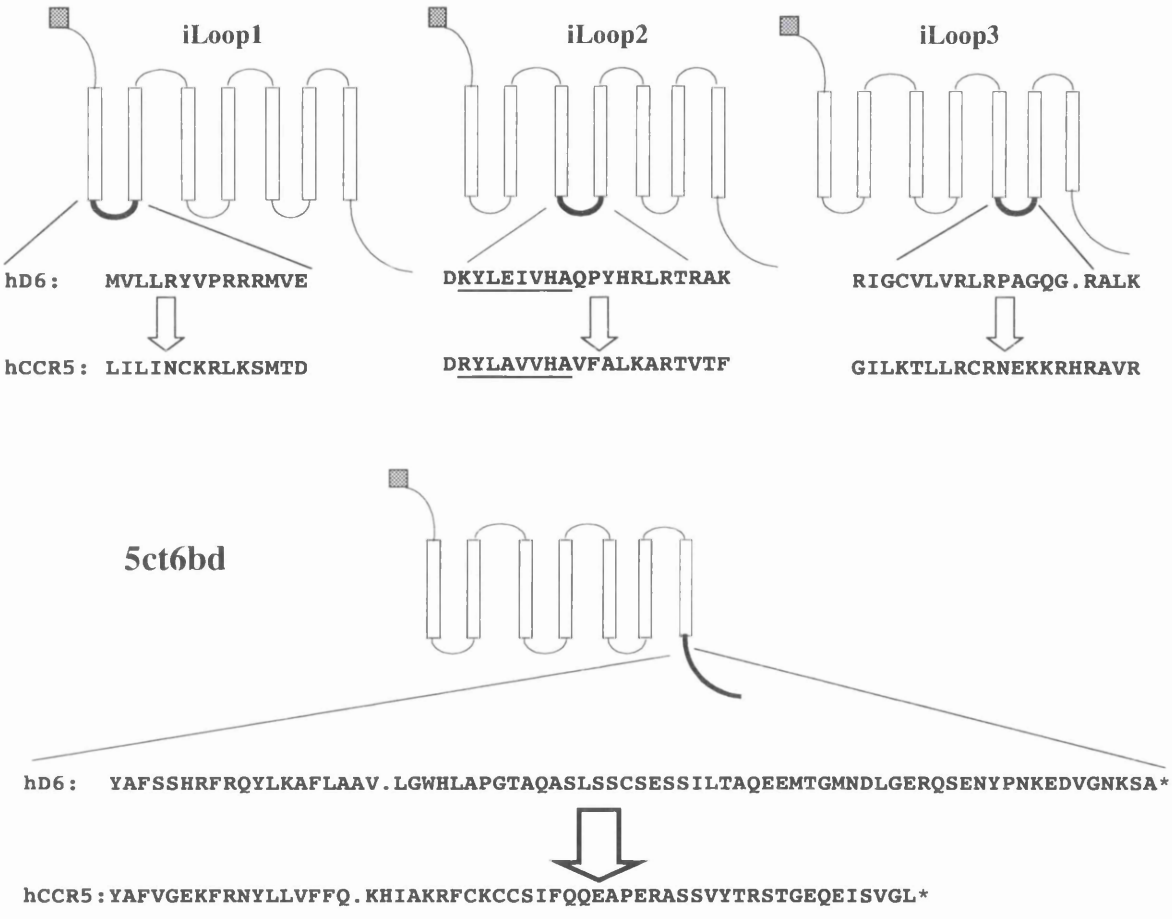


Figure 3.35. Schematic diagram of chimaeric constructs. Intracellular loops of hD6 were individually replaced by the corresponding domains of hCCR5. Constructs were generated by overlap extension PCR. Grey square indicates HA tag (grey square) at the N-terminus. hD6 domains are represented by thin black lines and white rectangles. Thick black lines represent hCCR5 sequence. The amino acid sequence of the domains is indicated using the single letter amino acid code.

The second intracellular loop of hD6 is important for surface expression

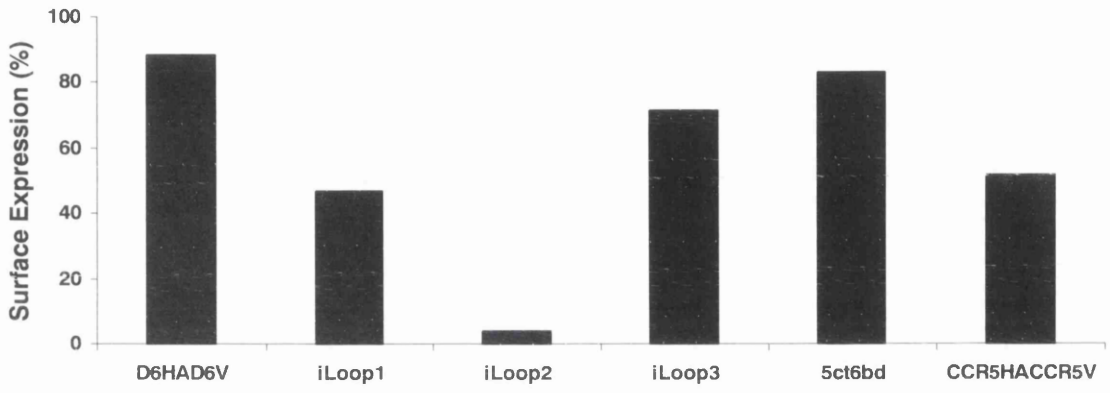


Figure 3.36. Surface expression of wild type and chimaeric receptors. HEK.293 cells were stably transfected with D6HAD6V, iLoop1, iLoop2, iLoop3, 5ct6bd and CCR5HACCR5V cDNAs and examined for expression of the HA tagged constructs by flow cytometry, following incubation with the FITC-coupled HA tag monoclonal antibody HA.11. Fluorescence of each sample was determined as a percentage of positively stained cells over background fluorescence registered for untransfected HEK.293. The results shown are representative of the surface expression for the various tagged constructs.

Chimaeric construct iLoop2 is expressed intracellularly

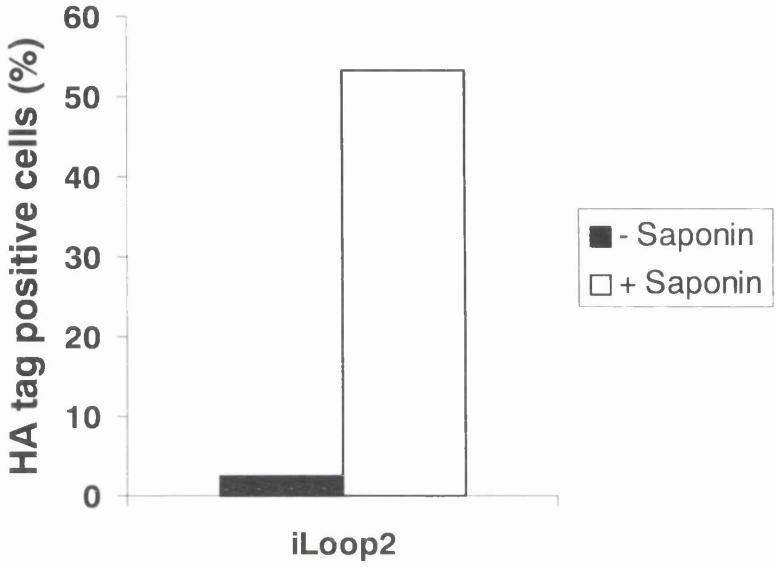


Figure 3.37. Expression of iLoop2 expressing HEK.293 cells. HEK.293 cells stably expressing iLoop2 were stained with HA.11 antibody in the presence (+) or absence (-) of saponin (0.01% v/v in PBS). Results shown are representative of the data observed in three independent experiments. Percentage of positively stained cells is determined in relation to untransfected HEK.293 cells also stained with the anti-HA antibody in the presence or absence of saponin.

^{125}I-PM2 binding to intracellular chimaeric constructs

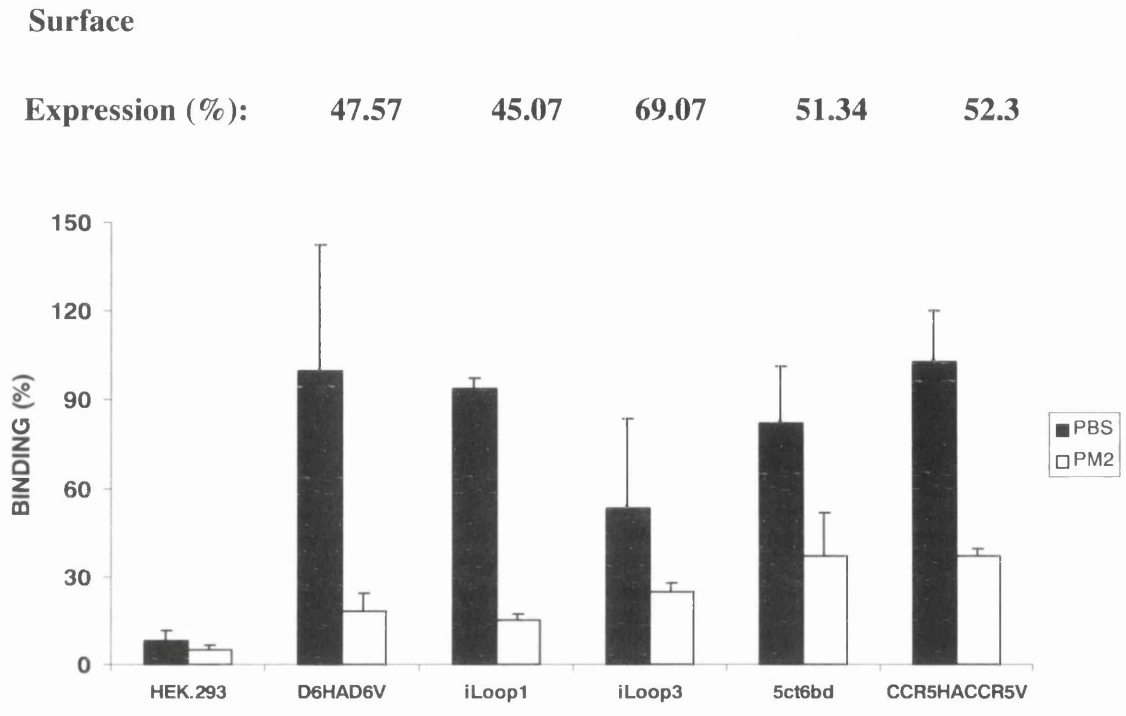


Figure 3.38. Displacement of ^{125}I-PM2 from cells expressing HA tagged wild type and chimaeric receptors. Approximately 1E6 HEK.293 cells stably expressing wild type hD6 (D6HAD6V), wild type hCCR5 (CCR5HACCR5V), iLoop1, iLoop3 and 5ct6bd were incubated with 6nM of ^{125}I-PM2 and 60nM of unlabeled PM2 or equivalent volumes of PBS. After 90 min incubation at room temperature, cells were washed with wash buffer and ice cold PBS. Remaining ^{125}I-PM2 activity was counted for 1 min in a Beckman Gamma S500B counter. The data presented is an average of two independent experiments carried out in triplicate. Percentage of binding was calculated in relation to binding registered for D6HAD6V in the absence of competitor (set arbitrarily at 100%). Error bars represent standard deviation. The percentage of cells expressing surface receptor was determined relative to untransfected HEK.293 cells using the anti-HA antibody as indicated above the graph.

Signalling by hCCR5 in response to PM2

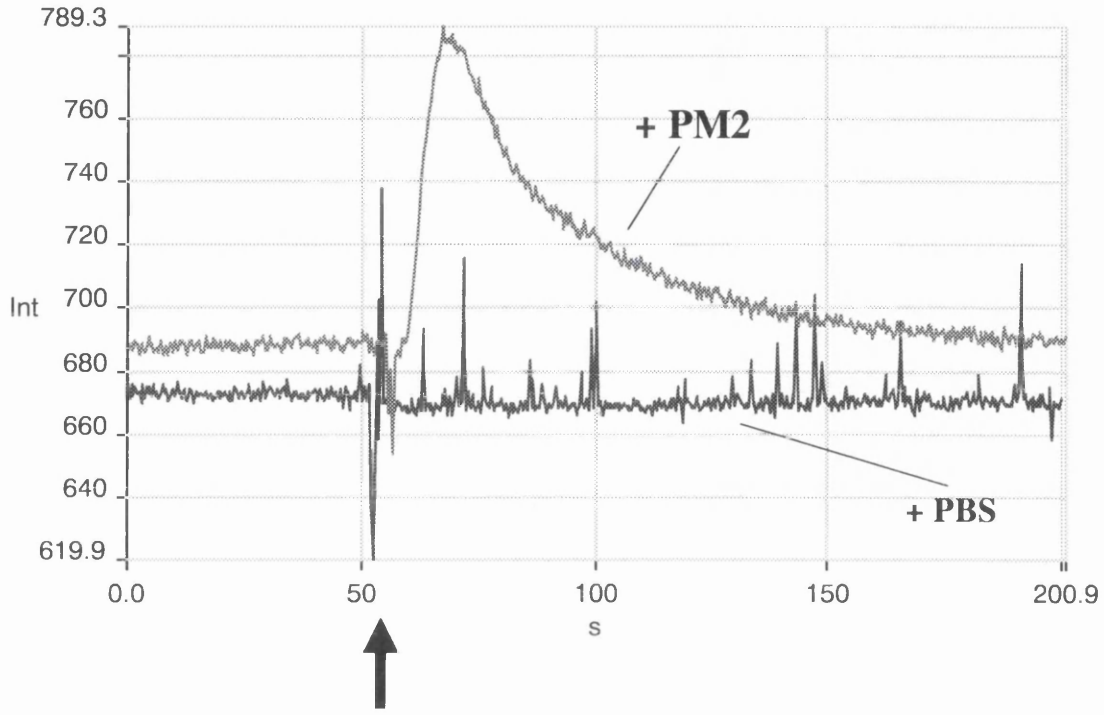


Figure 3.39. Detection of intracellular calcium in HEK.293 cells stably transfected with hCCR5 cDNA. A pool of G418-resistant HEK.293 cells expressing hCCR5 were loaded with Fura-2AM and assayed at 37°C in a continuously stirred cuvette in a Perkin-Elmer LS50 Spectrometer (340nm (λ_{ex}); 500nm (λ_{em})), with fluorescence emission recorded every 100ms for 300s as described in Section 2.2.5.6. PM2, or an equivalent volume of PBS, was added to a final concentration of 50nM at the time indicated by the black arrow. Int, intensity; s, seconds. Data shown is representative of the results observed in three independent experiments.

Schematic diagrams of iLoop2 mutants

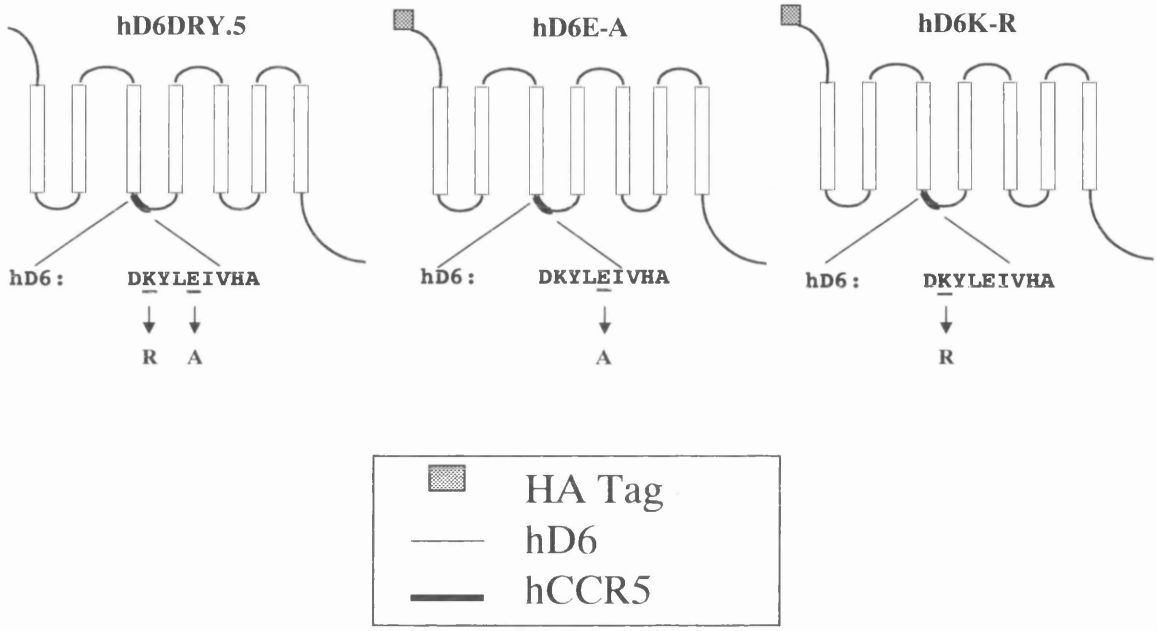


Figure 3.40. Point mutations in the second intracellular loop of hD6. hD6DRY.5 bears two point mutations that change the DKYLEI sequence of hD6 to DRYLAI as found in hCCR5. This plasmid was generated by PCR and constructed by Dr. R. Nibbs. Mutants hD6E-A and hD6K-R were generated by site directed mutagenesis to mutate residues Glu 134 to an Ala and Lys131 to an Arg, respectively. Grey square indicates HA tagged construct. Thick black lines represent area where the mutation has been introduced. Arrow points to the new residue.

