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ROLE OF NEUTROPHILS IN ALLERGIC ASTHMA

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The contribution of neutrophils to asthma pathogenesis has been mainly studied in the context of non-allergic neutrophilic asthma. However, neutrophils can also be rapidly recruited and are largely present in the airways of allergic eosinophilic asthmatic patients. Under these circumstances, they possess specific phenotypic features distinguishing them from resting blood neutrophils and are endowed with particular functions. The exact contribution of neutrophils to allergic asthma pathogenesis is still unclear, but growing experimental evidence supports the ability of neutrophils or neutrophil-derived products to influence the underlying allergic type 2 immune response and cardinal features of allergic asthma, thus shedding new light on neutrophil biology and functions in an allergic context.

Introduction

Neutrophils represent the most abundant immune cell type in the blood and are generated in the bone marrow under the control of key transcriptor factors such as C/ EBPα, PU.1, Gfi-1 and C/EBPε [1]. They have long been known as short-lived (half-life: 6-12 hours [2]) innate immune cells specialized in pathogen killing through their high phagocytic potential and the secretion of cytotoxic granules once recruited in the tissues [1]. Today, a more complex picture of the neutrophil is emerging, with immunoregulatory properties and implications in various non-infectious disorders [3]. Of note, unlike previously appreciated, neutrophils are now thought to encompass distinct phenotypic and functional subsets in humans [4,5] and mice [6,7], some of them exhibiting an extended half-life in certain inflammatory conditions [3]. Furthermore, in 2004, Brinkmann and colleagues have discovered the ability of neutrophils to form neutrophil extracellular traps (NETs), whose roles in health and disease are currently under close scrutiny (Box 1), and which has arguably contributed to the renewed interest in neutrophils [8].

Asthma constitutes a heterogeneous group of respiratory inflammatory disorders characterized by a similar clinical pattern of cough, wheeze and reversible airway obstruction [9]. Asthma phenotypes can be categorized according to clinical symptoms, specific triggers, inflammatory or immune status, or treatment response [10,11]. If one refers to an inflammatory phenotype classification [12,13], allergic asthma belongs to the 'eosinophilic' phenotype (Table 1). Notably,

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while patients with more than 3% sputum eosinophils are considered 'eosinophilic', up to 60% sputum neutrophils can also be present [10,12,13]. Immunologically, allergic asthma is characterized by the development of an aberrant immune response with a predominant adaptive type 2 T helper cell (Th2) profile directed against inhaled allergens [9]. Such Th2 response, via the secretion of cytokines such as interleukin(IL)-4, IL-5, IL-13, orchestrates many cardinal features of allergic asthma, such as eosinophilic inflammation, mucus hypersecretion, airway hyper-responsiveness and increased serum levels of type E immunoglobulins (IgE) [14].

Neutrophil recruitment in allergic asthmatic lungs

While neutrophils are not steadily present in the airways of allergic asthmatic patients, they are one of the first innate immune cells recruited into the lungs during specific asthma-related events such as allergenic challenges [15-17], virus-induced asthma exacerbations [18•• ,19,20] or nocturnal crises [21]. In mice, airway exposure to clinically relevant allergens that promote features of allergic asthma is also associated with the airway recruitment of neutrophils [18",22",23·,24].

Like in other tissues, the recruitment of neutrophils into the respiratory tract comprises several steps that are initiated by the endothelial expression of adhesion molecules [25] and followed by extravasation and migration according to a chemokine gradient. In a mouse model of ragweed pollen extract challenge, Hosoki and colleagues demonstrated that lung neutrophil recruitment was substantially lower in mice lacking the LPS receptor Toll like receptor (TLR)-4 [22"]. Similarly, inhibition of CXCR2, the receptor for CXCL1, CXCL2 and CXCL5 in mice, inhibited allergen-induced innate recruitment of neutrophils [22"], and the production of CXCL1, CXCL2 and CXCL5 was shown to be dependent on TLR-4 and its co-receptor MD2 in response to cat dander and other relevant pollens [22••,23•]. Epithelial cells might be the source of such chemokines, as they have been shown, in humans, to secrete CXCL8 (i.e. IL-8, the human analogue of CXCL1, CXCL2 and CXCL5) following allergenic challenges [23•,26]. In addition, alveolar macrophages may also deliver CXCL1 and CXCL2 in response to allergen-induced and antibody-mediated activation of FCγ III receptors [27]. In humans, sputum levels of CXCL8 have been shown to be increased during acute allergic asthma exacerbations [19] and following allergenic challenge [15], which was associated with increased sputum neutrophils and blood neutrophil chemotaxis [15].

