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REVIEW

Aspergillus fumigatus and Aspergillosis in 2019

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SUMMARY *Aspergillus fumigatus* is a saprotrophic fungus; its primary habitat is the soil. In its ecological niche, the fungus has learned how to adapt and proliferate in hostile environments. This capacity has helped the fungus to resist and survive against human host defenses and, further, to be responsible for one of the most devastating lung infections in terms of morbidity and mortality. In this review, we will provide (i) a description of the biological cycle of *A. fumigatus*; (ii) a historical perspective of the spectrum of aspergillus disease and the current epidemiological status of these infections; (iii) an analysis of the modes of immune response against *Aspergillus* in immunocompetent and immunocompromised patients; (iv) an understanding of the pathways responsible for fungal virulence and their host molecular targets, with a specific focus on the cell wall; (v) the current status of the diagnosis of different clinical syndromes; and (vi) an overview of the available antifungal armamentarium and the therapeutic strategies in the clinical context. In addition, the emergence of new concepts, such as nutritional immunity and the integration and rewiring of multiple fungal metabolic activities occurring during lung invasion, has helped us to redefine the opportunistic pathogenesis of *A. fumigatus*.

KEYWORDS antifungal agents, aspergillosis, *Aspergillus*, cell wall, diagnosis, glycans, immunity, microbiota, receptors

INTRODUCTION

Twenty years ago, a review entitled "*Aspergillus fumigatus* and Aspergillosis" was authored in *Clinical Microbiology Reviews* by J.-P. Latgé (1). Since then, significant scientific progress has been achieved, leading to the development of new antifungal strategies. In spite of these advances, aspergillosis remains a major health problem, with a rapidly evolving epidemiology and new groups of at-risk patients. The expanding threat posed by this mycopathogen stimulated this review, which addresses two main questions: what have we learned in the last 20 years of studying *A. fumigatus*, and why is it the most important aerial fungal pathogen to date?

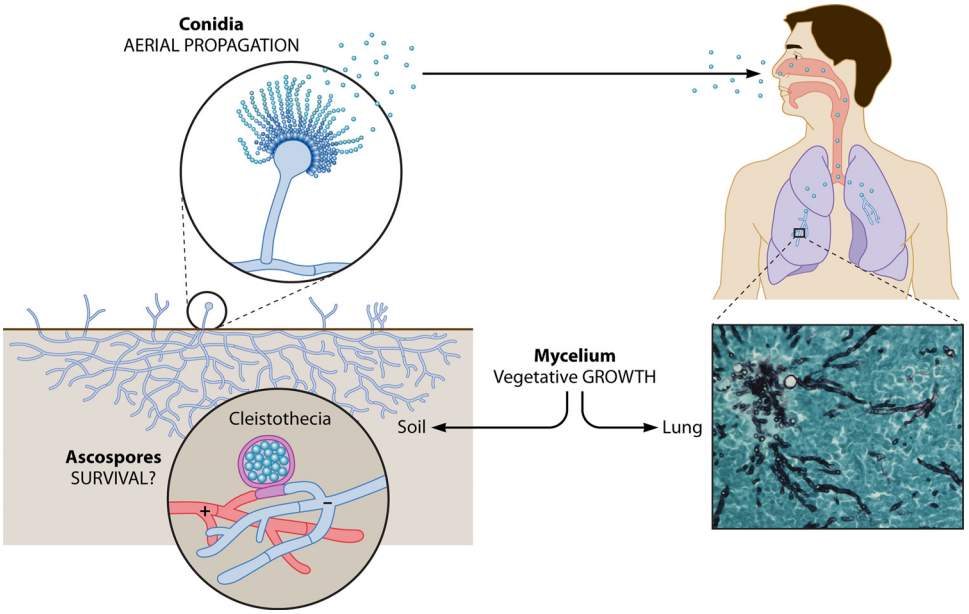

FIG 1 *Aspergillus fumigatus*, a trimorphic filamentous fungus with vegetative mycelium in nature and in patients, the asexual conidia produced after mycelial starvation, and the resting ascospores produced from two heterothallic strains of opposite sex.

ASPERGILLUS FUMIGATUS IN NATURE

A. fumigatus is a saprotrophic fungus with vegetative mycelial life occurring in the soil on decaying organic material (2). It spreads by asexual sporulation (Fig. 1). Asexual propagules (conidia) are produced in chains on separate phialides emerging from conidiophores borne on characteristic conidial heads. Conidia of *A. fumigatus* are pigmented and echinulate spherical structures of 2.5 μm in diameter. For most of the past 150 years, *A. fumigatus* was believed to reproduce exclusively by asexual means, and a parasexual cycle resulting from hyphal fusion was supposed to generate genetic diversity in the absence of meiosis (1, 3). However, accumulating evidence has implicated the presence of a cryptic sexual cycle. First, evidence for recombination came from population genetic studies (4, 5). Examination of the sequenced genome of *A. fumigatus* revealed over 200 annotated genes associated with sexual reproduction (6). Later, an *MAT1-2* family HMG gene and an *MAT1-1* (alpha domain) idiomorph were identified in strains of *A. fumigatus* in a 1:1 ratio, suggesting heterothallism in this species (7–9) (Fig. 2). Dyer and colleagues crossed *MAT1-1* and *MAT1-2* isolates and discovered that a sexual cycle could be induced when isolates of compatible mating

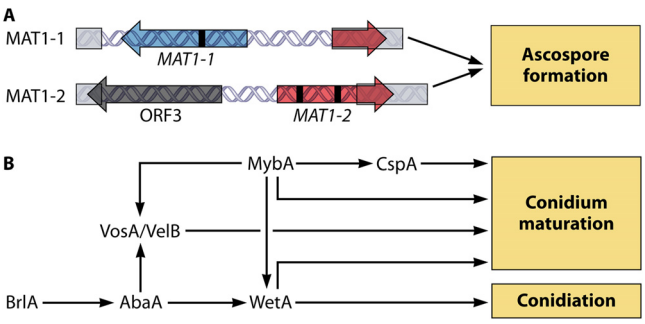

FIG 2 (A) MAT loci from the heterothallic species *Aspergillus fumigatus* (based on data from reference 13). Blue arrows indicate a MATα-domain gene, red arrows indicate a MAT high-mobility group gene, black bars indicate intronic sequences, and gray bars indicate homologous sequences. (B) Conidiation regulators in *A. fumigatus* (adapted from reference 428).

types were crossed (10). Light-yellow cleistothecia, typically 150 to 500 μm in diameter, formed singly or in small clusters, mainly along the junction where hyphae of the parental isolates came into contact. Asci contained heat-resistant ascospores (8 per ascus). Ascospores, the yellowish-white to greenish-white sexual propagules of *A. fumigatus* (4 to 5 μm in diameter), are lenticular with two equatorial crests. Although the teleomorph was named *Neosartorya fumigata* (10), the recent proposal to abandon dual nomenclature, termed "one name, one fungus," the name *A. fumigatus* will continue to designate this species (11). Formation of ascospores occurred when *MAT1-1* and *MAT1-2* isolates were crossed under very specific conditions: 30°C on Irish oatmeal agar for 6 to 12 months in the dark under poorly aerated conditions (10). However, growth conditions such as these required to trigger sex are rarely encountered in nature (12), which explains why the teleomorph of *A. fumigatus* has not been found in nature. Indeed, the biological function of the ascospores is unknown, even though it is tempting to speculate that they play a role in survival under adverse environmental conditions, such as those found in compost heaps where temperatures reach or surpass 75°C (13). Sugui et al. (721) have identified "supermater" isolates, which complete the sexual cycle within 4 weeks. Interestingly, the group of S. Krappman has shown that it is possible to switch the *MAT* locus in different strains. However, the artificially created homothallic strain with both *MAT* loci remains infertile, suggesting that additional factors (sexual pheromones?) are required to induce sex (14, 15). Moreover, sex is very specific and exclusive for the species *A. fumigatus*. Attempted crosses between isolates of *A. fumigatus* and the close relative *A. lentulus* failed to produce cleistothecia. This phenomenon (of sexual preference strictly between *A. fumigatus* spp.) is of medical significance, because most isolates of *A. lentulus* exhibit natural resistance to antifungal agents with potent activity against *A. fumigatus*, limiting the risk of gene flow between different *Aspergillus* species (16).

SPECTRUM OF *ASPERGILLUS* DISEASES: CLINICAL MANIFESTATIONS, DIAGNOSIS, AND EPIDEMIOLOGY

Lung infections due to *A. fumigatus* are caused by inhalation of airborne conidia, which are present in indoor and outdoor environments at concentrations ranging between 1 and 100 conidia per m^3 but can reach up to 10^8 conidia per m^3 in certain environments (17). Accordingly, isolation of *Aspergillus* spp. from cultures of the respiratory tract of asymptomatic patients with no evidence of invasive or allergic disease is common (18–20), with *Aspergillus* DNA found in 37% of lung biopsy specimens of healthy adults (21). Moreover, culture-proven *Aspergillus* colonization occurs in up to 30% of patients with chronic obstructive pulmonary disease (COPD) (22). It has been found recently that *Aspergillus* colonization has a strong genetic basis associated with impaired expression of the transcriptional factor *ZNF77* in bronchial epithelia, which leads to defective epithelial cell integrity and upregulation of extracellular matrix (ECM) proteins that facilitate conidial adhesion (23). Even though it is not always associated with infection, *Aspergillus* colonization is associated with a substantial increase in risk for development of invasive infection across a wide range of immunocompromised individuals (24). However, because invasive pulmonary aspergillosis occurs following inhalation of *Aspergillus* conidia, environmental exposures affect the epidemiology of the disease. In fact, a number of nosocomial clusters of invasive pulmonary aspergillosis (IPA) have been reported over the past 3 decades, often associated with hospital construction and defects in air-handling equipment (25).

Aspergillus species cause a wide spectrum of diseases in humans (26, 27). Depending on the underlying immune status of the host, *Aspergillus* diseases can be roughly classified into three groups with distinct pathogenetic mechanisms, clinical manifestations, and overlapping features, which are depicted based on their relative clinical importance in Fig. 3. These three groups of *Aspergillus* diseases will be discussed in the following sections.

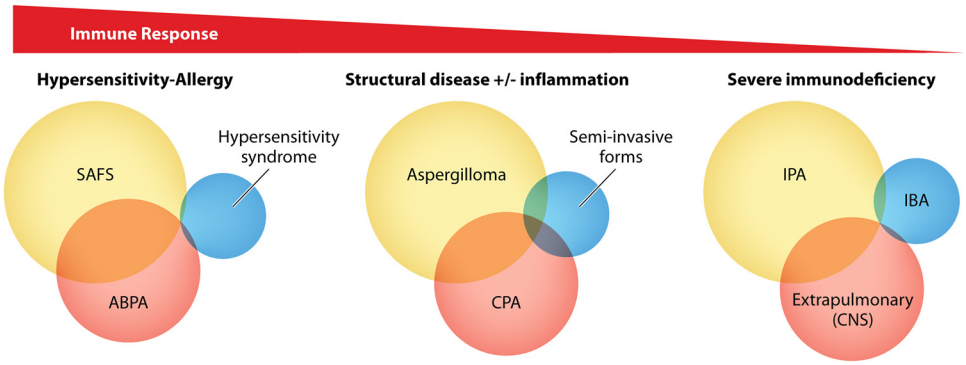

FIG 3 Syndromes associated with aspergillosis patients with different immune statuses include the following: SAFS, severe asthma with fungal sensitization; ABPA, allergic bronchial pulmonary aspergillosis; CPA, chronic pulmonary aspergillosis; IPA, invasive pulmonary aspergillosis; and IBA, invasive bronchial aspergillosis.

Aspergillosis in the Immunocompetent Patient

Aspergillus spp. can lead to chronic, noninvasive forms of infection with overlapping features, ranging from development of a fungus ball (aspergilloma) to a chronic inflammatory and fibrotic process currently classified as chronic pulmonary aspergillosis (28, 29). In particular, saprophytic colonization of a parenchymal lung cavity by *Aspergillus* is referred to as aspergilloma and consists of both dead and living mycelial elements, inflammatory cells, fibrin, mucus, and components of degenerating blood and epithelia. The mycelial mass may lie free within the cavity or be attached to the cavity wall by inflammatory/granulomatous tissue (25, 28–30).

The pathogenesis of aspergilloma usually involves colonization and proliferation of the fungus in a preexisting pulmonary cavity, most commonly due to pulmonary tuberculosis (TB). In fact, 20% of patients who recover from cavitary tuberculosis develop aspergillomas within 3 years (25, 29). In developing countries, tuberculosis is the predisposing factor for over 90% of cases of aspergillomas. Moreover, an aspergilloma may complicate a wide spectrum of cavitating pulmonary diseases, such as sarcoidosis, other fungal infections, and other chronic cavitary lung diseases (25, 29). Mild, self-limited hemoptysis, seen in 50% to 90% of patients, is the typical manifestation of aspergilloma; however, occasionally hemoptysis can be massive or even fatal (25, 29).

Other chronic, inflammatory forms of *Aspergillus* infection of the lung have been recognized in patients with chronic cavitary lung disease, which is characterized by an indolent clinical course of a chronic inflammatory disease evolving over months to years. This disease is characterized by constitutional symptoms, serum IgG-postitive antibodies (Abs) (precipitins) to *A. fumigatus*, elevated acute-phase markers of inflammation, and an immune status that ranges from normal to mild immunosuppression (25, 28–30). This is a very complex and heterogenous syndrome, which has been subclassified in the past as chronic necrotizing pulmonary aspergillosis (CNPA), chronic cavitary pulmonary aspergillosis (CCPA), and chronic fibrotic pulmonary aspergillosis (CFPA) (28, 29, 31). The entire spectrum of noninvasive diseases caused by *Aspergillus*, including aspergilloma, is now classified as chronic pulmonary aspergillosis (CPA) (28, 29). The exact incidence of CPA is not known, but it is considered an underrecognized disease entity. However, each year, an estimated >350,000 new CPA cases complicate treated pulmonary TB within 12 months of completion of anti-TB therapy (32). Locally invasive (semi-invasive) *Aspergillus* infection may be detected in histological preparations, whereas classical features of systemic fungal disease, including angioinvasion and dissemination, are absent from CPA patients. Defects in mucociliary clearance associated with structural lung disease and deregulated inflammatory responses appear to be a critical factor in the pathogenesis of CPA. Prior mycobacterial lung infection, emphysema, and COPD, or other chronic structural lung abnormalities, are dominant predis-

posing conditions for CPA. In addition, incompletely characterized defects in local and systemic innate immunity may play a role in the pathogenesis of CPA (29, 30, 33).

Diagnosis of aspergilloma and other forms of CPA is based on recently defined standardized criteria. Persistent (>3 months) symptoms of chronic inflammation, manifesting as chronic productive cough and weight loss, dyspnea, and fatigue, and/or the presence of mild hemoptysis are the usual presenting symptoms for CPA. Chest radiography remains the imaging modality of choice for diagnosis of aspergilloma and reveals a solid round mass within a cavity partially surrounded by a radiolucent crescent, typically located in the upper lung fields. However, aspergillomas can be bilateral and multiple. In many cases, computed tomography (CT) of the chest may further delineate the radiographic features of an aspergilloma that are not apparent on chest radiographs. CT is particularly useful in defining the precise pattern and extent of the disease. CPA differs from simple aspergilloma because of the presence of constitutional symptoms, the development of persistent pericystic lung nodules or pleural thickening, consolidations or ground glass opacities, and the development and/or progression of cavities.

Although sputum cultures are positive for *Aspergillus* in more than half of all patients with aspergilloma/CPA, culture is not a sensitive and specific diagnostic marker. The diagnosis of CPA is based on clinical symptoms, radiographic features, and serological evidence of IgG Abs to *Aspergillus* (25, 29, 34). A positive precipitin reaction for *Aspergillus* is particularly helpful in establishing the diagnosis of aspergilloma. The identification of anti-*Aspergillus* antibodies, developed 50 years ago, was originally achieved by the detection of antigen-antibody complexes precipitating in a gel (precipitins [35–37]). This robust and inexpensive method, available as a double immunodiffusion or counterimmunoelectrophoresis, continues as the reference method for serological diagnosis of CPA. One issue with this approach is the use of unstandardized, crude antigen mixture (38, 39). Immunoblots and enzyme-linked immunosorbent assays (ELISAs) have been implemented recently. With ELISA, it is possible to use recombinant antigens and to characterize the type of anti-*Aspergillus* antibodies (e.g., IgG) (38, 40, 41); recently, a kit was launched by Bio-Rad. However, the absence of the disclosure of the antigen molecule in this test prevents comparative studies. Western blotting, like the test from LDBIO Diagnostics, has the advantage of identifying the nature of the dominant antigenic molecule in a crude mixture, but it has the disadvantage of being semiquantitative. Detecting the presence of anti-*Aspergillus* IgG and IgM antibodies is quite easy today, but the level of antibody, the kinetics of the production, and the antibody isotypes have not been correlated to the severity of the disease. Many recombinant *Aspergillus* antigens have been produced in *Pichia pastoris* or in *Escherichia coli*, but there is a need for development of standardized methods for quantification of anti-*Aspergillus* antibody titers and correlation of their values with disease activity, severity, and response to therapy for CPA (38, 41–44).

Aspergillosis in the Atopic Patient

The most severe form of aspergillosis among atopic patients is allergic bronchopulmonary aspergillosis (ABPA) (33), which develops following sensitization to *A. fumigatus* allergens in a unique subset of atopic individuals: those patients with cystic fibrosis (45) or individuals with genetic predisposition for ABPA (27, 33, 46). ABPA is a hypersensitivity disease of the lung that is almost always related to *A. fumigatus*. The prevalence of *Aspergillus* sensitization in the United States, based on *Aspergillus* IgE levels, has been estimated to be at 6.4% (47), whereas the prevalence of ABPA has been estimated as 1% to 3.5% of all asthmatics, with about 4,000,000 patients affected worldwide (33, 48, 49). Seven to 14% of patients with poorly controlled asthma and 7% to 9% of those with cystic fibrosis (CF) meet the diagnostic criteria for ABPA. In fact, nearly all patients with ABPA have a history of chronic asthma. Clinically, ABPA symptoms include episodic wheezing, malaise, low-grade chronic fevers and cough, sputum containing brown mucus and plugs, chest pain, migrating pulmonary infiltrates, and sputum and blood eosinophilia. As ABPA progresses, central bronchiectasis becomes a dominant feature

of the disease and may result in chronic pulmonary secretions and, occasionally, hemoptysis, as well as characteristic radiographic abnormalities. In patients with CF, a specific feature of ABPA is hemoptysis, which might be complicated by lung collapse due to pneumothorax. The full spectrum of ABPA epidemiology, clinical manifestation, diagnostic criteria, and treatment has been addressed in recently published reviews (48–50) and guidelines (25, 33).

Criteria for the diagnosis of ABPA have evolved and now consist of (i) clinical and/or pulmonary function deterioration from baseline status (33), (ii) positive immediate cutaneous reaction to *A. fumigatus* antigens or elevated IgE *A. fumigatus* antibody serum level, (iii) elevated serum total IgE level of >1,000 U/ml, (iv) elevated serum IgG *A. fumigatus* antibody level or positive *A. fumigatus* precipitins, and (v) abnormal chest imaging findings or a change in baseline abnormalities (51). Pulmonary infiltrates in cases of ABPA initially are transient but ultimately progress to permanent radiographic changes indicative of bronchiectasis. The presence of proximal bronchiectasis, which is characterized by normal filling of bronchi distal to the sacular bronchial lesion, is considered a hallmark for diagnosis of ABPA. ABPA lesions are either focal or bilateral and tend to occur more frequently in the upper lobes. Late radiographic findings in patients with ABPA include cavitation, local emphysema, and extensive fibrosis. CT has been a significant aid in diagnosing ABPA because it is more sensitive than regular chest radiography; however, there are no pathognomonic CT findings for this entity. In cystic fibrosis patients with ABPA, there is a significant overlap in the radiographic features of both entities (33). Because no single immunological feature is diagnostic for ABPA, this disease is underdiagnosed. Use of purified recombinant *A. fumigatus* allergens (see the allergome database www.allergome.org for the recombinant *A. fumigatus* allergens, developed by R. Crameri [52, 53]) has the potential to substantially increase the specificity and sensitivity of diagnosis of *A. fumigatus*-related diseases, including ABPA. However, because the sensitivity of each recombinant *A. fumigatus* allergen is suboptimal, use of a panel of recombinant allergens is favored to increase the sensitivity of ABPA diagnosis. Further standardization and development of commercially available immunoassays containing a combination of these allergens holds promise for improving the diagnosis of ABPA.

Severe asthma with fungal sensitization (SAFS) is a distinct form of steroid-refractory asthma with evidence of sensitization to *Aspergillus* or to other fungal allergens in the absence of clinical and radiographic evidence of ABPA. The diagnostic criteria for SAFS include (i) poorly controlled asthma, (ii) a positive skin-prick test for fungal allergens (not necessarily to *Aspergillus* species) or *in vitro* demonstration of antifungal IgE of at least 0.4 IU/ml, and (iii) a total serum IgE concentration of 1,000 IU/ml (33, 54). The radiographic features of ABPA are important for diagnosis, as they may distinguish ABPA from SAFS. In addition, the levels of *Aspergillus*-specific IgE are typically <1,000 IU/ml in SAFS (33). The true prevalence of *Aspergillus*-related SAFS is difficult to estimate; however, mathematical modeling indicates that it affects millions of patients with asthma (55).

Invasive Pulmonary Aspergillosis in the Immunocompromised Patient

Trends in epidemiology of IPA. *Aspergillus* has emerged as one of the most common causes of infectious death in severely immunocompromised patients, with mortality rates up to 40% to 50% in patients with acute leukemia and recipients of hematopoietic stem cells transplantation (HSCTs) (55–59). Surveillance clinical studies in the late 1990s demonstrated a 3- to 4-fold increase in the frequency of *Aspergillus* infections at major cancer centers over the past 2 decades (60–64). Epidemiological studies in the mid-2000s report a significant decrease in the incidence and mortality rates of IPA in allogeneic HSCT and in solid-organ transplant (SOT) recipients, possibly associated with (i) changes in HSCT practices leading to shortened duration of preengraftment neutropenia and (ii) improved strategies to diagnose, prevent, and treat fungal diseases (65–68). Currently, IPA mostly occurs late (40 to 80 days) and very late (80+ days) after engraftment (56, 57, 62, 63, 65, 66, 69). These studies also reveal that

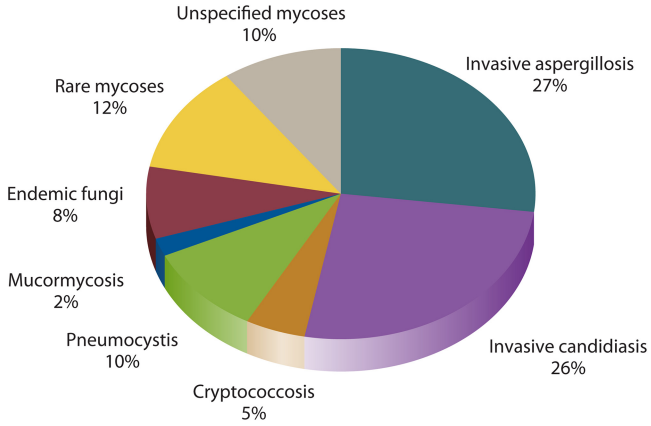

FIG 4 Direct health care cost of fungal diseases in the United States, showing the higher inpatient cost of invasive aspergillosis than all other fungal diseases (total inpatient cost of $4.6 billion per year, based on data from reference 75).

IPA is the most common invasive fungal infection (IFI) in HSCT and in solid-organ transplant recipients (43% and 59% of all IFIs, respectively), with a 12-month cumulative incidence of 1.6%. Recent large epidemiological studies in several European countries confirm the predominance of IPA over other IFIs in patients with leukemia and HSCT transplantation (56–59, 69–71). Even though no large epidemiological studies have been undertaken in recent years, all the data gathered showed that the situation in the clinics has not changed much in the last 20 years, and morbidity and mortality rates mentioned in the 2000s are still valid today. Since the late 1990s, IPA has actually surpassed invasive candidiasis as the most common fungal infection found at autopsy at several institutions; approximately 15% to 20% of patients with leukemia die of fungal pneumonia caused by *Aspergillus* spp. (72–74). Moreover, because of its prevalence and costly treatments, IPA has also become the most expensive fungal disease in the hospital setting (Fig. 4) (75).