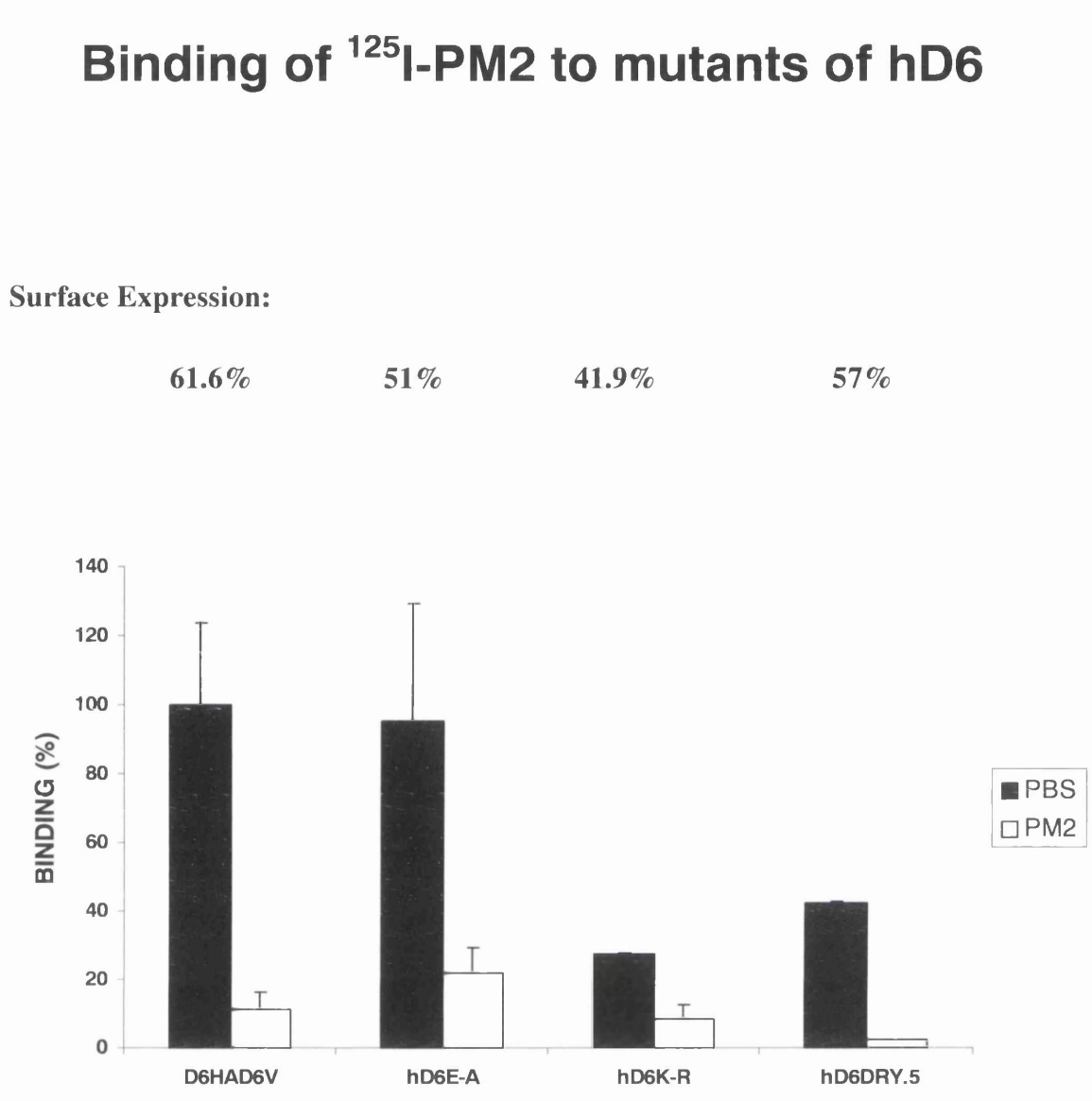
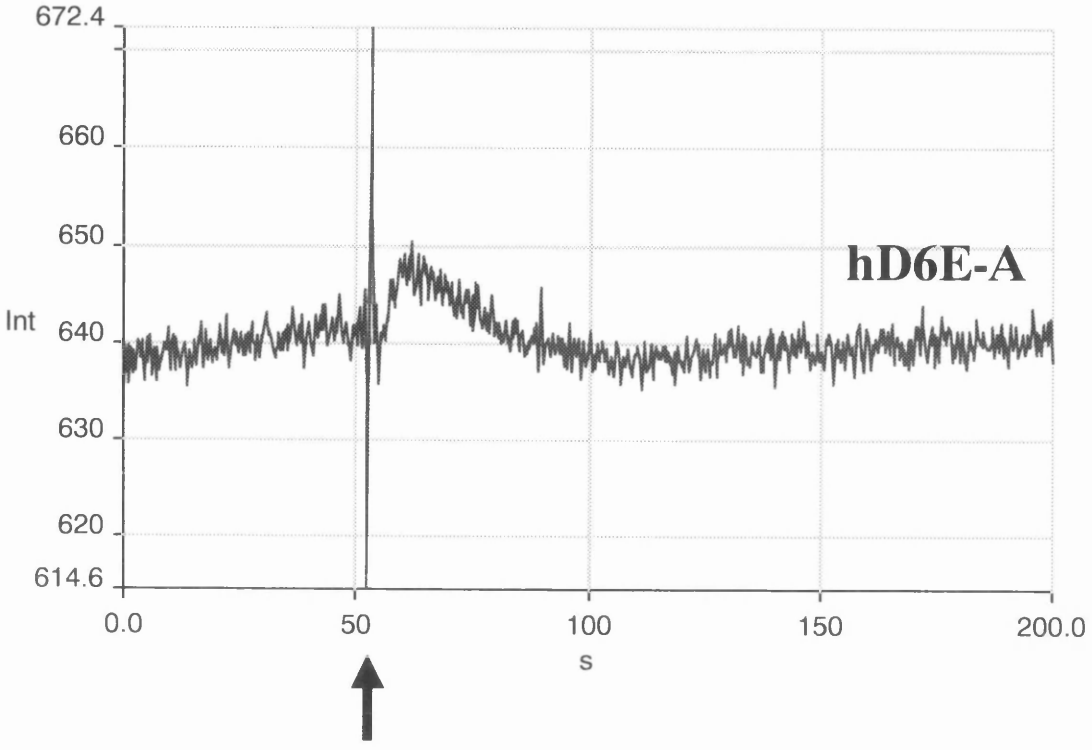


Figure 3.41. Surface expression and binding to ^{125}I-PM2 by mutant receptors of hD6. Pools of G418-resistant HEK.293 cells expressing HA tagged wild type hD6 (D6HAD6V), hD6DRY.5, hD6K-R and hD6E-A were assayed for their surface expression and ability to bind to ^{125}I-PM2. Cells were stained with the anti-hD6 specific antibody, 1D4, and surface expression determined as a percentage of positive cells in comparison to untransfected HEK.293. Transfected cells were incubated with ^{125}I-PM2 (6nM) and unlabeled PM2 (60nM) or equivalent volumes of PBS. Binding was calculated as an average of two experiments done in triplicate. Percentage of binding was calculated relative to ^{125}I-PM2 binding to D6HAD6V in the absence of competitor (set arbitrarily at 100%).

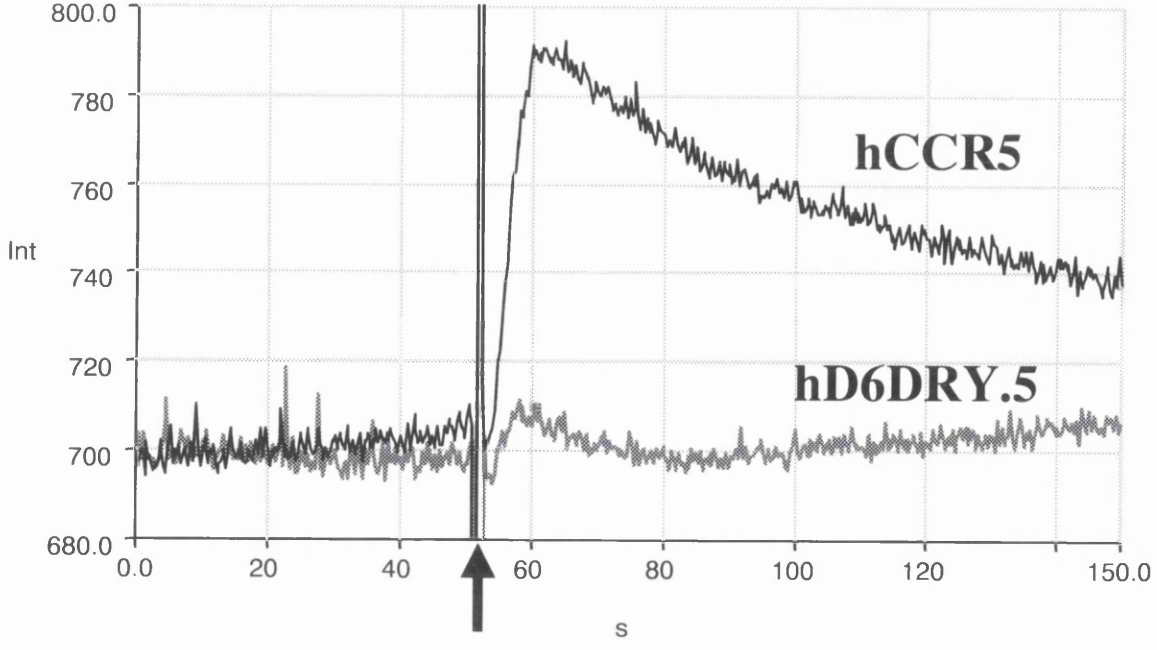
Figure 3.42. Detection of intracellular [Ca^{2+}] in HEK.293 cells stably expressing hCCR5 and the mutants hD6E-A and hD6DRY.5. Pools of G418-resistant HEK.293 cells transfected with hCCR5, hD6E-A or hD6DRY.5 cDNA, were loaded with Fura-2AM and assayed at 37°C in a continuously stirred cuvette in a Perkin-Elmer LS50 Spectrometer (340nm (λ_{ex}); 500nm (λ_{em})), with fluorescence emission recorded every 100ms for 150s. PM2 was added to a final concentration of 50nM at the time indicated by the black arrow. Data shown is representative of the observations from three independent experiments. Int, intensity; s, seconds.

Signalling by hDE-A and hD6DRY.5

A.



B.



Pertussis toxin abolishes signalling in hD6DRY.5 expressing HEK.293 cells

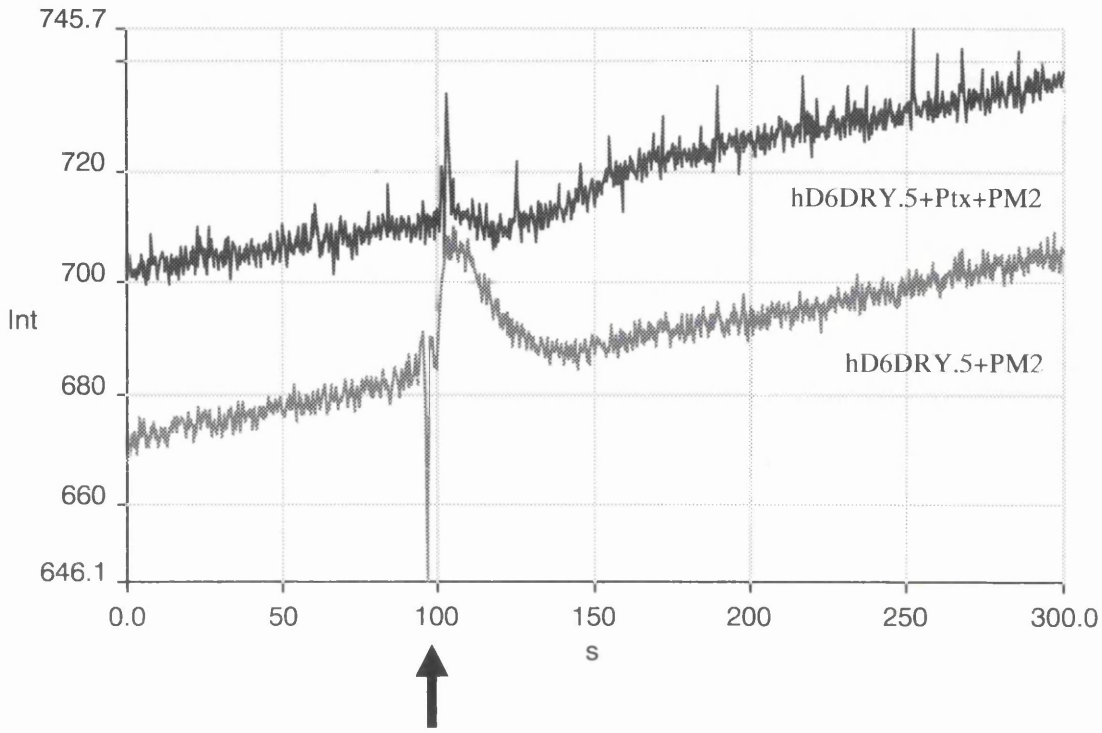


Figure 3.43. Detection of intracellular [Ca^{2+}] in HEK.293 cells stably transfected with hD6DRY.5 cDNA. HEK.293 stably expressing hD6DRY.5 were treated O/N with (+Ptx) or without 1μg/ml of Pertussis toxin the day before the calcium flux assay was performed. Untreated and treated cells were loaded with Fura-2AM and assayed at 37°C in a continuously stirred cuvette as described before. 50nM PM2 was added at the time indicated by a black arrow and fluorescence recorded every 100 ms for 300s. Int, intensity; s, seconds. Data shown is representative of the results observed in three independent experiments.

hCCR5 signalling mutants

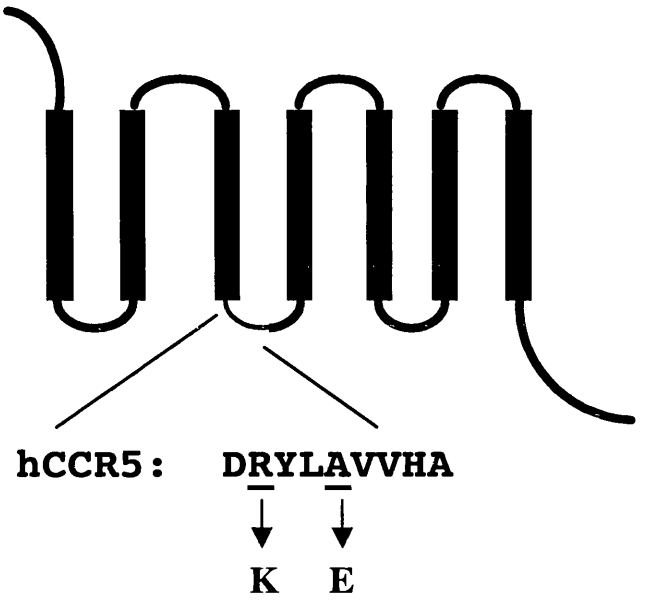
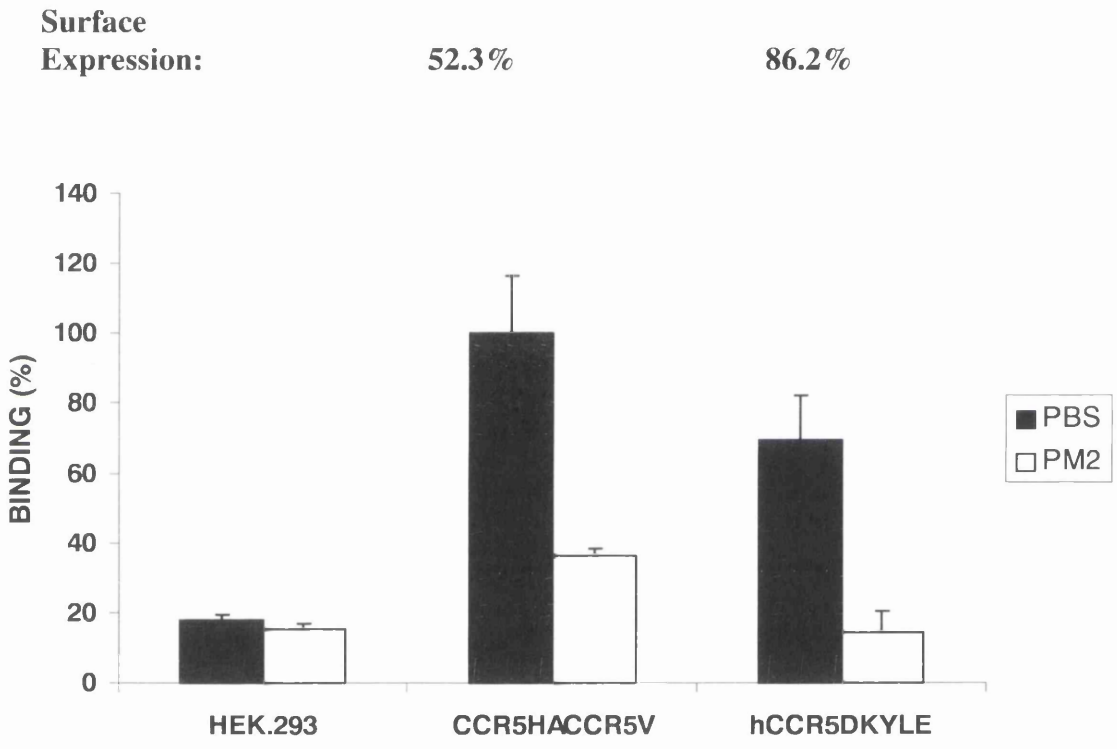


Figure 3.44. Schematic diagram of the hCCR5DKYLE mutant. Mutant hCCR5DKYLE bears two point mutations in the second intracellular loop of hCCR5. Mutated residues are underlined and arrow points to new residue(s). This mutant was generated by site directed mutagenesis.

Figure 3.45. Surface expression and ^{125}I-PM2 binding by wild type hCCR5, and hCCR5DKYLE in stably transfected HEK.293. A pool of G418-resistant HEK.293 cells expressing CCR5HACCR5V or hCCR5DKYLE were examined for surface expression by flow cytometry following incubation with the anti-hCCR5 antibody, 2D7. Fluorescence of each sample was compared to the background fluorescence given by untransfected cells and then expressed as a percentage. Untransfected and transfected HEK.293 expressing HA tagged wild type hCCR5 (CCR5HACCR5V) and hCCR5DKYLE, were incubated with 6nM ^{125}I-PM2 and 60nM PM2 or equivalent volumes of PBS. Data shown is the average of two experiments carried out in triplicate. Percentage of binding is calculated in relation to ^{125}I-PM2 binding registered for CCR5HACCR5V expressing cells in the absence of a competitor (set arbitrarily at 100%).

^{125}I-PM2 binding to hCCR5 signalling mutants



PM2 does not induce calcium flux in hCCR5DKYLE expressing HEK.293 cells

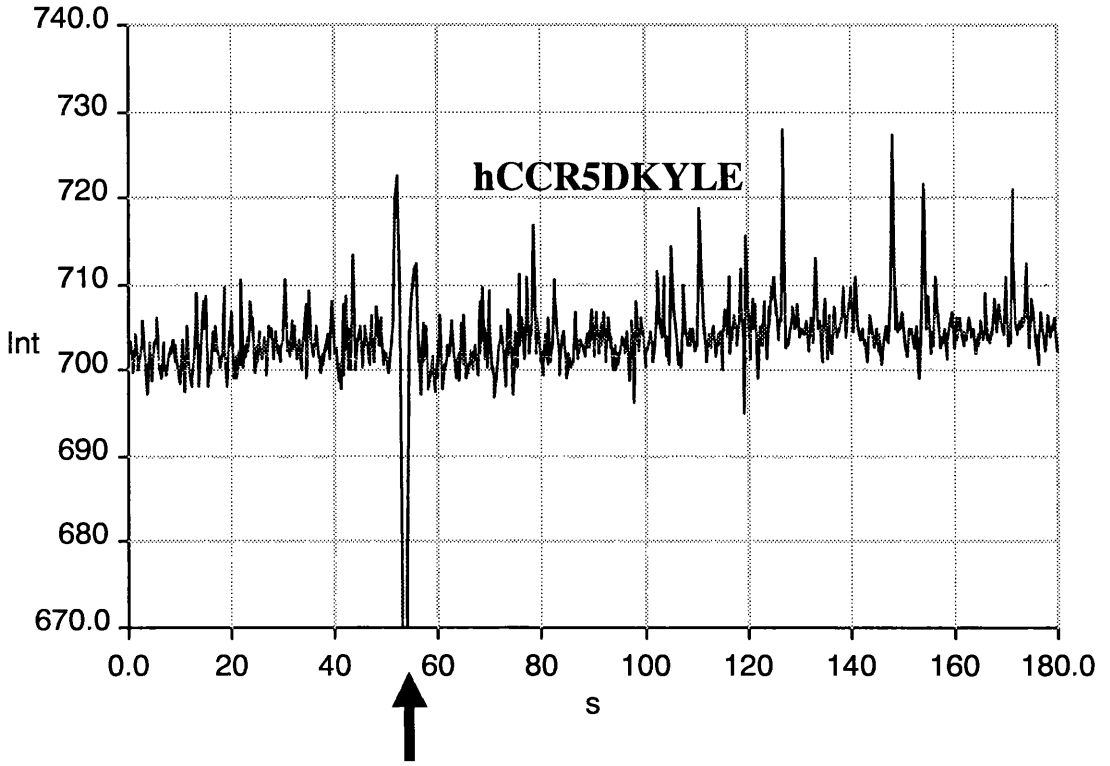


Figure 3.46. HEK.293 cells stably expressing hCCR5DKYLE do not generate a rise in intracellular [Ca^{2+}] upon stimulation with PM2. A pool of G418-resistant HEK.293 cells stably transfected with hCCR5DKYLE cDNA, were loaded with Fura-2AM and assayed at 37°C in a continuously stirred cuvette cuvette in a Perkin-Elmer LS50 Spectrometer (340nm (λ_{ex}); 500nm (λ_{em})), with fluorescence emission recorded every 100ms for 300s. PM2 was added to a final concentration of 50nM at the time indicated by the black arrow. HEK.293 cells stably expressing the signalling competent chemokine receptor hCCR5 were also tested and were seen to be capable of signalling (data not shown). Data shown is representative of three independent experiments. Int, fluorescence intensity; s, seconds.

