Review Article

Immunotherapy for fungal infections with special emphasis on central nervous system infections

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Opportunistic fungal infections are major causes of morbidity and mortality in the immunocompromized. Fungi have evolved complex and coordinated mechanisms to survive in the environment and the mammalian host. Fungi must adapt to "stressors" in the host, including nutrient scarcity, 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 immunocompromized states. Our review focuses on cryptococcosis and coccidioidomycosis because of the propensity of these diseases to involve the central nervous system (CNS). The CNS has long been considered "immunologically privileged" in the sense of being isolated from normal immune surveillance. This notion is only partially accurate. Immune-based therapies for fungal CNS disease are at an exploratory level and merit further evaluation in clinical trials.

Key words: Aspergillosis, central nervous system fungal infections, cryptococcosis, immunotherapy, opportunistic infections

Opportunistic fungal infections are a major cause of morbidity and mortality in immunocompromized patients. The major defects in host defense that render susceptibility to opportunistic fungal infections are summarized in Table 1. Deficits in host defense which render persons susceptible to fungal infections are complex, but can be broadly divided into the following categories: 1) neutropenia; 2) qualitative deficits in phagocyte function; 3) deficits in cell-mediated immunity; 4) deficits in humoral immunity; and 5) deficits in mucosal immunity.

Patients often have multiple defects in immunity predisposing to opportunistic fungal infections. There is now significant interest in several immunomodulatory strategies to combat opportunistic fungal infections, which of course need to be tailored to specific immunocompromised populations. These strategies broadly include colony growth factors, granulocyte transfusions (in neutropenic patients), recombinant cytokines, passive immunization with monoclonal antibodies and vaccine development [Table 2].

Opportunistic yeasts produce a spectrum of clinical disease that ranges from superficial and mucosal infections to disseminated disease. *Candida* species are endogenous flora that may gain access to the bloodstream through breeches in anatomical barriers. Oral mucosal candidiasis usually reflects severe T-cell depression commonly associated with AIDS or high-dose systemic corticosteroids. The bowel is the principal portal of entry in patients with acute leukemia receiving mucotoxic regimens.[1]

Host defense against *Cryptococcus neoformans* is also principally dependent on T-cell immunity. Thus, patients with AIDS, stem cell and solid organ transplant recipients and other patients receiving intensive immunosuppressive therapy are at risk for cryptococcal disease. Immunoglobulins directed against capsular epitopes and complement facilitate phagocytosis of the organism and play a role in host defense.[2-6] The principal portal of entry for C. *neoformans* is by inhalation, with subsequent spread to the blood and central nervous system.

Endemic dimorphic include *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Sporothrix schenkii* and *Penicillium marneffei*. Dimorphic fungi cause infections in immunocompetent persons, but severe infections with dissemination are more common in patients with compromised cellular immunity (e.g., AIDS, transplant recipients).

Filamentous fungi (moulds) are ubiquitous soil

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Table 1: Immunodeficiencies predisposing to opportunistic fungal infections

Immunodeficiency or patient group	Population(s) at highest risk	Fungal pathogen(s)
Neutropenia	Patients receiving cytotoxic chemotherapy for malignancy, conditioning regimen for hematopoietic stem cell transplantation or radiation therapy; patients with aplastic anemia	*Aspergillus* species and other filamentous fungi (e.g, zygomycetes, *Fusarium* and *Scedosporium* species), *Candida* species, *Trichosporon* species
Qualitative neutrophil dysfunction (inherited)	Patients with chronic granulomatous disease	*Aspergillus* species and other filamentous fungi
Mucosal immunity deficit	Patients receiving mucotoxic chemotherapy (e.g., anthracycline regimens for acute leukemia); patients with graft-versus-host disease of the gastrointestinal tract	*Candida* species
Defective cellular immunity	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	*Aspergillus* species and other filamentous fungi, *Candida* species, *Cryptococcus neoformans,* dimorphic fungi, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*)
Allogeneic hematopoietic stem cell transplant recipients, by time after transplantation		
<1 month	Patients with neutropenia and mucosal damage from conditioning regimen	*Aspergillus* species and other filamentous fungi, *Candida* species
1-6 months	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	*Aspergillus* species and other filamentous fungi, *Candida* species, *C. neoformans,* dimorphic fungi, *P. jirovecii*
>6 months	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[a]	*Aspergillus* species and other filamentous fungi, *Candida* species, *C. neoformans,* dimorphic fungi, *P. jirovecii*

[a]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.

