

MEETING REVIEW

Eosinophils in fungal diseases: An overview

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One-Sentence Summary: Eosinophil roles in fungal-associated allergic pulmonary disorders: contributions, mechanisms of fungal recognition and potential therapeutic relevance

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Abstract

Eosinophils are the prominent cells in asthma, allergic bronchopulmonary mycosis (ABPMs), and fungal-sensitization-associated asthma, but their roles in the immunopathology of these disorders are not well understood. Moreover, the immunological mechanisms underlying the molecular direct effector interactions between fungi and eosinophils are rare and not fully known. Here, we provide an overview of eosinophil contributions to allergic asthma and ABPMs. We also revise the major general mechanisms of fungal recognition by eosinophils and consider past and recent advances in our understanding of the molecular mechanisms associated with eosinophil innate effector responses to different fungal species relevant to ABPMs (*Alternaria alternata*, *Candida albicans*, and *Aspergillus fumigatus*). We further examine and speculate about the therapeutic relevance of these findings in fungus-associated allergic pulmonary diseases.

KEYWORDS

allergy, degranulation, DNA traps, eosinophils, fungus, inflammation

1 | INTRODUCTION

The incidence of fungal infections has become a critical worldwide health issue.¹ Demographic aspects, such as population aging, which is associated with several comorbidities including cancer development and autoimmune diseases, represent relevant risk factors for the development of fungal infections as a consequence of the use of immunosuppressive therapy and cytotoxic myeloablative drugs.² Consequences of immunosuppressive regimens include neutropenia and deficiencies in the effector functions of leukocytes and inflammatory response, which permit fungal colonization and tissue invasion, leading to invasive and systemic infections. Collectively, invasive fungal infections represent a mortality burden that approximates that observed in historically lethal infectious such as tuberculosis and malaria.³

Besides their roles as major pathogens, fungi also represent a source of major allergens.⁴ The incidence of asthma have increased, and global estimates indicate a global prevalence of 4.3–4.5%, varying from 1 to 21% of the adult worldwide population,⁵ with approximately 20% of 6–7-year-old children showing asthmatic episodes in

a period of 1 year.⁶ Considering a 30% increase in the global asthma burden in the past decades, allergic respiratory diseases constitute a major worldwide health problem.⁷ Climate changes associated with global warming have resulted in alterations in the patterns of sporulation of environmental fungi, with a general anticipation of fruiting periods and a delay of the final cycle of fruiting, in association with climate warming and rainy periods favoring the propagation of fungal spores such as small conidia.⁴ Sporulation of a major allergenic mold, *Alternaria alternata*, is increased by biomass growth upon incubation with increased CO₂ concentrations, consistent with future atmospheric predictions.⁸ Thus, the possible greater increase in allergic respiratory diseases associated with fungal sensitization represents a great public health concern, as a consequence of the progressive climate warming.

Exposure and sensitization to fungal allergens can promote the development and worsening of allergic diseases. Sensitization to fungi is an important factor in patients with allergic respiratory tract disorders, playing a major role in the development, severity, and persistence of lower airway disease, especially asthma.⁴ In humans, an association between fungal exposure and asthma or other allergic diseases (such as allergic rhinitis) is recognized clinically and epidemiologically.^{9,10} Historically, fungi have been long recognized as major triggers of asthmatic episodes. In 1930s, several studies have demonstrated asthma episodes attributed to mold environmental fungi, for example, *Aspergillus fumigatus*,¹¹ *Alternaria* spp.,¹² and *Tricophyton* spp.¹³ Clinical studies evaluating individuals showing unremitting asthma observed

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; ABPM, allergic bronchopulmonary mycosis; CLRs, C-type lectin receptors; CysLTs, cysteinyl leukotrienes; DAMPs, danger-associated molecular patterns; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EETs, eosinophil extracellular traps; EPO, eosinophil peroxidase; ETs, extracellular traps; MBP, major basic protein; NETs, neutrophil extracellular traps; PAMPs, pathogen-associated molecular patterns; PARs, protease-activated receptors; PRRs, pattern recognition receptors; ROS, reactive oxygen species

atopic reactivity in skin tests to fungal antigens in about 20–25% of the patients.^{14–17} Furthermore, fungal sensitization has been associated with a greater asthma severity as indicated by more frequent hospital and intensive care admissions, as well as more severe bronchial hyperreactivity.^{18–22} Association between severe asthma and fungal sensitization shows a stronger correlation than the sensitization to pollen or other antigens, as a risk factor for severe asthma.²⁰

Aspergillus fumigatus has a great capacity to colonize the bronchial tract of asthmatic patients, causing severe persistent asthma and poor lung function, potentially leading to allergic bronchopulmonary aspergillosis (ABPA).²³ The airways appear to be chronically or intermittently colonized by *Aspergillus* spp, commonly *A. fumigatus*, in patients with ABPA. ABPA is the most common form of allergic bronchopulmonary mycosis (ABPM), but other fungi, including *Candida albicans*, *Bipolaris* spp, *Schizophyllum commune*, *A. alternata*, *Penicillium* spp, and *Curvularia* spp, have also been implicated.^{4,24} The characteristics of ABPMs include severe asthma, eosinophilia, markedly increased total IgE and specific antifungal IgE levels, bronchiectasis, and mold colonization of the airways.²⁵ ABPMs are TH2 hypersensitivity lung diseases caused by bronchial colonization with fungi that affects asthmatic patients (~ 1% to 3%), individuals presenting allergic fungal sinusitis (more unusual) and patients with cystic fibrosis (~ 8%).^{26,27}

The roles of eosinophils in the immunopathology of ABPMs, as well as fungal sensitization-associated asthma, are not well understood. Eosinophils are known to be prominent cells in asthma and ABPMs; however, the immunological mechanisms that underlie the molecular interactions between fungi and eosinophils are poorly understood. In this review, we will link and ponder the principal findings demonstrating the roles of eosinophils in asthma and fungal-associated allergic pulmonary diseases, consider the known mechanisms of fungal recognition by eosinophils, and discuss potential therapeutic approaches to the treatment of these disorders.