Second generation of intracellular mutants

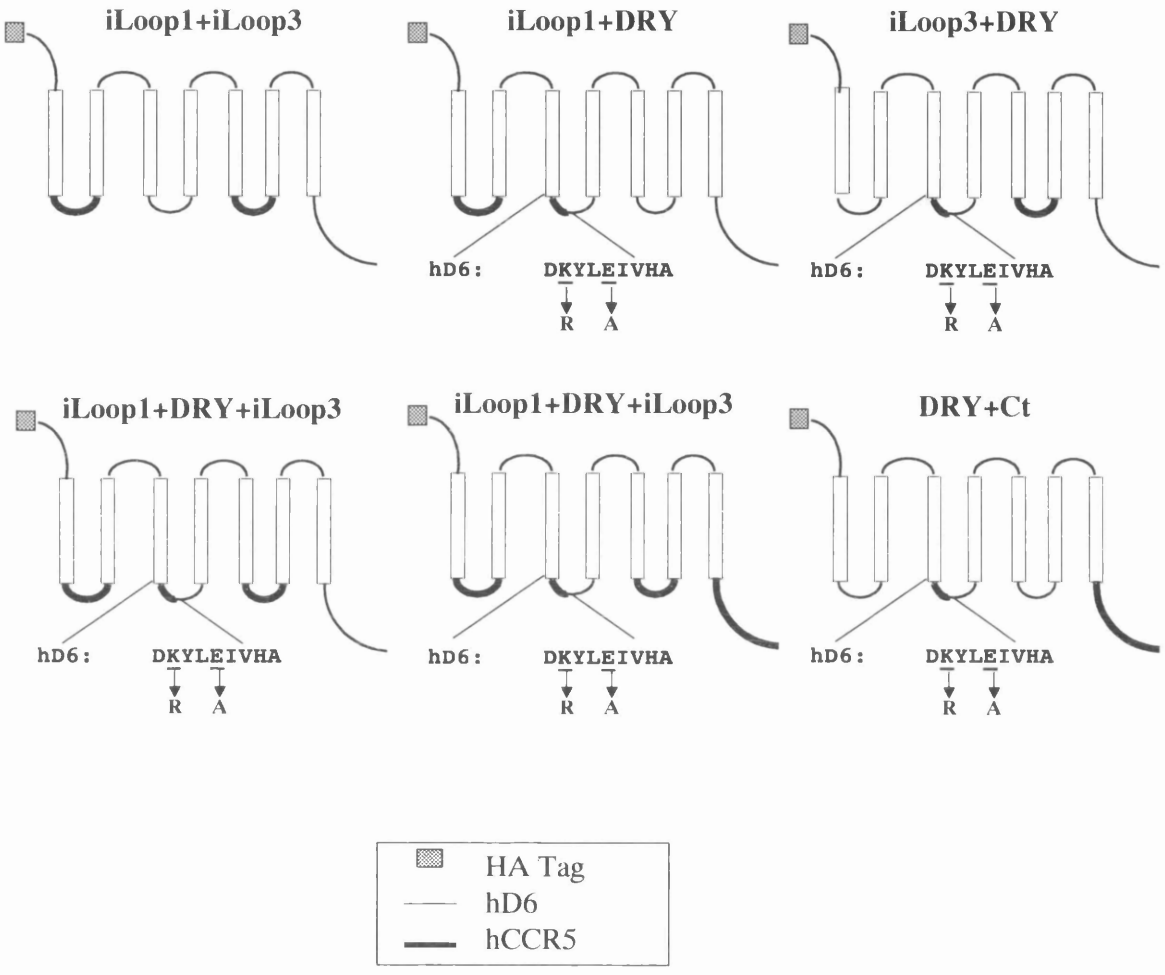


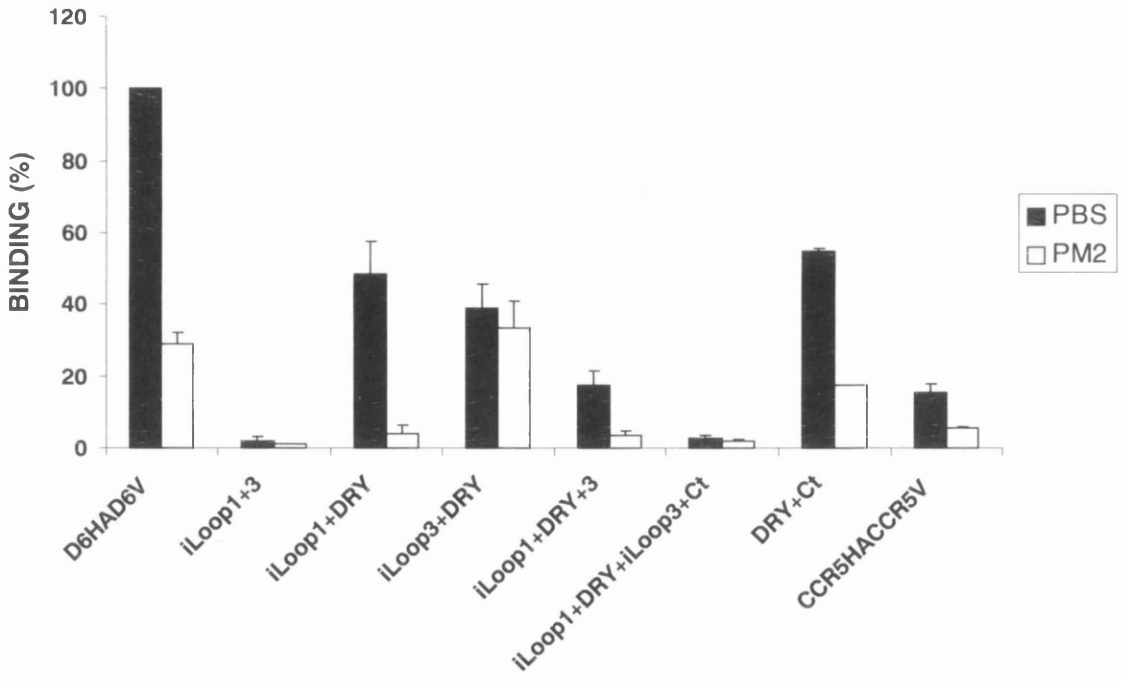
Figure 3.47. Schematic diagram of the second generation of hD6 intracellular mutants. Grey squares indicate HA tag at the N-terminus. Thin black lines indicate hD6 sequence. White rectangles indicate hD6 TM domains. Thick black lines indicate hCCR5 sequence. Arrows indicate point mutations. All cDNAs were stably transfected into HEK.293 cells. A pool of clones was obtained by selection with 800μg/ml G418.

Figure 3.48. Surface expression and ^{125}I-PM2 binding by HEK.293 cells stably expressing HA tagged hD6 (D6HAD6V), wild type hCCR5 (CCR5HACCR5V) and second generation of hD6 intracellular mutants. Surface expression of HA tagged constructs was assayed by flow cytometry after staining with the FITC-coupled anti-HA antibody, HA.11. Surface expression is shown as a percentage of positively stained cells in comparison to the background fluorescence of untransfected cells. Transfected cells were incubated with 6nM ^{125}I-PM2 and 60nM PM2 or equivalent volumes of PBS. Untransfected cells were also tested in the binding assay and showed no significant binding to ^{125}I-PM2 (not shown). ^{125}I-PM2 binding was calculated as a percentage of binding in relation to the values registered for ^{125}I-PM2 binding to D6HAD6V in the absence of PM2 (set arbitrarily as 100%). Data shown is the average of two experiments done in triplicate.

^{125}I-PM2 binding to the second generation of hD6 intracellular mutants

Surface expression (%):

88.26	20.81	49.91	13.13	14.16	33	43.48	51.76



Intracellular and surface expression of hD6 intracellular mutants

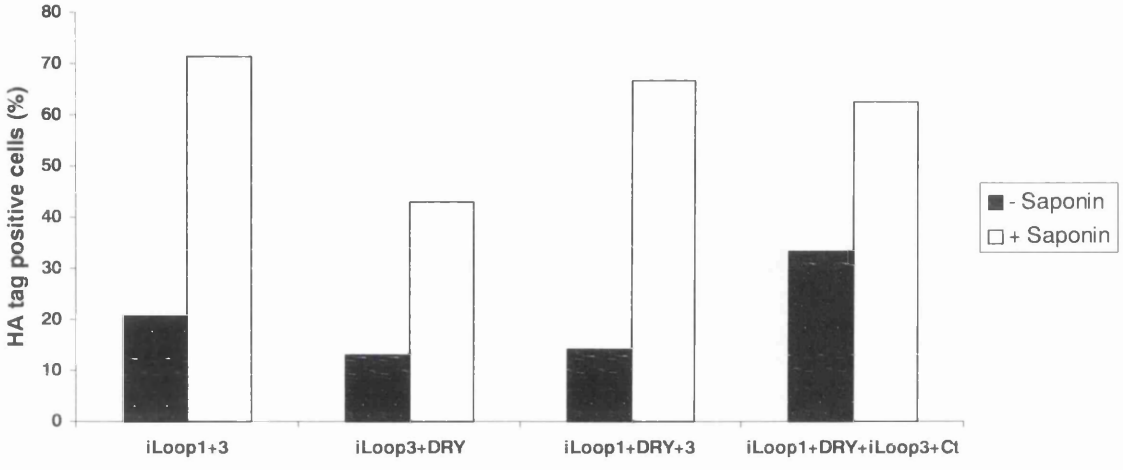
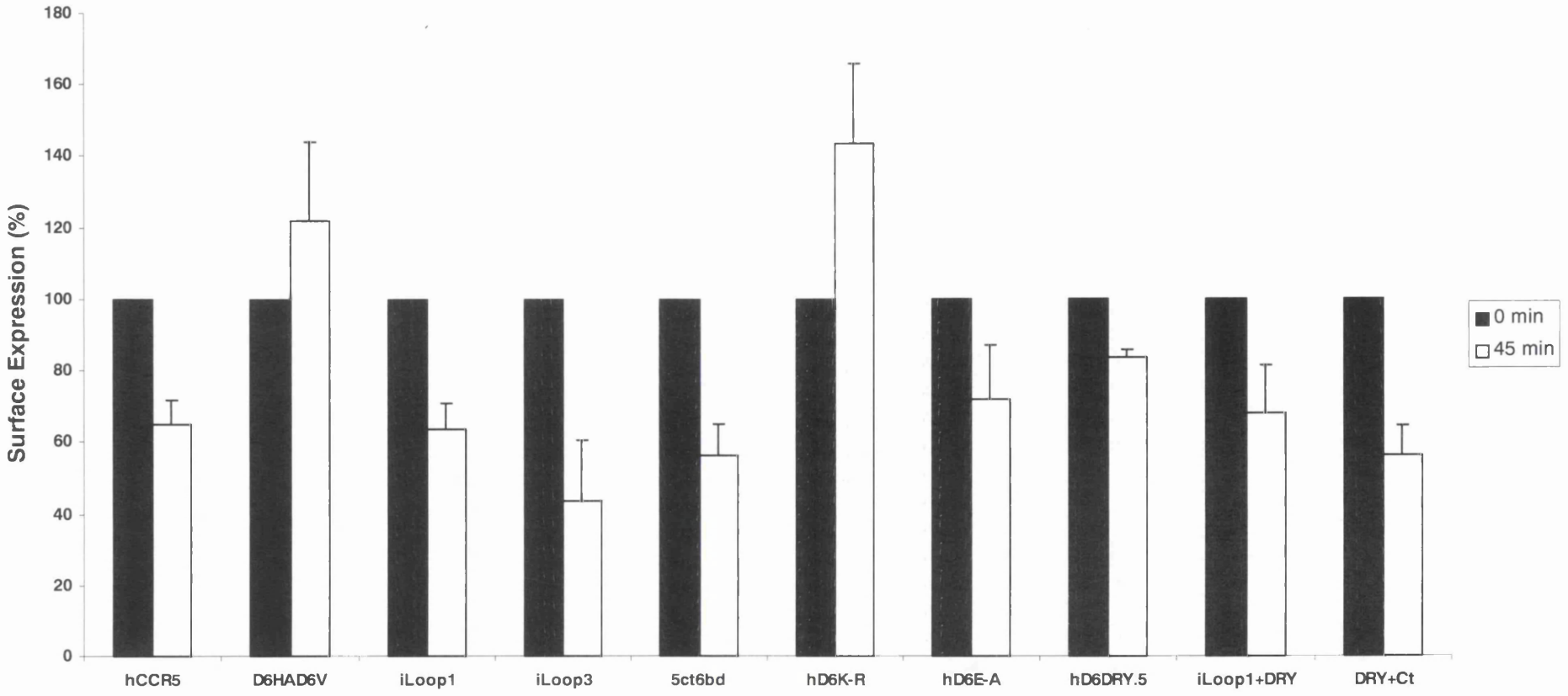


Figure 3.49. Detection of the HA tag in HEK.293 cells expressing second generation of hD6 intracellular mutants. HEK.293 cells stably expressing iLoop1+3, iLoop3+DRY, iLoop1+3+DRY or iLoop1+DRY+iLoop3+Ct were examined for expression of the HA epitope by flow cytometry. Cells were stained with the anti-HA antibody, HA.11, in the presence (+) or absence (-) of saponin. Surface expression is shown as a percentage of positively stained cells in comparison with background fluorescence of untransfected cells. Data shown is representative of the results observed in three independent experiments. Isotype controls were carried out as appropriate.

Figure 3.50. Internalization studies with HEK.293 cells expressing wild type hCCR5 (hCCR5), HA tagged hD6 (D6HAD6V) and the intracellular mutants of hD6. Cells were stained with the anti-hD6 antibody or with the anti-hCCR5 antibody (for hCCR5 expressing cells) before and after treatment with 100nM PM2 for 45 minutes at 37°C; appropriate control at 4°C was carried out and no internalisation was detected.. Surface expression of the receptors was assessed by flow cytometry and percentage of internalisation was determined according to the formula stated in Section 2.2.5.4. Data shown is the average of two independent experiments carried out in triplicate. Samples were incubated with the appropriate isotype controls and showed no unspecific staining.

Surface expression of hD6 intracellular mutants after incubation with PM2



Surface expression of hCCR5 intracellular mutants after treatment with PM2

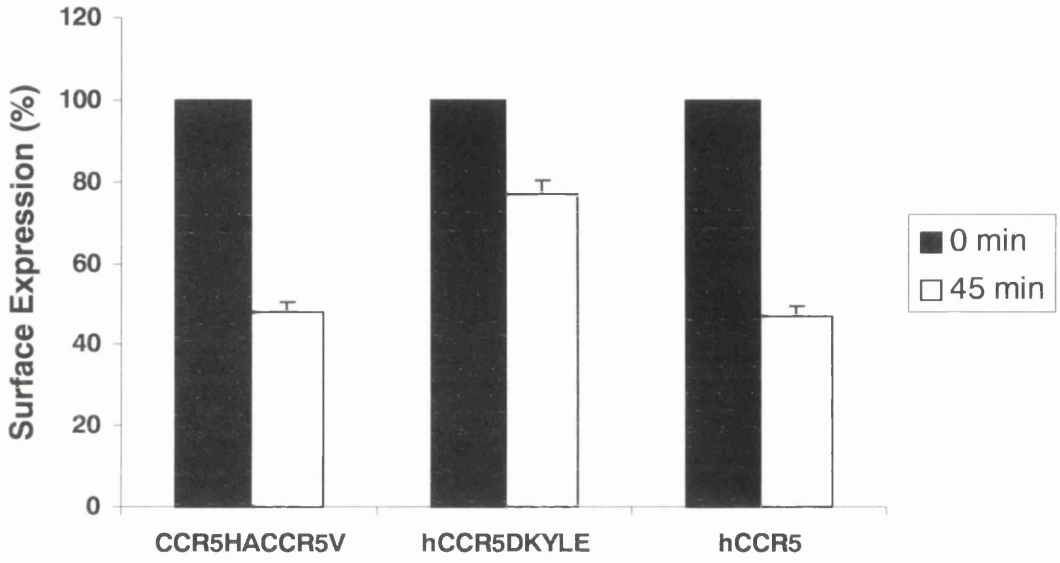


Figure 3.51. Surface expression of HEK.293 cells stably expressing wild type hCCR5 (hCCR5), HA tagged hCCR5 (CCR5HACCR5V) and the hCCR5 intracellular mutants and hCCR5DKYLE cDNAs after treatment with PM2. Cells were stained with the anti-hCCR5 antibody before (0 min) and after treatment with 100nM PM2 for 45 minutes at 37°C (45 min in graph). As a negative control, samples were also incubated in parallel at 4°C and no internalisation was detected (data not shown). Surface expression of the receptors was assessed by flow cytometry and percentage of internalisation was determined according to the formula described in Section 2.2.5.4. Data shown is the average of two independent experiments performed in triplicate. Isotype controls were carried out where appropriate (data not shown).

Chapter 4- Discussion

4.1. Identification of hD6 domains involved in ligand binding using chimaeric receptors

Generation of chimaeric constructs has been used extensively in structure-function studies of 7 TM G protein-coupled receptors. In most cases these chimaeras have been used to identify domains involved in ligand binding and receptor activation. This approach has also been shown to be useful in pinpointing segments of pharmacological importance. The high degree of homology between members of the G protein coupled-receptors has facilitated the construction of chimaeric receptors.

In order to identify the extracellular domains of hD6 responsible for its high surface expression and binding promiscuity, chimaeric receptors of hD6 and other chemokine receptors were generated. It was hoped that in these chimaeric proteins the gross structure of the receptor would be maintained allowing the role of primary sequence determinants in ligand binding to be assessed. However, as is clear from the results section, considerable technical problems involving the expression and detection of chimaeric receptors were encountered. This eventually led to the selection of small domain changes between hD6 and hCCR5 as the preferred method of analysis, using transient COS-7 cell transfection in combination with an HA epitope tag for detection of surface expression.

4.1.1. Optimising chimaeric receptor assays: construction, conformation and data interpretation

Epitope tag selection. The inclusion of an epitope tag on the N-terminus of expressed receptor was essential to permit consistent detection and quantitation of surface expression. Initially, a FLAG sequence (DYKDDDD) was used as this has been successfully employed in other similar studies (Gayle et al., 1993; Monteclaro & Charo, 1997; Pease et al., 1998). Binding of PM2 was consistently detected in several different cell lines transfected with tagged hD6 but using flow cytometry or ELISA, and two different anti-FLAG antibodies, it was not possible to detect the FLAG tag on the surface of these cells, despite it being readily detected by Western blotting. Without an adequate system to detect surface receptor, negative results in the binding assay were impossible to interpret. Moreover, there was an indication that the FLAG tag enhanced hD6 binding affinity for PM2. In fact, although some workers have used FLAG tags before with no effect on ligand binding, Perez and colleagues have shown that the FLAG epitope appears to increase spontaneous dissociation of the ligand from the FLAG tagged formyl peptide receptor when compared to the wild type receptor (Perez et al., 1993). In this study the authors suggest that the hydrophilic sequence of the FLAG might alter the positioning of the first extracellular domain causing a reduction in time of receptor occupancy. It is possible that the FLAG tag might subtly modify the position of hD6's domains involved in binding in a way that a higher affinity to PM2 is achieved. Alternatively, the highly acidic character of both the FLAG tag and the N-terminus of hD6 might increase charge in the N-terminus and possibly enhance hydrostatic interactions with the ligand.

These observations are not likely to be due to some cell-type specific phenomena since expression of these constructs in COS-7 and HOS cell lines, also shown to successfully express hD6 on their surface, did not result in successful detection of the FLAG epitope (data not shown).

The FLAG tag from D6FD6V was replaced by an HA tag (YPYDVPDYAGPG), a tag also widely used for tagging proteins specially receptors (Rucker et al., 1996, Verrall et al, 1997). Full displacement curves showed that HA tagged wild type hD6 stably expressing CHO cells (D6HAD6V) have the same affinity for PM2 as untagged wild type hD6 expressing CHO cells (hD6.1). Importantly, detection of the HA tag epitope was proven to be possible by immunocytochemistry and flow cytometry. For these reasons, it was concluded that this tag was much more useful and was therefore used in all subsequent experiments.

