

REVIEW

Chemokines and their receptors as potential targets for the treatment of asthma

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Asthma is a chronic and sometimes fatal disease, which affects people of all ages throughout the world. Important hallmarks of asthma are airway inflammation and remodelling, with associated bronchial hyperresponsiveness and variable airflow obstruction. These features are orchestrated by cells of both the innate (eosinophils, neutrophils and mast cells) and the adaptive (T_H2 T cells) immune system, in concert with structural airway cells. Chemokines are important for the recruitment of both immune and structural cells to the lung, and also for their microlocalisation within the lung tissue. Specific blockade of the responses elicited by chemokines and chemokine receptors responsible for the pathological migration of airway cells could therefore be of great therapeutic interest for the treatment of asthma.

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Abbreviations: ASM, airway smooth muscle; BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; FEV₁, forced expiratory volume in 1 second; HLMC, human lung mast cell; IL, interleukin; NKT cell, natural killer T cell; T_H1 , T helper type 1 cell; T_H2 cell, T helper type 2 cell; TGF- β , transforming growth factor- β

Introduction

The prevalence of asthma is increasing worldwide, particularly in young children, and is a significant cause of morbidity and mortality in all age groups (Braman, 2006). The word ‘asthma’ originates from the Greek word ‘aazein’, meaning panting and was clinically described as early as 500 BC in the Corpus Hippocraticum. However, asthma was not considered an inflammatory condition until the early twentieth century (Huber and Koessler, 1922; Becker, 1999).

Asthma pathophysiology

Asthma is a complex immunological and inflammatory disease characterized by the presence of airway inflammation, airway wall remodelling and bronchial hyperresponsiveness (BHR). Exactly how these three key features interact and whether they are dependent on each other for their expression remains unknown. The inflammatory response characteristically comprises activated T helper type 2 (T_H2) lymphocytes, eosinophils and activated mast cells, features that are remarkably consistent among cases with apparently different etiology for example, atopic asthma versus non-atopic asthma (Humbert *et al.*, 1996a, b; Ying *et al.*, 1997a).

Although BHR appears to be exacerbated by the presence of airway inflammation, it remains once the inflammation is controlled, and is remarkably absent in eosinophilic bronchitis (Brightling *et al.*, 1999). In addition to airway inflammation, a number of structural wall changes occur in asthma, generally described as ‘airway remodelling’. Features of airway remodelling include epithelial disruption, sub-basement membrane thickening and increased extracellular matrix deposition, goblet cell hyperplasia, increased vascularity, and smooth muscle cell hypertrophy and hyperplasia (Boxall *et al.*, 2006).

There is continued debate about the most important cell type mediating the airway changes in asthma. However, analysis of the current evidence indicates that most if not all elements of the asthmatic airway are dysfunctional. There is clear evidence of epithelial dysfunction with failure of healing and overproduction of growth factors and proinflammatory cytokines (Holgate, 1998), glandular dysfunction with hyperplasia of mucus glands and mucus hypersecretion (Carroll *et al.*, 2002), airway smooth muscle (ASM) dysfunction with resulting hypertrophy, hyperplasia, hyperresponsiveness and cytokine secretion (Ebina *et al.*, 1990, 1993; Brightling *et al.*, 2005a), and inflammatory cell activation with ‘overactive’ mast cells (Bradding *et al.*, 2006), T cells (Robinson *et al.*, 1992), eosinophils (Bradding *et al.*, 1994) and neutrophils (Carroll *et al.*, 2002). The current cornerstone of asthma management is the use of inhaled steroids, which are highly efficacious in about 90% of

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patients because they inhibit many diverse pathological aspects of the dysfunctional airway (Barnes and Adcock, 2003). However, for about 10% of patients, these drugs are of poor efficacy for reasons which are not really understood. These patients are therefore difficult to treat, suffer great morbidity and use up a disproportionate fraction of healthcare resources (Wenzel, 2005). Novel potential treatments for the management of asthma, targeted at specific aspects of the inflammatory response, are emerging, but to date these have been disappointing. An example is the use of anti-interleukin (IL)-5, which specifically targets eosinophil infiltration in the airway. However, in spite of marked inhibition of eosinophilic inflammation, clinical asthma persists unabated (O'Byrne, 2006). There is therefore an

unmet clinical need for novel modulators of inflammation and tissue remodelling with different mechanisms of action and/or adverse-effect profiles from existing drugs. Modulators of chemokines and their receptors have the potential to fill this gap.

Chemokines and their receptors

Chemokines represent a large family of 8–15 kDa chemotactic proteins expressed by many immune and non-immune cells, with an essential role in inflammatory reactions (Table 1). Although overall sequence homology between them is poor, they have a similar three-dimensional

