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Eosinophils and Anti-Pathogen Host Defense

Chapter 9.1

Introduction

Gerald J. Gleich and Kristin Leiferman

In the beginning, the eosinophil was a mystery leukocyte, brilliantly staining but without obvious function. The earliest clues came from the application of Paul Ehrlich's then new (in the latter part of the 19th century) technology for staining blood revealing associations with asthma and helminthiasis. By the end of Ehrlich's life in 1915, the eosinophil had been further linked to guinea pig anaphylaxis and, subsequently, human hypersensitivity reactions. However, for a time, the quest for more discoveries seemed stuck, and anaphylaxis, asthma, and helminth infections appeared to be the major diseases in which eosinophils had a role. This view was not to last. Once eosinophil granule proteins and reagents to localize them were available, it became clear that eosinophils participate in diseases that would not be predicted based on blood counts. Along with many inflammatory disorders, a role for eosinophils in infectious diseases beyond parasitosis has emerged. This section discusses eosinophil function relating to bacterial, fungal, and viral diseases, and strong cases are made for important eosinophil contributions to some of these diseases.

Observations dating to 1893 reported that blood eosinophils were reduced during bacterial infections. Much later, in the mid-1970s, studies in murine models showed that pyelonephritis caused by *Escherichia coli* and early subcutaneous pneumococcal abscesses produced eosinopenia, whereas trichinosis infections were accompanied by eosinophilia.¹ Remarkably, establishment of pyelonephritis or pneumococcal abscesses suppressed the eosinophilia induced by trichinosis infection.² A factor that caused eosinopenia was identified and partially characterized, but its molecular identity was not determined.³ Eosinopenia was produced by the injection of chemotactic factors, such as complement component C5a,⁴ so that they could account for at least part of the eosinopenia observed during bacterial infection. Numerous studies have compared the phagocytic and bactericidal activities of eosinophils and neutrophils,^{5–9}

and, overall, the eosinophil emerges as less able to ingest and kill bacteria than the neutrophil. However, experimental conditions in these studies varied, and the activation status of the cell, i.e., whether derived from a healthy, normal subject or a patient with eosinophilia, was important because cells from patients with eosinophilia were more active.¹⁰ Recognition that eosinophil granule proteins function as toxins prompted studies to determine their ability to kill bacteria, and results of the studies clearly showed that granule proteins kill both gram-negative and gram-positive bacteria.¹¹ Nonetheless, few investigations pointed to an important role for eosinophils in bacterial disease. More recent studies indicate a mechanism by which eosinophils are able to kill bacteria, summarized in this chapter by Simon and Yousefi. They focus, in particular, on the formation of extracellular DNA traps generated by eosinophils, and the demonstration that these traps are able to bind bacteria and kill them. The traps are formed by the extrusion of mitochondrial DNA, referred to as *catapult-like* because of its rapidity, and by the deposition of granule proteins on the extruded DNA. Earlier studies had shown that DNA avidly binds eosinophil granule major basic protein,¹² and, most probably, this complex is stable. Simon and colleagues found that eosinophil DNA traps are present in eosinophil-associated inflammatory diseases such as skin diseases and bronchial asthma. In other work, studies on eosinophil-deficient mice show reduced ability to clear *Pseudomonas* species from the peritoneal cavity and increased protection in the presence of added eosinophils.¹³ Therefore, the old literature's teaching that eosinophils are not important in bacterial diseases must be questioned on the basis of these new findings.

Concerning fungal diseases and eosinophilia and in contrast to the comments above on the relationship between bacterial diseases and eosinophils, the literature is bereft of reports on eosinophil-fungus interactions. While numerous observations show that mucin derived from the sinus cavities of patients suffering from chronic rhinosinusitis (CRS) contains fungal elements, eosinophils, and Charcot–Leyden crystals, no studies had explored the mechanisms of eosinophil-fungus interactions. In this chapter, Kita presents a summary of the mechanisms by which the

immune system responds to fungi. Extracts from numerous fungal species activate eosinophils from normal individuals with release of eosinophil-derived neurotoxin (RNase2); interestingly, *Alternaria* extracts do not correspondingly induce neutrophil activation. Investigation of the mechanism by which *Alternaria* extracts activate eosinophils concludes that the G protein-coupled protease-activated receptor is critical in the process. Eosinophils interact with living *Alternaria alternata* and release granule proteins onto the surface of the organism with death of the fungus. This interaction is mediated by the adherence of eosinophils through the β 2 integrin, integrin alpha-M (ITAM/CD11b), possibly through recognition of β -glucans. Thus, two key factors appear important for the interactions of eosinophils with fungi, namely PAR and ITAM (CD11b). Kita then reviews the mechanisms of fungal-mediated eosinophil inflammation *in vivo*, and he stresses the importance of chitin and fungal proteases possibly via respiratory epithelial-derived molecules, such as thymic stromal lymphopoietin, interleukin-33, and chemokines. He discusses diseases associated with eosinophilia and fungi, including allergic bronchopulmonary *Aspergillus*, severe asthma with fungal sensitization, allergic fungal sinusitis, and CRS. Treatment of certain eosinophil-associated diseases with anti-fungal agents has led to clinical improvement, whereas, in others, the response has been equivocal, especially in CRS, with the caveat that antifungal medications do not penetrate well into sinus cavities. Overall, this review is a summary of heretofore lacking information on immune responses to fungi and eosinophil participation and is a valuable summary of our current knowledge.

Interest in eosinophils and viruses stems from observations that respiratory syncytial virus (RSV) infection may be associated with eosinophilia. Further attention to a relationship emerged when a clinical trial of formalin-inactivated RSV vaccine resulted in strikingly more severe disease after subsequent RSV infection, with children developing *enhanced disease* and showing pronounced tissue eosinophilia. This raised the question whether eosinophils were responsible for the worsened outcome in the vaccinated children. The studies pertaining to these observations are reviewed in detail by Rosenberg and colleagues. Information supporting a protective role for eosinophils comes from studies of guinea pigs sensitized by allergen administration and then virus challenged. These animals showed a reduced parainfluenza/Sendai viral content, suggesting that the eosinophil, in an interleukin-5 (IL-5)-dependent manner, neutralized virus. The mechanisms by which this might occur still remain obscure. This chapter particularly is concerned with models of primary virus challenge in mice. Although the results from these models still leaves the role of the eosinophil in doubt, a caveat here is that the murine eosinophil seems to degranulate less readily than the human eosinophil and,

therefore, results in murine models may be misleading. In further exploring what is known about eosinophil-virus interactions, Rosenberg and colleagues allude to investigations with the pneumonia virus of mice (PVM), using human C-C motif chemokine 24 (CCL24/eotaxin-2)/mouse IL-5 double transgenic mice in which eosinophils in the respiratory tract demonstrate marked degranulation,¹⁴ and state that they observed accelerated PVM clearance in this model. Hence, the human CCL24 (eotaxin-2)/mouse IL-5 double transgenic mice may provide a unique insight into the potential maximal effects of the activated eosinophil. Overall, this chapter summarizes our current understanding of the ability of the eosinophil to neutralize respiratory viruses and the mechanisms by which this might occur.

Taken together, these three chapters highlight important information of the eosinophil's role in both innate and adaptive immune responses.

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Chapter 9.2

Eosinophil-Mediated Antibacterial Host Defense

Hans-Uwe Simon and Shida Yousefi

The primary function of eosinophils has previously been related to their interactions with helminthic parasites,¹ although this view has attracted some controversy. The cytoplasmic granules, which are believed to play an important role in host defense, consist of four distinct populations that can be identified by electron microscopy as primary granules, secondary granules, small granules, and lipid bodies.² The cytotoxic cationic proteins are stored in the secondary granules that consist of a core, which contains eosinophil granule major basic protein 1 (MBP-1), and a matrix composed of eosinophil cationic protein (ECP), eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO).³ MBP-1 is highly cytotoxic,⁴ and because of its cationic nature, it affects the charge of surface membranes resulting in disturbed permeability, and disruption and injury of cell membranes.⁵ Likewise, ECP can damage target cell membranes through the formation of pores or trans-membrane channels, but also has additional cytotoxic effects.⁶ Eosinophils have also been implicated in antiviral defense mechanisms.^{7–9}

Besides the antihelminthic and antiviral effects of at least some of the eosinophil granule proteins, antibacterial activities have also been demonstrated. By generating cytokines and chemokines, and by their ability to act as antigen-presenting cells, eosinophils may play different roles in antibacterial defense, although these topics are covered elsewhere in the book. In this chapter, we focus on our understanding of how eosinophils directly fight bacteria. For instance, ECP and MBP-1 can exhibit bactericidal activities by causing the permeabilization of the outer and inner membranes of *Escherichia coli*.¹⁰ Moreover, eosinophil-derived reactive oxygen species, in combination with EPO, are efficient in destroying *E. coli*,¹¹ and eosinophil granules have also been implicated in the destruction of *Pseudomonas aeruginosa*.¹² The antibacterial properties of eosinophils have also been demonstrated in hypereosinophilic interleukin-5 (IL-5) transgenic mice or following the adoptive transfer of eosinophils in wild-type or eosinophil-deficient mice,^{12,13} showing the importance of eosinophils in clearing bacteria *in vivo*. These data are supported by the observation that mice with congenital eosinophil deficiency (i.e., PHIL mice) show impaired bacterial clearance in an experimental model of *Pseudomonas* infection.¹²

The killing of bacteria might take place after phagocytosis (Fig. 9.2.1), which eosinophils are able to perform.¹⁴ Subsequently, phagocytosis of gram-positive

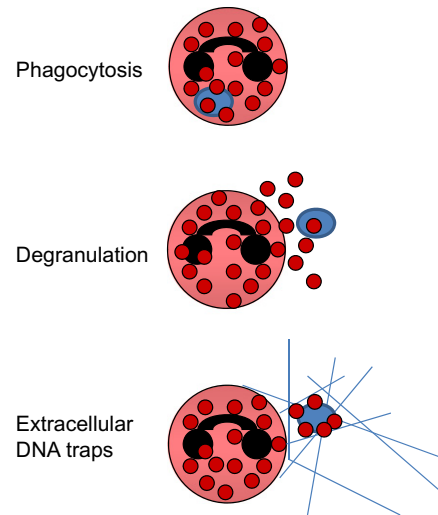


FIGURE 9.2.1 Antibacterial strategies used by eosinophils. *Phagocytosis*: Bacteria are ingested (blue). Granules (red) release cationic proteins into the phagosome. *Degranulation*: Granules and/or granule proteins are released in the extracellular space to kill the bacteria, but can also cause widespread tissue damage. *Extracellular DNA traps*: The incorporation of granule proteins into DNA traps, which also bind bacteria, likely increases the local concentration of antibacterial proteins and might limit tissue damage.

