Chapter 1

ANTIFUNGAL IMMUNOTHERAPY:
A REALITY CHECK

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ABSTRACT
In the last three decades, fungi have emerged as a major cause of human disease, especially among the immunocompromised. Conventional treatment and control measures have failed to significantly reverse rising trends of morbidity and mortality associated with Candida, Aspergillus, Cryptococcus, Histoplasma, Coccidioides and other fungal infections. Chemotherapy, the mainstay treatment of fungal diseases, has improved in recent years through the introduction of new derivatives (voriconazole, posaconazole, liposomal amphotericin B) of old antifungals and the introduction of whole new ones (echinocandins and terbinafine). However, chemotherapy-associated difficulties (toxicity, resistance, varied spectra of activity and inability to sterilize infected organs) will likely continue to pose limitations on its efficacy. Hence, the need to develop new approaches to prevent and treat fungal infections has long been recognized. Data pertinent to the development and testing of experimental immunotherapeutics against fungal infections have started to accumulate since the 1970s; new insights into fungal immunity and recent advances in genomics and proteomics are improving and accelerating the process. The majority of experimental antifungal vaccines described thus far tend to activate T cell-mediated immunity; others that target humoral immunity have been described as well. Furthermore, there exists a plethora of antifungal immunomodulators that activate, suppress or variably manipulate the immune response. These efforts and discoveries notwithstanding, there is little to show at the clinical level. In contrast with the long list of immunotherapeutics that are currently in use to prevent and/or treat viral and bacterial infections, cancer, transplantation, autoimmunity and allergy, few (if any) have been approved so far to treat fungal diseases. Problems hindering the progress of antifungal immunotherapy are persistent and numerous but the following examples should suffice to make the point: (i) serious fungal infections are primarily secondary to underlying diseases, (ii) tendency of fungal infections to elicit cellular rather than humoral immune responses, (iii) risks of blunting immunity in immunocompromised hosts, (iv) risks of vaccine-induced pathogen strain replacement (v) universalism versus species- (or even strain-) specific vaccine activity, (vi) increased immunoevasive potential of fungal pathogens owing to their eukaryotism and capacity to
switch form, (vii) lack of capital investment in developing, testing and translating laboratory bench findings into bedside immunotherapeutics, and finally (viii) overoptimism and dashed hopes. This extensive review aims at discussing recent developments in antifungal immunotherapy in the context of difficulties hindering its progress. It is hoped that, by realistically discussing where things stand right now, future work may proceed in a more guided and fruitful manner.

Key words: Adjuvants, antibodies, antifungal agents, antimicrobial peptides (AMPs), aspergillosis, B cells, candidiasis, cryptococcosis, cytokines, dendritic cells, fungi, immunotherapy, monoclonal antibodies, mycosis, neutrophils, probiotics, radioimmunotherapy, T cells, vaccines.

LIST OF ABBREVIATIONS

Antibody (Ab), antigen presenting cell (APC), antimicrobial peptide (AMP), calcineurin inhibitor (CI), cluster of differentiation (CD), dendritic cell (DC), granulocyte monocyte colony stimulating factor (GM-CSF), heat shock protein 90 (HSP90), interleukin (IL), killer toxin (KT), major histocompatibility complex II (MHC-II), monoclonal antibody (mAb), mononuclear (MN), natural killer cell (NK), pathogen-associated molecular pattern (PAMP), pattern recognition receptor (PRR), Pneumocystis carinii pneumonia (Pcp), polymorphonuclear (PMN), radioimmunotherapy (RI), sever combined immunodeficiency (SCID), T cell antigen receptor (TCR), T cytotoxic cell (Tc), T help cell (Th), T regulatory cell (Treg), toll-like receptor (TLR).

LITERATURE SEARCH AND REVIEW CRITERIA

Medline database was searched for articles published between the years 2006 and 2009. Search terms included, but were not limited to: adoptive cell transfer, antifungal biotherapeutics, antifungal immunotherapy, antifungal monoclonals, antifungal probiotics, aspergillosis, candidiasis, cryptococcosis, cytokines and fungal immunity, fungal immunity, fungal vaccines, novel antifungal agents, novel antimycotics. From more than 1300 articles initially chosen, a refined search for articles of relevance to the topic at hand was carried out on an article-by-article basis. Relevant work published before 2006 was also included where appropriate. Additionally, terms like fungal vaccines, antifungal therapy, antimycotic therapy, fungi, mycosis, mycotics, and other terms were used to search for completed or ongoing clinical trials at www.clinicaltrials.com and for approved antifungal therapies and those awaiting approval by the United States Food and Drug Administration (FDA) at www.fda.gov. This review is not a working research paper, nor is it an action report that warrants critical review of published work. It is rather a general review of where antifungal immunotherapy stands at the moment; what has been achieved, what is promising, and what is the future direction. Accordingly, claims made by cited works were taken at face value and their critique was kept to an absolute minimum.
INTRODUCTION

Fungi and fungal infections are responsible for a wide range of diseases in plants, animals, and humans. Although only a handful of the roughly 400 pathogenic fungi cause disease in humans, some can establish lifelong commensalism on human body surfaces and within the gastrointestinal and urogenital tracts putting humans at constant risk of exposure to and infection by fungi through inhalation, skin preaches and tissue injuries. Human diseases caused by or associated with fungi range from allergies and autoimmunity to invasive and life-threatening infections. Aspergillosis (Aspergillus fumigatus), histoplasmosis (Histoplasma capsulatum), candidiasis (Candida albicans), blastomycosis (Blastomyces dermatitidis), coccidiomycosis (Coccidioides immitis), paracoccidiomycosis (Paracoccidioides brasiliensis), Pneumocystis carinii pneumonia (Pcp) and cryptococcosis (Cryptococcus neoformans) are common human fungal infections. In clinical practice, the most commonly encountered yeasts are Candida and Cryptococcus species. Additionally, while Aspergillus species are the most frequently isolated filamentous molds, infection rates with Fusarium, Scedosporium, Penicillium species, zygomycetes, and other molds are on the rise [1-3]. Increased incidence of human fungal infections in recent years can be attributed to a multitude of predisposing and compounding factors. However, compromised immunity precipitated by complications of and chemotherapy against underlying disease states remains the major culprit in this regard. Despite aggressive antifungal chemotherapy, rates of opportunistic fungal infections in individuals on immunosuppressants (e.g. calcineurin inhibitors), antibiotics, antineoplastics (e.g. TNF-α inhibitors) and neutropenia-inducing drugs (dexamethasone) remain significantly high [4]. Better treatment and long-term management approaches of transplant recipients and patients with cancer, AIDS, diabetes, and other chronic diseases is further contributing to the already alarming expansion in the number of immunocompromised hosts at high risk of fungal infections.

To address these concerns, antifungal chemotherapy, the mainstay treatment of fungal infections, has improved significantly in the last two decades. This has been achieved through the introduction of more potent and less harmful derivatives of old azoles and polyenes (e.g. voriconazole, posaconazole, amphotericin B lipid complex (AMBLC) and liposomal AMB). Additionally, the introduction of new classes of antifungals like the echinocandins (caspofungin, micafungin and anidulafungin) and terbinafine has expanded the base of antifungal armamentarium available at our disposal. Notwithstanding these developments, resistance, narrow spectrum of activity, toxicity, and the inherent failure to fully clear sites of infection continue to limit the efficacy of antifungal chemotherapy. These longstanding considerations have been the driving force behind massive efforts to develop immune-based approaches to better deal with (prevent or treat) human fungal infections and diseases. New insights into fungal immunity, better understanding of fungal biology and host-pathogen interactions, new advances in vaccine design, genetic engineering, and B cell hybridoma technologies, the recent –omics revolution, and many other advances in related fields are all providing essential tools to search for, design, develop and test a wide array of immunomodulators and immunotherapeutics. In the last two decades, extensive efforts have been made to test and develop preventive and therapeutic vaccines, cytokines regimens, adoptive cell transfer procedures, monoclonal antibodies (mAbs), and antifungal peptides that could prevent and/or treat fungal infections. The immense complexity of fungal pathogens
being eukaryotic with abilities to switch form, change virulence profiles, commensalize hosts and so on has significant bearing on the progress (or lack of it thereof) of immunotherapeutics against fungal infections. Additionally, the sophisticated genetic makeup of fungi gives them great latitude at evading immunity and developing resistance not only to chemotherapy but probably to immunotherapy as well. It is perhaps a reflection of these complications that progress in antifungal immunotherapy has moved at a much slower pace than in other areas like bacterial and viral infections, cancer, transplantation, and allergy. The conspicuous scarcity of fungal vaccines and other immunomodulators approved for clinical use thus far clearly shows how slow progress in this field is.

**CONVENTIONAL TREATMENT OF FUNGAL INFECTIONS**

Chemotherapy against fungal infections relies on the capacity of various drugs to inhibit or disrupt the synthesis or integrity of cell wall components, plasma membranes, cellular metabolism, fungal proteins, mitotic activity (tables 1 and 5) [reviewed in 5, 6-8 and references therein] and signaling cascades [9-12]. The cell wall is the interface between cells and their environment; cell walls protect against osmotic pressure and control passage of molecules in/out of cells. Therefore, disruption of cell wall integrity or synthesis is detrimental to cell viability and survival. Antifungal agents active against fungal cell wall components (mannans, chitins, α- and β-glucans) are the candins (echinocandin and aminocandin), nikkomycin and aureobasidins. Echinocandins represent a new class of lipopeptides that has proven to be safe and effective against candidiasis when given intravenously. FDA-approved echinocandins (caspofungin, micafungin and anidulafungin) and related compounds (aminocandin) are effective fungistatic and fungicidal agents owing to their capacity to inhibit glucan synthesis by blocking the activity of (1,3)-β-D glucan synthase. Echinocandins are very active against *Candida* species; however other fungal pathogens including *Aspergillus* and *Cryptococcus* species are susceptible to echinocandin activity. The leading chitin synthesis inhibitor is the fungicidal agent nikkomycin, which is active against the dimorphic fungi *C. immitis* and *B. dermatitidis*. Aureobasidins alter chitin assembly and sphingolipid synthesis; the drug is mainly active against *Candida* species and *C. neoformans* but has some potency against *H. capsulatum* and *B. dermatitidis*.

Antifungal agents that disrupt plasma membrane synthesis and cellular metabolism remain the most commonly prescribed drugs to treat pre-systemic and systemic fungal infections. These include polyenes, azoles, allylamines and thiocarbamates. Polyenes (amphotericin B or AMB and nystatin or NY) form complexes with and induce autooxidation of ergosterol with the subsequent generation of free radicals that disrupt fungal plasma membranes and cause increased permeability and cell death. The development of lipid-based delivery systems (phospholipid vesicles or liposomes and cholesterol esters) of AMB has enhanced AMB’s therapeutic index by decreasing toxicity and increasing target specificity. Other lipid formulations like the AMB lipid complex (AMBLC) and the lipid-formulated AMB colloidal dispersion (AMBCD) have also been granted FDA approval for clinical use in the mid 1990s. NY, which binds ergosterol and causes alterations in membrane permeability that lead to cell death, is active against *Candida, Aspergillus, Histoplasma* species and *Coccidioides immitis*; the drug is relatively safe when administered intravenously. SPA-S-753
is a new water soluble polyene active against *Candida*, *Cryptococcus* and *Saccharomyces* species that yields higher survival rates, longer survival times and better sterilization of infected organs as compared with the parent molecule (AMB). Azole and azole derivatives represent a rapidly expanding group of antifungal drugs. Common examples include fluconazole and its derivatives (voriconazole and ravuconazole) itraconazole and its hydroxylated analogue posaconazole. Voriconazole inhibits ergosterol synthesis by inhibiting P450-dependent 14α-demethylase demethylation in *C. albicans*, *A. fumigatus*, *C. neoformans*, and other dimorphic fungi. Posaconazole is active against different species of *Candida*, *C. neoformans*, *Aspergillus*, *Fusarium*, zygomycetes, and some dematiaceous molds. Ravuconazole is an oral derivative of fluconazole with an expanded spectrum of *in vitro* activity. Its inhibitory potency and binding affinity to P-450-dependent 14α-demethylase is similar to that of itraconazole. The drug is active against *Candida* species, *A. fumigatus*, *C. neoformans*, hyaline hyphomycetes, dermatophytes, and dematiaceous fungi. The allylamines terbinafine and naftifine and the thio carbamates tolnaftate are synthetic inhibitors of squalene epoxidase, the enzyme responsible for the cyclization of squalene to lanosterol. They possess fungicidal activity against dermatophytes, *Aspergillus* species, *Fusarium* species, *Penicillium marneffei* and other filamentous fungi. Terbinafine has strong *in vitro* activity against different species of *Penicillium*, *Paecilomyces*, *Trichoderma*, *Acremonium*, and *Arthrobotrys*.

