

Innate and adaptive immune responses to fungi in the airway



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Fungi are ubiquitous outdoors and indoors. Exposure, sensitization, or both to fungi are strongly associated with development of asthma and allergic airway diseases. Furthermore, global climate change will likely increase the prevalence of fungi and enhance their antigenicity. Major progress has been made during the past several years regarding our understanding of antifungal immunity. Fungi contain cell-wall molecules, such as β -glucan and chitin, and secrete biologically active proteases and glycosidases. Airway epithelial cells and innate immune cells, such as dendritic cells, are equipped with cell-surface molecules that react to these fungal products, resulting in production of cytokines and proinflammatory mediators. As a result, the adaptive arm of antifungal immunity, including T_H1 -, T_H2 -, and T_H17 -type $CD4^+$ T cells, is established, reinforcing protection against fungal infection and causing detrimental immunopathology in certain subjects. We are only in the beginning stages of understanding the complex biology of fungi and detailed mechanisms of how they activate the immune response that can protect against or drive diseases in human subjects. Here we describe our current understanding with an emphasis on airway allergic immune responses. The gaps in our knowledge and desirable future directions are also discussed. (J Allergy Clin Immunol 2018;142:353-63.)

Key words: Fungi, innate immunity, adaptive immunity, airway

Fungi are ubiquitous organisms that make their homes both indoors and outdoors.¹ Human subjects are constantly exposed to environmental fungal spores, often at levels 1000-fold greater than levels of grass and tree pollens.² Virtually everyone in the United States is exposed to *Alternaria* species antigens at

Abbreviations used

ABPM:	Allergic bronchopulmonary mycosis
AFRS:	Allergic fungal rhinosinusitis
AHR:	Airway hyperreactivity
CLR:	C-type lectin receptor
CRS:	Chronic rhinosinusitis
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin
ILC2:	Group 2 innate lymphoid cell
MR:	Mannose receptor
PAR:	Protease-activated receptor
PRR:	Pattern recognition receptor
RAGE:	Receptor for advanced glycation end-products
SAFS:	Severe asthma with fungal sensitization
SPT:	Skin prick test
TLR:	Toll-like receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin

home.³ Molecular evidence suggests that animals and fungi have coevolved since diverging from plants more than 1 billion years ago.⁴ Since then, the immune system has been crucial in establishing a close relationship between the host and fungi by “keeping the peace” at barrier surfaces.⁵ In immunocompromised hosts fungi can colonize and infect the lungs and other organs, causing increases in morbidity and mortality. Alternatively, fungi and their products can cause exaggerated immune responses and pathologic changes in organs in certain subjects.

Although numerous environmental factors are associated with asthma and allergic diseases, allergen exposure likely plays a key role in triggering and exacerbating asthma and allergy symptoms.⁶ In particular, exposure to airborne allergens derived from animals, arthropods, and molds is considered an important risk factor.⁷⁻⁹ In human subjects an association between fungal exposure, sensitization, or both, in particular to *Alternaria* and *Aspergillus* species, and asthma is recognized in various countries.^{2,10} For example, *Alternaria* species sensitivity is linked closely to the development of allergic asthma.¹¹⁻¹³ Severe asthma and life-threatening acute asthma exacerbations have also been associated with increased airborne exposure to *Alternaria* species.^{14,15} Increased spore counts during crop harvest and after thunderstorms can contribute to asthma exacerbations as well.¹⁶ Furthermore, water damage and other environmental effects lead to increased growth of fungi, such as *Stachybotrys chartarum*, compromising air quality and promoting airway inflammation.¹⁷ In experimental animals the highly potent actions of *Alternaria* and *Aspergillus* species in stimulating innate and adaptive type 2 immunity have been recognized by a number of investigators.¹⁸⁻²⁵

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Terms in boldface and italics are detailed in the glossary on page 354.

In the coming years, global climate change might increase the incidence of fungal sensitization and allergic airway diseases because increased fungal colonization of plants occurs at higher CO₂ levels.²⁶ Indeed, *Alternaria alternata* grown on plants in a high-CO₂ environment showed increased spore production and higher antigen content per spore.²⁷ Higher air temperature was correlated with more days that *Cladosporium* species spore counts exceeded the allergenic threshold.²⁸ The likelihood of an increasing effect of fungi on human health provides an incentive to learn more about the mechanisms of infection and immune response to fungi.

Alterations in the bacterial microbiome have a dramatic effect on host immunity and contribute to a number of immune-mediated diseases.²⁹ Fungi are naturally present in the mammalian intestine,³⁰ and recent sequencing technologies have expanded our understanding of fungal communities at barrier surfaces.³¹ For example, intestinal fungal dysbiosis has been shown to influence gastrointestinal diseases, such as inflammatory bowel disease,³² as well as allergic inflammation of the lungs.³³ Furthermore, a large number of fungal species have been found in sputum specimens from asthmatic patients.³⁴

GLOSSARY

CHITIN: A long-chain polysaccharide similar to cellulose, chitin is a primary component of cell walls in fungi, exoskeletons of arthropods, insects, and the scales of fish and lissamphibians.

CpG SITES: Regions of DNA in which a cytosine nucleotide occurs next to a guanine nucleotide separated by only 1 phosphate. Methylation of the cytosine within a gene can turn the gene off.

CXCL10: A chemokine that elicits its effects by binding to the cell-surface chemokine receptor CXCR3, resulting in pleiotropic effects, including stimulation of monocyte, natural killer cell, and T-cell migration and modulation of adhesion molecule expression.

DUAL OXIDASE 1 (DUOX1): A member of the NADPH oxidase family. This protein generates hydrogen peroxide and thereby plays a role in the activity of thyroid peroxidase and lactoperoxidase and in lactoperoxidase-mediated antimicrobial defense at mucosal surfaces.

GALACTOMANNANS: Multifunctional macromolecular carbohydrates found in various albuminous or endospermic seeds.

GLUCAGON-LIKE PEPTIDE 1 (GLP-1): A 30-amino-acid peptide hormone produced in the intestinal epithelial endocrine L-cells by means of differential processing of proglucagon, the gene expressed in these cells.

β-GLUCAN: β-D-glucose polysaccharides found in the cell walls of bacteria, fungi, yeasts, algae, lichens, and plants, such as oats and barley.

