UNIVERSITÀ DEGLI STUDI DI MILANO

SCIENZE BIOMEDICHE CLINICHE E SPERIMENTALI

DIPARTIMENTO DI MEDICINA TRASLAZIONALE

CORSO DI DOTTORATO

PATOLOGIA E NEUROPATOLOGIA SPERIMENTALE

CICLO XXIII

CCRL2, A PUTATIVE CHEMOTACTIC RECEPTOR, PARTICIPATES IN THE LUNG DENDRITIC CELL TRAFFICKING

Settore scientifico disciplinare: MED/04

Dottorando: Safiyé Gonzalvo Feo

Tutore: Prof. Antonio Sica

Coordinatore del dottorato: Prof. Alberto Mantovani

A.A. 2009-2010

A mis brujas del más acá y el más allá

Acknowledgments

I thank all those who have positively influenced my life in these five years. Even to those who have welcomed me with a magical “Buongiorno” on a typical cloudy day in Milan.

A special thanks to Annunciata and Annalisa, who taught me everything wholeheartedly with much patience. Thanks for always being present not only at work. I thank Silvano for showing me that an iron hand can help (not always) to improve oneself. Thanks also for accepting me in your group, not only for the professional opportunity... Finally, I have to thank my ex-boss, Macias, I’ll never forget his proof of friendship in critical moments between the past and the present.

Of course, (and fortunately!) I don’t have to say: Thank you, only to “I capi”. I have a very special Thanks for the 3 “streghette”: Nadia, Marina and Barbara. Strict and sweet at the same time, always maternal. To Garlanda’s group (new and old), the group that adopted me…. Really they had no choice, it was the result of a random spatial distribution… but it worked: Ragazzi, tout le plaisir a été pour moi. To my real group, “I ragazzi di Brescia” for always being available.

Thanks also to my roomates of Via Sardegna, 7, some of them are now more than roomates, and all of them have been great company in the long and cold Milan nights. To my friends, the old ones, who have always been close no matter how far. To my new friends who have made me understand that it is possible to find a home anywhere. Last but not least, I thank all my family members , including those I have just rediscovered here. A special thanks to my wonderful mum, who taught me that happiness and respect for others are sacred obligations, and of course, to “mi Juanito”.

Summary

CCRL2 (chemokine CC motif receptor-like 2) is an orphan heptahelic serpentine receptor that is rapidly induced in dendritic cells (DC) during maturation. CCRL2 shares the highest homology with the inflammatory chemokine receptors CCR1 and CCR5 and the ligand, chemerin, with the chemotactic receptor ChemR23. Although both CCRL2 and ChemR23 are expressed by DC, they show an opposite regulation during DC maturation. In addition, CCRL2 is apparently unable to signal in response to chemerin and was shown to function as a presenting protein of chemerin to ChemR23-expressing cells.

In order to evaluate the biological relevance of this receptor, we used CCRL2 deficient mice in an established model of allergen-induced airway inflammation in which DC are known to play a crucial role. CCRL2-/- mice were protected in a model of OVA-induced airway inflammation with reduced leukocyte recruitment in the BAL (eosinophils and mononuclear cells) and reduced production of the Th2 cytokines IL-4 and IL-5 and chemokines CCL11 and CCL17. CCRL2-/- mice showed normal recruitment of circulating DC into the lung but a defective trafficking of antigen-loaded lung DC to mediastinal lymph nodes. This defect was associated to a reduction in lymph node cellularity and reduced priming of Th2 response. Chemerin was expressed by mouse lung vascular endothelial cells and by lymphatic endothelial cells and was found to be upregulated by retinoic acid. Retinoic acid stimulated endothelial cells (EC) promoted the transmigration of DC in a ChemR23-dependent manner, suggesting a role for membrane associated chemerin.

The results here reported define chemerin as a new relevant protein for DC trafficking across lymphatic and blood endothelial barriers. In addition, these results highlight a nonredundant role for CCRL2 in the migration of lung DC and in the induction of Th2-polarized airway allergic inflammation. Altogether, this study proposes the CCRL2/chemerin axis as a new potential target for therapeutic strategies aimed at controlling lung hypersensitivity.

Table of Contents

1. Introduction

1.1. Cell migration

Migration represents a crucial cellular function contributing to many important physiological and pathological processes. It plays a fundamental role during embryogenesis, and defects in this process can result in pathological outcomes such as congenital neurological defects. In the adult, cell migration is a key player in the organization of an effective immune response, in the repair of injured tissues and in angiogenesis, to cite a few examples. Deregulation of migration can lead to many types of pathological disorders including cardiovascular illnesses, chronic inflammatory diseases and metastasis [1, 2]. An important aspect of cell motility is the ability of cells to respond to directional cues with oriented movement. Gradients of diffuse chemicals give rise to directional cell migration, a phenomenon known as chemotaxis [3, 4]. Continuous trafficking of cells of immune cells throughout the body requires not only locomotion and chemotaxis, but also the ability to move out of the circulation toward target tissues where they exert their effector functions.

1.1.1. Extravasation

The vascular and lymphatic circulatory systems are lined by monolayers of endothelial cells (EC) that grow on an ablumenal layer of extracellular matrix (the basement membrane); these cells form organized intercellular junctional zones that include adhesion junctions, tight junctions and gap junctions [5-7] In this way, the endothelium serves as the principal barrier between the circulation and the underlying tissues. Leukocytes have to cross these barriers to reach its target tissues by a process known as extravasation.

1.1.1.1. Leukocyte extravasation across vascular endothelium

An inflammatory response is initiated by the extravasation of leukocytes across the blood vessel wall. This process involves a multistep cascade of successive interactions of adhesion receptors between leukocytes and EC [8, 9].

Circulating leukocytes in the bloodstream have to establish a contact (tethering) with the vascular wall and adhere to it, while withstanding the haemodynamic shear force. Tethering is largely mediated by selectins, a type 1 transmembrane glycoproteins.

Although interaction of endothelial E and P-selectin (expressed on EC and activated by proinflammatory stimuli) with their corresponding ligands on the leukocytes (L-selectin, PSGL1 (P-Selectin glycoprotein ligand-1)) are of low affinity, the high frequency of association-dissociation of interactions allows them to mediate labile and transient tethers between leukocytes and the endothelium [10, 11]. Tethering slows down the velocity of leukocytes in the bloodstreams and allows them to roll over the endothelial surface.

The slow rolling leukocytes encounter chemokines bound to glycosaminoglycans on the apical endothelial surface. Chemokines act by signaling via the G protein coupled receptor (GPCR) located in the microvilli of the leukocyte, inducing multiple conformational changes in leukocyte integrins [12-15], a family of heterodimeric receptors that actively changes their adhesive properties, leading to firm adhesion to the endothelium [15, 16]. The most relevant integrins for leukocyte adhesion to the endothelium are LFA-1 (lymphocyte function-associated antigen 1), Mac-1 and VLA-4 (very late antigen 4), while its main ligands on EC are VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1), both members of the immunoglobulin superfamily [17, 18]. ICAM-1 is scarcely expressed on the quiescent endothelium, whereas the expression of both molecules is induced after cell activation by proinflammatory cytokines [19, 20]. The presence of specific chemokines in different vascular beds helps orchestrate the selective recruitment of different leukocyte subpopulations to the inflammatory foci or to the secondary lymphoid organs [21].

After activation the integrins regulate different effectors of myosin contractility, actin remodeling GTPases, and molecules implicated in the regulation of the microtubule network leading to a marked morphological change. The rounded circulating cells are transformed into cells with polarized morphology, in which at least two regions can be identified: the cell front and the cellular uropod [22]. The polarization of the leukocytes allows the cell to coordinate the intracellular forces to produce the necessary cell crawling during the extravasation process [23]. Lymphocytes undergo ICAM-1 dependent lateral migration on the luminal surface of the endothelium, a movement that seems to allow then to sek out sites that are permissive for diapedesis (passage through intact capillary walls) [24, 25]. Once the leukocytes have reached an appropriate site for transmigration (preferably the intercellular junctions), they deploy exploratory pseudopods between two adjacent EC. The pseudopods then transform into a lamella that moves across the open space of the monolayer. During this process the LFA-1

molecule has a predominant role. This molecule is quickly relocalized to form a ring-shaped cluster at the contact interface between the leukocyte and endothelium, where it interacts with ICAM-1 [26] or JAM-A (junctional adhesion molecule A) [27]. When the transmigration process is over LFA-1 is finally concentrated in the uropod [27]. Other proteins localized at EC contacts, implicated in the transmigration process are ICAM-2, JAM-B, JAM-C, PECAM-1 (platelet endothelial cell adhesion molecule-1), ESAM (endothelium selective adhesion molecule) and CD99, many of which are able to interact both homophilically and heterophilically maintaining the interendothelial junctions or leukocyte-endothelial interactions [28, 29].

Diapedesis can occur also via the formation of a transcellular pore directly through an individual EC (transcellular diapedesis). This process takes place preferentially in the microvasculature, the blood brain barrier, or high endothelial venules (HEV) of the secondary lymphoid organs rather than in macrovasculature [30-32].

1.1.1.2. Leukocyte extravasation across lymphatic endothelium

During leukocyte trafficking they encounter also lymphatic endothelial barriers. A classical example is represented by the migration of dendrititc cells (DC) from peripheral tissues to draining lymph nodes. Current knowledge of the mechanisms involved in leukocyte trans-lymphatic migration is extremely sparse, however the few evidences available indicate that key events in the process are mediated by the same adhesion molecules that regulate blood vascular transmigration.

E-selectin is transiently upregulated on the surface of inflamed lymphatic endothelium [33]. E-selectin could have some role in the initial interaction between leukocytes and lymphatic endothelium, however since has been calculated that a leukocyte only has to contend with a shear stress of approximately 0.08 dynes/cm^2 (as opposed to 1–4 dynes/cm^2 in blood capillaries [34, 35] and considering that its engagement can trigger signals in selectin-expressing cell and the ligand-expressing cell (e.g. integrin's activation), the primary role of lymphatic E-selectin seems to be related to this second function, been a necessary prerequisite for VCAM-1 and ICAM-1 mediated adhesion. Similar to blood vascular endothelium, resting lymphatic EC express low levels of both ICAM-1 and ICAM-2, but not VCAM-1 [33]. However, after stimulation with TNF-α (tumor necrosis factor), both ICAM-1 and VCAM-1 are both upregulated. Also in lymphatic EC ICAM-1 and VCAM-1 mediate leukocyte adhesion steps [33]

DC show a relatively slow rate of transmigration across lymphatic EC by comparison with blood vessel endothelium (requiring hours rather than minutes) and a delay (1–3 h) between initial adhesion and the onset of transmigration [33]. Furthermore, the early stages of adhesion are insensitive to blockade by ICAM-1 and VCAM-1 antibodies. Possible explanations for these phenomena include pre-binding to other receptors, such as ICAM-2, successive rounds of transmigration events by DC or a lag period due to a requirement for chemokine-induced activation of integrins expression on DC [33, 36]

Similar observations for intercellular diapedesis across lymphatic and vascular EC have been reported, although in the case of lymphatic EC, both ICAM-1 and VCAM-1 appear to be present on both surfaces, whereas they are more polarized to the apical surface in blood EC [33]. Some studies suggests that leukocytes utilize also the transcellular pathway to transmigrate across lymphatic vessels, however it is not yet known whether it occurs *in vivo* [37, 38].

Despite the commonalities between endothelial lymphatic and vascular cells in terms of molecules expressed and the fact that they are morphologically indistinguishable, they are nevertheless specialized and adapted for their distinct functions, expressing some molecules characteristic of each kind of endothelium. Some lymphatic endothelial–specific receptors with a possible roles in regulating leukocyte adhesion and transmigration are mannose receptor, podoplanin and LYVE-1 (lymphatic vessel endothelial receptor 1)

Mannose receptor is expressed in lymphatic endothelium and in lymph node sinuses, where they have been shown to bind leukocyte-expressed L-selectin. Thus, this receptor may assist in adhesion of L-selectin+ lymphocytes prior to transmigration into afferent lymphatic vessels. Prodoplanin represents one of the most highly expressed genes in lymphatic endothelium [11, 12]. It contributes to the active recruitment of mononuclear leukocytes by lymphatic EC through its predominant expression on the basolateral surface, where it forms a complex with CCL21 that is subsequently shed into the stroma, thus establishing a peri-lymphovascular CCL21 gradient [39] . The key role that podoplanin plays in cell adhesion, migration, and vessel formation have been indicated by studies on podoplanin−/− mice, which exhibited cutaneous lymphedema, dilated cutaneous intestinal lymphatic vessels, and impaired lymphatic transport [40]. Furthermore, overexpression of podoplanin in vascular EC has been shown to promote the formation of elongated cell extensions and to increase cell adhesion, migration and tube formation [40]. The mechanisms through which podoplanin performs these functions

appear to be dependent upon interactions between a juxtamembrane cluster of basic amino acids in the short cytoplasmic tail of podoplanin and members of the ERM (ezrin, radixin, moesin)family [41]. ERM family proteins in turn bind to the cortical actin cytoskeleton and participate in a RhoA GTPase signal transduction pathway that regulates cell motility and adhesion. As podoplanin expression is concentrated at actin-rich microvilli and plasma membrane projections and can stimulate actin rearrangement, it is possible that this small membrane mucin may also play a role in regulating transcellular transmigration of cells across a monolayer of podoplanin-expressing cells, such as lymphatic endothelia. LYVE-1 is another abundant surface component of lymphatic EC [42, 43]. The similarity in amino acid sequence with the inflammatory homing receptor CD44 suggests that LYVE-1 might play an analogous role in lymphatic homing. Confocal microscopic observation that LYVE-1 is concentrated within the cleft-like openings of overlapping interendothelial junctions in initial lymphatics and only sparse within adjacent tight junction structures [5] is consistent with a role for the receptor in maintaining some aspect of junctional permeability.

1.1.2. Chemotactic factors

Every tissue site appears to be unique in its chemotactic property for immune cells. The chemotactic property of a tissue site is determined by its expression profile of chemotactic molecules. Chemotactic molecules include the chemokine protein family, inflammatory lipid mediators such as leukotrienes and prostaglandins, antimicrobial peptides such as defensins and chemerin, and cytokines. Also, microbial products such as N-formilmetionil-leucil-fenilalanina (fMLP) and complement proteins such as C3a and C5a can act as potent chemoattractants. Together, these chemotactic molecules form the greater chemotactic network important for precise and robust immune cell trafficking.

1.1.2.1. Chemokines

Chemokines are chemotactic cytokines that activate specific receptors expressed on cellular membranes. Chemokine receptors sense a chemical gradient and direct cells toward the gradient direction. The principal targets of chemokines are leukocytes and, as motility is an essential part of their function, chemokines play pivotal roles in coordinating leukocyte navigation. However, chemokines are not only simple chemotactic factors.

Accumulating evidence suggests that chemokines are important regulators in development, homeostasis and pathophysiological processes associated with osteoporosis [44], obesity and insulin resistance [45], viral infections [46], immune responses [47], mobilization of progenitors to the bone marrow [48], autoimmune encephalomyelitis. Moreover, nonhematopoietic elements, including epithelial cells, fibroblasts, and vascular elements have been shown to express receptors for and respond to CC chemokines.

The chemokine family comprises about 50 members (Table. 1); most are low molecular weight molecules (8-10 kDa), all consisting of roughly 70-130 amino acids, with four conserved cysteines [49-53]. Two main subfamilies, CXC and CC chemokines are distinguished, according to the position of the first two cysteines, which are separated by one amino acid (CXC) or adjacent (CC). The cysteines form two disulfide bonds, which confer to the chemokines their characteristic three-dimensional folding, with a flexible N-terminal loop connected to the more structured core of the molecule (3β-sheets) and a terminal α-helix. The CXC chemokine family can be further subdivided according to the presence or absence of a conserved tripeptide motif glutamic acid-leucine-arginine (ELR) at the N-terminus of the protein, before the CXC domain. This motif is not simply structural but seems to be linked to function, giving specificity for neutrophil chemotaxis and angiogenesis. The two members of the C (γ) family, lymphotactin-α (XCL1) and lymphotactin-β (XCL2), have two instead of the usual four conserved cysteines and fractalkine (CX3CL1) has three amino acids between the first two cysteines, giving rise to the one-member CX3C (δ) subfamily.

There are differences in the genomic organization of the chemokine families. Many of the genes for the CXC chemokines, which act on neutrophils are located on chromosome 4, while the majority of the genes for the CC chemokines, which act on monocytes are clustered on chromosome 17 [54]. These genes may have arisen by duplication and divergence from primordial chemokine genes but remained in clusters, supporting the idea that their functions are to some extent related. The remaining chemokines have a different chromosomal locations with respect to these two main clusters. These genes are more conserved between species and have highly specific functions, suggesting that they are older in evolutionary terms [54].

Like cytokines, chemokines are secreted proteins; only two of them, CXCL16 and CX3CL1 are synthesized with a typical transmembrane sequence, which anchors them to the cell membrane.

Chemokine Ligands	Other Names	Chemokine Receptors
CXC Subfamily		
CXCL1	GROα/MGSA-α	CXCR2
CXCL2	GROβ/MGSA-β	CXCR2R
CXCL3	GROγ/MGSA-γ	CXCR2
CXCL4	PF4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3A
CXCL10	IP-10	CXCR3A
CXCL11	I-TAC	CXCR3A
CXCL12	SDF1 α/β	CXCR4/ CXCR7
CXCL13	BCA-1	CXCR5
CXCL14	BRAK/bolekine	Unknown
CXCL15	Murine lungkine	Unknown
CXCL16	Bonzo ligand	CXCR6
C Subfamily		
XCL1	Lymphotactin/SCM-1α/ATAC	XCR1
XCL2	SCM-1β	XCR1
CX3C Subfamily		
CX3CL1	Fractalkine	CX3CR1
CC Subfamily		
CCL1	I-309	CCR8
CCL2	MCP-1/MCAF/TDCF	CCR2
CCL3	MIP-1α/LD78α	CCR1, CCR5
CCL4	MIP-1β	CCR5, CCR8
CCL5	RANTES	CCR1, CCR3, CCR5
CCL6	C10	CCR1
CCL7	MCP-3	CCR1, CCR2, CCR3, CCR5
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL9/10	MIP-1γ	CCR1
CCL11	Eotaxin	CCR3
CCL12	Murine MCP-5	CCR5
CCL13	MCP-4	CCR1, CCR2, CCR3
CCL14	HCC-1	CCR1, CCR5
CCL15	HCC-2/Lkn-1/MIP-1δ	CCR1, CCR3
CCL16	HCC-4/LEC/LCC-1	CCR1
CCL17	TARC	CCR4, CCR8
CCL18	DC-CK1/PARC/AMAC-1	Unknown
CCL19	MIP-3β/ELC/exodus-3	CCR7
CCL20	MIP-3α/LARC/exodus-1	CCR6
CCL21	6Ckine/SLC/exodus-2	CCR7
CCL22	MDC/STCP-1	CCR4
CCL23	MPIF-1/CKβ8-1	CCR1
CCL24	Eotaxin-2/MPIF-2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3	CCR3
CCL27	CTACK/ILC	CCR10
CCL28	MEC	CCR10

Table 1. Chemokine structural classification (adapted from Allavena et al, 2005)

Chemokines can also be grouped according to their function; in fact, they can be produced by leukocytes and tissue cells either constitutively or after induction, thus a former classification grouped chemokines into the functional subfamilies termed "inflammatory" and "homeostatic" (Fig. 1). Homeostatic chemokines usually guide the trafficking of leukocytes under steady state conditions, during immune surveillance of healthy peripheral tissues and control the architecture of secondary lymphoid organs; by contrast, inflammatory chemokines are produced and control the recruitment of effector leukocytes under conditions of inflammation, immune reactions, tissue injury and tumors [55].

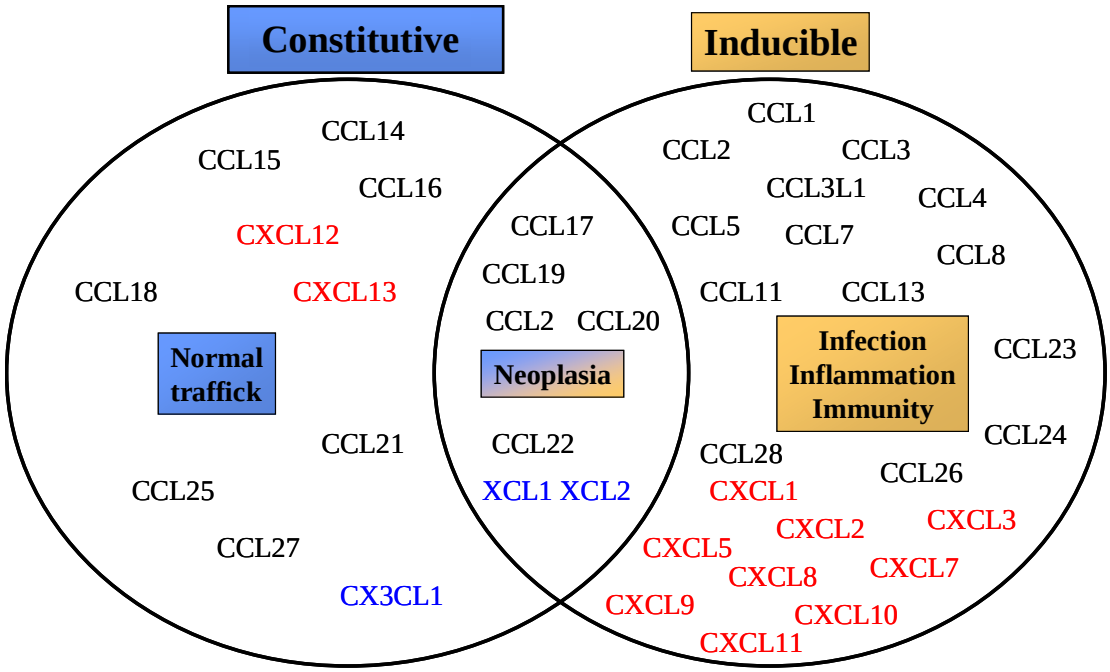


Figure 1. Chemokine functional classification. Chemokines can be classified as constitutive, regulating normal leukocyte trafficking, or inducible, associated to inflammatory and immune responses. Chemokine production by tumor cells may reflect constitutive gene expression or activation by autocrine/paracrine loops (Adapted from Locati et al, 2002)[55]

Many chemokines have broad target cell selectivity. In particular, expression of inducible chemokines can be elicited by almost any stimulus that alters cellular homeostasis; they can be thought of as vertebrate cellular "SOS response" that recruits leukocytes to areas of tissue injury. Recent findings, however, indicate that several

chemokines cannot be assigned unambiguously to either one of the two functional categories and therefore are referred to as “dual function” chemokines [56]. Dual function chemokines participate in immune defense functions (e.g. are upregulated under inflammatory conditions, examples include the interferon (IFN)-inducible chemokines CXCL9, CXCL10, CXCL11) and also target non-effector leukocytes, including precursors and resting mature leukocytes, at sites of leukocyte development and immune surveillance (for instance CCL1, CCL25, CXCL16) [56]. Many dual function chemokines are highly selective for lymphocytes and have a role in T-cell development in the thymus, as well as in T-cell recruitment to inflammatory sites. Remarkably, dual-function and homeostatic chemokines usually bind to a single receptor, expressed mainly on lymphoid cells, in contrast to inflammatory chemokines, which bind to multiple receptors.

In general, different chemokine classes tend to exhibit different ranges of leukocyte specificity; schematically, CXC-ELR+ chemokines (ELR is a highly conserved amino acid motif: Glu-Leu-Arg) are the major chemoattractants for neutrophils, whereas CXC-ELR- chemokines attract lymphocytes and monocytes but have little or no action on neutrophils. Chemokines belonging to the CC family act primarily on monocytes, but they can also attract lymphocytes, natural killer (NK) cells, basophils and eosinophils. CX3C and C chemokines act on lymphoid cells (T cells and NK cells) and fractalkine is also active on monocytes. Despite this specificity, redundancy in the action on target cells is an intriguing feature of chemokines: no chemokine is uniquely active on one leukocyte population and usually a given leukocyte population has receptors for and responds to different chemokines. This confers robustness to the system, as variations in the amount or quality of any chemokine or receptor would have bearable consequences for basal trafficking of leukocytes [57-59]. Moreover, the interaction of chemokines with their receptors is characterized by considerable promiscuity. Most ligands interact with more than one receptor and most known receptors have been reported to interact with multiple ligands; only CXCR4, CXCR5, CXCR6, CCR6, CCR9 and CX3CR1 bind to only one chemokine. Probably all cell types can produce chemokines under appropriate conditions and usually, a cell produces many chemokines concomitantly in response to the same stimulus (polyspeirism) Once again, this receptor promiscuity and polyspeirism contribute to the robustness of the chemokine network, essential features which act to retain the chemokine system function, even if genetic or epigenetic alterations affecting individual components occur.

1.1.2.2. Nonchemokine chemotactic factors

A group of heterogeneous molecules different from chemokines has been described to present also chemotactic activity. These include bacterial components, bioactive lipids and signals of "tissue danger". Among lipid mediators, leukotrienes [60] and prostaglandins [61] have been shown to induce directional migration of immune cells. Leukotriene B4 (LTB4), the most important chemoatractant produced during the early phases of inflammation is able to recruit neutrophils, eosinophils, Tcells and DC [19] while prostaglandin D2 (PGD_2) induces chemotaxis of eosinophils, basophils, and T-helper type 2 (Th2) cells [62]. Lysophospholipids, including lysophosphatidic acid and sphingosine 1-phosphate (S1P), also show direct chemotactic activity or modulate chemotaxis to other chemoattractants [22, 23]. Some defensins (small cationic and Cys-rich peptides with molecular masses ranging from 3 to 5 kD), such as defensin-1 and defensin-2 produced by activated neutrophils, induce chemotaxis of T cells [63] and DC [64]. Complement pathway products such as C3a and C5a are also chemotactic for leukocytes [65] e.g.. C5a promotes the recruitment of neutrophils and eosinophils. Some cytokines show chemotactic activity, e.g..interleukin-16 induces chemotaxis of CD4 T cells. Stem cell factor acts as a chemoattractant for mast cells and hematopoietic progenitor cells [66]. Serotonin, a neurotransmitter and inflammatory mediator released by mast cells, can induce the chemotaxis of eosinophils [67]. Chemerin is a recently identified protein with a chemotactic activity for DC, macrophages and NK cells [68-70].

1.1.2.2.1. Chemerin

Chemerin is a 163 aminoacids protein (Fig. 2) identified as the natural ligand of chemerin receptor (ChemR23), a GPCR related to chemokine receptors expressed in immature DC [70-72], macrophages [70] and the cytotoxic subset of NK cells [69]. Chemerin acts as a chemoattractant protein for ChemR23+ cells [68-70].

Chemerin mRNA (messenger RNA) is constitutively expressed at readily detectable levels in most tissues, primarily liver, lung, pituitary, and ovary. No chemerin transcripts are found in peripheral blood leukocyte populations [70 73]. The liver, appears to be a primary source of chemerin, and is likely to be responsible for the high levels of this protein in blood [72]. ChemR23 is also broadly distributed. ChemR23 transcripts have been found primarily in spleen, lymph nodes and lung [70]. It have been also found in cardiovascular system, brain, kidney, gastrointestinal tissues, and myeloid tissues (Fig. 2) [73].

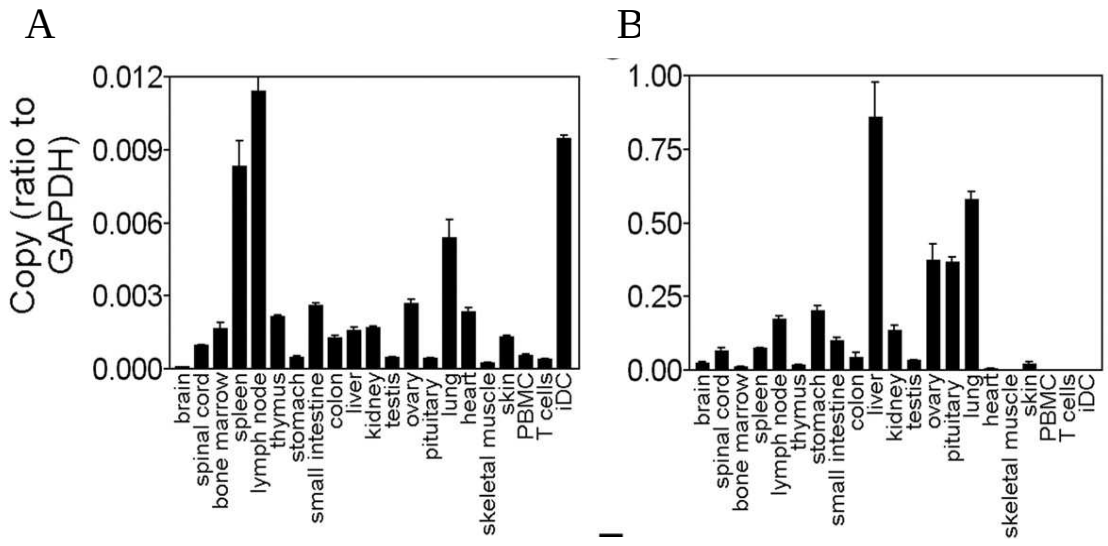


Figure 2. Expression of human ChemR23 (A) and chemerin (B) (From Wittamer et al, 2003)[70]

Chemerin is synthesized as a secreted 142 aminoacid precursor of low biological activity (prochemerin), which upon proteolytic cleavage of its C-terminal domain is converted into a potent and highly specific agonist of ChemR23 (Fig. 3 and 5) [70]. Chemerin lacking the last six (chemerin 157) or seven (chemerin156) aminoacids are the sole bioactive forms of chemerin on ChemR23, the former being more active than the latter [74]. Unlike chemerin, most chemokines are processed at their N-terminus. However the proteins closest structurally to prochemerin: cathelicidins (antibacterial peptides), cystatins (cysteine protease inhibitors), and the bradykinin precursor kininogen, are also activated by a C-terminal processing. [75-77]

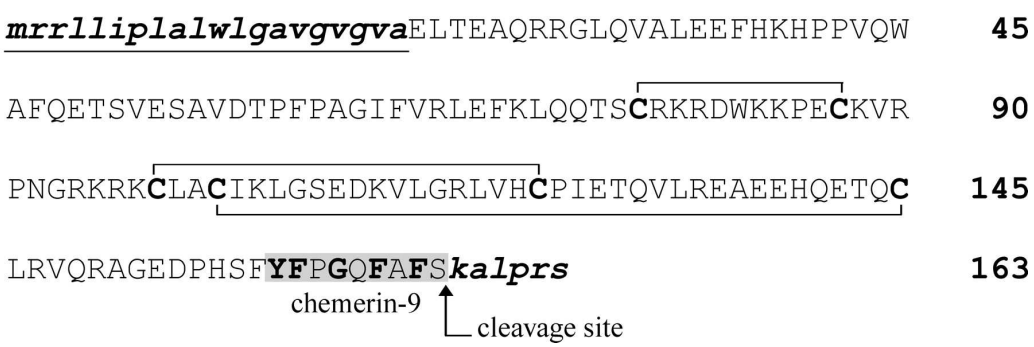


Figure 3. Aminoacid sequence of human prochemerin. The signal peptide is in bold lowercase italic characters and underlined, the cleaved C-terminal peptide is in bold lowercase italics and cysteins are in bold characters. The putative disulfide bonds are indicated. (From Wittamer et al, 2004)[74]

The production of chemerin is regulated both at transcriptional and posttranslational level. TIG2 (tazarotene-induced gene 2), chemerin encoding gene, has been found to be overexpressed in nonlesional psoriatic skin and at lower levels in psoriatic lesions, and to be up-regulated by the synthetic retinoid tazarotene in these lesions [78, 79]. Induction of this gene was also shown in the osteoclastogenic mouse bone marrow stromal cell line ST2 after stimulation by 1,25-dihydroxy-vitamin D3 and dexamethasone [78]. Therefore the transcriptional regulation of chemerin appears to vary according to cell types and tissue.

The clotting-associated serine proteases, factors XIIa and VIIa, the fibrinolysis-associated serine proteases plasmin and plasminogen activators, mast cell derived tryptase [80], serine proteases released from activated neutrophil azurophil granules (elastase (HLE) and cathepsin G (CG)) [80, 81] and Staphopain B, a cysteine protease secreted by *Staphylococcus aureus* [82] are all potent activators of chemerin. The activation of chemerin by different extracellular proteases associated with or induced by the clotting, fibrinolytic and inflammatory cascades, supports a role of chemerin in the recruitment of ChemR23+ cells to sites of tissue damage, infection or allergic inflammation to rapidly establish an appropriate immune response [72].

Chemerin is not only positively regulated at posttranscriptional level, there are also proteolytic events that negatively control its chemotactic activity. Proteinase 3 (PR3), a serin protease known to be abundant in polymorphonuclear (PMN) granules, and mast cell chymase are involved in the generation of specific chemerin variants, which are

inactive on ChemR23. PR3 specifically converts prochemerin into an inactive chemerin form, lacking the last eight carboxy-terminal amino acids (chemerin-155), whereas mast cell chymase converts active chemerin-157 and to a lesser extent, chemerin-156 (but not prochemerin) into the inactive chemerin-154 form [83].

In addition to transcriptional regulation, the post-translational processing by proteases constitutes an usual mechanism found also in the regulation of the biological activity of chemokines involved in inflammatory processes. Metalloprotease activity has been shown to lead to the inactivation of several chemokines e.g. the monocyte chemotactic proteins CCL2, 7, 8, and 13, and the general leukocyte attractant CXCL12 [84]. Proteolysis of CCL3/MIP-1α by HLE, CG, and PR3 inhibits its chemoattractant activity [85].

Given its homology with cystatins and cathelicidin precursors, as well as the similar organization of their genes, it is likely that chemerin adopts the so-called cystatin fold [70]. This fold is presumably stabilized by two disulfide bonds common to cystatins, and an additional bond specific to chemerin, but compatible with the cystatin fold. The C terminus of the protein, located after the last cysteine involved in the disulfide bond constitute an unstructured flexible domain crucial for the biological activity of the protein on ChemR23, with the aromatic residues Tyr149, Phe150, Phe154 and Phe156, as well as Gly152 playing a critical role in both ChemR23 binding and activation [74].

The fact that the same region (C terminal) of chemerin is involved in both, binding and activation of ChemR23 (a one site model), greatly differentiates it from the family of chemokines in the region involved and the way they interact with their cognate receptors. In most chemokines the flexible domain is located at the N-terminal domain [86], playing a similar role as C-terminus of chemerin [87]. Unlike chemerin, the interaction of chemokines with their receptors involves a two-site model: their binding involves first a high affinity binding site between the core of the chemokine and the N terminus and loops of the receptor, whereas activation of the receptor usually requires a second interaction between the flexible N terminus of the chemokine and the transmembrane helix bundle of the receptor [86].

Chemerin and ChemR23 are relatively well conserved between mammalian species (Fig. 4). The six cysteins presumably involved in disulfide bridging are strictly conserved in all specie. Importantly, the C-terminal end of chemerin (YFPGQFAFS), which is essential for the activation of ChemR23, is particularly well conserved . It

appears therefore that the chemerin-ChemR23 system has been well conserved in evolution, suggesting important and stable functions all along the vertebrate lineage [88]

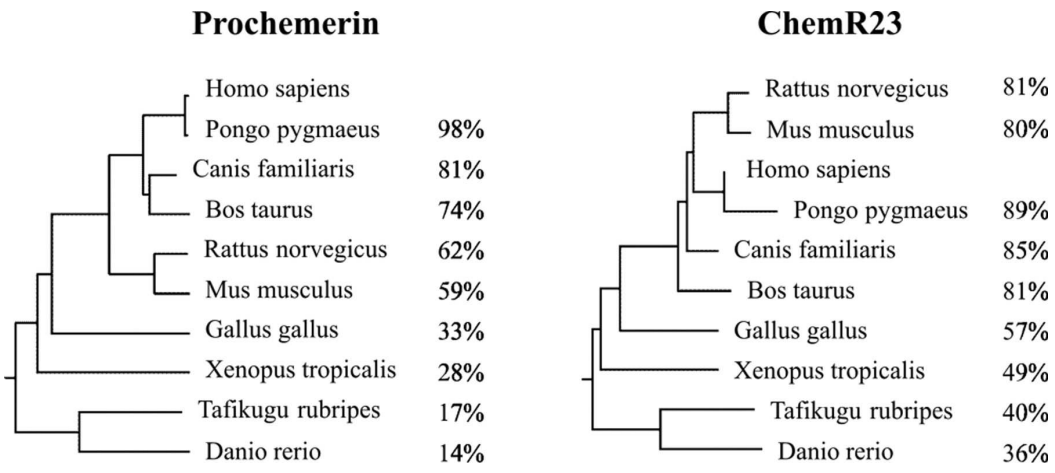


Figure 4. Dendogram of ChemR23 and prochemerin from different vertebrate species (From Luangsay et al, 2009) [88].

Although chemerin was first described as a chemoatractant, recent observations suggest that it can also display angiogenic and anti-inflammatory properties that are absolutely depending on proteolytic processing and act via ChemR23. Kaur et al [89] have reported that chemerin acts on EC through ChemR23 (the receptor is expressed in human EC (micro (HMECs) and macro(HUVECs and EA.hy926)-vascular human EC) and upregulated by pro-inflammatory cytokines (TNF-α, IL-1β and IL-6)), is able to promote EC proliferation, migration and capillary tube formation; and activation of endothelial gelatinases (metalloproteinase (MMP) 2/9).

Furthermore classically activated macrophages are capable of converting chemerin into the potent anti-inflammatory peptide C15 (140-AGEDPHGYFLPGQFA-154) (Fig. 5) [90]. C-15 enhances macrophage phagocytosis of microbial particles and apoptotic cells (efferocytosis) in a nonphlogistic manner.This is supported by *in vivo* studies where C15 ameliorated zymosan-induced peritonitis, reducing leukocyte recruitment by up to 65%, with a concomitant suppression of inflammatory mediator expression and a quantifiable reduction in the level of apoptotic cells suggesting a role in resolution of inflammation [91].

In addition, in a mouse model of acute lung injury chemerin instillation increased the mobilization of airway macrophages via ChemR23 and inhibited neutrophil recruitment

and the release of inflammatory cytokines (IL-6, TNF-α, IL-1β) and chemokines (KC/CXCL1) [88]

Besides ChemR23, orphan receptor GPR1 (G protein-coupled receptor 1) and CCRL2 have been identified as spare receptors for chemerin [92, 93]. However, only GPR1 is capable to support chemerin-derived signal transduction, therefore at least some of the activities of chemerin may be mediated through GPR1. CCRL2 functions as a not signaling receptor for chemerin. It has been suggested that could act by concentrating bioactive chemerin and facilitate its presentation to adjacent cells.

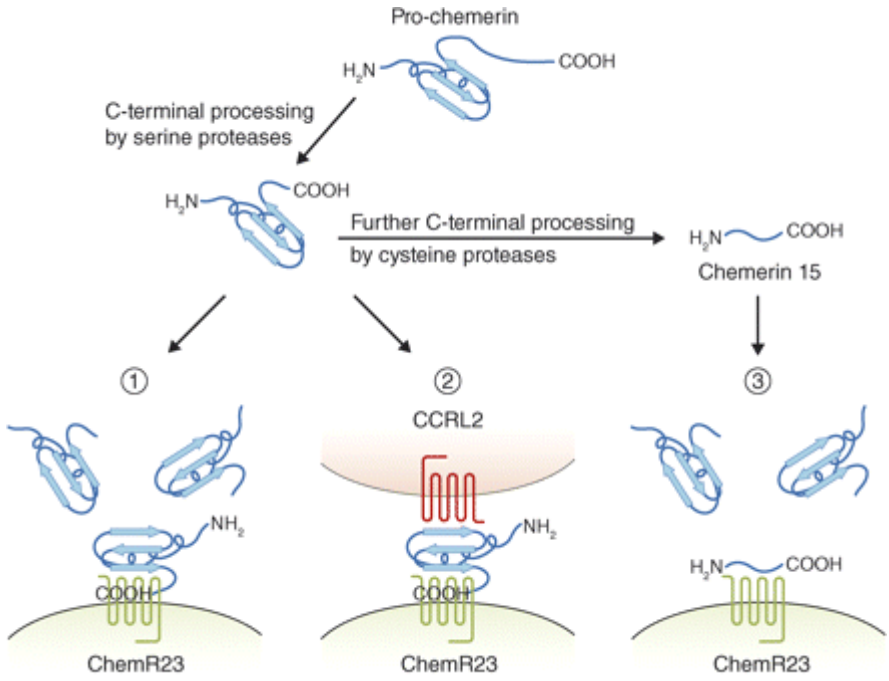


Figure 5. Regulation of the inflammatory response by chemerin. Prochemerin is processed by serin proteases at C-terminal region generating active chemerin, which induced cell migration through engagement of ChemR23 (1). Chemerin can binds also to CCRL2 by its N-terminal domain being presented in this way to adjacent ChemR23+ cells (2) Cystein protease processing of chemerin generates the anti-inflammatory peptide chemerin 15 which acts via ChemR23 inhibiting the generation of proinflammatory mediators (From Yoshimura and Oppenheim, 2008)[94]

1.1.3. Chemotactic receptors

The recruitment of different types of leukocyte subsets to tissues is governed by the regulated expression of chemotactic receptors. Signaling pathway of most

chemoattractant receptors is mediated by seven transmembrane domain GPCR. However an exception is represented by cytokines such as IL-16 and stem cell factor, which induce chemotaxis through CD4 and c-kit respectively [95, 96].

1.1.3.1. Chemokine receptors

Chemokines induce cell migration and activation by binding to specific seven transmembrane domain GPCR on target cells. Ten CC (CCR1-10), seven CXC (CXCR1-7), one CX3C (CX3CR1) and one XCR (XCR1) receptors have been identified (Fig.6) [51, 54]. Receptor expression is a crucial determinant of the spectrum of action of chemokines. The profile of chemokine-receptor expression on an individual cell is determined by its lineage, stage of differentiation and microenvironmental factors, such as chemokine concentration, the presence of inflammatory cytokines and low oxygen tension.

Some receptors are restricted to certain cells (e.g. CXCR1 is predominantly restricted to neutrophils); whereas others are more widely expressed (e.g. CCR2 is expressed on monocytes, T cells, NK, DC and basophils). Moreover, chemokine receptors can be constitutively expressed on some cells, whereas inducible in others (e.g. CCR1 and CCR2 are constitutively expressed on monocytes, but are expressed on lymphocytes only after IL-2 stimulation); again, the expression of some chemokine receptors can be restricted to a cell state of activation and differentiation (e.g. CXCR3 is expressed on activated T helper type 1 lymphocytes (Th1), whereas CCR3 is preferentially expressed on T helper type 2 lymphocytes (Th2) [97].

Although initially studied on leukocytes, some chemokine receptors are also expressed in nonhematopoietic cells, including neurons, astrocytes, epithelial and endothelial cells. These observations suggest that the chemokine system has other roles in addition to leukocyte chemotaxis.

As already pointed out for chemokines, the remarkable feature of the chemokine receptor family is their promiscuity as far as ligand binding is concerned; however, although relatively few receptors bind only one ligand, CC receptors bind only CC chemokines and CXC receptors bind only CXC chemokines. This ligand-receptor restriction may be related to structural differences between CC and CXC chemokines, which have similar primary, secondary and tertiary structures but different quaternary structures [98].

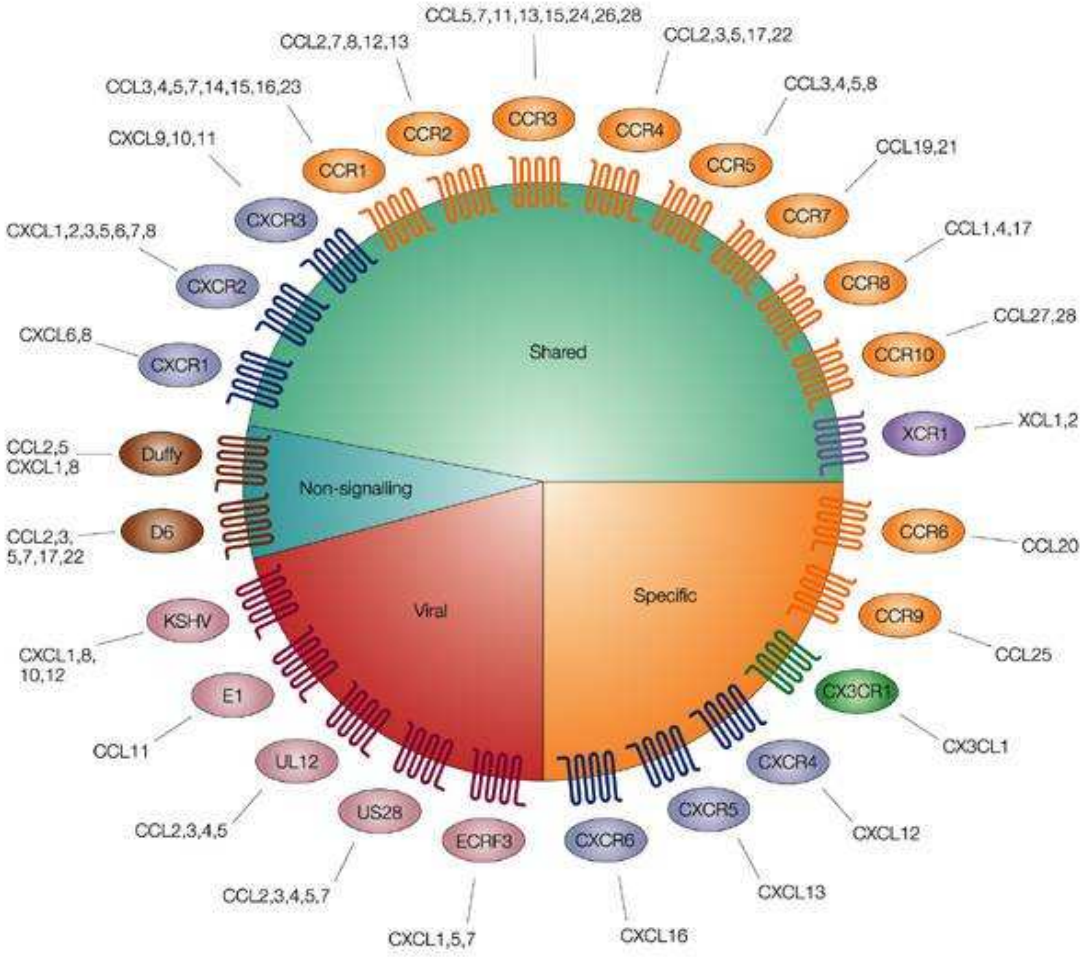


Figure. 6. Chemokine receptor family. Ligand specificity is represented, with many receptors binding more than one single ligand. (From Balkwill, 2004)[99]

1.1.3.1.1. Chemokine receptor signaling.

Although the details of the chemokine signalling pathways appear to vary depending on the cellular context, the general rule is that, upon chemokine binding, the G protein activated by Guanosine-5'-triphosphate (GTP) dissociates in α and βγ subunits, the latter activating the enzymes phospholipase C (PLC) [28] and phosphatidilinositol-3 kinase γ (PI3Kγ).

PLC catalyzes the cleavage of membrane bound phosphatydilinositol 4,5 biphosphate (PIP2) yielding two second-messengers: inositol triphosphate (IP3) that triggers the release of calcium from intracellular stores and diacylglycerol (DAG) that activates several isoforms of protein kinase C (PKC). While the rise in the concentration of intracellular calcium has been widely used to test the responsiveness of chemokine receptors to different chemokines, activation of PKC isoenzymes is stimulated by

almost any surface receptor and therefore is not special for chemokine-induced signal transduction. However, PKC activation by chemokines contributes to receptor phosphorylation which leads to desensitization and inhibition of functional responses [100].

PI3Kγ activation results in the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) that initiates the activation of protein kinase B (PKB) [101]. PI3K, PKB and PIP3 translocate to the leading edge of the cell where PP3 activate the small GTPase Rac, and all these mediators participate in local polymerization of the contractile protein filamentous actin (F actin), whereas the mediators of actomyosin contraction are recruited to the trailing edge [30] (Fig. 7). After chemotactic stimulation the phosphatase PTEN (phosphatase and tensin homolog, a PI3P phosphatase), which removes PIP3 by converting it back to PIP2, rapidly localizes to the cell membrane. Contrary to the former mediators PTEN localizes to the sides and rear of the cell, amplifying the intracellular PIP3 gradient [102]. Another important player is also activated: Cdc42, a small GTPase, which is recruited to the leading edge, where it is involved in PTEN exclusion from the leading edge, being essential for directional cell migration. [103-105]. Thus, upon chemokine stimulation, PI3K localizes anteriorly, whereas PTEN localizes posteriorly and this spatial and temporal regulation determines the polarity of the migrating cell [106].

