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Role of chemokines in asthmatic airway inflammation

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Summary: The incidence of asthma worldwide has increased significantly over the past two decades, especially in young children and in inner cities. Although there are several contributing factors that promote severe asthmatic responses, uncontrolled inflammation leads to the most severe consequences. Chemokines are an interesting target for decreasing the inflammatory response and therefore altering the pathogenesis of asthma reactivity in the lungs. However, it has become clear that there are a number of chemokines that play important roles in various aspects of asthmatic inflammation and reactivity. Identifying the functions each of these chemokines plays during the responses will be imperative to properly target different phases of the asthmatic condition. This review will outline what is known of the role of various chemokines that are produced during asthmatic responses and speculate on the appropriateness of targeting these mediators for therapy.

Chemokines in clinical asthmatic responses

Over the past several years a family of chemotactic cytokines has been identified that appears to have specificity for the type of leukocytes that are recruited to the site of inflammation (1–5). Chemokines have been primarily divided into two main subfamilies based upon their sequence homology and the position of the first two cysteine residues, C-×-C (α) and C-C (β). This group of chemoattractants has grown to include more than 40 different molecules. *In vitro* characterization would suggest that most of these chemokines have redundant or similar functions with other known chemokines. In addition to the chemokines, there are also numerous receptors that have been identified. The realization of the role of chemokines for mediating leukocyte accumulation and activation led investigators to begin to examine which chemokines are expressed at significant levels during human asthmatic disease. Bronchoalveolar lavage and sputum samples from asthmatics became the easiest method of determining chemokine levels in patient populations. Several studies have now identified that both C-×-C and C-C family chemokines are upregulated after allergen challenge, and the levels relate directly to the intensity of the airway reactivity responses (6–11). The airway epithelial cells and

alveolar macrophages produce significant levels of chemokines that can have an immediate impact on the environment of the airway and surrounding lung tissue. Initial investigations in human populations have centered on eosinophil-specific chemokines, as the eosinophil appears to be an important cell population in the pathology of asthma. Over the past several years, a number of chemokines, such as interleukin (IL)-8, RANTES, monocyte chemoattractant protein (MCP)-3 and MCP-4, have been identified that induce eosinophil recruitment (12–16). The one important question, however, is whether all of the chemokines function as eosinophil chemoattractants or whether a specific chemokine mediates the bulk of the chemotactic activity and can be targeted for therapy. Eotaxin is one of the only chemokines that appears to function primarily for eosinophil chemotaxis. In human asthma, eotaxin is produced at high levels and localized to the airway epithelium (17, 18). This concentrated expression may preferentially target eosinophils to the epithelium and induce degranulation leading to release of epithelium-damaging proteins. In addition, to recruiting and activating eosinophils, eotaxin can affect other cell populations. Basophils degranulate in the presence of eotaxin, while Th2-type cells can migrate toward eotaxin (19–21). Thus, eotaxin may be a primary target for therapeutic intervention. However, other chemokines and their receptors will also need to be examined, as there seems to be a great deal of functional overlap throughout the chemokine family.

Chemokines and early phase asthmatic responses

One of the first series of cells activated during allergen-induced airway reactivity and asthma are mast cells and basophils (22, 23). These cell populations are closely associated with the severity of the asthmatic reactivity and can be immediately activated for release of preformed mediators by IgE-induced degranulation. However, the chronic activation of these cells has recently been recognized and constitutes a possible component of the late phase asthmatic response (24). There are a number of chemokines that can induce chemotaxis of basophils and mast cells (25–28). Initial studies have shown that the CC chemokines MCP-1, 2 and 3, RANTES, macrophage inflammatory protein (MIP)-1α and eotaxin can all induce recruitment of basophils and mast cells. In addition to the recruitment of these cells, it is now evident that particular chemokines can also serve as activating factors that induce basophil and mast cell degranulation. In particular, MCP-1 and RANTES are able to induce significant histamine release from basophils. In fact, MCP-1 can induce nearly as much histamine release as IgE-mediated stimulation. This becomes important for chronic activation of these cells as the temporal production of MCP-1 indicates that it is produced at later phases of the allergic response, after allergen has been cleared, and therefore likely promotes continued cellular activation and mediator release without the presence of antigen (10, 29–31). Interestingly, eotaxin can potentiate basophil activation for the production of IL-4, and therefore, enhance the Th2 type environment created in the lung during allergic responses (32). This pathway of activation would allow prolonged activation and continuous degranulation of basophils during the allergen-induced responses.

