

Selective suppression of leukocyte recruitment in allergic inflammation

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Allergic diseases result in a considerable socioeconomic burden. The incidence of allergic diseases, notably allergic asthma, has risen to high levels for reasons that are not entirely understood. With an increasing knowledge of underlying mechanisms, there is now more potential to target the inflammatory process rather than the overt symptoms. This focuses attention on the role of leukocytes especially Th2 lymphocytes that regulate allergic inflammation and effector cells where eosinophils have received much attention. Eosinophils are thought to be important based on the high numbers that are recruited to sites of allergic inflammation and the potential of these cells to effect both tissue injury and remodelling. It is hoped that future therapy will be directed towards specific leukocyte types, without overtly compromising essential host defence responses. One obvious target is leukocyte recruitment. This necessitates a detailed understanding of underlying mechanisms, particularly those involving soluble chemoattractants signals and cell-cell adhesion molecules.

Key words: allergy - leukocytes - eosinophils - chemokines - interleukin-5

Allergic reactions appear to represent an aberration of a host defence reaction to helminth parasites. For reasons not well understood, agents present in the environment that would otherwise be innocuous, stimulate the immune system to produce a localised inflammatory response on re-exposure to allergen. This response is regulated by polarised CD4⁺ Th2 lymphocytes and involves the production of allergen-specific IgE and the recruitment and activation of the effector cells characteristic of host responses to parasitic worms. In allergic asthma, cross-linking of mast cell-bound IgE by allergens results in the release of preformed and *de novo* synthesised mediators that induce acute bronchoconstriction, mucus secretion and increased vasopermeability. Further, chemoattractants are produced by many cell types in the tissue and these substances induce the local accumulation of inflammatory cells. Of these, eosinophils and basophils have received the most attention because of their close association with allergic-type reactions. Eosinophils accumulate in very high numbers and have been under the spotlight for a number of years as potential targets for therapeutic intervention. This article describes some of the current knowledge of the role of eosinophils in allergic reactions, the mechanisms involved in their recruitment and the potential for inhibiting their accumulation.

Eosinophil trafficking

Eosinophils are derived from haematopoietic stem cells in the bone marrow. IL-5 is important for their differentiation, proliferation and maturation in the marrow: for re-

view see: (Kay et al. 2003, Zabeau et al. 2003). IL-5 is also able to induce the release of mature eosinophils into the blood by stimulating their migration across the marrow sinus endothelium (Collins et al. 1995, Palframan et al. 1998). Recruitment into tissues involves the action of locally-produced chemoattractants acting in concert with adhesion molecules. The $\alpha_4\beta_1$ integrin expressed on eosinophils appears to be particularly important in vivo (Weg et al. 1993), binding to VCAM-1 expressed on venular endothelial cells. The Th2 cytokines IL-4 and IL-13 are able to increase the expression of VCAM-1 by endothelial cells which will facilitate eosinophil attachment to the surface of the endothelium. Locally produced IL-5 does not appear to be particularly important as a chemoattractant for eosinophils; here chemokines have an important role (see below). However, locally-produced IL-5 does have an important function in increasing the survival of eosinophils once they have reached the tissues.

Chemokines as chemoattractants for eosinophils

None of the leukocyte chemoattractants identified up until towards the end of the 1980's was able to explain how phases of selective leukocyte accumulation occurred in different types of inflammatory reactions. Potent chemoattractants (originally identified by their chemotactic activity in Boyden chambers in vitro) were known, such as C5a, leukotriene B₄, formyl-methionyl peptides and platelet activating factor, but none of these exhibited leukocyte-type specificity. The discovery of the first chemokines led to the realisation that a large family of structurally-related small proteins exists. Approximately 50 human chemokines and 20 receptors have been identified to date (for reviews, see Luster 1998, Moser et al. 2004). Chemokines often stimulate several different receptors and a given leukocyte type often expresses more than one type of receptor. Moreover, a leukocyte can also change its chemokine receptor expression pattern when presented with different microenvironments. Despite these

