REVIEWS

Neutrophils and Asthma

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■ **Abstract**

Although eosinophilic airway inflammation is recognized as an important feature of some patients with chronic, stable asthma, evidence supports an important role for neutrophils in asthma. Neutrophils are the first cells recruited to the site of the allergic reaction. Their presence may influence clinical presentation and has been linked to the development of severe chronic asthma and sudden severe attacks. Neutrophils are eliminated by apoptosis during the resolution of the allergic response.

Key words: Asthma. Neutrophil. Allergy. Eosinophil.

■ **Resumen**

Aunque la participación de los eosinófilos y otras células del sistema inmune se reconoce como unos factores importantes de la inflamación de las vías aéreas, hoy existen sobradas evidencias de la participación de los neutrófilos en la fisiopatología del asma bronquial. Los neutrófilos son las primeras células que acuden al órgano de choque de una reacción alérgica. Su presencia puede influenciar la presentación clínica, están ligados a un desarrollo severo y crónico de la enfermedad, así como a una muerte súbita debido a la misma. Mediante mecanismos de muerte celular programada son eliminados de los procesos patológicos alérgicos.

Palabras clave: asma, neutrófilo, alergia, eosinófilo.

Introduction

Many types of cell are involved in the pathophysiology of asthma. The contribution of mast cells, lymphocytes, and eosinophils has been well established. Nevertheless, 1 review of the literature found that only around 50% of asthma cases were associated with eosinophilic inflammation, and that in most other cases asthma was accompanied by an increase in airway neutrophils and interleukin 8 (IL-8) [1].

Neutrophils are polymorphonuclear leukocytes that play an essential role in the immune system, acting as the first line of defense against bacterial and fungal infections. Their role in the inflammatory process was once thought to be restricted to phagocytosis and the release of enzymes and other cytotoxic agents, but it is now known that these cells can release diverse mediators that have profound effects on the airways of asthmatic individuals. There is increasing evidence of the participation of neutrophils in allergic processes in general, and in asthma in particular. In this review, we will analyze some of the diverse aspects of the important role played by these cells in bronchial asthma.

Neutrophil Activation and Asthma

Patients with symptomatic asthma have elevated levels of peripheral neutrophils that show signs of being activated. Both the numbers and activation levels of these neutrophils are lower in the absence of symptoms or after treatment and resolution of the allergic process [2].

The chemotactic activity of neutrophils induced by platelet activating factor (PAF) has been seen to be greater in patients with asthma than in a healthy reference population [3], and to be inversely related to the production of 5-HETE, a derivative of arachidonic acid produced by neutrophils [2]. This difference has not been found for neutrophil chemotaxis stimulated by histamine, substance P, vasoactive intestinal peptide, or somatostatin [3]. This chemotaxic activity in the serum is inhibited by immunotherapy, even in the presence of symptoms [4].

The number of neutrophils in induced sputum in control subjects is in some cases similar to that in patients with slight to moderate asthma [5], but in others it has been found to be greater [6]. Nonetheless, increased neutrophil levels have been

found in patients with acute [7] or persistent asthma compared with controls [8,9], especially in patients with low numbers of eosinophils and poor response to inhaled corticosteroids [10]. A positive correlation has also been reported between the number of neutrophils in sputum and both the concentration of hydrogen peroxide (H_2O_2) in the expired air of patients with asthma [9] and variations in peak flow [10]. A negative correlation, in contrast, has been reported between forced expiratory volume in 1 second (FEV_1) and neutrophil numbers in patients with active asthma [10]. One large study of 1197 patients with asthma that examined relationships between induced sputum total neutrophil and differential eosinophil cell counts, and prebronchodilator and postbronchodilator lung function found prebronchodilator FEV_1 to be associated with neutrophilic and eosinophilic airway inflammation, and sputum total neutrophil counts to be associated with postbronchodilator FEV_1 [11]. The authors concluded that their findings supported the hypothesis that neutrophilic airway inflammation had a role in the progression of persistent airflow limitation in asthma.

While some authors have found no differences in the number of neutrophils in bronchoalveolar lavage (BAL) fluid of patients with slight asthma compared to a control group [12], others have detected elevated levels of these cells in patients with asthma [13]. Such an increase, however, is more common in patients with persistent [14] severe [15] asthma, or acute asthma [16]. In patients with nocturnal asthma, the number of neutrophils has been found to be significantly increased at 4 AM compared to 4 PM, contrasting with results for patients whose asthma symptoms did not worsen during the night [17]. In 1 study, 8 out of 10 subjects with isocyanate-induced asthma had increased neutrophil levels in BAL fluid [18], whereas in another, these levels were the same in asthmatic patients sensitized to red cedar and in normal subjects, although higher in patients with symptoms than those without [19].

The number of neutrophils in biopsy specimens of patients with slight asthma is in some cases similar to that in normal subjects but in others it is greater, and it increases significantly in patients with severe asthma [15]. Cells in asthmatic patients also have a greater number of adhesion molecules. Increased neutrophils in biopsy material obtained from patients with slight asthma have been reported following exposure to seasonal allergens [12]. Greater numbers of neutrophils have also been found in the airways of asthma patients who died within 2 hours of the onset of an asthma attack than in patients with slow-onset fatal asthma, who were found to have a predominance of eosinophils [20]. Elevated neutrophil levels have also been found in the submucous glands of patients with asthma and fatal-onset asthma compared to healthy controls [21].

Although some authors have found an increase in the numbers of neutrophils in bronchial biopsy material but not in the BAL fluid of patients that developed a late allergic response following a bronchial challenge [22,23], others have found

increased numbers both in biopsy material and BAL fluid up to 6 hours post bronchial challenge [24].

Patients that have undergone specific bronchial challenge tests have shown increased numbers of neutrophils in peripheral blood [25], greater serum neutrophil chemotactic capacity [26], and elevated neutrophil numbers in induced sputum [27]. BAL fluid cells have shown signs of being activated (greater molecule adhesion expression on the cell surface) following antigen challenge [28] and similar findings have been reported for cells obtained in post-challenge bronchial biopsy specimens [16].