Staphylococcus aureus and gram-negative *E. coli* by eosinophils was demonstrated *in vitro*.¹⁵ At least in equine eosinophils, eosinophil granules discharge their contents into the phagocytic vacuole,¹⁴ providing one possible indication of how granule proteins could exhibit an antibacterial function. An alternative strategy to killing bacteria might be eosinophil degranulation¹⁶ (Fig. 9.2.1). In such a scenario, eosinophil basic proteins could be released in the vicinity of infection. While the activation of eosinophils can occur via multiple different receptors, immunoglobulin A (IgA) receptors are particularly efficient in eliciting eosinophil degranulation on cross-linking.^{17,18} IgA-mediated activation of eosinophils might be particularly important in the gastrointestinal mucosa, where secretory IgA is produced in high quantities.

Recently, a third strategy has suggested that extracellular DNA traps generated by eosinophils in the extracellular space are able to bind and kill bacteria was proposed (Fig. 9.2.1). Such mechanism might play a role in case of epithelial barrier defects of the gastrointestinal tract, avoiding the bacterial invasion of the body.¹³ Epithelial barrier defects may occur due to inflammatory responses. But how are DNA traps formed? Under *in vitro* conditions, eosinophils need to be stimulated with IL-5 or interferon gamma (IFN- γ) for 20 min before being stimulated with lipopolysaccharide (LPS). However, nonbacterial triggers, e.g., complement component 5a or eotaxin, are also able to promote efficient DNA release from eosinophils.¹³

Although DNA seems to be required for efficient bacterial destruction,¹³ it is unlikely that DNA carries out

this function. Indeed, extracellular ECP and MBP-1 were detected as colocalizing with DNA, as assessed by double immunofluorescence and confocal microscopy. Thus, it is likely that bacterial death is actually mediated by granule proteins within extracellular DNA traps. Time-lapse confocal imaging allowed the analysis of the kinetics of DNA release in single cells. Strikingly, DNA release happened within 1 s. The mechanism(s) of DNA release appear(s) to differ from the secretion of granule proteins that occur either by classical exocytosis or piecemeal degranulation. Time-lapse confocal imaging revealed that DNA is released from perinuclear structures. Combined two-color DNA and mitochondrial staining suggested that IL-5-primed and LPS-stimulated eosinophils release mitochondrial DNA, which was subsequently confirmed by using molecular biological techniques.¹³ Release of mitochondrial DNA was independent of cell death/apoptosis.

Extracellular DNA traps can also be generated by activated neutrophils.¹⁹ The DNA here is associated with granule proteins, such as elastase or myeloperoxidase.¹⁹ However, neutrophil DNA traps may additionally contain histones.¹⁹ In contrast to eosinophils, much more information is available regarding the pathogens trapped and killed by neutrophil DNA traps; these include gram-positive and gram-negative bacteria, fungi, and parasites.²⁰ The release of DNA can occur within minutes^{21,22} or hours;²³ in the latter case, cell death appears to be required.

Interestingly, eosinophil DNA traps were also seen in inflammatory skin diseases²⁴ and in bronchial asthma.²⁵ The primary function of these extracellular structures remains unclear under these conditions, although it is possible that eosinophils participate in anti-infection defense mechanisms in at least some of these subjects/diseases. On the other hand, the binding of released eosinophil cationic proteins to extracellular DNA may limit the collateral damage from granular contents in eosinophilic inflammatory diseases.

Taken together, there is accumulating evidence that eosinophils play a beneficial role in innate immune responses against bacteria. This suggests that therapies aiming to deplete eosinophils may cause increased susceptibility toward bacterial infections, although no such adverse effects were observed when treating patients with anti-IL-5 antibody.^{26,27} Clearly, many additional questions remain. For instance:

- Do eosinophils play a role in the fight against pathogens in asthma and other allergic diseases?
- Under which conditions do eosinophil cationic proteins exhibit antimicrobial properties?
- What are the exact molecular mechanisms of extracellular DNA release and how long do DNA traps remain in tissues?
- How do DNA traps correlate with other markers of inflammation? Can they be used as biomarkers of

eosinophil activation? Do they prevent exaggerated eosinophil-mediated tissue pathology?

The mechanisms of indirect protection against bacteria (e.g., promotion of epithelial repair and bridging innate and adaptive immunity) also remain largely unexplored.

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roles in asthma, allergy, and other disorders in which they are recruited in response to cytokines released by T-helper type 2 (T_H2) lymphocytes. Eosinophils do not ordinarily come to mind when one thinks generally of a respiratory virus infection. However, at least for one important respiratory virus, the human respiratory syncytial virus (RSV), eosinophils and their unique secretory mediators have been detected in lung tissue in response to primary infection and as a feature of a characteristic hypersensitivity response to inactivated vaccines and vaccine components. Interestingly, eosinophil recruitment and accumulation are almost always perceived in a negative light, as it is assumed that these cells contribute to tissue damage, bronchoconstriction, and respiratory dysfunction via degranulation of their cationic secretory proteins, enzymes, and cytokines. However, recent descriptions of antiviral activity both *in vitro* and *in vivo* suggest that eosinophil function may encompass both of these functions, and present more of a double-edged sword. Clearly, we do not have a complete understanding of the role of eosinophils in disease caused by RSV; here we highlight many of the questions that remain to be explored.

HUMAN RESPIRATORY SYNCYTIAL VIRUS DISEASE

RSV infection is a near universal affliction of infancy and childhood, accounting for approximately 50% of all pneumonia and up to 90% of the reported cases of bronchiolitis in infancy. Of those infants infected during the first year of life, one-third develops lower respiratory tract disease and 2.5% are hospitalized, accounting for more than 90,000 children in the United States every year. In many previously healthy infants, RSV disease is a mild and self-limited infection involving the upper and lower respiratory tract, with varying degrees of peribronchiolar and interstitial inflammation. In others, disease progresses to severe bronchiolitis and pneumonia, including submucosal edema and bronchiolar obstruction requiring oxygen, and in the worst cases, mechanical ventilation. Infants at particularly high risk for severe disease include those born prematurely, infants and children with cardiac or pulmonary anomalies, and immunocompromised infants and children, although a recent study by Hall and colleagues¹ noted that a substantial proportion of children with serious RSV disease had no pre-existing predisposing condition. Prophylactic monoclonal antibody therapy is available for high-risk infants only, and no vaccine has been approved for use. RSV has also recently been recognized as an important pathogen in the institutionalized elderly. The clinical features and pathology of RSV disease have been reviewed extensively, and the reader is

Chapter 9.3

Interactions of Eosinophils with Respiratory Virus Pathogens

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INTRODUCTION

Eosinophils, granule-bearing leukocytes found in peripheral blood and tissues, are best known for their

referred to these and other excellent sources of information.^{2,3}

BASIC BIOLOGY OF HUMAN AND MOUSE EOSINOPHILS

Eosinophils are leukocytes of the granulocyte lineage, as are neutrophils and basophils. Eosinophils differentiate in the bone marrow from CD34 antigen-positive pluripotent progenitor cells and are released into the bloodstream in a more or less completely mature state. Under normal, homeostatic conditions, very few eosinophils can be detected in peripheral blood (only approximately 2–3% of total leukocytes), as the vast majority reside in the tissues, primarily in the gastrointestinal tract. In response to as yet incompletely characterized stimuli, typically observed in allergic states, during infection with helminthic parasites, and in some idiopathic hypereosinophilic states, T_H2 lymphocytes are activated, which results in the production of a specific subset of T_H2 cytokines, including interleukin-5 (IL-5). IL-5 has a unique impact on the eosinophil lineage, as it induces the expansion of eosinophil progenitors in the bone marrow, it primes eosinophils in the periphery, and it prolongs eosinophil survival in the tissues. Eosinophils are capable of responding to a wide variety of other stimuli and can undergo chemotaxis in response to eotaxin (CCL11), MIP1-alpha (CCL3) and RANTES (CCL5), which are chemoattractant cytokines that interact with eosinophils via specific cell surface receptors CCR3, CCR1 and CCR5, respectively. Interestingly, despite years of research, there is still no absolute consensus on eosinophil physiology and function, even in well-characterized disease states. For example, while eosinophils and eosinophil secretory mediators can promote destruction of helminthic eggs and larvae in experiments performed *in vitro*, experiments performed *in vivo* with cytokine-deficient and eosinophil-deficient mice have yielded complex and inconsistent results. Similarly, although the weight of evidence suggests that eosinophils contribute to the pathophysiology of allergy and asthma, a chronic respiratory disease in which bronchoconstriction in response to environmental triggers is typically associated with production of T_H2 cytokines and recruitment of eosinophils to the airways, asthmatic responses are obviously negative sequelae of eosinophil function that alone cannot represent a direct evolutionary advantage to the host organism. Among the more recent hypotheses, several groups have focused on eosinophils as immunomodulatory mediators, as eosinophils can interact both directly and indirectly with T cells and mast cells, and can release a wide variety of preformed cytokines and other secretory mediators, primarily from cytoplasmic granules. Other chapters in this volume provide more extensive coverage of these subjects.

Mature eosinophils from all species are readily recognized by their eccentric bilobed nuclei and their characteristic red-staining cytoplasmic granules. As noted, human eosinophil granules are storage sites for cationic secretory mediators, including a unique eosinophil peroxidase, the eosinophil granule major basic protein 1 (MBP-1), eosinophil cationic protein (ECP/ribonuclease 3), eosinophil-derived neurotoxin (EDN/ribonuclease 2), and numerous enzymes and cytokines. Despite similar morphology, human and mouse eosinophils differ from one another and cannot be presumed to function identically in all circumstances. For instance, while there are several reports describing a high-affinity immunoglobulin E (IgE) receptor [high affinity immunoglobulin epsilon receptor subunit beta (FcεRI)] in human eosinophils from parasite-infected and asthmatic subjects,^{4–6} this finding and its functional significance has been questioned,⁷ and FcεRI has never been detected on eosinophils isolated from mice. Similarly, sialic acid-binding Ig-like lectin 8 (Siglec-8) can be detected on the surface of human eosinophils, while mouse eosinophils express the highly divergent functional orthologue, Siglec-F (Siglec-5). The mouse eosinophil ribonucleases are highly divergent orthologues of human ECP and EDN⁸ and Charcot–Leyden crystal protein, a major human eosinophil component, cannot even be identified in the mouse genome. Mouse eosinophils also display a profoundly reduced propensity to degranulate and undergo differential chemotaxis to known exogenous stimuli (reviewed in⁹). As such, eosinophils from humans and mice may look similar to one another, but they may not be formally identical to one another in their actions and in their capacity to cause, to ameliorate, or even to serve as biomarkers for disease.