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complications, it is now possible to begin to understand how leukocytes can traffick from one compartment to another, thus providing potential new targets for therapeutic intervention. In the early 1990's we realised that none of the known chemoattractants was selective for eosinophils and so could not account for the eosinophil-rich infiltration observed in allergic reactions. We therefore, began a project to identify such mediators generated in vivo. Sensitised guinea pigs were challenged with an aerosol of ovalbumin to produce acute bronchoconstriction followed by a delayed response associated with eosinophil infiltration. Bronchoalveolar lavage (BAL) fluid, taken at intervals after the allergen challenge, was bioassayed for chemoattractant activity by injecting it intradermally in naïve assay animals and measuring the local accumulation of circulating ^{111}In -eosinophils. We found that BAL fluid taken from 3-6 h after challenge contained eosinophil chemoattractant activity. This was purified using sequential HPLC systems, with bioassay of the fractions at each stage using the skin system in vivo. Protein sequencing revealed a novel 73 amino acid CC chemokine that we called "Eotaxin" (Griffiths-Johnson et al. 1993, Jose et al. 1994). Guinea pig Eotaxin was cloned using degenerate primers based on the sequence of the guinea pig protein (Jose et al. 1994, Rothenberg et al. 1995). Constitutive message was found in the lung and was upregulated on allergen challenge of sensitised animals. Subsequently, mouse (Rothenberg et al. 1995, Gonzalo et al. 1996), rat (Ishi et al. 1998, Williams et al. 1998) and human (Garcia-Zepeda et al. 1996, Kitauro et al. 1996, Ponath et al. 1996) Eotaxin homologues were cloned. These proteins have high sequence homology and all are potent eosinophil chemoattractants.

RANTES, some of the monocyte chemoattractant proteins (MCPs) and MIP-1 α are other CC chemokines that are also known to stimulate eosinophil responses. However, these proteins are non-selective and stimulate a wide variety of cell types besides eosinophils. More recently, two more "Eotaxins" with high functional similarity, but rather low sequence similarity, when compared with the originally-discovered Eotaxin have been identified (Forssmann et al. 1997, Patel et al. 1997, White et al. 1997, Shinkai et al. 1999). To distinguish these three distinct gene products, the names Eotaxin-1 (CCL11, formerly Eotaxin), Eotaxin-2 (CCL24) and Eotaxin-3 (CCL26) are used (Zlotnik et al. 2000).

The Eotaxin receptor, CCR3

The Eotaxin receptor, CCR3, is a 7-transmembrane-spanning receptor that is highly expressed on eosinophils. This has been cloned in man (Daugherty et al. 1996, Kitauro et al. 1996, Ponath et al. 1996) mouse (Post et al. 1995, Gao et al. 1996) and guinea pig (Sabroe et al. 1998). Human CCR3 binds Eotaxin-1, Eotaxin-2, Eotaxin-3 and MCP-4 with high affinity, whereas RANTES and MCP-3 bind with lower affinity. Guinea pig Eotaxin-1 is highly potent as a stimulator of human eosinophils (Jose et al. 1994) but, conversely, human Eotaxin-1 is inactive on guinea pig cells, although it is active on rat eosinophils (Sanz et al. 1998, Kudlacz et al. 1999). Despite the fact that human RANTES stimulates human eosinophils, RANTES

from other species has, in general, low activity on homologous eosinophils (Campbell et al. 1997). Unexpectedly, human RANTES binds to guinea pig CCR3 (Jose et al. 1994, Sabroe et al. 1998) and acts as an antagonist in vitro and in vivo (Marleau et al. 1996).

In the guinea pig, there is no evidence to date of endogenous CC chemokines, other than Eotaxin-1, acting on eosinophils. Anti-Eotaxin-1 antibodies completely block eosinophil accumulation induced by BAL fluid obtained from allergic and non-allergic inflammatory reactions (Humbles et al. 1997). A neutralising antibody to guinea pig CCR3 blocks responses of guinea pig eosinophils to Eotaxin-1 in vitro and prevents eosinophil accumulation in response to Eotaxin-1 in vivo (Sabroe et al. 1998). In mouse models of allergic airway inflammation, eosinophil recruitment appears to be mediated by a number of CC chemokines acting via CCR3 and, depending on the model and the mouse strain, MIP-1 α acting through CCR1 (Gonzalo et al. 1998, Ma et al. 2002). In man, MIP-1 α can also stimulate eosinophils in vitro but this is in only a subpopulation of individuals, whereas Eotaxin-1 is active on eosinophils from all donors (Sabroe et al. 1999).