Neutrophilic Mediators in Asthmatic Patients. Neutrophil Production of Compounds with Pathogenic Potential

The next section will describe factors produced by neutrophils that can contribute to early and late asthma responses. Mediators involved in early responses, for example, are released within 30 minutes of the in vitro allergen challenge of neutrophils.
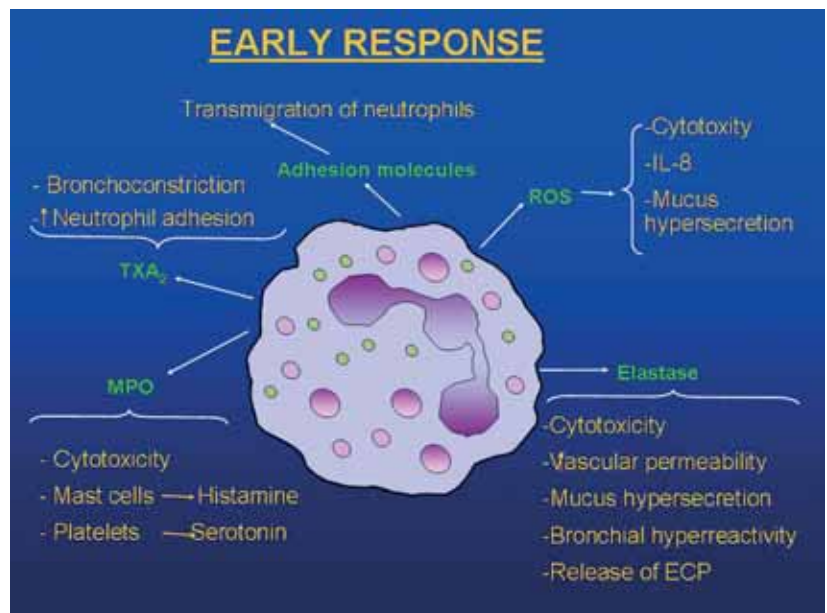

Figure 1. Neutrophils are important in the early response. TXA_2 indicates thromboxane A_2; MPO, myeloperoxidase; ROS, reactive oxygen species; IL, interleukin; ECP, eosinophil cationic protein.

Metalloproteinases

Matrix metalloproteinase-9 (MMP-9) is perhaps the best studied inflammatory mediator in asthma. Elevated levels of MMP-9 have been found in both BAL fluid and sputum from patients with asthma. These levels have been correlated with extent of cell infiltration [29] and asthma severity [30], and, although MMP-9 can be synthesized by diverse cells (macrophages, eosinophils, epithelial cells, and fibroblasts), its presence in these cases has been found to be due almost entirely to neutrophils [30]. Increased levels of MMP-9 mRNA and MMP-9 protein have been found in the bronchial walls of asthmatic patients [31]. A statistically significant increase in MMP-9 has been demonstrated in BAL fluid and biopsy

material obtained from the subepithelial basement membrane (SBM) of patients with severe asthma [32], and statistically significant elevated levels of neutrophils and macrophages, but not of eosinophils, have been found in patients with SMB levels of MMP-9, expressed in neutrophils. BAL fluid levels, however, were found to correlate strongly with the presence of eosinophils, not neutrophils, suggesting that the presence of MMP-9 in the SBM might promote the movement of eosinophils towards the lumen by allowing them to migrate more easily through the SBM and the epithelium, thus contributing to the worsening of pulmonary function. The role of neutrophils, in contrast, might be to clear the way for eosinophils, facilitating their passage to the lumen, where they would remain in the tissue to repair damage, releasing MMP-9 and transforming growth factor (TGF). The authors of the above study also found pulmonary function to be significantly decreased in patients with positive MMP-9 expression.

High levels of activated MMP-9 have been found in the BAL fluid of patients with asthma [30]. Such patients have also shown significantly increased levels of MMP-9 following a specific allergen challenge, contrasting with healthy subjects in whom no change was observed [34]. In the same study, postchallenge levels of MMP-9 were significantly correlated with changes in FEV_1 and sputum neutrophil percentage. In another study, neutrophils were found to contribute to MMP-9 levels following a specific allergen bronchial challenge [35].

One of the factors to consider with respect to MMP-9 is that it is released directly from neutrophils by IL-8 [36], meaning that the possible involvement of an autocrine mechanism in the production of MMP-9 by these cells cannot be ruled out.

It has been reported that the inhibition or lack of metalloproteinases, especially MMP-9, prevented the onset of asthma in a murine model [37]. MMP-2, for its part, might be involved in bronchial remodeling, as its presence is required for smooth muscle cell proliferation in vitro [36]. This protein has also been found in the sputum of patients with asthma [29].

MMP-8 has been reported as high in BAL fluid and biopsy specimens from asthmatic patients, and inversely proportional to FEV_1 values [39]. The presence of MMP-8 indicates the onset of irreversible injury rather than inflammation per se and the cells that preferentially express MMP-8 in such injuries are neutrophils.

Our group has shown that the treatment of neutrophils with anti-immunoglobulin E, N-formyl-methionyl-leucyl-phenylalanine (fMLP), and allergen causes the release of MMP-9 in a dose-dependent and time-dependent manner, and that the amount of MMP-9 released increases when neutrophils from allergic patients are incubated with monoclonal antibodies against FcεRI, FcεRII/CD23, and galectin-3 (manuscript in preparation).

Elastase

Elastase has many functions that might be involved in the pathophysiology of asthma, including epithelial damage, increased vascular permeability, hypersecretion of bronchial mucus, metaplasia of bronchial mucus glands, bronchoconstriction, and bronchial hyperreactivity [40]. In an allergen-specific nasal challenge, a significant increase in elastase was observed in patients that received the allergen as opposed to a buffer used as a negative control [41]. Increased levels of elastase have also been found in the nasal lavage fluid of patients with allergic rhinitis but not of those with nonallergic rhinitis [42]. It has been demonstrated that elastic fibers are disrupted in the bronchi of asthmatic patients, supporting the idea of an imbalance between proteases and antiprotease in this disease [43]. Neutrophilic elastase levels have also been seen to be elevated in the bronchial secretions of patients during asthma exacerbations [44,45] and in the induced sputum of asthmatic patients compared to healthy controls; these levels were negatively correlated with FEV_1 and positively correlated with the duration of the process. Elastase reproduces many of the pathophysiological situations that develop in asthma [40]; it promotes the recruitment of neutrophils to the lung when inducing IL-8 secretion and also produces eosinophil cationic protein (ECP) from eosinophils. Der p 1, a cysteine protease destroys α-1-antitrypsin, an elastase inhibitor, and thus increases the effects of elastase through its enzymatic action.

