Immunotherapy for Fungal Infections

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Opportunistic fungal infections are major causes of morbidity and mortality among immunocompromised individuals. Fungi have evolved complex and coordinated mechanisms to survive in the environment and in the mammalian host. Fungi must adapt to “stressors” in the host (including scarcity of nutrients, pH, and reactive oxygen and nitrogen intermediates) in addition to evading host immunity. Knowledge of the immunopathogenesis of fungal infections has paved the way to promising strategies for immunotherapy. These include strategies that increase phagocyte number, activate innate host defense pathways in phagocytes and dendritic cells, and stimulate antigen-specific immunity (e.g., vaccines). Immunotherapy must be tailored to specific immunocompromised states. Challenges exist in bringing promising immunotherapies from the laboratory to clinical trials.

AUGMENTATION OF NEUTROPHIL NUMBER

Colony-stimulating factors (CSFs). CSFs are mostly used to accelerate myelopoiesis in patients with neutropenia. Prophylaxis with a CSF can reduce the incidence of neutropenic fever by as much as 50%, which in some studies has translated into a reduction in hospitalization and use of antibiotics [8]. In a randomized study of patients receiving chemotherapy to treat acute myelogenous leukemia, prophylaxis with granulocyte-macrophage CSF (GM-CSF) led to a lower frequency of fatal fungal infections than that seen with placebo (1.9% vs. 19%) and reduced overall early mortality [9, 10]. However, CSF have not produced a survival advantage in other studies.

CSFs also augment phagocyte function. Granulocyte CSF (G-CSF), GM-CSF, and macrophage CSF increase the fungicidal activity of phagocytes in vitro against Candida and Aspergillus species [11–14]. G-CSF influences survival, proliferation, and differentiation of all cells in the neutrophil lineage and augments the function of mature neutrophils. Macrophage CSF increases...
<table>
<thead>
<tr>
<th>Immunodeficiency or patient group</th>
<th>Population(s) at highest risk</th>
<th>Fungal pathogen(s)</th>
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<tr>
<td>Neutropenia</td>
<td>Patients receiving cytotoxic chemotherapy for malignancy, conditioning regimen for hematopoietic stem cell transplantation, or radiation therapy; patients with aplastic anemia</td>
<td>Aspergillus species and other filamentous fungi (e.g., zygomycetes, Fusarium and Scedosporium species), Candida species, Trichosporon species</td>
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<tr>
<td>Qualitative neutrophil dysfunction (inherited)</td>
<td>Patients with chronic granulomatous disease</td>
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<td>Mucosal immunity deficit</td>
<td>Patients receiving mucotoxic chemotherapy (e.g., anthracycline regimens for acute leukemia); patients with graft-versus-host disease of the gastrointestinal tract</td>
<td>Candida species</td>
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<td>Defective cellular immunity</td>
<td>Patients with AIDS or with certain leukemias and lymphomas; patients receiving corticosteroids, calcineurin inhibitors, anti-lymphocyte immunoglobulin preparations, anti-TNF-α agents (e.g., infliximab), purine analogues (e.g., fludarabine), and alemtuzumab</td>
<td>Aspergillus species and other filamentous fungi, Candida species, Cryptococcus neoformans, dimorphic fungi, Pneumocystis jirovecii (formerly Pneumocystis carinii)</td>
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<td>Allogeneic hematopoietic stem cell transplant recipients, by time after transplantation</td>
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<td>&lt;1 month</td>
<td>Patients with neutropenia and mucosal damage from conditioning regimen</td>
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<td>1–6 months</td>
<td>Patients with cellular and humoral immunodeficiency; patients receiving high-dose steroids for graft-versus-host disease, which causes global immunosuppression and disables phagocyte and cellular immunity</td>
<td>Aspergillus species and other filamentous fungi, Candida species, C. neoformans, dimorphic fungi, P. jirovecii</td>
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<td>&gt;6 months</td>
<td>Patients with graft-versus-host disease; patients receiving a transplant from an HLA haplotype-mismatched or unrelated donor; patients with a T cell-depleted allograft, lymphopenia, or cytomegalovirus disease; patients with multiple stem cell transplantations*</td>
<td>Aspergillus species and other filamentous fungi, Candida species, C. neoformans, dimorphic fungi, P. jirovecii</td>
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* In allogeneic hematopoietic stem cell transplant recipients, partial reconstitution of cellular immunity is expected and the risk of opportunistic fungal infections is reduced at >6 months after transplantation in the absence of graft-versus-host disease. However, graft-versus-host disease requiring intensive immunosuppressive therapy (e.g., corticosteroids) disables neutrophil and macrophage function and prevents reconstitution of cellular and humoral immunity. Such patients are at high risk for invasive fungal infection late after transplantation.
phagocytosis, chemotaxis, and secondary cytokine production in monocytes and macrophages [15].

