

Molecular mechanisms in allergy and clinical immunology

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Chemokines and their receptors in allergic disease

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Mechanisms of chemoattraction underlie the spatial organization of the cells of the immune system under basal conditions and the localization of these cells to sites of inflammation. The chemokines, a family of around 50 small proteins, play a major role in these processes. Leukocytes are equipped with cell-surface sensors for chemokines. There are 19 such receptors that are differentially expressed on leukocytes: the repertoire of receptor expression depending on the type of leukocyte and its stage in maturation. From observations in animal models, clinical studies, *in vitro* cell biology, and molecular analysis, a working hypothesis has been established to explain the cellular interactions underlying allergic responses and the chemokines–chemokine receptors involved. Chemokines signal through G protein–coupled receptors that are used typically for sensory functions (eg, detection of olfactory signals in the nose). This type of receptor can be blocked selectively by small-molecule antagonists. This provides the opportunity for the development of therapeutic compounds designed to suppress the recruitment of particular leukocyte types in allergic reactions. (*J Allergy Clin Immunol* 2006;118:305-18.)

Key words: Chemokine, chemokine receptor, allergy, asthma, leukocyte migration, chemotaxis, antagonist

The discovery of chemokines and their corresponding receptors on the surface of leukocytes has greatly enriched our understanding of the molecular mechanisms by which cells of the immune system are selectively recruited to specific locations within the body.

Abbreviations used

α-GalCer: α-Galactosylceramide
AHR: Airways hyperresponsiveness
APC: Antigen-presenting cell
DC: Dendritic cell
GPCR: G protein–coupled receptor
LTB₄: Leukotriene B₄
MDC: Monocyte-derived chemokine
Mig: Monokine induced by IFN-γ
MIP: Macrophage inflammatory protein
NKT: Natural killer T lymphocyte
OVA: Ovalbumin
PI3K: Phosphatidylinositol 3-kinase
Treg cell: Regulatory T cell
WT: Wild type

CHEMOKINES

In human subjects, chemokines constitute a family of approximately 50 low-molecular-weight proteins that precisely coordinate leukocyte trafficking to lymphoid microenvironments and also regulate leukocyte recruitment to sites of inflammation.¹ Four chemokine groups have been defined on the basis of the arrangement of amino-terminal cysteine residues. The majority of chemokines are members of the CC or CXC classes, where the 2 N-terminal cysteines are adjacent or have a single amino acid separating them, respectively.² In addition, a C class featuring a single amino-terminal cysteine and a CX₃C class, in which the 2 cysteines are separated by 3 residues, have also been described.³

Identification of many of the chemokines was as a result of intensive research undertaken by several laboratories, which often resulted in their simultaneous discovery, with each laboratory affording the same protein a different name, usually descriptive of its activity. For example, pulmonary and activation-regulated chemokine was also discovered by other groups and given the alternative names macrophage inflammatory protein (MIP) 4, alternative macrophage activation-associated CC chemokine 1, and dendritic cell–chemokine 1. To avoid confusion, a

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systematic nomenclature to distinguish between group members was devised,² which has been embraced by the research community and used throughout this article. Chemokines are now given the prefix CCL (CC ligand), CXCL (CXC ligand), CX3CL (CX3C ligand), and XCL (C ligand), together with an identifying number.

All chemokines share the same common protein fold, known as a “Greek key” motif, in which 3 antiparallel β -pleated sheets are overlaid by a C-terminal α -helix. The 4 conserved (with rare exceptions) amino-terminal cysteine residues within chemokines form 2 intermolecular disulphide bonds, giving stability to the tertiary structure. The majority of CXC and CC chemokines are known to form dimers and higher-order oligomers, as deduced by using nuclear magnetic resonance–crystallographic studies. The biologic significance of this dimerization is unclear, although data suggest that the majority of chemokines are active as monomers *in vivo*.⁴ The chemokines CX3CL1 and CXCL16 are distinct in that they are expressed in a membrane-bound form on the end of a cleavable mucin-like stalk, which bestows dual functions on them.^{3,5} As stalk-bound forms, the chemokines function as effective adhesion molecules, binding leukocytes expressing their respective cognate receptors. Like the other chemokines, they can also function as soluble chemoattractants when cleaved from their stalk by the action of metalloproteinases, such as a disintegrin and metalloproteinase-17 (ADAM17).⁶

The excessive production of chemokines has been linked with the pathogenesis of several clinically important diseases, notably asthma, atherosclerosis, and multiple sclerosis,⁷ and consequently, much effort has been made to understand how they function at the molecular level with a view to therapeutic blockade, chiefly at the level of chemokine receptor antagonism.

CHEMOKINE RECEPTORS

As with many biological ligands, the effects of chemokines are mediated through cell-surface receptors of the G protein–coupled receptor (GPCR) superfamily, which is thought to make up almost 5% of the coding regions of the human genome.⁸ Chemokine receptors fall into the subfamily of class A, rhodopsin-like receptors, being approximately 350 amino acids long and with a distinctive motif of 7 hydrophobic regions, which, by analogy to rhodopsin, are thought to form transmembrane helices, leaving an extracellular N-terminal region and an intracellular C terminus. At the time of writing, 19 human chemokine receptors had been identified, with 10 CCRs, 8 CXCRs, and a solitary CX3CR and XCR1.^{9,10} Chemokines typically bind to their receptors with nanomolar affinity, and this binding is generally class restricted; that is, CC chemokine receptors are activated only by CC chemokines and CXC receptors by CXC chemokines. A well-known exception to this rule is the Duffy antigen receptor complex on erythrocytes, which binds both CC and CXC chemokines.¹¹ Likewise, the majority of chemokine receptors are promiscuous,

binding several different chemokine ligands. The activity of individual chemokines at each promiscuous receptor generally has a “pecking order,” with some ligands dominant over others. This is typically observed in the laboratory as differences in affinity, potency, and efficacy in assays such as competition binding, chemotaxis, and the induction of intracellular calcium flux, with this latter assay allowing rank orders of potency to be readily established by sequential stimulation of leukocytes with chemokines to induce heterologous desensitization (discussed in more detail later in this article). For example, the chemokines CCL11, CCL24, and CCL26 all activate the receptor CCR3 but with a distinct rank order of potency of CCL11 > CCL24 > CCL26, as deduced by using several biologic assays.¹²