Other studies have indicated that MCP-1 is also a potent activation factor for mast cells both *in vitro* and *in vivo* (30, 33). Subcutaneous injection of MCP-1 causes a dose-dependent accumulation of mast cells in the skin of rats (34) leading to local edema and leukocyte accumulation. The neutralization of MCP-1 during allergen-induced airway hyper-reactivity responses can significantly alter the adverse pathophysiologic events (30). The use of CCR2$^{-/-}$ mice during the allergen-induced responses further confirmed the relevance of this ligand-receptor activation pathway for the induction of airway hyper-reactivity. Interestingly, intratracheal injection of MCP-1 into the airways of mice directly induces mast cell degranulation and long-term airway hyper-reactivity. The latter study demonstrated that MCP-1 induced the activation and release into the airway of leukotriene C4 (LTC4), an arachidonic acid metabolite with the ability to induce airway hyper-reactivity. The fact that MCP-1 can induce LTC4-mediated pathways makes an important link between chemokine and arachidonic acid metabolite biology (30, 35). Thus, one potential target for alleviating certain phases of asthmatic inflammation linked to mast cell activation is MCP-1 and its receptor CCR2. Overall, chemokine-mediated exacerbation of chronic asthmatic disease may perpetuate activation of basophils and mast cell populations leading to a more severe outcome.

Recruitment and activation of leukocyte subsets during allergic responses

Neutrophils

The inflammation induced during allergic airway inflammation is mediated by the coordination of several immune specific activation events. The first leukocyte that appears to enter a site of allergic inflammation is the neutrophil (11). The activation and degranulation of local mast cell populations is an immediate response in the airway, mediated both by antigen-specific, surface-bound IgE and by cytokine-induced activation pathways. The relevance of neutrophils to airway hyper-reactivity

and subsequent late phase reactions and airway damage in atopic asthma has traditionally been a controversial topic. However, results collected over recent years have provided data that suggest that neutrophils, at the very least, have a role in inducing airway damage leading to lung dysfunction (36–39). The recruitment of neutrophils to the airway during allergic responses has been well documented. Neutrophils are a source of several inflammatory mediators as well as destructive proteases capable of damaging surrounding tissue in the lung (40). The family of chemokines containing C-×-C-ELR, which includes IL-8, epithelial cell-derived and neutrophil-activating 78-amino acid peptide (ENA-78) and growth-related oncogene product (GRO) α, β and γ, is primarily chemotactic for neutrophils. Several pieces of evidence have correlated C-×-C chemokines and neutrophils with the onset and maintenance of asthmatic inflammation. In particular, high levels of IL-8 have been observed in bronchoalveolar lavage fluid samples from asthmatics after allergen challenge (11, 41). In sudden onset fatal asthma, neutrophils were the predominant cell population found in the airspace with few eosinophils (42–44). To support the concept that neutrophils can initiate asthmatic responses, supernatants from degranulated neutrophils incubated with human bronchus increase the responsiveness to histamine (45). A number of studies have now begun to correlate the presence of neutrophils in asthmatics with severe chronic disease and peribronchial fibrosis (46). These latter issues have begun to take center stage in asthma as the more severe responses that are observed seem to relate to end-stage fibrosis and thickening of the large airways.

A number of studies have demonstrated the relationship of neutrophil accumulation, C-×-C chemokines and airway responsiveness. Studies examining the role of ozone in asthmatics have demonstrated that there is a significant increase in IL-8, sputum neutrophilia and an associated increase in airway reactivity (47–51). In animal models, ozone can increase airway hyper-reactivity responses that are correlated directly to the intensity of neutrophil infiltration observed. Additional animal studies have begun to further define a role for C-×-C chemokines and neutrophil influx during allergen-induced airway hyper-reactivity. Specifically, ENA-78, a molecule with similar function to IL-8, is found premade in mast cells and appears to be quickly released upon antigen stimulation *in vitro* and *in vivo* (52). The neutralization of this chemokine during allergen-specific responses in mice significantly reduces the neutrophil influx. In studies with guinea pigs, instillation of IL-8 can significantly induce granulocyte accumulation and airway reactivity responses (53). Thus, the C-×-C chemokines may have a prominent role in the late phase responses for recruitment and activation of neutrophils, leading to airway damage and physiologic dysfunction.