Note: This table is adapted from Table 1 in Segal *et al.* Immunotherapy for Fungal Infections, Clin Infect Dis, 2006; 42:507-15

Table 2: Goals and strategies for augmentation of the immune response to fungal infections

Goal	Strategies
Increase in neutrophil number	CSFs (G-CSF and GM-CSF); granulocyte transfusions; myeloid progenitors (common myeloid progenitors, granulocyte-monocyte progenitors); thymosin-α1
Activation of neutrophils	CSFs (G-CSF and GM-CSF); cytokines (e.g, recombinant IFN-γ); chemokines; TLR activation
Activation of macrophages and dendritic cells	Colony-stimulating factors (macrophage colony-stimulating factor and GM-CSF); cytokines (e.g, recombinant IFN-γ); TLR activation
Heightened cellular immunity	Cytokines (e.g, recombinant IFN-γ); TLR activation; pentraxin 3; thymosin-α1; vaccines
Heightened humoral immunity	Vaccines; antibody administration (e.g., monoclonal antibody 18B7 for *Cryptococcus neoformans*)

Most of the listed strategies are experimental and have not been evaluated among patients. G-CSF, granulocyte CSF; GM-CSF, granulocyte-macrophage CSF; TLR, toll-like receptor.

Note: This table is adapted from Table 2 in Segal *et al.* Immunotherapy for Fungal Infections, Clin Infect Dis, 2006; 42:507-15

inhabitants whose conidia (spores) we inhale on a regular basis. The sinopulmonary tract is the most common portal of entry. The respiratory mucosa and alveolar macrophages constitute the first line of host defense against conidia. At the hyphal stage, neutrophils are most important in controlling infection. The frequency of mortality from invasive aspergillosis has increased by several-fold over the past 10 to 20 years.[7,8] Patients at risk include those with neutropenia and leukemia, allogeneic hematopoietic stem cell transplant (HSCT) recipients, solid organ transplant recipients (particularly lung), chronic granulomatous

disease (CGD) and advanced AIDS.[8-10] Among allogeneic HSCT recipients, factors that increase the risk of invasive mould infections after neutrophil recovery include graft-versus-host disease (GVHD), human leukocyte antigen haplotype-mismatched or unrelated donors, receipt of T-cell depleted allografts, lymphopenia, receipt of systemic corticosteroids and cytomegalovirus disease.[11-15] Non-*Aspergillus* moulds including *Fusarium* species and zygomycetes have been observed with greater frequency among patients undergoing multiple stem cell transplants, a reflection of severe immunocompromise.[16]

Central Nervous System Fungal Infections

Any fungal infection can theoretically cause central nervous system (CNS) disease via hematogenous dissemination. *C. neoformans*, for reasons that are not well understood, has a tropism for the CNS. This yeast is ubiquitous in the environment and acquisition of infection is via inhalation. If the infection is not contained, hematogenous infection with distant seeding may occur. Meningitis is the most common manifestation of cryptococcal disease.

Dimorphic fungi can also cause CNS disease via inhalation and hematogenous dissemination. Of the dimorphic fungi, CNS disease is probably the most common in coccidioidomycosis. CNS coccidioidomycosis is not reliably eradicated even with prolonged antifungal therapy, necessitating the need for life-long treatment.[17]

Vaccine development is a priority for several fungal pathogens, including *Candida* spp., *C. neoformans*, *Aspergillus* spp., and the dimorphic fungi, *H. capsulatum*, *C. immitis* and *B. dermatiditis*.[18-21] This requires knowledge about host-pathogen interactions and mechanisms that the pathogen has evolved to evade host defense and survive in a hostile environment. Knowledge about specific mechanisms (virulence factors) that the pathogen uses to colonize, invade and proliferate in the host sheds light on potential targets for drug development and immunotherapy.

CNS disease is an uncommon complication of candidemia. Central nervous system disease complicating invasive aspergillosis and other mould infections can occur via direct extension from sino-orbital disease or via hematogenous dissemination. CNS mould infections are often rapidly fatal and will likely not be amenable to immunotherapy once CNS disease has occurred.[22,23] For these reasons, our review will focus on immunotherapeutic strategies targeted to CNS cryptococcosis and coccidioidomycosis.

