

Aspergillus fumigatus: Principles of Pathogenesis and Host Defense[∇]

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Aspergillus fumigatus is a ubiquitous saprophytic mold (67) that forms airborne spores (conidia). Humans inhale, on average, hundreds of these infectious propagules daily. In immune competent hosts, these encounters are of no further significance—conidia are killed and cleared by cells of the pulmonary immune system. However, disease occurs when the host response is either too strong or too weak. Thus, understanding how the host interacts with the organism to define this balance is a critical goal, the successful pursuit of which requires recognition of the dynamic nature of both fungal and host molecular participants.

In immunocompromised hosts, *A. fumigatus* represents a major cause of morbidity and mortality. This patient population is expanding due to the increasing use of transplantation for end organ disease, the development of immunosuppressive and myeloablative therapies for autoimmune and neoplastic disease, and the human immunodeficiency virus/AIDS pandemic (38). *A. fumigatus* is the most common invasive mold infection in these patients, and mortality rates exceed 50% in high-risk groups, such as leukemic patients and hematopoietic stem cell transplant recipients (74). Sensitivity to *A. fumigatus* antigens is associated with asthma, the prevalence of which is increasing in the developed world, though proving causation has been difficult (49, 54). Regardless, this increased prevalence brings a parallel rise in the number of individuals predisposed to allergic bronchopulmonary aspergillosis, a disease associated with aberrant responses to *Aspergillus* antigens in the setting of chronic inflammation. The spectrum of invasive, semi-invasive, and allergic disease caused by *A. fumigatus* is reviewed in several outstanding articles (9, 94).

The study of *A. fumigatus* molecules involved in virulence has been hampered by the lack of an identifiable sexual cycle, limiting classical genetic analysis (21). A recent study, however, indicates that *A. fumigatus* encodes distinct mating-type loci and the pheromone machinery required for sexual mating (103). Nonetheless, within the past decade, researchers have developed and refined experimental tools to generate mutant strains by homologous recombination (21, 35, 62, 152, 154), utilized RNA interference to repress endogenous transcripts (95), and expressed heterologous genes in *A. fumigatus* under the control of drug-inducible regulatory elements (145). The completion of the *A. fumigatus* genome (98) has accelerated gene structure and function studies and made possible comparative genomic analyses with other sequenced *Aspergillus*

species (*Aspergillus oryzae* and *Aspergillus nidulans*), as well as other genera of pathogenic (e.g., *Candida albicans* and *Cryptococcus neoformans*) and nonpathogenic (e.g., *Saccharomyces cerevisiae*) fungi. An important insight from the genomes has been that *A. fumigatus* does not share a common set of genes with other fungal pathogens (98).

The types of hosts that are susceptible to invasive aspergillosis and the lack of unique pathways conserved among pathogenic fungi underscore the importance of the host contribution to pathogenesis. Damage from *A. fumigatus* can result from fungal growth and tissue invasion or from inflammatory cells recruited to sites of infection (130). Included in the latter are responses that are ineffective in clearing the organism, occur in the process of immune reconstitution, or are associated with allergy. For example, in a murine model of chronic granulomatous disease, in which mice have defective phagocyte oxidase systems, administration of killed hyphae results in chronic inflammation due to persistence of fungal elements (92). From the perspective of the mammalian immune system, *A. fumigatus* represents an organism with continuous respiratory tract exposure that must be cleared from terminal airways with an immune response calibrated to avoid fungal tissue invasion, as well as inflammation-induced tissue damage. Here, we review our growing understanding of the interface between *A. fumigatus* and host defense mechanisms, with an emphasis on invasive disease in humans and small animal models.

A. FUMIGATUS IN THE MAMMALIAN ENVIRONMENT

Less than 20 of the ~200 *Aspergillus* species cause human disease, and among these, *A. fumigatus* is the predominant isolate, a finding that remains incompletely understood. The frequency with which *A. fumigatus* causes disease has been ascribed to external environmental prevalence or enhanced virulence (122, 137). Supporting the former are epidemiologic studies showing that *A. fumigatus* predominates over other genera of filamentous fungi and/or *Aspergillus* species in certain environments (5, 69) and those showing a high degree of genetic diversity among *A. fumigatus* isolates, without clustering between clinical and environmental isolates (36). Others, however, have not found this species to predominate (100). In a hospital survey, *A. fumigatus* represented <1% of airborne mold spores yet accounted for nearly 50% of patient isolates (122). In contrast, *A. niger* constituted more than half of the airborne isolates but only 17% of patient isolates, suggesting that *A. fumigatus*, compared to other aspergilli, is suited to colonize and persist within the human respiratory tree.

To survive within human hosts, *A. fumigatus* can bypass mucociliary clearance by virtue of its small airborne spores (2 to 3 μm in diameter), exhibits thermotolerant growth, and

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relies on biosynthetic pathways evolved to counter hostile environments within its ecological niche. *A. fumigatus* virulence is a polygenetic trait; single-gene virulence factors, such as *C. neoformans* phospholipase B (32), have not been identified. Polygenetic factors that influence the conidial size, rate of germination, and conidial resistance to host killing mechanisms likely shape the outcome of infection in permissive hosts. Once conidia have germinated, factors that influence hyphal growth rates and resistance to killing mechanisms, tissue invasion and dissemination, and production of secondary metabolites have the potential to influence pathogenesis. However, these factors are secondary to the immune status of the host.