Table 1 Human chemokines and their receptors

<i>CC chemokines</i>	<i>Source</i>	<i>Receptor</i>	<i>Responding cell type</i>
CCL1 (I-309)	Mo, T, MC	CCR8	Mo, T
CCL2 (MCP-1)	Mo, L, Fib, End, Epi, Neut, MC, DC	CCR2	Mo, DC, T, Neut
CCL3 (MIP-1 α)	Mo, L, Neut, Eos, Fib, MC	CCR1, CCR5	Mo, DC, Eos, T, NK
CCL4 (MIP-1 β)	Mo, L, Neut, Eos, Fib, MC, Bas, NK	CCR5	Mo, DC, T
CCL5 (RANTES)	Mo, T, Fib, MC	CCR5, CCR1, CCR3	Mo, Eos, T, MC, NK, DC
CCL7 (MCP-3)	Mo, MC, Fib, End, Epi	CCR1, CCR2, CCR5	Mo, DC, Eos, T, MC, NK,
CCL8 (MCP-2)	Mo, Fib	CCR2, CCR3, CCR5	Mo, DC, Eos, T, NK
CCL11 (Eotaxin)	Epi, End, Eos, MC	CCR3, CCR5, CXCR3	Eos, T, MC
CCL13 (MCP-4)	Epi, DC	CCR1, CCR2, CCR3	Mo, Eos, T, MC, NK,
CCL14 (HCC-1)	BM, SMC, Gut, Spleen, Liver	CCR1, CCR5	Mo, DC, Eos, T, NK, MC
CCL15 (HCC-2)	Mo, DC, L, NK	CCR1, CCR3	T, Mo, Eos, MC
CCL16 (HCC-4)	Mo	CCR1, CCR2, CCR5	T, Mo, Eos, MC
CCL17 (TARC)	Mo, DC, Epi, SMC, Fib	CCR4	T, DC, NK
CCL18 (PARC)	Mo, DC	CCR3	T, MC, Eos
CCL19 (MIP-3 β)	Neut, LN, Spleen, Thymus, Gut, MC	CCR7	T, fibrocytes, DC
CCL20 (MIP-3 α)	Liver, Lung, Thymus, Placenta, Appendix, Epidermis, Mo, T, Neut, End	CCR6	DC, T
CCL21 (SLC)	End, LN	CCR7	DC, T, B, NK, Fibrocytes
CCL22 (MDC)	Mo, Epi, DC, B, T, NK	CCR4	T, CD, NK
CCL23 (MIP-1)	DC, Mo, Lung, Liver	CCR1	Mo, DC, Eos, T, NK
CCL24 (Eotaxin-2)	Mo, T, Lung, Liver, Spleen, Thymus	CCR3	Eos, T, MC
CCL25 (TECK)	DC, Epi, End, Gut	CCR9	T
CCL26 (Eotaxin-3)	End, Heart, Ovary	CCR3, CCR2	Eos, MC, T
CCL27 (CTACK)	Placenta, Skin	CCR10	T
CCL28 (MEC)	Epi, End	CCR10	T
<i>CXC chemokines</i>			
CXCL1 (GRO- α)	Mo, Neut, End, Fib, MC	CXCR2	Neut, Mo
CXCL2 (GRO- β)	Mo, Neut, End, Fib, MC	CXCR2	Neut, Mo
CXCL3 (GRO- γ)	Mo, Neut, End, Fib	CXCR2	Neut, Mo
CXCL4 (PF-4)	Platelets	CXCR3B	End
CXCL5 (ENA-78)	End, Eos, MC	CXCR2	Neut, Mo
CXCL6 (GCP-2)	Mo, End, Fib	CXCR1, CXCR2	Neut, Mo, T
CXCL7 (NAP-2)	End	CXCR2	Neut, Mo
CXCL8 (IL-8)	Mo, T, Fib, Epi, End, Neut, Bas, NK, MC	CXCR1, CXCR2	Neut, Mo, T
CXCL9 (MIG)	Mo, Neut	CXCR3, CCR3	T, MC
CXCL10 (IP-10)	Mo, Neut, Fib, End	CXCR3, CCR3	T, MC
CXCL11 (ITAC)	Mo, Neut	CXCR3, CXCR7 , CCR3	T, MC
CXCL12 (SDF-1)	End, Epi	CXCR4, CXCR7	T, Mo, MC, NK, Eos, DC
CXCL13 (BLC)	Mo, End, DC	CXCR5	T, B
CXCL14 (BRAX)	Mo, Fib, B	Not known	
CXCL16 (SR-PSOX)	Mo, DC, B	CXCR6	T, NK
<i>C chemokines</i>			
XCL1 (Lymphotactin)	T, NK, MC	XCR1	T, NK
XCL2 (SMC-1 β)	T, NK	XCR1	T, NK
<i>CXC chemokine</i>			
CX3CL1 (Fractalkine)	DC, End, T	CXC3CR1	Mo, Neut, NK, T

Bold indicates antagonistic binding of a chemokine receptor.

Abbreviations: B, B cell; Bas, basophil; DC, dendritic cell; End, endothelial cell; Epi: epithelial cell; Eos, eosinophil; Fib, fibroblast; LN, lymph node; L, lymphocyte; MC, mast cell; Mo, monocyte/macrophage; Neut, neutrophil; NK, natural killer cell; T, T cell.

structure. Chemokines are derived from three or four ancestral genes and are, based on the position of two conserved cysteine residues, categorized into four subfamilies: the main -CC- and -CXC- groups and the less described -C- and -CX3C- groups (Zlotnik and Yoshie, 2000). Chemokines can also be classified according to function into the constitutive and the inducible chemokines. The constitutive group includes chemokines involved in the homeostatic trafficking, whereas the inducible chemokines are upregulated during inflammation. This is mediated mainly by cytokines produced at the site of inflammation, but also by endogenous molecules upregulated during infection and injury, for example, fibrinogen, elastase and defensins, which induce chemokine production through pattern recognition receptors (Smiley *et al.*, 2001; Devaney *et al.*, 2003).

Most chemokines are secreted proteins, but there are two important exceptions: CX3CL1 (fractalkine) and CXCL16. These chemokines are expressed in a membrane-bound form at the end of a cleavable mucin stalk, which allows for dual functions. In the membrane-bound form, these chemokines function as leukocyte adhesion molecules, and when the chemokine is cleaved from its stalk by metalloproteases, they then function as soluble chemoattractants (Bazan *et al.*, 1997; Matloubian *et al.*, 2000).