A number of recent studies have associated eosinophils and eosinophil degranulation products with various aspects of RSV infection in both human disease and in parallel mouse models, which need to be understood with the aforementioned caveats in mind. Here, we review some of these findings with an eye toward understanding what is and what is not known regarding the role of eosinophils, their role in vaccine-induced pathology, their interactions with respiratory virus pathogens, and the outcome of severe respiratory virus disease.

HYPERSENSITIVITY RESPONSES TO FORMALIN-INACTIVATED RESPIRATORY SYNCYTIAL VIRUS VACCINE—ARE THE EOSINOPHILS AT FAULT?

In the early 1960s, a number of children were enrolled in a clinical trial of a formalin-inactivated RSV vaccine. The negative outcomes of this trial, including records of the detailed responses of the vaccinated children who encountered natural RSV infection sometime thereafter,

have been documented and reviewed.^{10,11} Briefly, children immunized with the formalin-inactivated virus, upon encountering a natural RSV challenge, developed a hypersensitivity response, characterized by bronchoconstriction and severe pneumonia; this has been attributed in part to the development of non-neutralizing antibodies. Lung histology from two children that ultimately died as a result of this trial revealed the deposition of antibody–virus complexes and a pronounced tissue eosinophilia.¹² One or more of these features, which have collectively been termed *enhanced disease*, have been replicated and modeled with formalin-inactivated RSV in multiple species, including other primates, ferrets, cotton rats, and mice as well as with formalin-inactivated bovine RSV in cows.¹³ Interestingly, hypersensitivity responses of this nature are not unique to formalin-inactivated RSV; there are a limited number of reports describing aberrant responses to formalin-inactivated measles vaccine.¹⁴ This phenomenon has also been replicated experimentally to varying extents with formalin-inactivated versions of human metapneumovirus¹⁵ and parainfluenza virus,¹⁶ and in some reports even to carrier antigens.¹⁷ There is also a recent report of a severe hypersensitivity reaction, including T_H2 cytokine-mediated eosinophil infiltration into the lung tissue, in BALB/c mice immunized with

a vaccinia virus construct expressing the severe acute respiratory syndrome (SARS) coronavirus (CoV) N nucleocapsid protein.¹⁸ Our group has demonstrated that immunization of mice with formalin-inactivated pneumonia virus of mice (PVM) followed by intranasal virus challenge likewise results in pulmonary hypereosinophilia in the absence of a serum-neutralizing antibody response¹⁹ (Fig. 9.3.1). PVM is a natural rodent pneumovirus pathogen that is related to RSV; PVM replicates extensively in mouse bronchiolar epithelial cells tissue, eliciting a profound and potentially lethal inflammatory response, similar to the more severe forms of RSV disease.²⁰

Gene-deletion and cytokine depletion mouse model studies all point to T_H2 cytokines [interleukin-4 (IL-4), IL-5, and interleukin-13 (IL-13)] as crucial to eliciting pulmonary eosinophilia in response to formalin-inactivated RSV.^{21,22} A recent study by Moghaddam and colleagues²³ suggested that the oxidation of RSV antigens resulting from formalin exposure elicits a T_H2 response *in vivo*. Delgado and colleagues²⁴ and Cyr and colleagues²⁵ have both reported that independent toll-like receptor (TLR) stimulation in conjunction with RSV antigens results in a rebalancing of the T_H1/T_H2 cytokine responses, thereby reducing pathology. In initial studies aimed at

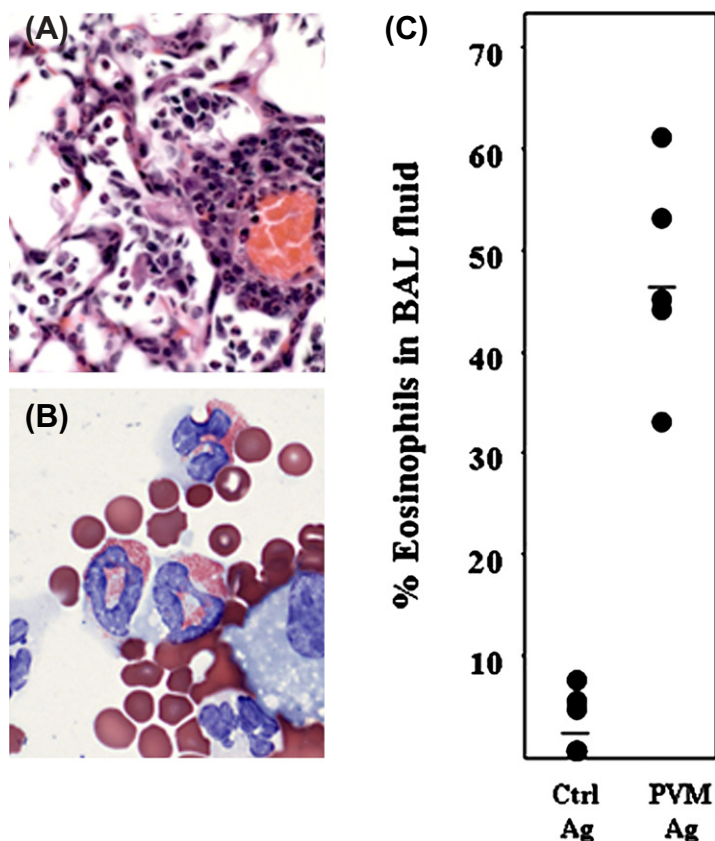


FIGURE 9.3.1 Hypersensitivity responses in mice vaccinated with formalin-fixed pneumovirus antigens. (A) Lung tissue from a mouse vaccinated with formalin-inactivated pneumonia virus of mice (PVM; a mouse pneumovirus related to human RSV) and then challenged intranasally with actively replicating virus. (B) Eosinophils detected in the bronchoalveolar lavage (BAL) fluid from the mouse described in (A). (C) Percentage eosinophils detected in the BAL fluid of mice vaccinated with formalin-inactivated PVM vs. control antigen. Ag, antigen; BAL, bronchoalveolar lavage; RSV, respiratory syncytial virus. Reprinted from¹⁹.

exploring the molecular mechanism of T_H2 -mediated immunopathology, pulmonary eosinophilia was observed in mice immunized with recombinant vaccinia virus expressing RSV-G protein followed by live RSV challenge in most, but not all published trials (reviewed in¹¹), results which initially suggested that the pathology related to formalin-inactivation might be attributed mechanistically to aberrant reactivity to this one virus protein alone. Interestingly, although the end point—pulmonary eosinophilia—looks more or less the same, recent analysis indicates that the pulmonary eosinophilia that develops in response to RSV-G protein and the eosinophilia that develops in response to formalin-inactivated RSV proceed via different molecular mechanisms. Among other distinctions, eosinophilia in response to RSV-G protein is not dependent on IL-4 and requires the actions of RSV-G protein-specific V β 14⁺ T cells; in contrast, eosinophilia in response to formalin-inactivated RSV antigens is IL-4 dependent, while not dependent on RSV-G protein-specific V β 14⁺ T cells (reviewed in¹¹).

Are Eosinophils Contributing to the Pathophysiology?

Much of the focus of the enhanced disease/hypersensitivity studies has been on the presence of eosinophils and the mechanisms of eosinophil recruitment to the lung tissue, yet it was never really clear whether or not eosinophils were directly responsible for the pathophysiological responses. In other words, it was unclear whether the vaccinated children became ill because of pulmonary eosinophilia, whether the eosinophils were engaged in altering future responses to virus infection, or whether eosinophilia was a neutral, secondary finding. These questions have been explored to some extent in mouse models using wild-type and eosinophil-deficient mice (including Δ dblGATA mice) immunized with vaccinia virus vectors expressing RSV-G and RSV-F proteins,^{11,26} but, as noted above, these experimental systems are now recognized as mechanistically unrelated to the pathology induced by formalin-inactivated RSV antigens. As such, although findings address a role for eosinophils, and they likewise suggest that eosinophils actually may not be contributing to systemic disease—specifically, that clinical symptoms, weight loss, and respiratory dysfunction measurements may be unrelated to the presence of absence of pulmonary eosinophilia—these conclusions may not be directly relevant to the way in which eosinophils contribute to pathology in the setting of formalin-inactivated RSV antigens. The role of eosinophils in modulating the pathology induced by formalin-inactivated vaccine antigens has not been explored and might be addressed in mouse models of explicit eosinophil deficiency with formalin-inactivated RSV antigens.

Thus, although our long-standing prejudices might make it easy to conclude that eosinophils contributed directly to the lung and systemic pathology observed in the initial vaccine trials and in the subsequent mouse modeling experiments, it is important to recognize that the presence of eosinophils in the lung may or may not lead to these outcomes. The data from mouse models are inconclusive on this point. Furthermore, the presence of eosinophils alone, even in human conditions, does not necessarily imply severe respiratory pathology. For example, in eosinophilic bronchiolitis, patients complain of only minimal respiratory symptomatology despite pronounced pulmonary eosinophilia.²⁷ Thus, at current writing, while eosinophils may serve as important biomarkers for aberrant hypersensitivity reactions, we can reach no conclusions regarding their contributions to pathophysiology from the published experimental data.

EOSINOPHIL RECRUITMENT IN RESPONSE TO PRIMARY RESPIRATORY SYNCYTIAL VIRUS INFECTION—A CAUSE FOR ALARM?