GM-CSF stimulates various neutrophil effector functions and prolongs neutrophil survival in vitro, increases antibody-dependent cytotoxicity of eosinophils, accelerates the proliferation of the monocyte-macrophage system, and is a potent activator of monocytes and macrophages [15]. Thus, GM-CSF may have a theoretical advantage against pathogens such as *Candida* and *Aspergillus* species, for which host defense is dependent on both neutrophil and macrophage function.

Some studies in vitro [16] and in animal models [17, 18] show that G-CSF and GM-CSF have additive antifungal activity when combined with antifungal agents. A phase II randomized study of G-CSF plus itraconazole for invasive candidiasis and candidemia in nonneutropenic patients showed the safety of G-CSF but was not powered to determine efficacy [19]. Currently, the clinical database concerning CSFs as adjunctive therapy for fungal infections is inadequate to assess efficacy.

In allogeneic hematopoietic stem cell transplant recipients, G-CSF (but not GM-CSF) results in Th2 skewing of lymphocytes and promotes the development of regulatory T cells [20, 21]. Administration of G-CSF after T cell–depleted, haploidentity-mismatched transplantation was associated with faster neutrophil recovery but prolonged cellular immune dysfunction [22]. Prospective, randomized trials are required to assess the short-term benefits versus long-term immune consequences of G-CSF in allogeneic hematopoietic stem cell transplantation.

**Myeloid progenitors.** The myeloid progenitors—common myeloid progenitors and granulocyte-monocyte progenitors—have recently been identified. The addition of these progenitors to hematopoietic grafts in mice that had been rendered neutropenic conferred protection against challenge with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* [23]. Novel strategies, such as this approach to accelerate neutrophil recovery, merit further study.

**Granulocyte transfusions.** The rationale for granulocyte transfusions is to provide supportive therapy for a patient with neutropenia who has a life-threatening infection by augmenting the number of circulating neutrophils until neutrophil recovery occurs. Today, the impetus to reevaluate granulocyte transfusions stems largely from improvements made in donor mobilization methods using therapy with G-CSF and corticosteroids [24]. In addition, the use of community donors for granulocytapheresis was shown to be safe in a phase I/II study, thus increasing the pool of potential donors [25].

We reserve granulocyte transfusions for patients with prolonged neutropenia and life-threatening infections refractory to conventional therapy. In allogeneic transplants in which the donor and recipient are seronegative for cytomegalovirus, using cytomegalovirus-seronegative granulocyte donors is advised [26]. The Transfusion Medicine and Hemostasis Network of the National Heart Lung and Blood Institute is currently in the planning stages of a randomized study of adjunctive granulocyte transfusions among neutropenic patients with severe bacterial and fungal infections.

Granulocyte transfusion in chronic granulomatous disease is supported by the principle that a small number of normal phagocytes may be able to complement the oxidative defect in a large number of chronic granulomatous disease phagocytes [27]. Alloimmunization caused by prior therapy with granulocyte transfusions was considered to be a possible mechanism for failure of donor cell engraftment in some patients with chronic granulomatous disease undergoing allogeneic hematopoietic stem cell transplantation [28].