IPA now predominantly manifests as a community-acquired pneumonia in nonneutropenic HSCT recipients late after engraftment (76), and it is associated with the use of corticosteroids and other immunosuppressive therapies for chronic graft-versus-host disease, metabolic abnormalities, acquired iron overload syndromes, aging, cytomegalovirus (CMV) coinfection, and other comorbidities (67, 68, 71). In solid-organ transplant recipients, lung, lung-heart, small bowel, and liver transplantation carry the highest risk for development of invasive aspergillosis (IA) (67, 68, 71, 77). Increasingly, IPA has been described in new groups of traditionally low-risk patients (Fig. 5) (71). These patients typically present with malignant, autoimmune, or inflammatory diseases and possess complex immune-metabolic abnormalities as a result of (i) an underlying disease, (ii) comorbidities, (iii) aging, (iv) immunosuppressive therapy, and (v) previous sepsis episodes, and they were treated with new biological therapies targeting immune-signaling pathways (71, 78, 79). In fact, particular attention should focus on the following groups: (i) patients with hematological malignancies of lymphoid origin who receive small-molecule kinase inhibitors (SMKIs) targeting BTK (78, 80), ERK (81), and JAK/STAT signaling (82); (ii) patients in the intensive care unit (ICU) recovering from bacterial sepsis (*Aspergillus* has now become a model pathogen for understanding mechanisms of sepsis-induced immune deactivation) (83–85); (iii) hematological malignancy patients who develop a cytokine storm syndrome following immunotherapy with chimeric antigen receptor T cells (CAR T cells) that requires immunosuppressive therapy with high doses of corticosteroids (86, 87); and (iv) critically ill patients admitted to the ICU with severe influenza (85, 88) or other viral infections (722, 723). Notably, the immunopathogenesis of IPA in all these patient groups remains unexplored, and the introduction of SMKIs targeting major antifungal pathways (e.g., syk kinase and NOX inhibitors) in phase II and phase III clinical trials is anticipated to result

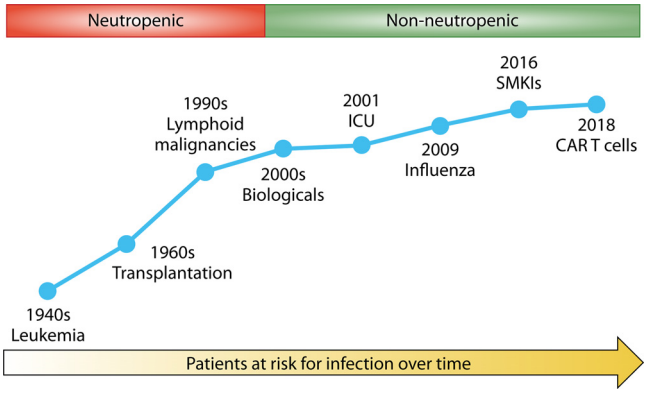

FIG 5 Epidemiological trends of IA. Evolving groups of nonneutropenic patients at risk for IA in the era of (i) targeted "precision-medicine" therapies for the management of malignant, inflammatory, and autoimmune diseases that impact the immune system and (ii) complex metabolic and immunological abnormalities in a large proportion of critically ill patients who survive severe infections in the intensive care unit. ICU, intensive care unit; SMKI, small-molecule kinase inhibitor; CAR T cells, chimeric antigen receptor T cells.

in additional cases of IPA. It also has been demonstrated that IPA has different pathophysiological mechanisms depending on the type of immunosuppression. The nonangioinvasive form of IPA has been increasingly recognized in a wide range of nonneutropenic hosts (89, 90). Histopathology in those nonneutropenic patients is characterized by extensive pyogranulomatous inflammatory reactions, inflammatory necrosis, and extensive cavitation. In lung transplant recipients, a distinct form of IA develops, characterized by necrotizing infection of the bronchial anastomosis. Ulcerative tracheobronchitis is the most aggressive form of invasive bronchial aspergillosis (IBA) and manifests with endobronchial plaques, nodules, or areas of ulceration and necrosis, which may extend to the adjacent pulmonary parenchyma and pulmonary vasculature. In fact, IBA is the most common infection within 3 months following lung transplantation, whereas IPA tends to occur much later in this patient population (25).

Once germination of conidia occurs in the lung, *Aspergillus* hyphae invade pulmonary arterioles and lung parenchyma, leading to ischemic necrosis. Hematogenous dissemination with thrombosis, hemorrhagic infarction, and invasion of distant organs (kidneys, liver, spleen, sinuses, and central nervous system) may result from invasion of arterioles by *Aspergillus* hyphae and is found in approximately one-third of cases of IPA at autopsy (72–74, 91).

Diagnostic approach for IPA. Timely diagnosis, especially for patients at high risk for IPA, is needed to improve disease outcome. Despite improvements during the last 20 years, finding an unambiguous molecular marker for early IPA diagnosis is one of the greatest challenges faced by scientists and clinicians today. Diagnosis of IA and discrimination from an infection without clinical syndrome and disease (colonization) or CPA remains challenging and imperfect and requires a combination of clinical, radiological, and microbiological features (25, 92, 93) (Fig. 6). The development of standardized diagnostic criteria for invasive aspergillosis has been a major improvement for IPA (94). However, a large proportion of IPA cases still are not detected by existing criteria, especially in nonneutropenic patients who possess atypical clinical and radiographic features of infection (95–97).

(i) Clinical and radiographic features for IPA diagnosis. Clinical and radiographic features for IPA diagnosis are subtle and nonspecific and become evident late in the course of the disease, especially in neutropenic patients (98). IPA may manifest with low-grade fever (which could be absent from patients receiving corticosteroids) triggered by lung inflammation, which may be followed by a mild, nonproductive cough suggestive of bronchitis. Pleuritic chest pain and progression to pneumonia occur as a clinical manifestation of angioinvasion and tissue necrosis induced by invasive fungal

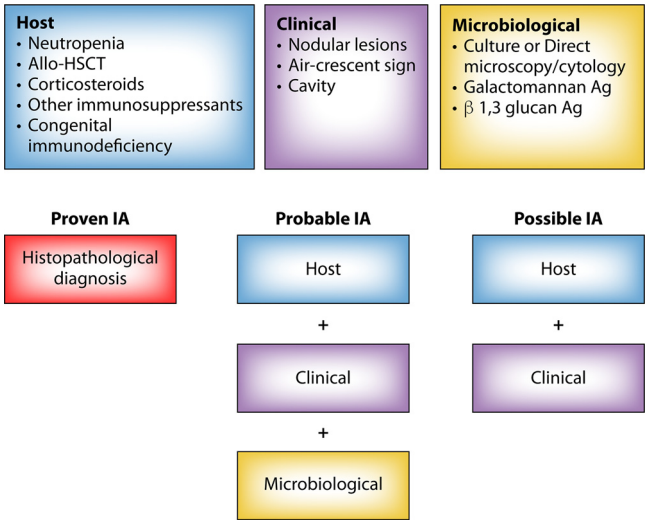

FIG 6 Standardized diagnostic criteria for invasive aspergillosis in hematological malignancy and in HSCT patients, based on data from reference 94. The concepts of proven, probable, and possible IA are introduced, depending on the presence of clinical and microbiological criteria (e.g., positive GM Ag). Adaptations of these criteria have been made for IA in nonneutropenic patients with ICU-related aspergillosis.

growth. Cavitation, the result of profound necrosis of lung parenchyma, tends to occur in patients if immunosuppression is reversed (25, 29). Cough, sputum production, and pleural effusion are either absent or minimal at the initial phase of the disease. Therefore, IA must be strongly considered in susceptible patients with respiratory disease that fails to respond to broad-spectrum antibiotics.

Chest radiographs are not sensitive enough to detect early forms of bronchopulmonary IPA, and up to 10% of patients with proven IPA have normal chest radiographs within a week of death (98, 99). High-resolution CT scans of the chest are recommended, since they may reveal a halo sign, which indicates hemorrhage and edema surrounding an infarct caused by thrombosis. This symptom is highly suggestive of acute IPA, especially in neutropenic patients with leukemia. However, the halo sign has been documented only in a limited number of patients. In addition, it is transient and initial halo signs may disappear within a week (93, 100, 101). With neutrophil recovery, these lesions cavitate, forming the "air crescent" sign, a classic sign of late filamentous invasive mold infection. Importantly, lesions frequently increase in size in the first 2 weeks of effective antifungal therapy. CT pulmonary angiography could represent a promising diagnostic tool with better performance than conventional CT for IPA (102). Nonetheless, clinical and radiologic presentation of IPA in the expanding group of nonneutropenic patients who develop infection is atypical, including cases with tree-in-bud opacities or diffuse ground glass appearance in CT (95). Novel imaging modalities combining the analytic capability of PET and magnetic resonance imaging (MRI) with *Aspergillus*-specific radiolabeled antibodies, termed immuno-PET/MRI, holds great promise to improve specificity and performance of diagnostics for IPA (103).

(ii) Microbiological diagnosis of IPA. Microbiological diagnosis of IPA remains a challenge. Respiratory cultures of *Aspergillus* from sputum, bronchial washings, or bronchiolar lavage (BAL) specimens have low sensitivity (<30%) for diagnosing IA but a high positive predictive value (>60%) in severly immunocompromised patients (93).

In contrast to immunocompetent patients, patients with IPA do not produce anti-*Aspergillus* antibodies as a result of the underlying immunosuppression. Detection of *Aspergillus* molecules circulating in the biological fluids is the only means to diagnose an invasive infection. The presence of large amounts of anti-*Aspergillus* antibodies indicates the presence of a latent, noninvasive form of *Aspergillus* infection (e.g., colonization or CPA) and should be monitored and evaluated radiographically. In-

creased antibody titer during the remission period of an infected patient would only indicate upregulation of an effective immune response to infection.

Despite significant advances in identification of surrogate markers for diagnosis of IA based on detection of circulating *Aspergillus* antigens, many issues remain unresolved after 20 years of research in this area. Specifically, the number of circulating biomarkers identified to date in biological fluids of IA patients is limited to 2 types of molecules: cell wall polysaccharide-based antigens (galactofuran-containing molecules and β1,3 glucans) and fungal DNA. The search for a circulating molecule that can serve as a disease biomarker is hampered by the following unresolved issues: (i) the low concentration of circulating fungal molecules, which requires detection by use of either a monoclonal antibody with high-affinity and -avidity characteristics (e.g., galactomannan [GM]) or by a process of signal amplification (β1,3 glucans and DNA), and (ii) an incomplete understanding of the kinetics of release of the various circulating fungal molecules. Biomarkers can be searched in serum urine, BAL fluid, and even sputum (104–106).

Galactomannan (GM) has been the antigen of choice for diagnosis of IPA since its discovery in the early 1970s. This has been due to the exquisite immunogenicity of the galactofuran side chain of the galactomannan, which has allowed the selection of monoclonal antibodies (MAbs) with high affinity and avidity. The method currently used is a sandwich ELISA based on a very efficient EB-A2 rat monoclonal antibody able to detect 0.5 to 1 ng of the galactofuran epitope (107, 108). This test is the most extensively used in clinics, has been validated in clinical studies, and has been endorsed in microbiological criteria for diagnosis of IA in all published guidelines (25, 93, 94). In addition, the decrease in GM values also can be used to monitor the efficacy of a treatment (109).

Initial studies evaluating the role of galactomannan serum assay in the diagnosis of IA have been conducted mainly in neutropenic patients undergoing chemotherapy for hematological malignancy or in recipients of HSCTs in the absence of antifungal prophylaxis. These studies have documented excellent sensitivity rates, ranging from 67% to 100%, and specificity rates, ranging from 86% to 99% (110, 111). When serially monitored, the galactomannan assay preceded the diagnosis of IA by an average of 6 to 14 days. However, in other studies the appearance of major lesions on high-resolution CT scans almost coincided with or even preceded the detection of the galactomannan antigen in serum (112). Furthermore, sensitivity rates as low as 30% can be seen in patients receiving mold-active antifungal prophylaxis, in pediatric populations, in nonneutropenic patients (including solid-organ transplant recipients), or in patients with low fungal burden, including those with invasive bronchial aspergillosis (110, 111, 113). Hence, sensitivity rates as low as 30% have been reported in nonneutropenic HSCT recipients receiving mold-active antifungal prophylaxis and in lung transplant recipients. However, in view of the lack of a gold standard for diagnosis of IPA, it is difficult to estimate the performance of the GM Ag test across different studies, which typically enroll a mixed population of patients with probable, possible, and proven disease. Recent studies showed that BAL fluid GM has improved sensitivity compared to serum GM in high-risk patients with IPA, including nonneutropenic patients (88, 114–119).

Initial studies have shown that the GM epitope recognized in the Platelia kit by the EB-A2 MAb consists of at least four β1,5-linked galactofuranose (Galf) units. Early studies also have shown that the MAb EBA2 binds to a galactofuranose epitope on the cell wall or secreted (galacto) mannoproteins of *A. fumigatus* (120). Recent data have indicated that the carbohydrate specificity of GM MAb is wider than that previously proposed, which could explain false-positive results of the assay. Even though this tetragalactofuran is the best epitope, the minimal recognized galactomannan fragment is a disaccharide, Galf-β1,5-Galf, which is recognized as the terminal part of the polysaccharide chain or as being within the internal part of the chain. In addition, no difference was observed between the isomeric pentasaccharides containing only β1,5 or alternating β1,5 and β1,6 linkages in their tetragalactofuranoside fragments. Ex-

panding the catalogue of epitopes recognized can explain the occurrence of some false-positive tests obtained with enzyme immunoassays (EIAs) using EB-A2 MAb (121, 122). Recently, other anti-galactofuran antibodies that seem more specific to longer β1,5-galactofuranose oligosaccharides have been produced (123), but the performance of these two MAbs requires clinical validation.

Another MAb recognizing a galactofuranose-rich peptidoglycan molecule that circulates in serum and other biological fluids of patients with IA has been selected (124, 125). The MAb recognizing this molecule has been used in an immunochromatographic point-of-care assay (lateral flow device [LFD]). The advantage of this LFD test is the lack of a boiling step in EDTA used for the detection of GM. However, the sensitivity is reduced 30-fold (detection threshold, 40 ng/ml) compared to that of the GM assay. In addition, the LFD test does not provide quantitative data. This test is used preferentially in BAL specimens (104, 126) and has several advantages over the GM Bio-Rad test: it is quick (15 min), it is easily adapted by routine microbiology laboratories, and it does not require a heat pretreatment of the serum. In view of the limited clinical data, comparative prospective clinical studies to determine the performance of the LFD versus the GM test are required before *in vitro* diagnostic (IVD) approval and commercial use. In addition, a recent meta-analysis (127) based on the eight retrospective clinical studies published since 2009 suggests that the LFD has a better performance in BAL samples than in sera. Importantly, the MAb has been adapted for immuno-PET/MRI (103, 125, 128).

Other proteins have been shown to be released *in vivo* in experimental models of IA. This is the case for small proteins (5 to 15 kDas) rich in disulfide bridges, which protect them from degradation in biological fluids (129, 130). The use of new systems based on the isolation of individual immune complexes on paramagnetic beads (131) may allow the use of proteins for the detection of aspergillosis.

The detection of β1,3 glucan, a fungal polysaccharide not specific for *Aspergillus*, is based on a very different approach (132–134). Detection of the circulating β glucans is a result of activation of a proteolytic cascade complex isolated from the horseshoe crab that is triggered by recognition of β1,3 glucans by factor G. This multicomponent cascade intensifies the detection due to several enzymatic steps, producing a chromogenic reaction. The sensitivity and specificity rates for this test have ranged from 67% to 100% and from 84% to 100%, respectively (133, 135). Several false-positive test results have been reported in patients with cirrhosis, in patients undergoing hemodialysis, in patients following abdominal surgery, and in those receiving chemotherapy with particular agents. Although the β1,3 glucan serum test lags in terms of sensitivity, specificity, and reproducibility compared to GM, it is considered an approved biomarker for diagnosis of IPA in all published guidelines.

The last circulating molecule considered for diagnosis of IPA is DNA. Many different protocols for PCR amplification have been published, but they remain heterogeneous, because they have been developed by specific laboratories (136–139) and have not been clinically validated (140). Two drawbacks of the PCR method for testing serum DNA is lack of sensitivity and of specificity. BAL specimen PCR is extremely sensitive but has issues of specificity because it cannot discriminate colonization from invasive growth. In contrast, PCR has value for diagnosis of CPA and IPA in BAL specimens.

PCR amplification targets either 28S RNA, the ITS1-5.8S region, or mitochondrial DNA. The DNA extraction protocol has been shown to be essential and is either manual or automated. Sources of the PCR samples are EDTA, blood, serum, or plasma. This is a very important issue, because the source of the detected DNA has not been localized, even though PCR detection was attempted $>$20 years ago (141). In contrast to viruses and bacteria, *A. fumigatus* cells do not circulate, and the origin and source of the DNA remains undefined. It is essential to know if the DNA comes from vesicles (exosomes), demonstrating that the release of the DNA is associated with active fungal growth, or if it results from the degradation of vegetative stages of the fungus. Although efforts were undertaken to standardize PCR protocols to be used for the detection of *Aspergillus* DNA in blood samples, the use of PCR remains controversial and has not been

included in the consensus IA definitions of the EORTC. Comparisons of PCR with antigen detection have been repeatedly undertaken (142). Meta-analyses have shown that the specificity of both GM and β1,3 glucan tests was significantly higher than that of PCR in serum samples. Recently, a combination of PCR/GM-EIA has been shown to be the most efficient diagnostic strategy, has reduced unnecessary antifungal therapy, and has improved fungal-free survival rates (104, 143).

RNA also has been discussed in the past, since the detection of fungal RNA, in contrast to DNA, indicates the presence of a growing, metabolically active fungus (144), but its use has to be pursued. Alterrnatively, microRNA has been proposed as a diagnostic tool (145). Another novel methodology is the detection of volatile compounds. A recent analysis of the volatile organic compounds of *A. fumigatus* showed that β bergamotene was released in higher concentrations *in vitro* and also was specifically identified in the exhaled breath condensates of patients with IA (146, 147).

Currently, there is not a single serological diagnosis which is 100% reliable for the diagnosis of IA (111, 148–150). A combination of diagnostic tests significantly increased the detection rate (109, 148, 151–153). Importantly, the performance of molecular diagnostic assays is hampered by the lack of a gold standard for diagnosis in the case of possible or probable IPA and the inability of these tests to distinguish between the different stages of the disease, from colonization to chronic noninvasive infection to IPA, all of which can coexist in the patient at risk.

TOOLS AND STRATEGIES TO STUDY THE PATHOBIOLOGY OF *A. FUMIGATUS* INFECTIONS

The Fungal Side

Thirty or forty years ago, the identification of a sexual stage would have opened the way to undertake classical genomic studies similar to the ones developed with *A. nidulans* (154). However, the development of genetic tools over the last 20 years has advanced molecular studies in *A. fumigatus*, obviating the need for classic genetic approaches based on sexual reproduction. Constructing a mutant of *A. fumigatus* is now easily accomplished using *ku80* and *ku70* backgrounds, which force the homologous recombination at the locus of interest and permit the use of electroporation (155, 156). Hygromycin is the best selection marker, but other markers, including the *PYRG* gene, pyrithiamine, phleomycin, or chlorimuron, have been used. This low number of selection markers can be compensated for by the use of recycling systems such as the β-Rec *six* system, developed by Krappmann's group, in which the use of the recombinase allows the insertion of one *six* copy inside the gene deleted and the possibility to reuse the same hygromycin marker (157, 158). The field lacks efficient conditional systems; most of them are leaky or use nonphysiological nutritional conditions, such as *ALCA* or *NIIA*, and cannot be used in animal models (159). However, the *TET*-on and *TET*-off systems are now operational to investigate the role of genes during infection in animal models (160, 161). Essential genes can be unequivocally identified by heterokaryon rescue (162). The system CRISP/CAS9 also has been adapted to *A. fumigatus* (163, 164) and allows one to manipulate clinical isolates. In the near future, a mutant library with all genes deleted will be available (M. Bromley, personal communication). However, this will not resolve the unannotated proteins (50% of total) encountered in the genome of the three sequenced strains (1163, 293, and Z5) of this species and the fact that only 5% of the annotated protein function has been verified. Annotation is complicated in that *A. fumigatus* is rich in protein families which have different functions in spite of similar sequences and in that the redundancy of certain gene families requires multiple deletions to assess a relevant phenotype (e.g., chitin synthase gene family) (165, 166). Transcriptome (167–170) and proteome (171–175) databases are becoming available and will be used to compare *omics* data obtained under different conditions. It should be noted that the genetic background of the parental strain is important and may complicate the *omics* studies, since parental fungal strains may differ in virulence (176).

The Host Side

An *in vivo* virulence screen of *Aspergillus* mutants is needed to evaluate the role of the corresponding genes in fungal disease. The mammalian animal models of IPA used to date to explore molecular aspects of *Aspergillus* pathogenicity have been developed largely in immunocompromised or transgenic mice. The animal models of IPA are imperfect and do not reproduce important physiological aspects of the infection. Specifically, they do not mimic the subacute infection course resulting from continuous exposure to very small amounts of inhaled conidia. However, the animal models have been useful in demonstrating the different pathophysiological mechanisms associated with various types of immunosuppression. In mice, a low *Aspergillus* tissue burden, extensive inflammatory injury, absence of angioinvasion, and localized infection have been observed in corticosteroid-immunosuppressed mice, whereas minimal inflammation, extensive angioinvasive *Aspergillus* growth, and disseminated infection are characteristic of IPA in cyclophosphamide-treated neutropenic mice (177–179). In corticosteroid-treated mice, host immune effector cells appear to have limited activity against *Aspergillus* hyphae, whereas chronic inflammation results in extensive alveolar damage and exudative bronchiolitis, which appears to be more important than the *Aspergillus*-induced injury (178). Apart from the mouse models of IPA, alternative minihost models of IPA in *Drosophila*, *Galleria*, and zebrafish have been used successfully to screen the virulence of mutants (180–184). Inhalation models of murine experimental aspergillosis rely on either immunocompetent mice using conidial exposures (107, 108), which are never reached in environmental situations, or immunocompromised mice with immunosuppression induced by either corticosteroids alone or cyclophosphamide and corticosteroids that results in neutropenia and severe immunodeficiency. In addition, these acute and lethal models of IPA (primary infection outcomes being animal survival of only a few days) do not permit the examination of the role of adaptive immunity in host defense. Establishment of invasive *Aspergillus* infection also can be attained in immunocompetent mice with intravenous administration of much lower inocula (10^5 to 10^7 conidia), bypassing the physiological route of infection, with the primary site of disease being the kidney (185). In immunocompromised mice, intranasal or intratracheal inoculation of conidia usually is undertaken with 10^5 to 10^6 conidia per mouse and is the favored mode of infection, since it leads to pulmonary infection that resembles IPA histopathologically. Aerosol infections in chambers also have been used (186) but have been abandoned because of their impractibility. Apart from studying IPA, establishment of other models of disease is important in order to elucidate host and pathogens' molecular attributes of pathogenicity. For example, a keratitis (eye) infection model in immunocompetent mice that largely depends on neutrophil-fungal interplay revealed the important role of anti-reactive oxygen species (ROS) enzymes in *Aspergillus* pathogenicity, while such enzymes do not favor fungal pathogenicity in the immunocompromised (e.g., neutropenic) mouse models of IPA (187). Lack of standardization in various parameters, including sex, age, and genetic background of mice (e.g., various strains of inbred versus outbred mice), makes comparison of results of virulence studies in mouse models of IPA very difficult, if not impossible. Bioluminescent strains have been constructed and can be used to analyze not only the dynamics of the infection process but also the variability in the outcome between individual mice (188). Finally, instead of survival as the only measure of fungal pathogenicity, other important parameters of infection outcome, including fungal burden and killing rates by innate immune cells, should be analyzed in immunocompetent mice. All of these different experimental conditions make comparison between studies very difficult to interpret.

Apart from dissecting fungal pathogenicity mechanisms, investigations in a mammalian host are required to understand the immune response during host-fungus interactions. Significant advances have been made in elucidating the main innate immune players and effector pathways for efficient control of *Aspergillus* infection in mouse models with the use of transgenic mice (189–202). Transgenic chronic granulomatous disease (CGD) mice having defects in NADPH oxidase components have been

used specifically to understand the pathobiology of aspergillosis. Unfortunately, transgenic mice are seldom used to analyze the virulence of mutant strains of *A. fumigatus* because of issues related to cost and availability. *In vitro* cell systems also have been employed, but the use of cell lines cannot be recommended, since they often give inadequate answers. Indeed, human cells have been the most appropriate for these pathogenic studies (203, 204). Studies of patients with primary immunodeficiency (e.g., chronic granulomatous disease, MonoMAC syndrome, and Card9 deficiency) and pharmacologically induced immunosuppression have been instrumental for understanding pathogenesis of IA (71, 205, 206).

PHYSIOLOGICAL IMMUNE RESPONSE TO *A. FUMIGATUS*

Innate Immunity

Physiologically, clearance of inhaled *Aspergillus* conidia occurs on a daily basis as a result of a multilayered, highly coordinated immune response that confers rapid pathogen elimination while preventing inflammatory lung damage (26, 205–210). Innate immunity at the level of the epithelial barrier and phagocytes (mainly alveolar macrophages and neutrophils) plays a key role in fungal eradication. It is increasingly recognized that cross talk between epithelia and phagocytes (211) and cell-to-cell interactions between different phagocytes (e.g., inflammatory monocytes and neutrophils) play a critical role in fine-tuning inflammatory responses to *Aspergillus* and in resolving infection (196).

Epithelial cells, a neglected portal of entry of *Aspergillus*. Lung epithelia comprise the first line of host defense during interaction with *Aspergillus* (212, 213). Due to their small size, some of the inhaled *Aspergillus* conidia can reach and interact with the lung alveolar epithelial cells (ECs) (214). Importantly, most inhaled conidia do not come in contact with the alveolar epithelium, as they are eliminated by mucociliary clearance (214). As opposed to bronchial epithelium, the alveolar epithelium comprises an extremely thin monolayer of type I ECs, which cover over 95% of the inner alveolar surface. Type I ECs are terminally differentiated cells in close contact with type II ECs, which secrete surfactant proteins. The role of the lung epithelium during *Aspergillus* infection has been insufficiently analyzed to date, and the published data are often contradictory. These discrepancies come from the use of epithelial cells of different origins (cell lines originating from type II ECs [e.g., A549] or bronchial epithelial adenocarcinoma, which are very different from primary ECs [204, 212, 213, 215, 216]) or the use of different mutants and morphotypes (resting conidia, swollen germinated conidia, or germ tubes) and the limited number of *in vivo* studies.