Our group has demonstrated that the neutrophils of patients with asthma release elastase via an immunoglobulin (IG) E–dependent mechanism [45]. We found that only patients with positive skin prick tests and positive serum IgE to the allergens studied had positive specific IgE to those allergens on the cell surface membrane. None of the subjects with negative skin prick tests and negative serum IgE to these allergens (patient and controls) had positive specific IgE on the cell surface. After an in vitro allergen challenge, only the neutrophils of patients with specific IgE on the cell surface released elastase. Because there was a possibility that the elastase might have been released as the result of an IgG mechanism, we tested for the presence of specific IgG on the cell surface of neutrophils in allergic patients but failed to find it, thus proving that the mechanism was IgE-dependent, with no involvement of IgG. We also found that an in vitro challenge of neutrophils with allergens to which the patients were sensitized caused a release of elastase that was dependent on the dose of allergen and the duration of the stimulus. This release was absolutely specific, since only the allergens responsible for clinical symptoms caused a release of elastase. There was no such release in either nonsensitized patients or in healthy subjects, although in such cases the neutrophils were functional because they released elastase when stimulated with a positive control (cytochalasin-B and fMLP). We also found an inversely proportional relationship between the amount of elastase released by the neutrophils and pulmonary function measured by FEV_1 [45].

Lactoferrin

Elevated peripheral serum lactoferrin and neutrophil levels have been observed in atopic patients compared to normal controls, although no correlation has been found with a fall in peak flow in asthma patients [46]. Although in vitro PAF, leukotriene B4 (LTB4), and phorbol myristate acetate induced a greater release of lactoferrin by neutrophils than a control buffer solution did, only PAF was able to induce a significantly greater release in atopic than in nonatopic subjects

and its activity was inhibited by the presence of a PAF receptor inhibitor. (LTB4 also induced a greater release of lactoferrin in atopic patients but the difference with nonatopic patients was not significant). No differences in neutrophil lactoferrin secretion have been observed between allergic patients with and without symptoms, although a greater amount of this substance has been found in the nasal lavage fluid of patients with symptomatic pollen-induced allergic rhinitis [47]. In another study, there was a statistically significant dose-dependent increase in the amount of lactoferrin found in nasal lavage fluid after an allergen challenge [48]. Lactoferrin levels in induced sputum and BAL fluid have also been found to be greater in patients with stable asthma than in control subjects [49]. One forensic autopsy study found that lactoferrin levels were significantly elevated in the bronchi of patients who had died of fatal asthma compared to the bronchi of patients who had died from nonrespiratory causes [50]. Lactoferrin plays a central role in the modulation of the inflammatory process in the airways because of its ability to combine with free ferric ions, preventing them from contributing to the catalysis of toxic oxygen radicals and thus allowing them to continue functioning. At the same time, however, lactoferrin amplifies inflammatory cell response by promoting the adhesion of leukocytes to endothelial walls [51]. The recent discovery that neutrophilic lactoferrin, in amounts similar to those found in airway fluid, induces several effects in eosinophils [52] might greatly contribute to the understanding of certain aspects of the pathophysiology of asthma and allergic processes in general. These effects include the production of superoxide, degranulation with release of eosinophil-derived neurotoxin, and the synthesis of leukotrienes with the subsequent secretion of LTC4 [53].

Our group has demonstrated that lactoferrin is secreted by the neutrophils of asthmatic patients through an IgE-dependent mechanism [unpublished observations]. We found that this enzyme was specifically released in response to antigens responsible for clinical symptoms as no effect was observed in nonsensitized or healthy subjects. We also found higher levels of lactoferrin in asthma patients compared to controls, and these levels were highest in patients with severe asthma. There was no significant difference in lactoferrin levels between patients with asthma and rhinitis although we did observe a significant inverse correlation between neutrophil lactoferrin release and lung function, measured by FEV_1, in the patients. We also detected a greater release of lactoferrin after bronchial allergen challenge but no statistically significant differences in secretion levels following bronchial challenge with serum or methacholine. Differences in the amount of lactoferrin released with respect to baseline figures were the same in patients with an early response only and with a dual response.

Myeloperoxidase

Myeloperoxidase (MPO) released from neutrophils can react with H_2O_2 generated during a respiratory burst, generating hypochlorous acid (HOCl) and similar compounds that can cause injury to surrounding tissue during the inflammatory process. MPO levels have also been found to be elevated in the BAL fluid of asthma patients compared to controls [54].

While significant differences have not been found for peripheral MPO/neutrophil levels between atopic patients and healthy controls [55], higher levels of MPO have been found in induced sputum and BAL fluid in patients with asthma than in control subjects, demonstrating that degranulation of primary granules takes place in asthma [56]. Antigen-specific nasal provocation has been seen to produce a delayed increase in MPO levels in nasal lavage secretions in atopic patients but not in healthy subjects [57]. In another study, a greater release of MPO was observed in allergic patients than in controls when neutrophils were stimulated with particles of Sephadex opsonized with serum [58]. MPO release has been seen to be greater in pollen-atopic patients at the end of spring than at times when these patients are asymptomatic [59]. Using the same opsonized particle method, a later study found that neutrophils released more MPO when preincubated with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in particular but that this effect was absent following incubation with IL-5 [60]. As occurs with eosinophils and ECP, the secretion of lactoferrin and MPO by neutrophils is not inhibited by the addition of cytochalasin-B [61].

Our group has performed various studies on the release of MPO by neutrophils. In 1 study, when these cells were stimulated with fMLP, a chemotactic factor activator of neutrophils, the release of MPO was greater in a group of asthmatic patients not receiving immunotherapy than in either immunotherapy-treated patients or healthy controls [62]. We also found a negative correlation between MPO release and pulmonary function, measured by FEV_1, in the patients. The release of MPO from the neutrophils of atopic patients is inhibited to varying degrees by different antihistamines (loratadine>terfenadine>cetirizine), sodium nedocromil, and corticosteroids (dexamethasone and budesonide) [unpublished observations]. On stimulating neutrophils with antigen, we detected a specific release of MPO that was not observed in nonsensitized allergic patients or healthy subjects [63,64]. The release occurred both in vitro [63] and in vivo after antigen-specific conjunctival provocation in asthmatic patients [64]. In the in vivo study, a significantly decreased amount of MPO was obtained in the tears of asthmatic patients who had received immunotherapy compared to those who had not.

