

Review

Eotaxin and the attraction of eosinophils to the asthmatic lung

Dolores M Conroy and Timothy J Williams

Leukocyte Biology Section, Biomedical Sciences Division, Imperial College School of Medicine, London, UK

Correspondence: Dolores M Conroy, Leukocyte Biology Section, Biomedical Sciences Division, Sir Alexander Fleming Building, Imperial College School of Medicine, London SW7 2AZ, UK. Tel: +44 20 7594 3118; fax: +44 20 7594 3119; e-mail: d.conroy@ic.ac.uk

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Abstract

Eosinophilic leukocytes accumulate in high numbers in the lungs of asthmatic patients, and are believed to be important in the pathogenesis of asthma. A potent eosinophil chemoattractant is produced in the asthmatic lung. This small protein, the chemokine eotaxin, is synthesized by a number of different cell types, and is stimulated by interleukin-4 and interleukin-13, which are produced by T-helper (Th)2 lymphocytes. Low molecular weight compounds have been developed that can block the eotaxin receptor C-C chemokine receptor (CCR)3, and prevent stimulation by eotaxin. This provides the potential for orally available drugs that can prevent eosinophil recruitment into the lung and the associated damage and dysfunction.

Keywords: allergy, asthma, chemokines, eosinophils, eotaxin

Introduction

The eosinophil was originally identified as a unique type of white cell in the blood, which is characterized by its bilobed nucleus and its cytoplasmic granules that stain pink with eosin [1]. An early observation was that this cell accumulates in the asthmatic lung [2], but its significance was not recognized. Subsequently, eosinophils have become identified through our defence system against helminth parasites. Parasitic worms characteristically induce an immune response that is polarized toward Th2 lymphocytes. These cells regulate the production of immunoglobulin E, which binds to mast cells to provide recognition for specific antigens, and are particularly involved in acute responses to worms in tissue. Th2 cells also regulate the accumulation of eosinophils, which are important effector cells for more chronic responses to worms. Eosinophils accumulate in high numbers around the parasites, where the cells become stimulated to release activated oxygen species and toxic granular proteins (major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin).

Allergy is believed to be a manifestation of the host response to worms that is inappropriately triggered by otherwise innocuous agents in the environment. Helminth parasites secrete enzymes in order to facilitate various stages in their lifecycle (eg digestion of tissue for access or nutrition). The immune system appears to be armed to detect such enzymes and, as a consequence, enzymes can be potent allergens (eg the digestive enzymes in house dust mite faeces).

The prominent role of eosinophils as effector cells in asthma and allergy has stimulated considerable interest in the mechanisms that are involved in the recruitment of these cells. Of particular importance are the chemical signals released in the lung that initiate and orchestrate the process of eosinophil recruitment from the blood microvessels in the airway wall. Until relatively recently, the nature of these chemoattractants was unknown. Mediators with chemotactic effects on eosinophils were recognized, but none of these could explain the selective accumulation of eosinophils that occurs in allergic-type

reactions, because the known substances were also chemotactic for other cell types, such as neutrophils (eg C5a, leukotriene B₄ and platelet-activating factor). The discovery of eotaxin has provided insights into the mechanisms that are involved in eosinophil recruitment *in vivo*.

Discovery of eotaxin

Exposure of sensitized guinea pigs to an aerosol of ovalbumin induces many features of human allergic asthma. Animals exhibit an acute bronchoconstriction, which results from mast-cell activation and the release of histamine and peptidoleukotrienes. This is followed by delayed bronchoconstriction associated with an accumulation of leukocytes, predominantly eosinophils. The airways also become hyperreactive to spasmogens (but not to the same extent as in human asthma).

We lavaged guinea pig airways at various intervals after allergen challenge, injected the bronchoalveolar lavage (BAL) fluid intradermally in assay guinea pigs that were previously injected intravenously with ¹¹¹In-eosinophils, and measured eosinophil accumulation in the skin sites. Using this technique, eosinophil chemoattractant activity was detected in BAL fluid with a peak at 3–6 h after challenge. This activity was purified in a series of high-performance liquid chromatography steps, using the *in vivo* skin bioassay at each stage in order to locate the chemoattractant. Microsequencing revealed a novel 73 amino acid C-C chemokine that we termed 'eotaxin' (condensed from eosinophil chemotaxin) [3,4]. The chemokine was present in three fractions at the final reversed phase high-performance liquid chromatography purification stage, which are believed to correspond to three glycosylation variants of eotaxin. The purified protein was potent in stimulating eosinophils *in vitro* and *in vivo*, but had no significant effect on neutrophils.