Few molecules have been identified to date in the initial interaction between the fungus and the epithelial cells: (i) the fucose lectin FleA, interacting with mucins favors mucociliary clearance (214), (ii) the suface invasin CalA, which interacts with $a_5b_1$integrin (217), (iii) the galactosaminogalactan adhesin binding nonspecifically to the epithelium (218), and (iv) a fibrinogen C domain-containing protein 1 (FIBCD1) binding to chitin (219).

Studies using cell lines suggested that ECs are capable of actin-dependent phagocytosis and efficient killing of *Aspergillus* conidia within mature ($LAMP1^+$ and cathepsin D^+) acidified phagolysosomes, with only a small (<3%) percentage of intracellular conidia being able to germinate and penetrate epithelia without lysis of the host cells (220). The Arp2/3 complex and the WAS-interacting protein family member 2 (WIPF2) are potentially responsible for internalization of conidia by airway epithelial cells (215). Nevertheless, intracellular survival via (i) phosphatidylinositol 3-kinase (PI3K)/Akt-dependent inhibition of apoptosis of infected cells and (ii) inhibition of phagolysosome fusion (221) have been proposed as mechanisms of intracellular persistence of the fungus even in the immunocompetent host (222). A recent study with the A549 cell line and mice identified a novel role of the pH-responsive transcription factor (TF) PacC in *Aspergillus* pathogenicity, linked to epithelial cell penetration. In this study, *A. fumigatus* conidia and germlings are internalized by epithelial cells in a Dectin-1-dependent manner, and *pacC* mutants, which aberrantly remodel the cell wall during germinative

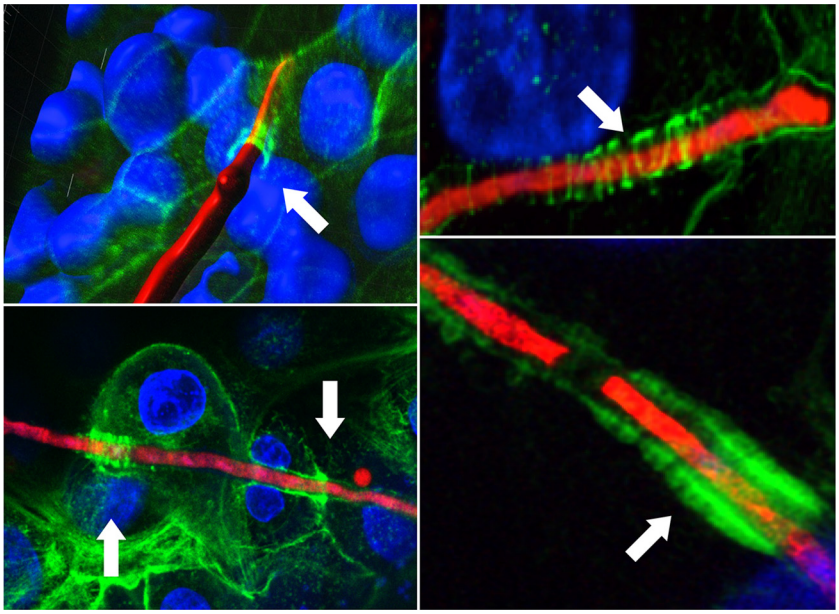

FIG 7 Crossing of the epithelial barrier by *A. fumigatus* germ tubes through an actin tunnel without disturbing the pulmonary lung epithelium. Note the actin sheath (see arrows) in response to the penetration of the germ tube, which occurs without perturbing epithelium integrity (adapted from reference 204 with permission from Oxford University Press).

growth, are able to enter efficiently into epithelial cells. Moreover, reduced epithelial cell wall damage due to defective protease secretion was observed with the *pacC* mutants (223). Mutations in CFTR receptors observed in cystic fibrosis were associated with defective uptake and killing of *Aspergillus* conidia by ECs and with subsequent deregulated inflammatory responses (224). Interestingly, defective phagosome acidification reported in $CFTR^{-/-}$ macrophages may underlie defective *Aspergillus* clearance by ECs (225). Importantly, recent *in vivo* studies in immunocompetent mice (226) and in primary human respiratory EC air-liquid cultures (204, 227) clearly demonstrated (i) the lack of phagocytic activity of ECs and (ii) the unique ability of germinating conidia to cross epithelia via an actin-mediated process in the absence of damage to the epithelial cell (Fig. 7).

Germinating conidia that escape from ECs or germinate on the epithelial surface trigger a host danger response pathway mediated by the release of antimicrobial effectors and alarmins to initiate inflammatory responses and timely neutrophil recruitment (212). Similarly, contact of germinating *Aspergillus* hyphae with bronchial epithelial cells selectively activates a p38/ERK1-2 pathway independently of MyD88/NF-κB to release interleukin-8 (IL-8) (228). The critical role of ECs in orchestrating *Aspergillus* immunity was convincingly shown by an elegant study of transient overexpression of *CXCL1* in lung epithelia of mice, which conferred protection in a neutropenic model of invasive aspergillosis (229). A critical role of IL-1α/IL-1R1/MyD88 signaling activation in lung-resident cells and ECs was associated with protective immunity against *Aspergillus* via early Cxcl1/Cxcl2-dependent neutrophil recruitment (191, 195). Interferon (IFN) types I and III, produced by ECs upon fungal recognition, could induce the recruitment of neutrophils (230, 231).

Even though the role of epithelial cells in the human setting has not been fully elucidated, epithelium as a portal of entry of *A. fumigatus* seems more important than was thought earlier. More work should be dedicated to the signals triggering actin recruitment on the epithelial cell. An accurate experimental setting to understand the role of ECs remains difficult to establish, because it should be (i) multicellular to specifically dissect cross talk of ECs and phagocytes and (ii) resume the important antifungal effector mechanisms mediated by bronchoalveolar fluid in order to inhibit

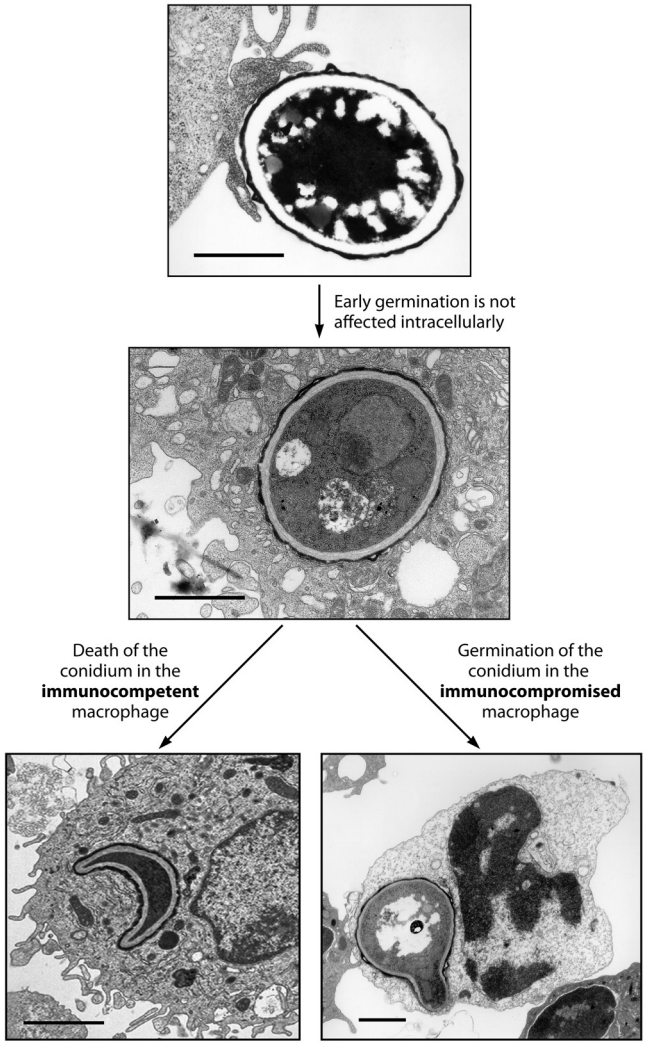

FIG 8 Phagocytosis of *A. fumigatus* conidia and intracellular fate of the conidia in the alveolar macrophage of an immunocompetent or immunocompromised host. Note the half-moon shape of the dead conidia inside the phagocyte (the middle and bottom panels are reprinted from reference 203).

hyphal overgrowth that is typically encountered in all of the existing models. Work on lung organoids with primary human ECs may help to better delineate the molecular features of epithelium-*Aspergillus* interplay (232, 233).

AMs: a major player in regulation of lung inflammation and killing of conidia. Alveolar macrophages (AMs) inside the alveolar lumen are the first immune cells to interact with inhaled conidia of *Aspergillus*. Apart from the major role of AMs in killing of conidia, these immune cells are endowed with unique anti-inflammatory functions essential for maintenance of immune homeostasis (206, 210). The different immunological functions of AMs are summarized in Fig. 8 and 9.

(i) Phagocytosis. Alveolar macrophages are professional phagocytes, characterized by a remarkable ability to engulf particulate material, ranging from resting conidia to apoptotic cells or even latex beads (234). Despite the lack of specificity of the phagocytosis process, certain macrophage receptors have been shown to facilitate engulfment of resting and germinating conidia: the C-type lectin II receptor DC-SIGN (CD209) (235), Dectin-1 (236), an unidentified macrophage glycoprotein-scavenger receptor recognizing the *Aspergillus* FleA fucose-binding lectin (214), and soluble pattern recognition receptors (PRR) that bind and opsonize conidia, including the long Pentraxin 3 (PTX3) (198) and the surfactant D (237). However, to date, the relative importance of

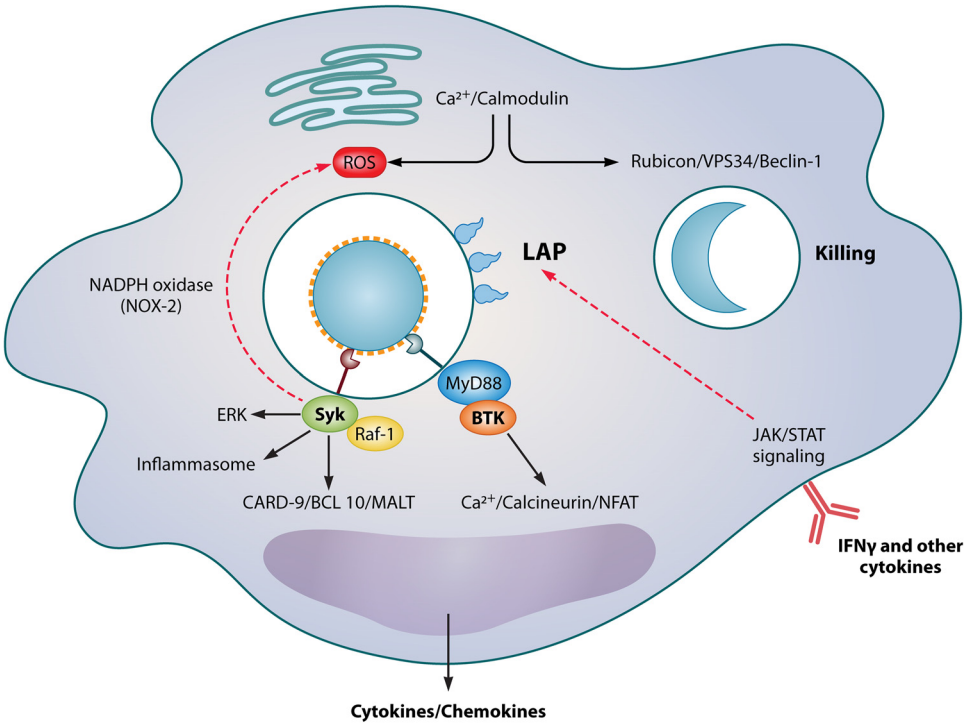

FIG 9 Major immune effector pathways in alveolar macrophages activated during phagocytosis of *A. fumigatus* conidia. Alveolar macrophages are the first immune cells that encounter *Aspergillus* conidia. Killing of conidia inside AMs occurs via NADPH oxidase-mediated activation of LC3-associated phagocytosis (LAP). Activation of Dectin-1 and other C-type lectin receptor signaling occurs during exposure to β-glucans and other immunostimulatory molecules on the cell wall surface of swollen conidia. This activation triggers Syk-dependent responses regulating (i) NADPH oxidase/ROS-dependent activation of LAP, (ii) the inflammasome, and (iii) CARD-9-, NF-κB-, and IRF1/5-dependent cytokine and chemokine induction. In parallel, (iv) Dectin-1/Raf-1 signaling activates NF-κB and (v) TLR/MyD88-dependent activation of BTK/calcineurin/NFAT signaling regulates TNF production and neutrophil chemotaxis, both via LAP-independent pathways. Certain cytokines (e.g., IFN-γ) modulate LAP and other macrophage responses via JAK/STAT-dependent or independent (e.g., DAPK1) pathways. A significant gap of knowledge exists regarding regulation of other antifungal effector mechanisms (e.g., antimicrobial peptides, iron homeostasis, and other nutritional immunity responses) independently of LAP, cytokines, and chemokines. Furthermore, the mechanisms of cross talk between macrophages and other myeloid cells in the physiological setting of granuloma formation is critical for infection control but remain completely uncharacterized.

these phagocytic receptors compared to nonspecific scavenger receptors in the phagocytosis of *A. fumigatus* is unknown.

(ii) *Aspergillus* phagosome biogenesis and mechanisms of killing by AMs. AMs efficiently eliminate phagocytosed *Aspergillus* conidia within 24 h in acidified phagolysosomes (203, 220, 238, 239). Initial studies on *Aspergillus* phagosome biogenesis demonstrated rapid acquisition of markers of the early (EEA-1, Rab5, and transferrin receptor) and late endosomal/lysosomal (Rab7, LAMP-1, CD63, and cathepsin D) pathways within 30 to 60 min of phagocytosis, which was regarded as a normal phagosome maturation process (203, 238). In contrast to the rapid acquisition of certain lysosomal markers, killing of *Aspergillus* conidia occurred after 6 h of phagocytosis and was associated with delayed phagosome acidification, intracellular swelling of *Aspergillus* conidia, and NADPH oxidase-dependent reactive oxidant species (ROS) production (203, 238). The killing of *Aspergillus* conidia was significantly compromised in AMs from mice with genetic defects in NADPH oxidase complex (p47phox$^{-/-}$) AMs and in wild-type AMs upon pharmacological or corticosteroid-induced inhibition of ROS (203). Although killing of *Aspergillus* conidia by AMs is undoubtedly dependent on both oxidative and nonoxidative mechanisms (240, 241), much of the discrepancy in the literature is related to differences in study endpoints (e.g., inhibition of growth versus killing), methodology of killing assessment (e.g., CFU plating versus conidial germination), moiety of infection, infection time points, NADPH oxidase knockout mouse strains, and method of immunosuppression used (203, 240–245).

Until recently, the molecular pathways regulating *Aspergillus* phagosome biogenesis were incompletely understood. The discovery of selective activation of Dectin-1/Syk signaling during germination of *Aspergillus* conidia due to stage-specific surface exposure of β-glucan (194, 246, 247) and the ability of PRR to regulate phagosome maturation (248) provided the conceptual framework of pathogen-associated molecular patterns (PAMPs) masking as a mechanism employed by fungi to escape macrophage killing. In line with this model, exposure of β-glucan and other immunostimulatory polysaccharides upon removal of the hydrophobic cell wall layer of rodlet proteins in *Aspergillus* conidia triggered Dectin-1 and Dectin-2 activation in both dendritic cells (DCs) and macrophages, resulting in potent neutrophil-dependent inflammatory responses and increased fungal clearance (249, 250). Furthermore, Dectin-1/Syk kinase signaling directly regulates maturation of β-glucan-containing phagosomes (251).

A noncanonical autophagy pathway, termed LC3-associated phagocytosis (LAP), links activation of certain PRR with phagosome biogenesis and inflammatory cytokine responses (252). LAP has an important role in immunity against *Aspergillus* (245, 253). Intracellular swelling of *Aspergillus* conidia and β-glucan-mediated activation of Dectin-1/Src/Syk kinase/NADPH oxidase signaling was a prerequisite for activation of LAP, phagosome maturation, and fungal killing (253). The physiological relevance of LAP in *Aspergillus* immunity is further supported by the ability of corticosteroids to block LAP via rapid inhibition of Src/Syk phosphorylation, a mechanism that explains the immunosuppressive action of these compounds on phagolysosomal fusion (254). Of interest, the role of LAP in immunity against *Aspergillus* (200, 245) has not been shown for other fungi (255–257). Melanin, which is a major component of the conidial cell wall, selectively inhibits LAP without interfering with Dectin-1/Syk signaling regulating cytokine responses. Physiologically, melanin-induced LAP blockade regulated fungal pathogenicity, since the melanin-deficient mutant restored its virulence upon conditional inactivation of Atg5 in myeloid cells *in vivo* (200). Of interest, melanin-induced LAP blockade depends on its Ca^{2+} binding properties and the ability of melanized conidia to inhibit a specialized Ca^{2+} calmodulin signaling pathway regulating LAP (239). Apart from LAP, identifying other phagosome biogenesis pathways regulating *Aspergillus* immunity should be exploited.

Notably, activation of LAP in macrophages also regulates inflammatory signaling pathways (258, 259). Canonical autophagy also regulates CXC-dependent neutrophil recruitment (257). In CGD, restoration of defective autophagy by pharmacological blockade of the IL-1 receptor (IL-1R) restored deregulated inflammasome-dependent neutrophil recruitment, Th17 inflammatory responses, and LAP, resulting in better control of invasive aspergillosis in mice (258). IL-37, another member of the IL-1 cytokine family, restricted *Aspergillus* immunopathology by preventing NLRP3-dependent neutrophil recruitment and detrimental immunopathology in murine aspergillosis in cystic fibrosis transgenic mice (260). Similarly, the anti-inflammatory activity of IFN-γ in CGD occurs via induction of the Ca^{+2}/calmodulin-regulated serine-threonine kinase DAPK1 in macrophages, which subsequently activates LAP parallel with the proteosomal degradation of the NLRP3 inflammasome (259). Thus, common anti-inflammatory mechanisms regulate phagosome biogenesis, conidial killing, and cytokine signaling in AMs following *Aspergillus* infection.

(iii) Activation of other signaling pathways by *Aspergillus* in macrophages. The full spectrum of signaling pathways regulating *Aspergillus* immunity is incompletely characterized. Apart from activation of Dectin-1, Dectin-2, and, recently, CD23 (261), C-type lectin receptors for *Aspergillus* β-glucans and mannans (206), a new C-type lectin receptor, MelLec, which specifically recognizes *A. fumigatus* DHN melanin, was identified recently in mouse endothelial and human myeloid cells (262). MelLec regulates inflammatory responses in human macrophages but appears to be not essential for killing of *A. fumigatus* conidia (262).

A newly identified signaling pathway in macrophages, following activation of Ca^{2+}-calcineurin-NFAT, regulates tumor necrosis factor (TNF)-dependent neutrophil

recruitment independently of Ca^{2+}-calmodulin-dependent LAP (239) and is defective in transplant patients receiving calcineurin inhibitors (202). Ca^{2+}-calcineurin signaling also orchestrates a novel process of lateral transfer of germinating conidia between macrophages via an actin-dependent process (201). Of interest, activation of Ca^{2+}-calcineurin-NFAT signaling is independent of Dectin-1/Syk kinase and MyD88 signaling, but it requires BTK kinase signaling activation downstream of TLR-9 and occurs upon conidial swelling in acidified phagolysosomes. BTK kinase regulates both NFAT and NF-κB signaling in macrophages (263) and has a nonredundant role in anti-*Aspergillus* immunity (80). Notably, treatment of patients with lymphoproliferative malignancies with inhibitors of BTK has resulted in a surge in cases of invasive aspergillosis (79, 264). TLR-9, the sole DNA-sensing endosomal TLR, is selectively recruited to *Aspergillus* phagosomes before conidial swelling independently of the presence of RodA, melanin, or fungal DNA, but the fungal cell wall component(s) that activate this receptor have not been identified (265).

Defective NOD-1/NOD-2 signaling in macrophages results in enhanced phagocytosis via upregulation of Dectin-1 expression without a pronounced effect in conidial killing and confers protection against invasive aspergillosis in mice and allogeneic HSCT patients (266, 267). Balanced activation of inflammatory responses followed the combined activation of the interferon-inducible protein AIM2 and NLRP3 inflammasomes in macrophages, and other myeloid cells in the lung play important roles in protection against invasive disease in a mouse model of infection with swollen *A. fumigatus* conidia (268). Of interest, inflammasome activation occurs via coordinated activation of C-type lectin receptors and TLRs following the release of fungal cell wall components liberated by the antifungal action of IRGB10 (269).

Activation of ERK signaling by *Aspergillus* resting conidia regulates killing and occurs via a pathway independent of TLR/MyD88 signaling (270). Similar to *Candida albicans*, activation of ERK signaling by *Aspergillus* may occur via C-type lectin/CARD9 and the RASGRF1–H-Ras downstream pathway (269, 271), although a CARD9-independent mechanism of ERK recently has been suggested (272). In humans, treatment of patients with solid tumors with ERK inhibitors resulted in development of invasive aspergillosis, illustrating the importance of mitogen-activated protein kinase (MAPK) signaling in anti-*Aspergillus* immunity (79). Better understanding of antifungal effector mechanisms in macrophages and their regulation by different signaling pathways is needed.

Circulating monocytes and mo-DCs. Other than resident lung macrophages, both $CCR2^+$ inflammatory monocytes and monocyte-derived dendritic cells (mo-DCs) mediate transport of *Aspergillus* conidia to the draining lymph nodes and initiate protective adaptive immunity (193). Importantly, inflammatory monocytes have a nonredundant role in innate defense against *Aspergillus* (192). Specifically, inflammatory monocytes differentiate rapidly to mo-DCs, which results in efficient uptake and killing of *Aspergillus* conidia *in vivo*. Furthermore, inflammatory monocytes regulate the conidicidal activity of neutrophils. In particular, $CCR2^+$ inflammatory monocytes are required for initiating a coordinated type I and type III interferon signaling pathway that is critical for optimal ROS production by neutrophils (196, 273). In humans, the classical $CD14^+$/$CD16^+$ human monocytes efficiently control growth of *Aspergillus* conidia (274) via Ca^{2+}/calmodulin-dependent activation of the LAP pathway (239).

Neutrophils. Undoubtedly, neutrophils are the most important immune effector cells against *Aspergillus*, since in humans, severe neutropenia is the major risk factor for development of invasive aspergillosis. Notably, neutrophil depletion within 3 h after *Aspergillus* challenge in immunocompetent mice results in lethal infections (275). Neutrophil chemotaxis through the induction of XCX chemokines is critical for control of *Aspergillus* infection. Accordingly, CXCR2-deficient mice that are defective in neutrophil chemotaxis are highly susceptible to development of invasive aspergillosis (242, 276). The different functions of neutrophils in fighting *A. fumigatus* are shown in Fig. 10.

(i) Neutrophil recruitment. In the lung of *Aspergillus*-infected mice, neutrophil recruitment occurs in two phases: an early phase via IL-1α/MyD88/IL-1R-dependent CXC chemokine release by lung epithelial cells and a late phase via a CARD-9-

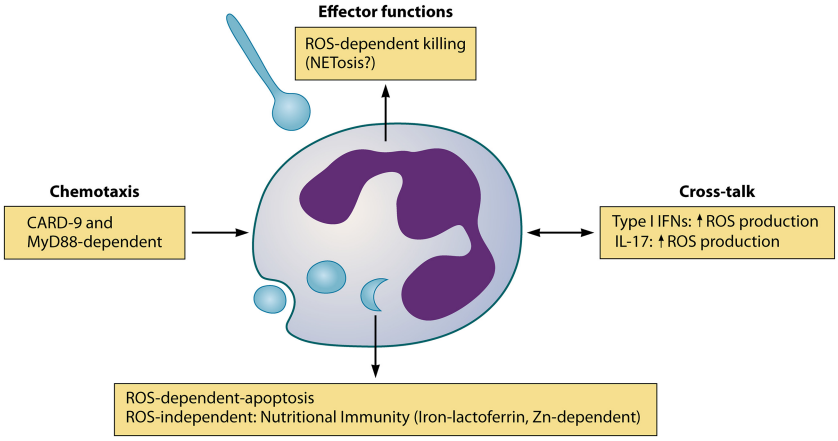

FIG 10 Neutrophils and their different anti-*A. fumigatus* activities. Neutrophil chemotaxis occurs in two waves and is regulated by (a) epithelium-dependent IL1R/MyD88 signaling and (b) inflammatory monocyte-mediated CARD9 signaling pathways. PTX-3 production by myeloid cells facilitates the anti-*A. fumigatus* killing by neutrophils. Neutrophils employ mainly ROS-dependent mechanisms of killing against intracellular (conidia) and extracellular (hyphae) fungal morphotypes. Intracellular killing of conidia is mediated by induction of apoptotic-type fungal cell death induced by ROS. Type I IFN and type III interferon signaling occuring via IFNLR1 in neutrophils is a pathway critical for optimal ROS production. IL-17A autocrine production via IL-6/IL-23 and Dectin-2 signaling is required for optimal ROS production. ROS-independent mechanisms of conidia leading to intracellular inhibition are mediated by Zn and Fe starvation by lactoferrin and other effectors. ROS-induced NETosis is another mechanism of inhibition of *Aspergillus* hyphae without a clear role in immunity *in vivo*.

dependent CXC chemokine response partially mediated by lung-infiltrating neutrophils (191, 195). CARD-9 signaling also regulates central nervous system (CNS) neutrophil chemotaxis, and patients with genetic deficiency of CARD-9 develop extrapulmonary CNS aspergillosis (199). Importantly, activation of the hypoxia inducible factor 1α (HIF1α) in lung-resident myeloid cells seems to be a master regulator of IL-1R-mediated early neutrophil influx in the lung during *Aspergillus* infection (211).