Eosinophils

There is no doubt that the overwhelming number of asthmatics that present with moderate to severe chronic disease are characterized by significant eosinophil accumulation and activation within the airway (38, 54, 55). Like neutrophils, eosinophils contain a number of products that when released directly impact on the function of the airways, either by damaging the structural cells or influencing other physiologic regulators. Determining the mechanisms of activation and recruitment of eosinophils in the airway is a focus of most researchers examining asthmatic disease. Although IL-8 can be shown to induce eosinophil accumulation, its relative efficacy compared to a number of C-C chemokines may not be as vital. Over the past several years a number of CC chemokines that bind to CCR3, the major receptor that is expressed on the circulating eosinophil population, have been shown to induce chemotaxis and activation of eosinophils (15, 56–60). These chemokines include RANTES, MCP-3, MCP-4, eotaxin and eotaxin-2. Of these chemokines only eotaxin and eotaxin-2 bind specifically to CCR3 and not to other receptors. Furthermore, eotaxin appears to be the most potent chemokine for movement of these cells into tissue. However, additional studies have now demonstrated that other non-CCR3-binding chemokines can cause the migration and activation of eosinophils. Some of the earliest studies using peripheral human eosinophils indicated that MIP-1α, a CCR1 ligand, could also induce chemotaxis of eosinophils (61). This observation has been reflected in murine systems that have demonstrated a function for MIP-1 a as an eosinophil recruitment factor both *in vitro* and *in vivo* (62–64). More recently, MDC, a CCR4 ligand, has also been identified as an eosinophil recruitment factor but does not depend upon CCR4 or CCR3 binding (65, 66). Therefore, MDC likely utilizes a different receptor than other known eosinophil chemotactic factors. Studies that have been performed in our laboratory have demonstrated that a complicated picture of eosinophil recruitment and activation may be emerging. Although peripheral eosinophils from mice follow what has previously been described, when elicited eosinophils are used the pattern of chemokine utilization changes drastically (*Table 1*). In peripheral eosinophils, only CCR1 and CCR3 ligands induce chemotaxis. However, when eosinophils are elicited to the peritoneum of sensitized mice, the isolated eosinophils from these lesions migrate to ligands specific for additional receptors. We have now shown that the CCR5 and CCR8 ligands MIP-1β and thymus-derived chemotactic agent

Table 1. **CC chemokine receptor function on eosinophils**

	Chemotaxis/mRNA expression			
Receptor (chemokine)	Control	IL-4	TNF	Ca flux
CCR1 (MIP-1[a])	++/++	–/+	+++/+++	++
CCR2 (MCP-1)	–/–	–/–	–/–	–
CCR3 (eotaxin)	+++/+	+++/++	+++/+++	+++
CCR4 (TARC)	–/–	–/–	–/–	–
CCR5 (MIP-1β)	–/–	++/++	++/++	–
CCR8 (TCA3)	–/–	+/++	+/++	–

[a]Determined by desensitization assays

(TCA) 3 can induce elicited eosinophils to migrate in chemotactic assays. Additional studies indicated that IL-4 and tumor necrosis factor (TNF), but not interferon (IFN), can upregulate the expression and function of CCR5 and CCR8 on eosinophils. Thus, once properly activated, such as in asthmatic patients, additional chemokines can affect the movement and activation of eosinophils. These concepts will be important for understanding the biology of eosinophil migration and activation in allergic tissues.