Regulation of chemokine production by Th2 cytokines

T-lymphocytes are critical elements in regulating allergic reactions (Basten et al. 1970). Allergy is generally associated with a polarisation of T-helper lymphocytes into the Th2 type (Robinson et al. 1992, Romagnani 1994), as first defined in the mouse (Mosmann et al. 1986). However, there is also evidence for a co-existence of Th1 and Th2 responses in mouse allergy models (Li et al. 1998, Randolph et al. 1999). Th2 cells characteristically produce IL-4, IL-5, IL-10 and IL-13. Neutralisation of IL-4 suppresses lung responses to allergen challenge when the antibody is administered before sensitisation. However, neutralisation of IL-13, but not IL-4, suppresses responses when the antibody is administered just before allergen challenge (Wills-Karp et al. 1998). Depletion of T-cells with an anti-CD3 antibody just before challenge suppresses Eotaxin-1 production and eosinophil accumulation (MacLean et al. 1996). Further, transfer of allergen-specific Th2 cells to naïve mice, followed by aerosol allergen challenge, induces eosinophil accumulation associated with Eotaxin-1 production in the lung (Li et al. 1998).

Although Th2 lymphocytes are critical for regulating eosinophil accumulation and activation in allergic inflammation, these cells do not appear to be a major source of eosinophil chemoattractant chemokines. Studies in guinea pigs (Gonzalo et al. 1996, Humbles et al. 1997, Li et al. 1997) and man (Lamkhioed et al. 1997, Mattoli et al. 1997, Ying et al. 1997, Nakajima et al. 1998), using in situ hybridisation and immunohistochemistry, show that the major sources of Eotaxin-1 are inflammatory cells such as macrophages, as well as eosinophils themselves, and also airway smooth muscle cells, vascular endothelial cells and, in particular, airway epithelial cells. Two Th2 cytokines, IL-4 and IL-13, have been shown to act synergistically with TNF α to induce Eotaxin-1 production in human cells in culture (Mochizuki et al. 1998, Terada et al. 2000). The first study linking Eotaxin-1 production to IL-4 was made in the mouse (Rothenberg et al. 1995) where it was shown

that tumours transfected with the IL-4 gene induced eosinophil recruitment associated with Eotaxin-1 mRNA upregulation *in vivo*. In addition, in IL-4 knockout mice and in anti-IL-4 treated mice a diminished Eotaxin-1 mRNA expression was observed (Chensue et al. 1997, Ruth et al. 1998). Similarly, it was shown in rats that intradermally-injected IL-4 induced eosinophil accumulation associated with Eotaxin-1 mRNA expression, which was suppressed by a neutralising antibody to Eotaxin-1 (Sanz et al. 1998).

Chemokines are also likely to be of critical importance in many of the upstream events involved in sensitisation to allergens. Thus, chemokines are involved in T and B cell trafficking and distribution under basal conditions and the recruitment of dendritic cells to tissues, followed by their movement to regional lymph nodes (Cyster 1999, 2003, Dieu et al. 1998, Ward et al. 1998, Kim et al. 1999, Moser et al. 2004).

Regulation of blood eosinophil levels

Eosinophils normally circulate in the blood in low numbers (1-4% of blood leukocytes) and there is evidence from guinea pig models that recruitment to sites of allergic inflammation is poor unless mechanisms exist to elevate circulating eosinophil numbers (Collins et al. 1995).

IL-5 (originally discovered in the mouse (Sanderson et al. 1985) is clearly important for eosinophilopoiesis, in stimulating differentiation and proliferation of eosinophils. It was shown in the guinea pig that intravenous IL-5 can also induce the acute release of a pool of mature eosinophils from the bone marrow and that this has a profound enhancing effect on eosinophil recruitment in the skin induced by intradermally-injected Eotaxin-1 (Collins et al. 1995). The mechanisms involved in this release process have been analysed in detail using a system where the microvasculature of the guinea pig femoral bone marrow is perfused *in situ* (Palframan et al. 1998). In this system IL-5 induces a massive migration of eosinophils across the endothelium into the sinuses, a process which involves β_1 and β_2 integrins acting in opposite directions.

Eotaxin-1 also releases eosinophils when infused into the arterial supply to the femoral bone marrow (Palframan et al. 1998). This appears to relate to the chemotactic effect of Eotaxin-1 across the sinus endothelium, as opposed to the chemokinetic effect of IL-5 (Palframan et al. 1998). A combination of the chemotactic effect of Eotaxin-1 and the chemokinetic effect of IL-5 acting synergistically induces very pronounced eosinophil release (Palframan et al. 1998).