The CNS has long been considered "immunologically privileged" in the sense of being isolated from normal immune surveillance. This notion is only partially accurate. Clearly, bacterial meningitis is associated with a robust cerebrospinal fluid (CSF) neutrophilic leukocytosis. Viral meningoencephalitides and chronic meningitis related to mycobacterial and fungal infections induce a variable CSF leukocytic pleocytosis. The brain is also a site where antigen display through major histocompatibility complexes can occur. Sensitized T-cells to myelin protein constituents are able to cross the blood-brain barrier and mediate multiple sclerosis in patients and experimental autoimmune encephalomyelitis.[24] This has raised interest in whether immunological manipulation can be used therapeutically when targeted against CNS tumors and infectious diseases. As we discuss, immunomodulatory strategies targeted to CNS fungal diseases are at an exploratory level.

Immunomodulatory Strategies

Immunomodulatory strategies can be applied in two different modes: prevention and therapy of established disease. In the prevention mode, a specific patient population at risk for an invasive fungal infection received an immunomodulator, such as a vaccine. Since cryptococcal disease occurs principally in persons with severe T-cell impairment (e.g., AIDS), an efficacy trial evaluating a hypothetical cryptococcal vaccine would focus on specific high-risk patients.

In contrast, the risk for coccidioidomycosis is principally geographic. Coccidioidomycosis is endemic in the southwestern United States and, thus, a prevention strategy against coccidioidomycosis would focus on persons living in this area. All persons living in this region are at risk for coccidioidomycosis, though the frequency and severity of disease are increased in specific persons, including those with impaired cellular immunity, the elderly, pregnant women and certain racial groups (e.g, Filipino, African).[25,26]

The second mode to use an immunomodulator is to treat invasive fungal disease. From a practical standpoint, the immunomodulator is used as adjunctive therapy in combination with an antifungal agent. Though several immunomodulatory strategies exist that target innate and antigen-specific immunity [Table 2], evidence of efficacy in well-designed clinical trials is sparse.[27] Adjunctive immune-based therapy for CNS fungal diseases must also address specific challenges related to neuro-immunolozgy.

Cryptococcus neoformans

C. neoformans is unique among the pathogenic fungi in having a polysaccharide capsule that is a major virulence factor that allows the pathogen to evade phagocytosis. Acapsular variants of *C. neoformans* have attenuated virulence. The *C. neoformans* capsule

also interferes with dendritic cell (DC) activation and maturation, a mechanism by which the fungus may suppress an effective T-cell response.[28] The capsule is composed primarily of glucuronoxylomannan (GXM); GXM has an alpha-1,3 linked mannose backbone that is O-acetylated and substituted with single side chains of xylose and glucouronic acid. The degree of acetylation and xylose substitution can vary, producing four serotypes. Serial isolates of *C. neoformans* from chronically infected patients show changes in virulence, karyotype and capsular polysaccharide structure, suggesting that structural changes occur during chronic infection.[29] "Phenotypic switching" (high frequency reversible changes in colony morphology) has been described in several strains of *C. neoformans*. For example, phenotypic switching in *C. neoformans* SB4 is associated with changes in colony morphology that included: 1) smooth, 2) wrinkled and 3) serrated.[30] The wrinkled phenotype was the most virulent in experimental infection. Infection of rats with serrated *C. neoformans* produced the most intense inflammatory responses characterized by granuloma formation and necrosis. Wrinkled *C. neoformans* produced a minimal inflammatory response. Thus, phenotypic switching affects *C. neoformans* virulence and the host inflammatory response, which may confer an advantage in establishing chronic infection and evading host defense.

Antibodies directed against capsular epitopes confer protection in murine cryptococcal infection.[31-35] Radioimmunotherapy, in which antifungal antibodies are conjugated to radioisotopes are being evaluated in animal studies.[36,37] Murine IgG1 (Mab 18B7) was well-tolerated in a Phase I dose-escalating trial in patients with cryptococcal meningitis.[38] Additional Phase II and III studies will be required to demonstrate if adjunctive therapy with anti-cryptococcal antibody confers additional benefit over standard antifungal therapy alone.

