Identification Of New Molecules Involved In Dendritic Cell Localization

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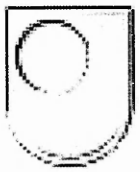

The Open University

IDENTIFICATION OF NEW MOLECULES INVOLVED IN DENDRITIC CELL LOCALIZATION

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Abstract

The present study was designed to investigate the expression and function of an orphan seven transmembrane receptor named CCRL2 (CC chemokine receptor like 2), a putative chemokine receptor. In human tissues CCRL2 was expressed mainly by lung, lymphoid tissues and fetal spleen. Among leukocytes CCRL2 was expressed by monocytes, neutrophils and dendritic cells (DC). Because chemokines play a fundamental role in DC trafficking, modulation of CCLR2 expression in this cell type was further investigated. Maturative stimuli like LPS and CD40L strongly up regulated CCRL2 mRNA and protein in DC. Culture of DC in the presence of inhibitors of maturation and function such as VitD3 and Dex had no effect on LPS-induced CCRL2 up regulation. On the contrary PGE2, that does not affect DC maturation, completely abolished LPS induction of CCRL2 expression. The effect of LPS and CD40L on CCRL2 expression was rapid (1.5h) and transient (maximal at 4h) and declined by 24h, conversely the upregulation of CCR7 that was slower and reached a plateau at 24h of stimulation.

Since CCRL2 gene is located in the main chemokine receptor cluster in the 3p21 chromosome, it is likely to be a conventional inflammatory chemokine receptor. In order to identify ligands CCRL2 transfectants were used in chemotaxis and calcium flux assays with a broad panel of inflammatory CC and CXC chemokines but no ligand was identified. The alterations in the DRYLAR/IV motif in the second intracellular loop suggest that CCRL2 may be a candidate for the family of chemokine decoy receptors like the receptor D6. This second hypothesis was evaluated performing chemokine scavenging assays. None of the chemokine tested was scavenged by CCRL2. However in parallel experiments two new ligands for D6, the CCR4 agonists CCL17 and CCL22 were identified. In summary these data suggest that CCRL2 might be involved in DC trafficking, through the regulation of the DC emigration from tissues following

stimulation. None of the chemokine tested was able to bind or activate CCRL2. Furthermore CCRL2 appears not to act as a chemokine scavenger receptor and its biological role is still elusive.

Acknowledgements

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Abbreviations

Ag	antigen
Ab	antibody
APC	antigen presenting cell
Ba	basophils
BCA	b-cell activating chemokine
bp	base pair
BRAK	breast and kidney chemokine
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDNA	complementary dna
CTACK	cutaneous t-cell–attracting chemokine
CX3CL	CX3C chemokine ligand
CX3CR	CX3C chemokine receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	dendritic cell
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELC	epstein-barr virus–induced receptor ligand chemokine
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial cell–derived neutrophil-activating factor (78 amino acids)

Eo	eosinophils
EST	expressed sequence tag
FCS	foetal calf serum
FDC	follicular dendritic cells
FL	flt-3 ligand;
FRET	Fluorescence Resonance Energy Transfer
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCP	granulocyte chemoattractant protein
GM-CSF	granulocyte / macrophage colony-stimulating factor)
GPRC	G protein coupled receptor
GRO	growth-related oncogene
HCC	hemofiltrate cc chemokine
HPC	haematopoietic progenitor cells
HPF	high power field
iDC	immature dendritic cells
IDC	interdigitans dendritic cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IN-DC	interstitial dendritic cells
IP	interferon-inducible protein
I-TAC	interferon-inducible t-cell a chemoattractant
kb	kilobase
kDa	kilodalton
KL	c-*kit* ligand

LC	Langherans cells
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MC	mast cells
MCP	monocyte chemoattractant protein
MDC	macrophage-derived chemokine
mDC	mature dendritic cells
M-DC	myeloid-related dendritic cells
MHC	major histocompatibility complex
Mig	monokine induced by γ interferon
MIP	macrophage inflammatory protein
Mo	monocytes
Mø,	macrophages
MPIF	myeloid progenitor inhibitory factor
mRNA	messenger rna
NAP	neutrophil-activating protein
NF-κB	nuclear factor kappa b
NK	natural killer
NK	natural killer cells
PARC	pulmonary and activation-regulated chemokine
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
P-DC	plasmacytoid dendritic cells
PFA	paraformaldehyde

PKB/C	protein kinase b/c
RANTES	regulated upon activation normal t cell–expressed and secreted
RNA	ribonucleic acid
rRNA	ribosomal rna
RT-PCR	reverse transcription polymerase chain reaction
SCM	single c motif
SD	standard deviation
SDF	stromal cell–derived factor
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLC	secondary lymphoid tissue chemokine
SSC	sodium chloride, sodium citrate buffer
T act	activated t cells
T muc	mucosal-homing t cells
T skin	skin-homing t cells
TAM	tumour associated macrophage
TARC	thymus and activation-related chemokine
TECK	thymus-expressed chemokine
TGF	transforming growth factor
TNF	tumour necrosis factor
Treg	regulatory t cells
tRNA	transfer RNA
VEGF	vascular endothelial growth factor
XCL	C chemokine ligand
XCR	C chemokine receptor

1 Chapter 1

1.1 Dendritic cells

1.1.1 Introduction

Dendritic cells (DC) are the most potent antigen-presenting cell in the immune system because they have unique capacity in capturing and processing antigens for presentation to T cells and because they express high levels of the co-stimulatory molecules for T-cell activation (1, 2). In addition to efficiently inducing the activation and proliferation of naive T cells, they fine-tune immune responses by instructing T-cell differentiation and polarization. DC transmit a distinct set of instructions to T cells that is based on their state of differentiation or maturation, and these instructions programme outcomes that range from humoral to cytolytic to suppressive (regulatory) T-cell responses (3).

Paul Langerhans in 1868 first described dendritic cells (DC) in human skin but thought these were cutaneous nerve cells. Steinman and Cohn (4) discovered these cells almost a century later in mouse spleen and called them "dendritic cells" on the basis of their unique morphology. Progress in the study of DC biology exploded in the 1990s when investigators developed cytokine-driven methods for expanding and differentiating DC ex vivo in both mouse and human systems (5-8).

1.1.2 Dendritic cell precursors

Human DC are all bone marrow-derived leukocytes. They are distinct from follicular DC (FDC), which are not leukocytes but are of stromal origin. (9). DC can originate from either common lymphocyte (CLP) or common myeloid progenitors (CMP) suggesting a redundancy in their developmental pathways (10) (Figure 1.1). These progenitors have been isolated from the bone marrow using a panel of markers. Both progenitors share a lack of the "lineage" markers of differentiated haematopoietic cells, but express IL-7 receptor in the case of lymphoid progenitors and c-kit in the case of myeloid progenitors. However the dual origin of DC is still controversial.

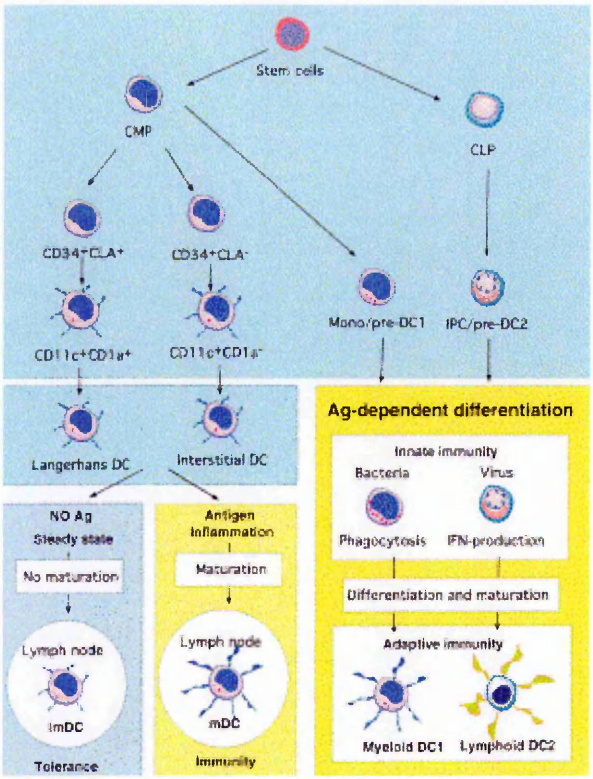

Figure 1.1 DC Development, Diversification, Maturation, and Function.

$CD34^+$ haematopoietic stem cells differentiate into common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP). The CMPs differentiate into $CD34^+CLA^+$ and $CD34^+CLA^-$ late progenitor cells that differentiate in blood into $CD11c^+CD1a^+$ Langerhans cell precursors and $CD11c^+CD1a^-$ interstitial DC precursors, respectively. These cells migrate into the skin or other tissues in an antigen independent way and may undergo a steady-state migration into the draining lymph nodes playing a critical role in immune tolerance. CMP and CLP also give rise to myeloid pre-DC1 and lymphoid pre-DC2 in bone marrow. They migrate into the blood and then to the lymphoid tissues. During bacterial infection, pre-DC1s ingest and kill bacteria and then differentiate into DC and initiate adaptive antibacterial immune responses. During viral infection, pre-DC2s rapidly produce large amounts of type-1 IFN and then differentiate into DC and initiate adaptive antiviral immune responses. Blue background is used for resting or tolerogenic cells, while yellow background is used for activated cells (figure courtesy of Silvano Sozzani, Università di Brescia, Italy).

1.1.3 Dendritic cell subsets

A large variety of DC subsets have been described in lymphoid and non-lymphoid organs. DC are mainly subdivided into myeloid-related DC (M-DC) and plasmacytoid DC (P-DC) (11). In the peripheral blood, M-DC precursors express CD11c but lack CD123, while the P-DC precursors display the $CD11c^{-}CD123^{+}$ phenotype. Both subsets are immature, since they are negative for co-stimulation molecules CD80, CD86 and CD40 (11). Based on in *vitro* data, there are different pathways for the development of mature DC from bone marrow $CD34^{+}$ precursors. Each pathway differs in terms of progenitors and intermediate stages, cytokine requirements, surface marker expression and, most importantly, biological function (10). M-DC are distinguished by at least two distinct pathways of maturation from $CD34^{+}$ progenitors since, after 5 days in culture with granulocyte-macrophage colony-stimulating factor, stem cell factor and tumor necrosis factor (TNF)-α, cells are sorted into either $CD14^{+}CD1a^{-}$ or $CD14^{-}CD1a^{+}$ populations (Figure 1.2) (12). In addition, it is likely that under certain conditions mature monocytes migrate from blood into tissues and differentiate into DC (13). Substantial diversity exists between M-DC and P-DC, supporting the possibility of different functional roles. M-DC have several features that allow them to capture antigens, exploiting a complex array of uptake mechanisms, including phagocytosis, micropinocytosis and receptor-mediated endocytosis (3), while P-DC have very limited phagocytic capacity (10, 11). M-DC represent the classic T cell-priming subset, but this function in P-DC is less clear, although there is definite evidence that P-DC play an important role in the defense against pathogens and neoplasms (14). Despite the experimental evidence that circulating and tissue P-DC can acquire the morphological and functional features of DC in *vitro* (15), the existence of fully mature P-DC in *vivo* is still controversial (14). Furthermore, M-DC and P-DC show marked disparity in tissue distribution and migration pathways. Immature M-DC are constitutively distributed in

peripheral tissues, especially in the skin and mucosal surfaces, which represent the areas of entry of exogenous antigens, where they are responsible for antigen capture and processing. Following antigen capture, M-DC undergo maturation into competent APC, bearing high levels of MHC and costimulatory molecules (e.g., HLADR, CD80, CD83, CD86, DC-LAMP/CD208), and migrate to lymphoid tissues, acquiring potent immunostimulatory activity (10), to become mature APC (e.g., interdigitating DC, IDC). In contrast to M-DC, P-DC are scarce or totally absent in skin, mucosae and other non-lymphoid tissues, while they typically occur in lymph nodes and tonsils, in close association with high endothelial venules (HEV) (16). The topographical association between P-DC and HEV reflects the migration pathway of this subset of DC, which leave the circulation and enter lymphoid tissue through HEV (17). Alternative ways, however, exist of migration of M-DC into lymph nodes. Even in the absence of inflammation, some DC are found in afferent lymph, suggesting that DC continuously traffic from normal tissues to lymph nodes. These rare steady-state migrating DC from skin to lymph nodes are phenotypically mature and might be important for immune tolerance, eliminating T cells with specificity for self antigens that have escaped the thymus during thymic selection (18). Finally, monocytes may undergo differentiation to DC upon migration to the lymph nodes (11).

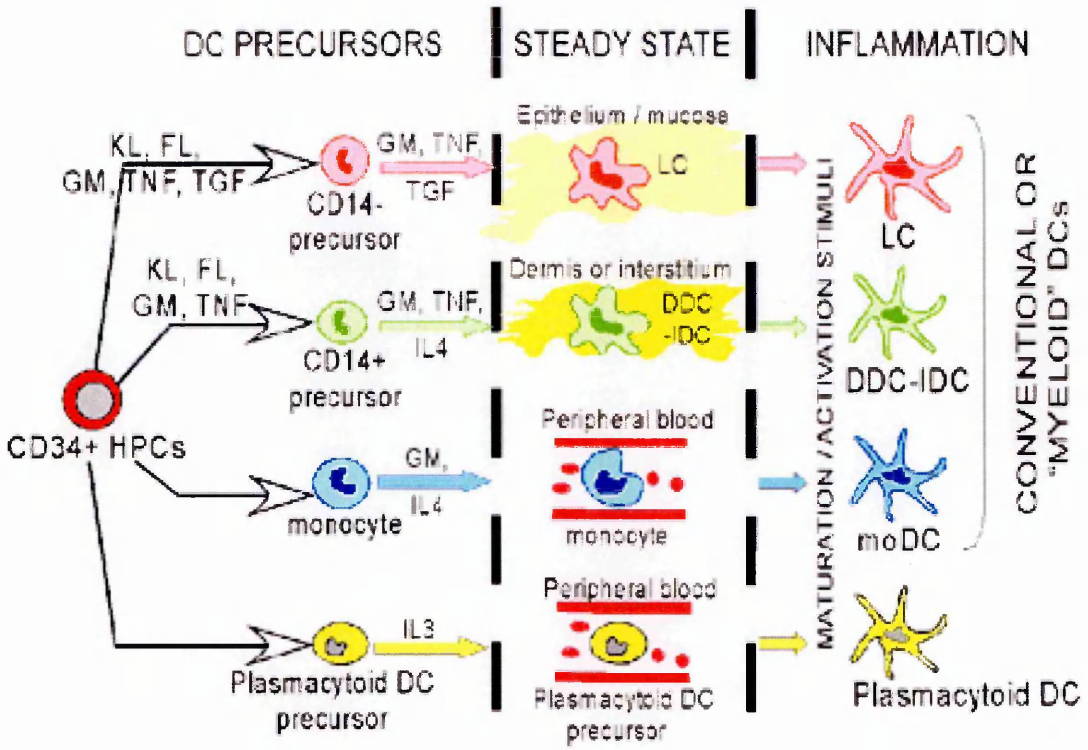

Figure 1.2 Development of human DC subsets.

Precursors in blood and bone marrow (left section) can give rise to four types of DC under cytokine-driven conditions ex vivo. Resident populations of immature, non activated DC (middle section) are normally found in the steady state in the periphery and/or the circulation. Semi-mature DC continuously present self-Ags, and probably harmless non-self-Ags, in secondary lymphoid organs to maintain peripheral tolerance and anergy. Counterparts for these various DC types develop in vitro in the presence of the indicated cytokines. Harmful pathogens or other dangerous insults to the steady state can lead to inflammation with full maturation and activation of each DC subset (right section). Taken from Rossi, M. et al. 2005 J. Immunol. (19).

1.1.4 Non lymphoid tissue DC

M-DC include intraepithelial Langerhans cells (LC) and interstitial DC (IN-DC). LC are present in the skin and mucosae, they have typical DC morphology and contain characteristic Birbeck granules (BG) seen on electron microscopy. Markers are HLA-DRY S-100 protein, CDla, E-cadherin and the LC-specific marker langerin (CD207), while they lack CD68 and factor XIIIa, and most antigens expressed by dermal IN-DC (Fig. 1.3). Moreover, LC lack several maturation antigens, such as DCLAMP/CD208, while expression of CD83 can be variable (20). In conditions associated with an increase or activation of intraepidermal LC, such as contact dermatitis, cells expressing a hybrid monocyte-LC phenotype ($CD1a^{+}CD11b^{+}CD36^{+}CD68^{+}$) can be observed (20); this observation supports the evidence that LC may derive from monocytes in *vivo* (13). IN-DC are present in the interstitial space of most tissues with the exception of the cornea and central nervous system. IN-DC express CD11c, CD68, factor XIIIa, macrophage-mannose receptor (CD206), along with the c-type lectin DC-SIGN (CD209) (Table 1.1). In analogy with LC, IN-DC lack DC maturation antigens (21) and, as with LC, dermal and mucosa IN-DC are strategically localized at the interface with the external surfaces where they can take up pathogens (22) and transport them to lymph nodes.

The migration of DC from peripheral tissues to lymph nodes is associated with changes in their phenotype. LC adhere to keratinocytes via homophilic interactions with E-cadherins, and down-regulate this adhesion molecule to leave the epidermis. In the lymph vessels and nodal sinuses migrating LC are identified as veiled cells, because of their sheet-like lamellipodia; similar to LC, veiled cells express CDla, S-100 protein and langerin/CD207, and are mostly immature (23).

	M-DC (myeloid-related DC)	
	LC (Langerhans cells)	IN-DC (interstitial DC)
CD1a	+	-
CD11c	-	+
CD68	-	+
CD206	-	+
CD207/langerin	+	-
CD208/DCLAMP	-	-
CD209/DC SIGN	-	+
E-cadherin	+	-
Factor XIIIa	-	+
S-100	+	-

Table 1.1: Epitopes that distinguish non lymphoid tissue DC

CD1a: Type I transmembrane protein related to the major histocompatibility complex (MHC) proteins. It forms heterodimers with beta-2-microglobulin and mediates the presentation of primarily lipid and glycolipid antigens.
CD11c: Integrin alpha X chain protein that combines with the beta 2 chain to form a leukocyte-specific integrin named CR4.
CD68: Transmembrane glycoprotein; member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family. It binds to tissue- and organ-specific lectins or selectins and it is also a member of the scavenger receptor family.
CD206: mannose receptor, C type 1 type I membrane receptor that bind high-mannose structures.
CD207: C-type lectin with mannose binding specificity, localized in the Birbeck granules, organelles present in the cytoplasm of Langerhans cells.
CD208: member of the lysosome-associated membrane glycoprotein (*LAMP*) family.
CD209: C-type lectin receptor acting as both cell-adhesion receptor and pathogen-recognition receptor
E-cadherin: Type I transmembrane glycoprotein that mediates calcium dependent cell-cell adhesion.
Factor XIIIa: A subunit of coagulation factor XIII, the last zymogen activated in the blood coagulation cascade
S-100: calcium binding protein localized in the cytoplasm and/or nucleus involved in the regulation of cell cycle progression and differentiation.

1.1.5 Lymphoid tissue DC

DC have been largely studied in lymph nodes and tonsils (24), where their distribution is rather complex, reflecting the occurrence of different subsets of DC, diversity of activation and maturation stages and pathways of migration (lymph *versus* blood borne). M-DC-related IDC, represent the majority of mature DC within the lymph node, showing bright expression of HLADR and DC-LAMP/CD208 (Fig. 1). IDC are predominantly found in paracortical T nodules, where they are intimately admixed with T lymphocytes. IDC are considered to descend for the most part from LC, and maintain the positivity for S-100 protein (Figure 1.3). However, antigens usually negative on LC (such as CD11c) are expressed by IDC, while langerin and CD1a are generally lost (24). P-DC are typically found in the 'traffic area' of lymph nodes and are better identified with the help of immunostains that show strong reactivity for CD68, CLA HECA-452, CD123, BDCA2 and TCL-1 (25).

Cortical B follicles contain two main DC, the germinal center DC (GCDC) and the follicular DC (FDC). GCDC express CD4, CD13 and CD11c, are strong APC for T cells, and can directly regulate B cell responses, producing IL-12 and inducing germinal center B cell expansion, plasma cell differentiation, and IL-10-independent isotype switching toward IgG1. The origin of GCDC is poorly studied; they might be related to the subset of dermal DC that express CXCR5 and traffic to B cell zones in lymph nodes (26). FDC do not represent bona fide DC, since they are non-hematopoietic in origin, but mesenchymal. In addition, they are not capable of activating naïve T cells, do not display a capacity for antigen capture and presentation, but do express preformed antigen-antibody complexes (antigen carrying cells) on their surface. FDC are typically located within primary and secondary B follicles, and interactions between CXC ligand 13 (B lymphocyte chemoattractant; CXCL13) expressed on FDC and CXCR5 expressed

by B cells and activated T cells play a role in B follicle development and organization. FDC express complement and Fc receptors, as well as a series of antigens that are useful for revealing them on sections, such as CD21, CD23, CD35, CNA.42, KiM4p, DRC1, nerve growth factor receptor, and clusterin (Figure 1.3). The functional role of FDC is still controversial (9). The close association with germinal center B cells has fostered the idea that B cell recognition of retained antigen on the surface of FDC is important for affinity maturation and memory B cell development. However, it is possible that FDC support B cell proliferation and differentiation in a nonspecific manner.

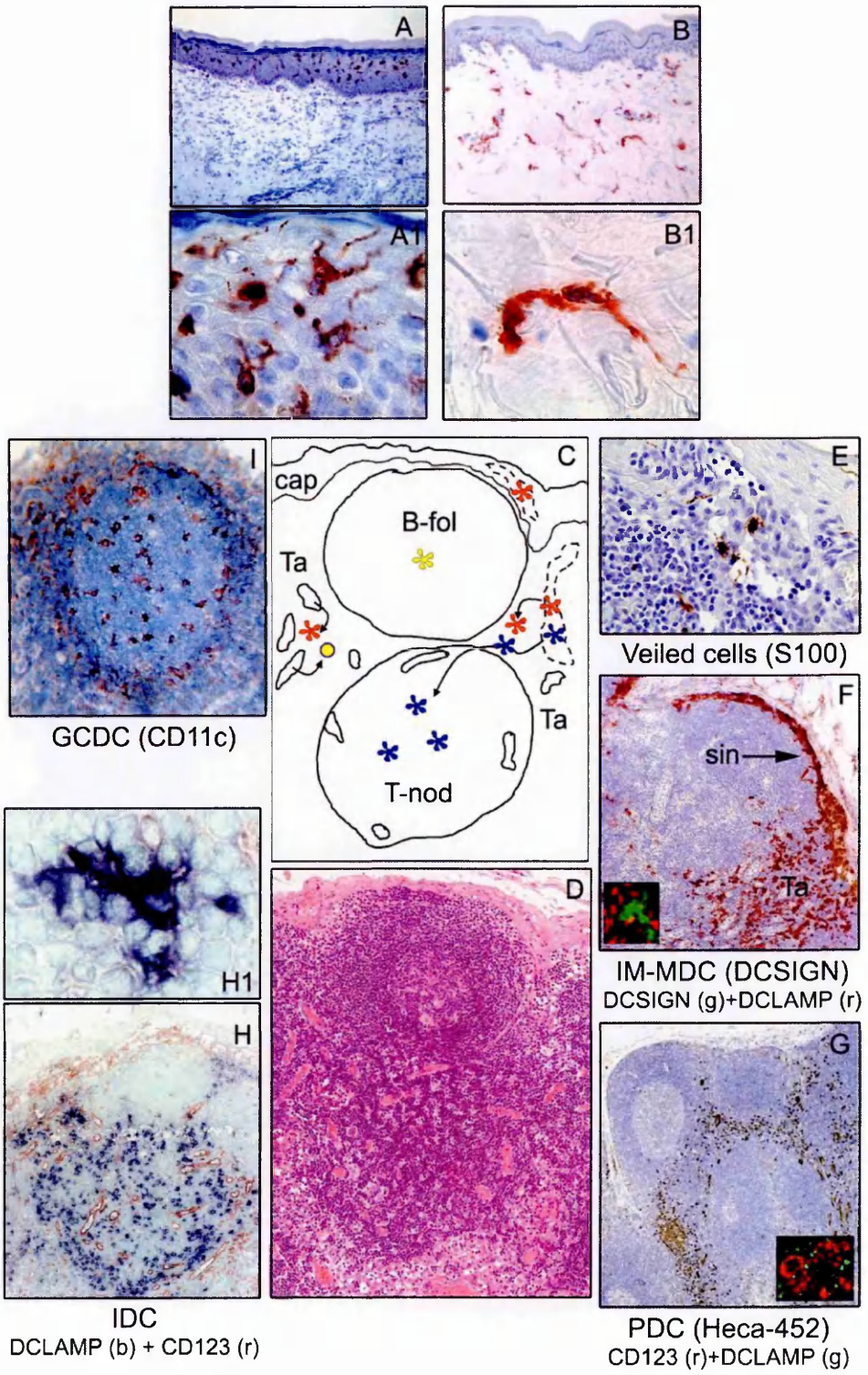
A
B
A1
B1
I
GCDC (CD11c)
C
cap
B-fol
Ta
Ta
T-nod
E
Veiled cells (S100)
F
sin
Ta
IM-MDC (DCSIGN)
DCSIGN (g)+DCLAMP (r)
H1
H
IDC
DCLAMP (b) + CD123 (r)
D
G
PDC (Heca-452)
CD123 (r)+DCLAMP (g)

Figure 1.3 Distribution and phenotype of DC subsets in peripheral tissues (skin) and lymph node.

In the skin LC are typically confined to the epidermis (A, A1), while IN-DC, here stained for macrophage mannose receptor, are found in the dermis (B, B1). In C and D, a drawing and the corresponding picture of a reactive lymph node is shown (cap: capsule; Bfol: B follicle; T-nod: paracortical T nodule; Ta: traffic area). Sinus vessels are illustrated with dashed lines, HEV as full lines; nodal DC are shown as star-shaped cells and include immature DC (red), mature DC (blue), and GCDC (yellow); P-DC are shown as round cells (yellow). In the marginal sinus some S-100 protein+ veiled cells are present (El; F shows staining for DC-SIGN, which identifies macrophages within the marginal sinus (sin), as well as the sinus lining cells and numerous DC along the traffic area (Ta); in the inset a double immunofluorescence for DC-SIGN and DCLAMP shows that the DC-SICN cells (green) do not express DCLAMP (red). In G, the traffic area at the periphery of B follicles and T nodules is clearly depicted by HECA-452 antibody, which stains P-DC and HEV; in the inset a double immunofluorescence for CD123 and DCLAMP shows that the CD123+ P-DC (red) do not express DCLAMP (green). The paracortical T nodule shown in H contains large numbers of DCLAMP' mature IDC (blue), that form close contacts with surrounding T cells (HI); CD123 in red (H) stains the HEV and scattered P-DC mainly localized at the periphery of the T nodule. In a secondary B follicle, CDl l c stains GCDC (I). (figure courtesy of Silvano Sozzani, University of Brescia, Italy).

1.1.6 Further distinctions in nomenclature

M-DC and P-DC have been, respectively, labeled DC1 and DC2 because of their propensity to stimulate Th1- vs Th2-type responses. This oversimplification, however, neglects stimulation of more varied T cell responses. The name DC1 does not take in account the function of LC or IN-DC as conventional DC. Skewing toward Th2 responses by plasmacytoid DC in the presence of IL-3 led to their being termed DC2. This has no bearing, however, on their role as tolerogenic DC in inducing $CD4^+$ and $CD8^+$ regulatory T cells (27). It also overlooks the major physiologic role of plasmacytoid DC as the most abundant source of type I IFNs after activation by viruses (15, 28). IN-DC and M-DC have been considered homologous, because both develop from a $CD14^+$ precursor. More recent phenotypic and functional data, however, indicate that these two types of DC are distinct (29). The specific descriptive term for each type of DC is therefore more useful than nomenclature like DC1 vs DC2, or myeloid vs lymphoid DC

1.1.7 Dendritic cell maturation

The process of DC maturation is now accepted as a crucial component of the induction of adaptive immune responses (Figure 1.4) . The term maturation refers to the differentiation process whereby DC respond rapidly to an environmental stimulus and become capable of eliciting adaptive immunity. The type of stimulus determines the program of DC differentiation and the subsequent immune response. DC can directly sense pathogens via TLRs, and respond to this recognition by up-regulating surface costimulatory molecules, secreting cytokines and chemokines, enhancing antigen presentation, and migrating to secondary lymphoid tissues (30). Some features of DC maturation, such as the up-regulation of CD86, can also be induced by proinflammatory cytokines, but cytokines alone seem insufficient for the activation of adaptive immunity

in vivo (31). Additional changes can be imparted to DC by CD40 ligation, which contributes to the generation of both CD4 and CD8 T cell immunity (32). So, the DC maturation process may be seen as the sensor that links innate immune responses to adaptive ones. During TLR-mediated DC maturation, distinct TLR ligands evoke distinct responses (30). This signaling complexity is further increased by the expression of a distinct TLRs profile by DC subsets, as well as by the differences in adaptor molecules used by single TLRs.

1.1.8 DC functions

1.1.8.1 Dendritic cells in T cell activation

DC express a variety of co-stimulatory molecules and produce several cytokines and chemokines which contribute to shape the quality of the T cell response. T cells establish contact with APCs by forming an immunological synapse, where TCRs and CD28 molecules are segregated together in a central area surrounded by a ring of adhesion molecules (33). In naïve T cells TCRs are inefficiently coupled to signal transduction pathways. Engagement of CD28 by B7 molecules expressed on APCs recruits membrane rafts containing kinases and adaptor proteins to the synapse and amplifies the signalling process initiated by the TCR. Sustained signalling is essential for naïve T cells to up-regulate anti-apoptotic cytokines, receptors for homeostatic cytokines, such as IL-7 and IL-15, and to induce T cell proliferation.(34). A short TCR signal leads to abortive T cell proliferation. In contrast a shorter TCR stimulation, even in the absence of CD28 mediated co-stimulation, is sufficient for effector T cells to induce their proliferation and activate their effector function (35). The balance between stimulatory and inhibitory signals in T cell activation is required for effective immune response to pathogens and for maintaining self tolerance (Figure 1.4).

Although the basic principles of DC physiology have been elucidated in considerable details, it is difficult at present to draw a general picture on how antigen presentation is carried out. A first variable to be considered in T cell priming is the relative contribution to antigen presentation of migrating versus resident DC. Indeed some antigens may reach the lymph node directly while others need to be ferried by migrating DC. This will impact on the frequency and activation state of the antigen presenting DC and ultimately on the strength of stimulation that will be delivered to the naïve T cell. Self antigens in pancreas, constitutively transported by migrating DC and presented in the draining lymph nodes, induce abortive proliferation and establishment of tolerance. In the presence of an infectious agent the same antigen delivered to the lymph nodes by high numbers of DC expressing co-stimulatory molecules and primed for IL-12 production induced productive T cell activation and differentiation to effector cells. A second variable is the nature of the activating signals received by DC. For instance CD40L expression by specific helper T cells can deliver to DC a licensing signal for effective CTL priming. DC that have been directly activated by microbial products have superior T-cell stimulatory capacity as compared to those that have been activated in a bystander fashion by inflammatory cytokines. The third variable is the kinetic of the DC activation. Migrating DC that secrete Th1 polarizing cytokines will be capable of driving Th1 differentiation, while the same DC at later time points, having exhausted IL-12 producing capacity, will prime T cells that either develop towards Th2 or remain non-polarized. The differentiation of naïve CD4+ T cells towards IFN-γ producing Th1 is promoted by IL-12 whereas differentiation towards IL-17 producing inflammatory T cells is promoted by IL-23. Mature DC that migrate to the lymph node induce rapid recruitment of NK cells and the IFN-γ produced by them is necessary for efficient Th1 polarization in vivo (36). Th2 polarisation is primarily driven by IL-4, but the source of this cytokine during T cell priming remains to be determined. Several studies indicated

that DC under appropriate stimulatory conditions can effectively prime Th2 responses in spite of not being able to make IL-4. DC with Th2 polarizing capacity can be generated either by maturation stimuli that do not induce IL-12 production or by exhausted DC. Since naïve T cells upon prolonged stimulation can produce low amounts of IL-4 that is sufficient to promote their own differentiation towards Th2, it is possible that Th2 differentiation would simply result from a lack of Th1 polarizing cytokines (37, 38). Plasmacytoid dendritic cells are capable of presenting endogenous antigens to CD8+ T cells, although their major function is the production of high amounts of type I interferons, following viral infection or TLR7 or TLR9 triggering by specific agonists (39). Mouse pDC, cultured from bone marrow precursors or isolated from spleen, can induce the development of both Th1 and Th2 effector cells depending on the dose of antigen (39). Thus, as observed for conventional DC, antigen dose, nature of maturation stimuli and state of pDC maturation will determine whether a Th1 or Th2 response develops.

