

# Pathophysiology of asthma

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Asthma is characterised by a specific pattern of inflammation that is largely driven *via* immunoglobulin (Ig)E-dependent mechanisms. Genetic factors have an important influence on whether atopy develops and several genes have now been identified [1]. Most of the genetic linkages reported for asthma are common to all allergic diseases [2]. However, environmental factors appear to be more important in determining whether an atopic individual develops asthma, although genetic factors may exert an influence on how severely the disease is expressed and the amplification of the inflammatory response.

## **Inflammation**

It had been recognised for many years that patients who die from acute asthma attacks have grossly inflamed airways. The airway lumen is occluded by a tenacious mucus plug composed of plasma proteins exuded from airway vessels and mucus glycoproteins secreted from surface epithelial cells. The airway wall is oedematous and infiltrated with inflammatory cells, which are predominantly eosinophils and lymphocytes. The airway epithelium is invariably shed in a patchy manner and clumps of epithelial cells are found in the airway lumen. Occasionally there have been opportunities to examine the airways of asthmatic patients who die accidentally and similar though less marked inflammatory changes have been observed [3]. More recently it has been possible to examine the airways of asthmatic patients by fiberoptic and rigid bronchoscopy, by bronchial biopsy and by bronchoalveolar lavage (BAL). Direct bronchoscopy reveals that the airways of asthmatic patients are often reddened and swollen, indicating acute inflammation. Lavage has revealed an increase in the numbers of lymphocytes, mast cells and eosinophils and evidence for activation of macrophages in comparison with nonasthmatic controls. Biopsies have revealed evidence for increased numbers and activation of mast cells, macrophages, eosinophils and T-lymphocytes [4, 5]. These changes are found even in patients with mild asthma who have few symptoms, and this suggests that asthma is an inflammatory condition of the airways.

Inflammation is classically characterised by four cardinal signs: calor and rubor (due to vasodilatation), tumour (due to plasma exudation and oedema) and dolor (due to sensitisation and activation of sensory nerves). It is now recognised that inflammation is also characterised by an infiltration with inflammatory cells and that these will differ depending on the type of inflammatory process. Inflammation is an important defence response that defends the body against invasion from microorganisms and against the effects of external toxins. The inflammation in allergic asthma is characterised by the fact that it is driven by exposure to allergens through IgE-dependent mechanisms, resulting in a characteristic pattern of inflammation. This allergic inflammatory response is characterised by an infiltration with eosinophils and resembles the inflammatory process mounted in response to parasitic and worm infections. The inflammatory response not

only provides an acute defence against injury, but is also involved in healing and restoration of normal function after tissue damage as a result of infection or toxins. In asthma, the inflammatory response is activated inappropriately and is harmful rather than beneficial. For some reason allergens, such as house dust mite and pollen proteins, induce an eosinophilic inflammation. Normally such an inflammatory response would kill the invading parasite (or *vice versa*) and would therefore be self-limiting, but in allergic disease the inciting stimulus persists and the normally acute inflammatory response becomes converted into a chronic inflammation which may have structural consequences in the airways and skin.

***Intrinsic asthma***

Although the majority of patients with asthma have atopy, in a proportion of patients with asthma there is no evidence of atopy with normal total and specific IgE and negative skin tests. This so-called "intrinsic" asthma usually comes on later in life and tends to be more severe than allergic asthma [6]. The pathophysiology is very similar to that of allergic asthma and there is increasing evidence for local IgE production, possibly directed at bacterial or viral antigens [7].

***Inflammation and airway hyperresponsiveness***

The relationship between inflammation and clinical symptoms of allergy is not yet clear. There is evidence that the degree of inflammation is related to airway hyperresponsiveness (AHR), as measured by histamine or methacholine challenge. Increased airway responsiveness is an exaggerated airway narrowing in response to many stimuli and is the defining characteristic of asthma. The degree of AHR is related to asthma symptoms and the need for treatment. Inflammation of the airways may increase airway responsiveness which thereby allows triggers which would not narrow the airways to do so. But inflammation may also directly lead to an increase in asthma symptoms, such as cough and chest tightness, by activation of airway sensory nerve endings (fig. 1).

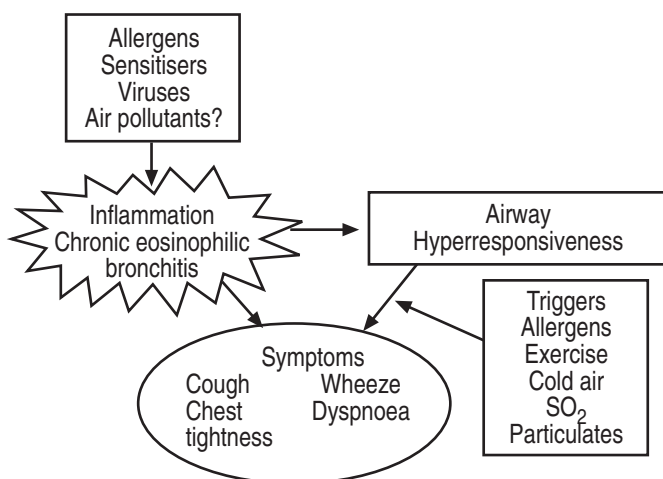


Fig. 1. – Inflammation in the airways of asthmatic patients leads to airway hyperresponsiveness and symptoms. Th2: T-helper 2 cells; SO<sub>2</sub>: sulphur dioxide.

### ***Persistence of inflammation***

Although most attention has focused on the acute inflammatory changes seen in asthma, this is a chronic condition, with inflammation persisting over many years in most patients. The mechanisms involved in persistence of inflammation in asthma are still poorly understood. Superimposed on this chronic inflammatory state are acute inflammatory episodes which correspond to exacerbations of asthma.

## **Inflammatory cells**

Many different inflammatory cells are involved in asthma, although the precise role of each cell type is not yet certain [4]. It is evident that no single inflammatory cell is able to account for the complex pathophysiology of allergic disease, but some cells predominate in asthmatic inflammation.

### ***Mast cells***

Mast cells are important in initiating the acute bronchoconstrictor responses to allergen and probably to other indirect stimuli, such as exercise and hyperventilation (*via* osmolality or thermal changes) and fog. Patients with asthma are characterised by a marked increase in mast cell numbers in airway smooth muscle [8]. Treatment of asthmatic patients with prednisone results in a decrease in the number of tryptase positive mast cells [9]. Furthermore, mast cell tryptase appears to play a role in airway remodelling, as this mast cell product stimulates human lung fibroblast proliferation [10]. Mast cells also secrete certain cytokines, such as interleukin (IL)-4 that may be involved in maintaining the allergic inflammatory response and tumour necrosis factor (TNF)- $\alpha$  [11].

However, there are questions about the role of mast cells in more chronic allergic inflammatory events and it seems more probable that other cells, such as macrophages, eosinophils and T-lymphocytes are more important in the chronic inflammatory process, including AHR. Classically mast cells are activated by allergens through an IgE-dependent mechanism. The importance of IgE in the pathophysiology of asthma has been highlighted by recent clinical studies with humanised anti-IgE antibodies, which inhibit IgE-mediated effects [12, 13]. Although anti-IgE antibody results in a reduction in circulating IgE to undetectable levels, this treatment results in minimal clinical improvement in patients with severe steroid-dependent asthma [14]. Interestingly, treatment with the anti-IgE monoclonal, did allow reduction of the dose of steroids requires for asthma control. This observation suggests that the mechanisms whereby IgE leads to airway obstruction are steroid sensitive, although corticosteroids do not reduce and may even increase, circulating levels of IgE [15, 16].

It is now increasingly recognised that mast cells may also release several other mediators that may play a role in the pathophysiology of asthma, including neurotrophins, proinflammatory cytokines, chemokines and growth factors. This has led to a re-evaluation of the role of mast cells, particularly during exacerbations [17].

### ***Macrophages***

Macrophages, which are derived from blood monocytes, may traffic into the airways in asthma and may be activated by allergen *via* low affinity IgE receptors (Fc $\epsilon$ R2) [18, 19]. The enormous immunological repertoire of macrophages allows these cells to produce

many different products, including a large variety of cytokines that may orchestrate the inflammatory response. Macrophages have the capacity to initiate a particular type of inflammatory response *via* the release of a certain pattern of cytokines. Macrophages may both increase and decrease inflammation, depending on the stimulus. Alveolar macrophages normally have a suppressive effect on lymphocyte function, but this may be impaired in asthma after allergen exposure [20]. One anti-inflammatory protein secreted by macrophages is IL-10 and its secretion is reduced in alveolar macrophages from patients with asthma [21]. Macrophages from normal subjects also inhibit the secretion of IL-5 from T-lymphocytes, probably *via* the release of IL-12, but this is defective in patients with allergic asthma [22]. Macrophages may therefore play an important anti-inflammatory role, by preventing the development of allergic inflammation. Macrophages may also act as antigen-presenting cells which process allergen for presentation to T-lymphocytes, although alveolar macrophages are far less effective in this respect than macrophages from other sites, such as the peritoneum [23].

There may be subtypes of macrophages that perform different inflammatory, anti-inflammatory or phagocytic roles in allergic disease. Immunological markers that can distinguish these subpopulations are beginning to emerge [24]. However, no differences in the macrophage population in induced sputum of allergic asthmatic compared to normal subjects have been detected [25].

### ***Dendritic cells***

Dendritic cells are specialised macrophage-like cells that have a unique ability to induce a T-lymphocyte mediated immune response and therefore play a critical role in the development of asthma [26]. Dendritic cells in the respiratory tract form a network that is localised to the epithelium and act as very effective antigen-presenting cells [27]. It is likely that dendritic cells play an important role in the initiation of allergen-induced responses in asthma [28]. Dendritic cells take up allergens, process them to peptides and migrate to local lymph nodes where they present the allergenic peptides to uncommitted T-lymphocytes and with the aid of co-stimulatory molecules, such as B7.1, B7.2 and CD40 they programme the production of allergen-specific T-cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF), which is expressed in abundance by epithelial cells and macrophages in asthma, leads to differentiation and activation of dendritic cells. This leads to production of myeloid dendritic cells which favour the differentiation of T-helper (Th)2 cells. [29]. Animal studies have demonstrated that myeloid dendritic cells are critical to the development of Th2 cells and eosinophilia [30]. Immature dendritic cells in the respiratory tract promote Th2 cell differentiation and require cytokines such as IL-12 and TNF- $\alpha$  to promote the normally preponderant Th1 response [31]. Dendritic cell based immunotherapy may be developed in the future for the prevention and control of allergic diseases.

### ***Eosinophils***

Eosinophil infiltration is a characteristic feature of allergic inflammation. Asthma might more accurately be termed "chronic eosinophilic bronchitis" (a term first coined as early as 1916). Allergen inhalation results in a marked increase in eosinophils in BAL fluid at the time of the late reaction and there is a correlation between eosinophil counts in peripheral blood or bronchial lavage and AHR. Eosinophils are linked to the development of AHR through the release of basic proteins and oxygen-derived free radicals [32, 33]. Experimentally activated eosinophils have been shown to induce airway epithelial damage, which is a characteristic of patients with asthma [34].

Several mechanisms involved in recruitment of eosinophils into the airways [35]. Eosinophils are derived from bone marrow precursors. After allergen challenge eosinophils appear in BAL fluid during the late response and this is associated with a decrease in peripheral eosinophil counts and with the appearance of eosinophil progenitors in the circulation [36]. The signal for increased eosinophil production is presumably derived from the inflamed airway. Eosinophil recruitment initially involves adhesion of eosinophils to vascular endothelial cells in the airway circulation, their migration into the submucosa and their subsequent activation. The role of individual adhesion molecules, cytokines and mediators in orchestrating these responses has been extensively investigated. Adhesion of eosinophils involves the expression of specific glycoprotein molecules on the surface of eosinophils (integrins) and their expression of such molecules as intercellular adhesion molecule (ICAM)-1 on vascular endothelial cells [37, 38]. An antibody directed at ICAM-1 markedly inhibits eosinophil accumulation in the airways after allergen exposure and also blocks the accompanying hyperresponsiveness [39], although results in other species are less impressive [40]. However, ICAM-1 is not selective for eosinophils and cannot account for the selective recruitment of eosinophils in allergic inflammation. The adhesion molecule very late antigen- (VLA)4 expressed on eosinophils which interacts with vascular cell adhesion molecule (VCAM)-1 appears to be more selective for eosinophils [41] and IL-4 increases the expression of VCAM-1 on endothelial cells [42]. GM-CSF and IL-5 may be important for the survival of eosinophils in the airways and for "priming" eosinophils to exhibit enhanced responsiveness.

Eosinophils from asthmatic patients show exaggerated responses to platelet-activating factor (PAF) and phorbol esters, compared to eosinophils from atopic nonasthmatic individuals [43] and this is further increased by allergen challenge [44], suggesting that they may have been primed by exposure to cytokines in the circulation. There are several mediators involved in the migration of eosinophils from the circulation to the surface of the airway. The most potent and selective agents appear to be chemokines, such as RANTES (regulated on activation T-cell expressed and secreted), eotaxins 1–3 and macrophage chemotactic protein (MCP)-4, that are expressed in epithelial cells [45]. There appears to be a cooperative interaction between IL-5 and chemokines, so that both cytokines are necessary for the eosinophilic response in airways [46]. Once recruited to the airways eosinophils require the presence of various growth factors, of which GM-CSF and IL-5 appear to be the most important [47]. In the absence of these growth factors eosinophils may undergo programmed cell death (apoptosis) [48, 49].

Recently a humanised monoclonal antibody to IL-5 has been administered to asthmatic patients [50] and as in animal studies, there is a profound and prolonged reduction in circulating eosinophils. Although the infiltration of eosinophils into the airway after inhaled allergen challenge is completely blocked, there is no effect on the response to inhaled allergen and no reduction in AHR. A clinical study with anti-IL-5 blocking antibody showed a similar profound reduction in circulating eosinophils, but no improvement in clinical parameters of asthma control [51]. These data question the pivotal role of eosinophils in AHR and asthma, but it is possible that eosinophils may be playing an important role in the structural changes that occur in chronic asthma through the secretion of growth factors, such as transforming growth factor- $\beta$  [52].

### *Neutrophils*

While considerable attention has focused on eosinophils in allergic disease, there has been much less attention paid to neutrophils. Although neutrophils are not a predominant cell type observed in the airways of patients with mild-to-moderate chronic asthma, they appear to be a more prominent cell type in airways and induced sputum of

patients with more severe asthma [53–55]. Also in patients who die suddenly of asthma large numbers of neutrophils are found in the airways [56], although this may reflect the rapid kinetics of neutrophil recruitment compared to eosinophil inflammation. The presence of neutrophils in severe asthma may reflect treatment with high doses of corticosteroids as steroids prolong neutrophil survival by inhibition of apoptosis [48, 57, 58]. However, it is possible that neutrophils are actively recruited in severe asthma. Neutrophils may be recruited to the airways in severe asthma and the concentrations of IL-8 are increased in induced sputum of these patients [54]. This in turn may be due to the increased levels of oxidative stress in severe asthma [59]. The role of neutrophils in asthma is also unknown and whether it plays a role in the pathophysiology of severe asthma needs to be determined, when selective inhibitors of IL-8 become available. The fact that patients with even higher degrees of neutrophilic inflammation, such as in chronic obstructive pulmonary disease (COPD) and cystic fibrosis, do not have the pronounced AHR seen in asthma makes it unlikely that neutrophils are linked to increased airway responsiveness. However, it is possible that they may be associated with reduced responsiveness to corticosteroids that is found in patients with severe asthma. Neutrophils may also play a role in acute exacerbations of asthma.

### ***T-Lymphocytes***

T-lymphocytes play a very important role in coordinating the inflammatory response in asthma through the release of specific patterns of cytokines, resulting in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways [60]. T-lymphocytes are coded to express a distinctive pattern of cytokines, which are similar to that described in the murine Th2 type of T-lymphocytes, which characteristically express IL-4, IL-5, IL-9 and IL-13 [61]. This programming of T-lymphocytes is presumably due to antigen-presenting cells, such as dendritic cells, which may migrate from the epithelium to regional lymph nodes or which interact with lymphocytes resident in the airway mucosa. The naive immune system is skewed to express the Th2 phenotype; data now indicate that children with atopy are more likely to retain this skewed phenotype than normal children [62]. There is some evidence that early infections or exposure to endotoxins might promote Th1-mediated responses to predominate and that a lack of infection or a clean environment in childhood may favour Th2 cell expression thus atopic diseases [63–65]. Indeed, the balance between Th1 cells and Th2 cells is thought to be determined by locally released cytokines, such as IL-12, which tip the balance in favour of Th1 cells, or IL-4 or IL-13 which favour the emergence of Th2 cells (fig. 2). There is some evidence that steroid treatment may differentially effect the balance between IL-12 and IL-13 expression [66]. Data from murine models of asthma have strongly suggested that IL-13 is both necessary and sufficient for induction of the asthmatic phenotype [67].

Regulatory T (Tr) cells suppress the immune response through the secretion of inhibitory cytokines, such as IL-10 and transforming growth factor (TGF) $\beta$ , and play an important role in immune regulation with suppression of Th1 responses [68, 69]. However, their role in allergic diseases has not yet been well defined.

### ***B-Lymphocytes***

In allergic diseases B-lymphocytes secrete IgE and the factors regulating IgE secretion are now much better understood [70]. IL-4 is crucial in switching B-cells to IgE production, and CD40 on T-cells is an important accessory molecule that signals through interaction with CD40-ligand on B-cells. There is increasing evidence for local production of IgE, even in patients with intrinsic asthma, as discussed above [6].

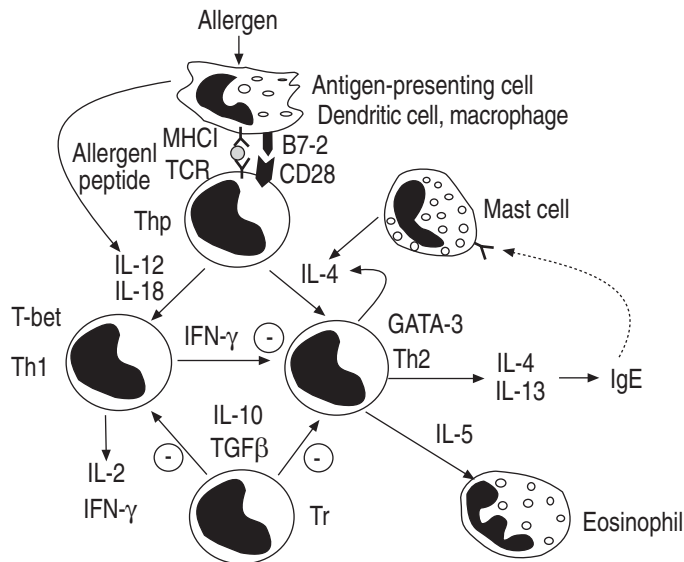


Fig. 2. – Asthmatic inflammation is characterised by a preponderance of T-helper (Th) 2 lymphocytes over Th1 cells. The transcription factors T-bet and GATA-3 may regulate the balance between Th1 and Th2 cells. Regulatory T-cells (Tr) have an inhibitory effect. IL: interleukin; IFN: interferon; TGF: tumour growth factor; IG: immunoglobulin.

### ***Basophils***

The role of basophils in asthma is uncertain, as these cells have previously been difficult to detect by immunocytochemistry [71]. Using a basophil-specific marker a small increase in basophils has been documented in the airways of asthmatic patients, with an increased number after allergen challenge [72, 73]. However, these cells are far outnumbered by eosinophils (approximately 10:1 ratio) and their functional role is unknown [72]. There is also an increase in the numbers of basophils, as well as mast cells, in induced sputum after allergen challenge [74]. The role of basophils, as opposed to mast cells, is somewhat uncertain in asthma [75].

### ***Platelets***

There is some evidence for the involvement of platelets in the pathophysiology of allergic diseases, since platelet activation may be observed and there is evidence for platelets in bronchial biopsies of asthmatic patients [76]. After allergen challenge there is a significant fall in circulating platelets [77] and circulating platelets from patients with asthma show evidence of increased activation and release the chemokine RANTES [78]. Chemokines associated with Th2-mediated inflammation have recently been shown to activate and aggregate platelets [79].

### ***Structural cells***

Structural cells of the airways, including epithelial cells, endothelial cells, fibroblasts and even airway smooth muscle cells may also be an important source of inflammatory mediators, such as cytokines and lipid mediators in asthma [80–83]. Indeed, because

structural cells far outnumber inflammatory cells in the airway, they may become the major source of mediators driving chronic inflammation in asthma. Epithelial cells may have a key role in translating inhaled environmental signals into an airway inflammatory response and are probably the major target cell for inhaled glucocorticoids (fig. 3).