Expression systems for analysing chimaeric receptors. Since the HA epitope tag allowed successful detection of receptor on the cell surface, it was then possible to confidently examine tagged mIL-8RL1 and the chimaeric constructs IL8HAD6V and D6HAIL8V. However, flow cytometry profiles using the anti-HA antibody showed that expression of IL8HAIL8V, IL8HAD6V and especially that of D6HAIL8V was not good. Cells expressing D6HAD6V were, on the other hand, strongly positive for expression of this receptor on the surface of the transfected cells. Binding assays showed that no binding to ^{125}I-PM2 was detected for the chimaeric constructs or for wild type HA tagged mIL-8RL1, although binding to tagged and untagged wild type hD6 was successfully detected. This of course is not surprising when only HA-tagged hD6 was found to be expressed on the surface of these cells. The HA tagged

constructs were stably transfected into HEK.293, COS-7 and HOS cells but as with the CHO cells only HA tagged wild type hD6 was ever detectable. The poor surface expression of the chimaeric constructs was disappointing and concerning. Even more concerning was the fact that continuous passaging of transfected cell lines lead to loss of receptor expression through time. Problems with stable cell lines losing chemokine receptor expression as a result from long maintenance of cells in culture has been reported before (Power & Meyer, 2000) and has been a common problem in this research group. It seemed possible that time in culture from transfection to analysis may be responsible for the absence of surface expression seen in cell lines stably transfected with IL8HAIL8V, D6HAIL8V and IL8HAD6V.

A transient transfection system was developed to attempt to circumvent this problem. The HA tagged constructs were transiently co-transfected into CHO cells with the Kk plasmid used here to produce a marker protein on transfected cells for sorting. This approach was shown to be effective in generating an enriched population of transfected cells but the 20-40% recovery of transfected cells made it technically difficult to generate large numbers of cells for full displacement binding curves. Unfortunately, as with the stable transfectants, only D6HAD6V was shown to be expressed on the surface of sorted CHO cells.

Nonetheless, this system did represent a rapid method for screening constructs for the production of surface expressed protein. However, frustratingly, it was found that transfection efficiency could be highly variable in these transient assays, most likely dependent on plasmid or transfection reagent batch, or the confluence of the cells. Finally, transient transfection of COS-7 cells was seen to be much more reproducible.

This cell line was therefore used as the optimal rapid expression system for future analyses.

Chimaeric receptor partners and the size of the swap. At the outset of this thesis only CCRs 1 to 5, DARC and D6 had been identified within the β-chemokine receptor family. At this time, it was thought that all these receptors bound ligands that also bound to D6 (later it was shown that CCR4 is the receptor for MDC/CCL22 (Imai et al., 1998) and TARC/CCL17 (Imai et al., 1997) rather than MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 as it was first thought (Power et al., 1995)). Thus it was felt that choosing these receptors as partners for D6 could prevent the identification of domains of D6 involved in ligand binding as a result of complementation. As a result, mIL-8RL1 was chosen.

The poor surface expression of the chimaeric constructs could possibly be attributed to the fact that these constructs were obtained by fusing the domains from chemokine receptors that belong to different families. However, surface expression of wild type mIL-8RL1 could not be demonstrated by flow cytometry, immunocytochemistry or by ELISA despite presumably being naturally presented on the surface of cells in which it is present endogenously. Variability between surface expressions of chemokine receptors in transfected cell lines has been reported before (Power & Meyer, 2000). For example, the chemokine receptor CCR3 is known to be very difficult to express on the surface of heterologous cell lines (Dairaghi et al., 1997) whereas hD6 is easily highly expressed on the surface of all of the cell lines tested so far (R. Nibbs, *pers. comm.*). The reasons for this remain unknown. It is possible that some receptors require accessory proteins, or post translational modifications for

optimal surface expression that are only available in certain cell types (Farzan et al., 1999, McLatchie et al, 1998, Blanpain et al, 1999). Nonetheless, it can be concluded that mIL-8RL1 is of little use as a partner for hD6 in chimaeric receptor studies.

Wild type receptors hD6 and hCCR5 bearing a HA tag at their N-terminus were expressed at similar levels on the surface of transiently transfected CHO cells. The first chimaeric receptors, CCR5HAD6V and D6HACCR5V, generally revealed much less surface expression. As before, binding of ^{125}I-PM2 to D6HAD6V expressing CHO cells was detectable but none of the other HA tagged constructs showed displaceable binding to ^{125}I-PM2. The fact that hCCR5 stably expressing CHO cells have been shown to bind to ^{125}I-PM2 in routine binding assays in this research group ((Nibbs et al., 1999) and R. Nibbs, *pers.comm.*), suggests that the transient expression assay is not sensitive enough to detect robust binding by this receptor despite its high surface expression.

The experiments with radioiodinated MIP-1αP binding did provide some potential insight into the binding activity of the chimaeras. Unfortunately, there was great difficulty in replicating this observation due to the consistently low surface expression of the chimaeras compared to the wild type tagged proteins not just in CHO cells but in other cell types too. This in itself suggested that the chimaeras were not folding correctly and led to the belief that the changes introduced into these chimaeras may have caused gross conformational changes in receptor structure. Other researchers have used large domain swaps to generate chimaeric constructs between not only chemokine receptors but also between other members of the GPCR family without disrupting the overall structure of the mutant receptor (Doranz et al,

1999; Quehenberger et al, 1997; Kobilka, 1988; Wu et al, 1997; Samson et al, 1997). However, the absence of binding by the hD6/hCCR5 chimaeras may not be due to the effects of the primary sequence of the N-terminal tail on ligand recognition, but rather due to a global disruption in receptor structure. It is possible that hD6 possesses specific sequence determinants that are important for the overall structure of the mature protein, which has been disturbed when the chimaeric constructs were generated. In this type of chimaeric receptor studies it is important that the overall structure remains constant so that the role of primary sequence determinants can be assessed. Disruption of the global structure of a chimaeric construct has been previously reported by Maggio and colleagues that showed that mixing of domains can indeed generate non-functional proteins (Maggio et al., 1993a; Maggio et al., 1993b) So, in order to provide more robust data about ligand recognition sites in hD6, more subtle swaps were employed.

Altogether the studies discussed above were of great value for improving many technical aspects of this project. They demonstrate the advantage of the HA tag over the FLAG tag epitope, they suggest that chimaeric constructs between members of two different families of chemokine receptors may be uninformative and lastly they allowed for the development of a transient system for rapid analysis of chimaeric constructs. Indeed, all the small single domain swaps that were made, with the exception of iLoop2, were extremely well expressed on the cell surface. However, the data from the experiments with the anti-hD6 antibody, suggest that in fact even small domain swaps may affect the overall structure of the mature chimaeric protein.

4.1.2. Extracellular ligand binding domains of hD6

Four hD6/hCCR5 chimaeras were generated where the N-terminus, or the first, second or third extracellular loops of hD6 were replaced by the corresponding domains of hCCR5 to obtain constructs 5nt6bd ExtLoopA, ExtLoopB and ExtLoopC respectively (Fig. 3.18).

COS-7 cells expressing 5nt6bd behaved similarly to those expressing wild type hD6, except for their ability to interact with RANTES/CCL5. Although ^{125}I-RANTES/CCL5 bound this receptor (with slightly less efficiency than it bound wild type hD6) unlabelled RANTES was completely unable to displace ^{125}I-PM2 from this chimaera when present in a 250-fold molar excess. One possible explanation for this observation is that inclusion of the N-terminus of hCCR5 to generate 5nt6bd creates a chimaeric receptor that preferentially attracts RANTES/CCL5 to bind to this domain away from the site it usually binds to in wild type hD6. Binding of different chemokines to separate domains of a given receptor has also been demonstrated for IL-8/CXCL8 and GROα/CXCL1 on CXCR1/CXCR2 chimaeric receptors and for MCP-1/CCL2 and MIP-1α/CCL3 on CCR1/CCR2 chimaeric constructs (Ahuja et al., 1996; Monteclaro & Charo, 1997).

These data suggest that the N-terminal of hD6, after the cysteine residue is not essential for high affinity PM2 binding, or promiscuous β-chemokine binding. Evidence corroborating this comes from the work with the anti-hD6 monoclonal antibody. This antibody recognises the N-terminus of D6 (evidenced by its inability to bind to 5nt6bd), yet does not compete with PM2 in binding to wild type D6. As mentioned before the importance of the N-terminus in receptor specificity has been

well demonstrated for many chemokine receptors (LaRosa et al., 1992; Monteclaro & Charo, 1996, Doranz et al, 1999; Mizoue et al, 1999; Zhao-hai et al, 1995; Hebert et al, 1993; Pease et al., 1998, Monteclaro, 1997).

It remains possible that the N-terminus of hCCR5 is compensating for this region of hD6 in 5nt6bd. The presence of acidic residues and sulphated tyrosines, creating an overall large negative charge in this region, may be sufficient to mediate weak interaction with positively charged areas on the chemokine, with selectivity and affinity then determined by other domains. N-terminal truncations of hD6 would be useful in identifying the indispensable function of this region. Indeed, early studies on chimaeric receptors suggested that the N-terminus of hCCR5 was of little importance in ligand binding but this was shown not to be the case when N-terminal truncations of this receptor were studied (Blanpain et al., 1999). I have discussed earlier that the results with the large domain swaps could be interpreted as a crucial role for the N-terminus in hD6 ligand binding. If this is correct, these studies suggest that the small domain between the cysteine residue and the first TM domain plays a role in ligand binding. Examining this domain could prove informative in the future.

Binding of ExtLoopA expressing cells to PM2, MCP-4/CCL13 or RANTES/CCL5 was not detected. This data indicates that the first extracellular loop of hD6, the hD6's extracellular domain most conserved between species, is important for hD6 binding profile. However, because binding of ^{125}I-PM2 and ^{125}I-RANTES/CCL5 was not detected for CCR5HACCR5V expressing cells, it is not possible to determine whether ExtLoopA has reduced affinity to these ligands like hCCR5 or whether this construct has totally lost its ability to bind to these ligands. Nevertheless, these data

clearly indicate that ExtLoopA no longer behaves like wild type hD6. The epitope for the anti-hD6 antibody is not disrupted in this chimaera giving some indication that this receptor is folded like wild type hD6. It would be interesting to see if cloning of the first extracellular loop into the corresponding region of hCCR5 would be sufficient to introduce hD6's promiscuous binding profile and affinity into hCCR5.

Swapping the second or third extracellular loop with the equivalent part of hCCR5 (the amino acid sequence of which is very different) produced receptors that appeared to have reduced affinity for PM2, MCP-4/CCL13 and RANTES/CCL5, yet retained their ligand promiscuity. However, the studies with the hD6 antibody show that the epitope for this antibody may be disturbed to some extent by these changes. This is indicative that the reduction in ligand binding may be caused by the changes disrupting the overall structures of the receptor. Thus, it is felt that one cannot definitely conclude that loops B and C interact directly with the ligand. Instead, they may play a role in maintaining the structure of the ligand binding domain. Finally, as discussed for the 5nt6bd chimaera, we cannot exclude that sequence determinants in loops B and C of hD6 important in binding to ligand are compensated for in the loops of hCCR5. However, it is of note that the sequences of these loops are quite different.

The second extracellular loop has been shown to be the major determinant of ligand specificity for hCCR5 (Samson et al., 1997). Samson *et al* have shown that this domain is involved in high affinity binding to MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 and in activation of hCCR5. Cloning of hCCR5's second extracellular loop into the corresponding domain of CCR2 is sufficient to confer high affinity binding by the chimaeric receptor to the hCCR5 ligands. However the data

presented above shows that cloning of hCCR5's second extracellular loop of hCCR5 into the corresponding region of hD6 is not sufficient to reduce levels of PM2 binding to an extent that they are no longer detectable as seen for hCCR5. Also, this receptor, ExtLoopB, still binds to non-hCCR5 ligands. Thus, the fact that ExtLoopB does not behave like wild type hCCR5 suggests that in the context of this construct the ligand binding domain of hD6 is dominant over the hCCR5 ligand binding domain.

4.1.3. Conclusions and future work

Considerable energy has been put into optimising the experiments to generate robust data. However, the methods finally used to generate information about the ligand binding domains of hD6 do have their limitations. First, the use of the transient COS (or CHO) cell expression, while allowing rapid screening of constructs, restricts the number of cells available for study preventing the generation of full displacement curves. Without this, changes in receptor affinity can only be implied by examining receptor surface expression in comparison to single point binding assays. Future work should provide more detailed assessment of binding affinity of all ligands by using stable transfection protocols. In fact, preliminary data suggests that the small domain swaps are adequately expressed on stably transfected HEK.293 cells. Second, small changes may miss important binding sites deeper within the receptors. However, it is felt that experiments targeting these residues would be difficult to interpret, as it is expected that such changes may disrupt the overall structure of the chimaera.

The data collected from the hCCR5/hD6 small domain swaps studies shows that the N-terminus, the second and the third extracellular loops of hD6 are not essential for hD6's promiscuous binding profile whereas the first extracellular domain of hD6 seems to play a central role in ligand binding. The fact that the constructs ExtLoopB and ExtLoopC bind less ligand than the wild type protein could be due to an abnormal conformation of the receptors that does not allow the ligand to properly interact with the receptors. Thus, perhaps not surprisingly the ability to bind ligand may be dependent on cooperative interactions between extracellular domains that contribute to forming binding determinants (Wang et al., 1999). The importance of the first extracellular loop of hD6 for ligand binding is compatible with the high divergence between hCCR5 and hD6 in this region: out of the 19 amino acids that constitute this domain only five are the same in the two receptors. hD6 has three Ser residues in this domain that may be important for appropriate interaction with the ligand. Three serine residues are present in the second extracellular loop of hCCR5 and this loop has been shown to play a central role in high affinity binding (Samson et al., 1997). Moreover, the third extracellular loop of hCCR3 that has been shown to be essential for ligand specificity (Monteclaro & Charo, 1996) has four Ser residues in this region. Recent studies have shown that O-glycosylation of hCCR5 at Ser 6 or 7 is required for high affinity binding of MIP-1α/CCL3 and MIP-1β/CCL4 (Bannert et al., 2001). hD6 mutants bearing smaller mutations in the first extracellular loop would most certainly help identification of important residues involved in ligand binding within this domain. Point mutations of the uncharged polar Ser residues to neutral Ala residues would clarify the role of these residues in terms of ligand binding. Cloning of hD6's first extracellular loop into the corresponding region of

hCCR5 would also be useful to study the role of this domain in the context of hCCR5.

Given that construct 5nt6bd only bears the hCCR5 N-terminal sequence up to the first Cys residues cloned into the corresponding region of hD6 it is possible that the remaining 10 amino acids that constitute the N-terminal domain of this receptor might in fact be important for ligand biding and promiscuity. Therefore, it would be useful to generate an extended version of 5nt6bd where the whole of the extracellular domain of hD6 was replaced by the corresponding region of hCCR5. In fact, it has been shown for CXCR4 that the distal N-terminus of this receptor (the first 27 amino acids up to the conserved Cys) was neither necessary nor sufficient for SDF-1/CXCL12 binding but the carboxy terminal to the conserved Cys residue of the N-terminus near first TM domain is indeed required for SDF-1/CXCL12 binding (Doranz et al., 1999). However, as proposed above the N-terminus of hCCR5 or indeed the extracellular loops B and C may functionally replace that of hD6. Therefore, truncations or more subtle targeted mutations may be a more productive and informative approach.

4.2. Signalling studies

Construction of chimaeric receptors has been shown to be useful to determine domains of the host receptor that are involved in ligand binding, receptor activation and signalling (Schwartz, 1994). It was decided that by constructing chimaeras of hD6 and hCCR5 it would be possible to investigate the function of intracellular domains. Initially, it was thought that by replacing the intracellular domains of hD6 by the corresponding domains of hCCR5 one could determine the roles of these domains in signalling through hCCR5. Moreover, it was thought that these constructs would also provide some information about the biochemistry and function of hD6. HEK.293 cells were used, as these have been show to provide a cell background that gives robust signalling to calcium ion fluxes through exogenous hCCR5, but no detectable signals when cells expressing hD6 were challenged with ligand. Furthermore, hCCR5 has been shown to be readily internalised into these cells upon ligand binding (R. Nibbs *pers. Comm.* and (Ling et al., 1999)) whereas hD6 is not (R. Nibbs *pers. comm.*).

4.2.1. The second intracellular loop: cell surface expression and signalling

Four chimaeric constructs were generated where the intracellular loops of hD6 were independently replaced by the corresponding domains of hCCR5. All constructs, except for iLoop2, produced proteins that were detected on the surface of HEK.293 cells. iLoop2 protein was only detected after permeabilisation of the transfected cells

with saponin. It should be noticed that this construct, iLoop2, is the only chimaera made using single small domain swaps, either intracellular or extracellular, which is not detectable on the cell surface. Given that hD6DRY protein, which has essentially the first nine amino acids of this loop from hCCR5 cloned into hD6 is trafficked as efficiently as wild-type hD6 to the cell surface it seems fair to suggest that the carboxyl-terminal portion of the second intracellular loop of iLoop2 is responsible for the lack of cell surface expression.

This observation suggests that iLoop2 has some kind of structural problem that affects folding of the mature protein to an extent that trafficking to the cell surface is affected. It is possible that the precise positioning of the TM domains is affected when the second intracellular loop of hD6 is replaced by the corresponding loop of hCCR5. Alternatively, as demonstrated for other GPCRs (Arai & Charo, 1996), interaction of accessory proteins with the second intracellular loop of hD6 might be necessary for cell surface expression of hD6. It should be noted that hD6 carries a conserved proline residue in the middle of this loop (Pro 140) that is only seen in two other receptors CCR10 (Pro 147) (Jarmin et al., 2000) and CCR11 (Pro 125) (Schweickart et al., 2000). Interestingly, CCR11 like hD6 is not capable of eliciting a calcium flux upon ligand binding when expressed on HEK.293 cells (R. Nibbs, *Pers. Comm.*). It is possible that this Pro residue may introduce a kink into the loop leading to alteration of the relative positions of the third and/or fourth TM domains. Alternatively, the high positive charge on this loop (four basic residues in hD6 and only two in hCCR5) might play a role in surface expression of the wild type protein. Further mutagenesis of this region would address these issues.

Although good surface expression and binding to PM2 was observed for iLoop1, iLoop3 and 5ct6bd none of these chimaeric constructs were shown to be capable of eliciting a calcium flux upon ligand binding. These data were curious given that previous reports have shown that the C-terminus and the third intracellular loop of a given chemokine receptor are important for coupling to G proteins and indicate that in the context of hD6 these domains of hCCR5 are not sufficient to independently allow coupling of the mutant receptors to the same G proteins that couple to hCCR5. For example, replacement of the third intracellular loop of CXCR1 by the corresponding domain of CCR2b is sufficient to make this new mutant receptor couple to Gα16 proteins known to couple to wild type CCR2b (Arai & Charo, 1996). Conversely, replacement of the same loop in CCR2b for the corresponding region of CXCR1 generates a mutant receptor that has lost its ability to couple to Gαq but retained its ability, albeit impaired, to couple to Gα16 (Arai & Charo, 1996). Moreover, studies with C-terminal truncations of hCCR5 have shown that this region of hCCR5 is important for the ability of this receptor to initiate signalling (Gosling et al., 1997).

To further investigate the role of the second intracellular loop in the context of hD6 and at the same time study the effects caused by introducing changes in the DKYLEIV motif, smaller changes were introduced in this region. Two constructs were generated: hD6K-R, where the residue Lys131 was mutated to an Arg, and hD6E-A, where the Glu134 residue was mutated to an Ala (mutant hD6E-A). Mutant hD6DRY.5 bears two point mutations that change Lys131 to an Arg and Glu134 to an Ala. Flow cytometry and radiolabelled ligand binding analysis revealed that HEK.293 cells transfected with these mutant cDNAs expressed abundant surface

protein, and that this protein bound ligand. It was noticed that the K to R change might possibly reduce ligand binding, however, without full displacement binding curves one cannot be certain of this observation.