Lymphocytes

The migration of lymphocytes into tissues appears to be dependent upon the expression of specific chemokines during the progression of inflammatory disease. Investigators have begun to define the association of certain chemokine profiles with particular types or phases of immune responses. The preferential expression of certain chemokines during immune responses likely dictates their function. For example, the CC chemokine family members RANTES, MIP-1α and MIP-1β are induced by IFN and TNF but regulated by IL-4, and appear to be closely associated with Th1-type responses (67–70). Likewise, the production of CxCR3 ligands (IFN-γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG) and IFN-inducible T-cell α chemoattractant (ITAC)) are specifically activated by IFN and may have critical roles in enhancing Th1-type lymphocyte recruitment and activation. Along with the preferential expression of chemokines during Th1-type responses, there is also the preferential expression of the associated chemokine receptors on Th1-type lymphocytes (21, 71, 72). A number of studies have shown that CCR1 and CCR5 (which bind RANTES and MIP-1α) as well as CxCR3 (which binds IP-10, MIG and ITAC) on Th1-type lymphocytes are favorably expressed. Thus, the chemokine expression during a Th1-type response correlates directly with the specificity of the chemokine receptors that are expressed on Th1-type lymphocytes.

As there are chemokines associated with Th1-type responses, there also appear to be certain chemokines that are closely associated with Th2-type responses. An impressive body of work has accumulated on CC chemokines and the expression of particular members of this family that are specifically activated by IL-4 and IL-13. The CC chemokines that are preferentially upregulated by Th2-type cytokines include MCP-1, eotaxin, TCA3, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) (73–76). Interestingly, these IL-4 and IL-13-induced chemokines appear to bind to a single chemokine receptor, which is unusual among chemokine family members, which normally have a promiscuous binding pattern to multiple chemokine receptors (77). Thus, the Th2 activation pathway, which has been associated with allergen-induced airway hyperreactivity, induces preferential chemokine production that is associated with allergic cell recruitment. This area will be of particular interest since studies have previously identified that these Th2-associated chemokines play significant roles in allergen-induced airway inflammation and airway hyper-reactivity (29, 65, 78–81). Analysis of *in vitro* derived Th2-type cells indicates preferential expression of CCR3 (eotaxin), CCR4 (MDC, TARC) and CCR8 (TCA3) (21, 71, 72, 82). This receptor expression pattern correlates well with the type of chemokines that are induced by Th2-type responses discussed above. Importantly, a recent study demonstrated that both CCR3 and CCR4 are utilized for sequential movement of Th2-type cells into allergen-challenged lungs (83). These results indicate that the expression of multiple receptors by lymphocytes functions to allow sequential migration of cells throughout chronic asthma-like responses. Verification of these specific responses will come from preclinical animal models and examination of receptor expression on circulating and infiltrating lymphocyte populations.

Regulation of cellular recruitment by airway epithelial cell-derived chemokines

The airway epithelial cells are one of the first lines of defense against invasion by infectious agents, primarily providing a barrier role in the lung (84–87). Over the past several years, airway epithelial cells have been identified as a source of a number of chemokines that may impact on the immune responses generated within the lung. The activation of chemokines in epithelial cells can dictate the type of cells that migrate in and therefore are responsible for creating a specific inflammatory environment in the lung that determines how the lung reacts to antigenic stimuli and disease. The activation of the epithelial

cells by TNF or IFN leads to the production of the chemokines IP-10, ITAC and RANTES which are associated with Th1-type responses (69). In contrast, the activation of these structural cells with IL-4 or IL-13 will lead to the production of chemokines such as eotaxin, MCP-3 and 4 that are related to allergic-type responses and can preferentially recruit Th2 cells and eosinophils (15, 59, 74, 88–90). Thus, the cytokine environment initiated by the structural cells of the airway can determine the type of cells and responses that are generated within the lung.