## Inflammatory mediators

Many different mediators have been implicated in asthma and they may have a variety of effects on the airways which could account for all of the pathological features of allergic diseases [84] (fig. 4). Mediators such as histamine, PG, leukotrienes and kinins contract airway smooth muscle, increase microvascular leakage, increase airway mucus secretion and attract other inflammatory cells. Because each mediator has many effects the role of individual mediators in the pathophysiology of asthma is not yet clear. The multiplicity and redundancy of effects of mediators makes it unlikely that preventing the synthesis or action of a single mediator will have a major impact in asthma. However, some mediators may play a more important role if they are upstream in the inflammatory process. The effects of single mediators can only be evaluated through the use of specific receptor antagonists or mediator synthesis inhibitors.

## Lipid mediators

The cysteinyl-leukotrienes, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, are potent constrictors of human airways and have been reported to increase AHR and may play an important role in asthma [85]. The introduction of potent specific leukotriene antagonists has recently made it possible to evaluate the role of these mediators in asthma. Potent LTD<sub>4</sub> antagonists protect (by ~50%) against exercise- and allergen-induced bronchoconstriction, suggesting that leukotrienes contribute to bronchoconstrictor responses. Chronic

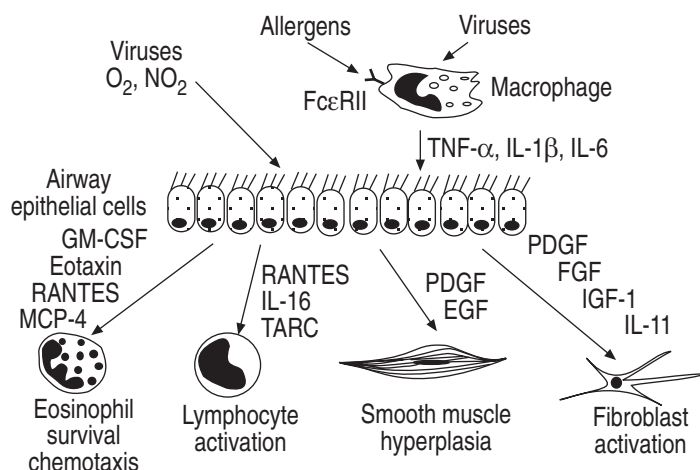


Fig. 3. – Airway epithelial cells may play an active role in asthmatic inflammation through the release of many inflammatory mediators, cytokines, chemokines and growth factors. O<sub>2</sub>: oxygen; NO<sub>2</sub>: nitrogen dioxide; TNF: tumour necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MCP: monocyte chemotactic protein; TARC: thymus and activation regulated chemokine; PDGF: platelet-derived growth factor; EGF: endothelial growth factor; FGF: fibroblast growth factor; IGF: insulin-like growth factor.



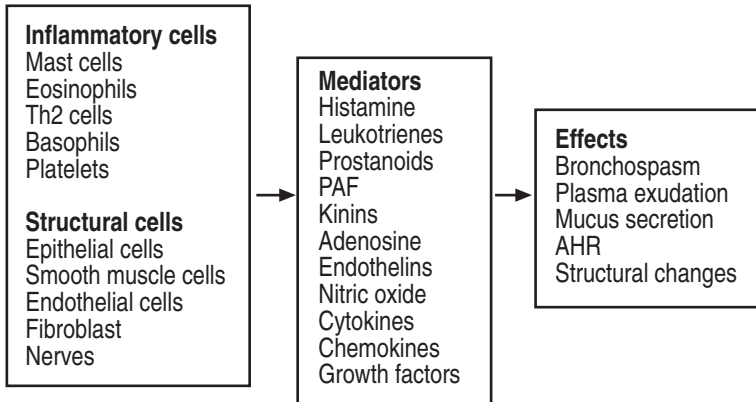


Fig. 4. – Many cells and mediators are involved in asthma and lead to several effects on the airways. PAF: platelet-activating factor; AHR: airway hyperresponsiveness.

treatment with antileukotrienes improves lung function and symptoms in asthmatic patients, although the degree of lung function improvement is not as great as with inhaled corticosteroids which have a much broader spectrum of effects [86, 87]. In addition to their effects of airway smooth muscle and vessels, cys-LTs have weak inflammatory effects, with an increase in eosinophils in induced sputum [88], but the anti-inflammatory effects of antileukotrienes are small [89].

PAF is a potent inflammatory mediator that mimics many of the features of asthma, including eosinophil recruitment and activation and induction of AHR [90], yet even potent PAF antagonists, such as modipafant, do not control asthma symptoms, at least in chronic asthma [91–93]. A genetic mutation that results in impaired function of the PAF metabolising enzyme, PAF acetyl hydrolase, is associated with presence severe asthma in Japan [94], suggesting that PAF may play a role in some forms of asthma.

PG have potent effects on airway function and there is increased expression of the inducible form of cyclooxygenase (COX-2) in asthmatic airways [95], but inhibition of their synthesis with COX inhibitors, such as aspirin or ibuprofen, does not have any effect in most patients with asthma. Some patients have aspirin-sensitive asthma, which is more common in some ethnic groups, such as eastern Europeans and Japanese [96]. It is associated with increased expression of LTC<sub>4</sub> synthase, resulting in increased formation of cys-LTs, possibly because of genetic polymorphisms [97]. PGD<sub>2</sub> is a bronchoconstrictor PG produced predominantly by mast cells. Deletion of the PGD<sub>2</sub> receptors in mice significantly inhibits inflammatory responses to allergen and inhibits AHR, suggesting that this mediator may be important in asthma [98]. Recently it has also been discovered that PGD<sub>2</sub> activates a novel chemoattractant receptor termed chemoattractant receptor of Th2 cells (CRTH2), which is expressed on Th2 cells, eosinophils and basophils and mediates chemotaxis of these cell types and may provide a link between mast cell activation and allergic inflammation [96].

### *Cytokines*

Cytokines are increasingly recognised to be important in chronic inflammation and to play a critical role in orchestrating the type of inflammatory response [99] (fig. 5). Many inflammatory cells (macrophages, mast cells, eosinophils and lymphocytes) are capable

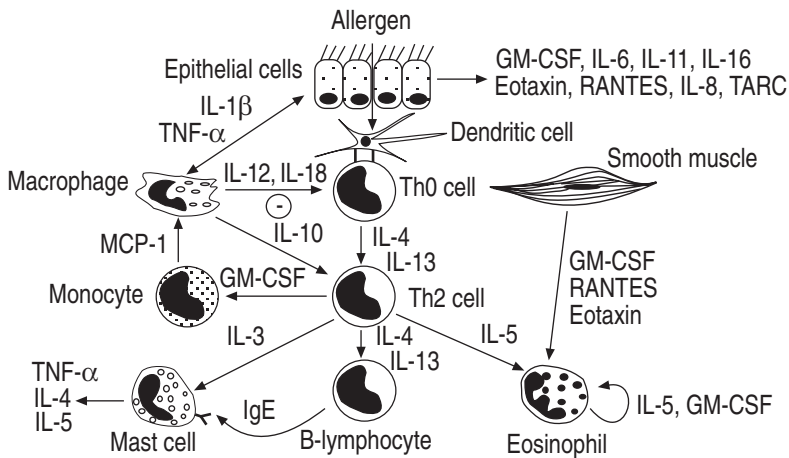


Fig. 5. – The cytokine network in asthma. Many inflammatory cytokines are released from inflammatory and structural cells in the airway and orchestrate and perpetuate the inflammatory response. TNF: tumour necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MCP: monocyte chemotactic protein; TARC: thymus and activation regulated chemokine; PDGF: platelet-derived growth factor; EGF: endothelial growth factor; FGF: fibroblast growth factor; IGF: insulin-like growth factor; Th: T-helper.

of synthesising and releasing these proteins and structural cells such as epithelial cells, airway smooth muscle and endothelial cells may also release a variety of cytokines and may therefore participate in the chronic inflammatory response [100]. While inflammatory mediators like histamine and leukotrienes may be important in the acute and subacute inflammatory responses and in exacerbations of asthma, it is likely that cytokines play a dominant role in maintaining chronic inflammation in allergic diseases. Almost every cell is capable of producing cytokines under certain conditions. Research in this area is hampered by a lack of specific antagonists, although important observations have been made using specific neutralising antibodies that have been developed as novel therapies [101].

The cytokines which appear to be of particular importance in asthma include the lymphokines secreted by T-lymphocytes: IL-3, which is important for the survival of mast cells in tissues, IL-4 which is critical in switching B-lymphocytes to produce IgE and for expression of VCAM-1 on endothelial cells, IL-13, which acts similarly to IL-4 in IgE switching and IL-5 which is of critical importance in the differentiation, survival and priming of eosinophils. There is increased gene expression of IL-5 in lymphocytes in bronchial biopsies of patients with symptomatic asthma and allergic rhinitis [102]. The role of an IL-5 in eosinophil recruitment in humans has been confirmed in a study in which administration of an anti-IL-5 antibody (mepolizumab) to asthmatic patients was associated with a profound decrease in eosinophil counts in the blood and induced sputum [50]. Interestingly in this study there was no effect on the physiology of the allergen-induced asthmatic response and this has been confirmed in a study in symptomatic asthmatic patients who showed no clinical improvement, despite a marked fall in circulating eosinophils [51]. These studies question the critical role of eosinophils in asthma. IL-4 and IL-13 both play a key role in the allergic inflammatory response since they determine the isotype switching in B-cells that result in IgE formation. IL-4, but not IL-13, is also involved in differentiation of Th2 cells and therefore may be critical in the initial development of atopy, whereas IL-13 is much more abundant in established

disease and may therefore be more important in maintaining the inflammatory process [67, 103]. Another Th2 cytokine IL-9 may play a critical role in sensitising responses to the cytokines IL-4 and IL-5 [104, 105].

Other cytokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF are released from a variety of cells, including macrophages and epithelial cells and may be important in amplifying the inflammatory response. TNF- $\alpha$  may be an amplifying mediator in asthma and is produced in increased amounts in asthmatic airways [106]. Inhalation of TNF- $\alpha$  increased airway responsiveness in normal individuals [107]. TNF- $\alpha$  and IL-1 $\beta$  both activate the proinflammatory transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) which then switch on many inflammatory genes in the asthmatic airway.

Other cytokines, such as interferon (IFN)- $\gamma$ , IL-10, IL-12 and IL-18, play a regulatory role and inhibit the allergic inflammatory process (see below).

### *Chemokines*

Many chemokines are involved in the recruitment of inflammatory cells in asthma [45]. Over 50 different chemokines are now recognised and they activate >20 different surface receptors [108]. Chemokine receptors belong to the seven transmembrane-receptor superfamily of G-protein-coupled receptors and this makes it possible to find small molecule inhibitors, which has not been possible for classical cytokine receptor [109]. Some chemokines appear to be selective for single chemokines, whereas others are promiscuous and mediate the effects of several related chemokines. Chemokines appear to act in sequence in determining the final inflammatory response and so inhibitors may be more or less effective depending on the kinetics of the response [110].

Several chemokines, including eotaxin, eotaxin-2, eotaxin-3, RANTES and MCP-4, activate a common receptor on eosinophils termed CCR3 [111]. A neutralising antibody against eotaxin reduces eosinophil recruitment in to the lung after allergen and the associated AHR in mice [112]. There is increased expression of eotaxin, eotaxin-2, MCP-3, MCP-4 and CCR3 in the airways of asthmatic patients and this is correlated with increased AHR [113, 114]. Several small molecule inhibitors of CCR3, including UCB35625, SB-297006 and SB-328437 are effective in inhibiting eosinophil recruitment in allergen models of asthma [115, 116] and drugs in this class are currently undergoing clinical trials in asthma. Although it was thought that CCR3 were restricted to eosinophils, there is some evidence for their expression on Th2 cells and mast cells, so that these inhibitors may have a more widespread effect than on eosinophils alone, making them potentially more valuable in asthma treatment. RANTES, which shows increased expression in asthmatic airways [117] also activates CCR3, but also has effects on CCR1 and CCR5, which may play a role in T-cell recruitment.

MCP-1 activates CCR2 on monocytes and T-lymphocytes. Blocking MCP-1 with neutralising antibodies reduces recruitment of both T-cells and eosinophils in a murine model of ovalbumin-induced airway inflammation, with a marked reduction in AHR [112]. MCP-1 also recruits and activates mast cells, an effect that is mediated via CCR2 [118]. MCP-1 instilled into the airways induces marked and prolonged AHR in mice, associated with mast cell degranulation. A neutralising antibody to MCP-1 blocks the development of AHR in response to allergen [118]. MCP-1 levels are increased in BAL fluid of patients with asthma [119]. This has led to a search for small molecule inhibitors of CCR2.

CCR4 are selectively expressed on Th2 cells and are activated by the chemokines monocyte-derived chemokine (MDC) and thymus and activation regulated chemokine (TARC) [120]. Epithelial cells of patients with asthma express TARC, which may then

recruit Th2 cells [121]. Increased concentrations of TARC are also found in BAL fluid of asthmatic patients, whereas MDC is only weakly expressed in the airways [122]. TARC may thus induce a sequence of responses resulting in coordinated eosinophilic inflammation (fig. 6). Inhibitors of CCR4 may therefore inhibit the recruitment of Th2 cells and thus persistent eosinophilic inflammation in the airways.

### ***Oxidative stress***

As in all inflammatory diseases, there is increased oxidative stress in allergic inflammation, as activated inflammatory cells, such as macrophages and eosinophils, produce reactive oxygen species. Evidence for increased oxidative stress in asthma is provided by the increased concentrations of 8-isoprostane (a product of oxidised arachidonic acid) in exhaled breath condensates [59] and increased ethane (a product of oxidative lipid peroxidation) in exhaled breath of asthmatic patients [123]. There is also persuasive epidemiological evidence that a low dietary intake of antioxidants is linked to an increased prevalence of asthma [124]. Increased oxidative stress is related to disease severity and may amplify the inflammatory response and reduce responsiveness to corticosteroids, particularly in severe disease and during exacerbations. One of the mechanisms whereby oxidative stress may be detrimental in asthma is through the reaction of superoxide anions with nitric oxide (NO) to form the reactive radical peroxynitrite, that may then modify several target proteins.

### ***Endothelins***

Endothelins are potent peptide mediators that are vasoconstrictors and bronchoconstrictors [125, 126]. Endothelin-1 levels are increased in the sputum of patients with asthma; these levels are modulated by allergen exposure and steroid treatment [127, 128].

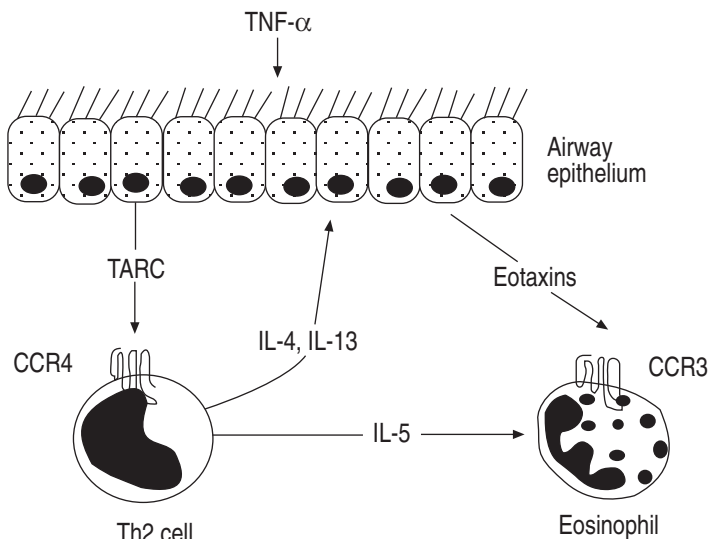


Fig. 6. – Chemokines in asthma. Tumour necrosis factor (TNF)- $\alpha$  releases thymus and activation regulated chemokine (TARC) from epithelial cells which attracts T-helper (Th)2 cells *via* activation of CCR4 receptors. These promote eosinophilic inflammation directly through the release of interleukin (IL)-5 and indirectly *via* the release of IL-4 and IL-13 which induce eotaxin formation in airway epithelial cells.

Endothelins are also expressed in the nasal mucosa in rhinitis [129]. Endothelins induce airway smooth muscle cell proliferation and promote a profibrotic phenotype and may therefore play a role in the chronic inflammation of asthma.

### Nitric oxide

NO is produced by several cells in the airway by NO synthases [130, 131]. Although the cellular source of NO within the lung is not known, inferences based on mathematical models suggest that it is the large airways which are the source of NO [132]. Current data indicate that the level of NO in the exhaled air of patients with asthma is higher than the level of NO in the exhaled air of normal subjects [133]. The elevated levels of NO in asthma are more likely reflective of an as yet to be identified inflammatory mechanism than of a direct pathogenetic role of this gas in asthma [134, 135]. Recent data suggest that the level of NO in exhaled air may increase in acute exacerbations of asthma due to a fall in pH (increased acidity) associated with inflammation [136]. The combination of increased oxidative stress and NO may lead to the formation of the potent radical peroxynitrite that may result in nitrosylation of proteins in the airways [137]. Measurement of exhaled NO in asthma is increasingly used as a noninvasive way of monitoring the inflammatory process [138].

### Effects of inflammation

The acute and chronic allergic inflammatory responses have several effects on the target cells of the respiratory tract, resulting in the characteristic pathophysiological changes associated with asthma (fig. 7). Important advances have recently been made in understanding these changes, although their precise role in producing clinical symptoms is often not clear. There is considerable current interest in the structural changes that occur in the airways of patients with asthma that are loosely termed "remodelling". It is believed that these changes underlie the irreversible changes in airway function that occur

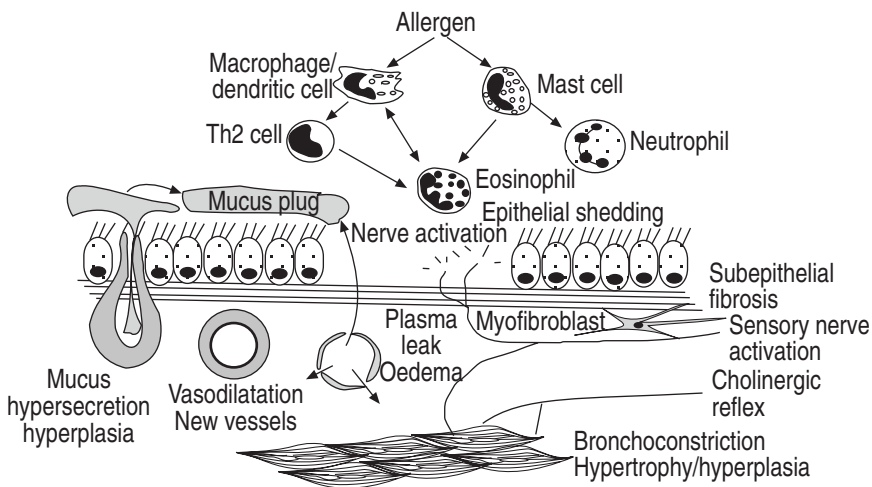


Fig. 7. – The pathophysiology of asthma is complex with participation of several interacting inflammatory cells which result in acute and chronic inflammatory effects on the airway. Th2: T-helper 2 cells.

in some patients with asthma [139, 140]. However, many patients with asthma continue to have normal lung function throughout life, so it is likely that genetic factors may determine which patients develop these structural changes in the airways.

### ***Epithelium***

Airway epithelial shedding is a characteristic feature of asthma and may be important in contributing to AHR, explaining how several different mechanisms, such as ozone exposure, virus infections, chemical sensitisers and allergen exposure, can lead to its development, since all these stimuli may lead to epithelial disruption. Epithelium may be shed as a consequence of inflammatory mediators, such as eosinophil basic proteins and oxygen-derived free radicals, together with various proteases released from inflammatory cells. Epithelial cells are commonly found in clumps in the BAL or sputum (Creola bodies) of asthmatics, suggesting that there has been a loss of attachment to the basal layer or basement membrane. Epithelial damage may contribute to AHR in a number of ways, including loss of its barrier function to allow penetration of allergens, loss of enzymes (such as neutral endopeptidase) which normally degrade inflammatory mediators, loss of a relaxant factor (so called epithelium-derived relaxant factor), and exposure of sensory nerves which may lead to reflex neural effects on the airway. Epithelial shedding may be a feature of more severe asthma and the airway epithelium may be largely intact in patients with asthma, although it does appear to be more fragile. It is not certain whether airway epithelial cells may be activated directly by inhaled allergens. Several inhaled allergens are proteases that may activate protease-activated receptor (PAR)-2, which shows increased expression in airway epithelial cells of asthmatic patients [141].

