ImmuCorrelates of Protection in Human Invasive Aspergillosis

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Protective immunity against Aspergillus depends on a highly coordinated interaction between the innate and adaptive arms of the immune system. Fungal recognition via pattern recognition receptors, such as pentraxin 3, dectin-1, and Toll-like receptors, leads to complement activation, phagocytosis, and killing of ingested fungi. Aspergillus-specific T-helper 1 and 17 cells produce cytokines such as interferon γ and interleukin 17, which facilitate macrophage activation and neutrophil recruitment, respectively. Genetic (or drug-induced) defects in components of these networks of antifungal immunity result in increased risk of invasive aspergillosis after chemotherapy or transplantation. We review the most important genetic, immunological, and pharmacological factors that influence human susceptibility to Aspergillus and discuss the potential role of immune biomarkers in risk stratification strategies that facilitate individualized antifungal therapy/prophylaxis in immunocompromised hosts.

Keywords. immune response to Aspergillus; human susceptibility; biomarker; Th17; PTX3.

In the face of its ever increasing incidence, there is currently no reliable method to accurately predict the risk of invasive aspergillosis (IA) after chemotherapy or transplantation. Although incorporation of fungal-derived biomarkers (eg, detection of galactomannan) into clinical algorithms has facilitated diagnostically driven strategies in high-risk populations [1, 2], the optimal approach remains controversial. It also remains debatable which patients benefit the most from antifungal prophylaxis [3]. Ideally, some of these clinical decisions should be individualized based on the net state of immunosuppression and underlying immunogenetic host factors that are unique to each individual. Discerning the immune response to Aspergillus is critical for the identification of host-derived biomarkers that predict the risk of invasive disease. Herein we review our current understanding of the immunological events that occur during Aspergillus infection and discuss the most important genetic and immune host factors that influence susceptibility to IA.

EPIDEMIOLOGY AND RISK FACTORS
Aspergillus fumigatus is a ubiquitous environmental fungus that causes potentially lethal infections in immunocompromised hosts. Individuals at high-risk for IA include chemotherapy and hematopoietic stem cell transplant (HSCT) recipients, solid organ transplant (SOT) recipients, and those with advanced human immunodeficiency virus (HIV)/AIDS, hyper-immunoglobulin E (Job’s) syndrome, and chronic granulomatous disease [4, 5]. The incidence of IA is highest in patients with primary immunodeficiencies eg, almost 30% of patients with Job’s syndrome [5]; however, owing to the increasing number of transplants, IA has become particularly a problem among transplant recipients.

IA occurs at a rate of 10% in patients with hematological malignancies [6] and is the most common cause of invasive fungal infection (IFI) among HSCT recipients in North America, with 12-week mortality rates as high as 36% [7, 8]. The incidence of IA ranges from 1% to 15% in SOT recipients [9], with the highest
Detection of β-glucans and other *Aspergillus* ligands by epithelial and phagocytic cells occurs via pattern recognition receptors (PRRs), which recognize highly conserved structures expressed by invading fungi, known as pathogen-associated molecular patterns (PAMPs) [4] and products released from damaged host cells, known as danger-associated molecular patterns (DAMPs) [16]. The main PRRs involved in recognition of *Aspergillus* are presented in Figure 1. Some of these PRRs are soluble, including pentraxin (PTX) 3 and mannose-binding lectin (MBL), both of which can trigger complement activation and promote phagocytosis [17]. Other PRRs, such as Toll-like receptors (TLRs) and dectin-1, are membrane bound and trigger intracellular signaling pathways that lead to cell activation [18].

### Role of T-helper 1 and T-helper 17 Cells

After fungal recognition, *Aspergillus* antigens are processed by dendritic cells (DCs) that migrate to the local lymph nodes and present fungal peptides to *Aspergillus*-specific CD4⁺-naïve T cells [19] and induce their differentiation (Figure 1). Polarization of T-helper response by DCs is largely dependent on the type of PRR involved in recognition of *Aspergillus*. Fungal recognition via TLRs induces interleukin 12 production and supports the differentiation of a T-helper (Th) 1 phenotype [19, 20], whereas recognition via dectin-1 [18] enables T cells to follow the Th17 differentiation pathway [21, 22].