Although respiratory virus infections are not among the diseases typically associated with T_H2 lymphocyte activation and profound pulmonary eosinophilia, eosinophils and/or eosinophil granule secretory proteins have been detected in lung washings or systemically in infants in need of supplemental oxygen secondary to severe RSV infection.^{28–30} As mentioned earlier, it is not at all certain whether pulmonary eosinophilia is uniquely related to the RSV pathogen, or whether eosinophilia is observed in response to RSV because it is the predominant severe respiratory pathogen among very young infants. Although not reported as frequently, the eosinophil granule protein ECP has been detected in nasopharyngeal secretions in response to other respiratory virus infections, including influenza and parainfluenza.^{31,32}

A number of recent studies have suggested that the age at which the individual experiences a first RSV infection has a profound impact on the nature of the primary response. In general, T_H2 cytokines (IL-4, IL-5) and evidence of eosinophilia (cells and/or degranulation products) are detected more readily in younger infants, although results are not completely consistent in all studies. For example, Kristjansson and colleagues³² examined the responses of infants diagnosed with RSV and found that those who were less than 3 months of age at the time of first infection had higher levels of IL-4 in their nasopharyngeal secretions than children who were older, although no differences were observed in analogous levels of ECP. Likewise, Sung and colleagues³³ documented elevated levels of both IL-4 and IL-5 in serum samples of RSV-

infected infants who were less than 18 months old at the time of primary infection than in older infants. Similarly, Kim and colleagues³⁴ examined eosinophils in the bronchoalveolar lavage fluid (BALF) from RSV-infected infants (ages 0.4–1.8 years) and found that the number of eosinophils detected in BALF correlated closely with IL-5 concentration, although interestingly, the age range of the group in which eosinophils were detected was not significantly different from the age range of the group in which eosinophils were absent.

Nasal eosinophilia has been detected in response to respiratory viruses other than RSV (including rhinoviruses and CoVs), although the circumstances tend to be limited and highly specific, such as in patients with pre-existing respiratory allergies.³⁵ Of particular interest, several groups have reported that influenza infection stimulates the production of the eosinophil chemoattractants eotaxin (CCL11) and CCL5 (RANTES) in normal nasal and airway epithelial cells in culture, which suggests the possibility of eosinophil recruitment.^{36–39} The role of eosinophils in acute SARS-CoV remains completely unexplored, but the eosinophil secretory ribonuclease, eosinophil-derived neurotoxin (RNASE2/EDN), was among the 52 signature genes that discriminated between individuals recovering from severe SARS-CoV infection and healthy controls.⁴⁰

Several large clinical studies have led to the consistent conclusion that infants who have recovered from severe RSV bronchiolitis are at significantly increased risk for both recurrent wheezing and childhood asthma.^{41–43} Given the presumed role of eosinophils in the pathogenesis of acute allergic asthma, it seems reasonable to ask whether the eosinophils recruited to the lungs during severe primary RSV bronchiolitis might cause, or at least predict, the progression to wheezing. Causation is of course difficult to ascertain in human subjects; however, a prospective study by Pifferi and colleagues⁴⁴ demonstrated that infants who were less than 1 year old and who had elevated serum ECP levels during a primary RSV infection were nearly 10 times more likely to have developed symptoms of wheezing in later childhood than older children and children without elevated levels of serum ECP. However, Sigurs and colleagues,⁴⁵ following much the same methodology, found that serum ECP was not predictive of progression to wheezing. In a more recent prospective study, Castro and colleagues⁴⁶ examined the outcomes of RSV-infected, <1-year-old infants; in this study, 48% of those enrolled went on to develop allergic symptomatology by age 6, with a significantly higher prevalence of asthma developing among the children who were infected with RSV at a younger age (below 6 months). Among those developing asthma, there were no differences in peripheral blood eosinophil counts at the time of acute RSV infection, nor were the cytokine profiles (as determined by phorbol myristate acetate stimulation of isolated mononuclear cells)

of children who developed allergic disease different from those who did not.

What Can We Learn from Mouse Models of Primary Virus Challenge?

Given the complexities of natural disease, and the fact that there is no human condition in which an individual is uniquely devoid of eosinophils, it is helpful (if not crucial) to have appropriate animal models to explore questions of association and causation. Inbred mice have been used extensively to study responses to RSV, although it is important to recognize that RSV inoculation of mice is formally a challenge-clearance model rather than an infection model, as RSV undergoes little if any replication in mouse lung tissue.

Schwarze and colleagues^{47,48} have explored RSV challenge in mouse models; in these studies, the authors describe T_H2 cytokine-dependent recruitment of eosinophils and associated airways hyperreactivity, a finding that has implicated eosinophils in the pathophysiological mechanism. Eosinophilia was also noted in a mouse model of secondary RSV challenge; Culley and colleagues⁴⁹ detected eosinophil recruitment to the airways on secondary RSV challenge among mice undergoing primary challenge at 1 day of age; eosinophil recruitment declined dramatically if primary challenge was delayed until mice were 1 week old, although the authors found no statistically significant difference in systemic disease, measured as weight loss, between these two sets of challenged mice. Dakhama and colleagues⁵⁰ likewise found that the extent of airway hyperresponsiveness induced by a secondary challenge was directly dependent on the age of first virus challenge, similarly associated with augmented eosinophil recruitment when the primary challenge occurred in mice <1 week of age. Tasker and colleagues⁵¹ performed a similar study, and identified T_H2 cytokine responses in neonatally primed mice that were associated with diminished virus replication in lung tissue. Finally, also noteworthy is the study by Harker and colleagues⁵² in which mice were primed with recombinant RSV (rRSV) expressing T_H1 [interferon gamma (IFN- γ)] or T_H2 (IL-4) cytokines prior to RSV challenge. In contrast to mice primed with rRSV/IFN- γ , mice primed with rRSV/IL-4 sustained airway eosinophilia in response to subsequent RSV challenge. Although IL-4 clearly functions to suppress antiviral $CD8^+$ T cell function in other experimental settings,^{53,54} challenge with RSV/IL-4 had no impact on the number of $CD8^+$ T cells recruited to the lung nor on the fraction producing IFN- γ when compared to mice challenged with wild-type RSV alone. The RSV/IL-4-primed mice had reduced lung virus titer and were protected against weight loss, a finding that correlated with the

recruitment of eosinophils. As is clear from these findings, the precise role of eosinophils remains uncertain, but one thing that is clear is the fact that eosinophils do not universally provoke lung pathology and systemic disease.

The role of RSV and PVM in enhancing asthmatic-type responses via an interplay with known allergens has also been explored;^{55–59} while the weight of evidence suggests that eosinophils play crucial roles in mouse models of asthma, what that precise role might be (promoting acute airways hyperreactivity vs. more chronic remodeling) is a complex and controversial issue that has been considered extensively by others and is beyond the scope of this chapter.

DO EOSINOPHILS PROMOTE ANTIVIRAL HOST DEFENSE?

One of the more curious aspects of eosinophil biology is, as discussed thus far in this review, that once they are detected, particularly in lung tissue, eosinophils are almost always considered as contributing in some negative way to the pathophysiology of disease. This is most intriguing, given our understanding of the role of their sister cell, the neutrophil, and the concept of the double-edged sword.⁶⁰ In other words, we know that neutrophils are recruited in response to bacterial and fungal infection, and serve to promote host defense against these invasive pathogens. However, if signals go awry, if neutrophil clearance does not proceed, and/or if neutrophil activation persists, pathology ensues. We were among the first groups to suggest that eosinophil function might encompass a positive, host-defense aspect as part of perhaps a more subtle double-edged sword,^{61,62} and to consider the possibility that eosinophils may be recruited in part to promote primary antiviral host defense, perhaps in situations in which acquired immune responses are less than immediately effective.⁶³ Interestingly, eosinophilia has been reported in association with T cell dysfunction in human

immunodeficiency virus (HIV) infection;^{64–66} this finding may in turn be related to the propensity for hypersensitivity reactions observed among HIV-infected patients.⁶⁷ However, correlations between eosinophilia and disease pathogenesis are often difficult to ascertain. In a primary study, Tietz and colleagues⁶⁴ found that elevated eosinophil counts among HIV-infected patients correlated with progression of disease and declining CD4⁺ T cell counts, while Chorba and colleagues⁶⁸ found no correlation between HIV viral loads, CD4⁺ T cell and eosinophil counts among more than 600 HIV-infected patients in sub-Saharan Africa, although concurrent helminthic parasite infection was clearly a confounding variable.

The first indication that eosinophils might have the means to function in promoting antiviral host defense came from a series of studies we performed in the late 1990s. In this work, we determined that eosinophils, acting at least in part via their secretory mediators, could reduce the infectivity of RSV for target epithelial cells *in vitro*;⁶⁹ Soukup and Becker⁷⁰ likewise demonstrated that eosinophils inhibit RSV infection in tissue culture. Shortly thereafter, Adamko and colleagues⁷¹ demonstrated that eosinophils elicited by allergen sensitization served to limit virus replication and/or promote virus clearance in guinea pigs challenged with parainfluenza/Sendai virus. In a more recent study, Phipps and colleagues⁷² demonstrated accelerated clearance of RSV from the lungs of the eosinophil-enriched IL-5 transgenic mice, and furthermore found that full antiviral activity was dependent on intact TLR signaling in eosinophils introduced exogenously (Fig. 9.3.2).

As discussed earlier, we have explored the responses of mice immunized with formalin-inactivated PVM followed by live virus challenge. In experiments using eosinophil-deficient Δ dblGATA mice, we found that eosinophils were not a crucial component of the (limited) protection resulting from this immunization strategy,¹⁹ a finding perhaps related to the virulence of this pathogen in inbred strains of

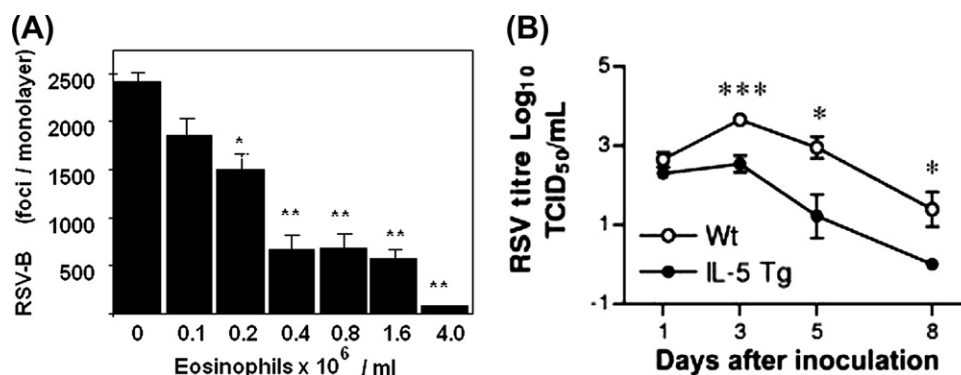


FIGURE 9.3.2 Eosinophils promote antiviral host defense. (A) Eosinophils reduce the infectivity of RSV for target epithelial cells *in vitro*. (B) Eosinophil-enriched interleukin-5 transgenic mice promote accelerated RSV clearance compared to wild-type mice, with reduced virus titers detected at all time points examined. IL-5, interleukin-5; RSV, respiratory syncytial virus; Wt, wild-type. Panels (A) and (B) reprinted from⁶⁹ and⁷² respectively.

mice, as well as its ability to infect eosinophils.⁷³ Nonetheless, we have recently observed accelerated clearance of PVM in eotaxin-2/IL-5 double-transgenic mice⁷⁴ in which eosinophils are undergoing profound and extensive degranulation (Percopo et al., unpublished data)

ARE EOSINOPHILS AMONG THE DIRECT TARGETS OF RESPIRATORY VIRUS INFECTION?