2 | ROLES OF EOSINOPHILS IN ALLERGIC ASTHMA AND ABPMs

Eosinophils are granulocytes characterized by their content of intracellular granules, also known as crystalloid or secondary granules, which contain preformed proteins including distinct cationic proteins such as eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN). Furthermore, eosinophils store within their granules substantial quantities of preformed chemokines, growth factors, and diverse cytokines with nominal Th1, Th2, and regulatory capacities.²⁸ Eosinophils may secrete their granule-stored proteins via classical exocytosis, compound exocytosis (characterized by the fusion of specific granules with each other and with the plasma membrane for granule content release), cytolysis (which enables the release of intact, membrane-bound, functional free extracellular granules), and piecemeal degranulation. Piecemeal degranulation is a process by which eosinophil granule contents are selectively mobilized into spherical and tubular vesicles that need to disengage from granules, cross the cytoplasm,

and fuse with the plasma membrane to release their specific granule-derived protein cargo.^{29,30}

Asthmatic patients show increased numbers of eosinophils in the bronchoalveolar fluid and peripheral blood when compared with control healthy individuals.³¹ The number of eosinophils in the blood and tissues is known to increase in allergic pulmonary inflammation, and the association between the presence of eosinophils and asthma associated with fungal sensitization has been long recognized.^{4,9,10} In ABPMs, blood and pulmonary eosinophilia has long been documented as clinical features. Higher eosinophil blood counts are positively correlated with bronchiectasis and the presence of mucus, in individuals presenting ABPA, which indicates that increased eosinophil amounts are associated with tissue inflammation and remodeling. Although there is a clear association between the presence of eosinophil and the pathological manifestations of ABPMs, the roles of eosinophils in the immunopathology of these disorders, as well as fungal sensitization-associated asthma, are not well understood. Considering the premise that fungal colonization would represent a trigger for the asthma development, clinical studies have evaluated the antifungal therapy in the asthma outcomes in patients presenting ABPA. In a clinical investigation, including an open-label phase and a randomized double-blind step, therapy with itraconazole resulted in an improvement in the outcome of ABPA, as indicated by a reduction in the prednisone doses, an improvement in pulmonary function tests and exercise tolerance, and a reduction in the IgE serum concentrations.³² Another double-blind study demonstrated that patients receiving itraconazole showed reductions in the eosinophil sputum counts and total serum IgE, as well as a decrease in the *A. fumigatus* specific IgG serum concentrations, in comparison with the placebo group. The improvement in the inflammatory parameters in the itraconazole group was associated with a reduction in the episodes of exacerbations in patients treated with oral corticosteroids.³³ Thus, antifungal therapy in association with oral corticosteroids has been defined as a valid therapeutic approach in the treatment of confirmed cases of ABPA, reinforcing a role for fungal colonization as a trigger for asthma exacerbation. In contrast, the use of antifungal therapy in cases of asthma associated with fungal sensitization, in the absence of fungal colonization, is still uncertain. Environmental exposure to fungal antigens is ubiquitous, which makes the development of immunomodulatory therapies the most plausible effective approach in these cases.

Eosinophils release several mediators that are responsible for bronchoconstriction including MBP,³⁴ cysteinyl leukotrienes (cysLTs),³⁵ and IL-13.³⁶ Thus, eosinophils have been speculated to be prominent cells involved in airway hyperreactivity. However, human studies evaluating anti-IL-5 neutralization, which resulted in extensive reductions of blood eosinophilia, and experimental models of eosinophil deficiencies have led to controversial conclusions concerning the role of eosinophils in airway hyperreactivity. A mouse strain (Δ dbl GATA) carrying a deletion in the high-affinity binding site for GATA in the promoter of GATA-1, on a BALB/c background, shows a complete absence of eosinophils, although other myeloid cells are unaffected.³⁷ The Δ dbl GATA mice exhibit similar airway hyperreactivity and mucus production in an ovalbumin experimental model of asthma when compared with wild-type mice. In contrast, Δ dbl GATA mice show decreased

collagen deposition, as well as impaired hyperplasia and proliferation of bronchial smooth muscle cells.³⁸ On a C57BL/6 background, the mouse strain PHIL, which expresses the diphtheria toxin A gene under the control of the *Epx* promoter, also shows a complete absence of eosinophils, while the other leukocyte populations are preserved. PHIL mice, in contrast to Δ dbl GATA mice, manifest an impaired airway hyperreactivity and a reduction in mucus production, in a similar OVA model of pulmonary allergic inflammation.³⁹ A possible explanation for these apparently conflicting findings might be related to the different mice backgrounds used. In a further study, Walsh and colleagues have performed a detailed analysis of the Δ dblGATA mice on C57BL/6 and BALB/c backgrounds.⁴⁰ They demonstrated that, in contrast to the results obtained on a BALB/c background, in a model of ovalbumin-induced allergic inflammation, eosinophils were crucial to the development of airway hyperresponsiveness and allergic inflammation by modulating chemokine and cytokine production in the lung, leading to T cell recruitment.⁴⁰