Of these three constructs only hD6E-A and hD6DRY.5 were shown to be able to elicit a calcium flux and become internalised in the presence of ligand. hD6E-A and hD6DRY.5 flux much less calcium than hCCR5 indicating that the response generated by the mutant receptors is much weaker than that registered for wild type hCCR5. Nevertheless, these studies show that a single point mutation in hD6 (E134A) introduces weak ligand-induced coupling to calcium ion fluxes. Moreover, the ability of hD6DRY.5 to signal was shown to be inhibited by Pertussis toxin. This indicates that this construct couples to Ptx-sensitive G proteins (most probably $G\alpha_i$) in order to flux calcium upon ligand binding. Additionally, when the DRYLAIV motif was changed to DKYLEIV, hCCR5 could no longer couple to calcium ion fluxes upon ligand binding and was not internalised as efficiently as the wild type receptor. Although, single point mutations were not generated, it is likely that the A to E change in the hCCR5DKYLE construct is principally responsible for lack of signalling. This evidence highlights, as shown before (Gosling et al., 1997), the importance of the DRYLAIV motif of hCCR5 in signalling and its sensitivity to mutation.

A second generation of hD6's intracellular mutants was generated in order to investigate the cooperative role of hCCR5 intracellular domains in the context of hD6; however, none of them produced detectable calcium ion fluxes upon ligand binding. In fact, the data from these mutants indicates that swapping of multiple

domains between receptors generates constructs that are often compromised in their ability to assemble correctly on the surface of the transfected cells. Thus, the lack of detectable calcium flux after stimulation of the transfected cells expressing combinations of the 'DRY' motif and one or more domains of hCCR5 cannot be confidently attributed to a loss of signalling by the mutant receptor but is probably a reflection of its poor surface expression. Given that signalling is detectable in hCCR5 transfectants, even at very low receptor occupancy, it seems fair to conclude that the additional domains of hCCR5 introduced along with the DRY mutation do not cause a dramatic enhancement of signalling.

4.2.2. Chimaeric receptor internalisation assays: evidence of alternative signalling pathways?

The studies discussed above generated mutants of hD6 to which a calcium ion flux is detectable (hD6E-A, hD6DRY.5) and a mutant of hCCR5 (hCCR5DKYLE) to which calcium ion flux is not detectable. As expected, these mutants behave differently from their wild type receptors counterparts when internalisation assays were performed. However, it was notable that hCCR5DKYLE was still internalised to some extent. What was even more surprising was that the hD6 mutants in which a single intracellular domain had been changed were also shown to be readily internalised after incubation with the ligand. Moreover, these mutants of hD6, were seen to be more efficiently removed from the cell surface than the signalling competent hD6 mutants, hD6E-A and hD6DRY.5. Furthermore, internalisation of hD6DRY.5 was increased by the addition of the first intracellular loop or the C-terminus from wild type hCCR5.

These intriguing and unexpected data suggest that ligand-induced signals must be coming from the chimaeric receptors in order to mediate internalisation. These signals appear to be independent from G-proteins regulated calcium ion fluxes although it is not possible to rule out the possibility that chimaeras like iLoop1 might be capable of inducing a calcium flux that is below the levels of detection of the assay. It is also possible that the chimaeric receptors might have acquired the ability of constitutively internalising ligand and recycling to the surface, and that the reduction in cell surface receptor upon stimulation with the ligand is actually due to the ligand preventing receptor recycling to the surface. Nevertheless, the data does show that any single intracellular domain of hCCR5 is sufficient to completely change the trafficking of hD6.

4.2.3. Conclusion and future work

In conclusion, the intracellular mutants of hD6 demonstrate the importance of the second intracellular loop for surface expression of this receptor and show that a single point mutation in this loop that changes the glutamine residue at position 134 to an alanine is sufficient to elicit calcium flux by this mutant receptor in the presence of ligand. Also, these studies emphasize again the importance of the DRY motif found in the second intracellular loop of hCCR5. Moreover, these studies show that any changes in the intracellular domains of hD6, except for the conservative mutation that changes the lysine residue at position 131 to an arginine, are sufficient to mediate ligand-induced downregulation of hD6 expression from the surface of the transfected cells. Furthermore, these studies provide evidence that suggests that

internalisation and calcium flux in response to ligand binding can be independent from each other.

These studies have generated a collection of data that deserves further investigation. Detailed analysis of the C-terminal part of the second intracellular loop should help to identify residues important in receptor conformation involved in surface presentation of hD6. These studies should be concentrated on making smaller mutations in this region (particularly altering the Pro residue at position 140 as well as neutralisation of basic residues) followed by surface expression assays.

Other studies should be carried out to identify signals coming from the chimaeric receptors, iLoop1, iLoop3 and 5ct6bd, which are responsible for ligand-induced internalisation. It is possible that these three chimaeric constructs are capable of inducing heterotrimeric G-protein complexes distinct from those measured by calcium flux assays. Internalisation of GPCRs requires phosphorylation of the receptor by serine-threonine kinases, like GRKs or PKC (Lefkowitz, 1998), and these might be activated upon ligand binding to these chimaeras. In fact, these chimaeric constructs are probably a much cleaner background to identify signals involved in receptor internalisation without the interference from other signals.

However, there is an alternative interpretaion of the results, which was raised earlier, and which requires further investigation. It is possible that the chimaeric receptors have actually become constitutively active. It is feasible that the reason why the ligand-induced calcium ion flux is so small in cells expressing hD6E-A or hD6DRY.5, is that these receptors already have a high basal signalling level and that only very small increases in this are possible. Likewise, the observed ligand-induced

internalisation of chimaeric receptor may actually be an indication that these receptors are constitutively active, and are therefore internalised in the absence of ligand and subsequently recycled back to the surface. It is possible that ligand treatment might in fact prevent recycling to the surface rather than be responsible for internalisation. This matter could be investigated in a transient transfection assay where the accumulation of intracellular second messengers, such as inositol phosphate would be measured. These types of studies have been used to show constitutive signalling by several viral chemokine receptors and mutated human receptors (Allen et al., 1991; Arvanitakis et al., 1997; Burger et al., 1999; Casarosa et al., 2001; Samama et al., 1993; Scheer, 1996).

Another important question that remains unanswered is whether wild type hD6 is capable of eliciting signals upon ligand binding. The results collected so far show that small changes in the intracellular domains of hD6 create signalling-competent receptors, as shown by calcium flux assays. It is possible that the changes introduced in order to generate the chimaeric constructs are sufficient to alter D6 structure in such a fashion that this receptor acquires the ability to undergo the conformational change(s) necessary for ligand-induced signalling. Perhaps a more likely explanation is that wild type hD6 is already capable of undergoing ligand-induced conformational change, and that the chimaeras allow this event to be detected by coupling to calcium ion fluxes and of internalisation. Nonetheless, identification of second messengers coupled to ligand-bound hD6 awaits further studies. This could be achieved by carrying out experiments where additional G proteins were introduced into HEK.293 cells previously transfected with hD6. Alternatively, analysis of cells that naturally express hD6 might be more useful. Recently published work describing the culture of

lymphatic endothelial cells from human dermis may prove extremely interesting in improving our understanding of D6 signalling and function (Makinen et al., 2001, Kriehuber, 2001).

Chapter 5- Concluding remarks

The studies described in this thesis highlight the importance of careful design and interpretation of work involving chimaeric chemokine receptors. Chimaeric constructs bearing domains of a CXC chemokine receptor and a CC chemokine receptor are unlikely to be appropriately transported to the cell surface of heterologous cell lines and therefore unlikely to be of use in the study of chemokine receptors. Moreover, in my hands, large domain swaps between members of the same CC chemokine receptor family are likely to also have conformational problems and for this reason have a poor surface expression. Small domain changes are less likely to dramatically alter the overall conformation of the receptor, allowing the investigation of primary sequence determinants on receptor function. Whilst these initial experiments proved disappointing and frustrating, they allowed for the optimisation of chimaeric receptor studies that should influence future interpretation of these kind of experiments.

The chimaeric receptors have demonstrated the importance of the receptor's first extracellular loop for ligand binding. The hD6 intracellular mutants have shown that the second intracellular loop is important for surface expression of this receptor and that a single point mutation within this loop is sufficient to elicit calcium flux upon ligand binding. The internalisation assays carried out for the many intracellular mutants indicate that receptor trafficking is altered by manipulating any of the intracellular domains. The hCCR5DKYLE mutant has once again emphasized the importance of the DRY motif for signalling transduction.

All in all these studies show that although chemokine receptors are thought to share the same structure and conformation, many domains and residues play fundamental roles in conformation, ligand binding, receptor trafficking and signalling of a given receptor. It is important to bear in mind at the time of constructing chimaeric proteins that any effect seen with a certain mutant may not be a direct reflection of the function of the primary sequence mutated, but could instead be a phenotype that reflects a new overall conformational adapted by the new construct in which many distant domains may be affected.

Bibliography

Abi-Younes, S., Sauty, A., Mach, F., Sukhova, G.K., Libby, P. & Luster, A.D. (2000). The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. *Circ Res*, **86,** 131-8.

Adema, G.J., Hartgers, F., Verstraten, R., de Vries, E., Marland, G., Menon, S., Foster, J., Xu, Y., Nooyen, P., McClanahan, T., Bacon, K.B. & Figdor, C.G. (1997). A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature*, **387,** 713-7.

Ahuja, S.K., Lee, J.C. & Murphy, P.M. (1996). CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. *J Biol Chem*, **271,** 225-32.

Ahuja, S.K. & Murphy, P.M. (1993). Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. *J Biol Chem*, **268,** 20691-4.

Aiuti, A., Webb, I.J., Bleul, C., Springer, T. & Gutierrez-Ramos, J.C. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med*, **185,** 111-20.

Alam, R., York, J., Boyars, M., Stafford, S., Grant, J.A., Lee, J., Forsythe, P., Sim, T. & Ida, N. (1996). Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients. *Am J Respir Crit Care Med*, **153,** 1398-404.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, j.D. (1989). Chapter 18- The Immune System. In *Molecular Biology of the Cell*, Robertson, M. (ed) pp. 1001-1058. Garland Publishing, Inc.: New York.

Albini, A., Ferrini, S., Benelli, R., Sforzini, S., Giunciuglio, D., Aluigi, M.G., Proudfoot, A.E., Alouani, S., Wells, T.N., Mariani, G., Rabin, R.L., Farber, J.M. & Noonan, D.M. (1998). HIV-1 Tat protein mimicry of chemokines. *Proc Natl Acad Sci U S A*, **95,** 13153-8.

Alcami, A. & Koszinowski, U.H. (2000). Viral mechanisms of immune evasion. *Trends Microbiol.*, **410,** 410-418.

Alcami, A. & Smith, G.L. (1995). Cytokine receptors encoded by poxviruses: a lesson in cytokine biology. *Imm. Today*, **16,** 474-478.

Alcami, A., Symons, J.A., Collins, P.D., Williams, T.J. & Smith, G.L. (1998). Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. *J Immunol*, **160,** 624-33.

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M. & Berger, E.A. (1996). CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science*, **272,** 1955-8.

Alkhatib, G., Locati, M., Kennedy, P.E., Murphy, P.M. & Berger, E.A. (1997). HIV-1 coreceptor activity of CCR5 and its inhibition by chemokines: independence from G protein signaling and importance of coreceptor downmodulation. *Virology*, **234,** 340-8.

Allen, L.F., Lefkowitz, R.J., Caron, M. & Cotecchia, S. (1991). G-protein-coupled receptor genes as protooncogenes: constituively activating mutation of the $\alpha_{1\beta}$-adrenergic receptor enhances mitogenesis and tumorigenicity. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 11354-11358.

Anborgh, P.H., Dale, L., Seachrist, J. & Fergusson, S.S.G. (2000). Differential regulation of β2-adrenergic and angiotensin II type 1A receptor trafficking and resensitization: role of the carboxylterminal domains. *Mol. Endocrinol.*, **14,** 2040-2053.

Arai, H. & Charo, I.F. (1996). Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem*, **271,** 21814-9.

Aramori, I., Ferguson, S.S., Bieniasz, P.D., Zhang, J., Cullen, B. & Cullen, M.G. (1997). Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor [published erratum appears in EMBO J 1997 Oct 1;16(19):6055]. *Embo J*, **16,** 4606-16.

Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M.C. & Cesarman, E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature*, **385,** 347-50.

Baggiolini, M. (1998). Chemokines and leukocyte traffic. *Nature*, **392,** 565-8.

Baggiolini, M. & Dahinden, C.A. (1994). CC chemokines and allergic inflammation. *Immunol. Today*, **15,** 127-133.

Baggiolini, M., Dewald, B. & Moser, B. (1997). Human chemokines: an update. *Annu Rev Immunol*, **15,** 675-705.

Baggiolini, M., Moser, B. & Clark-Lewis, I. (1994). Interleukin-8 and related chemotactic cytokines. The Giles Filley Lecture. *Chest*, **105,** 95S-98S.

Bais, C., Santomasso, B., Coso, O., Arvanitakis, L., Raaka, E.G., Gutkind, J.S., Asch, A.S., Cesarman, E., Gershengorn, M.C., Mesri, E.A. & Gerhengorn, M.C. (1998). G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator *Nature*, **391,** 86-9.

Balashov, K.E., Rottman, J.B., Weiner, H.L. & Hancock, W.W. (1999). CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci U S A*, **96,** 6873-8.

Bannert, N., Craig, S., Farzan, M., Sogah, D., Santo, N.V., Choe, H. & Sodroski, J. (2001). Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J. Exp. Med.*, **194,** 1661-1673.

Bao, L., Humbles, A., Bota, D., Gerard, C., Moser, B., Soler, D., Luster, A.D. & Gerard, N.P. (1999). Structure and function of the murine chemokine receptor CXCR3. *Eur. J. Immunol.*, **29,** 3804-3812.

Barak, L.S., Menard, L., Ferguuson, S.S., Colapietro, A.M. & Caron, M.G. (1995). The conserved seven-transmembrane sequence NP(X)2,3y of the G-protein-coupled receptor superfamily regulates multiple properties of the beta-2-adrenergic receptor. *Biochemistry*, **34,** 15407-15414.

Barlow, D.J. & Thornton, J.M. (1988). Helix geometry in proteins. *J. Mol. Biol.*, **201,** 601-619.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A. & Schall, T.J. (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature*, **385,** 640-4.

Belperio, J.A., Keane, M.P., Arenberg, D.A., Addison, C.L., Ehlert, J.E., Burdick, M.D. & Strieter, R.M. (2000). CXC chemokines in angiogenesis. *J Leukoc Biol*, **68,** 1-8.

Ben-Baruch, A., Bengali, K.M., Biragyn, A., Johnston, J.J., Wang, J.M., Kim, J., Chuntharapai, A., Michiel, D.F., Oppenheim, J.J. & Kelvin, D.J. (1995). Interleukin-8 receptor beta. The role of the carboxyl terminus in signal transduction. *J Biol Chem*, **270,** 9121-8.

Bennet, T.A., Maestas, D.C. & Prossnitz, E.R. (2000). Arrestin binding to the G protein-coupled N-formyl peptide receptor is regulated by the conserved 'DRY' sequence. *J. Biol. Chem*, **275,** 24590-24594.

Berg, E.L., Robinson, M.K., Warnock, R.A. & Butcher, E.C. (1991). The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J. Cell Biol.*, **114,** 343-349.

Berger, E.A., Murphy, P.M. & Farber, J.M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol*, **17,** 657-700.

Bertrand, C.P. (2000). Asthma: were beyond steroids? *Curr. Op. Chem. Biol.*, **4,** 407-411.

Blanpain, C., Doranz, B.J., Vakili, J., Rucker, J., Govaerts, C., Baik, S.S., Lorthioir, O., Migeotte, I., Libert, F., Baleux, F., Vassart, G., Doms, R.W. & Parmentier, M. (1999). Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein. *J Biol Chem*, **274,** 34719-27.

Blanplain, C., Wittamer, V., Vanderwinden, J., Boom, A., Renneboog, B., Lee, B., Poul, E.L., Asmar, L., Govaerts, C., Vassart, G., Doms, R.W. & Parmentier, M. (2001). Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellualr signaling pathways. *J. Biol. Chem.*, **276,** 23795-23804.

Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J. & Springer, T.A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature*, **382,** 829-33.

Bodaghi, B., Jones, T.R., Zipeto, D., Vita, C., Sun, L., Laurent, L., Arenzana-Seisdedos, F., Virelizier, J.L. & Michelson, S. (1998). Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. *J Exp Med*, **188,** 855-66.

Boisvert, W.A., Curtiss, L.K. & Terkeltaub, R.A. (2000). Interleukin-8 and its receptor CXCR2 in atherosclerosis. *Immunol Res*, **21,** 129-37.

Bokoch, G.M. (1995). Chemoattractant signalling and leukocyte activation. *Blood*, **86,** 1649-1660.

Boring, L., Gosling, J., Cleary, M. & Charo, I.F. (1998). Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*, **394,** 894-7.

Boshoff, C., Endo, Y., Collins, P.D., Takeuchi, Y., Reeves, J.D., Schweickart, V.L., Siani, M.A., Sasaki, T., Williams, T.J., Gray, P.W., Moore, P.S., Chang, Y. & Weiss, R.A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines [see comments]. *Science*, **278,** 290-4.

Bottazi, B., Luini, W., Govoni, D., Colotta, F. & Mantovani, A. (1992). Monocyte chemotactic cytokine gene transfer modulates macrophage inflitration, growth and susceptiblity to IL-2 therapy for a muirne melanoma. *J. Immunol.*, **148,** 280-285.

Boulay, F., Naik, N., Giannini, E., Tardif, M. & Brouchon, L. (1997). Phagocyte chemoattractant receptors. *Ann N Y Acad Sci*, **832,** 69-84.

Brelot, A., Heveker, N., Montes, M. & Alizon, M. (2000). Identification of residues of CXCR4 critical for human immunodeficiency virus coreceptor and chemokine receptor activities. *J Biol Chem*, **275,** 23736-44.

Broxmeyer, H.E. & Kim, C.H. (1999). Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. *Exp Hematol*, **27,** 1113-23.

Broxmeyer, H.E., Sherry, B., Cooper, S., Lu, L., Maze, R., Beckmann, M.P., Cerami, A. & Ralph, P. (1993). Comparative analysis of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells. Interacting effects involving suppression, synergistic suppression, and blocking of suppression. *J Immunol*, **150,** 3448-58.

Burger, M., Burger, J.A., Hoch, R.C., Oades, Z., Takamori, H. & Schraufstatter, I.U. (1999). Point mutation causing constitutive signaling of CXCR2 leads to transforming activity similar to Kaposi's sarcoma herpesvirus-G protein-coupled receptor. *J Immunol*, **163,** 2017-22.

Burgstahler, R., Kempkes, B., Steube, K. & Lipp, M. (1995). Expression of the chemokine receptor BLR2/EBI1 is specifically transactivated by Epstein-Barr virus nuclear antigen 2. *Biochem Biophys Res Commun*, **215,** 737-43.

Butcher, E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell*, **67,** 1033-1036.

Camarda, G., Spinetti, G., Bernardini, G., Mair, C., Davis-Poynter, N., Capogrossi, M.C. & Napolitano, M. (1999). The equine herpesvirus 2 E1 open reading frame encodes a functional chemokine receptor. *J Virol*, **73,** 9843-8.

Campbell, J. (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature*, **400,** 776-780.

Campbell, J.J., Bowman, E.P., Murphy, K., Youngman, K.R., Siani, M.A., Thompson, D.A., Wu, L., Zlotnik, A. & Butcher, E.C. (1998a). 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J Cell Biol*, **141,** 1053-9.

Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A. & Butcher, E.C. (1998b). Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*, **279,** 381-4.

Campbell, J.J., Pan, J. & Butcher, E.C. (1999). Cutting edge: developmental switches in chemokine responses during T cell maturation. *J Immunol*, **163,** 2353-7.

Casarosa, P., Bakker, R.A., Verzijl, D., Navis, M., Timmerman, H., Leurs, R. & Smit, M.J. (2001). Constitutive signaling of the Human Cytomegalovirus-encoded chemokine receptro US28. *J. Biol. Chem.*, **276,** 1133-1137.

Cerdan, C., Devilard, E., Xerri, L. & Olive, D. (2001). The C-class chemokine lymphotactin costimulates the apoptosis of human CD4(+) T cells. *Blood*, **97,** 2205-12.

Cerdan, C., Serfling, E. & Olive, D. (2000). The C-class chemokine, lymphotactin, impairs the induction of Th1-type lymphokines in human CD4(+) T cells. *Blood*, **96,** 420-8.