Antifungal compounds targeting protein synthesis include the sordarins and azosordarins. Sordarins target elongation factor EF2 and the large ribosomal subunit stalk rpPO. Hence they disrupt the displacement of tRNA from A site to P site and block the movement of ribosomes along the mRNA thread. Azosordarins target and disrupt polypeptide chain elongation with activity superior to that of sordarins especially in the case of candidiasis. Unlike the classical elongation factors EF1α and EF2 that are used by both fungal and mammalian cells for polypeptide chain growth, EF3 is a newly-described elongation factor involved in protein synthesis only in yeast cells. Description of EF3 may allow fungal protein translation to evolve as a more desirable target for development of new antifungal agents. 5-fluorocytosine (5-FC), which inhibits pyrimidine metabolism by interfering with RNA and protein synthesis, is the sole antimitotic antifungal agent in clinical use. The drug is active against *Candida*, *Aspergillus*, and *Cryptococcus* species as well as dematiaceous fungi that cause chromomycosis (*Phialophora* and *Cladosporium*). Combining 5-FC with various AMB formulations is useful in clearing the cerebrospinal fluid in non-HIV patients infected with cryptococcal meningitis, candidiasis, *Candida* endophthalmitis, renal and hepatosplenic candidiasis, *Candida* thrombophlebitis of the great veins, aspergillosis, and central nervous system (CNS) phaeohyphomycosis.

Interruption of cellular signaling cascades is a powerful tool to combat certain forms of cancer, viral infections, transplant rejection, autoimmunity, and other disease states. There is ample evidence to suggest that interruption of signaling cascades is effective against common forms of mycoses (candidiasis, cryptococcosis and aspergillosis) [9-13]. Calcineurin inhibitors cyclosporine A (CsA) and Fermentek catalogue number 506 (FK506), the mammalian target of rapamycin (mTOR) inhibitor sirolimus (rapamycin), the heat shock protein 90 (HSP90) inhibitor geldanamycin, the phosphatidylinositol 3-kinase inhibitor wortmannin and the angiogenesis inhibitors fumagillin and ovalicin are all associated with broad spectrum antifungal activity. For instance, the function of calcineurin, a Ca²⁺/calmodulin-dependent phosphatase that plays a role in T cell activation [14-16], can be compromised by the capacity
of FK506 or CsA to form drug-protein complexes [17, 18]. In turn, the proliferation and mitotic activity of target cells is halted. As fungi are eukaryotes, calcineurin inhibitors seem to disrupt signaling cascades within fungal cells and halt their growth [9-13]. It must be emphasized that this mode of treatment with regard to fungal infections is still in its infancy. So far, no inhibitor drugs that disrupt signaling cascades in fungal pathogens have been approved for clinical use. Though not directly related to this discussion, it is worth noting that work on fungal virulence factors and their inhibitors is becoming a rich source of new therapeutic concepts and strategies to prevent and treat fungal infections. One of the prime examples here is the development of inhibitors to the secreted aspartic proteinase of C. albicans [reviewed in 19]. However, like fungal cell signaling inhibitors, this approach is still in its early stages.

Table 1. Commonly used antifungal drugs

<table>
<thead>
<tr>
<th>Group (examples)</th>
<th>General mechanism of action</th>
<th>Susceptible fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Drugs active against cell wall components:</strong></td>
<td></td>
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<tr>
<td><strong>Echinocandins</strong> (caspofungin, micafungin, anidulafungin)</td>
<td>Inhibit cell wall glucan synthesis by blocking (1,3)-β-D glucan synthase activity</td>
<td>Candida species</td>
</tr>
<tr>
<td><strong>Nikkomycin and Aureobasidin</strong></td>
<td>Inhibit chitin synthesis and assembly</td>
<td>Candida and Cryptococcus species</td>
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<tr>
<td><strong>II. Drugs active against plasma membrane integrity or synthesis:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Polyenes</strong> (amphotericin B and nystatin)</td>
<td>Autooxidation of ergosterol and formation of free radicals which compromise plasma membrane integrity and increase permeability</td>
<td>Different species of Candida, Aspergillus, Histoplasma, Coccidioides, Cryptococcus, and Saccharomyces</td>
</tr>
<tr>
<td><strong>Azoles</strong> (voriconazole, posaconazole, itraconazole, and ravuconazole)</td>
<td>Inhibit ergosterol synthesis by blocking the activity of the P450-dependent enzyme 14 α-demethylase</td>
<td>Different species of Candida, Aspergillus, and Cryptococcus</td>
</tr>
<tr>
<td><strong>Allylamines</strong> (terbinafine and naftifine), Thiocarbamates (tolnaftate)</td>
<td>Inhibit the activity of squalene epoxidase which is responsible for the cyclization of squalene to lanosterol</td>
<td>Aspergillus, Fusarium, Penicillium, Trichoderma, Acremonium, and Arthrographis</td>
</tr>
<tr>
<td><strong>III. Drugs active against protein synthesis:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sordarins</strong> (sordaricin methyl ester)</td>
<td>Disrupt displacement of tRNA from A site to P site and movement of ribosomes S. cerevisiae</td>
<td>C. albicans and S. cerevisiae</td>
</tr>
<tr>
<td><strong>5-fluorocytosine</strong></td>
<td>Inhibits pyrimidine metabolism by disrupting RNA and protein synthesis</td>
<td>Different species of Candida, Aspergillus, and Cryptococcus</td>
</tr>
</tbody>
</table>

Notwithstanding the enormous benefits of antifungal chemotherapy, problems regarding toxic side effects, resistance, variable spectra of activity, inability to fully clear infections, and limited molecular targets persist. To overcome these limitations and make antifungal
chemotherapy more appealing to practitioners and patients, ideal antifungals have been sought. Ideal in the sense that they exhibit enhanced potency, broad spectrum of activity or equivalents (as will be discussed later), minimized mechanism-based host toxicity, flexible mode of administration, and favorable pharmacokinetics (bioavailability and effective tissue penetration) [10]. In the real world however, developing the ideal “magic bullet” is more of a hope than a possibility given the sophisticated and flexible genetic makeup of fungi (eukaryotes) that enables them to evade immunity and counter the adverse effects of drugs. Both features result in the replacement of dominant susceptible strains with dominant resistant ones. Therefore, regardless of the chemical composition or delivery system of chemotherapy, improvements on existing antifungals can only go so far to partially address some, but not all, of these aspects at once. The major lesson workers in this field have accumulatively learned from their long clinical experience with antifungal chemotherapy and their deep understanding of fungal pathogenesis and host immunity is that the tasks of managing fungal infections and lessening the harmful effects of chemotherapy are very challenging. So long as chemotherapy or any type of therapy for that matter continues to target the pathogen without enlisting the help of host immunity, conventional treatment-associated problems will persist [5, 20]. Hence, engaging host immunity in direct and indirect ways should be the rationale in the search for therapies and control measures that complement chemotherapy or do away with it altogether.

IMMUNOTHERAPY

The current state of antifungal immunotherapy speaks of a cocktail of immune-based strategies and approaches to manage, prevent, and/or treat fungal infections. However rational some of these approaches might be one can hardly see a coherent paradigm guiding their progress. It goes without saying that understanding fungal immunity is a prerequisite to developing a coherent understanding of how various components of innate and adaptive immunity can be enlisted to help deal with fungal infections. Defining the major players in fungal immunity is, more or less, the equivalent to defining pathogen immune vulnerabilities and points of attack. Therefore, before discussing the various modalities of antifungal immunotherapy in any meaningful way, an introduction to fungal immunity is in order.

IMMUNITY TO FUNGI

During their evolutionary histories, vertebrates in general and mammals in particular have evolved elaborate defense mechanisms to ward off, fight, and clear fungal infections. This is well demonstrated by the fact that infections in immunocompetent hosts elicit innate and adaptive responses that collectively clear the infection and protect against subsequent infections. In contrast, immunocompromised hosts are susceptible to a whole host of opportunistic infections (fungal and otherwise). Evidence supporting the notion that defective, deficient, compromised, or aged immunity predisposes to fungal infections are overwhelming. A majority of opportunistic fungal infections occur in patients with compromised immunity (transplant patients, patients with cancer, AIDS, diabetes and other
disease states) [1-3, 20-23]. Mice with autosomal or X-linked sever combined immunedeficiencies (SCIDs) and those with selective T cell (e.g. nude mutation nu/nu or Foxn1nu as it is now denoted) or granulocyte deficiencies are extremely susceptible to opportunistic fungal infections. Quantitative and qualitative defects of neutrophils, whether inherited or acquired, are associated with increased susceptibility to opportunistic fungal infections in animals and humans [24, 25]. NADPH-oxidase and myeloperoxidase deficiencies are associated with increased susceptibility to aspergillosis and pulmonary candidiasis [26]. Defects in proinflammatory Th1 responses that tip the balance in favor of Th2 responses are associated with deleterious effects on the outcome of fungal infections [27]. Weakened immunity as a result of old age is also known to predispose to opportunistic fungal infections [28, 29].

**SETTING THE STAGE**

There exist considerable temporal and spatial variations and, by consequence, variable immune response profiles against various forms of mycosis. Immunity against aspergillosis and pneumocystis depends on rapid pathogen recognition and deployment of effective innate responses followed by robust yet delayed adaptive responses. Immunity against *C. albicans* depends on the type of infection being mucosal or systemic. While resistance to *C. albicans* mucosal infections associates with Th1 responses, systemic infections typically associate with variable contributions of both Th1 and Th2 type responses. For dermatophytes, the role of CMI is to destroy the pathogen and render the host protected against re-infection. Resolution of diseases caused by dermatophytes is generally associated with delayed type hypersensitivity (DTH). Immunity to various species of *Cryptococcus* is disparate enough to allow for differentiation between species based on differences in the immunologic consequences that ensue following an infection. While *C. neoformans* mainly infects immunocompromised hosts, *C. gattii* tends to infect both immunocompromised and immunocompetent hosts. In general terms therefore, components of innate and acquired immunity converge to protect the host against fungal infections in spatial and temporal sequences that vary depending on the genus and/or species of the pathogen, its morphotype (mold, yeast, pseudoehypahe, hyphae, mycelium etc.), and site of infection (localized, mucosal, systemic and so on) [30-32]. Such sequential variations derive from differences in the sets of pathogenic determinants operative in different infectious agents and forms, the stage and site of infection, and the repertoire of expressed antigenic moieties and consequently the types of pathogen recognition systems involved.

Phenotype (morphotype or simply type) switching, a phenomenon common to many fungal species, has considerable bearing on the quantitative and qualitative features of fungal immunity [33-36]. For it is involved in shaping the profile of morphotype-specific pathogenic determinants and virulence factors as well as the array of antigenic structures recognized by the host. Common examples of fungi that undergo phenotype switching include the dimorphic fungi *H. capsulatum*, *P. brasiliensis*, *C. immitis*, and *B. dermatitidis* that transform from saprobic filamentous molds to unicellular yeasts in the host, the filamentous fungi *Aspergillus* species that transform into multicellular mycelia following inhalation as unicellular conidia and *Candida* species that grow into different phenotypes (yeast, blastospore, pseudoehypahe,
and hyphae). The dynamic changes that occur in *A. fumigatus* cell wall structure and synthesis significantly influence its ability to activate, suppress, or subvert host immunity [37]. This is well manifested in the differential ability of fungus-derived secreted or membrane-anchored proteins, glycolipids, and polysaccharides to induce vaccine-dependent protection in immunocompromised hosts or to induce cytokine production by human-specific CD4+ T cells. The effect of age is also worth considering here as the robustness of adaptive and innate immune components wane with age. For example, aged C57BL/6 mice intravenously infected with *C. albicans* hyphae experience significantly low survival rates, high fungal burdens, decreased frequency of IFN-γ-producing CD4+ T lymphocytes, and diminished *in vitro* ability of macrophages and splenocytes to respectively produce TNF-α and IFN-γ [22].

**The Participants**

Systemic and local adaptive cell-mediated immunity (CMI) has long been regarded as the major player in defending against common fungal infections. The role of antibody (Ab)-mediated immune responses was historically brushed aside as a negligible gig. Innate immunity has also been considered as passive, nonspecific, uninteresting, and uninformative aspect of fungal immunity. Historical emphasis on the role of adaptive CMI against fungal infections is based on overwhelming supportive evidence. T cells play a prominent protective role against systemic candidiasis, vaginal candidiasis, chronic mucocutaneous candidiasis (CMC), aspergillosis and cryptococcosis among many other fungal infections [24, 38, 39-43]. Depletion of CD4+ T cells is associated with increased susceptibility to *Pneumocystis pneumonia* (*P. pneumonia*) and other mycotic infections in rodents [42]. Differentiation of Th1 type responses is the determining factor of resistance to fungal infections [31]. Induction of Th1-mediated immunity by the secretion of IL-12, IFN-γ, and IL-6 confers significant protection against different forms of mycoses [44-46]. Positive lymphoproliferation characterized by overproduction of IFN-γ from cell cultures isolated from healthy individuals receiving cellular extracts of *A. fumigatus* or *A. fumigatus* antigens (the 88KD dipeptidase or the 90KD catalase) has been reported [45]. Increased levels of peripheral CD4+ and CD8+ memory T cells are detectable in patients infected with *P. brasiliensis* before and after chemotherapy [47]. In patients with relapsed infection, levels of IFN-γ are usually diminished, a finding that highlights the importance of IFN-γ in maintaining protective immunological memory against at least some fungal infections (e.g. *P. brasiliensis*) [47].