The role of eosinophils has also been defined as controversial in clinical studies employing IL-5 neutralization. IL-5 is a critical mediator of eosinopoiesis and eosinophilia,⁴¹ and it promotes eosinophil differentiation and survival from myeloid progenitors in the bone marrow.⁴² In this sense, IL-5 monoclonal antibody neutralization has been evaluated as a therapy for allergic pathologies, including asthma. IL-5 neutralization, despite a great reduction in circulating eosinophil numbers, does not impact the airway hyperreactivity in asthmatic patients.⁴³ However, a residual eosinophilia has been observed in clinical studies upon IL-5 neutralization, indicating that IL-5-independent mechanisms promote eosinophil differentiation and mobilization or even that complete IL-5 neutralization is not attained therapeutically.⁴⁴ Thus, it is possible that even small numbers of eosinophils must be sufficient to promote bronchoconstriction. Alternatively, other hypothesis is that eosinophils must contribute to the exacerbation but are dispensable for acute bronchoconstriction, since IL-5 neutralization has been reported to reduce the number of hospitalization episodes, and even the response to acute airway hyperreactivity is unaffected by IL-5 neutralization.^{43,45,46}

Another pathological aspect of allergic asthma is bronchial tree remodeling. Hyperplasia of the bronchial smooth muscle tissue and collagen deposition result in thickening of the bronchioles, contributing for the long-term alterations in respiratory function.⁴⁷ Eosinophils can contribute to many aspects of airway remodeling. Eosinophils release TGF- β 1 and constitute a major source of this cytokine in the lung tissue of asthmatic patients.⁴⁸ TGF- β 1 is a critical mediator of collagen deposition and fibrosis, acting as an inducer of collagen and extracellular matrix synthesis by fibroblasts.⁴⁹ Eosinophils also produce cysLTs, which promote smooth muscle cell proliferation and hyperplasia.⁵⁰ CysLTs particularly have been pointed as critical mediators of airway remodeling via multiple mechanisms. CysLTs are inducers of fibroblast proliferation and extracellular matrix synthesis.⁵¹ Moreover, cysLTs also promote airway muscle cell proliferation.⁵² LTC₄ promotes TGF- β 1 release by eosinophils and epithelial cells,^{53,54} and TGF- β 1 increases bronchial muscle cell responses to cysLTs,⁵⁵ indicating a crosstalk between these two critical mediators of the inflammatory response and tissue remodeling. Given the capacity of eosinophils

to produce large amounts of cysLTs and other mediators that promote smooth muscle cell hyperplasia and extracellular matrix deposition, these leukocytes have been postulated to be relevant for airway remodeling during asthma. The roles of eosinophil in airway remodeling have been postulated by the observation that Δ dbl GATA mice show impaired collagen deposition, smooth muscle layer thickening, and proliferation of smooth muscle cells in an OVA experimental model of chronic challenge. Furthermore, a clinical study employing IL-5 neutralization in asthmatic patients demonstrated a reduction in the expression of extracellular matrix proteins in the bronchial mucosa, in association with reduced numbers of eosinophils expressing TGF- β 1 and of TGF- β 1 concentrations in the bronchoalveolar lavage.⁵⁶ Thus, eosinophils must contribute to the chronic remodeling of the airways during allergic inflammation.

Fungal colonization of asthmatic airways in ABPMs results in a worsening of respiratory function, which is frequently associated with severe asthma.²⁵ A typical manifestation of the ABPM, particularly ABPA, is bronchiectasis, which is a consequence of the local tissue remodeling. In this sense, eosinophils must represent promoter cells for tissue remodeling and fibrosis in ABPMs. In an experimental model of allergic fungal sensitization employing intranasal challenge with *A. fumigatus* extracts, eosinophil deficiency resulted in decreased mucus production, reductions in the Th2 cytokines, IL-4 and IL-13, and impaired mononuclear cell recruitment.⁵⁷ In this work, Fulkeron et al. evaluated the impact of the genetic deficiency of CCR3, the receptor for the eosinophil chemoattractant chemokines CCL11 and CCL24 (eotaxin-1 and -2, respectively), the double-deficient CCL11/CCL24^{-/-} mice and the eosinophil devoid Δ dbl GATA mice. While all of these deficient mice showed an absolute absence of tissue eosinophilia, in this model, only the CCR3^{-/-} and Δ dbl GATA mice showed an impaired mononuclear cell mobilization and Th2 cytokine production, indicating a redundant role for other CCR3 ligands in these responses. In contrast, mucus production was reduced in all these genetically deficient mice, demonstrating a critical role for eosinophil recruitment in the mucus production in response to *A. fumigatus* allergic challenge. However, the role of eosinophils in the airway hyperreactivity in this model was not evaluated. Considering acute and chronic models of pulmonary *A. fumigatus* challenge, it is possible to speculate that the roles of eosinophil chemoattractant chemokines are different depending on the fungal sensitization and exposure protocol used.⁵⁸ In this work, CCL11 seems to have an importance in the reduction of airway hyperreactivity and lung eosinophilia in an acute model, whereas in the chronic model the airway hyperreactivity and eosinophilia were not different from the wild-type mice.

Although experimental models of fungal sensitization and challenge have been described, the role of eosinophils in the pathogenesis of ABPMs (with sights of airways fungal colonization) in animal models has not been established. Similarly, experimental models of ABPMs in eosinophil-deficient mice are lacking, and thus the mechanistic contribution of eosinophils to bronchoconstriction and airway inflammation remains uncharacterized. Moreover, the association between fungal colonization/sensitization and asthma has not been established in clinical studies using IL-5 neutralization therapy, and thus it seems uncertain whether IL-5-mediated eosinophilia contributes to the airway

hyperreactivity in ABPMs or fungal-associated asthma. Considering the diversity of asthmatic manifestations and the variety of allergenic triggers (e.g., fungi, pollen, dust mite antigens), more profound investigations are necessary for an understanding of the immunopathology of allergic conditions associated with fungal exposition since subgroups of fungal-sensitized asthmatic patients must benefit from therapeutic approaches that may not provide results in larger heterogeneous groups of asthmatic patients.