Oyoshi and colleagues demonstrated, in a model of skin allergy, that neutrophil-intrinsic leukotriene B4 (LTB4) synthesis and its receptor, BLT1, were involved in neutrophil recruitment [28]. Interestingly, LTB4 levels were increased in the BALF of asthmatic patients suffering from nocturnal asthma [29] and in exhaled breath condensate of asthmatic children [30] and adults [31]. In addition, the use of a LTB4 inhibitor in asthmatic patients triggered a substantial decrease in BALF neutrophils [32].

The Th2-associated cytokine IL-4 may control neutrophil recruitment during allergic asthma. Indeed, a recent study has demonstrated that IL-4 could dampen neutrophil expansion and migration through neutrophil-intrinsic IL-4 receptor signaling in mice [33••]. *Ex vivo* treatment of bone marrow neutrophils with IL-4 inhibited neutrophil migration in response to CXCL1 and

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CXCL2 by IL-4 receptor-dependent mechanisms [33••]. In a model of airpouch, CXCR2-dependent neutrophil recruitment was also inhibited when IL-4 biological half-life was prolonged [30]. The potential contribution of the IL-4/IL-4 receptor axis to the regulation of neutrophil numbers in the airways of allergic asthmatic patients or experimental animals will however require further investigations. Mast cells, whose allergen-dependent and IgE-dependent degranulation is thought to contribute to the acute allergic reaction [34], may also negatively regulate neutrophil influx in allergic asthmatics airways. Indeed, levels of mast cell-specific tryptase were found elevated in BALF of eosinophilic asthmatic patients [35], and such tryptase has been shown to be a strong chemorepellent for neutrophils *in vitro* [36].

Box 1 *Neutrophil extracellular traps.*

Neutrophil extracellular traps (NETs) are web-like structures composed of nuclear or mitochondrial chromatin associated to modified (e.g. citrullinated) histone proteins and decorated with 20-50 different proteins, such as neutrophil elastase, myeloperoxidase (MPO), LL37, cathepsin G, proteinase 3 or high mobility group protein B1. NETs can be released in the extracellular space in response to various microbial (e.g. bacteria, viruses, parasites, lipopolysacchar-ide [LPS]) and non-microbial (e.g. phorbol 12-myristate 13-acetate (PMA), crystals) stimuli. The molecular mechanisms of NET formation are not yet fully understood and may differ according to the stimuli (reviewed in [48]). Reactive oxygen species (ROS) formation, activation of the MEK/ERK pathway downstream of membrane receptors (PSGL1, RAGE, TLR2/4, Dectin 2, Fc-/R, Siglec 14), activation of autophagy (through the inhibition of mTOR pathway or the activation of the PI3K pathway) and induction of necroptosis have all been implicated in NET formation. If NETs were originally discovered for their role in bacterial killing [8], they have been more recently associated with non-infectious disorders like thrombosis, vasculitis, systemic lupus erythematosus, diabetes, cancer, asthma or chronic obstructive pulmonary disease (COPD) [60].

Table 1. *Asthma inflammatory phenotypes*

Phenotype	Inflammatory cells present in the airways	Immunological and inflammatory biomarkers	Severity	Triggers	Immune profile
Eosinophilic	Sputum eosinophils (>3%) Sputum neutrophils (<76%)	Specific IgE Th2-associated cytokines (IL-4, IL-5, IL-13) [61,62]	Mild to severe	Allergens (75%) Exercise Occupational (15%) Aspirin	Th2>»Th17
Neutrophilic	Sputum Neutrophils (>76%)	IL-8 Neutrophil elastase IL-1β TNF-α micro RNA-629-3p, 223-3p	Severe	Obesity Tobacco smoke Exposition to irritants	Th17>»Th2

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and 142-3p [62]

Paucigranulocytic	Levels of sputum eosinophils <3% and neutrophils <76%	?	Moderate	Not defined	?
Mixed granulocytic	High levels of sputum eosinophils (>3%) *and* neutrophils (>76%)	?	Severe	Not defined	Th2/Th17?