The immense importance of CMI in defending against fungal infections notwithstanding, this paradigm has been challenged at several fronts [24, 38, 43]. The ongoing appreciation of the role of dendritic cells (DCs) in recognizing fungal pathogens and initiating and orchestrating innate and acquired immunity to fungal infections is helping to reinstate innate immunity as an active and significant participant in fungal immunity. The heavy involvement of DCs in the immune response to fungal infections and their great potential as active vaccine delivery systems qualifies them as pivotal players with pathogen-specific recognition and handling credentials (figure 1). The role of neutrophils and other phagocytes in mounting effector responses against a wide range of local and disseminated fungal infections is now well recognized [24, 43, 48-50]. Nothing demonstrates the importance of neutrophils in fungal immunity more than the fact that high rates of opportunistic fungal infections almost
always associate with immunosuppression- or chemotherapy-induced neutropenia. Expression of innate immunity genes involved in recruitment, activation, and protection of neutrophils and monocytes (MNs) has been shown to significantly upregulate during the early phase of fungal infections [51, 52]. Using a cDNA microarray chip representing 42,421 genes, the expression of a large number of genes involved in cell function and survival significantly increased within 4-6 hours after incubation of human MNs infected with C. albicans [52]. Such genes included those of proinflammatory cytokines TNF-α, IL-1, IL-6, and leukemia inhibitory factor (LIF), chemokines IL-8 and macrophage inflammatory proteins (MIPs) 1, 3 and 4, MNs chemoattractant protein (MCAP1) and chemokine receptors CCR1, CCR5, CCR7, and CXCR5. The activity of MN viability-enhancing genes like BCL2-related protein, metallothioneins, CD71, and SOCS3 was also shown to upregulate within few hours of culture. Conversely, expression of genes adversely affecting MN function or viability (IL-15, IL-13Ra1, and CD14) was reported to downregulate during the 18 hour incubation period [52]. Upregulated expression of TLRs 2 and 4 was also reported following stimulation of human corneal epithelial cells (HCECs) with inactive Fusarium solani hyphae. Glucocorticoids (hydrocortisone) enhanced the expression of these TLRs on HCECs [51].

Despite the lack of a direct association between defective Ab production/function and susceptibility to fungal infections, a growing list of supportive roles of Abs against several fungal infections is reviving the interest in antifungal Ab immunity [53-61]. Systemic candidiasis (C. albicans), cryptococcosis (C. neoformans) and other fungal infections often result in the production of opsonizing and complement-fixing Abs. There is evidence to suggest that anti-Pneumocystis Ab therapy is effective at preventing and treating Pcp. Treatment with hyperimmune sera from mice immunized with P. carinii [62, 63] or with mAbs specific to P. carinii surface antigens [60, 64-66] resolved existing P. carinii infections in SCID mice and decreased hyperinflammatory reactions in immunoreconstituted mice. Complement fixation by the 4F11(G1) mAb was reported to be essential for optimal protection against infection with P. carinii and development of Pcp [67]. Infection by the melanin-producing Fonsecaea pedrosoi, the causative agent of chromoblastomycosis in humans, elicits production of anti-F. pedrosoi protective Abs [60]. Protective Abs can prevent biofilm formation by C. neoformans through interfering with capsular polysaccharide release from fungal cells [68]. Recovery from disseminated candidiasis in mice is associated with the presence of protective Abs against the molecular chaperone heat shock protein 90 (HSP90). Abs against pathogen-specific HSP90 epitopes and those against epitopes common to the pathogen and the host have been reported in patients recovering from systemic candidiasis [69, 70].

Humoral factors belonging to innate (mannose-binding lectins [MBLs], collectins, complement components) and humoral immunity (Abs and complement cascades) enhance antifungal innate defenses through facilitating complement activation and fixation as well as fungal cell opsonization and neutralization [43, 44]. For successful fungal commensalism and latency, immune responses against fungi need not be strong enough to eradicate the fungus nor weak enough to let the fungus run amok [24, 43]. To achieve this delicate balance between inflammation and tolerance, cooperation between various effectors and regulators deriving from the innate and adaptive arms of the immune system is needed (figure 2). One aspect of this coordinated process is the reciprocal relationship of neutrophil and T lymphocyte functions in protection against fungal infections [71, 72].
THE PROCESS

Intact surfaces (epithelia and endothelia), microbial antagonism, and antimicrobial peptides (AMPs) collectively provide a preliminary passive line of defense that tends to ward off fungal infections. Once the pathogen enters the host through breached skin, mucosal lining injuries or inhalation, innate immunity cells sense the pathogen and mount an effector response to kill the pathogen or hold it in check to prevent it from pathologically colonizing the tissue or disseminating to other tissues. Participants in this response include professional phagocytic cells; namely polymorphonuclear (PMN) cells (neutrophils), mononuclear (MN) cells (monocytes and macrophages) and DCs. Collectively, these cells recognize common fungal pathogen-associated molecular patterns (PAMPs), especially cell wall glucans, mannans and chitins. Such fungal antigens or PAMPs are specifically recognized by different sets of pattern recognition receptors (PRRs). Most notable are the toll-like receptor (TLR)/interleukin 1 receptor (IL-1R) family, complement receptors like CR3 or CD11b/CD18, some crystallizable fragment receptors (FcR), C-type lectin receptors (dectin-1, dectin-2, DC-SIGN, and galectins), and MBL. For example, glucuronoxylomannan (GXM) of C. neoformans is recognized by PAMP receptors expressed on the surface of DCs and macrophages [73]. Although dectin-1-knockout mice are more susceptible than wild-type counterparts to pneumocystis infections, both groups are equally susceptible to candidiasis suggesting that dectin-1 is required for immune responsiveness to some (but not necessarily all) fungal infections [74]. Recognition of carbohydrate moieties by innate immunity cells is now considered as an essential component of fungal immunity. Mincl, the C-type lectin expressed predominantly on macrophages and plays a significant role in innate immunity to C. albicans, is a case in point. Mice lacking this receptor show reduced levels of TNF-α production and high susceptibility to systemic candidiasis [75].

Phagocytes kill fungi by oxidative and nonoxidative processes. Oxidative burst-dependent killing involves the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase- and the inducible nitric oxide synthase-mediated pathways. Therefore, NADPH-oxidase deficiency initiates the development of chronic granulomatous disease (CGD) by blocking the formation of reactive oxygen radicals and increasing host’s susceptibility to aspergillosis [26]. The lysosomal hemoprotein myeloperoxidase that is present in abundance in the azurophilic granules of neutrophils and MNs represents another powerful oxygen-dependent mediator of fungal killing. Non-oxidative killing is mediated by collectins (pentraxin 3), defensins, neutrophil cationic peptides and numerous other AMPs that target and disrupt fungal cell membrane. Phagocytes can also restrict fungal growth and minimize infectivity through a whole host of mediators that deprive fungi of iron, inhibit dimorphism and block phenotype switching.

It is now recognized that DCs play a very pivotal role in initiating, linking and/or orchestrating innate and acquired immunity against fungal infections (figure 1). Although the heterogeneous population of tissue resident macrophages has the potential to function as APCs, its main function is mostly limited to phagocytosis and killing of fungi as well as immunomodulation through a number of cytokines they produce in response to fungal infections [reviewed in 76]. The importance of DCs derives from many aspects of their biology and function, the following however should suffice to make the point:
Figure 1. The central role of DCs in fungal immunity. Recognition and binding of fungal pathogen PAMPs by PRRs on DCs initiate both MyoD88-dependent and -independent signaling cascades that collectively activate DCs, induce them to produce cytokines and AMPs, and enhance their ability to express MHC-II and T cell costimulatory molecule ligands. Phagocytosis of pathogens into DCs is followed by processing and presentation of fungal antigenic peptides to Th cells so as to initiate Th cell differentiation. These two main events endow DCs with the ability to initiate, orchestrate, and link diverse sets of innate and acquired responses against fungal pathogens.
(i) DCs express a diverse set of PRRs that recognize and bind with a wide variety of fungal antigens expressed by most pathogenic fungi. The diverse repertoire of pathogen recognition and binding receptors expressed on DCs endows them with a pathogen-handling and -internalization plasticity unmatched by any other cell type. PRRs on DCs (TLRs, IL-1R, CR3 and MBLs) mediate distinct downstream signaling cascades that induce the synthesis of cytokines, AMPs and other signaling and effector molecules. The presence of conserved Toll/interleukin-1 receptor (TIR) domains in the cytosolic region of all TLR/IL-1R family of receptors on DCs allows them to generate signaling cascades similar to those that activate the transcription factor nuclear factor β (NFβ) and the stress-activated protein kinase family; both of which are involved in the activation and transcription of inflammatory and adaptive immune responses.

(ii) DCs are professional phagocytes; they internalize and process diverse sets of fungal peptides. Through different receptors and modes of phagocytosis, both human and mouse DCs can internalize A. fumigatus, C. albicans, C. neoformans, H. capsulatum, Malassezia furfur, and S. cerevisiae. DCs internalize yeasts and conidia predominantly by coiling phagocytosis, while hyphae internalization occurs by the more conventional zipper-type phagocytosis. Furthermore, recognition and internalization of unopsonized yeasts and conidia occurs through the engagement of DC-SIGN, dectin-1, CRs, and other cell wall sugar receptors. Therefore, the diverse set of PRRs expressed on DCs along with the capacity of DCs to cross-present internalized and exogenous fungal antigens released from dead phagocytes [77] enable them to decode and translate fungus-associated information into varied immune responses.

(iii) Signaling cascades initiated by TLR/IL-1R receptor engagement with PAMPs often recruit adapter proteins, most notably the Drosophila myeloid differentiation primary response gene 88 (MyoD88), which subsequently activates downstream kinases important to innate and adaptive immunity [78]. In other words, engagement of distinct receptors with distinct fungal morphotypes is translated by DCs into downstream MyoD88-dependent or – independent signaling cascades that lead to upregulated production of cytokines like IL-12, IL-4, and IL-10, and upregulated expression of MHC-II and T cell costimulatory molecule ligands. CD80/CD86 (B7.1/B7.2) is one of the prime ligands expressed on DCs to bind with the T cell costimulatory molecules CD28 and CTLA-4 (CD152). Upregulated expression of MHC-II on activated DCs restricts, to a large extent, their antigen presentation activity to CD4+ Th cells. Furthermore, in what could be described as a positive feedback loop, cytokines produced by DCs activate DCs and amplify their capacity to detect and phagocytose fungal pathogens. IL-10 secreted by DCs in a MyoD88-independent manner activates CD4+CD25+ T regulatory (Treg) cells, which are of prime importance in inducing and regulating antifungal resistance (figure 2) [24]. Increased IL-10 production tends to downregulate production of the proinflammatory cytokine IFN-γ and consequently dampens antifungal CMI against chronic candidiasis and endemic mycoses.

(iv) Cytokines produced by DCs, and other phagocytes combined with the direct physical engagement between DCs and Th cells through concomitant ligand/costimulatory molecule binding and fungal peptide-MHC-II/TCR interaction induces Th cell differentiation and cytokine production. Th cells differentiate into Th1, Th2, or T17 pathways depending on the kinetics of peptide-MHC-II/TCR interaction and the prevailing cytokine profile influencing the process. The consequences of Th differentiation into Th1, Th2, or T17 vary depending on the pathogen and site of infection. For the most part however, IL-12-driven differentiation of
Th cells into Th1 and the consequent production of proinflammatory cytokines (IFN-γ, IL-6, IL-12 and TNF-β) is protective against most fungal infections, mucosal ones in particular [24, 27-38, 43, 44]. IFN-γ and other Th1 cytokines enhance the production and function of Abs (Abs) and enhance the recruitment and activity of innate immunity effector components. Additionally, cytokines released by phagocytes and Th cells engage and activate effector Tc cells. Type 2 cytokines, IL-4 in particular, by acting to commit Th cells to the Th2 pathway at the expense of the Th1 pathway, dilute and counter Th1-mediated protective activity thus permitting fungal persistence and allergy. Elevated levels of IL-4 and other type 2 cytokines (IL-5, IL-10, IL-13) associate with diminished IFN-γ production, suppressed DTH responses, increased synthesis of nonprotective Abs (IgE, IgG4 and IgA), and eosinophilia [reviewed in references 43 and 79]. This profile positively correlates with fungal disease severity and poor prognosis. It must be noted however, that production of IL-4 or IL-5 is not always increased in patients with fungal infections (e.g. CMC infections) even if type 1 cytokine production is defective [80]. IL-23-driven differentiation of Th cells into Th17 pathway is believed to be a distinct lineage that is separate from the Th1 pathway. This is, of course, despite the close structural similarities between IL-12 and IL-23 and the fact that IL-23 functions through a receptor complex that consists of IL-12Rβ1 subunit plus components of the IL-23R chain. Like IL-12, IL-23 induces Th cells to produce proinflammatory cytokines (e.g. IFN-γ), however unlike IL-12, it induces Th cells to uniquely express IL-17. IL-17 is a negative regulator of Th1-mediated protective immunity; it measurably impairs immune resistance and promotes inflammation [24, 81]. Th17 responses against fungal infections (C. albicans and A. fumigatus) subvert neutrophil inflammatory responses, promote fungal virulence, and exacerbate inflammation and infection. Therefore, the IL-23/IL-17 pathway correlates far greater with severity and pathology of infectious diseases than the IL-12/IFN-γ pathway [82, 83]. Immunotherapeutic measures to counter the nonprotective and inflammatory tendencies of Th2 and Th17 responses in hosts with defective or compromised immunity may prove beneficial. The capacity of cytokine inhibitors (e.g. anti-IL-4 Abs) to block the differentiation of non-protective Th2 cells and enhance the differentiation of protective Th1 responses is a case in point [84].