3 | EOSINOPHILS AND FUNGAL RECOGNITION

The innate immune response to fungal pathogens is essential to assemble the protective immune response. Cells of the innate immune system detect fungal infection mainly through pattern recognition receptors (PRRs) expressed on cell surfaces or within intracellular compartments. The PRR classes that are more related to fungal recognition include the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs).^{59,60} PRRs recognize and respond to components of microbes called pathogen-associated molecular patterns (PAMPs) or to endogenous molecules produced by injured/dying cells called danger-associated molecular patterns (DAMPs).⁶¹ Upon recognition of PAMPs or DAMPs, PRRs trigger leukocyte effector mechanisms and induce adaptive immune responses and inflammation.

The human TLR family has 10 members (TLR1 to TLR10) that are located in different cellular compartments.⁵⁹ TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface where they primarily detect soluble/extracellular ligands. TLR2 recognizes bacterial lipoproteins, glycosylphosphatidylinositol anchors from protozoa, mycobacterial lipoarabinomannans, and lipoteichoic acid in Gram-positive bacteria.^{62–65} A critical aspect of the recognition mediated by TLR2 is its dimerization with TLR1 or TLR6. TLR2/TLR1 complexes recognize triacylated lipoproteins, whereas TLR2/TLR6 complexes recognize diacylated lipoproteins.^{66,67} TLR3, 7, 8, and 9 are positioned in endosomes where they detect internalized nucleic acids.⁵⁹ TLR3 is a receptor for internalized double-stranded RNA,⁶⁸ TLR9 recognizes DNA sequences containing nonmethylated CpG repetitions.⁶⁹ TLR7/TLR8 acts as receptors for single-stranded RNA.⁷⁰ TLRs have been implicated in the innate immune recognition of fungal pathogens. In *in vitro* experiments, TLR2, in cooperation with TLR6, recognizes zymosan, a cell wall component of *Saccharomyces cerevisiae*.⁷¹ TLR2 has also been implicated in innate immune recognition of fungal α -glucans,⁷² phospholipomannans from *C. albicans*,⁷³ and the major component of the *Cryptococcus neoformans* capsule, glucuronoxylomannan.⁷⁴ Based on *in vivo* and mostly on *in vitro* observations, TLR4 is involved in the recognition of fungal mannose-enriched glycoconjugates, such as fungal mannans, rhamnomannans, and glucuronoxylomannans.^{75–78} Although initially indicated as a receptor for bacterial nonmethylated CpG DNA sequences, *in vitro* and *in vivo* findings pointed that TLR9 has been described as a sensor for fungal DNA recognition upon internalization of *A. fumigatus*, *C. albicans*, and *C. neoformans*.^{79–81} Moreover, experimental models of fungal

infection in knockout mice deficient in TLRs or TLR-associated adaptor molecules have demonstrated a crucial involvement of TLR2 and TLR4 in the recognition of fungal pathogens such as *A. fumigatus*, *C. albicans*, and *C. neoformans* (for review, see Ref. 82). Corroborating the role of TLR2 and TLR4 in fungal recognition in experimental models, clinical studies have demonstrated the association of human polymorphisms in TLR1, TLR6, and TLR4 with the development of invasive pulmonary aspergillosis.^{83,84} Human eosinophils express mRNA or proteins for all TLRs except TLR8. However, the expression of these receptors may vary in intensity depending on the study. Moreover, there is some controversy regarding the expression of TLR3, TLR4, and TLR6. TLR7 is the only receptor that has been found to be highly expressed and functional in all studies.^{85–90}

The CLR family includes the dectin-1 receptor as its more prominent member, but other receptors such as DC-SIGN, mincle, langerin, siglecs, and mannose receptor, are also included. These are transmembrane receptors characterized by the presence of a carbohydrate-binding domain.⁶¹ Dectin-1 recognizes β -glucans, which are the major fungal cell wall component.^{91,92} The expression of dectin-1 in human eosinophils is controversial. A study has reported the expression of dectin-1 mRNA in human eosinophils⁹³ in accordance with previous published data.⁹⁴ However, Yoon and colleagues have not detected expression of dectin-1 protein in resting human eosinophils, either on the cell surface or in the intracellular compartment.⁹⁵ This finding is in accordance with the data from our group showing an absence of dectin-1 protein expression in fresh isolated human eosinophils.⁹⁶ We believe that differences in the experimental settings may be responsible for the controversial observations.