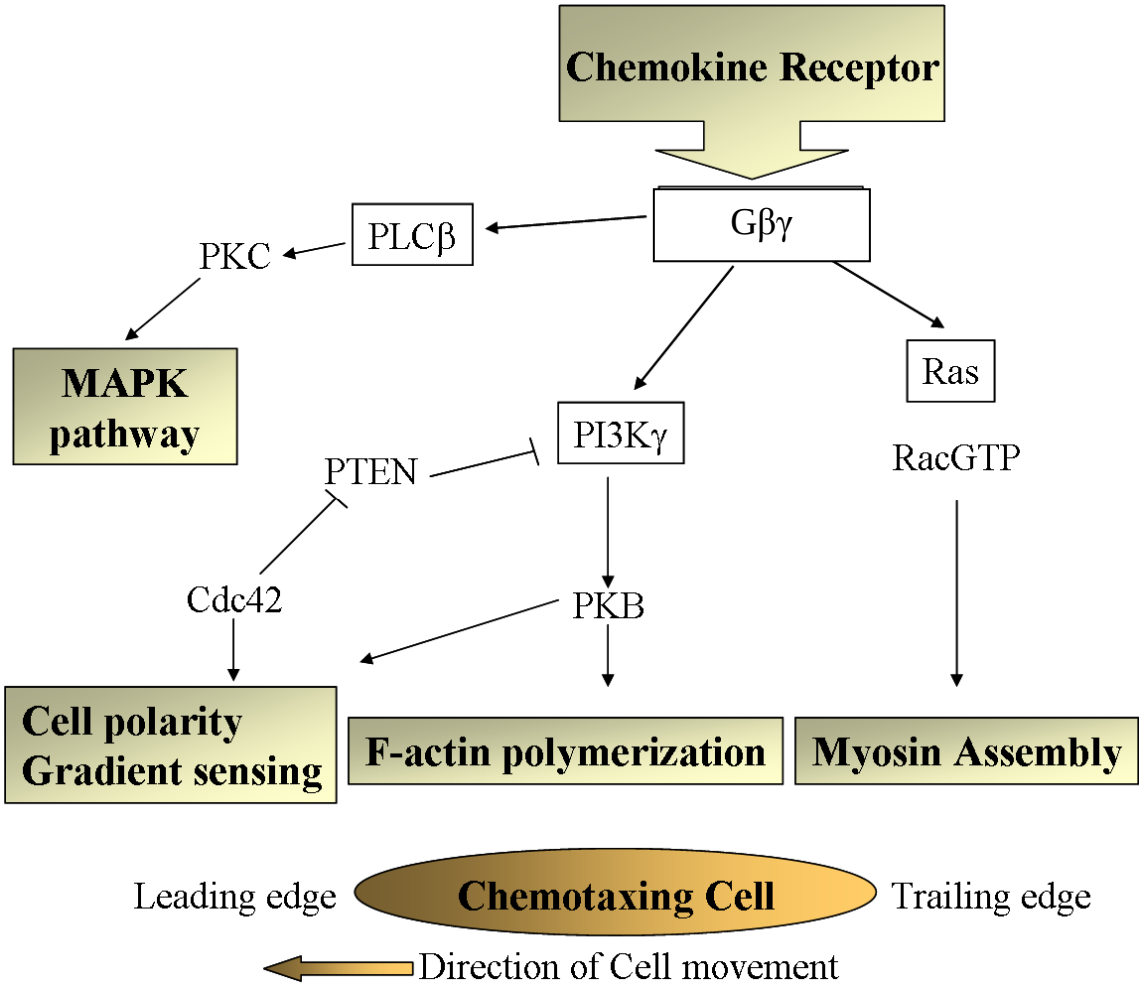


Figure 7. Signalling through chemokine receptors. Different signalling components localize either in the leading edge or in the trailing edge, determining cell movement (adapted from Tanaka et al, 2005)[107]

There are increasing evidence that chemokine receptors can also activate several intracellular effectors downstream of G coupling, including the low molecular weight protein Ras and Rho and the mitogen-activated protein kinase (MAPK) pathway [108, 109]. Moreover, it has been suggested that, like cytokine receptors, chemokine receptors are also able to signal through the JAK-STAT pathway [110, 111]. Upon ligand binding, the receptor dimerizes and catalyses the phosphorilation of the JAK kinases which in turn phosphorylate the receptor itself; this event brings to STAT molecule recruitment, dimerization and translocation to the nucleus where it triggers the expression of cytokine dependent genes.

Chemokine receptor signalling is transient, and the rapid termination of receptor activity is achieved by three mechanisms: receptor inactivation, desensitization and internalization. Receptor inactivation is mediated by the intrinsic GTPase activity of the Gα subunit, which hydrolyzes GTP and reunites with Gβγ to return to the initial conformation of inactive heterotrimer. Desensitization is caused by receptor phosphorilation, through G-protein-coupled receptor kinases, among which PKC and finally internalization is caused by β-arrestin or adaptin-2 mediated receptor sequestration and internalization, through clathrin-coated pits or caveolae. The speed of response recovery is determined by the fate of internalized receptors, either lysosomal degradation or dephosphorylation and cell surface recycling; the pathway leading to either degradation or recycling following receptor internalization are determined by the guardians of all vesicular machinery, Rab GTPases [112] and by the rate of *de novo* chemokine receptor synthesis.

Signal transduction downstream chemokine receptors is much more complicated than the one above described; indeed, a given receptor will activate signalling pathways influenced by those from other receptor systems.

1.1.3.2. Atypical chemokine receptors

Beside the conventional signalling chemokine receptors, other chemokine binding molecules with high structural similarity to chemokine receptors have been described, namely the Duffy antigen receptor for chemokines (DARC) [113-115], D6 [114-116] and ChemoCentryx chemokine receptor (CCX-CKR) [117]. These receptors do not elicit migration or conventional cellular responses, but still bind chemokines with high affinity. They are also referred to as "silent receptors" and have been suggested to favor transfer of chemokines across endothelial barriers and/or to act as decoy receptors which dampen inflammatory reactions [118]. Of note, sequence motifs critical for G protein coupling and signaling functions of chemokine receptors, namely the DRYLAIV in the second intracellular loop and the TXP motif in the second transmembrane domain, are not conserved in these receptors. It have been recently reported that similar to silent receptors, CCRL2 is able to bind chemerin without inducing an intracellular signaling[93].

1.1.3.2.1. C-C chemokine receptor-like 2 (CCRL2)

CCRL2 (C-C chemokine receptor-like 2), also known as L-CCR (LPS-inducible CC Chemokine Receptor), is a 7-transmembrane GPCR described as potential leukocyte chemoattractant receptor. CCRL2 shares over 40% amino-acid identity with CC-chemokine receptors and clusters on chromosome region 3p21-23 together with the most other CC-chemokine receptors (in particular, CCR1 and CCR5) [119]. However CCRL2 possesses an uncharacteristic intracellular loop2 sequence in place of DRY motif, similar to nonsignaling chemotactic receptors, that makes it considered as a candidate decoy receptor [120]. CCRL2 was originally cloned from the macrophage cell line RAW 264.7 [121]. Real time polymerase chain reaction (RT-PCR) and in situ hybridization experiments revealed CCRL2 mRNA expression by microglia, astrocytes and infiltrating macrophages in spinal cord and brain in a model of experimental autoimmune encephalomyelitis (EAE) [35-37]. The expression of CCRL2 on glial cells resulted induced upon LPS stimulation both *in vitro* and *in vivo* [122]. CCRL2 has been reported to be expressed in lung tissue and upregulated in macrophages and bronchial epithelium in a mouse model of ovalbumin (OVA) -induced airway inflammation [123]. Recent observation shows that CCRL2 is constitutively expressed in mouse mast cells [93].

Human CCRL2 counterpart is named HCR (human chemokine receptor) [124]. Mouse and human CCRL2 genes share 51% homology, which is low comparing to the homology usually observed for other chemotactic receptors (usually > 80%). HCR mRNA expression has been found in human liver, spleen, heart, lung, kidney, and skeletal muscle leukocytes [125]. In human blood and bone marrow CCRL2 is expressed at varying levels in all myeloid cells (monocyte, macrophages, PMN, and DC), lymphoid cells, and CD34+ myeloid precursors, with PMN expressing the highest levels. Unlike other lymphoid cells, B cells were initially reported to be negative for CCRL2 [125]. However a recent work demonstrates that HCR is clearly expressed on different B-cell types with a preferential expression in the early stages of differentiation and lack of expression in immature bone marrow B cells and IgM+-expressing B-cell lines [126]. Interestingly, it is upregulated in human T cells after *in vitro* stimulation and in DC stimulated with LPS, poly (I:C), IFNγ or CD40L [125]. In addition, human neutrophils constitutively express HCR [125], and its expression is shown to be upregulated in synovial neutrophils from rheumatoid arthritis patients [127, 128].

CCRL2 is rapidly induced after LPS treatment: it is induced by 30 min. in

RAW264.7 cells, reaching a peak at 2h, and decreases thereafter [121]. Similarly, in an OVA induced airway hyperesponsiveness model, CCRL2 is upregulated in the lung as early as 1-3 h after OVA challenge, returning to basal levels 6 h after [121, 123]

Recently it have been described the first biological function attributed to CCRL2 so far. Zabel et al found that mouse mast cells constitutively express CCRL2 [93]. Using CCRL2-deficient mice, they showed that the absence of CCRL2 did not affect the basic mast cell phenotype and function *in vitro* or T cell-mediated contact hypersensitivity *in vivo*. However, in a model of IgE-dependent passive cutaneous anaphylaxis *in vivo*, in which mast cells play a central role, they found that CCRL2-deficient mice had reduced inflammatory responses when a relatively low sensitization dose was used. The defective response was attributed to CCRL2 expression on mast cells, as mast cell-deficient mice reconstituted with CCRL2-deficient bone marrow progenitor cells showed an impaired response compared to those reconstituted with WT (wild type) cells[93].

Due to its homology with CC chemokines receptor family, CCRL2 has been considered a putative chemokine receptor and several attempts have been performede to identify the putative ligands. Recently Biber and colleagues reported that HEK 293 cells transfected with mouse CCRL2 showed pertussis toxin (PTX) sensitive chemotaxis and intracellular calcium signal in response to the chemokines CCL2, CCL5, CCL7 and CCL8 [129], suggesting that CCRL2 is a functional receptor. However these results remain controversial because radioligand binding of these chemokines to CCRL2-transfected cells could not induce any detectable signals and the results have never been confirmed (discussed by [120]}. Recent reports suggest that CCRL2 is a non-signaling receptor, belonging to atypical silent/decoy receptors family [93, 128].

Chemerin has been recently indicated as the natural non-signalling ligand for both human and mouse CCRL2. The binding affinity of chemerin for CCRL2 is similar to, if not slightly better than, chemerin binding to ChemR23 [93]. Leick et al [128] showed that CCL19 binds to CCRL2 with an affinity similar its classical receptor, CCR7, however this binding did not elicit any classical chemokine receptor response like MAPK phosphorylation, calcium mobilization or migration, confirming the 'silent' behavior of CCRL2. In spite of its phylogenetic homology with members of the CC chemokine receptor family, CCRL2 possesses an uncharacteristic intracellular loop 2 sequence in place of the DRYLAIV motif generally found in signaling chemokine receptors (QRYLVFL in human and QRYRVSF in mouse), a characteristic that might account for

its non-signaling characteristics after chemerin binding [130]. The presence of a glutamine (Q) at position 127 instead of aspartic acid (D), replacing an acidic residue with a neutral one rends coupling to the Gi protein highly unlikely.

Reports about CCRL2 internalization are conflicting. Zabel et al [93] have reported that CCRL2 show a low-level constitutive endocytosis and that chemerin binding does not trigger ligand internalization. On the other hand Leick et al have found that CCRL2 is constitutively recycled via clathrin-coated pits and quickly re-expressed at the cell surface and is able to internalize CCL19 as well as anti-CCRL2 antibodies [128]. However, even whether CCRL2 internalizes CCL19, the process is less efficient than CCL19 internalization mediated by the scavenger receptor CCX-CKR. It is likely that CCRL2 could act more as chemokine presenter (like DARC) rather than a scavenger (like D6). Zabel et al have proposed that CCRL2 could be able to bind the chemoattractant and increase local concentrations of bioactive chemerin which then become available for interaction with ChemR23 on adjacent cells, thus providing a link between CCRL2 expression and inflammation via the cell-signalling chemerin receptor ChemR23. A similar reasoning could be doing for CCL19. The identification of chemerin as a non-signalling ligand for CCRL2 introduces a novel functionality for atypical receptors: concentration and presentation, as opposed to internalization/degradation [9341]. Chemerin binds directly to the N-terminal domain of CCRL2 by its N-terminal region. The orientation of chemerin binding to CCRL2 leaves its critical cell-signaling C-terminus exposed, therefore available to bind to ChemR23 (Fig 8). In this regard, it is interesting to note that the first 16 aminoacids of human and mouse CCRL2 share 81% identity, compared with just 17% shared identity in the remaining 24 aminoacids of the N-terminal domain [9341]. In its role as a specialized molecule for concentrating extracellular chemerin, CCRL2 may operate like glycosaminoglycans, which are thought to bind, concentrate, and present chemokines to leukocytes and facilitate their chemotaxis.

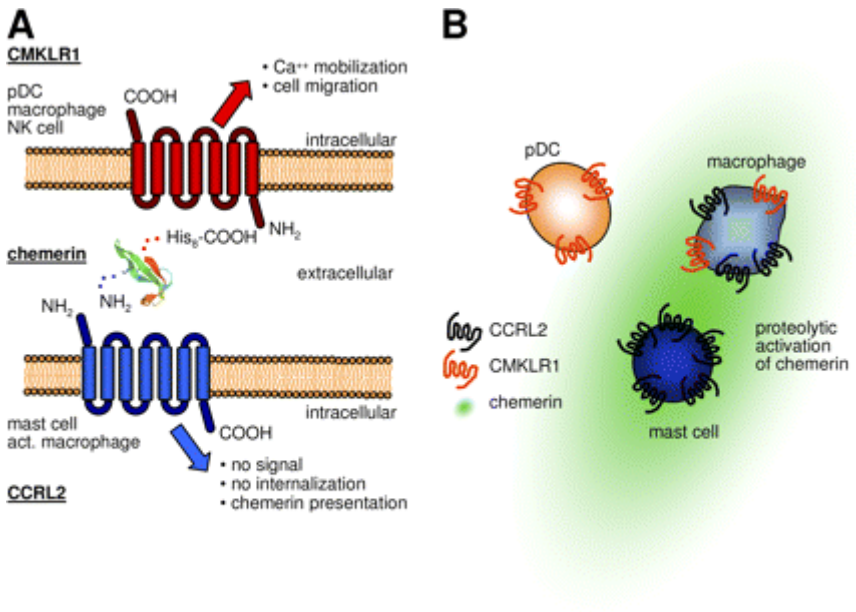


Figure 8. Presentation of chemerin by CCRL2. (A) Chemerin binds to CCRL2 leaving the C-terminal peptide sequence free to interact with adjacent ChemR23+ cells. (B) Alternatively, CCRL2 may concentrate the ligand for proteolytic processing, enhancing the local production of the active form which could then act as a chemoattractant after release from the cell surface. (From Zabel et al, 2008)[93].

1.1.4. Chemokines in physiology

The eponymous function of chemokines and their receptors is to mobilize cells in a gradient direction. This biological function is particularly important in leukocyte recruitment at sites of inflammation, although it is now well known that many different cells express chemokine receptors and are therefore able to respond to different chemotactic stimuli. The trafficking of DC and lymphocytes through secondary lymphoid organs is finely regulated by chemokine receptors. DC can take up antigens in peripheral tissues and migrate to draining lymph nodes where they present antigen to naïve T cells, triggering the T cells to proliferate and differentiate. These activated T cells can then migrate to the inflamed tissues to perform their effectors functions. The movement of T cells and DC between lymph nodes and the periphery may be controlled by differential chemokine receptor expression [131, 132].

Circulating monocytes and immature DC can express receptors for chemoattractants such as fMLP and C5a as well as chemokine receptors including CXCR4, CCR1, 2, 3, 4, 5 and 6 [133, 134]. Using these receptors, immature DC can migrate towards sites where there is a high concentration of inflammatory chemokines such as CCL2, CCL3 and

CCL5. Immature DC can also respond to constitutively expressed chemokines such as CXCL12, and this may be important for localizing DC in tissues under normal conditions. At sites of inflammation, immature DC are activated by inflammatory cytokines such as TNF-α and IL-1 [135], which results in DC maturation. This process causes a switch in their chemokine receptor expression. Maturing DC lose their responsiveness to CCL3, 4, 5, 7 and 20 [132, 136, 137], but concomitantly upregulate CCR7 and gain responsiveness to CCL19 and CCL21 which are constitutively expressed in T cell rich areas of secondary lymphoid organs including lymph nodes, spleen and tonsils.

Naïve T cells can express CXCR4 and CCR7, which may account for their localization in secondary lymphoid organs. Upon interaction with antigen-presenting DC, naïve T cells are activated and alter their chemokine receptor profile. Activated T cells can express CXCR3, CXCR4, CCR1, 2, 3, 4, 5, 6, 7 and 8 [131, 138]. Activated T cells can polarize to Th1 (expressing IFN-γ and IL- 12) or Th2 (expressing IL-4, IL-5 and IL-13) subsets, which differ in their cytokine production and function during an immune response and also express different chemokine receptors. CCR5 and CXCR3 are preferentially expressed on Th1 cells, while Th2 cells preferentially express CCR3, CCR4 and CCR8 [138, 139], although this differential chemokine receptor expression is not clear-cut. Finally, naïve B cells can express CXCR5 and CCR7 which direct them to the follicles of secondary lymphoid organs, where the ligands CXCL13, CCL19 and CCL21 are expressed. Upon maturation to plasma cells, B cells down-regulate CXCR5 [140] and CCR7 [141], but have increased sensitivity to CXCL12 (through CXCR4) which regulates plasma cell positioning in the spleen and lodgement in the bone marrow.

Several evidences suggest that chemokines have a role in the development of different biological responses that goes beyond cell recruitment. Chemokines have been shown to play a direct role also in definition of the cytokine milieu during both inflammatory and immune responses as well as in important mechanisms such as hematopoiesis. Thus, chemokines not only support differential leukocyte recruitment, but also directly affect target cell functions. Chip-based gene expression profile analysis in chemokine-activated monocytes revealed that CC chemokines induce specific transcriptional programs in target cells, demonstrating that chemokine effects on target cells include induction of transcriptional events [142].

Chemokines not only attract T cells, but they may also have roles in regulating T cell biology, influencing Th1/Th2 polarization [143]. CCL2 can suppress Th1 responses

and cause an increase in IL-4 (Th2 cytokine) production by activated and memory T cells *in vitro* [143, 144]. The addition of CCL2 to macrophages cultured *in vitro* has been shown to decrease IL-12 (Th1 cytokine) production [145]. CCL2 may therefore promote Th2 polarization both directly and indirectly by increasing IL-4 and decreasing IL-12 production, respectively. In contrast, addition of CCL3 to *in vitro* cultures of activated T cells promoted the development of IFNγ-producing cells and hence Th1 differentiation. Similarly, CCL3, 4 and 5 production by monocyte-derived DC can promote the development of IFNγ-producing cells [146]. However, experiments in mice deficient in the chemokines CCL2 and CCL3 and the chemokine receptors CCR1 and CCR2 resulted less conclusive, and in some instances gave opposite results, depending on the experimental protocols used [147-150]. More work is required to further elucidate the role of chemokines in the differentiation of T cells, and also the contribution of chemokines produced by T cells themselves. This aspect may have implications for the use of chemokine receptor antagonists in the treatment of inflammatory disease.

Chemokines play also an important role in hematopoiesis. Stem cells and hematopoietic progenitor cells in the bone marrow are subjected to the influence of a variety of different cytokines, resulting in either stimulation or inhibition of proliferation. Chemokine receptor expression by hematopoietic progenitor cells may regulate the homing of these cells within the bone marrow during differentiation and maturation, and their mobilization into the circulation. In particular, hematopoietic progenitor cells express CXCR4 and can migrate in response to CXCL12 [151]; therefore, this chemokine/receptor pair plays an important role in the balance between retention and mobilization of progenitor cells in the bone marrow and this function is accomplished through a mechanism of receptor desensitization and downregulation, with CXCL12 being constitutively produced in high amounts in bone marrow. Moreover, the CXCL12/CXCR4 axis can also be important for the retention of hematopoietic progenitor cells in the bone marrow, during B lymphopoiesis; retaining B cell precursors in the bone marrow would enable their regulated differentiation into mature B cells [152]. Beside hematopoiesis, CXCR4 and CXCL12 are important in development and embryogenesis, as clearly demonstrated by knockout mice [153, 154]. Both CXCR4 and CXCL12 deficient mice die in utero, pointing out their role in development and embryogenesis; this may also explain why CXCL12 is such a highly conserved chemokine. These mice have severely reduced numbers of B cell progenitors and myeloid progenitor cells, suggesting that the CXCL12/CXCR4 pair is responsible

for B cell lymphopoiesis and bone marrow myelopoiesis. Surprisingly, T cell lymphopoiesis is unaffected in these mice. They also have severe heart defects, including defective cardiac ventricular septum formation and a disorganized cerebellum. Evidences have been collected showing that mice deficient in either CXCR4 or CXCL12 have defective formation of the large blood vessels supplying the gastrointestinal tract, possibly due to defective regulation of vascular branching and/or remodeling processes in EC [153].

1.1.5. Chemokines in Pathology

The *in vivo* functions of chemokines are not limited to immunity and inflammation; given their broad spectrum activities, it is not surprising that they play a role in several types of human pathologies, including asthma, cardiovascular diseases, transplantation, neuroinflammation, HIV (human immunodeficiency virus)-associated diseases and neoplasia.

Multiple sclerosis (MS) is a demyelinating autoimmune disease mediated by CD4+ T cells specific for one or more autoantigens expressed in the central nervous system, where a variety of destructive inflammatory mediators are produced. Beside auto-reactive T lymphocytes, MS lesions contain a variety of cell types, including monocytes/macrophages and T cells which are subsequently recruited, activate microglia and cerebrovascular endothelium. During active MS attacks, significantly increased levels of CXCL9, CXCL10, and CCL5 are detected in cerebrospinal fluid [155] and CCL2, CCL7 and CCL8 have been found immunohistochemically in MS lesions [156]. Various chemokine receptors are also expressed in MS lesions: CCR2 and CCR5 are found expressed on macrophages, microglia and T cells, and CCR3 is also found on reactive astrocytes [157]. MS patients heterozygous or homozygous for the CCR5D32 allele (which encodes a non-functional form of CCR5) show delayed disease onset of approximately 3 years compared with affected siblings [158]. CXCR3 is expressed by more than 90 % of CD3+ T cells in cerebrospinal fluid, and >99 % of T cells in perivascular accumulations in active lesions [155]. The presence of CXCL9 and CXCL10 in MS lesions may account for the recruitment of CXCR3 positive T cells [159]. CCR5 and CXCR3 may represent a target for therapeutic intervention in MS

Rheumatoid arthritis is characterized by the presence of a mixed inflammatory cell infiltrate into synovium-lined joints, in response to autoantigens. The success of anti-TNF-based therapy has indicated the critical role played by this cytokine in arthritis,

presumably mediated by the induction of chemokines. Synovial fluid from pathological joints contains high levels of CCL2, CCL3, CCL5 and CXCL10 and both synovial-lining cells and leukocytes are the source. Leukocytes are also the target, expressing CCR2, CCR5, CCR2 and CXCR3 [160, 161].

Accumulating evidence indicates that chemokines play a central role in cardiovascular disease and in particular in atherosclerosis, a chronic inflammatory disease of the blood vessel wall, characterized by the accumulation of mononuclear cells. Atherosclerosis is a multifactorial disease, with many risk factors including smoking, hypertension, hypercholesterolemia, family history and diabetes; however, there is consensus on the origin of atherosclerotic plaques from an inflammatory response occurring in the arterial damage, either because of hypercholesterolemia or shear stress. CCL2 has been found to be involved in this pathology. CCL2 and CCR2 deficient mice show 60-85% less arterial lipid deposition than WT mice, in hypercholesterolemia models and this is consistent with the role of CCL2 in leukocyte recruitment. Recent evidence demonstrate that a broad spectrum CC-chemokine blockade reduces atherosclerosis in an Apo-E -/- mice [162, 163]. Another important chemokine in atherosclerosis is CX3CL1 (fractalkine). This chemokine is produced by EC after inflammatory cytokine stimulation and can mediate leukocyte adhesion and infiltration into the vascular wall, as well as NK-cell-mediated endothelium injury. High levels of mRNA for CX3CL1 and other 16q13-chromosome-linked chemokines have been observed in human arteries with advanced atherosclerotic lesions [164]. The ability to recruit leukocytes and its expression in vascular cells strongly suggest a pivotal role for fractalkine in the pathophysiology of atherosclerosis.

Chemokines and their receptors are also involved in rejection of allogeneic transplants. In particular, after an early non specific release of inflammatory chemokines attracting neutrophils and monocytes, CXCR3 and CCR5 ligands appear, several days after the transplants, consisting with their orchestrating the movement of cells involved in acute rejection [165]. Moreover, it has been demonstrated that CX3CL1 expression is enhanced in rejecting cardiac allografts [166]; in addition, the treatment of organ recipients with polyclonal anti-CX3CR1 blocking antibodies markedly prolonged survival of major histocompatibility complex (MHC)-mismatched heart allografts [166]. As far as bone marrow transplant is concerned, CXCR4 and its ligand chemokine CXCL12 are very important. As comprehensible given their role in the balance between stem cell precursor retention and mobilization in the bone marrow, CXCR4/CXCL12

play a role in bone marrow engrafment of CD34+ cells and this may have clinical implications with regard to therapeutic stem cell transplantation.

CD4 is the primary cell surface receptor used by HIV to penetrate T lymphocytes and macrophages. The chemokine receptors CXCR4 and CCR5 function as co-receptors which, along with CD4, allow viral envelope fusion and entry. A range of chemokine receptors have now been shown to have co-receptor activity *in vitro*, including CCR1, 2, 3, 4, 5, 8, 9, CXCR2, 4, 5, 6 and CX3CR1 [167], but so far only CXCR4 and CCR5 have been shown to act as co-receptors *in vivo*, hence X4 tropic and R5 tropic viruses are described. Since chemokine receptors act as co-receptors for HIV entry, endogenous chemokine production can regulate HIV replication. In 1995, Cocchi et al published that CCL3, CCL4 and CCL5 have CD8+ T-cell-derived HIV inhibitory activity [168]. Moreover, individuals homozygous for the D32 allele of CCR5, who are deficient in the cell surface expression of CCR5, can remain uninfected despite exposure to HIV [169, 170]. These observations suggest that chemokines and their receptors, which are involved in HIV infection, are potential targets for the development of new drugs to treat HIV.

Chemokines have also an important role in asthma, a disease detailed described in section 1.3

1.1.5.1 Chemerin/ChemR23 axis in pathology

Elevated levels of active chemerin have been found within the active range (33–358 ng/ml, corresponding to 2–23 nM) in ascitic fluids resulting from ovary and liver cancer, from an ovary hyperstimulation syndrome, as well as in a pool of synovial fluids from arthritic patients. Therefore, it is likely that chemerin represents an important signal involved in the recruitment of ChemR23+ cells during inflammation and autoimmune diseases, and at tumor sites [70].

Zheng et al [171] proposed a possible role for chemerin in the development of skin squamous cell carcinoma (SCC). Various stresses seem to activate prochemerin, which is highly expressed in normal epidermis [79] in the early stages of SCC. The bioactive chemerin could chemoattract antigen-presenting cells (DC and macrophages) to the target sites, resulting in immune responses and the release of cytokines and chemokines, which promote invasive tumors. Later, the transcription of TIG2 (chemerin codifying gene) is inhibited, resulting in the loss of chemerin in SCC.

On the other hand TIG2 has been reported as one of the most differentially expressed genes reported as deregulated (underexpressed) in adrenocortical carcinoma [172, 173] and

has been shown to present excellent diagnostic accuracy for distinguishing benign from malignant adrenocortical tumors [174].

ChemR23 has been found expressed, by immunohistochemistry, in inflammatory cells accumulating in the leptomeninges and in perivascular cuffs of MS lesions whereas chemerin was confined to vascular EC in the meninges and white matter lesions [175]. Additionally, ChemR23 has shown an important contribution in the pathogenesis of EAE, with potential involvement in the regulation of DC dynamics and macrophage accumulation during central nervous system inflammation [176]. A role for chemerin/ChemR23 axis has been suggested in the recruitment of inflammatory cells into the central nervous system in MS probably by the concerted action of other chemokines.

Plasmacytoid DC (P-DC) are normally absent from the skin. However, they accumulate in some inflammatory dermatoses, where they organize local immune responses. The best characterized examples of these conditions are lupus erithematosus (LE) [177] and psoriasis [178].

Psoriasis is a type I IFN-driven T cell–mediated disease characterized by the recruitment of P-DC into the skin [178, 179] . Prochemerin is expressed in all the layers of normal epidermis where might maintain the normal differentiation of epidermal keratinocytes [180]. In the context of psoriasis activated neutrophils and mast cells can convert prochemerin into active chemerin through the release of CG, HLE, and tryptase contributing to P-DC influx to psoriatic skin [181, 182]. Albanesi et al [182] have found that chemerin expression characterizes the early phases of evolving skin psoriatic lesions and is temporally strictly associated with P-DC, supporting a role for the chemerin/ChemR23 axis in the recruitment of P-DC in the early phases of psoriasis development.

Parolini et al [69] found that chemerin is expressed by inflamed EC lining blood vessels in oral lichen planus (OLP), a pathologic condition characterized by the accumulation of NK cells and DC. Additionally they observed that ChemR23 + myeloid and plasmacytoid DC and NK cells colocalize in areas where chemerin was detected, suggesting a role for chemerin in NK cells and P-DC recruitment and colocalization in OLP lesions.

LE is a complex disorder showing a broad spectrum of clinical manifestations, ranging from pure cutaneous form to a severe and progressive systemic disease. The hallmark of LE pathogenesis is represented by loss of tolerance to variable nuclear

antigen which leads to tissue deposition of immune complex followed by widespread inflammation and tissue damage. Recent evidences point to a potential role of P-DC in the pathogenesis of LE via production of type I IFN, a key protein in the pathogenesis of this disease [177, 183-185]. P-DC blood content resulted decreased in systemic LE patients; however, these cells have been found to infiltrate extra-lymphoid lesional tissues and to locally produce type I IFN [186-188]. Vermi et al [71] have reported a significant induction in chemerin expression in LE skin biopsies (this positivity was restricted to EC of dermal blood vessels and in the granular layer keratinocytes) with the concomitant presence in close proximity of ChemR23+ P-DC. This finding sustains the role of chemerin as cutaneous P-DC chemoattractant in LE.

1.2. Dendritic cells.

Dendritic cells (DC) are a heterogeneous population with unique properties in antigen presentation, and play a crucial role in inducing T and B cell responses as well as immune tolerance [135]. DC are bone marrow derived and migrate through the blood stream to peripheral tissues throughout the body, where they reside as immature DC with high ability to capture antigens. Immunological as well as inflammatory danger signals induce a functional maturation process into DC, transforming them from poor immunostimulatory cells that function as sentinels of the surrounding environment into potent T cell stimulators. The current paradigm of DC immunobiology states that maturation is associated with DC mobilization from the periphery to the T cell rich areas of lymphoid organs [189]. There, DC present the processed antigens in the context of MHC class I and class II molecules and elicit specific T cell responses [190, 191]. Recent evidences indicate that migration of DC into the lymph nodes also occurs in normal conditions and may serve to tolerize T cells against self and non-dangerous antigens [192]. However, the mechanisms involved in the mobilization of DC in the steady-state need to be better elucidated.

DC are very rare cells both in tissues and in biological fluids, and this aspect has made difficult the study of their functional properties, since the first description of this cells by Steinman and Cohn in 1973 [193]. However, in the last 15 years several procedures have been developed to generate large numbers of fully functional pure human and mouse DC from circulating and bone marrow precursors, including peripheral blood CD14+ monocytes and CD34+ stem cells. Using these *in vitro*

cytokine-driven populations, much work has been accumulated in the understanding of DC immunobiology.

DC constitute a heterogeneous population in terms of origin, morphology, phenotype, function and homing properties [189, 194, 195]. Since the nomenclature of circulating DC has become quite confusing because of the use of different antibodies for their identification, very recently, a group of experts drafted a nomenclature proposal under the auspices of the International Union of Immunological Societies and the World Health Organization [196]. In blood a subdivision into 3 subsets is suggested, that is, P-DC and 2 types of myeloid DC (M-DC).

P-DC in tissues were originally described as T-associated plasma cells, plasmacytoid T cells, or plasmacytoid monocytes. P-DC were found to have the capacity to produce high levels of interferon α (IFNα) in response to viruses, and these cells were named natural type 1 IFN-producing cells [197]. In human the CD68 marker can discriminate the P-DC from the 2 types of M-DC, but this requires combination with additional markers because CD68 is also strongly expressed on monocytes [198]. While CD123 expression is only incrementally higher on P-DC compared with monocytes, CD303 is a marker with good signal-to-noise ratio for the P-DC [199]. In mice P-DC selectively express Siglec-H (Sialic acid-binding immunoglobulin-like lectin H) [200] and bone marrow stromal antigen (BST2) [201]. However, expression of the BST2 antigen on plasma cells under steady state conditions and on many other cells after induction by type I IFN needs to be considered [196]. P-DC are mainly found in blood in the steady-state, from where they can enter into the lymph nodes upon inflammatory stimuli. P-DC are poor antigen presenting cells (APC) but upon activation they produce large amounts of cytokines, particularly type I IFN, that affect NK, B and T cell activity, and in this way can regulate inflammation and link innate and adaptive immunity [202].

In human blood, both the CD1c+ and CD141+ M-DC express myeloid markers CD13 and CD33, suggesting their direct derivation from the myeloid lineage. For proper identification of the CD1c+ DC in blood, CD19+CD20+B cells need to be excluded, because the latter cells also show strong expression of the CD1c molecule. A fraction of the CD1c+ blood DC expresses low-level CD14 [203]. No comparative data between CD14+ and CD14- subsets are available, however, in support to their uniqueness functional studies have shown a specific pattern of chemokines production in these cells. The CD141+DC represent a very minor subset of blood leukocytes. Costaining with CD14 is recommended to exclude low-level signals from monocytes.

These CLEC9A+CD141+ cells (but not CD1c+blood DC) are also major producers of IFN-β and cross-present antigen for CD8 class 1–restricted cytotoxic T lymphocyte responses in response to TLR-3/CD283 ligation. These and their other properties suggest that they are homologous to the mouse CD8α + DC subset [204]. It is recommended that the CD1c+ and CD141+ blood DC are defined separately and are not addressed as one population of myeloid blood DC [196]. Looking at M-DC the CD11c+CD11b+CD45RA- cells identified in mouse blood share features with the splenic CD8- DC subset [205], and they may be homologous to human CD1c+ myeloid blood DC [206]. The CLEC9A+ DC in mouse blood also stain for CD24 and may thus be representative for the CD8+splenic DC subset. Because anti-CLEC9A also strongly stains the human blood CD141+ cells [207] and because human CD141+ blood DC and mouse CD8+ spleen DC share specific transcriptional signatures [206], the CLEC9A+ mouse blood DC are potential homologues of the human CD141+CLEC9A+ myeloid blood DC.

M-DC constitute discrete population in tissues that play specific functions and express distinct markers depending on the anatomical location. Langerhans cells (LC), that were described more than a century ago and were later on recognized as the DC of the epidermis, express langerin and E-cadherin, which are down-regulated during maturation. However, LC do not express CD11b [208, 209]. Dermal DC localize at the subepithelial tissues of the dermis and specifically express CD13, and similar to the DC of the epithelial mucosa and the interstitial DC present in the solid organs, they express CD11b, mannose receptor and DC-SIGN [195]. All conventional DC express the β_2 integrin CD11c and CD1a, and except LC, also express the other CD1 isoforms [208]. Furthermore, DC are also observed in the afferent lymphatic vessels, where are called veiled cells. They represent DC emigrating from peripheral organs into lymphoid tissues [210].

Splenic DC are the best studied lymphoid organ DC. They are divided into different subpopulations based on the expression of classical lymphoid and myeloid markers. The main population is represented by the CD8α-CD11b+F4/80+DEC205-/low33D1+ cells, which are located mainly within the marginal zone of the spleen, while the CD8α+CD11b-F4/80-DEC205+33D1- cells are located mainly in the T cell-rich paracortical areas of the spleen and are termed interdigitating DC [195]. Regardless of the expression of myeloid or lymphoid surface markers, both populations can arise from a common precursor, revealing a high degree of plasticity that is a peculiar characteristic

of DC development [211, 212]. Lymph nodes contain fairly similar DC populations. In addition, a distinct subset with phenotypical markers of interstitial DC has been described [213-215]. This population most likely is represented by DC emigrated via the afferent lymphatics from their associated peripheral tissues. In fact, they display all the characteristics of a mature phenotype. Apart from this latter population, the majority of DC in the spleen and lymph nodes are in an immature state [216], a fact that apparently contradicts the current paradigm which states that DC migrate to secondary lymphoid organs upon maturation. Moreover, they have the capability to mature and acquire the phenotypic features of mature DC [216-218]. Endovenous injection of LPS has been shown to promote phenotypical as well as functional DC maturation in the spleen, as assessed by down-regulation of antigen processing capacity and up-regulation of immunostimulatory properties, a process that is accompanied with the migration of most splenic DC from the marginal zone to the T cell areas [217]. Unlike *in vitro*-generated immature bone marrow-derived DC (BMDC), DC isolated from the spleen do not respond to inflammatory CC chemokines [219]. Conversely, ex-vivo maturated DC subsets express CCR7 and migrate to its ligands CCL19 and CCL21 [219]. There is still not consensus about functional distinction of these two subsets [220]. Splenic CD8α- DC seem to be the most immature DC cell population in this organ [221]. CD8α- DC have a higher endocytic and phagocytic capacity than CD8α+ DC, which correlates with its preferential localization at the marginal zone and the subepithelial dome, which are typical antigen-uptake zones . On the other hand, CD8α+ DC are uniquely able to cross-present antigens associated to MHC I [222]. During *in vivo* T-cell responses, CD8α- DC mainly induce Th2 cell responses, whereas CD8α+ DC strongly elicit Th1 cell responses [223-226]. However, *in vitro* studies and *in vitro* stimulation followed by *in vivo* transfer experiments have demonstrated that under appropriate conditions both subsets can mediate all DC effector functions with the same efficiency [227, 228]. At present it is not known if this functional plasticity exists *in vivo* [220].

The above findings suggest a fundamental similarity between resident lymphoid organ DC and peripheral tissue interstitial DC. Immature DC in lymphoid organs may also act as sentinels for soluble or shed antigens arriving via the blood or the lymph. The latter is further supported by the recent characterization of the conduit system that transports soluble antigens from the afferent lymph to the resident DC in the lymph nodes [229]. Alternatively, lymphoid DC could receive antigens carried on by migratory

DC from the periphery resulting in amplification of the response [230]. Moreover, they may contribute to peripheral tolerance [231, 232]. Their unresponsiveness to inflammatory chemokines, although requiring further confirmation and extension of the studies to the known immature DC chemoattractants, may indicate that they have already achieved the proper tissue localization for these functions.

1.2.1. Dendritic cell activation

Maturation is a complex process that leads to differentiation of DC, by reducing their antigen uptake capability and transforming them into potent T cell stimulators. DC prime T cell responses in secondary lymphoid organs such as lymph nodes, spleen and mucosal-associated lymphoid tissues, where naïve T cells continuously recirculate seeking their cognate antigens. Functional maturation culminates with DC localization in T cell-rich areas of lymphoid tissues, where DC present peptide antigens acquired in the periphery in the context of MHC to circulating T cells. So, in order to mount an effective immune response, a coordinated regulation of DC migratory and functional properties must occur.

Maturation is induced by microbial, tissue damage as well as immune signals. DC express a large array of receptors for pathogen-associated molecular patters (PAMPs) and damage- associated molecules patterns (DAMP). DAMPs include heat shock proteins (HSP), high mobility group box 1 (HMGB1) protein, uric acid and adenosine triphosphate (ATP), all mediators released upon damage to the tissues by pathogens, trauma, vascular damage, or necrosis [233, 234].

The nature of an antigen can instruct the DC to influence the polarization of T cells by triggering specific receptors during uptake [235, 236]. DC recognize a wide array of inhaled antigens through expression of ancient pattern-recognition receptors (PRR) such as toll like receptors (TLR), NOD-like receptors (NLR), and C-type lectin receptors that recognize motifs on virtually any inhaled pathogen, allergen, or substance [237]. TLR ligands comprise a variety of microorganism-derived molecular structures, such as bacterial lipopolysaccharide (LPS), unmethylated cytosine-phosphate-guanosine (CpG) motifs in bacterial and viral deoxyribonucleic acid (DNA), and viral double-stranded ribonucleic acid (RNA). On the other hand, the binding of endogenous ligands such as heparan sulfate, hyaluronic acid and heat-shock protein 60 by TLR has been reported to be a signal of damage in case of abnormal release and accumulation of those products in

tissues [238]. Distinct TLR ligands provide distinct activation status and cytokine production patterns, resulting in the induction of differential immune responses.

In vitro-generated immature DC also express the inhibitory TLR family member SIGIRR (single immunoglobulin IL-1R)/TIR8, which is down-regulated during maturation [239]. Bone marrow-derived DC from TIR8-/- mice show increased responsiveness in terms of cytokine production to some but not all TLR agonists. It would be interesting to evaluate if DC subpopulations *in vivo* differentially express TIR8 or if its expression is related to the local microenvironment where they are located. It could be hypothesized that immature DC present in mucosal tissues like the lung or the intestine, which are exposed to harmless antigens, would profit from inhibitory receptor expression that are turn off once the cells are activated by pathogens or inflammatory stimuli.

Host-derived inflammatory stimuli able to induce DC maturation include molecules such as TNF-α, IL-1, IL-6, INF type I and II, and the T cell ligands CD40L and Receptor Activator for Nuclear Factor κ B Ligand (RANKL) [55-60]. Multiple activating signals coexist *in vivo*, and their effects on DC activation and function are not merely additive. In this regard, TLR triggering and CD40-CD40L engagement have been reported to be necessary for fully DC capability to elicit effective T cell responses [240, 241]. Simultaneous triggering of TLR can have synergistic effects. In addition, recent evidences suggest that non-TLR pattern-recognition receptors can also cooperate to optimize the outcome of the maturation process. DC-SIGN is a DC-specific C-type lectin that binds a broad panel of pathogens, including viruses, bacteria and *Schistosoma mansoni* parasite [242-244], and endogenous molecules like tumor glycoproteins [245]. DC-SIGN engagement of some pathogens, like *Mycobacterium tuberculosis* and *Helicobacter pilori*, appears to inhibit DC maturation as a strategy to favour pathogen survival [246, 247]. Importantly, DC-SIGN is able to mediate cellular adhesion between DC and activated neutrophils through the specific binding to Mac-1, which would allow neutrophils to induce DC maturation through the paracrine production of pro-inflammatory cytokines [248]. Dectin-1 is a C-type lectin specific for β-glucan that is highly expressed on DC and macrophages. In addition to promote β-glucan-containing microbes phagocytosis, it synergizes with TLR2 to produce TNF-α, IL-12 and reactive oxygen species. Conversely, engagement of the endocytic mannose receptor by a specific mAb (monoclonal antibody) and by some natural ligands is able to elicit an anti-inflammatory secretory program characterized by high production of IL-10, IL-

1Ra, IL-1R type II, and low level of IL-12 [249, 250]. It has been recently shown that scavenger receptors SREC-I (scavenger receptor expressed by EC) and LOX-1 (lectin-like oxidized LDL receptor-1) collaborate with TLR2 in cell responsiveness to outer membrane protein (OmpA), a major component of the outer membrane of Enterobacteriaceae [63]. SREC-I and LOX-1 bind OmpA while TLR2 transduces the signal. This mechanism may be functional in DC since they express LOX-I and probably SREC-I [251]. TLR2-activated functional program includes the production of pentraxin 3 (PTX3), a soluble pattern recognition receptor that in turn binds OmpA and amplify the inflammatory response [252 253]. DC produce high amounts of PTX3 upon activation by different TLR agonists, which is further enhanced by IL-10 and INF type I and inhibited by INFγ and the anti-inflammatory agents dexamethasone, Vitamin D_3 and prostaglandin E2 (PGE_2) [253].

DC share most features of antigen processing and presentation with other APC [254]. Exogenous protein antigens are processed in endosomes into peptides and loaded onto MHC class II that are displayed on the cell surface for recognition by antigen-specific T cells. Mature DC are ideally suited for presentation of the antigen they have captured at the time of activation. This is possible because maturation triggers the up-regulation of MHC II-complexes expression at cell membrane level while the overall antigen uptake capacity is inhibited, precluding the processing of antigens encountered after on. This property ensures that the antigens presented by a mature DC match with the environmental situation that triggers its maturation, and therefore is crucial for the effective transduction of information that DC undertake from the periphery to the lymphoid organs. Immature DC express significant levels of MHC II with a short half-life. Maturation down-regulates MHC II endocytosis and, after a transient increase, down-regulates MHC II synthesis, thereby preventing turnover of MHC II- peptide complexes and promoting their stable expression at the cell surface [218, 255, 256].

DC also express significant quantities of MHC I that is further up-regulated upon maturation [257] and therefore can present their own endogenous antigens after they have been degraded into peptides in the cytosol by the proteosome system. In addition, certain exogenous protein antigens may also be processed by DC in the context of MHC I, a unique DC process known as cross-presentation, that allows DC to cross-present antigens to cytotoxic CD8+ T cells [258-260]. Moreover, DC can present in the surface lipid moieties associated with CD1 molecules, a family of receptors structurally related with MHC I [261].

In addition to MHC, mature DC strongly up regulate the expression of adhesion as well as co-stimulatory molecules that are involved in the formation of the immunological synapse, an area encompassing sites of contact between DC and T cells. Up-regulated molecules include the B7 family members CD80 and CD86, CD40 and OX40L. [135, 189, 262, 263]. As mentioned before, maturation induces DC to secrete a distinct set of cytokines that help to determine the type of ensuing immune response, depending on the type of maturation stimulus [264, 265]. In addition, TLR-agonist-, but not CD40L-activated DC produce reactive oxygen radicals that are important for intracellular microbe killing [266]. Accumulating evidences indicate that the phenotypic and functional changes undergone by maturing DC are also associated with the specific population of activated DC [267, 268].

P-DC express MHC II and the costimulatory molecules CD80 and CD86 and are able to present antigens to CD4+ T cells and cross-present antigens to CD8+ T cells, although less efficiently than classical DC [269]. Besides producing type I IFNs, P-DC also secrete IL-12, IL-6, TNF-α, and inflammatory chemokines. Through secretion of these cytokines and type I IFNs, P-DC may influence both innate and adaptive immune responses. Type I IFN and IL-12 promote multiple T-cell functions including long-term T-cell survival and memory [270, 271], Th1 polarization of CD4+ T cells [272], CD8+ T-cell cytolytic activity, and IFN-γ production [271]. Moreover, type I IFN and IL-12 increase NK cell-mediated cytotoxicity and IFN-γ production *in vitro* and *in vivo* [273]. IFN-α and IFN-β also promote the differentiation and maturation of DC allowing them to effectively present and cross-present antigen to naive T cells [274]. By producing IL-6 and type I IFN, P-DC can also induce the differentiation of B cells into immunoglobulin-secreting plasma cells [275] and instruct plasma cells to preferentially secrete IgG rather than IgM. By producing chemokines such as CXCL9 (MIG), CXCL10 (IP-10), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) [276], mouse and human P-DC can attract activated CD4+ and CD8+ T cells to sites of infection.

1.2.2. Dendritic cell recruitment to peripheral tissues

Classical DC are present in non-lymphoid peripheral tissues in an immature state. Immature DC are the immunological sentries of peripheral tissues and their recruitment at sites of inflammation in response to chemotactic stimuli is critical for optimal immune response. The expression and regulation of functional chemotactic receptors account for the different distribution of DC subsets *in vivo*. Immature DC express a

unique repertoire of inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, CCR6) [132, 277-279]. These receptors bind a pattern of "inflammatory" chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL20 that are produced at peripheral sites of infection and immune reaction. In addition, immature DC also express functional CXCR4 [280]. Since CXCL12, the CXCR4 ligand, is constitutively expressed in both lymphoid and non-lymphoid tissues, this chemokine may play a role in the recruitment of DC in such tissues. Similarly, myeloid blood DC can migrate in response to a wide array of inflammatory chemotactic agonists [281, 282].