As discussed above, epithelial cells appear to be an important source of mediators in allergic inflammation (fig. 3). Release of mediators from epithelial cells may be stimulated by various inhaled stimuli, resulting in an increased inflammatory response. Epithelial cells may also release growth factors that stimulate structural changes in the airways, including fibrosis, angiogenesis and proliferation of airway smooth muscle. These responses may be seen as an attempt to repair the damage caused by chronic inflammation [142].

### ***Fibrosis***

The basement membrane in asthma appears on light microscopy to be thickened, but on closer inspection by electron microscopy it has been demonstrated that this apparent thickening is due to subepithelial fibrosis with deposition of Type III and V collagen below the true basement membrane [143, 144]. Several profibrotic cytokines, including TGF $\beta$  and platelet-derived growth factor (PDGF), and mediators such as endothelin-1 can be produced by epithelial cells or macrophages in the inflamed airway [143]. Even mechanical manipulation can alter the phenotype of airway epithelial cells to release profibrotic growth factors [145]. The role of fibrosis in asthma is unclear, as subepithelial fibrosis has been observed even in mild asthmatics at the onset of disease, so it is not certain whether the collagen deposition has any functional consequences. These changes may lead to irreversible loss of lung function in patients with asthma, although it may be that these changes are not functionally important as they are not correlated with disease severity [146, 147]. There is also evidence for fibrosis in airway smooth muscle and deeper in the airway and this is more likely to have functional consequences [148]. However, the fact that asthmatic patients are subject to chronic inflammation over many decades without gross fibrosis of the airways argues that there must be powerful

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inhibitory mechanisms that prevent a fibrotic reaction to the multiple profibrotic mediators produced.

### ***Airway smooth muscle***

There is still debate about the role of abnormalities in airway smooth muscle in asthmatic airways. Airway smooth muscle contraction plays a key role in the symptomatology of asthma and many inflammatory mediators released in asthma have bronchoconstrictor effects. More recently it has been recognised that airway smooth muscle cells may also have other functions in asthmatic airways [149]. *In vitro* airway smooth muscle from asthmatic patients usually shows no increased responsiveness to spasmogens. Reduced responsiveness to  $\beta$ -adrenergic agonists has been reported in *post mortem* or surgically removed bronchi from asthmatics, although the number of  $\beta$ -receptors is not reduced, suggesting that  $\beta$ -receptors have been uncoupled [150]. These abnormalities of airway smooth muscle may be a reflection of the chronic inflammatory process. For example, chronic exposure to inflammatory cytokines, such as IL-1 $\beta$ , downregulates the response of airway smooth muscle to  $\beta_2$ -adrenergic agonists *in vitro* and *in vivo* [151–153]. The reduced  $\beta$ -adrenergic responses in airway smooth muscle could be due to phosphorylation of the stimulatory G-protein coupling  $\beta$ -receptors to adenylyl cyclase, resulting from the activation of protein kinase C by the stimulation of airway smooth muscle cells by inflammatory mediators and to increased activity of the inhibitory G-protein ( $G_i$ ) induced by proinflammatory cytokines [152, 154, 155].

Inflammatory mediators may modulate the ion channels that serve to regulate the resting membrane potential of airway smooth muscle cells, thus altering the level of excitability of these cells. Furthermore, modulation of the activation kinetics of other ion channels by key inflammatory mediators can lead to altered contractile characteristics of smooth muscle.

In asthmatic airways there is also a characteristic hypertrophy and hyperplasia of airway smooth muscle [156], which is presumably the result of stimulation of airway smooth muscle cells by various growth factors, such as PDGF, or endothelin-1 released from inflammatory cells [143, 157]. Airway smooth muscle also has a secretory role in asthma and has the capacity to release multiple cytokines, chemokines and lipid mediators [83].

### ***Vascular responses***

Allergic inflammation has several effects on blood vessels in the respiratory tract. Vasodilatation occurs in inflammation, yet little is known about the role of the airway circulation in asthma, partly because of the difficulties involved in measuring airway blood flow. Recent studies using an inhaled absorbable gas have demonstrated an increased airway mucosal blood flow in asthma [158]. An increased rise in temperature of exhaled breath has been reported in patients with asthma, which presumably reflects the increased vascularity associated with inflammation [159]. The bronchial circulation may play an important role in regulating airway calibre, since an increase in the vascular volume may contribute to airway narrowing. Increased airway blood flow may be important in removing inflammatory mediators from the airway, and may play a role in the development of exercise-induced asthma [160]. There may also be an increase in the number of blood vessels in asthmatic airways as a result of angiogenesis due to the release of growth factors such as vascular-endothelial growth factor (VEGF) and TNF- $\alpha$  [161, 162]. There is increased expression of VEGF in asthmatic airways, particularly in macrophages and eosinophils and this is related to increased vascularity [163].

Microvascular leakage is an essential component of the inflammatory response and many of the inflammatory mediators implicated in asthma produce this leakage [164, 165]. There is good evidence for microvascular leakage in asthma and it may have several consequences on airway function, including increased airway secretions, impaired mucociliary clearance, formation of new mediators from plasma precursors (such as kinins) and mucosal oedema which may contribute to airway narrowing and increased AHR [166, 167].

### ***Mucus hypersecretion***

Mucus hypersecretion is a common inflammatory response in secretory tissues. Increased mucus secretion contributes to the viscid mucus plugs which occlude asthmatic airways, particularly in fatal asthma. There is evidence for hyperplasia of submucosal glands which are confined to large airways and of increased numbers of epithelial goblet cells in asthmatic airways [168, 169]. This increased secretory response may be due to inflammatory mediators acting on submucosal glands and due to stimulation of neural elements [170]. Th2 cytokines IL-4, IL-13 and IL-9, have all been shown to induce mucus hypersecretion in experimental models of asthma [168, 171–173]. The mediators that result in mucus hyperplasia are not yet fully understood, but recent evidence suggests that epithelial growth factor (EGF) play an important role in mucus secretion of upper and lower airways [173]. Indeed EGF may be the final common pathway for many stimuli that stimulate mucus secretion, including IL-13 and oxidative stress [174, 175]. EGF may stimulate the expression of the mucin gene MUC5AC which shows increased expression in asthma [168, 169]. The functional role of hypertrophy and hyperplasia of the muco-secretory apparatus in asthma is not yet known as it is difficult to quantify mucus secretion in airways. Recent experimental data indicate that AHR and mucus hypersecretion, together with MUC5AC expression is associated with the expression of a specific calcium-activated chloride channel in goblet cells designated gob-5, which has a human counterpart hCLCA1 [176]. Overexpression of gob-5 induced marked AHR and mucus hypersecretion in mice, indicating that mucus hypersecretion may play a role in AHR.

### ***Neural effects***

There has been a revival of interest in neural mechanisms in asthma and rhinitis, particularly in the context of symptomatology and AHR [177]. Autonomic nervous control of the respiratory tract is complex, for in addition to classical cholinergic and adrenergic mechanisms, nonadrenergic noncholinergic (NANC) nerves and several neuropeptides have been identified in the respiratory tract [178, 179]. Several studies have investigated the possibility that defects in autonomic control may contribute to AHR in asthma, and abnormalities of autonomic function, such as enhanced cholinergic and  $\alpha$ -adrenergic responses or reduced  $\beta$ -adrenergic responses, have been proposed. Current thinking suggests that these abnormalities are likely to be secondary to the disease, rather than primary defects [177]. It is possible that airway inflammation may interact with autonomic control by several mechanisms.

There is a close interaction between nerves and inflammatory cells in allergic inflammation, as inflammatory mediators active and modulate neurotransmission, whereas neurotransmitters may modulate the inflammatory response. Inflammatory mediators may act on various prejunctional receptors on airway nerves to modulate the release of neurotransmitters [180]. Inflammatory mediators may also activate sensory nerves, resulting in reflex cholinergic bronchoconstriction or release of inflammatory neuropeptides.



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Bradykinin is a potent activator of unmyelinated sensory nerves (C-fibres) [181], but also sensitises these nerves to other stimuli [182].

Inflammatory products may also sensitise sensory nerve endings in the airway epithelium, so that the nerves become hyperalgesic. Hyperalgesia and pain (dolor) are cardinal signs of inflammation, and in the asthmatic airway may mediate cough and chest tightness, which are such characteristic symptoms of asthma. The precise mechanisms of hyperalgesia are not yet certain, but mediators such as PG, certain cytokines and neurotrophins may be important. Neurotrophins, which may be released from various cell types in peripheral tissues, may cause proliferation and sensitisation of airway sensory nerves [183, 184]. Neurotrophins, such as nerve growth factor (NGF), may be released from inflammatory and structural cells in asthmatic airways and then stimulate the increased synthesis of neuropeptides, such as substance P (SP), in airway sensory nerves, as well as sensitising nerve endings in the airways [185]. Thus, NGF is released from human airway epithelial cells after exposure to inflammatory stimuli [186]. Neurotrophins may play an important role in mediating AHR in asthma [187].

Bronchodilator nerves which are nonadrenergic are prominent in human airways and it has been suggested that these nerves may be defective in asthma [188]. In animal airways vasoactive intestinal peptide (VIP) has been shown to be a neurotransmitter of these nerves and a striking absence of VIP-immunoreactive nerves has been reported in the lungs from patients with severe fatal asthma [189]. However, no difference in expression of VIP has been reported in bronchial biopsies from asthmatic patients [190]. It is likely that this loss of VIP-immunoreactivity in severe asthma is explained by degradation by tryptase released from degranulating mast cells in the airways of asthmatics. In human airways the single bronchodilator neurotransmitter appears to be NO [191].

Airway nerves may also release neurotransmitters which have inflammatory effects. Thus neuropeptides such as SP, neurokinin A and calcitonin-gene related peptide may be released from sensitised inflammatory nerves in the airways which increase and extend the ongoing inflammatory response [192, 193] (fig. 8). There is evidence for an increase in SP-immunoreactive nerves in airways of patients with severe asthma [194], which may be due to proliferation of sensory nerves and increased synthesis of sensory neuropeptides as a result of NGF released during chronic inflammation, although this has not been confirmed in milder asthmatic patients [190]. There may also be a reduction in the activity of enzymes, such as neutral endopeptidase, which degrade neuropeptides such as SP [195]. There is also evidence for increased gene expression of the receptors which mediate the inflammatory effects (NK1) and bronchoconstrictor effects (NK2) of SP [196, 197]. Thus chronic asthma may be associated with increased neurogenic inflammation, which may provide a mechanism for perpetuating the inflammatory response even in the absence of initiating inflammatory stimuli. At present there is little direct evidence for neurogenic inflammation in asthma, but this is partly because it is difficult to make the appropriate measurements in the lower airways [193].

## Transcription factors

The chronic inflammation of asthma is due to increased expression of multiple inflammatory proteins (cytokines, enzymes, receptors, adhesion molecules). In many cases these inflammatory proteins are induced by transcription factors, deoxyribonucleic acid (DNA) binding factors that increase the transcription of selected target genes [198] (fig. 9). One transcription factor that may play a critical role in asthma is NF- $\kappa$ B, which can be activated by multiple stimuli, including protein kinase C activators, oxidants and

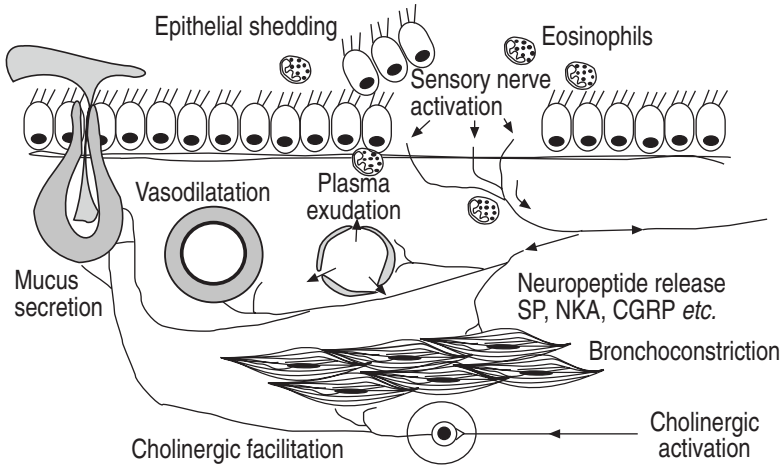


Fig. 8. – Possible neurogenic inflammation in asthmatic airways *via* retrograde release of peptides from sensory nerves *via* an axon reflex. Substance P (SP) causes vasodilatation, plasma exudation and mucus secretion, whereas neurokinin A (NKA) causes bronchoconstriction and enhanced cholinergic reflexes and calcitonin gene-related peptide (CGRP) vasodilatation.

proinflammatory cytokines (such as IL-1 $\beta$  and TNF- $\alpha$ ) [199]. There is evidence for increased activation of NF- $\kappa$ B in asthmatic airways, particularly in epithelial cells and macrophages [200, 201]. NF- $\kappa$ B regulates the expression of several key genes that are overexpressed in asthmatic airways, including proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , GM-CSF), chemokines (RANTES, MIP-1 $\alpha$ , eotaxin), adhesion molecules (ICAM-1, VCAM-1) and inflammatory enzymes (cyclooxygenase-2 and iNOS). The c-Fos component of AP-1 is also activated in asthmatic airways and often cooperates with NF- $\kappa$ B in switching on inflammatory genes [202]. Many other transcription factors are involved

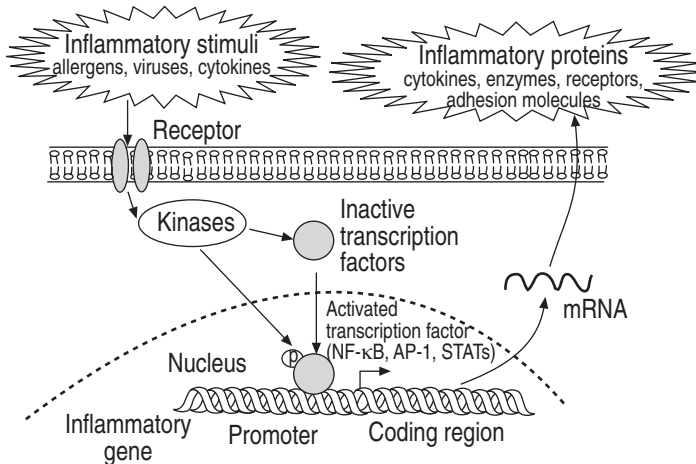


Fig. 9. – Transcription factors play a key role in amplifying and perpetuating the inflammatory response in asthma. Transcription factors, including nuclear factor kappa-B (NF- $\kappa$ B), activator protein-1 (AP-1) and signal transduction-activated transcription factors (STATs) are activated by inflammatory stimuli and increase the expression of multiple inflammatory genes. mRNA: messenger ribonucleic acid.

in the abnormal expression of inflammatory genes in asthma and there is growing evidence that there may be a common mechanism that involves activation of co-activator molecules at the start site of transcription of these genes that are activated by transcription factors to induce acetylation of core histones around DNA is wound in the chromosome. This unwinds DNA, opening up the chromatin structure, and allows gene transcription to proceed [203, 204].

Transcription factors play a critical role in determining the balance between Th1 and Th2 cells. There is persuasive evidence that GATA-3 determines the differentiation of Th2 cells [205] and shows increased expression in asthmatic patients [206, 207]. The differentiation of Th1 cells is regulated by the transcription factor T-bet [208]. Deletion of the T-bet gene is associated with an asthma-like phenotypes in mice, suggesting that it may play an important role in regulating against the development of Th2 cells [209].

## Anti-inflammatory mechanisms

Although most emphasis has been placed on inflammatory mechanisms, there may be important anti-inflammatory mechanisms that may be defective in asthma, resulting in increased inflammatory responses in the airways [210]. Endogenous cortisol may be important as a regulator of the allergic inflammatory response and nocturnal exacerbation of asthma may be related to the circadian fall in plasma cortisol. Blockade of endogenous cortisol secretion by metyrapone results in an increase in the late response to allergen in the skin [211]. Cortisol is converted to the inactive cortisone by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase which is expressed in airway tissues [212]. It is possible that this enzyme functions abnormally in asthma or may determine the severity of asthma.

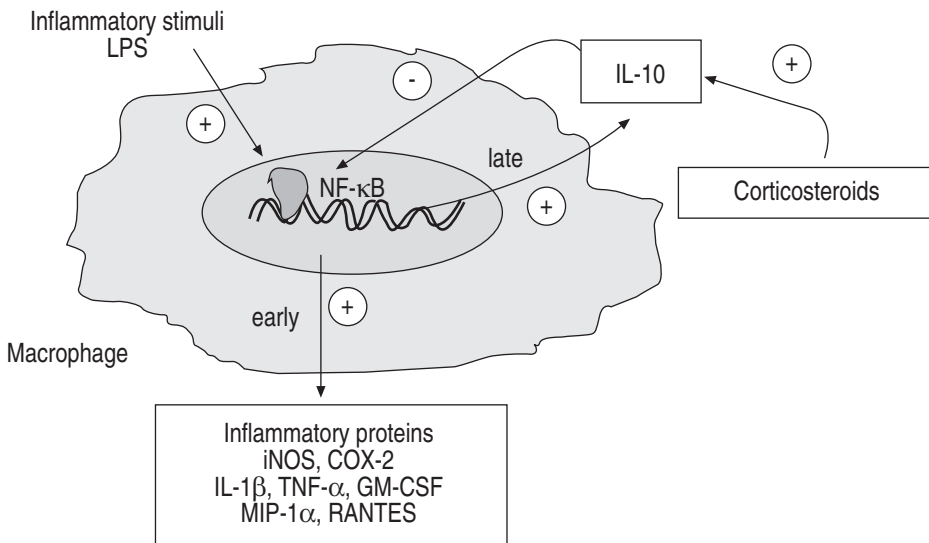


Fig. 10. – Interleukin (IL)-10 is an anti-inflammatory cytokines that may inhibit the expression of inflammatory mediators from macrophages. IL-10 secretion is deficient in macrophages from patients with asthma, resulting in increased release of inflammatory mediators. NF- $\kappa$ B: nuclear factor kappa-B; LPS: lipopolysaccharide; inducible nitric oxide synthase; COX: cyclooxygenase; TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; RANTES: regulated on activation T-cell expressed and secreted; MIP: macrophage inflammatory protein.

Various cytokines have anti-inflammatory actions [213]. IL-1 receptor antagonist (IL-1ra) inhibits the binding of IL-1 to its receptors and therefore has a potential anti-inflammatory potential in asthma. It is reported to be effective in an animal model of asthma [214]. IL-12 and IFN- $\gamma$  enhance Th1 cells and inhibit Th2 cells.

IL-12 promotes the differentiation and thus the suppression of Th2 cells, resulting in a reduction in eosinophilic inflammation [67]. IL-12 infusions in patients with asthma indeed inhibit peripheral blood eosinophilia [215]. There is some evidence that IL-12 expression may be impaired in asthma [66].

IL-10, which was originally described as cytokine synthesis inhibitory factors, inhibits the expression of multiple inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , GM-CSF) and chemokines, as well as inflammatory enzymes (iNOS, COX-2). There is evidence that IL-10 secretion and gene transcription are defective in macrophages and monocytes from asthmatic patients [21, 216]; this may lead to enhancement of inflammatory effects in asthma and may be a determinant of asthma severity [217] (fig. 10). IL-10 secretion is lower in monocytes from patients with severe compared to mild asthma [218] and there is an association between haplotypes associated with decreased production and severe asthma [219].

Other mediators may also have anti-inflammatory and immunosuppressive effects. PGE<sub>2</sub> has inhibitory effects on macrophages, epithelial cells and eosinophils and exogenous PGE<sub>2</sub> inhibits allergen-induced airway responses and its endogenous generation may account for the refractory period after exercise challenge [220]. However, it is unlikely that endogenous PGE<sub>2</sub> is important in most asthmatics since nonselective cyclooxygenase inhibitors only worsen asthma in a minority of patients (aspirin-induced asthma). Other lipid mediators may also be anti-inflammatory, including 15-hydroxy-eicosatetraenoic (HETE) that is produced in high concentrations by airway epithelial cells. 15-HETE and lipoxins may inhibit cysteinyl-leukotriene effects on the airways [221]. Lipoxins are known to have strong anti-inflammatory effects likely through modulation of the trafficking of key intracellular pro-inflammatory intermediates [222]. The peptide adrenomedullin, which is expressed in high concentrations in the lung, has bronchodilator activity [223] and also appears to inhibit the secretion of cytokines from macrophages [224]. Its role in asthma is currently unknown, but plasma concentrations are no different in patients with asthma [225].