Leukocytes typically express several different chemokine receptors at any one time, and these receptors can be broadly divided into 2 groups: those constitutively expressed by leukocytes and those induced under inflammatory conditions. The former are especially involved in homeostasis, such as CCR7, which is essential for the population of secondary lymphoid organs by leukocytes, whereas the latter are important for the recruitment of leukocytes to inflamed tissues. A good example of this is the differential display of chemokine receptors by T_H1 and T_H2 subsets of T lymphocytes after polarization, which allows the cells to selectively respond to multiple chemokines.^{13,14} This division into inducible and inflammatory subsets of receptors is not absolute because some receptors facilitate both basal leukocyte homing and also that observed in inflammatory environments. For example, CCR3 mediates the recruitment of eosinophils to the allergic murine lung and also instructs them to populate the gut under noninflammatory conditions.^{15,16} Fig 1 shows the chemokine receptor repertoires displayed on the surface of cells principally involved in allergic inflammation.

CHEMOKINE–CHEMOKINE RECEPTOR INTERACTIONS AND DOWNSTREAM SIGNALING

Conserved between the majority of CC chemokine receptors (and class A GPCRs in general) is a DRY (aspartate–arginine–tyrosine) motif at the cytoplasmic end of the third transmembrane α -helix. This is analogous to the ERY motif of bovine rhodopsin, to date the only GPCR the crystal structure of which has been solved.¹⁷ This motif is thought to act as an ionic lock, holding GPCRs in an inactive state before their activation by ligand.¹⁸ On ligation, a conformation change in the GPCR is believed to result in rearrangement of the intracellular loops, facilitating the recruitment of G protein. The importance of the DRY motif in chemokine receptor structure and function was shown in a study in which nonconservative mutagenesis of this domain in CCR3 lead to a dramatic loss of function and poor cell-surface expression.¹⁹

Studies using chimeric chemokine receptor constructs have demonstrated that ligand binding typically takes place according to a 2-step model, whereby the acidic

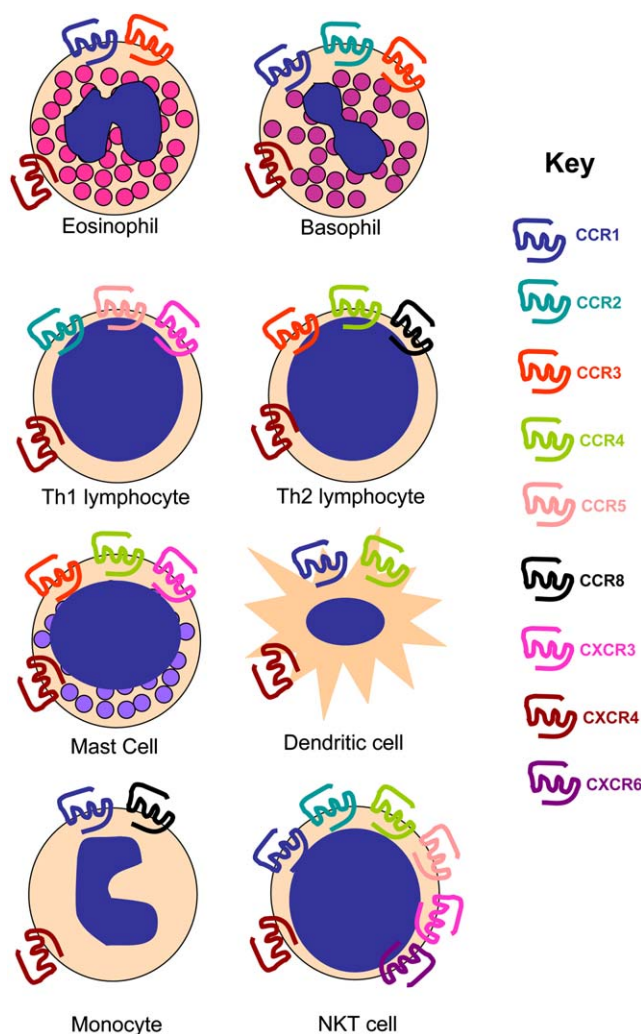


FIG 1. The chemokine receptor repertoires of leukocytes implicated in the pathogenesis of allergic disease. As in Table I, those illustrated represent commonly agreed attributions. The near-ubiquitous expression of the homeostatic receptor CXCR4 is noteworthy.

N-terminus of the receptor binds the basic chemokine with high affinity and delivers it to the remaining extracellular regions of the receptor.^{20,21} The former interaction can be facilitated further by glycosylation or sulfation of residues within the N-terminus,^{22,23} whereas the latter interaction is thought to result in conformational changes in the receptor, resulting in G protein-mediated intracellular signaling. This activation process is thought to be catalytic in nature because a single activated GPCR can in turn activate several G proteins.²⁴

Recent mutagenesis studies of CCR5 have further refined this model, highlighting an interaction between the N-terminus of the chemokine and transmembrane helices of the receptor.^{25,26} In the inactive receptor the side chains of helix II and helix III are believed to make contact through hydrophobic interactions, the perturbation of which by the chemokine amino-terminus is thought to induce the conformational changes needed for receptor

activation. Although it remains to be seen whether all chemokine receptors are activated in this manner, the model fits well with data regarding the chemokine N-terminus. For example, changes in chemokine sequence at the N-terminus typically result in altered capacities to bind receptors, activate receptors, or both (eg, truncation of CCL5 by 4 residues at the N-terminus [9-68] produces a receptor antagonist).²⁷ Likewise, elongation of the CCL5 N-terminus either naturally (with a methionine residue) or chemically (with an aminoxypentane group) produces potent receptor antagonists.^{28,29} The importance of the chemokine amino-terminus for receptor activation allows the body to easily regulate chemokine-mediated signaling. The type II membrane protein CD26/DPP IV has a unique aminopeptidase activity, which selectively cleaves dipeptides from the amino-terminus of proteins with a proline or alanine, common features of chemokines, especially those of the CC class. Several CC chemokines have greatly

impaired responses at their receptors after CD26 processing, including CCL5,³⁰ CCL22,³¹ and CCL11.³²