It is generally believed that DC precursors in the peripheral blood migrate into peripheral tissues and differentiate to become immature DC. Although it is conceivable that the same classes of molecules that regulate DC migration in inflammatory situations also direct the migration of these cells under normal conditions (e.g. adhesion molecules and chemoattractants), the mechanisms that regulate the homing of DC or their precursors, in steady-state conditions need to be better elucidated [279, 283]. The relevance of chemotactic receptors in DC travelling *in vivo* has been clearly documented in mice lacking PI3Kγ. PI3Kγ is located downstream seven-transmembrane chemotactic receptors and plays a non-redundant role in cell migration in response to chemotactic agonists [284]. 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 [285]. Therefore, PI3Kγ may represent a new valuable target to control inappropriate activation of specific immune responses.

A number of studies have established a role for CCR2 in the localization of LC precursors. It is of note that transgenic mice overexpressing CCL2, under the keratin promoter, have local accumulation of cells with DC morphology in the basal layer of the epidermis [286]. However, LC in the skin of CCR2-/- mice is normal and CCL2 expression has only been demonstrated in inflamed skin, indicating that it may not play a role in steady-state conditions. Following differentiation, LC precursors start to express CCR6 and respond to the cognate ligand CCL20 [287-289]. Since CCR6-/- mice have a normal numbers of skin DC [290], and CCL20 is principally up-regulated in inflammation, it is likely that the CCR6/CCL20 axis plays a role mainly in inflammatory skin disorders that are characterized by the expression of CCL20 by inflamed keratinocytes. CCL20 plays also a role for the homing of DC to mucosal

surfaces. In particular, CCL20 is constitutively expressed in murine intestinal Peyer's patches, and CCR6-/- mice lack for DC in the subepithelial dome of Peyer's patches within the intestinal mucosa, which correlates with a defect in humoral immune response to oral antigens in these mice [290, 291]. Recently, Shaerli and colleagues have proposed that CXCL14, may be involved in the homeostasis of CD14+ LC precursors [292]. CXCL14 is expressed in the superficial dermal plexus as well as within epidermis in normal human skin. It vitro, CXCL14 has a restricted chemoattractant ability for CD14+ DC precursors and not other myeloid or lymphoid cell subpopulations. Using an artificial epidermal model that closely resembles normal human skin and spontaneously expresses CXCL14 , they demonstrated that CD14+ DC precursors migrate into the suprabasal layer of keratinocytes, where they indeed acquired LC phenotype and function [292]. Further work with gene deficient mice is needed to mechanistically demonstrate its involvement in the recruitment of LC precursors. It had been previously reported that CXCL14 is chemotactic for monocytes [293] and immature DC [294]. CXCL14 is also express in other epithelial tissues, such as intestinal tract and the airways [293, 295], suggesting a broader role for this chemokine in mucosal DC homeostasis

Under inflammatory conditions an array of chemokines are expressed by resident cells and presented by activated EC, these include CCL2, CCL5, CCL7 CCL13, CCL20 and CCL22 [15, 296]. DC near the site of immune reaction represent also an important source of inflammatory chemokines. Pro-inflammatory mediators, such as TNF-α and IL-1 produced by tissue macrophages further contribute to the expression of inducible chemokines by virtually all cells resident in the area. As previously mentioned, immature DC express a repertoire of chemokine receptors that can interact with inflammatory chemokines. Interestingly, immature DC constitutively produce high levels and respond to the orphan human chemokine CCL18 [297]. CCL18 levels are high in human plasma and may contribute to the physiological homing of DC. In addition, CCL18 production has been demonstrated in several diseases, including various malignancies and inflammatory disorders, and may have a role in immature DC retention at the tissues in these conditions [298].

Immature DC express a wide variety of receptors for chemotactic agonists different from chemokines. These chemotactic stimuli are rapidly produced (within minutes) at the site of inflammation and represent an early signal for the recruitment of DC or their precursors, that can precede chemokines action. Early work documented that myeloid immature DC, but not mature DC, express functional receptors for fMLP and

chemotactic components of the complement cascade (e.g. C5a) [133]. The expression of C5a receptors was also confirmed *in vivo* in LC [299]. The formyl peptide receptor family includes multiple proteins, two of them FPR and FPR3 were found to be expressed by immature DC [300]. FPR is the fMLP receptor, whereas FPR3 is activated by the WKYMVM hexapeptide originally identified from a combinatorial peptide library for its ability to stimulate phophoinositide hydrolysis in lymphocytes [301]. Recently, F2L an endogenous high affinity ligand for FPR3 was isolated from porcine spleen [302]. F2L is a highly conserved acetylated 21-aminoacid peptide derived from the cleavage of the N-terminus of the intracellular heme-binding protein that activates FPR3 in the low nanomolar range. The acetylation of the N-terminal methionine of F2L is a modification that is reminiscent of the formylated methionine of bacterial and mitochondrial peptides active on FPR. F2L is able to induce calcium mobilization and chemotaxis of monocytes and immature DC. Therefore, F2L could be involved in the response of these cells to infection, inflammation or cell death. Recent evidences indicate that DC are attracted by dead or dying cells [303]. The first self-molecules shown to represent danger signals were the HSP gp96 or hsp70. These proteins are released from necrotic cells and are able to potently activate antigen-presenting cells [304]. Intracellular nucleotides released under conditions of hypoxia, ischemia, inflammation or mechanical stress, and crystalline uric acid derived from dead cells, were also shown to stimulate DC [305, 306].

Human and mouse DC express functional receptors for platelet activating factor (PAF) [64, 65]. Since PAF is synthesized at the site of inflammation following PLA2 activity, it is conceivable that it functions to recruit DC to the pathological site. Using a FITC (fluorescein isothiocyanate) skin painting assay, it was recently reported that the migration of LC to skin draining lymph nodes is increased in PAFR-/- mice and in WT animals administered with a PAFR antagonist. Similarly, the PAFR antagonist promoted the egression of LC from human skin explants, *in vitro* [307]. These results suggest that PAF normally acts as a negative regulator of DC mobilization from skin to lymph nodes. The retention ability of PAF into peripheral tissues may be relevant in the retention of DC at pathological sites, such as atherosclerotic plaques [307].

One of the effector's mechanisms of innate immunity relies on the generation of antimicrobial substances; these include inorganic cytotoxic molecules (e.g. hydrogen peroxide and nitric oxide) and antimicrobial peptides, like defensins and cathelicidins. The latter, in addition to their antimicrobial function, possess chemotactic activity for immature DC. Human α-defensins and β-defensins are chemotactic for immature, but

not mature DC [308]. The chemotactic activity of β-defensins is mediated by the interaction with CCR6, the receptor for CCL20. Although the migration in response to α-defensins is inhibited by PTX, the identity of the receptor for these proteins has not yet been identified [308]. Sharing a receptor with chemotactic factors is not unique to β-defensins, because LL-37, an antimicrobial peptide, promotes the migration of human neutrophils, monocytes and T lymphocytes through the interaction with FPRL1 [309]. Eosinophil-derived neurotoxin (EDN), a protein belonging to the ribonuclease A superfamily was found to be a selective chemotactic agonist for DC. Its effect is sensitive to the action of PTX; however, the nature of the chemotactic receptor engaged on DC membrane is still unknown [310].

P-DC are confined to primary and secondary lymphoid organs, a characteristic consistent with the fact that P-DC from human blood express an extensive profile of chemotatic receptors, however, with the exception of CXCR4 these receptors are apparently non functional [194]. Although unable to directly induce P-DC migration, CXCR3 ligands increase P-DC chemotactic response to CXCL12 [311]. Furthermore, CXCR3 ligands are fully competent in inducing P-DC adhesion and migration when immobilized on heparan sulphates expressed by EC [312]. Two recent reports showed that P-DC purified from patients with chronic hepatitis C, or exposed *in vitro* to IFN-α, acquire the ability to respond to CCR2, CCR5 and CXCR3 ligands [313, 314]. These results suggest that under appropriate stimulatory conditions, additional chemokine receptors may be involved in human P-DC recruitment. P-DC infiltrate have been found in peripheral tissues associated with diverse pathologies such as autoimmune diseases (e.g. LE disease, psoriasis and rheumatoid arthritis) [187, 315-317 318], allergic diseases (e. g. contact dermatitis and nasal mucosa polyps) [318] and tumors [319, 320].

In addition to chemokines, adenosine and F2L have the ability to induce P-DC migration through the engagement of the adenosine receptor A1 and FPR3 (the formyl peptide receptor formerly known as FPRL2), respectively [321, 322]. It was also reported that like M-DC, P-DC have functional receptors for the anaphylatoxins C3a and C5a [323]. Finally, IL-18 induces the migration of P-DC [324]. All these data suggest that, in addition to chemotactic cytokines, signals associated with inflammation and tissue damage may contribute to recruitment of P-DC to pathological tissues.

1.2.3. Dendritic cell recruitment to secondary lymphoid organs

In order to activate T lymphocytes, maturing DC migrate from the peripheral tissues to the draining lymphoid organs. As discussed before, inflammatory chemokines, acting through chemokine receptors such as CCR1, CCR2 and CCR5, function as signals to localize immature DC in inflammatory sites. Maturation is associated with downregulation of receptors for inflammatory chemokines and *de novo* expression of the chemokine receptor CCR7 that renders the cells responsive to CCL19 and CCL21, two chemokines that are expressed at the luminal side of high EC and in the T cell rich areas of secondary lymphoid organs, like tonsils, spleen and lymph nodes [325-327]. Within the T cell area, CCL19 is expressed by mature interdigitating DC, whereas CCL21 is expressed by stromal cells [328]. The "DC-chemokine receptor switch" paradigm is thought to be responsible for the migration of DC from the periphery to the draining lymph nodes. Crucially, the same signals that promote DC maturation virtually trigger the chemokine receptor switch, such as IL-1, TNF and LPS [132, 327, 329, 330]. Remarkably, CCR7 can also bee induced in partially-matured DC, like DC exposed to apoptotic cells, or even in steady-state conditions, as demonstrated for skin DC; this mechanism is thought to be important to allow DC to carry on self-antigens to the lymphoid organs for tolerance induction [331, 332].

In vitro exposure of DC to LPS, IL-1, TNF, and CD40 ligand, induce a rapid (< 1 h) inhibition of chemotactic response to CCL3, CCL4, CCL5, CCL7, C5a and fMLP [137, 327, 329, 333, 334]. 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 [137, 329]. As previously observed in phagocytic cells[335, 336], during maturation inhibition of chemotaxis is followed, with a slower kinetics, by the reduction of membrane receptors and by the down-regulation of mRNA receptor expression [327, 329]. Concomitantly, the expression of CCR7 and the migration to CCL19 and CCL21 is strongly upregulated, with a maximal effect at 24 h.

CCR7 expression by DC was shown to be required for the entry of DC into lymphatic vessels at peripheral sites both in steady state and inflammatory conditions [331, 337]. 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 [331]. During inflammation, the entry of DC into lymphatic vessels is boosted

by the up-regulation of CCL21 on lymphatic EC. 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 [337].

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. It has been reported that LC up-regulate CD44 and the integrin $\alpha_V\beta_3$ which are receptors for osteopontin, an important factor in LC migration to draining lymph nodes [338]. More recently it was proposed that CCR8 and its cognate ligand CCL1 are involved in the migration of mouse monocyte-derived DC out of the skin [339]. *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 the entry of DC into the lymphatics by regulating the entry of the afferent DC in the subcapsular sinus of the lymph nodes [339]. Mature DC are also known to express chemotactic receptors other than CCR7, although their biological relevance is still unclear. Earlier studies have shown that the expression of CXCR4, the CXCL12 receptor, is retained during DC maturation and mature monocyte-derived DC were shown to migrate in response to CXCL12 [137, 334]. However, blood DC matured *in vitro* apparently do not express functional CXCR4 [194].

Mice defective in β2 or α6 integrin have showed a reduced ability in the migration of cutaneous DC to the draining lymph nodes, whereas β3 integrins appear to be not important [340, 341]. Similarly, cutaneous DC from CD47 (integrin-associated protein)-deficient mice show an impaired migration to draining lymph nodes [342]. *In vitro*, mature BM-DC from these mice showed impaired migration to CCL19 despite normal expression of CCR7. CD47 is an ubiquitous G protein-coupled 5 transmembrane receptor that interacts in *cis* with β1 and β3 integrins and promotes cell activation [343]. CD47 is also a ligand of SIRPα (signal regulatory protein α), a glycoprotein expressed in certain DC subsets. Interestingly, SIRPα-deficient mice showed reduced migration of LC to the draining lymph nodes [344]. Conversely, DC generated from mice defective for JAM-A, showed a selective increase in their capacity to transmigrate across lymphatic EC monolayers *in vitro*. *In vivo*, JAM-A-/- mice have an enhanced migration of skin DC to lymph nodes and an increased response in a contact hypersensitivity model [345]. In the same way, *in vivo* DC migration was increased in SPARC (secreted protein, acidic and rich in cysteine) null mice [346]. SPARC is a calcium-binding matricellular

glycoprotein that binds a range of extracellular matrix components. The facilitated migration of DC in SPARC$^{-/-}$ mice was associated to an increased ability to mount an antigen specific immune response [346]. Consistently with the notion that the interaction with extracellular matrix components may represent an obstacle for the migration of DC, MMP-9 and –2 are required for the migration of skin DC *in vivo*. MMP-9-/- mice present a severe defect in the egression of LC from skin ex-vivo and *in vivo* [347], and for the recruitment of DC into the airways of antigen-sensitized mice [348]. CD44 is a heterogeneous multifunctional protein that functions as a major cell surface receptor for hyaluronate, an extracellular matrix component. CD44 isoforms are differentially modulated during DC maturation and participate in their migration out of the skin and in the induction of the sensitization phase of contact hypersensitivity response [349].

Overall these findings provide a model for DC trafficking in which activation of inflammatory chemokine receptors and the regulation of adhesion molecules and protease secretion, function as signals to localize immature DC or their precursors to peripheral tissues. After antigen 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 chemokines expressed in lymphoid organs, where priming of T lymphocytes takes place.

In homeostatic conditions P-DC are not present in peripheral tissues, been present mainly in secondary lymphoid organs, where migrate directly from the blood compartment, across HEV [350]. Indeed, P-DC express lymph node homing molecules such as L-selectin and PSGL1, the counter-ligand of P- and E-selectins [312]. In addition, P-DC express CXCR4, the receptor for CXCL12, which is a homeostatic chemokine expressed by HEV [350, 351]. L-selectin and CXCL12 are apparently central in inducing P-DC migration, as shown by the reduced number of P-DC in the secondary lymphoid organs of mice defective for L-selectin and DOCK2 (dedicator of cytokinesis), a molecule involved in CXCR4 signalling in P-DC [352]. Under reactive conditions, additional molecules are involved in mouse P-DC homing to lymph nodes, such as E-selectin and the chemokine receptors CCR5 and CXCR3 [351, 353]. Consistent with *in vivo* data, mouse P-DC migrate *in vitro* in response to CCL3 and CCL5, and to CXCL9 and CXCL10, the respective CCR5 and CXCR3 ligands. CCR7 is upregulated following

activation, and it's likely to be involved in mature P-DC migration through CCL19 and CCL21 presented by HEV.

1.2.4. 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 signalling level [194, 354]. For instance, the simultaneous exposure of DC to maturation factors and anti-inflammatory cytokines (e.g. IL-10) uncouples inflammatory chemokine receptors from chemotaxis and converts them in scavenging chemokine receptors [354]. Recent findings revealed that eicosanoids, such as cysteinyl leukotrienes (cysLT) and PGE_2 regulate CCR7-dependent migration of DC to lymph nodes [355, 356]. Experimental evidence on the role of cysLTs in DC migration emerged from studies in mice lacking the lipid transporter multidrug resistance protein 1 (MRP1) and by the use of MRP1 blocking antibodies [357, 358]. In the absence of MRP1 the migration of epidermal DC to the draining lymph nodes was impaired and the exogenous administration of LTC4 or LTD4 could rescue the defect. DC express the cysLT receptor CysLT1, and *in vitro*, cysLT promoted DC migration in response to the CCR7-ligands CCL19 and CCL21. Therefore, the MPR1-mediated efflux of cysLT and the autocrine or paracrine activation of cysLTR promote the migration of maturing DC. More recently, two studies have addressed the role of cysLT on DC functions, in particular the modulation of the cytokine profile and the antigen presentation capacity of DC [359, 360]. They have shown that inhibition of cysLT signalling suppresses antigen-inhalation-induced eosinophilic lung inflammation but did not explore the lymph nodes homing capacity of lung DC. However, Randolph GJ and colleagues have reported that airway DC migration to the mediastinal lymph nodes is not modulated in MRP1-deficient mice, suggesting a tissue-specific regulation [361].

FTY720 is a synthetic derivative from ISP-1 (sphingosine-like immunosuppressant 1), a compound from traditional chinese medicine. FTY720 was recently shown to prolong allograft survival by increasing the migration of T lymphocytes to CCR7 ligands and by promoting their sequestration in secondary lymphoid organs [362, 363]. FTY720 is phosphorylated *in vivo* to its active metabolite FTY720-P that is analogous to S1P and acts through the S1P (1-5) receptors [364]. SP1 was shown to induce the selective migration of human immature DC [365] and to inhibit the production of Th1 cytokines [366, 367]. Conversely, in the mouse system, both immature and mature DC were

reported to migrate to S1P [368], while in another study only mature DC were responsive [369]. However, more systematic studies are needed to demonstrate that these differences are not simply due to the DC tissue origin or culture conditions

PGE_2 modulates multiple aspects of DC biology, such as maturation, cytokine production, T cell activation and apoptosis [356]. Furthermore, PGE_2 promotes the migration of mature human monocyte-derived DC to the CCR7 ligands CCL19 and CCL21 [370, 371]. The effect of PGE_2 on these cells is mediated by two of the four PGE_2 receptors, namely prostaglandin E receptor (EP) 2 and 4 and the cAMP (cyclic adenosine monophosphate) pathway. Interestingly, blood M-DC, matured *in vitro*, did not require PGE_2 for an optimal migration in response to CCR7 ligands [371]. These results suggest that the coupling of CCR7 to chemotaxis is regulated by the state of activation/maturation of DC. The importance of PGE_2 for DC migration has been highlightened *in vivo* by the use of mice that are genetically defective for EP4 [372]. EP 4 -/- mice displayed a reduced migration of skin LC 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 LC migration was further confirmed in WT mice by the use of an EP4 antagonist, and correlated with an impaired induction of contact-type hypersensitivity responses [372]. PGE_2 is secreted by monocytes, macrophages, fibroblasts activated by inflammatory stimuli [373]. This suggests that immature DC are exposed to PGE_2 in situ at the site of inflammation. Recently, it has been reported that nitric oxide can also give a second signal to promote mature DC migration to the CCR7 chemokine CCL19 [374].

PGD_2 is released by mast cells during allergic response that interact with two different receptors, the D prostanoid receptor 1 and 2 [375]. Opposite to PGE_2, PGD_2 was shown to inhibit LC migration to the skin draining lymph nodes during percutaneous infection with the helminth parasite *Schistosoma mansoni* [375] and the migration of lung DC *in vivo* [376].

New anti-inflammatory lipid mediators were recently identified during the resolution of inflammation and following aspirin treatment [377]. These compounds were shown to inhibit leukocyte migration by interacting with chemotactic receptors. Among them, lipoxin A4, an autacoid generated from arachidonic acid, was found to inhibit DC mobilization in the spleen through the interaction with FPRL1 [378]. Similarly, resolvin E1, a new bioactive oxygenated product of eicosapentaenoic acid, a component of fish oils, reduced dermal inflammation, IL-12 production and DC migration through the

interaction with ChemR23, the chemerin receptor [73]. Therefore, the engagement of the same receptor by alternative ligands may result in an opposite biological output.

CD38 is an ectoenzyme that catalyses the synthesis of cyclic adenosine biphospate ribose, a potent second messenger for calcium release, as well as a receptor that initiates transmembrane signalling upon engagement with its counter-receptor CD31. CD38 was found to be upregulated during DC maturation and to promote a DC membrane and functional phenotype *in vitro* [379]. CD38 apparently controls DC migration, since mice lacking CD38 have a defect in the recruitment of DC precursors to peripheral tissues and in DC mobilization of mature DC to the draining lymph nodes [380]. In human monocyte-derived DC, CD38 is closely associated with CD11b and CCR7 at the membrane, and its engagement is necessary for efficient *in vitro* chemotaxis and trans-endothelial migration to CCL21 [381].

The lipid mediator LTB4 is involved in the regulation of DC traffic, attracting immature and maturing DC to the sites of inflammation and to the draining lymph node via the LTB4 receptor BLT1. It controls DC traffic also indirectly by inducing CCR7 expression and function [382]

1.3. Asthma. General characteristics

Asthma is a chronic inflammatory disorder of the airways arising as a result result of a dysregulated immune response to commonly encountered antigens in genetically predisposed individuals. It is characterized by the clinic pathologic symptoms of intermittent and reversible airway obstruction, attacks of wheezing and breathless due to broncocostriction, mucus hypersecretion, infiltration of Th2 cells, eosinophils and mast cells in the airway wall, airway hyperresponsiveness (AHR) to non specific stimuli and thickening of submucosa, eventually leading to impaired epithelial repair and airway remodelling [383].

Under physiological conditions, activated Th2 cells are able to induce an immune response against extracellular or parasitic infections. In allergic asthma, genetically susceptible individuals mount a chronic Th2 cell type immune response to common allergens, like house dust mite (HDM), plant pollen, molds and animal dander by secreting cytokines like IL-4, IL-5, IL-9, IL-10, IL-13 and granulocyte macrophage-colony stimulating factor (GM-CSF), whereas mast cells and eosinophils exert important effector functions [384] (Fig. 9). Individually, these cytokines can already

explain many of the salient features of asthma as IgE synthesis, growth and activation of eosinophils and mast cells and expression of EC-adhesion molecules [385].

IL-4 and IL-13 induce the expression of cell adhesion molecules on inflamed endothelium and epithelial production of chemokines, leading to the recruitment of inflammatory cells, and act on epithelia inducing globet cell metaplasia. IL-4 stimulate the production of IgE by B cells and IL-13 acts on bronchial smooth muscle cells causing bronchial hyper-reactivity (BHR). IL-5 is important for the growth, differentiation and activation of tissue eosinophils while IL-9 is important for mast cell growth and activation. GM-CSF stimulates the growth of eosinophils and the activation of APCs [386]. Moreover, cytokines produced by Th2 cells and inflammatory cells can affect the airway epithelium, subepithelial (myo-) fibroblasts and smooth muscle cells in the lungs, thus leading to structural abnormalities [385].

Mast cells play a key role in asthma through the release of several bronchoconstrictors, including histamine, which is preformed and stored in granules, and the lipid mediators leukotriene C_4, leukotriene D_4, leukotriene E_4 and prostaglandin D_2 (PGD_2}, which are synthesized following mast-cell activation. The release of these mediators may account for the variable bronchoconstriction observed in asthma, as these mediators are released by various environmental triggers, such as allergens, and an increase in plasma osmolality as a result of increased ventilation during exercise. Mucosal mast cells are recruited to the surface of the airways by stem-cell factor (SCF; also known as KIT ligand} released from epithelial cells, which acts on KIT receptors expressed by the mast cells [387]. Mast cells also release cytokines that are linked to allergic inflammation, including interleukin IL- 4, IL- 5 and IL- 13 [388].

The inflammation that occurs in asthma is often described as eosinophilic. The functional role of eosinophils in asthma is not clear and the evidence that links their presence to airway hyper-responsiveness has been questioned by the finding that the administration of IL-5-specific blocking antibodies that markedly reduce the number of eosinophils in the blood and sputum does not reduce airway hyper-responsiveness or asthma symptoms [389]. Eosinophilic bronchitis is not associated with AHR, but subepithelial fibrosis does occur, which suggests a role for eosinophils in airway fibrosis. Interestingly, the presence of eosinophils seems to be a good marker of steroid responsiveness [390].

B cells have an important role in asthma, through the release of allergen-specific IgE which binds to high-affinity Fc receptors for IgE (FcεRI) expressed by mast cells and

basophils, and to low-affinity Fc receptors for IgE (FcεRII) expressed by other inflammatory cells, including B cells, macrophages and possibly eosinophils [391]. The Th2-type cytokines IL-4 and IL-13 induce B cells to undergo immunoglobulin class switching to produce IgE.

Th2 cells do not react directly to inhaled antigens, as their T cell receptor can only recognize antigens that are processed into peptides for presentation in the context of MHC molecules [392]. Like in other epithelia and skin, the lung is equipped with an elaborate network of DC that can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes [393, 394]

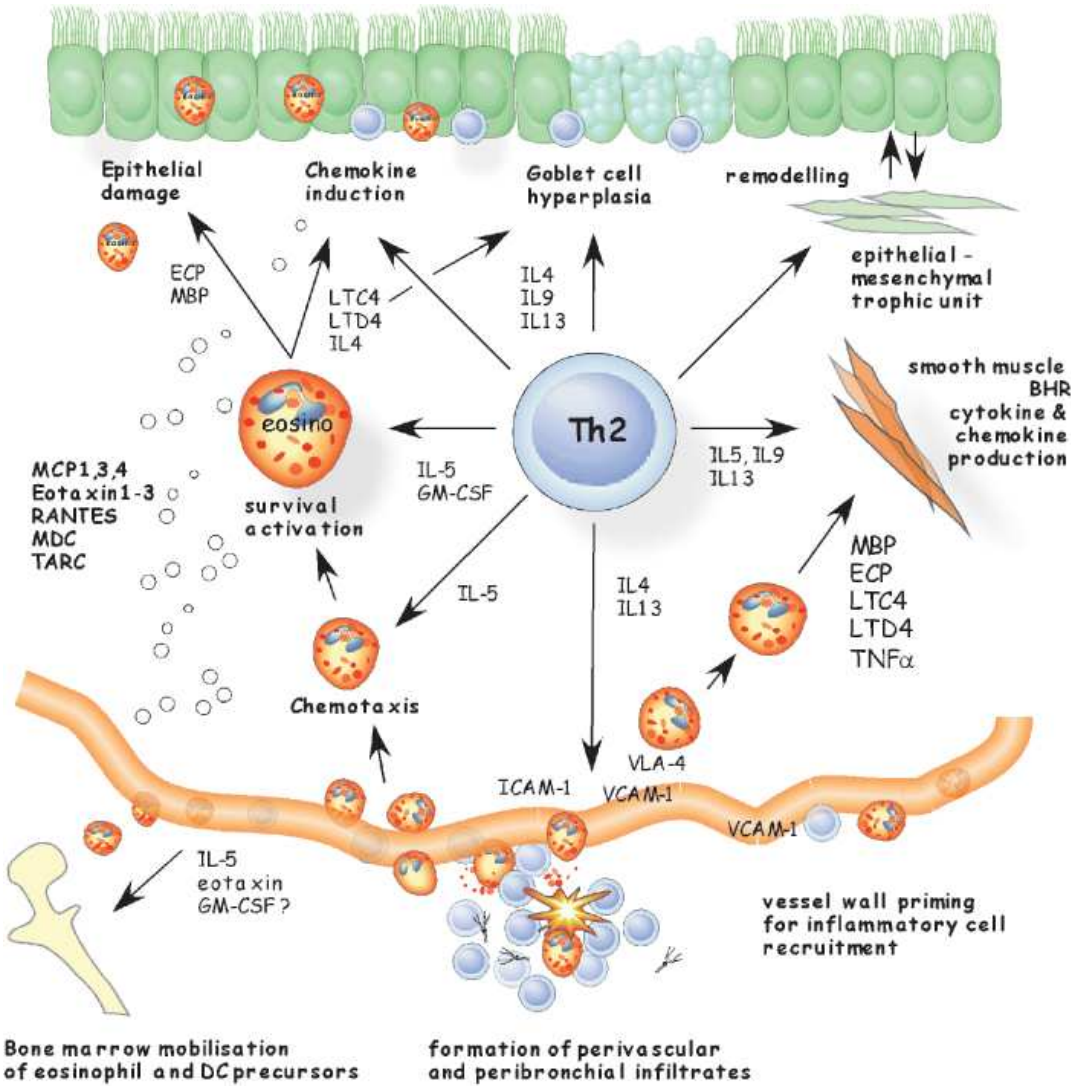


Figure. 9. Effector functions of Th2 cells. Effector Th2 cells produce several cytokines and chemokines that exert their effects on eosinophils, airway smooth muscle, epithelium and endothelium, resulting in several pathologic characteristics of asthma. (From Van Rijt and Lambrecht, 2005) [395]

1.3.1. Chemokines in asthma

Chemokines are key players in asthma because they promote the recruitment of both immune and structural cells to the lung and direct specific cell populations into different compartments within the lung tissue and control leukocyte activation [396, 397]. Cellular sources of chemokines within the asthmatic airway include inflammatory cells and structural cells like epithelial cells, fibroblasts, and EC [398].

Local Th2-cell dominance during allergic asthma is maintained, to a large degree, through production of the Th2 chemokines CCL17 and CCL22 [399], although the mechanism initiating expression of these CCR4-binding chemokines is not yet fully understood [400]. CCL17 is constitutively expressed in epithelial bronchial cells from healthy donors, but is overexpressed in subjects with asthma [401]. Regarding CCL22, some studies report a weak constitutive expression in healthy donors, with no evidence of its regulation in patients with asthma [402], while other demonstrate a high concentration of CCL22 in broncheoalveolar lavage (BAL) from individuals with asthma [403]. After activation and differentiation Th2 cells produce CCL1 and CCL22 [15], both chemoattractants for Th2 cells, favoring a Th2 environment by a positive loop.

Degranulated eosinophils release CCL5, CCL7, CCL11 and CCL13. Eosinophils are mainly recruited through the CCR3 receptor whose agonists: CCl1, CCL5, CCL7 and CCL13 present at high levels in asthma. This concentrated expression might preferentially target eosinophils to the airway. However other non-CCR3-binding chemokines can cause the migration and activation of eosinophils. CCL3, a CCR1/CCR5 ligand and CCL22, a CCR4 ligand, could induce chemotaxis of eosinophils through a CCR3 independent mechanism [404, 405]. An increase in CCL3 production has been observed in the lungs of patients with asthma [406] and in the nasal secretions of subjects with allergic rhinitis after allergen challenge [407]. Blocking of CCL3 in several animal models suggests a role for this chemokine in mild eosinophils mobilization during the first stage of airways allergic and in BHR-associated disease [408].

Mast cells release CCL2, CCL3 and CCL11, all of which are involved in leukocyte attraction during inflammation, thus connecting mast cell secretion with the late phase response in asthma [409, 410]. CCL11 binding to CCR3 expressed on mast cells surface after Fc-receptor for IgE crosslinkage, provide a second signal, leading to amplification of Fc-receptor for IgE-dependent IL-13 production [411]. CCL2 is able to recruit and activate mast cells. Instillation of CCL2 into the airways induces marked and prolonged airway hyper-response in mice, which is associated with mast cell degradation [412].

Macrophages in the lung insterstitium produce CCL3, CCL7 and CCL22 that address eosinophil migration out of the blood vessels and into the lung tissue [409], while DC produce the CCR4 ligands CCL17 and CCL22, suggesting that these cells are capable of selectively recruit Th2 cells and/or CD4+ CD25+ regulatory cells (Treg) at sites of inflammation [413].

CXCL12 expressed in HEV contributes to the migration of naïve T cell to draining lymph nodes via CXCR4. Hoshino et al. suggested that CXCL12 could play a role in airway remodelling by promoting angiogenesis [414]

The two IFN-inducible chemokines, CXCL10 (IP-10) and CXCL9, have a role in modulating allergy phenotype [415, 416]. Expression of CXCL9 is localized primarily to airway epithelial cells and functions by simultaneously diminishing IL-4 and enhancing IL-12 levels, suggesting that CXCL9 is able to direct activated T cells toward a Th1-cell phenotype significantly reducing AHR and eosinophil accumulation in allergen-sensitized and challenged mice [417]. CXCL10 has be shown to largely induce the opposite effect [415]. CXCL10 can induce a degree of eosinophil chemotaxis through CXCR3, however CXCL9 has no chemotactic effect and actually exerts a blocking effect on eosinophil migration toward potent chemotactic stimuli such as CCL11 [416]. Both CXCL9 and CXCL10 bind to CXCR3, been capable of differentially regulating allergic asthmatic responses through a single receptor, indicating a mechanism by which Th1 and Th2 cytokine regulation can be implicated in this CXCL9/CXCL10 axis.

There is little information on the role of CXCL13 in airways. Overexpression of CXCL3 has been detected by microarrays in an asthma mouse model [418] and in bronchial biopsies of patients with asthma [419]. Neutralization of CXCL13 employing specific antibodies decreased the number of infiltrating cells, including lymphocytes, macrophages, eosinophils, and neutrophils, as well as the formation of cells surrounding inducible bronchus-associated lymphoid tissue suggesting some role in migration of leukocytes associated with asthma [397, 419]

As discussed above, the structural cells of the lung might be a significant source of chemokine mediators, allowing localization of specific leukocyte populations into and around the airway. Epithelial cells of asthmatic people strongly upregulate CCL5, CCL11 and CCL13 [420, 421]. CCL11 is beside the most potent degranulator agent. This concentrated expression might preferentially target eosinophils to the airway. In addition to recruiting and activating eosinophils, the same chemokines can affect other asthma-related leukocyte populations, such as basophils and Th2 cells [138, 143]. Airway

EC are also a source of chemokines that contribute to the recruitment and the activation of basophils, eosinophils and lymphocytes into the airways during inflammatory responses. For instance, a combination of TNF-α and IFN-γ induced the production of CCL5 from EC isolated from nasal mucosa of subjects with allergic rhinitis. Interestingly, this cytokine-induced CCL5 production was higher in nasal mucosal EC recovered from atopic subjects as compared to non-atopic controls [422]. In parallel with observations in epithelium, cytokines, generated in response to respiratory virus infection, which is frequently associated with asthma exacerbation, can increase vascular permeability and might provide a mechanism by which respiratory viral infection can lead to leukocyte migration out of the circulation, leading to airway inflammation and immune modulation in the lung [423].. The production of CX3CL1 (fractalkine) by EC during inflammation also represents as a potential important initiator of CD4+ T-cell recruitment during allergic asthma. CX3CL1 is constitutively expressed by pulmonary EC of allergic asthmatics and in bronchoalveolar lavage after segmental allergen challenge accompanied by an increase in CX3CR1 function by CD4T cells in the peripheral blood in a manner that correlates positively with infiltrating cell numbers [424].

Beside to contribute to the integrity of the lung tissue and the repair process during inflammation, lung fibroblasts participate in the local immune response by secreting an array of inflammatory cytokines and chemokines. For instance, after stimulation with TNF-α *in vitro*, fibroblasts stimulated the migration of eosinophils by the release of CCL5 and CCL11 [425]. In addition, both IL-4 and IL-13, strongly involved in allergic asthma, have been shown to upregulate CCL2 [426], which recruits macrophages and basophils, hereby contributing to the asthmatic phenotype. Moreover, during airway inflammation, structural cells can also be activated by certain chemokines, which may contribute to the airway remodelling observed in asthma; e.g. chemokines might play a role in the peribronchial smooth muscle hypertrophy observed in asthmatics, as is illustrated by CCL1, which directly induces smooth muscle cell activation *in vitro* [427] and CCL2, a competence factor for fibroblast activation [428].

1.3.2. Dendritic cells in asthma

The mouse lung is grossly divided into large conducting airways and lung interstitium containing alveloar septa and capillaries where gas exchange is taking place [429]. Like in other epithelia and skin, the lung is equipped with an elaborate network of

DC that can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes. At least five different subsets of DC can be found in the lungs (Fig. 10) [393, 430]. In steady-state conditions, the conducting airways are lined with an intraepithelial highly dendritic network of $MHCII^{high}CD11c^{high}$ cells that are mostly CD11b- and, at least in the mouse and rat, express langerin and the mucosal integrin CD103 (αEβ7). These cells have the propensity to extend dendrites into the airway lumen through formation of tight junctions with bronchial epithelial cells [393, 431-433]. Immediately below, the lamina propria of the conducting airways contains $MHCII^{high}CD11c^{high}$ cells that are highly expressing CD11b and are a rich source of proinflammatory chemokines [433, 434]. The CD11b+CD103- subset also expresses the SIRPα molecule, a binding partner to CD47 involved in DC migration [435]. A similar broad division into CD11b+ and CD11b- can also be applied to lung interstitial CD11c+ DC [393, 436]. Both CD11b+ and CD11b- subsets express high amounts of CD11c, so they can best be denominated as conventional DC, to contrast this with another population of $CD11c^{int}$ P-DC that express Siglec-H, and BST1 and some markers shared with granulocytes and B cells [223, 393, 437]. The exact anatomical location of lung P-DC is unclear although they can be found to line alveolar septa *in situ* and have been recovered from digests of large conducting airways [223, 429]. The alveolar space also contains $CD11c^{high}MHCII^{high}$ DC. At least in the rat and man, alveolar DC are highly enriched in CD103+ subsets that resemble LC in man. Under inflammatory conditions, such as viral infection, allergen challenge, or LPS administration, there is recruitment of additional subsets of CD11b+ monocyte-derived DC that rapidly upregulate CD11c and retain expression of Ly6c as a remnant of their monocytic descent, and are easily confused with resident CD11b+ conventional DC [393, 438, 439].

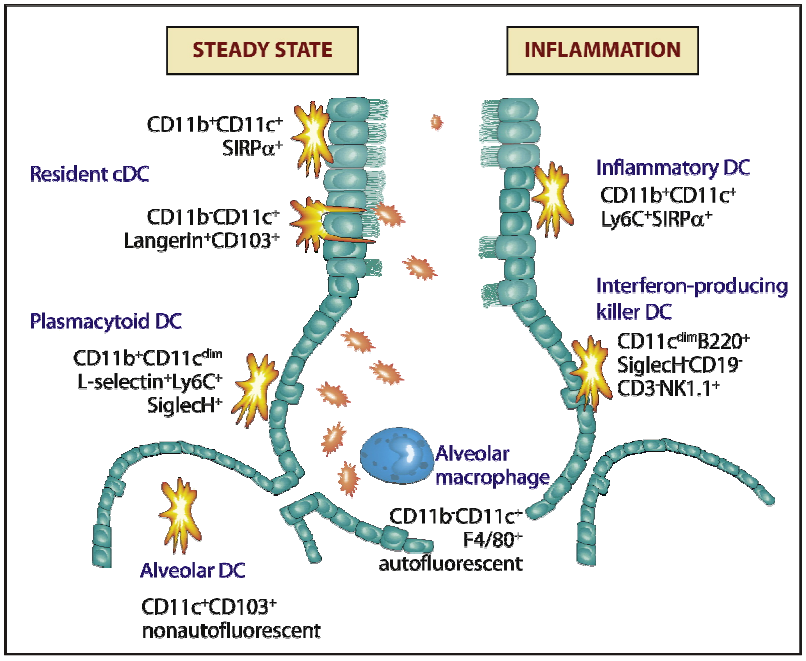


Figure 10. Lung DC subsets. In steady-state conditions, conventional DC (CD11b+ and CD11b− subsets) line the conducting airways. They are also found in the interstitial compartments. P-DC are found in both compartments with a slight preference for the interstitial compartment. The alveolar space contains DC, autofluorescence has to be considered to differentiate them from macrophages. Under inflammatory conditions, there is recruitment of CD11b+ monocytes that rapidly become DC. During viral infections as well as in some cancers, there is also recruitment of IFN-producing killer DC, a subset of NK cells CD11c+B220+, can occur. To distinguish them from P-DC staining for NK1.1 is needed (From Lambrecht and Hammad, 2009)[394]

Based on the anatomical distribution of even the most exposed DC, it is clear that DC are basically always covered by a layer of epithelial cells that seal off the inhaled air by formation of tight junctions [440]. Nevertheless, DC are able to sample the epithelium for inhaled antigens by forming long extensions throughout the epithelium to the airway lumen while maintaining the epithelial barrier function intact [441, 442]. Recently, published results suggest that an activating signal is needed for the extension of dendrites by DC across epithelial barriers [439]. Hammad et al [438] have identified airway epithelial cells as instructive in causing such DC sentinel behavior and activation in the lungs. Which epithelial signals are able to program this scanning response are largely unknown, but one possibility is the regulated expression or display of chemokines along the extracellular matrix of epithelial cells, followed by the secretion of DC-activating cytokines. Additionally, some clinically relevant allergens have the potential to cross the lung epithelium and gain access to DC [392]. For example Derp1, a major allergen of HDM has been shown to increase the permeability of the bronchial epithelium by cleaving the tight junctional proteins laudin and occludin [443].

Mucosal DC show an immature phenotype, meaning that they have exceptional allergen uptake and processing capability, but lack the power to stimulate naïve T cells [444]. Local conditions contribute to maintain DC in a such immature state. After antigen uptake in the presence of a danger signal airway DC migrate to the T cell rich area of the draining mediastinal lymph nodes where naïve T lymphocytes continuously pass by [445]. On their way to the mediastinal lymph nodes, DC process these captured antigens, display them as peptides in the context of MHC class II molecules and subsequently present them to naïve CD4 Th cells in the paracortex of the draining node [135]. During this process, DC acquire a mature phenotype, meaning that they upregulate their expression of costimulatory molecules and chemokines necessary for optimal naïve T cell activation, and they acquire the capacity to stimulate an effector response [446]. Upon encountering specific T cells, DC undergo a stable interaction with these cells, ultimately leading to the formation of an immunological synapse and T cell activation, division and differentiation [447].

The DC therefore become a reporter of their earlier microenvironment and have the potential to influence polarization of T cells by specific expression and secretion cytokine pattern depending on their lineage [224], their maturation status and the consequent costimulatory molecule pattern [448] and the environment they are in [449] as the

type of antigen captured, the presence of microbial patterns or endogenous danger signals or the route of exposure and the genetic background of the host [450, 451].

Airway is continuously exposed to airborne antigens. In order to prevent the continual induction of immune responses and stimulation of memory effector cells, which would support chronic inflammation in the airways and damage to the epithelial barrier and would impair gaseous exchange, a number of specialized control mechanisms exist. An absolute requirement is that these mechanisms discriminate harmless airborne antigens entering via the airways from pathogens [452, 453]. Given these risks, it is not surprising that multiple interactions between leukocytes and cells of the lung stroma are central to this process of homeostatic pulmonary regulation. The epithelium plays a central role via the production of molecules such as mucins, surfactants, complement products, and antimicrobial peptides. In addition, a unique population of macrophages termed alveolar macrophages, which reside outside the body in the airway spaces, are likely to play an important role. These cells exhibit immunosuppressive properties, dampening down local DC and T cell activation by secreting different products (e.g. nitric oxide, that contribute to keep lung DC in an immature antigen-capturing mode (reviewed by Holt et al, 2008)[453].

Most inhaled particles do not have any intrinsic activating properties, and therefore the default immune response to the inhalation of inert antigens is tolerance (Fig. 11). Unresponsiveness to immunological inert antigens is regulated at different levels.

In contrast with the gut mucosal immune system, where intestinal mucosa-derived DC continuously sample antigens and migrate into afferent lymphatics even in the steady state, conducting airway DC show very little migration to mediastinal lymph nodes. This difference might result from the fact that the gut is heavily colonized by microbes, whereas the (deeper) lung of unmanipulated animals is a relatively sterile environment. It follows that most of the lung DC migration to the mediastinal lymph nodes results from some form of insult to the lung, be it microbial, physical, or toxic in nature [439].

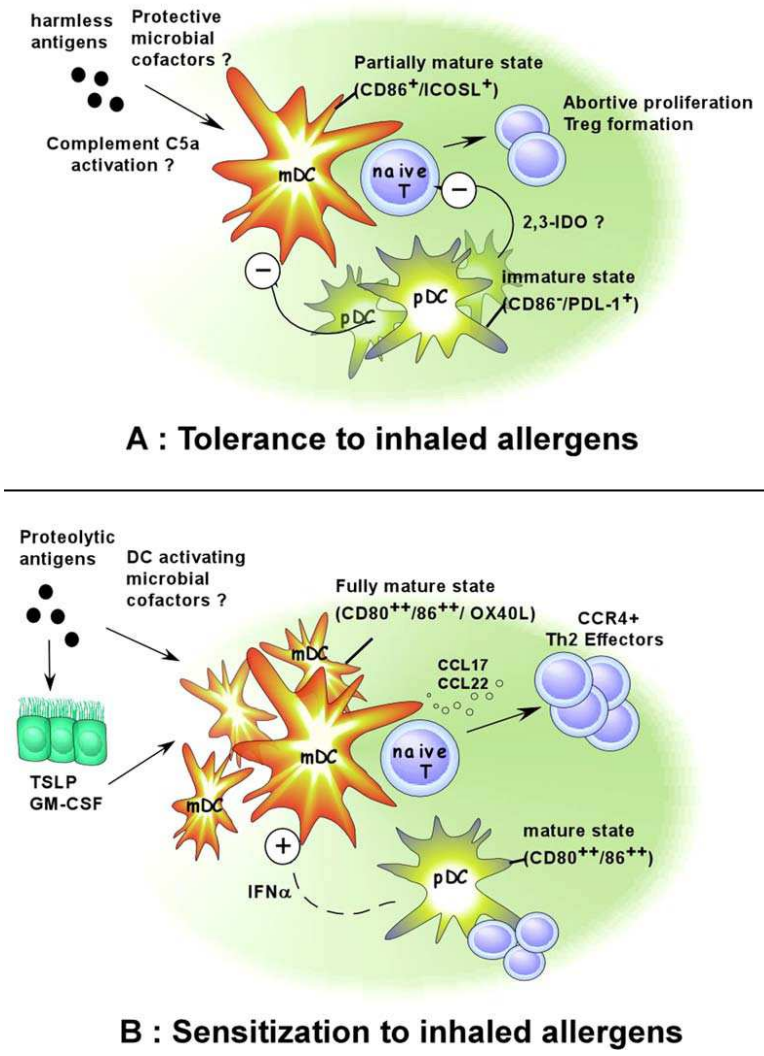


Figure 11. Immune regulation by DC in the lung. Under steady-state conditions (top), in the absence of danger signals, inhaled antigens are picked up by M-DC and P-DC, which take the antigen to the mediastinal lymph nodes. Here partially mature M-DC stimulate specific T cells, but these T cells fail to differentiate into effector cells and die. Some T cells might also differentiate into Tregs. P-DC in the draining lymph nodes give negative signals (IDO (indoleamine 2,3-dioxygenase) and PDL (programmed death ligand)-1)) to T cells and M-DC. At the same time, they also generate Tregs. Under inflammatory conditions (bottom), M-DC arrive in the draining node as fully mature cells. Antigen-specific T cells undergo proliferation, this time generating effector cells. On the other hand, P-DC also acquire a mature phenotype and prime antigen-specific T cells to become effector cells as well. (From Hammad and Lambrecht, 2006)[450].

In the absence of inflammatory triggers, DC that take up these immunologically inert antigens do not properly express costimulatory molecules, and consequently fail to reach the threshold necessary to induce T cell activation (leading to anergic T cells or T cells that are not able to survive) inducing an abortive T cell response [454]. Partially mature DC could stimulate the induction of IL-10- and/or transforming growth factor-β (TGF-β) producing Tregs in an IL-10 and/or inducible T cell costimulator ligand (ICOSL)-dependent manner [455].

In another hand, it have been proposed a role of P-DC in respiratory tolerance [223, 456]. The negative signal that is delivered by P-DC is mediated through programmed death (PD)-1-PD ligand 1 interactions [437] delivering a negative signal to T cells or to M-DC directly [223, 457]. Additionally, P-DC can produce the tryptophan-metabolizing enzyme IDO, which has a strong inhibitory activity on T-cell proliferation [458] and inhibits inflammatory airway disease [459]. In addition to IDO, the expression of transcription factors such as glucocorticoid-induced leucine zipper protein (TSC22 domain family protein 3, TSC-22R) is also considered a feature of tolerogenic DC. TSC-22R expression in DC, which is induced by glucocorticoids such as dexamethasone, leads to a decreased expression of costimulatory molecules such as CD80, CD83 and CD86, while the expression of "coinhibitory" molecules such as immunoglobulinlike transcript 3 (ILT-3) and PD-L1 is increased [460]. TSC-22R expressing DC produce IL-10 and elicit the development of Tregs [461]. Another explanation for the tolerogenic properties of P-DC is related to their immature phenotype [223, 456].