## Summary

Asthma is a complex inflammatory disease that involves many inflammatory cells, over 100 different inflammatory mediators and multiple inflammatory effects, including bronchoconstriction, plasma exudation, mucus hypersecretion and sensory nerve activation. Mast cells play a key role in mediating acute asthma symptoms, whereas eosinophils, macrophages and T-helper 2 cells are involved in the chronic inflammation that underlies airway hyperresponsiveness. There is increasing recognition that structural cells of the airways, including airway epithelial cells and airway smooth muscle cells become an important source of inflammatory mediators. Multiple inflammatory mediators are involved in asthma, including lipid and peptide mediators, chemokines, cytokines and growth factors. Chemokines play a critical role on the selective recruitment of inflammatory cells from the circulation, whereas cytokines orchestrate the chronic inflammation. This chronic inflammation may lead to structural changes in the airways, including subepithelial fibrosis, airway smooth muscle hypertrophy/hyperplasia, angiogenesis and mucus hyperplasia. Proinflammatory

transcription factors, such as nuclear factor- $\kappa$ B and activating protein-1 and GATA-3 play a key role in orchestrating the expression of inflammatory genes. There are several endogenous mechanisms that may counteract the inflammation of asthma and some evidence that these may be deficient in asthma. Because of the complexity of asthma drugs that target a single cell or mediator are unlikely to provide significant clinical benefit; the most effective drugs are those that target many mechanisms.  $\beta_2$ -Agonists are not only the most effective bronchodilators, but they also inhibit mast cells and plasma leakage, whereas corticosteroids inhibit multiple inflammatory effects and the production of cytokines and chemokines.

**Keywords:** Airway hyperresponsiveness, eosinophil, epithelial cell, mast cell, sensory nerve, T-helper 2 cells.

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# Chronic inflammation in asthma

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It is now widely accepted that chronic airway inflammation plays a key role in asthma [1]. This fundamental feature has been included in the most recent definitions of the disease: hence the Global Strategy for Asthma Management and Prevention reports that "asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T-lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli" [2]. Based on this consensus all treatment guidelines focus on the importance of anti-inflammatory drugs (mainly inhaled corticosteroids) to control the disease process [2, 3].

## Eosinophilic airways inflammation in asthma

Eosinophils are potent inflammatory cells which secrete a number of lipid mediators and proteins relevant to the pathophysiology of asthma including leukotrienes (LT)<sub>C<sub>4</sub></sub>, D<sub>4</sub>, and E<sub>4</sub>, platelet-activating factor (PAF) and basic proteins (major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO)) [4]. LT play an important role in asthma through their ability to induce a variety of effects including bronchoconstriction and inflammatory cell recruitment. Basic proteins induce direct damage to the airway epithelium [5] and promote bronchial hyperresponsiveness [6]. Eosinophils are also able to produce pro-inflammatory cytokines and thereby amplify the inflammatory reaction (transforming growth factor (TGF) $\beta$ , tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-4, -5, -6, -8, granulocyte-macrophage colony-stimulating factor (GM-CSF), RANTES (regulated on activation, T-cell expressed and secreted, eotaxin) [4, 7].

Derived from myeloid progenitors in the bone marrow, mature eosinophils circulate briefly in the peripheral blood and home to the site of inflammation under the action of several factors including cytokines and chemokines (see below) [4]. Eosinophil production and maturation are regulated by eosinophil-active cytokines IL-5, IL-3 and GM-CSF [4, 8]. Eosinophils may be activated by factors such as IL-5, PAF, GM-CSF and release toxic basic proteins (MBP, ECP, *etc.*) [4].

There is strong circumstantial evidence that eosinophils are important pro-inflammatory cells in the asthma process, irrespective of the patient's atopic status

[9, 10]. It is well known that blood and sputum eosinophilia are commonly associated with asthma. Moreover, the numbers of eosinophils in peripheral blood, bronchoalveolar (BAL) fluid and bronchial biopsies in a group of asthmatics were elevated when compared to normal controls and it was possible to demonstrate an increasing degree of eosinophilia with clinical severity [9]. Furthermore, immunostaining of the bronchial mucosa of patients who had died from severe asthma revealed the presence of large numbers of activated eosinophils and considerable amounts of MBP deposited in the airways [11]. Increased concentrations of MBP were found in BAL fluid from atopic asthmatics when compared to normal controls and correlations were found between the concentrations of MBP and the numbers of denuded epithelial cells in BAL fluid [12]. In atopic asthmatics, late-phase bronchoconstriction was accompanied by an influx of eosinophils in BAL fluid [13]. This was not observed in individuals developing an isolated early-phase response. Lastly, airway eosinophils are very sensitive to corticosteroid therapy, and their disappearance is associated with an improvement in bronchial hyperresponsiveness [14].

## T-lymphocytes in asthma

T-cells have a central role to play in an antigen-driven inflammatory process, since they are the only cells capable of recognising antigenic material after processing by antigen-presenting cells [15]. CD4+ and CD8+ T-lymphocytes activated in this manner elaborate a wide variety of protein mediators including cytokines which have the capacity to orchestrate the differentiation, recruitment, accumulation and activation of specific granulocytes at mucosal surfaces. T-cell derived products can also influence immunoglobulin production by plasma cells. There now exists considerable support for the hypothesis that allergic diseases and asthma represent specialised forms of cell-mediated immunity, in which cytokines secreted predominantly by activated T-cells (but also by other leukocytes such as mast cells and eosinophils) bring about the specific accumulation and activation of eosinophils [10].

Antigenic peptides are presented to T-cell receptors as a high-affinity complex of peptide and major histocompatibility complex (MHC) molecules. T-cells can be broadly divided into two groups based on their recognition of peptide in the context of either MHC class I gene or MHC II gene products. T-cells recognising endogenously generated peptides, presented with class I molecules, express the CD8 molecule which binds to class I molecules thus increasing the avidity of the interaction. Most cytotoxic T-cells have the CD8+ phenotype. The expression of CD4 by T-cells indicates recognition of peptides in the context of class II molecules to which CD4 is able to bind. Peptides presented in the context of class II MHC proteins generally elicit a T-helper (CD4+ T-lymphocyte) response. T-helper cells can be further subdivided according to the pattern of cytokines elaborated following activation [16, 17]. Great progress in the knowledge of phenotypic and functional activities of different T-cell subsets in mice and humans has been made recently. T-cells secreting cytokines such as IL-2, interferon (IFN)- $\gamma$  and TNF- $\beta$  are referred to as T-helper (Th)1 cells. The cytokines produced by these cells promote cytotoxic T-cell and delayed-type responses and inhibit allergic reactions. T-cells producing IL-4, IL-5, and IL-10 but not IFN- $\gamma$  are referred to as Th2 cells. These cells are critical to allergic diseases and asthma, as they provide B-cell help for isotype switching to immunoglobulin (Ig)E and eosinophil maturation, survival and activation. T-lymphocyte activation and expression of Th2-type cytokines is believed to contribute to tissue eosinophilia and local IgE-dependent events in allergic diseases and asthma [10].

The demonstration of primed circulating blood T-lymphocytes in acute severe asthma



is interesting as it presumably reflects the presence of activated cells in the bronchial mucosa, the major site of the asthmatic inflammatory process [18]. In allergic individuals, circulating blood CD4+ T-lymphocytes produce high levels of Th2-type cytokines including IL-5, GM-CSF and IL-3 and may therefore promote eosinophilic inflammation [10, 19]. Moreover, elevated numbers of CD4+ T-lymphocytes expressing IL-5 messenger ribonucleic acid (mRNA) have been demonstrated in the airways from asthmatics compared with nonasthmatic controls [20]. More precisely a Th2-like cytokine profile has been identified in bronchial samples from atopic and nonatopic asthma [21, 22]. This is in agreement with the demonstration that CD4+ and to a lesser extent CD8+ T-cell lines grown from BAL cells from atopic asthmatics produce more IL-5 protein than in control subjects [23].

Activated T-lymphocytes are usually sensitive to corticosteroid therapy and improvement of bronchial hyperresponsiveness and reduction in airway eosinophils parallels reduction of activated CD25+ T-lymphocytes and Th2-type cytokines including IL-4 and IL-5 [14]. However some patients with chronic severe asthma are refractory to corticosteroids [24]. Pharmacological targeting of T-cells has been proposed as a novel approach to the treatment of corticosteroid-dependent or corticosteroid-resistant asthma. A 12-week randomised, double-blind, placebo-controlled, crossover trial established that cyclosporin A improves lung function in patients with corticosteroid-dependent chronic severe asthma [25]. Compared with placebo, a 36-week treatment with cyclosporin A resulted in a significant reduction in median daily prednisolone dosage and total prednisolone intake [26]. In addition morning peak expiratory flow rate improved significantly in the active treatment group but not in the placebo group [26]. Using placebo-controlled, double-blind conditions SIHRA *et al.* [27] showed that cyclosporin A inhibited the late, but not the early, bronchoconstrictor response to inhaled allergen challenge of sensitised mild atopic asthmatics. These data support the concept that T-cells play a crucial role in asthma, as cyclosporin A exerts its immunosuppressive action primarily by inhibition of antigen-induced T-lymphocyte activation and the transcription and translation of mRNA for several cytokines including IL-2, IL-5 and GM-CSF [28]. More recently a single intravenous infusion of a chimeric monoclonal antibody that binds specifically to human CD4 antigen has been evaluated in severe corticosteroid-dependent asthmatics. This randomised double-blind, placebo-controlled trial demonstrated significant increases in morning and evening peak flow rates in the highest dose cohort [29]. Additional experiments showed that keliximab infusion induced a rapid and effective binding to all CD4+ T-cells with a transient reduction in numbers of circulating CD4+ T-cells and modulation of CD4 expression, further suggesting that therapy aimed at the CD4+ T-cell may be useful in asthma [30].

## Other inflammatory cells

### *B-cells*

Th2-type cytokine-induced B-cell activation and subsequent IgE production is believed to be a critical characteristic of patients with atopy, a disorder characterised by sustained, inappropriate IgE responses to common environmental antigens ("allergens") encountered at mucosal surfaces [31, 32]. Interaction of environmental allergens with cells sensitised by binding of surface Fc receptors to allergen-specific IgE is assumed to play a role in the pathogenesis of atopic asthma. Stimulation of IgE synthesis by B-cells is mainly driven by IL-4 [31]. It has recently been shown that in both atopic and nonatopic asthmatics, airways CD20+ B-cells have the potential to switch in favour of

IgE heavy-chain production, supporting the concept that local IgE production may occur in these patients [33]. These changes may be at least in part under the regulation of IL-4.

### ***Mast cells and basophils***

Mast cells and basophils have long been recognised as major effector cells of allergic reactions by virtue of their high affinity surface receptors for IgE (FcεRI) [1]. The early phase bronchoconstrictor response to allergen challenge of sensitised atopic asthmatics can probably be accounted for by mast cell and basophil products, mostly histamine. Mast cells can produce and store several cytokines which may play a role in the chronic asthmatic process, including TNF-α, IL-4, IL-5, and IL-6 [34]. Mast cells are also believed to be responsible at least in part of airways remodelling through fibroblasts activation. Mast cells have been described in the airways of asthmatics, and, although not necessarily increased in number, are in the activated state (degranulated) [35]. BB1+ basophils were identified in baseline bronchial biopsies of asthmatics, although eosinophils and mast cells were 10-fold higher. Similarly basophils increased after allergen inhalation in atopic asthma, but again basophils were <10% of eosinophils [36].

### ***Macrophages***

Macrophages are phagocytic cells derived from bone marrow precursors. They play a fundamental role as accessory cells and they also produce several mediators and cytokines promoting chronic inflammation. Macrophages infiltrate the asthmatic airways, especially in nonatopic patients, but also in atopics where allergen challenge activates macrophages [32, 37, 38]. This cell type may also play a role in airway remodelling through the production of growth factors such as platelet-derived growth factor (PDGF), bFGF and TGF-β [1].

### ***Dendritic cells***

Dendritic cells are specialised in antigen processing and presentation. IgE presumably plays a role in their function as they express high numbers of FcεRI. These cells are essential in the induction of immune responses within the airways and their numbers are increased in asthma. Their role in human asthma is still a matter of debate [1].

### ***Fibroblasts***

Fibroblasts are responsible for the production of collagen and reticular and elastic fibres. Myofibroblasts numbers in the submucosa correlate with subepithelial collagen deposition, supporting a role in airway remodelling [1].

### ***Neutrophils***

These cells are recruited in the airways after allergen challenge and are found in elevated numbers in cases of fatal asthma [39]. Their role in asthma however remains unclear.

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## Cytokines in asthma

### *Pro-eosinophilic cytokines*

Accumulating evidence tends to show that the combined effects of a wide array of cytokines produced by different cell types including activated T-lymphocytes could play a major part in regulating the successive steps leading to a characteristic eosinophil-rich airways inflammation [10]. It is well established that tissue recruitment of eosinophils from the bloodstream requires rolling and firm adhesion of circulating cells under the control of cytokine-induced adhesion molecules (mostly of selectin and integrin families) and migration following a gradient of chemotactic substances in which the newly described family of chemokines are of utmost importance [40, 41]. In addition eosinophils can be activated by several environmental factors including eosinophil-active cytokines (IL-5, GM-CSF and IL-3) [42–44]. As a result, tissue damage is due at least in part to the release of toxic granule proteins from activated infiltrating eosinophils [5, 6].

***Eosinophil active cytokines (IL-5, GM-CSF, IL-3).*** T-lymphocytes are thought to orchestrate eosinophilic inflammation in asthma through the release of cytokines including "eosinophil-active" cytokines (IL-5, GM-CSF and IL-3) which promote eosinophil maturation, activation, hyperadhesion and survival. The relevance of IL-5 to asthma has been highlighted by the demonstration of elevated numbers of bronchial mucosal activated (EG2+) eosinophils expressing the IL-5 receptor  $\alpha$ -chain mRNA in asthmatics and by positive correlations between the numbers of cells expressing IL-5 mRNA and markers of asthma severity such as bronchial hyperresponsiveness and asthma symptom (Aas) score [9, 45, 46]. Moreover aerosolised *Ascaris suum* extract-induced airways inflammation and bronchial hyperresponsiveness in nonhuman primates are dramatically reduced by the intravenous infusion of an anti-IL-5 monoclonal antibody (TRFK-5) prior to parasite extract inhalation [47]. However, preliminary data in human asthma indicate that anti-IL-5 dramatically reduces allergen-induced eosinophilia although no significant effect was observed on the magnitude of the late-phase reaction and bronchial hyperresponsiveness [48].

Using double immunohistochemistry and *in situ* hybridisation, 70% of IL-5 mRNA+ signals co-localized to CD3+ T-cells, the majority of which (>70%) were CD4+, although CD8+ cells also expressed IL-5 [20]. The remaining signals co-localized to mast cells and eosinophils [20]. In contrast double immunohistochemistry showed that IL-5 immunoreactivity was predominantly associated with eosinophils and mast cells. However, numbers of IL-5+ cells detected by immunohistochemistry were relatively low, raising the possibility that insufficient protein accumulated within T-cells to enable detection by immunohistochemistry [20].

GM-CSF and IL-3 are also thought to participate to the bronchial pro-eosinophilic cytokine network in asthma [43, 44]. T-cell lines grown from BAL cells in patients with atopic asthma have the capacity of producing elevated quantities of GM-CSF [23]. Recently, IL-5, IL-8 and GM-CSF immunostaining of sputum cells in bronchial asthma and chronic bronchitis has shown that the numbers of IL-5 and GM-CSF immunostained cells was increased in asthma, a condition characterised by elevated sputum eosinophilia, compared to chronic bronchitis where elevated IL-8 expression paralleled sputum neutrophilia [49]. Others have shown that bronchial epithelial cells are also able to participate to the production of GM-CSF in asthma, emphasising that noninflammatory cells can participate actively to the local inflammatory process [50]. Interestingly, inhaled corticosteroid attenuates both epithelial cell GM-CSF expression and the numbers of epithelial activated eosinophils, suggesting that inhaled corticosteroids could

attenuate airways inflammation partly by down-regulating epithelial cell cytokine expression [51]. Lastly, GM-CSF could also act on macrophages, as suggested by elevated  $\alpha$ GM-CSF receptor expression on CD68+ macrophages in nonatopic asthmatics [32, 52].

***Cytokine-induced upregulation of adhesion molecules.*** To migrate from the bloodstream to the bronchial mucosa, eosinophils must adhere to vascular endothelial cells, extracellular matrix components and tissue cells. Cell recruitment to the inflamed tissue consists of at least three events: rolling, firm adhesion and transendothelial migration [40]. Granulocyte margination and diapedesis at sites of inflammation seem to be principally under the control of the cytokine-induced upregulated expression of several endothelial adhesion molecules including P-selectin, E-selectin (ELAM-1), intercellular adhesion molecule (ICAM)-1 (CD54) and vascular cell adhesion molecule (VCAM)-1. The leukocyte receptors for the P- and E-selectins exist on most leukocytes [53]. The leukocyte receptors for ICAM-1 are LFA-1a (CD11a/CD18) and Mac-1 (CD11b/CD18) and those for VCAM-1 are VLA-4.

In the asthmatic airways, "pro-inflammatory" cytokines such as TNF- $\alpha$  can upregulate ICAM-1 and E-selectin expression and therefore granulocyte recruitment [40, 54]. More specifically, the expression of VLA-4 on lymphocytes and eosinophils but not on neutrophils, has led to the hypothesis that VCAM-1 may be the predominant endothelial regulator of the chronic asthmatic bronchial mucosal inflammation. VCAM-1 is upregulated by several cytokines including IL-4 and IL-13 [55, 56]. IL-4 and IL-13 have been detected in the asthmatic airways [46, 57], emphasising the fact that specific eosinophilic recruitment through an IL-4 (and/or IL-13) induced upregulation of VCAM-1 endothelial expression could participate to chronic bronchial mucosal inflammation. Animal models of asthma and IL-4-deficient mice have shown that this cytokine might be critical to the development of an allergic eosinophilic response [58].

***Eosinophil chemokines.*** Classical chemoattractants such as C5a act broadly on neutrophils, eosinophils, basophils and monocytes. The past few years have seen the discovery of a group of chemoattractive cytokines (termed chemokines) with similarities in structure whose principal activities appear to include chemoattraction and activation of leukocytes including granulocytes, monocytes and T-lymphocytes [41]. Chemokines are polypeptides of relatively small molecular weight (8–14kDa) which have been assigned to different subgroups by structural criteria. The  $\alpha$ - and  $\beta$ -chemokines, which contain four cysteines, are the largest families. The  $\alpha$ -chemokines have their first two cysteines separated by one additional amino acid ("CXC chemokines": IL-8, *etc.*), whereas these cysteines are adjacent to each other in the  $\beta$ -chemokine subgroup ("CC chemokines": eotaxins, monocyte chemoattractant proteins (MCPs), RANTES). Interestingly chemokines are distinguished from classical chemoattractants by a certain cell-target specificity: the CXC chemokines tend to act more on neutrophils, whereas the CC chemokines tend to act more on monocytes and in some cases basophils, lymphocytes and eosinophils [59]. Owing to the effects of some CC-chemokines on basophils and eosinophils, their ability to attract and activate monocytes, and their potential role in lymphocyte recruitment, these molecules have emerged as the most potent stimulators of effector-cell accumulation and activation in allergic inflammation [41]. The CC chemokines interacting with the "eotaxin receptor" CCR3 (eotaxin-1, eotaxin-2, RANTES, MCP-3, MCP-4) are potent pro-eosinophilic cytokines which are believed to play an important role in asthma [60]. Since eosinophil chemokines all stimulate eosinophils *via* CCR3, this receptor is potentially a prime therapeutic target in asthma and other diseases involving eosinophil-mediated tissue damage. Antagonising CCR3 may be particularly relevant to asthma, as this

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receptor is also expressed by several cell types playing a pivotal role in this condition, including Th2-type cells, basophils and mast cells.

Eotaxin mediates eosinophil (but not neutrophil) accumulation *in vivo*. Recently, eotaxin and CCR3 mRNA and protein product have been identified in the bronchial submucosa of atopic and nonatopic asthmatics [61]. Moreover eotaxin and CCR3 expression correlate with airway responsiveness. Cytokeratine-positive epithelial cells and CD31+ endothelial cells were the major source of eotaxin mRNA whereas CCR3 co-localized predominantly to eosinophils [61]. These data are consistent with the hypothesis that damage to the bronchial mucosa in asthma involves secretion of eotaxin by epithelial and endothelial cells resulting in eosinophil infiltration mediated *via* CCR3.