In contrast to the N-terminus, the C-terminus of chemokine receptors is typically rich in serine and threonine residues, which undergo phosphorylation by protein kinases after engagement of the receptor with ligand. This serves to render the chemokine receptor insensitive to further signaling by facilitating the recruitment of β -arrestins to the C-terminus. This process of desensitization simultaneously hinders G protein activation and also facilitates receptor endocytosis through clathrin-coated pits. Desensitization can be subdivided into either homologous desensitization or heterologous desensitization. Homologous desensitization occurs after repeated exposure of leukocytes to the same ligand and is mediated by specific G protein-coupled receptor kinases after occupation of the receptor by ligand. In contrast, heterologous desensitization does not require direct activation of the chemokine receptor itself and can be mediated by protein kinases, such as protein kinase A and protein kinase C, after the activation of downstream signaling pathways by other receptors.^{33,34} These can be either distinct chemokine receptors expressed on the same leukocyte or other GPCRs, such as those for N-formyl-methionyl-leucyl-phenylalanine (N-fmlp) and complement fragment 5 anaphylatoxin (C5a), which cross-desensitize signals through the chemokine receptor CXCR2 on human neutrophils.³⁵

A most unexpected finding by Fong et al³⁶ was that T and B cells from β -arrestin 2-deficient mice had impaired chemotactic responses to CXCL12.³⁶ Until this report, as their name implies, arrestins were thought to solely provide a stop signal. The same β -arrestin 2-deficient mice also exhibit reduced T-lymphocyte accumulation in their airways after allergen challenge.³⁷ To accommodate these findings, arrestins are now thought to additionally function as an adaptor scaffold, allowing the docking of kinases, such as c-Jun N-terminal kinase 3,³⁸ hematopoietic cell kinase (Hck), and the cellular homolog of the oncogene of Gardner-Rasheed feline sarcoma virus (c-Fgr),³⁹ these latter 2 molecules associating with β -arrestin to induce neutrophil degranulation after CXCL8 treatment. The recent finding that arrestins can also modulate histone acetylation and gene transcription suggest that they might also play a role in the transcriptional profiles of leukocytes after their recruitment to inflamed tissues.⁴⁰

In leukocytes, signaling downstream of chemokine receptors is typified by a chemotactic response, a process requiring the coordinated activation of adhesion molecules at the front of the cell and their inactivation at the rear as the cell moves up the chemoattractant gradient. Signaling in response to chemokines is mediated through the $G\alpha_i$ subunit, with preincubation of leukocytes with *pertussis* toxin generally ablating directed migration.⁴¹ $\beta\gamma$ subunits of the G protein activate phosphatidylinositol 3-kinase (PI3K), leading to the generation of phosphatidylinositol (3,4,5) triphosphate. Akin to *pertussis* toxin treatment, the use of PI3K inhibitors *in vitro* ablates lymphocyte migration,⁴² although recent studies of T-lymphocyte migration in response to CCL21 suggest that PI3K is not

an absolute requirement for all chemokine-mediated chemotaxis.⁴³

CHEMOKINES AND THEIR RECEPTORS IN ALLERGIC INFLAMMATION

In the following sections we will address some of the chemokines and chemokine receptors implicated in the pathogenesis of allergic disease, discussing relevant *in vitro* and *in vivo* data and, where available, translation of this research into human disease. Although the availability of animal models of allergic disease has allowed us to dissect signaling pathways involved in leukocyte recruitment (indeed allowing the identification of some chemokines, such as CCL11), the physiologic differences between the human and the rodent mean that caution should always be used when making extrapolations from the rodent to the human and vice versa. Moreover, genetic differences between animal strains can lead to conflicting reports in the literature when gene-deficient mice are used. For example, BALB/c mice deficient in CCL11 exhibit a reduction in ovalbumin (OVA)-induced lung eosinophilia,⁴⁴ whereas CCL11-deficient mice of the outbred ICR strain exhibited no difference in the numbers of bronchoalveolar lavage eosinophils after allergen challenge.⁴⁵ Likewise, the ability of mice deficient in the CCL11 receptor CCR3 to experience airways hyperresponsiveness (AHR) to methacholine after antigen inhalation is dependent on the route of sensitization, be it intraperitoneal⁴⁶ or epicutaneous.¹⁵

It is a mistake to view chemokines in isolation of other *in vivo* chemoattractant systems because leukocytes are equipped with receptors for chemoattractants other than chemokines that have a potentially important role in allergic reactions. Nonchemokine attractants exhibit low specificity for particular cell types but might be especially important in the early stages of an allergic response to be replaced by the more specific chemokines as the response develops. C5a, a fragment of the complement component C5 generated during complement activation, is a potent chemoattractant for neutrophils and other leukocytes. Similarly, Leukotriene B₄ (LTB₄) is a potent chemoattractant. LTB₄ is produced rapidly by activated mast cells, and recent research has highlighted its potential to recruit lymphocytes⁴⁷ and mast cell progenitors.⁴⁸

Fig 2 gives an example of the links between the cytokine, chemokine, and cellular adhesion systems mediating the recruitment of effector cells at sites of allergic inflammation, in this case eosinophils. T_H2 lymphocytes activated by antigen-presenting cells (APCs) release IL-4, IL-13, and IL-5, of which IL-4 and IL-13 induce CCL11 synthesis by, for example, airway epithelial cells. CCL11 acts on CCR3 receptors on eosinophils residing within microvessels, resulting in the upregulation of integrins, such as $\alpha_4\beta_1$. The integrins bind to complementary receptors on the venule wall, resulting in the arrest of eosinophils, followed by migration through the vessel wall. IL-5 increases the survival of accumulated eosinophils and also primes them for enhanced responses to diverse