It has been recognized that an additional role for the airway epithelial cells may be as a regulatory cell that modulates potentially harmful immune responses to innocuous antigens encountered in the lung. The constitutive production of IL-10, a potent cytokine suppressor factor, appears to regulate the resident cells of the airway to avoid local activation (91). Under normal circumstances the airway epithelial cells will want to protect themselves against damage to maintain lung function. It is only when an infectious or noxious agent is introduced into the lung that damages the airway epithelial cells that an immune response should occur. Thus, one could take the view that any cytokine that is made in a constitutive fashion by the airway epithelial cells may be produced in an effort to modulate the responses to environmental antigens. Eotaxin is one of the cytokines that is made constitutively by airway epithelium (18, 92). We have recently begun asking the question of whether eotaxin may normally have a protective role and only under atypical circumstances, such as allergy and peripheral eosinophilia, would promote an adverse response. Recent studies have identified the presence of CCR3 on neutrophils after cytokine stimulation, either IFN or TNF (93), suggesting a possible role during inflammation and cell-mediated immune responses. In studies using a neutrophil-dependent acute lung injury model, the neutralization of eotaxin in the airway significantly exacerbated the neutrophil influx and lung injury (P. A. Ward, personal communication). Likewise, the administration of exogenous eotaxin into the circulation of challenged mice significantly reduced neutrophil influx and acute lung damage. In additional studies in our labs, eotaxin could significantly reduce IL-8-mediated migration of neutrophils through an endothelial cell layer, suggesting that it may have a role in regulating the extravasation of neutrophils into a site of inflammation (S. Cheng, S. L. Kunkel, N. W. Lukacs, unpublished data). These latter ideas have been further examined *in vivo* using eotaxin$^{-/-}$ mice, which were found to have significantly increased recruitment of neutrophils into acute inflammatory responses. Thus, a function of eotaxin may be to regulate the local homeostasis of the lung by controlling the influx of neutrophils that can directly damage the lung. In agreement with this concept, the Th2 cytokine-induced overexpression of eotaxin in allergic asthma would promote the influx of circulating eosinophils but not neutrophils. This mechanism may help to explain why asthmatics with a predominant Th2 phenotype, peripheral eosinophilia and significant levels of eotaxin have primarily an eosinophil infiltration with few neutrophils present. It is likely that other chemokines have similar regulatory functions depending upon the cell populations involved.

Exacerbation of chemokines and asthmatic responses by viral infections

A number of clinical studies have established that the most common stimulus that exacerbates asthmatic responses is viral infections of the airway. Common viral infections include rhinovirus, respiratory syncitial virus (RSV), adenovirus and influenza (94–97). A number of cytokine mediators have been shown to be induced by viral infections in both local macrophage populations and airway epithelial cells. In most of the cases the viruses provide a means for a general upregulation of inflammatory responses that leads to excerbated lung damage and dysfunction. However, certain chemokines appear to be specifically upregulated in cells during viral infections. Using isolated airway epithelial cells many of these viruses appear to be able to upregulate IL-8 and RANTES (98–101). These chemokines may provide for a common defense pathway to begin the antiviral immune response through the recruitment of neutrophils (IL-8) and mononuclear cell populations (RANTES). Although their function in this regard remains unproven, clinical samples verify that these chemokines are upregulated (102). However, in an individual with a previous history of asthma, the accumulation and activation of these leukocyte populations can exacerbate the airway reactivity. A recent study has linked the level of chemokines induced by upper respiratory viral infections with the intensity of the asthmatic exacerbation as well as to eosinophil activation products in the airway (103). An important issue to discern is the fact that Th1-type antiviral responses induce several chemokines that augment the intensity of "Th2-type" inflammatory cell infiltration. For example, although RANTES has been identified as being primarily induced by acute and Th1-type mediators, it was one of the first and most potent eosinophil chemotactic factors identified that can facilitate eosinophil accumulation when injected *in vivo* (104, 105). Thus, asthmatic inflammation and exacerbation should not be considered only a Th2 cytokine-induced disease. This issue has recently been examined in experimental models where investigators co-transferred Th1 and Th2-type specific lymphocytes into a naïve mouse and

***Table 2*. Chemokine receptor targets in asthma**

Receptor	Chemokine ligands	Function
CCR2	MCP-1 to 4	Mast cell/basophil actvation
		T-lymphocyte recruitment
CCR3	Eotaxin, eotaxin-2	Eosinophil chemotaxis/activation
	MCP-3, MCP-4, RANTES	Th2-cell recruitment
CCR4	MDC, TARC	Th2-cell recruitment
		Dendritic cell recruitment
CCR8	I-309	Th2-cell recruitment
		Smooth muscle cell contraction

found that the presence of Th1-type cells did not suppress development of pulmonary disease, but rather intensified the inflammation and exacerbated the lung pathophysiology (106, 107). The mechanism of direct viral induced chemokine expression is not completely clear in all cases. However, the work performed examining the induction of chemokines suggests that a primary pathway be via oxidative burst leading to NFκβ activation induced by the infection of epithelial cells (108). It appears that the virus is required to infect and replicate in the cells in order to induce the chemokine expression (88, 100, 108). This mechanism of chemokine activation may be similar in RSV, rhinovirus and influenza virus.