Adhesion Molecules

Neutrophils are equipped with sensors for soluble signals generated in tissues in response to cell injury, and with sensors for surface molecules [65]. These receptors control communication between neutrophils and their external environment and are capable of recognizing endothelium activation, which promotes neutrophil adherence to endothelial cells or to the surface of bacteria or damaged cells for phagocytosis and subsequent destruction. At the present time, it is well documented that the molecules CD11b (variable chain M of the 2 integrins, which forms part of the receptor for complement CR3) and CD35 (the receptor for complement CR1) not only form part of the cell surface of neutrophils, but also that their lesser or greater expression can trigger immunodeficiency or cell activation. The expression of CD35 on the surface of neutrophils has been found to be higher in

asthmatic patients than in a healthy reference population [65], with levels increasing after bronchial challenge with antigen or histamine, or after exercise [66]. Elevated CD11b expression has also been detected in these cells following in vitro stimulation with fMLP [65]. The modulation of these molecules in allergic patients might not only mediate the phagocytosis of opsonized particles but also play a role in cell adhesion, allowing them to bind to vascular endothelial cells and subsequently migrate to the target organ (skin, nasal mucosa, airways, etc.) [65].

Increased adhesion of E-selectin and intracellular adhesion molecule-1 (ICAM-1) to neutrophils in peripheral blood has been observed in patients with greater variability in peak expiratory flow (PEF). When compared to patients with low PEF variability, the increases were significant for ICAM-1 but not for E-selectin [67].

Our group has investigated modifications in the following adhesion molecules and membrane receptors in the neutrophils of atopic patients: CD11a (variable chain αL of the β_2 integrins), CD11b, CD11c (variable chain αX of the β_2 integrins), CD18, CD62L (L-selectin), ICAM-1 (CD54), CD32 (receptor II of IgG: FcγRII), and CD16 (receptor III of IgG: FcγRIII) [68-70]. Patients stimulated with an allergen to which they were sensitized showed a neutrophil surface decrease in CD62L levels. This decrease was not found in healthy controls or in allergic patients stimulated with an antigen to which they were not sensitized. On stimulating the neutrophils of allergic patients with anti-IgE antibodies, we observed an increase in CD16 expression and a decrease in CD62L expression, measured as a percentage of positive cells. Immunofluorescence staining showed an increase in CD11b and CD18 expression, but a decrease in CD62L expression, and all the changes observed were dependent on the dose of anti-IgE added to the culture. We also observed that the effect of anti-IgE was more powerful than that of the antigen, since it was associated with a higher number of stimulated IgE receptors. As the amount of CD62L on the cell surface was reduced, there was an increase in soluble CD62L in the culture supernatant. Immunotherapy significantly inhibits the release of CD62L from the cell surface, restraining the passage of these cells from the bloodstream towards the site of the allergic response. As the results obtained for CD62 were consistent in the different analyses performed, we continued our study of this marker and found that both antihistamines (loratadine, terfenadine, and cetirizine) and disodium cromoglycate were unable to reverse the effects of anti-IgE, while budesonide and dexamethasone inhibited the downregulation of CD62L.

The loss of L-selectin mediated by anti-IgE antibodies is dependent on phospholipase A_2, protein kinase C, and phosphatidylinositol triphosphate kinase, and has some dependency on tyrosine and serin-treonine kinases; it is, however, totally independent of phosphatidylinositol and phosphatidylcholine phospholipases C [66-70].

Lipid Mediators

Neutrophils do not contain preformed lipid mediators, but they are able to synthesize them (mainly PAF and LTB4). Although there has been some debate in the past, it has now

been demonstrated that neutrophils are able to synthesize prostaglandins and thromboxanes through the enzyme cyclooxygenase (COX) [71,72].

While some authors have not found any differences between asthmatic patients and a healthy reference population in terms of the release of LTB4 by neutrophils stimulated with calcium ionophore A23187 [73], others have found statistically significant differences [2,71]. A possible explanation for these divergent results is that in the first case the patients were asymptomatic and receiving immunotherapy [73] while in the other 2 cases, the patients were not receiving immunotherapy [2,71]. Moreover, there were no such differences between patients with and without clinical symptoms either in or outside the pollen season [71]. There are also conflicting results with respect to research into another metabolite of 5-lipoxygenase, 5-HETE. In 1 study, the group reported a greater amount of 5-HETE in patients with asthma than in healthy controls [2], while in another they found that the opposite was true [72]. They suggested that the differences were due to the fact that the patients were asymptomatic in the first case and symptomatic in the second one.

COX metabolizes arachidonic acid into prostaglandins and thromboxanes in a series of steps. The enzyme has 2 isoforms: type I (COX-1), which is constitutive and present in many cells, and type II (COX-2), which is normally absent in basal conditions but can be induced in certain cells by mitogens, cytokines, and other factors. Several normal human cell types, including neutrophils, can express COX-2 and its eicosanoid derivatives following suitable stimuli. The neutrophils of asthmatic patients have been seen to synthesize COX-2 after in vitro challenge with PAF, TXB2, 9α-11β-PGF2, PGF2α, PGD2, and PGE2 [74], although a comparison with a healthy reference population has not been made.

Using flow cytometry, our group detected intracellular COX-2 in the neutrophils of allergic patients after 20 minutes of stimulation with an anti-IgE antibody [75]. We also found COX-2 in the cells of symptomatic patients during antigen stimulation, but not in those of healthy subjects or patients not sensitized to a particular allergen. Using immunoblotting, we detected a greater presence of COX-2 in neutrophils stimulated with anti-IgE antibodies (at a concentration of 25 μg/mL) than when stimulated by phorbol 12-myristate 13-acetate (PMA) (at 100 nM) or bacterial lipopolysaccharide (at 100 μg/mL). We also observed increased levels of thromboxane B_2 in the supernatant after stimulation with anti-IgE antibodies and increased levels of prostaglandin PGE_2 after stimulation with a specific antigen.

Reactive Oxygen Species

Neutrophils are the major source of superoxide anion (superoxide radical O_2^-), H_2O_2, and HOCL, and it has been well established that oxidants can act jointly with neutrophilic proteases to increase the degree of tissue damage by inactivating the participation of antiproteases (through oxidation of the methionine of α-1-antitrypsin, for example) and/or perhaps by altering the protein structure and thus making molecules more susceptible to proteolytic attack [2,76].