Source: Adapted from [9,12,13].

Phenotypic and functional features of neutrophils in allergic asthma

Several pieces of evidence support that neutrophils undergo profound changes in the blood and lungs of allergic asthmatic individuals. A recent clinical study compared the innate immune responses of two populations of children (i.e. Amish and Hutterite) sharing same ancestry, lifestyles but exposed to different levels of endotoxins [37••]. Briefly, Hutterite children, who are raised in an environment poor in endotoxins, are susceptible to allergic asthma, whereas Amish children live in endotoxin-rich homes and are protected against allergic asthma development [37••]. Notably, blood neutrophils from asthma-prone Hutterite children expressed higher levels of CXCR4 and CDllb, as well as lower levels of CD11c, as compared to the ones isolated from Amish children [37••]. Blood and nasal lavage neutrophils of atopic asthmatic patients have also been shown to express higher surface levels of CD49d than neutrophils of healthy subjects [5]. CD49d expression is further increased six hours after allergen challenge [5], suggesting a possible interaction between neutrophils and the specific allergens. Such interaction could be achieved by allergen-induced crosslinking of the high affinity IgE receptor (FcεRI), whose expression is higher in blood neutrophils from asthmatic patients [38] and increased during the pollen season [39]. Interestingly, the interaction between specific allergens and IgE/FcεRI on the neutrophil surface has been shown to enhance functional responses by increasing secretion of neutrophil products in asthmatic patients, like matrix metalloproteinase 9 (MMP-9) [40], neutrophil elastase (NE) [41], myeloperoxidase [42], IL-8 [43], eosinophil cationic protein [44] and reactive oxygen species (ROS) [45]. Moreover, as compared to neutrophils from healthy individuals, neutrophils from asthmatic patients exhibited boosted functional responses *in vitro,* such as an enhanced migratory capacity, a higher digestion phase in phagocytosis assays [46] and increased secretion of tumor necrosis factor (TNF)α, GM-CSF or interferon γ [47].

Recently, Toussaint and colleagues have shown, in a human experimental model of rhinovirus-induced allergic asthma exacerbations, that rhinoviruses promoted the release of host double-stranded DNA (dsDNA) and NE, two major NET components [48], supporting that NET-prone neutrophils were specifically recruited during asthma exacerbations.

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Contribution of neutrophils to allergic asthma pathogenesis

Given their large presence, their specific phenotype and their ability to secrete a wide range of products during allergic asthma, neutrophils are appealing candidates that may contribute to disease pathogenesis (Figure 1).

Using a model of airway allergy, Hosoki and colleagues showed that impaired neutrophil recruitment in TLR-4-deficient mice or in wild-type mice treated with a CXCR2 inhibitor was associated with decreased eosinophilic inflammation, IgE, Th2 cytokines and mucus secretion [22••]. Transfer of wild-type neutrophils in the trachea of TLR-4-deficient mice was sufficient to restore the Th2 immune response and features of airway allergy in this model, supporting that neutrophils could facilitate allergic sensitization and inflammation [22••]. Surprisingly, in a clinical study including Japanese children with primary autoimmune neutropenia, the incidence of asthma in 'neutrophil-deficient' children was found to be substantially lower as compared to a group of control children, and returned to the control levels with the resolution of neutropenia [49], supporting that neutrophils may promote asthma onset in humans, too.