Our studies with PVM⁷⁵ and the examination of pathology specimens from RSV patients^{76,77} demonstrate that pneumovirus replication *in vivo* takes place primarily in respiratory epithelial cells. However, it is clear that other cells, including human monocytes, support the replication of RSV and PVM in culture, and release proinflammatory cytokines in response to virus infection.^{78–80} Given the questions regarding the role of eosinophils and their interactions with respiratory viruses, we set out to determine whether pneumoviruses could infect and replicate within eosinophils, and to determine what the outcome of this infection might be. Kimpen and colleagues^{81,82} originally demonstrated that RSV could be taken up by purified human eosinophils, and virions were identified in phagolysosomal compartments, but virus replication was not examined. To explore PVM

replication in mouse eosinophils, we used our recently described method for generating sustained cultures of >95% pure mature eosinophils from unselected bone marrow progenitors.⁸³ With eosinophils generated by this culture method, we demonstrated a dramatic increase in virus titer within 7 days of inoculation (Fig. 9.3.3), associated with the replication-dependent release of infectious virions accompanied by the cytokine interleukin-6. Among others who have explored the direct interactions of pneumoviruses with eosinophils, Davoine and colleagues⁸⁴ determined that human eosinophils were unable to release granule proteins in response to RSV challenge without coincident exposure of virus to CD4⁺ T cells and antigen-presenting cells. Among the questions left to be explored are:

- How often are infected eosinophils detected *in vivo*?
- At what point during an acute infection are they detected and under what specific circumstances?
- Does virus infection and intracellular replication induce eosinophil apoptosis or disable eosinophils in some other, more subtle way, and thereby reduce their ability to promote virus clearance and antiviral host defense?

Answers to these questions may shed some light on the differential responses observed in the aforementioned experiments.

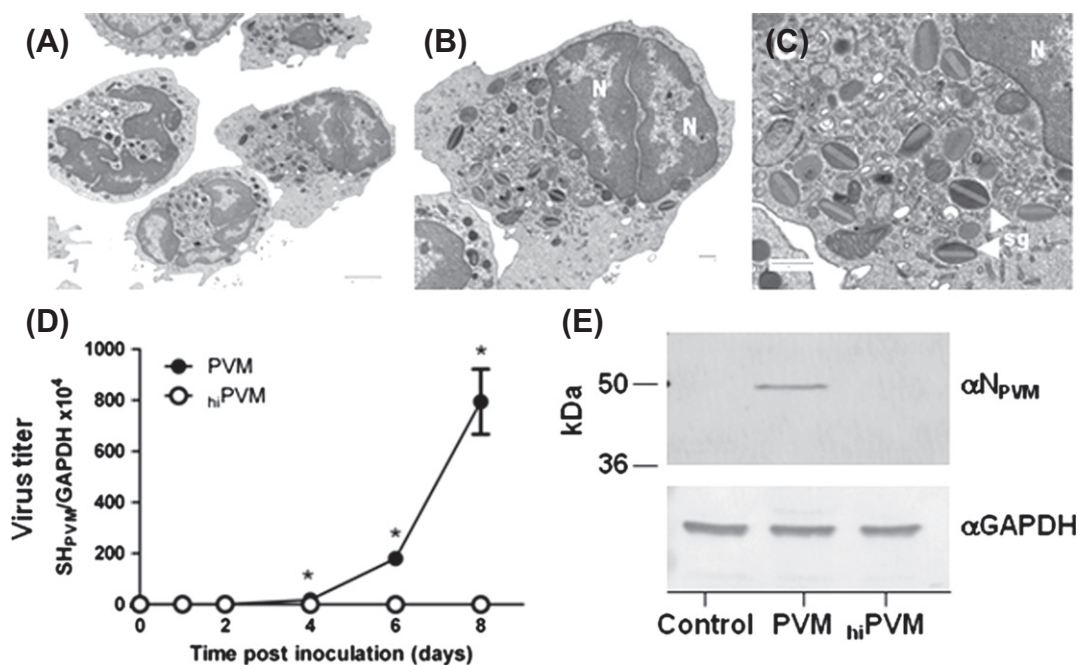


FIGURE 9.3.3 Pneumovirus replication in eosinophils. (A–C) Electron micrographs of cultured eosinophils derived from unselected mouse bone marrow; the symbol N denotes the characteristic bilobed nucleus, and sg (at arrows) denotes the cytoplasmic-specific granules. (D) Replication of PVM in cultured mouse eosinophils; filled symbols, replication-competent PVM; open symbols, heat-inactivated PVM. PVM is detected by quantitative RT-PCR targeting the virus *small hydrophobic* (SH) gene. (E) Detection of PVM replication in cultured mouse eosinophils with anti-PVM N protein-specific antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PVM, pneumonia virus of mice; RSV, respiratory syncytial virus; RT-PCR, reverse transcription polymerase chain reaction. *Reprinted from*⁷³.

CONCLUSIONS AND FUTURE PERSPECTIVES

The manner in which eosinophils respond to and participate in respiratory virus infection is very far from clear. While pulmonary eosinophilia is a hallmark, or biomarker, of the aberrant hypersensitivity response to formalin-inactivated RSV, there is no clear indication that eosinophils actually contribute to the negative sequelae of disease. Likewise, while severe primary RSV is associated with pulmonary eosinophilia and progression to asthma, these two features have not been linked clearly to one another mechanistically or pathophysiologically. Finally, several groups have shown that eosinophils can promote virus clearance, but this interesting and positive feature of eosinophil function is not observed in all circumstances or in all situations. Among the possibilities that have yet to be explored, eosinophil function may be less dependent on numbers elicited, and may be more closely related to the quality and extent of activation, to the unique nature of the cytokines eliciting recruitment, and/or to the strength of the signals sustaining viability *in situ*. These are all issues that are worthy of consideration as we attempt to improve our understanding of the true nature of the eosinophilic leukocyte and strive to achieve some clarification and sense of balance between their perceived negative and their incompletely characterized positive contributions to homeostasis and host defense.

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Chapter 9.4

Antifungal Immunity by Eosinophils: Mechanisms and Implications in Human Diseases

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INTRODUCTION

Eosinophils are implicated in the pathophysiology of allergic diseases, such as bronchial asthma and atopic dermatitis, and in host immunity to helminth infections.¹ During such inflammatory reactions, soluble mediators released by immune cells induce eosinophil recruitment from the bloodstream into the sites of inflammation, where as yet unknown stimuli trigger the release of proinflammatory mediators.² Marked extracellular deposition of released eosinophil granule proteins has been detected in specimens from patients with asthma, chronic rhinosinusitis (CRS), and atopic dermatitis.^{3,4,5} However, the presence of eosinophils *per se*, as in normal intestinal mucosa,⁶ does not lead to disease pathology. Thus, a fundamental and important question still remains: what *triggers* eosinophil activation and proinflammatory mediator release in human disease? The occurrence of eosinophilic inflammation in such disparate conditions as parasitic infections (presumably for the benefit of the human host) and hypersensitivity diseases (perhaps to the detriment of the patient) has become better understood as a consequence of recently reported information.

Fungi are ubiquitous in the environment, and as saprophytes or commensals, they may coexist without effect in the host with normal cellular immunity.⁷ Nonetheless, these airborne fungi and their products may contribute to the development and exacerbation of human airway diseases. For example, fungal products induce immunological and inflammatory reactions, resulting in a T-helper type 2 (T_H2)-like cytokine response and the destruction of mucosal barrier functions.^{8,9} Clinically, an association between fungal exposure and asthma has been widely recognized.¹⁰ Therefore, antifungal immunity may provide a valuable clue toward improved understanding of eosinophil biology and the mechanisms of human diseases. This chapter describes the recent development of our understanding of the biological and immunological properties of eosinophils and the mechanisms of eosinophilic inflammation with specific focus on antifungal immune

responses. The potential roles of eosinophils and antifungal immune responses in human diseases are discussed.

OVERVIEW OF ANTIFUNGAL IMMUNE RESPONSES

The host immune mechanisms against fungi range from protective mechanisms that were present early in the evolution of multicellular organisms (*innate immunity*) to adaptive mechanisms, which are specifically induced during infection and disease (*adaptive immunity*).¹¹ The life cycle of fungi starts with *conidia* (often called spores) that germinate and produce filaments (*hyphae*); these grow and branch in all directions to form a mass, collectively called the *mycelium* or colony. Colony formation only occurs if there are sufficient nutrients and moisture in the substrate. When the mycelium is mature, specialized hyphae called conidiophores are formed. On the conidiophores, thousands of conidia are produced, which can easily become airborne because of their small size and buoyancy.