In the absence of a stimulus, neutrophils have been

seen to produce more superoxide in atopic than in nonatopic individuals [76]. While it might seem that this is due to alterations in atopic patients, this is not the case as it was seen that the difference remained significant following the stimulation of neutrophils with calcium ionophore A23187 and the chemoattractant fMLP. Similar results have been seen for fMLP in other studies [77,78]. Neutrophils have also been found to produce more toxic oxygen radicals in asthmatic patients compared to controls when stimulated with PMA [78] or opsonized zymosan [76-78]. The production of O_2^- has been seen to be inversely proportional to FEV_1 values [79] and to bronchial hyperreactivity induced by methacholine [78] and histamine [77] (in both cases at the amount required to cause a 20% drop in FEV_1) . Furthermore, greater amounts of O_2^- have been found in patients who present a worsening clinical picture (determined by the presence of asthmatic attacks) than in patients with stable asthma [77].

In contrast, no difference has been found between atopic patients and healthy controls in terms of the production of toxic oxygen radicals when neutrophils have only been stimulated with PAF and LTB4 [76]. However, both of these substances prime the production of O_2^- following neutrophil stimulation with fMLP, and more so in allergic patients than in healthy controls [76-80]. Dexamethasone and azelastine have a greater inhibitory effect on O_2^- production in healthy controls than in allergic patients, suggesting a greater resistance to the effect of these medicines in atopic processes either because of a greater and more persistent presence of PAF and LTB4 in allergic patients or because of an intrinsic alteration in the patients themselves [79,81].

Neutrophils from the BAL fluid of asthmatic patients have been seen to produce greater amounts of toxic oxygen radicals than those from a reference group of healthy controls [81]. While platelets inhibited the formation of O_2^- in the neutrophils of healthy controls, they did not do so in a patient with severe asthma and an increased platelet count. The authors of the study suggested that there might be an abnormal relationship between platelets and neutrophils in certain types of asthma.

Our group has observed that the in vitro challenge of neutrophils from allergic patients with an antigen responsible for their clinical picture led to an increase in the respiratory burst of these cells [82]. The increase observed was directly dependent on the concentration of the antigen and the time during which it acted. The activation was specific since it did not take place when an antigen to which the patients were not sensitized was added or when cells from healthy controls were used. Following stimulation with the antigen, we detected the incorporation of the 2 cytoplasmic components of the NADPH oxidase, p47 and p67, into the cell membrane to form the active NADPH oxidase. In our model, stimulation of the antigen-specific respiratory burst in allergic patients was inhibited when antihistamines (loratadine, terfenadine, cetirizine
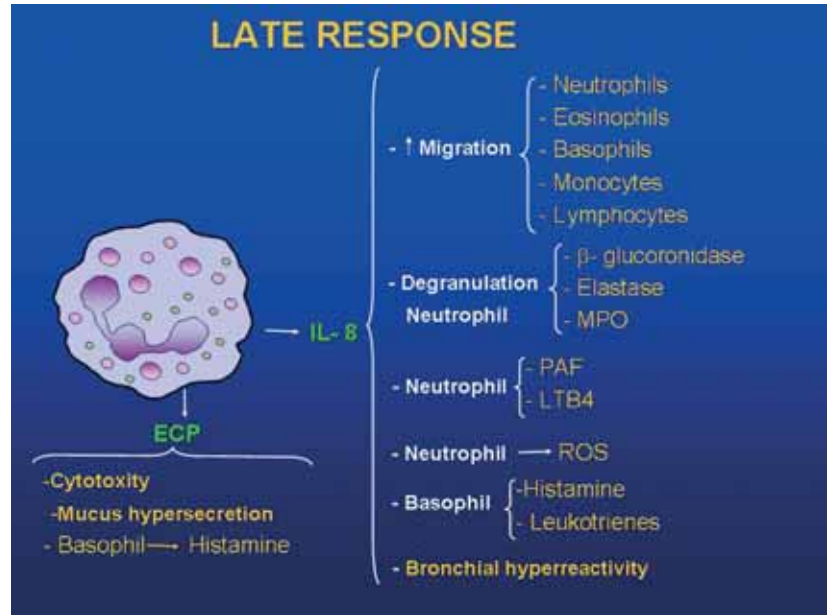

Figure 2. Neutrophils are also important in the late response. ECP indicates eosinophil cationic protein; IL, interleukin; MPO, myeloperoxidase; PAF, platelet activating factor; LTB4, leukotriene B4; ROS, reactive oxygen species.

and carebastine), sodium nedocromil, and corticosteroids (budesonide and dexamethasone) were added [unpublished observations]. In another study, we saw a greater production of respiratory burst in patients with asthma than in healthy controls following stimulation with anti-IgE antibodies [83]; the asthmatic patients receiving immunotherapy, in contrast, showed a reduction in respiratory burst to levels comparable to those of healthy subjects [83].

Neutrophil factors involved in the initiation and maintenance of late allergic reactions are released at 18 hours after the challenge of these cells.

Eosinophil Cationic Protein

ECP is a powerful cytotoxic molecule with a capacity to kill diverse cells in mammals and a wide variety of other organisms, including parasites, bacteria, and viruses. This protein plays an important role in resistance to parasites and in allergic reactions. ECP has traditionally been reported as being specific to eosinophils, with levels correlating with eosinophil activity in allergic rhinitis, asthma, conjunctivitis, and atopic dermatitis [84]. Recent studies, however, have also demonstrated the presence of ECP in human neutrophils [85], and others have found ECP levels to be more closely related to the presence/activation of neutrophils than to that of eosinophils in various allergic processes [86]. Our group, for example, demonstrated that ECP was found in and could be released and synthesized from the neutrophils of asthmatic patients [85]. Firstly, ECP can be measured by enzyme-linked immunoabsorbent assay and immunoblotting in the supernatants of stimulated neutrophils. Secondly, an increase in intracellular ECP can be detected by means of flow cytometry and fluorescent microscopy, and thirdly, when neutrophils are stimulated, ECP mRNA levels can be quantified using real-

time polymerase chain reaction. As with other mediators, these results are very specific, and only take place with allergens to which patients are sensitized and not in nonsensitized patients or healthy subjects. There are several differences between the release of ECP by eosinophils and that by neutrophils. In the first case, it takes place very quickly, in 10 to 30 minutes, whereas in the second case it requires between 3 and 18 hours. Many soluble stimuli, such as IL-5, GM-CSF, and PAF, practically do not release mediators from eosinophils by themselves, but they do in neutrophils. Neutrophils, unlike eosinophils, release ECP if stimulated with allergen or anti-IgE antibodies [85]. ECP participates in the pathophysiology of asthma through its cytotoxic capacity, which stimulates the release of histamine from basophils and lactoferrin, and causes an increase in bronchial mucus [84].