Thermotolerance. Thermotolerance facilitates *A. fumigatus* growth over less thermotolerant aspergilli not only within decaying organic matter, its ecological niche, but also within the mammalian respiratory tree. The molecular basis of thermotolerance remains unknown. Deletion of the *cgrA* gene, which encodes a nucleolar protein involved in ribosome biogenesis, impairs growth in vitro at 37°C but not at 22°C (16). A $\Delta cgrA$ strain is hypovirulent in a murine model of invasive aspergillosis but not in a fruit fly model of fungal disease at ambient temperature. Deletion of the *thtA* gene impairs growth at 48°C but does not affect virulence, an outcome that is not surprising, given that growth is maintained at 42°C (30). Both *cgrA* and *thtA*, whose function is unknown, have homologs in nonpathogenic and nonthermophilic fungi. To date, DNA microarray analyses have failed to identify a conserved set of genes that confer thermotolerance or facilitate fungal growth at different temperatures (98).

The conidial surface. The outer conidial surface contains protrusions, termed rodlets, that impart hydrophobic properties important in conidial dispersal. $\Delta rodA$ conidia lack rodlets and display enhanced sensitivity to alveolar-macrophage killing. However, deletion of the *rodA* gene does not impact virulence in a murine pulmonary-infection model (104, 138). Thus, in a complex system, lack of rodlets does not translate into a difference in pathogenicity compared to wild-type controls or reconstituted mutants. The molecular features of the conidial surface are represented in Fig. 1, together with significant interactions with mammalian factors that impact fungal growth and survival.

Surface-exposed sialic acid residues may be important for conidial dispersal and pulmonary deposition, as sialidase treatment results in conidial agglutination (149, 150). Sialic acid residues partly mediate conidial binding to fibronectin, the concentration of which is increased by lung damage (150). This interaction may be important for the establishment of aspergillosis or invasive disease. Higher density of sialic acid residues on *A. fumigatus* conidia might play a role in the predominance of human disease associated with *A. fumigatus* (149, 151), though limited density testing of other aspergilli has been performed. Removal of sialic acid residues diminishes conidial phagocytosis, but not surface binding, by cultured macrophages and type II pneumocytes (149). Thus, sialylation may either enhance immune function or provide a protected reservoir for conidia.

The cell wall. The cell wall consists predominately of a polysaccharide matrix comprised of α -(1,3) glucan; β -(1,3) glucan, some of which contains β -(1,6) branches; linear β -(1,3), β -(1,4) glucan; chitin; and galactomannan (68). All three predicted

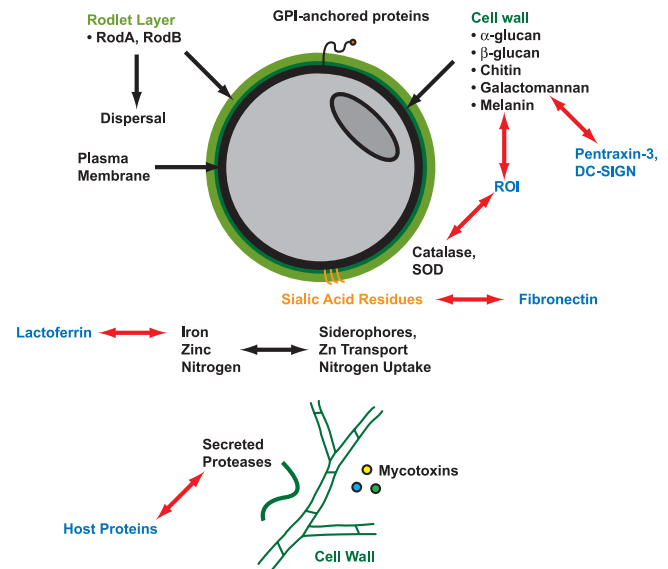


FIG. 1. Molecular features of *A. fumigatus* conidia and hyphae. Schematic representation of resting conidia (top) and hyphae (bottom). The organization of the conidial cell wall is depicted, together with specific conidial and hyphal cell wall and secreted components. Host receptors and products that interact with fungal molecules are listed in blue.

α -glucan synthase genes (*ags1*, *ags2*, and *ags3*) have been deleted. While the $\Delta ags1$ mutant displays the most profound α -glucan synthetic deficit (10), the $\Delta ags3$ strain causes enhanced virulence in a murine model of invasive aspergillosis (80). $\Delta ags3$ conidia and hyphae contain α -glucan levels similar to those of wild-type cells, suggesting that the mutant phenotype is due to other effects of *ags3* disruption; these include increased conidial melanin content, rapid germination, and relative conidial resistance to reactive oxygen species (80). The $\Delta ags3$ mutant and two other hypervirulent mutants characterized to date exhibit one or more of these conidial phenotypes (Fig. 2).

The conidial cell wall also contains at least nine glycosylphosphatidylinositol (GPI)-linked proteins connected to the polysaccharide skeleton (25). Disruption of the *Afpg-a* gene, which encodes a homolog of the catalytic domain that carries out the first step of GPI anchor biosynthesis in *S. cerevisiae*, results in the absence of GPI-linked proteins in *A. fumigatus* (71). Unlike *S. cerevisiae* GPI3, *Afpg-a* is nonessential but is required for normal hyphal growth, as disruptants undergo cell lysis more readily and have a thickened cell wall with increased mannoprotein and β -glucan content. Despite more rapid conidial germination, the $\Delta Afpg-a$ strain is hypovirulent in a neutropenic mouse model of pulmonary aspergillosis, a phenotype that may reflect slower growth. However, the absence of melanin and inability to traffic GPI-linked proteins to the plasma membrane and cell wall may contribute, as well (71).