Chemokine and receptor targets in asthma

The initial excitement of targeting chemokines during inflammation was fueled by the realization that these molecules mediated the localization and accumulation of leukocytes to a site of inflammation. In the early days of chemokine biology, when there were only a few chemokines identified, this prospect seemed straightforward: target IL-8 for neutrophilic infiltration and RANTES or MCP-1 for mononuclear infiltration. However, now there are between 45 and 50 different chemokines, which overlap in function and are differentially induced during various diseases. Therefore, targeting the correct or most important chemokine in a disease may not be feasible. Instead, the pharmaceutical industry has turned to attempting to inhibit chemokine function at the receptor level. The pharmacologics directed against G protein-coupled serpentine receptors have previously been successful. Data available from asthma patients and from models of allergic asthma suggest that there may be a number of viable chemokine receptor targets that will be prosperous (*Table* 2).

Perhaps the most sought after target has been CCR3. This receptor is highly expressed on eosinophils, and ligands that bind to CCR3, including eotaxin, MCP-3, MCP-4 and RANTES, are potent agonists that are expressed during asthma and mediate recruitment and accumulation of eosinophils at a site of inflammation (15). Reports have also identified CCR3 on subsets of Th2 lymphocytes derived *in vitro*. It is unclear whether this observation will hold *in vivo*; however, at least one report has shown that CCR3 is involved in Th2-cell accumulation during allergic responses in the lungs of mice (21). Other receptors have also been identified on Th2-type cells, including CCR4 and CCR8 (76, 82, 83). The objective of targeting these latter two receptors will be to inhibit the recruitment of Th2 type cells to the airway and therefore block the long-term detrimental effects of the allergic responses. Interestingly, TCA3, a CCR8 ligand, can also directly induce smooth muscle cell contraction and, therefore, may be additionally attractive as a target (109). Yet another chemokine receptor target that appears to be promising is CCR2, which binds all of the MCP-1 family members. The data for justifying CCR2 as a target centers around its ability to recruit and activate basophils and mast cells (as discussed above), as well as its effects on lymphocyte recruitment and skewing towards Th2-lymphocyte maturation (30, 33, 34, 110–113). Whether targeting any of these receptors will make an impact on allergic asthmatic inflammation remains unclear and will need to be tested in viable preclinical models before reaching patient populations.

An aspect that will need to be explored for targeting these receptors over a long-term period is the effect on infectious responses of the lung. Will inhibiting a particular chemokine receptor pathway lead to increased susceptibility to infectious agents? Recent studies using receptor "knockout" animals would suggest that some of these responses may be affected in a detrimental way. Using a model of live *Aspergillus*-induced allergen responses in $CCR2^{-/-}$ mice, the results suggest that these mice have a prolonged and more severe airway hyperreactive response (114) and also have a lack of clearance of the infectious organism from the airway. Since CCR2 appears to play an important role in mediating macrophage activation for increased pathogen phagocytosis and clearance, targeting this receptor may be very detrimental over a long-term period and allow the colonization of opportunistic organisms. Although relatively little is known about the function of other receptors, we do have some data on the role of the ligands that bind to those receptors. For example, MDC (a CCR4 ligand) also appears to mediate bacterial clearance and attenuate lethality in a bacterial sepsis model in mice (115). Targeting CCR4 with a pharmacologic may alter the host response to specific pathogens. These considerations must be taken into account and specifically tested as reagents for these receptors become available.

Conclusions

Chemokines play an important part in asthmatic disease progression at multiple levels. The coordinated production of specific chemokines and the expression of a distinct subset of receptors likely dictate the intensity and severity of the asthmatic response. The exacerbation of asthmatic responses by viral infections may directly or indirectly induce multiple chemokines during viral replication in the resident cells of the lungs. Chemokines were originally described as mediators of leukocyte recruitment; however, recent evidence suggests that they can also influence the outcome of the immune response by altering the cytokine profile and by activating/degranulating multiple effector cell populations. In view of the diversity of chemokine production and the promiscuous binding pattern for multiple receptors, it is unlikely that a single chemokine or chemokine receptor-induced mechanism will be identified for therapy, but rather a multimechanistic approach may be the most promising. Only after determining the function of specific chemokine and chemokine receptors during different disease phases can their true functions be identified and specific molecules targeted.

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