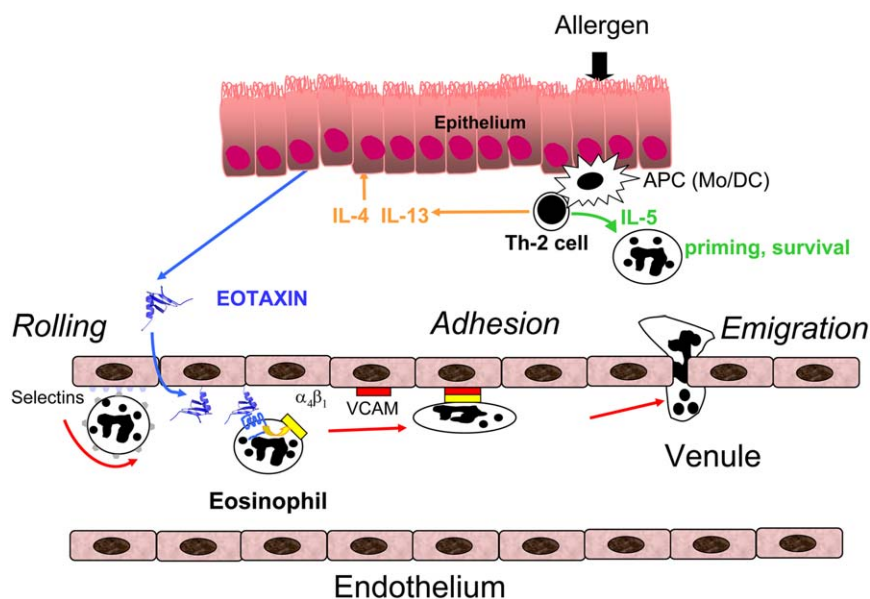


FIG 2. An example of the links between the cytokine, chemokine, and cellular adhesion systems mediating the recruitment of effector cells at sites of allergic inflammation. *Mo*, monocyte; *VCAM*, vascular cell adhesion molecule.

chemoattractants, such as LTB₄,⁴⁹ N-formyl-methionyl-leucyl-phenylalanine,⁴⁹ and CCL11.⁵⁰ Likewise, chemokine-mediated signaling can influence the downstream signaling pathways of other cell-surface receptors. For example, although the chemokine CCL11 alone is unable to induce IL-4 production by basophils, it can readily potentiate IL-4 production after their stimulation with an allergen, such as cat dander.⁵¹

CHEMOKINES ACTING ON DENDRITIC CELLS

Dendritic cells (DCs) are perceived as sentinels of the immune system, and as the most potent APCs of the immune system, the migration of activated DCs to lymph nodes after encounter with antigen is of paramount importance to the adaptive immune response. It therefore comes as no surprise that this migration is closely regulated at the molecular level by chemokines. Recruitment of immature DCs to inflamed tissues is mediated by a repertoire of receptors, including CCR2, CCR5, and CXCR4, although the use of these receptors appears to be selective, depending on whether the cells belong to the myeloid or plasmacytoid subsets.⁵² CCR6 (previously the orphan receptor STRL-22) was identified as being expressed by lung-derived DCs⁵³ and mediates responses to the ligand MIP-3 α /CCL20.⁵⁴ This responsiveness is apparently lost on maturation of either CD34⁺ or monocyte-derived DCs and is replaced by responsiveness to MIP-3 β /CCL19^{55,56} and secondary lymphoid-tissue chemokine (SLC)/CCL21⁵⁷ mediated by the receptor CCR7.⁵⁸ This maturation is accompanied by the production of chemokines, such as monocyte-derived chemokine (MDC)/CCL22, which serves to recruit activated T cells expressing CCR4.⁵⁹ Defects in either CCL19 and CCL21

expression⁶⁰ or deletion of CCR7^{61,62} result in mice with an impaired capacity for the recruitment of DCs to draining lymph nodes. Likewise, neutralization of CCR7 in an SCID mouse reconstituted with PBMCs from allergic human subjects impaired DC homing to the draining mediastinal lymph nodes after antigen challenge, with a resultant decrease in both T_H2 cytokine production and T-cell recruitment.⁶³ Regulation of CCR7 appears to be mediated by the transcription factor runt-related transcription factor 3 because its deletion results in mice with enhanced expression of CCR7 on alveolar DCs and their increased migration to the lung-draining lymph nodes. The resultant accumulation of activated DCs within these lymph nodes is associated with features typical of asthma, including increased serum IgE levels and AHR to methacholine.⁶⁴ As the authors of this study point out, in human subjects the gene encoding runt-related transcription factor 3 lies within a region of chromosome 1p36, which has been previously linked to asthma and atopy in a total genome scan for susceptibility genes.⁶⁵

CHEMOKINES AND THEIR RECEPTORS SELECTIVE FOR T LYMPHOCYTES

As the principal orchestrators of adaptive immune responses, T lymphocytes have a critical requirement for directed migration to and from secondary lymphoid organs in addition to sites of inflammation. As is the case with DCs, migration to the former is controlled by the CCR7 ligands CCL19 and CCL21, and their absence results in the failure of naive T cells to home to lymph nodes.⁶⁰ Additionally, CCR7 appears to determine T-cell exit from peripheral tissues because T cells from mice

deficient in the receptor are unable to leave the allergic lung or skin and enter into draining lymph nodes.^{66,67}

Naive T lymphocytes have been shown to express CXCR4,⁶⁸ and importantly, cell-surface levels of this receptor are upregulated by IL-4,⁶⁹ suggesting that in the cytokine milieu typical of allergic inflammation, the CXCR4-CXCL12 axis might contribute to pathology. In a well-characterized murine model of allergic airway disease, neutralizing antibodies to both CXCR4 and CXCL12 were observed to reduce lung eosinophilia and AHR, which is supportive of this hypothesis.⁷⁰ In addition, work from another group demonstrated that treatment of allergic mice with the CXCR4 antagonist AMD3100 resulted in significant reduction in AHR, eosinophilia, and the production of T_H2-associated cytokines, such as IL-4, IL-5, CCL17, and CCL22.⁷¹