(ii) Effector mechanisms. These cells have unique effector mechanisms to kill *A. fumigatus*. Indeed, neutrophils utilize an array of different strategies, which are specialized against different infectious stages of the fungus, to combat *Aspergillus* infection. However, the recognition of the *A. fumigatus* morphotypes by neutrophils as well as the fungal molecules triggering the neutrophil degranulation are poorly understood. Specifically, murine neutrophils rapidly phagocytose and kill *Aspergillus* conidia *in vivo* via Dectin-1-, CARD-9-, and MyD88-independent signaling via pathways that depend on Syk-kinase signaling. Downstream NADPH oxidase-induced ROS production (191, 277) triggers activation of an ROS-dependent apoptosis-like cell death program in fungal conidia (189). In addition, neutrophils employ an array of nonoxidative intracellular and extracellular effector mechanisms that inhibit germination of *Aspergillus* conidia. These include the release of antimicrobial peptides (e.g., defensins and cathelicidin), neutrophil proteases (278), and nutritional immunity mechanisms via lactoferrin and lipocalin-1-mediated iron and fungal siderophore sequestration (279, 280). In addition, scavenging of essential nutritional elements (e.g., zinc and manganese) via calprotectin (190) and release of PTX3 (281) are additional oxidase-independent neutrophil effector antifungal pathways against *Aspergillus*.

ROS-mediated release of neutrophil extracellular traps (NETs), consisting of DNA filaments decorated with antimicrobial molecules, has been proposed as a putative protective mechanism against *Aspergillus*, particularly in the CGD setting. NETs are most readily induced upon genetic removal of *rodA* but are poorly induced by the conidia themselves (282). Moreover, the contribution of NETosis in killing of *Aspergillus* hyphae has a redundant role *in vivo* in the keratitis model (283) and requires additional clarification for systemic pulmonary infection (282, 284). The fungicidal activity of neutrophils is enhanced by an autocrine loop of IL-17A-induced ROS production by a

distinct population of neutrophils upon Dectin-2 activation (285). Of interest, the *in vivo* role of canonical autophagy and LAP in neutrophil anti-*Aspergillus* activity has not been characterized.

Importantly, a study on neutrophils obtained from patients with congenital immunodeficiencies revealed that human neutrophils are capable of discriminating and of mounting specialized immune responses against different growth stages of *Aspergillus* (286). In particular, human neutrophils inhibited germination of phagocytosed *Aspergillus* conidia via Dectin-1-independent, Syk kinase-independent, nonoxidative mechanisms, mediated via CR3 (CD11b/CD18)/PI3K signaling pathways (286), as opposed to the findings of *in vivo* studies in mice, which suggest an essential role of Syk-dependent ROS production in conidial killing by neutrophils (191, 277). In contrast, *Aspergillus* hyphal damage was strictly dependent on oxidative killing opsonization and Fcγ receptor-mediated signaling via Syk/PI3K/PKCα,β-induced ROS production and required functional NADPH oxidase and myeloperoxidase (286). In line with this study, a significant role of calprotectin-mediated zinc and manganese chelation in restriction of *Aspergillus* hyphal growth and infection control was evident in a model of fungal keratitis, whereas calprotectin was nonessential for intracellular killing of conidia in the mouse model of pulmonary aspergillosis (190). It was shown recently that CD11b regulates phagocytosis of conidia by neutrophils but not neutrophil recruitment in a mouse model of invasive pulmonary aspergillosis (287). In spite of their major importance in the *Aspergillus* defense, antifungal molecular mechanisms promoted by neutrophils have been insufficiently characterized due to the lack of physiologically relevant human neutrophil cell lines and molecular tools for genetic studies.

Other innate immune cells with nonredundant roles in *Aspergillus* immunity. Plasmacytoid dendritic cells (pDC), a rare dendritic cell subset specializing in antiviral immunity via TLR7/TLR9 sensing of nucleic acids and robust secretion of type I IFNs, was recently reported to play an essential role in immunity against *Aspergillus* (288). pDCs selectively attacked *Aspergillus* hyphae, inhibited fungal growth, and released cytokines (IFN-α and TNF) via a TLR7/TLR9-independent pathway mediated by Dectin-2 (289). Importantly, pDC-depleted mice were hypersusceptible to invasive aspergillosis without evidence of increase in fungal burden compared to normal infected mice, implying an important role of pDC in immune tolerance during fungal infection (288). In view of the major role of type I and type III IFNs for optimal ROS production by neutrophils (196), it will be important to define the role of pDC-neutrophil cross talk in immunity against *Aspergillus*.

Natural killer (NK) cells display IFN-γ-mediated direct cytotoxicity against *Aspergillus* hyphae and figure prominently in antifungal host defense in the setting of neutropenia (290–292). Invariant NKT cells (iNKT) also have a nonredundant role in control of invasive aspergillosis via indirect activation by IL-12-producing antigen-presenting cells upon Dectin-1 activation (293), whereas direct activation of iNKT cells by the fungal glycolipid asperamide drives the development of severe asthma (294).

Eosinophils also contribute to immunity against *Aspergillus* by exerting direct antifungal killing properties and by regulating IL-23/IL-17 inflammatory cytokine responses in the lungs (295, 296). Activation of platelets via sensing of melanin and GAG triggers the release of inflammatory mediators in *ex vivo* studies (297).

Essential components of humoral immunity against *Aspergillus*. Apart from extensive studies on anti-*Aspergillus* antibodies, which have shown that antibodies do not have any protective role in antifungal host defense, the role of other circulating humoral effectors has been incompletely analyzed. In particular, the long pentraxin 3 (PTX3), an innate humoral effector, is a key player in host defense against *Aspergillus*. PTX3, a soluble PRR released by epithelial cells and phagocytes, acts as an opsonin that selectively binds to the *Aspergillus* conidial surface and promotes phagocytosis via FcγRIIA (CD32) and CR-3-dependent mechanisms (198, 298). Notably, PTX3$^{-/-}$ mice are highly susceptible to development of invasive aspergillosis and display defective conidial uptake (198). The role of hypofunctional polymorphisms in PTX3 in COPD patients who develop colonization with *Aspergillus* and are prone to CPA has been

demonstrated recently (299). A strong direct binding of the PTX3 to the mycelium has been indeed shown (A. Inforzato and J.-P. Latgé, unpublished data). However, PTX3$^{-/-}$ mice also display major defects in tissue repair (300) and in phagosome biogenesis (301). Thus, the precise mechanism of susceptibility of PTX3$^{-/-}$ mice to invasive aspergillosis deserves further examination.

Members of the collectin family include mannan-binding protein (MBL) (302) and surfactant proteins SP-A and SP-D (303–305). A novel immune evasion strategy of *Aspergillus* is mediated via metalloprotease Mep1p that is present on the conidial surface and cleaves complement proteins, MBL, and ficolins (306).

T Cell and Adaptive Immunity against *Aspergillus*

Experimental data of acute pulmonary aspergillosis in mouse models and in patients with primary immunodeficiency with complete absence of cells of lymphoid origin demonstrate that adaptive immunity has a redundant role in protection from invasive disease (192, 196, 206). Both CD4 and CD8 T cells indeed have an essential role in mediating protective immunity against *Aspergillus* in HSCT patients and in mouse models (307, 308). In addition, adoptive transfer of *Aspergillus*-specific CD4 T cells confers protection against IPA (309, 310). Therefore, in the setting of innate immunodeficiency, T cell-mediated immunity becomes an important host effector pathway against *Aspergillus* (311, 312). Likewise, *Aspergillus*-specific T cells are generated in patients with IPA in response to specific antigens, and IFN-γ-producing T cells correlated with protection and favorable outcomes (308, 313) and have the potential to be used for immunotherapy (309). Notably, CD4$^-$/CD8$^-$ mucosa-associated invariant T cells (MAIT cells), an innate-like T cell type, are strongly activated following 4 h of stimulation of human peripheral blood mononuclear cells (PBMCs) exposed to *Aspergillus* conidia in a T cell receptor-specific way (314); however, the physiological role of MAIT cells in antifungal immunity has not been explored.

Antigen-presenting cells shape the development of *Aspergillus*-specific T cell responses (206). In mice, CCR2$^+$ inflammatory monocytes drive the development of adaptive immunity (193). Generation of Th1 and Th17 anti-*Aspergillus* immunity is regulated by TLR/MyD88 and Dectin-1 signaling, respectively. Calcineurin-NFAT signaling in CD103$^+$ DCs shapes optimal induction of Th17 responses in the lung via the production of IL-2 (315). In addition, defective calcineurin-NFAT signaling in CD11c$^+$ myeloid cells in the lungs, including AMs and DCs, results in defective PTX3 production and susceptibility to IA (197). Recent studies in humans demonstrated that *Aspergillus*-reactive Th17 cells are mainly derived from cross-reactive *Candida*-specific T cells (316). Notably, several *Candida* proteins (e.g., mannose protein Mp65) sharing cross-reactive immunogenic epitopes with *Aspergillus* proteins have been identified (316). Overall, a definitive role for Th17 cells in physiological anti-*Aspergillus* immunity has not been demonstrated.

Significantly, T cells play a critical role in immunopathogenesis of chronic noninvasive forms of *Aspergillus* disease, such as asthma, CF, ABPA (317), and possibly CPA. Specifically, *Aspergillus* antigen-specific T regulatory cells (Tregs) are critical determinants of immune homeostasis in the lungs, and asthma is associated with defects in their expansion, leading to aberrant Th2-mediated immunopathology (318). Importantly, *Aspergillus*-specific Tregs with proinflammatory properties can be induced in human cells (318, 319). Defective tryptophan catabolism in the CGD setting results in defective Treg activity and loss of the Th17/Treg balance driving lung immunopathology in response to *Aspergillus* (320).

A Th2/Th1 bias in ABPA patients that is partially dependent on the CR3 signaling pathway significantly contributes to immunopathology (321). In the case of Treg deregulation, the T cell response to *Aspergillus* allergens is then shifted toward a Th2 CD4$^+$ cell response (318). In addition, T cells, B cells, natural killer cells, and eosinophils may be hyperresponsive to IL-4, leading to a positive feedback amplification loop of Th2 CD4$^+$ cells, synthesis of IL-4, and polyclonal activation of IgE-producing B cells (321). Recent insight into pathogenesis of *Aspergillus* sensitization demonstrates the

central role of innate immune cells, including iNKT cells (294) and innate lymphoid cells (ILCs) of subtype II (ILC2) (322). Furthermore, the noncanonical activation of RelB in alveolar macrophages via polymorphisms in CARD9 signaling ($CARD9^{S12N}$) (323) induces allergy-related inflammation via the production of IL-33, IL-5, and IL-13 Th2 polarizing cytokines.

The role of the Th17 pathway is increasingly recognized in allergic lung disease pathogenesis (316). Specifically, in a mouse model of asthma, Dectin-1-dependent IL-22 production was important for disease development (324). In addition, the role of Th17 and IL-17 in severe steroid refractory asthma with neutrophilia has been demonstrated (46). Furthermore, *A. fumigatus*-specific Th17 cells are expanded in patients with CF, COPD, asthma, and ABPA, and increased IL-17 levels correlate with disease severity (316, 325, 326). In fact, the levels of *Aspergillus*-specific Th17 cells correlated with disease activity in ABPA and were normalized during therapy, whereas Th1 and Th2 *Aspergillus*-specific cells remained unaffected (316). IL-17-producing T cells drive pulmonary eosinophils and promote *Aspergillus*-specific Th2 cell expansion, which is a central pathogenetic event of allergic lung inflammation (327). Furthermore, IL-17-producing Th2 cell subsets participate in exacerbation of allergic asthma induced by *Aspergillus* (328). Notably, TNF production by lung DCs suppresses eosinophil- and promotes neutrophil-mediated airway inflammation in *Aspergillus*-induced asthma (329).

The mechanisms of T cell-induced immunopathology in chronic forms of *Aspergillus* infection (28) have not been evaluated in depth (330, 331). A proinflammatory phenotype of circulating monocytes of patients with CPA associated with increased expression of genes regulating IL-1 and IL-15 production (330), increased levels of neutrophil chemoattractant molecules, and decreased production of IL-10 by macrophages has been described in CPA patients (332). In parallel, PBMCs from CPA patients display a reduced ability to produce IFN-γ upon stimulation with *A. fumigatus* (332). Whether the proinflammatory phenotype of CPA represents intrinsic defects of innate immune cells or is an indirect effect of chronic immune deregulation as a result of alveolar epithelial dysfunction is currently unknown (332). Additionally, the signaling pathways regulating cross talk of epithelia, innate and adaptive immune cells, and lung immune homeostasis following physiological exposure to *Aspergillus* are underexplored yet vital to developing new strategies that suppress the rise in COPD.

FUNGAL VIRULENCE

In its natural environment, the soil, *A. fumigatus* represents a saprobe that has to survive many abiotic and biotic adverse conditions. Similarly, during the human infection process, *A. fumigatus*, like other fungal pathogens (724), has developed mechanisms to survive an aggressive environment and to fight stress-related changes in temperature, pH, water balance, oxidative damage, and antifungal host molecules. Moreover, the fungus has to access nutrients that are often not easily available. What are the strategies developed by *A. fumigatus* to acquire nutrients and to defend itself against host defense reactions?

Acquiring Food

Although *A. fumigatus* has evolved highly sophisticated homeostatic mechanisms to scavenge, take up, and store essential nutrients, nutrition also can diminish its virulence, since overload of some nutrients can be detrimental. In fact, blocking or overstimulating these mechanisms may modulate fungal virulence (333–336).

Carbon. For *Aspergillus*, the lung is a protein sponge that needs to be degraded to permit fungal growth. *A. fumigatus* is rich in a myriad of proteases that degrade protein and provide amino acids essential for both carbon and nitrogen acquisition (337, 338). Among these proteases, the most powerful are the alkaline protease Alp1 and the metalloprotease Mep1. However, a double *alp1 mep1* mutant remains virulent in murine models of aspergillosis, an indication of the compensatory activity of any of the plethora of proteases secreted by the fungus. The presence of any specific carbon

source is not required for *A. fumigatus* growth, since this fungus can access carbon from the degradation of proteins. Accordingly, *A. fumigatus* grows very well in a medium containing exclusively collagen or elastin, which are the major proteins of the lung, without any supplementation with carbohydrates (339). The small amount of hexoses in the lung is therefore not a limiting nutritional condition for this opportunistic pathogen. While the transcriptional repressor CreA, controlling carbon catabolite repression, is not required for the initiation of a pulmonary infection, it seems important for infection maintenance and disease progression under an environment that is poor in oxygen and in gluconeogenic carbon sources (340). However, CreA plays a major physiological role due to carbon catabolite repression and also mediates growth on various nitrogen and lipid sources, as well as playing roles in amino acid transport, nitrogen assimilation, and glycogen and trehalose metabolism (341, 342).

Nitrogen. In contrast to carbon, nitrogen starvation is a prominent host-imposed stress condition for *Aspergillus* during early stages of infection (343). Moreover, the maintenance of amino acid homeostasis is key to the survival of *Aspergillus* within the host, as disruption of the cross-pathway control mechanism via the deletion of the pathway-specific transcription factor CpcA increases sensitivity to amino acid starvation and causes attenuated virulence (344). Similarly, deletion of the *A. fumigatus* gene encoding the Ras-related protein RhbA, which plays a role in a nitrogen-regulated signaling pathway, reduces virulence (345). Enzymes involved in amino acid synthesis, such as methionine synthase or chorismate synthase (controlling the biosynthesis of all aromatic amino acids), are essential for *A. fumigatus* viability. Other amino acids, such as lysine, tyrosine, phenylalanine, tryptophan, and histidine, are important for *in vivo* growth, even though mutants in their biosynthetic pathways are viable *in vitro* (343, 346). Mutants lacking the GATA factor known as AreA in *A. fumigatus* also are less virulent than wild types in murine models of invasive pulmonary aspergillosis, suggesting that versatility in nitrogen utilization plays an important role in the pathogenesis of aspergillosis (347). However, AreA-like CreA controls many metabolic pathways, and it is difficult to associate the role of these TF mutants with specific aspects of amino acid metabolism. The biosynthesis of many amino acids remains unexplored in *A. fumigatus*, and such metabolism is worth studying, even though amino acid biosynthesis seems a difficult target to dissect *in vivo* due to their presence in the host. Alternatively, amino acid transporters that could be inhibited have not been investigated in *A. fumigatus* (348).

Water requirement. Water is needed for vegetative growth. However, joint gene deletions have shown that the two aquaglyceroporins and the single aquaporin of *A. fumigatus* identified *in silico* do not play any role in water entrance and in the control of osmotic turgor pressure (A. Beauvais, unpublished data). The influx of water and the mechanisms controlling water equilibrium in the cell remain totally unknown in filamentous ascomycetes, including *A. fumigatus*. Experiments are needed to establish whether the steady-state exchange of water molecules occurs by passive diffusion through the phospholipid bilayer via passage through membrane proteins or if the plasma membrane water exchange correlates with ATP-driven membrane transport activity by the electrogenic H^+ ATPase Pma1-like in yeast (349).

Divalent cations. Fungi, like all eukaryotes, require ions for enzymatic activity. These elements present in the lungs are in the parts per million range as free elements or are associated with proteins (e.g., calprotectin, ferritin, transferrin, lactoferrin, and calcium-binding proteins). The absence of the available elements, especially divalent cations, inhibits fungal growth. In response, *A. fumigatus* has developed sophisticated methods to obtain such elements from the host (334). An excess of these elements can be detrimental. For example, copper and iron are essential redox-active transition metals for *A. fumigatus*; excess transition elements via highly reactive oxygen species generated in the Fenton reaction may damage lipids, DNA, and proteins. Thus, this pathogen not only has developed systems to acquire cations but also needs to do so in a very controlled manner.

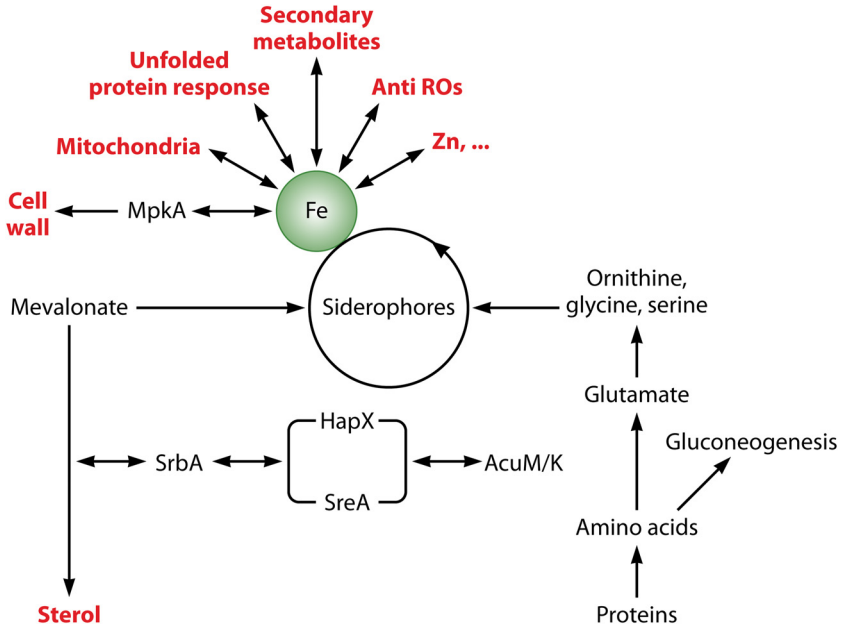

FIG 11 Iron metabolism as an example of the complex interactions occurring between multiple metabolic pathways.

(i) Iron. In *A. fumigatus*, siderophore-mediated iron uptake and high-affinity reductive iron assimilation (RIA) (350) are the two major iron uptake mechanisms identified to date (Fig. 11). *A. fumigatus* produces four siderophores: two extracellular fusarinine-type siderophores, fusarinine C (FSC) and its derivative, triacetylfusarinine C (TAFC), for iron uptake, as well as two intracellular ferrichrome-type siderophores, ferricrocin (FC) for storage of iron in hyphae and hydroxyferricrocin (HFC) for storage of iron in conidia. Iron acquisition from host tissues in *A. fumigatus* almost exclusively depends on the siderophore system. Blocking siderophore biosynthesis, mediated by the ornithine monooxygenase SidA, indeed rendered *A. fumigatus* avirulent (351). Deficiency in either extracellular (SidI, SidH, SidF, or SidC) or intracellular (SidC) siderophores only causes partial attenuation of virulence (352), indicating that lack of extracellular siderophores can only be partially complemented by RIA. Defects in the siderophore system decrease intracellular growth and survival after phagocytosis by macrophages and alters the phagocyte immune response (353, 354). Therefore, the siderophore system is crucial not only for extracellular but also for intracellular growth. Iron homeostasis and siderophore production are mediated by two counteracting transcription factors: SreA, which represses the siderophore system during iron sufficiency in order to avoid toxic effects (355), and HapX, which represses iron-consuming pathways during iron starvation to conserve iron (356). Other pathways independent of SreA or HapX also interact with iron metabolism. For example, the TF SrbA also activates siderophore-mediated iron uptake in response to hypoxia and iron starvation (357). Indeed, almost all biological functions are associated with iron metabolism in *A. fumigatus* and play some role in fungal virulence, gluconeogenesis, antigen secretion, unfolded protein response, the mitogen-activated protein kinase MpkA and signal transduction pathway, protein phosphatases, cell wall integrity, mitochondrial function, azole susceptibility, and even metabolism of other cations, such as Zn^{+2} (334) (Fig. 11). The essential role of iron suggests that inhibition of iron acquisition with the use of chelators is an alternative antifungal therapeutic strategy. However, despite initial success in preclinical studies, iron chelation therapy failed to improve the outcome of fungal disease in clinical trials (279, 358).

(ii) Copper. Everybody in agriculture and in the wine business knows that copper from the "Bordeaux mixture" has strong antifungal properties. However, it has been

shown that this other redox cation is necessary for *A. fumigatus* survival. Four high-affinity Cu^{+} importers of the Ctr family (CtrA1, CtrA2, CtrB, and CtrC) are responsible for copper uptake under Cu-depleted conditions in *A. fumigatus* (359, 360). Interestingly, *CTRC* deletion as well as combinatorial deletion of *CTRC* with *CTRA2* or *CTRB* resulted in hypersensitivity to iron starvation under copper-limited conditions, further highlighting the interconnection of copper and iron homeostasis. Three copper-binding TFs (AceA, MacA, and CufA) control copper homeostasis (359, 361–364). These TFs control not only the copper acquisition under copper limitation via activation of all Ctrs but also ROS detoxification genes, including copper-dependent superoxide dismutases and catalases. A defect in copper control leads to the accumulation of copper in the phagosome and to an increased killing by macrophages and attenuated virulence (362).

(iii) Zinc. After iron, zinc is the second most abundant transition metal in cells. Most TFs are zinc-dependent proteins, and almost 50% of all eukaryotic enzymes are zinc-containing proteins (365). Unlike iron and copper, zinc is redox inactive and, hence, does not mediate free radical-induced cellular damage but is nevertheless essential for fungal growth. The system developed by *A. fumigatus* to overcome zinc depletion is very different from the one used for iron. *A. fumigatus* possesses zinc importers as well as exporters to balance its zinc requirements (366). Eight genes that encode ZIP transporters (*ZRFA-H*) and eight genes that encode cation-diffusion transporters (*MSCA*, *ZRGA*, *ZRCA*, *ZRCB*, *ZRCC*, *MMTA*, *MTPA*, and *MTPB*) have been identified (366). In *A. fumigatus*, the zinc transporters ZrfA and ZrfB play roles under acidic, zinc-deficient conditions (367, 368). ZrfC is the major zinc transporter under alkaline conditions and counteracts the Zn/Mn chelating effect of calprotectin, a protein which provides major storage of Zn in living tissues (190, 369). The TF ZafA transcriptionally regulates the expression of ZrfA, ZrfB, and ZrfC (370). The *zafA* and *zrfA*/*zrfB*/*zrfC* mutants are unable to obtain zinc from the host and consequently are avirulent (279, 358). In addition to genes involved in the homeostatic response to zinc deficiency, ZafA influences the expression of many other genes involved in iron uptake and ergosterol biosynthesis, the adaptive response to oxidative stress, and the production of secondary metabolites (371). Importantly, the ZafA- and ZrfC-like proteins are distributed exclusively among fungi, and no orthologs have been found in mammals. Consequently, the ZafA and ZrfC proteins can be considered specific fungal targets, since zinc-chelating compounds can inhibit fungal growth both *in vitro* and *in vivo* (372–374).