RANTES, MCP-3, and MCP-4 have all the properties that are needed to mobilise and activate basophils and eosinophils and currently available evidence suggests a primary role for them in allergic inflammation [60]. A combined expression of eosinophil chemokines (eotaxins, MCPs and RANTES) together with eosinophil active cytokines (IL-5, GM-CSF and IL-3), has been demonstrated in asthma, indicating that these cytokines could act in synergy to promote the elaboration of an eosinophil-rich bronchial mucosal infiltrate [61, 62]. Indeed, priming eosinophils with IL-5 increases the chemotactic properties of RANTES on eosinophils [63]. The cell sources of RANTES and MCPs in asthma also include primarily epithelial and endothelial cells, as well as macrophages, T-lymphocytes and eosinophils [61]. Interestingly, bronchial epithelial cell production of RANTES is downregulated by inhaled corticosteroids [64].

Due to their cell-target specificity favouring neutrophil chemoattraction, a role for CXC chemokines in asthma is less likely although IL-8 has been shown to be a chemotactic factor for eosinophils [65]. Although eosinophils are most prominent in the airways of asthmatics, fewer eosinophils and more neutrophils have been identified in the airways of sudden-onset fatal asthma [39]. Elevated IL-8 expression has been reported in asthma [65]. In that setting, IL-8 could promote not only neutrophil accumulation but also eosinophil migration in synergy with IL-5.

### ***Pro-atopic cytokines in asthma***

Asthma is often, though not invariably, associated with atopy [66]. Since the clinical classification of asthma by RACKERMAN [67], it has been widely accepted that a subgroup of asthmatics are not demonstrably atopic, the so-called "intrinsic" variant of the disease [67]. Intrinsic asthmatics show negative skin tests and there is no clinical history of allergy. Furthermore, serum total IgE concentrations are within the normal range and there is no evidence of specific IgE antibodies directed against common aeroallergens. These patients are usually older than their allergic counterparts and have onset of their symptoms in later life, often with a more severe clinical course. There is a preponderance of females and the association of nasal polyps and aspirin sensitivity occurs more frequently in the nonatopic form of the disease. Whereas some authors suggest that only ~10% of asthmatics are intrinsic, the Swiss SAPALDIA survey (8,357 adults, aged 18–60 yrs) found that one-third of total asthmatics were nonallergic [68].

Ever since the first description of intrinsic asthma, there has been debate about the relationship of this variant of the disease to atopy [32, 66]. One suggestion is that intrinsic asthma represents a form of autoimmunity, or auto-allergy, triggered by infection as a respiratory influenza-like illness often precedes onset. Other authors have suggested that intrinsic asthmatics are allergic to an as yet undetected allergen. The present authors view is that although intrinsic asthma has a different clinical profile from extrinsic asthma it does not appear to be a distinct immunopathological entity [32]. This concept is supported by the demonstration of elevated numbers of activated eosinophils [37],

Th2-type lymphocytes [69], and cells expressing FcεRI [70] in bronchial biopsies from atopic and nonatopic asthmatics, together with epidemiological evidence indicating that serum IgE concentrations relate closely to asthma prevalence regardless of atopic status [66]. IL-4 expression is a feature of asthma, irrespective of its atopic status, providing further evidence for similarities in the immunopathogenesis of atopic and nonatopic asthma [32]. IL-4 mRNA is mainly CD4+ T- cell derived [20]. Expression of αIL-4 receptor mRNA and protein is significantly elevated in the epithelium and subepithelium of biopsies from atopic and nonatopic asthmatics compared to atopic controls [71]. Recent evidence of the effectiveness of nebulised soluble IL-4 receptors in atopic asthma further support the relevance of this cytokine in this disease [72].

In addition, IL-13 is a cytokine very close to IL-4 which exhibits activities possibly relevant to asthma: promotion of IgE synthesis, eosinophil vascular adhesion by VLA-4/VCAM-1 interaction and promotion of Th2-type cell responses [56, 57, 73]. Importantly IL-4 or IL-13 are absolutely required for IgE-switching in B-cells, a prerequisite for elevated IgE synthesis. The present authors have reported elevated expression of IL-13 mRNA in the bronchial mucosa of so-called atopic and nonatopic asthma [57]. Therefore, although intrinsic asthma have no demonstrable atopy, they have a biological pattern of airway inflammation strongly suggesting a possible "atopic-like" status which may be restricted to the bronchial submucosa [32]. As discussed above, local IgE synthesis in CD20+ B-cells has been demonstrated in the bronchial submucosa of

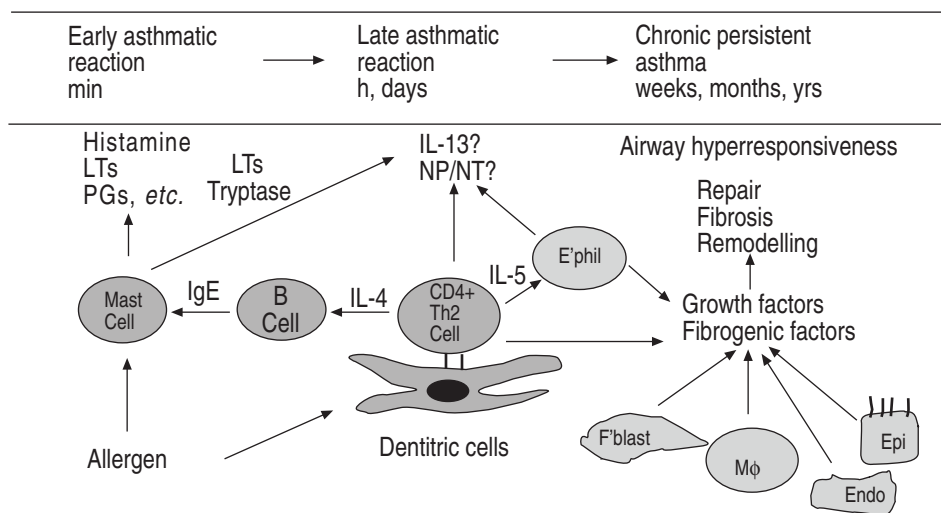


Fig. 1. – Proposed scheme for the progression of asthma in relation to pathogenesis. The progression of asthma with emphasis on the cells and mediators involved is shown diagrammatically. The early asthmatic reaction occurs within minutes and is largely due to the release of histamine and lipid mediators from mast cells following interaction of allergen with cell bound immunoglobulin (IgE). The late asthmatic reaction, which peaks 6–13 h after allergen challenge, is believed to be partially T-cell dependent. For example, the late-phase, but not the early-phase, was inhibited by cyclosporin A [27] and challenge with T-cell peptide epitopes induced an isolated late asthmatic reaction. CD4 cells may interact directly with smooth muscle to produce airway narrowing through the release of neurotrophins (NT) (which in turn activate neuropeptides (NP)). Interleukin (IL)-13 may also play a role in late-phase asthmatic reactions [74]. The role of the eosinophil remains uncertain. On the one hand there is much circumstantial evidence to incriminate eosinophils as pro-inflammatory cells in the asthma process. However, depletion of the eosinophils with anti-IL-5 did not influence the late-phase reaction or bronchial hyperresponsiveness in mild asthmatics [48]. Airway hyperresponsiveness is a feature of chronic persistent asthma and is due in part to airway thickening due to remodelling, fibrosis and other repair processes. These changes are brought about by the elaboration of growth factors and fibrogenic factors from various cell types including eosinophils (E'phil), fibroblasts (F'blast), monocytes (Mφ), epithelial (Epi) cells and endothelial (Endo) cells.

patients with atopic and nonatopic asthma (detection of elevated expression of  $\epsilon$  germ-line gene transcripts and mRNA encoding the  $\epsilon$  heavy chain of IgE) [33]. This along with the demonstration of elevated numbers of cells expressing the high affinity IgE receptor in intrinsic asthma suggests the possibility of local IgE-mediated processes in the absence of detectable systemic IgE production.

## Summary

There now exists considerable support for the hypothesis that asthma represents a specialised form of cell-mediated immunity, in which cytokines, chemokines and other mediators such as leukotrienes secreted by a wide range of inflammatory cells bring about the specific accumulation and activation of eosinophils in the bronchial mucosa (the progression of asthma is diagrammatically depicted in figure 1). These observations have important implications for future therapies, since it suggests that more selective drugs than corticosteroids should be of interest in asthma.

**Keywords:** Cell-mediated immunity, chemokines, cytokines, eosinophils, leukotrienes.

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# **Noninvasive assessment of airway inflammation in asthma**

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As bronchial asthma is currently considered to be and is defined as being an inflammatory disorder of the airways [1], it seems logical to include an assessment of this inflammation in the diagnosis and follow-up of the disease. In this assessment, a few factors need to be taken into account. Firstly, the inflammation underlying asthma is a complex phenomenon that displays characteristics, not only of the acute phase of an inflammatory process, with increased vascular permeability and plasma exudation, but also of a more subacute inflammatory phase with influx of inflammatory cells and in addition, characteristics of the chronic phase of an inflammatory response which is characterised by structural alterations, coined as airway remodelling [2]. The precise functional role of the various cells and mediators possibly involved in these different phases of the inflammatory process, still remain to be established. In addition, the exact relationship between the various components of the inflammatory process and the clinical characteristics of asthma are also uncertain. It has been argued that asthma symptoms mainly reflect the acute inflammation, caused by the release of pro-inflammatory mediators and resulting in widespread airway narrowing, whereas the functional abnormalities such as bronchial hyperresponsiveness are mainly due to airway remodelling [3, 4]. However, these assumptions are largely based on mathematical models that need to be further proven.

Most of the biopsy studies performed so far, have focused on the eosinophil as the prime marker of the (sub)acute phase of the inflammation. In general, these studies show a weak and inconsistent correlation between eosinophil counts and clinical or functional criteria of disease activity such as symptoms, baseline forced expiratory volume in one second (FEV<sub>1</sub>) or peak flow variability [5, 6]. This also applies to the degree of nonspecific bronchial hyperresponsiveness, especially towards a direct acting stimulus such as methacholine or histamine [7, 8]. The correlation with markers of indirect airway responsiveness such as exercise or adenosine is better, but still relatively weak [8]. Similarly, the degree of subepithelial fibrosis as a marker of airway remodelling is not consistently related to the degree of airway responsiveness [9, 10]. These observations imply that clinical and lung function criteria cannot be used as noninvasive indirect markers of the underlying inflammation, but that a more direct assessment is required reflecting the acute and the chronic phase of the inflammatory process. Endobronchial biopsies would enable a direct assessment of the inflammatory process, but the invasiveness of the technique precludes its use in daily clinical practice. Furthermore, it is difficult to evaluate the degree of cellular activation using histochemical techniques and the quantification of inflammatory cells is difficult. What also needs to be borne in mind is that the composition of the airway inflammation in asthma is a dynamic phenomenon,

that can be influenced by external factors such as intensity of allergen exposure or alterations in the treatment regimen [11, 12]. As a consequence, evaluation of the inflammation should not be a snapshot in time, but performed repeatedly. It would therefore seem that the ideal biomarker should not only offer a noninvasive way to quantify the airway inflammation, but in addition, should be cost-effective and easy to perform repeatedly in a clinical setting.

## Noninvasive markers of airway inflammation in asthma

A number of markers have been and are being considered as noninvasive markers of airway inflammation. Examples include blood eosinophil counts or serum eosinophil cationic protein (ECP), urinary eicosanoid metabolites, exhaled gasses, mediators in breath condensate or induced sputum (table 1).

As for any outcome measure, when considering the potential usefulness of any of these markers in the monitoring of disease activity, one of the first elements to be addressed is the reliability and the validity of the markers. Elements of reliability include interobserver consistency and repeatability. In the assessment of validity, a distinction is made between criterion validity or conformity to the gold standard measurement which is agreed to be airway inflammation as assessed in bronchial biopsies and content validity which includes evaluation of the disease specificity of a given marker and the responsiveness to intervention. These various elements have been evaluated to a variable degree for different possible markers of airway inflammation.

### *Blood markers*

***Eosinophils and eosinophil cationic protein.*** Measurement of blood eosinophil counts and serum ECP levels if correctly performed, are reproducible and consistent [13]. However, blood eosinophil counts have been shown to correlate weakly to eosinophil

**Table 1. – Biomarkers in asthma**

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Blood/serum
Eosinophils
Eosinophil cationic protein (ECP)
EPO
sIL-2R
Urine
9 $\alpha$ ,11 $\beta$ -PGF <sub>2</sub>
LTE <sub>4</sub>
EPX
Exhaled air
Nitric oxide
Carbon monoxide
Hydrocarbons
Breath condensate
Hydrogen peroxide
LT metabolites
8-isoprostane
Nitrotyrosine
Induced sputum
Cell differential
Soluble mediators (ECP, cysLT's)

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EPO: eosinophil peroxidase; sIL-2R: soluble interleukin-2 receptor; LT: leukotriene; cysLT's: cysteinyl leukotriene.

numbers in biopsies [14] and have a poor disease specificity. The correlation of serum ECP with the number of eosinophils in biopsies is variable: although in some studies, a correlation has been reported, this has not been invariably confirmed [14–16]. Serum ECP also lacks disease specificity. Increased levels of ECP can be found in various diseases including cystic fibrosis, whereas conversely, a large degree of overlap exists between normal and asthmatic individuals with varying severity [17–19]. Both eosinophil counts and ECP levels respond to factors known to influence the degree of airway inflammation such as changes in treatment or allergen exposure [20–22]. The sensitivity of ECP to these changes in comparison to other possible biomarkers has not been extensively investigated. From the data available, it would seem that ECP is somewhat more sensitive than mere eosinophil counts but less than sputum eosinophil counts [23]. A striking observation is that the response to treatment can be influenced by additional external factors, such as the smoking habits of the patients [24].

**Other markers.** Other circulating markers have been proposed, including soluble interleukin (IL)-2 receptor (CD25). However, these have not been extensively evaluated [25, 26].

### *Urinary markers*

Urinary eosinophil peroxidase (EPX) offers an even less invasive alternative to serum ECP [21, 27], especially for children. Another line of investigation is to measure eicosanoid metabolites in urine such as leukotriene (LT)E<sub>4</sub> or 9 $\alpha$ ,11 $\beta$ PGF<sub>2</sub> [28]. These measurements are reliable but require skilled expertise. How precisely they reflect ongoing inflammation in the airways needs to be further evaluated as increased urinary LTE<sub>4</sub> levels are not limited to asthma and they do not discriminate between asthma and normal subjects [29]. However, urinary markers respond to therapeutic interventions, illustrating their potential usefulness in the long-term monitoring of the disease [21, 30].

### *Exhaled air*

**Nitric oxide.** To date, of the gases present in exhaled air, nitric oxide (NO) has been most extensively investigated [31]. Recommendations have been issued on how to perform NO measurements, thus adding to the reliability of the technique [32, 33]. Weak correlations have been found between exhaled nitric oxide (eNO) and the number of eosinophils in biopsies or in sputum [34–36]. Exhaled NO is increased in nonsteroid treated asthma [37, 38], albeit the increase is not disease specific [39]. In established asthma, a relationship was found between eNO and asthma symptoms or  $\beta_2$ -agonist use [40, 41]. Exacerbations, both in children and in adults are also accompanied by increased NO levels [42]. In a comparative study, eNO proved to correlate better with asthma severity than serum ECP or soluble IL-2 receptor [43].

As part of the criterion validity assessment, eNO has been shown to respond to factors that are known to influence the degree of inflammation in asthma. Exhaled NO increases in response to allergen exposure. This response is sufficiently sensitive to detect naturally occurring changes in allergen exposure over the pollen season [44]. The response to nonallergic stimuli such as pollutants is less consistent [45, 46]. Treatment with short-acting inhaled  $\beta_2$ -agonists does not influence eNO [47]. This is consistent with the observation that these agents do not influence chronic inflammation in asthma, and validates the use of eNO to assess inflammation, independent of airway calibre. Anti-inflammatory compounds such as antileukotrienes, but especially inhaled steroids reduce eNO levels [48, 49]. Exhaled NO has proven to be extremely sensitive to steroid

treatment. Reduction in eNO may be seen within 6 h after a single dose of nebulised steroids [50] or within 2–3 days following treatment with inhaled steroids [49]. Concordantly, steroid-induced changes in NO precede improvement in symptoms, baseline FEV<sub>1</sub> or sputum eosinophilia [35, 49].

**Carbon monoxide.** Other gases that have been measured in exhaled air include carbon monoxide (CO) and hydrocarbons such as ethane and pentane [51, 52]. Both are considered to be representative of the level of oxidative stress. Similar to eNO, comparison with normal subjects indicates that exhaled CO is increased in nonsteroid but not in steroid-treated asthma [53, 54]. It is unclear to what extent exhaled CO and NO differ in their steroid sensitivity. The observation that children with persistent asthma, despite treatment with steroids which reduces their NO levels, have significantly higher exhaled CO compared with those with infrequent episodic asthma has led to the proposal that exhaled CO is less steroid-sensitive than eNO [52].

**Hydrocarbons.** The volatile hydrocarbons ethane and pentane are among the numerous end-products of lipid peroxidation of peroxidised polyunsaturated fatty acids that can be measured by gas chromatography from single breath samples. Increased levels have been measured in nonsteroid treated asthma. Others have described elevated pentane levels during episodes of acute asthma that returned to normal once the acute asthma subsided [55]. Of note is that smoking also increases ethane levels [56].

### **Breath condensate**

Another approach is to measure nonvolatile mediators in condensate of exhaled air. Exhaled breath condensate is collected by cooling or freezing of exhaled air. As the procedure is totally noninvasive and does not influence airway calibre, a major advantage of this technique is that it is extremely well tolerated even by patients with severe airway obstruction and children (figs. 1 and 2). The most common approach is for the subject to breathe *via* a mouthpiece through a nonbreathing valve block in which inspiratory and expiratory air is separated. During expiration, the breathing air flows through a condenser, which is cooled to -20°C. Preventing saliva contamination by swallowing or

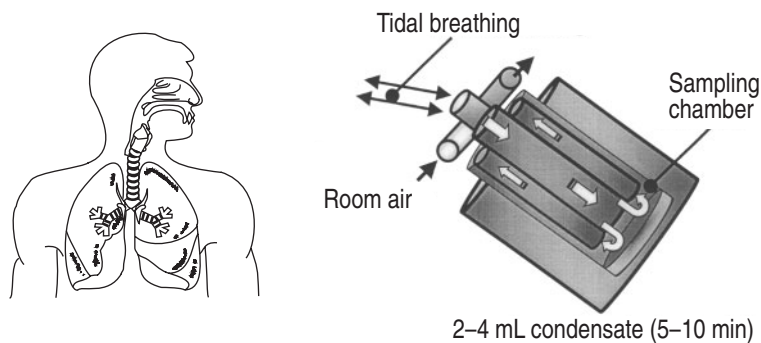


Fig. 1. – Diagram of the apparatus for measuring exhaled breath condensate.

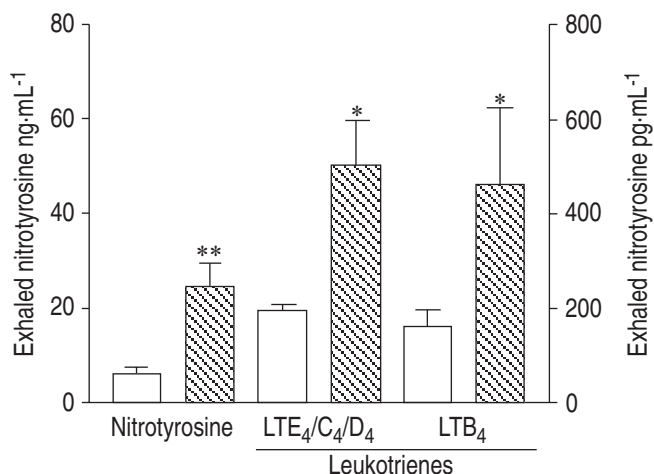


Fig. 2. – Exhaled nitrotyrosine and leukotrienes before and after steroid withdrawal in patients with moderate asthma. □: stable asthma; ▨: unstable asthma. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ .

rinsing the mouth and standardising condensate collection by volume or respiratory rate improves the reproducibility of the results. Exhaled condensate is usually analysed by gas chromatography and/or extraction spectrophotometry, or by immunoassays. Differences in condensate chemistry are thought to reflect changes in the airway lining fluid caused by inflammation and oxidative stress. Condensate from asthmatic subjects contains increased levels of leukotriene B<sub>4</sub>/C<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> in addition to several markers of oxidative stress including hydrogen peroxide, nitrotyrosine and 8-isoprostane [57–61]. Measurement of cytokines has proven less successful to date. Although the analysis of these various molecules in breath condensate remains to be fully validated, it would seem that they could provide useful information in the disease monitoring. Significant differences in hydrogen peroxide levels were observed between controlled and noncontrolled asthma [58], whereas others have shown that 8-isoprostane levels are less sensitive to steroid treatment than eNO or exhaled ethane [60]. As such, these markers might offer complementary information to the very sensitive NO measurements [59].