Several studies have implicated other receptors, such as the protease-activated receptors (PARs) and the β 2 integrin CD11b/CD18, as sensors for monitoring fungal PAMPs.^{95,97–99}

The surface receptor integrin CD11b/CD18 (also known as Mac-1, CR3) is a β 2 integrin composed of the common β 2 chain CD18 and the α M chain CD11b, and it is highly expressed by different leukocytes. CD11b/CD18 is known to bind iC3b-opsonized structures and to mediate different leukocyte responses such as leukocyte adhesion/diapedesis across the endothelium, phagocytosis, and degranulation.¹⁰⁰ However, *in vitro*, CD11b/CD18 has also been described to recognize β 1,3-glucans.^{98,99} In general, it has been reported that CD11b binds to β -glucans through a lectin site that differs from the I-domain known to bind to iC3b, ICAM-1, and fibrinogen.^{98,99,101} Studies in human eosinophils have demonstrated a role for the CD11b I-domain in the recognition of β -glucans present in *Alternaria* cell wall.⁹⁵

As previously mentioned, PARs have also been documented to be involved in the recognition of fungus-derived proteases.⁹⁷ PARs are seven-transmembrane G-protein-coupled receptors with an N-terminal extracellular domain that is cleaved and activated by proteases. After N-terminal domain cleavage, another protein domain is exposed and binds intramolecularly to a different site, thereby activating the receptor. PARs are expressed on epithelial cells, endothelial cells, and leukocytes and can be cleaved by host- or pathogen-derived proteases. Four PARs have been identified and cloned (PAR1, 2, 3, and 4).¹⁰²

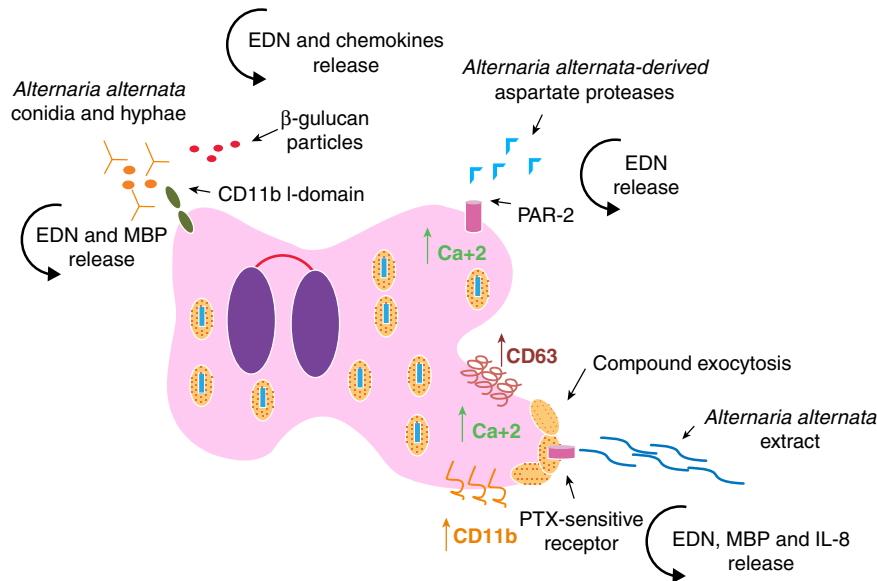


FIGURE 1 Innate effector eosinophil responses to *Alternaria alternata*. *Alternaria alternata* extract induces eosinophil-derived neurotoxin (EDN) release in a pertussis (PTX) toxin and calcium (Ca^{2+})-dependent manner. Human eosinophils also respond to *Alternaria alternata* extract by upregulating the plasma membrane expression of CD63 and CD11b and by releasing MBP and IL-8 via a secretory mechanism that might involve compound exocytosis.¹⁰⁴ Aspartate proteases isolated from *Alternaria alternata* induce EDN release from human eosinophils through PAR-2.⁹⁷ Moreover, human eosinophils release EDN and MBP in response to *Alternaria alternata* conidia and hyphae via the CD11b extracellular I-domain. Eosinophils also degranulate in response to β -glucan particles and release EDN and chemokines via the same mechanism.⁹⁵

In human eosinophils, the expression of PAR-2 and PAR-3 mRNA has been reported, but functional roles in human eosinophil activation (reactive oxygen species [ROS] production and degranulation) have only been described for PAR-2.¹⁰³ Moreover, in vitro studies revealed that PAR-2 expressed in eosinophils has been described to be capable of recognizing soluble protease-like molecules secreted by *A. alternata*.⁹⁷

4 | INNATE EFFECTOR EOSINOPHIL RESPONSES TO FUNGI

Although the association between eosinophilic inflammation and fungal sensitization or colonization has been long recognized, the immunological mechanisms underlying the direct molecular effector interactions between fungi and eosinophils have remained poorly understood. Among the most important fungi involved in the development of ABPMs, innate effector eosinophil responses have been described, especially for *A. alternata* and *A. fumigatus*.

In 2005, Inoue and colleagues showed that eosinophils incubated with extracts from environmental airborne fungi release EDN in response to *A. alternata* and *Penicillium notatum*, but not *Aspergillus versicolor*, *Bipolaris sorokiniana*, *C. albicans*, *Cladosporium herbarum*, and *Curvularia spicifera*. *Alternaria* also stimulates the release of MBP. Moreover, the authors reported that the process by which eosinophils degranulate in response to *Alternaria* extract involves compound exocytosis and calcium in a manner that is dependent on PTX-sensitive G-coupled receptors. These findings suggest the involvement of a G protein-dependent mechanism. Eosinophils also respond to *Alternaria* extract by releasing IL-8/CXCL8 and upregulating CD11b and CD63

expression on their plasma membrane (Figure 1). Interestingly, heat treatment of *Alternaria* extract at 56°C for 30 min abrogated its ability to induce eosinophil degranulation, indicating a role for complement in the eosinophil activation induced by *Alternaria* molecules.¹⁰⁴ Furthermore, Matsuwaki and colleagues proposed a mechanism by which *A. alternata* activates human eosinophils. The authors found that eosinophils released EDN in response to *A. alternata* extract via a calcium-dependent mechanism, but independent of TLR2 and TLR4. The authors also showed that the activity of aspartate protease secreted by *Alternaria* induces the activation and release of EDN from human eosinophils through PAR2⁹⁷ (Figure 1). EDN possesses ribonuclease activity and has been grouped under the RNase A superfamily.¹⁰⁵ It is known that EDN presents toxicity against helminth parasites^{106,107} and an antiviral activity against single-stranded RNA viruses^{108,109}; however potential roles of EDN against fungi are unknown. Of particular note is the more recent finding that EDN can initiate signal transduction via TLR2 interaction and act as an alarmin that activates the TLR2-MyD88 signaling pathway in dendritic cells, enhancing Th2 immune responses.¹¹⁰ Therefore, eosinophil secretion of EDN represents a capacity of eosinophils to provide a “warning signal” to nearby cells that potentially might be important in the immune response against fungi.