GLYCOSIDASES: Also called glycoside hydrolases or glycosyl hydrolases, an enzyme that catalyzes the hydrolysis of glycosidic bonds in complex sugars, thus degrading oligosaccharides and glycoconjugates.

GM-CSF: Also known as colony-stimulating factor 2 (CSF2), a monomeric glycoprotein secreted by macrophages, T cells, mast cells, natural killer cells, endothelial cells, and fibroblasts, which functions as a cytokine. GM-CSF functions as a white blood cell growth factor and stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes.

IFN-γ: A type II interferon, IFN-γ is a cytokine that is required for innate and adaptive immunity against viral, bacterial, and protozoal infections. IFN-γ has been shown to be an important activator of macrophages and inducer of class II MHC molecule expression. IFN-γ is produced predominantly by natural killer (NK) and NKT cells as part of the innate immune response and by CD4 T_H1 and CD8 cytotoxic T lymphocyte effector T cells once antigen-specific immunity develops.

IL-12: A cytokine produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation that has been shown to be required for differentiation of naive T cells into T_H1 cells.

IL-25: A proinflammatory cytokine that shares sequence similarity with IL-17 and has been shown to favor the T_H2-type immune response. IL-25 can induce NF-κB activation and stimulate IL-8 production.

IL-33: A member of the IL-1 family of cytokines that potently drives production of T_H2-associated cytokines.

MANNOPROTEINS: Antigenic proteins found in the yeast cell wall with many mannose groups attached.

OROSOMUCOID-LIKE 3 (ORMDL3): A gene on chromosome 17q21 highly linked to asthma that has been shown to upregulate airway smooth muscle proliferation, contraction, and Ca²⁺ oscillations in asthmatic patients.

REACTIVE OXYGEN SPECIES (ROS): A natural byproduct of oxygen metabolism that has a critical role in cell signaling and homeostasis. However, during times of environmental stress, ROS levels can increase significantly and cause damage to cell structures, which is known as oxidative stress.

THYMIC STROMAL LYMPHOPOIETIN (TSLP): An IL-2 family cytokine that stimulates maturation of T cells through activation of antigen-presenting cells, such as dendritic cells and macrophages. This cytokine is implicated in the T_H2-type immune response.

TLR1/TLR2 HETERODIMER: A complex in which TLR1 recognizes peptidoglycan and triacyl lipoproteins in combination with TLR2.

TLR2: A membrane surface receptor that recognizes many bacterial, fungal, viral, and certain endogenous substances and plays a fundamental role in pathogen recognition and the activation of innate immunity.

TLR3: A membrane surface receptor that recognizes double-stranded RNA associated with viral infection and induces activation of interferon regulatory factor 3 (IRF3), unlike all other Toll-like receptors that activate NF-κB.

TLR4: A transmembrane protein that belongs to the pattern recognition receptor family. Its activation leads to activation of the innate immune system through the intracellular signaling pathway NF-κB and inflammatory cytokine production. TLR4 recognizes LPS, which is a component present in many gram-negative bacteria and select gram-positive bacteria. Its ligands also include several viral proteins, polysaccharide, and a variety of endogenous proteins.

TLR9: An important receptor expressed in dendritic cells, macrophages, natural killer cells, and other antigen-presenting cells that preferentially binds bacterial and viral DNA and triggers signaling cascades that lead to a proinflammatory cytokine response.

TOLL LIKE RECEPTORS (TLRS): Members of the TLR family that play a fundamental role in pathogen recognition and activation of innate immunity.

UNFOLDED PROTEIN RESPONSE (UPR): A signaling network activated by stresses that compromise the endoplasmic reticulum (ER), which impair maturation, resulting in accumulation of misfolded proteins to alleviate this stress and restore ER homeostasis and promoting cell survival and adaptation. However, under irresolvable ER stress conditions, the UPR promotes apoptosis.

Thus there is clearly a need to understand the host's innate and adaptive immune responses to fungi and their implications in pathophysiology of human diseases. In this review article we will summarize current information in the field, identify gaps in our knowledge, and discuss the need for future clinical and basic science studies. Although a variety of topics could be covered, we will focus our discussion on asthma and allergic immune responses that are specifically relevant to the *Journal of Allergy and Clinical Immunology*. Readers are encouraged to refer to recent excellent reviews^{31,35,36} and other articles in this issue for other specific topics.

FUNGI AND THEIR PRODUCTS

Fungal biology

Understanding key biologic aspects of fungi is necessary to understand the host's immune responses to these organisms. Fungi are a kingdom of eukaryotes, and more than 100,000 species have been identified to date; this likely accounts for 5% of the predicted fungal species.¹⁶ Almost 98% of known fungal species belong to one of 2 phyla: the Ascomycota and the Basidiomycota.³⁷ The genera most commonly associated with the development of allergy in human subjects are *Alternaria*, *Cladosporium*, *Penicillium*, and *Aspergillus* species.³⁸ Fungi are heterotrophs and thus cannot make their own nutrients and must depend on other organisms to provide food.³⁹ Although most animals ingest food and then digest it internally, fungi secrete enzymes that digest their food extracellularly before it can be taken up as nutrients.

Fungi produce spores, or conidia, that can remain dormant until they are ready to germinate, at which time they swell and send out hyphae.⁴⁰ Dormant fungal spores likely evade the immune system through production of a protective hydrophobin layer.⁴¹ Fungal growth depends on temperature. Mesophilic fungi, such as *Alternaria* and *Cladosporium* species, grow best in moderate temperatures, typically between 20°C and 30°C. Because they are unable to grow at body temperature, mesophilic fungi rarely cause human infections; nonetheless, their airborne fragments and secreted products can cause respiratory allergies. In contrast, thermotolerant fungi, such as *Candida*, *Penicillium*, and *Aspergillus* species, are capable of growing at human body temperature (37°C), and they serve both as a part of the microbiome and as a cause of pathogenic infections.³⁷ Indeed, *Aspergillus* species is the most prominent fungus to infect the lungs.⁴² The ability of fungi to colonize and germinate in the respiratory mucosa makes them unique compared with other respiratory allergens (eg, house dust mite and grass and tree pollens) that do not grow within the host. Most fungal allergens are released during⁴³ and after¹⁶ spore germination, suggesting the importance of keeping the fungal lifecycle in consideration.