### ***Induced sputum***

To date, assessment of the reliability and validity of induced sputum has mainly focused on the percentage of eosinophils in the sample. In asthmatics, sputum eosinophil counts have a high interobserver consistency and repeatability, irrespective of the processing technique used [62–64]. In addition, a large number of studies have evaluated the validity of induced sputum. When assessing criterion validity, an element which needs to be borne in mind, is that sputum samples the airway lumen, extending from the central to the peripheral airways with increasing induction time [65–68]. Not unexpectedly therefore, sputum eosinophil counts correlate better with those in bronchoalveolar lavage (BAL) or bronchial wash than with eosinophil numbers in bronchial biopsies [69–72]. This probably reflects, at least in part, the kinetics of the inflammatory process in the airways. Due to differential cell trafficking, the inflammatory cell distribution in the airway lumen can be different from that observed in the airway mucosa. In addition, biopsies offer a snapshot in time of the mucosal inflammation, whereas sputum sample

cells that might have accumulated in the airway lumen over a longer time period. These differences need to be taken into account when using sputum for specific purposes. As for any sample derived from the airway lumen, sputum would seem less appropriate than biopsies for studies focusing on the pathogenesis of asthma. This does however not diminish the potential of sputum eosinophil counts in the diagnosis and clinical follow-up of asthma.

It has been shown that in subjects with obstructive airway disorders, an increased sputum eosinophil percentage has a higher sensitivity and specificity for the diagnosis of asthma than blood eosinophil counts or serum ECP [73]. The degree of sputum eosinophilia was shown to correlate with the clinical severity of the disease, in some studies [74]. In addition, preliminary reports indicate that analysis of induced sputum could help in diagnosing associated conditions such as gastrointestinal reflux by identifying lipid-laden macrophages [75] or associated left heart failure by screening for haemosiderine-laden macrophages recognised by Prussian-blue staining [76]. The possible role of sputum in diagnosing eosinophilic bronchitis as a cause of nonproductive cough has also been highlighted [77].

An important characteristic of induced sputum is its responsiveness to interventions known to affect the degree of inflammation in asthma. As for eNO, allergen exposure increases the per cent eosinophils and metachromatic cells in sputum. This was initially demonstrated following exposure to high doses of allergen given under laboratory conditions to elicit dual asthmatic reactions, which also caused an increase in circulating eosinophil counts [78]. Subsequent studies have illustrated that sputum analysis is sensitive enough to reflect more subtle changes in the degree of airway inflammation. It was shown that repeated exposure to an ~10-fold lower dose of allergen also induced an increase in sputum eosinophil numbers, whereas only a very small increase in blood eosinophil was noted on the last of the five challenge days [79]. Moreover, a significant increase in sputum eosinophils has been documented to occur over the pollen season in subjects with pollen-induced asthma and rhinitis [80]. Similarly, occupational exposure in the workplace can also influence the cellular composition of sputum, without effect on serum ECP [81]. Sputum also responds to nonallergen stimuli including pollutants, such as ozone or diesel exhaust, which have both been shown to increase the number of neutrophils in sputum [46, 82, 83].

Treatment can also influence sputum eosinophil percentages. Monotherapy with short-acting inhaled  $\beta_2$ -agonists has been shown to increase eosinophil counts [84]. However, this effect is not observed when  $\beta_2$ -agonists, either short- or long-acting, are given in combination with inhaled steroids [84, 85]. Anti-inflammatory asthma treatment decreases sputum eosinophil numbers. This has been illustrated for theophylline [86, 87], antileukotrienes [88], but especially for steroids [89–93]. Recent studies indicate that sputum eosinophil counts respond to modulations of the dose of steroids, which are insufficient to influence serum markers such as ECP [23]. An important aspect that needs to be fully established is the dose-response relationship of sputum eosinophilia compared with other biomarkers to changes in the steroid dose. JATAKANON *et al.* [94] compared the effect of a 4-week treatment with budesonide 100, 400 or 1600  $\mu\text{g}\cdot\text{day}^{-1}$  on exhaled NO, sputum eosinophilia and airway responsiveness to methacholine. The effect on exhaled NO reached a plateau from a dose of  $\geq 400 \mu\text{g}$ , whereas for sputum eosinophilia and airway responsiveness a significant dose-response relationship was observed throughout the different doses [94]. This aspect is of particular interest for disease monitoring. It can be argued that the very high sensitivity of eNO to the effect of steroids, combined with the nonspecific nature of the response limits the usefulness of exhaled NO in disease monitoring and that sputum eosinophilia offers more accurate information when titrating steroids to the minimal dose required to maintain asthma control.



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## Prospects for disease monitoring

Of particular interest is the observation that as for biopsies, the composition of sputum correlates poorly with other indices of disease activity such as symptom score, baseline FEV<sub>1</sub> or methacholine responsiveness. Although again, a better correlation is noted with indirect markers of airway responsiveness [35, 95–100]. Similarly, the response of sputum eosinophils to intervention is not always paralleled by changes in clinical outcome measures [89–91,101]. This implies that combining sputum analysis to other outcome measures could offer additional information, instead of merely reduplicating existing data, thus possibly improving disease monitoring.

However, before propagating the use of induced sputum in clinical asthma management, several elements need to be further clarified.

In line with the observation that sputum eosinophils correlate poorly with clinical characteristics of the disease, most cross-sectional studies conducted so far illustrate an important variability in sputum eosinophils, even when the samples are obtained from patients with a very similar clinical profile. To date, it is largely unknown whether sputum eosinophil counts in patients that otherwise seem well controlled, are important. Recent reports indicate that patients with high eosinophil counts in their sputum are more likely to lose asthma control, if their maintenance dose of steroids is reduced [102, 103]. Of note is that in these studies, eNO levels had no predictive value. In contrast, in a prospective study involving 31 steroid-treated well-controlled asthmatics the level of sputum eosinophilia was shown not to predict the likelihood of developing spontaneous exacerbations over a 1-yr follow-up period [104]. Based on these somewhat conflicting data, it is therefore unclear whether treatment strategies aimed at reducing sputum eosinophils in addition to controlling symptoms will result in long-term outcome of the disease.

Even if this would prove to be the case, a related question is as to what constitutes a clinically significant reduction in eosinophil counts. Recent studies in adults and children indicate that the normal range of sputum eosinophils does not exceed 2.5% [105–107]. Whether one of the goals of asthma treatment should be to maintain sputum eosinophils beneath this or another threshold value is again unknown. Preliminary data indicate that the level of clinical-asthma control over 1-yr is not significantly different in patients who irrespective of treatment have a median eosinophil count above or below 2.5% [108]. These issues need to be further evaluated in properly powered studies that examine in a prospective way whether treatment adaptations based on sputum eosinophils in addition to clinical criteria will result in a different level of long-term control. This type of study will also allow for the evaluation of whether following sputum eosinophilia is better or perhaps complementary to including bronchial hyperresponsiveness in the monitoring of the disease. Although bronchial hyperresponsiveness correlates weakly and inconsistently to inflammatory parameters in biopsies [8], recent data have rekindled interest in this parameter as a potentially useful overall marker of asthma severity. LEUPPI *et al.* [103] reported that in contrast to exhaled NO, the combined measurement of direct and indirect bronchial hyperresponsiveness predicted loss of asthma control when reducing the steroid dose [103]. In addition, SONT *et al.* [10] have shown that compared to standard treatment based on symptoms and lung function, including reduction of bronchial hyperresponsiveness as an additional treatment aim results in a reduced exacerbation rate. Short-term studies indicate that although both bronchial hyperresponsiveness and sputum eosinophils respond to a 1-month treatment with fluticasone propionate 1000 µg·day<sup>-1</sup>, the changes in both parameters are not correlated [91]. Whether both markers could therefore prove complementary, again needs to be evaluated.

Another question is whether treatment should be diversified based on the cellular

composition of sputum samples in asthmatics. An increasing number of reports indicate that in asthma roughly two different patterns of inflammation can be distinguished: one in which eosinophils predominate and another that is not eosinophilic, but neutrophilic. This has initially been described in asthma exacerbations [109, 110], but also seems to apply in persistent steroid-treated asthma, irrespective of severity [111–113]. It has been suggested that this has therapeutic implications, as sputum eosinophilia might predict a favourable response to steroids [114–116]. However, although tempting, these recommendations remain to be confirmed in larger scale studies.

## **Analyses on induced sputum**

To date, analysis of induced sputum has focused on the cell fraction, eosinophils in particular. In view of the complexity of the airway inflammation in asthma, complementing this analysis by the measurement of soluble mediators in the sputum supernatants might offer an even more accurate assessment of the inflammatory process. A range of molecules has been detected in supernatant including markers of increased vascular permeability such as fibrinogen or albumin [62, 63, 117], pro-inflammatory mediators including eicosanoid metabolites [118,119] and a variety of cytokines [113, 120, 121]. In general, the soluble markers have been less well validated. This is at least in part due to methodological problems associated with the collecting and processing of the sample as well as interference in the assay with mucus components [122].

What would seem to be of particular interest is to complement eosinophil counts with assessment of a marker that reflects airway remodelling, the more chronic phase of airway inflammation in asthma. As already indicated, theoretical models highlight the contribution of remodelling to the altered airway behaviour in asthma. Monitoring an index of remodelling in the follow-up of asthma therefore appears relevant. The ideal marker of remodelling remains to be identified. Possible candidates include growth factors or enzymes such as elastase or matrix metalloproteinase that are present in increased concentrations in the sputum supernatant of asthmatics [123, 124]. However, the exact functional role of these various molecules in the remodelling process is largely unknown, thus hampering the validation process.

A final point that needs to be further addressed is the feasibility of sputum processing. Provided proper attention is paid to the procedure, sputum induction has proven to be safe in asthma, even in the more severe forms of the disease [110, 125, 126]. Provided the sample is then processed according to a validated technique, the results are reliable [127]. However, it has to be realised that the samples need to be processed within 2 h after induction, in order to avoid deterioration of cell morphology. In addition, the overall procedure is time consuming and requires highly qualified laboratory technicians. Analysis of induced sputum is therefore expensive. Hence, if sputum analysis is to become a tool that is accessible to most clinicians, this technique needs to be simplified to become less labour intensive. Several approaches are currently being developed in this respect. This includes freezing or simultaneous homogenisation and fixation of sputum immediately after producing the sample [128]. This improves the preservation of cell morphology and allows for a longer time delay between induction and analysis of the sample. In addition, this would also enable automated cytometry. Another approach that has been proposed consists of lysing the cell pellet obtained after homogenisation and centrifugation of the sputum sample, in order to release eosinophil associated ECP. ECP in the cell lysate was found to correlate strongly with the absolute numbers of eosinophils in the cell pellet. The ratio of ECP in supernatant over ECP in the lysed pellet would even offer a marker of the degree of activation of eosinophils in the sputum sample [129]. This

technique has the advantage that the measurements can be automated, saving costs. Albeit theoretically appealing, these various approaches need further validation. Another potential disadvantage of induced sputum is that the induction process with nebulised hypertonic saline induces an inflammatory response, so that it is not advisable to make repeated measurements in less than 24 h [128]. While this may not be a limitation in long-term disease monitoring, it limits research studies of kinetic factors.

## Conclusion

Analysis of induced sputum offers a relatively noninvasive direct marker of airway inflammation in asthma. Including sputum analysis in the diagnosis but especially in the follow-up of the disease, could offer additional information to clinical-outcome measures. Measurement of exhaled biomarkers is also very promising and is feasible in children and patients with severe disease. Whether incorporating these new measurements into disease monitoring will result in improved long-term clinical control of asthma, or allow for the diversification of treatments now needs to be addressed in carefully designed studies.

## Summary

Bronchial asthma is currently considered and defined as an inflammatory disorder of the airways. It therefore seems logical to include a direct marker of airway inflammation in the diagnosis and follow-up of the disease. Several relatively noninvasive biomarkers have been and are being considered in this respect. Examples that have been most extensively investigated, as to their reliability and validity for the assessment of airway inflammation in asthma, include blood eosinophil counts or serum eosinophil cationic protein, urinary eicosanoid metabolites, exhaled gasses, mediators in breath condensate or induced sputum. From the studies performed to date, it would appear that biomarkers correlate poorly with other indices of disease activity, such as symptoms, baseline forced expiratory volume in one second or methacholine responsiveness. As such, including biomarkers in the diagnosis, but especially follow-up of the disease, could offer additional information to clinical-outcome measures. Whether this attitude in disease monitoring will result in improved long-term clinical control of asthma or allow for the diversification of treatments, now needs to be addressed. In addition, the routine use of these techniques in daily practice requires simplification of the methodology involved.

**Keywords:** Asthma, biomarker, breath condensate, exhaled nitric oxide, induced sputum.

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# Inflammatory cells in asthma: Mechanisms and implications for therapy

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Recent clinical studies have brought asthma's complex inflammatory processes into clearer focus, and understanding them can help to delineate therapeutic implications. Asthma is a chronic airway inflammatory disease characterized by the infiltration of airway T cells, CD<sup>+</sup> (T helper) cells, mast cells, basophils, macrophages, and eosinophils. The cysteinyl leukotrienes also are important mediators in asthma and modulators of cytokine function, and they have been implicated in the pathophysiology of asthma through multiple mechanisms. Although the role of eosinophils in asthma and their contribution to bronchial hyperresponsiveness are still debated, it is widely accepted that their numbers and activation status are increased. Eosinophils may be targets for various pharmacologic activities of leukotriene receptor antagonists through their ability to downregulate a number of events that may be key to the effector function of these cells. (*J Allergy Clin Immunol* 2003;111:S5-17.)

**Key words:** Asthma pathogenesis, T cells, mast cells, basophils, macrophages, eosinophils, cytokines, cysteinyl leukotrienes

A sensitized individual's initial response to allergen is dominated by products associated with mast cell activation, particularly histamine, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), leukotriene C<sub>4</sub> (LTC<sub>4</sub>), and tryptase. Within hours of the response, inflammatory cells are recruited from the circulation, including T cells, neutrophils, eosinophils, basophils, and monocytes. Understanding the complex mechanisms of asthma's inflammatory processes can help to delineate therapeutic implications, and recent clinical studies have highlighted mechanisms of this inflammatory process. For example, the effect of anti-IgE therapy on airway response to allergen bronchoprovocation has underlined the critical role of mast cells and basophils, which express the high-affinity IgE receptor. Studies with the leukotriene receptor antagonists (LTRAs) have demonstrated that cysteinyl leukotrienes (CysLTs) are important for most early and late physiologic responses to allergen bronchoprovocation and that CysLTs play a central role in the allergic airway and the recruitment of inflammatory cells.

## Abbreviations used

5-LO:	5-lipoxygenase
AA:	Arachidonic acid
BAL:	Bronchoalveolar lavage
CysLT:	Cysteinyl leukotriene
LTC <sub>4</sub> :	Leukotriene C <sub>4</sub>
LTD <sub>4</sub> :	Leukotriene D <sub>4</sub>
LTE <sub>4</sub> :	Leukotriene E <sub>4</sub>
LTRA:	Leukotriene receptor antagonist
MBP:	Major basic protein
PGD <sub>2</sub> :	Prostaglandin D <sub>2</sub>
SAC:	Segmental allergen challenge

This article reviews cellular mechanisms that are part of asthma's inflammatory processes and details recent clinical studies that shed light on those processes, providing a clearer understanding of specific roles played by therapeutic agents, particularly the leukotriene modifiers.

## INFLAMMATORY CELLS IN ASTHMA

Asthma is a chronic airway inflammatory disease characterized by infiltration of the airway T cells. In both normal and asthmatic airway mucosa, the prominent cells are the T lymphocytes, which are activated in response to antigen stimulation, or during acute asthma exacerbations, and produce high levels of cytokines. They are subdivided into two broad subsets according to their surface cell markers and distinct functions: the CD4<sup>+</sup> (T helper) and the CD8<sup>+</sup> (T cytotoxic) cells. CD4<sup>+</sup> cells are further subdivided into T<sub>H</sub>1 and T<sub>H</sub>2 cells, depending on the type of cytokines that they produce. Another subtype of CD4<sup>+</sup> cells has been identified, the T<sub>H</sub>3 cells, which produce high levels of transforming growth factor  $\beta$  and various amounts of IL-4 and IL-10.<sup>1</sup> The T<sub>H</sub>3 cells are associated with oral tolerance and are suggested to be regulatory T helper cells.<sup>1-5</sup> Other cells involved in the pathogenesis of asthma include mast cells, basophils, macrophages, and eosinophils. The interactions among all these cells and their products perpetuate the inflammatory response.

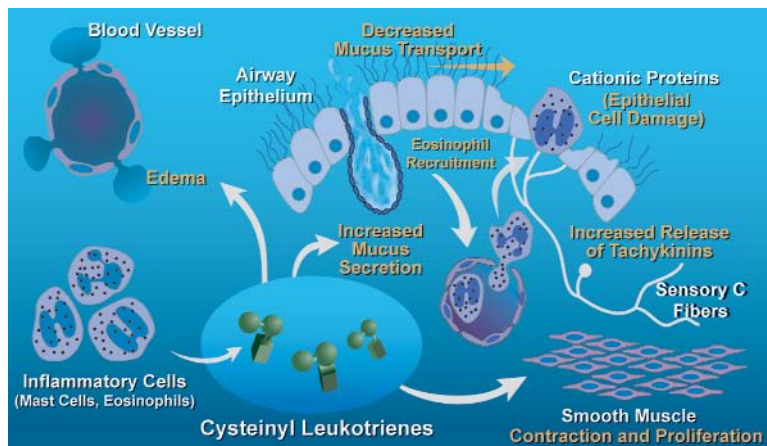
## CYTOKINES

The initial indication for cytokine involvement in the pathogenesis of asthma came from studies performed in the early 1990s showing that atopic asthma was associated with local T<sub>H</sub>2 cytokine expression. IL-3, IL-4, IL-5,

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**FIG 1.** Potential sites and effects of cysteinyl leukotrienes relevant to asthma pathophysiology. Adapted from Hay DW, Torphy TJ, Undem BJ. Cysteinyl leukotrienes in asthma: old mediators up to new tricks. *Trends Pharmacol Sci* 1995;16:304-9.

and GM-CSF were upregulated in asthmatic patients relative to control subjects. These cytokines were significantly upregulated after antigen challenge, and their receptors were identified locally on the surface of inflammatory cells.<sup>6</sup> Studies have confirmed the existence of the prominent  $T_H2$ -type cytokine profile not only in asthma but also in allergic rhinitis and atopic dermatitis.