It seems probable that primary functions of Treg cells in the airways are to limit the inflammatory consequences of infection and to maintain tolerance to harmless, inhaled aeroallergens. Treg cells represent a major mechanism of peripheral tolerance to ''self'' and in the regulation of the immune response to infectious organisms, both pathogens and commensals. Furthermore, Treg cells represent a major pathway proposed to contribute to the maintenance of immune homeostasis in the airways [462, 463]. A number of different mechanisms by which antigen-specific Treg cells inhibit the function of effector T cells, antigen-presenting cells, and innate cells have been described (reviewed in Vignali et al. [464]). A prominent inhibitory mechanism appears to be via the production of anti-inflammatory cytokines such as IL-10 and TGF-β [465, 466], but inhibitory molecules such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and PD1 are also likely to contribute [464]. IL-10 has broad immunosuppressive and anti-

inflammatory actions relevant to the inhibition of asthma pathology [466]. It is a potent inhibitor of proinflammatory cytokine production and acts on antigen-presenting cells to dampen T cell activation, including Th2 cells. The role of the cytokine TGF-β is more complex, with evidence for immune suppression and remodelling in the airways. Some of the functions of TGF-β that contribute to subepithelial fibrosis include amplification of fibroblast proliferation and differentiation as well as induction of the expression of collagen and other extracellular matrix proteins [467]. TGF-β induces apoptosis of airway epithelial cells and is potentially involved in the regulation of the adhesion properties of epithelial cells leading to damage of the epithelial layer. TGF-β has been shown to play a role in enhancement of goblet cell proliferation and mucus secretion [467, 468]. It also causes airway smooth muscle proliferation and contributes to increased airway smooth muscle cell mass [467].

During generation of an efficient effector immune response, lung DC have to overcome suppression by Tregs, and the dominant way in they seem to do this is by producing the cytokine IL-6, which counteracts the suppression by naturally occurring CD41CD25 Tregs. Resistance to allergen- driven AHR is mediated in part by Treg suppression of DC activation and the absence of this regulatory pathway contributes to susceptibility [469]. In human subjects with allergy, there is a reduction in the number and possibly function of Tregs [470] but it is unclear at present whether this would also lead to altered function of DC in these patients.

For immunological inert antigens additional signals from environmental exposures (e.g. respiratory viruses, air pollution or cigarette smoke) or endogenous danger patterns might pull the trigger on DC activation [471, 472].

DC will only start a T helper response if there is some sort of adjuvant activity on board at the time of exposure to the allergen. This activity is provided by the presence of PAMPs, DAMPs and cytokines released upon cell activation, necrosis or oxidative stress (e.g. cigarette smoking, ozone exposure, diesel particles). This adjuvant signal can also be found in the allergen itself. Indeed, Derp1, cockroach and many other allergens have proteolytic enzymes that can directly activate DC to promote Th2 cell responses through effects on polarizing cytokines, costimulatory molecules, and cell surface receptors [413, 473, 474].

Additionally, lung DC express numerous receptors for PAMPs and DAMPs. DC express protease-activated receptors (PAR) which are activated by proteolytic proteins like tryptase and thrombin [235]. Shortly after insult to the vascular compartment or after

pathogen entry in the mucosa, complement activation occurs, and lung DC can sense this ‘‘acute alert’’ through expression of the C5a and C3a anaphylatoxin receptors [457]. They also express neuropeptide receptor that can respond to neurotransmittors released in response to axon reflexes or efferent neural responses, supported by the fact that lung DC synapse with unmyelinated nerve endings in and beneath the airway mucosa and produce neurotransmittors [475]. Lung DC express receptors for prostaglandins and these acutely released inflammatory mediators can profoundly impact on the migration and maturation of the cell [476]. Endogenously released metabolites like extracellular ATP trigger purinergic receptors on lung DC and in this way relay information about allergen-induced platelet aggregation or metabolic cell stress to the cells of the immune system through widely expressed purinergic receptors [233, 234]. Eosinophil and mast cell degranulation can lead to the release of EDN and histamine that can feed back on DC and promotes further Th2 cell responses [477]. Triggering of these receptors activates an intracellular signalling cascade that influences the phenotype and functions of DC [478, 479] and lead to an inflammatory response [480, 481].

Completely active DC upregulate the expression of costimulatory molecules (CD80 and CD86, CD40 and PD-L1, and PD-L2) and produce pro-inflammatory cytokines involved in Th2 polarization (TNF-α, IL-1, IL-6, IL-10) [482-484]. The up-regulation of PDL-1 can have pro-inflammatory effects providing a costimulatory signal to T cells [485]. IL-6 produced by pulmonary DC blocks the development of Tregs. In addition IL-6 downregulate IL-12 and thus polarize naïve cells into a Th2 phenotype in the lungs [486]. The presence of IL-10 at the site of T cell differentiation diminishes the secretion of IL-12, thereby indirectly attenuating the differentiation of Th1 cells driving the differentiation of naïve T cells toward a Th2 phenotype [228, 487].

It is less clear whether airway DC are also required for the presentation of allergen to resting memory Th2 and/or effector Th2 cells during a secondary immune response but there are increasing findings that support a predominant role for DC in secondary immune responses in asthma. In favor of DC, it is known that the number and maturation state (expression of essential costimulatory molecules CD80, CD40, ICAM-1, PDL-1, PDL-2) of CD11b+ inflammatory DC is increased in the conducting airways and lung interstitium of sensitized and challenged mice during the acute phase of the response, where they are seen to form clusters with primed CD4 T cells, at areas of nerve endings [475, 488]. This accumulation of DC in the lung is supported by an almost threefold expansion of myeloid $CD31^{high}$ Ly-6c- haematopoietic precursor cells in the

bone marrow. Hu et al [444] have hypothesized that contact with primed T cells turns immature mucosal DC from an antigen uptake to an antigen presenting mode, leading to local antigen presentation to mucosal T cells. CCR2 is the predominant receptor for attracting Th2-cell-inducing DC to the lungs of mice after allergen exposure [489]. CCR2+ monocytes are recruited to the lung, followed by rapid differentiation to monocyte-derived inflammatory DC. Epithelial cells rapidly upregulate the expression of the CCR2 ligand CCL-2 (MCP-1) after challenge contributing to monocyte recruitment to the lung [438].

Many inflammatory cell types such as mast cells, basophils and eosinophils are recruited to the airways in chronic asthma. These cells release many mediators such as cytokines, neuropeptides, enzymes and lipid mediators that may also profoundly influence DC function and in this way might perpetuate ongoing inflammation [490]. As only one example, it is known that histamine and PGD_2, both released by mast cells upon cross-linking, reduce the potential of DC to produce bioactive IL-12, and contribute to Th2 polarization in this way [366, 376].

The presence of allergen specific IgE bound to the high-affinity receptor for IgE (Fc3RI) on DC can lead to a 1000-fold lowering of the threshold for allergen recognition and in this way IgE might sustain Th2 cell responses via effects on DC [491, 492].

Although M-DC preferentially induce a Th2 sensitization to inhaled harmless antigens leading to asthmatic features [223, 445, 482], M-DC are equally able to induce a Th1 response, under conditions of intense immune stimulation such as bacterial or viral infection [493, 494] or under environmental instruction by the presence of cytokines like IL-12 [495].

Whereas the epithelium was initially considered solely as a physical barrier, it is now seen as a central player in controlling the function of lung DC through release of Th2 cell-promoting cytokines. This situation by which stromal cells instruct the functional behavior of DC seems to be quite specific to the mucosal environment.

Chemokines produced by airway epithelium after allergen exposure, promote the recruitment of specific inflammatory cell types to the airways probably contributing to Th2 sensitization Ex. *In vitro* and *in vivo* studies have shown that exposure of airway epithelium to HDM resulted in the rapid secretion of CCL20, a chemokine attractant for immature DC [496]. Moreover, HDM exposure is also accompanied by an increased production of CCL2, a chemoattractant for monocytes, the precursors for inflammatory

DC [438]. Epithelial cells also secrete a variety of cytokines like GM-CSF, IL-33, Thymic stromal lymphopoietin (TSLP), and IL-25 that activate DC, mast cells and basophils [438].

GM-CSF has been shown to promote DC differentiation and maturation and to break inhalation tolerance [497]. IL-33 inducess Th2 cell differentiation by programming the function of DC and goblet cell hyperplasia [498]. TSLP is a 140 amino acid IL-7-like 4-helix-bundle cytokine that has potent DC-modulating capacities, by binding its receptor complex, composed of the IL-7 receptor (IL-7R) and the TSLP receptor (TSLPR) [87, 88] [499]. Epithelia cells, mast cells and basophils secrete TSLP upon allergen challenge. TSLP can directly activate DC to prime naive CD4+ T cells to differentiate into proinflammatory Th2 cells by inducing the Th2 cell-prone costimulatory molecule OX40L [499] and the production of the Th2 cell-attractive chemokines CCL17 and CCL22 by DC [500, 501]. In addition, TSLP might promote the production of Th2 cytokines by lymphocytes directly. The polarization of Th2 cells induced by TSLP-matured DC is further enhanced by IL-25, which is produced by epithelial cells, basophils, and eosinophils [502]. Interestingly, TSLP also induces DC maturation without production of IL-12 (an important regulator of Th1 cell development) [499] in contrast to most of the molecules known to cause DC maturation [189]. In addition to its effects on DC, TSLP can also activate human mast cells to produce Th2 cell-associated effector cytokines in the absence of T cells or IgE crosslinking [503]. Strikingly, IL-13 was shown to induce expression of TSLP, pointing out to an important feedback- loop acting via DC to enhance Th2 cell immunity [504].

Most known environmental risk factors that are associated with development of allergy like cigarette smoking, exposure to ambient particulate matter like diesel exhaust, exposure to high ozone concentrations, or exposure to RSV (respiratory syncytial virus) have the potential to interfere with the epithelial-DC crosstalk. For example, cigarette smoking enhances epithelial permeability and induces the production of TSLP by airway epithelial cells (reviewed in Robays et al, 2009)[505, 506], whereas the Th2 cell adjuvant effects of diesel depend on epithelial production of GM-CSF [507].

Epithelial-DC interactions are necessary for preventing overt Th2 cell responses. Epithelial cells modulate local DC differentiation (instruct DC to produce IL-12, IL-6, IL-10, and TNF-α), optimize antimicrobial defences in the airways and in the process downmodulate capacity for expression of potentially damaging Th2 cell immunity [508, 509]. A defective innate immune response of epithelial cells might therefore be at the heart of allergic sensitization.

Many of the Th2 cell effector pathways of allergic inflammation maintain the DC network in an activated state. Allergen challenge leads to enhanced production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in epithelial cells. Expression of TRAIL subsequently leads to enhanced production of CCL20, in this way attracting inflammatory CCR6+ DC and Th2 cells and perpetuating allergic inflammation [89]. Prototypical Th2 cytokines like IL-4 and IL-13 also feed back on the function of the airway DC and epithelial cells to directly or indirectly (via DC-activating cytokines like TSLP) stimulate DC in a STAT- 6-dependent way [504, 510, 511]. These feedback loops might be important in understanding the chronicity of allergic inflammation. Remodelling is a dynamic process that involves the laying down of extracellular matrix structures, which are also broken down by the action of tissue-degrading enzymes. How exactly collagen or extracellular disposition of matrix degradation products might influence DC function in remodelled airways is currently mainly unknown.

1.3.3. Dendritic cell recruitment to the airways

DC constantly migrate from the blood to the lungs and from the lungs to the draining lymph nodes. The migratory patterns of pulmonary DC are highly dependent upon inflammatory conditions in the lung. In steady-state, or non-inflammatory conditions, pulmonary DC undergo slow but constitutive migration to draining lymph nodes, where they remain for several days and confer antigen-specific tolerance. In the presence of inflammatory signals, airway DC trafficking increases dramatically, and these cells rapidly accumulate within draining lymph nodes. However, within a few days, the number of airway-derived DC in lymph nodes stabilizes or declines, even in the face of ongoing pulmonary inflammation. An important question for the understanding of airway DC biology is how DC populate the lung in the absence of inflammation, and how they are recruited there during inflammation. The understanding of the relative contribution of particular chemokines to the selective accumulation of leukocyte subsets to an organ site is made difficult by the simultaneous presence of multiple chemokines with partially overlapping functions as well as by the finding that chemokines often bind more than one receptor [57, 512].

It is not clear yet whether lung DC are recruited from the blood in a differentiated form or as early precursors. It is important to note that GM-CSF, a key growth factor for "DC-poiesis," is expressed in significant amounts in the lung. GM-CSF production in the airways is increased in an inflammatory context (e.g., respiratory viral infection or

exposure to environmental pollutants). DC can populate the lung through different mechanisms. DC present in the blood circulation could be directly recruited by means of appropriate pulmonary chemokine signals. Alternatively, monocytic DC precursors could first be chemoattracted from the blood into the lung and subsequently differentiate into DC under the influence of cytokines secreted by resident pulmonary cells (e.g., GM-CSF produced by respiratory epithelium). In addition, a hypothetical mechanism for maintaining lung DC homeostasis might be proliferation of putative intrapulmonary DC progenitors, induced by growth factors secreted by resident pulmonary cells (GM-CSF or Flt3L). Finally, indirect evidence points to the possibility of transdifferentation of pulmonary macrophages into DC under the influence of GM-CSF [513, 514].

In the absence of inflammatory signals, DC and their precursors are recruited from the bloodstream into the lung where they have a very rapid turnover of about 2–3 days [515]. Under steady-state conditions, there are at least two pathways to recruit DC precursors: one that depends on CCR1 and/or CCR5, and one that does not [516]. It is possible that the latter mechanism involves the chemokine receptor, CX3CR1, a receptor highly expressed on a population of monocytes that are recruited to noninflamed tissues, including the lung [517]. Because monocytes can differentiate into DC, CX3CR1-directed monocyte recruitment might be important in maintaining DC homeostasis under steady-state conditions. Steady state recruitment of DC into airway relies on constitutive chemokine production within the epithelium by one or more cell types which may include the resident DC [516].

In the presence of inflammatory signals, DC can be recruited very rapidly to the lungs as a response to the increased requirement for surveillance at the local site. Studies in allergic asthmatics have shown that circulating M-DC are reduced between 4 and 24 h after allergen inhalation [518], and start appearing in the airways 5–6 h after allergen [519]. Because the wave of DC arrives in the airways before the neutrophils and other mononuclear cells, DC contribute to the very early phase of the immune responses in the airways. DC are also found in increased numbers in the lung during secondary immune responses. Indeed, the number of airway CD11c+ CD11b+ M-DC is strongly increased within the airway epithelium following allergen challenge in sensitized animals [513]. This increase in the number of airway DC is accompanied with an increase in CD11c– CD11b+ MHCII+ monocytes that could be recruited to the airways and probably further differentiate into DC [520].

During bacteria-induced inflammation the rate of DC accumulation in the lung increases markedly [521]. Unlike a wave of incoming neutrophils, which rapidly moves into the airway lumen, the DC precursors remain within the epithelium during the acute inflammatory response and assume the dendritic form morphology typical of mature airway DC. During the 48 h. period after bacterial challenge, many of these DC migrate to draining lymph nodes. As in steady state conditions, CCR1/CCR5 are involved in DC recruitment in this inflammatory conditions. It is not excluded a function for other chemokine receptors in recruiting DC precursors to the lung. DC precursor recruitment during allergen challenge depends on CCR2/CCR6, differing mechanistically from the recruitment after a bacterial stimulus [522].

CCL20, the chemokine that acts on CCR6, is poorly expressed by bronchial epithelial cells and primary alveolar type 2 cells in steady state conditions; however is abundantly released in inflammatory conditions [523, 524]. Pichavant el al [525] have shown that bronchial epithelial cells of asthmatic patients stimulated with Derp1 showed an increased production of CCL20. CCL2 and CCL7 (two of the known murine CCR2 ligands) expression increases rapidly in lungs after antigen challenge in a antigen – induced lung inflammation model [522]. Hence ligands for both CCR2 and CCR6 are present in the lungs and could contribute to recruitment of DC. CCR2 ligands CCL2 and CCL7 are rapidly increase (peak at 1 day) after antigen stimulation, while CCL20 production is delayed. Chemokine expression is spatially segregated in resected human tonsils, since a CCR2 ligand localize to vascular endothelium, whereas CCL20 expression is restricted to epithelial cells bordering the exterior [90]. Osterhoelzer et al [522] suggest that an identical temporal and spatial pattern occur during antigen-driven lung inflammation with CCR2 mediating early DC precursors recruitment from the blood into lung interstitium, whereas CCR6 more centrally drives the later transit across epithelia into the airways (to enter the alveoli, the site where inhaled antigens first deposit).

It is possible therefore, that during secondary immune responses in the lung, CCR2 and CCR6 are important for DC recruitment, whereas CCR1 and CCR5 fulfil this function under steady-state conditions and after innate immune stimuli. Some of this difference might be due to the presence of antigen-specific T cells in the lung that can modify the responses of DC to antigen. During such secondary immune responses, the number of mature DC in the lung correlates with levels of inflammation, and with levels of various chemokines and cytokines [526, 527]. However, it is difficult to determine

whether increased levels of pulmonary DC lead to increased inflammation or whether the converse is true.

Monocyte chemokine-like protein, a new chemokine constitutively expressed in the lung, specifically attracts monocytes and DC *in vitro*; however, whether it has the same role *in vivo* remains to be elucidated [528].

Besides chemokines, other molecules can also favour the migration of DC into the lungs. The airway epithelium can attract DC by means of defensins, cationic peptides with bactericidal activity engaging CCR6 on immature DC [64, 529]. In this way, defensins may promote adaptive immune responses by recruiting DC to the site of microbial invasion. During inflammation, MMP-9-/- mice have been shown to have an impaired recruitment of DC into the airways, whereas the migration of DC to the draining lymph nodes was unaffected [213]. In summary, DC are recruited to the lungs via different mechanisms, and it seems very likely that these chemotactic agents may act sequentially to attract recently transmigrated DC, and position them at the inflamed site.

Whereas E- and P-selectin, VCAM-1, and L-selectin ligands have been extensively described in the transendothelial migration of skin DC, their involvement in the recruitment of precursors or DC to the lung has never been proven.

P-DC can also be recruited to nonlymphoid organs in inflammatory conditions. In asthma, there is an increased expression of peripheral node addressin (PNAd), ICAM-1, and VCAM on the vessels of affected tissues. Interactions with these adhesion molecules are likely to be involved in DC migration into this site, as circulating P-DC express the appropriate ligands, namely CD62L, β2, and α4 integrins, respectively [350, 530]. Moreover, P-DC express CXCR3, the receptor for CXCL10, which is upregulated under inflammatory conditions in the lung [415].

DC not only are responsive to the action of chemokines, but they also produce a wide range of chemokines in order to direct their environment. Depending on the stimulus, human DC are able to produce CCL2, CCL3, CCL4, CCL17, CCL22 and CXCL8 [281]. Interestingly, production of CCR4 ligands (CCL17, CCL22) by M-DC cells suggests that these cells are capable of selectively recruit Th2 cells and/or Treg cells at sites of inflammation [413]. This latter scenario may be especially important during chronic asthmatic responses that depend upon Th2 type cytokines.

1.3.4. Migration of airway dendritic cells to draining lymph nodes

It has been proposed that under steady-state conditions, M-DC slowly but continuously migrate to draining lymph nodes and present either (self)-auto antigens or harmless antigen in a tolerogenic form [531]. The mechanisms involved in the steady-state migration of airway DC to the mediastinal lymph nodes are unclear. Studies performed on skin DC suggest that semimature DC, which are immature DC with an intermediate phenotype, might use CCR7 to continuously migrate to the lymph nodes in the absence of inflammatory signals [331, 532]. It is not clear whether this would also happen in the lung as some studies have reported the absence of functional CCR7 expression by DC at mucosal surfaces [533].

The presence of ‘‘danger’’ signals in organs exposed to antigens is a strong stimulus for the migration of antigen-bearing DC toward lymph nodes. In a mouse model of allergic inflammation, ongoing airway inflammation was shown to cause a massive and accelerated flux of allergen-loaded DC from the airway mucosa to the mediastinal lymph nodes [444, 534]. The mechanisms behind this increased migration to the lymph nodes are not clear, but some likely mechanisms are described.

Factors driving DC migration from peripheral tissues to the T cell area of lymph nodes under inflammatory conditions is well documented in skin DC migration [535]. However very little is known regarding the involvement of this molecule in the trafficking of lung DC. CCR7 expression has been described on a subset of lung DC [536] and a series of experiments suggest that lung and skin DC use similar mechanism for their migration to the lymph nodes (e.g. *in vivo* neutralization of CCL21 prevents human DC migration to mediastinal lymph nodes of humanized severe combined immunodeficient (SCID) mice and the subsequent development of asthma features [537]. Moreover, the intranasal injection of latex beads into mice led to a CCR7-dependent accumulation of DC in the mediastinal lymph nodes [361]. DC migration from lung to lymph nodes is not only CCR7-dependent, but also involves another chemokine receptors, namely CCR8 which acts in concert with CCR7 [361]. CCL1, the chemokine that acts on CCR8 has been observed in the skin draining lymph nodes subcapsule. It is not known whether emigration of cells into the lymph nodes via afferent lymphatics requires chemokine signals to specifically enter the subcapsular sinus. If so, CCL1 is positioned to play a role at this point of entry during mobilization [33991].

CCR7 requires additional extracellular signals to become functionally coupled [538]. The responsiveness of CCR7 to CCL19 and CCL21 and the consequent lymph nodes

migration of DC is controlled by lipid mediators, such as the leukotrienes and prostaglandins. PGE_2 produced by epithelial cells after antigen exposure can stimulate DC emigration toward draining lymph nodes [372, 539]. The latter response is dependent on the EP4 receptor [371, 372, 539, 540]. In contrast, PGD_2 exerts an opposite effect: through the ligation of DP1, a receptor expressed by lung DC, inhibits the emigration of airway DC toward mediastinal lymph nodes and consequently prevents the induction of a primary immune response and of eosinophilic airway inflammation [376]. A stable analogue of PGI_2, Iloprost, could inhibit the migration of lung DC to the mediastinal lymph nodes, thereby abolishing the induction of an allergen specific Th2 response in these nodes [541]. The same effect was obtained with pharmacological agonists of the peroxisome proliferator-activated receptor-γ (PPAR-γ), an important intracellular mediator of prostaglandin signalling [542]. S1P, can modulate many different functions including migration, cytokine, and chemokine release [543, 544]. Inhalation of FTY720, a structural homologue of S1P, reduced the number of migrating M-DC in mediastinal lymph nodes of naive and allergen challenged mice and reduced asthma features [545].

Aim of the Thesis

CCRL2, is an orphan heptahelic serpentine receptor that shares high homology with the inflammatory chemokine receptors CCR5 and CCR1 [121]. It is rapidly induced during mouse DC maturation with a kinetics that precedes CCR7 induction [546]. In order to evaluate the relevance of this receptor in DC biology, CCRL2 deficient mice [121, 546] have been used in an established model of allergen-induced airway inflammation in which DC are known to play a crucial role [547].

Chemerin has been identified as a natural non-signaling protein ligand for CCRL2. CCRL2 is able only to bind the chemoattractant and increase local concentrations of bioactive chemerin which then is available for interaction with ChemR23, the functional chemerin receptor expressed on adjacent cells [93]. ChemR23 is expressed by human professional APC (monocyte/macrophages and DC) [70] and NK [69]. DC positive for ChemR23 have been found in close proximity of chemerin-positive human EC [71], suggesting a key role of the ChemR23/chemerin axis in directing DC trafficking. However limited information are presently available on chemerin/ChemR23 system in mouse, especially in DC-EC interactions.

Here we investigated the expression of functional ChemR23 in mouse bone marrow derived myeloid and plasmacytoid DC and its regulation during maturation. Moreover, since DC do transmigration and reverse-transmigration to cross the wall of vascular and lymphatic vessels, we investigated the possible presence and modulation of the ChemR23 functional ligand, chemerin, on lymphatic and vascular EC, taking advantages of specific cell mouse cell lines generated in the laboratory in the past.

2. Materials and Methods

2.1. Reagents

DMEM (high glucose), RPMI 1640 medium, heat-inactivated fetal bovine serum (FBS), ultraglutamine I, penicillin/streptomycin, non essential amino acids (NEAA) and sodium pyruvate were from Lonza (Verviers, Belgium). 1α,25 dihydroxyvitamin D3 (calcitriol), all trans retinoic acid (RA), bovine serum albumin (BSA, low endotoxin), gelatine from bovine skin, type B, aluminium hydroxide gel (alum), OVA; grade V, LPS and heparin were from Sigma (St. Louis, MO. USA). Low-LPS OVA lots were used. Recombinant mouse chemerin was from R&D Systems (Minneapolis, MN. USA), endothelial cell growth supplement (ECGS) from Biomedical Technologies, 2-βmercaptoetanol from Gibco (Scotland, UK), CpG from InvivoGen (San Diego, USA), mTNF-α, FMS-like tyrosine kinase 3 ligand (Flt3L), GM-CSF, CCl3, CCL5, CCL19, CXCL12 from Peprotech (Rocky Hill, NJ. USA). Sodium chromate Cr-51 (^{51}Cr) was from PerkinElmer.

2.2. Cell preparations

2.2.1. Isolation of mouse primary cells

Mediastinal lymph nodes cells were smashed to a single cell suspension. Lung tissue cell suspension was obtained by incubation with 1 mg/ml collagenase (type D; Sigma, St. Louis, MO. USA) and 20 μg/ml DNase (type I; Roche, Mannheim, Germany) for 1h at 37ºC. For flow cytometric analysis lung cells were depleted of red blood cells.

2.2.2. Endothelial cells

The mouse lung capillary endothelial cell line 1G11 [548] was grown in DMEM medium with 20% FBS, 1% NEAA, 1 mM Na pyruvate, penicillin (100 U/ml) and streptomycin (100 μg /ml), 1 mM ultraglutamine I, ECGS and heparin (100 μg/ml each).

The mouse lymphatic endothelial cell line (MELC) [549] was cultured in DMEM medium with 10% FBS, NEAA, sodium pyruvate, penicillin, streptomycin, heparin and ECGS as above, and 10% supernatant from sarcoma 180 cells.

In all cases cells at 6th-20th passage, grown to confluence on tissue culture plastic coated with 1% gelatine at 37°C in a humidified atmosphere with 5% CO_2, were used.

2.2.3. Bone marrow derived (BM-DC) and peripheral blood dendritic cells

Murine DC were generated from CD34+ bone marrow cells from C57Bl/6J, CCRL2-/- and ChemR23-/- mice (Charles River Laboratories) as previous described [93]. CD34+ cells were isolated from total bone marrow by positive immunoselection using the rat mAb MEC14.7 to mouse CD34 as a selecting agent. CD34+ cells were cultured in RPMI 1640 medium with penicillin/streptomycin, 5 % FBS and 2-βmercaptoetanol ($5x10^{-5}$M). To generate M-DC $2x10^5$/ml CD34+ cells were grown in medium with mGM-CSF (40 ng/ml) and Flt3L (100 ng/ml), while to generate P-DC $3x10^5$/ml CD34+ cells were grown in medium with Flt3L (200ng/ml). Cells were diluted 1:2–1:3 every 2 or 3 days and collected at day 8 (M-DC) or 9 (P-DC). DC were matured by incubation with TNF-α (20ng/ml) or LPS (100 ng/ml) (M-DC) or CpG (2μg/ml) (P-DC) for different times depending on the protocol.

Peripheral blood mononuclear cells were isolated from buffy coats of normal donors by Ficoll gradient (Ficoll-Paque™ PREMIUM, GE Healthcare, Life Sciences) and were magnetically sorted with blood DC antigen BDCA-1 (M-DC) and BDCA-4 (P-DC) cell isolation kits (Miltenyi Biotec), as described previously [194].

2.3. Generation of monoclonal antibody to CCRL2

10^7 X-rayed CCRL2/L1.2 cells in PBS (phosphate buffered saline) were injected intraperitoneally (i.p) every other week for 6 weeks into CCRL2-deficient mice. Three days after the last challenge, spleen cells were fused following conventional protocols. Hybridomas recognizing CCRL2/CHO-K and CCRL2/L1.2 transfectants but not parental cells were cloned and tested for specificity on CCRL2, CCRs and CXCRs transfectants. One hybridoma (hybr 4, IgG2a) was selected for these studies, purified and biotinylated.

2.4. Fluorescence activated cell sorting (FACS) analysis

BM-DC, BAL, mediastinal lymph nodes and lung cell suspensions were obtained as indicated above. Cells were incubated with rat anti-mouse CD16/32 mAb 2.4G2 hybridoma supernatant for 30 min. on ice to prevent nonspecific binding of antibodies to Fc receptors. For CCRL2 staining cells were then incubated with biotinylated anti CCRL2 mAb (3 μg/ml) in PBS buffer containing 1% FBS for 30 min. on ice. After washing, cells were incubated with streptavidin-FITC or -PE (BD Pharmingen). For

analysis of cell subpopulations, cells were stained with directly conjugated Abs to CD3e (145-2C11), CD4 (A15.1.17), CD8α (53-6.7), CD11b (M1/70), Gr-1 (RB6-8C5), I-Ab (2G9), CD11c (HL3), CD 80 (16-10A1), CD86 (GL1) from BD (Franklin Lakes, NJ, USA), CD103 (2E7), ST2 (DJ8) and Siglec H (440c) from eBioscience (San Diego, CA, USA), PDCA-1 (JF05-1C2.4.1) from Miltenyi (Bergisch Gladbach, Germany) and DEC 205 (hybridoma supernatant kindly provided by Dr A Vecchi)

Staining was analyzed by a FACS Canto flow cytometer (BD Biosciences). Live cells were gated based on forward and side-scatter and no staining for propidium iodide. Flow cytometry analysis of lung single cell suspensions was performed as described by Jakubzick and Randolph [550]. To obtain absolute numbers of each leukocyte subtype in BAL and lung tissue, percentages were multiplied by the total number of cells. Cell number in lung are referred to cell number/mg of tissue. All differential counts were performed in random order by the same observer

2.5. Quantification of gene expression by Real-Time PCR (RT-PCR)

Mouse EC lines (MELC and 1G11) were grown in 6 well culture plates (Becton Dickinson Labware) precoated with 1% gelatin. When confluent, serum and growth factors (ECGS, heparin, S180) supplemented medium was replaced by 0.2% BSA culture medium. Cells were stimulated with RA, 5 μM, 1α,25 dihydroxyvitamin D3 (Calcitriol, 1 μM), mTNF-α (20 ng/ml) and LPS (100 ng/ml), for the times indicated in each experiment. Supernatants of culture were collected, centrifuged and stored at -20°C until ELISA assays. The cells were used to determine the expression of different genes.

Dendritic and EC total RNA was isolated using Trizol®reagent (Invitrogen) according to the manufacturer's specifications. For real time PCR (RT- PCR) analysis, total RNA samples were treated with DNAse I (Invitrogen) in order to remove contaminating genomic DNA. Total RNA (1 μg) was reverse-transcribed using a High capacity cDNA (complementary DNA) reverse transcription kit from Applied Biosystems, following manufacturer's instructions. RT-PCR was performed on cDNA samples using a 7900HT Fast Real-Time PCR System machine according to manufacturer's guidelines (Applied Biosystems). The primers/fluorogenic (FAM) probes sets Mm 00503581_gH and Hs 99999901_s1 from Applied Biosystems were used to amplify chemerin and 18S respectively in a TaqMan gene expression assay. The other genes were amplified using a SYBR Green PCR master mix (Applied Biosystems,

Warrington, UK) and specific primers (Table 2). Human 18 S ribosomal and mouse β-actin and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene were used to normalize PCR results.

Gene product	Forward (5'-3')	Reverse (5'-3')
CCRL2	TGTGTTTCCTGCTTCCCCTG	CGAGGAGTGGAGTCCGACAA
ChemR23	CCATGTGCAAGATCAGCAAC	GCAGGAAGACGCTGGTGTA
CCR1	CTGCCCCCCCTGTATTCTCT	GACATTGCCCACCACTCCA
CCR2	CTACGATGATGGTGAGCCTTGTC	AGCTCCAATTTGCTTCACACTG
CCR3	AGTGGGCACCACCCTGTG	GCCATGACCCCAGCTCTTT
CCR4	ACGAAAGCATGCCAAAGCC	CCCCAAATGCCTTGATACCTT
CCR5	TCAGCACCCTGCCAAAAAAT	CAGGAGCTGAGCCGCAAT
CCR6	GGCCTGTATCAGCATGGACC	GATTTGGTTGCCTGGACGAT
CCR7	TGGTGGTGGCTCTCCTTGTC	CCTCATCTTGGCAGAGAAGCACA
CCR8	ACCCTGATTTCTTCACCGCC	TGCCCCTGAGGAGGAACTCT
CCR9	TGATGCCCACAGAACTCACAA	TGAAGTCATCAAACATGCCAGG
CXCR1	TCCTGAGGTGACTTTGAGAAAG	GGCAGCATTCCCGTGATATTT
CXCR2	GTCATCTTCGCTGTCGTCCTT	GTTGTAGGGCAGCCAGCAG
CXCR3	TGGAAAACAGCACCTCTCCC	AGAAGTCGCTCTCGTTTTCCC
CXCR4	CCTGCTTCCGGGATGAAAA	TGGTGGGCAGGAAGATCCTAT
CXCR5	ACTCGGAGCTCAACCGAGAC	AAGGTCGGCTACTGCGAGG
CXCR6	TACGATGGGCACTACGAGGG	ATCACTGGAATTGTTGAAGAGCC
CXCR7	TGTAACAGCAGCGACTGCATT	CATGGTGGGACACTGCACAG
CX3CR	CTGTCCGTCTTCTACGCCCT	CAGATTTCCCACCAGACCGA
XCR1	TCTTCACCGTCGTGGTAGCA	TGAGGTTGTAGGGAGCCCAG
CCL3	CATATGGAGCTGACACCCCG	TCTTCCGGCTGTAGGAGAAGC
CCL4	GCCCTCTCTCTCCTCTTGCT	GAGGGTCAGAGCCCATTG
CCL5	TGCTCCAATCTTGCAGTCGT	ACACACTTGGCGGTTCCTTC
CXCL10	CAACTGCATCCATATCGATGAC	TTCATCGTGGCAATGATCTC
CXCL12	CTGTGCCCTTCAGATTGTTG	TAATTTCGGGTCAATGCACA
β-Actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG
GAPDH	CGTGTTCCTACCCCCAATGT	TGTCATCATACTTGGCAGGTTTCT

Table 2. Set of primers used for quantitative RT-PCR

2.6. ELISA

Cytokines and chemokines were measured in 4x concentrated BAL fluids, lung homogenates and lymph nodes culture supernatants obtained as explained before by standard sandwich ELISA assays. Specific capture and detecting antibodies were from Pharmingen (IL-4, IL-5, INF-γ) and R&D (IL-13, IL-2, MCP-1/CCL2, CCL5/RANTES, eotaxin/CCL11, TARC/CCL17 and MDC/CCL22), and were used according to the manufacturer's protocol.

Lung tissue was homogenized at 50 mg/ml in HBSS (Hanks' Balanced Salt Solution) (Invitrogen), centrifuged at 800 *g* for 10 min. and the supernatant was collected. Mediastinal lymph nodes were cultured in the conditions indicated in each experiment and the supernatants were collected at the indicated times.

Total and OVA-specific IgE were detected in serum. Total IgE were measured by standard sandwich ELISA from Pharmingen. OVA-specific IgE were measured according to following protocol. Microtiter plastic plates were coated with anti-mouse IgE mAb (capture antibody) (Pharmingen, 2 μg/ml) O.N at 4°C, and afterward blocked with 3% BSA in PBS. Serum samples were diluted in PBS 5% FBS and then incubated O.N at 4°C. After washing with PBS-Tween 0.1%, 2 μg/ml of biotinylated OVA (detection reagent) was added to the wells for 1.5 h at room temperature (RT). Biotin was conjugated to OVA with the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce Rockford, IL, USA), following manufacturer's instructions. Plates were washed and, following the addition of streptavidin-HRP (horseradish peroxidase) (Pharmingen), incubated for 1 h at RT. OVA-specific IgE were detected by adding peroxidase substrate solution (colorimetric substrate) and reading in a microplate reader (VersaMax. Molecular Devices, Sunnyvale, CA. USA) to 450 nm.

For chemerin determination EC supernatants were concentrated 10 times using a vivaspin concentrator unit (3000 MWCO (molecular weight cutoff) , Sartorius Stedim Biotech) and examined by ELISA according to manufacturers' instructions (Mouse Chemerin Quantikine ELISA kit, R&D Systems).

2.7. FITC-dextran uptake

Endocytosis was measured as the cellular uptake of FITC-dextran (Sigma Chemical Co.) as described [95]. Approximately $2x10^5$ cells/sample were incubated in media

containing FITC-dextran (1 mg/ml) for 60 min. Uptake of the label was determined by flow cytometry

2.8. *In vitro* cell migration assays

2.8.1. Chemotaxis

Migration of BM-DC in response to different chemoattractants was evaluated using a chemotaxis chamber (Neuroprobe, Pleasanton, CA) and polycarbonate filter (5 μm pore size, Neuroprobe) as previously described [133, 551]. 50 μl of 1.5 x 10^6 cell suspension were applied to the top wells and allowed to migrate to agonists for 90 min. at 37°C. Cells that migrated onto the underside of the filter were stained using Diff-Quik and counted in an average of 5 high-power fields. Each data point was performed in triplicate.

2.8.2. Transendothelial migration assay

EC were grown to confluence on 0.1% gelatine coated transwell inserts in 24-well costar chambers (5μm pore size, Corning Costar, Cambridge, MA). When indicated the EC were stimulated with RA (5μM) 18 h before the transmigration assays. For the transmigration assay, 100 μl of DC (0.5x10^6 cells/ml of ^{51}Cr labelled mouse DC; 2x10^6 cells/ml of human DC) were placed in the upper chamber and 600 μl of chemoattractant or control medium were added to the lower chamber. DC were allowed to migrate for 90 min. (mouse DC) or 4 h (human DC) at 37 ºC in a 5% CO_2 atmosphere. Serum free medium with 0.2% BSA (chemotaxis medium) was used in all migration experiments performed with chemerin.

Mouse DC migration was evaluated as the percentage of radioactivity recovered in the lower compartment relative to input. Values are expressed as net migration (% radioactivity sample - % radioactivity control).

Human DC were collected in the lower chamber and counted at microscopy using a Bürker chamber. The results were expressed as the percentage of cell input.

In some experiments, human P-DC were preincubated (30 min. at 4°C) with anti-ChemR23 mAb (clone 1H2 (IgG2a, 3 μg/ml) [70] and then added to the upper chamber. Chemerin (300 pM) added to EC for 90 min. at 37º C in the upper chamber was used as a control. Before addition of P-DC chemerin was removed, in order to eliminate the excess and leaving only chemerin bound to EC.

2.9. *In vivo* migration of murine dendritic cells

2.9.1. FITC skin painting

FITC (fluorescein isothiocyanate isomer I, Sigma, St Louis, MO) was dissolved in a 50:50 (vol/vol) acetone–dibutylphthalate (BDH) mixture just before application. Mice were painted on the shaved abdomen with 0.2 ml/5 mg/ml FITC. After 24 h, inguinal lymph nodes were disaggregated and treated with collagenase A/DNase mixture for 30 min. CD11c+FITC+ cells were analysed by FACS as described by [552].

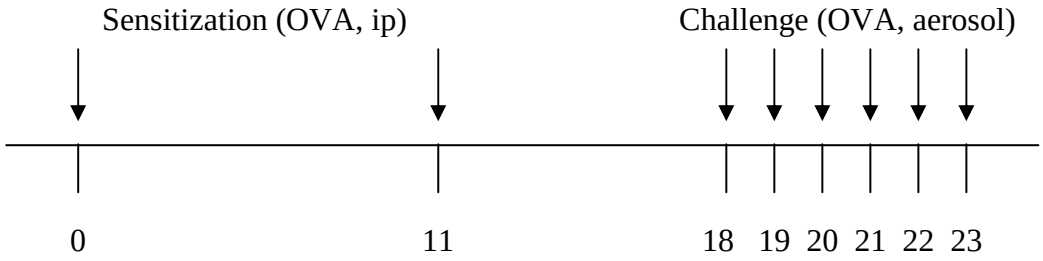
2.9.2. Migration of lung dendritic cells and dendritic cell adoptive transfer

To address the migration of lung DC to mediastinal lymph nodes, mice were sensitized with OVA (day 0 and 11) and 80 μl of FITC-OVA 10 mg/ml (Sigma, St Louis, MO) was administered intratracheally at day 18, under direct vision, using a 18-gauge polyurethane catheter connected to the outlet of a micropipette. CD11c+FITC+ DC were FACS counted in mediastinal lymph nodes as described by Jakubzick and Randolph [550]. Mediastinal lymph nodes were sampled 8, 24, 48 and 72 h after FITC-OVA administration

For adoptive transfer of bone-marrow-derived DC, cells at the end of the culture were pulsed overnight (O.N) with 100 μg/ml OVA, washed and instilled intratracheally (10^6/mouse) as described above.

2.10. Experimental protocol for induction of allergic airway inflammation

WT and CCRL2-/- mice were sensitized with OVA (LPS depleted by centrifugation using a centricom with cutoff of 100000 KDa), at a concentration of 0.01 mg/mouse i.p in 0.2 ml alum on day 0 and 11. Control mice received the same volume of PBS in alum. All groups were challenged daily with 5% OVA aerosol (for 20 min.) from day 18 to 23 (Fig. 12). The aerosol was generated by a PARI Master Nebulizer (Sapio Life) connected to the Plexiglas chamber. Mice were sacrificed by exsanguination under terminal anaesthesia at 24 h after the last aerosol.



Group	Sensitization	Challenge
PBS/OVA	**PBS**	**OVA**
OVA/OVA	**OVA**	**OVA**

Figure 12. Allergic airway hyperresponsiveness model protocol. Mice were sensitized with an intraperitoneal application of LPS-depleted OVA (0.01mg/ mouse in 0.2 ml alum) at day 0 and 11, and then challenged with 5% aerosolized OVA for 6 consecutive days from day 18. Controls were treated with PBS during sensitization. Lung inflammation and clinical manifestations as AHR and mucus production were assessed at different times after the final allergen challenge.

Sera were prepared by centrifugation and snap-frozen to –20°C until further use. Airways were washed three times with 1 ml of PBS via a tracheal cannula to obtain broncheoalveolar lavage (BAL). BAL was centrifuged (200 *g*, 5 min., 4˚C and then concentrated by using Vivaspin 2 concentrators (3000 MWCO, Sartorius Stedim Biotech), and stored at -20^0C for further analysis.

BAL cells were counted, then pelleted onto glass slides by cytocentrifugation ($5x10^4$ cells/slide). Differential cell counts were performed on Diff-Quik (Median Diagnostics AG, Bonnstrasse, Germany) stained cytospins. Percentages of eosinophils, lymphocyte/mononuclear cells, neutrophils and macrophages were determined by classifying at least 400 cells per slide under light microscopy visualization using standard morphological criteria. To obtain absolute numbers of each leukocyte subtype percentages were multiplied by the total number of cells obtained in the BAL fluid. All differential counts were performed in random order by the same observer.

Lung tissue cell suspension was prepared as indicated before. Cytocentrifuge preparations were prepared and Wright-Giemsa stained, and differential counts were performed as for BAL.

For lung tissue histopathology, lungs were fixed in 10% normal buffed formalin. 4-µm paraffin-embedded sections were stained with haematoxylin and eosin. For immunohistochemistry samples were snap frozen in dry ice and stored at -80 ℃

2.11. Measurement of airway hyperresponsiveness (AHR)

AHR was measured in mice 24 h after the final OVA challenge by recording respiratory pressure curves by whole body plethysmography (Buxco Technologies) in response to inhaled methacholine (Sigma) at concentrations of 30 mg/ml for 1 min. [96] as previous described [553]. Results are shown for Penh after allergen challenge. Penh results were confirmed by direct measurements of lung resistance and dynamic compliance in anaesthetized and tracheotomized mice in response to inhaled methacholine at concentrations of 30 mg/ml in a Buxco system (Buxco Technologies) as previous described [553].

2.12. Assessment of mucus production

After removal from the animal, one lobe of lung was inflated with PBS. Lungs were fixed in 10% normal buffered formalin. Paraffin embedded sections (4µm) were stained with Periodic Acid-Schiff (PAS). Globet cells were counted on PAS-stained sections and scored as previously described [553]. Briefly, the lungs were scored in the following way: score 0: 5% goblet cells; score 1: 5 to 25%; score 2: 25 to 50%; score 3: 50 to 75%; score 4: more than 75%. The sum of the airway scores from each lung was divided by the number of airways examined for the histologic goblet cell score (expressed as arbitrary units)

2.13. Immunostaining of lungs

For immunohistochemistry, sections were obtained from frozen lungs from three mice/experimental group. Immunostain was performed by using anti-CD11c (BD Franklin Lakes, NJ, USA), anti-Siglec H (rat anti-mouse, kindly provided by Prof. M. Colonna, Washington University, STL, MO, USA) and anti CD103 (eBioscience, San Diego, CA, USA). Primary antibodies were revealed using anti-hamster or anti-rat biotin-conjugated secondary antibodies (Vector, Burlingame, CA, USA) followed by HRP (for CD11c and Siglec H) or streptavidin-alkaline phosphatase for CD103) and 3,3' Diaminobenzidine (all from Dako, Denmark) or Ferangi Blue (Biocare Medical,

Concord, CA, USA). As a negative control, primary antibodies were omitted or replaced with an irrelevant isotype-matched mAb.

2.14. *In vitro* re-stimulation of draining lymph nodes

Mediastinal lymph nodes cell suspension was prepared as previously described. $5x10^5$ cells in 200 µl were seeded in U-bottomed plastic plates and OVA was added at varying concentrations. Plates were incubated for 96 h at 37°C. Supernatants were harvested and used to measure cytokine levels.

2.15. Dendritic cell-induced T cell proliferation

For alloantigen T cell proliferation, CD4+ T cells were purified from spleens of Balb/c mice (Charles River laboratories) using CD4 immunoconjugated microbeads (Miltenyi Biotech). Isolated T cells were labelled with 5 µM CFSE (Carboxyfluorescein diacetate succi- nimidyl ester) in RPMI containing 5% FBS for 20 min., washed and seeded at $2x10^5$ cells per well in U-bottom 96-well plates in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS and 10 mM Hepes pH 7.4 and antibiotics. DC from WT or CCRL2-/- mice were matured with 20 ng/ml of TNF-α for 24 h, washed and added to T cells at different ratios, ranging from 0.1 to 10 % relative to T cells quantity. After 72 h of co-culture cells were stained with a CD4 antibody and T cell proliferation was monitored by FACS in terms of CFSE dilution. T cells with CFSE content lower than T cells cultured in the absence of DC (and hence did not dilute the CFSE) were considered as proliferated cells. Antigen-specific T cell proliferation was tested with a similar protocol, by using CD4+ T cells purified from spleens of OT-II mice and by pulsing DC with 100 µg/ml OVA during maturation. Supernatants were frozen and the secreted cytokines were measured by ELISA in both cases.

2.16. Statistics

Statistic significance was calculated by Student T test, Mann-Whitney *U* test and one-way ANOVA as appropriate. Differences were considered significant at $p< 0.05$.

3. Results

3.1. Role of CCRL2 in lung leukocyte traffic

3.1.1. CCRL2 participates to inflammatory infiltrate/Th2 cytokine production evaluated in broncheoalveolar lavages (BAL) in a model of airway hyperresponsiveness

CCRL2 expression has been described during maturation of murine DC with kinetics that precedes CCR7 expression. To investigate the relevance of CCRL2 in DC biology, CCRL2 deficient mice (CCRL2-/-) [546] were used in an established model of allergen induced airway inflammation in which DC are known to play a crucial role in the transport of antigens to mediastinal lymph nodes [547]. OVA-immunized CCRL2-/- mice, challenged by aerosol for six consecutive days, showed a dramatic reduction in the total number of leukocytes and in particular of eosinophils and lymphocyte/mononuclear cells, with respect to WT animals (Fig. 13).

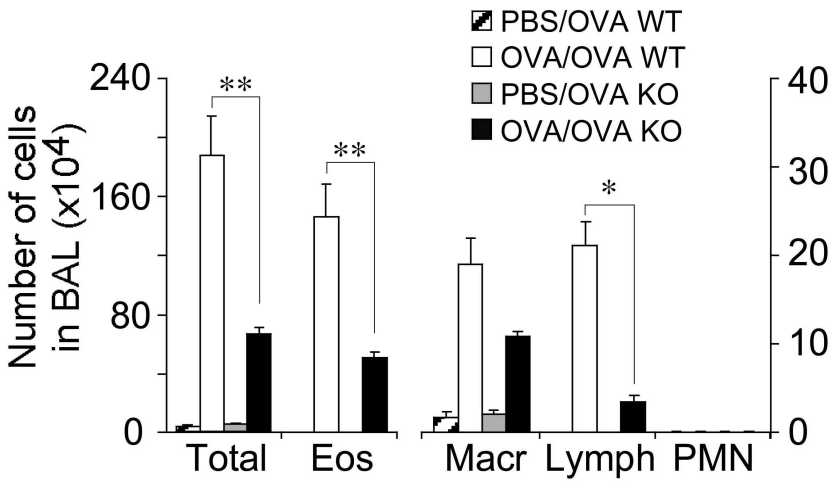


Figure 13. Leukocyte population is reduced in BAL from CCRL2-/- mice. Mice were sensitized and challenged with OVA by aerosol. BAL was collected 24 h after the last aerosol; total cell numbers were counted and differential cell counts performed. Data are mean ± SEM of one representative experiment of the five performed with 6 to 12 mice per group; *P<0.05; **P<0.01

T cell population is composed by different subsets with different patterns of cytokine production to drive different types of immune responses [554, 555]. To determine whether there was a selective deficiency of T subsets in BAL, cells were analyzed by FACS, using CD3 as a marker for T cell lineage in combination with CD4 (T helper cells) and CD8 (cytotoxic T cells). Since allergic asthma is characterized by a chronic immune response sustained by Th2 type cells, CD4+ST2+ Th2 subtype was also evaluated. A significant decrease in T cells was observed for all three subsets in CCRL2-/- mice compared to control ones (Fig. 14).