Inhalation of fungal spores, hyphae, fragments, or particles of secreted products is likely involved in the induction, exacerbation, or both of asthma and allergic diseases.⁴⁰ Up to 50,000 spores/m³ are detected at peak times of late summer and early fall¹⁶; theoretically, human subjects can be exposed to approximately 1 million spores per day. Fungal spores are highly variable in size, ranging from less than 2 µm to 250 µm in diameter. Spores larger than 10 µm, such as *Alternaria* species spores, can deposit in the upper airways (where the temperature is also less than 37°C), whereas *Aspergillus* and *Penicillium* species

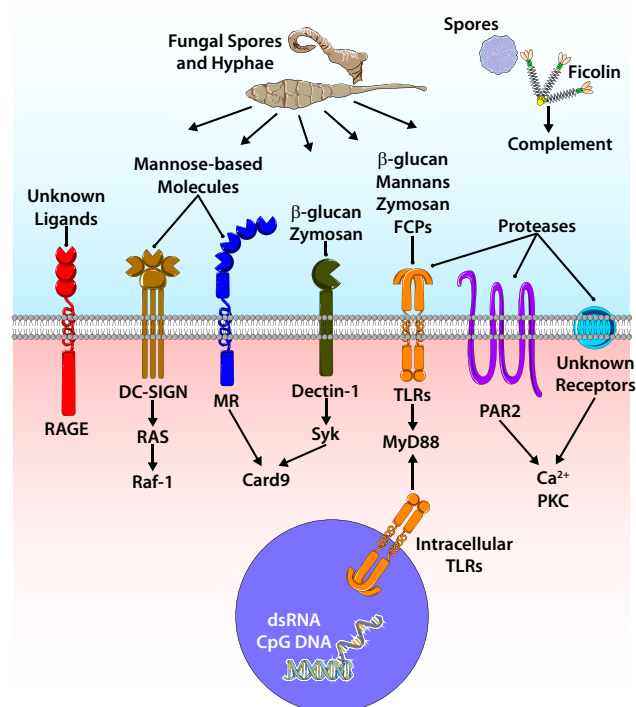


FIG 1. Fungal products and their receptors. Fungi and their products are recognized by several families of PRRs, including TLRs and CLRs, Dectin-1, and DC-specific ICAM3-grabbing non-integrin (*DC-SIGN*), and the MR. Fungi and spores can also be recognized by RAGE and ficolins, respectively. Fungus-derived proteases are recognized directly by PAR2 or indirectly through production of endogenous ligands, such as fibrinogen cleavage products (*FCPs*), which activate TLR4. *PKC*, Protein kinase C.

spores that are less than 10 µm can easily reach the lower airways.¹⁶ One should note that fungal fragments and submicron particles of fungal organisms can also serve as an aeroallergen source.⁴⁴ An extensive list of fungal allergens is available in a recent review article.³⁶

Fungal products

Biological molecules that are produced by fungi can be associated with their cell wall or secreted extracellularly. Major fungal products include *chitin*,⁴⁵ *β-glucan*,⁴⁶ proteases,⁴⁷ and *glycosidases*⁴⁸ (Fig 1). Chitin is intimately intertwined with β-glucans, *galactomannans*, and *mannoproteins* to form the structural foundation of the hyphal cell wall.³⁵ Chitin and β-glucans from *Aspergillus* species induce eosinophilic inflammation in mouse lungs.⁴⁹ Fungal proteases and glycosidases are presumed to degrade polymers and are used by fungi to capture nutrients from plant and mammalian hosts. The potential effect of these hydrolyases is likely significant because fungi contribute the majority of protease activity in house dust samples.⁵⁰ Certain fungal allergens are also proteases themselves, including Asp f 5, Asp f 6, and Asp f 11 from *Aspergillus* species.² Indeed, protease activities produced by fungi were sufficient^{18,51} and necessary⁵⁰ for induction of the robust allergic immune response induced by *Aspergillus* species in the airway.

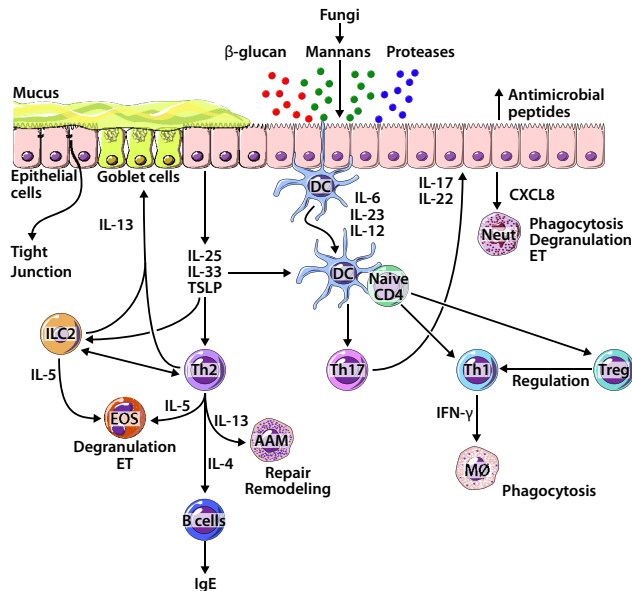


FIG 2. Innate and adaptive immune responses to fungi. Immune responses to fungi consist of several layers of adaptive and innate immunity. Epithelial tight junctions and mucus secreted by goblet cells deter and physically remove fungi. Fungal exposure to epithelial cells causes production of cytokines, such as IL-25, IL-33, and TSLP, which activate ILC2s and initiate T_H2 -type differentiation of naive $CD4^+$ T cells. DCs and their cytokines, such as IL-6, IL-23, and IL-12, also drive proliferation and differentiation of $CD4^+$ T cells. T_H1 cells activate macrophages through secretion of IFN- γ . T_H17 cells produce IL-17 and IL-22, which mediate CXCL8-dependent neutrophil recruitment and production of antimicrobial peptides by epithelial cells, respectively. ILC2s and T_H2 cells produce IL-5 and IL-13, which mediate eosinophilic inflammation, goblet cell hyperplasia, and airway remodeling. Effector cells, including macrophages, neutrophils, and eosinophils, use distinct strategies to provide antifungal immunity. AAM, Alternatively activated macrophages; EOS, eosinophils; ET, extracellular DNA trap; M ϕ , macrophages; Neut, neutrophils.