To date, almost 50 chemokines have been identified, which exert their effects through over 20 distinct G protein-coupled seven-transmembrane receptors. Chemokine receptors fall into the subfamily of class A rhodopsin-like receptors and take up almost 5% of the human genome (Murphy, 1994). Chemokines bind with nanomolar affinity and most chemokine receptors can bind multiple ligands, for example, CCR3 can bind CCL5, CCL7, CCL8, CCL13, CCL11, CCL24 and CCL26. Many chemokines are capable of binding to more than one receptor, such as CCL5 that binds to CCR1, CCR3 and CCR5. A few chemokines and chemokine receptors demonstrate exclusive binding, such as CCL25/CCR9 and CXCL12/CXCR4 (Zimmermann *et al.*, 2003). Recent reports show that chemokines can also act as antagonists for chemokine receptors as well as agonists. For example, CXCL10 is a natural antagonist of CCR3 (Loetscher and Clark-Lewis, 2001). In addition, Duffy antigen/receptor for chemokines (DARC) and D6 bind chemokines with high affinity without inducing any downstream signalling (Benecci *et al.*, 2004). Instead these membrane-bound proteins function as decoy receptors, which trigger internalization or degradation of chemokines. The function of certain chemokine receptors (CCR2, CCR5 and CXCR4) can further be modulated by homo- and/or heterodimerization of receptors, a phenomenon that has effects on the binding of ligands and the types and magnitude of the intracellular signalling pathway (Mellado *et al.*, 2001; Moriguchi *et al.*, 2005; Springael *et al.*, 2005).

Upon ligation of the chemokine to the extracellular portion of the chemokine receptor, the intracellular domain binds and activates the heterotrimeric G protein. In response, the $G\alpha_i$ subunit exchanges GDP for GTP, resulting in the dissociation of the heterotrimeric complex into the $G\alpha_i$ - and the $\beta\gamma$ -subunits. Downstream signalling triggers integrin activation, which enables firm adhesion of leuko-

cytes to endothelial cells. Focal actin polymerization of the leading edge of the cell leads to forward extension. At the rear end of the cell, focal activation of myosin II and formation and contraction of myosin-actin complexes retracts the cell, allowing migration in the direction of the chemotactic gradient (Rot and von Andrian, 2004).

In the multistep model of leukocyte extravasation, chemokines are important not only in the activation of integrins, leading to firm adhesion and subsequent diapedesis, but also in directing leukocytes into different compartments within the tissue. The lung is a unique organ in that it is supplied with blood from both the pulmonary and the systemic circulations that deliver blood to the parenchyma and the airways respectively. Due to the low pressure of the pulmonary system, leukocyte rolling and firm adherence are not required for extravasation, and it is suggested that a weak chemotactic signal alone can mediate migration into the underlying tissue (Doerschuk *et al.*, 2000).

T cells

As an integral part of the adaptive immune system, the T cell is often considered to play a crucial role in maintaining and regulating the allergic immune response in asthmatics. Depletion of T cells in some mouse models is associated with an inability to induce an 'asthmatic' response. In humans, $CD4^+$ T cells producing IL-4, IL-5 and IL-13 (putative T_H2 cells) have been identified in bronchoalveolar lavage (BAL) and bronchial biopsies of both atopic and non-atopic asthmatic patients (Robinson *et al.*, 1992; Brightling *et al.*, 2002b). Lung T cells are mainly memory cells and are found principally in two compartments: the bronchial lamina propria and epithelium, and the peripheral alveolar and interstitial regions of the lung (Wardlaw *et al.*, 2005).

Naive T cells leave the thymus expressing CCR7, essential for entering the high endothelial venules of lymph nodes, where the ligands CCL21 and CCL19 are produced. Naive T cells also express CXCR4, but the significance of this expression is unknown and CXCR4/CXCL12 are not necessary for entry into the lymph nodes (Weninger and von Andrian, 2003). Upon antigen presentation and activation, $CD8^+$ T cells proliferate and differentiate into cytotoxic T cells, and $CD4^+$ cells into either T helper type 1 (T_H1) or T_H2 effector cells, depending on the pathogenic insult. As a part of the differentiation into effector T cells, there is a drastic change in the chemokine receptor profile of the T cells, allowing them to migrate into the tertiary lymphoid tissues. It is thought that this migration is organ specific and that chemokines are important for providing a 'homing signal' for the lymphocytes expressing the correct chemokine receptor. The chemokines involved in lymphocyte migration to the skin and gut are well defined (Morales *et al.*, 1999; Kunkel *et al.*, 2000), but it is not known whether there is specific T-cell homing to the lung. If this is the case, the T cells recovered from lung would be expected to express adhesion molecules and chemokine receptors different than those found in other tissues. Indeed, lung-derived T cells from both BAL and peripheral lung tissue express a pattern of chemokine receptors distinct from the small intestine and

skin (CCR9⁺, CCR10⁺ and CCR4^{LO}), suggesting that there is a lung-specific pathway (Campbell *et al.*, 2001). When comparing chemokine receptor expression in T cells from blood and lung, three chemokine receptors were upregulated on the lung-derived cells: CXCR3 and CCR5, which appear to be highly expressed on all tissue T cells, and CXCR6, which is greatly enriched in BAL T cells compared with blood, especially in patients with sarcoidosis or interstitial lung disease (Morgan *et al.*, 2005a). High concentrations of the only ligand for CXCR6, CXCL16, is also found in BAL and is expressed in high amounts by alveolar macrophages, suggesting CXCR6 is a candidate lung homing receptor. However, there was no difference in the level of CXCR6 expression or CXCL16 production between normal subjects, patients with asthma, sarcoidosis or interstitial lung disease, suggesting that CXCR6 plays a role in the homeostatic trafficking of T cells to the lung rather than in the recruitment of T cells to the airways of asthmatics (Morgan *et al.*, 2005a). Interestingly, although ASM expresses CXCL10, and CXCR3 is highly expressed on lung T cells, there is no infiltration of CD3⁺ cells in the smooth muscle bundle of asthmatic airways (Brightling *et al.*, 2002a, 2005a).

In addition to tissue-specific migration, an inflammation-specific model has been proposed, which involves differential trafficking of T_H1 and T_H2 cells. Using either IL-12 or IL-4 to polarize naive T cells *in vitro* to a T_H1 or T_H2 phenotype, respectively, a distinct chemokine receptor expression pattern was demonstrated: the T_H1 cells expressed CCR5 and CXCR3, whereas the T_H2 cells expressed CCR3, CCR4 and CCR8 (Kim *et al.*, 2001). CCR4-deficient mice develop less airway eosinophilia and BHR following antigen challenge (Schuh *et al.*, 2002), but CCR8-deficient mice remain unaffected (Chung *et al.*, 2003; Goya *et al.*, 2003).