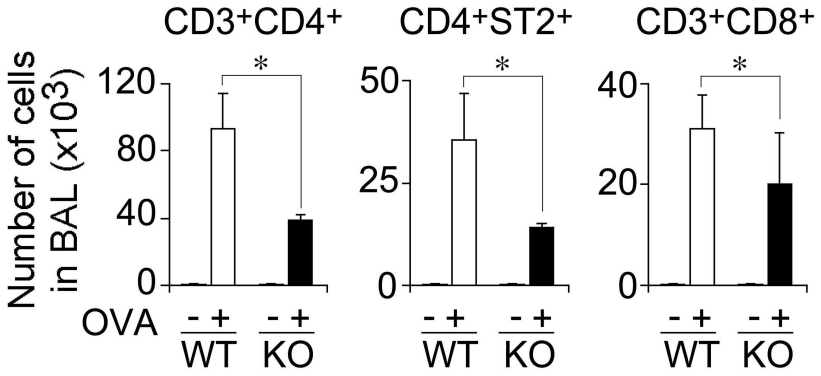


Figure 14. CCRL2 deficiency is associated with a reduction in T cell subsets in BAL. Mice were sensitized as reported in Fig.13, challenged and sacrificed. T cell subsets (CD3+CD4+: T helper cells, CD4+ST2+: Th2 cells and CD3+CD8+: cytotoxic T cells,) were determined by FACS. Data are mean ± SEM of one representative experiment of the three performed with 6 to 12 mice per group; *P<0.05.

However, a possible direct role for CCRL2 in the recruitment of eosinophils and T cells into the airways was ruled out by the lack of expression of CCRL2 in these two cell types; conversely BAL MHCII+ cells (macrophages and DC) expressed CCRL2 (Fig. 15).

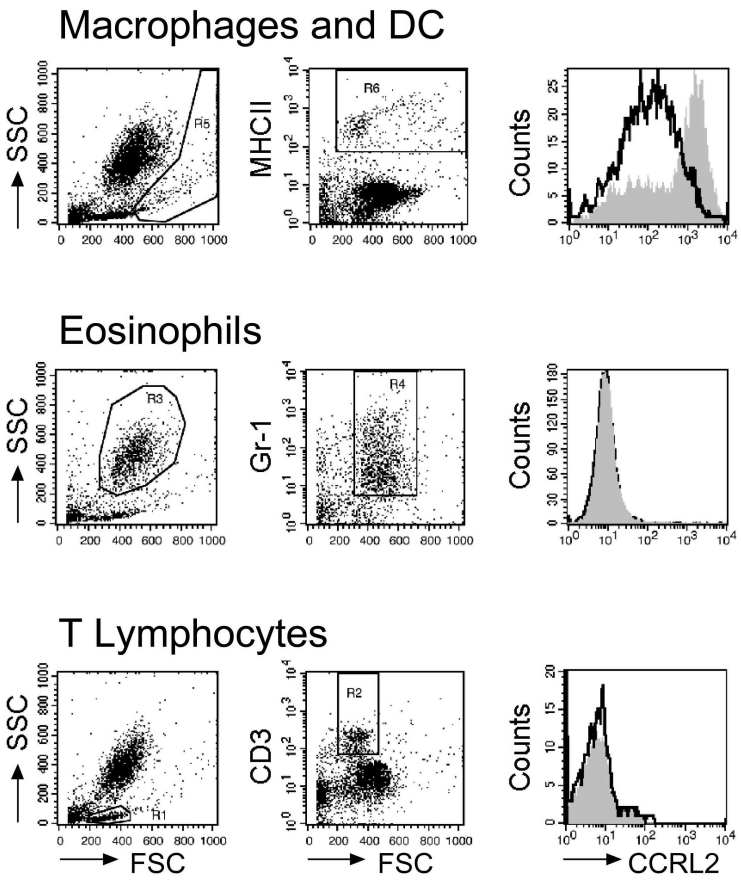


Figure 15. **CCRL2 membrane expression on BAL leukocytes by FACS analysis**. MHCII+ cells (macrophages and DC), eosinophils (Gr-1) and T lymphocytes (CD3) were analyzed. Histograms represent staining of cells from WT (open curves) and CCRL2-/- mice (filled curves) with anti-CCRL2 mAb. The data shown correspond to a representative experiment out of three performed with 6 to 12 mice per group

In allergic asthma, genetically susceptible individuals mount a chronic Th2 cell type immune response to common allergens, by secreting cytokines like IL-4, IL-5, IL-9, IL-10, IL-13 and GM-CSF [384]. Individually, these cytokines can already explain many of the salient features of asthma as IgE synthesis, growth and activation of eosinophils and mast cells and expression of cytokines and EC-adhesion molecules [385].

In parallel, Th2 and Th1 cytokines were measured in BAL from WT and CCRL2-/- mice. BAL of CCRL2-/- mice contained lower levels of the Th2 cytokines IL-4 and IL-5, and similar levels of IL-13 and of the Th1 cytokine IFNγ (Fig. 16A). Of note, although Th2 cells and eosinophils represent an important source for Th2 cytokines, IL-

13 can also be secreted by lung smooth muscle cells [556]. This may explain the different regulation of IL-13 versus IL-4 and IL-5 here observed.

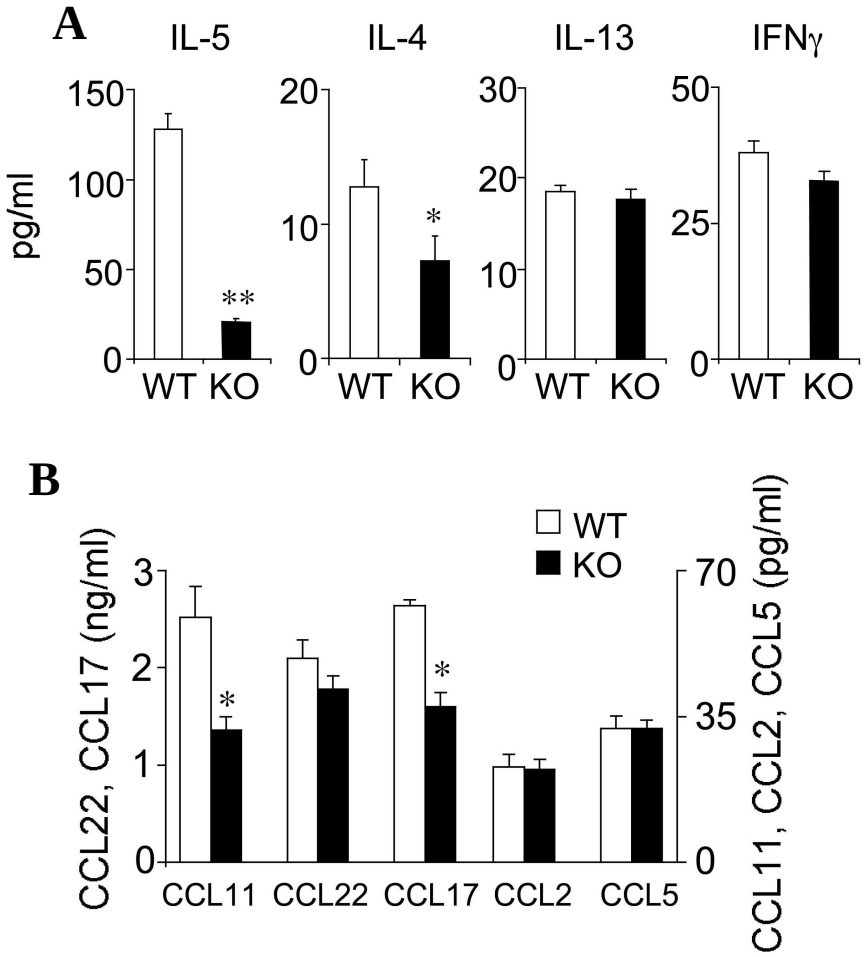


Figure 16. **Cytokine and chemokine levels in BAL.** (A) Cytokines and (B) chemokines, were measured in BAL. Data are mean ± SEM of one representative experiment of three performed with 6 to 12 mice per group; *P<0.05; **P<0.01

The development and maintenance of asthma correlates with the coordinated production of chemokines which guide leukocyte recruitment to different lung compartments [97]. To further examine the mechanisms associated with the defective leukocyte recruitment observed in CCRL2-/- mice, the levels of some relevant chemokines in allergic lung inflammation were evaluated. The eosinophil-attracting chemokine CCL11/eotaxin and the Th2-attracting chemokine CCL17/TARC levels were significantly decreased in BAL, while there were no differences for CCL22, CCL2

and CCL5 (Fig. 16B). This finding is consistent with the decreased number of eosinophils and Th2 cells observed in the airway lumen.

3.1.2. Minor role of CCRL2 in lung parenchyma inflammation.

The alterations observed in the BAL from CCRL2-/- mice were not paralleled by changes in the lung parenchyma, where normal degrees of infiltrating leukocytes (Fig. 17A) and Th cells (Fig. 17B) were detected.

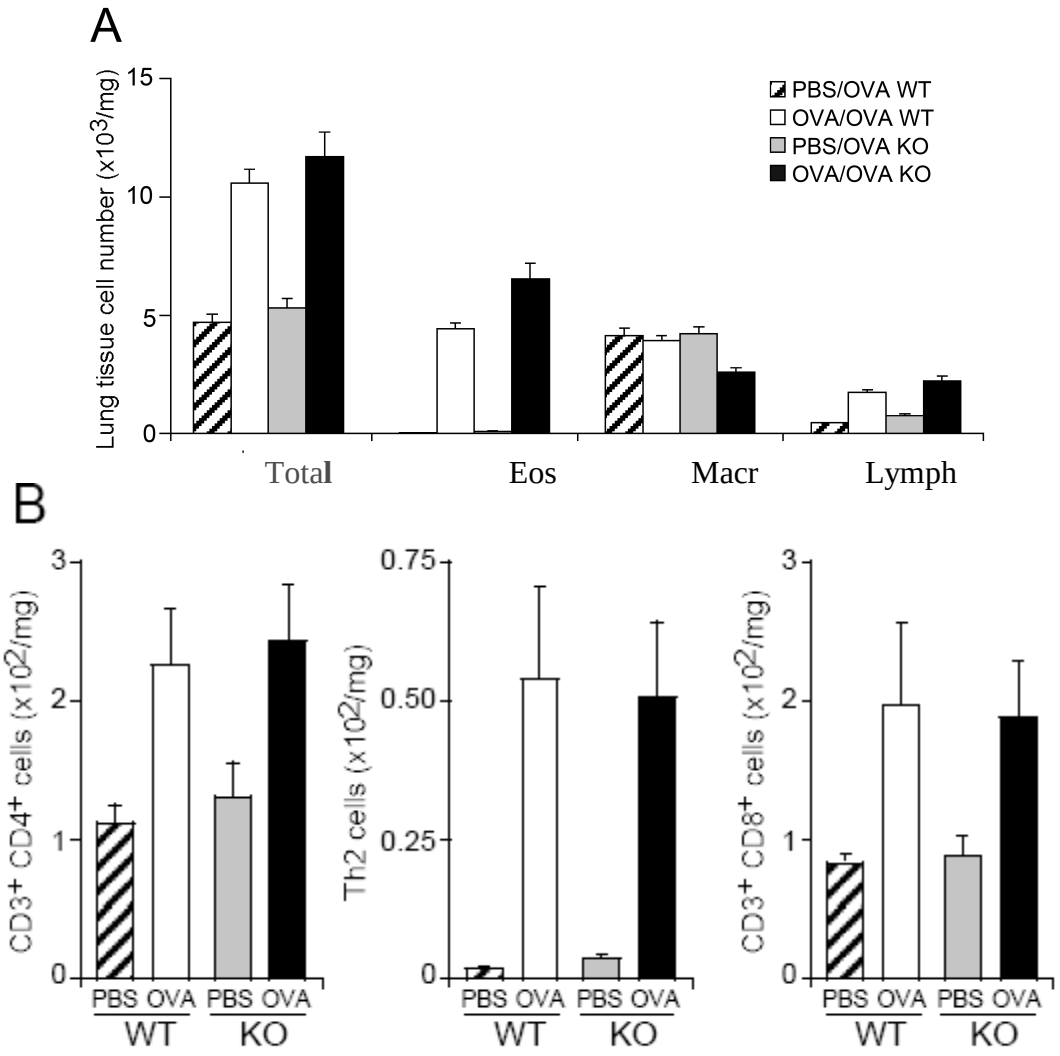


Figure 17. Leukocyte composition of lung parenchyma. WT and CCRL2-/- mice were sensitized and challenged with OVA by aerosol. Lungs were collected twenty-four h after the last aerosol and a cell suspension was obtained after treatment with collagenase/DNase. (A) Differential cell count was carried out. (B) T cell subsets (CD3+CD8+: cytotoxic T cells, CD3+CD4+: T helper cells and CD4+ST2+: Th2 cells)

were determined by FACS. Data are mean ± SEM of one representative experiment of two performed with 12 mice per group.

The levels of cytokines, IL-4, IL-5, IL-13 and IFNγ, measured in the BAL and expressed as pg/ml, resulted similar in CCRL2-/- mice compared to control ones (Fig. 18A). Similarly, no difference was observed in the expression of CCL11, CCL17 and CCL22 chemokines between CCRL2-/- and WT mice, while CCL2 and CCL5 levels were decreased in CCRL2-/- mice (Fig. 18B). The fact that the levels of chemokines recruiting eosinophils and Th2 cells are similar in WT and CCRL2-/- mice is consistent with the equal amounts of infiltrating leukocytes observed in the lung parenchyma of WT and CCRL2-/- mice.

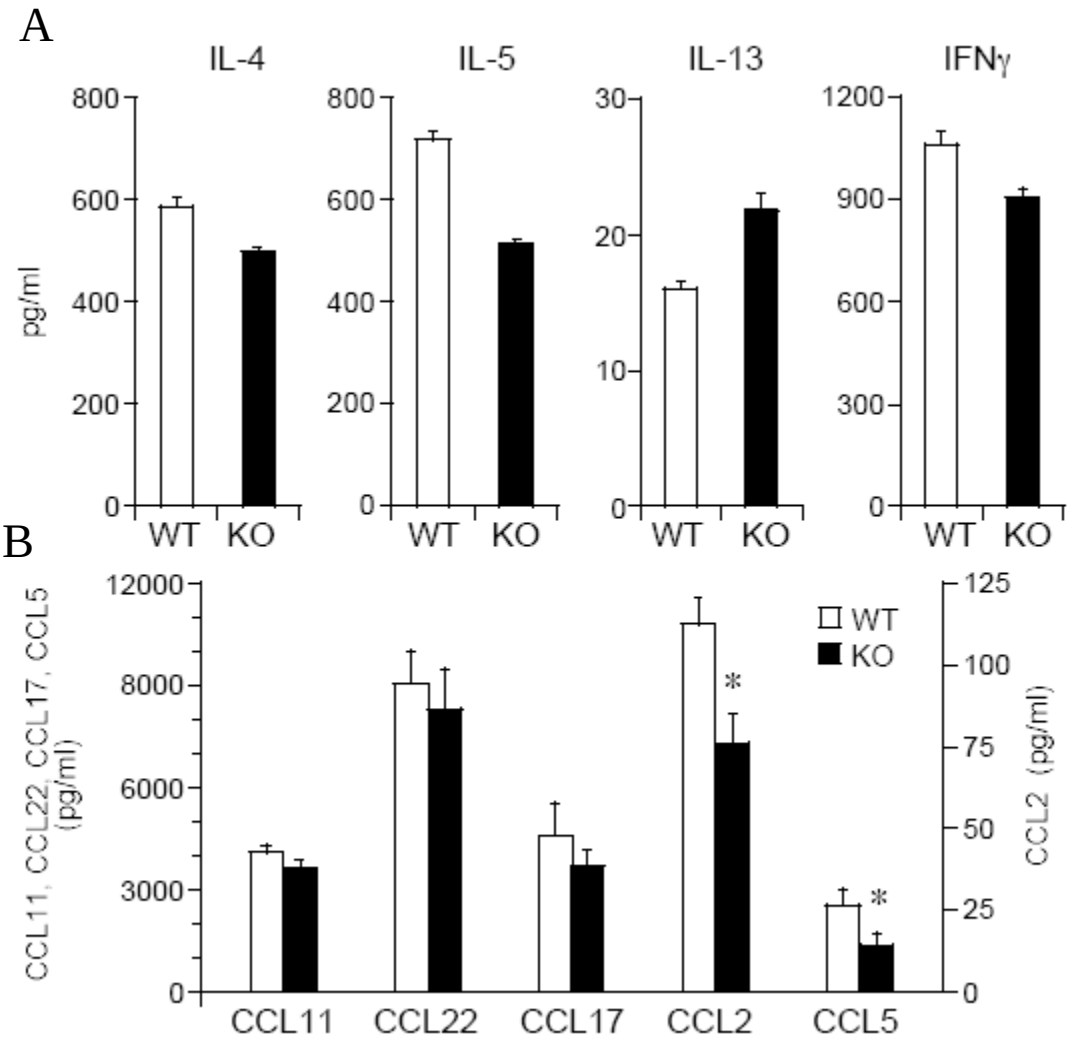


Figure 18. **Cytokine and chemokine levels in lung parenchyma.** (A) Cytokines and (B) chemokines were measured in lung parenchyma Data are mean ± SEM of one representative experiment of two performed with 12 mice per group; *P<0.05

3.1.3. CCRL2 does not modify typical features of asthma

Asthma is characterized by allergen specific IgE production by B cells [391]. To determine whether CCRL2 deficiency was associated with a defective Th2 dependent humoral response, circulating levels of total and OVA specific IgE were determined in serum. OVA-sensitized CCRL2-/- mice showed normal levels of circulating total and OVA-specific IgE (Fig. 19). This finding is consistent with the ability of CCRL2-/- mice to mount a normal primary and secondary humoral response following i.p. immunization with OVA/Alum or OVA/complete Freund's adjuvant (data not shown).

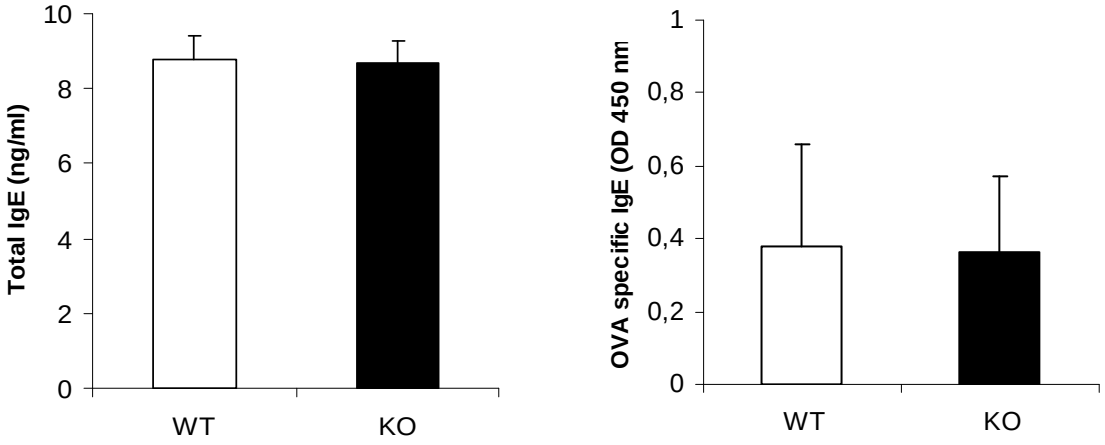


Figure 19. Total and OVA specific IgE in serum. Total IgE and OVA specific IgE were measured in serum of WT and CCRL2-/- mice. Data are mean ± SEM of one representative experiment of three performed with 6 to 12 mice per group.

Finally, the typical features of asthma, namely AHR and mucus hypersecretion were assessed in OVA-sensitized CCRL2-/- and WT mice. Both strains showed a significant increase in methacholine responsiveness evaluated as lung resistance (Fig. 20A, B) and dynamic compliance (Fig. 20C), that was however similar in the two mouse strains. Similarly, OVA-sensitized CCRL2-/- and WT mice displayed a comparable number of goblet cells (Fig. 20D).

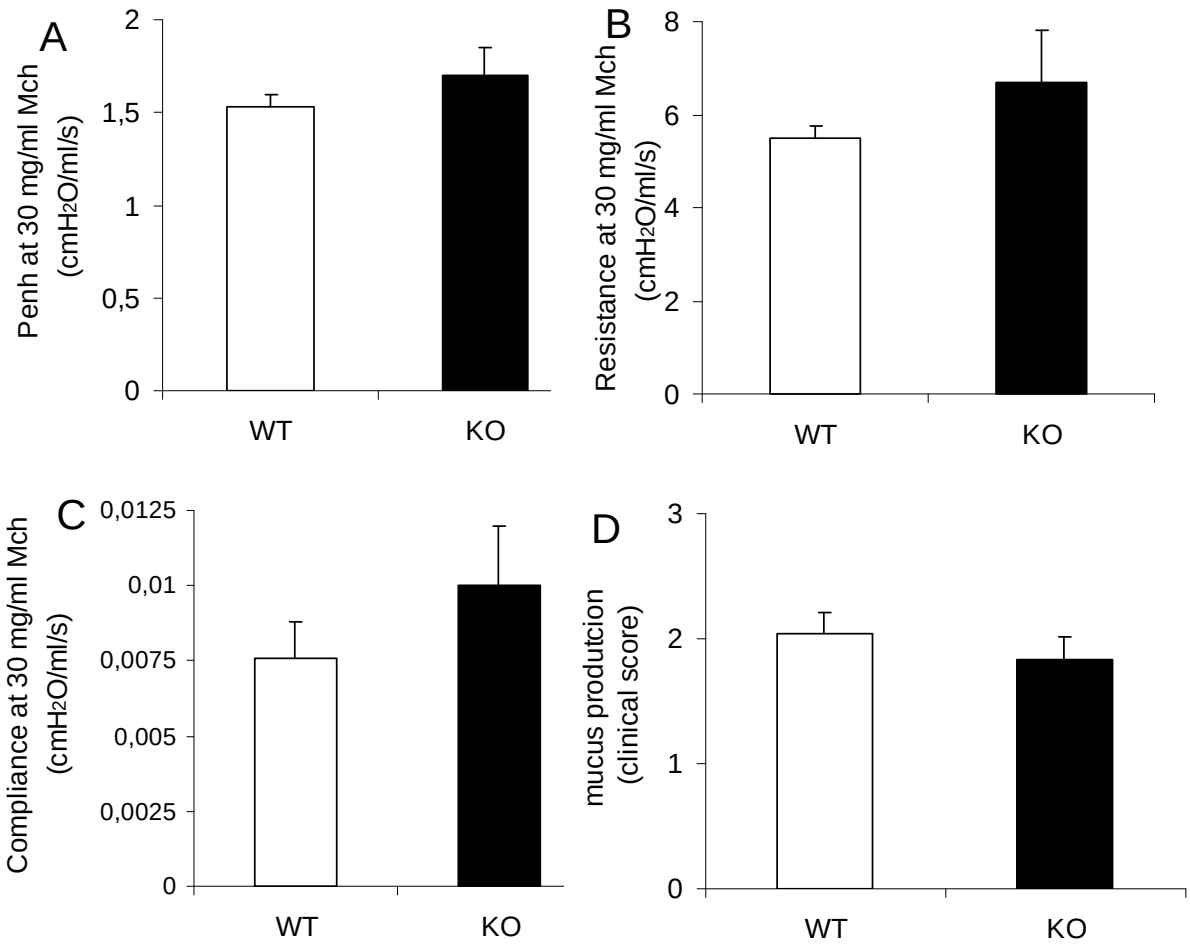


Figure 20. **Functional parameters measured in CCRL2-/- mice subject to an OVA-induced airway inflammation model**. (A) Penh (B) Resistance (C) Compliance and (D) mucus production. This last parameter was determined by scoring the samples according to the number of globet cells as described [553]. Data are mean ± SEM of one representative experiment of three performed with 6 to 12 mice per group.

3.1.4. CCRL2 dos not modify lung DC infiltrate after allergen challenge

The migration of lung DC from periphery to regional lymph nodes is a key step for the induction of immune response and tolerance [132, 531, 557]. Therefore, lung DC trafficking was investigated in OVA-challenged CCRL2-/- mice. Mice were sensitized with OVA at 0 and 11th day and then challenged with one 5% OVA aerosol at day 18. Lung tissue was collected at different time points after aerosol and tissue cell suspension was prepared as indicated in "Materials and Methods". Lung DC were identified by FACS as CD11c+MHCII+ cells. The alveolar space is rich in macrophages

that can be confused with alveolar DC for their high expression of CD11c, however, unlike DC, alveolar macrophages show high autofluorescence, that lets discriminate them from DC population [558]. In agreement with previous reports [213, 444], Figure 21 shows that the influx of DC into the lung was already detectable after 2 h of OVA challenge and reached peak levels at 24 h. No significant difference in the absolute number of DC (Fig. 21), neither in their activation phenotype (evaluated as CD80, CD86, CD40 and MHCII surface expression, data not shown) was found between CCRL2-/- and WT mice at any of the time points investigated.

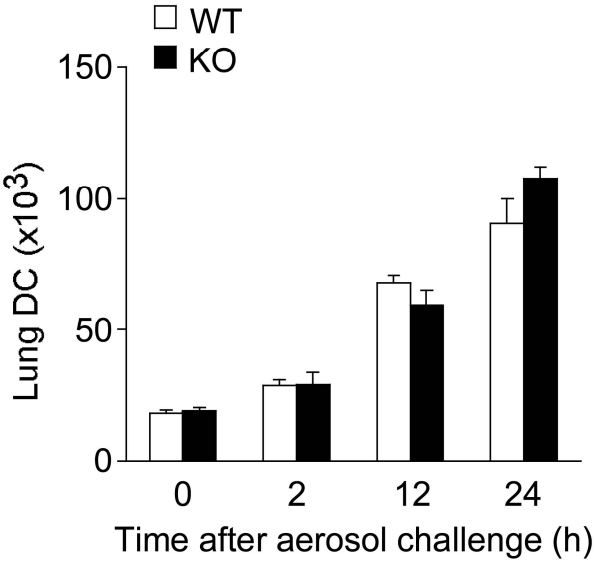


Figure 21. Lung DC number after allergen challenge. Lung tissue and trachea were isolated from sensitized mice after one OVA-aerosol challenge. DC (CD11c+MHCII+^{low} autofluorescence cells) were quantified by FACS analysis at different times points after aerosol Results are expressed as total number of DC per mouse. Data are mean ± SEM of one representative experiment of three performed with 6 mice per group.

Different subsets of DC can be found in the lungs. In steady-state conditions, conventional DC (CD11c^{high}MHCII^{high}) are found lining the conducting airways and in the deeper interstitial compartments, subdivided into CD11b+ and CD11b− subsets. CD11b- express langerin and the mucosal integrin CD103 (aEb7) at least in mouse and rat [393]. P-DC are found in both compartments with a slight preference for the interstitial

compartment. P-DC are CD11c^{int} and express Siglec-H, and BST-2 (recognized by PDCA1 antibody) and some markers shared with granulocytes and B cells [223, 437]. Under inflammatory conditions, additional CD11b+ monocytes are recruited to the lungs where they rapidly become DC (CD11c+CD11b+) and can easily be confused with resident CD11b+ conventional DC [81, 101, 102]. DC subsets show distinctive functions, thus an analysis of the subsets could be useful for the understanding of the differences seen between WT and CCRL2 -/- mice. Lungs of sensitized mice challenged by one OVA aerosol were thus analyzed by FACS Similarly to the results observed for total DC population in lung, no difference was observed in the relative proportion of the three major lung DC subsets, namely, myeloid CD103+ and CD11b+ DC and P-DC (Fig. 22).

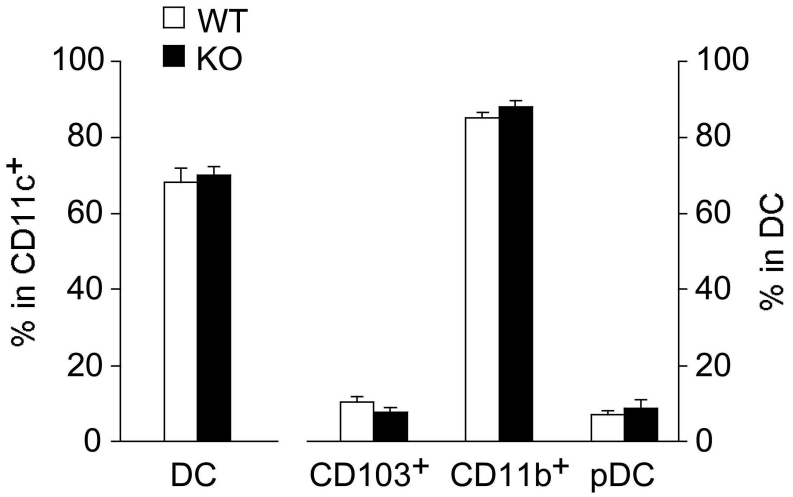


Figure 22. Lung DC subset composition after allergen challenge. Lung cell suspensions from OVA sensitized mice were obtained 24 h after one OVA-aerosol challenge. CD11c+MHCII+ cells, representing a mixture of lung macrophages and DC were initially gated. A further gate for the low autofluorescence population was drawn for pulmonary DC (left panel; results are expressed as the percentage of CD+/low autofluorescence DC in total lung CD11c+MHCII+ cells) and exclude lung macrophages. The three main pulmonary DC subsets (CD103+, CD11b+, and Siglec H+/PDCA1+ P-DC) were discriminated inside the DC population (right panel). Data are mean ± SEM of one representative experiment of three performed with 6 mice per group.

Furthermore, the *in vivo* distribution of DC subsets was investigated by immunohistochemistry of lung frozen tissue sections. CD103+ cells were mainly identified in the peri-bronchiolar space lining the conducting airways. Siglec H+ and CD11c+ cells were localized in the peri-bronchiolar space admixed to other immune cells as well as in the interstitial space of the lung parenchyma. CD11c stain was also observed in large cells located in the alveolar space likely reflecting expression by alveolar macrophages. However, no difference was observed between WT and CCRL2-/- mice in the distribution of these DC subsets in either resting or OVA-stimulated conditions (Fig. 23).

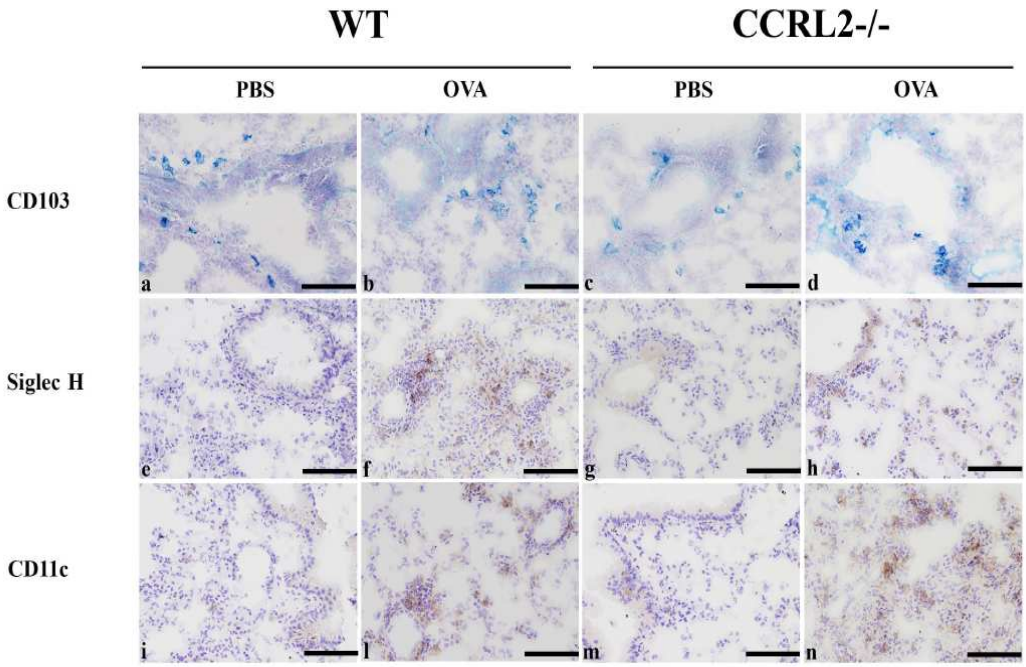


Figure 23. Localization of lung DC. CCRL2-/- and WT mice were sensitized with OVA and subsequently challenged with one OVA aerosol. Lung tissue was isolated 24h after aerosol. Representative sections are from frozen mouse lungs. Experimental conditions and antibody used are in the panels. CD103+ cells are regularly found observed in the peribronchiolar space (a-d). Round to oval Siglec H+ and CD11c+ cells increase significantly in number upon OVA stimulation in both WT and KO and are located either in the peribronchiolar space as well as in the interstitium of lung parenchyma. Scale bars: 100 micron.

3.1.5. CCRL2 affects the DC traffic to regional lymph nodes

Migration of pulmonary DC to draining lymph nodes is a key step in the initiation of the allergic response in the lung [547]. In order to study the migration of airway DC, OVA-sensitized animals were instilled intratracheally with a single administration of OVA conjugated with the fluorescent dye FITC. This treatment is known to induce lung DC maturation and mobilization [213]. Mice were sacrificed 0, 8, 24, 48 or 72 h later and CD11c+FITC+ DC were enumerated in mediastinal draining lymph nodes.

Figure 24A reports that the migration of FITC+ DC was already detectable after 8 h stimulation and reached a peak at 24 h, as previously reported [213]. CCRL2-/- mice showed a statistical significant reduction in FITC+ DC trafficking to lymph nodes at 48 and 72 h after antigen administration. Of note, airway-derived FITC+ DC expressed CCRL2, while FITC- DC, which correspond to resident mediastinal lymph nodes DC, did not (Fig. 24B). These results highlight a crucial role of CCRL2 in directing pulmonary DC migration to draining lymph nodes.

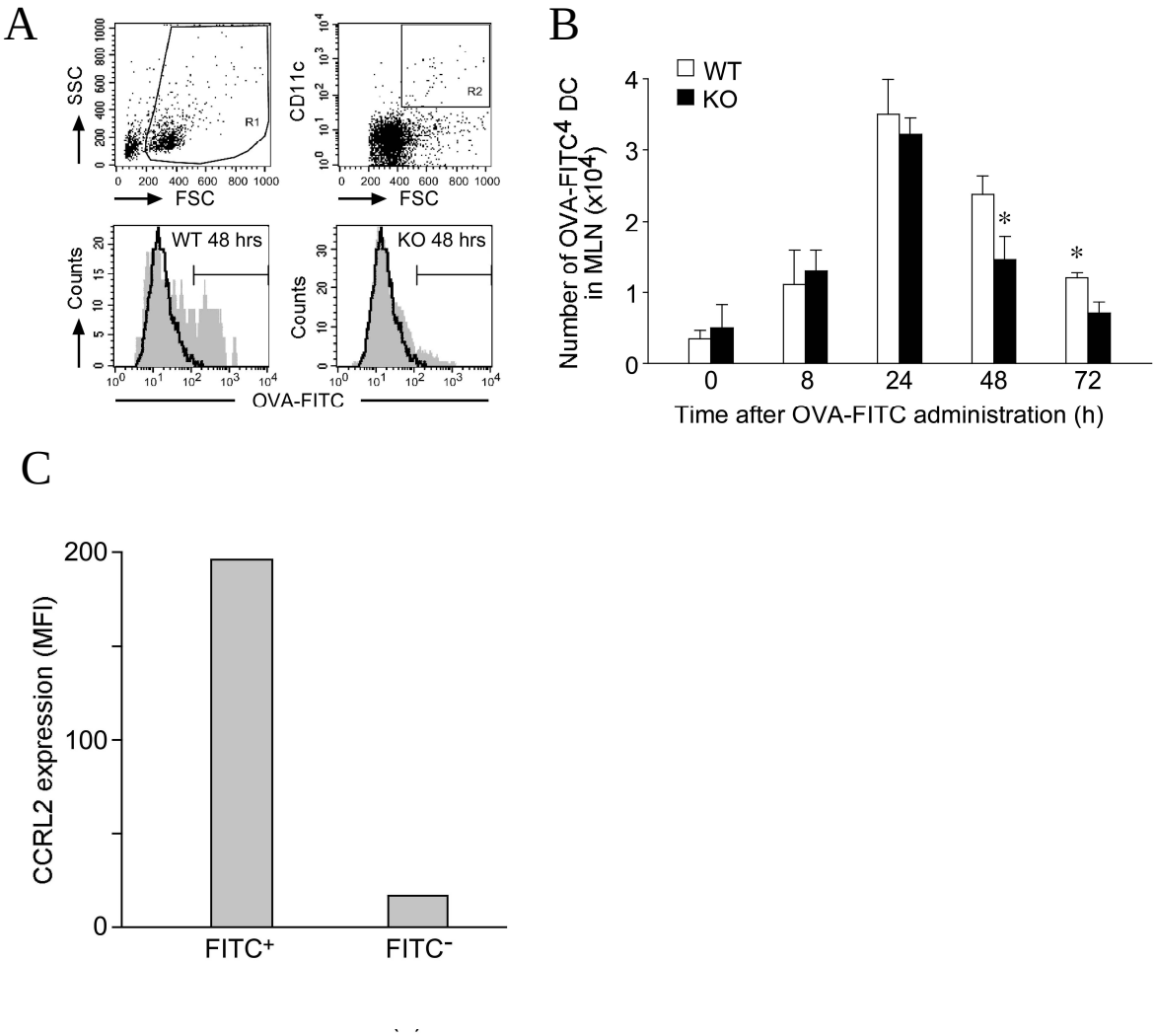


Figure 24. Lung DC migration to mediastinal lymph nodes. OVA-sensitized mice received i.t. 800 µg OVA FITC. (A) Representative FACS analysis of WT and CCRL2-/- mediastinal lymph nodes cells obtained 48 h after FITC-OVA administration. (B) Kinetics of FITC+ DC in lymph nodes. (C) Surface expression of CCRL2 in lymph node FITC+ and FITC- WT DC after OVA-FITC administration. Data are mean ± SEM of three (B) or two (C) representative experiments with 4 to 15 mice per group; * P <0.05.

3.1.6. CCRL2 does not affect generation and functions of bone-marrow *in vitro* derived DC.

In order to investigate the molecular basis for the defective migration of lung DC, experiments were performed using BM-DC. *In vitro*, DC generated from CCRL2-/- and WT bone marrow progenitors were similar in terms of cell yield (number of harvested

DC/number of seeded CD34+ progenitor cells), expression of membrane markers (CD11c, CD11b, MHCII, DEC205) and costimulatory molecule (CD80, CD86) (Fig 25A), and in their ability to take up antigens (Fig. 25B). These results postulate that CCRL2 deficiency does not interfere with DC development and maturation in vitro.

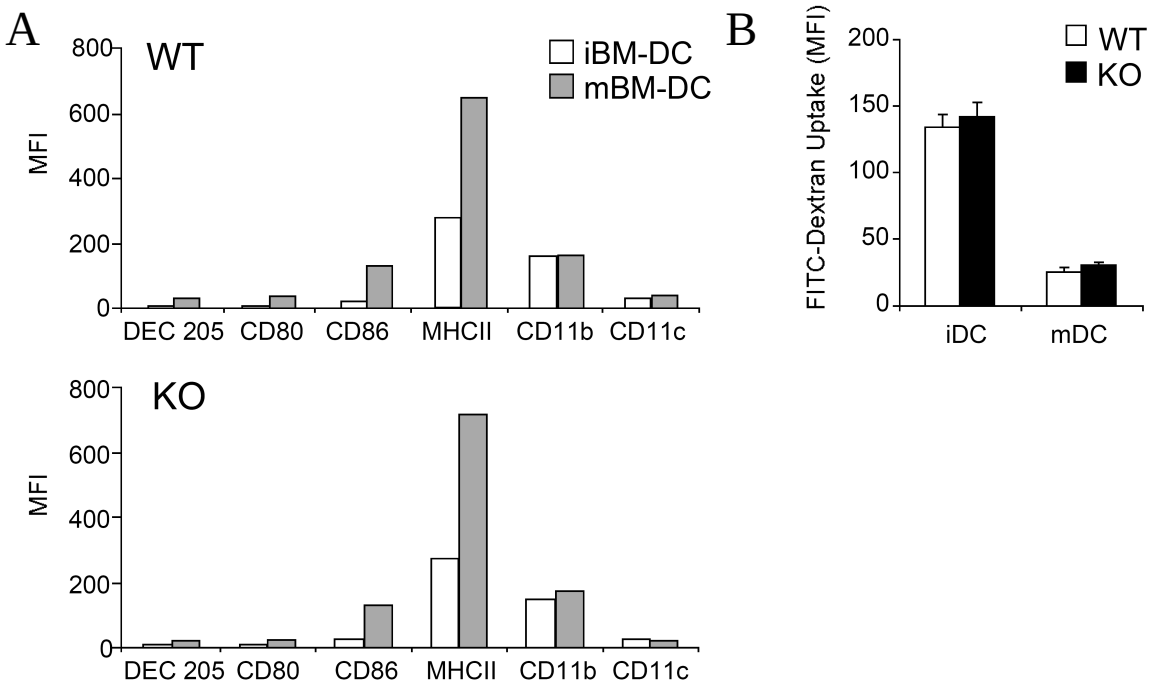


Figure 25. Characterization of CCRL2-/- bone marrow derived DC (BM-DC). Immature DC and mature DC (20ng/ml of TNF-α for 24 h) were generated from WT and CCRL2-/- mice.(A) Expression level of six different cell surface markers was determined by FACS. (B) Antigen uptake capacity was evaluated by pinocytosis of FITC-dextran. Data are representative of at least three independent DC cultures.

Considering the importance of chemokine/chemokine receptors in DC traffic and its consequent effect in the development of asthma, DC from CCRL2-/- mice were also tested for the expression and function of chemotactic receptors.

When evaluated at the mRNA level, CCRL2-/-DC showed a normal expression of the chemokine receptors CCR1, CCR2, CCR5, CXCR4 and CXCR6 and CCR7 (Fig.26). Expression of CCR-3, -4, -6, -8, -9, CXCR -1, -2, -3, -5, -7, CX3CR1 and XCR1 was low in both WT and CCRL2-/- DC (less than 500 mRNA molecules per 10^6 β-actin molecules) and was not modulated during maturation (data not shown).

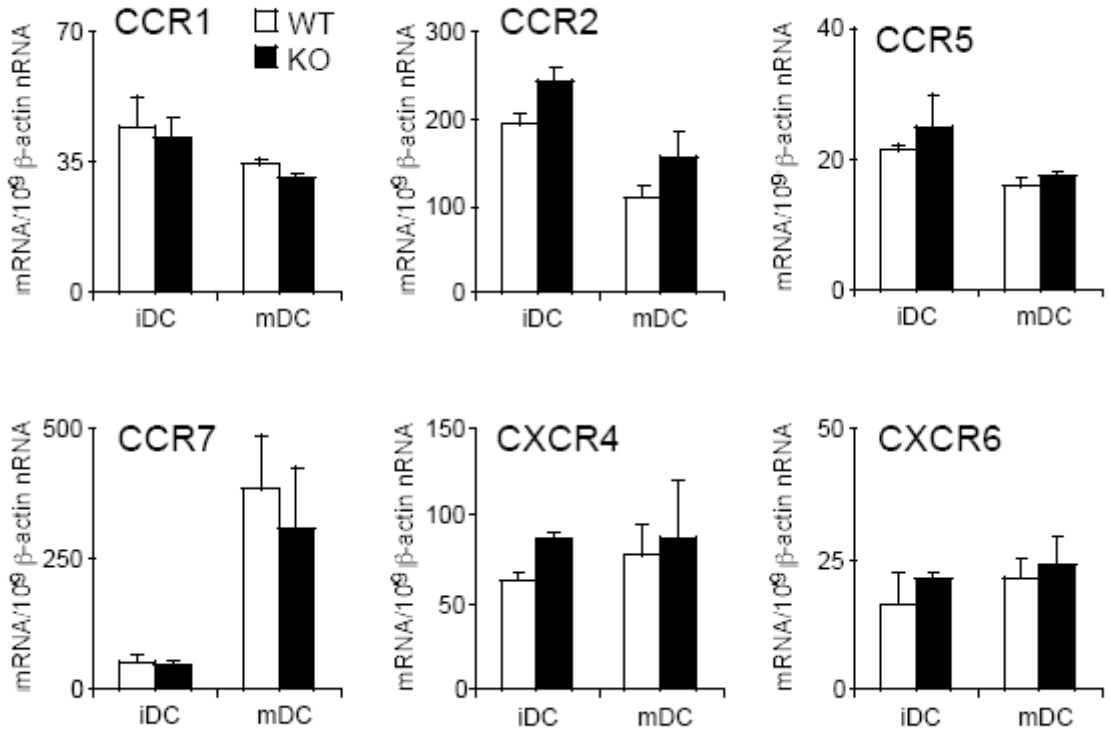


Figure 26. Expression of chemokine receptors by CCRL2-/- BM-DC. Bone marrow derived immature DC and mature DC (20ng/ml of TNF-α for 24 h) from CCRL2-/- and WT mice were evaluated for the expression of chemokine receptors by RT-PCR. Levels of β actin mRNA were used to normalize the measurements. Data are representative of at least three independent DC cultures.

When tested in chemotaxis assays, immature CCRL2-/-DC showed a normal *in vitro* migration to CCL5 and chemerin, and a slightly reduced response to CCL3 and CXCL12, a chemokine constitutively expressed in many tissues [559], although it is preferentially expressed in lymph nodes, lung, liver, and bone marrow [560]. Mature CCRL2-/- DC were competent in migrating to CCL19, a CCR7 ligand (Fig. 27)

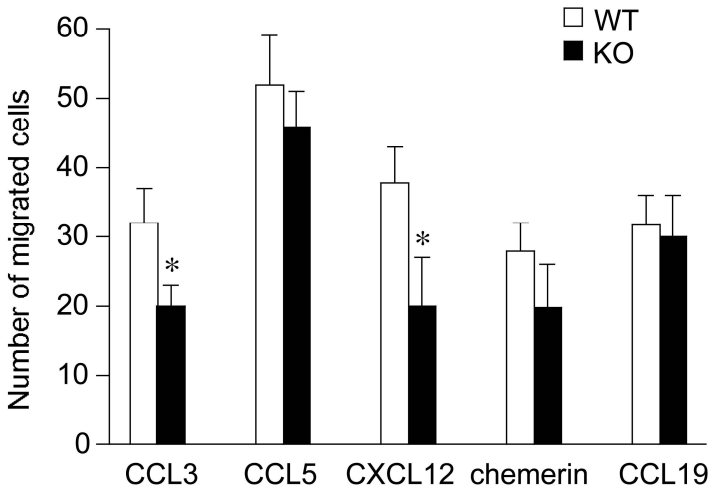


Figure 27. Chemotaxis of bone marrow-derived DC. One experiment representative of six, each one performed with independent DC cultures. CCL3, CCL5, CXCL12 and chemerin migration are shown for immature DC. CCL19 migration is shown for mature DC (20 ng/ml). Results are at the net of basal migration (10±3 and 8±2 cells respectively). Data are mean ± SEM of one experiment representative of six, each one performed with independent cell cultures, * $P < 0.05$.

Finally, CCRL2-/- mice DC were also evaluated for their ability to migrate *in vivo*. Skin painting experiments did not reveal any alteration in the *in vivo* migration ability of endogenous CCRL2-/- DC (Fig. 28). Altogether, these results exclude the possibility that CCRL2 deletion may be associated to a major alteration in the expression and function of DC chemotactic receptors.