IMMUNE RECEPTORS THAT RECOGNIZE FUNGAL MOLECULES

In general, allergens can be recognized by the cellular immune system through 3 major mechanisms: (1) engagement of pattern recognition receptors (PRRs), (2) molecular mimicry of *Toll-like receptor* (TLR) signaling complex molecules, and (3) recognition of the allergen's biological activities, such as proteases.^{52,53} Recognition of fungi and fungal allergens likely occurs through the first and third mechanisms. Several families of PRRs have been shown to be involved in interactions with fungi.

TLRs

TLRs are a family of PRRs that respond to pathogen-associated molecular patterns arising from a variety of pathogens, including bacteria, viruses, and fungi. To date, 12 TLRs have been identified in human subjects.⁵⁴ TLRs can act as surface receptors (TLR1, **TLR2**, **TLR4**, TLR5, TLR6, and TLR10) or intracellular receptors (TLR3, TLR7, TLR8, and **TLR9**), whereas TLR4 might also be intracellular, and TLR3 and TLR9 might also be extracellular.⁵⁵ Fungal products that interact with TLRs include zymosan (ie, a glucan), O-linked mannans, phospholipomannans, unmethylated CpG-rich DNA, and double-stranded RNA.^{36,56} The **TLR1/TLR2 heterodimer** recognizes β -glucan from fungal conidia and hyphae.⁵⁷ TLR2 also interacts with phospholipomannans

from *Candida albicans* and unidentified ligands from *Aspergillus fumigatus*.⁵⁷ Activation of TLR4 by *C albicans* and *Cryptococcus neoformans* occurs through O-linked mannans.⁵⁷ Intracellular TLRs, such as TLR3 and TLR9, are activated by double-stranded RNA from conidia and unmethylated CpG from fungal DNA,⁵⁷ respectively. The immunologic outcomes of activation of these TLRs could be either proinflammatory or anti-inflammatory, depending on the TLRs or target cell types.⁵⁷ For example, activation of TLR4 in conjunction with Dectin-1 and the mannose receptor (MR) in dendritic cells (DCs) induces a proinflammatory T_H17 -type response.⁵⁸ In contrast, engagement of TLR2 elicits a combined T_H2 -type and regulatory T (Treg) cell response, resulting in persistent fungal infection but diminished immunopathology.⁵⁹

C-type lectin receptors

C-type lectin receptors (CLRs) are a class of receptors that generally recognize carbohydrates, lipids, and proteins.³⁶ CLRs that recognize fungal products include Dectin-1 and Dectin-2, dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN), and the MR.⁶⁰ Dectin-1 binds β -glucan, whereas Dectin-2, DC-SIGN, and MR recognize mannose-based molecules.⁶⁰ Activation of these receptors triggers proinflammatory immune responses, including phagocytosis and production of cytokines and lipid mediators.⁶⁰ For example, Dectin-1 activation by β -glucan induces production of **IL-12** and IL-4/IL-13 by macrophages⁶¹ and rat mast cells,⁶² respectively.

Other PRRs

The receptor for advanced glycation end-products (RAGE) is a transmembrane receptor that belongs to the immunoglobulin superfamily and recognizes pathogen-associated molecular patterns, as well as a variety of glycated molecules. It still needs to be determined whether fungal products are directly recognized by RAGE or whether RAGE interacts with TLRs to mediate immune responses to fungi.⁶³ Nonetheless, in mice exposed to *Alternaria* species, deletion of RAGE diminished production of **IL-33** and protected the animals from airway inflammation.⁶⁴ Furthermore, increased RAGE levels have been positively correlated with asthma.³⁶ Ficolins are soluble lectins that can bind fungal conidia and activate the lectin pathway of complement. Clearance of *A fumigatus* was inhibited in ficolin-deficient mice.⁶⁵

Recognition of proteases

As described above, fungi produce a large amount of proteolytic and glycolytic enzymes. Proinflammatory activities of these fungal products have been recognized for a number of years.⁶⁶ Porter et al⁵⁰ compared the extent of allergic airway inflammation in mice infected intranasally with wild-type or protease-deficient *Aspergillus niger* conidia. Although wild-type conidia induced robust T_H2 cytokine production, eosinophilia, and airway hyper-reactivity (AHR), these pathologic changes decreased with protease-deficient conidia. Because other components, such as amounts of β -glucan, were similar between these strains, their observations point to the biological significance of fungal proteases in triggering allergic airway inflammation.

Several potential mechanisms can be considered as to the effects of fungal proteases. First, fungal proteases can disrupt

airway epithelial tight junctions, leading to increased recognition of fungal proteins by immune cells resident within mucosal tissues.³⁷

Second, fungal proteases can be recognized directly by cell-surface receptors of innate immune cells. Protease-activated receptors (PARs) comprise a family of four 7-transmembrane G protein-coupled receptors.⁶⁷ Within the structure of a PAR are both ligands and ligand-binding domains. On protease cleavage, the ligand is freed to interact with the ligand-binding domain to initiate signaling that drives Ca^{2+} flux and transcription of genes for inflammatory mediators. Indeed, *Alternaria* species extract activates human eosinophils and airway epithelial cells through PAR2, resulting in extracellular release of granule proteins⁶⁸ and production and secretion of *thymic stromal lymphopoietin* (TSLP) and IL-33,^{19,69} respectively. Furthermore, activation of epithelial cell PAR2 by *Aspergillus* species proteases suppresses *CXCL10* production by airway epithelial cells,⁷⁰ suggesting immunoregulatory roles for epithelial PAR2. Nonetheless, there are some controversies as to whether the increase in intracellular calcium concentrations in airway epithelial cells exposed to *Alternaria* species proteases is dependent on⁷¹ or independent of⁷² PAR2.

Finally, fungal proteases promote immune responses indirectly by facilitating production of molecules within the host, which in turn activate immune cells. For example, intranasal administration of proteases derived from *Aspergillus oryzae* to mouse airways induced allergic airway inflammation through cleavage of the clotting factor fibrinogen and generation of fibrinogen cleavage products that activate TLR4.⁷³ Therefore further studies are necessary to identify key fungal proteases and glycosidases and elucidate the cellular and molecular mechanisms involved in their activation of immune cells.