The physiological significance of CCR4 and CCR8 in humans is less certain and the studies investigating the chemokine receptor expression on T_H2 and T_H1 cells from human lung are somewhat contradictory. Using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR), Panina-Bordignon *et al.* (2001) reported that over 90% of T cells infiltrating the bronchial biopsies of allergen-challenged asthmatics produce IL-4 and express CCR4. They also reported that CCR8 was expressed on approximately 28% of infiltrating CCR4⁺ IL-4⁺ T cells, whereas no T cells were CCR3⁺. They also found that the two CCR4 ligands, CCL22 and CCL17, were expressed by airway epithelial cells and their expression was strongly upregulated after allergen challenge (Panina-Bordignon *et al.*, 2001). In contrast, when analyzing the relationship between cytokine production and chemokine receptor expression on freshly isolated blood T cells using single-cell flow cytometry, our group found that CCR3 and CCR4 are preferentially expressed by IL-4-secreting T cells in the lung in both healthy subjects and patients with mild asthma (CCR8 not studied) (Morgan *et al.*, 2005b). However, most lung T lymphocytes produced neither IL-4 nor interferon- γ (IFN- γ) after *in vitro* stimulation, and the ligands of CCR3 and CCR4 were not found in high amounts in BAL fluid. In addition, there was no polarization of the CXCR3⁺ CCR5⁺ T cells in terms of cytokine expression (Morgan *et al.*, 2005b). Together, the results from these studies suggest that the

distinct patterns of polarization seen in blood T cells do not apply in the physiological environment of the lung, and further work is needed to elucidate the mechanism by which T_H2 cells are recruited to the asthmatic lung.

Invariant natural killer T cells

Invariant natural killer T cells (NKTs) are a subpopulation of T cells that express a conserved canonical T-cell receptor that recognizes the glycolipid α -galactosylceramide. In response to stimuli, NKT cells respond very rapidly by expressing cytokines and have the capacity to produce both IL-4 and IFN- γ . In some animal models, pulmonary NKT cells regulate development of airway eosinophilia, BHR and IgE production (Lisbonne *et al.*, 2003). The role for NKT cells in human asthma has been debated. Akbari *et al.* (2006) claimed that 60% of all CD4⁺ lung T cells in asthmatics were NKT cells and that these cells were absent in the lungs of normal subjects and patients with sarcoidosis (Akbari *et al.*, 2006). However, these results have been severely criticised by several groups (Pham-Thi *et al.*, 2006; Thomas *et al.*, 2006), who suggest that the prevalence of these cells is nearer 1% of lung T cells. The extent to which NKT cells are involved in the pathogenesis of asthma is therefore unclear. Another study analyzing sputum from severe asthmatics found that NKT cells were increased as compared with normal controls and patients with mild asthma (Hamzaoui *et al.*, 2006). Most peripheral blood NKT cells express receptors for chemokines, which mediate homing to extra-lymphoid tissue or sites of inflammation (CCR2, CCR1, CCR5, CCR6, CXCR3, CXCR4 and CXCR6), whereas few NKT cells express lymphoid tissue-homing chemokine receptors (CCR7 and CXCR5) (Kim *et al.*, 2002; Motsinger *et al.*, 2002). Sen *et al.* (2005) found that there were significantly more NKT cells in peripheral blood of patients with asthma compared with normal subjects. Interestingly, NKT cells from asthmatics drove T-cell expression of IL-4 and IL-13, whereas NKT cells derived from healthy donors drove IFN- γ expression. Whereas CXCR3 and CCR6 were expressed at equal levels by NKT cells from normal and asthmatic individuals, CCR9 was upregulated by the asthmatic NKT cells, which migrated to CCL25 *in vitro*. Furthermore, immunohistochemistry and RT-PCR of endo-bronchial biopsies identified more CCR9⁺ NKT cells in the submucosa of the asthmatic than in the normal subjects (Sen *et al.*, 2005). CCR9 is widely recognized as a gut homing receptor, which is expressed on intraepithelial and lamina propria intestinal lymphocytes, and the authors suggest a role for CCR9 in NKT migration to the lung in asthma.

Dendritic cells

Dendritic cells play a potentially important role in asthma through their ability to process and subsequently present antigen to T cells. They form an intricate network in the airway epithelium and lamina propria of the airways, where they exist in an immature state capable of internalizing foreign antigens but unable to activate naive T cells. Once antigen has been captured, airway myeloid dendritic cells

mature and migrate to the regional lymph nodes, where they present processed antigen to T cells, preferentially inducing T_H2 responses. In mice, this process of migration to the lymph node is dependent on the activation of CCR7. Thus, on repeated exposure to aerosolized house dust mite, severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood mononuclear cells and pulsed with dendritic cells from allergic patients developed airway eosinophilia, an increase in IL-4 and IL-5 production in the lung lavage fluid and increased IgE production compared with that in mice primed with unpulsed dendritic cells. All these effects were reduced following *in vivo* neutralization of the CCR7 ligand CCL21 (Hammad *et al.*, 2002). Furthermore, the indirect upregulation of CCR7 in mice lacking the transcription factor Runx3 is associated with enhanced migration of alveolar dendritic cells to the mediastinal lymph nodes, and the development of elevated serum IgE, and BHR. These features are blocked by anti-CCR7 antibodies (Fainaru *et al.*, 2005). Interestingly, in *plt* mice, which lack the ability to generate both CCL19 and CCL21, there is attenuation of airway eosinophilia and BHR in the short term after ovalbumin challenge, and also failure for the inflammation to resolve in the longer term (Yamashita *et al.*, 2006). Targeting CCR7 therefore has the potential to do more harm than good.