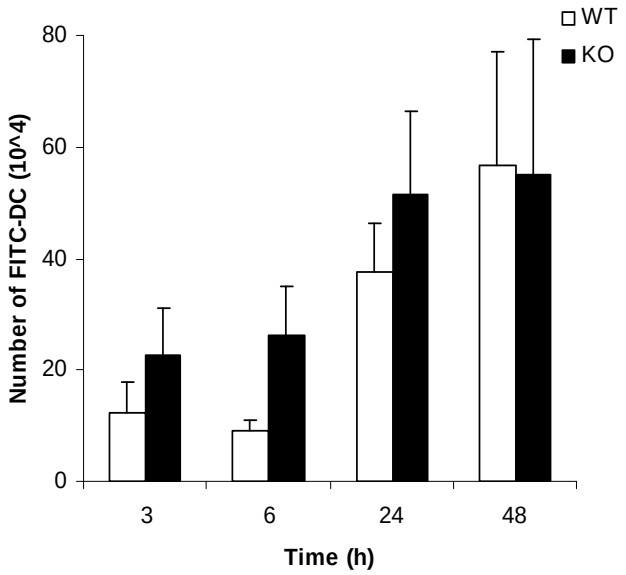


Figure 28. *In vivo* migration of CCLR-/- DC. WT and CCRL2-/- mice were sensitized with FITC on their shaved abdominal skin. Number of FITC+/CD11c+ DC in draining lymph nodes following FITC skin painting was determined by FACS at different times. Data are mean ± SEM of one representative experiments of three performed with 5 mice per group.

3.1.7. Defective lymphocyte priming in mediastinal lymph node of CCRL2-/- mice

The anti-OVA specific immune response was evaluated using cells collected from lung draining lymph nodes. For this purpose, lymph nodes from sensitized mice and challenged by treatment for 6 consecutively days with aerosolized OVA were collected 24 h after the last aerosol challenge, homogenized, and cell suspensions analyzed. Consistently with the reduced migration of antigen-loaded DC, mediastinal lymph nodes from CCRL2-/- mice showed a 2-fold reduction in total cellularity (Fig. 29A) and a significant reduction in the percentage of CD11c+MHCII+ cells (Fig. 29B).

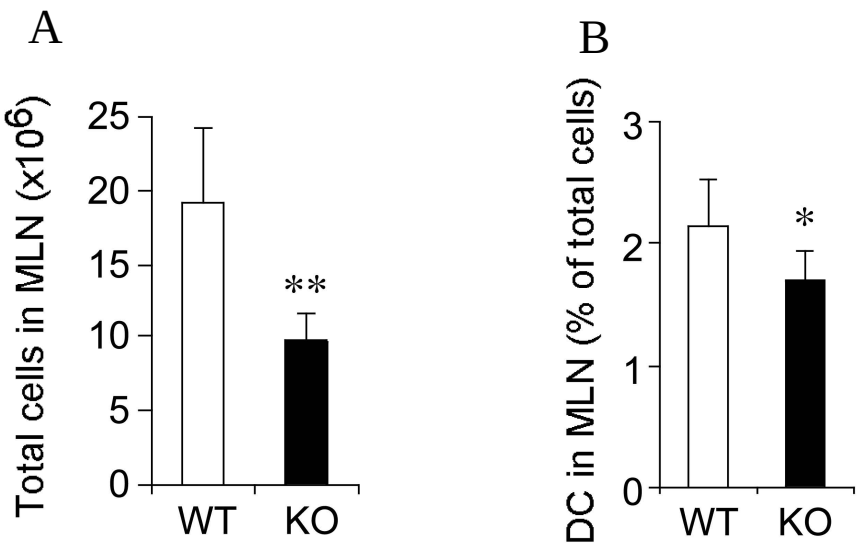


Figure 29. Impaired migration of CCRL2-/- DC to mediastinal lymph nodes. WT and CCRL2-/- mice were sensitized and challenged by treatment for 6 consecutively days with aerosolized OVA. Mediastinal lymph nodes were collected 24 h after the last aerosol. (A) Total cell and (B) Percentage of DC (CD11c+MHCII+) were determined. Data are mean ± SEM of 10 to 20 mice/group from 3 separate experiments. *, P <0.05; **, P <0.01.

In allergic asthma, activated DC produce pro-inflammatory cytokines as TNF-α, IL-1, IL-6, IL-10 [482-484, 561] that drive to differentiation of naïve CD4 Th cells in the paracortex of the draining node toward a Th2 phenotype [487]. The ability to prime naïve CD4+ T cells constitutes a unique and critical function of DC. The quality of T cell priming by DC is critical in asthma development since Th2 primed cells will secrete cytokines that will explain many of the salient features of asthma [385]. We then asked whether deficient CCRL2-/- DC migration to mediastinal lymph node has an impact on local type-2 cytokine production by T cells. Mediastinal lymph node cell suspensions were restimulated with OVA and Th1/Th2 cytokine levels assessed in culture supernatants. As shown in Figure 30, at the end of 4-day culture, WT cells secreted IL-5 and IL-13 at levels that were significantly higher than those detected using cells from CCRL2-/- lymph nodes. In the same experiments, IL-4 and IFNγ levels were below the assay detection limit.

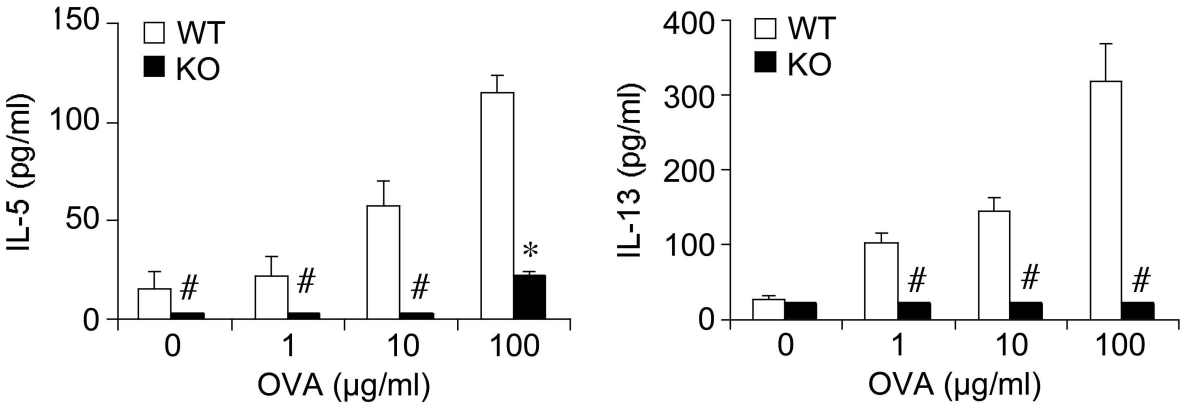


Figure 30. **Impaired cytokine production by restimulated lymph nodes from CCRL2-/- mice**. WT and CCRL2-/- mice were treated as explained in Fig. 29. Lymph node cell suspension was obtained 24 h after last aereosol exposure and cells were restimulated e*x vivo* with OVA for 96 h. Expression of Th1/Th2 cytokines was assessed by ELISA. Data are mean ± SEM of 10 to 20 mice/group from 3 separate experiments. *, P <0.05; #: undetectable levels.

To investigate the specific role of CCRL2 in elicitation of the Th2 response *in vivo*, WT mice were sensitized with OVA at days 0 and 11, and at days 18 and 20 received the i.t. injection of OVA-pulsed DC generated from either WT or CCRL2-/- mice. 48 h after the last DC instillation, mediastinal lymph node cells were collected and cultured *in vitro* in the presence of OVA for 96 h. As shown in Figure 31, cells obtained from mice that had received WT DC showed a strong Th2 response in terms of IL-5 and IL-13 production that was not further stimulated by the *in vitro* addition of OVA. The lack of response to OVA *in vitro* is possibly due to the high concentration of the antigen used to load DC before adoptive transfer. On the contrary, lymph nodes cells from mice that had received CCRL2-/- DC showed very modest response, that could be elicited in a dose-dependent manner by the *in vitro* addition of OVA. Cells from lymph nodes of mice instilled with unpulsed WT or CCRL2-/- DC did not produce IL-5 or IL-13 both under basal conditions and in response to OVA (data not shown).

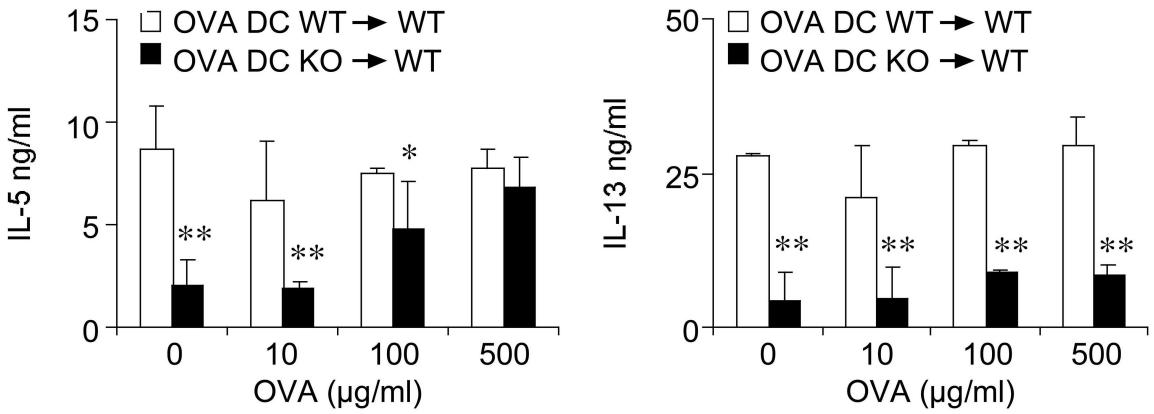


Figure 31. Impaired cytokine production by adopted transfer CCRL2-/- OVA-pulsed DC. WT or CCRL2-/- OVA-pulsed bone marrow DC were injected intratracheally to WT-OVA sensitized mice at days 18 and 20. 48 h later, lymph nodes were excised and cytokine production determined after *ex vivo* stimulation with OVA. Data are mean ± SEM of 10 to 20 mice/group from 3 separate experiments. *, P <0.05; **, P <0.01.

3.1.8. CCRL2 does not affect DC priming ability

The defective ability of CCRL2-/- DC to prime a Th2 response could not be ascribed to an intrinsic defect of CCRL2-/- DC, since they were fully active when tested for their allostimulatory capacity in mixed leukocyte reactions (MLR). Indeed, similar levels of CFSE-labelled CD4+ cell proliferation was observed when CD4+ T cells were cultured with either allogenic WT or CCRL2-/- DC (Fig 32A).

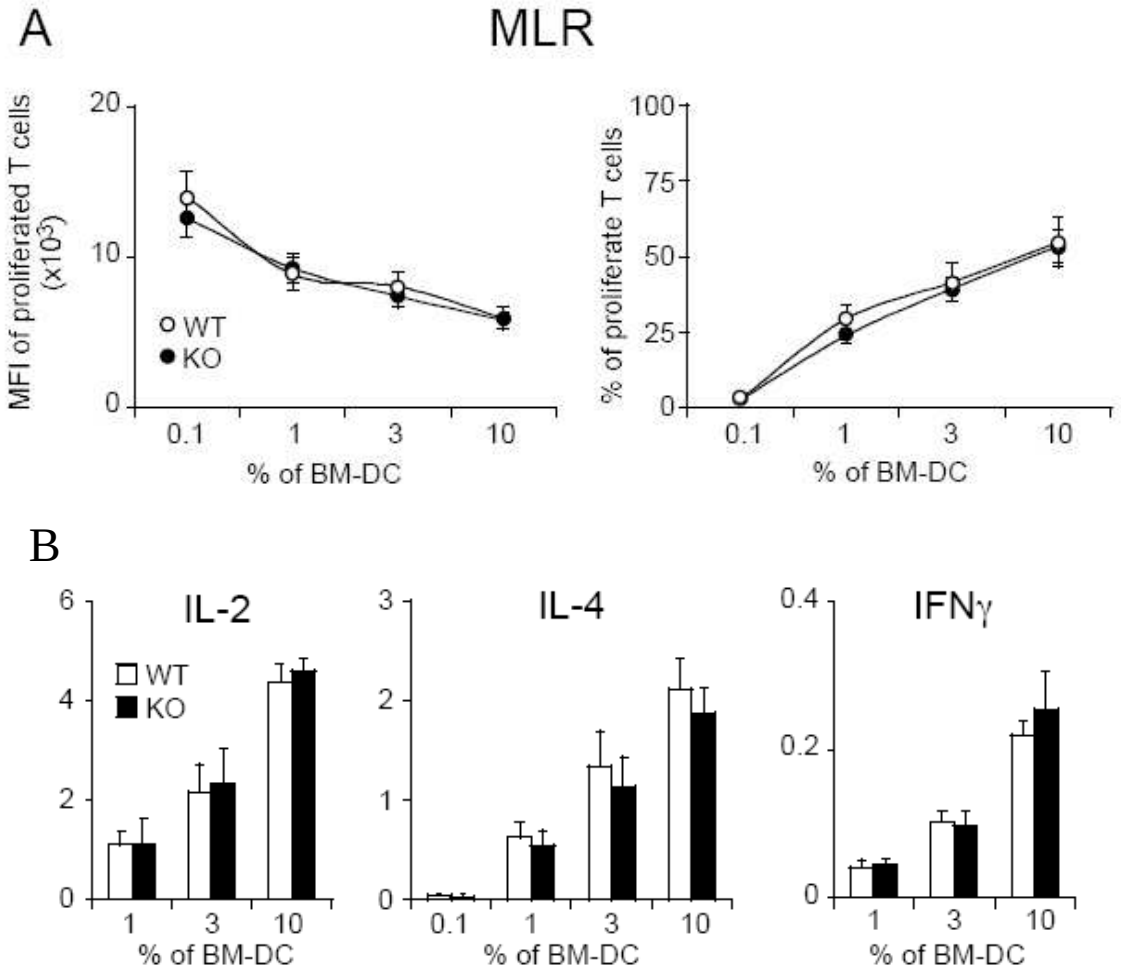


Figure 32. Induction of T cell proliferation by DC from WT and CCRL2-/- mice. BM-DC were maturated by incubation with 20 ng/ml TNF-α. CD4+ T cells were purified from naïve Balb/c mice. (A) CD4+T cells were CFSE-labelled and cultured with different ratios of DC for 72 h. The mean fluorescence intensity (MFI) and % of proliferative cells were analyzed by FACS as indicated in "Materials and Methods". (B) Th1/Th2 cytokines measurement in MLR supernatants. Data are mean ± SEM of 5 mice/group from 2 separate experiments (two independent DC cultures).

Similarly, CCRL2-/- DC induced a normal degree of OT-II OVA-specific TCR transgenic CD4+ T cell proliferation, *in vitro* (Fig. 33A). In agreement with these results, similar levels of cytokines (IL-2, IL-4 and IFN-γ) were detected in the supernatants of T cell cultures performed with WT and CCRL2-/- DC (Fig. 32B and 33B).

These results demonstrate that the defective ability of CCRL2-/- DC to prime a Th2 response could not be ascribed to an intrinsic defect of CCRL2-/- DC since they were fully able to induce T cell proliferation and cytokine production.

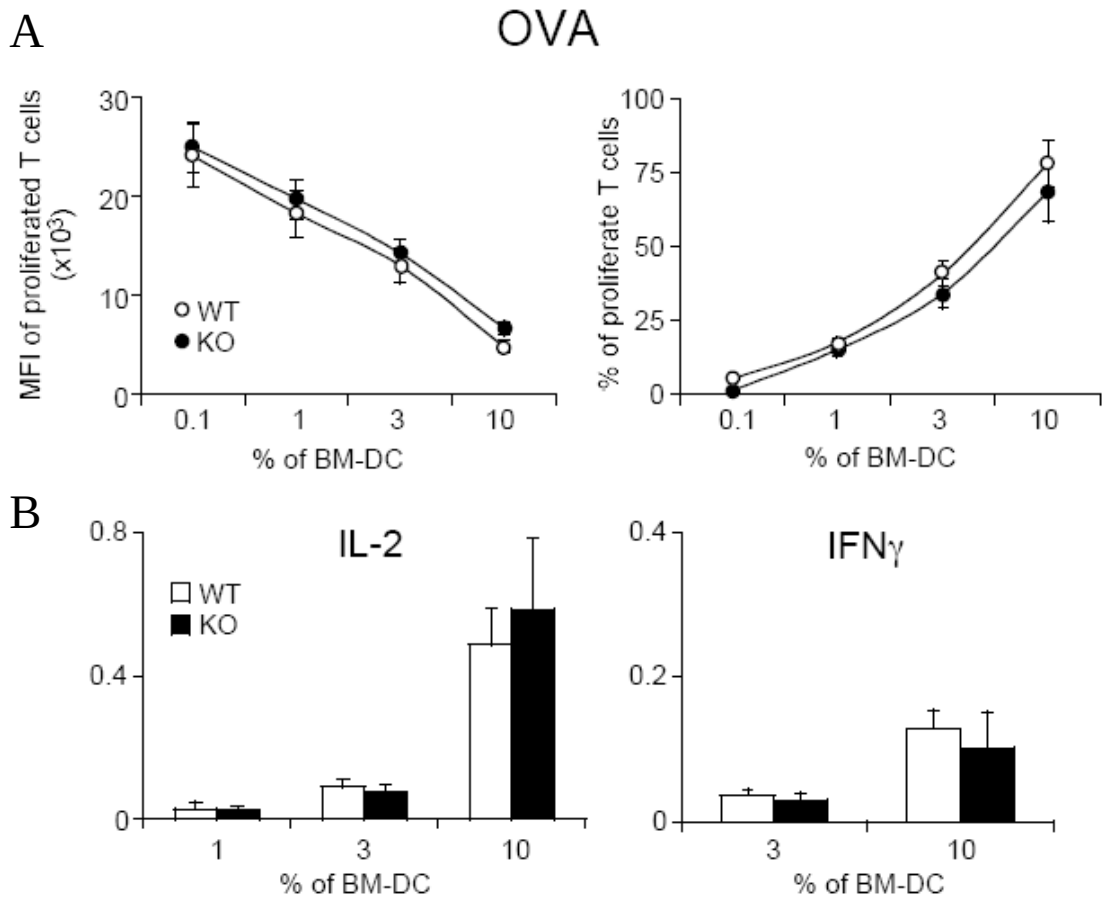


Figure 33. Induction of OVA specific T cell proliferation by DC from WT and CCRL2-/- mice. BM-DC were maturated by incubation with 20 ng/ml TNF-α. CD4+ T cells were prurified from naïve OT-II mice. (A) CD4+T cells were CFSE-labelled and cultured with different ratios of DC for 72 h. The MFI and % of proliferative cells were analyzed by FACS as indicated in "Materials and Methods". (B) Th1/Th2 cytokines measurement in MLR supernatants. IL-4 levels were below the detection limit. Data are mean ± SEM of 5 mice/group from 2 separate experiments (two independent DC cultures).

3.2. Role of a putative non-signalling ligand of CCRL2 in DC migration

Chemerin has been identified as a natural non-signaling protein ligand for CCRL2. Chemerin binding does not trigger ligand internalization, rather, CCRL2 is able to bind the chemoattractant and increase local concentrations of bioactive chemerin which then is available for interaction with ChemR23 on adjacent cells [93].

Chemerin is active as chemotactic factor when it binds its specific receptor ChemR23, a chemotactic receptor expressed by human professional APC (monocyte/macrophages and DC) [70] and NK [69]. DC positive for ChemR23 have been found in close proximity of chemerin-positive human EC [71]. This evidence suggests a key role of the ChemR23/chemerin axis in directing DC trafficking. However limited information are presently available on chemerin/ChemR23 system in mouse, especially in DC-EC interactions.

Here we investigated the expression of functional ChemR23 in immature mouse bone marrow derived myeloid and P-DC and its regulation during DC maturation. Moreover, since DC do transmigration and reverse-transmigration to cross the wall of vascular and lymphatic vessels, we investigated the possible presence and modulation of the ChemR23 functional ligand, chemerin, on lymphatic and vascular EC, taking advantages of specific cell mouse cell lines generated in the laboratory in the past.

3.2.1. Expression of functional ChemR23 by mouse DC

ChemR23 has been reported to be expressed by human M-DC and P-DC [71], while its presence in mouse DC is still controversial [68]. Thus its expression was first investigated in mouse BM-DC (M-DC and P-DC) by RT-PCR.

ChemR23 transcripts were found in immature M-DC and were downmodulated by O.N incubation with TNF-α or LPS (Fig. 34A). LPS induced a higher downregulation of ChemR23 than that caused by TNF-α. Maturation markers (downregulation of CCR1 and CCR2, and upregulation of CCR7) measured in parallel were strongly regulated by LPS treatment (Fig. 34). It is not surprising that ChemR23 is only partially downregulated by TNF-α, since TNF-α has been reported to induce a partial DC maturation (high expression of MHCII and costimulatory molecules, but low capacity to produce proinflammatory chemokines), while complete maturation is achieved only by strong stimuli, such as LPS [562].

To better understand the ChemR23 pattern of expression in M-DC, cells were exposed to the stimuli from 1 to 48 h. ChemR23 expression decreased progressively

during DC maturation, the lowest value being observed at 24 h. No further decrease was seen at 48 h (Fig. 34B).

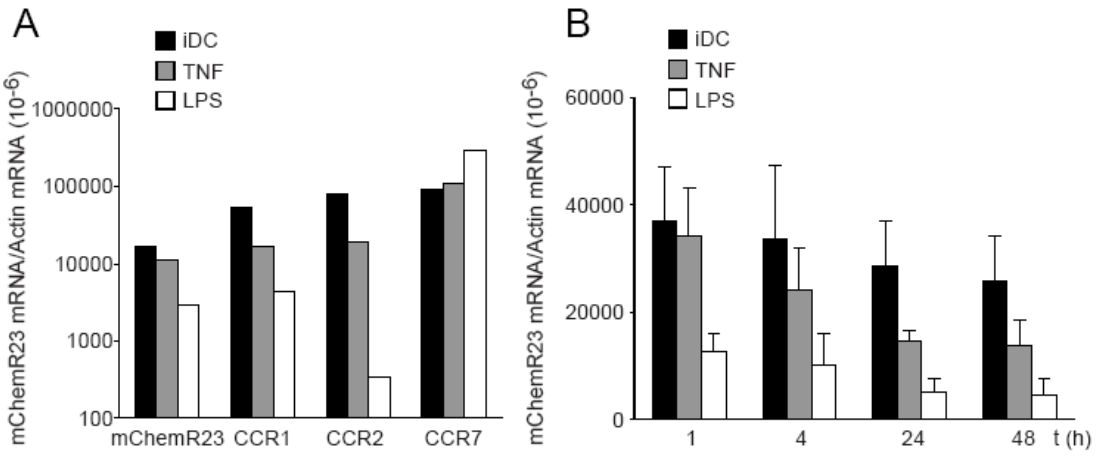


Figure 34. Expression of ChemR23 by mouse myeloid BM-DC. Mouse M-DC were generated from CD34+ bone marrow precursors as indicated in "Materials and Methods". (A) M-DCs were maturated by incubation with TNF-α (20 ng/ml) or LPS (100 ng/ml). CCR1, CCR2 and CCR7 expression was measured as control of maturation. Data are representative of two independent DC cultures. (B) Time-course expression of ChemR23 in M-DC stimulated with TNF-α (20 ng/ml) or LPS (100 ng/ml). Data are mean ± SEM of 2 experiments. ChemR23 and CCRs expression was determined by RT-PCR. Levels of βactin mRNA were used to normalize the measurements.

ChemR23 mRNA was also found in immature P-DC. In contrast with M-DC, ChemR23 expression in P-DC was not affected by CpG-induced maturation (Fig. 35), while DC maturation markers (CCR1, CCR2 and CCR7) were modulated as expected.

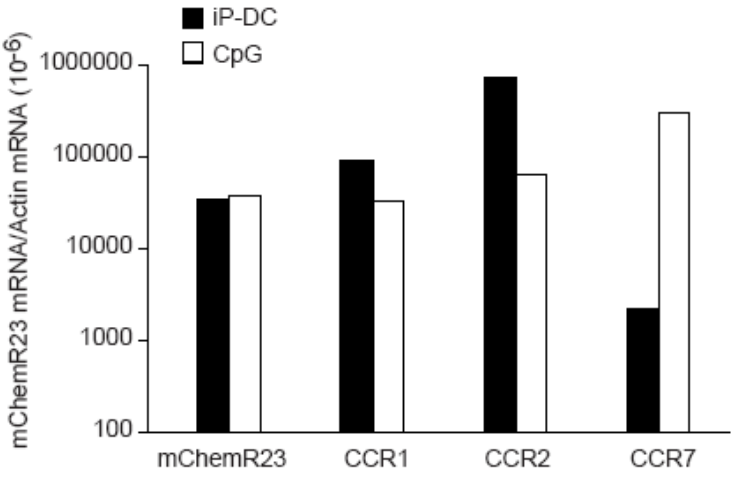


Figure 35. Expression of ChemR23 by mouse plasmacytoid BM-DC. Mouse P-DC were generated from CD34+ bone marrow precursors as indicated in "Materials and Methods". P-DC were maturated by O.N incubation with CpG (2µg/ml). ChemR23 expression was determined by RT-PCR. Levels of actin mRNA were used to normalize the measurements. Data are representative of one experiment of two performed.

To evaluated whether ChemR23 was functional, chemerin was used as chemotactic factor for mouse BM-DC. As shown in figure 36A and 36B mouse chemerin promoted a dose-dependent migration of immature M-DC (Fig. 36A) and P-DC (Fig. 36B) with an optimal dose of 100 pM for both DC subsets. P-DC did not migrate to chemerin when used bare filters (data not shown), however the interaction with an EC monolayer (MELC) favoured their migration. Mature DC did not migrated to chemerin but normally migrated in response to CCL19. The fact that mature mouse DC are unresponsive to chemerin, but still express some ChemR23 mRNA, might suggest that maturation provokes the uncoupling of ChemR23 from its signalling pathway. Alternatively, or in addition, M-DC might express on the membrane too few molecules of the receptor to bind enough chemerin to cause an effect. The percentage of M-DC migrated in response to chemerin was similar to that observed in response to an optimal concentration of CCL3, whereas for P-DC migration was half of that induced by CXCL12 (chemokines used as positive controls). Chemerin, being active in the pM range, showed a potency approximately 100 folds higher than that of most chemokines, which usually peak in the nM order. These results in mouse cells are in agreement with those described for human cells [71], indicating that also in the mouse chemerin represents a chemotactic factor for immature DC.

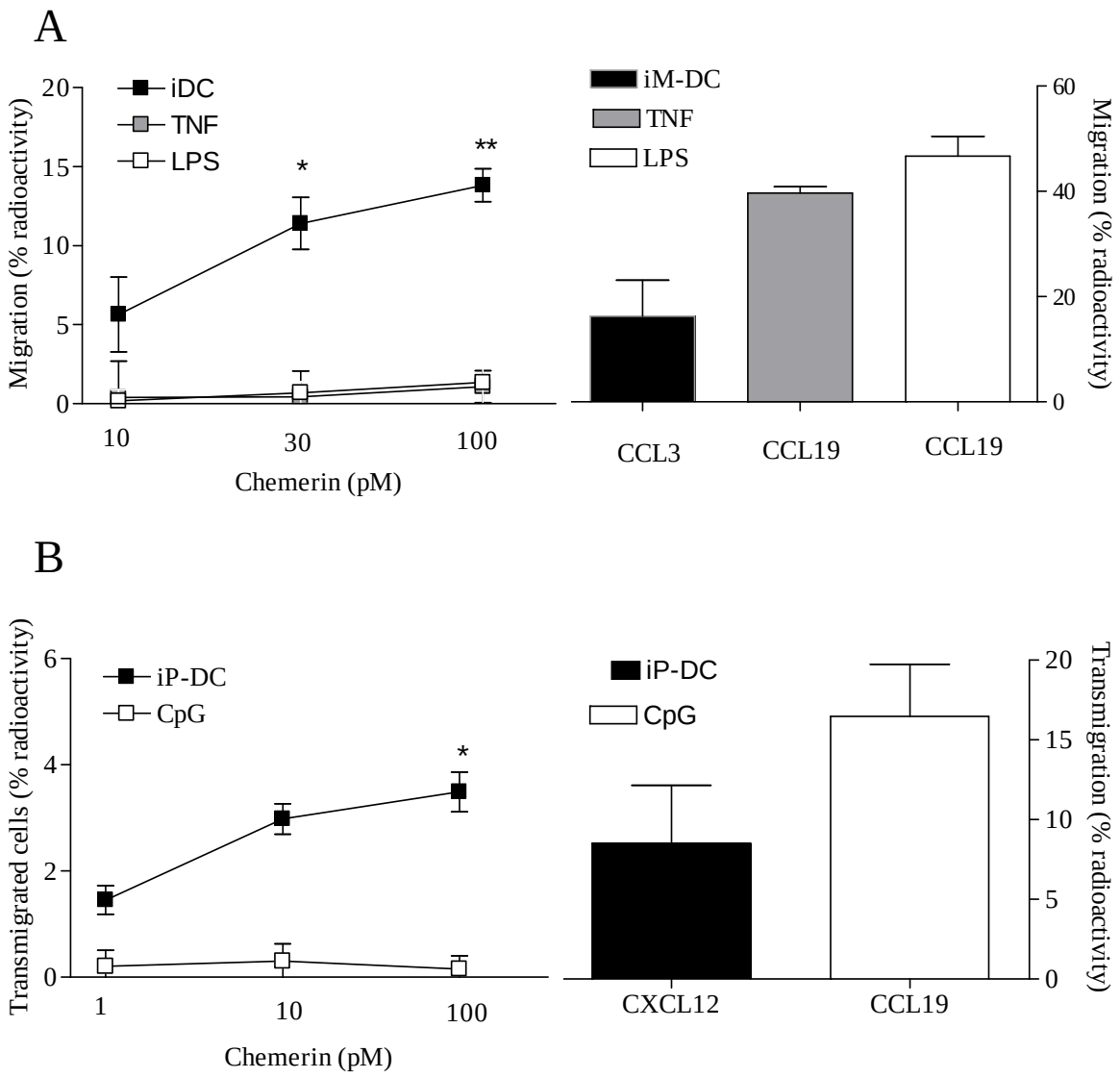


Figure 36. Mouse DC migration to chemerin. (A) M-DC and (B) P-DC were tested for their ability to migrate to recombinant mouse chemerin. Migration to an optimal concentration of CCL3 (immature M-DC), CXCL12 (immature P-DC) or CCL19 (mature DC) (100 ng/ml) was evaluated as a positive control of the experiment. ^{51}Cr labelled DC were allowed to migrate for 90 min. using transwell inserts. P-DC migration was assayed across a MELC monolayer. Values are expressed as net migration (% radioactivity sample - % radioactivity control). Data are mean ± SEM of 3 (A) or 2 (B) separate experiments (independent DC cultures). *, P <0.05; **, P <0.01.

To verify the role of ChemR23 in mouse DC migration, BM-DC from ChemR23-/- mice were generated. These DC failed to migrate in response to chemerin, but not to CCL3 (M-DC) and CXCL12 (P-DC) (data not shown), suggesting that the chemerin effect on DC migration is exclusively mediated by ChemR23.

3.2.2. Expression of chemerin by mouse haematic (1G11) and lymphatic (MELC) EC

Chemerin was found by immunohistochemistry in the interfollicular compartment of human secondary lymphoid organs close to HEV and in skin biopsies of LE patients on the EC lining dermal blood vessels [71]. Some chemokines detected on the surface of HEVs are produced by cells different from HEV cells (e.g., CCL19, CXCL12, and CCL2) and subsequently transported through the fibroblastic reticular cell network to HEVs [563, 564]. *In vitro*, human EC of different origin (e.g., cord blood, dermis, iliac artery, and bone marrow) did not show any production of chemerin in basal conditions and after cytokine stimulation (TNF-α, IL-1, LPS, IFNα, IFNγ and combinations of these agonists) [71]. Therefore it is unknown at the moment whether chemerin production is a characteristic of a specialized endothelium of an anatomical compartment or if is produced by nearby cells and afterward transported to EC

Using two mouse EC lines, MELC (a lymphatic EC line) and 1G11 (a lung capillary vascular EC line), a basal expression was observed at RNA level (data not shown). This result was confirmed at protein level by measuring the amount of chemerin in cell line supernatants by ELISA (Fig. 37C and 37D). 1G11 cells showed a basal production of chemerin higher than that produced by MELC.

Chemerin has been reported as a transcriptionally regulated gene, induced by calcitriol and dexamethasone in the bone stromal cell line ST2 [78] in psoriatic epidermis after topical application of tazarotene, an antipsoriatic synthetic retinoid [79], and in cultured fibroblasts by calcitriol [182]. Regulation of chemerin production by mouse EC was investigated at RNA level. Both cell lines were stimulated with RA and calcitriol and with different inflammatory stimuli (TNF-α, LPS, IFNγ, Il-1β)for different times (from 8 to 36 h). Maximal expression of chemerin mRNA was observed at 18 h (data not shown), therefore this time was used for all subsequent experiments.

As shown in figures 37A and 37B an up-regulation of chemerin mRNA was observed in both cell lines after 18 h of stimulation with RA. The up-regulation of chemerin

mRNA was paralleled by an increase of the protein detected in cell supernatants (Figs 37C and 37D). All the other stimuli assayed did not modulate chemerin production.

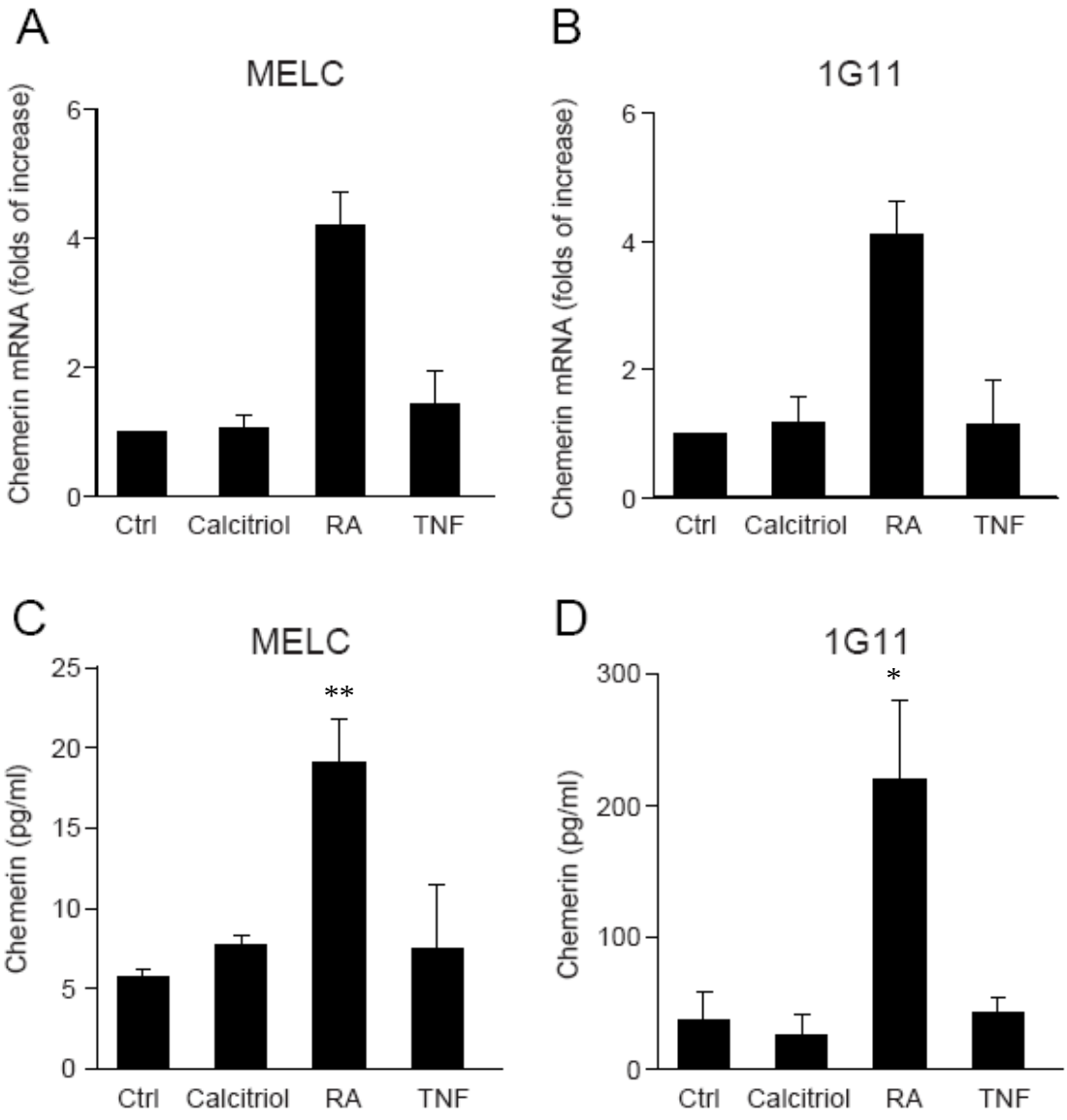


Figure 37. Expression of chemerin by mouse lymphatic MELC and haematic 1G11 EC. Confluent mouse EC, cultured as described in "Material and Methods", were stimulated with 1α,25 dihydroxycholecalciferol (calcitriol, 1μM), RA (5μM) and mTNF-α (20 ng/ml) in serum free medium, supplemented with 0.2 % BSA for 18 h. MELC (A) and 1G11 (B) chemerin mRNA levels were evaluated by RT-PCR. 18S mRNA gene was used to normalize the data. Values are reported as folds of induction over unstimulated cell levels (MELC:$4{,}035 \times 10^{-6} \pm 1{,}01$; 1G11:$113{,}80 \times 10^{-6} \pm 8{,}88$). MELC (C) and 1G11(D) mouse chemerin secretion in the supernatants was analyzed by ELISA. Data are mean ± SEM of 4 separate experiments. *, $P < 0.05$; **, $P < 0.01$.

3.2.3. Chemerin is involved in DC migration across EC

The presence of DC positive for ChemR23 in close proximity of chemerin positive EC, as reported [71], suggests a key role of the ChemR23/chemerin axis in directing DC trafficking across HEV under steady-state conditions and to peripheral tissues in inflammatory conditions [71].

To verify this hypothesis the ability of EC-derived chemerin to induce DC migration was evaluated *in vitro* in transmigration experiments. MELC and 1G11 EC were induced to produce chemerin by stimulation with RA for 18 h. Afterward, the stimulus was removed and BM-DC (M-DC and P-DC) migration in chemotaxis medium was assayed. Figures 38A-D show that unlike immature DCs, mature M-DC and P-DC were not able to migrate across RA stimulated EC lines or to chemerin, suggesting a specific role of chemerin in DC transmigration after EC exposure to RA. In line with this hypohtesis, DC from ChemR23-/- mice did not migrate in the same experimental conditions (Data not shown).

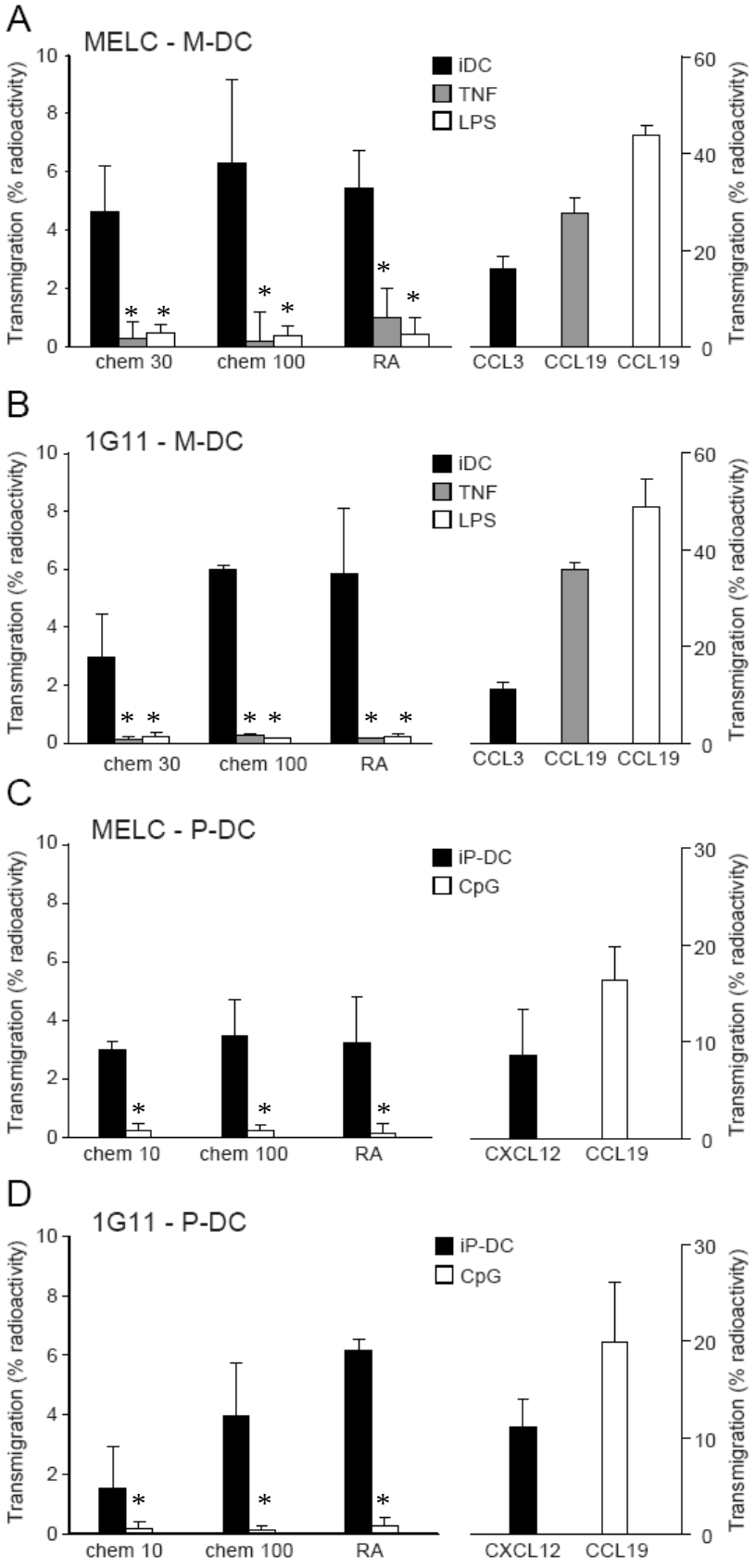


Figure 38. Transmigration of myeloid and plasmacytoid BM-DC across retinoic acid-activated EC. Panels (A) and (B) show M-DC migration across monolayers of

MELC (A) and 1G11 (B), stimulated with RA (5 µM) for 18 h. Migration in response to recombinant mouse chemerin (30 and 100 pM) or to an optimal concentration of CCL3 (immature DCs), CCL19 (mature DCs) (100 ng/ml) was evaluated as a positive control. Panels (C) and (D) show mouse P-DC migration across RA stimulated MELC (C) and 1G11 (D) monolayers. Migration in response to recombinant chemerin (10 and 100 pM) or to an optimal concentration of CXCL12 (immature DCs) or CCL19 (mature DCs) (100 ng/ml) was evaluated as a positive control. ^{51}Cr labelled DC were allowed to migrate across EC for 90 min. using transwell inserts. Values are expressed as net migration (% radioactivity sample - % radioactivity control). Data are mean ± SEM of 2 experiments. * $p < 0.05$ versus immature DC.

To exclude that BM-DC could have migrated in response to other chemokines produced by stimulated EC, chemokines known to be involved in immature DC migration (CCL3, CCL4, CCL5, CXCL10 and CXCL12) were measured by RT-PCR in RA stimulated 1G11 and MELC. No up-regulation of the mRNA for any of the chemokine tested was found (Fig. 39).

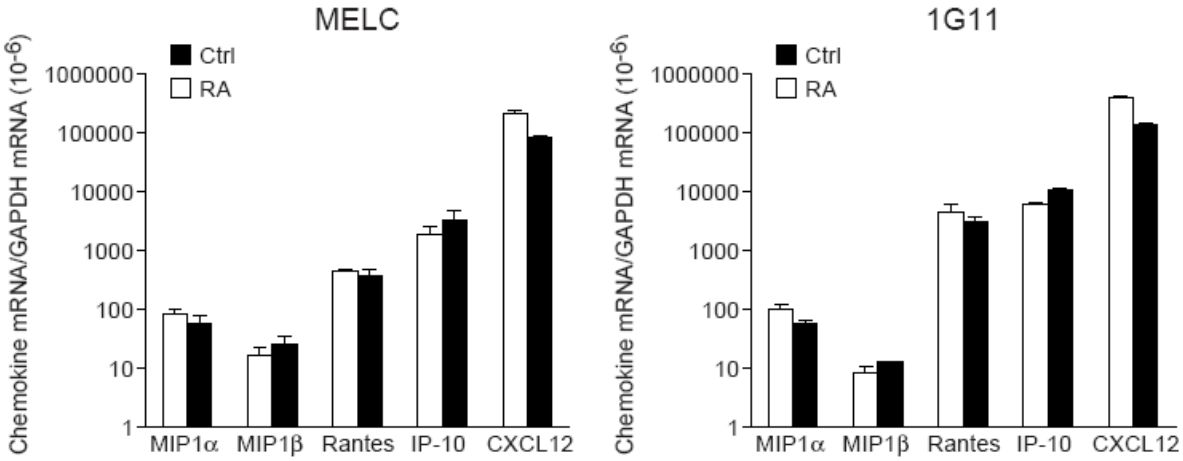


Figure 39. Chemokine expression by RA stimulated EC. Confluent mouse EC were stimulated for 18 h with RA (5µM) in serum free medium, supplemented with 0.2 % BSA. (A) MELC and (B) 1G11 mRNA levels for different DC attracting chemokines were evaluated by RT-PCR. GAPDH mRNA gene was used to normalize the data. Data are mean ± SEM of 2 independent experiments.

To definitively probe that DC migration to RA stimulated EC was mediated by ChemR23/chemerin system, migration using an anti-ChemR23 mAb was assayed. No blocking antibodies are available for mouse ChemR23, thus human DC were used in this part of the study. Human DC from peripheral blood (M-DC and P-DC) preincubated with the anti-human ChemR23 mAb 1H2 were assayed for their migratory capacity across a monolayer of RA stimulated EC (1G11). As shown in figures 40A and 40B, transmigration of both M-DC and P-DC across RA-stimulated endothelium was completely inhibited in the presence of antibody against ChemR23 (irrelevant antibody did not has such inhibitory effect) without affecting transmigration to CXCL12, demonstrating the specificity of ChemR23 in RA effects. Migration in response to chemerin pre-exposed 1G11 cells or to CXCL12, performed as controls, was also blocked by the antiChemR23 mAb (Fig. 40A and 40B).

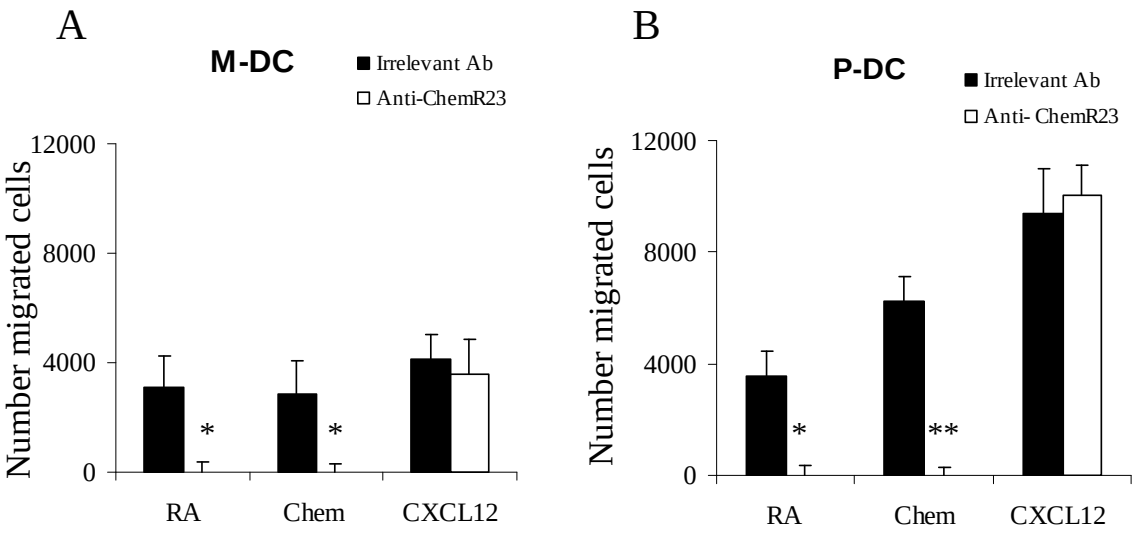


Figure 40. Transmigration of myeloid and plasmacytoid human DC across retinoic acid-activated EC is ChemR23 dependent. Blood DC subsets were isolated as described in "Materials and Methods". Migration of (A) M-DC and (B) P-DC across a monolayer of EC (1G11) stimulated with RA (5 µM) for 18 h was inhibited by the preincubation of DC with 3 µg/ml of the 1H2 anti-ChemR23 mAb for 30 min. at 4°C. Cells stimulation and transmigration were performed in serum free medium, supplemented with BSA 0.2%. Transmigration was evaluated after 4 h incubation in transwell inserts. Cells in the lower compartment of the transwell were recovered and microscopically counted. Migration in response to chemerin (300 pM) added to EC for 90 min. at 37 ºC in the upper chamber and to an optimal concentration (100 ng/ml) of CXCL12 was used as positive controls. Before DC addition, chemerin was removed, leaving only chemerin bound to EC. Results are reported as the net of basal migration: 9849±649 and 5062±919 for M-DC and P-DC respectively. Data are mean ± SEM of 2 experiments for M-DC and 4 separate experiments for P-DC. *, $P < 0.05$; **, $P < 0.01$.