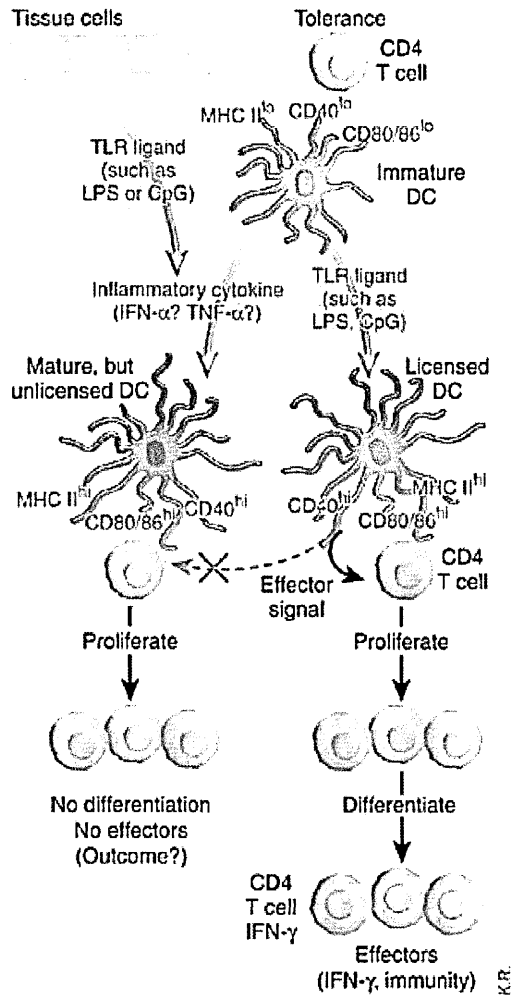

Figure 1.4 Inflammatory signals cause DC maturation but do not license DC to drive CD4 T cell effector functions.

Immature DC (top) expressing few MHC class II molecules and the T cell costimulatory molecules CD40, CD80 and CD86 are thought to induce tolerance. After recognition of pathogen products via TLR ligands (right) or inflammatory signals released by other cells (left), they become mature licensed DC or mature unlicensed DC, respectively. Both forms of mature DC express abundant MHC class II and costimulatory molecules, which enables them to induce the proliferation of naive CD4 T cells. However, only the licensed DC can provide an additional signal that drives $CD4^+$ T cell differentiation into effector helper cells (bottom right).(Heath, W.R., and al. 2005. *Nat Immunol* (40))

1.1.8.2 Dendritic cell role in the induction and maintenance of tolerance

Dendritic cells also play a fundamental role in the induction and maintenance of central and peripheral tolerance. Central tolerance in the thymus is achieved by inducing apoptotic cell death in potentially self-reactive T cell clones (41). Thymic medullary epithelial cells express MHC class I molecules and delete self reactive T cell clones with high affinity. Thymic DC represent a particular subset with a different life history, because most of them derive from an intra-thymic precursor, develop and die within the thymus. Several in vitro and in vivo experiments have demonstrated the ability of thymic DC to induce central tolerance. In contrast to their function in negative selection thymic DC are neither active, nor required for positive selection, which can be fully supported by cortical epithelial cells. The completely different outcome of the DC T cell interaction, which in the thymus induces apoptosis instead of T cell activation and proliferation, seems to be more related to the T cell differentiation stage rather than being a special feature of this DC subset. Central tolerance is efficient, but incomplete: self reactive T cells with lower affinity for self antigens, can escape negative selection (42). However, self-reactive T cell clones are not exclusively deleted by central tolerance mechanisms. Tolerance in vivo is achieved also by an active process of peripheral control of potentially self reactive T cells. Peripheral tolerance is important not only for self antigens but also for other peptides, like harmless environmental antigens. It is essential to acquire tolerance in order to avoid a chronic response. Most data show that DC are able to induce tolerance at an immature stage (43). Immature DC (iDC) can induce tolerance by killing T cells, by inducing T cell anergy, or by generating regulatory T cells. In this view continuous steady state DC migration from peripheral tissue to lymph-nodes in the absence of any microbial products might maintain peripheral tolerance. Indeed, several reports have also shown that low doses of soluble antigen induce peripheral tolerance when targeted to DC in steady state; T cell

proliferation takes place but reactive T cells are subsequently deleted after few days (43).

In addition to T cell deletion, DC can induce regulatory T cells. At least two different regulatory T cell subsets have been identified: naturally occurring regulatory T cells, which arise in the thymus and are characterized by the expression of CD4 CD25 and FoxP3, and T cells with suppressor function, which are induced in the periphery. This last subset of regulatory T cells is CD25 and FoxP3 negative. Both produce immunesuppressive cytokines, like IL10 and TGFβ and require cell contact for their regulatory function (43).

The presence of a *danger* signal, such bacterial products, inflammatory cytokines, lipid mediators and/or NAD+, released by necrotic cells, seems the major strength which drives DC to a fully mature phenotype with the ability to activate and induce the T cell proliferation and proper immune response. But many questions regarding the achievement of peripheral tolerance remain unsolved; when self antigens as well as microbial or viral products are present at the same time in the same environment. In this regard Medzhitov and Blander recently suggested that compartmentalization and presentation of Toll like receptor mediating antigen uptake differs from that mediating uptake of phagocytosed apoptotic cells (44). In this regard, even if still under debate, necrotic cells might also represent a danger signal, which differs from apoptotic cells. Another complex situation in terms of immune regulation is represented by the delicate equilibrium of tolerance and immunity against commensal bacterial. In the gut bacterial and bacterial products are physically separated from DC by the epithelial layers, which might influence the triggering of TLR. Only TLR expressed at the surface of DC might be able to sense the environment, whereas intracellular TLRs, like TLR9, 3 and 7, could not be engaged by TLR agonists. During an infection, bacteria are in direct contact with APCs, and this difference could determine the different outcome of the immune

response. Tumor transformation also represents a particular situation in terms of immune reaction. Neoplastic events might happen continuously throughout the life of an individual, but the role of immune surveillance is still not clear, since the incidence of transforming events in immune-deficient mice is very low, probably because the majority of mutations might induce apoptosis of the cell. Many open questions remain to be solved in order to understand the fine equilibrium between tolerance and immunity.

1.1.9 Clinical applications of human DC for active immunotherapy

There is great interest in altering the cytokine milieu that drives DC immunogenicity (45) or in using DC to expand Tregs for the control of autoimmunity (46). Most current clinical studies, however, use DC for active immunotherapy trials in cancer. Most tumor Ags are poor immunogens because they are self-Ags or self-differentiation Ags, to which there is considerable tolerance. DC provide a potential solution to this challenge by coupling tumor Ag with all of the requisite costimulatory ligands, cytokines, and chemokine-directed migration to secondary lymphoid organs. There they can stimulate incoming T cells to exit via efferent lymph into the periphery as cytolytic and helper T cell effectors.

Challenges to designing the optimal DC vaccine include the choice of DC subset or combination of subsets. For example, whether the functional distinctions between conventional DC subsets in vitro (29) have physiologic relevance in vivo is the subject of an ongoing vaccine trial in melanoma. The presumptive advantage of LCs has been the rationale for other investigators to include $CD34^+$ HPC-derived DC, which comprise LCs among the progeny, in vaccine preparations (47). The malleability of moDC precursors under certain cytokine conditions might also yield moDC progeny that function more like LCs (48). Other unknowns include optimal Ag-loading strategies

like peptide pulsing, overlapping polypeptide pulsing, cross-presentation of dying tumor cells, fused tumor-DC heterokaryons, DNA or RNA transfection with or without a vector construct, frequency and route of immunization, and cell dose. Finally for effective vaccination activated and terminally mature DC are needed to avoid any reversion to immature DC that may be inactive or even generate suppressive Tregs (49). The first human DC vaccine trial used the rare circulating DC isolated ex vivo from steady-state pheresis products and loaded with tumor-specific idiotypes to treat patients with follicular lymphoma (50). This approach is not selective for any one of several DC subsets in blood, and the yields are low. The advent of the cytokine-generated DC era has supported large-scale clinical evaluations, and a number of trials were performed. In the aggregate, these studies have shown that DC vaccinations are safe and that tumor-specific T cell responses can be generated by DC vaccination using standard immunologic assays in vitro. Although patients eligible for these early phase clinical trials have advanced disease, clinical responses have been achieved in some instances (51). Major challenges remain in terms of harnessing the capacity of DC for simultaneous presentation of multiple tumor Ags tailored to their own MHC molecules, rather than presentation of only a few peptides with defined MHC restrictions. Migration of DC to draining lymph nodes also requires optimization after vaccination.

1.2 Chemokines

1.2.1 Introduction

The first chemokine discovered was Platelet Factor 4 (now called CXCL4) in the late 1970s (52), but the existence of a distinct family of small secreted proteins, named *chemokine* because of their leukocyte *chemo*tactic and cyto*kine*-like activities, was evident only after the cloning of interleukin-8 (CXCL8) in 1987 as an 'anionic' neutrophil-activating factor and chemoattractant (53). Initially chemokines were identified on the basis of their biological activities in culture supernatants and were purified biochemically before being sequenced and cloned. More recently, completion of the human genome project has led to the final identification in humans of about 50 structurally and functionally related molecules and it is likely that most of the chemokines have now been discovered (54).

1.2.2 Chemokine classification

Chemokines are all small proteins (~8-14 kDa) frequently glycosylated. They bear a significant sequence identity to each other, and the protein structure is strictly dependent on 2 conserved disulfide bonds connecting conserved cysteine residues (55, 56). According to the position and spacing of these cysteine residues, 4 chemokine subfamilies have been defined: CXC, CC, C and CX3C. The largest group of chemokines has the first 2 cysteines in an adjacent position (CC chemokines). Most of these molecules, products of a large multigenic cluster on chromosome 17q11.2, act on monocytes, whereas other CC chemokines, products of different chromosomal loci, are active on different cell types (Figure 1.5). In the CXC family, the other large group of chemokines, the two amino terminal cysteine residues are separated by a single amino acid. Most of these molecules are coded by 2 large multigenic clusters. The first, located on chromosome 4q12-q13, includes CXC chemokines containing an ELR conserved

amino acid sequence on the N-terminus (ELR^+CXC chemokines) that act on neutrophils. The second, located on 4q21.21, includes CXC chemokines lacking the ELR sequence (ELR^- CXC chemokines) that act mainly on T lymphocytes (Figure 1.5). The third chemokine subfamily includes 2 highly related molecules with only 2 cysteine residues (C chemokines), encoded by a single cluster on chromosome 1q23, selectively active on T lymphocytes. The fourth family (CX3C chemokines) includes a single molecule with 3 intervening amino acids between the first 2 cysteine residues. This chemokine is coded by a gene located on 16q13 and acts on monocytes and T lymphocytes. It has a transmembrane domain that allows it to be tethered to the cell surface like the chemokine CXCL16.

Classically, chemokines have been named according to their expression patterns or functions, but due to the rapid discovery of many new chemokines, in 2000 Zlotnik and Yoshie (54, 57) proposed a new nomenclature that is based on the type of subfamily followed by a progressive number provided by the position of the corresponding coding gene in the cluster. Thus, chemokines now are identified by a name providing information on the respective structural subfamily, corresponding also to the type of receptor they engage, followed by a number provided by and referring to the respective coding gene (58).

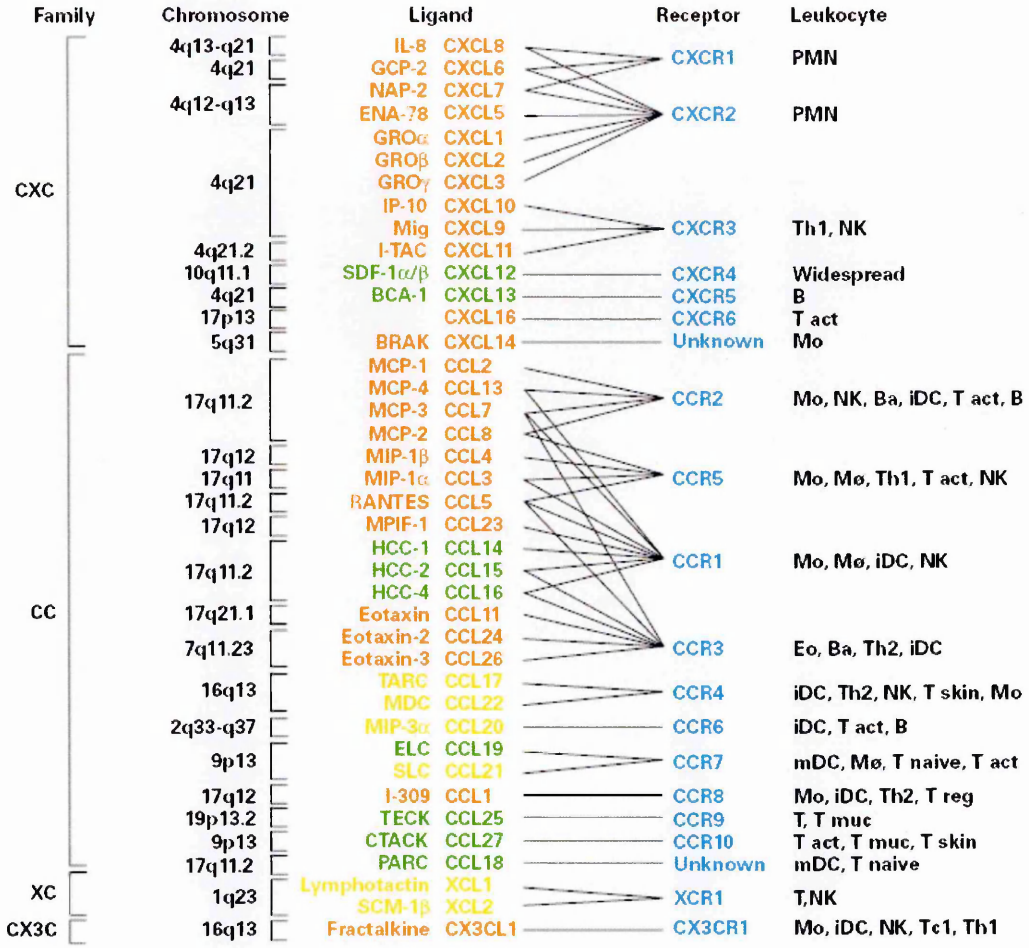

Figure 1.5 The chemokine system: an overview.

Chemokines, their receptors, and predominant receptor repertoires in different leukocyte populations are listed. The selected ligands are identified with one old acronym and the new nomenclature, in which the first part of the name identifies the family and L stands for "ligand," followed by a progressive number. Red identifies predominantly "inflammatory" or "inducible" chemokines; green, "homeostatic" agonists; yellow molecules belong to both realms. Taken from (59)

1.2.3 Chemokine structure

NMR spectroscopy and X-ray crystallography studies have provided high-resolution structures for a number of chemokines, revealing a conserved fold across subfamilies (Figure 1.6), despite low percentage of sequence similarities (60). This common fold is composed of a three stranded anti-parallel β-sheet covered on one face by a C-terminal α-helix and preceded by a disordered N-terminus (Figure 1.6). Most available structures support the formation of chemokine dimers but either absence of dimerization of specific chemokines and higher order oligomers have also been reported (61). Interestingly, the contact interface is different between the CC and CXC families. Dimerization of CC-chemokines involves their N-terminus, in such a way that this domain becomes buried in the β-sheet of the other protomer. In contrast, CXC-chemokines interact via the β1-strand, leaving the N-terminus accessible. On the basis of mutagenesis studies, it was demonstrated that the binding of chemokines to their cognate receptors involves the so-called N-loop, which immediately follows the first cysteine, and for some chemokines, the N-terminal segment as well. Truncation of the unstructured N-terminal domain of chemokines generates in most cases antagonists or very partial agonists, illustrating the role played by this domain in the activation of the receptor.

Although many studies support the formation of chemokine homodimers, CCL3/CCL4 and CXCL8/CXCL4 heterodimers have been reported as well (62). Chemokines were shown to be secreted by cells as preformed dimers but the dimer dissociation constants determined are in the micromolar range, concentrations significantly higher than the nanomolar concentrations required for their biological activities in vitro or in vivo. However, chemokines are also known to interact with glycosaminoglycans, and this interaction was reported to promote the aggregation of chemokines and to increase their

local concentration at the cell surface (63). Oligomerization might therefore be favored in vivo, even at relatively low concentrations. However, numerous studies have reported that chemokine mutants unable to form dimers retain their ability to bind and activate receptors, arguing that the monomers are the active forms (64). Although synergistic effects on cell chemotaxis were reported between Regakine and either CXCL8 or CCL8 (MCP-3) as well as between CXCL11 (I-TAC) and CXCL12 (SDF-1) (65), this phenomenon likely involves post-receptor events rather than chemokine dimerization. It is therefore largely accepted that, besides modifying indirectly the actual concentration of monomers, dimers do not act as ligands for chemokine receptors, and no solid elements exists that would support a link between oligomerization of ligands and oligomerization of receptors.

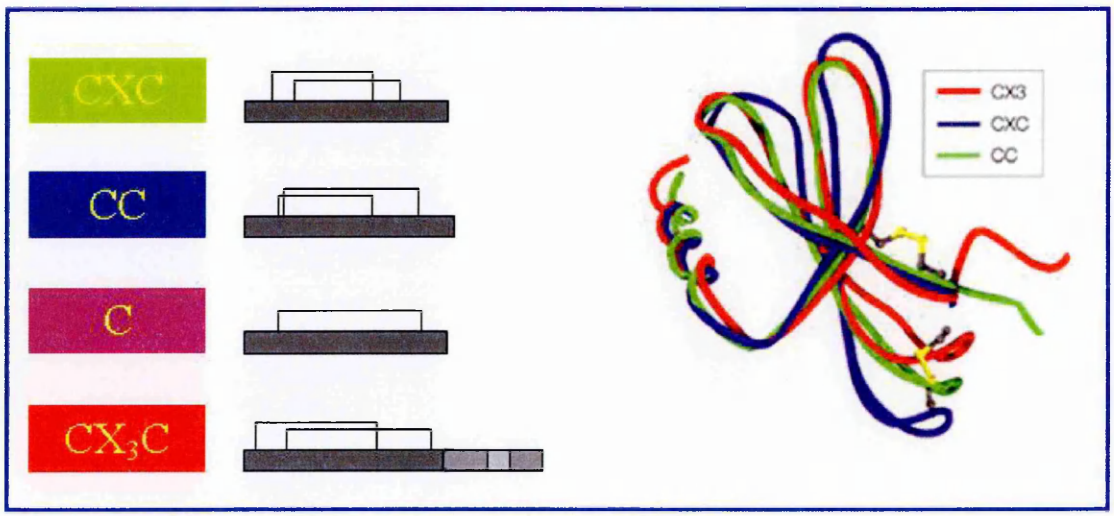

Figure 1.6 Chemokine subfamilies and chemokine fold

A. Chemokine primary sequences are represented with a black box, disulfide bonds connecting conserved cysteine residues are represented by a thin line. CX3C extended mucin-like stalk, transmembrane domain and intracellular domain are represented with gray boxes. B. The common chemokine fold is composed of a three stranded anti-parallel β-sheet covered on one face by a C-terminal α-helix and preceded by a disordered N-terminus.

1.3 Chemokine receptors

1.3.1 Nomenclature and classification

Chemokine receptors are defined by their ability to signal on binding one or more members of the chemokine superfamily of chemotactic cytokines (66). They define a distinct subfamily in the class A of the rhodopsin-like G protein–coupled receptor family (67, 68). At present, 18 receptors have been defined molecularly, 10 for CC chemokines (CCR1 to 10), 6 for CXC chemokines (CXCR1 to 6), and 1 for C chemokines and CX3C chemokines (XCR1 and CX3CR1, respectively). In most cases, each individual receptor binds multiple chemokines, but subclass restriction is strictly respected. Thus, a major functional correlate of chemokine subclassification is represented by the use of different receptors whose names include the chemokine subclass specificity followed by a number (Figure 1.5).

1.3.2 Tridimensional structure and activation mechanisms

The sequences of chemokine receptors have 25 to 80% aa identity, indicating a common ancestor. However, many other G protein-coupled peptide receptors also have ~25% aa identity to chemokine receptors, illustrating that the structural boundary is not sharp. Although they lack a single structural signature, there are several features that together are found more frequently among chemokine receptors than other types of GPCRs. These include a length of 340 to 370 aa; an acidic N-terminal segment; the sequence DRYLAIVHA, or a variation of it, in the second intracellular loop; a short basic third intracellular loop; and a cysteine in each of the four extracellular domains (68).

The three-dimensional structure of chemokine receptors is unknown, but a reasonable working model can be constructed for the transmembrane domains based on analogy with rhodopsin. Evidence has been reported that CCR2, CCR5, and CXCR4 form

homodimers, and in the case of CCR2, a dimer has been implicated as the functional form of the receptor, which may be needed for signaling (69).

As other GPCRs, chemokine receptors are integral membrane proteins that contain seven transmembrane α-helices (TM) linked by intracellular (ICL) and extracellular loops (ECL), an extracellular N-terminal domain and a cytosolic C-terminus (70). Most class A receptors share two conserved cysteines involved in the formation of a disulfide bond between ECL1 and ECL2. As mentioned before, chemokine receptors contain two additional conserved cysteines believed to form a second disulfide bond between the N-terminus and ECL3. These two disulfide bonds appear as necessary for the proper folding of chemokine receptors, the binding of their ligands and/or their ability to activate intracellular cascades even if cysteine mutants of CCR5, while strongly impaired in terms of chemokine binding, still support HIV infection (71).

Both the N-terminus and extracellular loops of chemokine receptors play a critical role in their interaction with chemokines. From mutagenesis experiments, the second extracellular loop (ECL-2) of CCR2, CCR5 and other receptors was reported to play an important role in the specificity of interaction with CC-chemokines (71, 72). Monoclonal antibodies that target this loop are also known to inhibit ligand binding (72). A number of acidic amino-acids and sulfated tyrosines located in the N-terminal domain of receptors also contribute to the high affinity binding of chemokines (73). All available studies converge to a model in which the core domain of the chemokine binds to the N-terminus and extracellular loops of the receptor, while the chemokine N-terminus interacts with the helix bundle and is involved in receptor activation. The proposed model for the binding of a chemokine to its receptor is a two-step mechanism (Figure 1.7), in which a first binding interaction of the chemokine with the receptor N-terminus allows a second interaction to take place with the extracellular loops and transmembrane bundle, resulting in receptor activation (74).

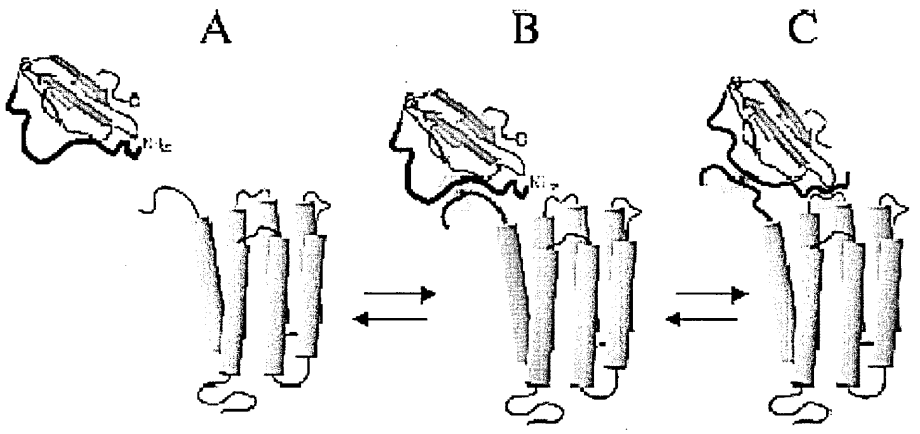

Figure 1.7 Model of chemokine receptor-ligand interaction.

A. Receptor is in an inactive conformation. B. Ligand binds to the N-terminus of the receptor, leading to a conformational change. C. The conformational change allows for ligand binding to the activation domain of the receptor.

During the past few years, our understanding of the activation mechanisms of GPCRs has greatly progressed, in part as a consequence of the availability of the 3D structure of bovine rhodopsin in its inactive state (75). Biochemical and biophysical approaches, supported by modeling studies, have allowed us to identify key structural motifs involved in the activation mechanism of GPCRs. Such motifs include the E/DRY box at the cytosolic border of TM3 (76) and the NPxxY motif in TM7, which are both highly conserved among class A receptors. It is believed that agonists induce relative movements of TM3 and TM6 of the GPCR activation by agonists, resulting in the opening of a binding pocket for the heterotrimeric G protein. Activation mechanisms require the disruption of intramolecular interactions that stabilize the inactive conformation of the receptor (77). It is now widely accepted that disruption of these constrains is induced by the binding of agonists, and that mutations affecting some of the key residues involved in these interactions can lead to the constitutive activity of receptors. Naturally occurring mutations leading to increased constitutivity of specific

receptors have been associated with pathological states (78). One of the best known constrains that maintains class A GPCRs in their inactive conformation is the so-called "ionic lock" that involves residue Arg in the E/DRY motif of TM3, the adjacent Asp/Glu residue, and a partly conserved Asp/Glu residue at the cytoplasmic end of TM6 (79). The substitution of the charge in one of these Asp/Glu results in an increase of the constitutive activity of rhodopsin, adrenergic and hormone receptors

1.3.3 Chemokine receptor signalling

Conventional (i.e. signalling) chemokine receptors, like all other members of the GPCR family, mainly transduce intracellular signals through the activation of heterotrimeric G proteins, and all chemokine receptors in particular mediate signalling through pertussis toxin-sensitive $G\alpha_i$ proteins (80). A common response of all chemokine receptors is, almost by definition, the stimulation of cell migration. Stimulation of chemotaxis by a chemokine requires the functional coupling of the receptor to $G\alpha_i$ because migration is completely inhibited by treatment of the cells with pertussis toxin. However, $G\alpha_i$ itself appears not to be necessary for cell migration. The essential step is the release of the heterotrimeric G protein $\beta\gamma$ subunits from $G\alpha_i$ and the G_i protein–coupled receptor (81). The release of $\beta\gamma$ subunits is required, but not sufficient, to induce chemotaxis because in addition to G protein–activation, seven-transmembrane-domain receptors generate accompanying signals that induce functional responses.

Stimulation of chemokine receptors rapidly activates phosphoinositide-specific phospholipase $C\beta_2$ (PLC-β_2) and PLC-β_3 isoenzymes, which leads to inositol-1,4,5-trisphosphate ($InsP_3$) formation and a transient rise in the concentration of intracellular free calcium ($[Ca^{2+}]_i$). This pathway has been widely used to test the responsiveness of chemokine receptors to different chemokines (82). The two PLC isoforms that are

involved in chemokine-signal transduction become activated by direct interaction with βγ subunits, which are released from the Gαβγ-receptor complex (83). However in neutrophils of mice that lack the genes encoding both isoforms chemokine-induced calcium elevation is suppressed, but do not show any defects in chemokine-stimulated migration (84), suggesting that calcium influx is not necessary for chemotactic response. Chemokine-mediated activation of PLC not only results in $InsP_3$ production but also leads to the formation of diacylglycerol and subsequent activation of protein kinase C (PKC).

Another well established effector of βγ subunits is the type I_B phosphatidylinositol 3 kinase γ (PI3Kγ) (85). Through this enzyme chemokines stimulate the rapid formation of phosphatidylinositol 3,4,5-trisphosphate PtdIns(3,4,5)P_3, which is subsequently dephosphorylated to become PtdIns (3,4)P_2. Mice that do not express PI3Kγ have severely impaired chemokine-stimulated signal transduction (85), which suggests that PI3Kγ is involved in distinct pathways downstream of G protein–coupled receptors. Among the best characterized downstream effectors of the PI3Ks is PKB (86). During G protein–coupled receptor-stimulated chemotaxis, PKB is rapidly activated and recruited to the membrane of the leading edge of the cell (87)

The involvement of βγ subunits in the activation of mitogen-activated protein kinases (MAPKs) by chemokines is less clear, although activation by chemokines is well documented (88). βγ subunits of the G protein–coupled receptors activate MAPK through a PI3Kγ-dependent pathway which, in turn, stimulates a Src-like kinase, initiating a "classical" growth factor signal transduction cascade that involves Shc, Grb2, SOS, Ras and Raf (89).

The heterotrimeric G protein α subunits activate Src family kinases (for example, Fgr, Lck or Lyn) (90). Stimulation of Src kinases by $G\alpha_i$ not only links chemokine receptors

to Ras activation by an alternative pathway, *via* Shc, Grb2 and SOS, but could also explain the activation of FAK and Pyk-2 (91) and of downstream effectors by chemokines (Figure 1.8)

A characteristic of most chemokine receptors is the induction of short transient signals and the rapid termination of receptor activity by receptor phosphorylation, desensitization and internalization. Homologous and heterologous desensitization of chemokine receptors is achieved by G protein–coupled receptor kinases (GRKs, mainly GRK2) PKA and PKC. Receptor phosphorylation causes the binding of arrestins, molecules that were previously shown to interrupt receptor activity and to couple to an adaptor protein 2 (AP-2) and clathrin-mediated internalization pathway (92). Receptor phosphorylation and internalization is agonist-stimulated but not inhibited by pertussis toxin. Phosphorylation of chemokine receptors occurs at multiple sites located at the cytoplasmic COOH-terminus and could involve more than one kinase.

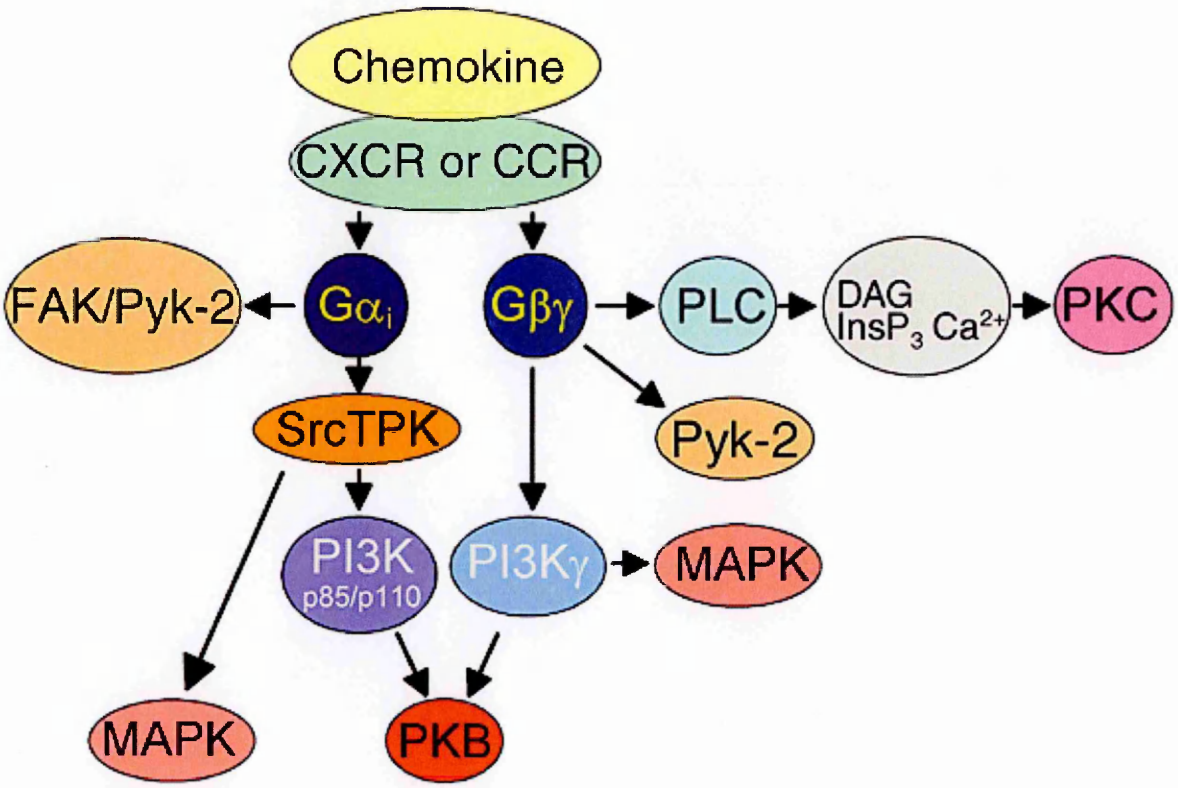

Figure 1.8 Chemokine receptor signaling.