Chaudhuri, A., Zbrzezna, V., Polyakova, J., Pogo, A.O., Hesselgesser, J. & Horuk, R. (1994). Expression of the Duffy antigen in K562 cells. Evidence that it is the human erythrocyte chemokine receptor. *J Biol Chem*, **269,** 7835-8.

Chaudhuri, A., Polyakova, J., Zbrzezna, V., Williams, K., Gulati, S. & Pogo, A.S. (1993). Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor *Plasmodium vivax* malaria parasite. *Proc. Natl. Acad. Sci. USA*, **90,** 10793.

Chauduri, A., Zbrzezna, V., Johnson, C., Nichols, M., Rubinstein, P., Marsh, W.L., Pogo, A.O. (1989). Purification and characterization of an erythrocyte membrane protein complex carrying the Duffy blood group antigenicity: possible receptor for *Plasmodium vivax* and *Plasmodium knowlesi malaria parasite. J Biol Chem*, **264,** 13770-4.

Cheng, Z.J., Zhao, J., Sun, Y., Hu, W., Wu, Y.L., Cen, B., Wu, G.X. & Pei, G. (2000). beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem*, **275,** 2479-85.

Chvatchko, Y., Hoogewerf, A.J., Meyer, A., Alouani, S., Juillard, P., Buser, R., Conquet, F., Proudfoot, A.E., Wells, T.N. & Power, C.A. (2000). A key role

for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J Exp Med*, **191,** 1755-64.

Clark-Lewis, I., Dewald, B., Geiser, T., Moser, B. & Baggiolini, M. (1993). Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg. *Proc Natl Acad Sci U S A*, **90,** 3574-7.

Clark-Lewis, I., Kim, K.S., Rajarathnam, K., Gong, J.H., Dewald, B., Moser, B., Baggiolini, M. & Sykes, B.D. (1995). Structure-activity relationships of chemokines. *J Leukoc Biol*, **57,** 703-11.

Clark-Lewis, I., Schumacher, C., Baggiolini, M. & Moser, B. (1991). Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *J Biol Chem*, **266,** 23128-34.

Cook, D.N. (1996). The role of MIP-1 alpha in inflammation and hematopoiesis. *J Leukoc Biol*, **59,** 61-6.

Cook, D.N., Beck, M.A., Coffman, T.M., Kirby, S.L., Sheridan, J.F., Pragnell, I.B. & Smithies, O. (1995). Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science*, **269,** 1583-5.

Cook, D.N., Prosser, D.M., Forster, R., Zhang, J., Kuklin, N.A., Abbondanzo, S.J., Niu, X.D., Chen, S.C., Manfra, D.J., Wiekowski, M.T., Sullivan, L.M., Smith, S.R., Greenberg, H.B., Narula, S.K., Lipp, M. & Lira, S.A. (2000). CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity*, **12,** 495-503.

Crump, M.P., Gong, J.H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J.L., Baggiolini, M., Sykes, B.D. & Clark-Lewis, I. (1997). Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *Embo J*, **16,** 6996-7007.

Cyster, J.G. (1999). Chemokines and cell migration in secondary lymphoid organs. *Science*, **286,** 2098-102.

Dairaghi, D.J., Fan, R.A., McMaster, B.E., Hanley, M.R. & Schall, T.J. (1999). HHV8-encoded vMIP-I selectively engages chemokine receptor CCR8. Agonist and antagonist profiles of viral chemokines. *J Biol Chem*, **274,** 21569-74.

Dairaghi, D.J., Oldham, E.R., Bacon, K.B. & Schall, T.J. (1997). Chemokine receptor CCR3 function is highly dependent on local pH and ionic strength. *J. Biol. Chem.*, **272,** 28206-9.

D'Ambrosio, D., Iellem, A., Bonecchi, R., Mazzeo, D., Sozzani, S., Mantovani, A. & Sinigaglia, F. (1998). Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J Immunol*, **161,** 5111-5.

Damon, I., Murphy, P.M. & Moss, B. (1998). Broad spectrum chemokine antagonistic activity of a human poxvirus chemokine homolog. *Proc Natl Acad Sci U S A*, **95,** 6403-7.

D'Apuzzo, M., Rolink, A., Loetscher, M., Hoxie, J.A., Clark-Lewis, I., Melchers, F., Baggiolini, M. & Moser, B. (1997). The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol*, **27,** 1788-93.

de Paulis, A., De Palma, R., Di Gioia, L., Carfora, M., Prevete, N., Tosi, G., Accolla, R.S. & Marone, G. (2000). Tat protein is an HIV-1-encoded ss-chemokine homolog that promotes migration and up-regulates CCR3 expression on human FcepsilonRI(+) cells . *J Immunol*, **165,** 7171-9.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R. & Landau, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, **381,** 661-6.

Didsbury, J.R., Uhing, R.J., Tomhave, E., Gerard, C., Gerard, N. & Snyderman, R. (1992). Functional high efficiency expressionof cloned leukocyte chemoattractant receptor cDNAs. *FEBS Lett.*, **297,** 275-279.

Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Briere, F., Zlotnik, A., Lebecque, S. & Caux, C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med*, **188,** 373-86.

Dilloo, D., Bacon, K., Holden, W., Zhong, W., Burdach, S., Zlotnik, A. & Brenner, M. (1996). Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat Med*, **2,** 1090-5.

Dittmer, D. & Kedes, D.H. (1998). Do viral chemokines modulate Kaposi's sarcoma? *Bioessays*, **20,** 367-70.

Doranz, B.J., Orsini, M.J., Turner, J.D., Hoffman, T.L., Berson, J.F., Hoxie, J.A., Peiper, S.C., Brass, L.F. & Doms, R.W. (1999). Identification of CXCR4 domains that support coreceptor and chemokine receptor functions. *J Virol*, **73,** 2752-61.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P. & Paxton, W.A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature*, **381,** 667-73.

Fan, G.H., Yang, W., Wang, X.J., Qian, Q. & Richmond, A. (2001). Identification of a motif in the carboxyl terminus of CXCR2 that is involved in adaptin 2 binding and receptor internalization. *Biochemistry*, **40,** 791-800.

Farzan, M., Choe, H., Martin, K.A., Sun, Y., Sidelko, M., Mackay, C.R., Gerard, N.P., Sodroski, J. & Gerard, C. (1997). HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5. *J Biol Chem*, **272,** 6854-7.

Farzan, M., Mirzabekov, T., Kolchinsky, P., Wyatt, R., Cayabyab, M., Gerard, N.P., Gerard, C., Sodroski, J. & Choe, H. (1999). Tyrosine sulfation of the amino-terminus of CCR5 facilitates HIV-1 entry. *Cell*, **96,** 667-676.

Feng, Y., Broder, C.C., Kennedy, P.E. & Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science*, **272,** 872-7.

Ferguson, S.S.G. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.*, **53,** 1-24.

Ferguson, S.S.G. & Garon, M.G. (1998). G protein-coupled receptor adaptation mechanisms. *Cell & Developmental Biology*, **9,** 119-127.

Fong, A.M., Robinson, L.A., Steeber, D.A., Tedder, T.F., Yoshie, O., Imai, T. & Patel, D.D. (1998). Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med*, **188,** 1413-9.

Foresi, A. & al, e. (1997). Eosinophils, mast cells, and basophils in induced suptum from patients with seasonal allergic rhinitis ans perennial asthma: reslationship to methacholine responsiveness. *J. Allergy Clin. Immunol.*, **100,** 58-64.

Forster, R., Mattis, A.E., Kremmer, E., Wolf, E., Brem, G. & Lipp, M. (1996). A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell*, **87,** 1037-47.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E. & Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, **99,** 23-33.

Foti, M., Granucci, F., Aggujaro, D., Liboi, E., Luini, W., Minardi, S., Mantovani, A., Sozzani, S. & Ricciardi-Castagnoli, P. (1999). Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. *Int Immunol*, **11,** 979-86.

Foxman, E.F., Campbell, J.J. & Butcher, E.C. (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J Cell Biol*, **139,** 1349-60.

Gao, J.L. & Murphy, P.M. (1994). Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. *J Biol Chem*, **269,** 28539-42.

Gao, W., Topham, P.S., King, J.A., Smiley, S.T., Csizmadia, V., Lu, B., Gerard, C.J. & Hancock, W.W. (2000). Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J Clin Invest*, **105,** 35-44.

Gayle, R.B.d., Sleath, P.R., Srinivason, S., Birks, C.W., Weerawarna, K.S., Cerretti, D.P., Kozlosky, C.J., Nelson, N., Vanden Bos, T. & Beckmann, M.P. (1993).

Importance of the amino terminus of the interleukin-8 receptor in ligand interactions. *J Biol Chem*, **268,** 7283-9.

Gerard, C. & Gerard, N. (1994). C5a anaphylatoxin and its seven trasnmembrane receptor. *Annu. Rev. Immunol.*, **12,** 775-808.

Gerard, C. & Rollins, B.J. (2001). Chemokines and disease. *Nature Immunol.*, **2,** 108-115.

Gerber, B.O., Zanni, M.P., Uguccioni, M., Loetscher, M., Mackay, C.R., Pichler, W.J., Yawalkar, N., Baggiolini, M. & Moser, B. (1997). Functional expression of the eotaxin receptor CCR3 in T lymphocytes co- localizing with eosinophils. *Curr Biol*, **7,** 836-43.

Godfrey, D. & Zlotnik, A. (1993). Control points in early T cell development. *Immunol. Today*, **14,** 547-53.

Godiska, R., Chantry, D., Raport, C.J., Sozzani, S., Allavena, P., Leviten, D., Mantovani, A. & Gray, P.W. (1997). Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. *J Exp Med*, **185,** 1595-604.

Gosling, J., Monteclaro, F.S., Atchison, R.E., Arai, H., Tsou, C.L., Goldsmith, M.A. & Charo, I.F. (1997). Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis and signal transduction from HIV-1 coreceptor activity. *Proc Natl Acad Sci U S A*, **94,** 5061-6.

Govaerts, C., Blanpain, C., Deupi, X., Ballet, S., Ballesteros, J.A., Wodak, S.J., Vassart, G., Pardo, L. & Parmentier, M. (2001). The TXP motif in the second transmembrane helix of CCR5. *J. Biol. Chem.*, **276,** 13217-13225.

Graham, G.J., MacKenzie, J., Lowe, S., Tsang, M.L., Weatherbee, J.A., Issacson, A., Medicherla, J., Fang, F., Wilkinson, P.C. & Pragnell, I.B. (1994). Aggregation of the chemokine MIP-1 alpha is a dynamic and reversible phenomenon. Biochemical and biological analyses. *J Biol Chem*, **269,** 4974-8.

Graham, G.J., Wright, E.G., Hewick, R., Wolpe, S.D., Wilkie, N.M., Donaldson, D., Lorimore, S. & Pragnell, I.B. (1990). Identification and caharacterization of an inhibitor of haematopietic stem cell proliferation. *Nature*, **344,** 442-444.

Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M. & Rollins, B.J. (2000). Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature*, **404,** 407-11.

Gunn, M.D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L.T. & Nakano, H. (1999). Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*, **189,** 451-60.

Gunn, M.D., Ngo, V.N., Ansel, K.M., Ekland, E.H., Cyster, J.G. & Williams, L.T. (1998a). A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature*, **391,** 799-803.

Gunn, M.D., Tangemann, K., Tam, C., Cyster, J.G., Rosen, S.D. & Williams, L.T. (1998b). A chemokine expressed in lymphoid high endothelial venules

promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci U S A*, **95,** 258-63.

Gupta, S.K., Hassel, T. & Singh, J.P. (1995). A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4. *Proc Natl Acad Sci U S A*, **92,** 7799-803.

Gutierrez-Ramos, J.C., Lloyd, C. & Gonzalo, J.A. (1999). Eotaxin: from an eosinophilic chemokine to a major regulator of allergic reactions. *Immunol Today*, **20,** 500-4.

Hadley, T.J., Lu, Z.H., Wasniowska, K., Martin, A.W., Peiper, S.C., Hesselgesser, J. & Horuk, R. (1994). Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy blood group antigen. *J Clin Invest*, **94,** 985-91.

Hadley, T.J. & Peiper, S.C. (1997). From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. *Blood*, **89,** 3077-91.

Hancock, W.W., Gao, W., Faia, K.L. & Csizmadia, V. (2000a). Chemokines and their receptors in allograft rejection. *Curr Opin Immunol*, **12,** 511-6.

Hancock, W.W., Lu, B., Gao, W., Csizmadia, V., Faia, K., King, J.A., Smiley, S.T., Ling, M., Gerard, N.P. & Gerard, C. (2000b). Requirement of the chemokine receptor CXCR3 for acute allograft rejection [In Process Citation]. *J Exp Med*, **192,** 1515-20.

Haque, N.S., Zhang, X., French, D.L., Li, J., Poon, M., Fallon, J.T., Gabel, B.R., Taubman, M.B., Koschinsky, M. & Harpel, P.C. (2000). CC chemokine I-309 is the principal monocyte chemoattractant induced by apolipoprotein(a) in human vascular endothelial cells. *Circulation*, **102,** 786-92.

Haribabu, B., Richardson, R.M., Fisher, I., Sozzani, S., Peiper, S.C., Horuk, R., Ali, H. & Snyderman, R. (1997). Regulation of human chemokine receptors CXCR4. Role of phosphorylation in desensitization and internalization. *J Biol Chem*, **272,** 28726-31.

Hayward, G.S. (1999). KSHV strains: the origins and global spread of the virus. *Semin. Cancer Biol.*, **9,** 187-199.

Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., Ponath, P.D. & Mackay, C.R. (1997). Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J Clin Invest*, **99,** 178-84.

Hedrick, J.A. & Zlotnik, A. (1997). Identification and characterization of a novel beta chemokine containing six conserved cysteines. *J Immunol*, **159,** 1589-93.

Hemmerich, S., Paavola, C., Bloom, A., Bhakta, S., Freedman, R., Grunberger, D., Krstenansky, J., Lee, S., McCarley, D., Mulkins, M., Wong, B., Pease, J., Mizoue, L., Mirzadegan, T., Polsky, I., Thompson, K., Handel, T.M. & Jarnagin, K. (1999). Identification of residues in the monocyte chemotactic protein-1 that contact the MCP-1 receptor, CCR2. *Biochemistry*, **38,** 13013-25.

Horuk, R. (2001). Chemokine receptors. *Cytokine and Growth Factor Reviews*, **12,** 313-335.

Horuk, R., Chitnis, C.E., Darbonne, W.C., Colby, T.J., Rybicki, A., Hadley, T.J. & Miller, L.H. (1993). A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. *Science*, **261,** 1182-4.

Horuk, R., Hesselgesser, J., Zhou, Y., Faulds, D., Halks-Miller, M., Harvey, S., Taub, D., Samson, M., Parmentier, M., Rucker, J., Doranz, B.J. & Doms, R.W. (1998). The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV- 1 strains. *J Biol Chem*, **273,** 386-91.

Hromas, R., Kim, C.H., Klemsz, M., Krathwohl, M., Fife, K., Cooper, S., Schnizlein-Bick, C. & Broxmeyer, H.E. (1997). Isolation and characterization of Exodus-2, a novel C-C chemokine with a unique 37-amino acid carboxyl-terminal extension. *J Immunol*, **159,** 2554-8.

Huang, L., Bosch, I., Hofmann, W., Sodroski, J. & Pardee, A.B. (1998). Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T- lymphotropic HIV-1 strains. *J Virol*, **72,** 8952-60.

Hub, E. & Rot, A. (1998). Binding of RANTES, MCP-1, MCP-3, and MIP-1alpha to cells in human skin. *Am J Pathol*, **152,** 749-57.

Imai, T., Baba, M., Nishimura, M., Kakizaki, M., Takagi, S. & Yoshie, O. (1997). The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J. Biol. Chem.*, **272,** 15036-15042.

Imai, T., Chantry, D., Raport, C.J., Wood, C.L., Nishimura, M., Godiska, R., Yoshie, O. & Gray, P.W. (1998). Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. *J Biol Chem*, **273,** 1764-8.

Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P.W., Matsushima, K. & Yoshie, O. (1999). Selective recruitment of CCR4-bearing Th2 cells toward antigen- presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol*, **11,** 81-8.

Isegawa, Y., Ping, Z., Nakano, K., Sugimoto, N. & Yamanishi, K. (1998). Human herpesvirus 6 open reading frame U12 encodes a functional beta- chemokine receptor. *J Virol*, **72,** 6104-12.

Izikson, L., Klein, R.S., Charo, I.F., Weiner, H.L. & Luster, A.D. (2000). Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2 [In Process Citation]. *J Exp Med*, **192,** 1075-80.

Jacobsen, S.E.W., Ruscetti, F.W., Ortiz, M., Gooya, J.M. & Keller, J.R. (1994). The growth response of Lin-Thy1$^+$ hematopoietic progenitors to cytokines is determined by the balance between synergy of multiple stimulators and negative cooperation of multiple inhibitors. *Exp. Haematol.*, **22,** 985-989.

Jarmin, D.I., Rits, M., Bota, D., Gerard, N.P., Graham, G.J., Clark-Lewis, I. & Gerard, C. (2000). Cutting edge: identification of the orphan receptor G-protein-coupled receptor 2 as CCR10, a specific receptor for the chemokine ESkine. *J Immunol*, **164,** 3460-4.

Ji, T.H., Grossman, M. & Ji, I. (1998).G protein coupled receptors. I. Diversity of receptor-ligand intreractions. *J. Biol. Chem.*, **273,** 17299-17302.

Jinquan, T., Quan, S., Jacobi, H.H., Jing, C., Millner, A., Jensen, B., Madsen, H.O., Ryder, L.P., Svejgaard, A., Malling, H.J., Skov, P.S. & Poulsen, L.K. (2000). CXC chemokine receptor 3 expression on CD34(+) hematopoietic progenitors from human cord blood induced by granulocyte-macrophage colony-stimulating factor: chemotaxis and adhesion induced by its ligands, interferon gamma-inducible protein 10 and monokine induced by interferon gamma. *Blood*, **96,** 1230-8.

Kasama, T., Strieter, R.M., Lukacs, N.W., Lincoln, P.M., Burdick, M.D. & Kunkel, S.L. (1995). Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis. *J Clin Invest*, **95,** 2868-76.

Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W., Schall, T.J. & et al. (1994). Lymphotactin: a cytokine that represents a new class of chemokine. *Science*, **266,** 1395-9.

Kim, C.H., Pelus, L.M., White, J.R. & Broxmeyer, H.E. (1998). Differential chemotactic behavior of developing T cells in response to thymic chemokines. *Blood*, **91,** 4434-43.

Kledal, T.N., Rosenkilde, M.M., Coulin, F., Simmons, G., Johnsen, A.H., Alouani, S., Power, C.A., Luttichau, H.R., Gerstoft, J., Clapham, P.R., Clark-Lewis, I., Wells, T.N.C. & Schwartz, T.W. (1997). A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma- associated herpesvirus. *Science*, **277,** 1656-9.

Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. & Lefkowitz, R.J. (1988). Chimeric alpha2-, beta2-Adrenergic Receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science*, **240,** 1310-1316.

Kolakowski, L.F., Lu, B., Gerard, C. & Gerard, N.P. (1995). Probing the ``Message:Address'' Sites for Chemoattractant Binding to the C5a Receptor. *J. Biol. Chem.*, **270,** 18077-18082.

Kon, O.M. & Kay, A.B. (1999). T cells and chronic asthma. *Int. Arch. Allergy Immunol.*, **118,** 133-135.

Kraft, K., Olbrich, H., Majousli, I., Mack, M., Proudfoot, A. & Opperman, M. (2001). Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization. *J. Biol. Chem.*, **276,** 34408-34418.

Krijgsveld, J., Zaat, S.A., Meeldijk, J., van Veelen, P.A., Fang, G., Poolman, B., Brandt, E., Ehlert, J.E., Kuijpers, A.J., Engbers, G.H., Feijen, J. & Dankert, J. (2000). Thrombocidins, microbicidal proteins from human blood platelets, are C- terminal deletion products of CXC chemokines. *J Biol Chem*, **275,** 20374-81.

Kuang, Y., Wu, Y., Jiang, H. & Wu, D. (1996). Selective G protein coupling by C-C chemokine receptors. *J Biol Chem*, **271,** 3975-8.