Guinea pig eotaxin was shown to be potent in inducing a calcium flux in human eosinophils [4], indicating the existence of a human homologue. Subsequently, primers based on the protein sequence have been used to clone cDNA for guinea pig [5,6], mouse [7], rat [8,9], horse [10] and human eotaxins [11–13]. All of the eotaxins are highly potent eosinophil chemoattractants, with greater than 60% protein sequence similarity, but with some functional cross-species restrictions (eg human eotaxin is inactive on guinea pig eosinophils [14], whereas human eotaxin is active in the rat, making this species useful for *in vivo* studies [15]).

More recently, two other human C-C chemokines have been discovered that have properties very similar to those of eotaxin. Thus, these functional homologues have been termed eotaxin-2 [16,17] and eotaxin-3 [18,19], although they exhibit sequence similarity of less than 40% and differ almost entirely in the amino-terminal region. The human

eotaxin gene is located on chromosome 17q11.2 in the C-C chemokine cluster, whereas eotaxin-2 and eotaxin-3 have been mapped to chromosome 7q11.2. The mouse homologue of eotaxin-2 has recently been described [20]. The promotor region of the C-C chemokines contain consensus-binding sites for the transcription factors nuclear factor- κ B and AP-1, which are known to be important in regulating inflammatory reactions.

The eotaxin receptor

More than 50 chemokines have been identified, signalling via at least 15 different seven-transmembrane-spanning, G-protein-coupled receptors. Chemokines are notorious for stimulating via several receptors, making analysis of their precise *in vivo* role difficult. The eotaxins are unusual (but not unique) in signalling via a single receptor: CCR3. The human receptor was cloned in several laboratories, and was found to be highly expressed on eosinophils [13,21,22]. Mouse [23] and guinea pig [14] CCR3 have also been cloned. Blocking monoclonal antibodies have been produced to both human [24] and guinea pig [14] CCR3. CCR3 is also found on basophils [25], mast cells [26] and a subpopulation of Th2 lymphocytes [27]. This provides a mechanism for recruitment of all of these cell types in the context of allergic inflammation.

The presence of CCR3 on Th2 lymphocytes is of particular interest, because these cells regulate eosinophil recruitment. In a mouse model of allergic airways inflammation, it was shown that eotaxin is particularly involved in early Th2-cell recruitment up to 4 days via CCR3, whereas subsequently another C-C chemokine, macrophage-derived chemokine, appears to be important, acting via CCR4 [28].

Following ligation of CCR3 on eosinophils by eotaxin, a series of events is triggered, including calcium mobilization, CD11b upregulation, mitogen-activated protein kinase activation, oxygen radical production, actin polymerization, and a rapid shape change that is associated with chemotaxis and granule release. CCR3 undergoes prolonged ligand-induced receptor internalization via clathrin-coated pits, which is not dependent on G-protein coupling, calcium transients or protein kinase C activation. Functional responses to eotaxin are inhibited by pertussis toxin, suggesting that the receptor is coupled to Gi α -type G proteins [29].

Eotaxin production *in vivo*

Early studies demonstrated constitutive eotaxin mRNA [5,6] and protein [30] in guinea pig lung, which are believed to be involved in maintaining the basal eosinophil population recognized in this species. Of more general importance is constitutive eotaxin expression in the gut [6]; under basal conditions the majority of eosinophils are localized to the gut, where these cells are believed to play

an important role in host defence against helminths. Eotaxin knockout mice have a selective reduction in eosinophils in the gut, which suggests that eotaxin may be important for basal trafficking of eosinophils into this tissue [31]. Gut eotaxin mRNA is elevated in ulcerative colitis and Crohn's disease [12], which may explain the molecular mechanisms of eosinophil recruitment in these diseases. In addition, the levels of eotaxin have been shown to correlate directly with the extent of tissue eosinophilia in Hodgkin's disease [32].

Several animal models have demonstrated local eotaxin production in allergic reactions, which correlate with eosinophil recruitment. A detailed study of the kinetics of eotaxin production and eosinophil accumulation [30] showed that, after allergen challenge of sensitized guinea pigs, a peak of eotaxin production was observed at 6 h in lung tissue, falling to low levels over 6–12 h. Similarly, eotaxin levels also peaked at 6 h in BAL fluid and fell over 6–12 h, but a significant low level persisted in the airway lumen up to 24 h. The eotaxin levels correlated with the number of eosinophils infiltrating the lung tissue, but the appearance of significant numbers of eosinophils in the BAL fluid occurred later (12–24 h), which may be because the persistence of eotaxin in the airway lumen resulted in a reversal in the direction of the chemoattractant gradient across the airway epithelium over this later period.