βγ subunits activated PLC, PI3Kγ and indirectly the FAK-related tyrosine kinase Pyk-2; GTP-bound $G\alpha_i$ directly activates Src-like kinases. Through intermediate proteins, the kinases stimulate the activation of the cytoskeleton-associated kinases FAK and Pyk-2, and, possibly *via* Shc, the MAPK cascade. These events include several tyrosine residue phosphorylations that direct the recruitment and activation of proteins that contain SH2 domains. Such mechanisms endorse the βγ subunit–independent activation of type I_A PI3Ks and lead to protracted PtdIns(3,4,5)P_3 formation. Taken from Thelen M. 2001, *Nat.Immunol.* (93)

1.3.4 Silent or decoy chemokine receptors

1.3.4.1 Introduction

As originally formulated at the end of the 19th century, a receptor is a "receptive substance which binds a ligand, usually with high affinity and specificity, and elicits a cellular response". Almost one century later, the first decoy receptor (the IL-1 type II receptor) was identified, and defined as "a receptor structurally incapable of transducing signal but able to recognize the agonist with high affinity and specificity" (94). The biological function of decoy receptors is to compete with signalling receptors for the ligand, sequester and target it to degradation. Decoy receptors are now recognized as a general strategy to negatively regulate primary inflammatory cytokines. In addition, at least the IL-1 decoy RII acts as a dominant negative, sequestering a key component of the signalling receptor complex, the IL-1R accessory protein (95). Interestingly, in phagocytes the IL-1 decoy RII targets the agonist for endocytosis and degradation, thus acting as a scavenger (96).

After the initial observation in the IL-1 system, decoy receptors have been identified for a large number of cytokines, in particular inflammatory cytokines (95) characterized by different structures and signalling properties, including the IL-1R family (IL-18 binding protein), the TNFR family (e.g. osteoprotegerin), the IL-10 family (IL-22 binding protein), the IL-4/IL-13R family (IL-13R2). In Drosophila, Argos was recently shown to act as a decoy for epithelial growth factor (97). The first evidence that a similar strategy could also exist in the chemokine system stemmed from the observation that under appropriate environmental conditions inflammatory chemokine receptors can be uncoupled from the signalling machinery retaining the ability to bind the ligand and targeting it to degradation. Under these conditions chemokine receptors have been

named "functional" decoy receptors, in that they are structurally identical to signalling receptors but act as decoys (98)

Subsequently three proteins that bind subsets of chemokines, but exhibit unusual properties compared to typical leukocytic chemokine receptors were identified. These are DARC (99), D6 (100) and CCX-CKR (101). Despite exhibiting structural homology to other chemokine receptors, and showing high affinity interactions with chemokines, these molecules do not couple to the major signaling pathways activated by other chemokine receptors upon ligand stimulation, and thus do not mediate cell migration. In fact, no alternative signals have been described from these receptors, leading to them often being referred to as 'silent'. Furthermore, they exhibit unusual expression patterns and, unlike typical chemokine receptors, are difficult to find on peripheral blood leukocytes. Recent data have provided further support that these molecules neutralize or transport chemokines.

Beside these three chemokine binding proteins another receptor for chemotactic molecules, named C5L2, with similar features was recently characterized. This receptor binds with high-affinity C5a and the desarginated forms of both C5a and C3a (C5adR[74] and C3adR[77]) (102). Moreover the receptor US28, encoded by the human cytomegalovirus, displays characteristics of a chemokine decoy receptor (103).

1.3.4.2 D6, a pro-inflammatory CC chemokine decoy receptor

Originally identified as a CCL3 binding molecule expressed in murine hemopoietic stem cells (104) and soon after in human cells (105) and (106), the D6 molecule is a typical chemokine receptor. The 7 TM domain organization is well conserved, the overall sequence identity to conventional chemokine receptors is in the 30–35% range, similar to the identity rate observed among chemokine receptors, and the N-terminal domain presents several charged residues, most likely involved in ligand recognition as

for other chemokine receptors. Radioligand binding experiments have demonstrated that D6 recognizes an unusual broad spectrum of ligands, being able to interact with most agonists of inflammatory CC chemokine receptors from CCR1 through CCR5 (105). While the ligand binding profile is unusually broad, receptor expression is fairly restricted, being D6 only detectable in placenta and on endothelial cells of lymphatic afferent vessels in skin, gut and lung (105, 106) (107).

A significant body of evidence has been gathered demonstrating that neither the human nor murine D6 sustain signalling activities typically observed after chemokine receptor triggering, such as calcium fluxes and chemotaxis (104, 105, 108) Sequence motifs critical for G protein coupling and signalling functions of chemokine receptors like the DRYLAR/IV in the second intracellular loop as well as the TXP motif in the second TM domain are not conserved in D6. Whether these modifications account for D6 loss of signalling function, while retaining high affinity ligand binding is presently unknown. D6 does not mediate chemokine transfer through endothelial barriers (108). When D6 was expressed on a lymphatic endothelial cell line (108) no evidence for facilitated chemokine transfer through the cell monolayer was obtained. Conversely, the presence of D6 consistently resulted in the degradation of appropriate ligands. Similar results were obtained in different D6 cell transfectants. Analysis of biochemical properties of D6 indicated that D6-internalized chemokines are readily released from the receptor during vesicle acidification, allowing subsequent ligand degradation and leaving D6 free to recycle to the cell surface. Consistently with this, prevention of vesicle acidification by pretreatment with ammonium chloride resulted in reduction of ligand degradation and accumulation of the receptor in intracellular compartments. Thus, in in vitro settings D6 does not mediate signalling activities or support chemokine transcytosis, but behaves as a decoy receptor that scavenges inflammatory CC chemokines (108).

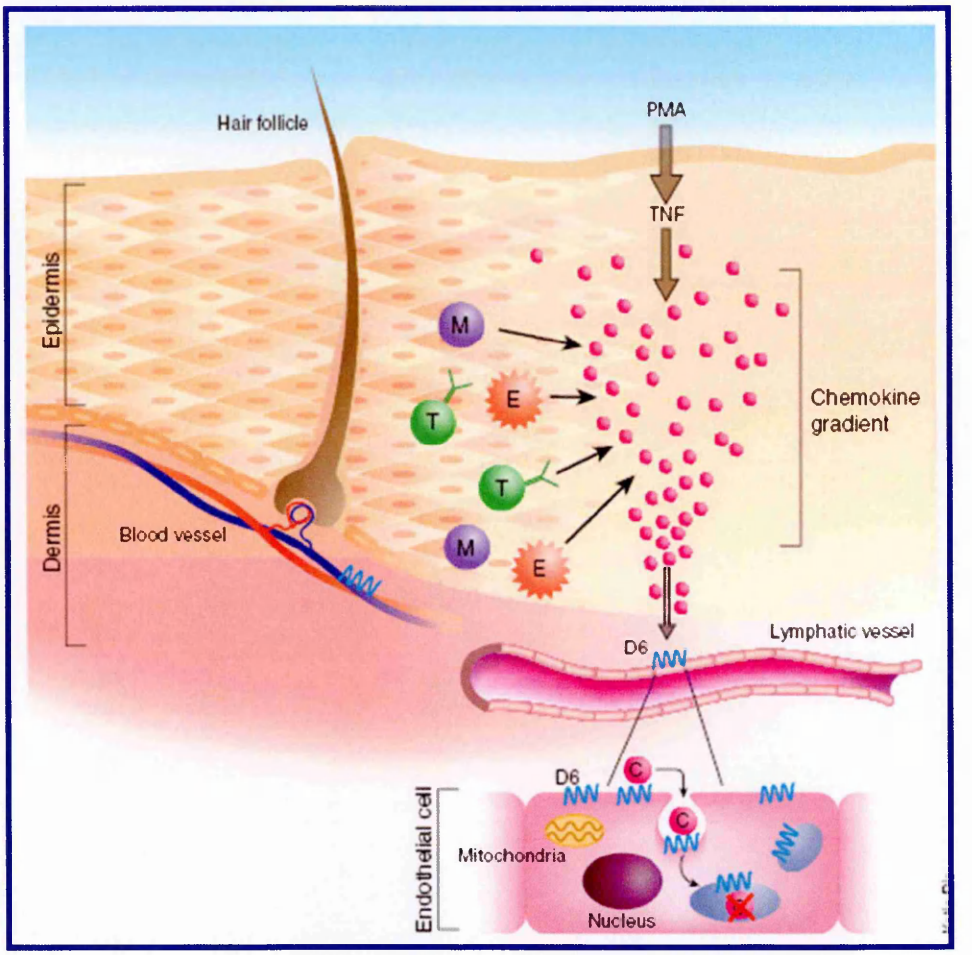

Figure 1.9 Model of D6 modulation of inflammatory responses in vivo.

In wild-type mice phorbol ester (PMA) initiates an acute and self limiting inflammation that is dependent on the generation of tumor necrosis factor (TNF). Later, genes encoding many β-chemokines are induced and transcribed, forming a chemotactic gradient that attracts CD4 and CD8 T lymphocytes (T), eosinophils (E) and mast cells (M). In D6-deficient mice, the inflammatory response is intense and prolonged, as the chemokine half-life is extended. As a result, the skin pathological lesions resemble those observed in the human psoriasis. The D6 decoy is found in lymphatic endothelium, where it acts as a 'conveyor belt' to 'mop up' and deliver the chemokines to endosomes. Taken from Gerard, C. 2005. *Nat Immunol* 6:366-368(109).

To test the potential role of D6 as a regulator of inflammatory chemokine biology in an in vivo settings, D6-null mice have been investigated in two different models of local inflammation. By using a model of inflammation induced by phorbol ester skin painting, Jamieson et al. recently demonstrated that D6-null mice had an exacerbated inflammatory response, initiated by TNF and then sustained by inflammatory chemokines, with a prominent inflammatory infiltrate that included T lymphocytes, mast cells and polymorphonuclear neutrophils (110). Keratinocyte proliferation and neovascularization were also observed, leading to the development of psoriasiform lesions. In an independent study, Martinez de la Torre et al. reported that D6 deletion resulted in an abnormal inflammatory response in a model of skin inflammation induced by subcutaneous injection of complete Freund adjuvant (111). In this latter model, inflammatory lesions had a faster apparence and showed a more severe evolution in D6-deficient animals, which also developed prominent necrosis and neovascularization. At short times (e.g. day 7) inflammation evolved in macroscopic granuloma-like lesions in a significant percentage of D6−/− animals, and only in a minority of wild-type littermates. Interestingly, differences were not evident at later time points (e.g. day 21). Increased levels of inflammatory CC chemokines were detected locally in both models, and pretreatment with chemokine receptors blocking antibodies was able to prevent lesion development, demonstrating that, in the absence of D6, the increased inflammatory response is caused by an inefficient control of the chemokine system. Although the specific role of individual CC chemokines in the recruitment of different leukocyte populations have not been defined, both reports described an unbalance restricted to inflammatory CC chemokines, consistently with D6 binding profile. Interestingly, some features were common at both experimental conditions, including the predicted derangement of CC chemokines and the unexpected effect on neovascularization, while others were apparently restricted to the specific experimental

model used, such as keratinocyte proliferation and the prominent neutrophil infiltrate, possibly sustained by a synergistic effect of CC chemokines on CXC chemokines-dependent neutrophil recruitment (110). In synthesis, the two experimental models highlighted a non-redundant role of D6 in the control of local inflammation in skin, but the molecular mechanisms involved in this effect are still ill defined and deserve further investigation, as well as the evaluation of the role of D6 in other tissues (Figure 1.9).

1.3.4.3 DARC: chemokine transport and/or neutralization?

The Duffy blood group antigen was first described in 1950. The same protein acts in erythrocytes as an entry receptor for some malarial parasites (112). Interestingly, its absence, caused by promoter mutation in Duffy negative individuals, provides erythrocyte resistance to malarial infection. It later transpired that Duffy antigen is a receptor for various pro-inflammatory chemokines of both CC and CXC subclasses (113, 114), leading to its renaming as Duffy antigen receptor for chemokines (DARC). Although being the broadest known chemokine receptor, being able to bind 16 inflammatory chemokines of the CC and CXC families, overall DARC expresses a fairly low homology rate with conventional chemokine receptors. Along with erythrocytes, DARC is also expressed on vascular ECs, where it is up-regulated during inflammation (115). It is particularly prominent at sites of leukocyte extravasation, including the high endothelial venules of lymph nodes (116). Importantly, DARC lacks canonical intracellular signaling motifs, and does not support any detectable ligand-induced signalling or migration. These features have lead to hypotheses that DARC is involved in the transcytosis, or neutralization, of chemokines at EC barriers and, on erythrocytes, that it may act to regulate plasma chemokine concentrations (99) (Figure 1.10). Immunoelectron microscopy studies in skin have demonstrated that chemokines can be internalized by ECs, transported across the cell, and presented on the tips of

luminal microvilli, presumably associated with GAGs moreover in vitro data have shown that CXCL8 can be transported across an EC monolayer in a DARC-dependent fashion (117).

Analysis of inflammatory reactions in DARC-deficient animals has lead to contrasting results (118, 119), possibly because DARC might exert a dual function, acting as mechanism that facilitates transfer of chemokines across vascular endothelium (120) and as a chemokine buffering system (113) (118, 121) under different circumstances.

Additional evidence for a decoy function of DARC has emerged from studies on mice engineered to over-express DARC in ECs. These mice show reduced angiogenic responses to certain CXC chemokines. Here, DARC may sequester these chemokines, preventing their binding to EC CXCR2, thus blocking angiogenic signals from this receptor (122). It has been proposed that DARC expressed by erythrocytes may function as a chemokine reservoir, maintaining plasma concentrations of certain chemokines. DARC negative humans display reduced plasma levels of CCL2 compared to DARC positive individuals and injected chemokines more rapidly disappear from the circulation in DARC *null* mice (99). Thus, erythrocyte DARC may act as a chemokine buffer, sequestering chemokines present at high levels in the serum, but maintaining a homeostatic level as their presence subsides. Because plasma chemokines desensitize circulating leukocytes, careful buffering by DARC may control leukocyte sensitivity to pro-inflammatory chemokines, limiting under- or over responsiveness.

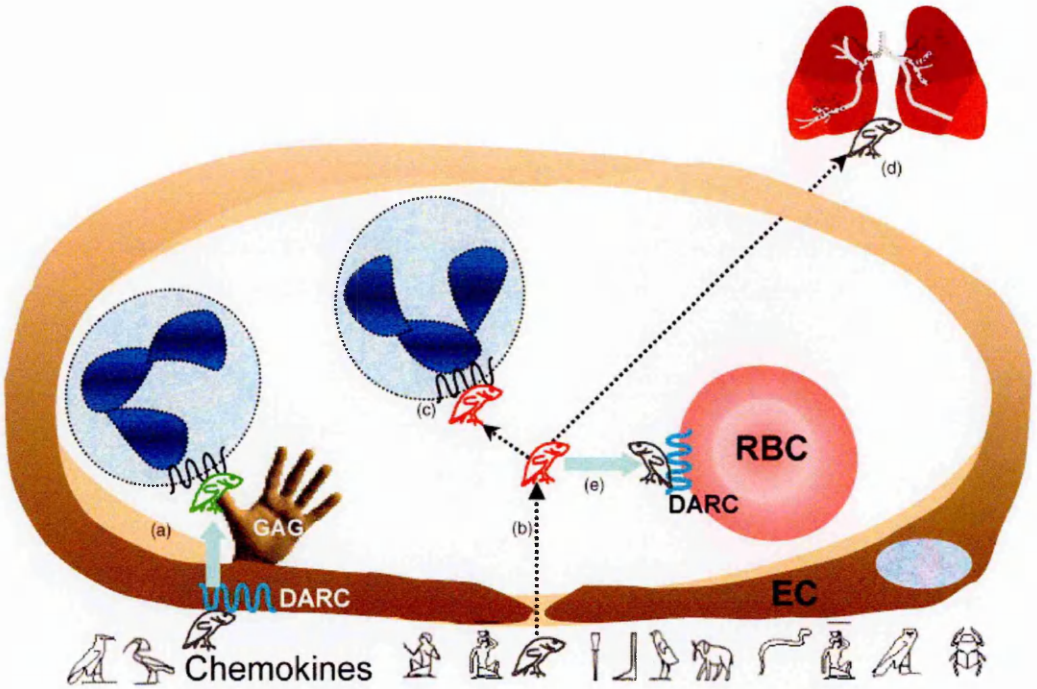

Figure 1.10 Contribution of EC and erythrocyte DARC to chemokine function.

Chemokines (depicted as hieroglyphs in black) are produced in the extravascular compartment by the tissue cells and are not "seen" by leukocytes in circulation. EC DARC transports chemokines in abluminal to luminal direction and are immobilized by the GAGs (a). These GAG-bound chemokines (green) can activate leukocyte integrins and convert leukocyte rolling into firm adhesion. Alternatively, chemokines may diffuse through the EC junction (b) bypassing the GAGs leading to free soluble chemokines in plasma (red) that trigger cognate receptors on blood leukocytes (c) resulting in leukocyte "desensitization". The free plasma chemokines (black) might bind to DARC on erythrocyte (RBC) surface (e) and cannot induce neither leukocyte adhesion nor desensitization. Thus, the same chemokine molecule (black, red or green) may play dramatically different functional roles in the process of leukocyte—EC adhesion and emigration and the interactions with DARC determine the microanatomical position of chemokines and through it their function. If not bound to the erythrocyte DARC, chemokines disappear from plasma into the lungs and kidneys (d) with yet unknown functional outcome.Taken from Rot, A. 2005 *Cytokine Growth Factor Rev* (99).

1.3.4.4 CCX-CKR, a decoy receptor for constitutive chemokines?

CCX-CKR binds the constitutive CC chemokines CCLs 19, 21, and 25 and also weakly to the follicular CXC chemokine, CXCL13, at least in humans (123). Similar to D6 and DARC, ligand-induced signals cannot be detected in response to ligand stimulation, and this receptor is therefore unlikely to mediate chemotaxis (101). There is little known regarding the biochemistry, expression, and function of CCX-CKR at present, but its ligand binding profile provides a compelling case for its involvement in developmental or homeostatic lymphocyte trafficking, or during the generation of immune responses. PCR and Northern blot analysis of mouse tissues show CCX-CKR to be widely expressed. It is expressed on T lymphocytes and immature dendritic cells, it binds selectively homeostatic CC chemokines and might represent the functional counterpart of D6 which selectively binds inflammatory CC chemokines. In this respect, the expression of CCX CKR in lymph nodes is particularly intriguing. CCX CKR does not transduce signalling activity after ligand engagement, and intriguingly also in this receptor the DRY motif in the second intracellular loop is not conserved. No information is available at present on the ligand internalization properties of CCX CKR.

1.3.4.5 The HCMV encoded receptor US28

The 7 TM domain receptor US28, encoded by the human cytomegalovirus, was originally recognized as a receptor for a large panel of CC inflammatory chemokines (124) and and more recently also for CX3CL1 (125). US28 is not a "silent" receptor, it is a constitutively signalling receptor (125),(126) that supports ligand scavenging by means of constitutive endocytosis and recycling (103). Molecular mechanisms involved in US28 cycling are presently unclear. While constitutive agonist-independent phosphorylation of serine/threonine residues on the C-terminal domain of the receptor have been proven to be required for receptor internalization (127), the role of

constitutive signalling activity has not been investigated, and the involvement and functional role of β-arrestin is presently controversial (128). Whatever the mechanism involved, clear evidence that US28 acts as a viral mechanism to blunt inflammation by sequestering inflammatory chemokines has been provided (129) , thus making US28 a candidate chemokine decoy receptor .

1.3.4.6 C5L2

The fourth "silent" receptor, called C5L2, is highly expressed in neutrophils and binds C5a, C5a des-Arg, and possibly other anaphylatoxins (130). As for other "silent" receptors, modifications in the DRY motif are responsible for C5L2 being structurally unable to couple G proteins and to sustain signalling activity (102). When investigated in cell transfectants, C5L2 was unable to undergo ligand-dependent internalization, although ligand-dependent receptor phosphorylation was observed, while more recently C5L2 internalization after C5a engagement has been reported in neutrophils (131). Whether C5L2 is capable of constitutive (i.e. ligand-independent) cycling and what is the fate of the ligand after receptor interaction have not been investigated, but in vivo results during experimental sepsis correlate C5L2 levels with positive prognosis (131), highlighting a possible role of C5L2 in blunting C5a proinflammatory effects. Thus, both in vitro and in vivo results candidate C5L2 as a chemoattractant decoy receptor.

1.3.4.7 Other chemokine decoy receptors?

Using these molecules as representatives, a molecular identikit of chemoattractant decoy receptors can be attempted (

Table 1.2). First absence of signalling activity that may be due to alterations in the DRYLAR/IV motif in the second intracellular loop, which is critical for G protein coupling and signalling functions in conventional receptors. A second common property

of decoy receptors appears to be their ability to act as scavenger receptors and it is interesting to note that at least in the case of some chemokine decoy receptors constitutive cycling has been demonstrated. It is presently unknown whether constitutive cycling is common to all chemokine decoy receptors, but it is tempting to hypothesize that this unusual feature may represent a second common property identifying this class of molecules. The third characteristic shared by chemoattractant decoy receptors is the ability to recognize broad panels of ligands. This task seems to have been achieved by shaping the N-terminal extracellular domain, that is the major determinant of multispecific chemokine binding both for DARC (132) and US28 (133). The prospect that other receptors may also fulfill this role must also be considered. These may emerge from the banks of orphan heptahelical receptors currently known, and in this prospective it is also noteworthy that the presently orphan chemokine receptor HCR also presents alterations in the DRYLAR/IV motif (134), and therefore may be considered a further candidate for this family.

	D6	DARC	CCX CKR	US28	CCRL2	C5L2
Structural features (DRYLAIV)	DKYLEIV	LGH	DRYVAVT	DRYYAIV	QRY	DLCFLAL
Ligands	CC chemokines (inflammatory)	CC and CXC chemokines	CC chemokines (homeostatic)	C, CX3C and CC chemokines	Unknown	C5a des-Arg and C5a
Expression	Lymphatic endothelial cells, placenta	Red blood cells, vascular endothelial cells	Lymph nodes, spleen, brain, placenta, kidney. Leukocytes: T, iDC	CMV-infected cells	Lymph nodes, spleen, thymus. Leukocytes: PMN, mDC, mono, MΦ, T	Placenta, spleen, kidney. leukocytes: PMN
Function	Decoy	Decoy; transporter	Unknown	Decoy	Unknown	Unknown (decoy?)

Table 1.2 Structural and functional properties of candidate chemoattractant decoy receptors.

The table summarizes the most relevant characteristics of the "silent" chemokine receptors D6 and DARC, the CMV-encoded chemokine receptor US28, the orphan chemokine receptor HCR, and the "silent" C5a receptor C5L2. In "Structural features" the sequence substituting the highly conserved DRYLAIV motif in the second intracellular loop is shown.

1.3.5 Orphan chemokine receptors

1.3.5.1 Introduction

The completion of the human genome sequencing project has identified approximately 720 genes that belong to the G-protein coupled receptor (GPCR) superfamily. Approximately half of these genes are thought to encode sensory receptors. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving 150 so-called orphan GPCRs with no known ligand or function. Between them there are few receptors that share features found more frequently among chemokine receptors than other types of GPCRs (Cytokine Receptor Database: http://csp.medic.kumamoto-u.ac.jp/CSP/Receptor.html) and for this reason they are tentatively classified as orphan chemokine receptors.

Phylogenetic clustering methods were also used to elucidate the chemical nature of receptor ligands, which led to the identification of natural ligands for many orphan receptors. Interestingly no *Drosophila* members belong to this group of receptors suggesting these receptors might have a recent evolutionary origin (135). Chemokine receptors are represented by two clusters (Figure 1.11). The first cluster contain CCR1-5, CCR8, CX3CR1, XCR1, D6 and the orphan receptor CCRL2, while the second cluster contains CXCR1-6, CCR6, CCR7, CCR9 and CCR10, CCX-CKR, DARC and the orphan receptors AMDR, RDC1, CML2 and Q96CH1. This analysis strongly suggests that CCRL2 might bind to CC- type chemokines.

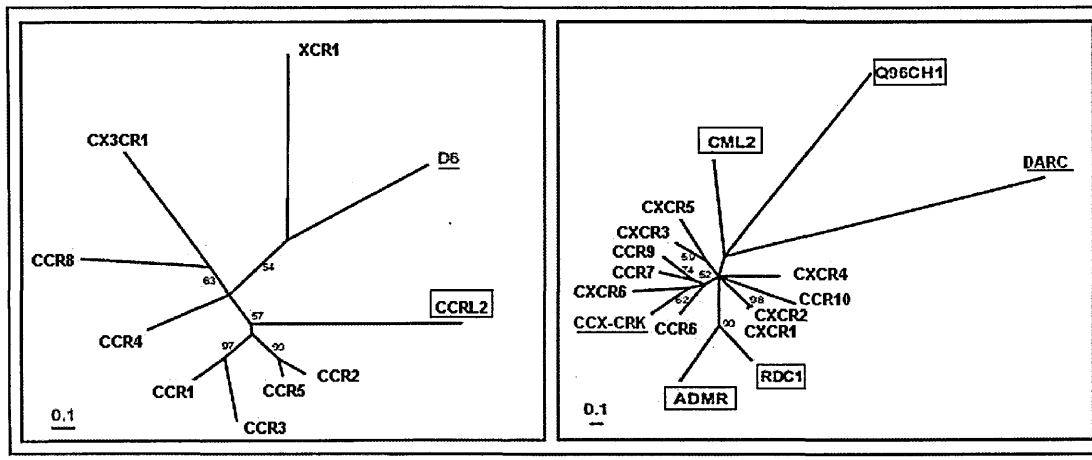

Figure 1.11 Phylogenetic trees of chemokine receptors

Trees were inferred as described in (135). The scale bars indicate a maximum likelihood branch length of 0.1 inferred substitutions per site. Silent/decoy receptors are underlined, orphan receptors are boxed. Modified from Metpally, R.P.et al. 2005 *BMC Genomics* (135).

1.3.5.2 CCRL2

CCRL2 was first cloned from a human neutrophil cDNA library and named HCR (Human Chemokine Receptor) (134). The deduced protein sequence of CCRL2, at amino acid level, is most closely related to CCR1 with 43% amino acid homology. CCRL2 gene is located at the edge of the main chemokine receptor cluster in the 3p21 region of the genome composed by XCR1, CCR1, CCR3, CCR2 and CCR5 (136, 137) . As mentioned above phylogenetic analysis has shown that CCRL2 is a typical member of the chemokine receptor family and have given the suggestion that the cognate ligand may be a CC chemokine (135). CCRL2 distribution in human leukocytes has been recently reported at the mRNA and protein level (138). Migeotte et al., using FACS analysis and a monoclonal antibody, found that CCRL2 is expressed by the majority of T lymphocytes ($CD3^+$), both on $CD4^+$ and $CD8^+$ cells and that it was present on the vast majority of memory T cells, and on about half of naïve T cells. CCRL2 is also

expressed on the majority of natural killer cells ($CD56^+$), but not on B cells. Moreover CCRL2 was found on neutrophils, monocytes and monocyte-derived dendritic cells with some donor-to-donor variability. CCRL2 expression is enhanced both on T cells after stimulation with OKT3 and IL-2 and in dendritic cells following stimulation by lipopolysaccharides, poly (I:C), IFN-γ or CD40L.

Yoshimura and collegues have recently found that CCRL2 is expressed by all infiltrating neutrophils and by some macrophages obtained from the synovial fluid (SF) of rheumatoid arthritis (RA) patients. In vitro studies of primary neutrophils revealed that CCRL2 mRNA is rapidly up-regulated following stimulation with lipopolysaccharide or tumor necrosis factor and that cells expressing CCRL2 migrated in response to a fraction of RA SF (139).

CCRL2 murine counterpart, named L-CCR, was originally described to be expressed in murine macrophages (140). More recently, L-CCR expression was also demonstrated in glial cells stimulated with LPS (141). A single publication reported functional activities after L-CCR engagement by CCL2, CCL5, CCL7 and CCL8, in the absence of any direct ligand/receptor interaction evidence (142).

1.4 Chemokine/chemokine receptor functions

1.4.1 Role in physiology

Although distinct chemokines exert several biologic functions, including regulation of hematopoiesis, fibrosis, and angiogenesis, their major (and eponymous) function is represented by the ability to induce directional cell migration, thus coordinating leukocyte recruitment in physiologic and pathologic conditions. Leukocyte contact with endothelium might be transient, reversible, and activation-independent. In this phase, cells roll across the endothelial surface through chemokine-independent interactions of selectins with counteradhesins. At inflamed sites, leukocytes enter a second phase

involving chemokine receptor engagement by chemokines immobilized to proteoglycans on the endothelial surface. Chemokine receptor engagement activates β2 integrins, allowing leukocyte high-affinity binding to endothelial cell counterreceptors and subsequent extravasation (143) (Figure 1.12). The simultaneous action of chemokines and integrins is also needed for full activation of leukocytes and, in synergy with primary cytokines, enhances phagocytosis, superoxide production, granule release, and bactericidal activity.

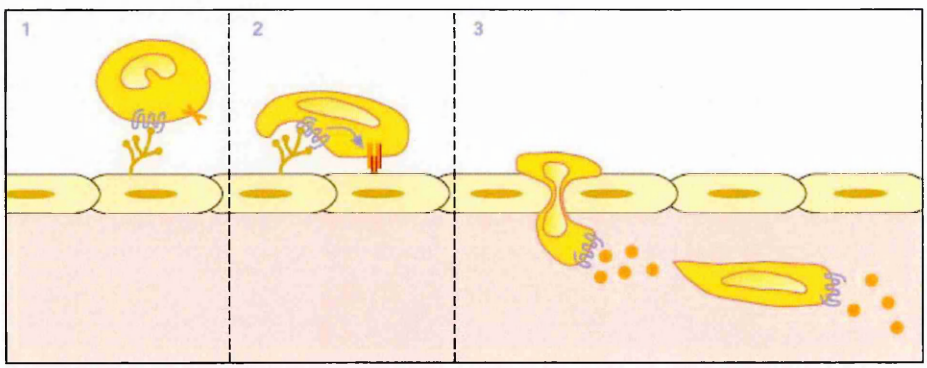

Figure 1.12 Chemokine biologic functions.

All chemokines share a common biologic property represented by leukocyte chemoattraction and recruitment during immune responses. (1) cell adhesion, (2) integrin activation, and (3) cell migration (figure courtesy of Alberto Mantovani, University of Milan, Italy).

Chemokines act as intercellular signals, being produced under appropriate conditions by virtually every cell type and acting on several target cells, including leukocytes. Some chemokines are produced constitutively, but most of them are inducible. In general, proinflammatory cytokines such as TNF, IL-1, or IFN-α. up-regulate inflammatory chemokines, whereas anti-inflammatory mediators, such as IL-10 and glucocorticoids, have an opposite effect. Most inducible chemokines are regulated at the transcriptional level, but some are stored in platelet granules for immediate release, as in the case of

CXCL4 and CCL5. Chemokine receptors also are subjected to expression control. It is interesting that receptors for inflammatory chemokines usually are regulated opposite to the ligands, and several receptors are detected (or functional) exclusively in specific cell states (eg CXCR3 on activated T cells). Although narrow- and broad-spectrum chemokines exist, the spectra of action of different proteins usually widely overlap, presumably to provide flexibility and specificity in leukocyte trafficking. Neutrophil-targeted chemokines are found mainly in the CXC subfamily, whereas monocyte/macrophages, eosinophils, and basophils are attracted mainly by CC chemokines (Figure 1.13). Both CC and CXC subfamilies also contain T lymphocyte–specific members. Specific chemokine receptors mark TH1 (CXCR3 and CCR5) vs TH2 (CCR3 and CCR4) subsets in resting conditions (144).