Interferon-γ

Exposure to various pathogens can stimulate at least two patterns of cytokine production by CD4+ T-cells. Th1 cells are defined by production of interferon (IFN)-γ, lymphotoxin and IL-2 and Th2 cells by production of IL-4, IL-5, IL-9, IL-10 and IL-13. Interferon-γ is produced by lymphocytes (CD4+, CD8+, NK cells) as well as macrophages and perhaps neutrophils.[39] It is induced by a number of signals, including IL-12 and IL-18[40,41] and in turn induces hundreds of genes, including its own inducers.[42,43]

Though several cytokines, including IL-12, IL-15 and TNF-α may also hold promise as adjunctive therapeutics, we will focus our discussion on IFN- γ because the database on rIFN-γ is the most developed. Several laboratories have shown that IFN-γ augments the antifungal activity of effector cells (macrophages and neutrophils) *ex-vivo* against a variety of pathogens, including *Candida albicans*, *Histoplasma capsulatum*, *Coccidiolides immitis*, *Cryptococcus neoformans* and *Aspergillus* species.[27,44] Data in mouse models using cytokine depletion, gene knockout mice and administration of exogenous cytokines have been instrumental in establishing the conceptual basis for immunotherapy in invasive mycoses and in paving the way to early clinical trials.

In murine cryptococcal infection, administration of IL-12, a potent inducer of the Th-1 type phenotype, resulted in ~10-fold decreases in the organism burden in the central nervous system.[45] In addition, the combination of fluconazole plus IL-12 significantly decreased the organism burden compared with either agent alone.[45] Lutz *et al.*[46] evaluated combination IFN-γ plus amphotericin B in murine cryptococcal infection. Interferon-γ alone was modestly effective but significantly potentiated amphotericin B in reducing infection in the brain. The efficacy was seen after both lethal and non-lethal challenges. In non-lethal infection, only the combination amphotericin B plus IFN-γ resulted in sterilization of the central nervous system. Potentiation of fluconazole was less impressive. *Ex-vivo* amphotericin B augmented IFN-γ induced killing of *C. neoformans* by mouse peritoneal macrophages.[47] This enhanced killing was associated with IFN-γ-mediated augmentation of IL-1, TNF-α and nitric oxide production by peritoneal macrophages

These and other studies led to a study of adjunctive IFN-γ in cryptococcal meningitis. Pappas *et al.*[48] conducted a Phase II, double-blind, placebo-controlled study to evaluate the safety and antifungal activity of adjuvant recombinant IFN-γ in AIDS-associated acute cryptococcal meningitis. Patients received 100 or 200 micrograms of rIFN-γ or placebo, thrice weekly for 10 weeks, plus standard therapy with intravenous amphotericin B, with or without flucytosine, followed by therapy with fluconazole. End points included conversion of cerebrospinal fluid fungal cultures from positive to negative at two weeks, resolution of symptoms and survival. Among 75 patients, two-week CSF sterilization occurred in 13% of placebo recipients, 36% of rIFN-γ (100 micrograms) recipients and 32% of rIFN-γ (200 micrograms) recipients. There was a trend toward improved combined mycologic and clinical success in rIFN-γ recipients (26% vs. 8%; $P=0.078$) and the drug was well tolerated.

AIDS-asssociated cryptococcal meningitis responds to standard antifungal therapy in approximately 70% of patients and 10-week survival following diagnosis is expected to be 90%.[49] At this point, it is premature to use adjunctive cytokine therapy as routine care for cryptococcal meningitis. A Phase III trial with efficacy as the primary endpoint is required to demonstrate

the benefits versus risk of rIFN-γ. It is reasonable to consider rIFN-γ in the setting of CNS disease refractory to standard antifungal therapy where options are very limited, though we emphasize that data in this setting are at the case report level.[50]