Altogether, these data suggest that EC release biologically active chemerin, that remains partially membrane-bound, promoting DC migration in a chemerin/ChemR23 dependent manner.

Since chemerin has been indicated as the natural nonsignaling ligand for CCRL2 [41], CCRL2 expression at basal level and after RA stimulation for 18h was determined both in MELC and in 1G11 EC by RT-PCR (Fig. 41).

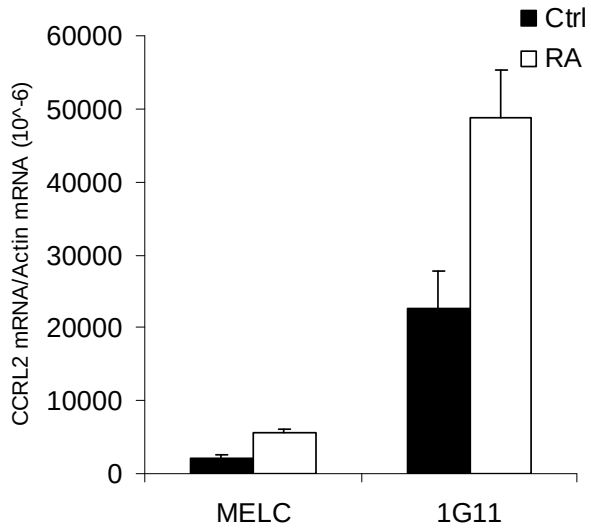


Figure 41. CCRL2 expression by mouse haematic (1G11) and lymphatic (MELC) EC stimulated *in vitro* with RA. Confluent mouse EC were exposed for 18 h to RA (5µM) in serum free medium, supplemented with 0.2 % BSA. MELC and 1G11 CCRL2 mRNA levels were evaluated by RT-PCR. Actin mRNA gene was used to normalize the data. Data are mean ± SEM of 2 separate experiments.

Both cell lines showed a basal expression of CCRL2, although 1G11 presented higher level. After stimulation with RA, CCRL2 expression increased two folds in both cases. The expression of CCRL2 in the same conditions in which chemerin is induced suggests a possible role of this receptor in chemerin presentation by EC.

To determine wether a reduced chemerin production in lymph nodes was the cause of the reduced CCRL2-/- DC migration observed, chemerin expression was determined in mediastinal lymph nodes of WT and CCRL2-/- mice after OVA immunization and challenge (Fig. 42). Chemerin expression was not modulated, excluding this possibility.

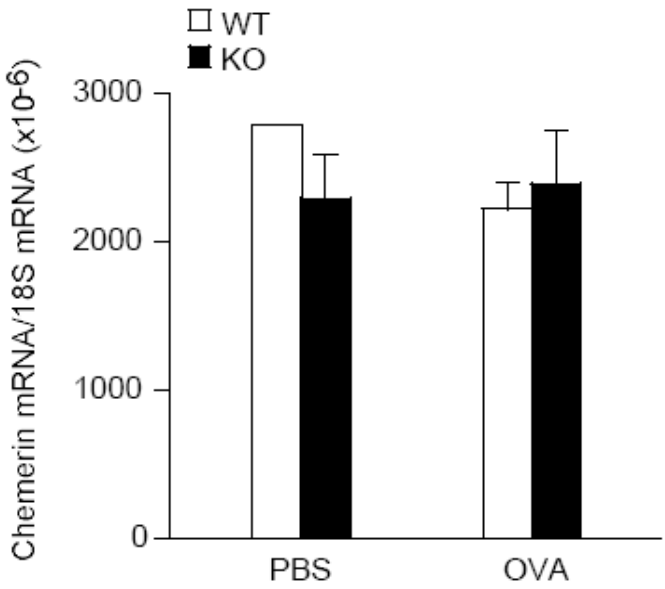


Figure 42. Chemerin expression in mouse mediastinal lymph nodes. CCRL2$^{-/-}$ and WT mice were subjected to the OVA-induced allergic airway inflammation model. Lymph nodes were obtained 24 h after the 6th aerosol challenge. Chemerin mRNA levels were evaluated by RT-PCR. 18S mRNA gene was used to normalize the data. Data are mean ± SEM of one representative experiment of 2 performed with 6 mice per group.

.

4. Discussion

The migration of lung DC from periphery to mediastinal lymph nodes is a key step in the development of airway allergy and inflammation, including asthma [361, 392, 398, 513]. DC rapidly induce CCRL2 during maturation with a kinetic that precedes the expression of CCR7, the main lymph nodes homing chemokine receptor [546]. To investigate the potential role of CCRL2 in DC biology CCRL2-deficient mice were used in an established Th2 model of OVA-induced airway inflammation in which DC migration was shown to play a prominent role [392, 557].

In this experimental model antigen-loaded DC revealed an impaired capacity to traffic to regional lymph nodes and this defect was associated with reduced T cell priming. CCL11 and CCL17 were also significantly reduced in the airways of CCRL2 deficient mice. CCL11 has been shown to be an important chemoattractant for eosinophils and Th2 cells whereas CCL17 is a chemoattractant for Th2 cells [565]. Therefore the decreased production of these two cytokines likely accounts for the reduced number of these two cell types in the airway lumen of CCRL2-/- mice. Indeed, lung eosinophils and T cells do not express CCRL2, ruling out a direct involvement of this receptor in their recruitment. In the lung, CCL11 and CCL17 are produced by epithelial and EC, whereas smooth muscle cells and alveolar macrophages are responsible for the production of other chemokines, such as CCL2, CCL12 and CCL22 [398, 566]. CCRL2 is expressed by bronchial epithelial cells and lung macrophages following OVA challenge [123]. Therefore it is possible that CCRL2 deficiency in these additional cell types might also contribute to the reduced airway inflammation observed in CCRL2-/- mice. On the contrary, the degree of leukocyte infiltration and the levels of CCL11 and CCL17 in lung tissue were not altered.

The levels of the Th2 cytokines IL-4 and IL-5 were reduced in both the airway lumen and the lung interstitium, highlighting a defect of CCRL2-/-mice in generating Th2 responses. Consistently with these results, T cell priming in mediastinal lymph nodes was almost abolished in CCRL2-/- mice. Since lung antigen-specific T cell response is initiated in the lymph nodes, these results suggest a critical role for CCRL2 in the generation of local primary immune responses. Indeed, adoptive transfer experiments of antigen-loaded CCRL2-/- DC into WT animals support the concept that the defective Th2 priming in mediastinal lymph nodes was strictly associated to a defect in DC trafficking. On the other hand, the recruitment of circulating DC to the lung of CCRL2-

/- mice exposed to OVA was not different from that of WT animals. This result suggests that this receptor is not involved in the tissue recruitment of peripheral DC. BM-DC generated from CCRL2-/- mice were indistinguishable from WT mice in terms of antigen uptake, membrane phenotype and antigen presentation capacity. Furthermore, CCRL2-/-DC expressed normal mRNA levels of all the chemokine receptors investigated and normal *in vitro* and *in vivo* migration properties, excluding the possibility that CCRL2 deletion might be associated to a more general alteration of the chemotactic receptor functions. Finally, it should be noted that *in vitro*, DC obtained from CCRL2-/-mice were fully competent in promoting the proliferation of allogeneic or syngeneic OT-II cells and in inducing Th2 cytokines production. Taking together, these results exclude an intrinsic defect of CCRL2-/- DC to promote T cell activation and support a role for CCRL2 in lung DC migration and function.

In spite of the reduced cell recruitment in BAL and of the reduced Th2 priming in lymph nodes, lung function, assessed as methacholine responsiveness, was only slightly decreased. These findings suggest that, in this model, lung function correlates better with the leukocyte content of lung parenchyma rather than the airway lumen. Indeed, previous studies have shown that blocking CCL12 or CCL22 reduces leukocyte recruitment to the lung interstitium but not to the airway lumen; this defect was associated to a decrease in AHR and mucus production [405, 567].

In the past few years, it has been proposed that CCL2, CCL5, CCL7 and CCL8 could bind and activate CCRL2 [129]. However, we (data not shown) and others [41, 107] have not been able to confirm these data. Of note, it have been reported that maturation of mouse DC *in vitro* causes a marked decrease in the chemotactic response towards CCL2 and CCL5 [330], whereas CCRL2 is induced during maturation [546]. CCRL2 presents a non-canonical DRYLAIV motif and the ability of this receptor to signal is still a matter of debate. It was recently shown that CCRL2 binds and presents chemerin in the absence of receptor internalization and signalling [93]. Chemerin is a chemotactic protein that has been recently purified and characterized as the ChemR23 ligand [70, 72]. This study reports that chemerin induces the *in vitro* migration of WT and CCRL2-/- M-DC and does not modify the migration to CCR7 ligands (data not shown).

The migration of DC from peripheral tissues to draining lymph nodes relies on the functional expression of CCR7 and CCR8 [132, 557, 568]. However, CCR7 proper functioning requires accessory signals. In particular, it was reported that CysLT and their transporter protein MRP1 were required for CCR7 activity [358]. It has been recently

reported that also LTB4, through the activation of its high affinity receptor BLT1, promotes CCR7 expression and function in migrating DC [382]. Recent work has proposed that DC trafficking from peripheral tissues is controlled in a tissue-specific manner, with lung DC egression being independent of the expression of MRP1 [361]. In this work we show that lung DC migration is impaired in CCRL2 deficient mice. However, we did not observe any defect in the migration of skin DC in a model of FITC-skin painting and using CCRL2/- bone marrow-derived DC injected in the footpad of WT mice (data not shown). Therefore, we propose that MRP1-independent lung DC trafficking to lymph nodes may require a still unknown accessory signal provided by CCRL2.

Our studies on the expression and function of the specific chemerin receptor ChemR23 in mouse immature DC and its downregulation during maturation suggest a prominent role of ChemR23 in homeostatic conditions. On the other hand this study reports that CCRL2 is rapidly induced during DC maturation, suggesting a role for this receptor in inflammation. Since CCRL2 was reported to bind and present chemerin to ChemR23 expressing cells [93], we can hypothesize a function in DC – EC interactions, favouring the processes that lead to DC transmigration across the endothelia. Our *in vitro* data on the basal and inducible expression of chemerin by lymphatic and blood EC are in favour of this hypothesis. Chemerin expression in mediastinal lymph nodes was not modulated *in vivo* by OVA immunization and challenge, excluding that the reduced migration of CCRL2-/- DC was the consequence of a reduced production of chemerin in lymph node. These results are in agreement with the hypothesis that the interaction with chemerin on EC is a relevant step for DC entry in lymphatic vessels. It has been recently reported [569] that, in addition to gut DC, also lung DC express the aldehyde dehydrogenase enzyme, that converts retinal to RA, especially when they are migrating DC. The fact that mouse EC lines exposed in vitro to RA increase the production of the mature active form of chemerin also fits with the hypothesis of the relevance of chemerin in lung DC-EC interactions.

In summary, this study reports that CCRL2 plays a nonredundant role in lung DC migration to peripheral lymph nodes and that this defective DC migration is responsible for the reduced Th2 response observed in CCRL2-/- mice. Our results on the expression and regulation of ChemR23 and chemerin in DC and EC suggest one possible mechanism, probably among others, of the defective migration of CCRL2-/- DC. Therefore, CCRL2 may represent a new target for the control of lung inflammatory responses.

List of Published Papers:

1. 2010. **Gonzalvo-Feo S**, Sironi M, Ruiz-Rosquete M, Bosisio D, Vecchi A, Sozzani S. Endothelial cell-derived chemerin promotes transmigration of mouse dendritic cell subsets. Manuscript in preparation
2. 2010. Otero K, Vecchi A, Hirsch E, Kearley J, Vermi W, Del Prete A, **Gonzalvo-Feo S**, Garlanda C, Azzolino O, Salogni L,. Lloyd C. M, Facchetti F, Mantovani1 A and Sozzani S. Non-redundant role of CCRL2 in lung dendritic cell trafficking. Blood. 116(16):2942-2949
3. 2009. Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosisio D, Madonna S, Fortugno P, **Gonzalvo-Feo S**, Franssen JD, Parmentier M, De Pità O, Girolomoni G, Sozzani S. Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment.. The Journal of Experimental Medicine. 206(1):249-258
4. 2007. Mantovani A, Garlanda C, Locati M, Veliz T, **Gonzalvo S**, Savino B, Vecchi A. Regulatory pathways in inflammation. Autoimmunity Reviews. 7(1):8–11

List of Abbreviations

1G11	Lung capillary endothelial cell line
^{51}Cr	Sodium chromate Cr-51
AHR	Airway hyperresponsiveness
Alum	Aluminium hydroxide gel
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BDH	Dibutylphtalate
BM-DC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
BST	Bone marrow stromal antigen
cAMP	Cyclic adenosine monophosphate
CCRL2	Chemokine CC motif receptor like 2
CCX-CKR	Chemo Centrix chemokine receptor
cDNA	Complementary DNA
CFSE	Carboxyfluorescein diacetate succi- nimidyl ester
CG	Cathepsin G
ChemR23	Chemerin receptor
CpG	Unmethylated cytosine-phosphate-guanosine
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CysLT	Cysteinyl leukotriene
DAG	Diacylglycerol
DAMP	Damage associated molecular patterns
DARC	Duffy antigen receptor for chemokines
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
ECGS	Endothelial cell growth supplement
EDN	Eosinophil derived neurotoxin

ELR	Tripeptide motif glutamic acid-leucine-arginine
Eos	Eosinophils
EP	Prostaglandin E receptor
ERM	Ezrin, radixin, moesin family
ESAM	Endothelium selective adhesion molecule
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FcεR	Fc receptor for IgE
FITC	Fluorescein isothiocyanate
Flt3L	FMS-like tyrosine kinase 3 ligand
fMLP	N-formilmetionil-leucil-fenilalanina
FPR	Formyl peptide receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycine
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G protein coupled receptor
GPR1	G protein-coupled receptor 1
GTP	Guanosine-5'-triphosphate
h	Hour
HBSS	Hanks' Balanced Salt Solution
HCR	Human chemokine receptor
HDM	House dust mite
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HLE	Human leukocyte elastase
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
HSP	Heat shock proteins
i.p	Intraperitioneal
ICAM	Intercellular adhesion molecule
ICOSL	Inducible costimulator ligand
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon

ILT-3	Immunoglonulinlike transcript 3
IP3	Inositol triphosphate
ISP-1	Sphingosine-like immunosuppressant 1
JAM	Junctional adhesion molecule
LC	Langerhans cells
LE	Lupus erythematosus
LFA-1	Lymphocyte function-associated antigen 1
LOX-1	Lectin-like oxidized LDL receptor-1
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
Lymph	Lymphocytes
LYVE-1	Lymphatic vessel endothelial receptor 1
mAb	Monoclonal antibody
Macr	Macrophages
MAPK	Mitogen activated protein kinase
M-DC	Myeloid dendritic cell
MELC	Mouse lymphatic endothelial cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Min.	Minute
MLR	Mixed leukocyte reaction
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRP1	Multidrug resistance protein 1
MS	Multiple sclerosis
MWCO	Molecular weight cutoff
NEAA	Non essential aminoacids
NK	Natural killer
NLR	NOD like receptor
O.N	Overnight
OLP	Oral lichen planus
OmPA	Outer membrane protein
OVA	Ovalbumin

PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PAR	Protease activated receptor
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
P-DC	Plasmacytoid dendritic cell
PDL	Programmed death ligand
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
Phe	Phenylalanine
PI3Kγ	Phosphatidilinositol-3 kinase γ
PIP2	Phosphatydilinositol 4,5 biphosphate
PIP3	Phosphatydilinositol 3,4,5 triphosphate
PKC/B	Protein kinase C/B
PLC	Phospholipase C
PMN	Polymorphonuclear
PNAd	peripheral node addressin
PPAR-γ	Peroxisome proliferator-activated receptor γ
PR3	Proteinase 3
PRR	Pattern-recognition receptor
PSGL1	P-selectin glycoprotein ligand-1
PTEN	phosphatase and tensin homolog
PTX	Pertussis toxin
PTX3	Pentraxin 3
R.T	Room temperature
RA	Retinoic acid
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Real time polymerase chain reaction
S1P	Sphingosine-1-phosphate
SCC	Skin squamous cell carcinoma
SCID	Severe Combined Immunodeficiency

SIGIRR	Single immunoglobulin IL-1 receptor
SIGLEC	Sialic acid-binding immunoglobulin-like lectin
SIRPα	Signal-regulatory protein α
SPARC	Secreted protein, acidic and rich in cysteine
SREC-I	Scavenger receptor expressed by EC
TGF-β	Transforming growth factor β
Th1	T helper type 1 cell
Th2	T helper type 2 cell
TIG 2	Tazarotene-induced gene 2
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Tregs	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule
VLA-4	Very late antigen 4
WT	Wild type

Bibliography

1. Ridley, A.J. *et al.* Cell migration: integrating signals from front to back. *Science* **302**, 1704-1709 (2003).

2. Vicente-Manzanares, M., Webb, D.J. & Horwitz, A.R. Cell migration at a glance. *J Cell Sci* **118**, 4917-4919 (2005).

3. Hoeller, O. & Kay, R.R. Chemotaxis in the absence of PIP3 gradients. *Curr Biol* **17**, 813-817 (2007).

4. Weiner, O.D. Regulation of cell polarity during eukaryotic chemotaxis: the chemotactic compass. *Curr Opin Cell Biol* **14**, 196-202 (2002).

5. Baluk, P. *et al.* Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med* **204**, 2349-2362 (2007).

6. Bazzoni, G. & Dejana, E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev* **84**, 869-901 (2004).

7. Pepper, M.S. & Skobe, M. Lymphatic endothelium: morphological, molecular and functional properties. *J Cell Biol* **163**, 209-213 (2003).

8. Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314 (1994).

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

10. Mehta, P., Cummings, R.D. & McEver, R.P. Affinity and kinetic analysis of P-selectin binding to P-selectin glycoprotein ligand-1. *J Biol Chem* **273**, 32506-32513 (1998).

11. Nicholson, M.W., Barclay, A.N., Singer, M.S., Rosen, S.D. & van der Merwe, P.A. Affinity and kinetic analysis of L-selectin (CD62L) binding to glycosylation-dependent cell-adhesion molecule-1. *J Biol Chem* **273**, 763-770 (1998).

12. Constantin, G. *et al.* Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13**, 759-769 (2000).

13. Sanchez-Madrid, F. & del Pozo, M.A. Leukocyte polarization in cell migration and immune interactions. *Embo J* **18**, 501-511 (1999).

14. Shamri, R. *et al.* Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nature immunology* **6**, 497-506 (2005).

15. Rot, A. & von Andrian, U.H. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* **22**, 891-928 (2004).

16. Alon, R., Grabovsky, V. & Feigelson, S. Chemokine induction of integrin adhesiveness on rolling and arrested leukocytes local signaling events or global stepwise activation? *Microcirculation* **10**, 297-311 (2003).

17. Elices, M.J. *et al.* VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* **60**, 577-584 (1990).

18. Marlin, S.D. & Springer, T.A. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* **51**, 813-819 (1987).

19. Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A. & Springer, T.A. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol* **137**, 245-254 (1986).

20. Carlos, T.M. & Harlan, J.M. Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068-2101 (1994).

21. Wong, K., Van Keymeulen, A. & Bourne, H.R. PDZRhoGEF and myosin II localize RhoA activity to the back of polarizing neutrophil-like cells. *J Cell Biol* **179**, 1141-1148 (2007).

22. del Pozo, M.A., Sanchez-Mateos, P., Nieto, M. & Sanchez-Madrid, F. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. *J Cell Biol* **131**, 495-508 (1995).

23. Geiger, B. & Bershadsky, A. Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell* **110**, 139-142 (2002).

24. Phillipson, M. *et al.* Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med* **203**, 2569-2575 (2006).

25. Schenkel, A.R., Mamdouh, Z. & Muller, W.A. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nature immunology* **5**, 393-400 (2004).

26. Shaw, S.K. *et al.* Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J Exp Med* **200**, 1571-1580 (2004).

27. Woodfin, A. *et al.* JAM-A mediates neutrophil transmigration in a stimulus-specific manner in vivo: evidence for sequential roles for JAM-A and PECAM-1 in neutrophil transmigration. *Blood* **110**, 1848-1856 (2007).

28. Vestweber, D. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunol Rev* **218**, 178-196 (2007).

29. Weber, C., Fraemohs, L. & Dejana, E. The role of junctional adhesion molecules in vascular inflammation. *Nature reviews* **7**, 467-477 (2007).

30. Engelhardt, B. & Wolburg, H. Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? *European journal of immunology* **34**, 2955-2963 (2004).

31. Carman, C.V. & Springer, T.A. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol* **167**, 377-388 (2004).

32. Carman, C.V. *et al.* Transcellular diapedesis is initiated by invasive podosomes. *Immunity* **26**, 784-797 (2007).

33. Johnson, L.A. *et al.* An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. *J Exp Med* **203**, 2763-2777 (2006).

34. Kannagi, R. Regulatory roles of carbohydrate ligands for selectins in the homing of lymphocytes. *Curr Opin Struct Biol* **12**, 599-608 (2002).

35. Sackstein, R. The lymphocyte homing receptors: gatekeepers of the multistep paradigm. *Curr Opin Hematol* **12**, 444-450 (2005).

36. McLaughlin, F. *et al.* Tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta down-regulate intercellular adhesion molecule (ICAM)-2 expression on the endothelium. *Cell Adhes Commun* **6**, 381-400 (1998).

37. Stoitzner, P., Pfaller, K., Stossel, H. & Romani, N. A close-up view of migrating Langerhans cells in the skin. *J Invest Dermatol* **118**, 117-125 (2002).

38. Stoitzner, P. *et al.* Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. *J Invest Dermatol* **120**, 266-274 (2003).

39. Kerjaschki, D. *et al.* Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol* **15**, 603-612 (2004).

40. Schacht, V. *et al.* T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *Embo J* **22**, 3546-3556 (2003).

41. Martin-Villar, E. *et al.* Characterization of human PA2.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. *Int J Cancer* **113**, 899-910 (2005).

42. Banerji, S. *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* **144**, 789-801 (1999).

43. Prevo, R., Banerji, S., Ferguson, D.J., Clasper, S. & Jackson, D.G. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J Biol Chem* **276**, 19420-19430 (2001).

44. Binder, N.B. *et al.* Estrogen-dependent and C-C chemokine receptor-2-dependent pathways determine osteoclast behavior in osteoporosis. *Nature medicine* **15**, 417-424 (2009).

45. Chavey, C. *et al.* CXC ligand 5 is an adipose-tissue derived factor that links obesity to insulin resistance. *Cell metabolism* **9**, 339-349 (2009).

46. Kohlmeier, J.E. *et al.* The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. *Immunity* **29**, 101-113 (2008).

47. Trifari, S., Kaplan, C.D., Tran, E.H., Crellin, N.K. & Spits, H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nature immunology* **10**, 864-871 (2009).

48. Pitchford, S.C., Furze, R.C., Jones, C.P., Wengner, A.M. & Rankin, S.M. Differential mobilization of subsets of progenitor cells from the bone marrow. *Cell stem cell* **4**, 62-72 (2009).

49. Baggiolini, M. Chemokines in pathology and medicine. *J Intern Med* **250**, 91-104 (2001).

50. Mantovani, A. *Chemokines. Chem Immunol vol. 72*. (Karger, Basel; 1999).

51. Rossi, D. & Zlotnik, A. The biology of chemokines and their receptors. *Annu Rev Immunol* **18**, 217-242 (2000).

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

53. Mackay, C.R. Chemokines: immunology's high impact factors. *Nature immunology* **2**, 95-101 (2001).

54. Murphy, P.M. *et al.* International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**, 145-176. (2000).

55. Locati, M. *et al.* The chemokine system: tuning and shaping by regulation of receptor expression and coupling in polarized responses. *Allergy* **57**, 972-982 (2002).

56. Moser, B., Wolf, M., Walz, A. & Loetscher, P. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* **25**, 75-84 (2004).

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

58. Baggiolini, M. Chemokines and leukocyte traffic. *Nature* **392**, 565-568. (1998).

59. Rollins, B.J. Chemokines. *Blood* **90**, 909-928. (1997).

60. Harvath, L. Neutrophil chemotactic factors. *Exs* **59**, 35-52 (1991).

61. Larsen, G.L. & Henson, P.M. Mediators of inflammation. *Annu Rev Immunol* **1**, 335-359 (1983).

62. Hirai, H. *et al.* Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* **193**, 255-261 (2001).

63. Chertov, O. *et al.* Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J Biol Chem* **271**, 2935-2940 (1996).

64. Yang, D. *et al.* Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**, 525-528 (1999).

65. Gerard, C. & Gerard, N.P. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu.Rev.Immunol.* **12**, 775-808 (1994).

66. Okumura, N. *et al.* Chemotactic and chemokinetic activities of stem cell factor on murine hematopoietic progenitor cells. *Blood* **87**, 4100-4108 (1996).

67. Boehme, S.A. *et al.* Cutting edge: serotonin is a chemotactic factor for eosinophils and functions additively with eotaxin. *J Immunol* **173**, 3599-3603 (2004).

68. Zabel, B.A. *et al.* Chemokine-like receptor 1 expression by macrophages in vivo: regulation by TGF-beta and TLR ligands. *Exp Hematol* **34**, 1106-1114 (2006).

69. Parolini, S. *et al.* The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. *Blood* **109**, 3625-3632 (2007).

70. Wittamer, V. *et al.* Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* **198**, 977-985 (2003).

71. Vermi, W. *et al.* Role of ChemR23 in directing the migration of myeloid and plasmacytoid dendritic cells to lymphoid organs and inflamed skin. *J Exp Med* **201**, 509-515 (2005).

72. Zabel, B.A., Silverio, A.M. & Butcher, E.C. Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. *J Immunol* **174**, 244-251 (2005).

73. Arita, M. *et al.* Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med* **201**, 713-722 (2005).

74. Wittamer, V. *et al.* The C-terminal nonapeptide of mature chemerin activates the chemerin receptor with low nanomolar potency. *J Biol Chem* **279**, 9956-9962 (2004).

75. Yang, D., Chertov, O. & Oppenheim, J.J. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cell Mol Life Sci* **58**, 978-989 (2001).

76. Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A. & Barrett, A.J. Human low-Mr kininogen contains three copies of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinases. *Biochem J* **234**, 429-434 (1986).

77. Verbanac, D., Zanetti, M. & Romeo, D. Chemotactic and protease-inhibiting activities of antibiotic peptide precursors. *FEBS Lett* **317**, 255-258 (1993).

78. Adams, A.E. *et al.* 1,25 dihydroxyvitamin D3 and dexamethasone induce the cyclooxygenase 1 gene in osteoclast-supporting stromal cells. *J Cell Biochem* **74**, 587-595 (1999).

79. Nagpal, S. *et al.* Tazarotene-induced gene 2 (TIG2), a novel retinoid-responsive gene in skin. *J Invest Dermatol* **109**, 91-95 (1997).

80. Zabel, B.A. *et al.* Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem* **280**, 34661-34666 (2005).

81. Wittamer, V. *et al.* Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* **175**, 487-493 (2005).

82. Kulig, P. *et al.* Staphylococcus aureus-derived staphopain B, a potent cysteine protease activator of plasma chemerin. *J Immunol* **178**, 3713-3720 (2007).

83. Guillabert, A. *et al.* Role of neutrophil proteinase 3 and mast cell chymase in chemerin proteolytic regulation. *J Leukoc Biol* **84**, 1530-1538 (2008).

84. McQuibban, G.A. *et al.* Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* **289**, 1202-1206 (2000).

85. Ryu, O.H. *et al.* Proteolysis of macrophage inflammatory protein-1alpha isoforms LD78beta and LD78alpha by neutrophil-derived serine proteases. *J Biol Chem* **280**, 17415-17421 (2005).

86. Clark-Lewis, I. *et al.* Structure-activity relationships of chemokines. *J Leukoc Biol* **57**, 703-711 (1995).

87. Clark-Lewis, I., Schumacher, C., Baggiolini, M. & Moser, B. Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *J Biol Chem* **266**, 23128-23134 (1991).

88. Luangsay, S. *et al.* Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* **183**, 6489-6499 (2009).

89. Kaur, J., Adya, R., Tan, B.K., Chen, J. & Randeva, H.S. Identification of chemerin receptor (ChemR23) in human endothelial cells: chemerin-induced endothelial angiogenesis. *Biochem Biophys Res Commun* **391**, 1762-1768 (2009).

90. Cash, J.L. *et al.* Synthetic chemerin-derived peptides suppress inflammation through ChemR23. *J Exp Med* **205**, 767-775 (2008).

91. Cash, J.L., Christian, A.R. & Greaves, D.R. Chemerin peptides promote phagocytosis in a ChemR23- and Syk-dependent manner. *J Immunol* **184**, 5315-5324 (2010).

92. Barnea, G. *et al.* The genetic design of signaling cascades to record receptor activation. *Proc Natl Acad Sci U S A* **105**, 64-69 (2008).

93. Zabel, B.A. *et al.* Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *J Exp Med* **205**, 2207-2220 (2008).

94. Yoshimura, T. & Oppenheim, J.J. Chemerin reveals its chimeric nature. *J Exp Med* **205**, 2187-2190 (2008).

95. Liu, Y. *et al.* Identification of a CD4 domain required for interleukin-16 binding and lymphocyte activation. *J Biol Chem* **274**, 23387-23395 (1999).

96. Nilsson, G., Butterfield, J.H., Nilsson, K. & Siegbahn, A. Stem cell factor is a chemotactic factor for human mast cells. *J Immunol* **153**, 3717-3723 (1994).

97. Luster, A.D. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* **338**, 436-445. (1998).

98. Lodi, P.J. *et al.* High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR. *Science* **263**, 1762-1767 (1994).

99. Balkwill, F. Cancer and the chemokine network. *Nat Rev Cancer* **4**, 540-550 (2004).

100. Richardson, R.M. *et al.* Regulation of human interleukin-8 receptor A: Identification of a phosphorylation site involved in modulating receptor functions. *Biochemistry* **34**, 14193-14201 (1995).

101. Burgering, B.M. & Coffer, P.J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).

102. Devreotes, P. & Janetopoulos, C. Eukaryotic chemotaxis: distinctions between directional sensing and polarization. *J Biol Chem* **278**, 20445-20448 (2003).

103. Meili, R. & Firtel, R.A. Two poles and a compass. *Cell* **114**, 153-156 (2003).

104. Li, Z. *et al.* Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* **114**, 215-227 (2003).

105. Weber, K.S.C., Klickstein, L.B., Weber, P.C. & Weber, C. Chemokine-induced monocyte transmigration requires cdc42-mediated cytoskeletal changes. *Eur.J.Immunol.* **28**, 2245-2251 (1998).

106. Xu, J. *et al.* Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell* **114**, 201-214 (2003).

107. Tanaka, T. *et al.* Chemokines in tumor progression and metastasis. *Cancer Sci* **96**, 317-322 (2005).

108. Knall, C. *et al.* Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J.Biol.Chem.* **271**, 2832-2838 (1996).

109. Majka, M., Ratajczak, J., Kowalska, M.A. & Ratajczak, M.Z. Binding of stromal derived factor-1alpha (SDF-1alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur J Haematol* **64**, 164-172 (2000).

110. Mellado, M. *et al.* The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J.Immunol.* **161**, 805-813 (1998).

111. Vila-Coro, A.J. *et al.* The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *Faseb J* **13**, 1699-1710 (1999).

112. Somsel Rodman, J. & Wandinger-Ness, A. Rab GTPases coordinate endocytosis. *J Cell Sci* **113 Pt 2**, 183-192 (2000).

113. Horuk, R. *et al.* A receptor for the malarial parasite Plasmodium vivax: the erythrocyte chemokine receptor. *Science* **261**, 1182-1184 (1993).

114. Nibbs, R.J., Wylie, S.M., Yang, J., Landau, N.R. & Graham, G.J. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem* **272**, 32078-32083 (1997).

115. Borroni, E.M. *et al.* The chemoattractant decoy receptor D6 as a negative regulator of inflammatory responses. *Biochemical Society transactions* **34**, 1014-1017 (2006).

116. Bonini, J.A. *et al.* Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. *DNA Cell Biol* **16**, 1249-1256 (1997).

117. Gosling, J. *et al.* Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol* **164**, 2851-2856 (2000).

118. Locati, M. *et al.* Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine Growth Factor Rev* **16**, 679-686 (2005).

119. Samson, M., Soularue, P., Vassart, G. & Parmentier, M. The genes encoding the human CC-chemokine receptors CC- CKR1 to CC-CKR5 (CMKBR1-CMKBR5) are clustered in the p21.3- p24 region of chromosome 3. *Genomics* **36**, 522-526 (1996).

120. Mantovani, A., Bonecchi, R. & Locati, M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature reviews* **6**, 907-918 (2006).

121. Shimada, T., Matsumoto, M., Tatsumi, Y., Kanamaru, A. & Akira, S. A novel lipopolysaccharide inducible C-C chemokine receptor related gene in murine macrophages. *FEBS Lett* **425**, 490-494 (1998).

122. Zuurman, M.W., Heeroma, J., Brouwer, N., Boddeke, H.W. & Biber, K. LPS-induced expression of a novel chemokine receptor (L-CCR) in mouse glial cells in vitro and in vivo. *Glia* **41**, 327-336 (2003).

123. Oostendorp, J. *et al.* Localization and enhanced mRNA expression of the orphan chemokine receptor L-CCR in the lung in a murine model of ovalbumin-induced airway inflammation. *J Histochem Cytochem* **52**, 401-410 (2004).

124. Fan, P. *et al.* Cloning and characterization of a novel human chemokine receptor. *Biochem Biophys Res Commun* **243**, 264-268. (1998).

125. Migeotte, I., Franssen, J.D., Goriely, S., Willems, F. & Parmentier, M. Distribution and regulation of expression of the putative human chemokine receptor HCR in leukocyte populations. *European journal of immunology* **32**, 494-501 (2002).

126. Hartmann, T.N. *et al.* Human B cells express the orphan chemokine receptor CRAM-A/B in a maturation-stage-dependent and CCL5-modulated manner. *Immunology* **125**, 252-262 (2008).

127. Galligan, C.L. *et al.* Up-regulated expression and activation of the orphan chemokine receptor, CCRL2, in rheumatoid arthritis. *Arthritis Rheum* **50**, 1806-1814 (2004).

128. Leick, M. *et al.* CCL19 is a specific ligand of the constitutively recycling atypical human chemokine receptor CRAM-B. *Immunology* **129**, 536-546 (2010).

129. Biber, K., Zuurman, M.W., Homan, H. & Boddeke, H.W. Expression of L-CCR in HEK 293 cells reveals functional responses to CCL2, CCL5, CCL7, and CCL8. *J Leukoc Biol* **74**, 243-251 (2003).

130. Haraldsen, G. & Rot, A. Coy decoy with a new ploy: interceptor controls the levels of homeostatic chemokines. *European journal of immunology* **36**, 1659-1661 (2006).

131. Sallusto, F. & Lanzavecchia, A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol Rev* **177**, 134-140 (2000).

132. Sozzani, S. Dendritic cell trafficking: more than just chemokines. *Cytokine Growth Factor Rev* **16**, 581-592 (2005).

133. Sozzani, S. *et al.* Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* **155**, 3292-3295 (1995).

134. Sozzani, S., Mantovani, A. & Allavena, P. Control of dendritic cell migration by chemokines. *Forum (Genova)* **9**, 325-338 (1999).

135. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245-252 (1998).

136. Sozzani, S. *et al.* Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J Immunol* **161**, 1083-1086 (1998).

137. Sallusto, F. *et al.* Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *European journal of immunology* **28**, 2760-2769 (1998).

138. Bonecchi, R. *et al.* Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1) and Th2. *J.Exp.Med.* **187**, 129-134 (1998).

139. Sallusto, F., Lenig, D., Mackay, C.R. & Lanzavecchia, A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* **187**, 875-883 (1998).

140. Kaiser, E. *et al.* The G protein-coupled receptor BLR1 is involved in murine B cell differentiation and is also expressed in neuronal tissues. *European journal of immunology* **23**, 2532-2539 (1993).

141. Hargreaves, D.C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med* **194**, 45-56 (2001).

142. Locati, M. *et al.* Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. *J Immunol* **168**, 3557-3562 (2002).

143. Sallusto, F., Lanzavecchia, A. & Mackay, C.R. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol.Today* **19**, 568-574 (1998).

144. Karpus, W.J. *et al.* Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J.Immunol.* **158**, 4129-4136 (1997).

145. Chensue, S.W. *et al.* Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma

formation: relationship to local inflammation, Th cell expression, and IL-12 production. *J Immunol* **157**, 4602-4608 (1996).

146. Zou, W. *et al.* Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12. *J Immunol* **165**, 4388-4396 (2000).

147. Lu, B. *et al.* Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J.Exp.Med.* **187**, 601-608 (1998).

148. Gao, J.L. *et al.* Impaired host defense, hematopoiesis, guanulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J.Exp.Med.* **185**, 1959-1968 (1997).

149. Cook, D.N. *et al.* Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* **269**, 1583-1585 (1995).

150. Gu, L. *et al.* Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* **404**, 407-411. (2000).

151. Aiuti, A., Webb, I.J., Bleul, C., Springer, T. & Gutierrez-Ramos, J.C. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* **185**, 111-120 (1997).

152. Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I. & Littman, D.R. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595-599 (1998).

153. Nagasawa, T. *et al.* Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**, 635-638 (1996).

154. Ma, Q. *et al.* Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A* **95**, 9448-9453 (1998).

155. Sorensen, T.L. *et al.* Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* **103**, 807-815 (1999).

156. McManus, C. *et al.* MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. *J Neuroimmunol* **86**, 20-29 (1998).

157. Simpson, J.E., Newcombe, J., Cuzner, M.L. & Woodroofe, M.N. Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. *J Neuroimmunol* **84**, 238-249 (1998).

158. Barcellos, L.F. *et al.* CC-chemokine receptor 5 polymorphism and age of onset in familial multiple sclerosis. Multiple Sclerosis Genetics Group. *Immunogenetics* **51**, 281-288 (2000).

159. Ransohoff, R.M. & Bacon, K.B. Chemokine receptor antagonism as a new therapy for multiple sclerosis. *Expert Opin Investig Drugs* **9**, 1079-1097 (2000).

160. Feldmann, M. & Maini, R.N. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol* **19**, 163-196 (2001).

161. Szekanecz, Z., Kim, J. & Koch, A.E. Chemokines and chemokine receptors in rheumatoid arthritis. *Semin Immunol* **15**, 15-21 (2003).

162. Bursill, C.A., Choudhury, R.P., Ali, Z., Greaves, D.R. & Channon, K.M. Broad-spectrum CC-chemokine blockade by gene transfer inhibits macrophage recruitment and atherosclerotic plaque formation in apolipoprotein E-knockout mice. *Circulation* **110**, 2460-2466 (2004).

163. Ali, Z.A. *et al.* Gene transfer of a broad spectrum CC-chemokine inhibitor reduces vein graft atherosclerosis in apolipoprotein E-knockout mice. *Circulation* **112**, I235-241 (2005).

164. Greaves, D.R. *et al.* Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* **21**, 923-929 (2001).

165. Hancock, W.W., Gao, W., Faia, K.L. & Csizmadia, V. Chemokines and their receptors in allograft rejection. *Curr Opin Immunol* **12**, 511-516 (2000).

166. Robinson, L.A. *et al.* A role for fractalkine and its receptor (CX3CR1) in cardiac allograft rejection. *J Immunol* **165**, 6067-6072 (2000).

167. Simmons, G. *et al.* Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol Rev* **177**, 112-126 (2000).

168. Cocchi, F. *et al.* Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**, 1811-1815 (1995).

169. Liu, R. *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367-377 (1996).

170. Benkirane, M., Jin, D.Y., Chun, R.F., Koup, R.A. & Jeang, K.T. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem* **272**, 30603-30606 (1997).

171. Zheng, Y. *et al.* Downregulation of tazarotene induced gene-2 (TIG2) in skin squamous cell carcinoma. *Eur J Dermatol* **18**, 638-641 (2008).

172. Velazquez-Fernandez, D. *et al.* Expression profiling of adrenocortical neoplasms suggests a molecular signature of malignancy. *Surgery* **138**, 1087-1094 (2005).

173. West, A.N. *et al.* Gene expression profiling of childhood adrenocortical tumors. *Cancer Res* **67**, 600-608 (2007).

174. Fernandez-Ranvier, G.G. *et al.* Identification of biomarkers of adrenocortical carcinoma using genomewide gene expression profiling. *Arch Surg* **143**, 841-846; discussion 846 (2008).

175. Lande, R. *et al.* Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta. *J Neuropathol Exp Neurol* **67**, 388-401 (2008).

176. Graham, K.L. *et al.* Chemokine-like receptor-1 expression by central nervous system-infiltrating leukocytes and involvement in a model of autoimmune demyelinating disease. *J Immunol* **183**, 6717-6723 (2009).

177. Banchereau, J. & Pascual, V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity* **25**, 383-392 (2006).

178. Nestle, F.O. *et al.* Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* **202**, 135-143 (2005).

179. Lande, R. *et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**, 564-569 (2007).

180. Zheng, Y. *et al.* [Alteration of tazarotene induced gene-2 expression in psoriasis vulgaris]. *Nan Fang Yi Ke Da Xue Xue Bao* **28**, 1792-1794 (2008).

181. Skrzeczynska-Moncznik, J. *et al.* Potential role of chemerin in recruitment of plasmacytoid dendritic cells to diseased skin. *Biochem Biophys Res Commun* **380**, 323-327 (2009).

182. Albanesi, C. *et al.* Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* **206**, 249-258 (2009).

183. Pascual, V., Farkas, L. & Banchereau, J. Systemic lupus erythematosus: all roads lead to type I interferons. *Curr Opin Immunol* **18**, 676-682 (2006).

184. Ronnblom, L. & Pascual, V. The innate immune system in SLE: type I interferons and dendritic cells. *Lupus* **17**, 394-399 (2008).

185. Cederblad, B. *et al.* Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-alpha- producing cells. *J Autoimmun* **11**, 465-470 (1998).

186. Blomberg, S. *et al.* Presence of cutaneous interferon-alpha producing cells in patients with systemic lupus erythematosus. *Lupus* **10**, 484-490 (2001).

187. Farkas, L., Beiske, K., Lund-Johansen, F., Brandtzaeg, P. & Jahnsen, F.L. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* **159**, 237-243 (2001).

188. Wenzel, J. *et al.* Enhanced type I interferon signalling promotes Th1-biased inflammation in cutaneous lupus erythematosus. *J Pathol* **205**, 435-442 (2005).

189. Banchereau, J. *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767-811 (2000).

190. Förster, R. *et al.* CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23-33 (1999).

191. Gunn, M.D. *et al.* Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* **189**, 451-460 (1999).

192. Steinman, R.M., Hawiger, D. & Nussenzweig, M.C. Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711 (2003).

193. Steinman, R.M. & Cohn, Z.A. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* **137**, 1142-1162 (1973).

194. Penna, G., Sozzani, S. & Adorini, L. Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells. *J Immunol* **167**, 1862-1866 (2001).

195. Shortman, K. & Liu, Y.J. Mouse and human dendritic cell subtypes. *Nature reviews* **2**, 151-161 (2002).

196. Ziegler-Heitbrock, L. *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* **116**, e74-80 (2010).

197. Perussia, B., Fanning, V. & Trinchieri, G. A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. *Nat Immun Cell Growth Regul* **4**, 120-137 (1985).

198. Strobl, H. *et al.* Identification of CD68+lin- peripheral blood cells with dendritic precursor characteristics. *J Immunol* **161**, 740-748 (1998).

199. Dzionek, A. *et al.* BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* **165**, 6037-6046 (2000).

200. Blasius, A.L., Cella, M., Maldonado, J., Takai, T. & Colonna, M. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood* **107**, 2474-2476 (2006).

201. Blasius, A.L. *et al.* Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J Immunol* **177**, 3260-3265 (2006).

202. Colonna, M., Trinchieri, G. & Liu, Y.J. Plasmacytoid dendritic cells in immunity. *Nature immunology* **5**, 1219-1226 (2004).

203. Thomas, R. & Lipsky, P.E. Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *J Immunol* **153**, 4016-4028 (1994).

204. Jongbloed, S.L. *et al.* Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J Exp Med* **207**, 1247-1260.

205. O'Keeffe, M. *et al.* Dendritic cell precursor populations of mouse blood: identification of the murine homologues of human blood plasmacytoid pre-DC2 and CD11c+ DC1 precursors. *Blood* **101**, 1453-1459 (2003).

206. Robbins, S.H. *et al.* Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* **9**, R17 (2008).

207. Caminschi, I. *et al.* The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement. *Blood* **112**, 3264-3273 (2008).

208. Ratzinger, G. *et al.* Mature human Langerhans cells derived from CD34+ hematopoietic progenitors stimulate greater cytolytic T lymphocyte activity in the absence of bioactive IL-12p70, by either single peptide presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells. *J Immunol* **173**, 2780-2791 (2004).

209. Valladeau, J. *et al.* Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity* **12**, 71-81 (2000).

210. Spry, C.J., Pflug, A.J., Janossy, G. & Humphrey, J.H. Large mononuclear (veiled) cells like 'Ia-like' membrane antigens in human afferent lympn. *Clin Exp Immunol* **39**, 750-755 (1980).

211. Martin, P. *et al.* Concept of lymphoid versus myeloid dendritic cell lineages revisited: both CD8alpha(-) and CD8alpha(+) dendritic cells are generated from CD4(low) lymphoid-committed precursors. *Blood* **96**, 2511-2519 (2000).

212. Traver, D. *et al.* Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science* **290**, 2152-2154 (2000).

213. Vermaelen, K.Y., Carro-Muino, I., Lambrecht, B.N. & Pauwels, R.A. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* **193**, 51-60 (2001).

214. Anjuere, F. *et al.* Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. *Blood* **93**, 590-598 (1999).

215. Henri, S. *et al.* The dendritic cell populations of mouse lymph nodes. *J Immunol* **167**, 741-748 (2001).

216. Wilson, N.S. *et al.* Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* **102**, 2187-2194 (2003).

217. De Smedt, T. *et al.* Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J Exp Med* **184**, 1413-1424 (1996).

218. Wilson, N.S., El-Sukkari, D. & Villadangos, J.A. Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis. *Blood* **103**, 2187-2195 (2004).

219. Colvin, B.L., Morelli, A.E., Logar, A.J., Lau, A.H. & Thomson, A.W. Comparative evaluation of CC chemokine-induced migration of murine CD8alpha+ and CD8alpha- dendritic cells and their in vivo trafficking. *J Leukoc Biol* **75**, 275-285 (2004).

220. Ardavin, C. Origin, precursors and differentiation of mouse dendritic cells. *Nature reviews* **3**, 582-590 (2003).

221. Kamath, A.T. *et al.* The development, maturation, and turnover rate of mouse spleen dendritic cell populations. *J Immunol* **165**, 6762-6770 (2000).

222. den Haan, J.M., Lehar, S.M. & Bevan, M.J. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-1696 (2000).

223. de Heer, H.J. *et al.* Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* **200**, 89-98 (2004).

224. Maldonado-Lopez, R. *et al.* CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* **189**, 587-592 (1999).

225. Maldonado-Lopez, R., Maliszewski, C., Urbain, J. & Moser, M. Cytokines regulate the capacity of CD8alpha(+) and CD8alpha(-) dendritic cells to prime Th1/Th2 cells in vivo. *J Immunol* **167**, 4345-4350 (2001).

226. Pulendran, B. *et al.* Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A* **96**, 1036-1041 (1999).

227. De Smedt, T. *et al.* CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo. *J Leukoc Biol* **69**, 951-958 (2001).

228. Manickasingham, S.P., Edwards, A.D., Schulz, O. & Reis e Sousa, C. The ability of murine dendritic cell subsets to direct T helper cell differentiation is dependent on microbial signals. *European journal of immunology* **33**, 101-107 (2003).

229. Sixt, M. *et al.* The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* **22**, 19-29 (2005).

230. Carbone, F.R., Belz, G.T. & Heath, W.R. Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. *Trends Immunol* **25**, 655-658 (2004).

231. Hawiger, D. *et al.* Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* **194**, 769-779 (2001).

232. Bonifaz, L. *et al.* Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* **196**, 1627-1638 (2002).

233. Idzko, M. *et al.* Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nature medicine* **13**, 913-919 (2007).

234. Willart, M.A. & Lambrecht, B.N. The danger within: endogenous danger signals, atopy and asthma. *Clin Exp Allergy* **39**, 12-19 (2009).