In contrast, disruption of the GPI-linked protein Ecm33p enhances virulence in cyclophosphamide-treated mice (119). This finding may depend on delayed host clearance of $\Delta ecm33$ conidia (29). Though $\Delta ecm33$ conidia germinate more rapidly than wild-type conidia (119), which could contribute to hypervirulence, this phenotype is also present in the hypovirulent

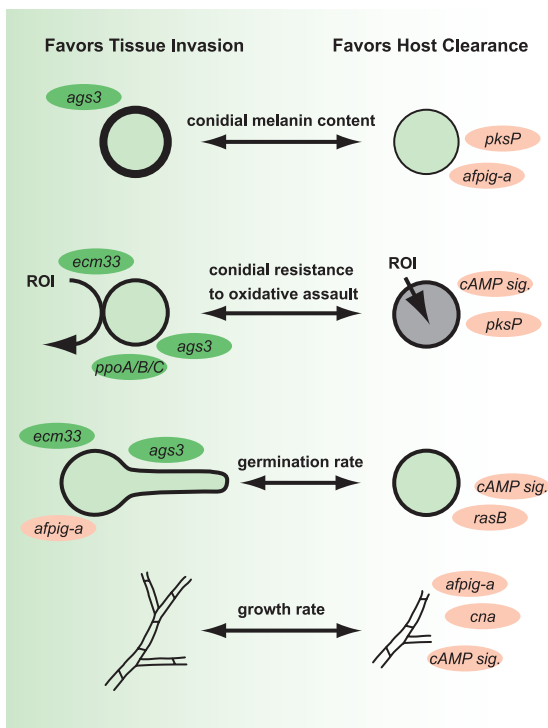


FIG. 2. Classification of *A. fumigatus* mutants based on phenotypic characteristics that favor fungal growth or clearance in immunocompromised hosts. *A. fumigatus* hypervirulent (green ovals) and hypovirulent (pink ovals) mutants show phenotypic differences in conidial melanin content, resistance to oxidative assault, germination rates, and hyphal growth. Differences in these phenotypic characteristics, either individually or in aggregate, likely contribute to either fungal tissue invasion or clearance in immunocompromised hosts.

GPI anchor-deficient strain, as noted above. The $\Delta ecm33$ strain gives rise to large, chitin-rich conidia that form linear chains with a separation defect (29, 119). Although $\Delta ecm33$ conidia are more resistant to killing by alveolar macrophages and neutrophils than control cells, $\Delta ecm33$ hyphae are more susceptible to neutrophil-dependent killing.

These examples demonstrate that fungal cell wall composition profoundly influences virulence in immunocompromised mice. While specific genetic deletions can yield hypervirulent *Aspergillus* strains that germinate and grow more rapidly in vivo, disruption of key biosynthetic pathways more commonly results in growth-delayed phenotypes and strains with reduced virulence in animal models of invasive aspergillosis (84, 85, 96). In addition, fungal cell wall composition has the potential to affect the host immune response, a finding that is discussed below.

Pigment biosynthesis. Many fungi synthesize melanin pigments that provide a protective barrier against UV radiation and serve to maintain the genomic integrity of long-lived cells, such as spores. In the context of infection, melanins counter host responses by diminishing fungal cell phagocytosis and intracellular trafficking to acidified compartments, as well as by increasing fungal resistance to reactive oxygen species and to cell lysis (22).

A. fumigatus conidia appear gray-green due to accumulation of 1,8-dihydroxynaphthalene-melanin (22, 156) and the loss of

polyketide synthase activity involved in pigment biosynthesis (PksP) yields strains with smooth, white conidia (65, 140, 141). In vitro, $\Delta pksP$ conidia induce neutrophils to release greater amounts of reactive oxygen species than wild-type conidia (65) and undergo phagocytosis (140) and trafficking to phagolysosomes more readily (56). In a murine model of invasive aspergillosis, the $\Delta pksP$ strain displayed reduced virulence compared to a wild-type strain (55, 140). Although *pksP* expression is triggered in conidia and hyphae recovered from infected mice (66), melanization of hyphae growing in vivo has not been demonstrated (156). Interestingly, loss of *pksP* function alters the conidial cell surface, resulting in enhanced exposure of β -(1,3) glucan, a polysaccharide that forms a major target of the mammalian innate immune system (discussed below) (76). Thus, *pksP*-deficient cells are likely to encounter more robust inflammatory responses that may account for the decrease in virulence observed with the mutant strain.

Resistance to oxidative stress. *A. fumigatus* detoxifies oxidative threats via glutathione synthesis and oxidoreductase activity, pathways conserved among pathogenic and nonpathogenic fungi (137). The protein products of four catalase and four superoxide dismutase genes catalyze the breakdown of H_2O_2 and superoxide radicals, respectively. Catalase deletion mutants (*catA*, *cat1*, and *cat2*) are more sensitive to H_2O_2 , but not to phagocyte killing in vitro (105). The $\Delta catA$ and $\Delta cat1$ mutants are as virulent as wild-type strains in immunocompromised mice (26), while the $\Delta cat1 \Delta cat2$ strain displays diminished histopathological lesions, suggesting that H_2O_2 may not be the predominant reactive oxygen species responsible for fungal killing.