**RECOMBINANT IFN-γ**

Several cytokines (e.g., IL-12, IL-15 [29, 30], and IL-18) and chemokines [31] hold promise as adjunctive therapeutics for invasive fungal infections. We focus on recombinant (r) IFN-γ, because it is associated with the most-developed clinical database.
IFN-γ augments the antifungal activity of effector cells (macrophages and neutrophils) ex vivo against a variety of pathogens, including Candida albicans, Histoplasma capsulatum, Cryptococcus neoformans, and Aspergillus species [32, 33]. Data obtained from mouse models using cytokine depletion, gene knockout mice, and administration of exogenous cytokines have been instrumental in establishing the conceptual basis for rIFN-γ immunotherapy in invasive mycoses [33].

rIFN-γ is licensed as a prophylactic agent for patients with chronic granulomatous disease on the basis of a randomized trial in which IFN-γ reduced the number and severity of infections (mostly bacterial) in patients with chronic granulomatous disease by ∼70% [34]. Administration of rIFN-γ to patients with chronic granulomatous disease augmented ex vivo neutrophil-mediated damage of Aspergillus hyphae, presumably through non—nicotinamide adenine dinucleotide phosphate oxidase–dependent pathways [35]. Despite the widespread use of prophylactic rIFN-γ in chronic granulomatous disease, invasive fungal infections have remained a persistent problem, with an incidence of 0.1 fungal infections per patient-year [36].

Pappas et al. [37] conducted a phase II, double-blind, placebo-controlled study of adjunctive rIFN-γ to treat patients with AIDS-associated cryptococcal meningitis. rIFN-γ was well tolerated and showed a trend toward improved clinical and mycological success. Neta et al. [38] reported 2 patients with idiopathic CD4 lymphopenia with refractory cryptococcal meningitis that responded to treatment with rIFN-γ.

It was disappointing that a randomized trial evaluating rIFN-γ as adjunctive therapy for invasive aspergillosis was prematurely terminated before any patient was enrolled. On the basis of a large volume of preclinical studies and phase I and II clinical trials, adjunctive rIFN-γ (in combination with anti-fungal agents) merits evaluation in trials powered to address efficacy. Invasive molds should be the highest-priority fungal pathogens on the basis of their associated mortality [39].

One concern about the use of rIFN-γ in treating allogeneic hematopoietic stem cell transplant recipients is the potential for worsening graft-versus-host disease. Although preliminary results suggest that rIFN-γ therapy is safe in allogeneic hematopoietic stem cell transplant recipients [40, 41], the safety of rIFN-γ therapy cannot be predicted on the basis of this limited database and therefore merits evaluation in a clinical trial with safety as the primary end point in this specific population.

**INNATE PATHOGEN RECOGNITION PATHWAYS**

**Toll-like receptors (TLRs).** TLRs are a conserved family of receptors that recognize common protein, carbohydrate, or DNA pattern motifs on microbes, leading to initiation of signaling for cytokine production and T cell and dendritic cell maturation. TLRs recognize motifs on Candida [42] and Cryptococcus species [43] and regulate the induced inflammatory responses. TLR4-defective mice are more susceptible to C. albicans infection, and this is associated with impaired chemokine expression and neutrophil recruitment [42].

Aspergillus conidia, but not hyphae, stimulate macrophages to produce the proinflammatory cytokines TNF-α and IL-1 in a TLR4-dependent fashion [44]. In contrast, Aspergillus hyphae, but not conidia, stimulated production of the anti-inflammatory cytokine IL-10 through TLR2-dependent mechanisms. This switch from proinflammatory to anti-inflammatory signals during germination may help Aspergillus to evade host defenses. Wang et al. [45] reported that TLR4, but not TLR2, mediated activation of human monocytes by A. fumigatus hyphae. Other investigators found that both TLR2 and TLR4 recognize Aspergillus hyphae, stimulate proinflammatory cytokines in effector cells, and stimulate neutrophil recruitment [46, 47].