IL-5 and Eotaxin-1 are generated in response to allergen in the sensitised lung. Eotaxin-1 is a powerful chemoattractant for eosinophils, but IL-5 has low activity as an eosinophil recruiting agent into tissues (Collins et al. 1995). Both mediators diffuse into the circulation and act synergistically to induce eosinophil release from the bone marrow into the blood. These cells are then available to be recruited into the lung (Humbles et al. 1997). These conclusions are consistent with the effects of a neutralising antibody to IL-5 in the guinea pig, which was shown to block bone marrow eosinophil release, blood eosinophilia and recruitment into the lung (Humbles et al. 1997). Eotaxin-1 has also been implicated in the acute re-

lease of eosinophil progenitors into the circulation (Palframan et al. 1998).

The bone marrow pool of mature eosinophils is also found in man but represents only a minor population in the mouse. In man, eosinophil progenitors have been detected in the circulation of atopic patients (Gibson et al. 1991) and, in asthma, Eotaxin-1 has been shown to have the capacity to mobilise the bone marrow pool of mature cells (Robinson et al. 1999). Eotaxin-1 gene-deleted mice have reduced circulating eosinophils (Rothenberg et al. 1997). This may relate to acute eosinophil release from the bone marrow, but is probably more closely connected with a reported role for Eotaxin-1 in leukopoiesis in this species (Peled et al. 1998).

In addition to its expression on eosinophils, CCR3 has also been shown to be expressed on basophils (Ugucioni et al. 1997), mast cells (Ochi et al. 1999, Romagnani et al. 1999) and some Th2 lymphocytes (Gerber et al. 1997, Sallusto et al. 1997, Bonecchi et al. 1998); all cells associated with the allergic response.

Chemokines in allergic airway inflammation

Eotaxin-1 generation has been detected in guinea pig (Jose et al. 1994, Rothenberg et al. 1995, Humbles et al. 1997) and mouse (Gonzalo et al. 1996, MacLean et al. 1996) models of allergic airway inflammation. However, the situation is more complex in mice where antibodies to Eotaxin-1, MIP-1 α , RANTES, MCP-3 and MCP-5 have all been shown, at least partially, to inhibit eosinophil recruitment (Gonzalo et al. 1996, Jia et al. 1996, Lukacs et al. 1997, Stafford et al. 1997). Mice with a targeted deletion of the Eotaxin-1 gene were shown by Rothenberg et al. (1997) to have a 70% reduction in lung eosinophils 18 h after allergen challenge, but this effect diminished at later time points. In contrast, Yang et al found no detectable effect of Eotaxin-1 gene deletion on eosinophil recruitment (Yang et al. 1998). These studies agree with the idea that other ligands, including some CC chemokines, may be involved in the mouse.

The mouse has provided invaluable information about allergic reactions particularly with respect to the role of T-cells (see below), but interpretation of the role of chemokines and eosinophils is complicated by variations dependent on strain and differences in sensitisation/challenge protocols (eg. single vs multiple challenge models). Marked differences were observed when responses of sensitised mice to one or two allergen challenges were compared (Campbell et al. 1998). A single challenge with cockroach antigen induced eosinophil accumulation associated with the production of Eotaxin-1 and MIP-1 α . Two challenges, separated by 2 days, induced a larger eosinophil infiltrate that was largely due to Eotaxin-1. Airway hyperresponsiveness in both protocols was also more dependent on Eotaxin-1 than on MIP-1 α . However, the antibody to Eotaxin-1 blocked hyperresponsiveness in response to two challenges. These results may, in part, relate to the ability of Eotaxin-1 to induce activation and degranulation of eosinophils (Tenschler et al. 1996, Elsner et al. 1996), a property not shared by MIP-1 α (Campbell et al. 1998). In some models, eosinophil activation correlates with airway hyperresponsiveness to spasmogens. How-

ever, there are clearly other routes to airway dysfunction and examples where hyperresponsiveness can be separated from eosinophil activation.

Eotaxin-1 and Eotaxin-2 expression is upregulated in many cell types in human asthmatic airways (Lamkhioued et al. 1997, Mattoli et al. 1997, Ying et al. 1997, 1999). Following allergen challenge of allergic asthmatic subjects, an increase in the percentage of Eotaxin-1 positive cells in induced sputum (Zeibecoglou et al. 1999) was found and also a time-dependent increase in Eotaxin-1 levels in BAL fluid (Brown et al. 1998). In contrast, RANTES was the only detectable eosinophil chemoattractant to be found in human BAL fluid after allergen challenge (Teran et al. 1996) even though this chemokine is less potent than Eotaxin-1 in chemotaxis assays. In the absence of a deliberate allergen challenge, increased levels of Eotaxin-1 have been found in extracts of induced sputum from asthmatic patients (Yamamoto et al. 2003, Dent et al. 2004). We believe that the majority of the Eotaxin-1 is bound to the mucus matrix as we found this chemokine in extracted sputum from asthmatic subjects but not in BAL fluid or extracts of sputum cells (unpublished data). Further, we have found that Eotaxin-1 binds more strongly than RANTES to components of the mucus matrix, which may account for the preferential detection of RANTES in BAL fluid and of Eotaxin-1 in sputum. The upregulation of CC chemokines with the ability to recruit eosinophils to the airways suggests that antagonists of CCR3 may provide novel therapy in asthma.