Interleukin 8

IL-8 is a powerful chemoattractant and activator of neutrophils. In the lungs, it seems to be the most potent chemoattractant for these cells. Although IL-8 can be produced by several cell types, it can also be synthesized by neutrophils in response to various inflammatory mediators. The production of IL-8 by neutrophils, thus, can contribute to an additional recruitment of neutrophils and, importantly, increase or prolong the activation of neutrophils in an autocrine form.

Increased IL-8 levels have been observed in induced sputum, BAL fluid, and tracheal suction specimens from patients with asthma compared to controls [87], and a greater release of both nasal and bronchial IL-8 following a specific antigen challenge has also been reported [87]. Asthma exacerbations following the withdrawal of inhaled corticosteroids have been associated with a significant increase in sputum IL-8 and neutrophil influx 2 weeks prior to the exacerbation [88]. This could have important clinical implications because there appears to be a window during which it might be possible to adjust asthma treatment [88]. In addition to demonstrating that IL-8 is produced by neutrophils in atopic patients, our group has also shown that IL-8 synthesis is stimulated in association with an increase in mRNA expression. The process is based on a mechanism dependent on the Ca^{2+}-calmodulin-calcineurin axis and the stimulation of the nuclear factor κB [89-91]. Antihistamines (loratadine >terfenadine>cetirizine), corticosteroids (budesonide and dexamethasone), and immunotherapy are able to inhibit the production and release of IL-8 by the neutrophils of allergic patients following IgE-dependent stimuli.

Although our group was initially unable to detect nuclear factor of activated T cell (NFAT) activity in neutrophils [91],
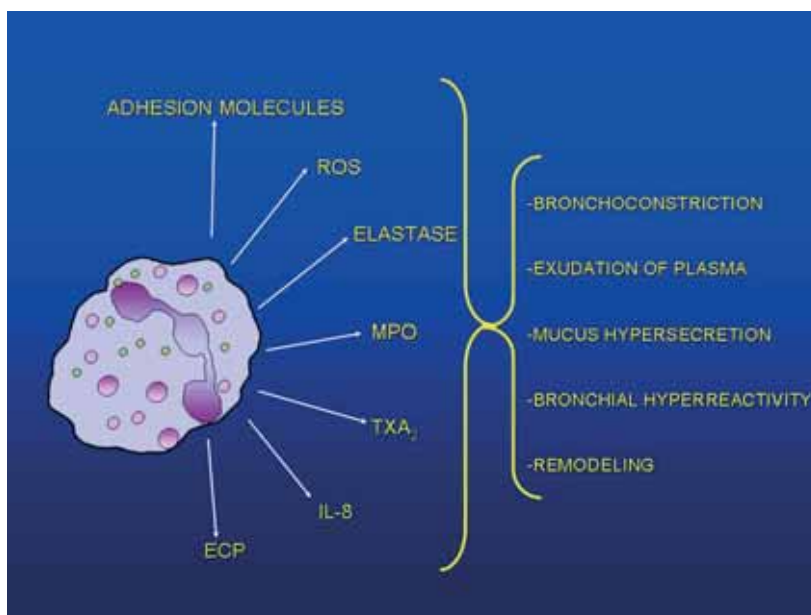

Figure 3. In asthma, the pathophysiological consequences of the immunoglobulin E–dependent activation of neutrophils are numerous. ROS indicates reactive oxygen species; MPO, myeloperoxidase; TXA2, thromboxane A2; IL, interleukin; ECP, eosinophil cationic protein.
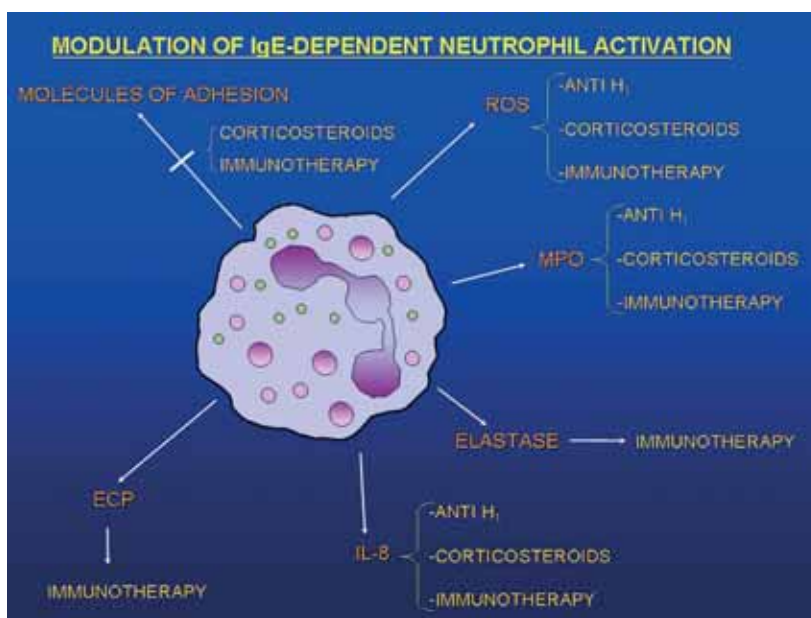

Figure 4. Different agents can modulate immunoglobulin (Ig) E-dependent neutrophil activation. The most important modulator is specific allergen immunotherapy. ECP indicates eosinophil cationic protein; ROS, reactive oxygen species; MPO, myeloperoxidase; IL, interleukin.

we have since verified the presence of mRNA for 4 of the 5 isoforms of NFAT in these cells: NFAT1 (NFATp), NFAT2 (NFATc), NFAT4, and NFAT5. Of these, only NFAT2 translocates to the nucleus when cells are stimulated with allergen or anti-IgE antibodies. This response is stimulus specific since it only occurred when allergens to which the patients were sensitized were used, indicating that this nuclear factor is activated by an IgE-dependent mechanism [92].

Evidence exists that NFAT2 plays an important role in the induction of T$_H$2 cytokines. In 1 study, a mutant NFAT2-deficient mouse was found to be unable to secrete IL-4 [93]. Our findings are consistent with these concepts, since an IgE-dependent mechanism would stimulate the production of T$_H$2 cytokines such as IL-4, which have also been found to be present in and to be released by neutrophils [94]. It would also contribute to a perpetuation of the allergic process from the originating regulation of the neutrophils.