**BALANCING ACT: INFLAMMATION VS. TOLERANCE**

In the rush to minimize tissue colonization and pathogen persistence, Th1 immunity recruits and activates effector immune components that clear the pathogen and cause significant inflammation. The activity of Th17, while dampening protective Th1 immunity, tips the balance in favor of further inflammation and tissue injury. Inversely, Th2 responses impair protective Th1 immunity by enhancing anti-inflammatory responses thus permitting the pathogen to colonize and persist within the host. Under normal circumstances, there seems to be some sort of a balancing act between proinflammatory and anti-inflammatory responses that ultimately leads to the optimal immunity. However, the “Th1/Th17/Th2 alone” paradigm may not convincingly explain the exquisite and subtle balance between proinflammatory and anti-inflammatory tolerogenic responses in hosts with competent immunity. In other words, by only considering Th1, Th17, and Th2 responses acting on their own, the routine transition from the proinflammatory phase to the anti-inflammatory phase of
the immune response would be rough and bumpy. This is particularly true given the massive and sudden changes in proinflammatory and anti-inflammatory cytokine concentrations and the minimal overlap between the two phases. Therefore, a new paradigm that could better explain the smooth generation of immune responses that are strong enough to fight and nearly clear infections without leading to pathologic inflammation and full tissue sterilization is needed (figure 2). It is worth mentioning here that full clearance of the pathogen within the timelines of the immune response is never desirable as it deprives the host of developing a level of tolerance sufficient to generate and maintain immunologic memory. Currently, it is postulated that the task of finely tuning and balancing protective (Th1) and nonprotective (Th17) proinflammatory responses on the one hand and nonprotective anti-inflammatory (Th2) responses on the other, falls upon T regulatory (Treg) cells [24, 43, 44, 72, 85]. Several subsets of the thymus-derived, naturally occurring Treg cells (CD4+CD25+FoxP3+) and the adaptively differentiated Tregs (CD4+CD25+FoxP3-) secrete sets of cytokines (IL-10, tumor growth factor-beta (TGF-β), and IL-4) that tend to keep Th1, Th17, and Th2 responses in check (figure 2) [24, 72, 80-82].

![Diagram](image)

Figure 2. The balancing act of Treg cells. Balancing the activity of proinflammatory and anti-inflammatory cytokines is essential to minimize inflammation and permit pathogen tolerance. To achieve this goal, Treg cells produce cytokines (IL-10, TGF-β, etc.) that differentially activate, subvert, or suppress the activity of innate and acquired cellular components.

As discussed previously, IL-10 that is primarily produced by DCs and Treg cells in response to fungal mannans and other cell wall polysaccharides, negatively affects IFN-γ production, weakens CMI, and dampens Th1 responses against chronic candidiasis, severe forms of endemic mycoses, and aspergillosis in neutropenic patients [43]. Indoleamine 2,3-dioxygenase (IDO), the enzyme that mediates tryptophan (Trp) catabolism to N-formylkynurenine, can cause Trp depletion in target cells and halt their growth and activity [72]. It has been postulated [24] that during the early phase of a fungal infection, IL-10 and CTLA-4, by acting on IDO, suppress the activity of recruited neutrophils to limit inflammation and tissue damage. In the late phase of the infection, IL-10 and TGF-β inhibit
Th2 responses in order to limit pathogen persistence and allergy. Specialized DC subsets like the granulocyte monocyte colony stimulating factor (GM-CSF)/IL-4-stimulated myeloid DCs and FLT3-ligand plasmacytoid (pDCs) were reported to fulfill the requirements for Th1/Treg antifungal priming and tolerization [reviewed in 24]. Thymosin-α (Tα1), a modulator of human pDCs that functions through TLR9, was reported to affect these processes in DCs by activating the IDO-dependent pathway resulting in Treg development and tolerization [71]. This theme in fungal immunity, while leaving the door open for measured manipulations through DC and cytokine treatments, highlights some of the risks that could associate with such therapies. For instance, disturbing the Treg-initiated and maintained cytokine balance could easily lead to very adverse consequences by tilting the immune response towards stronger inflammation and tissue damage or towards tolerance, pathogen persistence and allergy. Vaccination with cell wall polysaccharides that induce IL-10 production could also replicate similar adverse effects.

**AVENUES FOR ANTIFUNGAL IMMUNOTHERAPY**

Based on current understanding of fungal immunity (figures 1 and 2), two distinct classes of immune-based antifungal intervention can be identified (table 2). The first pertains to the wide array of agents and strategies that target the host to modulate and enhance its immunity to fungal infections, hence the term host-targeting immunomodulation or HTI. Under this general heading we can see vaccines (native, recombinant, synthetic, antigen-pulsed DCs, etc.), cytokines and immunostimulatory Abs and peptides, as well as cell transfer (phagocytes, NK cells, and sensitized T cells) therapies. This approach helps to shift the focus from countering pathogen colonization and infectivity to enhancing anti-pathogen host immunity.

**Table 2. Comparative summary of host-targeting immunomodulation and pathogen-targeting immunotherapy**

<table>
<thead>
<tr>
<th>Class: examples</th>
<th>Mechanism(s) of action</th>
<th>Potential advantages</th>
<th>Potential disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Host-targeting immunomodulation strategies:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccines</td>
<td>Prime the immune response and generate immunologic memory</td>
<td>Minimal toxicity.</td>
<td>Certain modalities (vaccines, mAbs and AMPs) require immune competence to be fully effective. Cytokine and cell transfer therapies can cause excessive inflammation and tissue injury and inhibit pathogen tolerance.</td>
</tr>
<tr>
<td>Cytokines and cell transfer therapies</td>
<td>Restore or reshape the balance of prevalent cytokines. Reverse the effects of neutropenia Direct killing of pathogens.</td>
<td>Minimal risk of resistance induction (vaccines may induce pathogen strain replacement). Broad spectrum of activity.</td>
<td></td>
</tr>
<tr>
<td>Monoclonal Abs and antimicrobial peptides</td>
<td>Activate innate and adaptive cellular components, Modulate cytokine synthesis, Modify Th cell differentiation and/or function and alter host cell gene expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal Abs</td>
<td>Fungicidal activity mediated by complement- and antibody-dependent cytotoxic reactions and pathogen opsonization. Fungistatic-like activities immunostatus mediated by neutralizing, blocking, and (rapid and suitable</td>
<td>Passive activity that occurs independent of host’s</td>
<td>Considerable risk of resistance induction.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Considerable risk of toxic side effects. Monoclonal Abs exhibit species-specific</td>
</tr>
</tbody>
</table>
Antifungal Immunotherapy: A Reality Check

Complementary to host-targeting immunomodulation, blocking, opsonizing and neutralizing Abs (whole or fragmentary, monoclonal or polyclonal, naked or conjugated with radioactive or cytocidal agents) and AMPs (natural or synthetic, native or modified) can be used to target and kill fungal pathogens, inhibit their growth or hinder their mobility and infectivity. This second mode of immune-based intervention, which by definition focuses on the pathogen, will be referred to thereafter as pathogen-targeting immunotherapy. Each of these two general approaches has advantages and disadvantages, they sometimes overlap, and each presents powerful tools to combat fungal infections. As discussed in previous studies [5, 20], modalities comprising host-targeting immunomodulation have, for the most part, broad spectrum of activity, reduced toxicity and minimal risk of inducing resistance. On the negative side however, issues like inflammation and allergic reactions, accelerated vaccine-induced pathogen strain replacement, and the need for competent immunity are of concern. Pathogen-targeting immunotherapy on the other hand acts independent of host immunostatus and its narrow spectrum of activity can be compensated for by the relative ease with which pathogen-specific Abs can be designed or by the generation of pan-fungi mAbs. However, serious side effects and the likelihood of resistance development are two major drawbacks. In the subsequent sections, it should become clear that vaccines and mAbs are the two most promising components of host- and pathogen-targeting immune-based interventions respectively.

HOST TARGETING IMMUNOMODULATION

Manipulating the immune system by derivatives from specific pathogens (vaccines), derivatives of the immune response against specific pathogens (Abs, phagocytes or T cells), non-pathogen-specific or general immune response derivatives (cytokines and AMPs) is becoming an integral part in preventing and treating viral and bacterial infections and in managing patients with cancer, allergy, autoimmunity and transplantation. In the case of fungal infections, these preventive and therapeutic modalities are still in the basic research and development stages. There is mounting experimental evidence however to suggest that manipulating the immune response holds considerable promise to better manage and treat serious fungal infections. There is also mounting evidence to suggest that host-targeting interventions can significantly enhance the outcome of conventional antifungal chemotherapy. The efficacy of these modalities in modulating immunity depends on their respective potential to direct and/or stimulate the immune response to better deal with fungal infections. In general terms therefore, their success in modulating immunity can only be limited by factors that block, dilute or undesirably amplify their activity. Hence, problems classically associated with chemotherapy (narrow spectrum of activity, toxicity and...
resistance) ought to be dramatically reduced. Nonetheless, compromised, blunted, overwhelmed, and derailed immunity are potential spoilers.

**Fungal Vaccines**

The hope vaccines hold for enhancing our ability to control and manage fungal infections far exceeds that of any other preventive or therapeutic immunomodulatory approach. The sheer number of studies carried out towards this endeavor in comparison with say, adoptive cell transfer or cytokine therapy, testifies to this effect. Although clinical trials of the coccidioidomycosis vaccine that were conducted in the 1980s by the Valley Fever Vaccine Study Group failed to yield significant differences in susceptibility to coccidioidomycosis between immunized and placebo subjects [86], these studies marked the true beginning of research work on fungal vaccines. Following these initial studies, the identification, development, and testing of various approaches and components related to fungal vaccine research has witnessed breathtaking advances. Unfortunately however, the vast majority of experimental fungal vaccines developed thus far have failed to fair any better than the coccidioidomycosis vaccine. Systematic review of approaches involved in the design, development and testing of experimental fungal vaccines is needed in order to define some of the obstacles hindering their progress. In attempting to address this issue, the following discussion will deal with classical and recombinant fungal vaccines as well as recent work on fungal vaccine adjuvants. Some of the foreseen contributions of genomics and proteomics to fungal vaccine design and development will also be glimpsed.

**Adjuvants**

Maximizing the potential of success of a vaccine is dependent upon a number of factors. Chief among these is vaccine immunogenicity and the use of compatible adjuvants. Use of adjuvants is essential to enhance the immune response to vaccines and induce long-term memory, especially when vaccines are prepared from whole killed organisms. Different classes of adjuvants that slowdown antigen degradation (depot adjuvants) like aluminum salts, deliver antigens to APCs (particulate adjuvants) like microparticles, iscoms, and liposomes, or stimulate immunity and promote cytokine production by activating APCs (immunostimulatory adjuvants) like lipopolysaccharides, microbial cytidylate-phosphate-guanylate DNA, and saponins are currently available. The efficacy of adjuvants is hard to predict; in other words, it can be said that achieving optimal vaccine/adjuvant combinations is a matter of trial and error. Developing new adjuvants or exploring different combinations from the depot/particulate pool and the immunostimulatory pool is complementary to the production of effective vaccines, fungal or otherwise. Overall, some of the adjuvants tested in conjunction with different fungal immunogenic moieties have proven useful. Subcutaneous immunization of *C. albicans* membrane antigen (CMA) mixed with incomplete Freund’s adjuvant induced resistance to infections caused by *C. albicans* and *A. fumigatus* in mice [87]. Immunoprotection of the Candida-derived rAls3p-N vaccine was enhanced when administered in combination with the depot adjuvant aluminum hydroxide [88].
Immunogenicity and efficacy of rAls3p-N plus aluminum hydroxide was greatly enhanced when diluted in phosphate-buffered saline as compared with that diluted in saline; this although the quantity of rAls3p-N bound to aluminum hydroxide was greater in the later diluent [89].

Use of immunostimulatory glycolconjugate adjuvants has been associated with some positive outcomes in experimental fungal vaccine studies [90-93]. Hence, the majority of adjuvants in current use with experimental fungal vaccines belongs to this class. Immunization with the tetanus toxoid conjugate of GXM resulted in the production of protective Abs in animal models of cryptococcosis [94]. When diphtheria toxoid CRM197 is conjugated with the algal antigen laminarin (Lam), a Laminaria digitata–derived poorly immunogenic cell wall β-glucan, the resultant vaccine was protective against murine systemic and mucosal candidiasis [95]. Inclusion of the Lam antigen enabled a novel polysaccharide-protein conjugate vaccine to induce the production of Abs against β-glucan and to protect against experimental candidiasis and aspergillosis [96]. Vaccination with the recombinant truncated form of the A. fumigatus antigen Asp f3 that lacks the IgE binding sites was protective against aspergillosis when mixed with TiterMax [97]. Thymosin α1 promoted DC activation and production of IL-12p70 and IFN-γ and enhanced the priming of Th1 responses against aspergillosis [85]. The ability of thymosin-α1 to activate A. fumigatus-pulsed DCs and in turn activate Th1-dependent fungal immunity, accelerate myeloid cell recovery, and protect highly susceptible mice should collectively qualify this TLR9 ligand as a strong candidate adjuvant that seems to coordinate the activation of innate and adaptive immunity [85, 98]. By using ovalbumin (OVA) expressed in bacterial and yeast vectors, it was demonstrated that fungal mannosylation enhances antigen immunogenicity in the context of CD4+ T cell responses [99]. O-linked mannosylated yeast-derived OVA antigens were reported to be more potent than unmannosylated counterparts at inducing antigen-specific T cell proliferation [100]. Although neither O-linked nor N-linked mannosylated OVA antigens succeeded in stimulating DCs to produce TNF-α or IL-12, the ability of mannosylation to engage adaptive CMI is nonetheless promising.