Kunkel, S.L. (1999). Through the looking glass: the diverse in vivo activities of chemokines. *J Clin Invest*, **104,** 1333-4.

Kunkel, S.L., Lukacs, N., Kasama, T. & Strieter, R.M. (1996). The role of chemokines in inflammatory joint disease. *J Leukoc Biol*, **59,** 6-12.

Lalani, A.S., Barrett, J.W. & McFadden, G. (2000). Modulating chemokines: more lessons from viruses. *Immunol Today*, **21,** 100-6.

Lalani, A.S., Graham, K., Mossman, K., Rajarathnam, K., Clark-Lewis, I., Kelvin, D. & McFadden, G. (1997). The purified myxoma virus gamma interferon receptor homolog M-T7 interacts with the heparin-binding domains of chemokines. *J Virol*, **71,** 4356-63.

Lalani, A.S., Masters, J., Zeng, W., Barrett, J., Pannu, R., Everett, H., Arendt, C.W. & McFadden, G. (1999). Use of chemokine receptors by poxviruses. *Science*, **286,** 1968-71.

Lalani, A.S. & McFadden, G. (1997). Secreted poxvirus chemokine binding proteins. *J Leukoc Biol*, **62,** 570-6.

Lalani, A.S. & McFadden, G. (1999). Evasion and exploitation of chemokines by viruses. *Cytokine Growth Factor Rev*, **10,** 219-33.

Lalani, A.S., Ness, T.L., Singh, R., Harrison, J.K., Seet, B.T., Kelvin, D.J., McFadden, G. & Moyer, R.W. (1998). Functional comparisons among members of the poxvirus T1/35kDa family of soluble CC-chemokine inhibitor glycoproteins. *Virology*, **250,** 173-84.

Laning, J., Kawasaki, H., Tanaka, E., Luo, Y. & Dorf, M.E. (1994). Inhibition of in vivo tumor growth by the beta chemokine, TCA3. *J Immunol*, **153,** 4625-35.

LaRosa, G.J., Thomas, K.M., Kaufmann, M.E., Mark, R., White, M., Taylor, L., Witt, D. & Navarro, J. (1992). Amino terminus of the Interleukin-8 receptor is a major determinant of receptor subtype specificity. *J. Biol. Chem.*, **267,** 25402-25406.

Lee, J., Horuk, R., Rice, G.C., Bennett, G.L., Camerato, T. & Wood, W.I. (1992). Characterization of two high affinity human interleukin-8 receptors. *J Biol Chem*, **267,** 16283-7.

Lefkowitz, R.J. (1998). G protein-coupled receptors: III. New roles for receptor kinases and β- arrestins in receptor signalling and desensitization. *J. Biol. Chem.*, **273,** 18677-18680.

Legler, D.F., Loetscher, M., Roos, R.S., Clark-Lewis, I., Baggiolini, M. & Moser, B. (1998). B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med*, **187,** 655-60.

L'Heureux, G.P., Bourgoin, S., Jean, N., McColl, R & Naccache, P.H. (1995). Diverging signal transduction pathways activated by interleukin-8 and related chemokines in human neutrophils: interleukin-8, but not NAP-2 or GRO alpha, stimulates phospholipase D activity. *Blood*, **85,** 522-531.

Lilly, C.M., Nakamura, H., Kesselman, H., Nagler-Andersson, C., Asano, K., Garcia-Zepeda, E.A., Rothenberg, M.E., Drazen, J.M. & Luster, A.D. (1997). Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J. Clin. Invest.*, **99,** 1767-1773.

Lindhout, E., Vissers, J.L.M., Figdor, C.G. & Adema, G.J. (1999). Chemokines and lymphocyte migration. *The Immunologist*, **7,** 147-152.

Ling, K., Wang, P., Zhao, J., Wu, Y.L., Cheng, Z.J., Wu, G.X., Hu, W., Ma, L. & Pei, G. (1999). Five-transmembrane domains appear sufficient for a G protein-coupled receptor: functional five-transmembrane domain chemokine receptors. *Proc Natl Acad Sci U S A*, **96,** 7922-7.

Loetscher, M., Gerber, B., Loetscher, P., Jones, S.A., Piali, L., Clark-Lewis, I., Baggiolini, M. & Moser, B. (1996). Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes [see comments]. *J Exp Med*, **184,** 963-9.

Luckacs, N.W., Prossera, D.M., Wiekowskia, M., Liraa, S.A. & Cooka, D.N. (2001). Requirement for the chemokine receptor CCR6 in allergic pulmonary infection. *J. Exp. Med.*, **194,** 551-556.

Lukacs, N.W., Oliveira, S.H. & Hogaboam, C.M. (1999). Chemokines and asthma: redundancy of function or a coordinated effort? *J Clin Invest*, **104,** 995-9.

Lukacs, N.W., Strieter, R.M., Chensue, S.W. & Kunkel, S.L. (1996). Activation and regulation of chemokines in allergic airway inflammation. *J Leukoc Biol*, **59,** 13-7.

Lukacs, N.W., Strieter, R.M., Warmington, K., Lincoln, P., Chensue, S.W. & Kunkel, S.L. (1997). Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J Immunol*, **158,** 4398-404.

Luster, A.D. & Leder, P. (1993). IP-10, a -C-X-C- chemokine, elicits a potent thymus-dependent antitumor response in vivo. *J Exp Med*, **178,** 1057-65.

Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T. & Springer, T.A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A*, **95,** 9448-53.

Ma, Q., Jones, D. & Springer, T.A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity*, **10,** 463-71.

MacDonald, M.R., Burney, M.W., Resnick, S.B. & Virgin, H.I. (1999). Spliced mRNA encoding the murine cytomegalovirus chemokine homolog predicts a beta chemokine of novel structure. *J Virol*, **73,** 3682-91.

Mach, F., Sauty, A., Iarossi, A.S., Sukhova, G.K., Neote, K., Libby, P. & Luster, A.D. (1999). Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest*, **104,** 1041-50.

Mack, M., Luckow, B., Nelson, P.J., Cihak, J., Simmons, G., Clapham, P.R., Signoret, N., Marsh, M., Stangassinger, M., Borlat, F., Wells, T.N.,

Schlondorff, D. & Proudfoot, A.E. (1998). Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med*, **187,** 1215-24.

Maggio, R., Vogel, Z. & Wess, J. (1993a). Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular 'cross-talk' between G-protein-linked receptors. *Proc. Natl. Acad. Sci. USA*, **90,** 3103-3107.

Maggio, R., Vogel, Z. & Wess, J. (1993b). Reconstituion of funtional muscarinic receptors by co-expression of amino- and carboxyl-terminal receptor fragments. *FEBS Lett.*, **319,** 195-200.

Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B., Nice, E.C., Wise, L., Mercer, A., Kowalski, H., Kerjaschki, D., Stacker, S.A., Achen, M.G. & Alitalo, K. (2001). Isolated lymphatic endothelial cells transduce growth, survival and migratory signalss via the VEGF-C/D receptor VEGFR-3. *The EMBO Journal*, **20,** 4762-4773.

Malkowski, M.G., Wu, J.Y., Lazar, J.B., Johnson, P.H. & Edwards, B.F. (1995). The crystal structure of recombinant human neutrophil-activating peptide-2 (M6L) at 1.9-A resolution. *J Biol Chem*, **270,** 7077-87.

Manome, Y., Wen, P.Y., Hershowitz, A., Tanaka, T., Rollins, B.J., Kufe, D.W. & Fine, H.A. (1995). Monocyte chemoattractant protein-1 (MCP-1) gene transduction: an effective tumor vaccine strategy for non-intracranial tumors. *Cancer Immunol Immunother*, **41,** 227-35.

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

Mayer, M.R. & Stone, M.J. (2001). Identification of receptor bidning and activation determinants in the N-terminal and N-loop regions of the CC chemokine eotaxin. *J. Biol. Chem.*, **276,** 13911-13916.

McQuibban, G.A., Gong, J.H., Tam, E.M., McCulloch, C.A., Clark-Lewis, I. & Overall, C.M. (2000). Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science*, **289,** 1202-6.

Melchers, F., Rolink, A.G. & Schaniel, C. (1999). The role of chemokines in regulating cell migration during humoral immune responses. *Cell*, **99,** 351-4.

Mellado, M., Rodriguez-Frade, J.M., Aragay, A., del Real, G., Martin, A.M., Vila-Coro, A.J., Serrano, A., Mayor, F., Jr. & Martinez, A.C. (1998). The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J Immunol*, **161,** 805-13.

Mellado, M., Rodriguez-Frade, J.M., Vila-Coro, A.J., Fernandez, S., Martin de Ana, A., Jones, D.R., Toran, J.L. & Martinez-A, C. (2001). Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO J.*, **20,** 2497-2507.

Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E. & Rot, A. (1997). Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell*, **91,** 385-95.

Miller, L.H., Mason, S.J., Dvorak, J.A., McGinniss, M.H. & Rothman, I.K. (1975). Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science*, **189,** 561.

Miller, M.D. & Krangel, M.S. (1992). Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines, CRC. *Crit. Rev. Immunol.*, **12,** 17-46.

Moatti, D., Faure, S., Fumeron, F., Amara, M.E.W., Skenadji, P., McDermont, D.H., Debre, P., Aumont, M.C., Murphy, P.M., de Prost, D. & Comabadiere, C. (2001). Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blodd*, **97,** 1925-1928.

Monteclaro, F.S. & Charo, I.F. (1996). The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1alpha receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *J Biol Chem*, **271,** 19084-92.

Monteclaro, F.S. & Charo, I.F. (1997). The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1. Receptor activation by a pseudo-tethered ligand. *J Biol Chem*, **272,** 23186-90.

Morales. (1999). CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc. Natl. Acad. Sci. USA*, **96,** 14470-5.

Morello, J.P. & Bouvier, M. (1996). Palmitoylation: a post-translational modification that regulates signalling from G-protein coupled receptors. *Biochem. Cell Biol.*, **74,** 449-457.

Moser, B. & Loetscher, P. (2001). Lymphocyte traffic control by chemokines. *Nature Immunol.*, **2,** 123-128.

Mueller, A., Kelly, E. & Strange, P.G. (2002). Pathways for internalization and recycling of the chemokine receptor CCR5. *Blood*, **99,** 785- 791.

Mule, J.J., Custer, M., Averbook, B., Yang, J.C., Weber, J.S., Goeddel, D.V., Rosenberg, S.A. & Schall, T.J. (1996). RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity in vivo: role of immune cell subpopulations. *Hum Gene Ther*, **7,** 1545-53.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., Barrera, J.L., Mohar, A., Verastegui, E. & Zlotnik, A. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410,** 50-6.

Murdoch, C. & Finn, A. (2000). Chemokine receptors and their role in inflammation and infectious diseases. *Blood*, **95,** 3032-43.

Murphy, P.M. (1996). Chemokine receptors: structure, function and role in microbial pathogenesis. *Cytokine Growth Factor Rev*, **7,** 47-64.

Murphy, P.M. (2001). Viral exploitation and subversion of the immune system through chemokine mimicry. *Nature Immunol.*, **2,** 116-122.

Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J. & Power, C.A. (2000). International Union of

Pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.*, **52,** 145-176.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H. & Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*, **382,** 635-8.

Nagira, M., Imai, T., Hieshima, K., Kusuda, J., Ridanpaa, M., Takagi, S., Nishimura, M., Kakizaki, M., Nomiyama, H. & Yoshie, O. (1997). Molecular cloning of a novel human CC chemokine secondary lymphoid- tissue chemokine that is a potent chemoattractant for lymphocytes and mapped to chromosome 9p13. *J Biol Chem*, **272,** 19518-24.

Nagira, M., Imai, T., Yoshida, R., Takagi, S., Iwasaki, M., Baba, M., Tabira, Y., Akagi, J., Nomiyama, H. & Yoshie, O. (1998). A lymphocyte-specific CC chemokine, secondary lymphoid tissue chemokine (SLC), is a highly efficient chemoattractant for B cells and activated T cells. *Eur J Immunol*, **28,** 1516-23.

Nakashima, E., Mukaida, N., Kubota, Y., Kuno, K., Yasumoto, K., Ichimura, F., Nakanishi, I., Miyasaka, M. & Matsushima, K. (1995). Human MCAF gene transfer enhances the metastatic capacity of a mouse cachectic adenocarcinoma cell line in vivo. *Pharm Res*, **12,** 1598-604.

Nakashima, E., Oya, A., Kubota, Y., Kanada, N., Matsushita, R., Takeda, K., Ichimura, F., Kuno, K., Mukaida, N., Hirose, K., Nakanishi, I., Ujiie, T. & Matsushima, K. (1996). A candidate for cancer gene therapy: MIP-1 alpha gene transfer to an adenocarcinoma cell line reduced tumorigenicity and induced protective immunity in immunocompetent mice. *Pharm Res*, **13,** 1896-901.

Nakayama, T.A. & Khorana, H.G. (1991). Mapping of the amino acids in membrane embedded helices that interact with the retinal chromophore in bovine rhodopsin. *J. Biol. Chem.*, **266,** 4269-4275.

Neote, K., DiGregorio, D., Mak, J.Y., Horuk, R. & Schall, T.J. (1993). Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell*, **72,** 415-25.

Neote, K., Mak, J.Y., Kolakowski, L.F., Jr. & Schall, T.J. (1994). Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. *Blood*, **84,** 44-52.

Ng, C.F., Sun, F.F., Taylor, B.M., Wolin, M.S. & Wong, P.I.-K. (1991). Functional properties of guinea pig eosinophil leukotriene Ba receptor. *J. Immunol.*, **47,** 3096-3103.

Ngo, V.N., Tang, H.L. & Cyster, J.G. (1998). Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med*, **188,** 181-91.

Nibbs, R.J., Kriehuber, E., Ponath, P.D., Parent, D., Qin, S., Campbell, J.D., Henderson, A., Kerjaschki, D., Maurer, D., Graham, G.J. & Rot, A. (2001).

The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol*, **158,** 867-77.

Nibbs, R.J., Salcedo, T.W., Campbell, J.D., Yao, X.T., Li, Y., Nardelli, B., Olsen, H.S., Morris, T.S., Proudfoot, A.E., Patel, V.P. & Graham, G.J. (2000). C-C chemokine receptor 3 antagonism by the beta-chemokine macrophage inflammatory protein 4, a property strongly enhanced by an amino- terminal alanine-methionine swap. *J Immunol*, **164,** 1488-97.

Nibbs, R.J., Wylie, S.M., Yang, J., Landau, N.R. & Graham, G.J. (1997a). Cloning and characterization of a novel promiscuous human beta- chemokine receptor D6. *J Biol Chem*, **272,** 32078-83.

Nibbs, R.J., Yang, J., Landau, N.R., Mao, J.H. & Graham, G.J. (1999). LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity. *J Biol Chem*, **274,** 17478-83.

Nibbs, R.J.B., Wylie, S.M., Pragnell, I.B. & Graham, G.J. (1997b). Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein- 1alpha receptors, CCR-1, CCR-3, and CCR-5. *J Biol Chem*, **272,** 12495-504.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.M., Clark-Lewis, I., Legler, D.F., Loetscher, M., Baggiolini, M. & Moser, B. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1 [published erratum appears in Nature 1996 Nov 21;384(6606):288]. *Nature*, **382,** 833-5.

Oliveira, L., Paiva, A.C.M., Sander, C. & Vriend, G. (1994). A common step for signal transduction in G protein-coupled receptors. *TiPS*, **15,** 170-172.

Oliveira, S.A. & Shenk, T.E. (2001). Murine cytomegalovirus M78 protein, a G protein coupled receptor homologue, is a constituent virion and facilitates accumulation of immediate-early viral mRNA. *Proc. Natl. Acad. Sc.*, **98,** 3237-3242.

Oppermann, M., Mack, M., Proudfoot, A.E. & Olbrich, H. (1999). Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus. *J Biol Chem*, **274,** 8875-85.

Oravecz, T., Pall, M., Roderiquez, G., Gorrell, M.D., Ditto, M., Nguyen, N.Y., Boykins, R., Unsworth, E. & Norcross, M.A. (1997). Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J Exp Med*, **186,** 1865-72.

Pachynski, R.K., Wu, S.W., Gunn, M.D. & Erle, D.J. (1998). Secondary lymphoid-tissue chemokine (SLC) stimulates integrin alpha 4 beta 7-mediated adhesion of lymphocytes to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) under flow. *J Immunol*, **161,** 952-6.

Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-

Ramos, J.C. & Gearing, D. (1997). Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation [published erratum appears in Nature 1997 Sep 4;389(6646):100]. *Nature*, **387,** 611-7.

Panina-Bordignon, P., Papi, A., Mariani, M., Di Lucia, P., Casoni, G., Bellettato, C., Buonsanti, C., Miotto, D., Mapp, C., Villa, A., Arrigoni, G., Fabbri, L.M. & Sinigaglia, F. (2001). The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J. Clin. Invest.*, **107,** 1357-1364.

Pease, J.E., Wang, J., Ponath, P.D. & Murphy, P.M. (1998). The N-terminal extracellular segments of the chemokine receptors CCR1 and CCR3 are determinants for MIP-1alpha and eotaxin binding, respectively, but a second domain is essential for efficient receptor activation. *J Biol Chem*, **273,** 19972-6.

Penfold, M.E., Dairaghi, D.J., Duke, G.M., Saederup, N., Mocarski, E.S., Kemble, G.W. & Schall, T.J. (1999). Cytomegalovirus encodes a potent alpha chemokine. *Proc Natl Acad Sci U S A*, **96,** 9839-44.

Percherancier, Y., Planchenault, T., Valenzuela- Fernandez, A., Virelizier, J.L., Arenzana-Seisdedos, F. & Bachelerie, F. (2001). Palmitoylation-dependent control of degradation, life span and membrane expression of the CCR5 receptor. *J. Biol. Chem.*, **276,** 31936-31944.

Perez, H.D., Holmes, R., Vilander, L.R., Adams, R.R., Manzana, W., Jolley, D. & Andrews, W.H. (1993). Formyl peptide receptor chimeras define domains involved in ligand binding. *J. Biol. Chem.*, **268,** 2292-2295.

Playfair, J.H.L. (1987). *Immunology at a Glance.* Blackwell Scientific Publications.

Power, C.A. & Meyer, A. (2000). Generation of stable cell lines expressing chemokine receptors. In *Methods in Molecular Biology: Chemokine Protocols*, Proudfoot, A.E.I., Wells, T.N.C. & Power, C.A. (eds), Vol. 138. pp. 99- 104. Humana Press Inc.: Totowa, NJ.

Power, C.A., Meyer, A., Nemeth, K., Bacon, K.B., Hoogewerf, A.J., Proudfoot, A.E. & Wells, T.N. (1995). Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. *J Biol Chem*, **270,** 19495-500.

Premack, B.A. & Schall, T.J. (1996). Chemokine receptors: gateways to inflammation and infection. *Nat Med*, **2,** 1174-8.

Proudfoot, A.E., Buser, R., Borlat, F., Alouani, S., Soler, D., Offord, R.E., Schroder, J.M., Power, C.A. & Wells, T.N. (1999). Amino-terminally modified RANTES analogues demonstrate differential effects on RANTES receptors. *J Biol Chem*, **274,** 32478-85.

Proudfoot, A.E., Power, C.A., Hoogewerf, A.J., Montjovent, M.O., Borlat, F., Offord, R.E. & Wells, T.N. (1996). Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J Biol Chem*, **271,** 2599-603.

Quesniaux, V.F., Graham, G.J., Pragnell, I., Donaldson, D., Wolpe, S.D., Iscove, N.N. & Fagg, B. (1993). Use of 5-fluorouracil to analyze the effect of

macrophage inflammatory protein-1alpha on long term reconstituting stem cells in vivo. *Blood*, **81,** 1497-1504.

Rawlison, W.D., Farrell, H.E. & Barrell, B.G. (1996). Analysis of the complete DNA sequence of murine cytomegalovirus. *J. Virol.*, **70,** 8833-8849.

Rodriguez-Frade, J.M., Mellado, M. & Martinez-A, C. (2001). Chemokine receptor dimerization: two are better than one. *TRENDS in Immunol.*, **22,** 612-617.