The relative contribution of endogenous eotaxin to eosinophil recruitment varies with the animal strain and species, the challenge and sensitization protocol, and the phase of the response. The reasons for this are probably related to contributions by other CCR3 ligands, other chemokine receptors on eosinophils (particularly CCR1 in mice) and nonchemokine chemoattractants. One study [33] showed a 70% reduction in eosinophils 18 h after allergen challenge in eotaxin gene-deleted mice, whereas at 48 h after challenge the eosinophilia was of a similar magnitude in wild-type and knockout mice. Another study in a different strain [34] found no difference in allergen-induced eosinophilic lung inflammation in eotaxin-deficient mice. Administration of neutralizing antibodies that are specific for eotaxin have been shown to reduce the accumulation of eosinophils in the lung [35]. In addition, an antibody to eotaxin was shown to abolish eosinophil chemoattractant activity in BAL fluid from allergen-challenged guinea pigs [30]. In multiple allergen challenge experiments in mice, an antibody to eotaxin reduced eosinophil recruitment and abolished hyperresponsiveness of the lung [36].

Several studies have demonstrated eotaxin expression in the lungs of asthmatic patients, either as increased mRNA or protein. In bronchial biopsies from asthmatic persons increased levels of mRNAs for eotaxin and its receptor CCR3 were observed, and were associated with airway

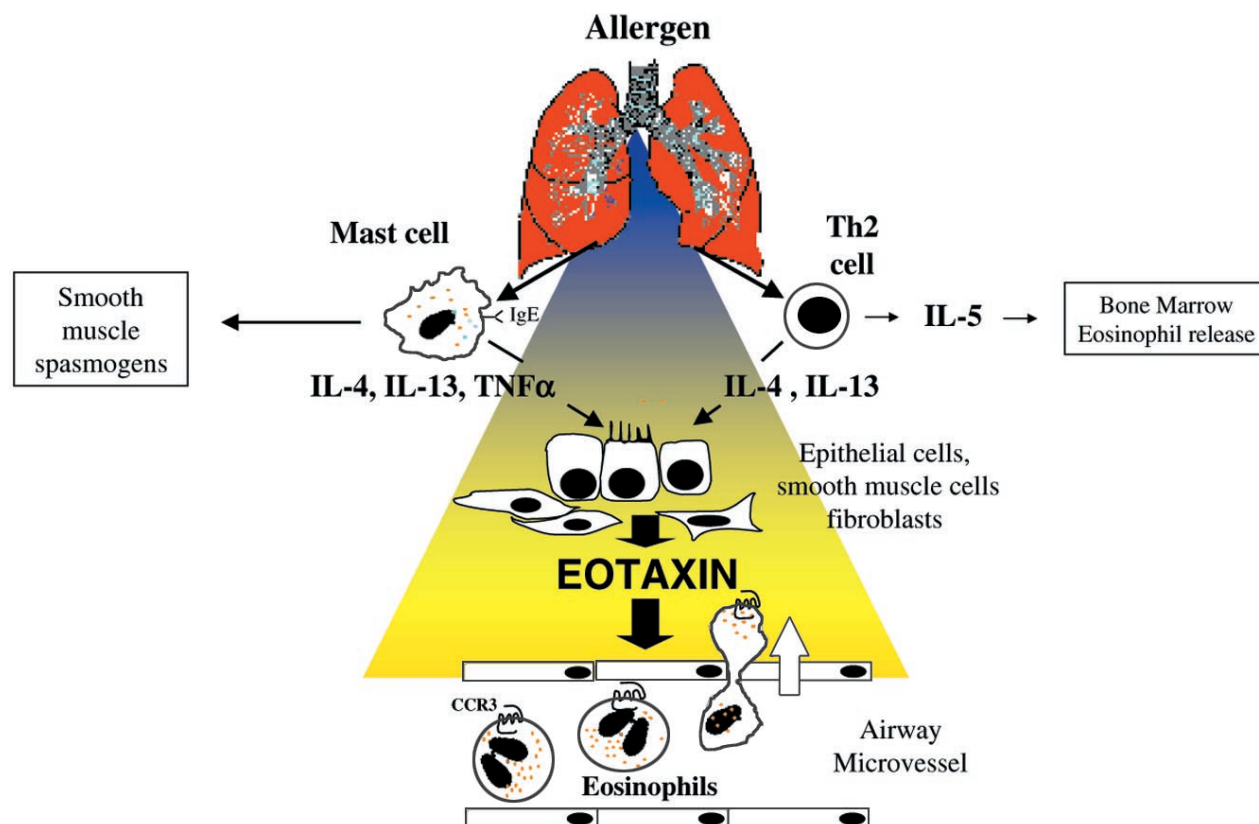
hyperresponsiveness and predominant colocalization of eotaxin mRNA to bronchial epithelial and endothelial cells in the submucosa [37]. Atopic asthmatic persons were reported to have high concentrations of eotaxin in the BAL fluid, and increased expression of eotaxin mRNA and protein in the epithelium and submucosa of their airways as compared with normal control individuals. In the BAL cells the eotaxin immunoreactivity colocalized mainly to macrophages, with a lesser contribution of T cells and eosinophils [38]. The number of cells that expressed mRNA for eotaxin in the bronchial mucosa of asthmatic persons significantly correlated with airway eosinophilia, bronchial hyperreactivity and symptom scores [39]. An association between plasma eotaxin levels, asthma diagnosis and compromised lung function has also been demonstrated [40]. In addition, levels of eotaxin are increased in the sputum of atopic and nonatopic asthmatic persons [41]. Increased eotaxin mRNA and protein expression has been observed in chronic sinusitis and allergen-induced nasal responses in seasonal allergic rhinitis [42]. In response to allergen challenge in the skin of atopic patients, eotaxin was associated with early (6 h) tissue eosinophilia, whereas eotaxin-2 and monocyte chemoattractant protein-4 appeared to be involved in later (24 h) infiltration of these CCR3⁺ cells [43].