Enhanced immunostimulatory effects of bacterial DNA, which stems from the presence of CpG dinucleotide-rich sequences, has been utilized to develop a class of adjuvants known as CpG-oligodeoxynucleotides (CpG-ODNs). The therapeutic value of CpG-ODNs is dependent upon their ability to enhance the differentiation of Th1-mediated immune responsiveness. As CpG-ODNs induce Th1-type cytokines, some investigators [101] have speculated that they may suppress Th2-type responses, a beneficial outcome in allergic, fungal diseases. Treatment of asthmatic patients with CpG-ODNs minimized injurious eosinophil-mediated inflammation [101]. CpG-ODNs have been used as adjuvants with experimental fungal vaccines. Enhanced capacity of the A. fumigatus Asp f16 antigen to protect against murine aspergillosis when mixed with unmethylated CpG-ODNs was attributed to the adjuvant’s ability to engage Th1-mediated protective immunity [85, 102-104]. Additionally, although treatment of mice with C. neoformans infection using fluconazole or unmethylated CpG-ODN improved survival, cleared the pathogen, and prevented dissemination, combined treatment using both agents significantly improved therapy outcome [105]. While the general consensus is that treatment with CpG-ODN confers protection and enhances resistance to a wide spectrum of fungal pathogens, some instances to the contrary have been reported. For example, treatment of normal mice with synthetic CpG-
ODNs was shown to increase susceptibility to infection with resistant strains of *C. albicans* [106].

The enormous potential of DCs to recognize, process and present fungal antigens and to orchestrate innate and adaptive immune responses against fungal infections has been employed in vaccine design and “adjuvanation” procedures [71, 98, 102, 103, 107, 108]. As discussed previously, numerous effective immunostimulatory adjuvants that can engage adaptive CMI are available; however, the appeal of DC-based vaccine strategies derives from their ability to package immunogenicity into immunostimulatory rather than idle molecular entities. Fungal vaccine design dependent upon the use of DCs pulsed with whole fungi, fungal cell extracts or fungal RNA has been tried with some success [102]. Vaccination with *A. fumigatus* antigen extract-pulsed DCs mixed with CpG-ODN protected mice against subsequent *A. fumigatus* infections in a Th1-mediated manner [104]. Upregulation of several TLRs on DCs following pulsation with fungal RNA suggests that fungal RNA can activate DCs via nucleotide receptor signaling cascades [103]. Administration of *A. fumigatus* RNA-pulsed DCs induced Th1-derived IFN-γ-dependent protective responses against subsequent challenges with *A. fumigatus* [103]. Interestingly, the cytosine deaminase (CD) activity of DCs, which converts the antifungal 5-FC into the potent anticancer compound 5-FU [109-110], has been utilized to generate libraries of recombinant Ab fragments (mainly, single-chain variable fragments), display them on phage surfaces, and select phage Abs against target antigens for possible clinical use [111]. Use of pharmacologically-activated invariant natural killer T (iNKT) cells [112] or rapamycin-induced autophagy [113] were shown to positively modulate DCs and enhance antigen-specific B and T cell responses in the presence of immunogenic proteins.

**CLASSICAL APPROACHES TO FUNGAL VACCINE DEVELOPMENT**

Notwithstanding the presence of considerable similarities between fungal and human cells, the list of fungal cell antigens that are immunogenic in mammalian hosts is extensive [5, 114]. As the cell wall plays an important role in host-fungus interactions, several cell wall components are major inducers of fungal immunity, hence the interest in such components as potential candidates for vaccine development. For instance, vaccination with *C. albicans* β-mercaptoethanol (β-ME) extracts prolonged survival and decreased fungal burden in mice upon subsequent infection with lethal doses of the pathogen [115]. Proteomic analysis has revealed the presence of complex polypeptide patterns with multiple immunogenic components associated with β-ME extracts that were used in the preparation of the vaccine. Immunization of rabbits with a glycolconjugate consisting of synthetic *C. albicans* β-1,2-mannan disaccharides clustered on a glucose core and conjugated with tetanus toxoid resulted in the generation of high titers of Abs reactive with the disaccharide cluster and the trisaccharide epitope [93]. Anti-sera from immunized rabbits cross-reacted with *C. albicans* β-mannan cell wall extracts. A glycolconjugate consisting of the *C. albicans*-expressed complement receptor 3-related protein (CR3-RP) has demonstrated significant immunogenic potential in rabbits [90].

Sugar modifications can alter immunogenicity of fungal antigens [99, 100]. For example, it was reported that mannosylation of an immunoreactive cryptococcal mannoprotein was
critical for optimal T cell responsiveness against *C. neoformans* [116]. Administration of a CR3-RP glycolconjugate triggered enhanced CMI by upregulating the expression of IL-2 receptor α-subunit (CD25) on B-lymphocytes, induced isotype switching, and increased the CD4⁺/CD8⁺ ratio in hosts. Vaccination of BALB/c mice with the *C. albicans* ecm33 mutants (RML2U), mutants that express cell walls rich in protein in the outermost layer, was protective against subsequent lethal doses of the virulent *C. albicans* strain SC5314 [91]. The *Candida* rAls3p-N vaccine, a variant of the rAls1p-N vaccine, was shown to be effective when combined with aluminum hydroxide adjuvant [88]. Although immunoprotection conferred by rAls3p-N vaccine was comparable to that induced by rAls1p-N against disseminated candidiasis, the new variant vaccine was superior to rAls1p-N in protecting against oropharyngeal and vaginal candidiasis [117]. While intraperitoneally-administered rAls1p-N modestly improved survival during disseminated candidiasis in mice, increased efficacy was achieved by using the subcutaneous route. In a mouse model of brain cryptococcosis, intracerebral administration of doubly-treated heat-inactivated *C. neoformans* cells enhanced survival after subsequent local challenge with lethal doses of *C. neoformans*. Survival was associated with local inflammation, reduced fungal growth within the brain and development of delayed-type hypersensitivity [118]. Immunization of BALB/c mice with radio-attenuated *P. brasiliensis* cells promoted lasting protection against virulent forms of the pathogen as >99% decrease in CFU recovery was evident three months post challenge [119]. The protective effects of this experimental vaccine were associated with Th1-mediated responses; namely increased levels of IgG2a and IFN-γ production. Treatment of dexamethasone-anergized BALB/c mice infected with *P. brasiliensis* with a 15mer peptide from the major diagnostic antigen gp43 (P10) in combination with chemotherapy reduced lung fungal burden and prevented fungal dissemination to liver and spleen [120]. Previously, it has been shown that vaccination with P13, a decapeptide mimotope of the cryptococcal capsular polysaccharide GXM protects against subsequent lethal *C. neoformans* challenges [121, 122]. In chronically infected mice, vaccination with P13 prolonged survival and upregulated the expression of IFN-γ, IL-6 and IL-10 in a manner dependent on mouse strain (BALB/c vs. C57BL/6), carrier protein type (tetanus toxoid vs. diphtheria toxoid) and route of infection (intraperitoneal vs. intravenous) [123]. In a related recent study [124] however, mAbs generated against a heptasaccharide representing the major structural motif of GXM (M2) that is present in common clinical isolates of *C. neoformans* failed to elicit protective Ab responses. Based on these findings it was concluded that the M2 motif of *C. neoformans* GXM moiety is not suitable for vaccination purposes. A 25KD cryptococcal deacetylace (d25) was able to induce cell proliferation and secretion of IL-2 and IFN-γ, but not IL-4, in spleen cells isolated from mice immunized with the d25 or infected with *C. neoformans* [125]. Secretion of IFN-γ, but not IL-2, was essential for anti-cryptococcosis activity of d25 in mice [125].

Synthetic chemistry has been a considerable source of chemically-defined immunogenes that can be used to design experimental fungal vaccines. A novel, immunologically neutral, linker methodology to prepare highly defined vaccine conjugates has been recently described [126]. This methodology relies on combining complex saccharide antigens with specific Th cell epitopes. Using this method, two glycoconjugates prepared from synthetic (1→2)-β-mannan trisaccharide with inter residue-S-linked mannopyranose residues and coupled to tetanus toxoid elicited the synthesis of Abs that recognize O-linked trisaccharide epitopes in native cell wall antigens of *C. albicans* [92]. Using this saccharide-peptide linker chemistry, T
cell peptides from *C. albicans* wall proteins were synthesized and conjugated to the cell wall beta-mannan trisaccharide (beta-(Man)(3). Out of six glycopeptide conjugates, three (beta-(Man)(3)-Fba, -Met6, and -Hwp1) were able to increase survival and reduce kidney fungal burden in mice with candidiasis [127]. The other three conjugates either moderately protected against (-Enol and -Gap1) or slightly enhanced disease (-Pgk1).

**CROSS-KINGDOM Fungal Vaccines**

Using antigenic components derived from one strain or species to vaccinate against a related strain or species is relatively common in preparing viral and bacterial vaccines. Vaccination across higher orders of classification is not that uncommon either. For example, CD-1 mice receiving weekly injections of heat-killed *S. cerevisiae* (HKY) via the subcutaneous or oral routs prior to the initiation of coccidioidomycosis experienced prolonged survival and reduced fungal burden [128]. In contrast, vaccination across different life kingdoms has been uncommon until recently [129]. The algal beta-glucan (laminarin) vaccine when conjugated with an appropriate protein component was protective against a wide array of fungal infections through the induction of Abs that inhibit fungal growth [130]. Based on the relative success of laminarin and other preparations in experimental animal models it has been argued that, with some fine-tuning, it may become possible to use preparations like laminarin to vaccinate against classes of fungal pathogens expressing the beta-glucan moiety [129]. Vaccination with the recombinant N terminus of the *Candida* adhesin Als3p (rAls3p-N) was protective against lethal candidemia [88, 131, 132] and *S. aureus* infections especially when combined with aluminum hydroxide [133]. The cross-kingdom protective potential of Als3p was attributed to shared similarities with the *S. aureus* -derived clumping factor. In the reverse direction however, the protective potential of *S. aureus* clumping factor against candidiasis is yet to be fully explored. Several putative MHC-II-binding proteins deduced from *E. coli* by proteomic and/or bioinformatic means were also reported to be protective against *C. posadasii* infections in mice [134]. Although the initial hype regarding rAg2/Pra, a bacterially-expressed recombinant proline-rich protein, as a potential *Candida* vaccine has subsided due to contradictory data, additional promising findings have been reported [135]. In that, subcutaneous vaccination of C57BL/6 or BALB/c mice with rAg2/Pra plus an adjuvant followed by intraperitoneal challenge with *C. posadasii* significantly reduced fungal burden. When mixed with an adjuvant, a combined vaccine consisting of rAg2/Pra and a *C. posadasii*-derived molecular homologue (Prp2) sharing 69% sequence identity with rAg2/Pra was able to induce significant T cell-mediated protection against intranasal challenge with *C. posadasii*. This protective effect was attributed to the capacity of the combined vaccine to stimulate a heterogeneous T-cell repertoire as compared with the relatively homogeneous response generated by rAg2/Pra vaccine alone [135].

**RECOMBINANT VACCINATION APPROACHES**

Work on recombinant fungal vaccines, which has been going on for sometime now, is extensive in terms of approaches, target pathogens and outcomes. However, the overall
success of this approach in delivering effective fungal vaccines still falls way short of the fan-fair with which it has started. That is not to say that the promise is totally lost. In fact, the following discussion will try to make this very point by borrowing random examples, albeit recent, from across the board. Vaccination with a recombinant strain of \textit{B. dermatitidis} lacking the WI-1 adhesin gene was reported to be protective against pathogenic strains of the fungus through Th1-mediated responses [136]. Significant humoral immune responses were noted following immunization with HSP90 constituents, recombinant HSP90 protein preparations or HSP90-encoding DNA vaccines administered via the intradermal, intranasal, or intravenous routes [137]. Priming by an intradermal injection of rHSP90 followed by intranasal or intradermal boosting with the same form significantly increased serum and vaginal fluid concentration of \textit{Candida} HSP90-specific IgG and IgA Abs and enhanced HSP90-specific splenocyte responses \textit{in vitro}. Immunization of C57BL/6J mice with a hybrid phage consisting of \textit{C. albicans} HSP90 DEPAGE specific epitope (SE-CA-HSP90) expressed on a filamentous phage surface and fused to a major phage coat protein (pVIII) was able to reduce kidney fungal burden, induce anti-SE-CA-HSP90 Ab responses, enhance DTH responses, enhance NK cell activity, and augment ConA-induced splenocyte proliferation [138]. Immunization with a \textit{C. albicans} HSP90 protein-expressing DNA vaccine cloned into the vaccination vector pVAX1 was able to protect against systemic candidiasis in BALB/c mice by eliciting the production of protective anti-\textit{Candida}-HSP90 IgG Abs [139]. A recombinant HSP90-related protein 60 (rHSP60) was able to reduce fungal burden and induce synthesis of IFN-\(\gamma\) and IL-12 in mice with \textit{P. brasiliensis} [140].