Rodriguez-Frade, J.M., Vila-Coro, A.J., de Ana, A.M., Albar, J.P., Martinez, A.C. & Mellado, M. (1999). The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc Natl Acad Sci U S A*, **96,** 3628-33.

Rollins, B.J. (1997). Chemokines. *Blood*, **90,** 909-28.

Ross, R. (1999). Atherosclerosis- an inflammatory disease. *New Engl. J. Med.*, **340,** 115-126.

Rossi, D. & Zlotnik, A. (2000). The biology of chemokines and their receptors. *Annu Rev Immunol*, **18,** 217-42.

Rossi, G.L. & Oliveira, D. (1997). Does the mast cell still have a key role in asthma? *Chest.*, **112,** 523-529.

Rothenberg, M.E., MacLean, J.A., Pearlman, E., Luster, A.D. & Leder, P. (1997). Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J Exp Med*, **185,** 785-90.

Rottman, J.B., Slavin, A.J., Silva, R., Weiner, H.L., Gerard, C.G. & Hancock, W.W. (2000). Leukocyte recruitment during onset of experimental allergic encephalomyelitis is CCR1 dependent. *Eur J Immunol*, **30,** 2372-7.

Rucker, J., Samson, M., Doranz, B.J., Libert, F., Berson, J.F., Yi, Y., Smyth, R.J., Collman, R.G., Broder, C.C., Vassart, G., Doms, R.W. & Parmentier, M. (1996). Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell*, **87,** 437-46.

Sabroe, I., Hartnell, A., Jopling, L.A., Bel, S., Ponath, P.D., Pease, J.E., Collins, P.D. & Williams, T.J. (1999). Differential regulation of eosinophil chemokine signaling via CCR3 and non-CCR3 pathways. *J Immunol*, **162,** 2946-55.

Salcedo, R., Ponce, M.L., Young, H.A., Wasserman, K., Ward, J.M., Kleinman, H.K., Oppenheim, J.J. & Murphy, W.J. (2000). Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood*, **96,** 34-40.

Sallusto, F., Kremmer, E., Palermo, B., Hoy, A., Ponath, P., Qin, S., Forster, R., Lipp, M. & Lanzavecchia, A. (1999a). Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur. J. Immunol.*, **29,** 2037-45.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. (1999b). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*, **401,** 708-12.

Sallusto, F., Lenig, D., Mackay, C.R. & Lanzavecchia, A. (1998). Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med*, **187,** 875-83.

Sallusto, F., Mackay, C.R. & Lanzavecchia, A. (2000). The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.*, **18,** 593-620.

Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., Forster, R., Burgstahler, R., Lipp, M. & Lanzavecchia, A. (1999c). Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol*, **29,** 1617-25.

Samama, P., Cotecchia, S., Costa, T. & Lefkowitz, R.J. (1993). A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.*, **268,** 4625-4636.

Samson, M., LaRosa, G., Libert, F., Paindavoine, P., Detheux, M., Vassart, G. & Parmentier, M. (1997). The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J Biol Chem*, **272,** 24934-41.

Samson, M., Libert, F., Doranz, B.J., Rucker, J., Liesnard, C., Farber, C.M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R.J., Collman, R.G., Doms, R.W., Vassart, G. & Parmentier, M. (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene [see comments]. *Nature*, **382,** 722-5.

Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M. & Czarnetzki, B.M. (1993). IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor [published erratum appears in J Immunol 1994 Oct 1;153(7):3360]. *J Immunol*, **151,** 2667-75.

Schall, T.J. (1994). The Chemokines. In *The Cytokine Handbook*, Thomson, A. (ed) pp. 419-460. Academic Press: New York.

Schaniel, C., Pardali, E., Sallusto, F., Speletas, M., Ruedl, C., Shimizu, T., Seidl, T., Andersson, J., Melchers, F., Rolink, A.G. & Sideras, P. (1998). Activated murine B lymphocytes and dendritic cells produce a novel CC chemokine which acts selectively on activated T cells. *J Exp Med*, **188,** 451-63.

Scheer A, Fanelli, F., Costa, T., De Benedetti, P.G. & Cotecchia S. (1996). Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *Embo J.*, **15,** 3566-3578.

Schraufstatter, I.U., Burger, M., Hoch, R.C., Oades, Z.G. & Takamori, H. (1998). Importance of the carboxy-terminus of the CXCR2 for signal transduction. *Biochem Biophys Res Commun*, **244,** 243-8.

Schwartz, T.W. (1994). Locating ligand-binding sites in 7TM receptors by protein engineering. *Current Opinion in Biotechnology*, **5,** 434-444.

Schweickart, V.L., Epp, A., Raport, C.J. & Gray, P.W. (2000). CCR11 is a functional receptor for the monocyte chemoattractant protein family of chemokines. *J Biol Chem*, **275,** 9550-6.

Senkevich, T.G., Bugert, J.J., Sisler, J.R., Koonin, E.V., Darai, G. & Moss, B. (1996). Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. *Science*, **273,** 813-6.

Sgadari, C., Angiolillo, A.L., Cherney, B.W., Pike, S.E., Farber, J.M., Koniaris, L.G., Vanguri, P., Burd, P.R., Sheikh, N., Gupta, G., Teruya-Feldstein, J. & Tosato, G. (1996). Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proc Natl Acad Sci U S A*, **93,** 13791-6.

Sgadari, C., Farber, J.M., Angiolillo, A.L., Liao, F., Teruya-Feldstein, J., Burd, P.R., Yao, L., Gupta, G., Kanegane, C. & Tosato, G. (1997). Mig, the monokine induced by interferon-gamma, promotes tumor necrosis in vivo. *Blood*, **89,** 2635-43.

Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T. & Honjo, T. (1995). Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF-1) gene. *Genomics*, **28,** 495-.

Simmons, G., Clapham, P.R., Picard, L., Offord, R.E., Rosenkilde, M.M., Schwartz, T.W., Buser, R., Wells, T.N.C. & Proudfoot, A.E. (1997). Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science*, **276,** 276-9.

Smith, C.A., Smith, T.D., Smolak, P.J., Friend, D., Hagen, H., Gerhart, M., Park, L., Pickup, D.J., Torrance, D., Mohler, K., Schooley, K. & Goodwin, R.G. (1997a). Poxvirus genomes encode a secreted, soluble protein that preferentially inhibits beta chemokine activity yet lacks sequence homology to known chemokine receptors. *Virology*, **236,** 316-27.

Smith, D.R., Polverini, P.J., Kunkel, S.L., Orringer, M.B., Whyte, R.I., Burdick, M.D., Wilke, C.A. & Strieter, R.M. (1994). Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. *J Exp Med*, **179,** 1409-15.

Smith, M.W., Carrington, M., Winkler, C., Lomb, D., Dean, M., Huttley, G. & O'Brien, S.J. (1997b). CCR2 chemokine receptor and AIDS progression [letter; comment]. *Nat Med*, **3,** 1052-3.

Sorensen, T.L., Tani, M., Jensen, J., Pierce, V., Lucchinetti, C., Folcik, V.A., Qin, S., Rottman, J., Sellebjerg, F., Strieter, R.M., Frederiksen, J.L. & Ransohoff, R.M. (1999). Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest*, **103,** 807-15.

Sozzani, S., Allavena, P., D'Amico, G., Luini, W., Bianchi, G., Kataura, M., Imai, T., Yoshie, O., Bonecchi, R. & Mantovani, A. (1998a). Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J Immunol*, **161,** 1083-6.

Sozzani, S., Luini, W., Bianchi, G., Allavena, P., Wells, T.N., Napolitano, M., Bernardini, G., Vecchi, A., D'Ambrosio, D., Mazzeo, D., Sinigaglia, F.,

Santoni, A., Maggi, E., Romagnani, S. & Mantovani, A. (1998b). The viral chemokine macrophage inflammatory protein-II is a selective Th2 chemoattractant. *Blood*, **92,** 4036-9.

Sozzani, S., Luini, W., Molino, M., jiLiek, P., Bottazzi, B., Cerletti, C., Matsushima, K. & Mantovani, A. (1991). *J. Immunol*, **147,** 2215-2221.

Sozzani, S., Molino, M., Locati, M., Luini, W., Cerletti, C., Vecchi, A. & Mantovani, A. (1993). Receptor-activated calcium influx in human monocytes exposed to monocyte chemotactic protein-1 and related cytokines. *J Immunol*, **150,** 1544-53.

Spprigs, M.K. (1996). One step ahead of the game: viral immunomodulatory molecules. *Ann. Rev. Immunol.*, **14,** 101-130.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*, **76,** 301-314.

Stein, J.V., Rot, A., Luo, Y., Narasimhaswamy, M., Nakano, H., Gunn, M.D., Matsuzawa, A., Quackenbush, E.J., Dorf, M.E. & von Andrian, U.H. (2000). The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, exodus-2) triggers lymphocyte function-associated antigen 1-mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J Exp Med*, **191,** 61-76.

Stine, J.T., Wood, C., Hill, M., Epp, A., Raport, C.J., Schweickart, V.L., Endo, Y., Sasaki, T., Simmons, G., Boshoff, C., Clapham, P., Chang, Y., Moore, P., Gray, P.W. & Chantry, D. (2000). KSHV-encoded CC chemokine vMIP-III is a CCR4 agonist, stimulates angiogenesis, and selectively chemoattracts TH2 cells. *Blood*, **95,** 1151-7.

Streblow, D.N., Soderberg-Naucler, C., Vieira, J., Smith, P., Wakabayashi, E., Ruchti, F., Mattison, K., Altschuler, Y. & Nelson, J.A. (1999). The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell*, **99,** 511-20.

Strieter, R.M., Polverini, P.J., Kunkel, S.L., Arenberg, D.A., Burdick, M.D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D. & et al. (1995). The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem*, **270,** 27348-57.

Strieter, R.M., Standiford, T.J., Huffnagle, G.B., Colletti, L.M., Lukacs, N.W. & Kunkel, S.L. (1996). "The good, the bad, and the ugly." The role of chemokines in models of human disease. *J Immunol*, **156,** 3583-6.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T. & Nagasawa, T. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract [see comments]. *Nature*, **393,** 591-4.

Tanabe, S., Heesen, M., Yoshizawa, I., Berman, M.A., Luo, Y., Bleul, C.C., Springer, T.A., Okuda, K., Gerard, N. & Dorf, M.E. (1997). Functional expression of the CXC-chemokine receptor-4/fusin on mouse microglial cells and astrocytes. *J Immunol*, **159,** 905-11.

Tang, H.L. & Cyster, J.G. (1999). Chemokine up-regulation and activated T cell attraction by maturing dendritic cells. *Science*, **284,** 819-22.

Tangemann, K., Gunn, M.D., Giblin, P. & Rosen, S.D. (1998). A high endothelial cell-derived chemokine induces rapid, efficient, and subset-selective arrest of rolling T lymphocytes on a reconstituted endothelial substrate. *J Immunol*, **161,** 6330-7.

Telford, E.A., Watson, M.S., Aird, H.C., Perry, J. & Davison, A.J. (1995). The DNA sequence of equine herpesvirus 2. *J. Mol. Biol.*, **249,** 520-528.

Teran, L.M. (2000). CCL chemokines and asthma. *Immunol Today*, **21,** 235-42.

Teruya-Feldstein, J., Jaffe, E.S., Burd, P.R., Kanegane, H., Kingma, D.W., Wilson, W.H., Longo, D.L. & Tosato, G. (1997). The role of Mig, the monokine induced by interferon-gamma, and IP-10, the interferon-gamma-inducible protein-10, in tissue necrosis and vascular damage associated with Epstein-Barr virus-positive lymphoproliferative disease. *Blood*, **90,** 4099-105.

Tonacchera, M., Chiovato, L., Pinchera, A., Agretti, P., Fiore, E., Cetani, F., Rocchi, R., Viacava, P., Miccoli, P. & Vitti, P. (1998). *J. CLin. Endocrinol. Metab.*, **83,** 492-498.

Townson, J.R., Graham, G.J., Landau, N.R., Rasala, B. & Nibbs, R.J.B. (2000). Aminooxypentane addition to the chemokine macrophage inflammatory protein-1αP increases receptor affinities and HIV inhibition. *J. Biol. Chem.*, **275,** 39254-61.

Tran, E.H., Kuziel, W.A. & Owens, T. (2000). Induction of experimental autoimmune encephalomyelitis in C57BL/6 mice deficient in either the chemokine macrophage inflammatory protein- 1alpha or its CCR5 receptor. *Eur J Immunol*, **30,** 1410-5.

Trapp, B.,Peterson, J., Ransohoff, R. M., Rudick, R., Mork, S., Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.*, **338,** 278-290.

Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., Cheng-Mayer, C., Robinson, J., Maddon, P.J. & Moore, J.P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co- receptor CCR-5. *Nature*, **384,** 184-7.

Upton, C., Mossman, K. & McFadden, G. (1992). Encoding of the interferon-gamma receptor by myxoma virus. *Science*, **258,** 1369- 1372.

Van Den Steen, P.E., Proost, P., Wuyts, A., Van Damme, J. & Opdenakker, G. (2000). Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood*, **96,** 2673-81.

Vicari, A.P., Ait-Yahia, S., Chemin, K., Mueller, A., Zlotnik, A. & Caux, C. (2000). Antitumor effects of the mouse chemokine 6Ckine/SLC through angiostatic and immunological mechanisms. *J Immunol*, **165,** 1992-2000.

Vicari, A.P., Figueroa, D.J., Hedrick, J.A., Foster, J.S., Singh, K.P., Menon, S., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Bacon, K.B. & Zlotnik, A.

(1997). TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. *Immunity*, **7,** 291-301.

Vichi, P., Whelchel, A. & Posada, J. (1999). Transmembrane Helix 7 of the Endothelin B Receptor Regulates Downstream Signaling. *J. Biol. Chem.*, **274,** 10331-10338.

Vieira, J., Schall, T.J., Corey, L. & Geballe, A.P. (1998). Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J Virol*, **72,** 8158-65.

Vila-Coro, A.J., Mellado, M., Martin de Ana, A., Martinez, A.C. & Rodriguez-Frade, J.M. (1999). Characterization of RANTES- and aminooxypentane-RANTES-triggered desensitization signals reveals differences in recruitment of the G protein-coupled receptor complex. *J Immunol*, **163,** 3037-44.

Walz, A., Peveri, P., Aschauer, H. & Baggiolini, M. (1987). Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Commun.*, **149,** 755-761.

Walz, D.A., Wu, V.Y., de Lamo, R., Dene, H. & McCoy, L.E. (1977). Primary structure of human platelet factor 4. *Thromb Res*, **11,** 893-8.

Wang, Z., Lee, B., Murray, J.L., Bonneau, F., Sun, Y., Schweickart, V., Zhang, T. & Peiper, S.C. (1999). CCR5 HIV-1 coreceptor activity- role of cooperativity between residues in N-terminal extracellular and intracellular domains. *J. Biol. Chem.*, **274,** 28413-19.

Wardlaw, A.J., Moqbel, R., Cromwell, O. & Kay, A.B. (1986). Platelet-activating factor: a potent chemotactic and chimokinetic factor for human eosinophils. *J. Clin. Invest.*, **78,** 1701-1706.

Webb, L.M., Ehrengruber, M.U., Clark-Lewis, I., Baggiolini, M. & Rot, A. (1993). Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc Natl Acad Sci U S A*, **90,** 7158-62.

Wells, T.N. & Peitsch, M.C. (1997). The chemokine information source: identification and characterization of novel chemokines using the WorldWideWeb and expressed sequence tag databases. *J Leukoc Biol*, **61,** 545-50.

Wess, J., Nanavati, S., Vogel, Z. & MAggio, R. (1993). Functional role ofproline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the M3 muscarinic receptor. *EMBO J.*, **12,** 331-338.

Williams, G., Borkakoti, N., Bottomley, G.A., Cowan, I., Fallowfield, A.G., Jones, P.S., Kirtland, S.J., Price, G.J. & Price, L. (1996). Mutagenesis studies of interleukin-8. Identification of a second epitope involved in receptor binding. *J Biol Chem*, **271,** 9579-86.

Witt, D.P. & Lander, A.D. (1994). Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr Biol*, **4,** 394-400.

Wong, L.M., Myers, S.J., Tsou, C.L., Gosling, J., Arai, H. & Charo, I.F. (1997). Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene. Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J Biol Chem*, **272,** 1038-45.

Woolfson, D.N. & Williams, D.H. (1990). *FEBS Lett.*, **277,** 185-188.

Wu, L., Gerard, N.P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A.A., Desjardin, E., Newman, W., Gerard, C. & Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature*, **384,** 179-83.

Wu, L., LaRosa, G., Kassam, N., Gordon, C.J., Heath, H., Ruffing, N., Chen, H., Humblias, J., Samson, M., Parmentier, M., Moore, J.P. & Mackay, C.R. (1997). Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *J Exp Med*, **186,** 1373-81.

Wuyts, A., Struyf, S., Proost, P. & Van Damme, j. (1999). Chemokines. *In (ed. Thize, J.) The cytokine network and immune factors. Oxford University Press*, 125-145.

Yang, D., Howard, O.M.Z., Chen, Q. & Oppenheim, J.J. (1999). Immature dendritic cells generated from monocytes in the presence of TGF-beta1 expres functional CC chemokine receptor 6. *J. Immunol.*, **163,** 1737-47.

Yoneda, J., Kuniyasu, H., Crispens, M.A., Price, J.E., Bucana, C.D. & Fidler, I.J. (1998). Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst*, **90,** 447-54.

Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M., Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H. & Yoshie, O. (1997). Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *J Biol Chem*, **272,** 13803-9.

Yoshida, R., Nagira, M., Kitaura, M., Imagawa, N., Imai, T. & Yoshie, O. (1998). Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. *J Biol Chem*, **273,** 7118-22.

Yoshida, T., Imai, T., Takagi, S., Nishimura, M., Ishikawa, I., Yaoi, T. & Yoshie, O. (1996). Structure and expression of two highly related genes encoding SCM-1/human lymphotactin. *FEBS Lett*, **395,** 82-8.

Yoshie, O., Imai, T. & Nomiyama, H. (1997). Novel lymphocyte-specific CC chemokines and their receptors. *J Leukoc Biol*, **62,** 634-44.

Yoshimura, T., Matsui, K., Ito, M., Yunohara, T., Kawasaki, N., Nakamura, T. & Okamura, H. (1987). Effects of highly purified eicosapentaenoic acid on plasma beta thromboglobulin level and vascular reactivity to angiotensin II. *Artery*, **14,** 295-303.

Youn, B.S., Jang, I.K., Broxmeyer, H.E., Cooper, S., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Elick, T.A., Fraser, M.J., Jr. & Kwon, B.S. (1995). A novel chemokine, macrophage inflammatory protein-related protein-2, inhibits colony formation of bone marrow myeloid progenitors. *J Immunol*, **155,** 2661-7.

Zaballos, A., Gutierrez, J., Varona, R., Ardavin, C. & Marquez, G. (1999). Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. *J Immunol*, **162,** 5671-5.

Zabel, B.A., Agace, W.W., Campbell, J.J., Heath, H.M., Parent, D., Roberts, A.I., Ebert, E.C., Kassam, N., Qin, S., Zovko, M., LaRosa, G.J., Yang, L.L., Soler, D., Butcher, E.C., Ponath, P.D., Parker, C.M. & Andrew, D.P. (1999). Human G protein-coupled receptor GPR-9/6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J. Exp. Med.*, **190,** 1241-56.

Zhang, Y.J., Rutledge, B.J. & Rollins, B.J. (1994). Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. Identification of a mutated protein that inhibits MCP-1-mediated monocyte chemotaxis. *J Biol Chem*, **269,** 15918-24.

Zhao, J., Ma, L., Wu, Y.L., Wang, P., Hu, W. & Pei, G. (1998). Chemokine receptor CCR5 functionally couples to inhibitory G proteins and undergoes desensitization. *J Cell Biochem*, **71,** 36-45.

Zlotnik, A., Morales, J. & Hedrick, J.A. (1999). Recent advances in chemokines and chemokine receptors. *Crit Rev Immunol*, **19,** 1-47.

Zlotnik, A. & Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity*, **12,** 121-7.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I. & Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*, **393,** 595-9.