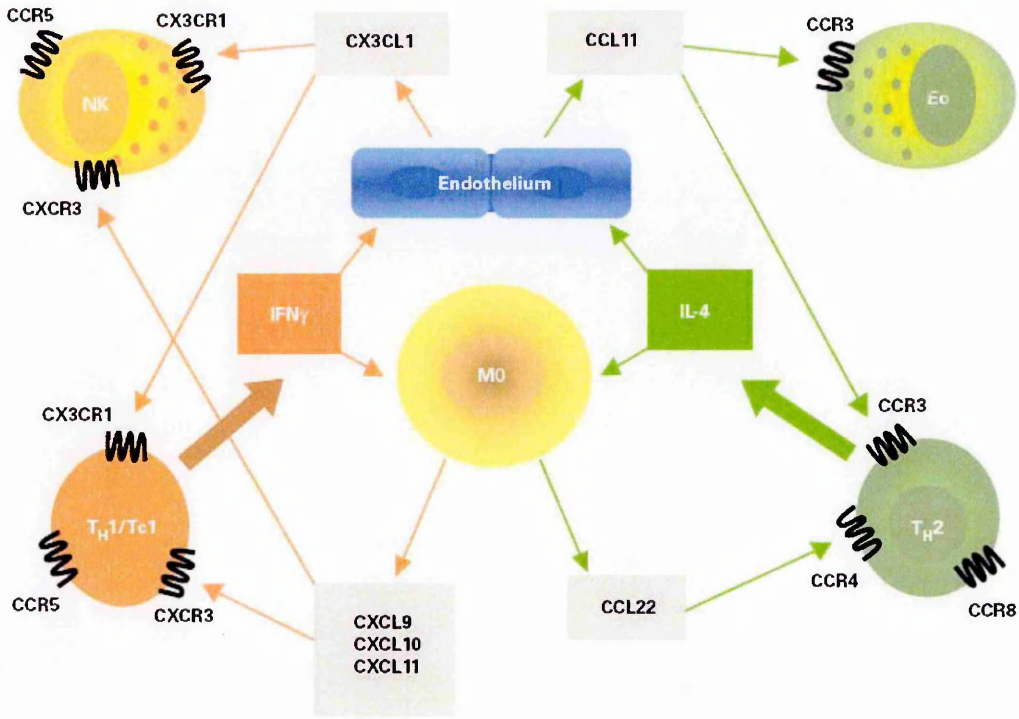

Figure 1.13 Role of chemokines in polarized immune responses.

During type I and type II immune responses, master cytokines, represented by interferon (IFN)-γ and interleukin (IL)-4, respectively, regulate chemokine production by stromal and inflammatory cells. Chemokines then support selective recruitment of polarized T cells and specific type I and type II effector cells expressing distinct panels of chemokine receptors (figure courtesy of Alberto Mantovani, University of Milan, Italy).

1.4.2 Role in disease

Considerable progress has been achieved in our knowledge of the function of the chemokine system and in understanding its role in the pathophysiology of human diseases. This complex system (approximately 50 cytokines and 20 receptors) coordinates leukocyte recruitment in a variety of human diseases, ranging from infections and inflammation to cancer. Leukocyte recruitment and activation are key steps in the pathogenesis of several human diseases.

1.4.2.1 The Chemokine System in Inflammatory and Infectious Diseases

Since the description of the first chemokine an impressive amount of information has been accumulated, correlating the chemokine system with the pathogenesis of inflammatory-based disorders (145). A nonredundant role of CXCL8 in neutrophil-mediated inflammatory disease has been demonstrated. CXCL8 neutralization results in almost complete protection from multiple inflammatory challenges, (146) by genetic deletion of CXCR2 that causes defective neutrophil recruitment, (147) and more recently by pharmacologic inhibition of CXCR1 (148). Gene-targeted studies also demonstrated the importance of CC inflammatory chemokines and respective receptors in monocyte recruitment. $CCR1^{-/-}$ and $CCR2^{-/-}$ mice present altered *Schistosoma* egg or purified protein derivative–induced granulomatous inflammation that correlates with abnormal TH1 and TH2 cell responses. $CCR1^{-/-}$ mice also have reduced pancreatitis-associated pulmonary infiltration, and $CCR5^{-/-}$ mice have enhanced delayed hypersensitivity reactions and increased humoral responses to T cell–dependent antigenic challenge. Collectively, these results demonstrate a nonredundant role for inflammatory chemokines in leukocyte recruitment associated with acute and chronic inflammatory responses. Studies with gene-targeted animals also have demonstrated a

role for chemokines in host defense. For example, $CXCR2^{-/-}$ and $CCR1^{-/-}$ mice have increased susceptibility to *Aspergillus fumigatus* inoculation, whereas $CCR5^{-/-}$ and $CCR2^{-/-}$ mice are more susceptible to *Listeria monocytogenes* infection (147). Although chemokines and chemokine receptors probably evolved to coordinate leukocyte recruitment to support antimicrobial responses, many have been exploited by infectious agents to facilitate infection. Two models have been identified. In a first scenario, pathogens interfere with the chemokine system by producing chemokine-binding molecules, such as the M3 protein secreted by cells infected with murine γ herpesvirus 68 (149), or by pirating chemokines or chemokine receptors and modifying them to generate antagonists or chemokine scavengers (150). A second mechanism is represented by exploitation of cellular receptors for cell entry. A first example of such a mechanism is represented by the malaria-causing protozoan *Plasmodium vivax,* which enters erythrocytes by using a chemokine promiscuous receptor called DARC (112) A second example is HIV-1, which gains access to immune cells using CD4 as a primary cellular receptor and a chemokine receptor, CXCR4 or CCR5, as strain-specific coreceptors. A variety of blocking agents, including agonists, antagonists, and antibodies, clearly have demonstrated a nonredundant role for CCR5 and CXCR4 in HIV infection. Moreover, a clear-cut role for CCR5 has also been demonstrated through the discovery that a mutant allele bearing a 32-base-pair deletion in the open reading frame (*CCR5-Δ32*), which encodes a truncated and inactive receptor not translocated to the membrane surface, is highly protective against initial infection in *CCR5-Δ32* homozygotes.(150)

1.4.2.2 Role of the Chemokine System in Autoimmune Diseases

Leukocyte recruitment, accumulation and activation are common events characterizing autoimmune diseases. The use of potent and cytotoxic immunosuppressive therapies for the treatment of these diseases reflects limited understanding of the mechanisms that allow leukocytes to be recruited to the chronic inflammatory reaction characteristic of autoimmune diseases. The importance of chemokines and chemokine receptors in the pathogenesis of autoimmunity was initially suggested by many animal models, and more recently obtained further support by genetic evidences and clinical studies. In animal models of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis (RA), chemokines levels correlate with disease progression (151, 152), and the treatment of affected mice with chemokine antagonists or blocking antibodies has provided the first proof of concept attesting the involvement of chemokines in autoimmune diseases (153). More recently, knockout mice have revealed that the absence of a chemokine or its receptor may prevent or attenuate the insurgence of autoimmune diseases. For example, the absence of CCR1 and CCR2 is protective in EAE (153), and CCL3 deficiency in non-obese diabetic mice is protective for induced insulitis and spontaneous diabetes (154). Consistently with results obtained in animal models, clinical studies have demonstrated that chemokines and chemokine receptor expression is significantly altered during the evolution of certain autoimmune diseases. In RA patients, clinical disease activity correlates with CCL2 and CCL5 levels (155), which have been proposed as clinical markers. Moreover, methotrexate treatment of RA patients correlates with a reduction of CCL5 levels (156). Elevated serum levels of CXCL10 have been associated with clinical activity of systemic lupus erythematosus disease (157). Elevated levels of inflammatory CC chemokines (CCL3, CCL4 and CCL5 in particular) and their receptors (CCR2, CCR3 and CCR5) have also been found in the central nervous system of MS patients (158, 159), and T cells from MS patients

treated with IFN-β showed reduced CCR5 expression and inhibition of cell migration to its ligands CCL5 and CCL3 (160). A relevant role of CCR5 in T cells recruitment to brain lesions correlates with recent genetic evidence showing that MS patients with the Δ32 *CCR5* allelic variant, are not protected in the initial phase but have a lower risk of recurrent clinical disease (161).

1.4.2.3 Role of Chemokines in Allergic Diseases

Allergic inflammation is a Th2 disease associated with the selective recruitment of eosinophils and allergen-specific Th2 lymphocytes. Selective expression of chemokine receptors on these leucocytes was postulated to be the mechanism by which they are selectively recruited to the allergic site (162-164). In vitro and in vivo studies have provided evidence that CCR3, CCR4 and CCR8 are involved in the recruitment of Th2 lymphocytes (165). Mouse models with blocking antibodies against CCR3 and CCR4 ligands (CCL11, CCL22 and CCL17) show decreased airway inflammation and airway hyperresponsiveness (AHR) (166-168) while neutralization of CCR8 ligand (CCL1) has no effect on the recruitment of Th2 cells in the lung (164). However genetically modified animals do not confirm in vivo data obtained with inhibitors: CCR3 $^{-/-}$ mice have reduced eosinophil recruitment to the lung after allergen challenge, but increased AHR (169), CCR4 $^{-/-}$ mice show no protection against development of allergic inflammation (170) and two studies out of three reported no effect of CCR8 deficiency on the development of allergen-driven airway inflammation (171). The number of $CCR4^{+}$ and $CCR8^{+}$ T cells in human lung biopsies was increased after allergen challenge. Moreover CCL22 and CCL17 levels were increased after allergen challenge, while until now CCL1 and CCL11 expression were not detected (172, 173)

1.4.2.4 Role of Chemokines in Neoplastic Diseases

Chemokines and chemokine receptors have been found expressed in almost all tumors, but at now no evidence exists about their involvement in cancer pathogenesis. It is likely that chemokines have important effects on cancer pathobiology because they affect different activities that impact cancer like leukocyte infiltration, metastatic potential, tumor growth and angiogenesis (174-176). The composition of the tumor infiltrate is related to tumor and stromal cell production of chemokines. In vitro and in vivo experiments suggest that the chemokine CCL2 can suppress tumor growth inducing a dense mononuclear infiltrate (177). Moreover it has been demonstrated that the same chemokine is able to act as adjuvant to enhance T-cell dependent host anti-tumor response (178). However most clinical and epidemiological studies suggest that chemokine expression might be advantageous for the tumor. In ovarian and breast cancer chemokine levels (CCL2 and CCL5) correlate with macrophage infiltration, lymph node metastasis and clinical aggressiveness (179-182). In contrast high serum levels of CCL2 in pancreatic cancer patients correlate with macrophage infiltration and with positive prognosis (183). Chemokines may also help the tumor to subvert the immune system by the polarization of the immune response to a Th2 type in order to suppress specific anticancer responses. Examples are given by Hodgkin's lymphoma in which there is a prominent production of Th2 chemokines by Reed-Sternberg cells (184) and by the human Kaposi sarcoma-associated herpes virus (KSHV) that encodes three chemokines (v-MIPI, II and III) that selectively attract Th2 lymphocytes (185). Cancer cells not only produce high levels of chemokines but also may express functional chemokine receptors. CXCR4 appears to be expressed by the majority of cancer cells (23 different types of cancer: for example breast, prostate, pancreatic, lung and ovarian carcinomas) (175). CCR7 is expressed by gastric and esophageal carcinoma cells and by melanoma (186). Experimental murine cancer models provide some proof

that cancer cells may use chemokine receptors in order to migrate to metastatic sites where their ligands are overexpressed (186). Data from 600 prostate cancer patients revealed that CXCR4 protein expression was higher in localized and metastatic prostate cancer compared to normal or benign prostatic tissue (187). Chemokines may also act as growth and survival factors in an autocrine and/or paracrine manner. For example melanoma cells express high levels of CXCR2 and also produce constitutively CXCL1 and CXCL8 that in an autocrine way stimulate proliferation and survival (188). Prostate cancer cells, glioblastoma cells express CXCR4 and CXCL12 stimulate their proliferation (187). Furthermore, chemokines may regulate angiogenesis within both primary and metastatic tumors. $ELR^{+}CXC$ chemokines promote angiogenesis while $ELR^{-}CXC$ chemokines are anti-angiogenic CXCL10 levels in lung cancers are inversely related to tumor progression (189), while CXCL5 levels in NSCLC are correlated with the vascularity of the tumor and angiogenesis (165).

1.4.2.5 Role of Chemokines in Vascular Diseases

Atherosclerotic plaques are thought to result from an inflammatory response to arterial damage (190). Chemokines may be involved in different steps of this inflammatory disease: first they can mediate monocyte firm adhesion to vascular endothelium and migration to subendothelium where they become the foam cells originating fatty streaks. Moreover chemokines may be involved in the later stage of the disease activating macrophages and migration of smooth muscle cells into the intima and at the end in the thrombus formation over the plaque (191, 192). Animal models of atherosclerosis have revealed a role for many chemokines such as CXCL8, CXCL12, CXCL10, CCL1 (193-196). Both $CCL2^{-/-}$ and $CCR2^{-/-}$ mice have 65-85 % less arterial lipid deposition than normal mice in hypercholesterolemia models. Disease reduction is correlated with decreased macrophage infiltration into the arterial wall, suggesting that

CCL2 attracts CCR2-bearing monocytes to the vessel wall (197, 198). Moreover proof of concept of the role played by CCL2 and CCR2 in this disease derives from the use of a CCL2 antagonist that is able to prevent monocyte recruitment in a coronary artery remodelling system (199). $CX3CR1^{-/-}$ mice are protected against diet-induced atherosclerosis (200, 201). Interestingly it has been reported that a polymorphism (V280) in this receptor correlates with protection from coronary artery disease (202, 203). The relevance of the chemokine system in the human disease is supported by data from human lesions: CCL2, CCL5, CCL3, CCL11 and CX3CL1 have been detected within atherosclerotic plaques (204, 205). Moreover clinical data reveal that members of the statin family inhibit expression of CCL2 (206), suggesting that these drugs may reduce atherosclerotic risk by inhibiting macrophage recruitment into the arterial wall.

1.4.3 Role of chemokines in dendritic cell biology

DC are highly mobile and differentially localized to tissues for the regulation of immunity. They are positioned as sentinels in the periphery, where they frequently encounter foreign antigens, and they readily relocate to secondary lymphoid organs, particularly lymph nodes, to position themselves optimally for encounter with naive or central memory T cells. The trafficking of DC to lymph nodes through afferent lymphatic vessels is crucial for the execution of their functions. Chemokines play a fundamental role in DC trafficking (185) even if recent work has documented that many chemotactic agonists, different from chemokines, play a relevant role in DC subset recruitment (16). Furthermore, chemokine receptor expression is not predictive of DC migration since multiple factors, including prostaglandins, leukotrienes, sphingosine1-phosphate, extracellular nucleotides and some membrane proteins (e.g. CD38) play an important role in the regulation of chemokine receptor function (16). Therefore, DC

migration in vivo is a tightly regulated process controlled at the level, of chemokine production and chemokine receptor expression and function.

1.4.3.1 Recruitment of myeloid dendritic cells

Immature DC express a unique repertoire of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, CCR6) (207). These receptors bind a pattern of "inflammatory" chemokines, including CCL5, CCL2, CCL3, CCL4 and CCL20 (Table 1.3). In addition, immature DC also express functional CXCR4 (208), the receptor for CXCL12, chemokine that is constitutively expressed in many lymphoid and non-lymphoid tissues.

Receptor	Ligand
CCR1	CCL3, CCL5, CCL7
CCR2	CCL2, CCL8, CCL13
CCR4	CCL17, CCL22
CCR5	CCL3, CCL4, CCL5
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12

Table 1.3 Ligand specificity of chemokine receptors expressed by dendritic cells

Taken from Sozzani, S. 2005.*Cytokine Growth Factor Rev* (16)

Little is known about the mechanisms that regulate the homing of DC or their precursors, in steady-state conditions (209). CCR2 plays a role in the localization of Langerhans cell precursors (210) and transgenic mice over expressing CCL2, under the keratin promoter, have local accumulation of cells with DC morphology in the basal layer of the epidermis (211). CCR6 and its ligand CCL20 seem to be important for the homing of DC to mucosal surfaces but not for the basal recruitment of Langherans cells in the skin. In fact *CCR6*$^{-/-}$ mice have normal numbers of skin DC cells while they have a defect in humoral immune response to oral antigens (212).

1.4.3.2 Migration of DC to lymphoid organs

DC maturation is associated with a dramatic change in their repertoire of chemokine receptors. Receptors for inflammatory chemokines are down-regulated while cells start to express CCR7 the receptor for CCL19 and CCL21, two homeostatic chemokines, highly expressed in secondary lymphoid organs, like tonsils, spleen and lymph nodes (213) In vitro exposure of DC to LPS, IL-1, and TNF, or the culture in the presence of CD40 ligand, induced a rapid (<1 h) inhibition of chemotactic response to CCL3, CCL4, CCL5, CCL7, C5a and formylated peptides (fMLP) (Figure 1.14) (208, 213-215). Receptor desensitization by endogenously produced chemokines is likely to be responsible for this effect, however, the reported desensitization to C5a and fMLP, two chemotactic factors that are not produced by activated DC, implicates additional agonist-independent mechanisms (208) (215). Inhibition of chemotaxis is followed, with a slower kinetics, by the reduction of membrane receptors and by the down regulation of mRNA receptor expression (208, 213). Concomitantly, the expression of CCR7 and the migration to CCL19 and CCL21 is strongly up regulated, with a maximal effect at 24 h

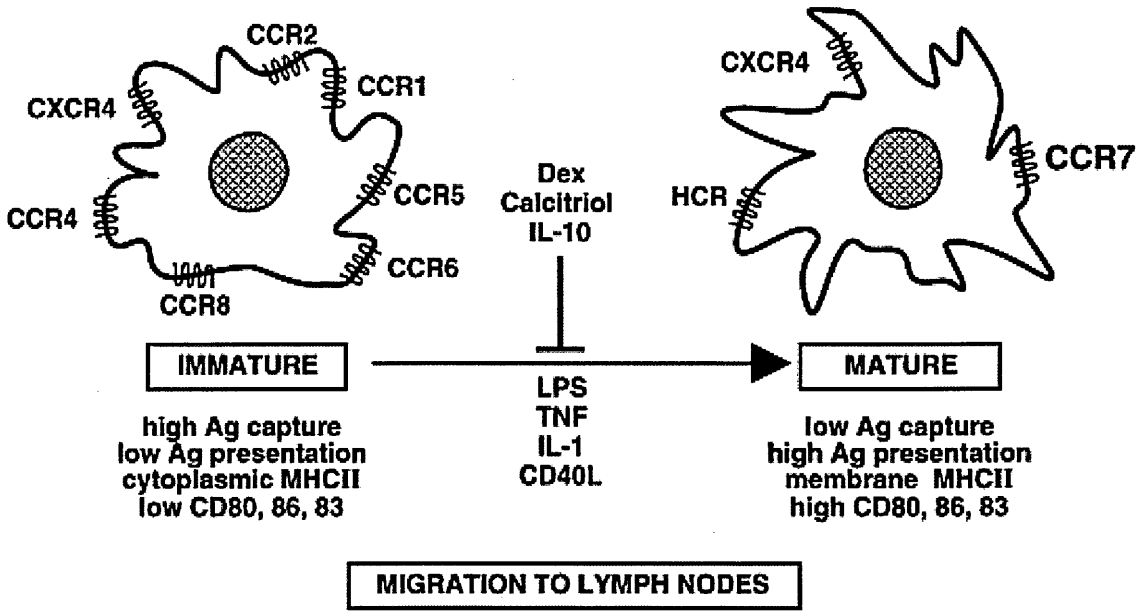

Figure 1.14 Chemokine receptors expressed by dendritic cells.

Immature DC express many chemokine receptors for inflammatory chemokines. Inflammatory signals (e.g. LPS, IL-1 and TNF) or immune stimuli (e.g. CD40 ligation) induce DC maturation that is associated to the downregulation of inflammatory chemokine receptors and the expression of CCR7. CCR7 plays a pivotal function in the migration of DC to draining lymph nodes. HCR is the original name of CCRL2 (figure courtesy of Silvano Sozzani, University of Brescia, Italy).

The crucial role of CCR7 and its ligands is clearly observed in vivo in mice deficient for these proteins. In mice homozygous for an autosomal recessive mutation, paucity of lymph node T cells (plt), naive T cells fail to home to secondary lymphoid organs. The plt mutation is associated with the lack of expression of one of the two forms of CCL21, named CCL21-Ser, present in the secondary lymphoid organs, and in a defect in the expression of CCL19 (216). As a consequence of the lack of CCL21 within secondary lymphoid organs, DC from these mice fail to accumulate in the spleen and in the T cell areas of lymph nodes (Figure 1.15). (217). Similarly, $CCR7^{-/-}$ mice showed a defective

architecture of secondary lymphoid organs and a defective homing of DC and lymphocytes (218).

CCR7 expression by DC was shown to be required also for the entry of DC into lymphatic vessels at peripheral sites both in steady state and inflammatory conditions (219). $CCR7^{-/-}$ mice are characterized by the absence of $CD11c^{+}MHCII^{high}$ DC, a subpopulation of DC that is postulated to migrate in a semimature state of activation, from the skin to the draining lymph nodes to maintain tolerance under steady-state conditions (219). During inflammation, the entry of DC into lymphatic vessels is boosted by the up-regulation of CCL21 on lymphatic endothelial cells. Therefore, inflammatory stimuli not only promote the recruitment of immature DC into tissues but also initiate their maturation process and boost the recruitment of maturing DC into lymphatics (36).

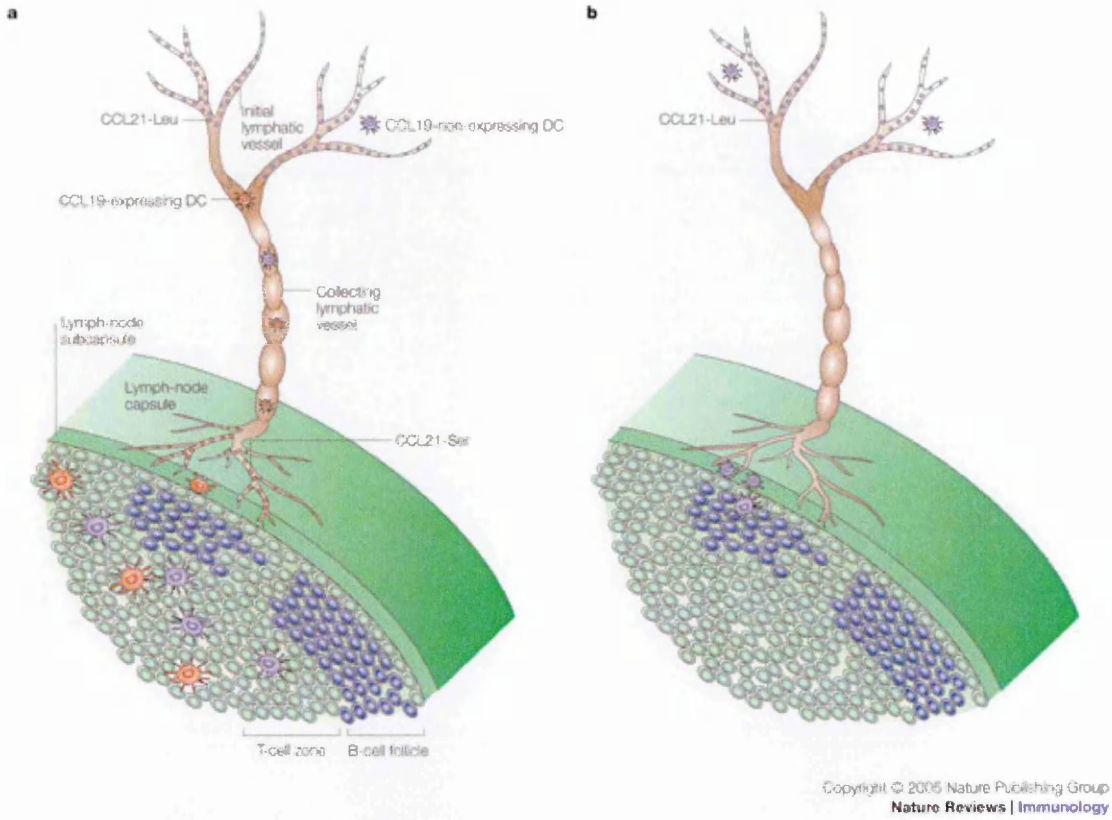

Figure 1.15 Expression of CCR7 ligands and fate of dendritic cells in wt and *plt* mice.

a) The CCR7 ligand CCL19 is expressed by DC after their maturation. In mice, there are two known functional genes that encode CCL21. One form of CCL21, CCL21-Leu (purple), is expressed in the periphery, at a minimum by initial lymphatic vessels. The other form of CCL21, CCL21-Ser (red), is expressed in lymph nodes, including in the terminal lymphatic vessels that are present in the subcapsular sinus. Which of these CCL21 gene products is expressed by collecting lymphatic vessels is not clear. **b)** Functional CCL19 expression by DC is abrogated in *plt* (paucity of lymph-node T cells) mice. In addition, CCL21-Ser, but not CCL21-Leu, is absent. Peripheral DC migrate poorly to the T-cell zone of lymph nodes in *plt* mice, but some DC aberrantly accumulate at the subcapsular sinus. Taken from Randolph, G.J. et al. 2005. *Nat Rev Immunol* 5:617-628.(220)

The migration pathway that leads DC from periphery to secondary lymphoid organs is still poorly understood and may involve multiple signals in addition to CCR7. In a recent study it was proposed that CCR8 and its cognate ligand CCL1 are involved in the emigration of mouse monocyte-derived DC out of the skin. Furthermore, in vitro, the reverse transmigration of human monocyte-derived DC was significantly inhibited by the presence of blocking CCR8 antibodies. Since CCL1 is expressed in the subcapsule of the lymph nodes, it is possible that CCL1/CCR8 may function downstream of the entry of DC into the lymphatic by regulating the entry of the afferent DC in the subcapsular sinus of the lymph nodes (221). Expression of CXCR4, the CXCL12 receptor, is retained during DC maturation and mature monocyte-derived DC were shown to migrate in response to CXCL12 (215), however, blood DC matured in vitro apparently do not express functional CXCR4 (222).

The relevance of chemotactic receptors in DC traveling in vivo has been clearly documented in mice lacking the gamma isoform of phosphoinositide-3 kinase (PI3Kγ) (223). DC generated from PI3Kγ null mice show a profound defect in the migration in response to both inflammatory and constitutive chemokines. A defect of DC migration was also observed in vivo in PI3Kγ$^{-/-}$ and most importantly, this defect was associated with a defective ability of PI3Kγ$^{-/-}$ mice to generate a specific immune response.

Overall these findings provide a model for DC trafficking in which activation of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR4, CCR5 and CCR6) function as signals to localize immature DC or their precursors to peripheral tissues. After Ag uptake, immune/inflammatory stimuli induce DC maturation and the loss of responsiveness to inducible chemokines locally produced. This unresponsiveness plays a permissive role for DC to leave peripheral tissues. Meanwhile the slower up regulation of CCR7, and possibly other chemotactic receptors, prepare DC to respond to CCL19 and CCL21 expressed in lymphoid organs.

1.4.3.3 Migration of plasmacytoid dendritic cells

The expression of chemokine receptors on sorted blood myeloid DC and plasmacytoid DC is, in general, fairly similar (222). Both subsets express relatively high levels of CC chemokine receptor CCR2 and CXCR4. Whereas CCR1, CCR3, CCR4, CXCR1, CXCR2, CCR6 and CXCR5 are very weakly, or not expressed, on both circulating myeloid DC and plasmacytoid DC. Conversely, CCR5 and CXCR3 expression is clearly divergent in the two subsets, being low on blood myeloid DC, but high on plasmacytoid DC (222, 224). In contrast with the overall similar pattern of chemokine receptor expression, circulating myeloid DC and plasmacytoid DC exhibit a profound difference in their capacity to migrate in response to chemokines with CXCL12 being the only chemokine active in a classic chemotaxis assay (222) or in transmigration assays across an endothelial cell monolayer (225). In classical chemotaxis assays, the ligands of CXCR3, namely CXCL9, CXCL10 and CXCL11, are inactive in inducing plasmacytoid DC migration but can promote plasmacytoid DC migration in response to CXCL12 (226). However, it was shown that CXCR3 ligands are fully competent in inducing plasmacytoid DC adhesion and migration when presented to plasmacytoid DC immobilized on the heparan sulfates present on endothelial cells membrane, a physiological relevant condition (14).

1.4.3.4 Regulation of dendritic cell migration

Multiple evidences have shown that chemokine receptor expression is not predictive of DC migration suggesting that the coupling of chemokine receptors to chemotaxis is also regulated at the signaling level (Figure 1.16) (98, 222). For instance the simultaneous exposure of DC to maturation factors and anti-inflammatory cytokines (i.e. IL-10) uncouples inflammatory chemokine receptors from chemotaxis and converts them in scavenging chemokine receptors (98) Recent findings revealed that eicosanoids, such as

cysteinyl leukotrienes (cysLTs) and prostaglandin E2 (PGE2) regulate CCR7-dependent migration of DC to lymph nodes (227). CysLTs derive from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. Experimental evidence about the role of cysLTs in DC migration emerged by the use of MRP1 blocking antibodies and from studies in mice lacking the lipid transporter multidrug resistance protein 1 (MRP1) (228). In the absence of MRP1 the migration of epidermal DC to the draining lymph node was impaired and the exogenous administration of LTC4 or LTD4 could rescue the defect. DC express the cysLTs receptor CysLT1, and in vitro, cysLTs promoted DC migration in response to the CCR7-ligands CCL19 and CCL21. Therefore, the MPR1-mediated efflux of cysLTs and autocrine or paracrine activation of cysLTR promote the migration of maturing DC.

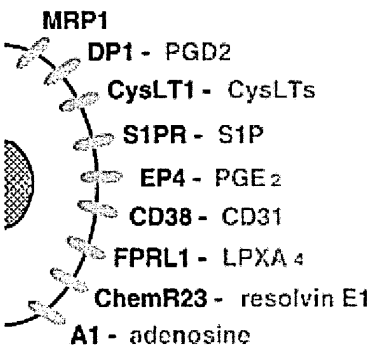

Figure 1.16 Factors that regulate dendritic cell migration.

Several agonists are known to promote (CystLTs, S1P, PGE2, CD31), or inhibit (PGD2, LPXA4 and resolvin E1) the migration of dendritic cells. A1, adenosine receptor A1; CystLTs, cysteinyl leukotrienes; S1P, sphingosine 1-phosphate; PGE2, prostaglandin E2; PGD2, prostaglandin D2; LPXA4, lipoxin A4.

Taken from Sozzani, S. 2005.*Cytokine Growth Factor Rev* (16)

PGE2 is an arachidonic acid metabolite generated by PGE2 synthases downstream of cyclooxygenases. PGE2 modulates multiple aspects of DC biology, such as maturation, cytokine production, T cell activation and apoptosis (227). Furthermore, PGE2 promotes the migration of mature human monocyte-derived DC to the CCR7 ligands CCL19 and CCL21 (229). The effect of PGE2 on these cells is mediated by two of the four PGE2 receptors, namely EP2 and EP4 and the cAMP pathway. Interestingly, blood myeloid (CD1b/c+) DC, matured in vitro, did not require PGE2 for an optimal migration in response to CCR7 ligands (230). These results suggest that the coupling of CCR7 to chemotaxis is regulated by the state of activation/maturation of DC. The importance of PGE2 for DC migration has been highlightened in vivo by the use of mice that are genetically defective for EP4 (231). *Ptger4*$^{-/-}$ mice displayed a reduced migration of skin Langerhans cells to regional lymph nodes after FITC sensitization, in vivo, and a reduced spontaneous emigration from skin explants, ex vivo. The nonredundant role of EP4 in Langerhans cell migration was further confirmed in wild-type mice by the use of an EP4 antagonist, and correlated with an impaired induction of contact-type hypersensitivity responses (231)

2 Chapter 2. Materials and methods

2.1 Cell culture

2.1.1 Isolation of PBMC

PBMC were purified by Ficoll-Hypaque density centrifugation. Briefly, heparinised venous blood from healthy donors, or buffy coat (through the courtesy of the Centro Trasfusionale, Ospedale Civile Fornaroli, Magenta, Italy) was diluted 1:1 with sterile PBS (without Ca2+ or Mg2+; Gibco BRL, Paisley, UK) and centrifuged on Ficoll (Biochrom) for 30 minutes at 500 *g*. The mononuclear cell layer was removed using a sterile pipette, and washed thoroughly by resuspension in sterile PBS (Gibco BRL). The PBMC were then used for RNA extraction or monocyte isolation.