235. Hammad, H. *et al.* Th2 polarization by Der p 1--pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* **98**, 1135-1141 (2001).

236. Kalinski, P., Hilkens, M.U., Wierenga, E.A. & Kapsenberg, M.L. T-cell priming by type-I and type-2 polarized dendritic cells: the concept of a third signal. *Immunol.Today* **20**, 561-567 (1999).

237. Barrett, N.A. & Austen, K.F. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* **31**, 425-437 (2009).

238. Rifkin, I.R., Leadbetter, E.A., Busconi, L., Viglianti, G. & Marshak-Rothstein, A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev* **204**, 27-42 (2005).

239. Garlanda, C. *et al.* Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. *Proc Natl Acad Sci U S A* **101**, 3522-3526 (2004).

240. Sporri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nature immunology* **6**, 163-170 (2005).

241. Fujii, S., Liu, K., Smith, C., Bonito, A.J. & Steinman, R.M. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* **199**, 1607-1618 (2004).

242. Geijtenbeek, T.B. & van Kooyk, Y. Pathogens target DC-SIGN to influence their fate DC-SIGN functions as a pathogen receptor with broad specificity. *Apmis* **111**, 698-714 (2003).

243. Ludwig, I.S. *et al.* Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J Virol* **78**, 8322-8332 (2004).

244. Meyer, S. *et al.* DC-SIGN mediates binding of dendritic cells to authentic pseudo-LewisY glycolipids of Schistosoma mansoni cercariae, the first parasite-specific ligand of DC-SIGN. *J Biol Chem* **280**, 37349-37359 (2005).

245. Aarnoudse, C.A., Vallejo, J.J., Saeland, E. & van Kooyk, Y. Recognition of tumor glycans by antigen-presenting cells. *Curr Opin Immunol* (2005).

246. Geijtenbeek, T.B. *et al.* Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* **197**, 7-17 (2003).

247. Bergman, M.P. *et al.* Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J Exp Med* **200**, 979-990 (2004).

248. van Gisbergen, K.P., Sanchez-Hernandez, M., Geijtenbeek, T.B. & van Kooyk, Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med* **201**, 1281-1292 (2005).

249. Chieppa, M. *et al.* Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol* **171**, 4552-4560 (2003).

250. Allavena, P., Chieppa, M., Monti, P. & Piemonti, L. From pattern recognition receptor to regulator of homeostasis: the double-faced macrophage mannose receptor. *Crit Rev Immunol* **24**, 179-192 (2004).

251. Delneste, Y. *et al.* Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* **17**, 353-362 (2002).

252. Jeannin, P. *et al.* Complexity and complementarity of outer membrane protein A recognition by cellular and humoral innate immunity receptors. *Immunity* **22**, 551-560 (2005).

253. Doni, A. *et al.* Production of the soluble pattern recognition receptor PTX3 by myeloid, but not plasmacytoid, dendritic cells. *European journal of immunology* **33**, 2886-2893 (2003).

254. Pierre, P. *et al.* Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* **388**, 787-792 (1997).

255. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782-787 (1997).

256. Villadangos, J.A. *et al.* MHC class II expression is regulated in dendritic cells independently of invariant chain degradation. *Immunity* **14**, 739-749 (2001).

257. Rescigno, M. *et al.* Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc Natl Acad Sci U S A* **95**, 5229-5234 (1998).

258. Ackerman, A.L., Kyritsis, C., Tampe, R. & Cresswell, P. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nature immunology* **6**, 107-113 (2005).

259. Albert, M.L., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-89 (1998).

260. Guermonprez, P. *et al.* ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* **425**, 397-402 (2003).

261. Joyce, S. & Van Kaer, L. CD1-restricted antigen presentation: an oily matter. *Curr Opin Immunol* **15**, 95-104 (2003).

262. De Smedt, T. *et al.* Ox40 costimulation enhances the development of T cell responses induced by dendritic cells in vivo. *J Immunol* **168**, 661-670 (2002).

263. Quezada, S.A., Jarvinen, L.Z., Lind, E.F. & Noelle, R.J. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* **22**, 307-328 (2004).

264. Pulendran, B., Banchereau, J., Maraskovsky, E. & Maliszewski, C. Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol* **22**, 41-47. (2001).

265. Pearce, E.J., Kane, C.M. & Sun, J. Regulation of dendritic cell function by pathogen-derived molecules plays a key role in dictating the outcome of the adaptive immune response. *Chem Immunol Allergy* **90**, 82-90 (2006).

266. Vulcano, M. *et al.* Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *J Immunol* **173**, 5749-5756 (2004).

267. Dalod, M. *et al.* Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *J Exp Med* **195**, 517-528 (2002).

268. Sundquist, M. & Wick, M.J. TNF-alpha-dependent and -independent maturation of dendritic cells and recruited CD11c(int)CD11b+ Cells during oral Salmonella infection. *J Immunol* **175**, 3287-3298 (2005).

269. Liu, Y.J. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* **23**, 275-306 (2005).

270. Le Bon, A. & Tough, D.F. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* **14**, 432-436 (2002).

271. Mescher, M.F. *et al.* Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev* **211**, 81-92 (2006).

272. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature reviews* **3**, 133-146 (2003).

273. Biron, C.A. Interferons alpha and beta as immune regulators--a new look. *Immunity* **14**, 661-664 (2001).

274. Le Bon, A. *et al.* Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nature immunology* **4**, 1009-1015 (2003).

275. Poeck, H. *et al.* Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. *Blood* **103**, 3058-3064 (2004).

276. Penna, G. *et al.* Cutting edge: differential chemokine production by myeloid and plasmacytoid dendritic cells. *J Immunol* **169**, 6673-6676 (2002).

277. Sozzani, S., Allavena, P., Vecchi, A. & Mantovani, A. The role of chemokines in the regulation of dendritic cell trafficking. *J Leukoc Biol* **66**, 1-9 (1999).

278. Allavena, P. *et al.* The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol Rev* **177**, 141-149. (2000).

279. Cavanagh, L.L. & Von Andrian, U.H. Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol* **80**, 448-462 (2002).

280. Power, C.A. *et al.* Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3alpha from lung dendritic cells. *J Exp Med* **186**, 825-835 (1997).

281. Penna, G., Vulcano, M., Sozzani, S. & Adorini, L. Differential migration behavior and chemokine production by myeloid and plasmacytoid dendritic cells. *Hum Immunol* **63**, 1164-1171 (2002).

282. Sallusto, F. & Lanzavecchia, A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. *J Exp Med* **189**, 611-614. (1999).

283. McColl, S.R. Chemokines and dendritic cells: a crucial alliance. *Immunol Cell Biol* **80**, 489-496 (2002).

284. Hirsch, E. *et al.* Central role for G protein coupled phosphoinositide 3-kinase γ in inflammation. *Science* **287**, 1049-1053 (2000).

285. Del Prete, A. *et al.* Defective dendritic cell migration and activation of adaptive immunity in PI3Kgamma-deficient mice. *Embo J* **23**, 3505-3515 (2004).

286. Nakamura, K., Williams, I.R. & Kupper, T.S. Keratinocyte-derived monocyte chemoattractant protein 1 (MCP-1): analysis in a transgenic model demonstrates MCP-1 can recruit dendritic and Langerhans cells to skin. *J Invest Dermatol* **105**, 635-643 (1995).

287. Vanbervliet, B. *et al.* Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. *European journal of immunology* **32**, 231-242 (2002).

288. Charbonnier, A.S. *et al.* Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. *J Exp Med* **190**, 1755-1768 (1999).

289. Yang, D., Howard, O.M., Chen, Q. & Oppenheim, J.J. Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-beta 1 express functional C-C chemokine receptor 6. *J Immunol* **163**, 1737-1741 (1999).

290. Varona, R. *et al.* CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J Clin Invest* **107**, R37-45 (2001).

291. Cook, D.N. *et al.* CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* **12**, 495-503 (2000).

292. Schaerli, P., Willimann, K., Ebert, L.M., Walz, A. & Moser, B. Cutaneous CXCL14 targets blood precursors to epidermal niches for Langerhans cell differentiation. *Immunity* **23**, 331-342 (2005).

293. Kurth, I. *et al.* Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development. *J Exp Med* **194**, 855-861 (2001).

294. Shellenberger, T.D. *et al.* BRAK/CXCL14 is a potent inhibitor of angiogenesis and a chemotactic factor for immature dendritic cells. *Cancer Res* **64**, 8262-8270 (2004).

295. Hromas, R. *et al.* Cloning of BRAK, a novel divergent CXC chemokine preferentially expressed in normal versus malignant cells. *Biochem Biophys Res Commun* **255**, 703-706 (1999).

296. Mantovani, A., Locati, M. & Sozzani, S. CC chemokines, in *The Cytokine Handbook; 4th Edition*. (eds. A.W. Thomson & M.T. Lotze) 1083-1100 (Elsevier Science Ltd., London; 2003).

297. Vulcano, M. *et al.* Unique regulation of CCL18 production by maturing dendritic cells. *J Immunol* **170**, 3843-3849 (2003).

298. Schutyser, E., Richmond, A. & Van Damme, J. Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. *J Leukoc Biol* **78**, 14-26 (2005).

299. Morelli, A., Larregina, A., Chuluyan, E., Kolkowski, E. & Fainboim, L. Expression and modulation of C5a receptor (CD88) on skin dendritic cells. Chemotactic effect of C5a on skin migratory dendritic cells. *Immunology* **89**, 126-134 (1996).

300. Yang, D. *et al.* Human dendritic cells express functional formyl peptide receptor-like-2 (FPRL2) throughout maturation. *J Leukoc Biol* **72**, 598-607 (2002).

301. Le, Y., Murphy, P.M. & Wang, J.M. Formyl-peptide receptors revisited. *Trends Immunol* **23**, 541-548 (2002).

302. Migeotte, I. *et al.* Identification and characterization of an endogenous chemotactic ligand specific for FPRL2. *J Exp Med* **201**, 83-93 (2005).

303. Pulendran, B. Immune activation: death, danger and dendritic cells. *Curr Biol* **14**, R30-32 (2004).

304. Binder, R.J., Anderson, K.M., Basu, S. & Srivastava, P.K. Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells in vivo. *J Immunol* **165**, 6029-6035 (2000).

305. la Sala, A. *et al.* Alerting and tuning the immune response by extracellular nucleotides. *J Leukoc Biol* **73**, 339-343 (2003).

306. Shi, Y., Evans, J.E. & Rock, K.L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-521 (2003).

307. Angeli, V. *et al.* Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* **21**, 561-574 (2004).

308. Yang, D., Biragyn, A., Kwak, L.W. & Oppenheim, J.J. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* **23**, 291-296 (2002).

309. De, Y. *et al.* LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-1074 (2000).

310. Yang, D. *et al.* Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells. *Blood* **102**, 3396-3403 (2003).

311. Krug, A. *et al.* IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation. *J Immunol* **169**, 6079-6083 (2002).

312. Kohrgruber, N. *et al.* Plasmacytoid dendritic cell recruitment by immobilized CXCR3 ligands. *J Immunol* **173**, 6592-6602 (2004).

313. Cicinnati, V.R. *et al.* Altered chemotactic response of myeloid and plasmacytoid dendritic cells from patients with chronic hepatitis C: role of alpha interferon. *J Gen Virol* **89**, 1243-1253 (2008).

314. Cicinnati, V.R. *et al.* Mycophenolic acid impedes the antigen presenting and lymph node homing capacities of human blood myeloid dendritic cells. *Transplantation* **88**, 504-513 (2009).

315. Lande, R. *et al.* Characterization and recruitment of plasmacytoid dendritic cells in synovial fluid and tissue of patients with chronic inflammatory arthritis. *J Immunol* **173**, 2815-2824 (2004).

316. Facchetti, F. *et al.* Neoplasms derived from plasmacytoid monocytes/interferon-producing cells: variability of CD56 and granzyme B expression. *Am J Surg Pathol* **27**, 1489-1492; author reply 1492-1483 (2003).

317. Sozzani, S., Vermi, W., Del Prete, A. & Facchetti, F. Trafficking properties of plasmacytoid dendritic cells in health and disease. *Trends Immunol* **31**, 270-277.

318. Wollenberg, A. *et al.* Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *J Invest Dermatol* **119**, 1096-1102 (2002).

319. Grouard, G. *et al.* The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* **185**, 1101-1111 (1997).

320. Vermi, W. *et al.* Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. *J Pathol* **200**, 255-268 (2003).

321. Devosse, T. *et al.* Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. *J Immunol* **182**, 4974-4984 (2009).

322. Schnurr, M. *et al.* Role of adenosine receptors in regulating chemotaxis and cytokine production of plasmacytoid dendritic cells. *Blood* **103**, 1391-1397 (2004).

323. Gutzmer, R. *et al.* Human plasmacytoid dendritic cells express receptors for anaphylatoxins C3a and C5a and are chemoattracted to C3a and C5a. *J Invest Dermatol* **126**, 2422-2429 (2006).

324. Kaser, A. *et al.* Interleukin-18 attracts plasmacytoid dendritic cells (DC2s) and promotes Th1 induction by DC2s through IL-18 receptor expression. *Blood* **103**, 648-655 (2004).

325. Ngo, V.N., Tang, H.L. & Cyster, J.G. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* **188**, 181-191 (1998).

326. Willimann, K. *et al.* The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7. *European journal of immunology* **28**, 2025-2034 (1998).

327. Dieu, M.C. *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* **188**, 373-386 (1998).

328. Ngo, V.N. *et al.* Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med* **189**, 403-412 (1999).

329. Sozzani, S. *et al.* Cutting edge: Differential regulation of chemokine receptors during dendritic cell maturation: A model for their trafficking properties. *J.Immunol.* **161**, 1083-1086 (1998).

330. Vecchi, A. *et al.* Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells. *J Leukoc Biol* **66**, 489-494 (1999).

331. Ohl, L. *et al.* CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **21**, 279-288 (2004).

332. Verbovetski, I. *et al.* Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. *J Exp Med* **196**, 1553-1561 (2002).

333. Yanagihara, S., Komura, E., Nagafune, J., Watarai, H. & Yamaguchi, Y. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J Immunol* **161**, 3096-3102 (1998).

334. Lin, C.L., Suri, R.M., Rahdon, R.A., Austyn, J.M. & Roake, J.A. Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. *European journal of immunology* **28**, 4114-4122 (1998).

335. Lloyd, A.R. *et al.* Granulocyte-colony stimulating factor and lipopolysaccharide regulate the expression of interleukin 8 receptors on polymorphonuclear leukocytes. *J Biol Chem* **270**, 28188-28192 (1995).

336. Sica, A. *et al.* Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J Exp Med* **185**, 969-974 (1997).

337. MartIn-Fontecha, A. *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* **198**, 615-621 (2003).

338. Weiss, J.M. *et al.* Osteopontin is involved in the initiation of cutaneous contact hypersensitivity by inducing Langerhans and dendritic cell migration to lymph nodes. *J Exp Med* **194**, 1219-1229 (2001).

339. Qu, C. *et al.* Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J Exp Med* **200**, 1231-1241 (2004).

340. Xu, H. *et al.* The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *European journal of immunology* **31**, 3085-3093 (2001).

341. Price, A.A., Cumberbatch, M., Kimber, I. & Ager, A. Alpha 6 integrins are required for Langerhans cell migration from the epidermis. *J Exp Med* **186**, 1725-1735 (1997).

342. Hagnerud, S. *et al.* Deficit of CD47 Results in a Defect of Marginal Zone Dendritic Cells, Blunted Immune Response to Particulate Antigen and Impairment of Skin Dendritic Cell Migration. *J Immunol* **176**, 5772-5778 (2006).

343. Brown, E.J. & Frazier, W.A. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol* **11**, 130-135 (2001).

344. Fukunaga, A. *et al.* Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 regulates the migration of Langerhans cells from the epidermis to draining lymph nodes. *J Immunol* **172**, 4091-4099 (2004).

345. Cera, M.R. *et al.* Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A-deficient mice. *J Clin Invest* **114**, 729-738 (2004).

346. Sangaletti, S., Gioiosa, L., Guiducci, C. & Colombo, M.P. Dendritic cell migration in absence of matricellular SPARC. *Mol Biol Cell* **14S**, 274A (2003).

347. Ratzinger, G. *et al.* Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J Immunol* **168**, 4361-4371 (2002).

348. Vermaelen, K.Y. *et al.* Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. *J Immunol* **171**, 1016-1022 (2003).

349. Weiss, J.M. *et al.* An essential role for CD44 variant isoforms in epidermal Langerhans cell and blood dendritic cell function. *J.Cell Biol.* **137**, 1137-1147 (1997).

350. Cella, M. *et al.* Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nature Med.* **5**, 919-923 (1999).

351. Yoneyama, H. *et al.* Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int Immunol* **16**, 915-928 (2004).

352. Gotoh, K. *et al.* Differential requirement for DOCK2 in migration of plasmacytoid dendritic cells versus myeloid dendritic cells. *Blood* **111**, 2973-2976 (2008).

353. Yoneyama, H. *et al.* Plasmacytoid DCs help lymph node DCs to induce anti-HSV CTLs. *J Exp Med* **202**, 425-435 (2005).

354. D'Amico, G. *et al.* Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nature immunology* **1**, 387-391. (2000).

355. Yopp, A.C., Randolph, G.J. & Bromberg, J.S. Leukotrienes, sphingolipids, and leukocyte trafficking. *J Immunol* **171**, 5-10 (2003).

356. Morelli, A.E. & Thomson, A.W. Dendritic cells under the spell of prostaglandins. *Trends Immunol* **24**, 108-111 (2003).

357. Randolph, G.J. *et al.* A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc Natl Acad Sci U S A* **95**, 6924-6929 (1998).

358. Robbiani, D.F. *et al.* The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell* **103**, 757-768 (2000).

359. Machida, I. *et al.* Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J Immunol* **172**, 1833-1838 (2004).

360. Okunishi, K., Dohi, M., Nakagome, K., Tanaka, R. & Yamamoto, K. A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. *J Immunol* **173**, 6393-6402 (2004).

361. Jakubzick, C., Tacke, F., Llodra, J., van Rooijen, N. & Randolph, G.J. Modulation of dendritic cell trafficking to and from the airways. *J Immunol* **176**, 3578-3584 (2006).

362. Pinschewer, D.D. *et al.* FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. *J Immunol* **164**, 5761-5770 (2000).

363. Henning, G. *et al.* CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. *J Exp Med* **194**, 1875-1881 (2001).

364. Mandala, S. *et al.* Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**, 346-349 (2002).

365. Idzko, M. *et al.* Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *Faseb J* **16**, 625-627 (2002).

366. Idzko, M. *et al.* Expression and function of histamine receptors in human monocyte-derived dendritic cells. *J Allergy Clin Immunol* **109**, 839-846 (2002).

367. Muller, H. *et al.* The immunomodulator FTY720 interferes with effector functions of human monocyte-derived dendritic cells. *European journal of immunology* **35**, 533-545 (2005).

368. Renkl, A. *et al.* Distinct effects of sphingosine-1-phosphate, lysophosphatidic acid and histamine in human and mouse dendritic cells. *Int J Mol Med* **13**, 203-209 (2004).

369. Czeloth, N., Bernhardt, G., Hofmann, F., Genth, H. & Forster, R. Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol* **175**, 2960-2967 (2005).

370. Scandella, E., Men, Y., Gillessen, S., Forster, R. & Groettrup, M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* **100**, 1354-1361 (2002).

371. Luft, T. *et al.* Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. *Blood* **100**, 1362-1372 (2002).

372. Kabashima, K. *et al.* Prostaglandin E2-EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells. *Nature medicine* **9**, 744-749 (2003).

373. Sato, T., Kirimura, Y. & Mori, Y. The co-culture of dermal fibroblasts with human epidermal keratinocytes induces increased prostaglandin E2 production and cyclooxygenase 2 activity in fibroblasts. *J Invest Dermatol* **109**, 334-339 (1997).

374. Giordano, D., Magaletti, D.M. & Clark, E.A. Nitric oxide and cGMP protein kinase (cGK) regulate dendritic cell migration toward the lymph node-directing chemokine CCL19a. *Blood* (2005).

375. Trottein, F., Faveeuw, C., Gosset, P. & Angeli, V. Role of the d prostanoid receptor 1 in the modulation of immune and inflammatory responses. *Crit Rev Immunol* **24**, 349-362 (2004).

376. Hammad, H. *et al.* Prostaglandin D2 inhibits airway dendritic cell migration and function in steady state conditions by selective activation of the D prostanoid receptor 1. *J Immunol* **171**, 3936-3940 (2003).

377. Serhan, C.N. & Chiang, N. Novel endogenous small molecules as the checkpoint controllers in inflammation and resolution: entree for resoleomics. *Rheum Dis Clin North Am* **30**, 69-95 (2004).

378. Aliberti, J., Hieny, S., Reis e Sousa, C., Serhan, C.N. & Sher, A. Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity. *Nature immunology* **3**, 76-82 (2002).

379. Fedele, G. *et al.* CD38 is expressed on human mature monocyte-derived dendritic cells and is functionally involved in CD83 expression and IL-12 induction. *European journal of immunology* **34**, 1342-1350 (2004).

380. Partida-Sanchez, S. *et al.* Regulation of dendritic cell trafficking by the ADP-ribosyl cyclase CD38: impact on the development of humoral immunity. *Immunity* **20**, 279-291 (2004).

381. Frasca, L. *et al.* CD38 orchestrates migration, survival and th1-immune response of human mature dendritic cells. *Blood* (2005).

382. Del Prete, A. *et al.* Regulation of dendritic cell migration and adaptive immune response by leukotriene B4 receptors: a role for LTB4 in up-regulation of CCR7 expression and function. *Blood* **109**, 626-631 (2007).

383. Bochner, B.S., Undem, B.J. & Lichtenstein, L.M. Immunological aspects of allergic asthma. *Annu Rev Immunol* **12**, 295-335 (1994).

384. Robinson, D.S. *et al.* Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* **326**, 298-304 (1992).

385. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).

386. Herrick, C.A. & Bottomly, K. To respond or not to respond: T cells in allergic asthma. *Nature reviews* **3**, 405-412 (2003).

387. Da Silva, C.A., Reber, L. & Frossard, N. Stem cell factor expression, mast cells and inflammation in asthma. *Fundam Clin Pharmacol* **20**, 21-39 (2006).

388. Galli, S.J. *et al.* Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* **23**, 749-786 (2005).

389. Leckie, M.J. *et al.* Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* **356**, 2144-2148 (2000).

390. Green, R.H. *et al.* Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* **57**, 875-879 (2002).

391. Gould, H.J., Beavil, R.L. & Vercelli, D. IgE isotype determination: epsilon-germline gene transcription, DNA recombination and B-cell differentiation. *Br Med Bull* **56**, 908-924 (2000).

392. Hammad, H. & Lambrecht, B.N. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nature reviews* **8**, 193-204 (2008).

393. GeurtsvanKessel, C.H. & Lambrecht, B.N. Division of labor between dendritic cell subsets of the lung. *Mucosal immunology* **1**, 442-450 (2008).

394. Lambrecht, B.N. & Hammad, H. Biology of lung dendritic cells at the origin of asthma. *Immunity* **31**, 412-424 (2009).

395. van Rijt, L.S. & Lambrecht, B.N. Dendritic cells in asthma: a function beyond sensitization. *Clin Exp Allergy* **35**, 1125-1134 (2005).

396. Hartl, D., Lee, C.G., Da Silva, C.A., Chupp, G.L. & Elias, J.A. Novel biomarkers in asthma: chemokines and chitinase-like proteins. *Current opinion in allergy and clinical immunology* **9**, 60-66 (2009).

397. Panina-Bordignon, P. & D'Ambrosio, D. Chemokines and their receptors in asthma and chronic obstructive pulmonary disease. *Current opinion in pulmonary medicine* **9**, 104-110 (2003).

398. Smit, J.J. & Lukacs, N.W. A closer look at chemokines and their role in asthmatic responses. *Eur J Pharmacol* **533**, 277-288 (2006).

399. Panina-Bordignon, P. *et al.* The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* **107**, 1357-1364 (2001).

400. Honda, K. *et al.* Prostaglandin D2 reinforces Th2 type inflammatory responses of airways to low-dose antigen through bronchial expression of macrophage-derived chemokine. *J Exp Med* **198**, 533-543 (2003).

401. Matsuoka, T. *et al.* Prostaglandin D2 as a mediator of allergic asthma. *Science* **287**, 2013-2017 (2000).

402. Sekiya, T. *et al.* Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* **165**, 2205-2213 (2000).

403. Lezcano-Meza, D., Negrete-Garcia, M.C., Dante-Escobedo, M. & Teran, L.M. The monocyte-derived chemokine is released in the bronchoalveolar lavage fluid of steady-state asthmatics. *Allergy* **58**, 1125-1130 (2003).

404. Lukacs, N.W. *et al.* Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J Immunol* **158**, 4398-4404 (1997).

405. Gonzalo, J.A. *et al.* The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* **188**, 157-167 (1998).

406. Alam, R. *et al.* Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients. *American journal of respiratory and critical care medicine* **153**, 1398-1404 (1996).

407. Bensch, G.W., Nelson, H.S. & Borish, L.C. Evaluation of cytokines in nasal secretions after nasal antigen challenge: lack of influence of antihistamines. *Ann Allergy Asthma Immunol* **88**, 457-462 (2002).

408. Pease, J.E. & Williams, T.J. Chemokines and their receptors in allergic disease. *J Allergy Clin Immunol* **118**, 305-318; quiz 319-320 (2006).

409. Lukacs, N.W., Prosser, D.M., Wiekowski, M., Lira, S.A. & Cook, D.N. Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J Exp Med* **194**, 551-555 (2001).

410. Scott, K. & Bradding, P. Human mast cell chemokines receptors: implications for mast cell tissue localization in asthma. *Clin Exp Allergy* **35**, 693-697 (2005).

411. Price, K.S. *et al.* CC chemokine receptor 3 mobilizes to the surface of human mast cells and potentiates immunoglobulin E-dependent generation of interleukin 13. *Am J Respir Cell Mol Biol* **28**, 420-427 (2003).

412. Campbell, E.M. *et al.* Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2-/- mice: the role of mast cells. *J Immunol* **163**, 2160-2167 (1999).

413. Hammad, H. *et al.* Monocyte-derived dendritic cells exposed to Der p 1 allergen enhance the recruitment of Th2 cells: major involvement of the chemokines TARC/CCL17 and MDC/CCL22. *Eur Cytokine Netw* **14**, 219-228 (2003).

414. Hoshino, M., Aoike, N., Takahashi, M., Nakamura, Y. & Nakagawa, T. Increased immunoreactivity of stromal cell-derived factor-1 and angiogenesis in asthma. *Eur Respir J* **21**, 804-809 (2003).

415. Medoff, B.D. *et al.* IFN-gamma-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. *J Immunol* **168**, 5278-5286 (2002).

416. Thomas, M.S., Kunkel, S.L. & Lukacs, N.W. Regulation of cockroach antigen-induced allergic airway hyperreactivity by the CXCR3 ligand CXCL9. *J Immunol* **173**, 615-623 (2004).

417. Andrew, D.P. *et al.* STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. *J.Immunol.* **161**, 5027-5038 (1998).

418. Fulkerson, P.C., Zimmermann, N., Hassman, L.M., Finkelman, F.D. & Rothenberg, M.E. Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma. *J Immunol* **173**, 7565-7574 (2004).

419. Velazquez, J.R. & Teran, L.M. Chemokines and Their Receptors in the Allergic Airway Inflammatory Process. *Clin Rev Allergy Immunol* (2010).

420. Devalia, J.L., Bayram, H., Abdelaziz, M.M., Sapsford, R.J. & Davies, R.J. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int Arch Allergy Immunol* **118**, 437-439 (1999).

421. Taha, R.A. *et al.* Eotaxin and monocyte chemotactic protein-4 mRNA expression in small airways of asthmatic and nonasthmatic individuals. *J Allergy Clin Immunol* **103**, 476-483 (1999).

422. Terada, N. *et al.* RANTES production in nasal epithelial cells and endothelial cells. *J Allergy Clin Immunol* **98**, S230-237 (1996).

423. Sedgwick, J.B., Menon, I., Gern, J.E. & Busse, W.W. Effects of inflammatory cytokines on the permeability of human lung microvascular endothelial cell monolayers and differential eosinophil transmigration. *J Allergy Clin Immunol* **110**, 752-756 (2002).

424. Rimaniol, A.C. *et al.* The CX3C chemokine fractalkine in allergic asthma and rhinitis. *J Allergy Clin Immunol* **112**, 1139-1146 (2003).

425. Teran, L.M. *et al.* Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. *Am J Respir Cell Mol Biol* **20**, 777-786 (1999).

426. Laberge, S. & El Bassam, S. Cytokines, structural cells of the lungs and airway inflammation. *Paediatr Respir Rev* **5 Suppl A**, S41-45 (2004).

427. Luo, Y., D'Amore, P.A. & Dorf, M.E. Beta-chemokine TCA3 binds to and activates rat vascular smooth muscle cells. *J Immunol* **157**, 2143-2148 (1996).

428. Blease, K. *et al.* Antifungal and airway remodeling roles for murine monocyte chemoattractant protein-1/CCL2 during pulmonary exposure to Asperigillus fumigatus conidia. *J Immunol* **166**, 1832-1842 (2001).

429. Wikstrom, M.E. & Stumbles, P.A. Mouse respiratory tract dendritic cell subsets and the immunological fate of inhaled antigens. *Immunol Cell Biol* **85**, 182-188 (2007).

430. Plantinga, M., Hammad, H. & Lambrecht, B.N. Origin and functional specializations of DC subsets in the lung. *European journal of immunology* **40**, 2112-2118.

431. Jahnsen, F.L. *et al.* Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus. *J Immunol* **177**, 5861-5867 (2006).

432. Lambrecht, B.N., Salomon, B., Klatzmann, D. & Pauwels, R.A. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. *J Immunol* **160**, 4090-4097 (1998).

433. Sung, S.S. *et al.* A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol* **176**, 2161-2172 (2006).

434. van Rijt, L.S. *et al.* Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of TH2 effector responses in a mouse model of asthma. *J Allergy Clin Immunol* **114**, 166-173 (2004).

435. Raymond, M. *et al.* Selective control of SIRP-alpha-positive airway dendritic cell trafficking through CD47 is critical for the development of T(H)2-mediated allergic inflammation. *J Allergy Clin Immunol* **124**, 1333-1342 e1331 (2009).

436. von Garnier, C. *et al.* Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract. *J Immunol* **175**, 1609-1618 (2005).

437. Kool, M. *et al.* An anti-inflammatory role for plasmacytoid dendritic cells in allergic airway inflammation. *J Immunol* **183**, 1074-1082 (2009).

438. Hammad, H. *et al.* House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nature medicine* **15**, 410-416 (2009).

439. Jakubzick, C. *et al.* Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes. *J Exp Med* **205**, 2839-2850 (2008).

440. Akbari, O. *et al.* Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nature medicine* **8**, 1024-1032 (2002).

441. Chieppa, M., Rescigno, M., Huang, A.Y. & Germain, R.N. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* **203**, 2841-2852 (2006).

442. Rescigno, M. *et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology* **2**, 361-367 (2001).

443. Wan, H. *et al.* Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* **104**, 123-133 (1999).

444. Huh, J.C. *et al.* Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. *J Exp Med* **198**, 19-30 (2003).

445. Lambrecht, B.N. *et al.* Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* **106**, 551-559 (2000).

446. Kool, M. & Lambrecht, B.N. Dendritic cells in asthma and COPD: opportunities for drug development. *Curr Opin Immunol* **19**, 701-710 (2007).

447. Maldonado, R.A., Irvine, D.J., Schreiber, R. & Glimcher, L.H. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature* **431**, 527-532 (2004).

448. Harris, N.L. & Ronchese, F. The role of B7 costimulation in T-cell immunity. *Immunol Cell Biol* **77**, 304-311 (1999).

449. Vieira, P.L., de Jong, E.C., Wierenga, E.A., Kapsenberg, M.L. & Kalinski, P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* **164**, 4507-4512 (2000).

450. Hammad, H. & Lambrecht, B.N. Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. *J Allergy Clin Immunol* **118**, 331-336 (2006).

451. Shinagawa, K. & Kojima, M. Mouse model of airway remodeling: strain differences. *American journal of respiratory and critical care medicine* **168**, 959-967 (2003).

452. Lloyd, C.M. & Hawrylowicz, C.M. Regulatory T cells in asthma. *Immunity* **31**, 438-449 (2009).

453. Holt, P.G., Strickland, D.H., Wikstrom, M.E. & Jahnsen, F.L. Regulation of immunological homeostasis in the respiratory tract. *Nature reviews* **8**, 142-152 (2008).

454. Gett, A.V., Sallusto, F., Lanzavecchia, A. & Geginat, J. T cell fitness determined by signal strength. *Nature immunology* **4**, 355-360 (2003).

455. Ostroukhova, M. *et al.* Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* **114**, 28-38 (2004).

456. Oriss, T.B. *et al.* Dynamics of dendritic cell phenotype and interactions with CD4+ T cells in airway inflammation and tolerance. *J Immunol* **174**, 854-863 (2005).

457. Kohl, J. *et al.* A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest* **116**, 783-796 (2006).

458. Fallarino, F. *et al.* Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *J Immunol* **173**, 3748-3754 (2004).

459. Hayashi, T. *et al.* Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J Clin Invest* **114**, 270-279 (2004).

460. Cohen, N. *et al.* GILZ expression in human dendritic cells redirects their maturation and prevents antigen-specific T lymphocyte response. *Blood* **107**, 2037-2044 (2006).

461. Hamdi, H. *et al.* Induction of antigen-specific regulatory T lymphocytes by human dendritic cells expressing the glucocorticoid-induced leucine zipper. *Blood* **110**, 211-219 (2007).

462. Hawrylowicz, C.M. & O'Garra, A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nature reviews* **5**, 271-283 (2005).

463. Roncarolo, M.G. *et al.* Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* **212**, 28-50 (2006).

464. Vignali, D.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nature reviews* **8**, 523-532 (2008).

465. Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. & Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146 (2006).

466. O'Garra, A., Barrat, F.J., Castro, A.G., Vicari, A. & Hawrylowicz, C. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev* **223**, 114-131 (2008).

467. Makinde, T., Murphy, R.F. & Agrawal, D.K. The regulatory role of TGF-beta in airway remodeling in asthma. *Immunol Cell Biol* **85**, 348-356 (2007).

468. McMillan, S.J., Xanthou, G. & Lloyd, C.M. Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* **174**, 5774-5780 (2005).

469. Lewkowich, I.P. *et al.* CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* **202**, 1549-1561 (2005).

470. Kuipers, H. & Lambrecht, B.N. The interplay of dendritic cells, Th2 cells and regulatory T cells in asthma. *Curr Opin Immunol* **16**, 702-708 (2004).

471. D'Hulst A, I., Vermaelen, K.Y., Brusselle, G.G., Joos, G.F. & Pauwels, R.A. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* **26**, 204-213 (2005).

472. Dahl, M.E., Dabbagh, K., Liggitt, D., Kim, S. & Lewis, D.B. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nature immunology* **5**, 337-343 (2004).

473. Charbonnier, A.S. *et al.* Der p 1-pulsed myeloid and plasmacytoid dendritic cells from house dust mite-sensitized allergic patients dysregulate the T cell response. *J Leukoc Biol* **73**, 91-99 (2003).

474. Jeong, S.K. *et al.* Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J Invest Dermatol* **128**, 1930-1939 (2008).

475. Veres, T.Z. *et al.* Dendritic cell-nerve clusters are sites of T cell proliferation in allergic airway inflammation. *Am J Pathol* **174**, 808-817 (2009).

476. Hammad, H. *et al.* Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells. *J Exp Med* **204**, 357-367 (2007).

477. Yang, D. *et al.* Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses. *J Exp Med* **205**, 79-90 (2008).

478. Chomarat, P., Dantin, C., Bennett, L., Banchereau, J. & Palucka, A.K. TNF skews monocyte differentiation from macrophages to dendritic cells. *J Immunol* **171**, 2262-2269 (2003).

479. Yadav, R., Zammit, D.J., Lefrancois, L. & Vella, A.T. Effects of LPS-mediated bystander activation in the innate immune system. *J Leukoc Biol* **80**, 1251-1261 (2006).

480. Janeway, C.A., Jr. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* **20**, 197-216 (2002).

481. Rubartelli, A. & Lotze, M.T. Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox. *Trends Immunol* **28**, 429-436 (2007).

482. Eisenbarth, S.C. *et al.* Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* **196**, 1645-1651 (2002).

483. Bortolatto, J. *et al.* Toll-like receptor 4 agonists adsorbed to aluminium hydroxide adjuvant attenuate ovalbumin-specific allergic airway disease: role of MyD88 adaptor molecule and interleukin-12/interferon-gamma axis. *Clin Exp Allergy* **38**, 1668-1679 (2008).

484. Tsan, M.F. & Baochong, G. Pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors. *J Endotoxin Res* **13**, 6-14 (2007).

485. Shin, T. *et al.* Cooperative B7-1/2 (CD80/CD86) and B7-DC costimulation of CD4+ T cells independent of the PD-1 receptor. *J Exp Med* **198**, 31-38 (2003).

486. Dillon, S. *et al.* Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J Clin Invest* **116**, 916-928 (2006).

487. Anderson, C.F., Lucas, M., Gutierrez-Kobeh, L., Field, A.E. & Mosser, D.M. T cell biasing by activated dendritic cells. *J Immunol* **173**, 955-961 (2004).

488. Beaty, S.R., Rose, C.E., Jr. & Sung, S.S. Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. *J Immunol* **178**, 1882-1895 (2007).

489. Robays, L.J. *et al.* Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J Immunol* **178**, 5305-5311 (2007).

490. Lambrecht, B.N. & Hammad, H. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nature reviews* **3**, 994-1003 (2003).

491. Maurer, D. *et al.* Fc epsilon receptor I on dendritic cells delivers IgE-bound multivalent antigens into a cathepsin S-dependent pathway of MHC class II presentation. *J Immunol* **161**, 2731-2739 (1998).

492. Grayson, M.H. *et al.* Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J Exp Med* **204**, 2759-2769 (2007).

493. Kuipers, H. *et al.* Lipopolysaccharide-induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells. *J Immunol* **171**, 3645-3654 (2003).

494. Kuipers, H. *et al.* Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization. *J Leukoc Biol* **76**, 1028-1038 (2004).

495. Fukao, T., Matsuda, S. & Koyasu, S. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-gamma production by dendritic cells. *J Immunol* **164**, 64-71 (2000).

496. Nathan, A.T., Peterson, E.A., Chakir, J. & Wills-Karp, M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J Allergy Clin Immunol* **123**, 612-618 (2009).

497. Cates, E.C. *et al.* Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* **173**, 6384-6392 (2004).

498. Rank, M.A. *et al.* IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol* **123**, 1047-1054 (2009).

499. Liu, Y.J. *et al.* TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* **25**, 193-219 (2007).

500. Ito, T. *et al.* TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* **202**, 1213-1223 (2005).

501. Headley, M.B. *et al.* TSLP conditions the lung immune environment for the generation of pathogenic innate and antigen-specific adaptive immune responses. *J Immunol* **182**, 1641-1647 (2009).

502. Wang, Y.H. *et al.* IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* **204**, 1837-1847 (2007).

503. Allakhverdi, Z., Comeau, M.R., Jessup, H.K. & Delespesse, G. Thymic stromal lymphopoietin as a mediator of crosstalk between bronchial smooth muscles and mast cells. *J Allergy Clin Immunol* **123**, 958-960 e952 (2009).

504. Miyata, M. *et al.* Thymic stromal lymphopoietin is a critical mediator of IL-13-driven allergic inflammation. *European journal of immunology* **39**, 3078-3083 (2009).

505. Robays, L.J., Maes, T., Joos, G.F. & Vermaelen, K.Y. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke-induced allergic airway sensitization. *Mucosal immunology* **2**, 206-219 (2009).

506. Monick, M.M. *et al.* Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* **278**, 53035-53044 (2003).

507. Bleck, B., Tse, D.B., Jaspers, I., Curotto de Lafaille, M.A. & Reibman, J. Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation. *J Immunol* **176**, 7431-7437 (2006).

508. Contoli, M. *et al.* Role of deficient type III interferon-lambda production in asthma exacerbations. *Nature medicine* **12**, 1023-1026 (2006).

509. Rate, A., Upham, J.W., Bosco, A., McKenna, K.L. & Holt, P.G. Airway epithelial cells regulate the functional phenotype of locally differentiating dendritic cells: implications for the pathogenesis of infectious and allergic airway disease. *J Immunol* **182**, 72-83 (2009).

510. Medoff, B.D. *et al.* CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol* **182**, 623-635 (2009).

511. Webb, D.C., Cai, Y., Matthaei, K.I. & Foster, P.S. Comparative roles of IL-4, IL-13, and IL-4Ralpha in dendritic cell maturation and CD4+ Th2 cell function. *J Immunol* **178**, 219-227 (2007).

512. Kunkel, S.L. Through the looking glass: the diverse in vivo activities of chemokines. *J Clin Invest* **104**, 1333-1334 (1999).

513. van Rijt, L.S. *et al.* In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* **201**, 981-991 (2005).

514. Wang, J. *et al.* Transgenic expression of granulocyte-macrophage colony-stimulating factor induces the differentiation and activation of a novel dendritic cell population in the lung. *Blood* **95**, 2337-2345 (2000).

515. Holt, P.G., Haining, S., Nelson, D.J. & Sedgwick, J.D. Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *J Immunol* **153**, 256-261 (1994).

516. Stumbles, P.A. *et al.* Regulation of dendritic cell recruitment into resting and inflamed airway epithelium: use of alternative chemokine receptors as a function of inducing stimulus. *J Immunol* **167**, 228-234 (2001).

517. Geissmann, F., Jung, S. & Littman, D.R. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19**, 71-82 (2003).

518. Upham, J.W., Denburg, J.A. & O'Byrne, P.M. Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects. *Clin Exp Allergy* **32**, 818-823 (2002).

519. Jahnsen, F.L. *et al.* Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax* **56**, 823-826 (2001).

520. van Rijt, L.S. *et al.* Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma. *Blood* **100**, 3663-3671 (2002).

521. McWilliam, A.S., Nelson, D., Thomas, J.A. & Holt, P.G. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J Exp Med* **179**, 1331-1336 (1994).

522. Osterholzer, J.J. *et al.* CCR2 and CCR6, but not endothelial selectins, mediate the accumulation of immature dendritic cells within the lungs of mice in response to particulate antigen. *J Immunol* **175**, 874-883 (2005).

523. Reibman, J., Hsu, Y., Chen, L.C., Bleck, B. & Gordon, T. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol* **28**, 648-654 (2003).

524. Starner, T.D., Barker, C.K., Jia, H.P., Kang, Y. & McCray, P.B., Jr. CCL20 is an inducible product of human airway epithelia with innate immune properties. *Am J Respir Cell Mol Biol* **29**, 627-633 (2003).

525. Pichavant, M. *et al.* Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* **115**, 771-778 (2005).

526. Hollingsworth, J.W. *et al.* TLR4 signaling attenuates ongoing allergic inflammation. *J Immunol* **176**, 5856-5862 (2006).

527. Whitehead, G.S. *et al.* The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *American journal of respiratory and critical care medicine* **175**, 243-249 (2007).

528. Pisabarro, M.T. *et al.* Cutting edge: novel human dendritic cell- and monocyte-attracting chemokine-like protein identified by fold recognition methods. *J Immunol* **176**, 2069-2073 (2006).

529. Cole, A.M. & Waring, A.J. The role of defensins in lung biology and therapy. *Am J Respir Med* **1**, 249-259 (2002).

530. Olweus, J. *et al.* Dendritic cell ontogeny: a human dendritic cell lineage of myeloid origin. *Proc Natl Acad Sci U S A* **94**, 12551-12556 (1997).

531. Steinman, R.M. & Nussenzweig, M.C. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* **99**, 351-358 (2002).

532. Sanchez-Sanchez, N., Riol-Blanco, L. & Rodriguez-Fernandez, J.L. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol* **176**, 5153-5159 (2006).

533. Kobayashi, H. *et al.* In situ demonstration of dendritic cell migration from rat intestine to mesenteric lymph nodes: relationships to maturation and role of chemokines. *J Leukoc Biol* **75**, 434-442 (2004).

534. Vermaelen, K. & Pauwels, R. Accelerated airway dendritic cell maturation, trafficking, and elimination in a mouse model of asthma. *Am J Respir Cell Mol Biol* **29**, 405-409 (2003).

535. Saeki, H., Moore, A.M., Brown, M.J. & Hwang, S.T. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7)

participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* **162**, 2472-2475 (1999).

536. Swanson, K.A. *et al.* Flt3-ligand, IL-4, GM-CSF, and adherence-mediated isolation of murine lung dendritic cells: assessment of isolation technique on phenotype and function. *J Immunol* **173**, 4875-4881 (2004).

537. Hammad, H. *et al.* Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J Immunol* **169**, 1524-1534 (2002).

538. Randolph, G.J., Angeli, V. & Swartz, M.A. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nature reviews* **5**, 617-628 (2005).

539. Scandella, E. *et al.* CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood* **103**, 1595-1601 (2004).

540. Bertho, N. *et al.* Efficient migration of dendritic cells toward lymph node chemokines and induction of T(H)1 responses require maturation stimulus and apoptotic cell interaction. *Blood* **106**, 1734-1741 (2005).

541. Idzko, M. *et al.* Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. *J Clin Invest* **117**, 464-472 (2007).

542. Angeli, V. *et al.* Peroxisome proliferator-activated receptor gamma inhibits the migration of dendritic cells: consequences for the immune response. *J Immunol* **170**, 5295-5301 (2003).

543. Graeler, M. & Goetzl, E.J. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *Faseb J* **16**, 1874-1878 (2002).

544. Spiegel, S. & Milstien, S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**, 397-407 (2003).

545. Idzko, M. *et al.* Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* **116**, 2935-2944 (2006).

546. Otero, K. *et al.* Non-redundant role of CCRL2 in lung dendritic cell trafficking. *Blood* (2010).

547. Lambrecht, B.N. Dendritic cells and the regulation of the allergic immune response. *Allergy* **60**, 271-282 (2005).

548. Dong, Q.G. *et al.* A general strategy for isolation of endothelial cells from murine tissues: characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. *Arterioscler.Thromb.Vasc.Biol.* **17**, 1599-1604 (1997).

549. Sironi, M. *et al.* Generation and characterization of a mouse lymphatic endothelial cell line. *Cell and tissue research* **325**, 91-100 (2006).

550. Jakubzick, C. & Randolph, G.J. Methods to study pulmonary dendritic cell migration. *Methods Mol Biol* **595**, 371-382 (2010).

551. Bonecchi, R. *et al.* Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by interferon gamma in human neutrophils. *J.Immunol.* **162**, 474-479 (1999).

552. Macatonia, S.E., Knight, S.C., Edwards, A.J., Griffiths, S. & Fryer, P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J Exp Med* **166**, 1654-1667 (1987).

553. Grunig, G. *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261-2263 (1998).

554. Dudley, E.C., Girardi, M., Owen, M.J. & Hayday, A.C. Alpha beta and gamma delta T cells can share a late common precursor. *Curr Biol* **5**, 659-669 (1995).

555. Petrie, H.T., Scollay, R. & Shortman, K. Commitment to the T cell receptor-alpha beta or -gamma delta lineages can occur just prior to the onset of CD4 and CD8 expression among immature thymocytes. *European journal of immunology* **22**, 2185-2188 (1992).

556. Grunstein, M.M. *et al.* IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **282**, L520-528 (2002).

557. Randolph, G.J., Ochando, J. & Partida-Sanchez, S. Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol* **26**, 293-316 (2008).

558. Vermaelen, K. & Pauwels, R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* **61**, 170-177 (2004).

559. Tashiro, K. *et al.* Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science* **261**, 600-603 (1993).

560. Muller, A. *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56 (2001).

561. Kadowaki, N. *et al.* Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* **194**, 863-869 (2001).

562. Granucci, F., Vizzardelli, C., Virzi, E., Rescigno, M. & Ricciardi-Castagnoli, P. Transcriptional reprogramming of dendritic cells by differentiation stimuli. *European journal of immunology* **31**, 2539-2546. (2001).

563. von Andrian, U.H. & Mempel, T.R. Homing and cellular traffic in lymph nodes. *Nature reviews* **3**, 867-878 (2003).

564. Miyasaka, M. & Tanaka, T. Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nature reviews* **4**, 360-370 (2004).

565. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **25**, 677-686 (2004).

566. Gutierrez-Ramos, J.C., Lloyd, C., Kapsenberg, M.L., Gonzalo, J.A. & Coyle, A.J. Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. *Immunol Rev* **177**, 31-42 (2000).

567. Gonzalo, J.A. *et al.* Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* **163**, 403-411 (1999).

568. Hammad, H. & Lambrecht, B.N. Lung dendritic cell migration. *Adv Immunol* **93**, 265-278 (2007).

569. Guilliams, M. *et al.* Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood* **115**, 1958-1968.