Antiretroviral therapy in patients with HIV infection

Without question, the most successful immune-based strategy to prevent cryptococcal disease in patients with HIV infection is the development of highly active antiretroviral therapy (HAART). In a large proportion of HIV-positive patients, HAART decreases the HIV viral load, enabling reconstitution of cellular immunity. McNeil *et al*[7] evaluated trends in mortality related to fungal diseases in the Unites States between 1980 and 1997. The number of cases of HIV-associated cryptococcal-disease-related fatalities increased in the mid and late 1980s, remained stable through the early and mid-1990s and then significantly decreased. The largest percentage decrease in *C. neoformans*-related mortality occurred in 1996-1997, coincident with the widespread use of HAART. Improved antifungal therapy and prophylaxis may have also influenced the reduction in cryptococcal disease-related mortality. Surveillance studies of HIV-associated cryptococcosis in the United States[51,52] and France[53] showed a reduction in cryptococcal disease associated with HAART, though, in some areas the decrease in the incidence of cryptococcosis predated the availability of HIV protease inhibitors.[51]

Immune reconstitution inflammatory syndrome (IRIS), results from an exuberant inflammatory response towards previously diagnosed or incubating pathogens (e.g, mycobacterial and cytomegalovirus disease).[54] Immune reconstitution inflammatory syndrome is well-described in AIDS-associated cryptococcal meningitis following initiation of effective antiretroviral therapy and manifests with meningismus and elevated CSF opening pressures, protein levels and white blood cell counts.[55,56] Repeat CSF cultures are required to distinguish IRIS from persistent or recrudescent cryptococcal disease. Immune reconstitution inflammatory syndrome does not represent treatment failure.

Studies have reported that in HIV-positive patients with prior cryptococcal meningitis who have an immunologic response to HAART, discontinuation of secondary antifungal prophylaxis is safe if patients are closely monitored for recrudescence of disease.[57,58] Our own practice is to continue secondary prophylaxis with fluconazole in these patients.

Coccidiodes immitis

The dimorphic fungi exist in the yeast form at body temperature and in the filamentous form at lower temperatures. *C. immitis* is endemic in southwestern United States. The tissue form is the spherule. Arthroconidia are inhaled from the environment where they convert into spherules. Spherules enlarge (20 to 100 um) and segment internally into hundreds of endospores. Mature endospores rupture through the spherule where they can extend the local infection or disseminate. The spherule prevents access of neutrophils to maturing endospores and following rupture, endospores are themselves covered by a matrix from the inner spherule wall which may also aid in evading phagoctyosis.[59] The potential for *C. immitis* to widely disseminate has prompted the search for extracellular fungal proteinases[60,61] that may be targets for inhibitors or vaccines.

In mouse models, IFN-γ plays a pivotal role in resistance to *C. immitis*, whereas IL-4 down-regulates protective immunity against *C. immitis*.[62] Adjunctive therapy of refractory coccidioidomycosis with IFN-γ in patients is anecdotal and cannot be routinely advised.[63]

Substantial effort has been devoted to developing a vaccine for coccidioidomycosis. In the 1980s, a randomized placebo-controlled study involving 2,867 subjects from endemic regions showed no benefit of the formalin-killed spherule vaccine in preventing coccidioidomycosis.[64] Additional candidate vaccines for coccidioidomycosis are being developed, with efforts focused on identifying immunodominant Coccidioides antigens to be used in subunit vaccines (http:www.valleyfever.com/). Studies in multiple laboratories have shown that antigen 2/proline-rich antigen (Ag2/PRA) as both protein and DNA vaccines provide significant protection in experimental coccidioidomycosis.[65-69] The completed and genomic sequence of *C. immitis* may facilitate identifying new candidates antigens for vaccine development.

Conclusions

CNS fungal diseases are important causes of morbidity and mortality, particularly in the severely immunocompromized. Novel immune-based therapies are required both to prevent fungal diseases and as adjunctive therapy. Our review focused on cryptococcosis and coccidioidomycosis because of the propensity of these diseases to involve the CNS. Several immune augmentation strategies exist [Table 2]. Several challenges exist regarding bringing promising immune-based therapies developed in the lab to clinical trials (reviewed in).[27] So far, clinical trials on CNS fungal diseases have involved Phase I and II trials in AIDS-associated cryptococcal meningitis. Such studies address safety and are not powered to evaluate efficacy. The widespread use of HAART in HIV-infected patients has dramatically decreased the incidence of cryptococcal meningitis, such that large pivotal clinical trials of cryptococcal meningitis may no longer be

feasible in the United States because of inadequate patient numbers; collaboration with international study sites will likely be required. Several candidate vaccines against coccidioidomycosis are at pre-clinical stages of development.

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