Eosinophils

Airway eosinophilia is a common feature of asthma. About 80% of asthmatic subjects have an increased number of eosinophils in BAL, endobronchial biopsies and induced sputum compared with normal controls. Sputum eosinophilia correlates with asthma severity and treatment directed at normalization of the sputum eosinophil count reduces asthma exacerbations and admissions without the need for additional anti-inflammatory treatment (Green *et al.*, 2002a). Eosinophils are end-stage granulocytes that derive from the bone marrow under the influence of IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rothenberg, 1998). Under baseline conditions, they leave the bone marrow and migrate to the gastrointestinal tract, where they reside within the lamina propria (Mishra *et al.*, 1999). In asthma and other inflammatory conditions, however, eosinophils migrate to extraintestinal tissues such as the lung, where they have been thought to affect the airways in a number of ways (Wardlaw, 1999). *In vitro*, toxic granule proteins such as major basic protein, eosinophilic cationic protein and eosinophil peroxidases have a direct cytotoxic effect on the respiratory epithelium, enhance ASM responsiveness and trigger mast cell degranulation (Gundel *et al.*, 1991; Piliponsky *et al.*, 2001). Eosinophils also secrete a number of proinflammatory interleukins, including IL-2, IL-4, IL-5, IL-10, IL-12 and IL-13, which enhance the T_H2 response, and the profibrotic cytokine transforming growth factor- β (TGF- β), which is involved in airway remodelling (Martin *et al.*, 1996; Ohno *et al.*, 1996).

IL-5 is a key cytokine for the growth, activation and survival of eosinophils (Sanderson, 1992; Dewson *et al.*, 2001), and together with IL-4 and IL-13, regulates the

trafficking of these cells into sites of inflammation (Sher *et al.*, 1990; Horie *et al.*, 1997). IL-4 and IL-13 control eosinophil trafficking directly by upregulating adhesion molecules on endothelial cells, particularly vascular cell adhesion molecule 1 (Schleimer *et al.*, 1992; Ying *et al.*, 1997b) or by inducing chemokine expression by airway epithelial cells (Wardlaw, 2001). However, as discussed above, the ability of anti-IL-5 to markedly deplete eosinophils but have no effect on asthma symptoms or experimental allergen challenge means there is doubt as to their importance in asthma pathophysiology.

Several studies have shown the role of the CCR3 binding chemokines in eosinophil migration to the lung, particularly CCL5 and the eotaxins CCL11, CCL24 and CCL26. CCR3 is the chemokine receptor, which is most highly expressed by eosinophils (Ponath *et al.*, 1996), and CCR3 expression is relatively abundant on these cells. CCL11, CCL5 and CCR3 mRNA and protein have been detected in bronchial biopsies and BAL from asthmatic patients, and the number of CCR3 mRNA-positive cells in the bronchial mucosa correlated with airway eosinophilia and BHR (Ying *et al.*, 1997c). CCL26, which is upregulated in bronchial epithelial cells after stimulation with IL-4, is important for eosinophil transepithelial migration *in vitro* (Cuvelier and Patel, 2001; Yuan *et al.*, 2006). Various mouse models have been used to further elucidate the specific role of CCR3 in eosinophil migration and airway physiology. Results of two studies using CCR3 knockout mice demonstrated reduced eosinophil recruitment, but were contradictory with respect to the development of BHR and seemed to depend on the allergen sensitization protocol. Humbles *et al.* (2002), who used a systemic ovalbumin (OVA)/alum sensitization followed by respiratory OVA challenge to mimic the asthmatic response, found a reduction in lung eosinophils in the CCR3^{-/-} mice. The eosinophils appeared to be able to migrate through the endothelial cells, but not the endothelial basement membrane, suggesting that in this model, CCR3 binding chemokines are not essential for rolling, adhesion and transmigration through endothelium, but important for migration into the lung parenchyma (Humbles *et al.*, 2002). Perhaps surprisingly, this reduction in eosinophil recruitment was accompanied by a marked increase in BHR. In contrast, Ma *et al.* (2002), using epicutaneous OVA sensitization, were able to significantly reduce BAL eosinophils, with a parallel decrease in BHR (Ma *et al.*, 2002). A proportion of human donors have CCR1⁺ eosinophils and thus respond to CCL3. CCL3 has also been detected in the lungs of asthmatics (Sabroe *et al.*, 1999; Phillips *et al.*, 2003), and an *in vivo* blockade of murine CCL3 using monoclonal antibodies resulted in reduced airway eosinophilia and BHR in the early allergic response (Campbell *et al.*, 1998).

Lukacs (2001) proposed a mechanism for murine eosinophil migration in response to chemokines, where migration from blood into the lung tissue is regulated by CCL3, CCL7 and CCL22, produced by macrophages in the lung interstitium (Lukacs, 2001). Once in the lung, a gradient of CCL5 and CCL11 is responsible for guiding the eosinophils to the airway epithelium. CCL11 is also an important eosinophil degranulator (Fujisawa *et al.*, 2000) and it would be logical if eosinophils did not degranulate before having reached their

final destination. This mechanism of recruitment is supported by data from another mouse model demonstrating that initial eosinophil localization was dependent on CCL3, whereas secondary recruitment and degranulation was CCL11 dependent and CCL3 independent (Campbell *et al.*, 1998). The relevance of these observations in human asthma needs to be further clarified.

The role for CCR6 in human eosinophil recruitment is uncertain. Sullivan *et al.* (1999), using RT-PCR and polyclonal anti-human CCR6 serum to detect receptor expression, declare that 20% of blood eosinophils from asthmatic patients express low levels of CCR6, and that these eosinophils respond to CCL20 (Sullivan *et al.*, 1999). Other more recent studies using flow cytometry and monoclonal antibodies found CCR6 to be undetectable in BAL and blood (Liu *et al.*, 2003).