Fungi are multicellular organisms and express various biological molecules during different stages of their life-cycle. The fungal cell wall is composed of several components, including a fibrillar layer, mannoprotein, chitin, and β -glucan.¹² Chitin is a (β 1-4)-linked polymer of *N*-acetyl-D-glucosamine (NAG), and produced in the cytosol by chitin synthase. Glucan has a large group of D-glucose polymers having glycosidic bonds; of these, the most common glucans composing the fungal cell wall have the α -configuration, such as the (β 1-3)- and (β 1-6)-linked glucosyl units. Up to 60% of the dry weight of the cell wall of fungi consists of glucans.¹³ Fungi also produce many proteases that are required for their germination, growth, and survival.¹⁴

Fungal cell wall components are recognized by cognate receptors, such as the soluble receptor, pentraxin-related protein PTX3 (PTX3),¹⁵ and membrane-bound receptors, such as Toll-like receptors (TLRs). PTX3 binds to the yeast cells of *Paracoccidioides brasiliensis*, but not to *Candida albicans*, suggesting that PTX3 recognizes specific fungal species and morphological forms.¹⁶ In *Aspergillus*-induced asthma, mannan-binding lectin A (MBL-A) appears to enhance the T_H2-type immune responses, suggesting that this opsonin enhances fungus-specific T-cell responses.¹⁷ Fungal killing and host immunity often depend on multiple TLR- and non-TLR-mediated pathways.¹⁸ Because the composition of the fungal cell wall is complex, the cell wall constituents can activate more than one TLR-dependent signaling pathway. For example, macrophage tumor necrosis factor (TNF- α) and interferon gamma (IFN- γ) secretion triggered by *C. albicans* yeast cells depend on

TLR2 and TLR4, whereas hyphal cells trigger only TLR2-dependent responses.¹⁹

The receptors for β -glucans are expressed by several immune cells including macrophages²⁰ and neutrophils.²¹ The nonopsonic recognition of β -glucans by these cells has been ascribed to multiple receptors, including dectin-1,²² lactosylceramide,²³ and CD11b/CD18 (integrin β 2/CR3).²⁴ Dectin-1 possesses a single nonclassical C-type carbohydrate recognition domain (CRD) connected to the transmembrane region by a stalk, and a cytoplasmic tail, possessing an immunoreceptor tyrosine-based activation (ITAM) motif, which induces the production of reactive oxygen species and inflammatory cytokines.²⁵ Lactosylceramide (CDw17) is a glycosphingolipid found in the plasma membranes of many cells and was identified as a β -glucan receptor from the biochemical analyses of the interactions between poly-¹⁻⁶-D-glucopyranosyl-¹⁻³-D-glucopyranose glucan (PGG glucan) and isolated human leukocyte membrane components.²³ CD11b/CD18 (CR3) is a heterodimeric integrin, consisting of the α_M (CD11b) and β_2 (CD18) chains.²⁶ CD11b is an adhesion molecule but it also serves as a phagocytic receptor for iC3b-opsonized particles, including opsonized particulate glucans,²⁷ through its I-domain. Importantly, CD11b also possesses a lectin domain, which maps to a site C-terminus to the I-domain. The lectin domain recognizes selected monosaccharides and a variety of β -glucans, including zymosan, although the polymeric ligand with the highest affinity contained very little β -glucan and consisted mostly of mannose.²⁸

EOSINOPHIL RESPONSES TO FUNGI AND FUNGAL PRODUCTS *IN VITRO*

Activated human eosinophils defend against large, non-phagocytosable organisms, most notably the multicellular helminthic parasites. Therefore, one could reasonably speculate that eosinophils may be involved in host defense and/or immune responses to other organisms with a large surface, for example filamentous fungi. We incubated human eosinophils with extracts from several environmental airborne fungi (*Alternaria alternata*, *Aspergillus versicolor*, *Bipolaris sorokiniana*, *C. albicans*, *Cladosporium herbarum*, *Curvularia spicifera*, and *Penicillium chrysogenum*).²⁹ *Alternaria* and *Penicillium* induced calcium-dependent exocytosis [e.g., eosinophil-derived neurotoxin (EDN) release] in eosinophils from normal individuals (Fig. 9.4.1). *Alternaria* also strongly induced other activation events in eosinophils, including increases in intracellular calcium concentration, cell surface expression of CD63 and CD11b, and production of interleukin-8 (IL-8). Interestingly, *Alternaria* did not induce neutrophil activation.

How do eosinophils recognize and respond to fungal products within the *Alternaria* extracts? An extensive analysis of eosinophil expression of TLRs and their responses to TLR ligands showed that eosinophils constitutively expressed TLR1, TLR4, TLR7, TLR9, and TLR10 mRNAs and that the TLR7 ligand, R848, can activate eosinophils.² However, eosinophil response to *Alternaria* is unlikely to be dependent on TLRs and their ligands.³⁰ Rather, the eosinophil-stimulating activity in *Alternaria* extract was highly heat-labile and had a molecular mass of about 60 kDa, suggesting the involvement of proteinaceous molecules and their receptors.

Proteases, especially serine proteases, activate hematological and interstitial cells through a family of G protein-coupled proteinase-activated receptors (PARs) and induce the production of several proinflammatory mediators.³¹ Four members of this receptor family have been cloned and are designated PAR-1, PAR-2, PAR-3, and PAR-4. Protease cleavage of these receptors creates a neo-NH₂ terminus, which acts as a tethered ligand and activates the seven transmembrane segments of the PAR. Human eosinophils constitutively transcribe mRNA for PAR-2 and PAR-3, but not for PAR-1 and PAR-4.³² Trypsin, an authentic agonist for PAR-2, induces superoxide anion production and degranulation of eosinophils; 5 nM trypsin induces responses that are 50–70% of those induced by 100 nM PAF, a positive control. Similarly, a cysteine protease, papain, induces isolated human eosinophils to degranulate and to produce superoxide anion.³³ *A. alternata* produces aspartate protease(s) and recognition of this protease(s) by PAR-2 is likely responsible for the activation of eosinophils in response to *Alternaria* extracts. Indeed, when stimulated with *Alternaria* extracts, eosinophils show an increased intracellular calcium concentration that is desensitized by peptide and protease ligands for PAR-2 and inhibited by PAR-2 antagonistic peptide(s). *Alternaria*-derived aspartate protease(s) cleaves PAR-2 to expose *neo-ligands*; these *neo-ligands* bind to the seven transmembrane segments of PAR-2 and activate eosinophil degranulation (Fig. 9.4.2). Treatment of *Alternaria* extract with aspartate protease inhibitors, which are conventionally used for human immunodeficiency virus 1 (HIV-1) and other microorganisms, attenuates the eosinophils' responses to *Alternaria*. Thus, fungal aspartate protease and eosinophil PAR-2 appear critical for the eosinophils' innate immune response to certain fungi, suggesting a novel mechanism for eosinophil activation, for pathological inflammation in asthma and for host–pathogen interaction.

Eosinophils respond not only to fungal extracts but also to living fungal organisms. Human eosinophils react vigorously to *A. alternata* grown in tissue culture dishes³⁴ (Fig. 9.4.3). Eosinophils release their cytotoxic granule proteins, such as EDN and eosinophil granule major basic protein 1 (MBP-1), into the extracellular milieu and onto

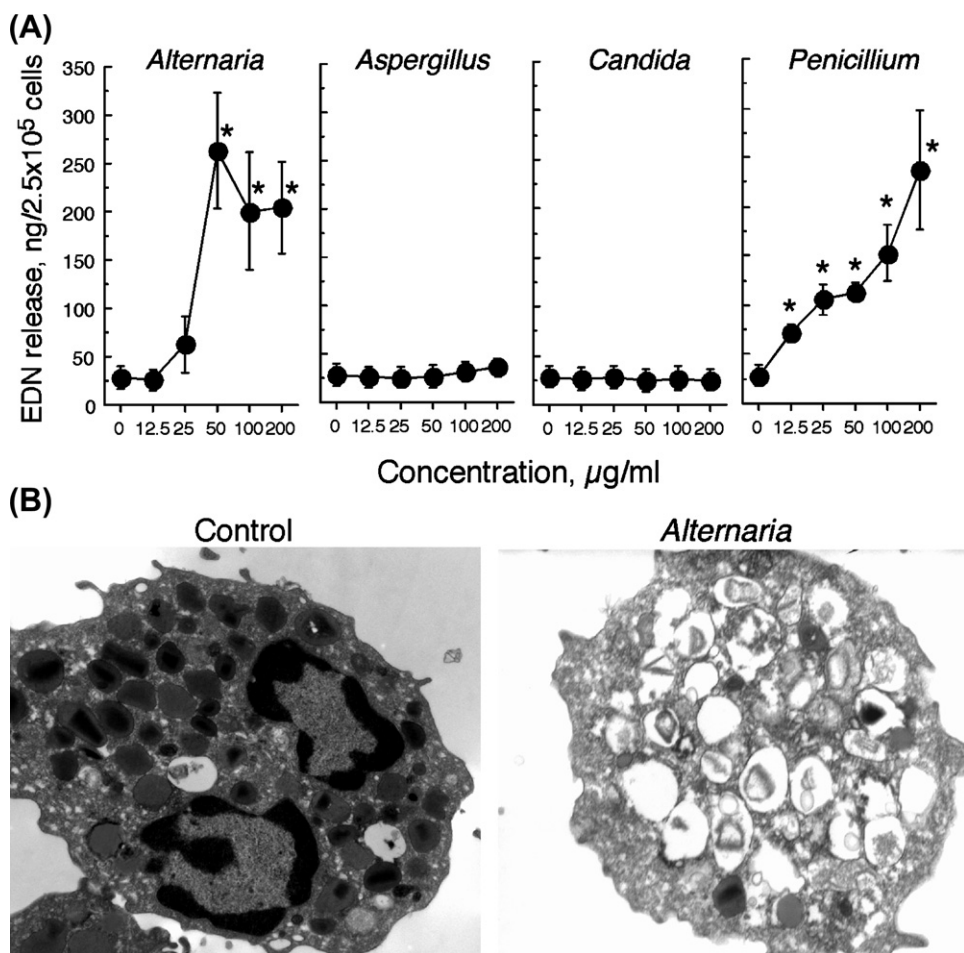


FIGURE 9.4.1 Eosinophil degranulation induced by fungal extracts. (A) Eosinophils were incubated with fungal extracts and the concentrations of eosinophil-derived neurotoxin (EDN) in the supernatants were measured by immunoassay. (B) Photoelectron micrographs of eosinophils stimulated with medium alone (control) or *Alternaria* extract. Note the granule fusions and electron lucent granules in eosinophils stimulated with *Alternaria*.