(iv) Calcium. Calcium participates in the regulation of almost all fungal processes controlling growth and morphogenesis (375). The calcium-based signaling system is able to change the concentration of free calcium in the cytoplasm in response to environmental stimuli and especially in response to the fluctuations of the Ca concentration in the phagocyte phagolysosome during infection (376). Ca metabolism is controlled by a set of organized and sequential biochemical features involving calcium channels, pumps, calcium transporters, or binding proteins. High-affinity plasma membrane calcium channels Cch1 and Mid1 have been characterized (377). The fungal vacuole serves as a major storage compartment, alongside the endoplasmic reticulum, the Golgi apparatus, and mitochondria. Direct communication exists between these different storage organelles. A vacuolar calcium channel, YvcA, vacuolar Ca^{2+}-ATPases PmcA, PmcB, and PmcC, the Golgi apparatus-located Ca^{2+}/Mn^{2+}-ATPase PmrA, a mitochondrial Ca^{2+} uniporter, MucA, and a mitochondrial carrier protein, AgcA, all have been identified as playing essential roles in calcium efflux and influx in *A. fumigatus* (378–380). Deletions of the genes coding for these different transporters usually affect hyphal growth; susceptibility to oxidants, azoles, and cell wall drugs; cation homeostasis; cell wall integrity; septum localization; and virulence, thereby defining the central role of Ca^{2+} in *A. fumigatus* biology. The Ca^{2+} signal transduction mechanisms have been analyzed in *A. fumigatus*. They are similar to the ones found in other fungal systems and classically involve the Ca^{2+}-binding protein calmodulin, the serine/threonine phosphatase calcineurin, and the transcription factor CrzA. A *crzA* mutant is impaired in germination, cell wall integrity, and virulence, confirming the essential role of Ca metabolism in *A. fumigatus* life (381–386).

(v) Manganese and magnesium. Despite the fact that manganese and magnesium are essential for *A. fumigatus* growth and essential for bacterial pathogenesis (364, 387), it is striking to see that neither manganese nor magnesium homeostasis has been studied in this fungus. In bacterial pathogens, magnesium transporters such as MgtC constitute essential virulence factors. A eukaryotic homolog was found in *A. fumigatus*, but this MgtC homolog is not involved in magnesium metabolism and has no function in fungal virulence. It only suggests that horizontal transfer of bacterial genes can occur in *A. fumigatus* (388). This is another example of the necessity to be careful in considering *in silico* homologies and annotations. In yeast, CorA proteins have been associated with the transport of magnesium ions at the plasma membrane (389). CorA orthologous genes are Afu5g05830, Afu2g08070, and AFUB_101430. Orthologous genes of the yeast magnesium transporter Alr1 (Afu4g00930 and Afu2g08070) also have been identified in the genome of *A. fumigatus*. In baker's yeast, external manganese is taken up in yeast via the Nramp transporters Smf1 and Smf2, and an orthologous solute:proton symporter (Afu4g10990) also has been identified. Despite the presence of these numerous orthologs, their roles in the manganese and/or magnesium metabolism have not been investigated in *A. fumigatus*.

(vi) Phosphate. The metabolism of phosphate has not been investigated in *A. fumigatus*. This element is present at low free concentrations *in vivo* and is essential for fungal survival. In *A. fumigatus*, three phosphate permeases orthologous to *Saccharomyces cerevisiae* Pho84, active at alkaline/neutral pH, are upregulated during infection. Moreover, under phosphate limitation, the expression of neutral phosphatases is induced, while acidic phosphatases, including the major phosphatase Afu1g03570, identified *in vitro* when the fungus is grown in the presence of P_i at low pH, are downregulated (390). This is another example of the dependence of gene expression on the combination of pH and nutrient availability. A link between calcineurin and calcium metabolism, protein kinase A, and phosphate transport has been established during P_i acquisition (391). The role of the cyclin-dependent kinases, such as the homologs of the yeast PHO84/PHO80, has yet to be fully elucidated.

Fighting against Unfavorable Host Conditions

Adaptation to hypoxia *in vivo*. During growth in soil, compost heaps, or mammalian lungs, *A. fumigatus* encounters low oxygen concentrations. Sites of local tissue hypoxia are commonly observed in experimental models of *A. fumigatus* infection (356). *Aspergillus* itself can contribute to pulmonary hypoxia by inhibition of angiogenesis, thereby preventing neovascularization in damaged tissues (392). During infection, the continuous activation of the inflammatory response contributes to further development of hypoxia due to destruction of the pulmonary tissue as collateral damage (393). Therefore, aerobic *A. fumigatus* has developed systems to adapt to low-oxygen environments. Indeed, strain fitness in low oxygen correlates well with *A. fumigatus* virulence (176). Hypoxic fungal growth is under the control of the common hypoxia regulator Hif1α (211, 393), but it is also associated with other major cellular pathways, such as sterol and siderophore biosynthesis and mitochondrial respiration (357, 394–396). The sterol regulatory element-binding protein gene *SRBA* is induced by both hypoxia and iron starvation; it subsequently regulates genes involved in ergosterol and siderophore biosynthesis. Major transcriptional and metabolic changes have been identified during growth under limited oxygen concentrations (397–399). Specific enzymes and proteins associated with fungal virulence are produced during hypoxic growth (400–404). All of these data indicate that hypoxia is an essential environmental parameter for fungal growth *in vivo*. However, it remains to be determined if the low concentration of oxygen or the presence of a high level of CO_2 influences fungal growth (405, 406). Four β-carbonic anhydrases (CafA-D), which metabolize CO_2, have been identified in the genome of *A. fumigatus*, but their roles in fungal virulence await the construction of a quadruple Caf mutant (407).

Do pH changes affect fungal growth environment? *A. fumigatus* is not highly sensitive to pH changes, since it is able to grow at very low pH ($\sim$3.5) but also can

survive at very alkaline pH. The mechanisms which ensure that the cell senses and responds to sudden extracellular shifts have been analyzed in *A. nidulans*. The PacC family of transcription factors is responsive to an ambient alkaline pH. Two putative transmembrane pH sensors at the plasma membrane, the arrestin-interacting PalH and PalI, assist the proteins PalA, -B, -C, and -F via interaction with endosomal pathway components to induce proteolysis of the transcription factor. The cleaved transcription factor enters the nucleus to effect pH-dependent gene expression (408). In the case of *A. fumigatus*, this response to pH is essential, since the pH of the phagolysosome can be around 5 (409). Indeed, mutants in the PacC pathway are not pathogenic, and *pacC* null mutants assumed a compact colonial phenotype (223). That *A. fumigatus* resists low pH means that the low pH putatively encountered by the fungus in the phagolysosome is not a factor that counteracts fungal growth *in vivo*. However, PacC controls more than pH adaptation, since the *pacC* mutant has an altered cell wall associated with differences in susceptibility to cell wall drugs (223) (S. Raj, Z. Liu, M. Bromley, and J.-P. Latgé, unpublished data).

Resistance to heat. One of the virulence traits of *A. fumigatus* is its thermophilicity. This species grows better at 40 to 41°C (fever temperature) than at 25°C. However, no specific pathway controlling the thermophilicity of this species has been identified (410–412). Furthermore, no virulence factor of *A. fumigatus* has been linked to growth at fever temperature (7). Several metabolic pathways have been associated with the resistance to high temperature. For example, trehalose has been found as a major metabolite controlling the resistance to high temperature (413). The secretion of secondary metabolites such as trypacidin and gliotoxin has been shown to vary with growth temperature (414). The role of temperature in fungi has been better studied to understand heat shock rather than adaptation to constant high temperatures. Approximately 50 heat shock proteins (HSPs) have been reported in the genome of *A. fumigatus*, but these HSPs are chaperones mostly associated with resistance to stress rather than to heat (415–417). HSPs of small molecular weight, such as Awh11, Hsp30, Hsp20, and Hsp12, seem specific to both *A. fumigatus* and high temperature without being involved in *in vivo* growth, as seen with the Awh11 mutant (unpublished observations). The multigenic resistance to high temperature needs more investigation.

Resistance to ROS. ROS have been claimed to be essential in the defense of the host against *A. fumigatus* (2). The proposed role of ROS originates from CGD patients who have an impaired neutrophil cytotoxic response. The lack of a functional NADPH oxidase system results in failure to produce antimicrobial ROS (418, 419). Moreover, increased ROS production is associated with disease severity in CF patients (420). Unlike ROS, reactive nitrogen species do not have an impact on fungal virulence in immunocompromised murine aspergillosis models (187, 203). *A. fumigatus* has many systems which are able to counteract ROS. Among them are the enzyme catalases (421), superoxide dismutases (422), and the antioxidants thioredoxin (187, 423), glutathione (424), and their associated enzymes. The deletion of many anti-ROS enzymes as well as the several transcription factors which control the response to oxidative stress (NapA, RsrA, AtfA, Yap1, Skn7, and MybA) (421, 422, 425–428) do not result in a significant (if any) loss of virulence. This result suggests that corticosteroid-treated murine models which are characterized by phagocytes producing very low ROS amounts cannot be used to identify the role of fungal anti-ROS molecules during infection. In contrast, in an eye model of infection in immunocompetent mice where an infection is associated with active ROS production, fungal anti-ROS enzymes such as catalases and superoxide dismutases are important for fungal virulence (187).

The role of ROS is more complex than originally thought. The fungus has anti-ROS systems that also control the increase of intracellular ROS occurring when the fungus is stressed (429). For example, antifungals have an impact on fungal redox homeostasis by causing intracellular accumulation of ROS, and the induction of ROS production contributes to the ability of antifungals to inhibit fungal growth (430). Interestingly, the elevated toxic intracellular ROS levels produced by mitochondrial complex I in response to antifungals were abolished by the inhibition of the mitochondrial respiratory com-

plex I by rotenone. Similarly, superoxide dismutase mutants are susceptible to minute amounts of menadione, a compound used to induce superoxide ion production intracellularly in the fungus. In conclusion, it remains unknown if the anti-ROS systems developed by the fungus are more important to detoxify intracellular ROS produced by stress or to counteract extracellular toxic ROS produced by the host phagocytes.

Secondary metabolites: true virulence factor? Secondary metabolites often have been mentioned as playing a role in infection (431–434). Thirty-nine clusters (https://www.jcvi.org/smurf) have been identified (435), and the production of most secondary metabolites is controlled by LaeA, a central transcriptional regulator acting on chromatin structure. However, only a limited impact of the deletion of the LaeA was seen in experimental murine aspergillosis trials with a *laeA* mutant (436–438). In contrast, some secondary metabolite clusters responsible for the synthesis of ferricrocin (described above), DHN-melanin (described below), or hexadehydroastechrome (439) have shown an impact on fungal virulence in murine models of invasive aspergillosis, even though the molecules produced by the different clusters are not toxic *per se*. Toxic secondary metabolites produced by *A. fumigatus* (fumigaclavin, trypacidin, helvolic acid, gliotoxin, fumitremorgin, fumagillin, and pseurotin) do impact host cells (433, 440–443). How these toxic secondary metabolites affect virulence of *A. fumigatus* is not clear, as shown by experimental aspergillosis undertaken with mutants not producing targeted secondary metabolites (444, 445). Moreover, secondary metabolite toxicity usually is not assessed at the concentration produced *in vivo*. The putative synergistic manner of contributions of these metabolites with other fungal factors to virulence has not been investigated, and the effect of these secondary metabolites on the lung microbiota has not been investigated, since the production of certain fungal secondary metabolites can be induced or modified by the presence of other bacteria or by other members of the microbiota (446). More *in vivo* studies are required to assess the impact of secondary metabolites during infection.

Response to light. The role of circadian rhythms, which have been studied mainly in the model system *Neurospora*, are beginning to be analyzed in *A. fumigatus* (447). Light-induced and -repressed genes have been identified by transcriptome analysis, and the photoadaptation of this species to constant or short light stimuli has begun to be analyzed (448, 449). In contrast to that in *A. nidulans*, the asexual sporulation process was not regulated by light in *A. fumigatus* despite the presence of both LreA and FphA orthologs in the genome (448). Instead, LreA drove the synthesis of mycelial pigmentation, and FphA was required for germination, suggesting that light served as a stress signaling mechanism in *A. fumigatus* rather than having a developmental role. The response to light was, however, able to disclose strain heterogeneity in this species (450). Circadian rhythms also govern immune cell function and have an impact on recognition and clearance of bacterial or viral pathogens. Expression of the important fungal pattern recognition receptor as well as the phagocytic activity of macrophages against conidia of *A. fumigatus* was shown to be independent of light stimulation. However, the clearance of *A. fumigatus* from the lungs of infected mice is clearly influenced by the day or night time of inoculation (449), suggesting that other specific processes are responsible for the time-of-day differences in conidial clearance from the lung.

The Cell Wall: a Protective Coat and the Home of Virulence Determinants

The cell wall protects *A. fumigatus* from external aggression and is a major and unique organelle of the fungal cell. In the case of fungal pathogens such as *A. fumigatus*, it also plays an active role in infection, since it harbors components that are virulence factors influencing host response. Recent reviews have been published on the fungal cell wall, focusing on *A. fumigatus*, which now serves as a model to understand cell wall biosynthesis (26, 451, 452). The cell wall is composed of >90% polysaccharides, with overall composition varying with the morphotype analyzed (Fig. 12). The core skeleton is a branched β1,3 glucan linked to chitin, galactomannan, and β1,3-β1,4 glucans. Other polysaccharides, such as α1,3 glucan and mannans, act as a cement,

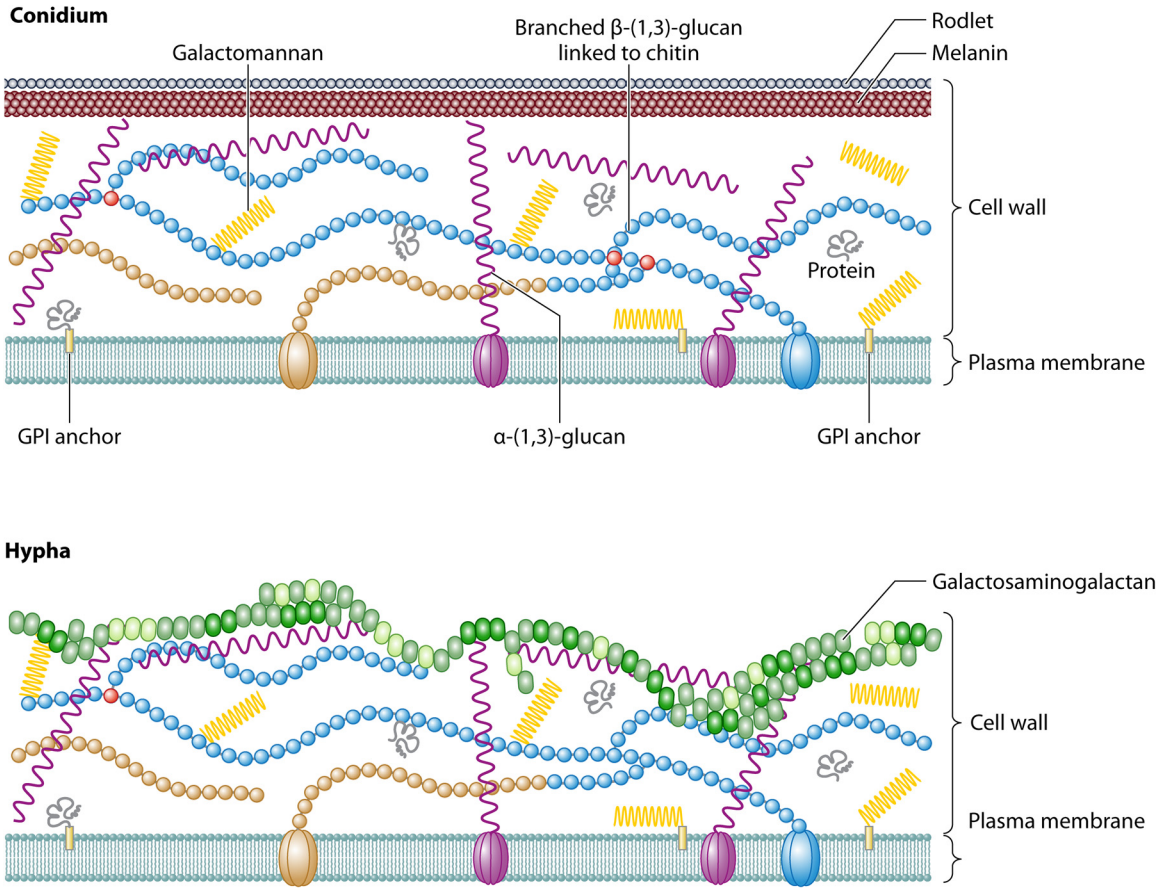

FIG 12 Schematic representation of the conidium and hyphal cell walls. Note that even though the major core polysaccharide components of the cell wall are the same in both morphotypes, the surface composition is different between the conidium and the mycelium, with rodlets and melanin on the conidium cell wall, whereas the mycelium is covered by galactosaminogalactan (GAG).

filling the pores between fibrillar polysaccharides (453, 454). In contrast to prior reports, recent surface nuclear magnetic resonance findings suggest that the association between α1,3 glucans and chitin is the skeletal core of the *A. fumigatus* cell wall, with β1,3 glucans being the filling material (455). Moreover, it is now known that the composition and structural organization of the cell wall depends on the position of the cell (apical, subapical, or distal) in the hypha but is altered in response to changes in the external environment (*in vivo* versus *in vitro* conditions, pH, antifungals, hypoxia, and microbiota) (456, 457). Cell wall is recognized as an organelle continuously changing over time; the roles these changes play in *in vivo* fungal growth are not fully understood. For example, the role of the wall stress sensors (Wsc1-3 and MidA) present on the hyphal surface during infection are unknown (458–461). In addition, various signaling pathways associated with these sensors have been implicated in the regulation of cell wall biosynthesis. Among these are the cell wall integrity/protein kinase C pathway, the Ca^{2+}/calcineurin pathway, a second MAPK cascade, the HOG pathway, and the pH-sensing PacC pathway (461–463). Our understanding of how the compensatory (such as chitin synthesis) or salvage mechanisms in *A. fumigatus* are integrated with the environment remains elusive. Many studies have analyzed the role of the major constitutive cell wall component β1,3 glucan in the immune response after the discovery of its recognition by the C-type lectin Dectin 1 (464). The other major cell wall component, chitin, also induces an immune response; however, the immune chitin receptor has not been identified, although the Fc-γ receptor seems to be important to induce a Syk kinase-dependent pathway (465). The immune roles of these cell wall components, common to all yeasts and filamentous fungi, have been discussed in many reviews and

will not be discussed here (466–470). In contrast, the other cell wall components specific to the *A. fumigatus* cell wall outer layer, which are melanin, rodlets, β1,3 glucans, and galactosaminogalactan (GAG), will be discussed below.

Melanin, α1,3 glucans, and hydrophobins of conidia protect the fungus against host defense. The outer layer of the conidium is composed of melanin covered by a rodlet layer that confers hydrophobic properties to *A. fumigatus* conidia, which is a prerequisite of their buoyancy in the air. However, the rodlet has puzzled immunologists, since it masks the recognition of the conidia by the immune system (249). This rodlet layer is exclusively composed of hydrophobins encoded by the *RODA* gene. This protein is characterized by the presence of eight cysteine residues capable of forming four disulfide bridges and organizing the protein crystal in an amyloid configuration that waterproofs the conidial cell wall (471). How the individual proteins aggregate to form a rodlet layer remains unknown. Point mutations in any of the Cys residues lead to the disappearance of the rodlet layer, indicating that the three-dimensional structure of the protein is absolutely required to obtain the rodlet configuration (472). Other point mutations in the hydrophobic residues of one or preferably the two amyloid regions (I115S/I146G point mutation) of the RodA protein have modified the size of the rodlet fibers and delayed the production of the rodlet, leading to partial coverage of the conidial surface by rodlets. Even though the rodlets are present in these mutants, the conidia of these mutants remain hydrophilic, like the conidia of the *rodA* mutant or the cysteine point-mutated mutant. The reason is that the conidial outer layer of these mutants is covered by a layer of glycoproteins responsible for conidial hydrophilicity. In contrast to the parental conidia, mutant conidia lacking disulfide bridges within RodA or expressing RodA carrying the double (I115S/I146G) mutation activated dendritic cells with the subsequent secretion of proinflammatory cytokines. Interestingly, the immune reactivity of RodA mutant conidia was not due to a modification in the RodA structure, since none of the mutated RodA protein or folded, unfolded, reduced, or nonreduced configurations of parental RodA protein failed to induce an immune response. The presence of different carbohydrate and protein molecules on the conidial surface, appearing as compensatory reactions to rodlet mutation, was responsible for dendritic cell activation. Another cell wall protein, CcpA, found on the surface of the conidium, also played a role in immunomasking the conidium (473).

The pigment located below the rodlet layer is the hydrophobic polymer dihydroxynaphthalene (DHN) melanin. Deletion of the genes in the melanin cluster has shown that the DHN melanin is important for the structure and stiffness of the conidial cell wall (452). However, the chemical structure of the full melanin molecule remains unknown due to the difficulty of solubilizing melanin. In a seminal study, Kwon-Chung and colleagues identified the different enzymes acting on the biosynthesis of melanin (474–476). It is now known that all of the genes involved in the production of the melanin molecule belong to the secondary metabolite cluster 33 in the SMURF database. However, it was recently shown (V. Aimanianda and J.-P. Latgé, unpublished results) that the first molecule on the pathway can autooxidize, producing an insoluble melanin pigment without any enzyme intervention. Loss of melanin in the *pksP* mutant results in the production of hydrophilic white conidia accompanied by the deposition of glycoproteins on the rodlet surface (477). A *pksP* mutant is less virulent than the wild type; melanin blocks NADPH oxidase-dependent activation of LC3-associated phagocytosis by inhibiting a master upstream regulatory Ca^{2+}/calmodulin pathway (200, 376). A specific DHN-melanin C-type lectin receptor that triggers immune activation has been found on host immune cells (262), but its role during infection is not yet clear.

The α1,3 glucan is located below the melanin. Similar to the *pksP* mutant, the *ags1-ags2-ags3* mutant, devoid of the cell wall α1,3 glucan, produces hydrophilic conidia covered by glycoproteins. The mutant was less virulent than the parental strain due to exposed glycoprotein pathogen-associated molecular patterns (PAMPs), which induces a higher sensitivity to killing by phagocytes (241).

In our search for a minimal cell wall, the successive deletion of *AGS1-3*, *RODA*, and *PKSP* (*apr* mutant) was recently achieved (472). Mycelial growth of this mutant was not

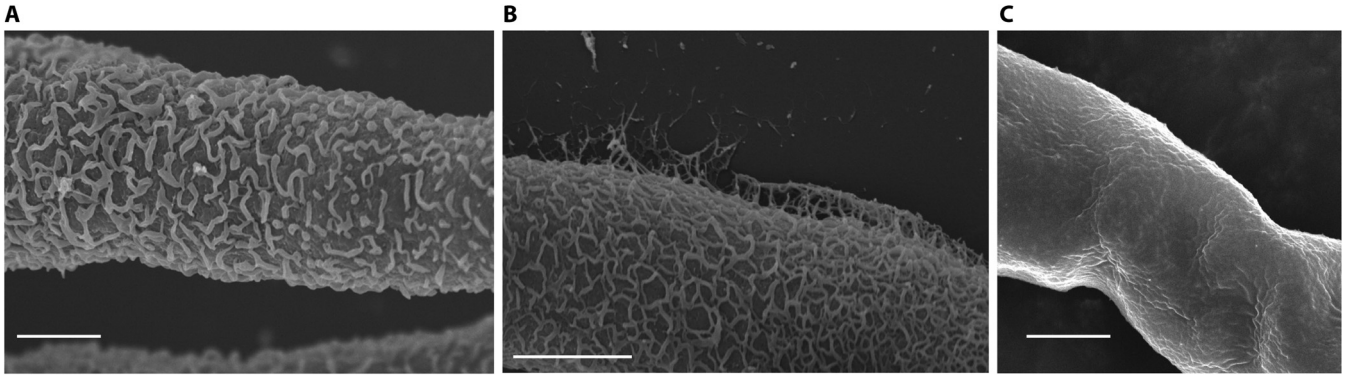

FIG 13 Galactosaminogalactan present on the surface of the mycelium (A and B) is responsible for adhesion to an inert surface (B) or to host cells. (C) Note the glabrous mycelium in a GAG-minus mutant.

affected, indicating that the alkali-insoluble fibrillar polysaccharides are the only essential polymers of the mycelial cell wall. However, the production of hydrophilic white conidia of the quintuple mutant was reduced, suggesting that the components of the outer layer are essential for normal conidiogenesis. However, the construction of the conidial outer layer is unclear, as are the compensatory reactions resulting from these multiple deletions (472). The conidial survival of the *apr* quintuple mutant is not affected, and conidia do not germinate more quickly than the triple *ags* mutant. Similarly, the susceptibility to the phagocyte defense reaction of the *apr* mutant is not higher than that of the *pksP* mutant, confirming the major role of the pigment in the defense reaction. In contrast, the joint deletion of the α1,3 glucan and melanin led to the activation of the synthesis of the galactosaminogalactan (GAG), which normally is found only in the mycelium of the parental strain. All of these single and multiple mutants are characterized by the emergence on the conidial surface of a layer of (glyco)proteins covering the rodlets, which is responsible for conidial hydrophilicity, even though the rodlets are present. The presence of these molecules on the conidial surface also is responsible for stimulation of the immune response (241, 477, 478). The complex metabolic network integrating the various compensatory reactions and rewiring remains unknown. Moreover, even though RodA tightly overlaps the α1,3 glucans and melanin, our recent data suggested that α1,3 glucan, melanin, and rodlets associate on the conidial surface without the establishment of covalent linkages.