Therapeutic intervention to block eosinophil recruitment selectively

The role of eosinophils in the pathology of allergic reactions remains highly contentious, despite intensive study in terms of basic cell biology, animal modelling and clinical investigations: (for a recent review see (Williams 2004). The major therapeutic targets that have been explored are IL-5 and Eotaxin-1/CCR3.

Deletion of the IL-5 gene (Foster et al. 1996) or use of antibodies to neutralise IL-5 (Hamelmann et al. 1999) have been shown to suppress eosinophil recruitment to the lung and in many, but not all cases (Corry et al. 1996), inhibit hyperresponsiveness of the airways in animal models of allergic airways disease. Eotaxin-1/IL-5 double knockout mice exhibited a profound suppression of eosinophil recruitment into lung and hyperresponsiveness (Mattes et al. 2002). Studies in allergic cynomolgus monkeys showed that a single dose of anti-IL-5 antibody could suppress eosinophil recruitment and airways hyperresponsiveness for several months. These results encouraged the initiation of clinical trials in mild atopic asthmatics (Engler et al. 1980) and chronic severe asthmatics (Kips et al. 2003). Unfortunately, although blood eosinophil levels were much reduced, no effects on lung function were observed. This argued against a significant role for eosinophils in airway hyperresponsiveness, although later studies revealed that the antibody treatment only depleted lung eosinophils by 55%, despite the marked depletion of blood eosinophils (Flood-Page et al. 2003).

Eosinophils can release products such as cationic proteins and activated oxygen species that can damage the

airways as well as products, such as leukotrienes, that cause bronchoconstriction. Eosinophils can also release cytokines and growth factors that provide them with a potential role in immunoregulation and tissue remodeling. Of particular interest as eosinophil products are TGF α and TGF β_1 . Interestingly, the suppression by anti-IL-5 (Blyth et al. 2000) or IL-5 gene deletion (Trifileff et al. 2001, Cho et al. 2004) of lung tissue eosinophils was associated with an attenuation of tissue remodelling in chronic murine models of repetitive allergen challenge, and with a decrease in the content of TGF β_1 in the lung (Cho et al. 2004) was observed. This has also been observed in man: here treatment with anti-IL-5 has been shown to reduce indices of tissue remodelling in allergic reactions in the skin (Phipps et al. 2002) and lung (Flood-Page et al. 2003). The importance of eosinophils in tissue remodelling has recently been brought into focus in GATA-1 knockout mice lacking eosinophils. These mice exhibited convincing evidence of suppression of tissue remodelling after chronic allergen challenge of the lung (Humbles et al. 2004) but in this case no change was seen in TGF β_1 expression suggesting that other eosinophil-derived reagents are involved.

The major effects of therapy aimed at IL-5 are attenuation of eosinophil production by the bone marrow and reduction of eosinophil survival. An alternative is to inhibit eosinophil recruitment into tissue, where the Eotaxins are thought to play a major part, acting via CCR3. Several low molecular weight CCR3 receptor antagonists have been developed that can effectively block eosinophil migration (White et al. 2000, Sabroe et al. 2000, Naya et al. 2001, Varnes et al. 2004). Some of these compounds have reached the early stages of clinical trials.

Conclusions

Eosinophils are a prominent feature of allergic inflammation, notably in asthma and these cells are believed to be major effector cells of tissue damage. The search for eosinophil-selective chemoattractants led to the discovery of Eotaxin-1 and related CC chemokines that act via the subsequently-discovered Eotaxin receptor, CCR3. The evidence accumulated has provided a working hypothesis to explain mechanisms involved in eosinophil recruitment and the links between Th2 lymphocytes regulating allergic inflammation and eosinophils. Small molecule antagonists of CCR3 may provide a new generation of therapeutic compounds for allergy and asthma.

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