Local delivery of cytokine guanine (CpG) oligodeoxynucleotides (which signal through TLR9) and the Aspergillus allergen Asp f16 resulted in activation of airway dendritic cells capable of inducing Th1 priming and resistance to the fungus [48]. Thymosin-α1, a naturally occurring thymic peptide, induced maturation and IL-12 production in dendritic cells pulsed with Aspergillus, an effect mediated by distinct TLRs [49]. Thymosin-α1 augmented Th1 immunity against Aspergillus, accelerated myeloid recovery in neutropenic mice, and was protective against Aspergillus challenge in murine bone marrow transplant recipients.

Recognition of Aspergillus motifs and activation of neutrophils are coordinated by distinct members of the TLR family, each likely activating specialized antifungal effector functions and inflammatory responses [50]. Indeed, liposomal amphotericin B, in addition to its intrinsic antifungal activity, may stimulate antifungal resistance by activating TLR-4 in neutrophils [51]. These studies provide a rationale to stimulate or inhibit specific classes of TLRs as a means of enhancing both innate and antigen-specific immunity to fungi.

**Pentraxins.** Pentraxins are a superfamily of conserved proteins characterized by a cyclic multimeric structure. Pentraxin 3 is an innate pathogen recognition protein that binds to specific motifs on P. aeruginosa, Salmonella typhimurium, and A. fumigatus. Pentraxin 3-deficient mice were highly susceptible to Aspergillus infection [52]. These mice demonstrated defective recognition of conidia by alveolar macrophages and dendritic cells, as well as inappropriate induction of type 2 cytokine responses. Administration of pentraxin 3 protected against Aspergillus challenge in murine T cell–depleted allogeneic bone marrow transplant recipients [52] and potentiated the protective effect of treatment with subtherapeutic levels of amphotericin B [53].
**ANTIBODY-BASED THERAPY**

Antibody-based therapy has seemed promising in a variety of experimental fungal infections, and pilot clinical trials are underway. *C. neoformans* has a polysaccharide capsule that facilitates evasion of phagocytosis. Antibodies directed against capsular epitopes confer protection in murine cryptococcal infection [54–58]. A murine IgG1 (monoclonal antibody 18B7) has shown acceptable safety in a phase I dose-escalating study of patients with treated cryptococcal meningitis [59].

In addition to functioning as immunomodulators (e.g., facilitating phagocytosis), antibody therapy may function as a drug (e.g., by neutralizing a key secreted enzyme). Mycograb is a human genetically recombinant antibody against the *Candida* heat shock protein 90. Mycograb conferred protection in cases of murine systemic candidiasis in mice [60] and is currently being evaluated in clinical trials. Mycograb also demonstrated in vitro synergy with antifungal agents against *Aspergillus* species [61] and *C. neoformans* [62].

Antibodies targeted against secreted aspartyl proteases and fucosidic anti-idiotypic antibodies with yeast killer toxin activity [63] have also shown promise in animal models. Cenci et al. [64] showed protection of killer anti-idiotypic antibodies against early invasive aspergillosis in murine allogeneic T cell-depleted bone marrow transplant recipients.

Protective immunity to *Candida* and *Aspergillus* species is mediated by antigen-specific Th1 cells. T cells coordinate humoral responses, including antibody class switching. B cell–deficient mice were able to control primary *Candida* and *Aspergillus* infection but were unable to control reinfection with *C. albicans*. B cell–deficient mice failed to generate IL-10–producing dendritic cells and regulatory CD4+CD25+ T cells [65]. Antifungal opsonizing antibodies restored IL-10 production by dendritic cells, a finding suggesting that antibodies may modulate dendritic cell and T cell responses to fungal antigens.