The frequency of circulating *Aspergillus*-specific T cells in healthy individuals has been estimated to range from 0.05% to 0.4% [23]. Production of interferon (IFN) γ, interleukin 4, interleukin 10, and interleukin 17 (IL-17) in response to *Aspergillus* antigens has been demonstrated in vitro [23–25]. In general, Th1 and Th17 cytokines are considered to confer protective immunity against *Aspergillus* (Figure 1), whereas Th2 responses are deleterious [26].

### IMMUNE RESPONSE TO ASPERGILLUS

#### Innate Immunity

Although humans inhale several hundred to thousands of *A. fumigatus* spores per day, invasive disease is rare because of the efficient immune response that controls fungal growth [4]. Bronchial epithelial cells are the first cells to recognize *Aspergillus* conidia through receptors that bind β-glucans exposed on the fungal surface [13, 14] and respond by generating reactive oxygen species, antimicrobial peptides, and cytokines. Once in the alveoli, *Aspergillus* conidia are primarily killed by alveolar macrophages, while neutrophils attack hyphae germinating from conidia that escape macrophage surveillance. Because hyphae are too large to be engulfed, neutrophils possess an array of extracellular killing mechanisms, including the formation of neutrophil extracellular traps, which have a fungicidal effect and prevent further spreading [15].

### CANDIDATE IMMUNE CORRELATES OF PROTECTION IN IA

Several immune biomarkers have been validated for the prediction of infectious complications and clinical outcomes in immunocompromised hosts. These include monitoring of CD4⁺ cell count in HIV-infected individuals [27] and measurement of cytomegalovirus (CMV)–specific cell-mediated immunity in SOT [28]. Although to date there has been a relative paucity of validated biomarkers for prediction of IFI, encouraging progress has been made in the identification of genetic factors implicated in the development of IA in patients with hematological malignancies and those undergoing HSCT [13, 29–32] (Table 2). A detailed discussion on all the genetic variants that influence susceptibility to IA is beyond the scope of this article and has been provided elsewhere [16, 26, 33]. It should be noted, however, that susceptibility to IA is multifactorial and
the phenotype associated with these genetic variants is virtually exclusive of individuals with underlying immunosuppression. Small sample size, heterogeneity of cohorts, selection bias, and statistical flaws are common limitations of genetic association studies on IA [33]. Thus, data on genetic susceptibility to IA should be interpreted with caution. The following are candidate immune correlates of protection that, in our opinion, have the strongest evidence in the literature suggesting a potential role as biomarkers for IA in the evaluation of patients at risk (Table 3). However, their true clinical value remains to be evaluated in well-designed prospective studies.

**Pentraxin 3**

PTX3 belongs to the same family of acute-phase reactants as C-reactive protein, but it is synthesized locally, not systemically, at inflammatory sites. It binds *Aspergillus* ligands such as galactomannan and zymosan [18]. PTX3 is stored in neutrophil granules and is rapidly released in response to inflammatory signals. After release, PTX3 localizes in neutrophil extracellular traps, where it acts as an opsonin facilitating the phagocytosis and killing of *Aspergillus* hyphae [34]. PTX3 also activates different pathways of the complement system [17].

Genetic deficiency of PTX3 affects the antifungal function of neutrophils and is associated with increased risk of IA in HSCT recipients [30] (Table 2). Similarly, PTX3-knockout mice exhibit defective recognition of *Aspergillus* conidia, have a skewed Th2 profile, and are highly susceptible to IA [35]. Administration of PTX3 is effective for both prevention and treatment of *Aspergillus* in vivo [36]. In rodents, the protective effect of PTX3 is similar or superior to that observed with liposomal amphotericin B or voriconazole, suggesting a possible role for PTX3 as adjuvant therapy in the treatment of IA [36, 37].

Circulating levels of PTX3 are elevated in patients with IFI and normalize with successful antifungal therapy [38]. PTX3 levels in bronchoalveolar lavage specimens of lung transplant recipients are significantly higher in patients with IA than in those with *Aspergillus* colonization (S. Husain, unpublished data). However, PTX3 also binds other pathogens [18, 39] and it is not highly specific for *Aspergillus* infection. High levels of PTX3 predict bacteremia and septic shock in patients with hematological malignancies [40]. PTX3 levels are also elevated in ventilator-associated pneumonia [41], meningococcal disease [42], dengue fever [43], and leptospirosis [44]. In most of these studies, high PTX3 levels had predictive value for disease severity and increased mortality.