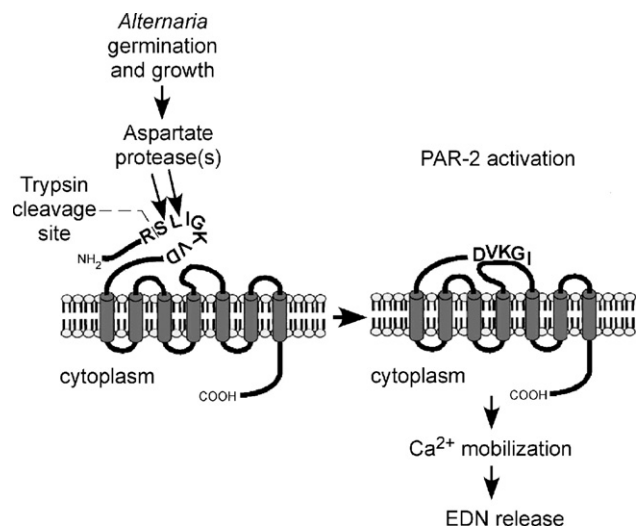
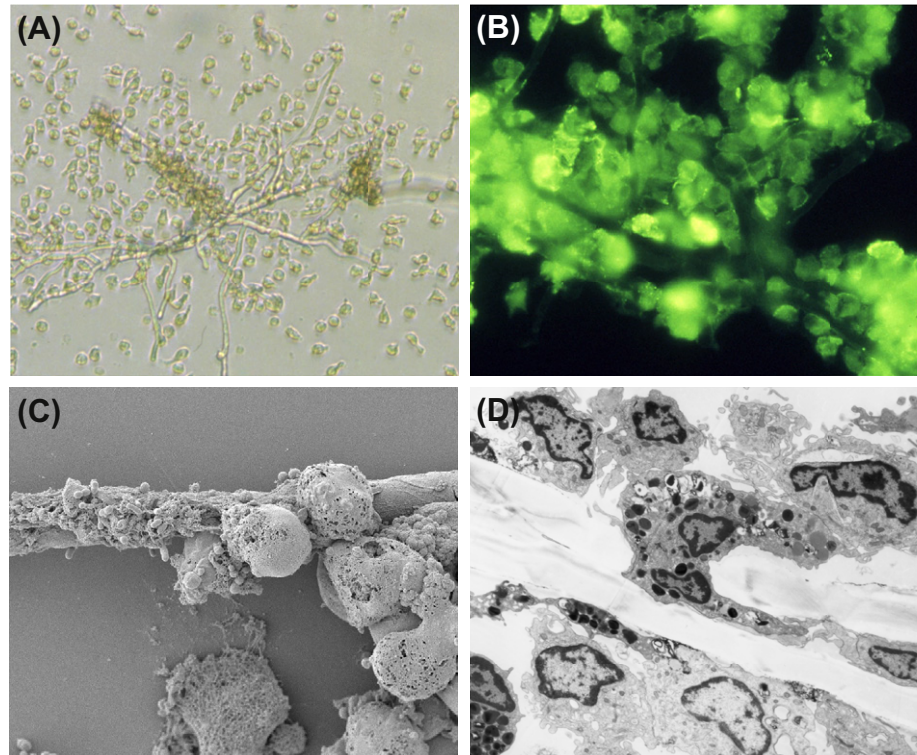


FIGURE 9.4.2 Mechanisms of PAR-2 activation and eosinophil EDN release in response to *Alternaria* extract. EDN, eosinophil-derived neurotoxin.

the surface of fungal organisms and kill the fungus. While human eosinophils do not express common fungal receptors such as dectin-1 and lactosylceramide as described earlier, eosinophils do express and use their integrin $\beta 2$ molecule, CD11b, to adhere to a major fungus cell wall component, β -glucan. More specifically, the I-domain of CD11b is distinctively involved in how eosinophils interact with β -glucan. It has been known that eosinophils express CD11b constitutively and that the level of expression is increased by incubating eosinophils with various agonists, such as fMet-Leu-Phe (fMLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), or platelet-activating factor (PAF).¹ Furthermore, compared with neutrophils, eosinophils have a larger total pool of CD11b within the cells.³⁵ Historically, receptor ligands immobilized to relatively large surfaces [such as immunoglobulin G (IgG)-coated sepharose beads and parasites], though not particulate ligands (such as aggregated IgG and bacteria), are effective stimuli for eosinophil

FIGURE 9.4.3 Interaction of eosinophils with living *Alternaria* organisms. Isolated human eosinophils were incubated with germinating *Alternaria* for 24 h. The culture mix was analyzed by the following: (A) inverted light microscopy, (B) anti-MBP-1 staining and fluorescence microscopy, (C) scanning electron microscopy, and (D) transmission electron microscopy. Note that fungal hyphae are covered with eosinophils and their cellular components in (C). In (D), eosinophils adhere tightly to fungal hyphae and their granules are lost in >50% of the cells.



degranulation.¹ Eosinophil CD11b plays a pivotal role in eosinophil adhesion to these large surfaces and degranulation.³⁶ Thus, eosinophils likely use their versatile CD11b molecules to react to microorganisms (e.g., helminths, fungi) as well as other cellular targets with large surfaces.

Altogether, eosinophil response to filamentous fungi is mediated by at least two major pathways, recognition of fungal proteases by PARs, and cellular adherence to the cell wall β -glucan by integrin β 2 integrin, CD11b/CD18. This two-pronged approach may have important implications in the mechanisms of antifungal immunity. *Drosophila* detects fungal infections by recognizing pathogen-associated molecular patterns (PAMPs) and by monitoring the effects of fungal virulence factors. Specifically, the receptor gram-negative bacteria-binding protein 3 on *Drosophila* recognizes β -glucan, and the secreted fungal virulence factor PR1 protease cleaves *Drosophila* serine protease Persephone, activating the downstream immune response to the fungi.³⁷ Similarly, eosinophil CD11b may recognize fungal structures by monitoring the presence of β -glucan. Eosinophil PARs may act like sensors to monitor fungal protease activities or putative virulence factors and provide a cue to immune and inflammatory systems once the activities reach a certain threshold. Therefore, recognition of fungi by both their structures and activities may be a conserved trait during the evolution of immunity. Such immune responses by eosinophils to fungi may benefit the host, but in turn, it may also play a role in the development

and/or exacerbation of eosinophil-related human airway diseases, such as asthma.

MECHANISMS INVOLVED IN FUNGUS-MEDIATED EOSINOPHILIC INFLAMMATION *IN VIVO*

Recently, major progress has been made regarding the mechanisms involved in eosinophilic inflammation in response to fungi *in vivo*. As described earlier, chitin is one of the major cell wall components and provides structural rigidity to fungi, as well as to crustaceans, helminths, and insects. Airway administration of chitin induces the accumulation of interleukin-4-expressing innate immune cells, including eosinophils and basophils.³⁸ Tissue infiltration of eosinophils is unaffected by the absence of TLRs but is reduced when the injected chitin is pre-treated with mammalian chitinase, acidic mammalian chitinase (AMCase), or when the chitin is injected into mice over-expressing AMCase. Chitin induces macrophage activation and the production of leukotriene B₄ (LTB₄), which is required for optimal recruitment of eosinophils. Similarly, cell wall preparations of *Aspergillus* isolated from house dust induce the recruitment of eosinophils into mouse lungs, and the effects are attenuated by enzymatic degradation of cell wall chitin and β -glucans.³⁹ Thus, chitin and likely β -glucan are recognized by innate immune cells in

the airway and are implicated in eosinophil infiltration induced by fungal exposure.

Fungal proteases also likely play critical roles in eosinophilic airway inflammation induced by fungi. The protease activities in *Aspergillus* extracts correlate with their activities to induce immunoglobulin E (IgE) response and eosinophilic inflammation when they are administered to mouse lungs.⁹ Furthermore, active proteases are present in almost all houses and many of these activities are derived from fungi, especially *Aspergillus niger*.⁴⁰ The conidia of *A. niger* readily establish airway mucosal infection, eosinophilic inflammation, and IgE antibody production, while protease-deficient *A. niger* has reduced ability to promote airway eosinophilia. Importantly, in mice infected with *A. niger*, IL-5 and interleukin-13 are required for optimal clearance of lung infection *in vivo*, suggesting T_H2-type airway inflammation and/or that eosinophils are protective against fungal infection *in vivo*.

The immunological pathways that link airway fungal exposure and development of T_H2-type eosinophilic inflammation have been an active area of research for many years. The airway epithelium is no longer just a structural barrier;⁴¹ it can respond to environmental insults, such as allergens, microorganisms, cigarette smoke, and pollution, by secreting inflammatory mediators and antimicrobial peptides, and by recruiting immune cells.⁴² Our current understanding emphasizes the importance of epithelial-derived cytokines, particularly the C-C chemokine receptor family, interleukin-25 and -33, and thymic stromal lymphopoietin (TSLP), in initiating T_H2-type airway inflammation.^{43,44}

TSLP is an interleukin-7-like cytokine that is produced by epithelial cells in the lungs, gut, and skin.⁴⁵ Expression of TSLP in the airways of patients with asthma correlates with the severity of the disease, suggesting that it is involved in the development of allergic airway inflammation.⁴⁶ TSLP activates dendritic cells to polarize naive T cells toward the T_H2 cells that produce T_H2-type cytokines as well as TNF- α .⁴⁷ Mice expressing TSLP in the lungs develop a spontaneous airway inflammation with characteristics similar to human asthma.⁴⁸ Conversely, mice deficient in the TSLP receptor show decreased airway inflammation when they are challenged with allergens.⁴⁹ TSLP mRNA and protein are induced when a human airway epithelial cell line, BEAS-2B, is exposed to prototypic proteases, namely trypsin and papain. TSLP induction by trypsin requires intact protease activity and PAR-2. Importantly, when BEAS-2B cells or normal human bronchial epithelial cells are exposed to *Alternaria* extract, TSLP is also induced. The TSLP-inducing activity of *Alternaria* is partially blocked by treating the extract with a cysteine protease inhibitor, E-64, or by infecting BEAS-2B cells with small interfering RNA for PAR-2, suggesting roles for fungal protease(s) and their receptor, PAR-2.

Among the most potent molecules of the innate immune system are the interleukin-1 (IL-1) family members;⁵⁰ these cytokines, such as IL-1, interleukin-18, and interleukin-33 (IL-33), are evolutionarily ancient and are involved in regulating both innate and adaptive immune responses. IL-33 was first described as a nuclear factor abundantly expressed in the nucleus of endothelial and epithelial cells.⁵¹ *In vivo* systemic administration of IL-33 to mice induces lung and gastrointestinal eosinophilia and increased levels of serum IgE and immunoglobulin A (IgA).⁵² IL-33 drives the production of cytokines and chemokines by T_H2 cells, mast cells, basophils, eosinophils, natural killer T (NKT) cells, and natural killer (NK) cells.⁵² Airway exposure of naive mice to *Alternaria* extracts induces the rapid release of IL-33 into the airway lumen, followed by innate T_H2-type responses.⁵³ Biologically active IL-33 is constitutively stored in the nuclei of human airway epithelial cells, and exposing these cells to *Alternaria* releases IL-33 extracellularly. Pharmacological inhibitors of purinergic receptors or deficiency in the purinergic receptor P2Y, G-protein coupled, 2 (P2Y2) gene abrogate IL-33 release and T_H2-type responses in the *Alternaria*-induced airway inflammation, suggesting essential roles for adenosine triphosphate and the P2Y purinoceptor 2 receptor in epithelial responses to airborne fungi. Altogether, these findings suggest that airway exposure to fungi or their products activate the innate immune cells (e.g., macrophages, epithelial cells) and facilitate their production of immunological molecules that are involved in eosinophilic inflammation, such as IL-33, LT_B4, and TSLP. Fungal chitin and proteases likely are key components in triggering the immune response.