Although *A. alternata*-secreted proteases activate eosinophils, likely through the mediation of the PAR-2, another study reported that eosinophils can also directly recognize *A. alternata* cell wall components (β -glucan) and activate and release granule proteins.⁹⁵ In a study conducted by Yoon and colleagues, human eosinophils were found to respond to *A. alternata* by releasing their granule proteins via the β 2 integrin molecule CD11b.⁹⁵ In this work, and in contrast to previous studies, the authors did not use *A. alternata* extract or derived

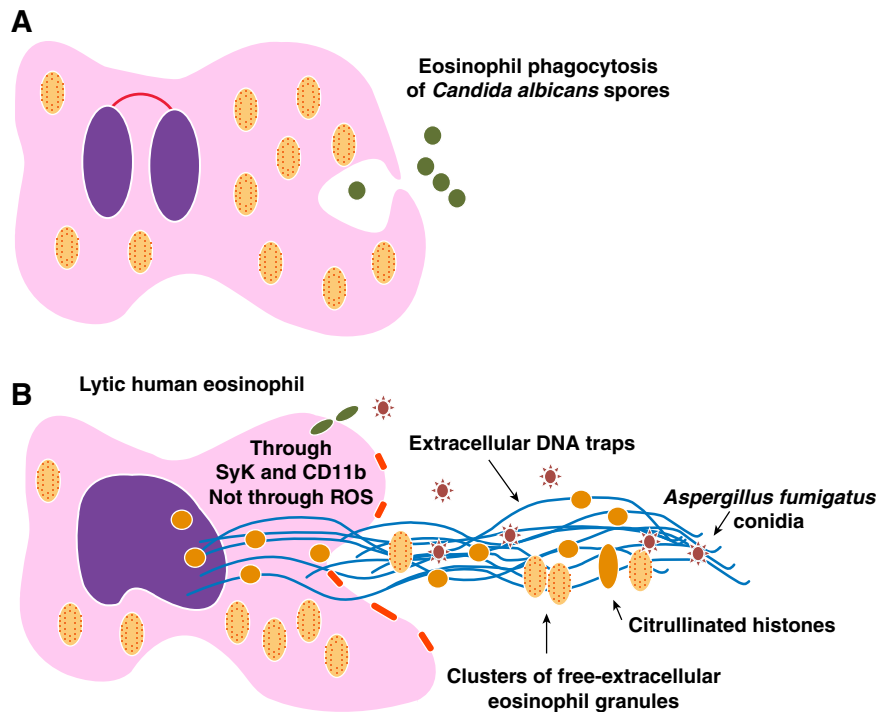


FIGURE 2 Innate effector eosinophil responses to *Candida albicans* and *Aspergillus fumigatus*. (A) Human eosinophils phagocytose *Candida albicans* spores.¹²⁰ (B) Human eosinophils cytolytically release extracellular DNA traps (EETs) in response to *Aspergillus fumigatus* in a process that is dependent of CD11b and the Syk kinase pathway, which does not involve ROS. *Aspergillus fumigatus* conidia are entrapped by EETs associated with citrullinated histones and clusters of free-extracellular eosinophil granules

proteases but *A. alternata* conidial and hyphal morphotypes. They showed that eosinophils secrete EDN after 3 h of incubation with *A. alternata* hyphae in a contact- and time-dependent manner. MBP was also released and appears to localize to eosinophils and to hyphae. Importantly, eosinophils present fungicidal activity against *A. alternata* hyphae. Eosinophils also respond to β -glucan particles by releasing EDN and different chemokines, but not to the other fungal cell wall component, chitin. Intriguingly, the authors verified that β -glucan particles or *A. alternata* hyphae promote eosinophil degranulation via an interaction that involves the I domain, but not the lectin domain, of the CD11b integrin (Figure 1). Interestingly, human neutrophils also degranulate in response to β -glucan particles, but via a mechanism involving both the lectin and I domains of CD11b. These findings are intriguing because the lectin domain has generally been indicated to be crucial for glucan recognition by phagocytes. Thus, we can speculate that (i) the recognition of β -glucans by eosinophils without the participation of the lectin domain is exclusive to eosinophils (since the studies postulating that the lectin domain is essential for β -glucan recognition were performed in other cells); (ii) CD11b might have unique interactions with other proteins on the surface of eosinophils (since leukocytes express different plasma membrane molecules that can potentially interact with CD11b); (iii) in the absence of dectin-1, eosinophils might use distinct mechanisms to interact with β -glucans. Eosinophils lack dectin-1 expression (although there are some controversial data). Since dectin-1 has been described to cooperate with CD11b/CD18 in neutrophils and macrophages in the binding and recognition of β -glucans,^{111,112} its absence might configure a new way by which eosinophils interact with β -glucans.

Taken together, these data are interesting because they reveal at least two independent mechanisms involved in the eosinophil response to *A. alternata*, which can involve the recognition of (i) fungal-secreted proteases and (ii) β -glucans expressed on the fungus cell wall. Whether these observations can be extended to other fungi or other cells is still not known. More studies in this field are still needed to improve our understanding.