The protective potential of a genetically engineered \textit{C. albicans} tet-NRG1 strain-derived antigen tested as an experimental live, attenuated vaccine against disseminated candidiasis was shown to be dependent upon T cell immunity both in immunocompetent and immunodeficient mice [141]. Immunization with a recombinant antigen of \textit{A. fumigatus} (rAsp\textsubscript{f3}) was protective against invasive aspergillosis [142]. Three truncated versions of rAsp\textsubscript{f3}, spanning amino acid residues 15-168, 1-142 and 15-142 (all lacking sequences required for IgE binding) were also protective. Interestingly, vaccination with rAsp\textsubscript{f3} (1–142) or rAsp\textsubscript{f3} (15–168) drastically diminished the production of antigen-specific Abs compared with the full-length rAsp\textsubscript{f3} (1–168) or the double-truncate rAsp\textsubscript{f3} (15–142). These findings suggest that it is possible to design vaccines that target cellular immunity and activate T cells capable of enhancing the function of macrophages or other cellular elements involved in fungal pathogen clearance. In search for additional \textit{Aspergillus} immunogenic antigens, a \(\lambda\)-phage cDNA expression library prepared from \textit{A. fumigatus} mRNA was used to screen for \textit{A. fumigatus} antigens reactive with anti-sera from rabbits infected with conidiospores of \textit{A. fumigatus}. Abs against several antigens including the glycosylhydrolase Asp-f-16, enolase, and HSP90 were produced by recipient rabbits [143]. A cDNA library constructed from \textit{A. fumigatus} mycelia using the \(\lambda\)-ZAP expression vector and tested against sera from patients with allergic bronchopulmonary aspergillosis (ABPA) revealed the presence of a novel gene encoding the 43KD \textit{A. fumigatus} allergen Asp\textsubscript{f16}. Asp\textsubscript{f16} was capable of reacting with IgE from ABPA patients but not with sera from patients with allergic asthma, asymptomatic \textit{Aspergillus} skin test-positive asthmatics or normal controls [144]. The Asp\textsubscript{f16} allergen was also able to induce significant proliferation in peripheral blood mononuclear (PBM) cells isolated from patients with ABPA. The deduced amino acid sequence of Asp\textsubscript{f16} shares extensive sequence homology with a 31KD antigen known as Asp\textsubscript{f9} [144-146]. Vaccination with a recombinant form of Asp\textsubscript{f3} or with a truncated form of Asp\textsubscript{f3} lacking the IgE binding
sites was protective against aspergillosis [97]. The protective potential of truncated Asp3 was dependent upon the presence of the adjuvant TiterMax. Vaccination with a recombinant N-terminal domain of A1s1p (rA1s1p-N) was protective against disseminated candidiasis in BALB/c and other outbred mouse strains [147]. As Asp3 and related molecules and truncates can stimulate adaptive CMI and as these molecular entities exist in other Aspergillus species and other molds (Coccidioides posadasii and Penicillium citrinum), and yeasts (C. albicans, C. boidinii and S. cerevisiae), it is worth testing the capacity of Asp3 and related structures and homologues to act as pan-fungal (or cross-genera) vaccines.

A genetically-engineered, live, attenuated vaccine constructed by disrupting two chitinase genes (CTS2 and CTS3) of C. posadasii or C. immitis was recently shown to be protective against coccidioidomycosis in BALB/c and C57BL/6 mice [148]. Vaccinated animals mounted immune responses involving the production of Th1 and Th2 type cytokines that collectively helped to establish well-formed granulomas, diminish infection-associated inflammation and reduce lung fungal burden. As mentioned previously, Pcp is a prevalent opportunistic infection among AIDS patients worldwide. Alveolar macrophages recognize Pce through a Pce β-glucan C-type lectin receptor known as dectin-1. Treatment of SCID mice with Pcp using a fusion protein consisting of the extracellular domain of dectin-1 plus murine IgG1 Fc fragment reduced lung fungal burden and tissue damage [149]. The protective effect of dectin-1-Fc was related to its ability to enhance macrophage recognition of β-glucan and its capacity to opsonize Pce cells for killing by alveolar macrophages. DCs and macrophages from dectin-1-deficient mice failed to produce cytokines in response to stimulation by β-glucan in vitro [149].

**THE -OMICS PROMISE**

The availability of complete genome sequences of more than 90 different fungal species including C. albicans, A. fumigatus, and S. cerevisiae among others is making it possible to revise previously published gene sequences of fungal proteins currently available at different data bases (Genebank and IUIS). A study that has compared old published sequences of many proteins with updated genome sequences concluded that there could be a core set of allergen-like proteins in each fungal species [150]. The study identified a number of inconsistencies in the approved allergen list available at Genebank and IUIS data bases. It concluded that Asp 56KD (Asp15) and (Asp16) ought to be replaced by the larger protein motif (Asp17). Additionally, the study suggested that Asp12 ought to be classified as HSP90, Mala s 10 as hsp88 and (Alt a 3, Cla h 4 and Pen c 19) as HSP70. Immunoproteomic and bioinformatic approaches to deduce pathogen-derived proteins capable of binding with MHC molecules (MHC-II in particular) and able to stimulate Th cell-mediated protective immunity has resulted in the discovery of Escherichia coli–derived putative proteins protective against Coccidiomycosis in C57BL/6 mice [134]; another potential cross-kingdom vaccine. Twenty nine C. albicans–derived immunoreactive proteins that specifically react with Abs from vaccinated mouse sera were identified using immunoproteomic approaches [91]. Six of these proteins (Gnd1p, Cit1p, Rpl10Ep, Yst1p, Cys4p, Efb1p) were described as immunogenic. Proteins that were exclusively identified in the mutant surfome (Met6p, Eft2p, Tk11p, Rpl10Ep, Atp1p, Atp2p) were also immunogenic [91]. In summary, while still in its infancy
with regard to fungal pathogens, diseases and immunotherapeutics, the –omics revolution may prove valuable in identifying infinite numbers of fungal antigens and peptides as potential immunotherapeutic candidates. Devising high contrast screening strategies to drastically minimize the number of molecules worthy of further evaluation is essential should the voluminous data generated by fungal genomic and proteomic research not become a major distraction.

**CELL TRANSFER THERAPY**

Cells of innate and adaptive immunity are pivotal in mediating protection against fungal infections. Using cellular components to prime, modulate, and enhance immunity against fungi is one of the approaches being developed and tested to treat fungal infections. Work on this front has focused on using mixed or purified pools of lymphocytes (DCs, NK cells, CD4+ T cells and CD8+ T cells) either isolated from infected animals or manipulated *in vitro* prior to their application as non-adaptive or adaptive immunotherapeutic modulators. Work on the transfer of DCs has already been described in the previous section; therefore, the discussion below will mainly deal with experimental work pertinent to mixture cell populations, phagocytes, NK cells, and T cells.

It has been reported that pharmacologically-activated iNKT cells can positively modulate DCs and enhance antigen-specific B and T cell responses in the presence of antigenic proteins [112]. Human lymphocytes cultured with IL-2 were shown to conjugate with and directly inhibit the growth of various strains of *C. neoformans* [159]. Highly purified populations of human lymphocytes cultured with encapsulated *C. neoformans* exhibited strong fungistatic activity involving NK, CD4+, CD8+, and CD16/56+ T cells [160]. Besides the capacity of Asp16 antigen to induce humoral and allergic responses against *Aspergillus* [142-144], it can induce effector T cell responses. In that, *Aspergillus*-specific CTLs were generated from PBM cell cultures stimulated by DCs pulsed with the complete pool of pentadecapeptides (PPCs) spanning the coding region of Asp16 or stimulated by APCs consisting of Epstein-Barr virus (EBV)-transformed B lymphoblastoid cells (BLCL) [107]. PPC-DC-only-primed cell lines induced effector cell phenotype and peptide specificity at levels comparable to those of DC/BLCL primed cell lines. However, the later approach was more efficient in generating Asp16-specific IFN-γ-producing cells at higher frequencies. Administration of DCs pulsed with the overlapping pentadecapeptides spanning the entire 427-aa coding region of Asp16 induced strong proliferative responses [161]. A CD4+ IFN-γ producing cell line isolated from immunized donors was cytotoxic to autologous pool-pulsed and *Aspergillus* culture extract-pulsed targets. Furthermore, two lines isolated from HLA-B*3501+ donors following challenge with DCs pulsed with the overlapping pentadecapeptides were strongly cytotoxic to autologous target cells pulsed with either Asp16-peptide pool or *Aspergillus* culture extracts. Culture supernatants from these cell lines exhibited considerable killing activity against *Aspergillus* conidia. Moreover, cells from these cultures exhibited direct cytotoxic activity against *Aspergillus* hyphae. MHC I (HLA-B*3501)-restricted CD8+ T cells were exclusively responsible for the cytotoxic activity and IFN-γ production [162]. While use of B cells or serum from animals immunized with *Candida*-derived rAls3p-N antigen failed to transfer protection to naive animals as Ab titers were lower than baseline values (> or = 1:6400), adoptive transfer of CD3+, CD4+ or CD8+ T cells from immunized mice was protective against candidiasis in naïve mice [88]. Based on the assumption that Th cells are of
significance in inducing anti-aspergillus immunity, especially in hematopoietic stem cell transplant (HSCT) patients, a method of large-scale generation of anti-aspergillus T cells has been recently described [163, 164]. In this study, about $10^9$ white blood cells were stimulated with *Aspergillus* antigens; IFN-γ producing cells were selected and expanded. Within two weeks, a median number of $2 \times 10^7$ CD3⁺CD4⁺ cells exhibiting an activated memory Th phenotype were obtained. Upon re-stimulation, there was significant *in vitro* Th1 differentiation as evidenced by the presence of significant numbers of viable T cells secreting IFN-γ, but no IL-4 or IL-10. This technique may be useful in prospective trials for large-scale adoptive transfer of fungal antigen-primed T cell.

Adoptive transfer of CD4⁺ splenocytes from mice sensitized with *A. fumigatus* into naïve mice was shown to prolong survival following intravenous challenge with viable *A. fumigatus* conidia [145]. Following stimulation with cell wall or membrane components of *B. dermatitidis*, CD4⁺ T cells expressing Vα2*J*α49⁺ and Vβ¹⁺β1⁺ TCR antigens but not those expressing Vβ8.1⁺ or Vβ8.2⁺ were able to adoptively transfer protective immunity and produce high levels of IFN-γ. This finding suggests that the generation of protective T cell clones against immunodominant antigens of *B. dermatitidis* is greatly influenced by the TCRα and TCRβ combinations that favor the production of Th1 cytokine responses [165]. Along these lines, we have reported a pattern of time-dependent overexpansion of specific TCRβ clones in a mouse model of estrogen-dependent vaginal candidiasis (EDVC) [166]. In that, T cell clones expressing TCRβs 1, 6, 10 and 15 predominated during the intermediate phase (week 2) post *C. albicans* infection while those expressing TCRβs 1, 4, 6, 8, 3, 10, 13, 14 and 18 dominated the vaginal T cell pool at week 3 post infection. Moderately reduced tissue (lungs, spleen and liver) fungal burden was reported in nude C57BL/10 mice and 5Gy-irradiated heterozygous nude (nu/C57BL/6) mice intranasally challenged with *H. capsulatum* upon receiving an intravenous inoculation of resting 2.3H3 cells (a CD4⁺ T cell clone) [167]. The effect was species-specific as mice challenged with *C. immitis* failed to clear the infection. These findings suggest that nude mice are permissive for the expression of adoptively-transferred anti-*H. capsulatum* T cell-mediated immunity. These and numerous similar findings call for further exploration of the potential of adoptively-transferred antigen-specific Th cells to modulate and restore protective immunity, especially in the immunocompromised.

CD8⁺ T cell involvement in protection against infection is generally dependent upon help from Th cells. However, there is evidence to suggest that CD8⁺ T cells play a significant role in protection against fungal infections in the absence of Th cell help. For instance, although CD4 deficiency impairs CD8⁺ T cell activation and function in viral and bacterial infections [168-169], generation of *C. neoformans*-specific effector CD8⁺ T cells capable of producing protective levels of IFN-γ was reported to occur in CD4-deficient mice with pulmonary cryptococcosis [39]. The functionality of CD8⁺-derived IFN-γ was demonstrated by the finding that neutralization of IFN-γ in CD4⁺CD8⁺ mice increased macrophage susceptibility to infection by *C. neoformans*. Furthermore, IFN-γ, as the signature protective cytokine in the majority of fungal infections, seems to derive not only from Th1 cells but also from cells of phenotypes other than CD4⁺ altogether. For example, depletion of CD4⁺ T cells in mice treated with anti-CD40/IL-2 did not significantly diminish the concentration of serum IFN-γ [170]. Hence, the potential of CD8⁺ cells to manipulate immunity against fungal infections is worth considering.
In addition to cell transfer work on lymphocyte, several studies have indicated that transfusion of donor granulocytes (neutrophils) is effective in alleviating the side effects of neutropenia and in enhancing the repopulation of granulocyte cell subsets [171-176]. Limitations on the potential benefits of GTX treatment in neutropenic cancer patients derive from the fact that this form of treatment is often delayed for concerns of pulmonary reactions. However, these concerns have been recently challenged. In that, treatment of three children suffering from hematological malignancies and proven fungal infections (cerebral mold infection, disseminated candidiasis or nasopharyngeal mucormycosis) with combination antifungal therapy plus G-CSF-mobilized granulocyte transfusion (GTX) during subsequent neutropenia effectively resolved the infections thus permitting anticancer therapy completion without delay [171]. A similar approach was successfully used to treat thirty two cancer patients with fungal infections [172]. A prospective phase II clinical trial was conducted to test the safety and efficacy of early-onset therapy using G-CSF-elicited cross-matched GTX therapy given at two doses consisting each of 8x10^8 granulocytes/Kg body weight in neutropenic children with severe infections [173]. Results from the study suggest that early-onset GTX therapy is well-tolerated, does not result in notable pulmonary reactions, and is effective (>92% response rate) in clearing associated infections. Short-term activation (≤3 days) and low dose irradiation of the human myeloid cell line (HL-60) was shown to generate viable, poorly replicative cells with candidacidal activity suitable for use as a novel GTX therapy [174]. Infusion of HL-60 cells treated in this manner significantly improved survival of neutropenic mice with candidemia. Reconstitution of neutrophil count and function by infusing G-CSF-activated GTX was reported to be effective at clearing Zygomycete infections [175]. A phase III clinical trial sponsored by the National Institutes of Health (Heart, Lung, and Blood Institute) to evaluate the safety and efficacy of GTX in treating patients with bacterial and fungal infections during neutropenia is currently underway (www.clinicaltrials.gov, Identifier: NCT00627393).