Neutrophils

Several clinical studies have observed a correlation between airway neutrophilia and asthma severity, as measured by forced expiratory volume in 1 second (FEV1), and some investigators have suggested that a predominantly neutrophilic recruitment pattern may be a feature of a subgroup of severe asthmatics (Sur *et al.*, 1993; Fahy *et al.*, 1995; Pavord *et al.*, 1999). There are several reasons why neutrophils could be important in severe asthma: activated neutrophils release tissue damaging enzymes such as neutrophil elastase, reactive oxygen compounds, cytokines and lipid mediators. Neutrophil elastase acts as a secretagogue for goblets cells and may thus contribute to the prominent mucus hypersecretion in asthma. The importance of neutrophils in severe asthma is further evidenced by postmortem investigations of patients with sudden-onset fatal asthma, which demonstrated absence of eosinophils and relatively more neutrophils in the airway submucosa (Sur *et al.*, 1993) and airway mucosal glands (Carroll *et al.*, 2002).

Sputum neutrophilia combined with normal numbers of eosinophils is a characteristic of non-atopic asthmatics, who have an impaired response to steroid treatment (Green *et al.*, 2002b). This supports the idea already proposed by Wenzel *et al.* (1999) that neutrophilic asthma represents a distinct inflammatory phenotype. By thoroughly characterizing severe asthmatics, the authors distinguished between the non-eosinophilic and the eosinophilic severe asthmatics, which both had high levels of neutrophils in their airways. The non-eosinophilic airways exhibited a lower degree of sub-basement membrane thickening, less infiltration of mast cells, lymphocytes and macrophages (Wenzel *et al.*, 1999).

Neutrophils express two main chemokine receptors: CXCR1 and CXCR2, and it is thought that CXCR2 is the major receptor involved in recruitment (White *et al.*, 1998), whereas CXCR1 is important in neutrophil activation (Chuntharapai and Kim, 1995; Sabroe *et al.*, 1997). CXCL8, which is known to be expressed by activated airway epithelium, fibroblasts, macrophages, mast cells and by the neutrophils themselves, binds both receptors and is a potent chemoattractant for neutrophils. When analyzing the tracheal aspirates of acute severe asthmatics, Ordonez *et al.*

(2000) detected high amounts of both neutrophils and CXCL8, and that the levels of CXCL8 correlated significantly and positively with the length of the acute asthma exacerbation (Ordonez *et al.*, 2000). Gibson *et al.* (2001) confirmed these results when studying sputum from persistent asthmatics of all severities, where they found that levels of CXCL8 correlated with neutrophil numbers and was upregulated in non-eosinophilic asthmatics (Gibson *et al.*, 2001).

Recently, different antagonists of CXCR2 have been developed to prevent neutrophil accumulation; in particular, a selective non-peptide antagonist of CXCR2 has been shown to exhibit significant anti-inflammatory effects in acute and chronic models of arthritis in rabbits (Podolin *et al.*, 2002). However, in a recent study, Pignatti *et al.* (2005) show a downregulation of CXCR1 and CXCR2 in airway neutrophils, suggesting that activation of the neutrophils with CXCL8 in the airways would downregulate the receptors once the cells were recruited to the lung (Pignatti *et al.*, 2005). This phenomenon might render antagonistic therapies aimed at CXCL8 receptors redundant. CXCL8 expression is regulated by various cytokines such as tumor necrosis factor- α (TNF- α) and the relatively recently discovered IL-17, expressed by CD4⁺ cells. Stimulation of a human bronchial epithelial cell line with IL-17 upregulated expression of CXCL8, and a recent study found that sputum IL-17 was associated with neutrophilia in severe asthma (Sun *et al.*, 2005).

Mast cells

Mast cells play a significant role in the pathophysiology of asthma through their ability to release a plethora of autacoid mediators, proteases and cytokines in response to their activation by both allergen and other non-immunological stimuli (Bradding *et al.*, 2006). Importantly, mast cells are present in a chronically 'activated' secretory state within the asthmatic bronchial mucosa, with evidence of ongoing mediator release and cytokine synthesis (Wenzel *et al.*, 1988; Broide *et al.*, 1991; Bradding *et al.*, 1994; Ying *et al.*, 1997a). Mast cells present in the BAL fluid of asthmatic subjects exhibit increased spontaneous and IgE-dependent mediator release, and strong correlations have been observed between the severity of BHR and mast cell numbers, histamine concentrations and constitutive histamine release in BAL fluid (Flint *et al.*, 1985; Casale *et al.*, 1987; Wardlaw *et al.*, 1988). Mast cells are resident in normal airways, and their number in the lamina propria is similar in both normal subjects and patients with asthma. However, in asthma, mast cells infiltrate three key structures: ASM (Figure 1), the airway submucosal glands and the airway epithelium (Bradding *et al.*, 1994; Carroll *et al.*, 2002; Brightling *et al.*, 2002a). Since mast cells in the asthmatic airway are activated and their mediators have profound effects on these structural elements, this redistribution of mast cells is likely to be of great importance for the development and propagation of this disease. This concept is supported by the observation that the number and activation state of mast cells in the submucosal glands correlate strongly with the extent of mucus plugging, whereas the number of mast cells in the



Figure 1 Bronchial biopsy specimen from a patient with asthma showing mast cell infiltration of airway smooth muscle.

ASM correlates with the severity of BHR. Furthermore, in eosinophilic bronchitis, the mucosal immunopathology is virtually identical when compared with asthma, in terms of cellular infiltrates and T_H2 cytokine expression, but mast cells are not found in the ASM in eosinophilic bronchitis (Brightling *et al.*, 2002a). This further suggests that the mast cell myositis in asthma is a key requirement for the expression of BHR and variable airflow obstruction.