Neutrophils can be activated through the 3 IgE receptors, but primarily through galectin-3 and—especially—FcεRI. According to studies by our group, all these IgE-dependent neutrophil effects can be modulated by antihistamines, corticosteroids, and immunotherapy, the last of which has the greatest effect on the IgE-dependent activation of neutrophils.

The Neutrophil as a Regulatory Cell in Asthma

Atopy is a pathologic process in which, following the recognition of allergens by antigen-presenting cells (APCs), an immunological response develops under the influence of T cells that induces the synthesis of IgE by B cells, which—when bound by their Fc portions to the different cell receptors, and after recognition of the antigen responsible for the clinical disease—induce the release of mediators and the accumulation of inflammatory cells at the site of inflammation. Various inflammatory cells (macrophages, lymphocytes, mast cells, basophils, eosinophils, neutrophils, and platelets) have been found to be involved in the allergic reaction, but the roles played by each of them have not been fully elucidated.

APCs play a key role in the immunological response as they present antigen to T cells, which is necessary to eventually achieve a complete response. In allergic processes, such as asthma, the processing of an allergen and its presentation to the T cell receptor (TCR) by the HLA-II complex (signal 1) are needed. The complete development of the process requires costimulation (signal 2) mediated by the union of CD28 in the T cells with CD80 or CD86 in the APCs. This interaction allows CD28 to combine with the TCR, leading to an additional activation of the complex. Other accessory molecules are also required to complete the activation of T cells such as leukocyte function-associated antigen-1 (LFA-1) in the T cells, and its main receptor, ICAM-1 in the APCs. Another pair of adhesion receptors is formed by CD2 in the T cells and CD58 (LFA-3) in the APCs. Various cytokines, such as TGF-ß and IL-10 can regulate this activation [95,96].

Immature dendritic cells (DCs) are distributed throughout the different tissues of the body, where they come into contact with different stimuli (necrotic cells, microorganisms, etc.) and are subsequently presented to T cells. This process can last from minutes to hours, depending on the tissue where the reaction takes place.

As DCs mature, they change their phenotype and their functional activity is influenced by varying factors such as the nature of antigens (microorganisms, apoptotic/necrotic cells), the microenvironment, and endogenous signals, all factors that can lead to antigen-specific T-cell activation or the induction of immunological tolerance [97-106].

Neutrophils do not return to circulation but are either eliminated by secreting mucosa or die in the tissues within 1 to 2 days. One means by which neutrophils are destroyed is apoptosis, or genetically programmed cell suicide. After apoptosis, they are eliminated by both professional phagocytes (macrophages) and non-professional phagocytes (eg fibroblasts). Neutrophil life span can be prolonged by different signals in inflamed tissues via the suppression of apoptosis. Obviously, this is not only an effect of extrinsic mediators, but also an action of the intrinsic neutrophil resources of autocrine/paracrine regulation [107]. The mitochondrial pathway of apoptosis is dependent on the B-cell CLL/lymphoma 2 (BCL-2) protein family (whose members include the BCL-2 associated x protein [BAX]) for the efficient release of pro-apoptotic factors such as the second mitochondria-derived activator of caspase (SMAC) from the mitochondrial intermembrane space. These factors induce caspase activation, which is necessary to elicit the phenotypes associated with apoptosis. The BCL-2 family also contains antiapoptotic proteins such as myeloid cell leukemia-1 (MCL-1). In patients with atopic asthma, human IgE delays neutrophil apoptosis but this effect is not dependent on either FcεRI crosslinking or the autocrine release of soluble mediators. It is, however, associated with MCL-1 activation and BAX and SMAC retention, which induces a reduction in caspase-3 activity. IgE-dependent delayed neutrophil apoptosis in allergic patients may therefore contribute to persistent neutrophilic inflammation in atopic asthma [108].

The fact that neutrophils play an immediate role in immune defense requires their early arrival at the inflammation site, making them ideal candidates for controlling the recruitment of other cell types to this site. Activated neutrophils produce several chemokines, including IL-8, growth-related oncogene-α (GRO-α), macrophage inflammatory protein 1-α (MIP-1α), and MIP-1ß [109]. IL-8 and GRO-α are chemoattractive for neutrophils and therefore form a positive feedback loop that induces the accumulation of large numbers of neutrophils. MIP-α and MIP-ß attract immature DCs in addition to T cells, monocytes, and macrophages [110], while α-defensins, which are released during neutrophil degranulation, have been found to chemoattract both T cells and immature DCs [111]. Immature DCs also produce IL-8 soon after stimulation, thereby attracting neutrophils and contributing to their colocalization [112]. As a result, crosstalk between neutrophils and DCs in different pathogenic challenge situations is enabled as the cells are located in the same place at the same time. Neutrophils play a direct role in adaptive immunity via various mechanisms. They recruit immune cells such as T cells and DCs to the inflammation site, instruct these cells directly, and induce adaptive immune responses. During inflammation, neutrophils are able to travel from the inflammation site to the nearest lymph node [113], where they undergo apoptosis and are taken up by DCs. As a consequence, DCs can present neutrophil-derived antigen to T cells. Neutrophils have also been found to acquire antigen-presenting functions, thereby enabling them to directly activate T cells [114]. Finally, they can directly transfer antigens to DCs, which subsequently activate T cells [115].

Like other cells such as lymphocytes, macrophages, and natural killer cells, neutrophils are able to synthesize and release a great variety of cytokines that play a key role in the

development of the immune response. Examples include IL-1, IL-3, IL-6, IL-8, TNF-α, IL-12, IFN-γ, GM-CSF, MIP, and TGF-ß.

The surface-expressed lactoferrin released from neutrophils after contact with autologous CD4$^+$ T cells has been seen to suppress T_H1 cytokine but with a tendency to enhance T_H2 cytokine production [116]. Puellmann et al [117] have demonstrated the presence of TCR in neutrophils. Activation of the neutrophil immunoreceptor by known TCR agonists increases IL-8 secretion and inhibits neutrophil apoptosis. These results suggest that the activation of TCR in neutrophils and the subsequent release of IL-8 may contribute to neutrophil inflammation in patients with atopic asthma.