Enhanced resistance to reactive oxygen species was observed in a strain in which three fatty acid oxygenase genes (*ppoA*, *ppoB*, and *ppoC*) are transcriptionally repressed by RNA interference (142). The triple-*ppo*-silenced strain was hypervirulent in a murine model of invasive aspergillosis. The mechanism underlying its phenotype remains unclear but does not appear to involve changes in the synthesis of prostaglandins or gliotoxin, a mycotoxin.

Gliotoxin. Gliotoxin is readily detected in human aspergillosis (70) and exerts immunosuppressive properties on host leukocytes by blocking phagocytosis and transcription of inflammatory mediators (99) and inducing apoptosis of neutrophils and monocytes (126, 147). In vitro, gliotoxin exhibits ciliostatic properties on respiratory epithelial cells (1). Strains of *A. fumigatus* that do not produce gliotoxin appear less virulent than gliotoxin-producing strains (132). Thus, gliotoxin has long been suspected to contribute to the pathogenesis of invasive aspergillosis.

Three independent groups have disrupted a nonribosomal peptide synthetase encoded by *gliP* within the putative gliotoxin biosynthetic cluster (33, 63, 131). The $\Delta gliP$ strains failed to produce gliotoxin, and no morphological or developmental phenotypes were detected. The first two groups reported no difference in survival after infection with the $\Delta gliP$, parental, or complemented strains in neutropenic mice (33, 63). Similar results were obtained with a $\Delta gliZ$ strain that lacked a transcription factor required for gliotoxin biosynthesis (17). However, the third group recently demonstrated reduced virulence of a $\Delta gliP$ strain in a low inoculum model of pulmonary aspergillosis in corticosteroid-treated mice (131). These data

suggest that gliotoxin is dispensable for *A. fumigatus* virulence in some hosts, but not others, and do not preclude a role in virulence for numerous other mycotoxins produced by this organism.

Pathways that regulate fungal growth and morphogenesis.

Hyphal growth underlies tissue invasion in susceptible hosts and is subject to regulatory control by pathways that sense and respond to stress conditions, nutrient availability, and other environmental conditions. For example, the Ca^{2+} -calmodulin-activated protein phosphatase calcineurin, a regulator of eukaryotic stress responses, is required for virulence in *C. albicans* and *C. neoformans*. In *A. fumigatus*, deletion of the calcineurin catalytic subunit (ΔcnaA) is associated with a conidiation defect and slowed hyphal growth at 37°C (129). In vivo, the ΔcnaA strain was markedly hypovirulent in two models of invasive aspergillosis.

Cyclic-AMP-dependent signals regulate the growth, development, and morphogenesis of several fungal pathogens and underlie, in part, the synthesis of virulence factors, such as the pigment and capsule in *C. neoformans*. In *A. fumigatus*, this pathway includes a regulatory heterotrimeric G protein (*gpaB*) (72), adenylylase cyclase (*acyA*) (72), and the regulatory (*pkaR*) (159) and catalytic (*pkaC1*) subunits of protein kinase A (PKA) (73). All mutants in this signaling pathway (ΔgpaB , ΔpkaC1 , and ΔpkaR) examined in murine models of invasive aspergillosis were less virulent than wild-type strains. Germination was delayed in the ΔgpaB , ΔacyA , ΔpkaC1 , and ΔpkaR strains, while hyphal growth was impaired in the ΔacyA , ΔpkaC1 , and ΔpkaR strains. The ΔgpaB , ΔacyA , and ΔpkaR strains were more sensitive to oxidants in vitro than the parental strains, though all mutant strains contained melanin. In sum, these results implicate the cyclic AMP-dependent PKA signal cascade as a critical regulator of conidiation, development, growth, and stress responses.

Filamentous growth depends on fungal homologs of the Ras family of proteins with GTPase activity. In *A. fumigatus*, the nonessential *rasB* gene regulates germination, radial growth, and hyphal branching (41). ΔrasB conidia germinate slowly, and ΔrasB hyphae display increased and aberrant branching. As expected, the ΔrasB strain was less virulent in vivo than control and complemented strains (41).

Nutrient uptake. Acquisition of essential nutrients within the host environment is required for invasive growth. *A. fumigatus* acquires iron in mammalian hosts through the action of siderophores, negatively charged molecules that bind ferric iron and facilitate iron uptake and storage. The ΔsidA mutant lacks the enzyme that catalyzes the first committed step in siderophore biosynthesis and displays reduced virulence in immunosuppressed mice (124). Deletion of the zinc-responsive transcriptional activator gene *zafA* impaired zinc homeostasis and uptake during in vivo fungal growth, resulting in loss of virulence in the mutant strain (91). Nitrogen uptake is another essential process for fungal growth within mammalian tissues. The *A. fumigatus* *rhbA* gene enhances growth on nitrogen-poor sources (101, 113) and its deletion diminished virulence in vivo (102).