**Mannose-Binding Lectin**

MBL is another soluble PRR with opsonin and complement activating properties. Several studies have suggested an association between MBL deficiency and susceptibility to a broad range of infectious diseases [45]. MBL haplotypes associated with low MBL expression are more common in patients with chronic necrotizing pulmonary aspergillosis than in ethnically matched control subjects [46]. Serum MBL levels <500 ng/mL are characteristic of patients with IA but not immunocompromised
Table 2.  Selected Genetic Polymorphisms With Strong Association for Risk for IA in Immunocompromised Hosts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Patient Population</th>
<th>SNP Position</th>
<th>Nucleotide Substitution</th>
<th>Risk of IA OR (95% CI)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBL2</td>
<td>HSCT</td>
<td>See comment</td>
<td></td>
<td>7.3 (1.9–27.3)</td>
<td>Donor MBL-low genotypes: single-point mutations at codons 52, 54, or 57 and/or polymorphisms in the promoter region at positions −950 and −221</td>
<td>[31]</td>
</tr>
<tr>
<td>TLR4</td>
<td>HSCT</td>
<td>−2604, +1363</td>
<td>A/G, C/T</td>
<td>3.22 (1.02–10.16)</td>
<td>Association observed when polymorphism(s) present in donors; data adjusted for CMV status and GVHD</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.96 (1.52–16.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+1063</td>
<td>A/G, C/T</td>
<td>6.16 (1.97–19.26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLEC7A</td>
<td>Hematological malignancies</td>
<td>c.714</td>
<td>A/C</td>
<td>3.89 (1.51–9.99)</td>
<td>Amino acid change Y238X results in decreased dectin-1 expression; OR for the presence of risk allele on donor and recipient after adjustment for GVHD and HLA haplotype mismatch</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c.255 + 813, c.375–1404</td>
<td>G/T, C/G</td>
<td>5.59 (1.37–22.77)</td>
<td>Study included chemotherapy-treated patients and HSCT recipients; OR for patients homozygous for risk allele</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.91 (1.52–15.89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX3</td>
<td>HSCT</td>
<td>+281, +734, +281G, +734A</td>
<td>A/G, A/C</td>
<td>2.92 (1.69–5.05)</td>
<td>Association observed when polymorphism(s) present in donors; OR for patients homozygous for risk allele; data adjusted for HLA-matching status, use of total-body irradiation, and antifungal prophylaxis</td>
<td>[30]</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Hematological malignancies</td>
<td>c.2797</td>
<td>A/G</td>
<td>2.75 (1.27–5.95)</td>
<td>Study included chemotherapy-treated patients and HSCT recipients</td>
<td>[29]</td>
</tr>
</tbody>
</table>

Abbreviations: c., refers to complementary rather than genomic DNA; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-vs-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IA, invasive aspergillosis; MBL, mannose-binding lectin; OR, odds ratio; SNP, single-nucleotide polymorphism.

patients with febrile illness not due to *Aspergillus* [47]. Low serum concentrations of MBL are also associated with increased incidence of bacterial infections [48] and IFI [49] in patients with hematological malignancies who are undergoing chemotherapy.

Despite this evidence, the fact that MBL-knockout mice do not exhibit increased susceptibility to IA [50], along with the high prevalence of MBL deficiencies in the general population [51, 52], indicates that the role of this pathway in host defense against fungal infections might not be clinically significant in otherwise immunocompetent hosts. Although MBL deficiency is more common in patients with IA, this biomarker lacks predictive value for clinical outcomes [51]. In subjects with chronic pulmonary aspergillosis, worse respiratory symptoms are associated with high, not low, serum levels of MBL, suggesting that MBL level is merely a marker of inflammation [53]. Similarly, MBL levels do not discriminate between patients with chronic pulmonary disease due to *Aspergillus* and the more benign form, allergic bronchopulmonary aspergillosis [53].