2.1.2 Isolation of monocytes

Isolation of monocytes from PBMC was performed using Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Briefly, PBMC resuspended in osmolarized RPMI were centrifugated on osmolarized Percoll diluted with osmolarized RPMI at the ratio for 30 minutes at 550 g. The monocyte monolayer was removed using a sterile pipette, and washed thoroughly by resuspension in sterile PBS (Gibco BRL). Purity of the monocyte population was always >95 % as assessed by flow cytometry using anti-CD14 conjugated to FITC.

2.1.3 Generation of monocyte-derived dendritic cells

Monocytes were cultured for 6 days at 1 x 10^6/ml in six-well tissue culture plates (Falcon; BD Biosciences, Franklin Park, NJ) in RPMI 1640 supplemented with 10% FCS, 50 ng/ml GM-CSF, and 20 ng/ml IL-13. Where indicated, DC were further cultured in the presence of 100 ng/ml LPS and 20 ng/ml TNF for 48 h or as otherwise specified. CD40 ligand (CD40L)-transfected J558L cells or mock-transfected control

cells were cultured with DC at a 1:4 ratio. Incubation of DC with the J558L mock-transfected cells did not induce cell maturation or chemokine production (data not shown).

2.1.4 Peripheral blood DC purification and culture

PBMC were isolated from buffy coats by Ficoll gradient (Pharmacia Biotec, Uppsala, Sweden), and peripheral blood myeloid (M-DC) and plasmacytoid (P-DC) DC were magnetically sorted with BDCA-1 and BDCA-4 cell isolation kits (Miltenyi Biotec, Bergisch Gladblach, Germany), respectively to a purity of 95–98%. Blood M-DC and P-DC (2×10^4 cells/well) were cultured in 96-well plates (Costar, Cambridge, MA) in RPMI 1640 culture medium supplemented with 5% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, 1 mM sodium pyruvate, and 1% nonessential amino acids plus 1000 U/ml GM-CSF and 10 ng/ml IL-4 (BD PharMingen, San Diego, CA) or 20 ng/ml IL-3 (BD PharMingen), respectively.

2.1.5 Cell lines and culture

The mouse L1.2 lymphoma cell line was grown in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FCS (HyClone Laboratories, Logan, UT), 10 mM Hepes pH 7.4, 50 μM 2-mercaptoethanol. Mouse lymphatic endothelial cells (MELC) were obtained following a previously described procedure (232). Briefly, hyperplastic vessels were induced by injection of IFA in DBA/2 mice and were isolated from liver and diaphragm. After collagenase treatment, the single cell suspension was cultured at 37°C and 5% CO_2 in gelatin-treated plastic dishes in DMEM (Life Technologies) supplemented with 10% FCS, 10% sarcoma 180 cell-conditioned medium, 1 mM sodium pyruvate, 100 μg/ml heparin, 100 μg/ml endothelial cell growth supplement, and antibiotics. CHO-K1 cells were cultured in DMEM-HAM F12 medium supplemented with 2 mM L-glutamine, 10% FCS, 10 mM Hepes pH 7.4.

2.2 Cell transfectants

2.2.1 cDNA cloning

The human CCRL2A and B coding sequences were amplified by PCR from genomic DNA using standard methodology and cloned into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). The insert was fully sequenced and shown to be identical to Gene Bank sequence xxx

2.2.2 Cell transfection

The mouse L1.2 lymphoma cell line was transfected by electroporation with linearized CCRL2A and B/pcDNA3 and selected with 800 μg/ml G418 (Life Technologies), resistant cells were cloned by limiting dilution. Chinese hamster ovary (CHO)-K1 cells were transfected with CCRL2A and B/pcDNA3 with lipofectamine 2000 (Invitrogen), selected with 500 μg/ml G418 and cloned by limiting dilution. D6/L1.2 and D6/CHO-K1 transfectants were obtained as previously described (108), CCR4/L1.2 were a kind gift of Dr. Daniele D'Ambrosio (BioXell, Milan, Italy). Clones MELC-2 and D6/MELC-2, selected for these studies, have been described previously (108). CCR4-D6/L1.2 were obtained transfecting CCR4/L1.2 with the plasmid D6/pcDNA6 encoding the HA-tagged human D6 receptor, and selected for the stable expression of both receptors in growing medium in the presence of hygromycin and G418

2.3 Methods for analysing RNA expression

2.3.1 RNA extraction

Total RNA was prepared from all samples using TRIZOL® Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 5-10x106 cells were centrifugated and pellet was lysed with 1 ml of TRIZOL® by repetitive pipetting. Samples were incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes and 0.2 ml of chloroform per 1 ml of TRIZOL was added. Samples were shaken vigorously by hand for 15 seconds and then incubated at 15 to

30°C for 2 to 3 minutes. Samples were centrifugated the samples at 12,000 × *g* for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization. The aqueous phase is transferred to a fresh tube, and RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization). Samples were incubated at 15 to 30°C for 10 minutes and centrifugated at 12,000 × *g* for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. After having removed the supernatant, the RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. The sample was shaked by vortexing and centrifugated at no more than 7,500 × *g* for 5 minutes at 2 to 8°C. At the end of the procedure, briefly the RNA pellet was dried (air-dry or vacuum-dry for 5-10 minutes). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C. For RT-PCR, total RNA was DNase-treated to remove contaminating genomic DNA, using RNase-free DNase I (Pharmacia Biotech, St. Albans, UK).

2.3.2 RT-PCR

2.3.2.1 cDNA synthesis

cDNA was synthetised from 5 μg of DNase-treated total RNA using the SuperScript. III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Briefly, the volume of 5 μg of DNase-treated total RNA was adjusted to 8 μl with DEPC-treated water, and 1 μl of oligo dT/random examers 50 μM and 1 μl of

dNTPs 10mM were added. The RNA was incubated at 65 °C for 5 min, then on ice for at least 1 min. Then 10 µl of cDNA Synthesis Mix (10X RT buffer 2 µl, 25 mM MgCl2 4 µl 40, 0.1 M DTT 2 µl, RNaseOUT. (40 U/µl) 1 µl, SuperScript. III RT (200 U/µl) 1 µl) were added to each sample, mixed gently and collected by brief centrifugation. Tubes were incubated for 50 min at 50°C, then at 85°C for 5 min and chilled on ice in order to terminate reaction. 1 µl of RNAse H was added to each tube and incubated for 20 minutes at 37°C, cDNA synthesis reaction can be stored at -20°C or used for PCR. 2µl (equivalent to 200 ng of total RNA) were used for PCR.

2.3.2.2 Primers

The primers for CCRL2 were designed from sequences submitted to Genbank. The primer sequences and product size are: forward gatgaggcagagcaatgtga; back ggcagggtaagcaagaaaca; product size: 209 bp. The 'housekeeping' gene β-actin was used in all PCR reactions to control for reverse transcription of the total RNA.

2.3.2.3 Polymerase chain reaction (PCR)

PCR was performed using a GeneAmp® PCR System 9700 thermal cycler (Perkin Elmer, Beaconsfield, UK). For each primer pair, a master mix was prepared containing all reagents except for the cDNA. The final volume of each PCR reaction was 25 µl,containing 200 ng cDNA, 1 U AmpliTaq DNA polymerase, GeneAmp PCR buffer, GeneAmp dNTPs (all from Perkin Elmer) and 4 µM each primer. The following protocol was used for the PCR reaction: 94 °C (5 min); 35 cycles 94 °C (30 s), 60 °C (30 s), 72 °C (30 s); 72 °C (7 min). 15 µl of each PCR reaction was added to 5 µl of loading buffer and electrophoresed through a 1.2 % agarose gel containing 0.5 µg/ml ethidium bromide (Sigma). Bands were visualised by UV transillumination and their sizes were estimated using a co-migrated DNA size marker (123 bp marker; Gibco

BRL, Paisley, UK). PCR products were gel extracted (QiaQuick Gel Extraction kit, Qiagen, UK) and sequenced to confirm their identity.

2.3.2.4 Real-time PCR

Real-time PCR was done with SYBR Green dye and GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City CA). The sequences of primer pairs specific for each gene (Invitrogen, Milan, Italy) were designed with Primer Express Software (Applied Biosystems). Two µl of cDNA were used as the template; 12.5 µl of 2x SYBR Green PCR Master Mix (Applied Biosystems) were mixed with template and primers. The total reaction volume was 25 µL. Cycling conditions were 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Experiments were done in triplicate for each sample. Absolute quantification was performed using standard curves for both CCRL2 and β-actin plasmids. Extrapolated number of CCRL2 copies of mRNA were normalized to 10^6 copies of ß-actin mRNA.

2.3.3 Northern blotting

2.3.3.1 cDNA probes

cDNAs for CCRL2A and β-actin were obtained from expression plasmids. Appropriate restriction enzymes were used to cut out each cDNA insert of CCRL2A and B-actin; each digest reaction was added to 5 µl of loading buffer (40 % w/v sucrose; 0.25 % w/v bromophenol blue; 0.25 % xylene cyanol [all from Sigma, Poole, UK]; made up in distilled water) and electrophoresed through a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. Insert cDNA bands were visualised by UV transillumination and gel extracted (QiaQuick Gel Extraction kit, Qiagen, UK). The purified cDNA insert was subsequently used for probe labelling (Section 2.4.4.iii).

2.3.3.2 Northern blotting

10 μg of total RNA was mixed with 5 μl of loading buffer (48 % deionised formamide [Gibco BRL, Paisley, UK], 6 % formaldehyde [Merck/BDH, Lutterworth, UK], 5 % glycerol, 20 mM MOPS [3-*N*-morpholinopropanesulphonic acid], 5 mM sodium acetate and 1 mM EDTA pH 8.0 [all from Sigma, Poole, UK] made up in DEPC-treated water). Each RNA sample was heated to 65 °C for 5 minutes, then placed on ice prior to loading. The RNA was subjected to electrophoresis through a 1 % agarose-formaldehyde gel (1 % agarose [Gibco], 6 % formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 8.0, 0.5 μg/ml ethidium bromide) then blotted by capillary transfer onto nylon membrane (Hybond N_+, Amersham, UK). After transfer, the membrane was UV crosslinked (1200 J) in a Stratalinker (Stratagene, La Jolla, CA, USA).

2.3.3.3 Probe labelling and hybridisation

The membrane was placed in a suitable hybridisation tube and pre-hybridised for 1-2 hours at 42 °C with 20 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 1 % BSA, 7 % SDS, 45 % formamide, made up in distilled water). cDNA probes were labelled by random priming using the Stratagene Prime-It II kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Unincorporated [$\langle^{32}$P]CTP nucleotide was removed by passing the radiolabelled probe through a Clontech Chromaspin TE 100 spin column (Clontech, Basingstoke, UK), following the manufacturer's instructions. Prior to use, the radiolabelled probe was heated to 100 °C for 5 minutes, then quenched on ice for up to 30 min. Probe was then added to 20 ml of hybridisation buffer (approximately 1 x 10^6 cpm/ml of buffer), which was poured on to the membrane in place of the pre-hybridisation buffer. Hybridisation was performed overnight at 42 °C. Following hybridisation, membranes were washed

twice with 2 x SSC, 0.1 % SDS for 5 min at room temperature, twice with 0.1 x SSC, 0.1 % SDS for 15 min at 68 °C and finally once with 2 x SSC for 10 min at room temperature. The membrane was then wrapped in Saran wrap and exposed overnight to Kodak Biomax MS film with an intensifying screen, at –70 °C. Densitometry was performed using NIH Image 1.61. Radiolabelled probe was stripped from the membrane by washing the membrane in boiling 0.1 % SDS. The membrane was left on a shaker until the solution cooled down; it could then be re-probed.

2.4 Flow cytometry

2.4.1 Monoclonal antibodies

Monoclonal anti-human CCRL2A/B antibody (catalog number: MAB2350 clone: 152211) was from R&D System (Minneapolis, MN). This antibody was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with NS0 cells transfected with CCRL2A isoform. The IgG fraction of the tissue culture supernatant was purified by Protein G affinity chromatography. It detects both the CRAM-A and CRAM-B isoforms. Monoclonal anti-human D6 (Clone 196124) and anti-CCR4 (Clone: 205410) antibodies were from R&D System.

2.4.2 Immunofluorescent staining protocol

For staining, cells were washed in PBS supplemented with 1 % BSA and 0.01 % NaN3 (FACS buffer). Approximately 5 x 10^5 cells were resuspended in 200 µl of FACS buffer in a microfuge tube, then 10 µg of human IgG was added to block Fc receptors. After 15 min incubation at room temperature, primary antibody was added to a final concentration of between 2-20 µg/ml. Cells were incubated with the primary antibody for 30 min on ice. The cells were then washed twice by addition of 1 ml of cold FACS buffer, centrifugation at 13,000 rpm for 10 seconds in a microfuge at 4 °C, and resuspension in cold FACS buffer. Cells stained with FITC- or PE-conjugated

antibodies were then ready for flow cytometric analysis. For unconjugated antibodies, cells were resuspended in 200 µl of FACS buffer and FITC-conjugated secondary antibody was added (at an appropriate dilution). Following a further 30 minute incubation on ice, the cells were washed twice as before, then analysed by flow cytometry. For two-colour flow cytometry, cells were incubated with the first antibody, washed, then incubated with the second antibody, washed, and analysed. Cells were analysed on a FACScan® flow cytometer using Cellquest software (Beckton Dickinson, Oxford, UK).

2.5 Measurement of intracellular Ca^{2+} concentration

Changes in $[Ca^{2+}]$, were monitored using the fluorescent probe fura-2 according to the technique reported by Grynkiewicz et al. (233). Briefly, cells (10^7/ml) were resuspended in RPMI 1640 and incubated with 1 µM fura-2 acetoxymethyl ester (Calbiochem, San Diego, CA) at 37°C for 20 min. After incubation, cells were washed and resuspended in HBSS (Biochrom) containing 1.2 mM CaCI, and kept at room temperature until used. Fura-2 fluorescence was measured in a Perkin-Elmer LS 50B spectrophotometer (Perkin-Elmer Instruments, Norwalk, CT) at 37°C with cells (3-5 X IO'/ml) continuously stirred. Samples were excited at 340 and 380 nm, and emission was continuously recorded at 487 nm.

2.6 Chemotaxis

Migration of D6/L1.2 and CCR4/L1.2 cells was evaluated using 5 µm pore size Transwell filters (Corning Costar, Cambridge, MA). 600 µl of binding buffer supplemented with different concentrations of chemokines were placed into the lower chamber. CCRL2A/B 11.2, D6/L1.2 or CCR4/L1.2 cells were resuspended in the same buffer at the concentration of 1 x 10^7 cells/ml, and 100 µl of cell suspension were placed onto the upper chamber. After 4 h of incubation at 37°C and 5% CO_2, the upper

chamber was removed and the cells in the lower chamber were counted in a Bürker chamber.

2.7 Binding

CCL2 competitive binding was performed by incubating 7.5 x 10^5 D6/L1.2 cells with 50 pM ^{125}I-CCL2 in the presence of different concentrations of unlabeled CCL2, CCL4, CCL17, CCL19 or CCL22 in 200 µl binding buffer (RPMI 1640, 4 mM Hepes pH 7.4, 1% BSA) at 4°C for 2 h. After incubation, the cell-associated radioactivity was measured. To estimate the K_d (i.e. the equilibrium dissociation constant) and the B_{max} (i.e. the maximum number of binding sites), CCL2 homologous competitive binding data were analyzed by non-linear fitting using the equation of the "homologous competitive binding curve" (GraphPad Prism 3.0a; GraphPad Software Inc., San Diego, CA). Inhibition curves were analyzed using the equation of the "one site competitive binding equation" (GraphPad Prism 3.0a) to estimate the IC_{50} value, from which the K_i value was then calculated according to the Cheng-Prusoff equation (234).

2.8 Chemokine scavenging

D6/CHO-K1 (2 x 10^5), D6/MELC-2 (1 x 10^5) or D6/L1.2 (1 x 10^6) cells were incubated for indicated time periods at 37°C in 200 µl of binding buffer supplemented with 1.2 nM of indicated chemokine. At the end, the chemokine concentration in the supernatant was measured by specific ELISA (R&D Systems).

2.9 Receptor internalization.

CCR4/L1.2 or D6/L1.2 cells were resuspended at 5 × 10^6 cells/ml in binding buffer and incubated with the appropriate receptor-specific primary antibody at 4°C for 1 h. After washing in ice-cold PBS containing 1% FCS, cells were incubated for various times at 37°C in the presence or absence of 60 nM CCL22. After washing in ice-cold PBS containing 1% FCS and 1% sodium azide, cells were incubated at 4°C with fluorescein

isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Laboratories) for the anti D6 monoclonal antibody or streptavidin - FITC for the anti-CCR2 antibody, respectively. In recycling experiments, cells were restained with the primary antibody after the internalization step and before the labeling with the appropriate secondary antibody was performed as described above. After staining, cells were resuspended at 5 x 10^5/ml in ice-cold PBS containing 1% FCS and 1% sodium azide and analyzed in a FACSCalibur™ flow cytometer (BD Biosciences) and data were analyzed with CellQuest software (BD Biosciences). Relative receptor surface expression was calculated as 100× [mean channel of fluorescence (stimulated) - mean channel of fluorescence (negative control)/mean channel of fluorescence (medium) - mean channel of fluorescence (negative control)] (%). L1.2 cells not expressing D6 or CCR4 and irrelevant monoclonal antibodies were used for negative controls with similar results.

2.10 Statistical analysis

Standard deviations were calculated and statistical significance assessed by Student t test for paired samples. Values of $p < 0.05$ were considered to be significant.

3 Chapter 3. CCRL2 expression in tissues and leukocytes

3.1 Introduction

A small number of orphan receptors, presenting structural similarities with known chemokine receptors, still await functional characterization. Among these CCRL2 is an interesting candidate as it shares over 40% amino acid identity with CCR1, CCR2, CCR3 and CCR5. Furthermore its gene is located in the main cluster of CC-chemokine receptor genes in the 3p21-23 region, together with CCR1 to 5, CCR8 to 10, XCR1 and CX3CR1 (235)

The functions of a given receptor are dictated by its expression pattern across leukocyte subclasses, the regulation of its expression as well as the regulation of the expression of the chemokines acting on this receptor. Determining the distribution and regulation patterns of an orphan receptor may therefore greatly contribute to the understanding of its potential roles. It may also allow us to tentatively classify the receptor as responding to inflammatory or constitutive chemokines, and may raise hypotheses regarding its contribution to the various steps of an immunological response. Finally it may suggest in which tissues and conditions the cognate ligand(s) should be searched for.

CCRL2 was first cloned from a human neutrophil cDNA library (134), and data obtained by Northern blotting showed expression in lymphoid (spleen, lymph node, fetal liver, bone marrow) and non-lymphoid (lung, heart) organs (134).

3.2 Aim of the chapter

The aim of this chapter was to examine the expression of CCRL2 in different tissues and main circulating leukocyte populations. The subsequent aim was to study the regulation of CCRL2 expression in DC.

3.3 Results

3.3.1 CCRL2 expression in tissues

CCRL2 tissue expression was assessed by mean of a commercial multiple tissue array (Clontech) containing polyA$^+$ RNA from 76 different human tissues. Densitometric analysis (Figure 3.1) revealed that CCRL2 transcripts were present at highest levels in lung and fetal spleen while moderate levels were present in bone marrow, testis, fetal liver, lymph node, spleen, fetal lung, the descending part of the colon, mammary gland and fetal thymus. Low levels of CCRL2 were also detectable in brain (cerebellum), heart, ilocecum, thymus, placenta, and liver. All the other tissues present on the array did not express CCRL2 transcripts. As previously reported by others (134), this analysis suggests that CCRL2 is highly expressed in almost all lymphoid tissues; however our results show highest levels of transcript in lung and fetal tissues suggesting a specific role for this receptor during embryogenesis.

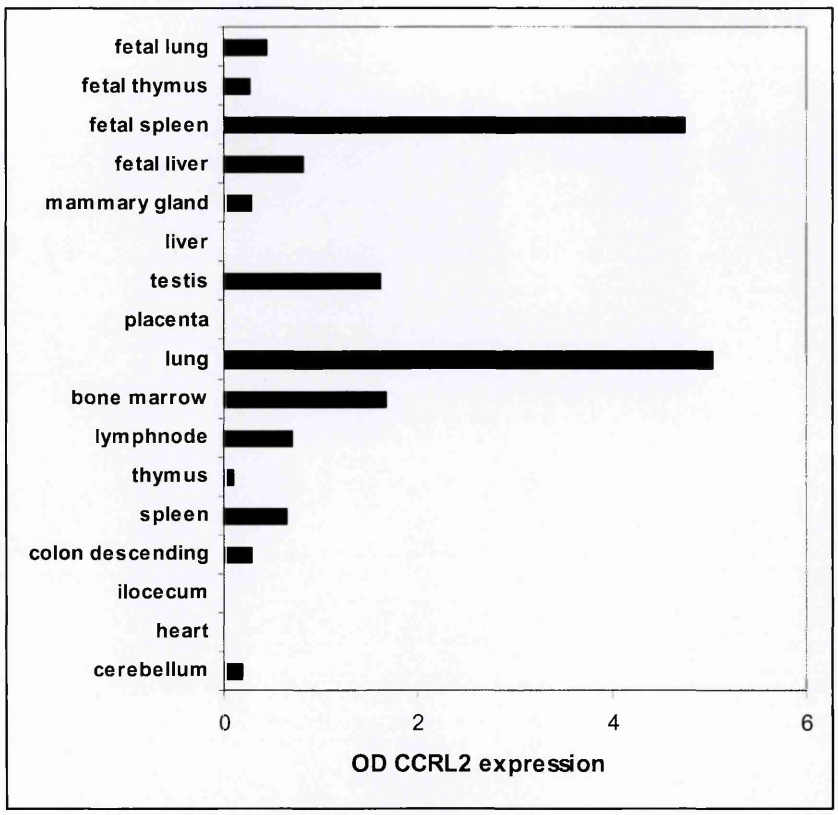

Figure 3.1. CCRL2 expression in human tissues.

Multiple tissue array (Clontech) was hybridized with CCRL2 specific probe. Results shown were obtained from densitometric analysis of one autoradiography.

3.3.2 CCRL2 expression in leukocytes

To study CCRL2 expression in leukocyte subsets, real-time quantitative polymerase chain reaction was performed with T naïve, Th1, Th2, Tc1, Tc2 and B lymphocytes, NK cells, immature and mature DC (treated 4 hours with LPS). Mature dendritic cells expressed the highest copy number of CCRL2 molecules, while the remaining leukocytes analyzed expressed low levels of CCRL2 transcript (Figure 3.2). In agreement with previous results (236) resting monocytes and neutrophils expressed CCRL2 transcripts as assessed with Northern blot analysis (Figure 3.3). FACS analysis on peripheral blood mononuclear cells (PMBC) labeled with a monoclonal antibody

against CCRL2, indicated that almost 50% of monocytes were CCRL2 positive while only 15% of lymphocytes expressed the receptor (Figure 3.4). Our analysis suggests that CCRL2 expression is restricted to cells of the myelomonocytic lineage and indicates that CCRL2 expression is strongly up-regulated in dendritic cells after maturation.

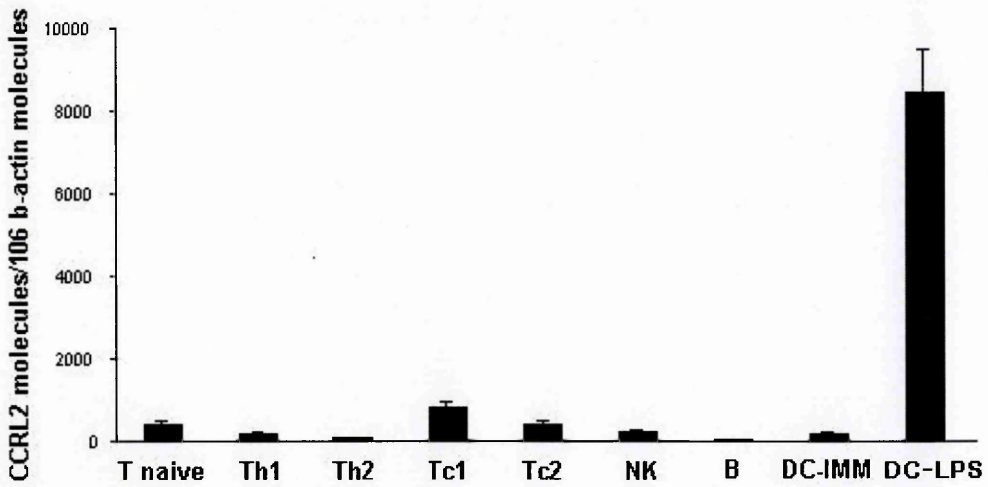

Figure 3.2. CCRL2 expression in human leukocytes.

Real-time quantitative PCR was performed with cDNA obtained from the retrotranscription of 2 μg of RNA of the indicated leukocytes. Results shown were obtained from statistical analysis of three different experiments. Absolute quantification was performed using standard curves for both CCRL2 and β-actin.

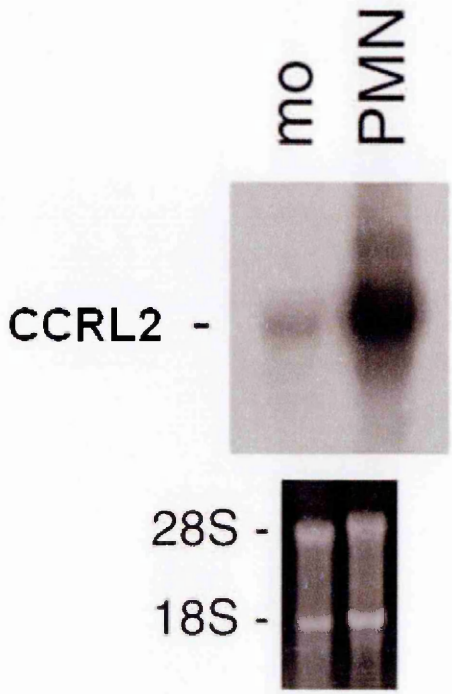

Figure 3.3 CCRL2 expression in human monocytes, neutrophils.

10 μg of total RNA were purified from monocytes and PMN obtained from buffycoats of healty donors and used in Northern blot analysis with CCRL2 probe. The autoradiography shown was obtained after 12 h of exposure. Etidium bromide staining is reported below. The figure shows a representative experiment of three performed with similar results.

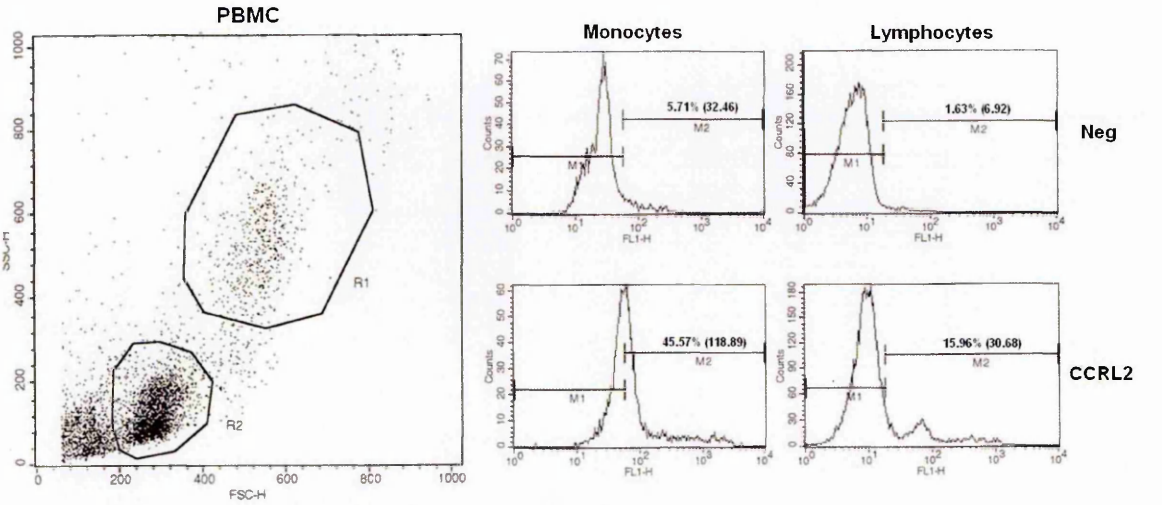

Figure 3.4 CCRL2 expression in human leukocytes.

Human PBMCs were isolated as described in Materials and methods and stained for CCRL2 expression. PBMC subsets were analyzed by FACS analysis. One experiment representative of four independent ones is shown. Each panel contains the percentage of positive cells and the mean fluorescence intensity of CCRL2 expression. R1: monocytes, R2: lymphocytes.

3.3.3 Regulation of CCRL2 expression in DC

CCRL2 over-expression in monocyte derived DC (Figure 3.2) was also investigated at the protein level. DC were differentiated in vitro from blood monocytes (>95% $CD14^{+}$), obtained by Ficoll and Percoll gradients. Monocytes were cultured for 6 days with GM-CSF and IL-13 and then FACS analysis was performed in order to assess the correct DC differentiation. As depicted in Figure 3.5, at the end of the culture cells were 90% CDla positive, nearly 90% MHC class II positive, <10% CD14 positive and 11% CD83 positive.

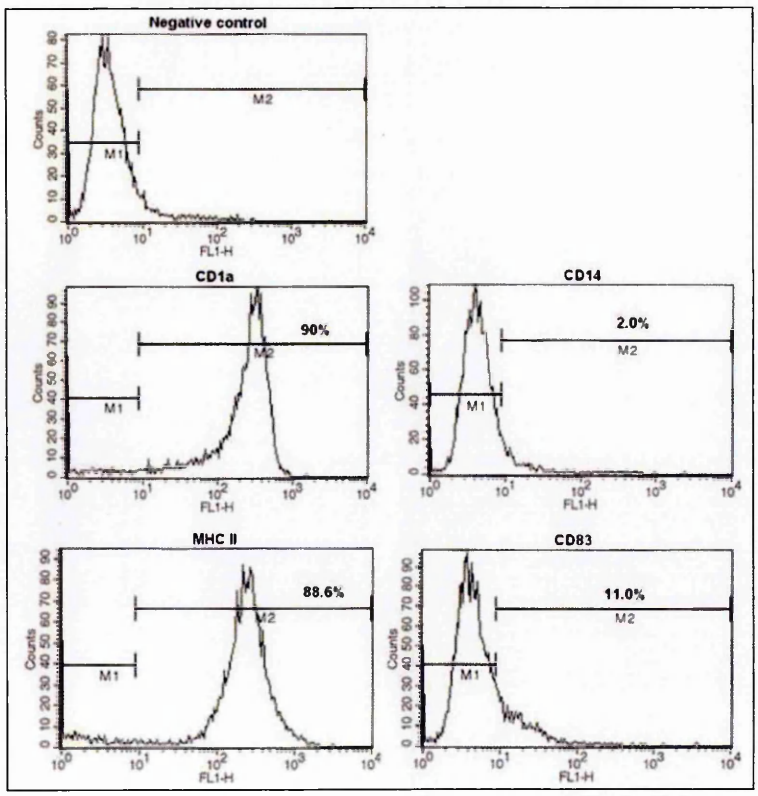

Figure 3.5 Phenotype of immature DC

Monocytes after 6 days of culture in the presence of 50 ng/ml GM-CSF and 10 ng/ml IL-13 were subjected to FACS analysis for CD1a, CD14, MHCII and CD83 expression using specific monoclonal antibodies. Each panel contains the percentage of CCRL2 positive cells. One experiment representative of four independent ones is shown.

DC maturation was obtained by stimulation with LPS, TNF-α or admixing with the irradiated cell line J558 CD40L-transfected for 4 hours. Figure 3.6 showed that all stimuli tested were able to induce DC maturation, as assessed with FACS analysis for CD83 protein expression. CD83 was not expressed by immature DC while was strongly upregulated by all the maturative stimuli used. Incubation of DC with the J558 mock cell line did not induce cell maturation (data not shown).