Galactose-containing polymers, an insufficiently studied mycelial weapon. (i) The immunosuppressive adhesin galactosaminogalactan. The surface of the vegetative morphotype, the mycelium, is covered by a major polysaccharide, adhesin (absent from conidia) (Fig. 13). This polysaccharide is a galactosaminogalactan, which is a linear and water-insoluble heterogeneous polymer with an average size of 100 kDa, composed of galactose (in a pyranose form), galactosamine, and *N*-acetylgalactosamine (GalNAc) residues linked through α1,4 glycosidic linkage and in a random distribution along the polysaccharide chain (479). As with other fungal polymers, the synthesis of GAG involves the nucleotide sugars UDP-galactose and UDP-GalNAc. Both UDP-galactose and UDP-GalNAc serve as substrate donors for one or more specific glycosyltransferases to polymerize the GAG chain (480, 481). A putative α-glycosyltransferase from the CAZy family Gt4 has been shown to be essential to GAG synthesis (Fig. 14). The protein topology and the presence of 14 transmembrane domains suggest that this protein is localized at the plasma membrane and is the GAG synthase or one member of the GAG synthase (481). However, to date, no *in vitro* activity of GAG synthase has been demonstrated; the way this protein makes these different types of linkages in GAG is unknown. Several enzymes associated with a putative GAG synthesis cluster in chromosome 3 have been identified recently (480, 482, 483). However, their role in the synthesis or remodeling of GAG has not been fully elucidated. GAG is an adhesin involved in biofilm formation (450) that binds to epithelial cells and helps germ tubes

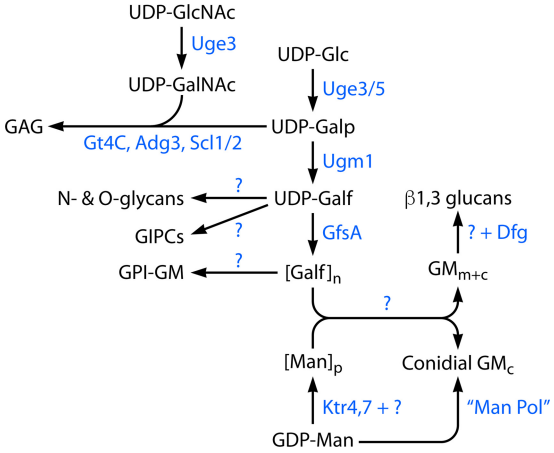

FIG 14 Galactose-containing molecules and their synthesis, an understudied pathway that is essential for *in vivo* fungal growth. GM, galactomannan; GAG, galactosaminogalactan; Ugm1, UDP galactospyranose mutase; Uge3/5, UDP glucose epimerase; GfsA, galactofuranosyltransferase; Gt4C, Scl1/2, and Adg3 are members of the biosynthetic cluster for galactosaminogalactan; Dfg, orthologs of the *DCW5/DFG1* pathway of yeast involved in the binding of the GM to β1,3 glucans; m, mycelium; c, conidium; ManPol, a complex of 11 mannosyltransferases involved in the synthesis of the conidial mannan (based on data from references 158, 452, 479–482, 488, and 493), indicates the unknown members of this biosynthetic pathway.

cross through actin tunnels (204, 218). GAG facilitates binding of hyphae to epithelial cells, macrophages, neutrophils, and platelets (297). It binds to pentraxin 3 (PTX3), an essential anti-*A. fumigatus* component of the humoral system (A. Inforzato, T. Fontaine, and J.-P. Latgé, unpublished). It masks PAMP exposure and the subsequent release of CXC chemokines regulating neutrophil recruitment (482). In addition, GAG has direct immunosuppressive properties: it triggers polymorphonuclear neutrophil (PMN) apoptosis via an NK cell-dependent mechanism (484) and inhibits PMN chemotaxis (485). In addition, cell wall-associated GAG mediates resistance to NADPH oxidase-dependent neutrophil killing and increased resistance to neutrophil extracellular traps (485). In mouse models of invasive aspergillosis, GAG inhibits Th1 protective antifungal immunity and skews immune responses toward Th2 to facilitate fungal infections (260). In human PBMCs, GAG dampened protective Th1 and Th17 cytokine production by selectively inhibiting IL-1 bioactivity via the induction of IL-1Ra (260). In a model of pulmonary aspergillosis in immunocompetent mice, GAG-induced increases of the fungal burden were largely dependent on the induction of IL-1Ra and subsequent blockade in PMN chemotaxis. These results demonstrated that IL-1Ra has an important role during *A. fumigatus* infections and support the concept that GAG induction of IL-1Ra has important clinical consequences (260). Within the complex GAG, the oligosaccharide responsible for the immune induction of IL-1Ra is an oligogalactosamine with at least 6 osamine units (725). Another cluster very homologous to the GAG cluster located in chromosome 4 is highly expressed *in vivo*, but its function is currently unknown (J.-P. Latgé, unpublished results).

(ii) Galactomannan, a galactose polymer with incompletely characterized biological and immunological properties. Galactose also can be present in *A. fumigatus* in the form of galactofuranose (Galf), the five-membered ring form of this hexose. It is present either as a galactomannan (GM) bound to cell wall glucans or is anchored to the membrane by glycosylphosphatidylinositol (GPI) (485) or as a single residue at the terminal, nonreducing end of a few proteins (120, 486, 487). The Galf side chains of galactomannan are synthesized from UDP-Galf (488). Galactomannan is stepwise assembled during its transit through the secretory pathway (489) (Fig. 14). The biosynthesis of galactomannan is likely involved in the synthesis of a galactomannan-anchor precursor in the endoplasmic reticulum, followed by further elongation in the Golgi complex and covalent binding to β1,3 glucans (490).

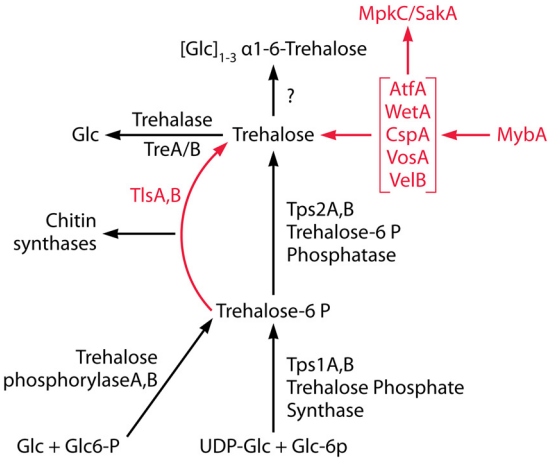

FIG 15 Trehalose biosynthesis, a key pathway for survival of *A. fumigatus*. In red are the regulators (transcription factors) controlling trehalose biosynthesis (*Glc*, glucose). Note that trehalose biosynthesis has not been shown biochemically but is deduced from gene deletion or from *in silico* analysis (based on data from references 428, 497, 499, 508, and 510).

Even though GM circulates in the human body during infection and galactofuranose metabolism has been shown to be essential in the virulence of many bacteria and parasites (491, 492), its immunological function has been poorly investigated in *A. fumigatus*. This polysaccharide induces a Th1 response in the presence of PBMCs (P. Robinet and J.-P. Latgé, unpublished results; S. Wong, V. Krylov, D. Argunov, A. Karelin, J.-P. Bouchara, T. Fontaine, J.-P. Latgé, and N. Nifantiev, submitted for publication) but at concentrations much higher than circulating titers. In addition, it has not been evaluated in the immunosuppressive situation. However, galactofuranose oligosaccharides seem nonessential for pathogenicity during infection, since galactose-minus mutants are as pathogenic as their parental strains (488). In contrast to galactofurane, the mannan moiety of the galactomannan molecule is essential for fungal survival and virulence. Among all the genes coding for mannosyltransferases, it is now known that mannan synthesis is under the exclusive control of Ktr4 and Ktr7, two out of three members of the family homologous to the KRE2 family. The synthesis of the *A. fumigatus* mannan is very different from the mannan synthesis in yeast, another example of major differences occurring between yeast and molds, even though *in silico* analysis predicts almost identical sequences in the mannosyl transferases in both fungal groups (158, 493).

Some Unexplored Biological Features of *A. fumigatus*

Quiescence and dormancy. Although the mycelium of *A. fumigatus* is very short-lived in the laboratory, conidia can survive for many months at room temperature and as ascospores for years. This survival capacity and extreme resistance to environmental insults is a major biological characteristic of this fungal species. The genes responsible for conidial quiescence in this species and for their survival have been investigated only partially, mainly by transcriptomic and proteomic analysis (410, 494, 495). Conidial quiescence has been associated with cytoplasmic viscosity and ergosterol levels and the occurrence of heat shock proteins (496) and other chaperones, as well as osmolytes, such as mannitol and trehalose. Trehalose, a disaccharide formed by a 1-to-1 glycosidic bond between two α-glucose units, plays an essential role in the induction and maintenance of quiescence, since trehalose-deficient mutants are unable to survive (497–499). Two trehalose biosynthesis pathways have been identified in *A. fumigatus* (Fig. 15). The first pathway consists of two main enzymes: trehalose-6-phosphate synthase (encoded by Tps1A-B) and trehalose-6-phosphate phosphatase (encoded by Tps2A-B). Tps1 converts UDP-glucose and glucose-6-phosphate (G6P) into UDP and trehalose-6-phosphate (T6P). T6P is converted by Tps2 into trehalose and into free

inorganic phosphate (P_i). The second pathway uses a trehalose phosphorylase enzyme (encoded by genes for TrepA-B) that converts glucose-1-phosphate (G1P) and glucose into trehalose. This noncanonical pathway of trehalose biosynthesis has not been fully characterized. Even though the studies are scarce, it seems that mannitol and other polyols are not involved in controlling conidium survival (500). Early electron microscopy studies also have shown that the conidia of *Aspergillus* are rich in glycogen and in lipids (501). This association with lipids fits recent transcriptomic data, which have shown that glyoxysome was the only enriched gene ontology (GO) term in an RNA-sequencing (RNA-seq) analysis of the conidia of three *Aspergillus* species (363), but deletion in the genes controlling the biosynthesis of these reserve nutrients has not been undertaken. Functional classes related to adaptation to the intracellular oxidative state, such as the oxidation-reduction process, oxidoreductase activity, including catalase and superoxide dismutase, and response to oxidative stress, were found in all *Aspergillus* species and are also supposed to play a role in conidial quiescence (422, 494). Many mutants that are affected in cell wall biosynthesis after deletion of chitin synthases or mannosyltransferases (158, 166, 452) have conidia with reduced survival during storage. More generally, the deletion of several transcription factor genes involved in conidiogenesis, such as *MYBA*, *ATFA*, or *WETA*, or in regulation of G protein signaling, impair conidial quiescence (427, 428, 498, 502). However, all of these mutants have been insufficiently analyzed to identify the mechanisms of initiation of quiescence in *A. fumigatus*. Such a molecular definition of quiescence could have a major impact on therapeutic intervention.

Mechanisms controlling the termination of quiescence are also unclear. Conidia of *A. fumigatus* do not germinate or swell in distilled water. Early transcriptome analyses have shown that the conidia contain one-third of the genome with active transcripts and display a silent fermentative metabolism (410, 503). These data suggest that resting conidia, like a time bomb, are ready to explode and germinate as soon as they encounter a favorable aqueous environment (one which is nutrient and oxygen rich) without any need of *de novo* transcription to initiate germination (504, 505). Accordingly, recent RNA-seq identification of the transcripts regulated during germ tube emergence showed that the genes upregulated during germination are highly similar to those observed in vegetative mycelium (170, 503, 505).

In contrast to conidia, the ascospores, which are the sexual propagules of *A. fumigatus*, enter a timed dormancy program. The viability of ascospores increased with the age of the fruiting body, being maximal after 20 weeks. Breaking this dormancy requires a thermal shock. *A. fumigatus* ascospores are able to germinate after treatment at 70°C for 90 min (10), while conidia are killed after exposure at 65°C. Most of the studies have been performed with *Neosartorya fischeri*, a species very closely related taxonomically to *A. fumigatus*, which produced ascospores abundantly, in contrast to *A. fumigatus* (506). In this species, ascospores can survive at 85°C in an aqueous environment for more than 10 min or for more than 7 days in a dry state at a temperature of 60°C (507, 508). *Neosartorya* ascospores are characterized by a thick cell wall, low water content, high viscosity, and the accumulation of protective compatible solutes (496, 500, 509). High cytoplasmic viscosity slows down the metabolic rate; therefore, it produces only small amounts of reactive metabolites, such as deleterious oxygen radicals. Synthesis of trehalose-based oligosaccharides composed of a trehalose core with 1 to 3 α-1,6 glucose units, with a putative antioxidant function, have been identified (508, 510). These account for three times the concentration of trehalose, but the enzymes responsible for their synthesis have yet to be identified. Establishment of dormancy is a two-phase process. The first phase includes the accumulation of compatible solutes (from a total of 0.45 to 1.0 M), a large increase of viscosity, disappearance of bulk water, acquisition of stress (especially heat) resistance, and an increase of redox stability. In contrast to conidia, the polyol mannitol, present in the same amount as trehalose, is one of the essential solutes for the maintenance of dormancy (500). The deletion of the mannitol 1-phosphate dehydrogenase gene (*MPDA*) responsible for the production of mannitol led to aborted ascospores (500). The second phase is charac-

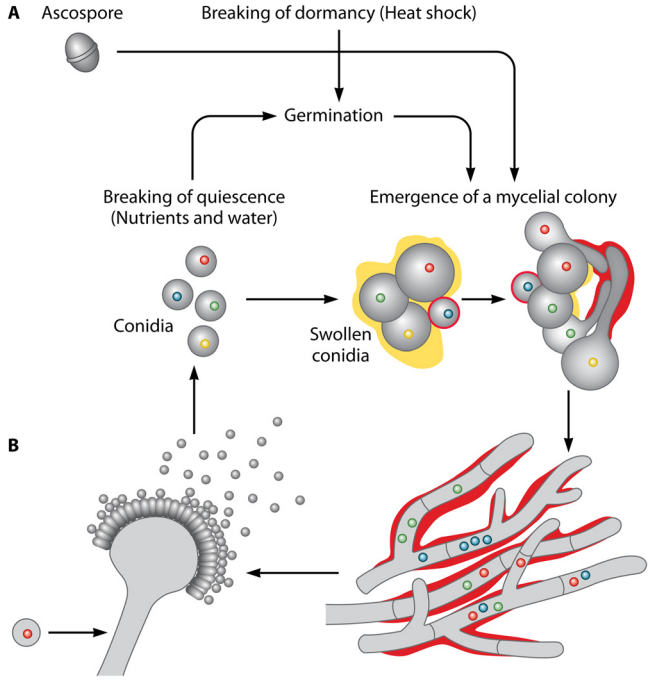

FIG 16 Schematic representation of key biological characteristics of *A. fumigatus* essential for the survival of the species. (A) Quiescence of resting conidia and dormancy of ascospores; role of extracellular polysaccharides (α1,3 glucans in yellow and galactosaminogalactan in red) in the formation of a fungal multihyphal biofilm. (B) Colony heterogeneity of nuclear organization, schematized by different colors of the nuclei, leading to the absence of synchrony in this organism that may result from genetic and/or epigenetic changes occurring during nucleus divisions within the expanding mycelium.

terized by a decrease of mannitol and an increase of trehalose and trehalose oligosaccharides, accompanied by a further increase of redox stability (511). Although the intracellular composition and biophysical parameters of the ascospores have been analyzed, the structural organization of the ascospore cell wall and its role in permeability have not been studied. Preliminary data have shown that the composition of the cell wall of the ascospores is indeed very different from that of the conidial and mycelial cell walls, since they are extremely rich in chitosan (A. Neiman and A. Beauvais, unpublished).

What are the molecular mechanisms leading to quiescence and dormancy in *A. fumigatus*? In plants, the induction of seed dormancy is controlled by a diverse group of regulators involved in seed maturation, hormonal action, dormancy, and chromatin regulation (512). Concerted actions of TFs play an essential role in the regulation of seed maturation and the phase transition from embryo to seedling. Moreover, several genes controlling seed dormancy have been identified (513, 514). Finally, a number of chromatin factors are required for a proper regulation of seed dormancy (512, 515). Antioxidants also play a pivotal role in the regulation of dormancy (516). In *A. fumigatus*, very few studies have been undertaken. Even though the conidia of at least two TF mutants have lost their capacity to enter into quiescence (M. Blatzer, I. Mouyna, M. Bromley, R. Beau, and J.-P. Latgé, unpublished data), triggering and other mechanisms controlling fungal quiescence and dormancy remain unknown.

Cellular heterogeneity of a colony. Although they originate from a conidium with a single nucleus, *Aspergillus* cells in a colony are heterogeneous (Fig. 16). Heterogeneity in growth, secretion, and RNA composition can be found between and within zones of colonization, and the quantitative composition of the secretome of the different zones differs (517, 518). Heterogeneity in protein secretion is accompanied by heterogeneous gene expression in the colony (519). In *A. niger*, at least two populations of hyphae at the periphery of a colony can be distinguished by their transcriptional and translational

activity. In an analysis of gene expression in individual hyphae, up to 300 genes were differentially expressed (520). Heterogeneity in gene expression between hyphae is a surprising finding, considering that a fungal mycelium originates from a single nucleus, ensuring that similar nucleic material is embedded in each nucleus after mitosis. In addition, hyphae share a cytoplasm due to the presence of porous septa that allow cytoplasmic streaming. However, apical compartments of growing hyphae behave unicellularly, while older compartments have a multicellular organization and stream material between cellular compartments due to the heterogenous and reversible closure of septa by Woronin bodies (521). Moreover, epigenetic processes are involved in heterogeneous expression of hyphae (520). Although heterogeneity has been studied mainly for biotechnological purposes within *A. niger* and *A. oryzae* (517), cellular heterogeneity also has been reported in *A. fumigatus*, in which significant differences exist in germination, cell wall organization, and survival of progeny conidia originating from the same parent conidium (R. J. Bleichrodt, P. Foster, G. Howell, J.-P. Latgé, and N. D. Read, submitted for publication; F. Danion, A. Dufour, A. Beauvais, and J.-P. Latgé, unpublished data). The concept of nuclear autonomy is now accepted for multinucleate organisms (522–525) but has not been investigated in *A. fumigatus*; potentially, it plays an essential role during infection or the emergence of drug resistance. Epigenetics in this organism should be analyzed, especially since the genome of *A. fumigatus* has been shown to be rich in epigenetic markers and in DNA modification enzymes (http://www.aspgd.org). In addition, if the reproduction of this species is predominantly clonal and the sexual stage rarely encountered in nature, could it be that the main source of genetic diversity and gene flow in the genus *Aspergillus* arises from a parasexual cycle (3–5)?

Biofilms. *A. fumigatus* grows as a multihyphal colony (Fig. 16). These hyphal communities are often called biofilms because cells are in contact with each other, as in a classical bacterial or yeast biofilm. It is a unique and relevant morphotype for *A. fumigatus*, which also allows the survival of the mycelium in spite of environmental stresses due to the presence of persister cells in the colony (456, 526, 527). However, to date, no signaling pathways associated with hypha-to-hypha contact have been identified. In colonies, an extracellular matrix (528) surrounds the three-dimensional hyphal structures characteristic of a colony, and its adhesive properties hold adjacent hyphae together (456, 529, 530). The composition of the ECM has been analyzed mainly *in vitro*. Extracellular matrix levels increase during the maturation of *Aspergillus* biofilms (529, 531). Initial studies of *Aspergillus* biofilm matrix content utilized biofilms grown under aerial-static conditions (529, 532). The matrix was found to contain mainly polysaccharides (galactomannan, α-1,3 glucan, and galactosaminogalactan), proteins, and small amounts of polyols, lipids, DNA, and melanin (452, 479, 486, 533). The ECM composition varied between studies that were performed under different experimental conditions and media. Major secreted antigens were detected in the matrix (533). As in biofilms of other fungal species, extracellular DNA has been identified but has not been consistently found. The origin and role of this extracellular DNA in the construction of the colony remains unknown (529, 534, 535). Importantly, *Aspergillus* biofilms are more resistant to antifungal drugs than are planktonic cells (415, 529, 535–538), but again, the reasons for a reduced permeability to drugs has not been explored in detail.

Aspergillus biofilms have been found *in vivo*, using aspergillomas from both human and murine models of invasive pulmonary aspergillosis (456). *A. fumigatus* extracellular matrix produced in biofilms *in vivo* contained galactomannan, α1,3 glucans, and GAG (in relatively much higher levels than those *in vitro*). The biofilm serves as a barrier against the detrimental effects of the host defense response and/or of antifungal drugs. However, many questions remain unanswered. Does the presence of biofilm impact the production of secondary metabolites, since it is known that environmental conditions play a key role in secondary metabolite synthesis (539, 540)? What is the role of host DNA and proteins in the organization of the *Aspergillus* matrix? The addition of exogenous material (DNA and proteins) resulted in greater structural integrity of the carbohydrate matrix (541). What is the exact role of the biofilm in the formation of the

granuloma? Finally, is distinguishing between the terms "colony" and "biofilm" only a semantic problem in *Aspergillus* since, in contrast to yeast (542), a multicellular colony is the hallmark of filamentous fungi? However, it is unknown if specific signaling pathways control the communication between neighboring hyphae (like in a true yeast biofilm) and subsequently influence colony morphology.

***A. fumigatus* lives in habitats containing mixed microbial populations.** For many years, the healthy lung was considered a sterile organ. It should be noted that adult human airways have a surface area of 70 m^2 (which is 40 times larger than the surface area of the skin), that we inhale 14 m^3 of air daily, and that this air is rich in microorganisms (bacteria, fungi, including *A. fumigatus*, and viruses) (543). The sterile-lung dogma has been challenged with the application of culture-independent genomic methods. Today, it is accepted that populations of bacteria, fungi, and viruses coexist in the lungs of healthy as well sick patients (543–545). In the air, concentrations of bacteria and fungi in the air that reach the lung are 10^5 to $10^6/m^3$ and 1×10^5 to $2 \times 10^5/m^3$, respectively (546–548).

Analyzing lung microbiota remains a technical challenge, since it is difficult to avoid aspiration or experimental contamination when identifying the "true" inhabitants of the lung (549). Most lung microbiota studies have focused on bacteria without accounting for the virome and mycobiome components (546, 550–552). In spite of the limited number of published studies, it is clear that a diversity of microbiota is synonymous with healthy lungs; in these samples, *Prevotella*, *Veillonella*, and *Streptococcus* were the predominant bacteria, with only a minimal contribution from potential pathogens (545). In healthy lungs, it is believed that the microbiota is a community of mostly transient microorganisms that are derived from the upper respiratory tract. Alternatively, patients with chronic respiratory diseases support thriving resident microbial communities (543, 553).

Lung microbiota studies that discuss *Aspergillus* diseases have been conducted mainly on samples from patients with noninvasive forms of aspergillosis, including patients with cystic fibrosis (45), asthma, allergic bronchopulmonary aspergillosis (33), and chronic obstructive pulmonary disease (COPD). Nonetheless, it has been increasingly realized that the majority of invasive aspergillosis patients have bacterial and viral coinfections that suggest a putative role of microbiota in these populations (24). Cystic fibrosis infections are frequently polymicrobial, with *Pseudomonas*, *Staphylococcus*, and *Burkholderia* being the genera of the pathogenic species most frequently detected; the low species diversity is associated with decreased lung function and increased inflammation (554, 555). The fungal biota is indeed complex in CF patients and includes mostly members of *Eurotiales* (*Aspergillus* and *Penicillium*), *Candida*, and, surprisingly, *Malassezia*, a skin inhabitant that appears to be more ubiquitous than originally described (550, 556, 557). In addition, these studies have revealed that the composition of bacterial and fungal communities varies greatly among patients.

The composition of the lung microbiome in asthmatics differs significantly from that of healthy controls (558). Accordingly, modulation between airway dysbiosis and allergen response has been associated with the worsening of allergy symptoms (559, 560). Even though *Aspergillus* is recognized as a major contributor to fungal-sensitized asthma, no study has correlated the severity of ABPA symptoms to a specific lung microbiota.

A. fumigatus is now recognized as a major component of the microflora of COPD patients. Early studies (23, 561, 562) have shown that *A. fumigatus* can be isolated from up to 20% of COPD patients. Common bacterial colonizers in a stable COPD state are *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*. Bacterial colonization in COPD patients is more dynamic than that in other respiratory infections. However, to date, increased *Aspergillus* titers do not correlate to specific changes in the bacterial and/or viral lung microbiota in COPD patients.