Regulation of eotaxin production by T-helper 2 lymphocytes

Early studies suggested that eosinophil recruitment in allergic reactions is regulated by Th2 lymphocytes. In accord with this, it has been shown that eotaxin production is T-cell-dependent using a mouse allergy model [44]. Many cell types in the lung appear to be capable of synthesizing eotaxin (eg airway epithelial cells, airway smooth muscle cells, vascular endothelial cells and macrophages, as well as eosinophils themselves) [30,37,38]. Thus, cytokines that are synthesized by Th2 lymphocytes, such as interleukin-4, interleukin-13 and interleukin-5, have been investigated as potential intermediaries in eotaxin production.

The first study to link eotaxin production to interleukin-4 was performed in the mouse [45], in which it was shown that tumours transfected with the interleukin-4 gene induced eosinophil recruitment associated with eotaxin mRNA upregulation. In addition, an anti-interleukin-4 antibody inhibited eotaxin mRNA expression in a model of type 2 cell-mediated lung granulomas [46]. Similarly, eosinophil accumulation induced by intradermal interleukin-4 in the rat was found to be mediated in part by endogenously generated eotaxin [15]. In addition to inducing eotaxin, interleukin-4 has recently been shown [20] to upregulate eotaxin-2 mRNA expression in mouse lung. Interleukin-13, another cytokine that is generated by Th2 cells, was shown [47] to be more potent than interleukin-4 in inducing eotaxin expression by lung epithelial cells and promoting lung eosinophilia *in vivo*. In addition, pulmonary

Figure 1



Eotaxin-induced eosinophil recruitment in asthma. Inhaled allergen activates mast cells and Th2 lymphocytes in the lung to generate the cytokines interleukin (IL)-4, IL-13 and tumour necrosis factor (TNF)- α . These cytokines stimulate the generation of eotaxin by lung epithelial cells, fibroblasts and smooth muscle cells. Eotaxin acting on CCR3 on eosinophils then stimulates the selective recruitment of these cells from the airway microvessels into the lung tissue. Ig, immunoglobulin.

expression of interleukin-13 induced eotaxin production and eosinophil infiltration, as well as mucus hypersecretion, subepithelial fibrosis and bronchial hyperreactivity [48]. Both interleukin-4 and interleukin-13 can induce eotaxin-3 mRNA expression in human vascular endothelial cells [18]. Studies *in vitro* [49] have demonstrated that interleukin-4 synergizes with the pro-inflammatory cytokine tumour necrosis factor- α to increase eotaxin production from lung fibroblasts. Interleukin-5 does not appear to mediate eotaxin generation, because neutralization of this cytokine *in vivo* has no effect on eotaxin production stimulated by allergen [30]. Thus, these observations have established a mechanism that links Th2 cells to eosinophil recruitment *in vivo* (Fig. 1).