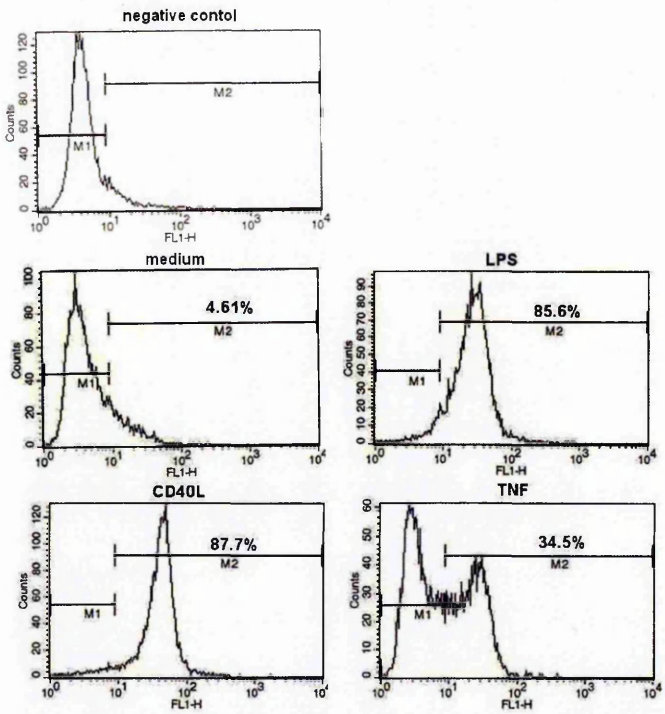

Figure 3.6 Upregulation of CD83 expression by maturative stimuli.

Immature DC were cultured in the presence of LPS (100 ng/ml), TNF-α (50 ng/ml) and CD40L (5:1, DC:CD40L-transfectants) for 4 hours and then subjected to FACS analysis for CD83 expression by the use of a monoclonal antibody. Each panel contains the percentage of positive cells and the mean fluorescence intensity of CCRL2 expression. The figure shows a representative experiment of three performed with similar results

Northern blot experiments revealed that in immature DC CCRL2 expression varied among donors (data not shown). However stimulation with maturative stimuli like LPS (100ng/ml) and CD40L expressing cell line (1:5) for 4h up regulated CCRL2 expression of 28.5 ± 7.9 fold (n=4) and 18.8 ± 10.9 fold (n=2) respectively, over the control evaluated with densitometrical analysis. In an opposite way stimulation for the same time with TNF-α (50 ng/ml), a cytokine able to induce DC maturation, had no effect on CCRL2 basal expression (Figure 3.7A). Upregulation of CCRL2 expression by LPS was then measured at protein level by FACS analysis. As shown in figure 3.7B 35% of immature DC expressed CCRL2 while after LPS stimulation the percentage of CCRL2 positive cells raised to 70%.

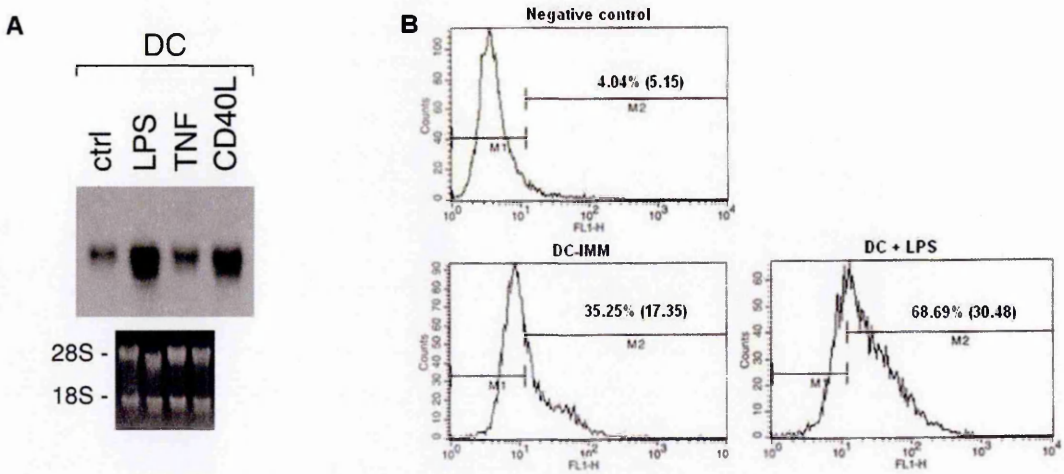

Figure 3.7 CCRL2 expression in human dendritic cells.

A 10 μg of total RNA were purified from DC incubated for 4h in the presence or absence of LPS (100 ng/ml), TNF-α (50 ng/ml) and CD40L (5:1, DC:CD40L-transfectants) and used in Northern blot analysis. B Immature DC and DC stimulated with LPS were subjected to FACS analysis for CCRL2 expression by the use of monoclonal antibody. Each panel contains the percentage of positive cells and the mean fluorescence intensity of CCRL2 expression. The figure shows a representative experiment of three performed with similar results

In order to understand if CCRL2 mRNA upregulation was associated with DC maturative process, we cultured DC in the presence of inhibitors of maturation and function. Figure 3.8 panel A shows that both VitD3 (10^{-7} M) and Dex (10^{-5} M), two drugs able to block DC maturation in terms of CD83 expression and cytokine production (IL-12) had no effect on LPS-induced CCRL2 up regulation; on the contrary PGE_2 (10^{-5} M), that does not affect DC maturation but similarly to VitD3 and DEX is able to inhibit IL-12 production, completely abolished LPS effect on CCRL2 expression. These compounds had no effect on CCRL2 basal expression (data not shown). Figure 3.8 B shows the mean of densitometric analysis of 4 Northern blot experiments in which cells were treated for 4h as indicated. LPS induced an increase of 28.5 ± 7.9 fold ($p<0.001$ by paired Student's T-test) fold over control. When DC were stimulated with LPS together with PGE_2 CCRL2 expression was reduced to 0.97 ± 0.3 ($p<0.001$) fold over the control, while stimulation with LPS together with DEX and $VitD_3$ did not inhibit CCRL2 up regulation (24.7±17.6 and 54.8±24.2, respectively, over the control). Thus, these results indicated that only PGE_2 is able to reverse the stimulatory effect of LPS.

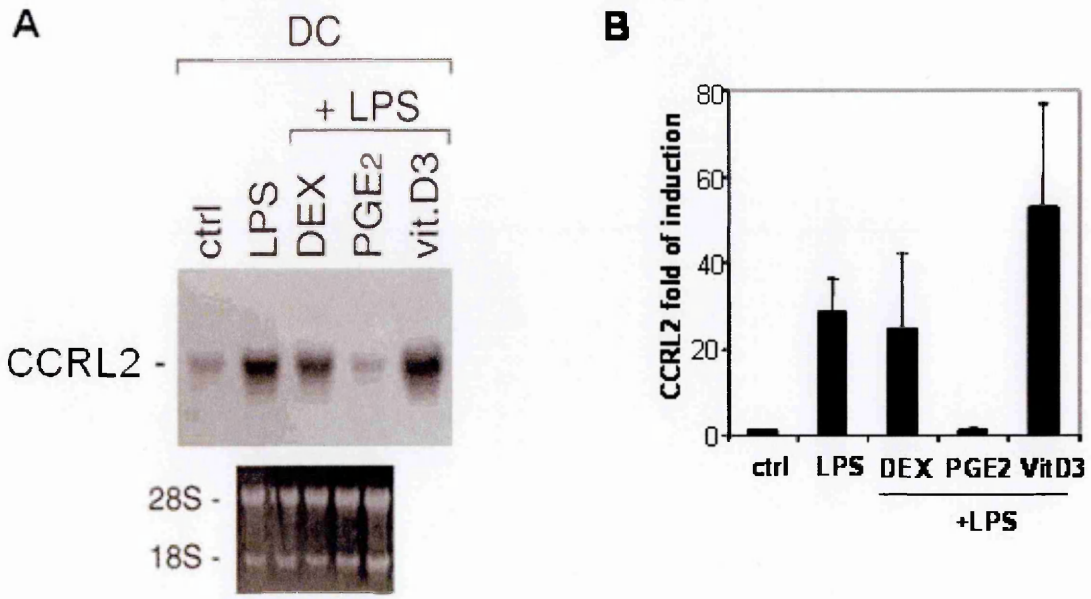

Figure 3.8 Effect of DEX, PGE2, VitD3 on CCRL2 expression in LPS stimulated DC.

A Northern blot analysis was performed with 10 μg of total RNA purified from DC incubated for 4h in the presence or absence of LPS (100 ng/ml) and in combination with DEX (10^{-6} M), PGE2 (10^{-5} M) and VitD3 (10^{-7} M). Results are representative of at least three different cell preparations. Autoradiographies shown were obtained after 12 h of exposure. Etidium bromide staining is reported below. B Mean of the densitometric analysis of four different experiments.

Figure 3.9 shows that the effect of LPS on CCRL2 expression by DC was detectable after 1.5 h stimulation, reached a maximum at 4 h and was completely abolished at 24h. In a similar way, when DC were stimulated with CD40L-transfected cell line, CCRL2 expression was upregulated following 4h stimulation but not at 24h. In the same experimental conditions CCR7 was strongly up regulated only after 24h stimulation with both LPS and CD40L, in agreement with previous published results (185).

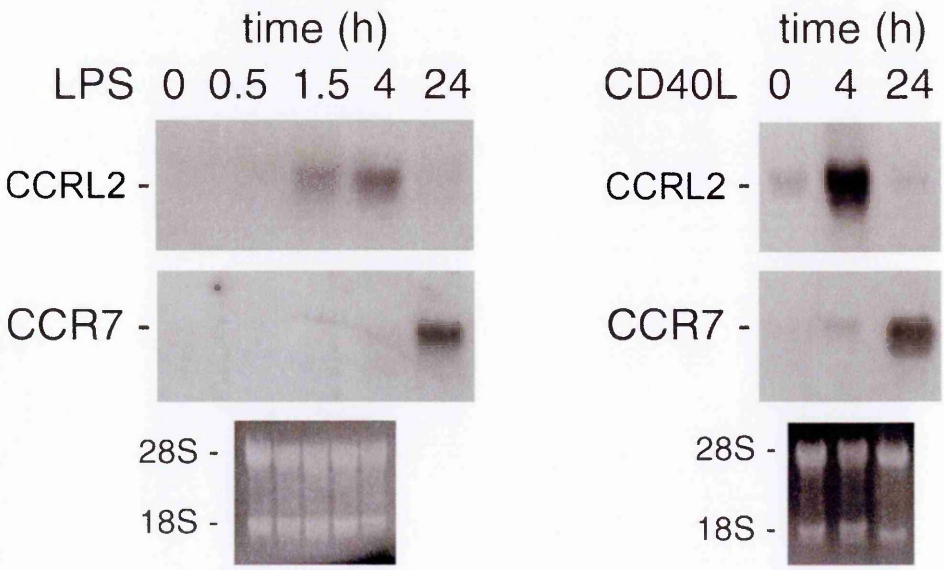

Figure 3.9 Time-course of CCRL2 expression in LPS and CD40L stimulated DC.

DC were incubated for different times with LPS (100 ng/ml) or with CD40L (4:1, DC:CD40L-transfectants) as indicated. 10 µg of total RNA were used in Northern blot analysis. Results of one experiment, representative of two performed are shown. Autoradiographies were obtained after 12 h of exposure. Etidium bromide staining of the membrane is shown in the lower part of the figure.

3.3.4 CCRL2 expression in plasmacytoid dendritic cells

In order to understand whether CCRL2 expression was restricted to myeloid DC, RT-PCR analysis was performed on RNA extracted from blood DC subsets isolated on the basis of the expression of specific membrane markers BDCA-1 for myeloid-DC and BDCA-4 for plasmacytoid-DC. Figure 3.10A shows that the CCRL2 transcript was present in both subsets. FACS analysis for hCCRL2 showed that it was expressed by ~ 40% of the circulating mDC population and by virtually all pDC.

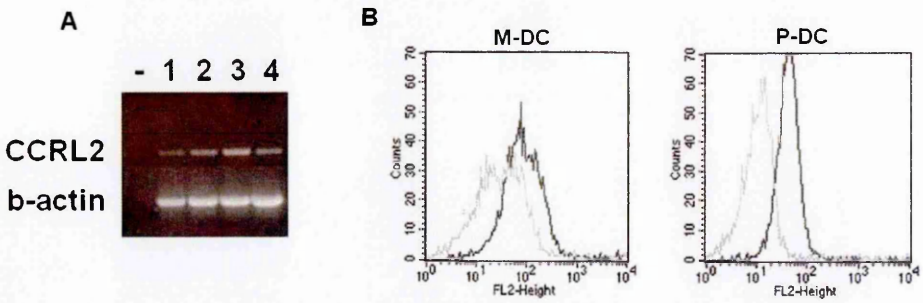

Figure 3.10 Expression of CCRL2 in blood DC subsets.

Blood DC subsets were isolated as described in materials and methods **A.** 2μg of total RNA were used in RT-PCR experiment using specific primers for CCRL2 and β-actin. (-: negative control, 1: P-DC, 2: M-DC, 3: circulating monocytes, 4: monocyte derived DC. Ethidium bromide staining for both genes is shown. **B**. Blood DC subsets were stained for CCRL2 expression with a monoclonal antibody. Data are representative of at least two different experiments performed with independent donors. Thin lines represent negative control, bold lines represent CCRL2 staining.

3.4 Discussion

To gain insight into the putative biological role of CCRL2, its expression was investigated in a variety of tissues and specific cell types. Over all tissue expression demonstrate that CCRL2 is highly expressed in fetal tissues, suggesting a role in embryogenesis. Furthermore it is highly expressed by both adult and fetal lung and, in this context, recent results published by Oostendorp et al. (237) suggest a specific role for murine CCRL2 in a model of ovalbumin (OVA)-induced airway inflammation. CCRL2 is also expressed by adult tissues where hematopoiesis occurs like bone marrow, lymphatic tissues, fetal spleen and fetal liver.

Analysis of leukocytes has revealed a unique profile for CCRL2. It is expressed by neutrophils and monocytes, as other inflammatory chemokine receptors. Interestingly it is also highly expressed by mature DC, a characteristic shared with the homeostatic chemokine receptor CCR7. Moreover inflammatory chemokine receptors like CCR1, CCR2 and CCR5 are downregulated during DC maturation while CCRL2 is rapidly and transiently upregulated, suggesting a function between the recruitment of leukocytes to inflammatory sites, and their redirection to lymphoid organs. Moreover the important up-regulation of CCRL2 following stimulation by CD40L suggests a possible role in DC-T cell interaction.

Data presented in this chapter indicate that CCRL2 upregulation by LPS is not part of DC maturation because it is not reversed by coincubation of DC with inhibitors of maturation process like $VitD_3$ and DEX. Conversely PGE_2, an arachidonic acid metabolite that plays an essential role in DC migration to draining lymph nodes, completely abolishes LPS-induced CCRL2 upregulation. The role of PGE_2 in regulating DC migration is still controversial: it appears from in vitro and in vivo data that PGE_2 has a dual role in supporting migration of DC (229, 238), (230) regulating the expression and the activity of CCR7. Our data suggest that the downregulation of

CCRL2 by PGE_2 may be involved in the effective migration of DC to the two CCR7 ligands.

Plasmacytoid DC secrete high levels of type I interferon following activation. The production of type I interferon is believed to play a crucial role in anti-viral immune responses and in the activation of other leukocyte populations, like B lymphocytes and NK cells. Plasmacytoid DC are normally absent from peripheral tissues and they are believed to migrate constitutively from the blood to the lymph nodes, through high endothelial venules (17). Recruitment of plasmacytoid DC to non-lymphoid tissues is observed in some pathological conditions, such as autoimmune diseases, allergic diseases and in tumors (16). However, the mechanisms leading to the recruitment of plasmacytoid DC to inflammatory sites remain unresolved.

Blood myeloid DC and plasmacytoid DC express similar pattern of chemokine receptors but they exhibit a profound difference in their capacity to migrate in response to chemokines with CXCL12 being the only chemokine active in a classic chemotaxis assay or in transmigration assays across an endothelial cell monolayer (225). In classical chemotaxis assays, the ligands of CXCR3, namely CXCL9, CXCL10 and CXCL11, are inactive in inducing plasmacytoid DC migration but can promote plasmacytoid DC migration in response to CXCL12 (226, 239). However, it was shown that CXCR3 ligands are fully competent in inducing plasmacytoid DC adhesion and migration when presented to plasmacytoid DC immobilized on the heparan sulfates present on endothelial cells membrane, a physiological relevant condition (14, 224, 240). For all these reasons the full characterization of chemoattractant receptors expressed by plasmacytoid DC versus myeloid DC will help in the understanding the different migratory capacity of this subset. Our results indicate that P-DC express CCRL2 both at protein and mRNA level. It will be helpful to study CCRL2 regulation by inflammatory stimuli in pDC.

Finally our analysis reveals that CCRL2 is not expressed by T or B-lymphocytes and by NK cells. These results are different from data published by Migeotte et al. (138) that reports CCRL2 expression on the majority of T lymphocytes and on the majority of natural killer cells.

Conclusions from this chapter:

- CCRL2 is expressed by different tissues: lung, fetal spleen and lymphoid tissue.
- Among leukocytes CCRL2 is expressed by monocytes, dendritic cells and neutrophils
- CCRL2 expression in DC is up regulated by maturative stimuli like LPS and CD40L
- CCRL2 upregulation is reversed by PGE2 costimulation
- The upregulation of CCRL2 by LPS and CD40L is transient and complementary to CCR7 induction.
- CCRL2 is expressed by both plasmacytoid and myeloid DC

4 Chapter 4. Generation of CCRL2 transfectants and screening for CCRL2 agonists

4.1 Introduction

The general strategy for the identification of ligands for orphan GPCRs is the use of a recombinant assay system. Following receptor expression, candidate ligands are screened against the receptor to identify molecules capable of specific regulation of that receptor. Such ligands can include tissue extracts, expressed or purified proteins or small peptides, natural and synthetic small molecules, and lipids.

It is important to choose of the right expression system and to find the best conditions to use for the screening because the success of this kind of experiment is entirely dependent upon the receptor being expressed at the cell surface and being able to couple to the signal transduction machinery of that cell. For these reasons it is not sufficient to demonstrate the presence of mRNA in the cell that is indicative of receptor expression but it does not always follow that receptor protein is expressed or is indeed at the cell surface. So it is important to use an antibody to demonstrate the presence and site of receptor expression through the use of studies such as fluorescence activated cell sorting (FACS) (241).

CCRL2 was transfected in two different cell lines, CHO-K1 and L1.2, that are frequently used to test chemokine receptor functions. As described in the introduction, phylogenetic analysis of GPCR receptor relationships suggested that CCRL2 constitutes a separate branch related to the chemokine receptors CCR1, 2, 3 and 5 and for this reason the analysis presented in this chapter was focused on CC chemokines (242).

4.2 Aim of the chapter

This chapter describes the generation of CCRL2 transfectants and their use in screening assays such us chemotaxis and calcium fluxes.