In terms of migration to inflammatory environments, it is well documented that T cells can dynamically regulate their cell-surface levels of several different chemokine receptors, allowing them to respond to a variety of signals.⁷² This is perhaps best illustrated by studies examining the expression profiles of T cells polarized *in vitro* to either the T_H1 or T_H2 subsets.^{13,14} IFN- γ -producing T_H1 cells typically express CCR5 and CXCR3, whereas IL-4-producing T_H2 cells express CCR3, CCR4, and CCR8. Such polarization has also been observed to some degree *in vivo*, with IL-4-producing cells recovered by means of bronchoalveolar lavage shown to preferentially express CCR3 and CCR4.⁷³ Consistent with a role for the CCR4 axis in T-cell recruitment to the allergic lung, CCL22 and CCL17 have been observed to be upregulated in the human lung after allergen challenge,^{74,75} with another study demonstrating coexpression of CCR4 and CCR8 on a significant percentage of T cells.⁷⁶ In mice neutralization of both eotaxin-1/CCL11 and MDC/CCL22 by specific mAbs blocked early-stage recruitment of T_H2 cells to the allergic lung, whereas only MDC blockade proved effective in the long-term blockade of T_H2 cell recruitment after repeated antigen stimulation.⁷⁷

Studies with CCL2-deficient mice suggest that this chemokine is critical for the *in vitro* polarization of T cells to the T_H2 subclass because OVA challenge of deficient mice led to reduced IL-4 and IL-5 production and an inability to undergo Ig class switching.⁷⁸ Supportive of this, depletion of CCL2 in murine models of allergic airways disease has been demonstrated to reduce AHR,^{79,80} and mice deficient in CCR2, the major receptor for CCL2, show reduced pulmonary granuloma formation after injection of *Schistosoma* species egg antigen.⁸¹ However, studies by other groups have shown enhanced T_H2 responses to OVA⁸² and *Aspergillus* species⁸³ in CCR2-deficient mice, suggesting that the relationships among the chemokine, its receptor, and pathology are complex. A more recent study comparing both CCR2- and CCL2-deficient mice has reported intact T_H2-mediated responses and lung fibrosis in both animals after challenge with *Aspergillus* species.⁸⁴ In the context of the other studies, it thus appears that there is considerable variation in the importance of either chemokine or ligand in allergic

pathology depending on the experimental model used, making the relative importance of either CCL2 or CCR2 in human allergic disease difficult to gauge.

Attempts to demonstrate an absolute requirement for CCR4 or CCR8 in allergic inflammation have also met with mixed results. CCR4-deficient mice revealed little difference from their wild-type (WT) counterparts in terms of OVA-induced allergic airway inflammation,⁸⁵ although attenuation of chronic AHR was observed in the same mice treated with *Aspergillus fumigatus* spores.⁸⁶ Likewise, neutralization of CCR4 in guinea pigs with an mAb was ineffective in terms of modulation of the allergic response.⁸⁷ The role of CCR8 in allergic inflammation is also less than clear cut. Mice deficient in CCR8 exhibit similar airways inflammation as WT mice after OVA challenge.^{88,89} Additionally, neutralization of the CCR8 ligand CCL1 reduced eosinophil migration to the murine lung but had no effect on T_H2 cell recruitment after allergen challenge.⁹⁰ In contrast to these reports, defects in T_H2 responses have been reported in CCR8-deficient mice after both OVA- and cockroach antigen-induced airways inflammation.⁹¹

Intriguingly, the CXCR3 ligand CXCL10/IFN- γ -inducible protein 10 has been observed to be upregulated in the allergic lung after allergen challenge.⁷⁵ This correlates with data from a study of the allergen-challenged murine lung, in which expression of the CXCR3 ligand CXCL9/monokine induced by IFN- γ was observed.⁹² Moreover, intravenous administration of low doses of CXCL9 was demonstrated to inhibit allergen-induced eosinophil recruitment. We and others have shown *in vitro* that CXCR3 ligands are natural antagonists of CCR3-mediated responses.^{93,94} Such counterplay between T_H1 and T_H2 subsets might serve to finely tune leukocyte recruitment *in vivo*.

Recent studies have focused on regulatory T (Treg) cells as a means of suppressing allergic inflammation. CD25⁺ Treg cells have been observed to suppress the differentiation of murine CD4⁺ T cells toward T_H2 cells through a contact-dependent mechanism,⁹⁵ and in human subjects defects in CD4⁺CD25⁺ Treg cell suppression have been postulated to result in allergic diseases, such as asthma.^{96,97} CD4⁺CD25⁺ Treg cells purified from peripheral blood make up around 10% of the CD4⁺ population and have been reported to express CCR4 and CCR8, which might facilitate their migration toward APCs and activated T cells, inhibiting APC function or suppressing responding T cells, respectively.⁹⁸ Nickel-specific skin-homing CD4⁺ Treg cells have been shown to express CCR3, CCR4, CCR5, CXCR3, and CCR8, although functional responses to most ligands were lost on activation, with the exception of responses to CCL17, CCL2, and CCL1.⁹⁹ Similarly, in mice immunized with *Schistosoma mansoni* egg antigen-coated beads to elicit a T_H2 response, CD4⁺CD25⁺ IL-10-producing cells were shown to selectively express CCR8, and their infiltration within the granuloma coincided with CCL1 production.¹⁰⁰