**Dectin-1**

Dectin-1 is a PRR expressed on the surface of bronchial epithelial cells and myeloid cells that binds β-glucan [54]. Dectin-1 binding to germ tubes augments production of inflammatory cytokines such as tumor necrosis factor (TNF) α [54] and boosts Th17 differentiation of *Aspergillus*-specific CD4+ T cells [22]. Dectin-1 deficiency results in impaired cytokine production, insufficient neutrophil recruitment/activation, impaired fungal killing and uncontrolled growth of *A. fumigatus* [55]. Dectin-1-deficient mice exhibit increased mortality after challenge with *A. fumigatus* [55].

In humans, the Y238X polymorphism in the gene encoding dectin-1 (*CLEC7A*) generates a truncated dectin-1 protein [56], and its presence in either donor or recipient is associated with increased susceptibility to IA after HSCT [13]. Other polymorphisms in *CLEC7A* with negative effect on dectin-1 expression/function have also been associated with increased susceptibility to IA among patients with hematological diseases [29] (Table 2).

**DC-SIGN**

DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) is involved in recognition of *Aspergillus* and other important human pathogens, such as HIV and mycobacteria. Human DCs bind and internalize *A. fumigatus* conidia via DC-SIGN [57]. Galactomannan seems to be the main DC-SIGN ligand.
Th17 cells promote neutrophil recruitment via IL-17 and epithelial barrier functions via IL-22. Among the pool of T cells that participate in immune response against *Aspergillus*, IL-17–producing cells are perhaps the most important T-cell subset. Qualitative or quantitative neutrophil defects are undoubtedly the main risk factor for IA, and Th17 cells play a pivotal role in recruiting and activating neutrophils at the sites of fungal infection (Figure 1). Because human neutrophils do not express a functional IL-17 receptor, Th17-dependent neutrophil recruitment/activation likely occurs indirectly via chemokine secretion by IL-17–activated epithelial and endothelial cells, and directly, via the release of CXCL8 and other cytokines by Th17 cells [64].

The antifungal effect of IL-17 is such that some strains of *A. fumigatus* have evolved to sense and respond to IL-17 simultaneously [66]. TNF-α and IFN-γ recruit neutrophils to the site of infection and promote neutrophil/macrophage activation, whereas interleukin 22 promotes epithelial renewal and enhances the expression of antimicrobial peptides by epithelial cells [66] (Figure 1). Thus, the role of polyfunctional Th17 cells in host defense against *Aspergillus* goes beyond IL-17 production, because they can regulate several aspects of antifungal immunity.
Deficient production or antibody-mediated neutralization of IL-17 makes mice highly susceptible to *A. fumigatus* [55]. In humans, genetic variation in proteins involved in Th17 differentiation influences susceptibility to IA. Interleukin 1β has recently emerged as an important cytokine in the generation of mucosal Th17 cells [67], and a polymorphism in the promoter region of *IL-1β* was strongly associated with increased risk of IA in an European cohort of hematological patients [68]. Phosphorylation of the signal transducer and activator of transcription (STAT) 3 occurs in response to interleukin 6 and interleukin 23 (Figure 1) and this pathway regulates many aspects of Th17 differentiation [67]. Polymorphisms in the interleukin 23 receptor gene seem to influence the risk of IFI after HSCT [69]. Patients with mutations in *STAT3* (ie, hyperimmunoglobulin E [Job’s] syndrome) have selective impairment of IL-17–producing T cells and are prone to *Aspergillus* infections [5, 70].

**ROLE OF IMMUNOSUPPRESSIVE AND ANTIFUNGAL AGENTS**

In addition to myelotoxicity, immunosuppressive agents influence antifungal immunity by several mechanisms. Steroids [71], tacrolimus [72], cyclosporine [73], and mycophenolic acid [74] inhibit IL-17 production by human T cells. Glucocorticoids also inhibit the expression of PTX3, dectin-1, and DC-SIGN in myeloid cells [75–77]. Cyclosporine and tacrolimus inhibit TLR signaling after liver transplantation [78]. Interestingly, although cyclosporine and tacrolimus are known to inhibit the nuclear factor of activated T cells pathway on lymphocytes, recent data indicate that calcineurin inhibitors also block dectin-1– and TLR4-dependent activation of this pathway in myeloid cells [79]. Similarly, rapamycin derivate not only inhibit lymphocyte response to interleukin 2, but they also decrease the expression of TLR2, TLR4, and dectin-1 on myeloid cells [80]. Thus, these immunosuppressive medications can influence the risk of IA independently of their capacity to block T-cell activation. Host defense against *Aspergillus* is consequently affected at multiple levels in individuals receiving combination immunosuppressive therapy.