Despite the fact that eosinophils directly activate in response to *Alternaria* in vitro, in vivo studies show that when *Alternaria alternata* extract (but not necessarily other allergen extracts) is administered to mice, an augmented expression of IL-33 is detected in epithelial cells^{113–115}; or increased levels of cysLTs are present in bronchoalveolar fluids.¹¹⁶ IL-33 or cysLTs activate group 2 lung innate lymphoid cells (ILC2) promoting IL-5 and IL-13 rapid production and subsequent accumulation of eosinophils.^{116–118} In fact, we can speculate that the production of epithelium-derived cytokines (such as IL-33, thymic stromal lymphopoietin (TSLP), or IL-25) in response to fungi might be via engagement with PRRs (TLR4, PAR2) or other mechanisms (e.g., proteolytic activity).¹¹⁹ For instance, some studies suggest that *Alternaria* possessed serine protease activity that elicited the rapid release of IL-33 into the airways of mice through a mechanism that was dependent upon the activation of PAR2 and the adenosine triphosphate signaling.¹¹⁵

Roles for eosinophils have also been proposed against *C. albicans*. Utilizing transmission electron microscopy methods, it has been suggested that human eosinophils are capable of phagocytosing *C. albicans* spores¹²⁰ (Figure 2A). Nevertheless, little is still known regarding the functional roles of eosinophils in this fungal infection.

Eosinophils are the predominant cells in the lungs of mice repetitively challenged with *A. fumigatus* in an experimental model of fungal asthma.¹²¹ During acute challenges, neutrophils have been reported to be the dominant lung cell type, although eosinophils are also present. In 2014, Lilly and colleagues showed that eosinophils are important contributors to the clearance of *A. fumigatus* from the lungs of mice after an acute challenge in vivo.¹²² They showed that eosinophils are recruited to the lungs 24 h and 48 h after a single intratracheal challenge with *A. fumigatus* conidia. Using dβGATA-1-deficient mice, they verified an enhanced susceptibility of the animals to infection. Moreover, exclusively in the lungs of eosinophil-deficient mice, they observed an impairment of fungal clearance and the presence of germinating conidia and some hyphae. dβGATA-1-deficient mice also showed lower levels of different cytokines and chemokines compared with the wild type control. Interestingly, eosinophils generated from mouse bone marrow exhibited killing activity against *A. fumigatus* in vitro, which did not require cell contact, suggesting that eosinophil-secreted factors were deleterious to *A. fumigatus* conidia. Although the susceptibility of dβGATA-1-deficient mice might be attributed only to eosinophil deficiency effector functions against the fungus, other mechanisms must not be excluded. For instance, eosinophils have been reported to modulate dendritic and T cells functions during allergen challenge¹²³ and contribute to bone marrow homeostasis.¹²⁴ Thus, the lack of these immunomodulatory eosinophil roles in other immune cells might contribute to a deficient or altered immune response that could be implicated in the impaired *A. fumigatus* clearance in dβGATA-1-deficient mice. Furthermore, to date, the exact mechanism by which murine bone marrow-derived eosinophils kill *A. fumigatus* conidia in vitro is not recognized.¹²² Additional studies are needed to clarify this point.

Interestingly, we have recently reported that human eosinophils release extracellular DNA traps in response to *A. fumigatus* in vitro. The process of extracellular release of DNA traps (ETs) by leukocytes (including neutrophils, eosinophils, mast cells, macrophages) has been described as an important mechanism of the innate immune response to different infectious diseases, including fungal infections.^{125,126} The process of ET release was first described by Brinkmann and colleagues in 2004 in neutrophils as an antimicrobial mechanism that is capable of immobilizing, trapping, and potentially killing pathogens in a microenvironment enriched with cytotoxic molecules.¹²⁷ The process of extracellular DNA trap release by neutrophils (neutrophil extracellular traps [NETs]) in response to fungi, including *Aspergillus* spp.,^{128–130} *C. albicans*,^{131,132} *Cryptococcus gatti*,¹³³ and *Paracoccidioides brasiliensis*,¹³⁴ has been described in the literature. However, whether eosinophils respond to fungal infection by releasing ETs is not known. In 2008, the first evidence was published that human eosinophils rapidly and noncytolytically release mitochondrial DNA to form eosinophil extracellular traps (EETs) in response to bacteria and other stimuli.¹³⁵ Furthermore, a different study demonstrated that eosinophils respond to thymic stromal lymphopoietin to release mitochondrial EETs associated with eosinophil granule cationic proteins.¹³⁶ More recently, Ueki and colleagues provided the first evidence that eosinophils undergo cytolysis to liberate EETs (in a process named EETosis) in a ROS-dependent manner.¹³⁷ Citrullinated histones and

clusters of eosinophil granules are found associated with these EETs. In fact, in leukocytes and especially in neutrophils, the process of ET release is frequently accompanied by cell death and ROS production, but controversial findings have been reported. For instance, in neutrophils, the contribution of ROS to NET release in response to important fungi, including *Aspergillus* spp.,^{128,130} *C. albicans*,¹³¹ and *C. gatti*,¹³³ has been documented. In contrast, Mejia and colleagues have recently indicated that NET production against *Paracoccidioides brasiliensis* conidia is ROS independent, but it is partially involved in NET induction against yeast.¹³⁴ The primary source of the released DNA (mitochondrial or nuclear) is still debatable.^{138,139} These differential findings may be related to the different stimuli used and the distinct mechanisms of activation of different immune cells. As mentioned, roles in the clearance of *A. fumigatus* have been previously described for murine eosinophils,¹²² but the interactions between *Aspergillus* and eosinophils are poorly understood. Moreover, the occurrence of EETosis and EETs in asthma,¹⁴⁰ eosinophilic rhinosinusitis and otitis¹⁴¹ has been reported in the literature. However, no information has been published for ABPA.