A. fumigatus secretes catabolic enzymes, including peptidases, to degrade macromolecular polymers for nutrient uptake. Since the pulmonary parenchyma contains large quantities of elastin and collagen, the proteolytic breakdown of these polymers may represent a fungal virulence mechanism. Two secreted collagenolytic peptidases have been isolated, an

alkaline serine protease of the subtilisin subfamily (AfAlp) that is expressed in the lungs of neutropenic mice (133) and a metalloprotease (MEP) (87). Single and double gene deletion mutants all display virulence similar to that of control strains in immunosuppressed mice (57, 88, 134). Similar results have been obtained with strains defective for a secreted aspartic peptidase (112) and an intracellular metallopeptidase (MepB) (52). Although a number of other proteases have been characterized (4, 11, 12, 98, 110, 111), there is no evidence to date that loss of specific protease genes impacts *A. fumigatus* virulence, a feature that may reflect redundancy.

THE HOST RESPONSE TO *A. FUMIGATUS*

Cellular constituents of innate immune defense. Inhaled conidia pose an invasive threat and trigger innate and adaptive immune responses. Conidia interact not only with leukocytes, but with epithelial cells in the respiratory tree and, upon hyphal tissue invasion, with endothelial cells. The role of conidial and hyphal escape from these cellular barriers during the process of dissemination and angioinvasion was recently reviewed in an outstanding article (40).

Experimental studies performed in the early 1980s in immunocompromised mice indicate a critical role for macrophages in conidial defense and postulate that neutrophils act primarily against products of germination (121). In these studies, corticosteroid treatment interfered with macrophage killing of resting conidia, suggesting that macrophage function is a critical conidial clearance mechanism. In contrast, nitrogen mustard-induced neutropenia rendered mice highly susceptible to intravenous administration of swollen conidia (121). The animals were relatively less susceptible to resting conidia, implying that neutrophils play a predominant role in killing hyphae. Neutrophils attach to and degranulate on hyphal surfaces in vitro (39).

Neutropenia has long been an associated risk factor for the development of invasive aspergillosis in humans, and several animal models have been utilized to study invasive aspergillosis under these conditions. Recent work has expanded the view of neutrophils in host defense against *A. fumigatus* and has indicated a key role for these cells in anticonidial defense (Fig. 3). In mice treated with a blocking antibody to CXCR2, the receptor for the neutrophil chemoattractants CXCL2/MIP-2 and CXCL3/KC, or that are genetically deficient in it, neutrophil recruitment into the airways is delayed, enabling conidia to escape alveolar macrophage control, germinate, and establish invasive disease (18, 81). Neutrophils form aggregates around conidia in the airways of immune competent mice in vivo; these structures prevent conidial germination without requiring conidial uptake (18).

Other innate immune cell subsets contribute to antifungal defense. For example, adoptively transferred NK cells and CCR6⁺ myeloid dendritic cells confer a protective benefit in neutrophil-depleted mice (93, 108). Pulmonary dendritic cells transport conidia to draining mediastinal lymph nodes to activate fungus-specific adaptive immune responses (19).

Molecular recognition of fungal cells. Microbial recognition through secreted and membrane-bound germ line-encoded receptors is central to the function of innate immune cells and triggers effector responses, such as the release of microbicidal molecules, cytokines, and chemokines. Conidia, but not hy-

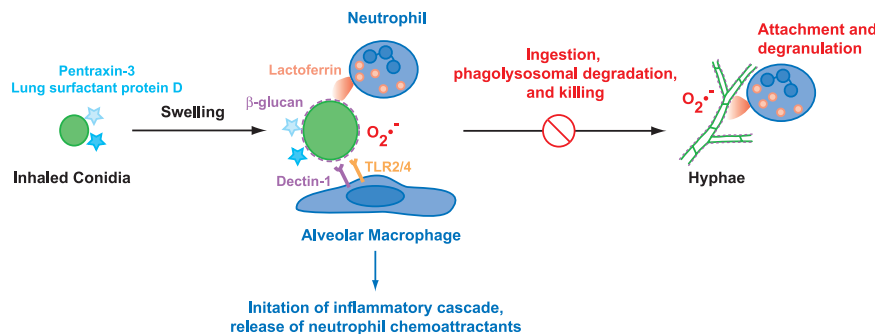


FIG. 3. Stage-specific innate immune responses to *A. fumigatus*. Inhaled conidia bind soluble receptors, for example, pentraxin-3 and lung surfactant protein D, that enhance inflammatory responses. Conidial swelling can occur in the bronchoalveolar space or within alveolar macrophage phagosomes and results in β -glucan surface exposure, triggering inflammatory and fungicidal responses through the mammalian β -glucan receptor, dectin-1, TLR2- and TLR4-dependent signals contribute to host recognition of germinating conidia and hyphae, as well. Recruited neutrophils cooperate to inactivate conidia and, together with alveolar macrophages, prevent conidial germination in immune-competent hosts. The generation of fungicidal ROIs, for example, superoxide anions ($O_2^{\cdot-}$), is critical to this process. The release of neutrophil granules activates ROI-independent mechanisms to limit fungal growth; these include lactoferrin, a molecule that sequesters iron. In the setting of hyphal tissue invasion, neutrophils attach to fungal cells and degranulate, thus attacking hyphae with ROI-dependent and -independent mechanisms. Dendritic cells and NK cells (not shown) contribute to innate immune defense in specific circumstances, particularly in the setting of defective neutrophil activity (see the text).

phae, bind the soluble receptor pentraxin-3 within terminal airways via galactomannan, an interaction that enhances uptake by alveolar macrophages and dendritic cells (43). Pentraxin-3^{-/-} mice are highly susceptible to invasive aspergillosis, even without exogenous immune suppression (43). In vitro, recombinant pentraxin-3 enhances conidial killing by alveolar macrophages. In vivo, pentraxin-3 drives the pulmonary production of protective Th1-biased cytokines, such as interleukin 12 (IL-12) and gamma interferon (IFN- γ), and enhances murine survival in a bone marrow transplantation model of invasive aspergillosis (44). Lung surfactant protein D interacts with conidia to augment neutrophil oxidative responses and confers protective effects in a corticosteroid-induced model of invasive aspergillosis (77) (Fig. 3).