The constitutive expression of MHC class II antigens is restricted to professional APCs, such as DCs, B cells, and cells of the monocyte/macrophage lineage. Such antigens are not expressed in the neutrophils of healthy individuals but their induction has been described in both mature and precursor neutrophils [118] in response to interferon (IFN)-γ GM-CSF, and/or IL-3 [119,120]. The expression of MHC class II on the surface of neutrophils has been found to be donor dependent [121] and has also been reported in vivo in Wegener´s granulomatosis [122], rheumatoid arthritis [123], tuberculosis pleural effusions [124], and following the administration of GM-CSF, granulocyte CSF, and IFN-γ [125-127].

The only known function of MHC class II antigen is the presentation of antigens to cells. Costimulatory signals are required, however, for the full activation of T cells; such signals are delivered via the interaction between APC receptors and T-cell counterreceptors. Multiple pairs of costimulatory molecules, ICAM-1 (CD54), and LFA-3 (CD58) are constitutively expressed on neutrophils [128]. Other molecules, such as CD80 and CD86, which are both ligands for CD28 in T cells, are not expressed on naïve neutrophils, but are synthesized de novo in response to IFN-γ, GM-CSF, or a combination of both [118,129].

There is experimental evidence that neutrophils themselves act as APCs. When neutrophils are cultured with staphylococcus enterotoxin—also called superantigen—and T cells, the latter proliferate. The use of staphylococcus enterotoxin in this type of experiment has several advantages: *a)* the antigen does not need to be processed, *b)* there is only a relative dependency on the haplotype DR, and *c)* antigen-specific T cells are not needed because stimulation by heterologous cells is possible. As most T cells respond to superantigen, there is a significant proliferation of these cells, making these experiments easy to perform and reproduce. However, presentation of superantigen is very different from the more complex presentation of peptide antigens (which is what occurs in the development of allergic responses). In contrast to the presentation of superantigens, that of peptides to T cells requires processing of the antigenic protein by the APCs, followed by the union of the processed peptide molecules to HLA II and the transfer of the HLA II-peptide complex to the cell surface. Because the clefts formed by the 2 chains of HLA to which the peptides are joined vary with the haplotype DR, there is a certain selectivity with respect to the peptides that may be presented/displayed. It has also been found, using tetanic toxin, that neutrophils can act as APCs [129].

Our group has shown that neutrophils from allergic patients modulate the expression of their cell-surface HLA-DR molecules after cytokine activation in different amounts to neutrophils from healthy controls do [unpublished observations]. We found that the expression of MHC II on neutrophils was significantly higher in asthmatic patients not receiving immunotherapy than in a healthy group. In agreement with our results, other authors have shown that allergic patients have a greater expression of HLA-DR in different cells such as basophils [130], B cells [131], and in cellular infiltrates at the sites of allergen-induced late-phase cutaneous reactions [132]. The mechanisms leading to the described findings are complex. Upregulation of HLA-DR in allergic patients compared to healthy subjects can be partially explained by changes to the cytokine microenvironment in neutrophils from atopic patients. Because neutrophils are able to produce and store IL-4, an autocrine stimulation is possible. IL-4 is also responsible for MHC II expression in mast cells [133]. The same effect (IL-4-induced MHC II expression) can be assumed for neutrophils. We have previously shown that allergens have direct effects on neutrophils in vitro [unpublished observations] but these effects in vivo may also contribute to the observed modulations in HLA-DR surface expression.

Immunotherapy has been seen to negatively modulate IgE-dependent upmodulation in HLA-DR surface expression in neutrophils from atopic patients who had received allergen immunotherapy [unpublished observations]. Immunotherapy produces a downmodulation of HLA-DR molecules on the surface of other cells, such as B cells [131,134], basophils [130], and T cells [135]. HLA-II is the key molecule for APCs. The fact that immunotherapy in asthmatic patients has been associated with a reduction in the percentage of neutrophils expressing the HLA-DR$^+$ marker suggests that immunotherapy acts directly on these cells to reverse their APC status.

Functions based on IgE-mediated mechanisms, such as the production of superoxide and LTC4, or the release of EDN and ECP, have not been observed in human eosinophils, although these show a vigorous response to IgG3- and IgG1-mediated stimuli through the FcγRII receptor [86,136-142]. These findings have led some authors to conclude that factors other than IgE and its receptors must act as inducers of the activation/release of eosinophils in allergic diseases [136]. It is possible that neutrophils might be responsible for the activation/release of eosinophils in IgE-mediated atopic processes [143].

The mediators secreted by neutrophils have the following effects on eosinophils:

– They attract eosinophils to the focus of inflammation causing their chemotaxis, either directly [144], or through IL-8, which is a powerful chemoattractant for these cells [145].

– Elastase released by neutrophils causes degranulation of eosinophils, releasing considerable amounts of ECP, depending on the dose of elastase [146].

– Neutrophilic lactoferrin, in quantities similar to those found in airway fluid [143], has several effects on eosinophils: degranulation with the release of EDN, superoxide production, and the synthesis of leukotrienes with the subsequent secretion of LTC4 [148].

– In addition to its normal functions, ECP can release lactoferrin from the serous glands of the airway mucosa [149].

– More recently it has been shown that neutrophils, in addition to IL-8, MMP-9, LTB4, PAF, and TNF-α, induce eosinophil trans-basement membrane migration [150].

– Murine neutrophils produce IL-17 [151] and this cytokine mediates eosinophil activation via differential intracellular signaling cascades in allergic inflammation [152]. IL-23 and IL-6 promote the survival and differentiation of IL-17–producing cells. Two families of lipid agonists control the magnitude and duration of inflammation: protectins and resolvins. Resolvin E1 has been seen to decrease the production of the proinflammatory cytokines Il-23, IL-6, and IL-17, and to increase that of the counter-regulatory mediators IFN-γ and lipoxin A4 to promote the resolution of allergic airway inflammation [153].

– Neutrophil proteases (elastase, cathepsin G, and proteinase-3) may enhance airway inflammation in asthma through the activation of eosinophils to produce superoxide and neutrophilic cytokines and chemokines. The mechanism may underlie part of the pathogenesis of severe asthma, and effective inhibition of these proteases could be a future therapeutic target [154].

In conclusion, although eosinophilic airway inflammation is recognized as an important feature of certain forms of chronic, stable asthma, evidence also supports an important role for neutrophils in this disease. Because neutrophils are the first cells recruited to the site of an allergic reaction, they may influence clinical presentation and play a role in the development of severe chronic asthma and the onset of sudden severe attacks. Neutrophils are removed by apoptosis during the resolution of the allergic response.

Funding Sources

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