Polarization of T-lymphocytes into Th1/Th2 phenotypes involves cytokines that amplify one pathway while down-regulating the other. There is recent evidence that chemokines may also have a role in this process. It has been shown in mouse models [50] that monocyte chemoattractant protein-1 is involved in regulating inflammation

polarized toward Th2 cells. Moreover, eotaxin has been shown to act as an antagonist of the CXC chemokine receptor-3, which is preferentially expressed on Th1 cells [51], whereas ligands that stimulate CXC chemokine receptor-3 (IP-10, MIG and I-TAC) are potent antagonists of CCR3 [52].

Release of eosinophils from bone marrow

Eosinophils normally circulate in the blood in low numbers (1–2% of blood leukocytes), so mechanisms are necessary to increase circulating cells when required. An intravenous injection of interleukin-5 in guinea pigs induces an acute increase in circulating eosinophils, and this amplifies tissue recruitment induced by locally administered eotaxin [53]. In accord with this, an antibody that neutralizes interleukin-5 inhibits both allergen-induced blood eosinophilia and the recruitment of eosinophils to the lung [30]. In experiments in which the microcirculation of the femoral bone marrow was perfused *in situ*, it was shown [54] that intra-arterial injection of interleukin-5 induced the release of eosinophils into the draining vein. Eotaxin was also

shown [55] to stimulate eosinophil release, and a marked synergism with interleukin-5 was observed. In addition, eotaxin, but not interleukin-5, released eosinophil progenitors from the bone marrow, which may be relevant to the presence of such cells in the peripheral circulation of allergic individuals [55].

Thus, a combination of interleukin-5 and eotaxin produced by the allergen-challenged lung and released into the circulation may be involved in producing the observed blood eosinophilia by acting remotely on the bone marrow *in vivo* [55]. This is in addition to the other important effects of interleukin-5, such as stimulation of differentiation and proliferation of bone marrow eosinophils and suppression of eosinophil apoptosis at sites of inflammation.

C-C chemokine receptor-3 as a therapeutic target

Helminth parasites have evoked many strategies to avoid host defence mechanisms. Interestingly, hookworms secrete an enzyme that selectively cleaves and inactivates eotaxin [56]. Prevention of the effects of eotaxin is also the aim of therapy developed to interfere with leukocyte trafficking to the lung. Neutralization of eotaxin by an antibody is a possible route to therapy in allergic disease. An alternative route is by blocking the receptor. Early studies [57] showed that human RANTES (regulated upon activation, normal T-lymphocyte expressed and secreted) acted as an antagonist to CCR3 on guinea pig eosinophils, and could be used to block eotaxin-induced eosinophil recruitment *in vivo*. Met-RANTES, which has an additional methionine at the amino-terminus, inhibited eosinophil recruitment in a late allergic reaction in the mouse skin [58], and attenuated leukocyte infiltration and airway hyperresponsiveness in a model of allergic airways inflammation in the mouse by antagonizing both CCR3 and CCR1 [35]. Another chemokine mutant, Met-CK β 7, a modified form of the C-C chemokine macrophage inflammatory protein 4, has been shown to be a potent and selective CCR3 antagonist [59]. Furthermore, guinea pig CCR3 cDNA has been cloned, and a blocking antibody to the receptor has been produced [14]. This antibody has also been shown to block eotaxin-induced eosinophil recruitment *in vivo* [14].

Significantly, it has been shown recently that low molecular weight compounds can block human CCR3, and now several pharmaceutical companies have small molecule antagonists in development. The first report of one such antagonist (the compound UCB 35625, based on a Japanese patent from Banyu Pharmaceuticals, Tokyo, Japan) [60] indicated that this compound effectively blocks both CCR3 and CCR1. This may be advantageous, because it has been shown that about one in 10 individuals have eosinophils that respond to macrophage inflammatory protein-1 α acting via CCR1, in addition to responses to eotaxin acting via CCR3 that are seen in all individuals

[61]. Another compound, SB-328437, has been described that acts selectively as a CCR3 antagonist [62].

Conclusion

The discovery of eotaxin has led to a greater understanding of the mechanisms that are involved in eosinophil recruitment at sites of allergic inflammation. Small molecule antagonists that block CCR3, perhaps taken as a once daily tablet, may prove to be effective therapeutic compounds that are aimed at suppressing important effector mechanisms involved in the pathogenesis of asthma and allergy.

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