INNATE IMMUNE RESPONSES

Exposure to microbes, such as bacteria and viruses, induces innate immune responses in the host followed by development of adaptive immune responses. Exposure to fungi and their products also provokes analogous immunologic pathways (Fig 2).

Epithelial barrier and epithelial cells

The first line of immune response against fungi is the epithelial barrier, which provides protection by excluding microbes and removing them physically.⁷⁴ Epithelial cells form tight junctions to prevent invasion of environmental pathogens into the host. Mucus provides a layer of protection, in which cilia and mucus work together to physically remove microbes.⁷⁵

Epithelial cells express PRRs and respond to microbes by producing antimicrobial peptides, cytokines, and other proinflammatory molecules.⁷⁶ In particular, a triad of cytokines that promote type 2 immune responses, specifically IL-25, IL-33, and TSLP, is produced and released by airway epithelial cells in response to various environmental factors and microbial stimuli.⁷⁷ They are also produced by airway epithelial cells in response to *A Alternata*, proteases, or both *in vitro*.^{69,78,79} Notably, IL-33, which was preformed and stored in nuclei, was released quickly into the airway lumen when animals were exposed to *Alternaria* species extract.^{19,22,78,80} Other common aeroallergens did not have such properties,^{19,78} suggesting a unique biological activity of the fungus *A Alternata*.

The molecular mechanisms that mediate IL-33 release are an active area of current research. IL-33 was described initially as a damage-associated molecular pattern released from damaged or necrotic cells.⁸¹ However, recent studies suggest that cells can respond actively to fungal exposure. For example, *Alternaria* species-induced IL-33 release from normal human bronchial epithelial cells involves rapid extracellular accumulation of ATP, followed by autocrine and paracrine activation of P2 purinergic receptors and increases in intracellular calcium concentration levels.⁷⁸ Administration of Ca^{2+} chelators, as well as pharmacologic or genetic inhibition of P2 purinergic receptors, inhibited *Alternaria*-induced IL-33 release.^{22,78} In addition, in airway epithelial cells exposed to *A Alternata*, oxidative stress pathways were activated and played a pivotal role in initiation of IL-33 release.^{22,82} The *unfolded protein response* can also play a role in epithelial response to *Alternaria* species because mice deficient in *ORMDL3* did not develop airway inflammation.⁸³ Interestingly, an agonist for *glucagon-like peptide 1* inhibited *Alternaria*-induced expression of *dual oxidase 1* and IL-33 release *in vivo* in mice.⁸⁰ Collectively, these findings suggest that oxidative stress, purinergic receptors, and intracellular calcium are likely to be key players in the signaling cascade involved in IL-33 release from airway epithelial cells and that it can be controlled by pharmacologic agents, such as glucagon-like peptide 1 agonists.

Group 2 innate lymphoid cells

In the early 2000s, group 2 innate lymphoid cells (ILC2s) were first described in mice infected with the parasite *Nippostrongylus brasiliensis* or those exposed to the fungus *Aspergillus* species as non-B/non-T cells that secrete IL-5 and IL-13 in response to IL-25.^{84,85} ILC2s were later isolated and characterized as a population of innate immune cells by several investigators.⁸⁶⁻⁸⁸

When naive mice were exposed to *A Alternata* extract, levels of $\text{T}_\text{H}2$ cytokines in the airway, including IL-5 and IL-13, increased as early as 6 hours after exposure.⁷⁸ Subsequent studies established that the IL-33-activated lung ILC2 population drives this rapid type 2 cytokine response.^{20,21} Intranasal administration of *A Alternata* also induced rapid production of cysteinyl leukotrienes that can activate ILC2s through cysteinyl leukotriene type 1 receptor, suggesting that an IL-33-independent pathway for fungus-induced ILC2 activation also exists.⁸⁹

Accumulating evidence points to a critical role for ILC2s in fungus-mediated immunopathology in the airway. For example, *A Alternata* exposure increased peribronchial infiltration of inflammatory cells and promoted epithelial hyperplasia, mimicking the observations in human patients with asthma.^{20,90} IL-7 receptor-deficient mice that lack ILC2s and adaptive immune cells (eg, T cells) were protected from these pathologic changes, even when they were exposed to *A Alternata*. Conversely, adoptive transfer of ILC2s to these mice restored lung pathology,^{20,90} suggesting that ILC2s are necessary and sufficient to mediate fungus-induced airway inflammation. *Alternaria*-induced activation of ILC2s further enhanced lung inflammation in mice that had been sensitized to an irrelevant allergen.⁹¹ Nevertheless, in mice exposed to *A Alternata*, ILC2s also produced amphiregulin, which can play a role in epithelial repair,²¹ suggesting that ILC2s can provide tissue protection during allergic immune responses to fungi. Further studies will be necessary to elucidate contributions

of ILC2s to allergic airway inflammation and pathologic changes in animal models and human subjects.

DCs

DCs take up antigen at local tissue sites, undergo maturation through upregulation of costimulatory molecules, and migrate to the lymph nodes, where they promote proliferation and differentiation of naive T cells. These DCs likely play a pivotal role in antifungal immunity. For example, pulmonary infections of *C neoformans* and *A fumigatus* induce predominantly T_H1-type responses that are orchestrated by monocyte-derived DCs.^{92,93} In mice depleted of these monocyte-derived DCs, T_H2 or T_H17 responses were generated, suggesting a key role for DCs in controlling T-cell responses.

DCs recognize fungal mannans directly through Dectin-2, DC-SIGN, and MR, triggering production of proinflammatory and immunoregulatory cytokines, including IL-1, IL-2, IL-6, IL-10, and IL-23.⁹⁴ Importantly, DCs distinguish between conidia and hyphae of fungi. For example, *C albicans* conidia induce IL-12 production by DCs, resulting in a T_H1-type immune response, whereas hyphae induce IL-4 and IL-10, leading to a T_H2-type response.⁹⁵ Thus the lifecycle of fungi likely affects the characteristics of the DC response, resulting in differential immunologic outcomes.