It is clear from the preceding discussion that the search for immunotherapeutics in this area has focused on localized and systemic components of secondary, but not primary, lymphoid organs. It is well established that primary lymphoid organs are subject to significant infection-related structural and functional alterations. For instance, different types of pathogens infect the thymus and cause changes in the development and behavior of thymus-derived T cells [reviewed in 177]. During viral [178-180], bacterial [181, 182], fungal [183, 184], and protozoal [185, 186] infections, the thymus experiences atrophy, depletion of immature CD4^+CD8^+ cells, diminished thymocyte proliferation, epithelial network densification, and increased extracellular matrix deposition. Potential consequences of this process involve the induction of central tolerance to pathogen-derived antigens [177, 187] and the consequent generation of perturbed T cell repertoires containing autoreactive T cells and/or lacking T cell specificities needed to recognize and handle specific pathogens. In such cases, adoptive transfer of pathogen-specific T cells is an option; with that however comes potential cross-reactivity and immune responsiveness. The introduction of genetically-engineered syngeneic T cells expressing TCR specificities capable of handling a specific pathogen may be a more guided approach. However, this requires the identification of pathogen specific TCRαβ and/or TCRγδ T cell repertoires, a task which has already started [165, 166] but yet to be fully explored.
Based on current understanding of T cell immunity against fungal infections, it is postulated [5] that the relative significance of different T cell subsets in protection against fungal infections is dependent more on the cytokine profile they produce and less on their static phenotypic profile, vis-à-vis CD4 vs. CD8 or, for that matter, lymphocytes vs. phagocytes. Consequently, the use of proinflammatory, anti-inflammatory, regulatory or suppressor cytokines to modulate the immune response during fungal infections seems the more logical. The ease with which optimal doses of cytokine therapy can be determined and effectively administered and the relative accessibility in managing potential adverse consequences adds to the fundamental appeal of cytokines as effective yet relatively benign agents with antifungal potential. Cytokine therapy may however be hampered by the need to maintain a delicate balance between inflammatory and anti-inflammatory cytokines so as to effectively fight infections without causing injurious inflammation [24, 43, 44, 72, 85].

Cytokines are signaling molecules that specifically induce the proliferation, differentiation, activation, or suppression of target cells. In turn, cytokines specifically modulate host immunity in varied ways depending on the specific cytokine(s) involved, the subset of target cells expressing the respective receptor(s), the kinetics of interaction between the cytokine and its receptor, and the subsequent signaling cascade(s) generated by the interaction. Although the efficacy of antifungal cytokine therapy varies depending on the level and nature of immunosuppression or immunodeficiency and the antifungal agent being used to treat the infection, the benefits of cytokine therapy on the general outcome of managing mycotic infections is tangible. The pharmaceutical industry, having realized this, is hot on the trail of cytokine-based therapies against invasive fungal infections. For example, Leukine® (sargramostim; Bayer HealthCare Pharmaceuticals) is a GM-CSF preparation that is currently undergoing expanded evaluation (phase III of clinical trial) as an antifungal agent. Administration of IFN-γ, IL-12 or anti-IL-4 can enhance Th1-dependent immunity against opportunistic fungal infections [84]. In an experimental model of cytokine gene therapy [188], it was demonstrated that J774 macrophages transfected with a single-chain cDNA encoding the p40 and p35 subunits of IL-12 construct can secrete biologically active IL-12 in vitro. Treatment of C. immitis-infected BALB/c mice (highly susceptible to coccidioidomycosis) with IL-12-transduced J774 cells inhibited C. immitis growth. The protective effects of IL-12-transduced J774 cells were correlated with a significant increase in IFN-γ synthesis in the lungs and blood of infected/treated mice. The immunotherapeutic role of IL-12 against P. carinii infection was demonstrated in IL-12 (p35)-deficient mice or mice depleted of CD4+ cells [189]. Pathogen challenge followed by treatment with local IL-12 instillation or IL-12 gene therapy resulted in a rapid release of IL-12 into lung tissues and accelerated pathogen clearance from the lungs in normal mice. IL-12-deficient mice and mice depleted of CD4+ cells showed delayed clearance of infection. Treatment of deficient or depleted mice with intranasal recombinant IL-12 accelerated clearance of the P. carinii infection. This response was associated with increased recruitment of inflammatory cells into lavage fluid and increased release of TNF-α, IL-12 and IFN-γ. Treatment with anti-CD40 in combination with IL-12 prolonged survival and protected mice against systemic cryptococcosis [170]. Protection in this model was correlated with decreased kidney and brain fungal burden and increased IFN-γ and TNF-α production. A similar treatment approach
failed to protect IFN-γ knockout mice (IFN-γ−/−) against *C. neoformans* infections suggesting that protection against cryptococcosis is IFN-γ-dependent. In a subsequent study, it was demonstrated that similar treatment regimens augmented host immunity against *C. neoformans* brain infections [190]. Protection in this case was correlated with upregulated MHC-II expression on brain CD45<sup>high</sup>CD11b<sup>+</sup> cells and overall activation of microglial cells. Once again, IFN-γ was shown to be critical for microglial cell activation and anticytococcal activity following treatment with anti-CD40/IL-2 combination albeit independent of CD4<sup>+</sup> T cell involvement [190].

Besides the well established protective roles of IFN-γ and IL-12 against fungal infections, some studies have suggested that IL-17 plays a positive role in regulating innate and adaptive immune responses against some forms of mycosis [reviewed in 191 and references therein]. IL-17, as a proinflammatory cytokine, was shown to induce the synthesis of downstream cytokines such as GM-CSF and CXCL8/IL-8 by localized differentiated neutrophils. Additionally, IL-17 can induce the synthesis β-defensins and other AMPs. CD4<sup>+</sup> TCRαβ<sup>+</sup> or TCRγδ<sup>+</sup> T cells (mainly residents of the epithelium of the skin, GI and reproductive tracts) produce significant quantities of IL-17 [192, 193]. It has been reported that the duration of neutropenia was shortened following the administration of recombinant G-CSF and GM-CSF [194, 195]. Additionally, the phagocytic and killing activities of neutrophils, monocytes and macrophages were enhanced following the administration of recombinant G-CSF, M-CSF and GM-CSF [194, 195].

Administration of cytokines has proven beneficial when used in combination with chemotherapy. For example, cytokine adjunctive therapy of cancer patients with corticosteroid-induced neutropenia reduced the duration of neutropenia and enhanced granulocyte antifungal activity, which minimized the risk of invasive fungal infections and permitted aggressive cytotoxic therapy [196]. Intranasal administration of GM-CSF into immunosuppressed mice suffering from pulmonary aspergillosis significantly reduced lung fungal burden suggesting that lung-targeted immunotherapy may complement antifungal chemotherapy and improve treatment outcome [23]. In mice with 5-FC-induced neutropenia, treatment with G-CSF augmented the antifungal activity of 5-FC. GM-CSF significantly enhanced PMNs fungicidal activity and enhanced the collaboration between PMNs and voriconazole or fluconazole to kill *C. albicans* [48]. However, the synergistic effect of GM-CSF was more evident in the case of voriconazole given that it is a more potent (>10-fold) anti-*Candida* azole than fluconazole. G-CSF was shown to reverse neutrophil dysfunction against *Aspergillus* hyphae in HIV-infected non-neutropenic patients [49]. In cancer patients with therapy-refractory neutropenia, the response to antifungal therapy or even donor-derived GTX therapy is often suboptimal. Immune enhancement using recombinant IFN-1β in neutropenic cancer patients on G-CSF-stimulated GTX therapy successfully cleared or stabilized associated opportunistic fungal infections with minor rIFN-1β-related side effects [176].

In this context, it is worth noting that conventional antifungals like polyenes (AMB and NY) and azoles (voriconazole and ketokonazole) can induce proinflammatory cytokine production and alter gene expression in different tissues. For example, upon recognition of AMB by TLR-2, several proinflammatory cytokines are released by TLR<sup>+</sup> phagocytes [50, 197]. Furthermore, recognition and binding of NY by TLR-1- or TLR-2 has been reported to induce phagocytes to release several proinflammatory cytokines (IL-1β, IL-8 and TNF-α)
As discussed previously, following recognition and binding of A. fumigatus-, C. albicans-, or C. neoformans-derived PAMPs by TLRs 2, 4, 9 or CD14 on professional phagocytes, an inflammatory response that induces Th1-mediated protective immunity ensues [85]. The capacity of polyenes to mimic fungal antigens and engage innate immunity in a TLR-dependent manner should provide new means to further explore the utility of cytokine therapy against fungal infections. While stimulation of THP-1 human MNs by A. fumigatus hyphal fragments alone caused significant upregulation in the activity of few cytokine genes (e.g. CCL4), co-stimulation with the fungal fragment and voriconazole resulted in upregulation of a larger number of cytokine/chemokine genes (CCLs: 3, 4, 5, 7, 11 and 15 as well as CXCL6). This finding indicates that voriconazole enhances the capacity of fungal antigens to upregulate the expression of a wider and presumably more protective profile of cytokine genes [28]. Using a toxicogenomic microarray platform comprised of cDNA probes generated from mouse livers exposed to different hepatotoxicants, it was shown that out of 2364 genes assessed, ketoconazole at hepatotoxic doses upregulated nine genes encoding enzymes involved in phase I metabolism and one phase II enzyme (glutathione S-transferase) [199].

Immunosuppressants that target proinflammatory cytokines interfere with normal inflammatory and immune responses leading to increased incidence of opportunistic infections. Tissue availability and concentration permitting, some cytokine inhibitors bind target cytokines with high enough affinity to block their binding with respective receptors. Treatment with the TNF-α antagonists infliximab, adalimumab and the receptor fusion protein etanercept (FDA-approved drugs for the treatment of Crohn's disease, rheumatoid arthritis, psoriasis and other inflammatory diseases) has been associated with increased risk of bacterial and fungal infections [53, 200, 201]. The potential of certain cytokines to induce immunosuppression and other adverse effects has also been reported with regard to fungal infections. For example, administration of recombinant human G-CSF prior to infection with A. fumigatus in outbred ICR mice pretreated with hydrocortisone enhanced the action of the SCH56592 azole derivative [202]. However, it failed to be protective as treated mice developed large lung abscesses with significant PMN cell infiltration and high fungal burden. By removing G-CSF from the treatment regimen, reduced lung fungal burden and longer survival rates were observed.

**IMMUNOSTIMULATORY ANTIBODIES AND ANTI FUNGAL PEPTIDES**

As will be discussed in the next section, Ab- and AMP-based therapies, for the most part, target and neutralize and/or kill pathogens. However, there are cases where such molecules, whether generated as part of the immune response or administered as immunotherapeutics, can modulate host immunity. Development and use of molecules that can modulate and enhance protective immunity is desirable especially when Th differentiation does not move along the protective Th1 pathway, excessive inflammation, or selective immunodeficiency among other scenarios. In such cases, these and other molecules like cytokine inhibitors [53, 200], ligand-receptor inhibitors [170], and signaling molecules can be considered as constituents of the general class of host-targeting immunomodulatory agents [20, 53, 170, 200].
A protective natural mAb (3B4) that recognizes a surface antigen on *C. albicans* germ tubes was generated using B cells from *C. albicans*–infected mice [203]. Besides its pathogen-targeting role of suppressing germ tube formation, 3B4 was able to modulate phagocytosis and bind with the self-antigen keratin, which was shown to enhance the selection of B cells into the B1 B cell compartment, the site of antifungal Ab synthesis. An IgG1 mAb generated against a 70KD glycoprotein antigen secreted by the thermally dimorphic fungus *Sporothrix schenckii* significantly reduced CFU counts in an IFN-γ-dependent manner when injected before, during or after *S. schenckii* infection in immunocompetent or T cell-deficient mice [204]. The protective potential of anti-CD40 treatment against systemic *C. neoformans* infections has been correlated with increased IFN-γ and TNF-α production and upregulated expression of MHC-II on brain microglial cells [170].

With regard to AMPs, it is well established that ribotoxin-derived peptides, cathelicidins and other cationic peptides can modulate cytokine production, alter host gene expression profiles and minimize proinflammatory responses against microbial antigens. The ribotoxins comprise a family of fungal extracellular ribonucleases capable of inactivating ribosomes by specifically cleaving the single phosphodiester linkage at the conserved sarcin/ricin loop of the large rRNA. Subsequent inhibition of protein biosynthesis is followed by apoptosis-induced cell death. Peptides derived from the ribotoxin of *A. fumigatus* modulate immunity in infected mice in a manner consistent with Th1 type responses [146]. An engineered synthetic killer peptide (KP), which functionally mimics fungal killer toxins, binds with β-glucan on fungal cells and mediates direct microbicidal therapeutic effects [205]. Additionally, KP was shown to selectively bind to DCs through MHC-II and CD16/32 and modulate the expression of MHC-II and costimulatory molecules on DCs, thus enhancing their capacity to induce lymphocyte proliferation. Echinocandins, especially caspofungin, have proven potent at variably altering innate immunity against a variety of fungal infections (e.g. *C. parapsilosis*). The hematoregulatory peptide SK&F107647 is known to elicit a time-dependent increase in CD11b⁺ MNs and neutrophils in healthy animals [206]. Although administration of SK&F107647 alone into neutropenic rabbits with disseminated candidiasis did not significantly reduce fungal burden, use of the peptide in combination with low doses of AMB resulted in a significant reduction in lung, liver, spleen, and kidney fungal burdens. Cathelicidin and other cationic peptides, whether constitutively expressed or induced by inflammatory mediators like IL-6 and TNF-α, can modulate cytokine production, alter host gene expression, and minimize inflammation [207-209].