4.3 Results

4.3.1 Analysis of CCRL2 A and B receptor variants

CCRL2 cDNA was cloned by three different groups and deposited in Gen-Bank under different names. We observed that one of these sequences, named CRAM-A (accession n°AF015524), differed from the others named CCRL2B (NM_003965), CRAM-B (AF015525) and CRKX (U95626), in the deduced protein sequence by the presence of 12 additional amino acid at its N-terminus as shown in Figure 4.1. Comparison of the CRAM-A and CCRL2 sequences with the sequences from an extended region of chromosome 3 revealed that the 70 bp insertion in CRAM-A was due to alternative mRNA splicing as shown in Figure 4.2. The predicted initiation codon of CRAM-A was within an exon 360 bp upstream from the major coding exon. In the genomic sequence the 70-bp fragment found in CRAM-A was delineated by the canonical intronic splice acceptor and splice donor dinucleotides. These observations suggested the existence of two different proteins differing in their N-terminus and hence in their ligand selectivity or affinity, because this part of the receptor is important for ligand binding. The possible presence of two alternative transcripts suggested another level of regulation for this gene; for other chemokine receptors this mechanism is known to extend the range of concentrations over which a cell can respond (243). Due to these observations we decided to clone both variants.

```
NM_003965       ------------MANYTLAPEDEYDVLIEGELESDEAEQCDKYDAQALSAQLVPSLCSAV 48
AF015524        MIYTRFLKGSLKMANYTLAPEDEYDVLIEGELESDEAEQCDKYDAQALSAQLVPSLCSAV 60
                             ************************************************

NM_003965       FVIGVLDNLLVVLILVKYKGLKRVENIYLLNLAVSNLCFLLTLPFWAHAGGDPMCKILIG 108
AF015524        FVIGVLDNLLVVLILVKYKGLKRVENIYLLNLAVSNLCFLLTLPFWAHAGGDPMCKILIG 120
                ************************************************************

NM_003965       LYFVGLYSETFFNCLLTVQRYLVFLHKGNFFSARRRVPCGIITSVLAWVTAILATLPEFV 168
AF015524        LYFVGLYSETFFNCLLTVQRYLVFLHKGNFFSARRRVPCGIITSVLAWVTAILATLPEFV 180
                ************************************************************

NM_003965       VYKPQMEDQKYKCAFSRTPFLPADETFWKHFLTLKMNISVLVLPLFIFTFLYVQMRKTLR 228
AF015524        VYKPQMEDQKYKCAFSRTPFLPADETFWKHFLTLKMNISVLVLPLFIFTFLYVQMRKTLR 240
                ************************************************************

NM_003965       FREQRYSLFKLVFAIMVVFLLMWAPYNIAFFLSTFKEHFSLSDCKSSYNLDKSVHITKLI 288
AF015524        FREQRYSLFKLVFAVMVVFLLMWAPYNIAFFLSTFKEHFSLSDCKSSYNLDKSVHITKLI 300
                **************:*********************************************

NM_003965       ATTHCCINPLLYAFLDGTFSKYLCRCFHLRSNTPLQPRGQSAQGTSREEPDHSTEV 344
AF015524        ATTHCCINPLLYAFLDGTFSKYLCRCFHLRSNTPLQPRGQSAQGTSREEPDHSTEV 356
                ************************************************************
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Figure 4.1 ClustalW Protein sequence alignment of human CCRL2A (AF015524) with CCRL2B (NM_003965).

The alignment shows that the two proteins are identical, as indicated by asterisks, differing only in their N-terminal part. The following symbols denote the degree of conservation observed in each column: "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. Red means small+ hydrophobic (incl.aromatic -Y) residues; blue means acidic residues; magenta means basic residues, green means hydroxyl + amine + basic – Q residues.

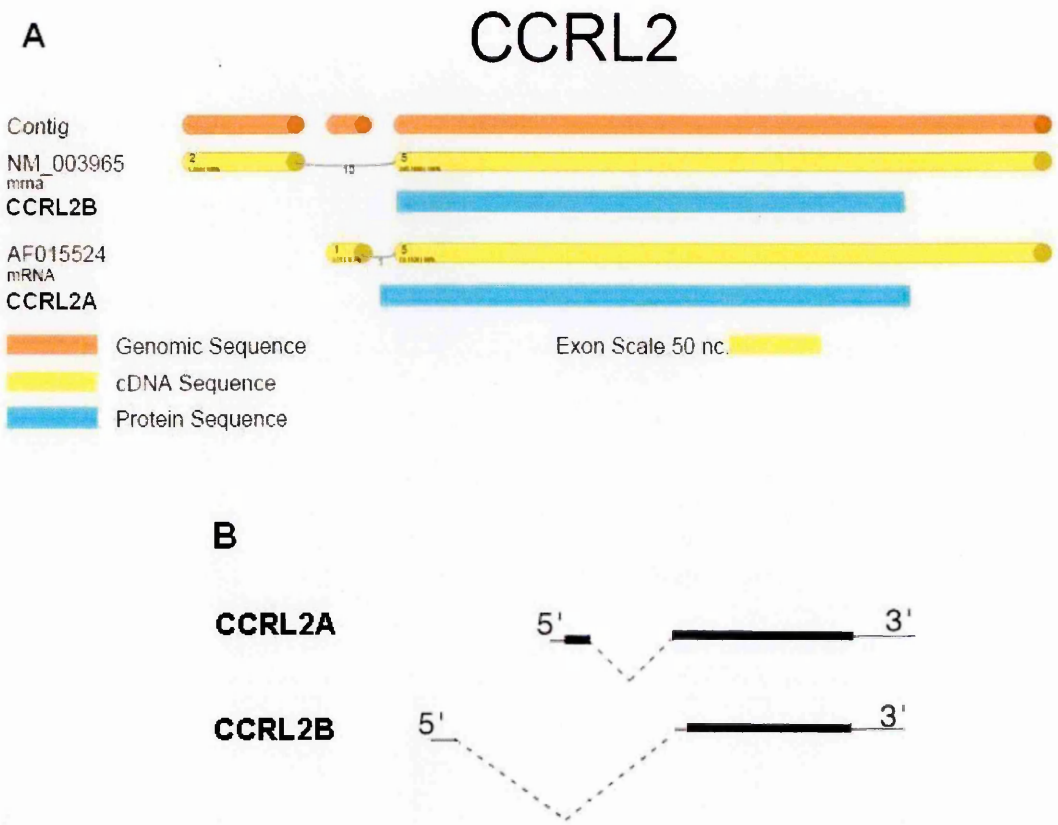

Figure 4.2 Schematic representation of CCRL2 gene.

A Red colour represents sequence of exons on chromosome 3p21 coding for CCRL2, yellow are alignment of the two different mRNA sequences present in the databases and blue represent the protein sequence of the two CCRL2 splicing variants. **B** Structure of cDNA encoding CCRL2A and CCRL2B. Thin lines represent non translated regions; dashed diagonal lines represent introns; thick lines represent coding regions. The drawing is not to scale.

4.3.2 Cloning of CCRL2 A and B receptor variants and transfection in L1.2 and CHO-K1 cell line

CCRL2A and B were cloned from cDNA derived from LPS treated dendritic cells. Primers to amplify the full-length sequence for CCRL2 were chosen according the two sequences AF015524 and NM_003965 for CCRL2A and B respectively. The resulting PCR products were cloned into the *Bam*HI - *Not* I sites of pcDNA 3.1 and sequenced. The two plasmids were transfected with Lipofectamine 2000 in CHO-K1 cells according to the manufacturer's instructions. After 24 hours cells were detached and studied with FACS analysis with a monoclonal antibody against CCRL2. As shown in Figure 4.3 nearly 20% of transfected cells expressed CCRL2 on their membrane. Similar transfection efficiency was obtained with CCRL2A (data not shown).

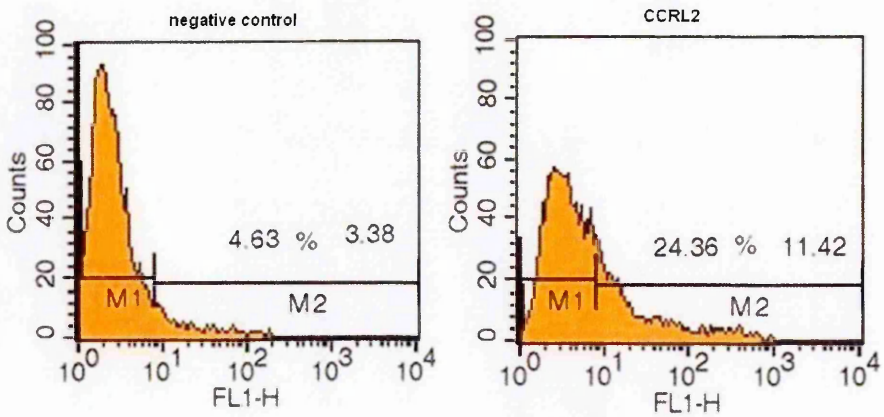

Figure 4.3 FACS analysis of CCRL2B bulk transfection.

CHO cells after 24 hours from transfection with CCRL2B expression plasmid were subjected to FACS analysis for CCRL2 expression by the use of specific monoclonal antibodies. Each panel contains the percentage of positive cells and the mean fluorescence intensity of CCRL2 expression.

Cells were then selected with G418 at a concentration of 500ug/ml for approximately 2 weeks in order to produce stable transfectants. Single cell cloning was performed using limiting dilution technique and the resulting cell clones were checked by RT-PCR for CCRL2 mRNA expression. Mock transfections were performed with empty vector. Figure 4.4 shows RT-PCR of 10 CHO clones obtained from CCRL2B transfection. Clones G7 as negative and clones D7, G10 and H6 that express high levels of CCRL2B mRNA were further subjected to FACS analysis in order to check protein expression. As expected the negative clone G7 did not express CCRL2 protein while two out of three CCRL2B mRNA positive clones (G10 and H6) showed high level of CCRL2 protein expression (Figure 4.5).

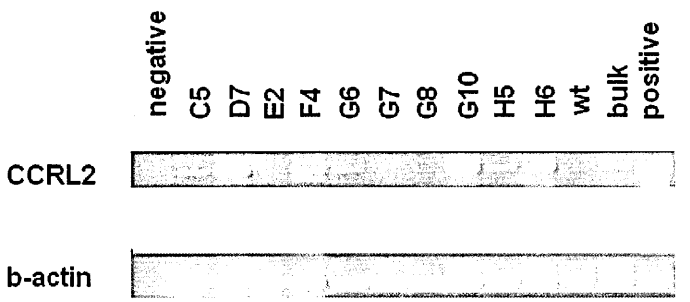

Figure 4.4 Expression of CCRL2B in CHO clones.

2μg of total RNA was used in RT-PCR experiments using specific primers for CCRL2B and b-actin. Number of cycles for CCRL2B and β-actin were 32 and 28 respectively Ethidium bromide staining for both genes is shown.

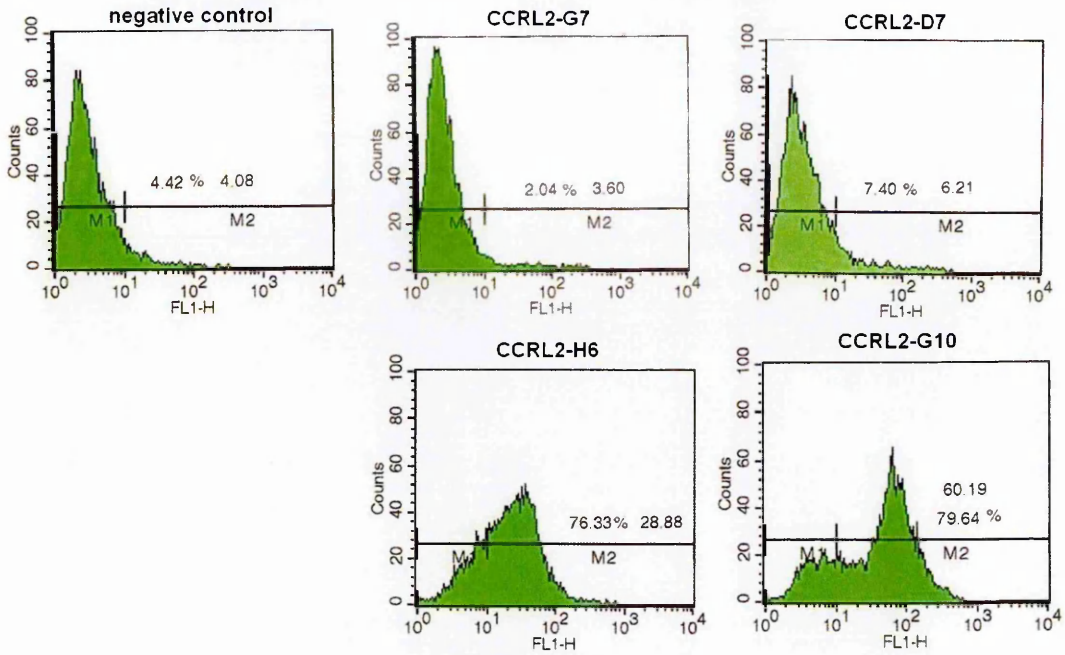

Figure 4.5 FACS analysis of CCRL2B expressing clones.

CCRL2B/CHO transfectants were subjected to FACS analysis for CCRL2 expression by the use of specific monoclonal antibodies. Each panel contains the percentage of positive cells and the mean fluorescence intensity of CCRL2 expression. Results are representative of two separate experiments.

Due to the correlation between mRNA and protein data we proceeded with the screening of other clones of both CCRL2A and B using only FACS analysis as shown in Figure 4.6.

From CCRL2B cloning we obtained six clones and only three of them were positive with weak immunoreactivity, indicative of low surface expression of the receptor (Figure 4.6A), while we were able to obtain more clones with higher levels of receptor expression from CCRL2A transfection (Figure 4.6B).

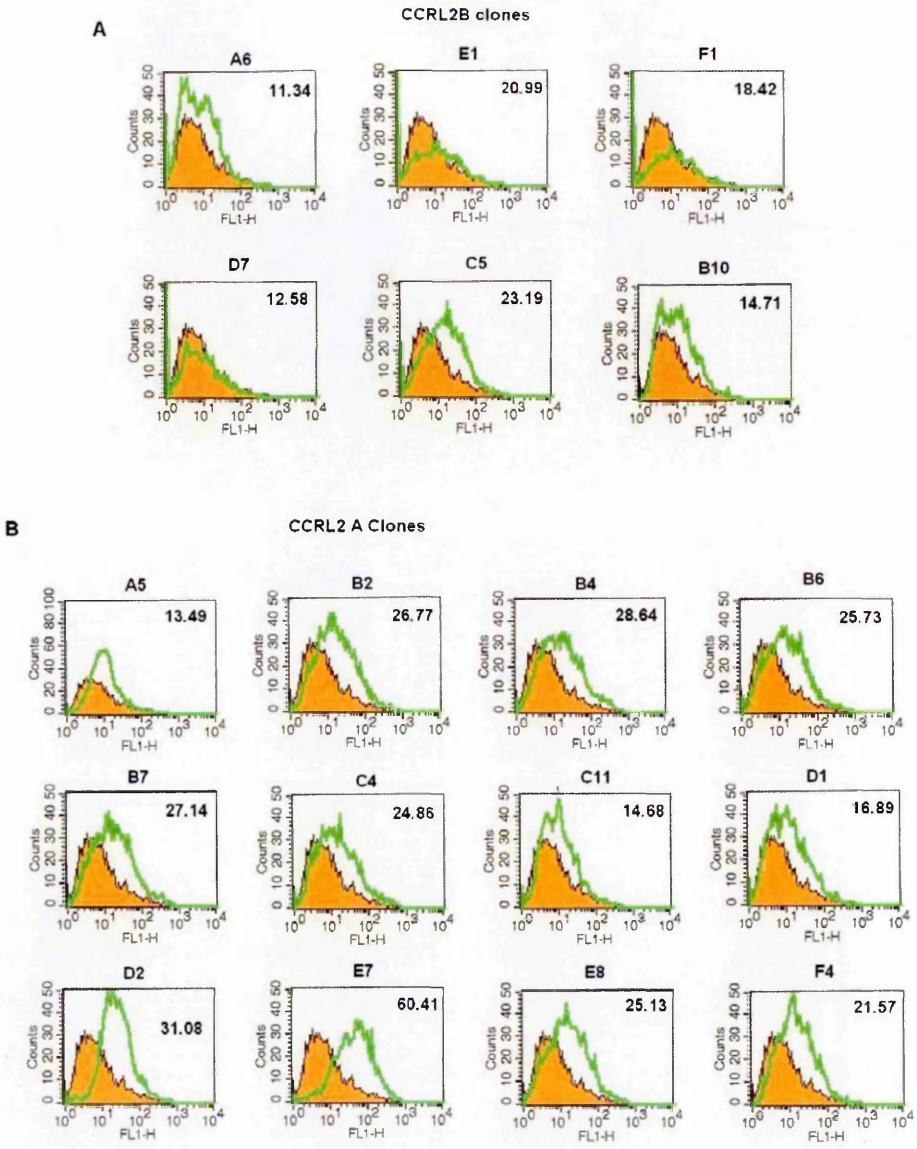

Figure 4.6 Screening of CCRL2A and B/CHO clones.

CCRL2A and B/CHO transfectants were subjected to FACS analysis for CCRL2 expression by the use of specific monoclonal antibodies. Orange histograms represent negative control, green histogram represent CCRL2 staining. Each panel contains the mean fluorescence intensity of CCRL2 expression.

In order to maximize expression of the vector-expressed CCRL2 we treated cells with sodium butyrate, an inhibitor of histone deacetylases (HDACs) that induces gene expression. In fact acetylation correlates with nucleosome remodeling and transcriptional activation while deacetylation of histone tails induces transcriptional repression through chromatin condensation. Relaxation of the chromatin structure induced by HDACs facilitates the accessibility of a variety of factors to DNA (244).

We focused our attention on three clones of CCRL2A/CHO (D2, E7, E8) and on three clones of CCRL2B/CHO (A6, C5, B10) measuring membrane protein levels by FACS analysis before and after treatment with sodium butyrate over night. Figure 4.7 shows that there was at least 3-fold increase in cell surface CCRL2 in all clones following butyrate treatment, indicating that this is the best condition to use for our screening analysis.

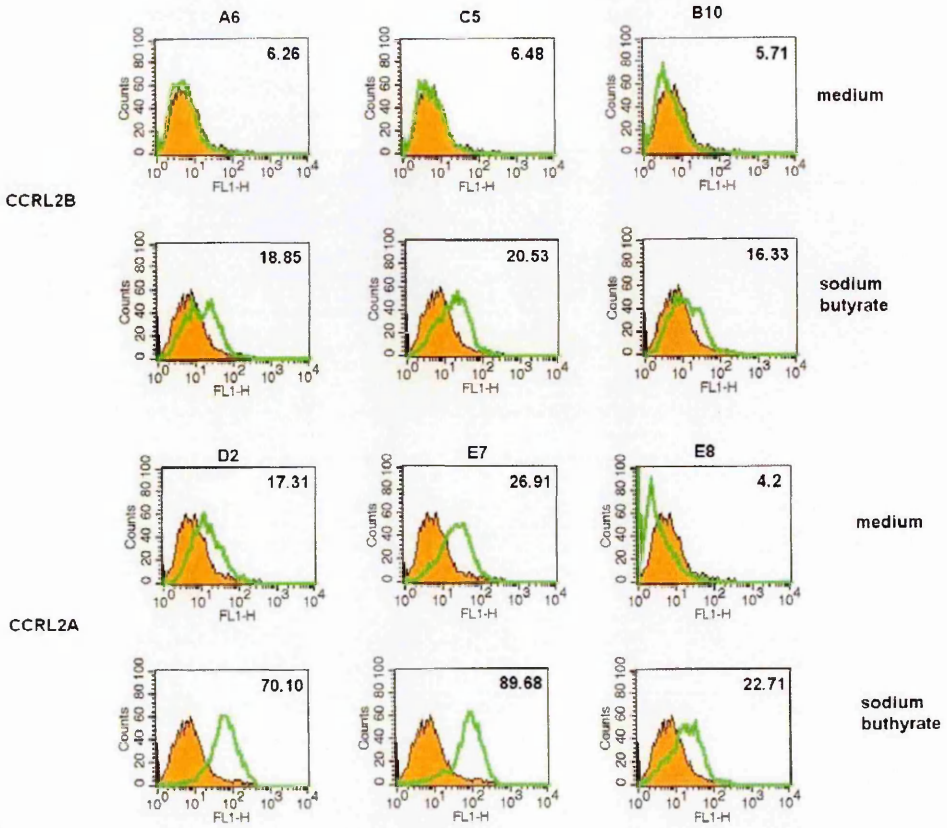

Figure 4.7 Effect of sodium butyrate treatment on CCRL2A and B/CHO clones.

CCRL2A and B/CHO transfectants were cultured at different concentrations in the absence or presence of sodium butyrate and then subjected to FACS analysis for CCRL2 expression by the use of specific monoclonal antibodies. Orange histograms represent negative control. Each panel contains the mean fluorescence intensity of CCRL2 expression.

During our analysis we noticed that CCRL2 expression in clones was not stable. For example CCRL2B expression in clone B10 had a MFI of 14.71 in the experiment shown in fig 4.6A that was reduced at 5.71 in the experiment shown in fig.4.7. The same problem was noticed for CCRL2A levels: for example CCRL2A expression of clone E8 that was MFI=25.13 in figure 4.6 and MFI=4.2 in figure 4.7.

In order to identify the best conditions in which the transgene was expressed we cultured cells at different concentrations. So cells were plated at different densities (20.000, 100.000 and 500.000 cells/well) and the day after detached and stained for CCRL2 expression. As shown in Figure 4.8 there was a 3-fold increase in cell surface CCRL2A and B levels when cells were plated at lower density (20.000 cells/well) in comparison with cells cultured at higher density (500.000), suggesting a correlation between plasmid expression and cell cycle or cell confluence. This behaviour was present also when cell were pre-treated with sodium butyrate (Figure 4.8), even if the difference is lower being nearly 2 fold. There are reports that correlate chemokine receptor expression with cell cycle phases (245) and it is possible that expression of CCRL2 is higher when cell are duplicating and is downregulated when cells arrest their cycle after reaching confluence.

The mouse L1.2 lymphoma cell line was transfected by electroporation with linearized vectors and selected with 800 μg/ml G418; resistant cells were cloned by limiting dilution. Screening of positive clones was done using the same approach shown before for CHO transfectants (data not shown).

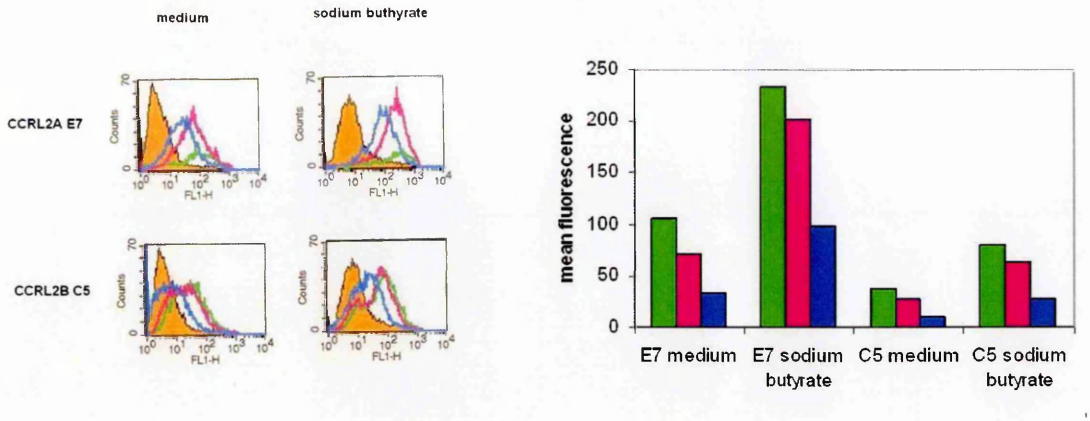

Figure 4.8 CCRL2 expression in CHO transfectants cultured at different concentrations.

CCRL2A and B/CHO transfectants were cultured at different concentrations in the absence or presence of sodium butyrate **A** FACS analysis for CCRL2 expression by the use of specific monoclonal antibodies. Orange histograms represent negative control, green histograms 20.000 cells/well, pink 100.000 cells/well and blue 500.000 cells/well. **B** CCRL2 mean fluorescence intensity of the same FACS analysis.

4.3.3 Chemotaxis and Calcium flux

L1.2 cells transfected with CCRL2A and CCRL2B were chosen to perform chemotaxis and calcium flux experiments because they represent a very useful cell model for this functional assay. Cells were treated overnight with sodium butyrate in order to maximize CCRL2 expression and cell migration was performed with Transwell system with a broad panel of human chemokines. Figure 4.9 shows that CCRL2B/L1.2 were not able to migrate in response to all chemokine tested: CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL13, CCL15, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL25, CXCL8 and CXCL10. As expected, cells were able to migrate in response to CXCL12, the ligand of the endogenous receptor CXCR4. Moreover transfectants did not migrate in response to supernatants obtained from different tumor

cell lines (data not shown). Similar results were obtained with CCRL2A/L1.2 cells (data not shown).

Another consequence of chemokine receptor stimulation is the activation of G-protein–sensitive PLC isoforms, resulting in the generation of DAG and inositol 3,4,5-triphosphate, which leads to the release of Ca^{2+} from intracellular stores. Hence, we analyzed chemokine-induced Ca^{2+} mobilization in CCRL2A and B transfectants. CCRL2B/L1.2 did not flux in response to all chemokines tested (CCL4, CCL3, CCL1, CCL2, CCL16, CCL15, CCL5 and CCL13 as shown in Figure 4.10). Moreover Ca^{2+} mobilization experiments were negative using other CC chemokines (CCL7, CCL11, CCL17, CCL18, CCL19, CCL22, CCL25; data not shown). As shown in Figure 4.10 CCRL2B transfectants exhibited a small flux in response to CCL2 and CCL5 that were due to endogenously expressed murine CCR2 and CCR5 (246, 247). In fact the same calcium fluxes were detectable in untransfected cells (data not shown). As shown in Figure 4.10A, CXCL12 was able to induce a strong calcium flux due to the endogenous murine CXCR4 expression.

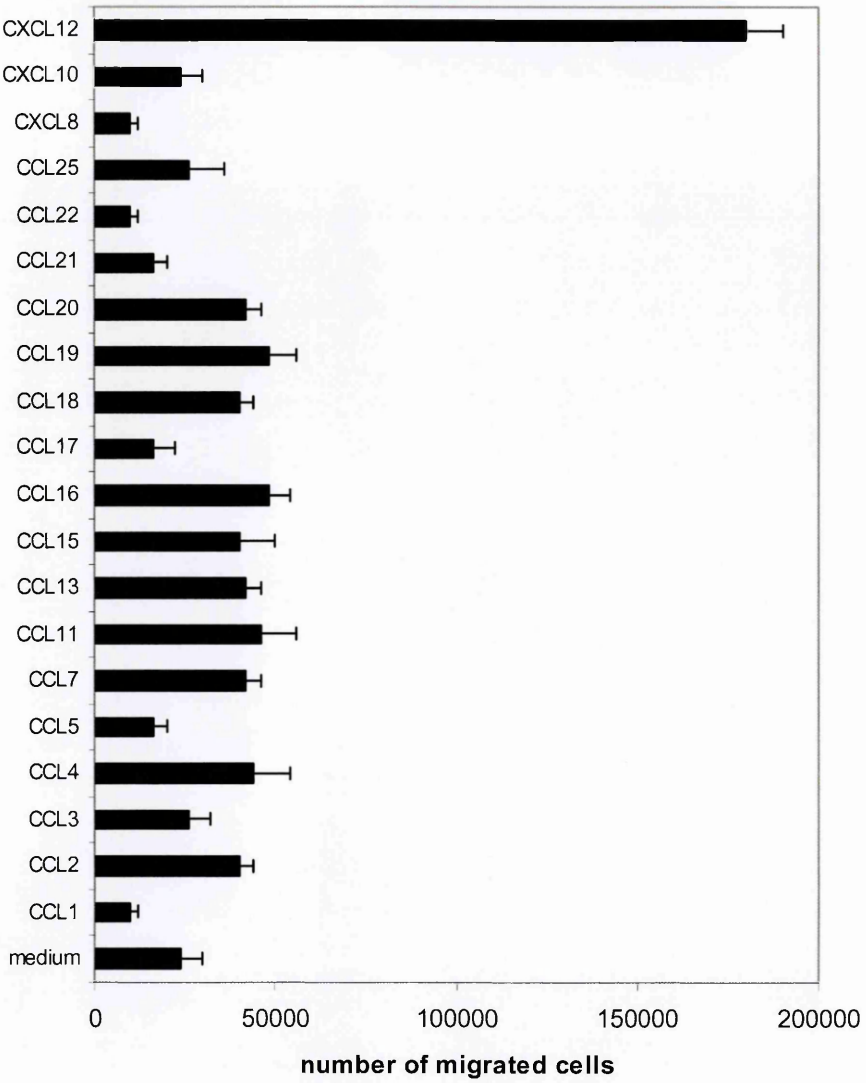

Figure 4.9 Chemotaxis assays with L1.2 cells stably transfected with CCRL2B.

Cells were stimulated in separate experiments with the different chemokines at the concentration of 10 nM. The graph represents the number of cells that migrated with the corresponding SD values.

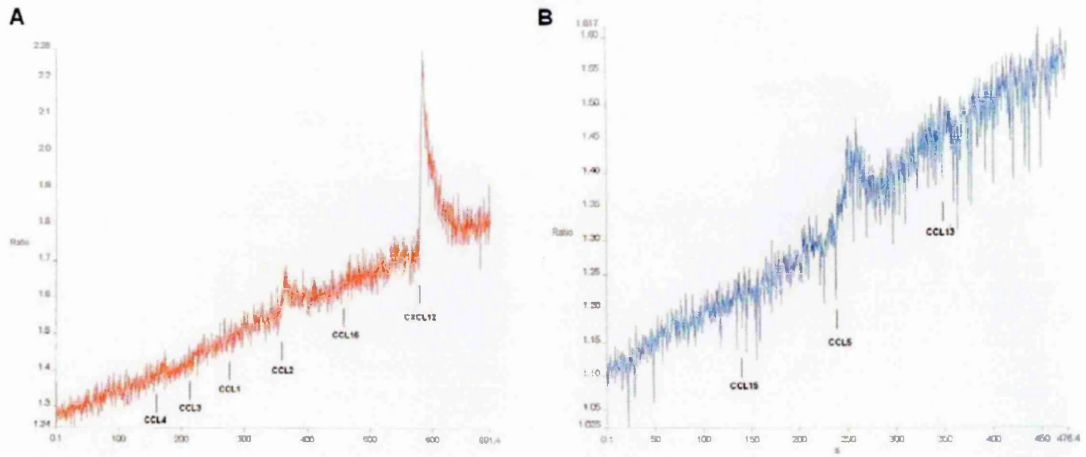

Figure 4.10 Intracellular calcium measurements in L1.2 cells stably transfected with CCRL2B.

Cells were stimulated with 100nM recombinant human CCL4, CCL3, CCL1, CCL2, CCL16 and CXCL12 (**A**) and with CCL15, CCL5 and CCL13 (**B**). The arrows indicate the moment of stimulus. The experiments were repeated at least 2 times. Note the different scale of y axis.

4.4 Discussion

Alternative splicing produces two CCRL2 mRNA variants, predicted to encode two different receptors CCRL2A and CCRL2B. CCRL2A is predicted to contain 12 additional amino acids at its N terminus as compared with CCRL2B. Alternative splicing is well described as a mechanism of generating diversity among G protein-coupled receptors, resulting in changes in the C termini and intracellular loops as well as truncated receptors, sometimes resulting in changes in receptor activities (reviewed in Ref. (248)).Although the coding regions of chemokine receptor genes are often found on single exons, there are now examples of exceptions to this rule, including, the genes for CCR2 (249) and mCXCR4 (250). Alternative splicing produces two forms of human CCR2, CCR2A and CCR2B, which differ in their C-terminal regions (249), and two forms of mouse CXCR4, which differ by 2 aa within the N-terminal region (250).

With regard to activities for chemokine receptor variants arising from alternative splicing, functional studies have revealed differences in the responses to ligands between the two forms of CCR9 (243). The splicing that gives origin to the two forms of CCR9, CCR9A and CCR9B, is identical to that of CCRL2. Functional studies have revealed that CCR9A is the more efficient receptor in terms of calcium fluxes, chemotaxis and binding affinity

Starting from this observation, in order to identify potential ligands we cloned both receptor variants. CCRL2A and B were cloned and expressed in CHO and L1.2 cell lines and stable clones were generated. In order to maximize CCRL2 expression cells were treated overnight with the histones deacetylases inhibitor sodium butyrate, probably because this treatment caused relaxation of the chromatin structure facilitating the accessibility of a variety of factors to DNA (244). Moreover cells had to be cultured at low densities conditions because we observed a decrease of 3-fold in basal conditions

and of 2-fold after sodium butyrate treatment of CCRL2 surface expression after reaching confluence. This observation may suggest that CCRL2 expression is related to cell cycle. It was published that in primary cultures of human microvascular endothelial cells (HMVECs) CXCR3 expression is limited to the S/G2-M phase of their cell cycle (245) and that CXCR3 agonists block HMVEC proliferation explaining angiostatic effect of these molecules. So it will be interesting to study in primary cells if CCRL2 expression is related to cell cycle.

Having found the best conditions to use in order to maximize CCRL2 expression we performed screening assays using L1.2 transfectants. Chemotaxis and calcium flux experiments suggested that both CCRL2 splicing variants were not able to respond to most CC chemokines (fig 4.9). Our data are in contrast with previously published paper of Biber et al (142) that demonstrated that the murine homolog of CCRL2, named LCCR was a functional chemokine receptor for the chemokines CCL2, CCL5, CCL7, and CCL8, although the authors did not show evidence for a direct ligand-receptor interaction such as radioligand binding.

We are not able to explain such differences, our experiments were performed with a different cell line, L1.2, while Biber group used HEK293 cells. L1.2 is a mouse pre-B cell line frequently used to express chemokine receptors (251-253) because chemotaxis and calcium flux assays are easy to perform. Moreover this cell line expresses endogenously high level of CXCR4 used as positive control. We cannot exclude that this cell line does not contain the right G protein to transduce a signal different from chemotaxis after CCRL2 engagement, although Yoshimura et al. (139) describe that cells expressing either CCRL2 splice variants migrated in response to a fraction of rheumatoid arthritis synovial fluid, indicating that CCRL2 is a functional receptor.

Taken together our results do not address the question whether CCRL2 is a signalling receptor.

Conclusions from this chapter:

- CCRL2 exists in two spicing variants, CCRL2A and B, that differ for their N terminal domain
- Transfectants for both splicing variants express the receptor on membrane
- Levels of CCRL2A and B expression positively correlate with low density conditions of culture
- CCRL2A and B/L1.2 transfectants do not migrate or calcium flux in response to a broad panel of CC chemokines

5 Chapter 5. Comparison of CCRL2 and D6: is CCRL2 a second chemokine scavenger receptor?

5.1 Introduction

Although there are numerous examples of the identification of ligands for orphan GPCRs, there has been a decrease in the number of published orphan receptor/ligand pairings in the past two years. This suggests that traditional approaches for the identification of ligands for orphan GPCRs, largely based on the screening of putative GPCR ligands, will not be successful for the identification of ligands of the remaining 160 or so orphan GPCRs and alternative approaches are required. Indeed, this also raises the possibility that not all orphan GPCRs require a ligand, and some may play an alternative role in cell biology (241).

Referring to chemokine receptors, beside "classic" conventional signalling receptors, other chemokine binding molecules with high structural similarity to chemokine receptors have been described, namely the Duffy antigen receptor for chemokines (DARC) (112), D6 (106) and (105), and CCX CKR (123). In addition a chemoattractant receptor, C5L2, with similarity to C5aR andC3aR, has been cloned; it binds C5a and the desarginated forms of both C5a and C3a (102). These molecules are characterized by distinct patterns of tissue distribution and different ligand specificities, but they share the ability to bind chemokines with high affinity in the absence of any demonstrable signalling function, and therefore are now indicated as "silent" receptors. "Silent" receptors have been suggested to favour transfer of chemokines across endothelial barriers and/or to act as decoy receptors which dampen inflammatory reactions (95).

It is interesting to note that all "silent" chemokine receptors present alterations in the DRYLAR/IV motif in the second intracellular loop, which is critical for G protein

coupling and signalling functions in conventional receptors, and maybe such alteration may represent a first hallmark of decoy receptors. The analysis of the CCRL2 amino acid sequence reveals the presence of two main alterations: a substitution of a highly conserved aspartic acid residue to asparagine, in the second transmembrane domain (N82), a residue present in almost all seven transmembrane receptors (58). Moreover CCRL2 has an alteration of the sequence in the DRYLAIV motif of the third intracellular loop (QRYLVFL). Because of sequence similarities between CCRL2 and silent or decoy chemokine receptors, we decided to perform internalisation assays in both CHO-CCRL2 and CHO-D6 transfectants.

5.2 Aim of the chapter

This chapter investigated the ability of CCRL2 transfectants to scavenge CC chemokines. The screening was performed in parallel with D6 transfectants and allowed the identification of new ligands for this receptor. Subsequently internalization of both CCRL2 and D6 was studied. Finally murine D6 transfectants were functionally characterized.

5.3 Results

5.3.1 Analysis of CCRL2-dependent chemokine scavenging: comparison with D6

CCRL2B and D6/CHO-K1 transfectants were incubated for 3 h with 1.2 nM of various chemokines. At the end of the incubation, the chemokine concentration in the supernatant was measured by ELISA. As shown in Figure 5.1, no chemokine was scavenged by CCRL2B while among known D6 ligands, the inflammatory CC chemokines CCL5, CCL11 and CCL22, were scavenged with the highest efficiency (94.3 ± 0.2%, 92.5 ± 2.1% and 91.99± 1.2% of the initially seeded chemokine, respectively). CCL7, CCL4, CCL2, CCL3L1 and CCL17 were scavenged with intermediate efficiency (85.1 ± 0.5%, 83.9 ± 2.9%, 76.5 ± 2.2%, 76.0 ± 6.6% and 61.2±11.9% of the initially seeded chemokine, respectively). Interestingly, CCL3 and CCL3L1, which only differ for the presence of a serine or a proline residue in position 2, were scavenged with different efficacy (29.0 ± 4.1% and 76.0 ± 6.6% of the initially seeded chemokine, respectively), in agreement with previous results reporting that only the CCL3L1 variant is a high affinity D6 ligand (254). CCL1, which does not bind D6 (105), was the only inflammatory CC chemokine tested not scavenged by D6 (3.1 ± 1.4% of the initially seeded chemokine). None of the chemokines tested was scavenged by untransfected CHO-K1 cells. Unlike CC inflammatory chemokines, the homeostatic chemokines CCL19 and CCL20, agonists at CCR7 and CCR6 respectively, were not scavenged (8.2 ± 9.2% and 6.7 ± 12.5% of the initially seeded chemokine, respectively). As expected (105), the CXC chemokine CXCL8 was not scavenged by D6 transfectants (2.4 ± 7.3% of the initially seeded chemokine).

Thus, D6 scavenges with variable efficacy agonists of the inflammatory chemokine receptors CCR1 (CCL3, CCL3L1, CCL5, and CCL7), CCR2 (CCL2 and CCL7), CCR3

(CCL5 and CCL11), CCR4 (CCL17 and CCL22) and CCR5 (CCL3, CCL3L1, CCL4 and CCL5).

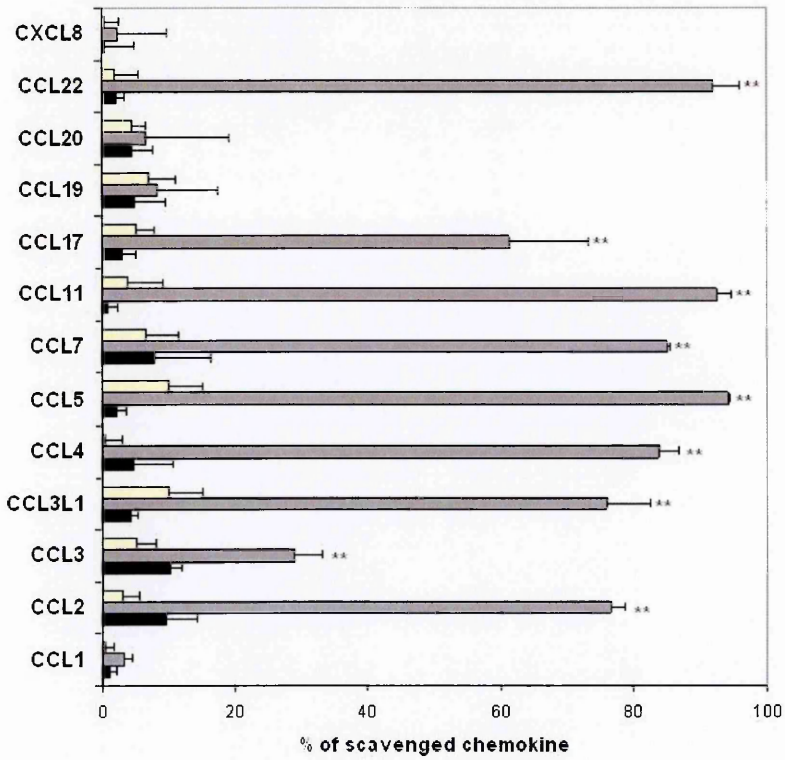

Figure 5.1 CCRL2B and D6-mediated chemokine scavenging.

Untransfected CHO-K1 cells (white bars) or CCRL2B/CHO-K1 cells (black bars) or D6/CHO-K1 cells (gray bars) were incubated for 3 h with 1.2 nM of different chemokines. At the end of the incubation, the chemokine concentration in the supernatants was measured by ELISA. Results are expressed as % of scavenged chemokine, mean ± SD, 3 replicates, at least 3 experiments. **, $p < 0.01$ compared to untransfected CHO-K1 cells.

5.3.2 Identification of new D6 ligands

Among inflammatory chemokines, the CCR4 agonists CCL22 and CCL17 were not previously investigated for their ability to interact with D6 (105). As shown in Figure 5.1, both chemokines were efficiently scavenged by D6. Figure 5.2 shows that CCL17 was less efficiently removed than CCL22 by D6/CHO-K1 transfectants at short time points (69.7 ± 3.0 % and 86.4 ± 1.3% of the initially seeded chemokine at 1 h for CCL17 and CCL22, respectively), while at longer times of incubation, the two CCR4 agonists were scavenged with comparable efficacy (81.8 ± 2.6 % and 89.6 ± 2.6% of the initially seeded chemokine at 6 h, respectively). CCL17 and CCL22 scavenging was also analyzed in D6/L1.2 and D6/MELC-2 transfectants, with similar results (data not shown).

Since ELISA may detect partially degraded forms of chemokines, we tested the effective biological inactivation of CCL22 by analyzing the chemotactic activity of CCL22 preincubated with untransfected CHO-K1 or D6/CHO-K1 transfectants on CCR4/L1.2 transfectants (Figure 5.3A). After 3 h preincubation with untransfected CHO-K1 cells, the chemotactic activity of CCL22 was unaffected, whereas incubation with D6/CHO-K1 transfectants drastically reduced (> 100 times) the CCL22-mediated chemotactic activity, in agreement with ELISA results.

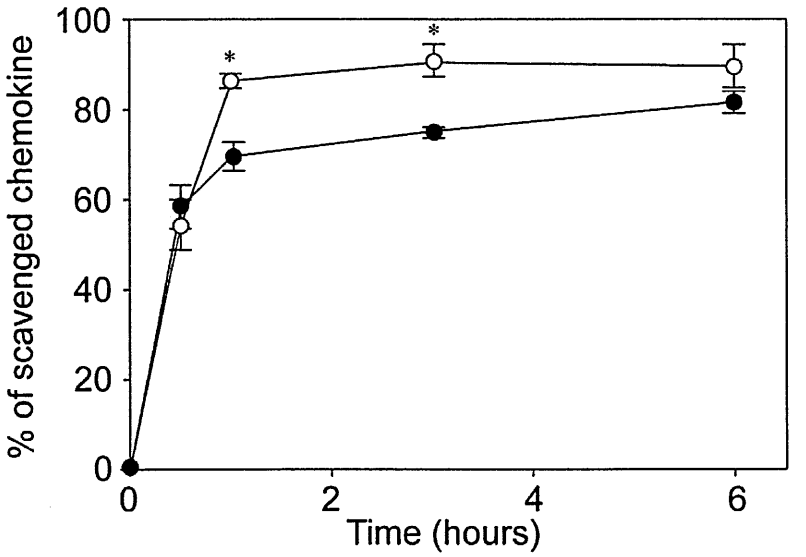

Figure 5.2 Kinetics of D6-mediated CCL17 and CCL22 scavenging

D6/CHO-K1 cells were incubated with 1.2 nM of CCL17 (•) or CCL22 (○) at 37°C for the indicated periods. At the end of the incubation, the chemokine concentration in the supernatants was measured by ELISA. Results are expressed as % of scavenged chemokine, mean ± SD, 3 replicates, at least 3 experiments. *, $p < 0.05$ compared to CCL17 scavenging.

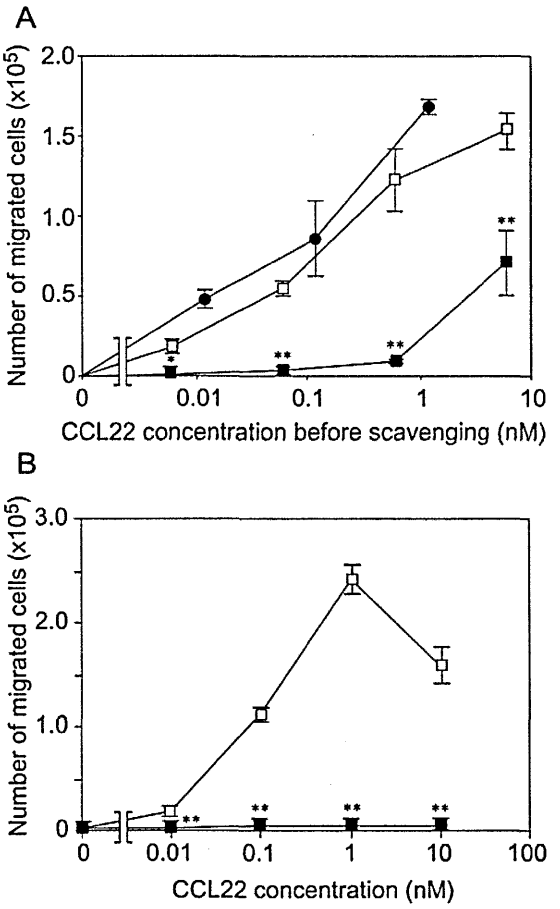

Figure 5.3 D6-mediated inactivation of CCL22 chemotactic activity

(A) Migration of CCR4/L1.2 cells in response to increasing concentrations of CCL22 (●) or CCL22 preincubated with parental CHO-K1 (□) or D6/CHO-K1 cells (■). *, $p < 0.05$; **, $p < 0.01$, compared to CCL22 activity after preincubation with untransfected cells. (B) Migration of CCR4/L1.2 (□) and CCR4-D6/L1.2 cells (■) in response to increasing concentrations of CCL22. Figures show a representative experiment of three performed with similar results. Values are the number of migrated cells (mean ± SD). **, $p < 0.01$ compared to CCR4/L1.2 cells.

In order to define the functional outcome of coexpression of CCR4 and D6 in the same cell context, L1.2 transfectants stably co-expressing both CCR4 and D6 (CCR4-D6/L1.2) have been generated. Flow cytometry analysis showed comparable expression of the two receptors (data not shown). When tested in chemotaxis assay, CCR4-D6/L1.2 cells completely lost ability to migrate in response to CCL22 (Figure 5.3B). On the contrary, cell migration in response to CXCL12, a chemokine not scavenged by D6 acting on the endogenous CXCR4, was unaffected by D6 coexpression (data not shown).