Natural killer T-lymphocyte (NKT) cells are important regulators of the innate arm of the immune system and are a

subpopulation of $\alpha\beta$ T cells, expressing a conserved canonical TCR (V α 24J α 18-V β 11 in human subjects) that recognizes the glycolipid α -galactosylceramide (α -GalCer).¹⁰¹ The majority of NKT cells express CCR1, CCR2, CCR5, CXCR3, CXCR4, and CXCR6,^{102,103} whereas in contrast expression of CCR7 is significantly less than that of other T-cell subsets. This distinct expression profile is thought to allow them to be recruited to extralymphoid tissues through the production of chemokines, such as CCL2, CCL3, and CXCL10, whereas the lack of CCR7 expression excludes the majority of cells from populating secondary lymphoid organs. The NKT subsets can be further divided on the basis of their cytokine expression. A CD4⁺ IL-4/IL-2-producing subset was found to express CCR4 and respond to CCL17 in chemotaxis assays, whereas low cytokine-producing subsets, both CD8⁻ and CD4⁻CD8⁻ expressed CCR1, CCR6, and CXCR6 and responded chemotactically to CCL20 and CCL3.¹⁰³ As is the case for the differential expression of chemokine receptors on T_H1 and T_H2 lymphocytes, it is envisaged that such flexible programs of expression allow for the fine tuning of NKT cell recruitment *in vivo*. Using CD1-deficient mice (lacking NKT cells) in a ragweed-induced model of allergic airways disease, Bilenki et al¹⁰⁴ were able to show that the *in vivo* stimulation of NKT cells with α -GalCer resulted in enhanced CCL11 and IL-4 production and subsequent eosinophilia in WT but not CD1-deficient mice, which is suggestive of a proactive role for NKT cells in allergic airways inflammation. This contrasts with their suppressive role in bleomycin-induced models of pulmonary fibrosis, in which administration of α -GalCer resulted in increased survival of mice, correlating with increases in pulmonary IFN- γ levels and decreases in TGF- β levels.¹⁰⁵

Patients with allergic asthma have been documented as having significantly more peripheral blood NKT cells than healthy volunteers, which express significant levels of CCR9 and are subsequently responsive to CCL25 *in vitro*.¹⁰⁶ Histochemical analysis identified NKT cells as having infiltrated the bronchial mucosa of asthmatic subjects, and after purification, these cells were identified as having the potential to drive *in vitro* cocultures of CD3⁺ T cells into the expression of IL-4 and IL-13, unlike those isolated from healthy volunteers, which drove IFN- γ production. This ability to induce a T_H2 bias was dependent on cross-talk between activated CCR9 and CD226, as shown by independent blockade of CD226 expression by shRNA and transfection of NKT cells from nonasthmatic subjects with CCR9 cDNA, suggesting that blockade of CCR9 might prove fruitful in the treatment of asthma.¹⁰⁶

EOSINOPHIL-SELECTIVE CHEMOKINES AND THEIR RECEPTORS

The characteristic accumulation of large numbers of eosinophils in allergic reactions suggests an important function, but the precise relationship between eosinophils

and symptoms, particularly in asthma, has proved elusive. Some animal studies, in which IL-5 was neutralized with antibodies or the gene was deleted to suppress eosinophil production, suggested a strong link between activation of eosinophils in the lung and AHR.^{107,108} However, IL-5 neutralization in asthmatic patients did not show an effect on airway function.¹⁰⁹ More recent studies in animals^{110,111} and human subjects¹¹² suggest that eosinophils are important in airway remodeling, with eosinophil-derived TGF- β being implicated.

In early studies RANTES/CCL5 was shown to be chemotactic for eosinophils, as well as other cell types. The first potent eosinophil chemoattractant with high selectivity was eotaxin-1/CCL11, which was discovered by means of protein purification of bronchoalveolar lavage fluid and peptide sequencing.¹¹³ Two related chemokines, eotaxin-2/CCL24 and eotaxin-3/CCL26, were subsequently discovered and found to be encoded on a different gene than that of CCL11.¹¹⁴⁻¹¹⁸ The 3 eotaxins (CCL11, CCL24, and CCL26) have similar activity on eosinophils, although they exhibit relatively low sequence similarity. They signal through a single receptor, CCR3, that is highly expressed on human eosinophils at around 50,000 receptors per cell¹¹⁹ and also on basophils,¹²⁰ mast cells,¹²¹ and a subpopulation of T_H2 lymphocytes.¹²² CCR3 is a very promiscuous receptor, with upward of a dozen different chemokines able to mediate a signal through the receptor in addition to CCL11, CCL24, CCL26, and CCL5, albeit with varying potency and efficacy. Many studies have demonstrated that CCL11, CCL24, and CCL26 are generated in allergic reactions and that their production correlates with eosinophil recruitment.^{123,124} There is also evidence that both CCL11 and IL-5 are able to release eosinophils acutely from the bone marrow, which provides them with another potentially important role *in vivo*.¹²⁵⁻¹²⁷

In our hands, eosinophils from all individuals express CCR3. However, in around 15% to 20% of individuals, CCR1 is also expressed at high levels,^{128,129} rendering the eosinophils of these individuals highly responsive to CCL3 and suggesting that the CCR1-CCL3 axis has the potential to recruit eosinophils in allergic disorders affecting a significant proportion of the population. Supportive of this postulate, expression of CCL3 in the lungs of human asthmatic subjects has been reported,¹³⁰⁻¹³² and increased CCL3 levels have also been observed in the sera of patients with atopic dermatitis.¹³³ Moreover, *in vivo* blockade of murine CCL3 with a specific mAb resulted in reduced AHR and eosinophil recruitment inflammation in the initial stages of disease after challenge with cockroach allergen.¹³⁴ As with the data on AHR in CCR3-deficient mice, the allergen-sensitization protocol used appears to be important because similar neutralization of CCL3 in an OVA-induced model resulted in only a partial reduction in AHR and eosinophil recruitment to the lung.⁷⁹ An additional role for the CCR1-CCL3 axis in airways remodeling observed during chronic lung inflammation has also been put forward based on data obtained after challenge of CCR1-deficient mice with the fungal pathogen *A fumigatus*.¹³⁵ Significantly lower levels of T_H2

cytokines were found in the lungs of knockout mice compared with those of WT mice, which also correlated with significantly less observable fibrosis.