Binding and uptake of conidia occurs through a variety of receptors (19, 58, 59, 107) with distinct leukocyte expression patterns. The signaling receptor DC-SIGN on human alveolar macrophages and lung dendritic cells binds conidial galactomannan and participates in conidial uptake, though the significance of this interaction for subsequent immune responses remains unclear (125). Host immune responses to fungal cells depend on several classes of transmembrane receptors that trigger signaling cascades. The mammalian Toll-like receptor (TLR) family consists of 11 members, and at least 2, TLR2 and TLR4, are thought to mediate inflammatory responses to *A. fumigatus* conidia. TLR2 and TLR4 both signal through the common soluble adaptor protein MyD88 to induce the production of cytokines and chemokines (e.g., tumor necrosis factor [TNF] and macrophage inflammatory protein 2/CCL2) and reactive oxygen intermediates (ROIs) (Fig. 3).

TLR2-deficient alveolar macrophages secrete ~30 to 40% less TNF, a key cytokine mediator of anti-*Aspergillus* responses in mice and humans (82, 148), than control cells stimulated with resting or swollen conidia (7, 128). This has also been observed for macrophages isolated from other sources (50, 78, 97). Killed hyphae and soluble hyphal antigens stimulate TLR2-dependent inflammatory responses, as well (20). In vivo, TLR2^{-/-} mice treated with vinblastine are more susceptible to

invasive aspergillosis than wild-type counterparts (6). TLR2^{-/-} mice treated with cyclophosphamide demonstrate an elevated lung fungal burden compared to control mice (14).

Human monocytes recognize hyphae in a TLR4-dependent manner, as judged by antibody blockade (146). Both TLR4-independent and -dependent inflammatory responses to hyphae have been reported for murine macrophages (78, 83, 97). However, loss of TLR4 function increases mortality in cyclophosphamide-treated mice compared to control animals (14). TLR2- and TLR4-dependent signaling pathways activate neutrophil antifungal responses (15), though the fungal ligands recognized by these receptors remain unknown.

Although conidia and hyphae activate TLR-dependent signals, loss of the TLR adaptor protein MyD88 is not associated with enhanced susceptibility to invasive aspergillosis in otherwise immune-competent mice (14). Cyclophosphamide-treated MyD88^{-/-} mice die more rapidly of invasive aspergillosis than control animals. These data suggest that TLR/MyD88-independent signaling is critical for murine antifungal defense.

Antibody-blocking experiments and in vitro studies with genetically deficient macrophages demonstrate a TLR-independent *A. fumigatus* conidial and hyphal recognition mechanism via dectin-1 (45, 50, 128, 136), an NK-like C-type lectin-like receptor with an intracellular immunoreceptor tyrosine-based activation motif-like motif (3, 23, 24). Dectin-1 binds β -(1,3) glucans from a variety of other human-pathogenic fungi (42, 127, 144), is phosphorylated, and signals through p72^{Syk} kinase and CARD9, a caspase recruitment domain protein, to trigger inflammatory responses that include the release of TNF and IL-12, neutrophil chemoattractants, and ROIs (117, 143). CARD9^{-/-} mice are highly susceptible to systemic infection with *C. albicans* (47), while dectin-1^{-/-} mice are susceptible to *Pneumocystis carinii* (120) and, in one of two reported studies, to *C. albicans* (136).

Inhaled *A. fumigatus* conidia contain little surface β -glucan and trigger marginal inflammatory responses (Fig. 3). The process of conidial germination alters the cell wall composition, and β -(1,3) glucan is exposed during conidial swelling and

hyphal growth (45, 50, 128). Thus, dectin-1 signaling is triggered in a stage-specific manner and provides a mechanism to focus fungicidal responses on spores that pose an invasive threat and to ignore resting conidia. This division may represent a mechanism to restrict inflammatory responses spatially, for example, to phagocytosed swollen conidia, and temporally in the face of ongoing exposure to airborne *A. fumigatus* spores.

Killing of fungal cells. Following conidial phagocytosis, swelling proceeds within alveolar macrophages and is required to activate NADPH oxidase-dependent killing mechanisms (109). This process may depend on fungal β -(1,3) glucan exposure in the phagosome. Consistent with this notion, conidial killing by alveolar macrophages occurs independently of MyD88 function (79). Phagosomes that contain conidia fuse with endosomes and mature into phagolysosomal compartments. Phagolysosomal acidification leads to killing of internalized conidia, a process disrupted by the inhibitor baflomycin A (53).