Antibody-based therapy has largely been applied to extra-cellular pathogens. However, antibodies to a cell surface histone-like protein of *H. capsulatum* enhanced phagocytosis and were protective in murine histoplasmosis [66]. The effectiveness of antibody-based therapy in experiments involving histoplasmosis is particularly intriguing, because *H. capsulatum* is capable of survival and replication in host cells.

**VACCINATION**

Vaccine development is a priority for several fungal pathogens, including *C. albicans* [67], *C. neoformans* [68], *A. fumigatus* [69], and dimorphic fungi [70]. Many challenges confront vaccine development for fungi, including different host risk factors and modes of fungal pathogenesis. No single antigen can be expected to be used in a “pan-fungal” vaccine; rather, specific tailored vaccines will be required for the major fungal pathogens [71].

One impediment to fungal vaccine development is that the patients who are most susceptible to opportunistic fungal infections are those least able to mount protective responses. Wuthrich et al. [72] showed that CD4+ T cells were dispensable in vaccine immunity against pulmonary blastomycosis (an extracellular pathogen) and histoplasmosis (a facultative intracellular pathogen) in immunocompromised mice. CD8+ T cells, in the absence of CD4+ T cells, mediated vaccine-induced protection against these fungi, and protection by Blastomyces-immune CD8+ T cells could be adoptively transferred. These results contradict the dogma that induction of CD8+ T cell responses against exogenously processed antigens requires CD4+ T cells, and they provide encouragement for vaccine development for patients with impaired CD4+ T cell immunity (e.g., patients with advanced AIDS).

Another impediment relates to the limited number of licensed vaccine adjuvants. Candidate adjuvants that act on multiple innate and antigen-specific host defense pathways are likely to be the most effective in protecting against opportunistic fungal infections. The definition of adjuvants has mostly been restricted to those that stimulated antibody titers (e.g., pneumococcus) or, in the case of the bacillus Calmette-Guérin vaccine, delayed-type hypersensitivity responses. More recently, the concept of adjuvants has been expanded to include soluble mediators and antigenic carriers (e.g., endotoxin, Flt3 ligand, and heat-shock protein) that activate antigen-presenting cells and stimulate innate and cellular immunity [73].

Heat-shock proteins are an example of naturally produced proteins that have been exploited as vaccine adjuvants in cancer and infectious diseases [73–79]. Heat-shock proteins exhibit powerful immunostimulatory effects on dendritic cells in a TLR2- and TLR4-dependent fashion [80, 81] and induce antibody and type I cellular immunity that may be promising in fungal vaccine development [82]. Fungi also produce heat-shock proteins that may be targets for vaccine development. Long et al. [83] identified heat-shock protein 60 as the ligand on *H. capsulatum* that mediates binding to CD18 receptors on human macrophages. Immunization with recombinant heat-shock protein 60 from *H. capsulatum* conferred protection from a subsequent challenge in mice [84].

Paradoxically, vaccination may be useful to attenuate pathological inflammatory responses or induce tolerance. Allergic bronchopulmonary aspergillosis develops from sensitization to airway *A. fumigatus* antigens, leading to a Th2 CD4+ cell response characterized by secretion of IL-4, IL-5, and IL-13 [85]. T cells are the key components mediating allergic responses to *A. fumigatus* antigens in mouse models of allergic bronchopulmonary aspergillosis [86]. There is significant interest in immunotherapy for allergic bronchopulmonary aspergillosis,
including the use of CpG sequences [87], recombinant allergens, and peptides to induce tolerance, as well as antigenic and DNA-based vaccines aimed at controlling the Th2-mediated responses in allergic bronchopulmonary aspergillosis [88].

Adoptive immunotherapy. In mice, the importance of cell-mediated immunity against Aspergillus infection (an extracellular pathogen) has become well established [89, 90]. Immunization of immunocompetent mice with an Aspergillus crude filtrate resulted in memory responses mediated by antigen-specific, Th1-committed CD4+ T cells [91]. Adoptive transfer of these cells conferred protection to neutropenic mice, thereby establishing a “proof of principle” regarding cellular immunity as a target for immune augmentation in invasive aspergillosis [91]. This study also showed the overly simplistic nature of the generalization that host defense against extracellular pathogens (e.g., Aspergillus) is humoral, whereas defense against intracellular pathogens is cellular.