In allergic immune responses fungi can serve as an adjuvant to promote immune responses to fungal components, as well as to bystander antigens, and DCs likely play a key role in recognition of the adjuvant activities of fungi. For example, airway exposure to innocuous allergens, such as ovalbumin, is generally tolerogenic.⁹⁶ When these antigens were coadministered into the airway with fungal products, a strong T_H2 response developed.⁹⁷ Bone marrow–derived DCs cultured with *A alternata* extract up-regulated expression of costimulatory molecules and increased production of IL-6 but not IL-12; when transferred to naive mice, these *Alternaria*–stimulated DCs promoted development of T_H2-type CD4⁺ T cells and IgE antibody production.⁹⁷ In addition, in a mouse model of protease-induced allergic immune responses, DCs were activated by ILC2-derived IL-13 to mediate T_H2-type memory T cells.⁹⁸ Altogether, DCs can be activated directly by fungal antigens or collaborate with other innate immune cells, such as ILC2s, to mediate innate immune responses to fungi and regulate adaptive immunity.

Neutrophils and eosinophils

Neutrophils and eosinophils are considered effector immune cells that mediate antifungal immunity. These granulocytes likely accomplish the task through both distinct and overlapping mechanisms. Neutrophils use several mechanisms to control fungi, including production and/or extracellular release of granule proteins, antimicrobial peptides, and **reactive oxygen species**, as well as formation of neutrophil extracellular DNA traps.⁹⁹ The smaller conidia are generally phagocytized by neutrophils, whereas larger hyphae trigger neutrophil extracellular DNA traps.¹⁰⁰ IgG opsonization of fungi can further enhance neutrophil phagocytosis and promote antibody-directed cell toxicity.¹⁰¹

Eosinophils are not professional phagocytes; therefore they respond to fungi in different ways than neutrophils. For example, human eosinophils released granule proteins extracellularly in

response to *A Alternata* conidia and hyphae *in vitro*; the process was dependent on eosinophil adhesion to fungi through eosinophil CD11b and fungal β -glucans.¹⁰² Human eosinophils also responded to *A Alternata* proteases through PAR2, resulting in extracellular release of granule contents.^{68,103} Furthermore, murine bone marrow–derived eosinophils killed *A fumigatus* through a contact-independent mechanism *in vitro*,¹⁰⁴ and eosinophils released their extracellular DNA traps, targeting fungal hyphae.¹⁰⁵ Eosinophil extracellular DNA traps might contribute to the formation of the viscous and sticky mucus seen in patients with allergic bronchopulmonary aspergillosis and other related disorders.¹⁰⁵ Further information will be necessary to better understand the roles of neutrophils and eosinophils in antifungal immunity in human subjects, as well as the pathologic outcomes of patients with fungus-associated diseases.

ADAPTIVE IMMUNE RESPONSES TO FUNGI

Adaptive immune cells, such as T cells and B cells, express genetically rearranged surface receptors that are exquisitely specific for a particular antigen. Innate and adaptive immune responses are inextricably intertwined, and a successful adaptive immune response is a coordinated effort requiring activation of tissue-resident cells, antigen-presenting cells, and antigen-specific T and B cells.

T_H1/T_H17 cell response

Successful clearance of fungal organisms by the host and recovery of tissue homeostasis involves a balance of T_H1- and T_H17-type responses and Treg cells. For example, activation of CLR_s *in vivo* leads to development of T_H1- and T_H17-type CD4⁺ T cells.⁶⁰ The T_H1 response provides protective immunity against fungi through enhancing the functions of phagocytic cells through production of *IFN- γ* and promotion of B-cell production of opsonizing antifungal antibodies.¹⁰⁶ T_H17 responses are also critical because they activate tissue cells, such as epithelial cells and fibroblasts, resulting in production of chemokines that recruit phagocytes to the site of immune response.¹⁰¹ In patients with primary aspergillosis, *Aspergillus*–specific T cells from the lungs displayed a T_H17-type phenotype, whereas circulating specific T cells showed a T_H1-type profile.¹⁰⁷ Furthermore, airway exposure to fungal β -glucans enhanced allergen-driven T_H2-type immune responses, resulting in a mixed T_H17 and T_H2 response that is resistant to glucocorticoid treatment.¹⁰⁸

Protective T_H1- and T_H17-type antifungal immunity is both promoted and downregulated by innate immunity. For example, commensal gut microbiota influence CD4⁺ T-cell polarization to fungal infection. In mice infected with *Aspergillus* species, depletion of gut microbiota by the antibiotic vancomycin suppressed T_H17 cell development.¹⁰⁹ In pulmonary *Paracoccidioides brasiliensis* infection, mice deficient in TLR4 had more Treg cells and showed diminished T-cell activation and impaired clearance of fungal organisms.¹¹⁰ In addition, as described above, the same fungi might produce different types of adaptive immunity depending on the context. For example, in *Aspergillus* species infection, factors from dormant conidia promoted T_H1-type response, whereas those from hyphae or swollen (ie, germinating) spores favored T_H2-type immunity.⁹⁵ Such differential T-cell responses to fungi are likely regulated by innate immunity because in macrophages *Aspergillus* species conidia mediated production

of proinflammatory cytokines through TLR4 activation, whereas hyphae mediated IL-10 production through TLR2.¹¹¹ Furthermore, in the absence of TLR2, mice infected with *P brasiliensis* showed decreases in numbers of Treg cells and enhanced numbers of T_H17 cells, resulting in efficient resolution of infection but increased pathologic outcomes.⁵⁹ Thus T_H1- and T_H17-type immune responses to fungi are generally protective, but they need to be fine-tuned to provide effective immunity while minimizing damage to the host.

T_H2 cell response

T_H2-type immunity to fungi is generally characterized by an inability to clear fungal pathogens accompanied by detrimental allergic inflammation, including tissue eosinophilia, goblet cell hyperplasia, and airway remodeling.¹¹² Furthermore, unlike classical macrophage activation induced by T_H1 cell-derived cytokines, IL-4 and IL-13 induce an alternate form of macrophage activation (alternatively activated macrophages) that is less efficient at controlling fungal growth.¹⁰¹

Major progress has been made during the past several years to understand the mechanisms involved in the T_H2-type response to fungi. Generally, epithelial cells are triggered by exposure to allergens to release cytokines, such as IL-25, IL-33, *GM-CSF*, and TSLP,¹¹³ which promote development, differentiation, or both of T_H2-type CD4⁺ T cells. These cytokines might not be required for priming of naive CD4⁺ T cells in draining lymph nodes but are necessary for effector functions of primed T_H2-type CD4⁺ T cells at the site of inflammation.¹¹⁴ Additional factors, such as prostaglandins, can also be involved in regulation of type 2 immunity to fungi because PGE₂-deficient mice showed reduced levels of IL-33, as well as diminished eosinophilic inflammation in mice exposed to *A Alternata*.²⁴ Furthermore, IL-33 impaired clearance of *Aspergillus* species by suppressing PGE₂ expression, resulting in diminished IL-17A and IL-22 production.¹¹⁵