Most lung microbiota studies are descriptive and list the different microbial communities, but they have not addressed the role(s) that specific microbiota play in triggering aspergillosis. Recent data have suggested that the pulmonary microbiota can

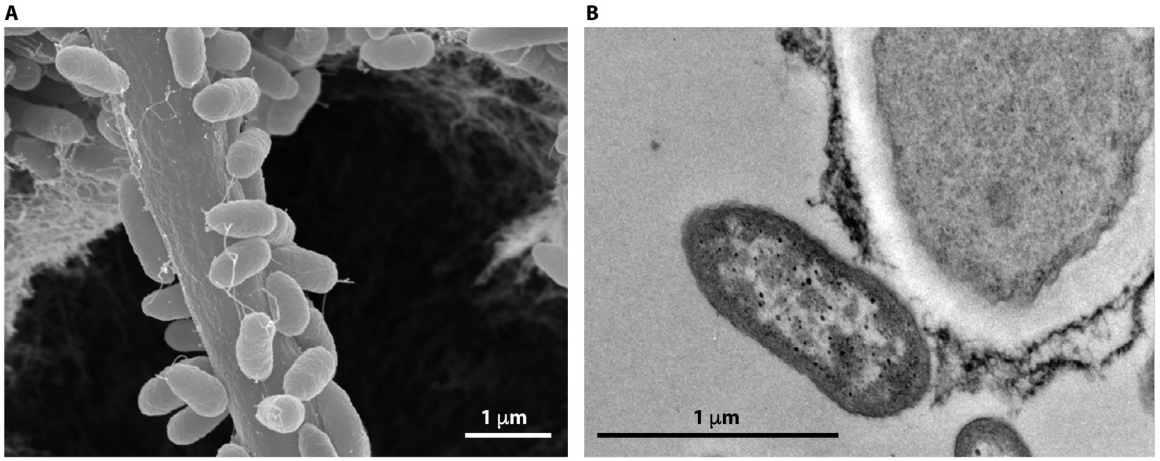

FIG 17 Binding of *Pseudomonas aeruginosa* to the mycelium of *Aspergillus fumigatus* (adapted from reference 572).

impact the establishment of aspergillosis infections. For example, risk of infection is higher with concurrent infection, such as with CMV or flu viruses (85, 88, 563–565). Mechanistic insight into the pulmonary microbiome on the immunopathogenesis of *Aspergillus*-related lung diseases is emerging (566, 567). The analysis of the impact of the soil microbiota on *A. fumigatus* potentially could identify factors important for fungal survival and growth in a hostile environment. This information could lead to the discovery of true virulence factors for fungal growth in the lung.

Studies aimed at understanding the molecular interactions between members of the microbiota with *A. fumigatus* (Fig. 17) have been focused on *Pseudomonas aeruginosa* and *A. fumigatus*. It is known that *Aspergillus* and bacteria coinhabit CF-infected pulmonary tissue. *A. fumigatus* has been isolated in 60% of the CF patients with *Pseudomonas* infections (568, 569). Under *in vitro* and *in vivo* conditions, *P. aeruginosa* releases molecules such as homoserine-lactones, phenazines, siderophores, and quinolones that interfere with the growth of *A. fumigatus* (570–572). Most studies have found that these bacterial molecules inhibit the fungal biofilm (573–576). Homoserine-lactones reduce the growth of *A. fumigatus* (570), while the effect of the other signaling molecule, 4-quinolone (577), has not been tested against *A. fumigatus*. Four phenazines (pyocyanin, phenazine-carboxamide, phenazine-carboxylic acid, and 1-hydroxy-phenazine) are produced by *P. aeruginosa*, and recent studies have shown that their effect is strictly dependent on the nature of the molecule and the environment (572). Most of the effects of the phenazine are deleterious except at low concentration, where pyocyanin, phenazine-carboxamide, and phenazine-carboxylic acid are able to reduce Fe^{3+} in an iron-starved environment and to favor *A. fumigatus* growth. In contrast, at high concentrations, the four phenazines can penetrate into fungal cells and induce the production of reactive oxygen species (specifically $O_2^{\cdot -}$) and reactive nitrogen species, leading to fungal death (572). The superoxide dismutase produced by Sod2 is essential for *A. fumigatus* to resist the antifungal effect of bacterial oxidants. In addition to the intracellular production of ROS, 1-hydroxy-phenazine has a supplementary chelating function which is responsible for iron starvation associated with growth inhibition (572). Two other molecules produced by *P. aeruginosa*, the siderophores pyoverdine and pyochelin, are strong chelators of divalent cations and inhibit fungal growth by deprivation of iron and zinc from the external medium. Like phenazine, pyoverdine and pyochelin are able to penetrate the fungal cells and induce detrimental ROS species, especially under hypoxic conditions (578, 579). In addition to molecules able to both chelate cations and induce intracellularly damaging ROS, *P. aeruginosa* also secretes rhamnolipids (F-diRhls), which specifically inhibit the fungal β1,3-glucan synthase activity (580). These rhamnolipids induce the formation of short, multibranched, chitin-rich, thick-walled hyphae. Phenazines and rhamnolipids were

detected in the sputum of cystic fibrosis patients at concentrations that impact the growth of *A. fumigatus in vitro* (572, 581, 582), suggesting that these inhibitory molecules have a true role during pulmonary infections. If the impact of bacterial molecules on the growth of *A. fumigatus* has begun to be understood, the influence of *A. fumigatus* on *Pseudomonas* has been almost totally neglected to date. However, secreted fungal glycoside hydrolases can affect bacterial biofilm (583), and other fungal metabolites can impact bacterial quorum sensing and c-diGMP signaling (584). Recent unpublished studies have shown that some *A. fumigatus* transcription factors either positively or negatively affect bacterial growth in mixed biofilms (J.-P. Latgé and M. Bromley, unpublished data).

The above-mentioned water-soluble bacterial molecules have a rather negative influence on the fungus. In addition, *P. aeruginosa* produces volatile chemicals (VOCs) that stimulate *A. fumigatus* growth (585). At physiological concentrations, the bacterial dimethyl sulfoxide (DMS) significantly augmented the growth of *A. fumigatus*, and this effect was amplified when the fungus was grown under sulfur starvation. These findings were consistent with the observation that organic S compounds are essential for the growth of *A. fumigatus* (586). The capacity of volatile bacterial organic compounds to promote *A. fumigatus* vegetative growth is not restricted to *P. aeruginosa*, since other Gram-negative bacteria, such as *Escherichia coli* and *Burkholderia cepacia*, are able to stimulate the growth of *A. fumigatus*. The interactions between *P. aeruginosa* and *A. fumigatus* during pulmonary infection can be seen as a two-step process. Initially, when *P. aeruginosa* and *A. fumigatus* are separated, volatiles released by *P. aeruginosa* favor fungal growth in the lung parenchyma. At later stages of the microbial infection, when the two microorganisms come in direct contact, the mutualistic interaction becomes antagonistic. *A. fumigatus* also produces a multiplicity of volatile organic compounds, which are predominantly terpenes (146). However, the impact(s) of fungal VOCs on bacteria needs further study, especially since the volatome of *A. fumigatus* varies under different environmental situations (587). These studies show the very complex positive and negative interactions that can occur between a single bacterial inhabitant of the lung and *A. fumigatus*. We are only beginning to realize the complexity of the interactions between the full microbiota and mycobiota during aspergillosis.

Other types of interactions that occur at larger distances result from the modification of the immune response by the microbiota that impact host response to the fungus. These interactions can be extremely complex (588). When mice were exposed to *Aspergillus* conidia, no allergic response was seen. In contrast, repeated exposure of *Aspergillus* spores to mice treated with antibiotics that disrupt the gut microbiota led to a strong CD4 T-cell-mediated allergic response. This is the first evidence that defined the role of antibiotics and gut microbiota in promoting the development of allergy airway disease particularly associated with *Aspergillus*. It shows the complexity of the interactions, since events in distal mucosal sites in the gastrointestinal tract play a role in regulating immune response in the lung (561, 589). Having been undertaken in mice, such studies should be now initiated in humans. It also is likely that the composition of the microbiome and its effect on immune response are important determinants of the susceptibility to and severity of *Aspergillus*-related diseases. For example, the role of *Aspergillus* in the induction of the Th17 response, including the production of IL-22 and release of antimicrobial peptides, could affect the composition of the microbiome. Likewise, metabolites produced by the microbiota could have an important immunoregulatory function specifically targeting the IL-1 axis (590, 591).

A. fumigatus is regularly reported to be infected by viruses (592, 593). The presence of these viruses may have a role in fungal virulence, since the sequenced strain Af293, which is infected with a mycovirus, is less pathogenic than other wild-type strains (176). In addition to biochemical interactions, the presence of viruses and horizontal gene transfer events (388) suggest the exchange of genetic material between members of the microbiota. Finally, association of *Aspergillus* conidia with environmental nanoparticles modulates host response and virulence and deserves further research (594).

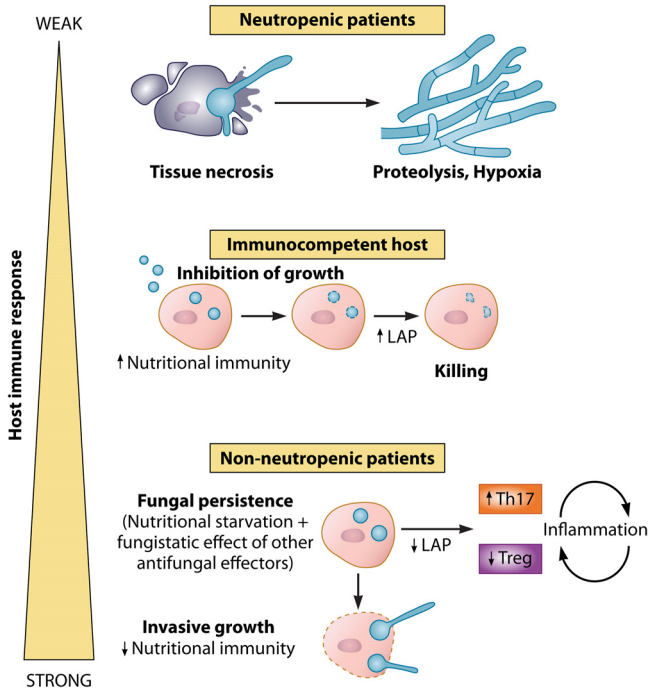

FIG 18 New hypothetical scheme showing the nutritional immunity governed by LAP responsible for the persistence and the infective propagules in nonneutropenic patients. In neutropenic patients, the lack of host response is associated with rapid growth, which induces necrosis of host tissues and facilitates fungal nutrition.

NUTRITION, A KEY CONCEPT TO UNDERSTANDING *A. FUMIGATUS* INFECTIONS

Nutritional Immunity To Explain the Immunopathogenesis of Invasive Aspergillosis

It is apparent that cross talk and cell-to-cell interactions between professional phagocytes (e.g., inflammatory monocytes and neutrophils) and between AMs and nonimmune cells (endothelia, epithelia, and fibroblasts) play a critical role in optimal control of *A. fumigatus* infection and resolution of inflammation. Therefore, apart from understanding the individual mechanisms of host-*Aspergillus* interaction, future research also should aim to dissect mechanisms of temporal and spatial cross talk of different immune cell subsets at the site of infection. For example, invasive mold infections are histopathologically characterized by granulomatous inflammatory responses in the nonneutropenic host (595). The mechanisms of granuloma formation in response to *Aspergillus* remain completely unknown.

In neutropenic patients with acute myelogenous leukemia, the degree and severity of leukocytopenia increases the risk of IA (58, 59). In neutropenic hosts, cell destruction and angioinvasive fungal growth of hyphae culminates in tissue necrosis and infection dissemination. In this disease setting, the ability of the fungus to grow under various nutritional conditions, especially those resulting from proteolysis in a hypoxic environment, is advantageous for invasive hyphal growth (Fig. 18). From the host site, intracellular and extracellular pathways of nutritional immunity, an ancient host defense strategy of iron limitation to pathogens, becomes essential once other immune effector mechanisms are compromised (239, 279, 596). Accordingly, the relative rarity of IA in patients with congenital neutropenia or aplastic anemia compared to the neutropenic patients with HSCT or acute leukemia implies that deregulated iron and nutrient homeostasis in the latter group of patients facilitates vegetative hyphal development. In line with this concept, acquired iron overload and increased systemic labile iron triggers unrestricted hyphal growth in serum and markedly increases the risk for IA in high-risk neutropenic patients with HSCT (597, 598). Importantly, apart from inhibiting pathogen growth, iron and other components of nutritional immunity (e.g.,

zinc) regulate essential metabolic and inflammatory pathways in immune cells that are largely uncharacterized (365, 599).

In nonneutropenic immunocompromised models of IA with genetic (CGD) or pharmacological inhibition of NADPH oxidase (e.g., corticosteroid-induced immunosuppression), the consequences of the lack of ROS production and downstream activation of the LAP pathway compromise the fungicidal ability of myeloid cells and lead to the persistence of *Aspergillus* conidia (Fig. 18). Activation of compensatory metabolic and nutritional immunity mechanisms prevents *Aspergillus* conidia outgrowth in this setting of fungal persistence, which is supported by the lack of evidence of profound tissue invasion. Of interest, the persistence of fungal conidia in nonneutropenic models of IA results in lack of efficacy of conventional antifungal agents (178, 600). Eventually, prolonged downregulation of antifungal effectors that physiologically eliminate conidia and/or restrict fungal growth inside these patients' phagocytes leads to conidial germination, especially in the setting of compromised nutritional immunity. It is tempting to speculate that, in a model analogous to cancer evolution, unabated chronic inflammation induced by prolonged fungal persistence triggers metabolic reprogramming of macrophages and results in nutritional immunity deregulation favoring fungal growth. The predominant role of iron deregulation in the development of IA has been demonstrated recently in a nonneutropenic model of lung transplantation (596) and in the *Aspergillus* keratitis model (279). These findings support a model of sequential immune defects in phagosome biogenesis and iron homeostasis in phagocytes, leading to invasive *Aspergillus* growth (Fig. 18). The mechanisms that drive iron deregulation in the setting of chronic inflammation are uncharacterized and might affect essential antifungal effector pathways, apart from increasing nutrient availability to the pathogen. Therefore, the concept of nutritional immunity, an ancient host defense strategy of iron limitation to pathogens, needs to be better understood at the molecular level and reidentified to include a broader range of effector mechanisms and metabolic pathways (601). In particular, the hypothesis of lowered concentrations of antifungal nutritional effectors (e.g., lactoferrin, lipocalins, and calprotectin) and/or other antimicrobial molecules/metabolites that become fungistatic rather than fungicidal cannot be ruled out. Finally, dissecting the mechanisms of nutritional immunity defects triggering invasive infections is essential; it is expected that the number of nonneutropenic patients with immunometabolic defects (e.g., patients with iron overload syndromes and/or chronic metabolic diseases) at risk for IA will increase.

Nutritional Flexibility Controls the Opportunistic Virulence of *A. fumigatus*

Each of the virulence determinants discussed above were considered separately for an easier understanding of fungal requirements. However, a full understanding of the pathobiology requires the integration of all of the anti-*A. fumigatus* host defense and metabolic pathways in the host environment (Fig. 19). The tremendous adaptability of *A. fumigatus* in terms of nutrient-balancing strategies is key to understanding its pathogenicity. Even though RNA-seq data have shown that major metabolic changes occur during vegetative growth *in vivo*, the key pathways are still undefined. This is a consequence of the incomplete metabolomic data and very poor annotation of the genome. Half of the *A. fumigatus* proteins are unknown, and fewer than 5% of the annotated proteins have been verified. Many of these have shown an important rewiring of the *Aspergillus* protein function compared to yeast homologues. Its nutritional flexibility coming from its adaptation to a saprophytic life in nature is a major condition for *A. fumigatus* growth in the host. Its large number of hydrolytic enzymes allows *A. fumigatus* to easily obtain carbon, nitrogen, and required ions. It is clear that this fungus has evolved many strategies to acquire the nutrients required for its vegetative growth *in vivo*. Under certain conditions, such as in the phagolysosome, some key nutrients may be lacking due to the host scavenging process, with the consequence of arrested fungal growth. Indeed, the virulence of *A. fumigatus* results more from a global physiological adaptation to its environment rather than from the expression of specific virulence factors, as is the case for bacterial pathogens, namely,

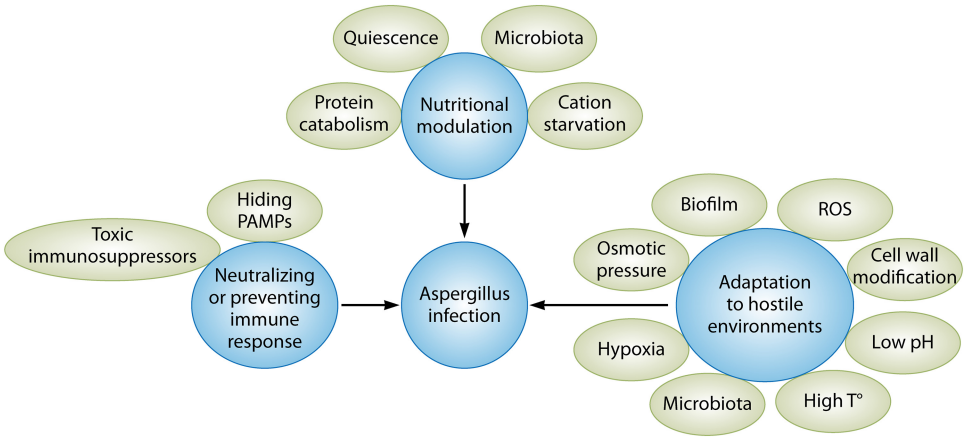

FIG 19 Integration of all potential fungal virulence determinants and pathways in *A. fumigatus*. Favorable and unfavorable environmental and physiological conditions framing the development of *A. fumigatus in vivo* show the extreme nutritional flexibility and the multiple compensatory reactions of the stress response characterizing this opportunistic pathogen. Toxic immunosuppressors can be GAG and secondary metabolites.

factors which do not affect the growth *in vitro* but are exclusively expressed and required for invading the host.

Nutrient acquisition for *A. fumigatus* requires efficient interconnections between metabolomic pathways (Fig. 19) that are, to date, unexplored and poorly understood. *Aspergillus* species are well known for their high-capacity protein secretion. However, their secretion capacity, which is vital for the fungus, must be regulated to avoid the accumulation of toxic unfolded proteins when the organism encounters adverse environmental conditions (602). The unfolded protein response (UPR) is a stress response pathway that is charged with maintaining the fidelity of the protein folding activities of the endoplasmic reticulum and control protein secretion and associated access to food (603, 604). Loss of HacA, the major transcription factor of the UPR, and of the stress sensor, IreA, impairs protein secretion, which results in attenuated virulence of these strains, which are then unable to extract sufficient nutrients from host tissues (605). Interestingly, iron assimilation is affected in these mutants, demonstrating again that nutritional versatility is a complex phenomenon resulting from interconnections of pathways not logically related (605). The central metabolic role of iron shows the complexity of these connections. Iron regulation is interconnected with other regulatory circuits not expected to occur in eukaryotes and includes pH regulation, gluconeogenesis, zinc and other cation metabolism, sterol biosynthesis, amino acid biosynthesis, and oxidative stress response (334) (Fig. 11). For example, deficiency in the transcription factor AcuM was shown to impair both gluconeogenesis and high-affinity iron uptake in *A. fumigatus*, whereas only the latter was suggested to be responsible for the virulence defect caused by AcuM deficiency (606). Genetic inactivation of *HISB*, being essential for histidine biosynthesis, displays increased sensitivity to limitation of various metals, including Mn, Cu, and Fe, along with attenuated virulence, indicating that histidine is required not only for protein biosynthesis but also for host and metal adaptation (346). Similarly, a transcription factor regulating leucine biosynthesis also controls iron acquisition (607). In addition, can the fungus retrieve nutrients from its own reserves? Even though autophagy seems dispensable for the virulence of *A. fumigatus*, the putative intracellular recycling of nutrients, which could represent a salvage pathway for the growth of the fungus in a sudden stress or starvation situation, should be better investigated (608, 609).

Many examples have shown that the role of a gene in fungal growth *in vitro* or *in vivo* may not be due to the function originally associated with the targeted gene but rather to an unsuspected function in a different and unexpected pathway. To think that the phenotype of an *A. fumigatus* mutant is due only to the deletion of the targeted

gene and not to the result of this deletion on the overall physiology of the organism is an incomplete conclusion.

In the case of *A. fumigatus*, the virulence mechanisms developed by this fungus are centered on the acquisition of nutrients that are essential for both *in vitro* and *in vivo* growth. This is the reason to assign the attribute of opportunistic fungus to this pathogen. Future work should attempt to address the complex mechanisms of metabolic adaptation of the fungus inside the phagocyte and the cross talk of host and pathogen metabolites that determine fungal resistance to host effectors, intracellular persistence, and invasive fungal growth.

ANTIFUNGAL DRUGS AND THERAPY

Antifungal Drugs and Associated Problems

The current arsenal of antifungal compounds used for the treatment of aspergillosis consists of three classes of antifungal agents, two of them targeting ergosterol, the functional fungal analogue of cholesterol and the major component specific to fungal membranes, and a third class that targets the synthesis of β1,3 glucan, the major component of the fungal cell wall (610–614) (Fig. 20). Amphotericin B (AMB), a polyene class of antifungal drug, irreversibly binds to ergosterol, resulting in fungal cell death. Binding of AMB to sterols causes membrane leakage, the proposed mechanism leading to cell death (615). Detailed structural and biophysical studies have demonstrated that polyenes act like an ergosterol sponge, forming large, extramembranous aggregates that extract the essential membrane-lipid ergosterol from the plasma membrane. It also has been proposed that AMB has an oxidative killing mechanism of action (616). A main advantage of AMB as an anti-*A. fumigatus* drug is the absence of resistance emergence, due in part to its cidal activity (617). Less toxic, lipid-associated drugs have been formulated. Amphotericin B lipid complex (ABLC) consists of amphotericin B in a complex with two lipids: L-α-dimyristoyl phosphatidylcholine and L-α-dimyristoyl phosphatidylglycerol (618). The large molecular size of the compound results in rapid uptake of the drug by macrophages. The other formulation is liposomal amphotericin B (L-AmB), which is composed of a small, unilamellar vesicle consisting of hydrogenated soy phosphatidylcholine, cholesterol, and distearoyl phosphatidylglycerol (619). These compounds have comparable activity, are far less toxic, and exhibit pharmacokinetics equal to that of the mother compound.

The synthesis of ergosterol begins with acetyl-coenzyme A (CoA) and involves 20 steps that have been subjected to multiple reviews (620, 621). The antimycotic azoles have a five-membered nitrogen-containing heterocyclic moiety, which binds to an iron atom in the heme group located in the active site of the lanosterol 14-α-demethylase encoded by *CYP51A* and *CYP51B*. Consequently, azoles block the demethylation of lanosterol, resulting in ergosterol depletion and the accumulation of a toxic sterol produced by Erg3. This toxic sterol exerts a severe membrane stress on the cell. The fungicidal activity is the result of a compensatory cell wall stress pathway following ergosterol depletion that results in carbohydrate patch formation with subsequent penetration of plasma membrane and fungal killing (622). Because of favorable bioavailability, pharmacokinetics, and lack of host toxicity, triazoles (primarily voriconazole and posaconazole) have become the primary choice of treatment for IA (25). The azoles have a drawback, since they also inhibit cytochrome P450 (CYP 450) enzymes that are responsible for the metabolism of various chemicals, including numerous other drugs. In addition, they serve as substrates of the CYP 450 enzymes; therefore, drugs that inhibit or induce the activity of these enzymes also can lead to clinically significant changes in azole concentrations. To overcome the problem of drug-drug interactions associated with the azoles, attempts have been undertaken to replace the triazole metal-binding group with a tetrazole.

The echinocandins are the only other class of antifungals to reach the clinic in decades. Three echinocandins are currently available for clinical use: caspofungin, micafungin, and anidulafungin. These compounds are cyclic hexapeptides that act as noncompetitive inhibitors of β1,3 glucan synthase, a key enzyme for cell wall synthesis.

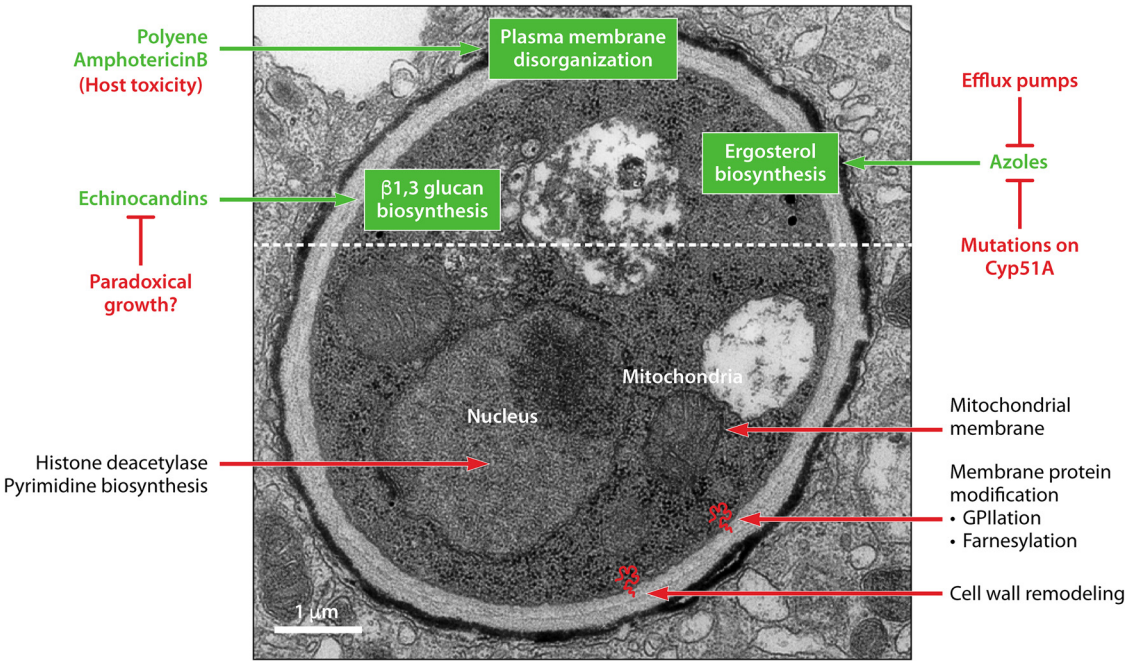

FIG 20 Current antifungal targets and drugs (in green) and issues associated with drug therapy (in red). New drug targets are indicated in black and their target localization by red and white arrows.