Many of these cytokines have been found in human beings and have been shown to be associated with pathologic changes of asthma.<sup>6</sup> For example, IL-13 is associated not only with IgE synthesis and chemoattraction of eosinophils but also with mucus hypersecretion, fibroblast activation, and the regulation of airway smooth muscle function.<sup>7</sup> Another  $T_H2$  cytokine, IL-9, is upregulated preferentially and is associated with airway hyperresponsiveness, mucus hypersecretion, eosinophil function, IgE regulation, and the upregulation of calcium-activated chloride channel.<sup>8-10</sup>

The list of chemokines associated with asthma has expanded and includes eotaxin, monocyte chemoattractant protein 4, and RANTES. Although transforming growth factor  $\beta$  has long been considered the major profibrotic cytokine associated with subepithelial fibrosis and production of extracellular matrix proteins, other cytokines such as IL-11 and IL-17 have also demonstrated profibrotic activity in association with severe asthma.<sup>11-13</sup>

## LEUKOTRIENES

Although not cytokines, the CysLTs have emerged as important mediators in asthma and as modulators of cytokine function. Leukotrienes are lipid mediators resulting from the catabolism of the arachidonic acid (AA) released from the cell membrane by phospholipase  $A_2$  after cell activation. After its release, AA is metabolized either by the cyclooxygenase pathway, generating prostaglandins and thromboxanes, or by the 5-lipoxy-

genase (5-LO) pathway, which in association with 5-LO-activating protein as a helper protein produces the leukotrienes: leukotriene  $B_4$ ,  $LTC_4$ , leukotriene  $D_4$  ( $LTD_4$ ), and leukotriene  $E_4$  ( $LTE_4$ ), with the last three forming the CysLT group.  $LTC_4$  is metabolized enzymatically to  $LTD_4$  and subsequently to  $LTE_4$ , which is excreted in the urine. The CysLTs are produced in eosinophils, monocytes, macrophages, mast cells, basophils, and, to a lesser extent, endothelial cells and T lymphocytes.<sup>14</sup>

Increased production of CysLTs has been detected in bronchoalveolar lavage (BAL)<sup>15,16</sup> and urine<sup>17</sup> samples from patients with asthma, especially after allergen challenge<sup>18</sup> or during an acute asthma attack.<sup>19</sup> In allergic airway inflammation, the expressions of 5-LO and 5-LO-activating protein enzymes are increased; their mRNA is present in endothelial and inflammatory cells after allergen challenge in mice.<sup>20</sup> Furthermore, an overexpression of  $LTC_4$  synthase has been demonstrated in bronchial biopsy specimens from asthmatic patients.<sup>21-23</sup>

The CysLTs also have been implicated in the pathophysiology of asthma by way of multiple mechanisms, including mucus hypersecretion, increased microvascular permeability, ciliary activity impairment, inflammatory cell recruitment, edema, and neuronal dysfunction (Fig 1).<sup>24-29</sup> The CysLTs also induce eosinophil recruitment into the airways of guinea pigs *in vitro*<sup>30</sup> as well as in patients with asthma *in vivo*.<sup>31,32</sup> Most important, these molecules increase airway hyperresponsiveness and cause smooth muscle hypertrophy in both healthy subjects and asthmatic patients.<sup>32,33</sup>

## MAST CELLS

Mast cells make up a small proportion of cells recovered by BAL, but within the airway tissue as many as 20% of inflammatory cells are mast cells.<sup>34,35</sup> In BAL specimens, normal mast cell numbers range from 0.02%

to 0.48%.<sup>36,37</sup> Normal mast cell numbers<sup>36,38-41</sup> or increases of 2- to 6-fold, have been reported in patients with atopic<sup>34,37,42-47</sup> as well as nonatopic asthma.<sup>48</sup> On the airway surface and in the submucosa, mast cells are mostly the mucosal type, containing tryptase in secretory granules designated  $MC_T$ , as opposed to the tissue-type mast cell containing both tryptase and chymase, designated  $MC_{TC}$ .

Present on the surface of and within the airway, mast cells are well positioned to respond to a provocative stimulus. Normally, they are the only resident cells in the airway that can interact with allergen by way of the IgE bound to the high-affinity receptor FcεRI. On allergen challenge of the airways, the mast cells respond within minutes, releasing both preformed mediators such as histamine and tryptase and newly synthesized products such as PGD<sub>2</sub> and LTC<sub>4</sub>.<sup>16,38,40,49-52</sup> Clearly, the immediate response to allergen challenge is dominated by products that are found with the mast cell. These products are potent bronchoconstrictors and may induce alterations in vascular permeability. Mast cell numbers in the bronchial mucosa also may increase after the late-phase response to allergen challenge.<sup>53</sup> In addition to allergen, such other stimuli as exercise, aspirin, and chemicals may invoke mast cell degranulation, leading to bronchoconstriction and vascular changes.

Ongoing mast cell degranulation has been shown to be present in chronic asthma, as evidenced by increased levels of the mast cell mediators histamine, PGD<sub>2</sub>, and tryptase,<sup>34,36,39,41,54,55</sup> although BAL histamine levels may be elevated in persons with allergic rhinitis without asthma.<sup>36,45</sup> In vitro both spontaneous and IgE-mediated release of histamine has been enhanced in BAL mast cells of asthmatic versus nonasthmatic persons,<sup>46</sup> and spontaneous histamine release has been increased in patients with symptomatic versus asymptomatic asthma.<sup>41</sup>

Mast cells may further participate in asthma's inflammatory changes through the elaboration of cytokines. In response to IgE-dependent stimuli, mouse mast cell lines have been shown to produce a profile of cytokines, including IL-3, IL-4, IL-5, and IL-6, similar to the T<sub>H</sub>2 profile produced by T lymphocytes. Human lung mast cells have been shown to release IL-4,<sup>56</sup> IL-5,<sup>57</sup> and IL-13<sup>58</sup> in vitro, and mucosal biopsy specimens from asthmatic persons have revealed positive staining by immunohistochemical means for IL-4, IL-5, IL-6, and TNF-α in mast cells.<sup>59</sup> In IgE-mediated reactions, mast cells are most likely an important immediate source of TNF-α. Unlike other sources of TNF-α, such as macrophages, resting mast cells contain preformed stores available for immediate release.<sup>60</sup> Further localization of cytokines to mast cell subsets reveals preferential IL-4 expression by  $MC_T$  mast cells, with predominantly IL-5 and IL-6 expression by the  $MC_{TC}$  subset.<sup>61</sup>

## BASOPHILS

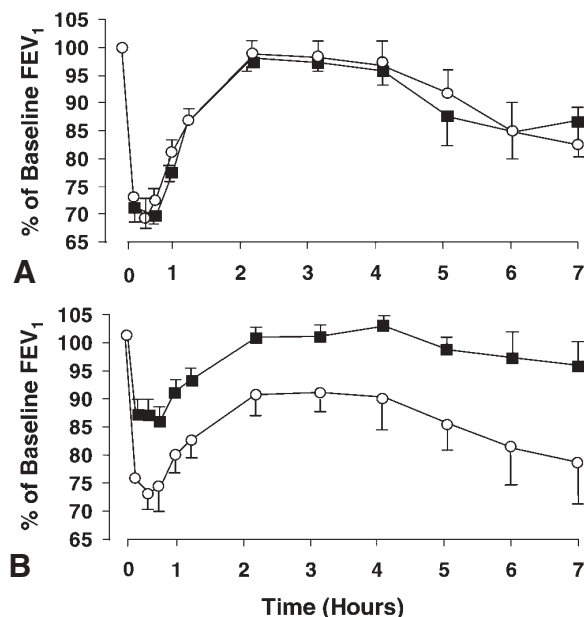
Basophils possess high levels of the FcεRI receptor and are capable of an immediate response to allergen.

Although basophils are not present in healthy airways, they are present in the airways of asthmatic persons under a variety of circumstances. Basophils have been reported in the sputum of patients with symptomatic asthma,<sup>62</sup> and recent studies have demonstrated basophil infiltration of airways in cases of fatal asthma<sup>63,64</sup> and in bronchial biopsy specimens from patients with asthma.<sup>65,66</sup> During the late response to allergen challenge, large numbers of basophils have appeared in BAL specimens after segmental allergen challenge (SAC)<sup>38,67</sup> and have been noted in airway tissue after inhalation bronchoprovocation.<sup>66,68</sup> Like mast cells, basophils release histamine on activation; unlike mast cells, however, they do not produce PGD<sub>2</sub>. The major product of AA metabolism in the basophil appears to be LTC<sub>4</sub>. On a per cell basis, basophils produce as much LTC<sub>4</sub> as do mast cells and much more than do eosinophils. Recently, basophils have also been found to be a rich source of IL-4 and IL-13, demonstrating both spontaneous release and response to IgE-mediated stimuli.<sup>69,70</sup> In fact, basophil production of these cytokines rivals that reported for T-cell clones. Mixed lymphocyte populations produce only 10% to 20% as much IL-4 as do basophils.<sup>71</sup>

## MACROPHAGES

Macrophages are the predominant cell recovered by BAL in both nonasthmatic and asthmatic persons. Although most macrophages are recovered from alveoli, small volume lavage or lavage of isolated airway segments<sup>72</sup> supports macrophage predominance in conducting airways as well as alveoli. Thus, macrophages are well positioned to respond to and regulate inflammation along the airway. Although the prominence of macrophages along the airway surface and their diverse functions strongly implicate macrophages as playing a role in asthma, it is unclear whether that role is one of promoting or preventing inflammatory responses. On the one hand, macrophages can perform accessory cell functions by presenting antigen and providing secondary signals (eg, IL-1) required for the differentiation and proliferation of specific lymphocyte responses. These functions may play a role in sensitizing the airway to respond to further exposures. On the other hand, in some systems, alveolar macrophages have been found to be poor antigen-presenting cells, and in the large proportions of macrophages to lymphocytes (5:1 to 10:1) found on the airway surface, macrophages most likely suppress lymphocyte responses.<sup>73</sup> Thus, the role of the resident macrophage in initiating immune responses remains unclear. Adding to this complexity are the findings that blood monocytes are better antigen-presenting cells than are macrophages and may be recruited to inflammation sites. In addition, dendritic cells are present in the airways and appear to be much more potent antigen-presenting cells than are macrophages.<sup>74</sup>

Nevertheless, airway macrophages may participate in airway inflammation through multiple mechanisms. Alveolar macrophages express the low-affinity receptor



**FIG 2.** Effect of anti-IgE therapy on allergen bronchoprovocation. FEV<sub>1</sub> is shown as a percentage of baseline in the first 7 hours after allergen challenge. The early phase is during hour 1, and the late phase is from hours 2 to 7. The responses to placebo (**A**) and rhuMAB-E25 (**B**) are depicted at baseline (*open circles*) and at the end of 9 weeks of treatment (*closed squares*). RhuMAB-E25 significantly reduced allergen-induced bronchoconstriction during both early- and late-phase responses to allergen bronchoprovocation. From Fahy JV, Fleming HE, Wong HH, Liu JT, Su JO, Reimann J, et al. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 1997;155:1828-34.

for IgE FcεRII,<sup>75</sup> and expression appears to be increased in asthmatic persons relative to healthy subjects.<sup>76</sup> Macrophage release of lysosomal enzymes in response to SAC has been demonstrated *in vivo*.<sup>77</sup> *In vitro* studies have revealed that alveolar macrophages can respond to antigen through IgE to release leukotriene B<sub>4</sub>, LTC<sub>4</sub>, PGD<sub>2</sub>, superoxide anion, and lysosomal enzymes.<sup>78-81</sup> Macrophages also produce other inflammatory mediators, such as platelet-activating factor, prostaglandin F<sub>2α</sub>, and thromboxane.<sup>82,83</sup> These mediators may play important roles in producing bronchoconstriction or in causing inflammatory changes, including cell recruitment and altered vascular permeability.

Pro-inflammatory cytokines produced by macrophages include IL-1, TNF-α, IL-6, and GM-CSF, which may induce endothelial cell activation, cellular recruitment, and prolonged eosinophil survival. Interleukin-6 and TNF-α may be released by IgE-dependent stimulation.<sup>84</sup> Macrophages also elaborate histamine-releasing factors that appear to act on the basophil and mast cell by way of binding to surface IgE.<sup>85-87</sup> Thus, macrophages may play a role in perpetuating mast cell activation in asthma and late-phase responses independently of repeated exposures to specific allergen.

## CLINICAL STUDIES SUPPORTING THE ROLE OF MAST CELLS AND BASOPHILS IN ASTHMA

### Anti-IgE

In the pathogenesis of allergic disease, IgE plays a central role. Mast cells and basophils are the primary cells that bear the high-affinity IgE receptor, and a critical role for these cells is supported by the effect of anti-IgE therapy on the airway response to allergen bronchoprovocation. Omalizumab is a humanized murine monoclonal antibody (rhuMAB-E25) that binds to the same portion of IgE as the high-affinity IgE receptor. Because the anti-IgE antibody and the cell surface receptor compete for the same site (FcεR, binding domain) on IgE, the anti-IgE antibody can only bind free IgE and will not cause mediator release by cross-linking IgE on the cell surface. Results from 2 clinical studies have demonstrated that treatment with omalizumab inhibits the early- and late-phase responses to allergen challenge. In one study, shown in Fig 2, treatment with omalizumab reduced free serum IgE by nearly 90%, increased the dose of allergen required for an early response, and inhibited the maximum early and late changes in FEV<sub>1</sub> by about 40% and 60%, respectively.<sup>88</sup> In a separate study, omalizumab was given intravenously at an initial dose of 2 mg/kg, followed by 1 mg/kg at 1 week and every 2 weeks thereafter for a total of 10 weeks.<sup>89</sup> Free serum IgE was reduced by 89%, and the dose of inhaled allergen causing a 15% fall in FEV<sub>1</sub> was significantly increased by 2.3 to 2.7, doubling concentrations on days 20, 55, and 77. Methacholine reactivity was also significantly decreased by day 76.

### Antihistamine and antileukotriene therapy

The concept that histamine and leukotrienes are responsible for most of the early and late physiologic responses to allergen bronchoprovocation is supported by a study in which identical bronchoprovocations were performed after 1 week of pretreatment with the LTRA zafirlukast (80 mg twice daily), the antihistamine loratadine (10 mg twice daily), or both in combination.<sup>90</sup> As shown in Fig 3, the results clearly demonstrated the inhibition of both the early and late responses with each agent alone. Zafirlukast was more effective than loratadine in the early ( $P < .05$ ) but not the late response. Combination therapy inhibited the early response by 75% and the late response by 74% and was more effective than either drug alone during the late response ( $P < .05$ ). These results clearly support a role for mast cell-derived and basophil-derived mediators in both the early and late bronchoconstrictive responses to allergen.

On the basis of the effects of multiple antileukotriene therapies on both the early- and late-phase responses, the central role of leukotrienes in this allergic airway reaction is firmly established. Whereas antileukotriene therapy may affect cell recruitment airway edema, and during the late-phase response blocking smooth muscle contrac-



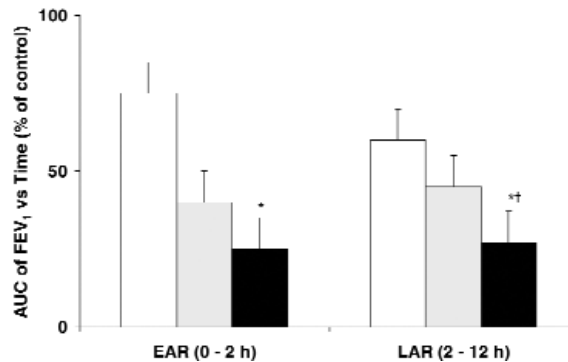
tion, may be a major mechanism of antileukotriene therapy. In a recent study, increasing doses of the  $\beta$ -agonist terbutaline were administered intravenously 7 hours after allergen bronchoprovocation, when FEV<sub>1</sub> was 57% of baseline. At the end of infusion at 45 minutes, FEV<sub>1</sub> had returned to 100% of baseline and 84% of the maximal attainable value.<sup>91</sup> The reversal of late-phase airway obstruction by a  $\beta$ -agonist supports the role of smooth contraction in this response to allergen challenge, in which leukotrienes (and histamine) appear to play major roles in the contractile responses.

## SAC

The cellular, mediator, and cytokine responses underlying the physiologic response to allergen exposure have been examined with the SAC model, in which allergen is instilled directly into an airway segment during bronchoscopy and events occurring within minutes, hours, or days can be examined, generally by BAL. This model also has been used to examine the effects of prednisone and leukotriene modifier therapy on inflammatory changes. Cellular effects of these asthma medications on BAL cells after SAC are summarized in Table I.

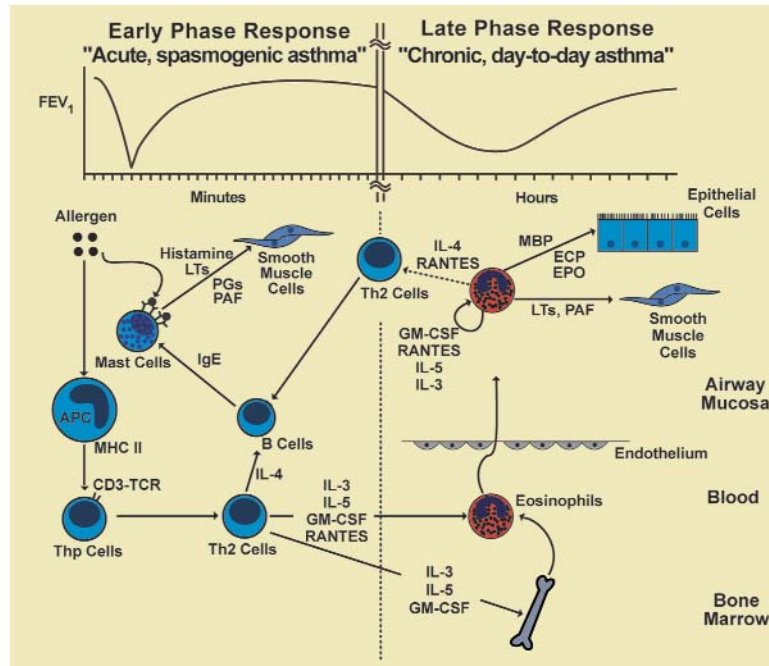
The initial response to allergen in the sensitized individual is clearly dominated by products associated with mast cell activation, particularly histamine, PGD<sub>2</sub>, LTC<sub>4</sub>, and tryptase.<sup>38,51,52,97</sup> These products are released within minutes and appear in the absence of cellular changes. Lipid products not produced by mast cells are also present, however, which implies the activation of other resident cells within the lung by the initial events. Within hours, immune and inflammatory cells are recruited from the circulation. This recruitment involves virtually all cell types including granulocytes (neutrophils, eosinophils, and basophils) and mononuclear cells (monocytes and lymphocytes); however, a remarkable selectivity of cells characterizes the allergic response, specifically eosinophils, basophils, and helper T cells. The T cells are further characterized as memory T cells and express a cytokine profile (eg, IL-4, IL-5) consistent with T<sub>H</sub>2 cells.<sup>91</sup> These cells have in common the expression on their surface of the adhesion molecule very late antigen 4, which binds to vascular cell adhesion molecule 1 on endothelial cells, suggesting a shared pathway for recruitment. IL-4 and IL-13 are T<sub>H</sub>2 cytokines that specifically induce vascular cell adhesion molecule 1 expression.<sup>97,98</sup> Whether there is a sequential recruitment of granulocytes followed by a mononuclear infiltration is not clear, but within 24 hours, all cell types are present. Furthermore, the mononuclear cell population shifts from one dominated by mature alveolar macrophages to one characterized by monocytes and monocytoïd cells expressing blood dendritic cell-specific antigens<sup>99</sup> (Liu MC, personal unpublished data).

The effects of leukotriene-modifying medications on inflammation induced by SAC have demonstrated inhibition of multiple cell types recruited during SAC. An examination of pretreatment with the 5-LO inhibitor



**FIG 3.** Effect of antihistamine and antileukotriene therapy on allergen bronchoprovocation. Results demonstrate inhibition of both early (EAR) and late (LAR) responses to all treatments and supported a role for mast cell-derived and basophil-derived mediators in both the early and late bronchoconstrictive responses to allergen. The early- and late-airway responses after the different treatments are expressed as area under the curve in percentage of the response during the control bronchoprovocation performed in the absence of drugs. All treatments caused significant reductions of both phases ( $P < .05$ ). Bar heights represent mean; error bars represent SE. Asterisk denotes significant difference between zafirlukast plus loratadine (black bars) and loratadine alone (white bars); dagger denotes significant difference between zafirlukast plus loratadine and zafirlukast alone (shaded bars). From Roquet A, Dahlen B, Kumlin M, Ihre E, Anstren G, Binks S, et al. Combined antagonism of leukotrienes and histamine produces predominant inhibition of allergen-induced early and late phase airway obstruction in asthmatics. *Am J Respir Crit Care Med* 1997;155:1856-63.

zileuton (600 mg four times daily) for 8 days demonstrated a significant increase in eosinophils in BAL at 24 hours during placebo treatment but no significant increase in eosinophils during active treatment. A study that examined a 7-day pretreatment with the LTRA zafirlukast (20 mg twice daily) demonstrated significant decreases in lymphocytes and alcian blue-positive cells in BAL at 48 hours.<sup>93</sup> TNF in BAL was also inhibited. Finally, researchers examined pretreatment for 6 weeks with zileuton (600 mg four times daily versus placebo) in a parallel design protocol.<sup>94</sup> On the basis of the initial response to SAC, the group could be divided into low and high leukotriene producers according to leukotriene levels in BAL at 24 hours after SAC. High leukotriene producers had greater eosinophil, total protein, and cytokine (TNF- $\alpha$ , IL-5, IL-6) levels than did low leukotriene producers. Eosinophil influx was significantly inhibited only in the group producing high levels of leukotrienes. Whereas the changes in airway obstruction inhibited by antileukotriene therapy probably represent effects on smooth muscle function, the results of SAC studies support an additional role of leukotrienes in the recruitment of inflammatory cells. This is further supported by studies with inhaled leukotrienes demonstrating both eosinophil and metachromatic cell (probably basophil) recruitment. Increased numbers of eosinophils and neutrophils appeared in the airway mucosa 4 hours after inhalation challenge with LTE<sub>4</sub>. Numbers of eosinophils were 10-fold greater than those of neutrophils.<sup>100</sup> A



**FIG 4.** A scheme of putative immune and inflammatory events associated with the pathophysiology of asthma, with emphasis on early- versus late-phase asthmatic responses. Sites of potential anti-inflammatory action of LTRAs are shown by *large arrows*. ECP, Eosinophil cationic protein; EPO, eosinophil peroxidase; PG, prostaglandin; PAF, platelet activating factor; APC, antigen presenting cell; TCR, T-cell receptor.