CXCR4 mRNA has been shown by Nagase et al¹³⁶ to be expressed by freshly isolated eosinophils, with protein detection only apparent after culture for 24 hours, a phenomenon that was attenuated by inclusion of IL-5 in the culture medium at concentrations with known antiapoptotic properties. Thus, the *in vitro* data suggest that it is unlikely that CXCR4 is a major mediator of eosinophil recruitment in allergic disorders, but the upregulation of CXCR4 as they age might mark eosinophils for clearance from the circulation, akin to the manner in which senescent neutrophils preferentially home to the bone marrow.¹³⁷

BASOPHIL-SELECTIVE CHEMOKINES AND THEIR RECEPTORS

In an effort to understand the repertoire of chemokine receptors expressed by human basophils, Iikura et al¹³⁸ detected mRNA transcripts for CCR1, CCR2, CCR3, and CCR5, although only CCL2 and CCL11 induced *in vitro* migration, with CCL11 clearly the most potent chemokine. In keeping with this, CCL11 has also been reported to be a potent inducer of basophil migration *in vivo* in a humanized SCID mouse model grafted with autologous human skin.¹³⁹ Likewise, CCL5, a ligand for CCR1, CCR3, and CCR5 has been reported to recruit basophils into the nasal mucosa of allergic patients after challenge¹⁴⁰ and to induce basophilic cell recruitment after intradermal injection in rats.¹⁴¹ In this report, downstream signaling was also reported to induce mRNA levels of histidine decarboxylase mRNA, potentially exacerbating inflammation by increasing histamine production. Transcripts for CXCR4 have also been reported in basophils coupled with functional data linking stromal cell–derived factor 1–induced $[Ca^{2+}]_i$ flux and chemotaxis, although there is debate as to whether the receptor is present on resting cells.^{138,142}

In addition to its chemotactic activity, CCL11 can also potentiate the production of basophil IL-4 production, acting through CCR3.⁵¹ However, CCL11 has little ability to induce basophil degranulation, unlike CCL3,¹⁴³ CCL2,^{138,144} CCL7,¹⁴⁵ and CCL13.¹⁴⁶ CCL13 also exhibited a biphasic response in assays of basophil shape change, with CCR2 and CCR3 cooperating to mediate responses across the concentration range examined.¹⁴⁷ Collectively, these data suggest a division of labor in both the activation of basophil chemokine receptors and the signaling pathways lying downstream. Curiously, few data exist to suggest that CCR4 is expressed by basophils, although its cDNA was originally cloned from a cDNA library prepared from the immature basophil line KU-812,¹⁴⁸ suggesting that it might be downregulated on maturation.

In the clinical setting, intradermal injection of CCL11, but not CCL5 or CCL3, has been reported to lead to an acute wheal-and-flare reaction thought to be mediated by the degranulation of mast cells.¹⁴⁹ Supportive of this,

numbers of detectable mast cells were also observed to decrease in the 24 hours after chemokine injection. Moreover, the ability of the basophil to produce CCL3 after IgE cross-linking suggests that it might constitute a positive feedback mechanism in the allergic setting.¹⁵⁰

MAST CELL-SELECTIVE CHEMOKINES AND THEIR RECEPTORS

Mast cells characteristically express c-kit, and the ligand for this receptor, stem cell factor (SCF), induces their chemotaxis,¹⁵¹ in addition to other important effects, such as proliferation, differentiation, and inhibition of apoptosis. Several chemokine receptors have been identified on mast cells or mast cell lines, including CXCR2,¹²¹ CXCR3,¹⁵² CXCR4,^{121,153} CCR1,¹⁵⁴ CCR3,^{121,155} CCR4,¹⁵⁴ and CCR5,¹²¹ and their recognized chemokine ligands have been shown to be chemotactic for these cells. Notably, CCR3 is expressed on both mature human mast cells and their progenitors.¹²¹ Attempts to ascertain the role of CCR3 in mast cell trafficking have proved paradoxical. For example, CCR3^{-/-} mice infected with *Trichinella spiralis* exhibited a normal jejunum and cecum mast cell hyperplasia and unaffected worm expulsion.¹⁶ However, CCR3^{-/-} mice sensitized with OVA intraperitoneally and challenged with aerosolized OVA had increased numbers of tracheal intraepithelial mast cells and an increased hyperresponsiveness compared with those seen in WT mice.⁴⁶ Recently, CXCR2^{-/-} mice have been reported to have reduced numbers of intestinal mast cell progenitors, as determined by using limiting dilution assays, suggesting that mediators such as keratinocyte-derived chemokine (KC)/CXCL1 and MIP-2/CXCL2 are involved in trafficking, whereas there was no reduction in mast cell progenitors in CCR2^{-/-}, CCR3^{-/-}, or CCR5^{-/-} mice.¹⁵⁶ Work in our laboratory has shown that immature, but not mature, murine and human mast cells express the receptor BLT1.⁴⁸ This suggests that circulating progenitors can migrate toward LTB₄ generated by activated mast cells in tissues. This provides a potential mechanism for mast cell hyperplasia associated with allergy.

A role for the CCR1-CCL3 signaling pathways leading to mast cell degranulation has also been proposed, with studies by Toda et al¹⁵⁷ demonstrating decreased chemotaxis of mast cells to CCL3 after costimulation of both FcεRI and CCR1. This, they postulate, focuses the allergic inflammatory response by maintaining cell numbers at the site of allergen accumulation.¹⁵⁷ Recent *in vivo* observations by the same group have also shown that costimulation of FcεRI and CCR1 appears to optimize the degranulatory response in a murine model of allergic conjunctivitis.¹⁵⁸ As would be predicted from their earlier work, neutralization of CCL3 by the use of CCR1-deficient mice or mAb blockade of CCL3 was sufficient to inhibit disease score.¹⁵⁸ A more recent publication from the same group suggests that antagonism of CCR3 in the same murine model by small-molecule antagonists is also