The safety profile of these compounds is impressive and attributable to a fungus-specific target that is not conserved in mammals. However, a precise biochemical mode of action of echinocandins and their membrane target(s) remain elusive. Moreover, these drugs, which are active at 0.5 μg/ml, show a paradoxical effect *in vitro*; higher concentrations (>4 μg/ml) result in incomplete growth inhibition against *A. fumigatus*. This paradoxical growth also seems to exist *in vivo* (623–625). The slow-growth phenotype resulting from high-dose exposure is characterized by hyperbranching, occasional lysis of hyphal apical compartments, translocation of the β1,3 glucan synthase to vacuoles, disappearance of cell wall β1,3 glucan, and compensatory increase of cell wall chitin. After approximately 2 to 3 days, paradoxically growing hyphae emerge from the β1,3 glucan-depleted and growth-inhibited microcolonies. These paradoxically growing hyphae are characterized by fast growth, normal morphology, renewed localization of the β1,3 glucan synthase to the hyphal tips, reconstitution of β1,3 glucan synthesis, and normalization of the cell wall chitin levels (625). In addition to solubility problems of this drug at high concentration, the stability of the echinocandin concentration or the presence of a high concentration of serum (50%), which may play a role in this paradoxical effect (626), the genetic and molecular mechanisms responsible for this phenomenon remain speculative. The MpkA-RlmA-CWI pathway has been shown to be involved in the control of paradoxical growth (627). The echinocandin-induced increase in cell wall chitin is thought to at least partly mediate this paradoxical growth. Moreover, calcineurin also plays an important role in this paradoxical growth, since *cnaA* and *crzA* deletion mutants showed increased caspofungin susceptibility and no paradoxical growth (628). The TF ZipD, responding to Ca^{2+}/calcineurin signaling, has been shown to be involved in paradoxical growth via regulation of chitin biosynthesis genes (627). Inhibition of Hsp90 function (involved in *A. fumigatus* calcineurin-dependent stress response to echinocandins), which results in increased caspofungin susceptibility, also abolishes paradoxical growth (629). In spite of the association between paradoxical growth and many fungal proteins and pathways, paradoxical growth remains inadequately understood.

The CLSI (Clinical & Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) methods allow the determination of

MICs and can identify strains resistant to drugs. To date, no resistance problems associated with echinocandins and amphotericin B have been identified in clinics. However, echinocandin-resistant mutants can be induced easily *in vitro* (630, 631), and hot spots for point mutations inducing the resistance to amphotericin were identified in *A. fumigatus* after use of natamycin (632–634) and are starting to be seen in laboratories (635, 636). However, resistance to AMB is only anecdotal. In contrast, resistance to azoles has been reported with increased frequency (538, 637, 638). Importantly, there was not an absolute association between *in vitro* MIC and clinical response (681).

Azole resistance in *A. fumigatus* reflects an increase in drug use for prophylactic and long-term treatment regimens, and acquired azole resistance was reported in patients who received long-term treatment with azoles (528, 538, 639–641). Although resistance to azoles has become an important concern, global surveillance studies reveal 3.2% of *A. fumigatus* isolates are resistant to one or more azoles, with large discrepancies between regions, from 25% to less than 1% in the multicenter epidemiological SCARE study with 22 centers from 19 countries (637, 642–644). The azole target is the 14-α sterol demethylase encoded by cyp51A and -B genes in *A. fumigatus* (645), and the main mechanisms of resistance involve mutations in the *CYP51A* gene (646–651). A common mechanism of azole resistance found in environmental and clinical isolates of *A. fumigatus* now involves modification of *CYP51A* and its promoter (TR34/Leu98His; TR46/Tyr121Phe/Thr289Ala). Complex regulation is expected, since TR34 was bound by both the sterol-regulatory binding protein SrbA and the CCAAT binding complex (652). Other proven mechanisms of resistance in *A. fumigatus* are Cyp51A amino acid substitutions at Gly54, Gly138, Met220, and Gly448. A 53-bp tandem repeat in the promoter region of *A. fumigatus* has also been reported, as well as a triple mutation in the *CYP51A* gene (653, 654). Indeed, the heterogeneity of the *A. fumigatus* population has been linked to azole resistance by genome sequencing, leading to 4 to 7 different clusters, depending on the methodology used (655, 656).

The insertion of repeated sequences in the promoter region was speculated to be related to the extensive use of fungicides in agriculture (657). Azoles are the most widely used class of agricultural fungicides, and it is unlikely that the pesticide industry will cease marketing fungicides; these chemicals provide protection to plants against phytopathogenic fungi and are a key tool in world food production (658, 659). Moreover, the role of these azoles used in agriculture as the source for emergence of the azole-resistant strains has not been demonstrated conclusively (660). For example, in agriculture, the worldwide emergence of azole-resistant phytopathogens occurred 2 to 6 years after commercial application of these fungicides. However, this was not the case with *A. fumigatus*. Similarly, the cereal pathogen *A. flavus*, a producer of foodborne mycotoxins that has been exposed yearly to fungicide applications, has no reported azole resistance. It was shown that no clear fitness has been associated with the induction of azole resistance (in contrast to plant pathogens) (661). The mechanisms of acquiring resistance are also more complex than the integration of 34 or 46 repeated sequences in the *CYP51A* promoter. Many resistant isolates do not have amino acid substitutions in Cyp51A (662) but use alternative mechanisms. For example, the upregulation of ABC and MFS transporter genes (663); the modification of HapE (652); the involvement of mitochondrion metabolism, especially the mitochondrial complex 1 and the cytochrome b_5-CybE redox systems (664, 665); the role of TF SrbA and AtrR, regulating *CYP51A* (666, 667); and *Cyp51B* overexpression (646) have been associated with azole resistance. Resistance to azoles is a fashionable topic (>600 references in PubMed), but does it deserve so much attention? Resistance to antifungals in *A. fumigatus* has not been recognized as a substantial clinical problem in medical mycology (668). However, case reports show that patients who are infected with azole-resistant strains have a higher mortality incidence than patients who are infected with susceptible strains (638, 669, 670). It is necessary to take into account the emergence of these forms of resistance when one considers the overarching problem of multidrug-resistant bacteria. The paucity of fungus-specific targets is an additional problem

because of the prevalence of cross-resistance to all drugs with a common target. Therefore, there is a need to identify additional antifungal drugs to overcome future challenges that will be posed by emerging antifungal resistance. A major concern is that there are few discovery programs devoted to developing new antifungal therapeutics, because pharmaceutical companies expect limited financial returns due to the costliness of clinical trials. Indeed, the major pharmaceutical companies selling antifungal drugs each have an azole and an echinocandin molecule and seem to be reluctant to invest money on the search for new lead compounds.

Regardless, many new drug targets have been identified or revisited, and some of them have led to the discovery of new antifungal drugs (671, 672). Below is a nonlimitative list of putative drugs and targets followed by startup companies which may reach the clinics in the near future. Lysine deacetylases and acetyltransferases, which catalyze the removal or addition of acetyl groups from core histones, resulting in changes in chromatin structure and gene expression, have been selected recently (673). Farnesyltranferase, which catalyzes posttranslational lipidation on the C terminus of many important signaling proteins, also has been identified as a promising target. Inhibitors of GPI anchor biosynthesis as well as of GPI-anchored proteins, essential in the construction of the cell wall and, accordingly, fungus specific (162), have been spotted. An inhibitor of inositol acyltransferase (AX001) that blocks fungal glycosylphosphatidylinositol biosynthesis is currently being developed. Fungal pyrimidine biosynthesis also has been recognized as a target for a long time, and the compound F901318, which inhibits the oxidoreductase enzyme dihydroorotate dehydrogenase, was launched recently. Inhibitors of the glyoxylate cycle also look promising. The arylamide T-2307, which is structurally similar to aromatic diamidines, causes collapse of fungal mitochondrial membrane potential. The cyclic metallohexapeptide VL-2397 is structurally related to the siderophore ferrichrome; a rapid antifungal effect follows its transport into fungal cells via siderophore iron transporter 1, which is absent from mammalian cells. Additional inhibitors of β1,3 glucan synthase, such as the echinocandin rezafungin, exhibit a prolonged half-life, allowing weekly treatments, and rezafungin has completed phase II studies. A new molecule, different from echinocandin SCY-078, is available in oral and in intravenous formulations and has recently received fast-track FDA approval. Even though chitin synthesis is an evident target, no clinically relevant inhibitors of this enzyme have been identified. Nikkomycins and polyoxins are specific chitin synthase inhibitors of chitin synthases, and although they potently inhibit enzyme activity *in vitro*, they are not efficiently taken up *in vivo* and consequently often are not effective antifungals (674). Figure 20 represents the old and new molecules based on the inhibition of old ones and new, promising drug targets.

Ongoing Therapies of Human Diseases Caused by *Aspergillus*

Guidelines have been published for the treatment of ABPA (25, 93, 675), aspergilloma (25, 28, 93), CPA (25, 28, 29), and IPA (25, 93) and will not be detailed here. Only key points will be addressed in the following section.

ABPA treatment. Current therapeutic modalities for ABPA have been addressed in recently published guidelines, and they include oral and inhaled corticosteroids that suppress the inflammatory immunopathology induced by *Aspergillus* infection rather than eradicate the fungus, while systemic antifungal agents have a complementary role and are given in selected cases (25, 93, 675).

Recommended corticosteroid doses for acute exacerbations of ABPA include an induction phase with dosages of 0.5 to 1.0 mg/kg of body weight of prednisone equivalent daily for 1 to 2 weeks, followed by a maintenance phase with a dose of 0.5 mg/kg every other day for 6 to 12 weeks and a tapering phase for a total duration of at least 6 to 12 months (25, 93, 675). However, some patients (especially CF patients) cannot be successfully weaned off corticosteroids and require a relatively low daily maintenance dose of $<$10 mg. The role of immunotherapy with anti-IgE monoclonal antibodies (omalizumab) to replace steroids holds promise for ABPA (676).

Increasingly, use of itraconazole and now of the newer triazoles voriconazole,

posaconazole, and isavuconazole has been employed as adjunctive fungal allergen-reducing therapy for ABPA as a corticosteroid-sparing agent in patients with frequent relapses or corticosteroid dependence (677, 678). However, in view of the need for prolonged courses of therapy, there is concern about toxicity and development of secondary resistance to azoles. The benefit from preemptive antifungal therapeutic strategies to decrease flares and inhibit disease progression in ABPA remains uncertain.

Aspergilloma. Guidelines on the management of aspergilloma represent expert opinions due to the lack of controlled studies (25, 28, 93). The definitive treatment of aspergilloma is surgical resection, which often is contraindicated because of severe underlying pulmonary dysfunction, and it is often associated with significant complications. An alternative treatment in patients who are not candidates for surgery is intracavitary or CT-guided percutaneous instillation of amphotericin B deoxycholate (d-AMB). Bronchial arterial embolization (BAE) has been extensively used as a bridging therapy in the management of hemoptysis in patients with aspergilloma until surgical resection of the aspergilloma can be performed.

Chronic pulmonary aspergillosis. CPA treatment is challenging, as it requires prolonged use of systemic antifungal agents (25, 28, 29). In addition, response to therapy is difficult to assess and is based on quality-of-life scoring systems that evaluate weight gain and improved energy levels. Inflammatory markers tend to improve slowly, and they usually remain elevated even during long-term therapy. Relapse months or years after discontinuation of treatment is reported frequently. In the literature, most patients with CPA have received treatment with oral itraconazole, whereas intravenous d-AMB has been successfully used in refractory cases (675, 679). Both itraconazole and voriconazole are regarded as the treatment of choice for CPA according to the guidelines, which is supported by data from large, controlled, nonrandomized studies (25, 28, 29). The newer triazole, isavuconazole, has not been studied in CPA but is recommended as an alternative option in recently endorsed guidelines (25, 28, 29). However, emerging rates of azole resistance in *Aspergillus* isolates recovered from patients with CPA have been reported, although there is controversy on whether *in vitro* resistance of *A. fumigatus* to azoles correlates with worse clinical outcomes (680, 681). In addition, serious side effects necessitating discontinuation of treatment with azoles occurs in up to one-third of the patients. Alternative treatment strategies include intravenous use of polyenes or echinocandins or combination of azoles with echinocandins (25, 28, 29, 682). Inhaled AMB also can be successfully used for CPA treatment and provides the advantage of tolerability and ease of administration.

Invasive aspergillosis. Over the past 2 decades, we have witnessed improved outcomes of patients with IA as a result of better diagnostic and therapeutic modalities (56, 65, 67, 96, 683). However, the mortality rate of IA remains high, particularly in patients with complex and persistent underlying immune defects who develop breakthrough infections while receiving mold-active antifungal therapy (684). Several therapeutic strategies have been applied in an attempt to improve the outcome of IA. These include the introduction of potent antifungal agents, such as the newer triazoles voriconazole, posaconazole, and, most recently, isavuconazole; early initiation of treatment; use of drug delivery formulations to improve the therapeutic index of currently available antifungals; combination antifungal therapy; surgical excision of sequestered necrotic lesions; and, in selected patients, use of immunomodulating agents. Recommendation of and systematic evaluation of the value of these therapeutic modalities has been comprehensively addressed in recently published guidelines (25, 93). However, there is a lack of data from prospective controlled clinical studies on the values of these antifungal therapeutic approaches for patients with refractory relapsed underlying disease, breakthrough IA in patients who have been on mold-active antifungals, or IA caused by azole-resistant *Aspergillus* species, because these patients are typically excluded from clinical studies (684).

The introduction of the broad-spectrum triazole voriconazole has been a major therapeutic advance in the management of IA. The improved survival observed in a large, randomized trial in patients with definite or probable aspergillosis made vori-

conazole the preferred drug for first-line therapy for IA in the guidelines (685). However, a recent study has shown that isavuconazole was equally efficacious and better tolerated than voriconazole (686). Based on this study, both isavuconazole and voriconazole have an IA indication in the management of IA (93). Posaconazole, a newer triazole with broad-spectrum activity against *Aspergillus* and other molds, is regarded as an alternative therapeutic option in the management of IA (92, 93). Although there are no comparative studies, L-AmB appears to have activity comparable with that of the newer triazoles in the management of IA (28, 29). The echinocandins caspofungin and micafungin have been used as primary therapy in noncomparative studies of IA, with response rates ranging from 33% to 57% for caspofungin (687–690) and 45% to 71% for micafungin (54, 479, 691). Both echinocandins have also shown activity as salvage therapy in patients who are intolerant or have IA refractory to other antifungal therapies (93).

Future Therapeutic Strategies

Combination treatment. The concept of combination therapy with antifungals that possess different mechanisms of action (e.g., azoles plus echinocandins, AMB plus echinocandins, and AMB plus azoles and echinocandins) as a means to improve suboptimal long-term survival of high-risk patients with IA treated with azole monotherapy is theoretically appealing. Such an approach has been supported by *in vitro* data, preclinical studies, and, in a very limited number of patients, with IA (692–695). The first study to demonstrate an impact of combination therapy on the outcome of patients with IA and positive galactomannan was seen with a combination of voriconazole and anidulafungin (696). Another study demonstrated the benefit of a combination of L-AmB and caspofungin (697). Recent guidelines and expert opinions suggest the option of combination therapy with different classes of antifungals in patients with IA breaking through a mold-active antifungal and in cases of infections caused by azole-resistant *Aspergillus* species. In view of the significant interpatient pharmacokinetic variability, monitoring of plasma drug levels is recommended on an individualized basis (92, 93, 684, 698). Targeting antifungal liposomes with Dectin 1 exhibits enhanced efficacy (699).

Surgery. The role of adjunctive surgery in the management of IA has not been demonstrated in controlled studies. Pulmonary infarcts and tissue sequestration compromise antifungal drug activity and are common causes of therapy failure, infection relapse, and fatal hemorrhage in patients with IA. Thus, resection of infected pulmonary tissue is beneficial only for selected patients (25, 92, 93, 700).

Immunotherapy. Because the net state of the underlying immunodeficiency is the critical determinant of IA outcome, host-directed therapeutic strategies aiming for the restoration of the underlying immune defects are appealing. Similar immunotherapeutic strategies have been successfully implemented in the clinical management of immunocompromised patients with viral infections (e.g., CMV) (701). Many preclinical studies over the past 30 years have successfully employed different immunotherapeutic approaches, with the use of (i) cytokines, (ii) myeloid hemopoietic growth factors (M-CSF) (iii) phagocyte transfusions, (iv) generation of *Aspergillus*-specific T cells, (v) engineered CAR T cells targeting β glucan, (vi) checkpoint inhibitors, (vii) NK cells, and (viii) vaccines that enhance *Aspergillus*-specific immune responses (702–711). Other investigators implemented anti-inflammatory therapies to restrict detrimental inflammatory immunopathology induced by the fungus (712). Unfortunately, none has succeeded in clinical trials. Granulocyte transfusion therapy has become more feasible since the introduction of G-CSF/corticosteroid mobilization into the donor leukocyte collection process. However, the only randomized clinical trial assessing the efficacy of granulocyte transfusions for IA was not conclusive because of the limited number of patients enrolled in the study (25, 45, 92, 93). Therefore, high-quality randomized clinical studies on adjunctive immunotherapy are urgently needed in patients with IA. More importantly, better understanding of the heterogenicity in underlying immune defects and risk stratification strategies for tailored, host-directed therapy in distinct

groups of patients with IA is needed. The proposed need for precision immunotherapy for IA originates from failures of multiple immune modulatory studies that targeted sepsis. These failures were attributed to incomplete understanding of immune deactivation mechanisms in different patient groups (712).

Vaccination projects have been undertaken with either the injection of antigens or the use of MAbs. These projects have not produced significant results; antigen vaccination is not applicable for immunocompromised patients (713), and the inhibitory effects of anti-β-(1,3) glucan and enolase monoclonal antibody are transitory (714–716).

Prophylaxis in high-risk patients. Patients with hematological malignancy at high risk for IA are managed with either (i) primary antifungal prophylaxis or (ii) biweekly monitoring of biomarkers such as the galactomannan antigen (25, 92, 93). The decision as to which strategy to choose depends on local epidemiology, access to rapid diagnostics, and patient characteristics (684). Posaconazole has been endorsed by several guidelines as the prophylactic azole of choice. Antifungal prophylaxis with the echinocandin micafungin in HSCT recipients was shown to reduce the incidence of IA; however, the fact that echinocandins are parenteral drugs limits their ability to be used in extended antifungal prophylaxis during the postengraftment period, an interval when the patient is susceptible to IA. One benefit of prophylaxis is the reduction of relapses (717). However, a significant proportion of breakthrough invasive mold infections occur in high-risk hematological malignancy patients on primary and secondary prophylaxis with all classes of approved antifungal agents (684).

THE FUTURE OF *A. FUMIGATUS* AND ASSOCIATED ASPERGILLOSIS

What will be the diseases caused by *Aspergillus* in 2030? The number of immunocompromised patients with more complex immune and metabolic abnormalities will increase in the near future as a result of (i) the unprecedented success of precision medical therapies targeting immune signaling pathways for malignant and autoimmune diseases, (ii) improved antimicrobial therapies and better life-support measures for bacterial and viral infectious diseases in the intensive care setting, and (iii) higher rates of inflammatory and metabolic diseases as a result of aging and environmental exposures. For example, the number of COPD patients at risk for aspergillosis will continue to rise with the expansion of heavily polluted industrialized areas. Consequently, the number of *Aspergillus* infections will continue to increase, especially if worldwide antifungal resistance emerges. Although the morbidity of tuberculosis and malaria is much higher (10 million and 200 million patients worldwide, respectively) than that of invasive aspergillosis, with aspergillus, 50% or more of the patients die of the disease, whereas only 12% and 0.2% die from tuberculosis and malaria, respectively (www.who.int). For this reason, new antifungal treatment strategies must be developed. Even though it is not a scientific issue, an economic component of aspergillosis certainly exists that has hampered efforts to search for new antifungals by the big pharmaceutical companies. Even though the number of IPA cases is rather low compared to those of other infectious diseases, aspergillosis is a very expensive disease, especially among transplant patients. Consequently, the development of new drugs will be in the hands of startup companies and should benefit from fast-track treatment and less expensive approval of these new drugs by regulatory agencies.

In addition, invasive aspergillosis remains an underdiagnosed disease, whereas the true epidemiology of chronic forms of *Aspergillus* infection is unknown and underestimated. Progress in diagnosis is needed in the near future, especially since early diagnosis in IPA is associated with improved cure rates. However, the future of diagnosis is challenging if one thinks in terms of the improvement of a single diagnostic parameter. Indeed, all existing consensus definitions already jointly use microbiology, clinical, and serological parameters, which reflect on both host and pathogen determinants in discriminating disease from asymptomatic *Aspergillus* infection (colonization). Therefore, multiparametric diagnosis may be the way to proceed in the future. This has led our laboratory to successfully implement a multivalent, combined approach for a predictive diagnosis of this disease: clinical and biological information,

especially immunosuppressive drugs and kinetics of the immunosuppressive regimen; blood parameters; SNPs for the production of various cytokines and chemokines upon *Aspergillus* challenge; presence of coinfections; type and duration of bacterial treatment; antibody response and circulating antigen titers; PCR data; etc., were parameters included in such analysis (M. Garcia, J. Springer, P. Y. Bochud, H. Einsele, J. Loeffler, and J.-P. Latgé, unpublished data). The aforementioned high mortality rate has its root in both the imperfect diagnosis and in the limited efficiency of antifungals used in the clinics. Aspergillosis entails a spectrum of disease that develops as a result of deregulated host response to the pathogen. Therefore, manipulating the immune system to reverse underlying immunodeficiency and prevent inflammatory damage will be the key to tackling aspergillosis.

Poor outcomes of aspergillosis patients also is due to a lack of understanding of the establishment of the fungal infection (e.g., colonization) in the host. Certainly, the adaptation of the fungus to its environment and its ability to cope with a milieu of poor nutritional conditions are essential. In contrast to *Candida*, which is maintained in the gut or vagina as a commensal and is usually a beneficial member of the microbiota, the ecological niche of the saprophyte *A. fumigatus* is the soil. To date, no studies have examined the molecular adaptation of this fungus to the soil and its capacity to resist microbial enemies and to survive the nutritional stress of this niche. Growing in such a competitive environment has served as an evolutionary virulence school for this fungal pathogen (2, 718). It has been repeatedly suggested that encountering constant protozoal predation in the soil has led to the development of cellular strategies by *A. fumigatus* to counteract phagocytic uptake or to gain intracellular passage (719, 720). *A. fumigatus* also has mastered how to compete with the myriad of soil bacteria and how to use microbial metabolites as nutritional inputs (585). An aspect that has not been considered is the putative positive role of antibacterial activity of this fungus in limiting the number of harmful bacterial pathogens, such as *Haemophilus*, *Mycobacterium*, and/or *Klebsiella*, in the lung. The ability to resist and/or process multiple antagonistic molecules in the soil and the high number of putative efflux pumps in the genome (approximately 5% of the genome) may be the raison d'etre for the low occurrence of drug-resistant strains (in contrast to phytopathogenic fungi). Besides resistance to environmental stresses, the fungus has to access nutrients that often are not easily accessible. As a consequence, the fungus has evolved multiple strategies to acquire nutrients required for its vegetative growth. This nutritional demand and ability to resist toxic metabolites has been coupled to its capacity to defend itself against host immune reactions.

Evolving in an inhospitable environment, this pathogen developed compensatory mechanisms and pathways to counterbalance the effect of lethal molecules or harmful mutations. The fungal virulence also is facilitated by one of the molecular characteristics of the *A. fumigatus* genome, that being the repeated occurrence of gene families with close or interchangeable functions. The study of the cell wall and antifungal drugs has been a paradigm to identify the occurrence of compensatory mechanisms and their role in fungal life. Seen in cell wall biosynthesis, this metabolic rewiring has been associated with many other salvage pathways. A better metabolic understanding of *A. fumigatus* physiology should be mastered now in order to better grasp its opportunistic virulence.

A final thought is the need to reconsider the often negative value associated with the word "opportunistic," which clearly does not reflect the true burden of human diseases caused by this unique fungus. Opportunistic clearly means that this fungus is not an obligate pathogen, one that requires the human host to develop or that has selected specific virulence factors for its unique growth *in vivo*. In this case, the term opportunistic does not translate to low pathogenicity. In contrast, this fungus has developed many redundant or independent strategies required for growth and survival in very different hostile environments. As a consequence, the multigene virulence that characterizes the pathogen *A. fumigatus* provides barriers to host killing/clearance of fungal propagules. It is very difficult to eliminate because it is necessary to knock down

all of the pathogenic determinants, which are continuously reshaped as a function of the environment. Perhaps the term "chameleon" rather than opportunistic better describes *A. fumigatus*.

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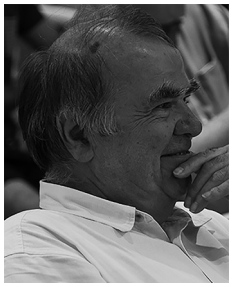

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