POTENTIAL ROLES FOR EOSINOPHIL ANTIFUNGAL IMMUNITY IN HUMAN DISEASES

Information regarding the roles of eosinophils in protecting against fungal infection in humans is scarce. Coccidioidomycosis, caused by the fungus *Coccidioides immitis*, may be accompanied by an increase in peripheral blood eosinophils.⁵⁴ Marked deposition of MBP-1 is observed in lesions of patients with paracoccidioidomycosis.⁵⁵ A number of studies suggest potential detrimental roles for eosinophils and fungi in T_H2-mediated airway diseases, such as allergic bronchopulmonary aspergillosis (ABPA), severe asthma associated with fungal sensitivity (SAFS), and CRS.

Allergic asthma is generally considered a chronic inflammatory disorder of the airways mediated by T_H2 cells, which drives the cardinal features of asthma, including airway eosinophilia, airway hyperreactivity, and excessive production of mucus. These pathological immune responses

have long been recognized to mirror the beneficial immune responses to helminthic infection. Although the etiology of asthma could be complex, a number of epidemiological studies suggest that fungi are involved in asthma. For example, direct associations between increased fungal exposure and worsening of asthma have been demonstrated repeatedly.⁵⁶ Fungal sensitivity, particularly to *Aspergillus*, *Alternaria*, and *Cladosporium*, is associated with asthma.^{57,58} In a large survey of US housing, exposure to *A. alternata* antigens was correlated with active asthma symptoms.⁵⁹ Sensitization to *Alternaria* at age 6 correlated with chronic asthma at age 22,⁶⁰ and sensitization to *Alternaria* and other species has been associated with severe and potentially fatal episodes of asthma.⁵⁶ In addition, epidemics of asthma caused by increased airborne fungal spores that occur during thunderstorms further illustrate the association between fungal exposure and worsening of asthma symptoms.⁶¹

ABPA is a T_H2 hypersensitivity lung disease caused by bronchial colonization of *A. fumigatus* and is characterized by exacerbations of asthma, recurrent transient chest radiographic infiltrates, and peripheral and pulmonary eosinophilia, especially during exacerbation.⁶² The minimal criteria required for the diagnosis of ABPA are:

- asthma or cystic fibrosis with deterioration of lung function;
- immediate *Aspergillus* skin test reactivity;
- total serum IgE \geq 1000 ng/ml (416 IU/ml);
- elevated *Aspergillus*-specific IgE and IgG antibodies; and
- chest radiographic infiltrates.

ABPA is likely the most common form of allergic bronchopulmonary mycosis (ABPM). Other fungi, including *Candida*, *Penicillium* and *Curvularia*, are occasionally responsible for a syndrome similar to ABPA.⁶³ The characteristics of ABPM include severe asthma, blood and pulmonary eosinophilia, markedly elevated IgE and specific IgE, bronchiectasis, and mold colonization of the airways.

The term SAFS has been coined to illustrate the high rate of fungal sensitivity in patients with severe asthma.⁵⁶ It is possible that ABPA represents one manifestation of a spectrum of fungus-associated airway diseases. From a fungal perspective, the human lung is not sterile. The conidia of *A. fumigatus*, *Penicillium* and *Cladosporium*, and presumably other fungi, are often present, and they induce an immune response when germination is initiated.⁶⁴ Excess mucus and airway architecture distortion may allow fungal germination and escape from host defense. In approximately 50% of patients with severe asthma, fungal cultures are positive without evidence of IgE fungal sensitization, suggesting that fungal colonization of the airways is common even in the absence of an

allergic component.⁶⁵ There is a significant association between *A. fumigatus* IgE sensitization, colonization, and impaired postbronchodilator forced expiratory volume in one second (FEV₁).⁶⁶ Patients with SAFS respond to oral antifungal therapy, implicating fungi in the pathophysiology of asthma.⁶⁷

CRS is chronic inflammation of the upper airway and is often associated with nasal polyps. CRS is defined as symptoms and signs of sinus inflammation persisting for more than 8–12 weeks.⁶⁸ In the past, a number of studies have attempted to elucidate the mechanisms of CRS. Several hypotheses concerning its pathogenesis have been proposed and tested, including chronic bacterial infection, inhalant or food allergies, and T-cell disturbance caused by aerodynamic factors.⁶⁹ Although its cause is still unclear, there are several distinctive features in CRS. For example, the histological hallmark of CRS is marked tissue eosinophilia, which is seen in almost all patients.^{70,71} These eosinophils likely play a major role in the pathophysiology of CRS via the release of their cytotoxic granule proteins, such as MBP-1.⁷² In fact, a highly significant correlation was noted between the extent of disease, as examined by computed tomography (CT) scan, and the peripheral blood eosinophil count.⁷³ Consequently, MBP-1 levels in mucus exceeding the concentrations that cause damage to the upper airway respiratory epithelium are detected in sinus mucus from patients with CRS.⁴

In 1981, Millar and colleagues first described sinus specimens from five patients with CRS that showed histological similarities to ABPA.⁷⁴ Since then the majority of reports have shown non-*Aspergillus* fungi, such as *Curvularia*, *Alternaria*, and *Bipolaris*, causing similar findings, promoting a change to the more general term, allergic fungal sinusitis (AFS). The diagnostic histological feature of AFS is the presence of *allergic mucin*, characterized by sloughed respiratory epithelial cells, accumulations of intact and degenerating eosinophils, Charcot–Leyden crystals, and cellular debris containing eosinophils and their products; noninvasive fungal hyphae are found within the *allergic mucin* and are best demonstrated with impregnated silver stain. AFS prevalence ranges from 4% to 7%⁷⁵ of all the CRS cases; however, it is also speculated that AFS is underdiagnosed because of confusion concerning the criteria for diagnosis⁷⁶ and less than adequate techniques to detect fungi. Indeed, fungi are ubiquitous and found in the mucus of a majority of patients with CRS as well as in healthy individuals.^{77,78} Furthermore, in some patients with CRS, eosinophils sometimes form clusters around fungal organisms in the mucus (Fig. 9.4.4), reminiscent of the accumulation of eosinophils around helminth parasites. These findings suggest that the eosinophilic immune response to fungal organisms may be implicated in the pathophysiology of CRS.^{77,78} Interestingly, systemic antifungal treatment in patients with SAFS not only

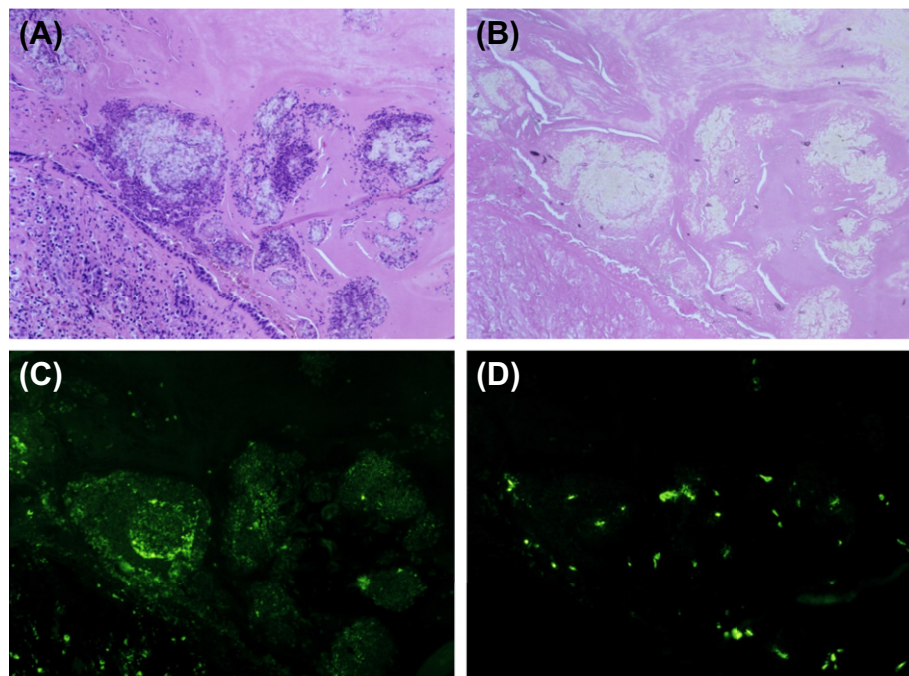


FIGURE 9.4.4 Eosinophilic inflammation and fungal localization in the mucus of a CRS patient. A surgical tissue specimen from a patient with CRS was stained by hematoxylin and eosin stain (A), Gomori–Grocott Methenamine Silver GMS stain (B), anti-MBP-1 antibody (C), or anti-*Alternaria* antibody (D). Note several clusters of eosinophils within the mucus (A and C). Fungi were not visualized well by GMS stain (B) but were clearly shown by the antibody (D). CRS, chronic rhinosinusitis; MBP-1, eosinophil granule major basic protein 1.

improved asthma symptoms but also rhinological symptoms.⁶⁷ Two other randomized controlled trials of CRS patients with antifungal agents have demonstrated a benefit while other trials have not.⁷⁹ The controversy over antifungal therapy for CRS and the involvement of fungi in CRS continues, requiring further investigations.

CONCLUSION AND FUTURE PERSPECTIVES

Evidence exists that eosinophils respond to fungal organisms and their products, resulting in the production of proinflammatory mediators and killing of fungi. Immune cells in the airways recognize carbohydrate molecules and proteases that are produced by fungal organisms and initiate T_H2 immune responses and eosinophilic inflammation. There are a number of epidemiological findings that link the exposure or sensitivity to fungi, eosinophilic airway inflammation, and human airway diseases. However, several major questions still remain. For example: are eosinophils protective against fungal colonization or infection in humans? How do human eosinophils become activated in diseased tissues or at the site of the immune response, resulting in tissue damage? Which molecules in fungi and host receptors play a pivotal role in antifungal immune responses? In SAFS or CRS, do antifungal immune responses and the aberrant activation of eosinophils play major roles in disease pathophysiology? Careful analysis of recent genetically engineered eosinophil-deficient mice *in vivo* and further characterization of immunobiology of eosinophils *in vitro* will be necessary to answer these critical

questions. Well-designed clinical studies and pharmacological trials in patients with SAFS and CRS will be necessary to dissect the roles for eosinophils and fungi in human diseases. Although these are challenging tasks, future studies have a promise to reveal the true importance of eosinophils in human health and disease.

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