Human lungs mast cells (HLMC) express numerous chemokine receptors. In particular, CCR3, CXCR1, CXCR3 and CXCR4 are highly expressed by *ex vivo* HLMC, and the respective ligands for these receptors CCL11, CXCL8, CXCL10 and CXCL12 mediate lung mast cell chemotaxis (Brightling *et al.*, 2005a, b). It is not clear whether the mast cell infiltration of ASM in asthma involves the recruitment of progenitors, which then differentiate locally, or the local migration of resident differentiated cells. However, it is well recognized that mature differentiated cells have the ability to migrate within the airway wall. Interestingly, we found that both CXCR3 and CCR3 expression were increased on *ex vivo* HLMC compared with human bone marrow-derived mast cells, suggesting that they may be important for the migration of mature differentiated cells (Brightling *et al.*, 2005b). In bronchial biopsies from patients with asthma, approximately 50% of mast cells expressed CXCR3 compared with nearly 100% of mast cells within the asthmatic ASM. In contrast, CCR3 was almost absent on ASM mast cells (Brightling *et al.*, 2005b). We also found that there was increased expression of the CXCR3 ligand CXCL10 in asthmatic ASM compared with that from normal subjects in both biopsies and when activated *in vitro* (Brightling *et al.*, 2005a). Taken together, these observations suggest that CXCR3 and CXCL10 play a crucial role in determining the distribution of mast cells within the lung, especially migration to the ASM in asthma.

These experiments also showed that CXCR1 and CXCR4 contribute to HLMC chemotaxis induced by asthmatic ASM-conditioned media, but CCR3 did not in spite of there being high concentrations of its ligand CCL11 present (Brightling *et al.*, 2005a). This may be because CXCL10 is a natural

antagonist of CCR3 (Loetscher and Clark-Lewis, 2001). However, when asthmatic ASM is activated by T_H2 cytokines, CCL11 contributes to the HLMC migration, which occurs in response to ASM-conditioned medium. There also seems to be an added level of complexity in that normal ASM appears to secrete an inhibitor of HLMC migration (Sutcliffe *et al.*, 2006). Romagnani *et al.* (1999) suggested that CCR3 was involved in the homing of tryptase–chymase double positive cells (MC_{TC}), which they found to be the predominant mast cell phenotype expressing this receptor (Romagnani *et al.*, 1999). They also showed that CCL11 and CCL5 mediated mast cell migration through CCR3, thereby suggesting that the CCR3/CCL11/CCL5 pathway is important in mast cell migration in connective tissues (Romagnani *et al.*, 1999). However, MC_{TC} mast cells within ASM rarely express this receptor (Brightling *et al.*, 2005a). Furthermore, Humbles *et al.* (2002) surprisingly found increased baseline numbers of submucosal mast cells in the trachea and large bronchi of CCR3 knockout mice. Furthermore, following antigen challenge, intraepithelial mast cell numbers increased significantly more in the CCR3-deficient mice compared with normal littermates (Humbles *et al.*, 2002). The mechanism behind this is unclear but indicate a multifaceted and poorly understood role for CCR3 in mast cell migration in the airway.

Juremalm *et al.* (2000) showed that CCL5, a recognized agonist for CCR1, also appeared to induce cord blood mast cell chemotaxis through CCR4, whereas the recognized ligands CCL17 and CCL22 did not (Juremalm *et al.*, 2000). They also demonstrated that both CCL17 and CCL22 significantly inhibited the migration toward CCL5 by 54 and 74%, respectively. In contrast, CCL2, CCL3, CXCL10 and CXCL12 did not affect CCL5-induced migration. The authors concluded that CCL17 and CCL22 act as antagonists for CCL5-mediated migration induced through CCR4. The role this has in determining the specificity of the mast cell migratory response in asthmatic airways requires further work, particularly as expression of CCR4 on HLMC is relatively low.

Structural lung cells

As described above, structural lung cells such as airway epithelium, smooth muscle and fibroblasts are important sources of chemokines, and contribute to the recruitment of leukocytes both to and within the lung. However, structural cells also express certain chemokine receptors and can therefore be activated by chemokines produced by inflammatory cells infiltrating the lung, or in an autocrine manner, which may potentially contribute to airway remodelling.

One of the main features of airway remodelling is increased ASM mass (Kay, 1996). Mathematical models have suggested that an increase in ASM volume is enough to induce BHR through local mechanical effects alone (Macklem, 1996). ASM mass correlates with asthma severity and is thought to be the result of hypertrophy and/or hyperplasia (Ebina *et al.*, 1993; Benayoun *et al.*, 2003; Woodruff *et al.*, 2004). In an *ex vivo* study of asthmatic ASM, increased proliferation has been suggested to be the main cause of

hyperplasia in asthmatics (Johnson *et al.*, 2001), but several studies have failed to show that this is the case *in vivo* (Benayoun *et al.*, 2003; Woodruff *et al.*, 2004). Another explanation is that smooth muscle progenitors, either located within the airway or recruited from peripheral blood, such as CD34⁺ collagen I⁺ fibrocytes, are recruited to the ASM and differentiate into ASM. Chemokines might therefore be involved in this migration. This theory is supported by a recent study from our group demonstrating the functional expression of CCR7 on ASM and fibroblasts from both asthmatic and normal donors (Kaur *et al.*, 2006). In the bronchial mucosa, one of the ligands for CCR7, CCL19, was expressed by blood vessels, but it was also expressed by mast cells within the asthmatic ASM bundles, and in severe asthma by the ASM itself. *In vitro*, CCL19 expression by ASM was increased in response to injury as shown by a wound healing assay. Furthermore, peripheral blood-derived fibrocytes have been shown to express CCR3, CCR5, CCR7 and CXCR4, but only CCR7 and CXCR4 have been proven to be functional. It is therefore plausible that these cells are recruited to the airway through CCL19⁺ blood vessels in the lung and then recruited to the ASM bundle by mast cell- and ASM-derived CCL19. Coupled with the observation that the number of mast cells in the ASM bundle is increased in asthma, these results focus attention on a potential mechanism by which mast cell-derived CCL19 binds to and activates CCR7⁺ ASM, myofibroblasts and/or fibroblasts, and thereby contributes to the increased ASM mass in asthmatic airways (Kaur *et al.*, 2006).