Several mouse models exposed naive animals to fungal extracts, sometimes in combination with other allergens, to mimic recurrent and chronic exposure to fungi in human subjects. For example, airway exposure of mice to combined extracts of house dust mite, ragweed, and *Aspergillus* species broke tolerance and established chronic features of asthma, including eosinophilic inflammation, mast cell and smooth muscle hyperplasia, mucous production, and airway remodeling.¹¹⁶ Interestingly, the pathologic changes persisted for several weeks, even after cessation of allergen exposure, and antibodies to IL-5 or IL-13 showed minimal effects, suggesting the presence of a pathway independent of T_H2 cells.

In another study chronic and multiple exposures of naive mice to *A Alternata*, *A fumigatus*, and house dust mite extracts induced a robust increase in airway eosinophils, tissue remodeling, and increased plasma concentrations of allergen-specific IgE and IgG₁ antibodies that also lasted for a prolonged period.¹¹⁷ Notably, a marked increase in lung levels of IL-33 was observed, and the pathologic changes were nearly abolished in mice deficient in the IL-33 receptor ST2. Consistent with this, IL-33 that is produced in mice exposed to *A Alternata* dysregulated tolerogenic Treg cells and drove them to a proinflammatory phenotype.¹¹⁸ Thus animals that are exposed to fungal products for a prolonged period appear to provide robust models that mimic human asthma. Although the IL-33 pathway might play a major role

in the model, further studies are necessary to elucidate the mechanisms.

As described above, ILC2s provide rapid and innate immune responses to fungi. Notably, recent findings suggest that ILC2s in conjunction with CD4⁺ T cells or ILC2s alone contributed to persistent immune responses to fungi. For example, in mice exposed to fungal antigens for a prolonged period (eg, 4 weeks), ILC2 numbers increased by greater than 2-fold, and both the proportion and absolute number of IL-5- or IL-13-producing ILC2s and CD4⁺ T cells increased by approximately 4-fold, suggesting both ILC2s and T_H2-type CD4⁺ T cells can provide antifungal immunity.¹¹⁷ Furthermore, intranasal administration of protease stimulated both ILC2s and T_H2 cells, causing allergic airway inflammation and increased levels of IgE antibodies^{98,119}; this process was severely impaired in ILC2-deficient mice. Moreover, ILC2s that are responsive to TSLP contributed to airway inflammation and AHR in mice that had been exposed to multiple allergens, including *Aspergillus* species.¹²⁰ In a similar model IL-33-responsive ILC2s played a role in the persistence of airway remodeling and AHR.¹²¹ Importantly, epithelial IL-33 activated ILC2s to produce IL-13, which in turn promoted production of IL-33 and ST2 expression by airway epithelial cells.¹²¹ Thus, during a chronic phase of fungus-induced airway inflammation, tripartite interactions among airway epithelial cells, T_H2 cells, and ILC2s can form a positive feedback loop, resulting in continued and persistent immunopathology.

CLINICAL IMPLICATIONS

Detailed information on the clinical aspects of fungus-associated airway diseases is provided in recent excellent review articles.^{16,37} Here we will highlight some of the wide-ranging implications of fungi in airway diseases, such as asthma and chronic rhinosinusitis (CRS). As discussed in the introduction, epidemiologic studies suggest that asthma is induced or exacerbated by exposure to fungi and/or by increased immune reactivity to fungal organisms. Fungi likely contribute to asthma through 2 distinct mechanisms. In the case of thermotolerant fungi, such as *Aspergillus* species, fungal colonization might provide an ongoing source of fungal allergens, leading to a persistent immune response, airway inflammation, and tissue damage. Alternatively, temperature-sensitive fungi, such as *Alternaria* species, can cause disease exacerbations that are tied closely to increased exposure to fungal spores or fragments and to fungal products (eg, proteases).

IgE antibodies to fungi are associated with increased asthma severity; patients with severe asthma are often sensitized to multiple fungal species.¹⁶ Furthermore, greater lung colonization with *A fumigatus* is observed in asthmatic patients than in healthy subjects.¹²² The combination of severe asthma and fungal sensitivity, as documented by skin prick tests (SPTs) or serum IgE measurements, is now recognized as a phenotype known as severe asthma with fungal sensitization (SAFS).^{2,123} Patients with SAFS experience more bronchiectasis and fixed airflow obstruction than patients with other types of asthma of comparable severity, suggesting more advanced disease pathology.¹⁶

In other phenotypes of asthma, fungi colonize the airway. For example, in allergic bronchopulmonary mycosis (ABPM), fungal colonization of the asthmatic airway leads to IgE sensitization to fungi, recurrent and transient radiographic infiltrates, peripheral

and pulmonary eosinophilia, and bronchiectasis.¹²⁴ Initially called allergic bronchopulmonary aspergillosis because of increased serum levels of *Aspergillus* species-specific IgE and IgG, this syndrome was subsequently named ABPM when other fungi were found to be involved. Although the cause of ABPM is not fully understood, poor airway clearance, airway remodeling, and mucus overproduction might allow the spores of thermotolerant fungi, such as *A fumigatus*, *Aspergillus flavus*, *A niger*, and *Bipolaris* species, to remain in the airways long enough to germinate, produce hyphae, and release their biological products (eg, proteases).³⁷

In the upper airway CRS can be comorbid with eosinophilic mucin (thick mucus with degranulating eosinophils and fungal hyphae) in the sinus cavity and IgE antibodies to fungi,¹²⁵ a condition known as allergic fungal rhinosinusitis (AFRS). AFRS occurs in approximately 5% to 10% of patients with CRS⁴²; approximately 50% of patients with AFRS also have asthma.¹⁶ AFRS is associated with various fungi, including *Bipolaris*, *Curvularia*, *Cladosporium*, *Alternaria*, and *Aspergillus* species,¹²⁵ and is more severe than other forms of CRS.³⁷ Thus both upper and lower airway subtypes of asthma and CRS clearly involve increased immune sensitivity to fungi or colonization of the respiratory mucosa with fungal organisms. However, a fundamental question still remaining is the proportion of patients with asthma and CRS whose diseases are affected by fungi themselves or by immune responses to them.