Deletion of the gp91 subunit of the NADPH oxidase complex results in a murine model of X-linked chronic granulomatous disease, and gp91^{phox-/-} mice are uniformly susceptible to intratracheal challenge with a low-dose ($<10^4$) conidial inoculum (92), highlighting the critical importance of the superoxide anion ($O_2^{\cdot-}$) and its derivatives, for example, the hydroxyl radical ($OH\cdot$), in host defense against *A. fumigatus*. Neutrophils deficient in Rac2, a hematopoietic cell-specific Rho family GTPase, have multiple deficits that include cytoskeletal abnormalities and deficiency in superoxide production. Accordingly, Rac2^{-/-} mice are more vulnerable to intravenous *A. fumigatus* challenge than wild-type mice (116), although this experimental model does not recapitulate the natural route of infection.

Myeloperoxidase (MPO)-deficient mice survived intranasal administration of 2×10^5 conidia, while gp91^{phox-/-} mice did not (2). However, MPO^{-/-} mice showed a higher lung fungal burden than wild-type mice, suggesting that MPO-dependent HOCl production may contribute to host defense. The in vivo contributions of reactive nitrogen intermediates to anti-*Aspergillus* defense remain undefined. Although conidia stimulate macrophage nitric oxide production in vitro (46), neutrophils, monocytes, and alveolar macrophages may kill conidia in a nitric oxide-independent manner (86).

Human neutrophils display an NADPH oxidase-independent conidial defense mechanism through the release of lactoferrin, a chelator that sequesters bioavailable iron and thus limits conidial germination (158) (Fig. 3). In light of ongoing conidial exposure, this pathway may be particularly important in patients with chronic granulomatous disease or high-risk patient populations with increased systemic iron stores (61).

Adaptive responses: T lymphocytes. T-cell responses to *Aspergillus* antigens are present in healthy individuals, likely due to universal exposure to the organism. Lymphoproliferative responses to conidia and hyphae reside mainly in the CD4⁺ T cell fraction (48, 106). While the full antigenic specificity of these cells has not been defined, responses to recombinant catalase and DPP V proteins, both of which are potent stimuli of antibody production, have been detected (11, 48, 75). However, neither nude nor SCID mice, which lack functional T cells or both T and B cells, respectively, are more susceptible to

invasive aspergillosis than wild-type mice (153; M. Feldmesser, unpublished data), demonstrating the primacy of innate immune responses in preventing this disease. Nonetheless, more recently, roles for adaptive immunity have been examined.

Several lines of evidence support T cell participation in host defense. Allogeneic hematopoietic stem cell transplantation results in protracted susceptibility to invasive aspergillosis. After transplantation, the time to recovery of specific T cell responses is prolonged in comparison to that for other pathogens, such as cytomegalovirus (48). The presence of Th1-associated responses correlates with resistance to disease in animal models of pulmonary and systemic infection (27, 28, 48). Correlation of cytokine stimulation patterns from peripheral blood mononuclear cells with clinical outcome in human disease suggests that a high ratio of IFN- γ to IL-10 may be beneficial (48). In the first demonstration of clinical benefit of adoptive immunotherapy for invasive aspergillosis in humans, transfer of CD4⁺ T cell clones was associated with not only earlier recovery of *Aspergillus*-responsive CD4⁺ and CD8⁺ T cells, but a higher ratio of IFN- γ to IL-10 than was present in spontaneously recovering CD4⁺ T cell populations in untreated patients (106).

The mechanism by which these T cell responses benefit the host is incompletely understood. However, hyphal damage is significantly higher when the organism is incubated with T cells, antigen-presenting cells (APCs), and neutrophils than with neutrophils alone or with either T cells or APCs (13, 48). These findings support the ability of *Aspergillus*-responsive T cells to enhance neutrophil function.

The development of T cell responses following pulmonary infection has been examined in murine models and has yielded conflicting results. Following intratracheal administration, CD4⁺ T cells were detected in lung and mediastinal lymph nodes 3 days after infection, with peak responses at 7 to 10 days postinfection. While heat-killed conidia primed CD4⁺ T cells for Th2 development, with induction of IL-13 and IL-4 following subsequent stimulation, Th1-associated skewing of the response was seen following inoculation with live organisms, marked by a predominance of IFN- γ and TNF production (115). Dendritic cells exposed to hyphae in the lung primed CD4⁺ T cells in the thoracic lymph node and spleen to produce IL-4, a Th2-biased cytokine (19). These results suggest that the development of a beneficial Th1- or deleterious Th2-biased response depends, in part, on the fungal growth stage encountered by APCs.

However, in a second model examining Th polarization, also in immunocompetent mice, infection with live conidia via aerosol resulted in IL-25 expression in the lung. This Th2 cell- and mast cell-derived cytokine, which may be an important mediator of allergic responses, resulted in upregulation of other Th2-associated cytokines and development of eosinophilia, but through stimulation of a CD4⁻ cell population (51). The variance in finding the development of Th1- or Th2-like environments following infection with live conidia may reflect differences in mouse strains, inocula, routes of administration, or *A. fumigatus* strains. Further, in the second study, CD4⁺ T cell phenotypes following infection were not directly studied.

In elegant studies using an *A. fumigatus*-specific T cell transgenic mouse, Rivera and coworkers demonstrated that development of CD4⁺ T cell responses is incremental following

pulmonary infection, with rapid recruitment to regional lymph nodes and, following extensive proliferation, trafficking to the airways via MyD88-independent mechanisms (114). Though MyD88-mediated signaling enhances Th1 differentiation of CD4⁺ T cells in the regional lymph nodes, as determined by identification of cells that secrete IFN- γ upon further stimulation, IFN- γ secretion by Th1 cells in the airway is MyD88 independent (114). The contribution of dectin-1 signaling to the priming and differentiation of *A. fumigatus*-specific CD4⁺ T cell responses remains unknown.