Another characteristic of airway remodelling is subepithelial collagen deposition, which is a result of deposition of collagen I, III and V, fibronectin and tenascin in the lamina

reticularis (Roche *et al.*, 1989). The cells responsible for producing these proteins are thought to be myofibroblasts positioned directly beneath the airway epithelium (Brewster *et al.*, 1990). After *in vitro* stimulation with the fibrogenic cytokines endothelin-1 (ET-1) and TGF- β 1, which are produced in the airways of asthmatic patients, peripheral blood fibrocytes differentiate into myofibroblast like cells, suggesting that fibrocytes are potential myofibroblast precursors (Schmidt *et al.*, 2003). Fibrocytes located underneath the epithelial layer were also found in biopsies from asthmatic patients. The recruitment of fibrocytes to the epithelium is therefore important for the development of airway remodelling in asthma. In addition to CCR7, CXCR4 is functionally expressed by fibrocytes and its ligand, CXCL12, is produced by bronchial epithelium, and has been proven to attract human peripheral blood fibrocytes (Phillips *et al.*, 2004; Kaur *et al.*, 2006). Taken together, this suggests that CXCL12 is involved in the migration of fibrocytes toward the epithelium, thus contributing to the increase in subepithelial and luminal myofibroblasts observed in asthma.

Future perspectives

The discovery of chemokines as important regulators of the immune system has led to intense research of over the last 20 years. Since chemokines are involved in the abnormal migration patterns of leukocytes and structural cells within the lung, they make attractive drug targets. However, designing novel pharmacological agents targeting chemokines and their receptors as treatments for asthma raises a

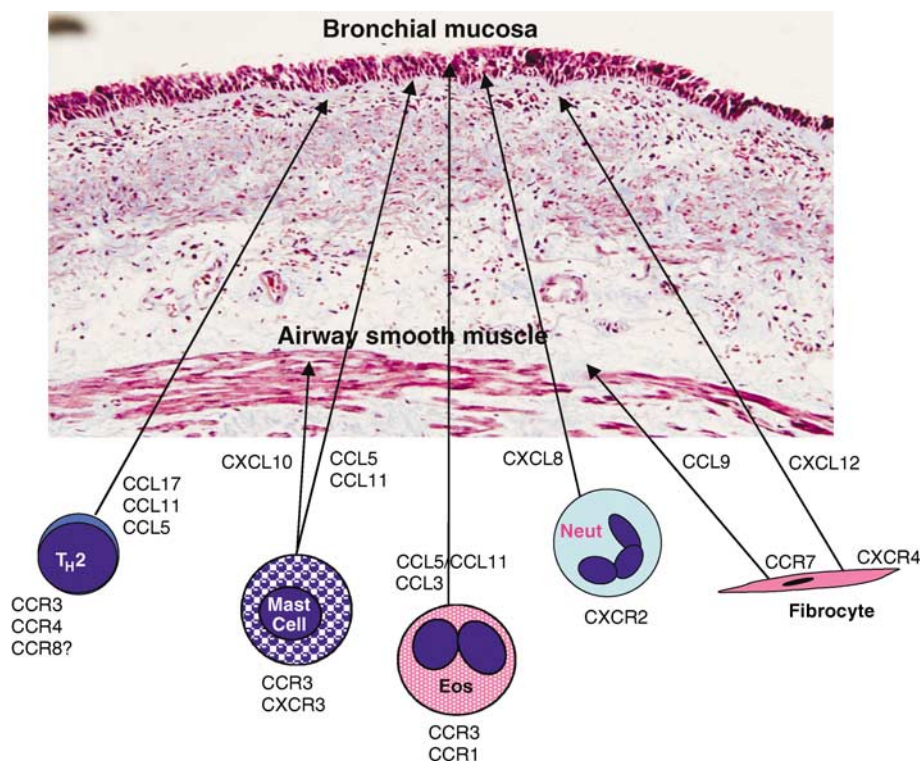


Figure 2 Chemokines and chemokine receptors with potential as therapeutic targets for the treatment of asthma.

number of questions. Firstly, inhibition of which chemokines/chemokine receptors would be most effective in order to obtain a significant reduction in asthma symptoms? Secondly, how could selective inhibition be achieved? The situation is complicated by the fact that numerous chemokines are upregulated in airway inflammation and that chemokines bind several receptors leading to marked redundancy. Inhibition of chemokine receptor activity can be achieved either by using modified chemokines or small-molecule chemokine receptor antagonists, which function as antagonists, or by using neutralizing antibodies against the receptor directly. CCR3 is a potential target, since this chemokine receptor has proven to be involved in migration of eosinophils, certain T_H2 cells and possibly airway mast cells. A recent mouse study used two types of small-molecule antagonists to block CCR3, which significantly and selectively reduced eosinophil infiltration to the lung of mice (Das *et al.*, 2006). The CXCL10/CXCR3 axis also looks interesting as it appears to target mast cells to the ASM in asthma. Very few chemokine receptor antagonists have been developed for humans to date, but there is great interest from the pharmaceutical industry in this area, and further antagonists are likely to emerge in the future. A schematic summary of chemokines potentially involved in distribution of cells in the asthmatic airway is depicted in Figure 2. Lastly, bearing in mind the obvious redundancy in the chemokine network, more general approaches aimed at inhibiting cell migration in asthma might ultimately prove more fruitful. For example, HLMC express the K⁺ channel K_{Ca}3.1 (Duffy *et al.*, 2001, 2004) and blockade of this inhibits HLMC migration to a number of diverse chemoattractants (Cruse *et al.*, 2006).

Conflict of interest

The authors state no conflict of interest.

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