Among the large range of neutrophil products, some of them have been identified to be present at higher levels in allergic asthmatic airways and to contribute in disease pathogenesis (Figure 1). Upon *ex vivo* stimulation with a relevant allergen, MMP-9 release and respiratory burst (i. e. associated with the production of ROS) were found to be higher in neutrophils of allergic asthmatic patients as compared to control neutrophils [40,45]. To address the role of MMP-9 and ROS *in vivo,* transgenic mice in which MMP-9 expression is lacking or ROS production is impaired have been subjected to a model of allergic asthma based on administration of ovalbumin (OVA) and the Th2 adjuvant alum [50-52]. Upon allergenic challenge, mice deficient in MMP-9 had an impaired recruitment of inflammatory cells, which was accompanied by a lower bronchial hyperreactivity, less IL-13 and fewer OVA-specific IgE [50,52]. Furthermore, Vermaelen and colleagues observed that the migration and maturation of dendritic cells, the main antigen-presenting cells responsible for Th2 sensitization, were compromised in the lungs of MMP-9-deficient mice, which could explain the altered development of the Th2 immune-mediated airway allergy [52]. Similarly, ROS-impaired mice failed to develop eosinophilic inflammation, mucus secretion and IL-13 secretion [51], and the ability of splenocytes to secrete IL-13 upon stimulation was also impaired in those animals, supporting that the Th2 response was facilitated by ROS [51].

Neutrophils were found to be a major source of oncostatin M, an important player in mucosal barrier dysfunction [53], in allergic asthmatic patients [54]. The exact contribution of oncostatin M to allergic asthma is still unclear, but endotracheal treatment of mice with adenovirus expressing mouse oncostatin-M has been shown to be sufficient to promote features of allergic asthma [55].

During the challenge phase of a OVA/alum model, NE inhibition was associated with decreased mucus cell numbers, eosinophil recruitment, bronchial hyperreactivity and Th2 immune responses [56]. In addition, mice chronically exposed to NE developed mucus cell metaplasia and lung eosinophilic inflammation [57]. iln vitro, NE has been shown to induce expression of the mucin protein MUC5AC by human epithelial cells [58,59].