This question is complicated by difficulties in establishing diagnostic criteria that detect involvement of fungi, both in terms of pathophysiology and diagnostic tools. For example, SAFS can include both subjects whose sensitization to fungi does not contribute to the pathophysiology of their diseases and subjects with sensitization to fungi that colonize the lungs and cause damage to their airways. Fungal sensitization can occur even in subjects without allergic symptoms or evidence of airway inflammation.³⁷ Furthermore, detection of fungal organisms in biological specimens (eg, sputum) is complicated by species-associated variations in optimal culture conditions, poor recovery of organisms or spores, and possible contamination from environmental fungi. Assessment of fungal sensitivity is not straightforward either, with SPTs and serum IgE tests often producing conflicting results.³⁷ Fungal extracts are not standardized and are highly variable because of strain differences, batch-to-batch variations, diverse source materials (eg, spore, hyphae, and secreted proteins), and enzymatic degradation.¹²⁶

Progress has been made in addressing these current shortcomings. For example, fungi were detected in more than 60% of patients with CRS with nasal polyps when sterile sinus lavage fluid was collected during endoscopic surgery, even in those not given a diagnosis of AFRS.¹²⁷ Fungi were also detected in approximately 20% of control subjects. Measurement of an *in vitro* cellular immune response to fungi by using an IL-4 production assay identified sensitivity to fungi in up to 60% of patients with CRS with nasal polyps,¹²⁷ suggesting that the prevalence of both fungal organisms and fungal sensitivity in patients with T_H2-type chronic airway diseases might be greater than previously anticipated. These data indicate the need for further refinement of tools for evaluating the involvement of fungi in patients with allergy and chronic airway diseases, as well as studies to elucidate the immunologic mechanisms by which fungi promote or modulate immunopathology.

CONCLUSIONS

Fungi, like helminth parasites, are complex organisms and produce a wide variety of molecules depending on their life cycles. Mammalian immune responses to fungi are also complex, as demonstrated by protective T_H1/T_H17-type responses to cell-wall β -glucan and pathologic T_H2-type responses to secreted proteases. Furthermore, fungi provide not only allergens but also potent “adjuvants,” and they promote or modulate immune reactions to bystander allergens. However, we recognize several major gaps in our current knowledge.

First, our knowledge regarding fungal products and their “receptor(s)” in immune cells is still limited. For example, although fungal proteases likely play a key role in mediating type 2 immunity, we still do not know which specific proteases from each fungal species are involved. Furthermore, although certain fungal products, such as chitin, have been used successfully to dissect the mechanisms of type 2 immunity,¹²⁸ our understanding of how they are recognized by “receptor(s)” in the mammalian immune system is limited. Questions of whether and how other fungal biological molecules (eg, glycosidases) contribute to immune responses remain unanswered.

Second, as described in Fig 2, a variety of cell types and their products, in particular cytokines, are involved in the host's immune responses to fungi. Although DCs and conventional CD4⁺ T cells have been studied extensively, recent studies suggest potential roles for tissue cells (eg, airway epithelial cells) and innate lymphoid cells. The hierarchy and importance of these cell types needs to be elucidated. Is there a specific cell type that plays a “central” role in regulating antifungal immunity? Alternatively, do mammalian immune cells form a network in which several cell types contribute together? How do these cell types or networks adjust to the changing life stages and products of fungal organisms?

Third, clinically, the diagnosis and treatment of fungal sensitivity has been a challenge. Fungal sensitization often occurs without IgE-mediated type I hypersensitivity responses, and results are inconsistent between SPTs and serum IgE measurements.³⁷ As discussed earlier, fungal extracts are highly variable.³⁹ New diagnostic approaches, such as those monitoring the CD4⁺ T-cell response to fungi, might improve detection.¹²⁷ Furthermore, better treatment options for patients with hypersensitivities to fungi are needed. Avoidance is impractical because of the ubiquitous nature of these organisms.

The efficacy of antifungal immunotherapy has been controversial.¹²⁹ Would conventional glucocorticoids or antifungal agents work? In this regard it is imperative to develop better diagnostic tools to identify specific cohorts of patients with fungal involvement and to develop safe and efficacious treatment for them. Newer agents with a safer pharmacologic profile and novel therapeutic routes and regimens might also need to be considered.

Finally, as described in recent studies,³⁰ fungi are likely a part of the mammalian microbiome. A major question still remains regarding the overall significance of fungal exposure and colonization to human disease and health at the population level. Because asthma is associated with polymorphisms in certain immune molecules,¹³⁰ it will be important to identify genetic variants that are associated with increased infection/colonization by fungal organisms or increased immune reactivity to them. Furthermore, because global climate change is imminent, it is imperative to expand our body of knowledge regarding how fungi

interact with our immune systems and how immune responses will mediate and modulate disease processes in various organs, such as the respiratory, gastrointestinal, and cutaneous immune systems. Future efforts that involve collaborations among clinicians, immunologists, and fungal experts will be necessary to address these questions.

What do we know?

- Fungal allergen exposure is closely linked to asthma development, and fungal sensitivity is associated with more severe asthma.
- Fungi are unique among airway allergens because they can cause both acute and chronic responses in the airway through inhalation and colonization.
- Fungi express pathogen-associated molecular patterns and secrete proteases, glycosidases, and other enzymes; these molecules can promote type 2 immune responses in animals and human subjects.
- Various PRRs expressed by immune cells are activated by fungal products.
- Immune responses to fungi are mediated by a network of innate and adaptive immune cells, including but not limited to airway epithelial cells, ILC2s, DCs, and CD4⁺ T cells.

What is still unknown?

- Which specific fungal components and secreted products are potent triggers of immune responses?
- Which specific cell types and which specific cellular receptors are involved in responding to the fungal products?
- How can we identify patients who are immunologically sensitive to fungi and their products?
- How can we treat patients with increased fungal sensitivity safely and effectively?
- Which genetic signatures are associated with fungus-mediated diseases?
- What role do fungi play in the human microbiome and in regulation of the immune system?

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