In addition to controlling fungal replication, antifungal agents exert an immunomodulatory effect in host response to *Aspergillus* and colonizing fungi. Liposomal amphotericin B induces TLR4 expression/signaling and promotes the degranulation and fungicidal activity of human neutrophils against *Aspergillus* conidia and hyphae [59]. Voriconazole induces TLR2 expression/signaling and promotes chemokine and inflammatory cytokine gene expression in human monocytes challenged with *A. fumigatus* hyphae [81]. Echinocandins exert an immunomodulatory effect by “unmasking” β-glucans in the fungal cell wall, thus enhancing dectin-1–mediated neutrophil and macrophage activity against *Aspergillus* hyphae [82]. It has also been proposed that antifungal therapy can ameliorate graft-vs-host disease in dectin-1–deficient individuals, who have higher rates of *Candida* colonization, by down-regulating the persistent activation of the Th17 inflammatory pathway in response to colonizing fungi [83].

**CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS**

Antimold agents can be used to treat established (targeted therapy) or suspected disease (empirical therapy), to prevent infection (prophylaxis), and to prevent invasive disease (diagnostically driven strategy). Although no single laboratory test should under any circumstance replace clinical judgment, host-derived biomarkers can potentially facilitate clinical decision making in the three later scenarios. First, empirical treatment for *Aspergillus* is frequently given to high-risk neutropenic patients with suspected IA because of persistent fevers despite treatment with broad-spectrum antibiotics. The shortcomings of this strategy include breakthrough IFI with non-*Aspergillus* molds, toxicity and drug interactions, and the emergence of antifungal resistance [2]. In this setting, incorporation of immune biomarkers into clinical algorithms could help decreasing the overtreatment of febrile, but otherwise stable, patients by stratifying individuals based on the presence of a protective immune phenotype (eg, preserved Th17 function).

Second, targeted antimold prophylaxis in hematological patients (which can be associated with increased risk of drug toxicity [3]) and universal antimold prophylaxis (of questionable utility in lung transplant recipients [84]) are more likely to benefit individuals with an immunological profile associated with increased susceptibility to IA (eg, PTX3 or dectin-1 deficiency). Analogous to the monitoring of CMV-specific cell–mediated immunity to predict CMV disease in high-risk SOT recipients [28, 85], it is interesting to speculate that assessment of *Aspergillus*–specific T cells might be useful in stratifying individuals at risk of IA.

Finally, fungal biomarker–based strategies facilitate timely diagnosis and early treatment of IA [2], but the frequency of testing varies from one center to another and the optimal interval for measurement of serum galactomannan (or *Aspergillus* polymerase chain reaction) and lung imaging remains to be defined. Host-derived biomarkers could help identify patients who are likely to require more frequent or longer testing in diagnostically driven strategies.

Similar to their clinical applicability, the turnaround time of testing for these biomarkers remains to be defined. Considering that expression levels can be measured by flow cytometry or enzyme-linked immunosorbent assays (Table 3), the turnaround time for most of these markers might be short enough to allow monitoring in real-life clinical practice. As an analogy, the CD4+ cell counts used in monitoring HIV-infected individuals are

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obtained by flow cytometry. Similarly, the average turnaround time for serum/bronchoalveolar lavage galactomannan (measured by immunoassay) at our institution is 3 business days.

A better understanding of antifungal immunity and human susceptibility to IA has led to the identification of potential immune correlates of protection. Although incorporation of these biomarkers into clinical algorithms may potentially guide clinical decision making and lead to better outcomes, their clinical value remains to be defined. Clinical trials designed to evaluate the role of immune biomarkers in risk stratification strategies that facilitate individualized antifungal therapy/prophylaxis are urgently needed.

Notes

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