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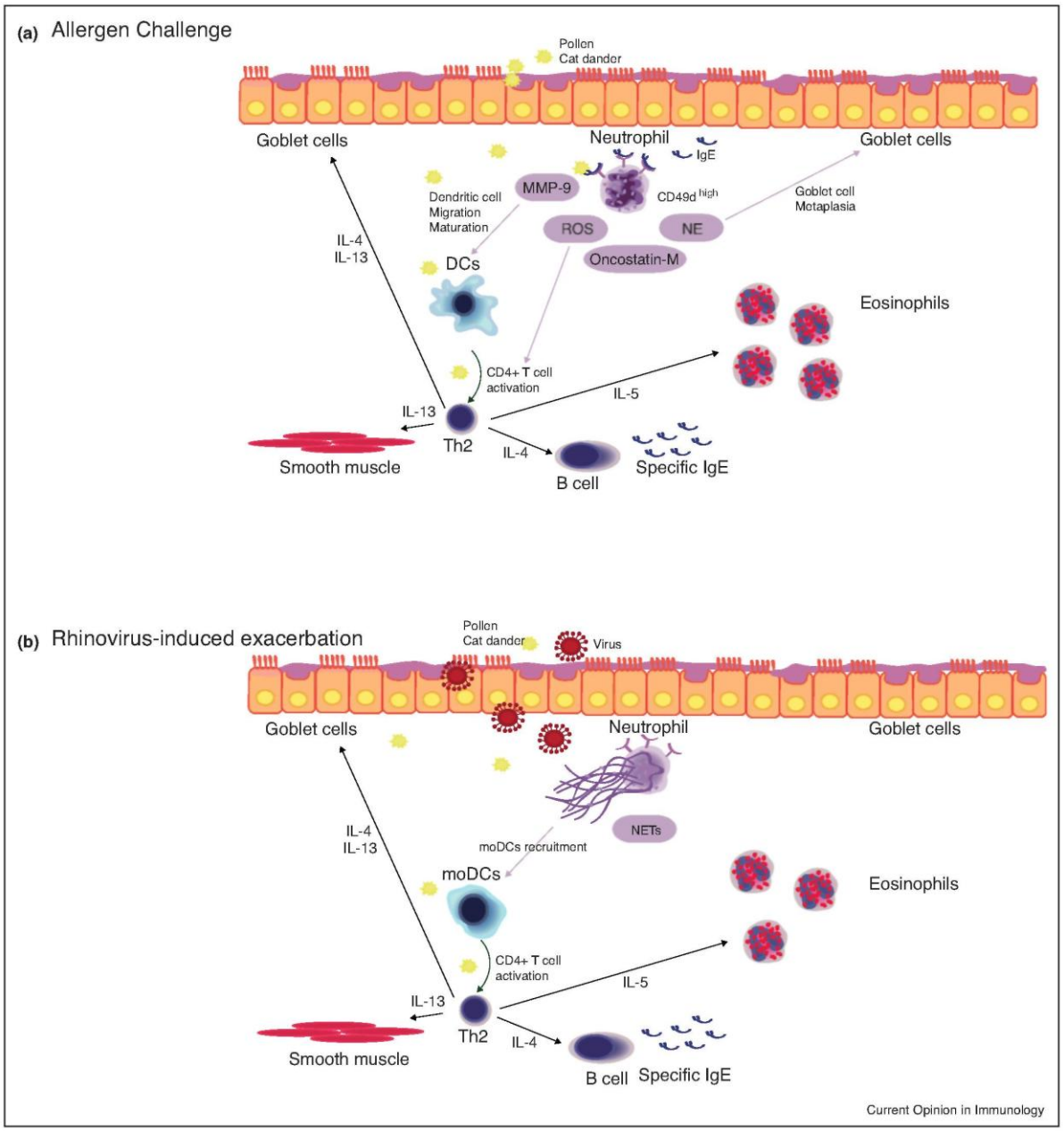
In a recent study, NETs have been shown to be essential in mediating allergic asthma exacerbations elicited by rhinoviruses [18••] (Figure 1). Indeed, NETs were massively released in the lungs of rhinovirus-infected mice, and inhibition of NETs formation or degradation of NETs strongly diminished all the cardinal features of asthma exacerbations [18••]. Furthermore, in this model, injection of mouse dsDNA, a major NET component, was sufficient to recapitulate most of the hallmarks of exacerbation. Mechanistically, NETs were found to promote the recruitment of inflammatory dendritic cells to the lung, which mediate allergic response to house dust mites in mice. During rhinovirus-induced asthma exacerbations in humans, dsDNA levels strikingly correlated with NE BALF levels, with the levels of type 2 cytokines detected in nasal lavages and BALF, and with the clinical severity of the exacerbation [18••].

Figure 1 **Putative contributions of neutrophils to allergic asthma**



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(a) Upon allergenic challenges, neutrophils are one of the first innate immune cells recruited to the airways. They are able to secrete matrix metalloproteinase 9 (MMP-9), reactive oxygen species (ROS), oncostatin-M, and neutrophil elastase (NE), among others via IgE-dependent mechanisms. MMP-9 induces the recruitment and maturation of lung dendritic cells, which mediate Th2 sensitization. ROS can interact with the activation of Th2 cells. Oncostatin-M has a global facilitating effect on asthma hallmarks. NE can stimulate mucus cell metaplasia, (b) During allergic asthma exacerbations provoked by respiratory viral infections, neutrophils can release NETs, which attract monocyte-derived dendritic cells (moDCs) into the lung and promote type 2-mediated exacerbations.

Conclusions and perspectives

Clinical evidence supports that neutrophils are recruited to the lungs of allergic asthmatic patients, especially during asthma symptomatic manifestations. In this context, the few studies that have looked at their potential implication in allergic asthma pathogenesis have underscored a potential pro-inflammatory, deleterious role, which facilitates type 2-mediated disease development. However, the contribution of neutrophils to allergic asthma and type 2 responses may have been understudied so far. Indeed, historically, they have been merely considered as first-line innate responders and effector cells in the context of type 17 host defence responses. Today, it is increasingly clear that neutrophils encompass distinct subsets and are endowed with many immunoregulatory properties. The use of high dimensional unbiased technologies, combined with novel transgenic tools that target specific neutrophil subsets should help uncovering their complex contributions to health, host defence and diseases in general, and to allergic disorders in particular

Conflict of interest statement

Nothing declared.

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