TABLE I. Chemokine receptors and their ligands implicated in allergic disease

Receptor	Principal ligands	Cell types that express receptor	Clinical evidence for a role in allergic disease
CCR1	CCL3/MIP-1 α CCL5/RANTES CCL7/MCP-3	<u>Mo</u> , DC, Eo, Bs, T, Mc, NK, NKT	Increased expression of CCL3 in the asthmatic lung ¹³⁰⁻¹³² and in the serum of patients with AD ¹³³
CCR3	CCL5/RANTES CCL11/eotaxin-1 CCL13/MCP-4 CCL24/eotaxin-2 CCL26/eotaxin-3	<u>Eo</u> , <u>Bs</u> , T, Mc	Increased expression of CCL11, ¹⁷⁵ CCL24, ¹⁷⁵ and CCL26 ¹⁷⁶ in the allergic lung and also in the sputum ^{177,178} ; increased expression of CCL5 in the bronchial mucosa of atopic patients with mild asthma ^{179,180} and release into BAL fluid after endobronchial allergen challenge ¹³² ; increased CCL5 levels in the nasal mucosa of atopic patients with seasonal rhinitis after allergen provocation ¹⁸¹
CCR4	CCL17/TARC CCL22/MDC	<u>T</u> , DC, Bs, Mc, NK, NKT	Increased serum levels of CCL17 in patients with AD ¹⁸² and CCR4 ⁺ T lymphocytes in skin biopsy specimens ¹⁸³ ; colocalization of CCR4 ⁺ T lymphocytes with CCL22 and CCL17 in bronchial biopsy specimens from asthmatic subjects ⁷⁶
CCR8	CCL1/I-309	<u>T</u> , Mo, NK	CCR8 ⁺ T lymphocytes observed in bronchial biopsy specimens from asthmatic subjects ⁷⁶
CXCR3	CXCL9/Mig CXCL10/IP-10 CXCL11/I-TAC	<u>T</u> , B, Mc, NKT	CXCL9, CXCL10, and CXCL11 expressed by activated lung epithelial cells ¹⁸⁴ ; resident lung T lymphocytes are CXCR3 ⁺ ¹⁸⁵ ; CXCL10 produced by apoptotic keratinocytes in patients with AD ¹⁸⁶
CXCR4	CXCL12/SDF-1 α	<u>T</u> , B, DC, Eo, Bs, Mc, Mo, NKT	Increased CXCR4 expression on eosinophils in the BAL fluid of patients presenting with lung eosinophilia ¹³⁶

The table highlights the cellular distribution of chemokine receptors implicated in the clinical manifestations of allergic disease, together with their principal ligands. Although there are reports of chemokine receptors on nearly every type of cell, those shown represent commonly agreed on attributions, with the main reservoir of expression underlined.

MCP, Monocyte chemoattractant protein; Mo, monocytes; Eo, eosinophils; Bs, basophils; T, T lymphocytes; Mc, mast cells; NK, natural killer cells; AD, atopic dermatitis; BAL, bronchoalveolar lavage; TARC, thymus- and activation-regulated chemokine; I-309, inducible-309; Mig, monokine induced by IFN- γ ; IP-10, IFN- γ -inducible protein; B, B lymphocytes; I-TAC, IFN- γ -inducible T-cell chemoattractant; SDF, stromal cell-derived factor.

beneficial, with an impaired early-phase reaction thought to be due to the inhibition of mast cell degranulation.¹⁵⁹

BLOCKADE OF CHEMOKINE RECEPTORS BY SMALL-MOLECULE ANTAGONISTS

A key point in turning our current understanding of leukocyte recruitment into a future therapy for the treatment of allergic disease is the question of which receptor or receptors to target. As a key effector of eosinophil migration in allergic inflammation, CCR3 was the major focus, with early *in vitro* and *in vivo* proof-of-principle studies suggesting that CCR3 blockade was feasible.¹⁶⁰⁻¹⁶² Subsequent work by the pharmaceutical industry using high-throughput screens identified several potent small-molecule antagonists of CCR3 with activities in the low nanomolar range.¹⁶³⁻¹⁶⁷ Enthusiasm for CCR3 antagonists appeared to wane with the report that IL-5 blockade had little effect on lung function, as recently reviewed by Wells et al.¹⁶⁸ However, as mentioned above, subsequent clinical and animal studies have also highlighted a role for eosinophils in the remodeling of the airways associated with

asthma. Consequently, interest in CCR3 as a therapeutic target has been rekindled, particularly because it is also expressed by basophils, T_H2 cells, and mast cells, and blockade on these cells might also be therapeutically beneficial.

A major obstacle to overcome is that fact that there is often limited homology between human and rodent orthologues of chemokine receptors, and consequently, many small molecules generated against human receptors are ineffective at crossing the species barrier. This makes their validation in well-established rodent models of allergic inflammation impossible, and reports describing the *in vivo* efficacy of such compounds lag well behind the earlier *in vitro* studies. The Yamanouchi Pharmaceutical Company has recently described compounds with efficacy in an assay of eosinophil recruitment to the macaque lung after bronchoprovocation with CCL11¹⁶⁹ and in a murine model of cutaneous inflammation.¹⁷⁰ Likewise, Abbot Laboratories have described the antagonist A-122058, which was effective in reducing the number of eosinophils after intraperitoneal injection of CCL11 into mice.¹⁷¹ To date, the only small-molecule antagonist in phase II trials is the compound from GlaxoSmithKline, GSK766994, but no reports have been published regarding its efficacy

in an allergic asthma and rhinitis study.¹⁷² This follows on from the reports of efficacy of their GW701897B compound in vagally mediated bronchoconstriction in anti-gen-challenged guinea pigs.¹⁷³

One point worth making is the fact that chemokine antagonists, like many compounds targeting GPCRs, appear to function by binding to the transmembrane helices, a region often highly conserved between different receptors.¹⁷⁴ Thus it is not surprising that some of these compounds have selectivity for more than one receptor, such as the compound UCB 35625 (a transomer of BANYU J113863), which has nanomolar activity at both CCR1 and CCR3.¹⁶³ It might be envisaged that targeting of 2 or more receptors with such compounds might provide the therapy of the future for allergic diseases.¹⁶³

SUMMARY

Our knowledge of the chemokine family has greatly enhanced the understanding of the mechanisms underlying allergic reactions. Small-molecule antagonists can now be designed to block particular chemokine receptors. Converting this knowledge into effective therapy for allergic diseases presents major challenges for the future. One important factor is the complexity of the chemoattraction mechanisms, particularly the number of different receptor types on a given cell and their plasticity, and the fact that chemokines can often stimulate more than one receptor. We are beginning to obtain detailed information on these systems (Table I),^{76,129-133,136,175-186} but this, in turn, is highlighting the lack of knowledge of some of the fundamental mechanisms involved in allergic reactions, especially the roles of particular cell types and how their interactions give rise to symptoms. There is still much to learn.

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