**TABLE I.** Effects of prednisone and leukotriene-modifying agents on BAL cells after SAC

Medication	Cellular effect	Reference
Zileuton	No significant increase in eosinophils after SAC	Kane et al, <sup>92</sup> 1996
Zafirlukast	Inhibition of lymphocytes and basophils	Calhoun et al, <sup>93</sup> 1998
Zileuton	Inhibition of eosinophils in high leukotriene producers	Hasday et al, <sup>94</sup> 2000
Prednisone	Inhibition of eosinophils, basophils, and lymphocyte subsets	Dworski et al, <sup>95</sup> 1994; Liu et al, <sup>96</sup> 2001

recent study also demonstrated increased sputum eosinophils, as well as increased airway eosinophils and metachromatic cells (probably basophils), after inhalation of LTE<sub>4</sub>.<sup>101</sup>

The effect of systemic steroid therapy with prednisone has also been examined in this model. Pretreatment with prednisone had no effect on the release of mast cell-derived mediators in the initial response,<sup>92,93</sup> but subsequent events were inhibited.<sup>93</sup> In particular, significant decreases in cell recruitment of eosinophils, basophils, and T-cell subsets (CD4, CD45RA, and CD45RO cells) but not neutrophils occurred. Increases in airway permeability, kinins, the adhesion molecule E-selectin, and cytokine (IL-4, IL-5, IL-2, and transforming growth factor  $\alpha$ ) gene expression or protein induced by SAC were also inhibited. Increases in GM-CSF were not inhibited. Clearly, steroid therapy suppressed multiple components of the allergic airway response.

## EOSINOPHILS

Activated T cells and eosinophils are important pathophysiological elements in asthma (Fig 4). The numbers of

these cells have been correlated broadly with disease severity.<sup>102</sup> Mucosal damage in chronic asthma has been shown to be associated with cytotoxic and pro-inflammatory mediator release from activated eosinophils.<sup>103,104</sup> These products include reactive oxygen species and cytotoxic granule and vesicular proteins: major basic protein (MBP), eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin, as well as cytokines and chemokines together with phospholipid-derived, pharmacologically active mediators. Cytokines released from T<sub>H</sub>2-type cells, particularly IL-3, IL-5, and GM-CSF, are thought to regulate eosinophil priming, activation, and survival.<sup>105,106</sup>

## CysLTs

Eosinophils are a rich source of CysLTs<sup>103</sup> that are derived from native AA by the action of phospholipase A<sub>2</sub>.<sup>107</sup> Human eosinophils synthesize and release relatively large concentrations of LTC<sub>4</sub> (as much as 70 ng/10<sup>6</sup> cells) after stimulation with the calcium ionophore A23187.<sup>108</sup> In general, eosinophils obtained from asthmatic subjects appear to produce more LTC<sub>4</sub>



than do those from healthy control donors.<sup>109,110</sup> Experimentally, coculture of eosinophils with endothelial cells<sup>111</sup> or exogenous addition of cytokines (eg, IL-3, IL-5, GM-CSF, and TNF) has resulted in the upregulation of ionophore-induced release of LTC<sub>4</sub>.<sup>106,111,112</sup>

Leukotriene C<sub>4</sub> metabolism produces LTD<sub>4</sub> and LTE<sub>4</sub>, which are rapidly degraded in the body; small amounts of LTE<sub>4</sub> can be measured in the urine.<sup>113</sup> In human beings the 5-LO pathway is expressed only in myeloid cells, including mast cells, basophils, neutrophils, eosinophils, and alveolar macrophages.<sup>114</sup> The CysLTs appear to have selective eosinophilotactic activity<sup>27,100</sup> and promote transmigration of these cells through endothelial barriers.<sup>115</sup>

There are two receptors for CysLTs on smooth muscle cells, CysLT<sub>1</sub> and CysLT<sub>2</sub>. The CysLT<sub>1</sub> receptor is the regulator for bronchial smooth muscle contraction and thus is directly relevant to asthma treatment.<sup>116</sup> On the other hand, CysLT<sub>2</sub> appears to be involved mainly in pulmonary vein contraction.<sup>117</sup> In addition to LTD<sub>4</sub>, both LTC<sub>4</sub> and LTE<sub>4</sub> bind to CysLT<sub>1</sub>, although LTE<sub>4</sub> exhibits a greatly reduced binding capacity.<sup>116</sup> Evidence at the level of mRNA expression has suggested that eosinophils may have the capacity to synthesize CysLT<sub>1</sub> receptors.<sup>115</sup> Recent studies have also suggested that eosinophils may express CysLTs, which may have implications for the capacity of these cells to respond to LTRAs in the setting of allergic and airway inflammation.<sup>118</sup>

## LEUKOTRIENE MODIFIERS

The contribution of the CysLTs in bronchoconstriction has been inferred from recent developments in the field of leukotriene-modifying therapies.<sup>33,119,120</sup> LTD<sub>4</sub> receptor antagonists (montelukast, zafirlukast, and pranlukast) inhibit the biologic activities of LTD<sub>4</sub> and the other members of the CysLT family by competing for their receptors on smooth muscle cells.<sup>121</sup> Clinical trials of LTRAs, including montelukast and zafirlukast,<sup>122-126</sup> have significantly controlled asthma symptoms in a substantial proportion of patients. Although the role of eosinophils in asthma and their contribution to bronchial hyperresponsiveness are still debated,<sup>127</sup> it is widely accepted that in asthma the number and activation status of eosinophils are increased and that eosinophil granule-derived products cause mucosal injury that may promote irreversible changes (tissue remodeling). The actions of LTRAs go beyond their potent bronchodilatory effects, particularly those that appear to downregulate various eosinophil effector parameters. Both montelukast and zafirlukast decrease peripheral blood and airway eosinophil numbers.<sup>93,128</sup> In cellular infiltrate obtained from induced sputum, reductions in the number of sputum eosinophils were significantly less after treatment with montelukast.<sup>129</sup> These findings provide further support to the notion that CysLTs have eosinophilotactic properties.<sup>27,100,128</sup>

In a rat model of allergic airway responsiveness after allergen challenge, eosinophil (MBP positive) and IL-5 mRNA-positive cell numbers were significantly reduced

in both BAL and lung tissue from rats that received montelukast compared with untreated animals.<sup>130</sup> In contrast, there are negligible data on the activation status of eosinophils in human airway tissue in LTRA-treated versus untreated subjects. Such data would expand current understanding and better define the anti-inflammatory properties of LTRAs in vivo. It is becoming clear that although LTRAs such as montelukast and zafirlukast dampen the eosinophilic response, the precise pathways underlying such an effect remain to be established. There is a need for a better identification of the spatial and temporal targeting of the effects of LTRAs during the life cycle of the eosinophil in inflammation, from eosinopoiesis to its demise in a given inflammatory site.

The first potential site for the effect of LTRAs is in early T-cell events associated with the development of the late-phase response. The LTRAs may interfere with the capacity of T<sub>H</sub>2 cells to release critical cytokines (eg, IL-3, IL-5, GM-CSF) as well as chemokines (eg, RANTES) required for bone marrow stimulation of eosinopoiesis. Additionally, LTRAs may downregulate receptors or critical elements for these cytokines. More likely, LTRAs may exert their effect on the growth, differentiation, and efflux of bone marrow eosinophil progenitors (Fig 4).<sup>131</sup> A currently ongoing study is aimed at examining the LTRAs' mode of action on sequential growth and differentiation steps from CD34<sup>+</sup> progenitor to the fully differentiated and activated cell. In addition, the effects of these agents on bone marrow levels of eosinophil-sensitive chemokines (eg, RANTES and eotaxin) are to be ascertained. The results of this study will determine whether there is a potential blocking action of LTRAs on the proximal arm of cell accumulation in tissue and related events associated with the egress of eosinophils from hematopoietic sites.

Montelukast inhibits eosinophil transmigration across human umbilical vein endothelial cells.<sup>115</sup> In addition, the demonstration that human eosinophils express mRNA for the CysLT<sub>1</sub> receptor strongly supports the hypothesis that these inflammatory cells may accumulate and traffic to sites of allergic inflammation as a result of chemotactic activity exerted by CysLTs. Thus, LTRAs may interfere with eosinophil recruitment by way of effects on adhesion molecules that facilitate rolling, tethering, flattening, and transmigration by diapedesis (Fig 4).

Previous studies have concluded that LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> have little if any chemotactic activity for human eosinophils<sup>132,133</sup> and no upregulatory effects on eosinophil effector function (including cytotoxicity)<sup>134</sup> in comparison with leukotriene B<sub>4</sub>. In light of the fact that these results achieved more concrete evidence for a chemotactic function of CysLTs, it is crucial that these data be revisited to appreciate the magnitude and mechanisms regulating and reducing eosinophils in the sputum of LTRA-treated asthmatic subjects. In addition, the effect of LTRAs may be the outcome of an indirect effect on bystander immune, inflammatory, and structural cells in the bronchial mucosa. For instance, the lower numbers of eosinophils in sputum may occur as a result of puta-

tive CysLT receptor-mediated effects on epithelial cells, which in turn may influence the synthesis, storage, and release of eosinophilotactic chemokines, including RANTES and eotaxin.<sup>71,135</sup> These proteins have been shown to be present in the bronchial tissue in asthma.<sup>136,137</sup> In addition, epithelial cell-induced eosinophil chemotaxis also may be influenced by cytokines such as IL-16, a potent T-cell and eosinophilotactic cytokine and a major product of bronchial epithelial cells. IL-16 has been shown to use CD4 receptors expressed on eosinophils to exert its chemotactic activity,<sup>138,139</sup> and IL-16 expression has been shown to be a pathologic feature of human bronchial asthma.<sup>140</sup>

Little is known about the influence of LTRA treatment on IL-5 bioactivity both locally and systemically, but inhalation of LTD<sub>4</sub> causes an increase of IL-5.<sup>30</sup> This cytokine is a critical factor in eosinophil terminal differentiation and, together with IL-3 and GM-CSF, prolongs eosinophil survival in the tissue. Production of IL-5 by mononuclear cells under stimulation with mite antigen was markedly suppressed when they were exposed to the LTRA pranlukast. The data may provide clues to the mechanism by which LTRAs, including zafirlukast and montelukast, can reduce airway, sputum, and blood eosinophil counts in clinical asthma.<sup>141</sup> Such data could provide further support for the possibility that LTRAs may exert their influence on eosinophilic responses by interfering with IL-5 protein synthesis and turnover in asthmatic airways.

A well-recognized function of chemotactic factors relates to their ability to upregulate inflammatory cell function by increasing the expression of various receptors as well as inducing and enhancing their capacity to synthesize and release their de novo synthesized and preformed, stored mediators. Eosinophils are major secretory cells in airway inflammation, and a better understanding of the effects of LTRAs on eosinophil degranulation and exocytosis and on eosinophil mediator secretion is needed (Fig 4).

Among the inflammatory cells, eosinophils may be targets for various pharmacologic activities of LTRAs through the ability of these agents to downregulate a number of extracellular and intracellular events that may be key to the effector function of these cells. Much remains to be studied in the pursuit of a clearer understanding of the range of activities of these anti-inflammatory agents. The spectrum of their anti-inflammatory effects may range from dampening chemotactic activities that are key to cell trafficking to and accumulation in relevant tissues to the interruption of intracellular events regulating granule and secretory vesicle exocytosis and mediator release.

## CONCLUSIONS

The sensitized reaction to an allergen includes responses from T cells, mast cells, basophils, macrophages, and eosinophils. In the case of asthma and allergic rhinitis, the mediators released from these cells perpetuate the asthmatic inflammatory response, and the CysLTs have emerged as one of the important mediators.

In human beings, the 5-LO pathway is expressed only in myeloid cells, including mast cells, basophils, neutrophils, eosinophils, and alveolar macrophages. Eosinophils from asthmatic subjects produce more LTC<sub>4</sub> than do those from healthy control donors, and there is an overexpression of LTC<sub>4</sub> synthase in bronchial biopsy samples obtained from asthmatic patients. Mast cells and basophils are primary cells that bear a high affinity IgE receptor and, during their key involvement in the early and late phases, CysLTs are released. Basophils produce as much LTC<sub>4</sub> as do mast cells and much more than do eosinophils.

The CysLTs have the potential to cause the cardinal symptoms of asthma: mucus hypersecretion, increased microvascular permeability, and edema, as well as impaired ciliary activity, inflammatory cell recruitment, and neuronal dysfunction. They are able to induce smooth muscle hypertrophy and hyperplasia, and their increased production can be measured in BAL and sputum. With their selective eosinophilotactic activity, the CysLTs can also promote transmigration of eosinophils through endothelial barriers.

Clinical trials with LTRAs have shown them to exert significant control over asthma symptoms and to modulate cytokine function. Consequently, these agents have been implicated in the pathophysiology of asthma, acting through multiple mechanisms. Basic and clinical studies will continue to deepen our understanding of the complex inflammatory processes in asthma and related conditions. The results of such studies hold important therapeutic implications.

## QUESTIONS AND DISCUSSION

*Marc Peters-Golden:* Qutayba, is the epithelium capable of differentiating into mesenchymal-like cells, as they appear to do in the kidney? Could the smooth muscle cells that appear to be pushing up into the epithelial layer actually be the basal epithelial cells that are differentiating into smooth muscle cells instead?

*Qutayba Hamid:* We did not see any evidence that epithelial cells go to something that is not a cytochromes-positive cell or what would probably look like a smooth muscle. Epithelial cells from asthmatic patients certainly behave differently from normal epithelial cells in the way that they are differentiated. I have not seen any evidence that they differentiate into mesenchymal cells.

*Stephen Holgate:* There is a most remarkable organization underneath the epithelium. People talk about leukocytes coming up through the epithelium into the lumen as if the leukocytes are eating their way through. As you strip off the epithelium and look down at it, you see that it is full of channels. From electron micrographs, we can see leukocytes and dendritic cells traffic through these channels. These channels are highly organized, and nothing is eating its way through. What we have shown recently is that the attenuated fibroblasts, or whatever we want to call them, actually extend tendrils through so that they are in contact with the basement membrane underneath the epithelium itself. These "flat cells" extend their

feelers up through to the epithelium and form an integrated unit, just as is seen in the developing fetal lung.

*Qutayba Hamid:* Not to be forgotten are the neuroendocrine cells in the lung epithelium, which can secrete a number of growth factors. They appear to change with age and to regulate the repair process.

*Stephen Holgate:* In a children's biopsy study we did collaboratively with colleagues in northern Siberia, there were cells that stained like these neuroendocrine cells, and children with asthma had many more of these. They may be relevant to the differentiation process that you described.

*Redwan Moqbel:* How does the presence or absence of brush border in airway epithelial cells compared with gut epithelium impinge on their functions?

*Stephen Holgate:* The gut is designed to shed its epithelium and the whole machinery of the crypt, and the way the epithelium turns over is to get rid of it all the time. It has got a fantastic turnover rate. The airway epithelium is not designed for that. There is a fundamental difference in the turnover rate of epithelial cells. I think that it would be quite dangerous to extrapolate too much from the gut, even though the lung is an outgrowth of the gut.

*Qutayba Hamid:* When you take stem cells from subjects with severe asthma and grow them, they differentiate into epithelial cells that do not have cilia. If you take them from healthy individuals, the cells have a lot of cilia. The cells from the patients with the most severe asthma have changed their phenotype so that they cannot produce any more cilia. It is difficult to say whether there are any structural changes in the cell, but if there are any changes, they are phenotypic rather than structural.

*Stephen Holgate:* Christopher Britling in Leicester recently had quite a nice study showing that patients who have asthma with variable air flow obstruction and bronchial hyperresponsiveness had the same number of eosinophils and mast cells in the submucosa and epithelium, but it was the smooth muscle that showed a marked increase in the mast cell population. Is studying BAL and mucosal specimens actually looking at the right compartment if we are interested in smooth muscle responses? Mast cells or other inflammatory cells that are in the smooth muscle may be more important, and the mast cells that are present in the smooth muscle are ones that contain kinase rather than tryptase and seem to be resistant to steroids. What do you think?

*Mark Liu:* Cells that are on the airway surface or within the epithelium initially can respond and change the surface permeability so that the antigen can get to the cells that are deeper in the tissue. Whether the mechanism has to do with directly activating those deeper mast cells that are next to smooth muscle or whether neural phenomena or other factors are involved in the bronchoconstriction is not clear to me. We are clearly limited in terms of what biopsy specimens tell us. In the Kepley study of fatal asthma,<sup>64</sup> many sections were available. There were basophils all through the lung, in alveoli or alveolar structures and around smooth muscle, not just lined up along the surface of the airway.

*Redwan Moqbel:* Mark, how does the fact that the IgE receptors are expressed beyond basophils and mast cells affect your conclusions about how these cells function?

*Mark Liu:* Whether IgE receptors are functionally significant or not is open to discussion. There is no question that mast cells and basophils are the most responsive cells to antigen and to IgE-mediated stimuli. I am not even sure what the physiologic stimulus is for the eosinophil, for example, or for the macrophage for that matter. Even though the receptors are there in other cells, I just do not know what to make of them in terms of mediator release.

*Qutayba Hamid:* Why does the release of mediators from mast cells seem to be resistant to steroids?

*Mark Liu:* The mast cell is not responsive to steroids directly, but products from the mast cell and mechanisms that the mast cell may initiate would be responsive to steroids. If you look only at the mast cell and its mediator release, cytokine generation, and those sorts of effects, they are not affected by treatment of steroids. But many of the cascades are initiated by the mast cell, such as cell recruitment, the consequences of TNF- $\alpha$  action on the endothelium, histamine release, and permeability changes. All these would be steroid responsive, because the steroids would affect the downstream events initiated by the mast cell.

*Anthony Sampson:* Redwan, do you think that the leukotrienes released from the eosinophils act on the CysLT<sub>1</sub> receptors of eosinophils to control their maturation, proliferation, and migration?

*Redwan Moqbel:* I do not know. Eosinophils contain lipid bodies that are a major source of many of the cysteinyl phospholipids, and eosinophils can generate their own chemotactic factors. Whether these function in an autocrine manner through the CysLT<sub>1</sub> or CysLT<sub>2</sub> receptors is important to know but has not yet been studied.

*Qutayba Hamid:* Are the cells obtained in sputum fully activated cells? Do they reflect cells that have gone all the way through the activation processes and are capable of pouring out all the mediators?

*Redwan Moqbel:* Yes. Most, but not all, of the cells are measurable, because as we section the sputum plugs, a high percentage of the cells show activation.

*Emilio Pizzichini:* Can we be sure that the major source of the degranulation products is eosinophils? For example, we have observed neutrophilic exacerbations in which the size of inflammation is 50 million cells/mL, whereas the stable state is 4 million cells/mL. Could the neutrophils be secreting the MBP and eosinophil cationic protein that is damaging the epithelium?

*Redwan Moqbel:* We have made observations similar to what you have described. The MBP is stored primarily in eosinophils, and to a lesser extent basophils, whereas neutrophils have been shown to express eosinophil cationic protein and eosinophil-derived neurotoxin. The only other MBP-like source is found in trophoblasts during pregnancy. The MBP from eosinophils acts on neutrophils, and there is always the possibility that once eosinophils undergo cytolysis or necrosis, the MBP may

become sequestered in neutrophils. We use MAb BMK-13 to detect the human MBP.

*Qutayba Hamid:* What are the half-lives of these eosinophil products in the tissue?

*Redwan Moqbel:* These products have long-term effects, and because of their cationic nature they bind strongly to the target cells, which makes it difficult to get rid of them. These basic proteins are extremely "sticky" and rich in arginine. Peroxidase and MBP are the two that are most cytotoxic. Eosinophil cationic protein and eosinophil-derived neurotoxin have potent ribonuclease activity and lesser cytotoxic capacity. I am not aware of any studies on the half-lives of these proteins in the tissue, but I assume that they may be long.

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