The interaction of CCR4 agonists with D6 was further investigated on D6/L1.2 transfectants in competition binding experiments with ^{125}I-CCL2 (Figure 5.4). D6 binds CCL22 more strongly (K_i = 0.33 nM), similarly to CCL2, while it binds CCL17 more weakly (K_i = 2.9 nM), similarly to CCL4. Similar results were obtained using ^{125}I-CCL22 (data not shown). As expected, CCL19 did not bind to D6. Since for some G-protein-coupled receptors the apparent affinity of ligands can vary depending on the tracer, competition binding experiments have also been performed using ^{125}I-CCL4, with similar results (data not shown).

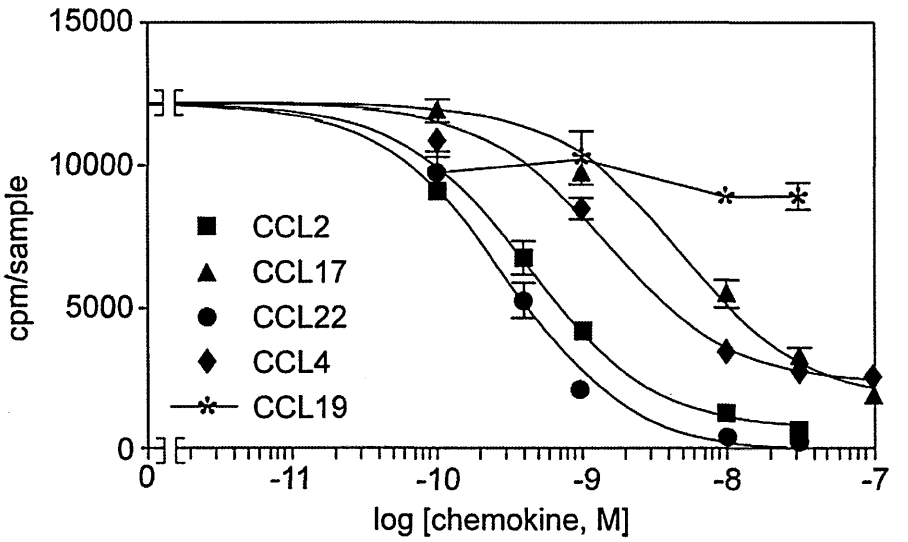

Figure 5.4 Binding of CCL17 and CCL22 to D6

Competitive binding of ^{125}I-CCL2 (mean ± SD) to D6/L1.2 cells in the presence of different concentrations of unlabelled CCL2 (■), CCL4 (♦), CCL17 (▲), CCL19 (*) or CCL22 (●). Binding to untransfected L1.2 cells was 320±59 cpm/sample. The figure shows a representative experiment of at least three performed with similar results.

As for other D6 ligands, treatment of D6/L1.2 transfectants with either CCL17 and CCL22 was unable to elicit any detectable calcium flux (data not shown) or cell migration (Figure 5.5).

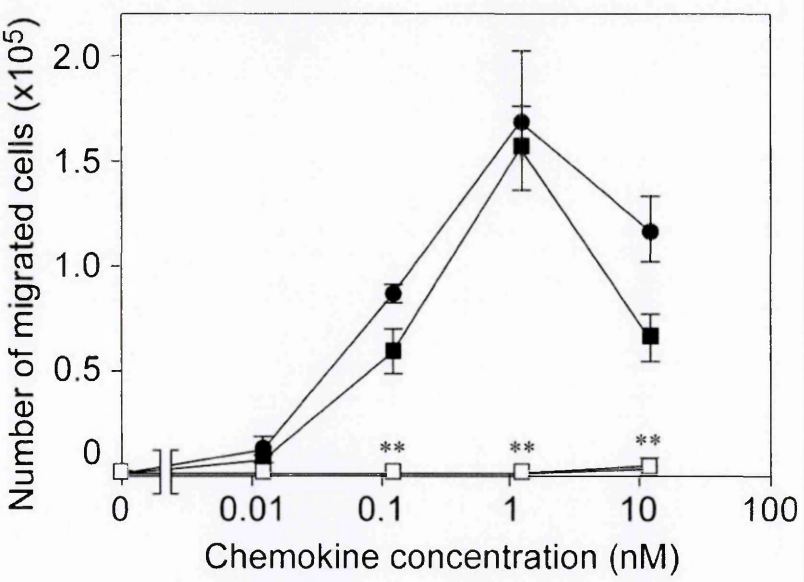

Figure 5.5 CCL17 and CCL22 chemotactic activity on CCR4 and D6 transfectants

Migration of CCR4/L1.2 (closed symbols) and D6/L1.2 (open symbols) in response to increasing concentrations of CCL17 (square) and CCL22 (circle). The figure shows a representative experiment of at least three performed with similar results. Values are the number of migrated cells (mean ± SD). **, $p < 0.01$ compared to CCR4/L1.2 cells migration.

CCL22 is processed by the dipeptidyl-peptidase IV (CD26) to produce the truncated forms CCL22 (3-69) and CCL22 (5-69), that lack, respectively, the first two and four amino acids at the NH_2 terminus and lose their agonist activity on CCR4 transfectants (255, 256). It was important to assess whether the promiscuous CC chemokine D6 receptor was able to interact with processed CCL22. As shown in Figure 5.6A, after three hours of incubation, D6 expressing cells scavenged unprocessed CCL22 (94.6 ± 1.8% of the initially seeded chemokine), but not the processed variants CCL22 (3-69) and CCL22 (5-69) (1.3 ± 3.6% and 3.5 ± 12.0% of the initially seeded chemokines, respectively). In order to understand whether the lack of scavenging of the NH_2-truncated molecules was due to their lost ability to interact with D6, competition binding analysis on D6/L1.2 transfectants using ^{125}I-CCL2 as tracer were performed. As shown in Figure 5.6B, CCL22 (3-69) and CCL22 (5-69), unlike native CCL22, were unable to displace ^{125}I-CCL2, indicating that both CCL22 processed forms were not recognized by D6.

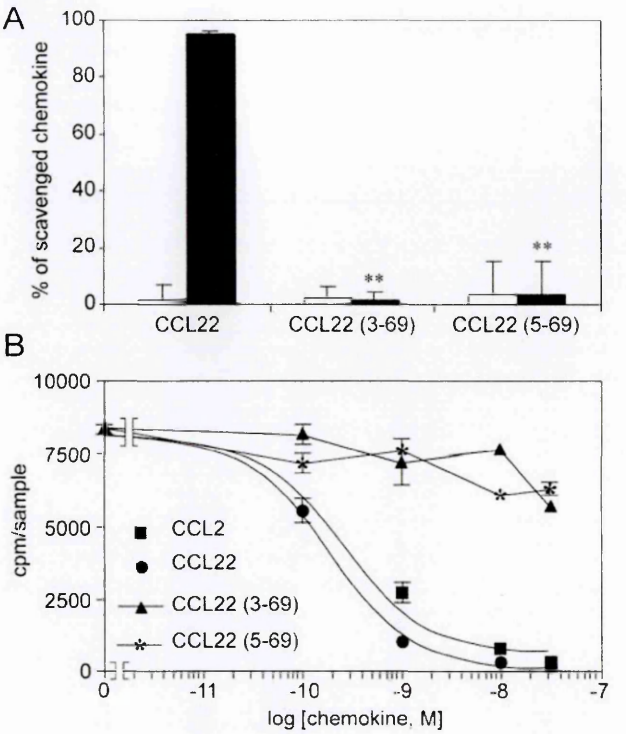

Figure 5.6 Differential binding and scavenging of native and truncated CCL22 by D6.

(A) D6-mediated scavenging of CCL22, CCL22 (3-69) and CCL22 (5-69). Untransfected (white bars) or D6-transfected CHO-K1 cells (black bars) were incubated for 3 h with 1.2 nM of CCL22, CCL22 (3-69) or CCL22 (5-69). Results (mean ± SD) are the percentage of scavenged chemokine as assessed by ELISA. The figure shows a representative experiment of at least three performed with similar results. **, $p < 0.01$ compared to CCL22 scavenging. (B) Competitive binding of ^{125}I-CCL2 (mean ± SD) to D6/L1.2 cells in the presence of different concentrations of unlabelled CCL2 (■), CCL22 (●), CCL22 (3-69) (▲) or CCL22 (5-69) (*). The figure shows a representative experiment of at least three performed with similar results.

5.3.3 Analysis of CCRL2 internalization: comparison with constitutive and ligand independent internalization of D6

To determine whether ligand binding would induce D6 internalization, D6/L1.2 and CCR4/L1.2 cells were labelled with the appropriate receptor-specific monoclonal antibody at 4°C, incubated at 37°C in the presence or absence of the ligand (60 nM CCL22) for indicated time periods, and labelled with appropriate secondary antibody. Receptor expression levels were analyzed by flow cytometry as described in methods. As shown in Figure 5.7, CCL22 induced a significant and rapid decrease of cell surface CCR4 expression levels, in agreement with previous reports (257). On the contrary, a significant fraction of D6 receptors underwent internalization both in the presence and in the absence of the ligand, and the treatment with the ligand (60 nM CCL22) was unable to induce any further receptor internalization. Similar results were obtained using an anti-HA monoclonal antibody, recognizing an HA tag inserted at the N-terminus of D6 and CCR4 (data not shown).

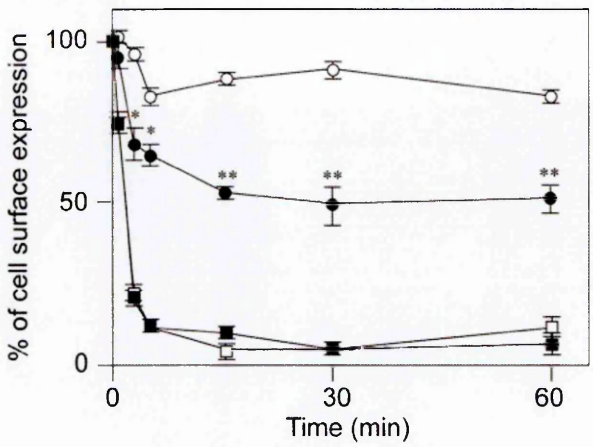

Figure 5.7 D6 and CCR4 internalization

D6 internalization. D6/L1.2 (square) and CCR4/L1.2 (circles) cells were firstly labelled with primary antibody at 4°C for 1h and then incubated in medium (open symbols) or medium containing 60 nM CCL22 (closed symbols) for the indicated time periods at 37°C. Cells were cooled on ice, washed with cold medium, and then labelled with secondary antibody at 4°C to determine the cell surface receptor levels. *, $p < 0.05$; **, $p < 0.01$ compared to CCR4/L1.2 cells incubated with medium alone.

GPCR recycling may be determined by measuring surface reappearance at various times after inducing internalization by agonist. To determine D6 surface expression following internalization, D6/L1.2 cells were labeled with anti-D6 monoclonal antibodies either only before or before and after incubation at 37 °C for the indicated time periods, and the surface expression of D6 was analyzed. As above, cells stained only before incubation displayed a significant rate of receptor internalization (Figure 5.8). Labeling of the cells with anti-D6 monoclonal antibodies after the internalization step revealed that a significant fraction of internalized receptors was replaced on the cell membrane. Taken together, these results indicate that D6 behaves as a constitutively active receptor, undergoing rapid and ligand-independent internalization and re-expression on

membrane. Similar experiments were performed with CCRL2A and B transfectants, in order to understand if also these receptors are constitutively internalized. As shown in Figure 5.8 CCRL2B trasfectants, differently from D6 did not undergo significant internalization and similar stainings were obtained labeling cells with anti-CCRL2 monoclonal antibodies either only before or before and after incubation at 37 °C

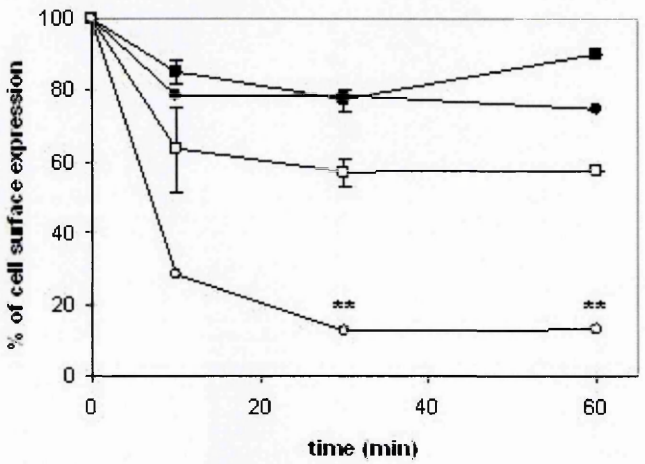

Figure 5.8 CCRL2 and D6 internalization

D6/L1.2 and CCRL2B cells were stained with anti-D6 or anti-CCRL2 primary antibody and incubated at 37 °C for 1 hour. After transfer at 4 °C, cells were stained again (restaining) or not with the primary antibody and then with the secondary antibody. The symbols are as follows: anti-D6, no restaining (○), anti-D6, restaining (●), anti-CCRL2, no restaining (□), anti-CCRL2 restaining (■). Data are representative of at least three independent experiments. **, $p < 0.01$ compared to CCR4/L1.2 cells incubated with medium alone.

5.3.4 Cloning and characterization of murine D6

Previous studies reporting lack of signaling and decoy activity of D6 were focused on the human molecule (105) (108). It was therefore important to assess whether mouse D6 shares these key properties with its human orthologue. For this reason murine D6 was cloned in pcDNA3 expression vector and then expressed in L1.2 and CHO cell lines. Similarly to the human counterpart, mouse D6 behaves as a silent receptor, in terms of calcium fluxes and chemotaxis (Figure 5.9A, and data not shown), and as a promiscuous, efficient scavenger of inflammatory CC chemokines with the expected ligand selectivity (Figure 5.9B).

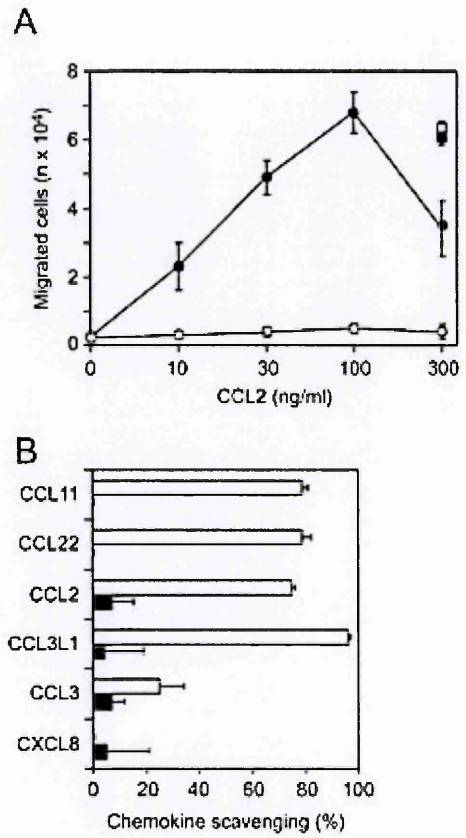

Figure 5.9 Functional analysis of murine D6.

(A) Chemotactic activity. Migration of mD6/L1.2 (open symbols) and mCCR2/L1.2 (closed symbols) transfectants in response to CCL2 (circles) and to the homeostatic chemokine CXCL12 (squares), which is not recognized by D6 but acts via the endogenously expressed receptor CXCR4, was evaluated on 5-μm pore-size Transwell filters (Corning, New York, USA). Migrated cells were counted on a Burker chamber. Results are means±SD of triplicate samples of one experiment that is representative of three performed. (B) Chemokine scavenging. Either mD6/CHO-K1 (open bars) or untransfected CHO-K1 (closed bars) cells were incubated at 37°C for 5h with the indicated chemokines. Results are means±SD of triplicate samples of one experiment that is representative of three performed.

5.4 Discussion

Results presented here indicate that both CCRL2 variants were not able to internalize/scavenge a broad panel of CC chemokines. In contrast, the same screening analysis on D6 transfectants confirmed and extended previous observations on the promiscuous binding (105) and scavenging (108) of CC chemokines by this receptor. Although chemokine classification in homeostatic and inflammatory is not absolute (258, 259), it was found that D6 only recognizes and scavenges inflammatory chemokines, including CCR1 through CCR5 agonists.

Conversely D6 did not interact with CC chemokines such as CCL19 (CCR7 ligand) and CCL20 (CCR6 ligand), usually behaving as homeostatic chemokines. Thus, the spectrum of ligands recognized by D6 contrasts with that of CCX-CKR, which binds the homeostatic chemokines CCL19, CCL21 and CCL25 but not inflammatory chemokines (123). It is at the moment unclear whether CCX-CKR internalizes and scavenges homeostatic CC chemokines, as D6 does for the inflammatory ones (226).

In addition to already known ligands, the results presented here indicate that the spectrum of chemokines recognized by D6 also includes the CCR4 agonists CCL22 and CCL17. CCL22 and CCL17 are constitutively expressed in lymphoid organs, in particular in the thymus, spleen, lymph nodes and to a lesser extent in the gut (260). Immature myeloid DC constitutively express low levels of CCL22 (261). However, as inducible chemokines, CCL22 and CCL17 are part of regulatory circuits of polarized Th1 and Th2 responses. IL-4 and IL-13 induce CCL22 production, whereas IFN-γ inhibits it (185). Moreover, inflammatory signals (e.g. LPS) augment CCL22 production (262, 263). Hence, CCL22 and CCL17 belong to both realms of homeostatic and inflammatory chemokines, and their recognition by D6 is therefore consistent with the general preferential interaction of this decoy receptor with inflammatory proteins.

DC express CCR4 and respond to CCR4 agonists. CCL22 has been suggested to play a role in the trafficking of epidermal Langerhans cells at the inflammatory site (264) and in the formation of T cell-DC clusters in both inflamed skin and lymph nodes (265, 266). D6 is strategically located on endothelial cells lining afferent lymphatics (107) and has been suggested to act as a gatekeeper to prevent excessive transfer of inflammatory chemokines to lymph nodes. By recognizing CCR4 agonists, D6 may regulate DC migration to lymph nodes via afferent lymphatics.

CCL22 is processed by dipeptidyl peptidase IV (CD26) to produce the processed variants CCL22 (3-69) and (5-69), which loose the capacity to interact with CCR4 (255, 256). Dipeptidyl peptidase IV is widely expressed in cells and tissues and is more abundant in Th1 compared to Th2 cells (255, 260). Interestingly, processed CCL22 forms are not recognized by the promiscuous receptor D6. The selective recognition of CCL22 versus CCL22 (3-69) and CCL22 (5-69) may represent a strategy to focus the decoy function on the CCR4 agonists, without interference from inactive processed forms.

Results presented here also demonstrated that the chemokine receptor D6, differently from CCR4, is constitutively internalized in a ligand independent way. Ligand binding of a typical GPCR results in a series of events including G-protein activation, receptor phosphorylation, desensitization, b-arrestin association, sequestration, and/or internalization. D6 might be in a constitutively active conformation but unable to transduce G-protein-mediated signals due to specific mutations in regions of the receptor essential for G-protein as an asparagine in place of an aspartic acid in the second transmembrane domain and a change in the canonical DRYLAIV motif in the second cytoplasmic loop to DKYLEIV in D6. Both regions were shown to be important in G protein-dependent signaling in chemokine receptors (95). It is also possible that

multiple mutations may have occurred to generate D6 as a non-signaling receptor. Indeed, two other chemokine receptors, US28, a cytomegalovirus-encoded chemokine receptor with broad ligand specificity (103), which has also been proposed as a chemokine scavenger receptor, and a constitutively active mutant of CXCR4 (CXCR4-CAM) are known to undergo constitutive internalization (267, 268). However, unlike D6, these receptors transduce G-protein-mediated signals. A second possible mechanism is that the cytoplasmic tail of D6 contained a structural element allowing it to constitutively internalise.

Analysis of CCRL2 internalization with antibody feeding experiments, showed very little if any, constitutive internalization of this receptor (Figure 5.9). Collectively from results presented in this chapter we can conclude that CCRL2, despite structural similarities with chemokine silent or decoy receptors, does not scavenge any CC chemokine tested and does not constitutively internalise in a ligand-independent way.

Conclusions from this chapter:

- CCRL2 does not mediate CC chemokine scavenging
- D6 scavenges CC chemokines that bind receptors from CCR1 to CCR5
- D6 mediates inactivation of CCL17 and CCL22 chemotactic activity
- CCL17 and CCL22 bind D6 with high affinity but do not induce chemotactic activity
- Native and truncated CCL22 are differentially bound and scavenged by D6
- D6 but not CCRL2 is constitutively internalised in a ligand independent way and rapidly replaced on cell surface

6 Chapter 6 Summary and future plans

6.1 Summary

6.1.1 The elusive orphan receptor CCRL2

Chemokines and their receptors have important roles in directing leukocyte traffic but also in several other functions from haematopoiesis to cancer metastasis. In 1998 Fan et al. (134) cloned an orphan seven transmembrane receptor with high homology and several characteristics common to chemokine receptors, such as peptide length, positioning of 4 extracellular cysteines and genomic localization in a chemokine receptor cluster.

For all these reasons we thought it important to try to deorphanize it. First we studied the sites of the orphan GPCR expression as a primary indication of its biological role. In human tissues CCRL2 is expressed at high levels in lung and lymphoid tissues. Among leukocytes CCRL2 is expressed mainly by cells of the myelomonocytic lineage, PMN and monocytes. Immature monocyte-derived DC express low levels of CCRL2 but maturative stimuli like LPS and CD40L strongly upregulated its expression in a transient way, with maximal expression at 2 hours and return to basal levels at 24h of stimulation. Another interesting aspect of CCRL2 expression in maturing DC is the downregulation by PGE2, an arachidonic acid metabolite that promotes the migration of mature human monocyte-derived DC to the CCR7 ligands CCL19 and CCL21. Several aspect of DC migration are still not clear, in particular which molecules determine DC entry to lymphatic vessels. Being CCRL2 expressed by maturing DC and regulated by PGE2 is likely to be involved in this process.

In order to identify CCRL2 ligands we have tried different approaches. CCRL2 exists in two splicing variants differing for the presence of 12 aminoacid in the N terminal region. Because this part of the receptor plays an important function in ligand binding

we decided to clone and transfect both variants. First we performed functional assays as chemotaxis and calcium fluxes focusing our attention on CC chemokines because phylogenetic analysis strongly suggests that the CCRL2 putative ligand might be a chemokine of this family, but our effort was unsuccessful.

CCRL2 displays some characteristics typical of chemokine decoy receptors like a variation of the DRYLAIV motif in the second intracellular loop and an N/D substitution in the second transmembrane domain. Following the hypothesis that CCRL2 might be a decoy or silent chemokine decoy receptor we performed scavenging experiments with most of the CC chemokines but again unsuccessfully.

Finally we tried to understand if CCRL2 is constitutively internalized, a characteristic shared by chemokine decoy receptors. In contrast to D6 that is constitutively internalized in absence of ligands, CCRL2 is expressed on the cell membrane and is not internalized.

The expression profile of CCRL2 suggests that this receptor might have an important role in DC biology in particular in the migration from the periphery to lymph nodes. Despite the use of different approaches we have not been able to find a ligand for this receptor. We can assume that both CCRL2 variants, despite structural homologies with CC-receptors, are not able to induce migration and calcium fluxes to all CC chemokines tested. Moreover our analysis suggests that although CCRL2 shares structural characteristics with chemokine decoy receptors, it does not behave like a member of this family in terms of chemokine scavenging and constitutive internalization.

6.1.2 The inflammatory chemokine decoy receptor D6

We have identified two new ligands for D6 that are the CCR4 agonists CCL22 and CCL17. These chemokines are not able to induce migration of D6 expressing cells, but are rapidly scavenged by D6 and double transfectants CCR4/D6 lose the ability to

migrate to both chemokines. Moreover we have found that D6 is not able to bind and scavenge both truncated versions of CCL22 named CCL22 (3–69) and CCL22 (5–69). These two proteins do not interact with CCR4 and have lost their ability to induce recruitment of Th2 cells. So our data suggest that D6 is able to scavenge inflammatory chemokine ligands from CCR1 to CCR5 but is a blind receptor not scavenging inactive forms of truncated chemokines.

Further work was done on the ability of D6 to constitutively internalize. D6 membrane levels, differently from CCR4, do not decrease following chemokine stimulation because the receptor is constitutively cycling in a ligand-independent manner. This behaviour is similar to that previously described for the human CMV-encoded chemokine receptor US28, which has also been proposed as a chemokine scavenger receptor.

6.2 Future plans

There are a number of questions arising from this thesis that should be answered by further work. The following sections will outline some of these issues.

6.2.1 Does CCRL2 form heterodimers with other chemokine receptors?

One hypothesis not investigated about the role of CCRL2 in chemokine biology is heterodimerization. There is now extensive literature describing the phenomena of GPCR dimerization (61). A number of techniques have been developed to demonstrate that GPCRs are capable of forming both heterodimers and homodimers. The functional relevance of this remains unclear and is the subject of intense speculation. It may be that GPCR heterodimers or homodimers are required for the processing or trafficking of the receptor. A well-characterized example of this is the GABA-B-R2 receptor, which appears to function as a trafficking protein to deliver the functional GABA-B-R1

receptor to the cell surface (269). The prominent current hypothesis is indeed that GPCRs assemble as dimers shortly after synthesis in the endoplasmic reticulum, and traffic as such throughout their life in the cell.

Chemokine receptors make no exception to this new rule and both homo- and heterodimerization were demonstrated for CC and CXC receptors. Oligomerization was reported for four chemokine receptors so far: CCR2, CCR5, CXCR2 and CXCR4 (61). Co-immunoprecipitation, BRET and FRET experiments have shown unambiguously that CCR2 and CCR5 are able to form both homo- and heterodimers. Functional analyses demonstrated negative binding cooperativity between the two subunits of a dimer. The consequence is that only one chemokine can bind with high affinity onto a receptor dimer.

In addition to their ability to form homodimers, or to associate with close structural relatives, chemokine receptors were also reported to form oligomers with receptors belonging to other families like AMPA glutamate receptors and opioid receptors but the exact molecular mechanisms and physiological consequences of cross-families dimerization events remain to be elucidated.

Although there is little published evidence for this, it remains possible that some orphan GPCRs may act as accessory proteins, which in combination with other orphan or liganded GPCRs, may modulate responses to known ligands or confer new ligand binding characteristics. This issue can be best addressed by taking a proteomics approach. CCRL2 could be epitope tagged, transfected into cell lines, and immunoprecipitation experiments to isolate receptor complexes performed. Alternatively, receptor combinations could be studied in mammalian cell lines using techniques such as bioluminescence resonance energy transfer (BRET) or time-resolved fluorescence resonance energy transfer (TR-FRET).

6.2.2 Does CCRL2 activate G-protein independent pathways?

It has been assumed that all signaling events mediated by GPCRs occur as a consequence of G-protein activation. As a consequence, all orphan GPCR ligand screening experiments have relied upon the detection of the ability of candidate ligands to activate one of the classical heterotrimeric G-protein signaling pathways. There are now a number of examples of GPCRs that activate signal transduction pathways through G-protein-dependent and -independent signaling pathways. Perhaps the first example of this phenomenon was the demonstration that in addition to classical G-protein-mediated signaling, the C-terminal tails of the β_2-adrenoceptor and the P2Y1 purinergic receptor interact with the Na^+/H^+ exchanger regulatory factor (NHERF) to directly regulate Na^+/H^+ exchange (270). Further examples of G-protein-independent signaling include the coupling of metabotropic glutamate receptors directly to intracellular calcium stores through homer proteins and GPCR activation of phospholipase D as a consequence of activation of the small G-proteins Arf and RhoA (271). This raises the possibility that there may be examples of the GPCR family that do not require G-proteins for signaling and that some of the remaining orphan GPCRs also signal through G-protein-independent mechanisms. If so, novel screening approaches will have to be established in order to identify activating ligands.

6.2.3 Role of D6 in DC migration

D6 expressed by lymphatic endothelial cells (LECs) might block the cellular responses to inflammatory chemokines and might enable the cells to leave tissues through receptors such as CCR7. This would facilitate the movement of antigen-presenting cells and lymphocytes from inflamed tissues to the draining LNs by desensitizing responses to inflammatory but not constitutive chemokines like CCL21 that promote lymphatic entry of maturing DC that express CCR7 and preventing recruitment of immature DC.

Moreover DC express the chemokine receptor CCR4 and respond to CCR4 agonists. CCL22 has been suggested to play a role in the trafficking of epidermal Langerhans cells at the inflammatory site (264) and in the formation of T cell-DC clusters in both inflamed skin and lymph nodes (265, 266).

In vivo approaches with D6 $^{-/-}$ mice will be necessary to test the hypotesis that D6 recognizing the majority of inflammatory CC chemokines may be important in the regulation of DC migration to lymph nodes via afferent lymphatics.

6.2.4 Is D6 expressed by leukocytes and is it able to dimerize with other chemokine receptors?

It will be necessary to further characterize D6 expression in leukocytes because its co-expression with signalling chemokine receptors might be important for halting migrating cells following their arrival at an inflammatory site. This would enable leukocytes to accumulate at centres of chemokine production, and enable them to perform their functions efficiently, clear infectious agents, release anti-microbial compounds, further induce the inflammatory response, or promote resolution and healing. For dendritic cells and other antigen-presenting cells, pausing at inflammation sites could also enable more-complete exposure to the full range of antigens that can be phagocytosed and processed, ready for presentation to lymphocytes.

Data presented in this thesis suggest that D6 might also be able to function in a cell-autonomous manner to limit responses to chemokines. As shown in chapter 5 cells coexpressing CCR4 and D6 completely lost ability to migrate in response to CCL22. On the contrary, cell migration in response to CXCL12, a chemokine not scavenged by D6, acting on the endogenous CXCR4, was unaffected by D6 coexpression.

Future experiments have to be performed in order to understand if D6 forms dimers with CCR4 and if this is the mechanism responsible for the abolished functional response to CCR4 ligands in double transfectants.

7 Publications

Parts of this thesis have been published; reprints are enclosed at the back.

Published work

1: Locati M, Torre YM, Galliera E, Bonecchi R, Bodduluri H, Vago G, Vecchi A, Mantovani A. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. Cytokine Growth Factor Rev. 2005;16:679-86.

2: Martinez de la Torre Y, Locati M, Buracchi C, Dupor J, Cook DN, Bonecchi R, Nebuloni M, Rukavina D, Vago L, Vecchi A, Lira SA, Mantovani A. Increased inflammation in mice deficient for the chemokine decoy receptor D6. Eur J Immunol. 2005 May;35(5):1342-6.

3: Galliera E, Jala VR, Trent JO, Bonecchi R, Signorelli P, Lefkowitz RJ, Mantovani A, Locati M, Haribabu B. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. J Biol Chem. 2004;279(24):25590-7.

4: Bonecchi R, Locati M, Galliera E, Vulcano M, Sironi M, Fra AM, Gobbi M, Vecchi A, Sozzani S, Haribabu B, Van Damme J, Mantovani A. Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. J Immunol. 2004;172:4972-6.

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