A role for CD8⁺ T cells in the naturally occurring host response has not been demonstrated. In ovalbumin-specific T cell-transgenic mice, a commercial *A. fumigatus* extract can function as a selective CD8⁺ cell adjuvant, with induction of proliferation, IFN- γ production, and cytolytic activity (135). However, the demonstration that gliotoxin suppresses perforin-mediated cytolytic activity by a CD8⁺ cell clone in vitro suggests a potential means by which the organism may limit the efficacy of this cell population (155).

At present, our knowledge regarding the roles of regulatory T cells in host response is very limited, though given the prominent role played by inflammation in pathogenesis, these cells likely are very important. Lung CD25⁺ regulatory T cells reduce neutrophil TNF and ROI production in response to conidia (118). In murine models of invasive disease or allergy, two distinct CD4⁺ CD25⁺ T cell populations in the lung and thoracic lymph nodes play different roles in dampening inflammation (90). An early population in the lung, whose development is dependent upon the presence of the costimulatory molecule B7-2 (CD86), dampens the inflammatory response via IL-10 production and reduces neutrophil antihyphal activity at the expense of allowing fungal growth in response to invasive disease. In contrast, a late transforming growth factor β -producing population found in the lymph nodes, whose development is dependent upon the presence of B7-1 (CD80), decreases allergic Th2-associated responses. Dendritic cell production of indolamine-2,3-dioxygenase, an enzyme involved in tryptophan catabolism in response to regulatory T cell conditioning, contributes to the suppressive activity of regulatory T cells and to development of the late regulatory T cell population (90). Thus, T cell populations may be important both for mounting optimal defenses against invasion that is associated with Th1-like polarization and for regulation, so that inflammation is limited when the organism is present in a form that can be handled by resident defenses. Much remains to be uncovered about the mechanisms for both functions.

B cells and antibody responses. Antibody responses to *A. fumigatus* result from environmental exposure in the absence of disease, occur in the course of either aspergilloma or invasive disease, or participate in hypersensitivity responses. Serum antibodies that bind *Aspergillus* components, particularly those of the immunoglobulin G (IgG) and IgM subclasses, are broadly found in patients without aspergillosis (8). In addition to systemic antibodies, isolation of *Aspergillus*-specific IgA with secretory component from bronchial lavage fluid suggests that significant mucosal responses also are made in those without aspergillosis (123). Many *A. fumigatus* hyphal molecules are potent B cell antigens, which are present among secreted, cell wall, and cytosolic fractions (37, 64, 157). Serum antibodies that bind these molecules are more commonly seen among

patients with aspergilloma than among those with invasive disease, a finding that likely reflects differences in the predisposing conditions between these groups. In addition, over 80 proteins can bind IgE from sensitized patients (34), demonstrating the degree to which *A. fumigatus* can induce allergy, further discussion of which is beyond the scope of this review. Killed conidia do not elicit IgG responses to hyphal antigens following intratracheal inoculation of mice (115), a finding that could reflect the host's ability to limit the immune response when the organism cannot pose the threat of invasive disease but could also indicate differences in specificity. Conidial surface-specific antibody responses have not been studied.

However, antibodies are not believed to play a role in protection against invasive disease, and study of B cell-deficient μ MT mice following induction of neutropenia suggests that antibody may be harmful (60, 89). Nonetheless, immune serum administered to B cell-deficient mice prolongs survival, suggesting a potential role for antibody in the initial host defense. More recently, monoclonal antibodies to laminarin [a β -(1,3) glucan] and to an unidentified \sim 97-kDa glycoprotein have demonstrated that antibody can prolong survival in experimental murine infection (31, 139).

CONCLUSIONS

A. fumigatus exploits qualitative or quantitative deficiencies in the host immune defense to cause invasive disease. To invade mammalian tissues, *A. fumigatus* relies on the coordinated expression of a multitude of genes involved in many aspects of fungal growth, including conidial germination, cell wall assembly, hyphal growth, nutrient acquisition, and resistance to adverse conditions, such as oxidative stress. These pathways are likely to have evolved in response to competitors within the ecological niche of the organism and are unlikely to reflect specific adaptations to counter mammalian host defense mechanisms. Specific genetic changes enhance the ability of the fungus to cause invasive disease within immunocompromised mice and appear to confer one of the following attributes: rapid germination (and growth) or increased melanin content and heightened resistance to oxidative damage. Whether these mutant genotypes confer advantages in fungal growth in external environments remains unclear.

The fungal cell wall composition represents a critical interface for the host innate and adaptive immune responses. Both the fungal surface and the products secreted by the organism change substantially during the course of germination, resulting in dynamic interactions that modulate the host response. One very important example, and perhaps, the best understood, is that of stage-specific recognition of fungal β -(1,3) glucan. This polysaccharide functions as an immunologic indicator of microbial growth and enables the immune system to focus potentially tissue-damaging inflammatory responses on fungal cells with invasive potential. Host defense mechanisms show striking redundancy at the levels of molecular recognition, antifungal effector functions, and cellular components. This redundancy likely contributes greatly to the requirement for host damage for development of invasive disease due to *A. fumigatus*.

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