In this context, we have provided the first evidence that isolated human eosinophils release DNA extracellular traps in response to *A. fumigatus* in vitro via a cytolytic mechanism that depends on the Syk tyrosine kinase pathway (described to mediate cell signaling via different classes of receptors involved in fungal recognition) and the integrin CD11b, but that it does not involve ROS production. In addition, we demonstrated the colocalization of DNA and citrullinated H3, which suggests that the EETs contain nuclear-derived genomic DNA.⁹⁶ Microscopy studies have revealed that *A. fumigatus* conidia and cell-free extracellular eosinophil granules are associated with EETs⁹⁶ (Figure 2B). We also characterized the presence of EETs in the mucus plugs of patients with ABPA with positive cultures for *A. fumigatus*.⁹⁶ Interestingly, our in vitro observations were consistent with observations in bronchial mucus samples obtained from ABPA patients. An analysis of the bronchial secretions of the patients revealed a massive infiltration of eosinophils and significant numbers of eosinophils exhibiting signs of EETosis (decondensed nuclei or loss of lobulation). Moreover, our data are consistent (except for ROS dependency) with previous findings describing a cytolytic mechanism that leads to EET release that is mainly associated with clusters of secretion-competent eosinophil granules.¹³⁷

Interestingly, our in vitro findings revealed that released EETs did not have fungicidal/fungistatic activities. These findings are consistent with clinical observations showing persistent fungal colonization in ABPA individuals, even in the presence of abundant EETosis-derived EETs. In fact, previous studies using neutrophils have not identified an influence of NET formation on the killing of *A. fumigatus* conidia, but only inhibition of its germination.¹²⁹

Taken together, these observations allow us to postulate that excessive release of DNA extracellular traps by eosinophils may contribute to the formation of sticky and viscous mucus, which may trigger breathing difficulty and airway obstruction and contribute to the deleterious pulmonary inflammatory process typical of ABPA.

5 | CONCLUDING REMARKS AND FUTURE QUESTIONS

While the roles of eosinophils in the airway hyperreactivity in asthma associated with fungal sensitization or ABPMs is still debatable or even unknown, their contributions to pulmonary airway remodeling are more accepted. Regarding the innate effector eosinophil responses to fungi, a functional role in the killing of the fungus were observed for *A. alternata* (human eosinophils) and *A. fumigatus* (murine eosinophils). However, based on our recent findings for human eosinophils and *A. fumigatus* in vitro and in ABPA patients, we believe that the released EETs may be less related to the fungal killing and more involved in the development of the pathological features of ABPA. In this way, excessive EET release may be more related to the formation of sticky mucus, which may trigger breathing difficulty and airway obstruction, and less with the capacity of EETs to contribute to fungal clearance. Moreover, free granules entrapped in EETs may act as proinflammatory agents, causing persistent pulmonary inflammation. Cell-free extracellular granules have been characterized as cell-free secretory functional entities that express functional receptors on their membranes with ligand-binding domains oriented toward the outer granule membrane.¹⁴² Thus, the entrapment of these functional secretory granules by EETs might contribute to the exacerbation of the inflammatory response. Moreover, in concert with this hypothesis and considering that eosinophils release citrullinated EETs, it is possible that the abundant EET deposition during ABPA must represent a pathological mechanism of the exacerbation of airway remodeling and injury. DNA extracellular traps released by neutrophils have been reported to have roles in fibrosis,¹⁴³ activation of the coagulation cascade,¹⁴⁴ and tissue injury.¹⁴⁵ A recent work has demonstrated that NETs are responsible for the development of fibrosis in elderly mice since PAD4-deficient mice showed a decrease in collagen deposition and fibrosis during aging.¹⁴³ PAD4 is responsible for arginine deimination, leading to the formation of citrulline residues in histones, which has been demonstrated to be an essential process in chromatin relaxation and NET extrusion.¹⁴⁶ Thus, EET release during ABPA or even allergic asthma associated with fungal sensitization must represent an immunopathogenic mechanism that contributes to airway inflammation, hyperreactivity, and remodeling. Thus, approaches targeting EETs or their formation might represent new therapeutic targets. In this sense, recombinant DNase I has already been employed for the treatment of cystic fibrosis, making EET degradation a viable approach for clinical studies in ABPA and potentially for other ABMPs or fungal asthma.

Furthermore, there remain important questions about the mechanisms underlying the process of EET release in response to *A. fumigatus*, as well as about whether other important fungi responsible for other forms of ABPMs are also capable of inducing EET release. Current knowledge is only beginning to elucidate the different responses of eosinophils to the diversity of fungi involved in ABPMs and the involved mechanisms. For example, as observed for *A. alternata*-induced eosinophil degranulation, does *A. fumigatus*-induced EET release involve the recognition of soluble factors or proteases

secreted by the fungus? Considering that it is already known to involve CD11b, is the I-domain crucial? Mechanisms of fungal recognition and the potential roles of TLR in *A. fumigatus*-induced EET release should also be better addressed. Whether TLR2 or TLR4 are involved remains to be revealed. Other questions include (i) which signaling pathways are involved in the process of EET release induced by *A. fumigatus*, (ii) whether calcium is involved, and (iii) whether there is a role for PAD4 as observed for neutrophils. These important questions remain to be answered.

AUTHORSHIP

J.S.N. and R.T.F. equally reviewed the literature and critically wrote the paper.

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