Cellular adoptive immunotherapy may include not only adoptive transfer of specific Th1 cells but also active vaccination with dendritic cells. Dendritic cells pulsed with C. albicans or A. fumigatus activated CD4+ Th1 cell responses on adoptive transfer into immunocompetent mice. The infusion of fungus-pulsed dendritic cells accelerated the recovery of functional antifungal Th1 responses in mouse allogeneic hematopoietic stem cell transplant recipients and conferred protection against experimental aspergillosis [92].

Dendritic cells also have key functions in containing and dampening inflammatory responses by tolerization through the induction of regulatory T (Tr) cells [93]. These studies in mice demonstrate that the remarkable functional plasticity of dendritic cells in response to fungi can be exploited for the deliberate targeting of cells and pathways of cell-mediated immunity in response to fungal vaccines in transplantation [93–95] (figure 1).

CHALLENGES IN DESIGNING MYCOLOGICAL IMMUNOTHERAPY TRIALS

Immunotherapy can be evaluated as preventive or as adjunctive therapy. Prevention should be targeted to patients at significant risk for the infection of interest and should focus on infections with significant morbidity or mortality that are inadequately covered by standard therapies.

One challenge relates to accrual of adequate numbers of patients in trials involving uncommon infections. Assuming a vaccine with 80% efficacy in preventing invasive aspergillosis and a 5% frequency of invasive aspergillosis in a population of interest (e.g., allogeneic hematopoietic stem cell transplant recipients), subjects receiving the vaccine would be expected to
have a 1% rate of invasive aspergillosis. Assuming a power of 0.8, \( \alpha < .05 \), and a 1-sided analysis designed to show superiority of vaccination, a sample size of 544 patients would be required. This number is, in fact, an underestimate, because it does not consider false-positive diagnoses or differences in antifungal prophylaxis and diagnostic evaluation between centers, which would reduce the ability of the analysis to detect a protective effect of vaccination. Selecting a patient population with a higher risk of invasive aspergillosis (e.g., T cell–depleted allogeneic hematopoietic stem cell transplant recipients) would reduce the required sample size.

The paradigm for clinical trial design aimed at preventing infection with dimorphic fungi (e.g., by vaccination) will be different, because these pathogens affect both immunocompetent and immunocompromised persons and are geographically restricted. In the 1980s, a randomized placebo-controlled study involving 2867 subjects from regions of endemicity showed no benefit of the formalin-killed spherule vaccine in preventing coccidioidomycosis [96]. The frequency of definite coccidioidomycosis was \( \sim 1\% \), emphasizing the need for large numbers of subjects to demonstrate vaccine efficacy. Additional candidate vaccines for coccidioidomycosis are being developed [97].

Studies of adjunctive immunotherapy for established infection should target specific well-defined patient groups to maximize the likelihood of detecting a treatment effect. Kullberg et al. [98] reasonably suggest that phase I and II studies of immunotherapies should focus on laboratory surrogates that are likely to predict efficacy (e.g., augmenting Th1 responses), which would pave the way to larger studies that evaluate clinically relevant end points (e.g., survival and resolution of infection).

Funding for clinical trials of novel antifungal therapeutics may be the most important hurdle. According to the US Food and Drug Administration, developing a new drug costs an average of $500 million and takes \( \sim 8.5 \) years. Vaccines targeted to pathogens that affect a broad segment of the general population have more attractive marketing potential than do vaccines for opportunistic fungal pathogens that affect only those individuals with severe defects in the immune system. Bringing promising, novel antifungal immunotherapeutics to clinical trials and to market will likely require creative partnerships between academia, industry, and government.

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References