**PATHOGEN-TARGETING IMMUNOTHERAPY**

The mainstay treatment of fungal infections relies on the capacity of drugs (polyenes, azoles, echinocandins ...etc.) to target the pathogen and block its infectivity, stop or slow its growth, or kill it altogether. The same rationale has been applied in the search for immune molecules that could mimic the action of antifungal drugs minus the limiting side effects associated with chemotherapy. Therefore, great efforts were put into developing and testing mAbs, AMPs, signaling cascade inhibitors and other immune molecules that could neutralize, block, opsonize, complement-fix, halt the growth or altogether kill fungal pathogens. The term “inhibitory” that could be used to describe this class should pertain not to their effect on
the immune response but rather to their detrimental effect on the pathogen. By definition, such agents mediate their activity in a passive manner (rapid), and do not require competent immunity to be effective (suitable for use in immunocompromised hosts). However, as pathogen-targeting agents, these modalities remain prone, albeit with a twist, to some of the limitations associated with conventional chemotherapy. For example, they are pathogen-specific with very narrower spectrum of activity especially in the case of mAbs. This limitation can be detoured by the ease with which pathogen-specific mAbs can be designed and the potential to generate mAbs which recognize antigens common to different fungal species. Induction of fungal resistances as another limitation, is by no means unique to pathogen-targeting immunotherapies, it is common among all pathogen- and to some extent host-targeting treatment approaches. The focus therefore should shift from whether an agent can induce resistance to the mechanisms and differential abilities of various therapies in inducing resistance. In other words, the enigma of microbial resistance can only be managed but probably never solved as will be discussed later. Additional problems that may potentially associate with mAbs and AMPs like cross-reactivity, immune responsiveness, and undesirable side effects are not totally unique to mAbs and AMPs either.

**MONOClonAL ANTIBODY THERAPY**

Ever since the introduction of plasma cell hybridoma technology in the mid 1970, an astonishingly large number of hybridomas generated against a whole host of antigens from all kinds of species to produce mAbs for use in research, diagnosis and therapy have been developed. The era of therapeutic mAbs has started in the 1990s with the introduction and approval of several mAbs to treat different forms of cancer. Prominent examples include rituximab (rituxan or MabThera), an anti-CD20 on human B cells, was approved by the FDA in 1997 to treat lymphomas, leukemias and some autoimmune diseases. Infliximab (remicade), a TNF-α inhibitor, received FDA approval in 1998 for the treatment of psoriasis, Crohn’s disease, ulcerative colitis, and other autoimmune diseases. Following these and similar successes, development of therapeutic mAbs for the treatment of cancers, autoimmunity, allergy and other diseases has picked up considerable speed. The 2006 Pharmaceutical Research and Manufacturers of America report (Medicines in Development Biotechnology) has indicated that there are about 160 different mAbs in clinical trial or awaiting FDA approval; the same group reported that the number of therapeutic mAbs grew to 192 in 2008. The same outlook was echoed by the World Health Organization in its 2008 report [210], which estimated that more than 20 mAb-based drugs have been approved for marketing and a further 160 are in the pipelines. Though, the scope of mAb-based experimental antifungal therapy as reported by different research groups around the world is considerable and promising, stories of success are rare. In that, of the 192 therapeutic mAbs described in the 2008 Medicines in Development Biotechnology report noted above, only one (the anti-HSP90 Mycograb®) is intended to treat a fungal infection (candidiasis).

It is now well accepted that Abs are part of the protective immune response against fungal infections. Ever since the 1960s, it has been known that administration of serum Abs can enhance the outcome of antifungal chemotherapy in cryptococcosis [211]. Fungal infections elicit significant Ab responses; the extent to which such Abs confer protection
Antifungal Immunotherapy: A Reality Check

varies depending on their isotype, subisotype, and titer, the presence or absence of nonprotective competing Abs, and the MHC background of the host [54, 55, 212, 213]. IgVH gene usage variations have also been reported to influence the specificity and protective efficacy of Abs [212]. The appeal of mAb immunotherapy partly derives from its potential to accommodate some of the limitations imposed by vaccine. Additionally, it is possible to engineer mAbs with predetermined fine specificity at the isotype/subisotype and complementarily determining region (CDR) levels. It is possible to prepare Ab therapeutic regimens in pure form and mix them with whatever enhancing additives required. It is also possible to administer them in optimal doses via selected routes. Unlike vaccines, therapeutic mAbs provide immediate protection irrespective of the immune status of the host, which makes them good candidates for use in immunocompromised hosts in particular. Based on the fungal molecular component targeted, experimental antifungal mAbs can be classified into those that target cell wall components, cytoplasmic and signaling pathways, and naturally occurring antifungal antibodies (the anti-idiotype class). Interestingly, this classification echoes with that of conventional antifungals as discussed earlier in this chapter.

ANTIBODIES TARGETING FUNGAL CELL WALL AND ASSOCIATED COMPONENTS

The development of protective mAbs against C. albicans [56, 57] and C. neoformans [58, 214, 215] has marked the beginning of a fervent search for protective and therapeutic mAbs against common fungal infections. Generation of effective and protective mAbs against fungi relies on the identification of immunogenic fungal antigens. The fungal cell wall contains several molecular structures involved in fungal pathogenesis and virulence. Therefore, like vaccines, the search for protective mAbs against fungi has focused on those raised against fungal cell wall constituents. mAbs produced against capsular polysaccharide antigens of C. neoformans prolonged survival and decreased fungal burden in C. neoformans-infected mice [59, 216]. Fungal beta-glucans have been used as potential targets to develop effective conventional and novel antifungal agents such as echinocandins, yeast killer toxins (KT) and protective Abs [217]. Anti-beta-glucan Abs are detectable in sera of healthy humans and mice. When elicited by glucan-based vaccines, they can exert fungicidal protective activity. Additionally, receptors for beta-glucan cell wall KT can induce the synthesis of fungicidal protective Abs in natural and experimental infections. In a recent study, it was demonstrated that sera from a random population of healthy human subjects contained detectable amounts of anti-laminarin Abs, though at levels lower than that of Abs directed at C. albicans beta-(1,6)-glucan (pustulan), branched beta-(1,3/1,6)-glucan (Pool 1), and mannan [218]. The prevalent isotype of anti-laminarin Abs, like all anti-beta-glucans, was IgG and the prevalent subisotype was IgG2 [217, 218]. Anti-laminarin (anti-beta-glucan) mAbs, were previously shown capable of inhibiting the synthesis of beta-glucan on C. albicans cells and hence protect against candidiasis [95]. In a subsequent study [219], it was reported that 2G8 was able to reduce brain and liver fungal burden in mice systemically infected with highly virulent, encapsulated strains of C. neoformans. 2G8 was able to reduce C. neoformans capsule thickness without interfering with protease or phospholipase production. The Ab was capable of opsonizing acapsular, but not encapsulated, forms of C. neoformans for further phagocytosis and killing.
by moocytes and macrophages. An anti-C. albicans Ab prepared in chicken egg yolk (anti-CA IgY) significantly reduced adherence of C. albicans to cells of the human pharynx carcinoma cell line FaDu in a dose-dependent manner [220]. Treatment with anti-CA IgY Abs reduced fungal burden in tongue lesions and reduced systemic dissemination in mice with oral candidiasis. The protective potential of anti-CA IgY was attributed to its ability to block adhesion of C. albicans to host target cells [220]. In a related study [221], a C. albicans-specific chicken egg yolk Ab (IgY) was reported to inhibit the growth of both fluconazole-sensitive and fluconazole-resistant strains of C. albicans.

An IgG1 mAb (9) generated against cell wall extract preparations of A. fumigatus was reported to bind with significant affinity to surface peptides on hyphal and yeast forms of A. fumigatus and to inhibit hyphal development and reduce spore germination time. A9 was able to protect against murine invasive aspergillosis by reducing fungal burden and enhancing survival rates [222]. Growth of Fusarium species was inhibited in the presence of a fusion protein consisting of recombinant chicken-derived single chain Abs specific against Fusarium surface antigens [223]. Expression of the fusion protein in transgenic Arabidopsis thaliana plants was protective against F. oxysporum. Furthermore, administration of mAbs raised against the glucosylceramide (GlcCer) antigen N-2'-hydroxyhexadecanoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadenum of F. pedrosoi reduced fungal growth and enhanced phagocytosis and killing of fungal cells by murine macrophages [224]. G15, a human IgM mAb raised in xeno-mice transgenic for human IgM, IgG2 and Igκ chain, which recognizes epitopes on the capsular polysaccharide GXM of C. neoformans, was able to prolong the survival of D strain 24067-sensitized mice following challenge with a lethal dose of virulent C. neoformans [212]. G5, an anti-C. albicans IgA mAb identified from a hybridoma produced by fusing lymphocytes from C. albicans-immunized mouse with Sp2/O cells, exhibited potent in vitro candidacidal activity and in vivo prophylactic activity [225]. An IgM mAb (C7) raised against C. albicans–derived protein glycosyl moieties prolonged survival and enhanced macrophage opsonization in C. albicans-infected mice [225]. C7 was reported to ably inhibit C. albicans germination and adhesion to HEP2 and oral epithelial cells [227]. Passive treatment with Abs against P. carinii demonstrated partial protection against Pcp [228]. Additionally, passive treatment with anti-P. carinii IgM mAbs (4F11 and its IgG1 switch variant 4F11G1) protected SCID mice against the development of Pcp [64]. 4F11G1, which recognizes multiple epitopes on the surface of P. carinii isolates in mice, also recognizes P. carinii isolates from humans, rhesus macaques, rats, and ferrets [64, 66]. Experiments carried out on the protective capacity of 4F11G1 and its (Fab')2 derivative in an intranasal immunoprophylaxis SCID mouse model of Pcp have established that Fc-mediated functions of 4F11G1 (complement fixation in particular) are required for maximum effect of these Abs against P. carinii especially when administered passively [60].

**ANTIBODIES TARGETING CYTOPLASMIC AND SIGNALING COMPONENTS**

In addition to cell wall components, fungal cytoplasmic and signaling moieties have been used to generate therapeutic antifungal mAbs. Anti-gp70, a mAb raised against the 70KD intracellular/secreted glycoprotein component of P. brasiliensis, was able to abolish lung
granulomas in mice infected with *P. brasiliensis* [229]. The molecular chaperon HSP90 and related proteins are perhaps the most promising and extensively explored targets for development of antifungal mAbs. This is because these proteins help to maintain eukaryotic cell viability; they also represent a class of immunodominant antigens that elicit significant Ab responses in animals and humans. One of the heavily studied and talked about HSP90 variant is that which belongs to *Candida* species (the 47KD HSP90). This variant is expressed by typical pathogenic morphotypes of *Candida* species (pseudohyphae and hyphae) during mucosal and systemic candidiasis. In fact, the appearance of anti-HSP90 Abs in humans and animals with invasive candidiasis is closely associated with recovery from infection [31, 57, 69, 70, 230-232]. A human recombinant anti-HSP90 mAb called Efungumab (trade name is Mycograb®, Novartis Pharmaceuticals Corp., UK) was reported to be protective against murine *C. tropicalis* but not *C. albicans, C. krusei, C. glabrata* or *C. parapsilosis* infections [31, 230, 231, 233, 234]. In a second study using Mycograb® in combination with AMB to treat candidiasis in mice, it was reported that synergy, defined as a significant increase (FET *P* < 0.05) in the number of negative biopsy specimens compared with those obtained using AMB alone, was evident against fluconazole-resistant strains of *C. albicans* (kidney), *C. krusei* (spleen), *C. glabrata* (spleen), and *C. parapsilosis* (liver and spleen) [233]. Hence it was concluded that complete resolution of infection with *C. albicans, C. krusei*, or *C. glabrata* is achievable by combining Mycograb® with AMB. Synergy between Mycograb and AMB in human invasive candidiasis was further confirmed using lipid-associated forms of AMB [61]. Combination therapy with Mycograb® plus AMB, caspofungin, or fluconazole against 8 clinical isolates of *C. neoformans* has indicated that Mycograb® efficiently synergizes with AMB against such infections [234].

**ANTIFUNGAL ANTI-IDIOTYPES**

Use of whole or partial anti-idiotypic mAbs that represent internal images of fungal antigens or toxins to treat invasive fungal infections has been tried with some success. Several yeast genera synthesize and export proteins or glycolproteins with toxic effects (killer toxins or KTs) against sensitive yeast species. Yeast KTs recognize and bind to specific KT receptors on the surface of susceptible microorganisms and form ionic channels in the cytoplasmic membrane or inhibit DNA synthesis [235]. Thus, KT producing fungi gain competitive nutrient and niche advantages over sensitive microorganisms. The first yeast killer toxin was described in *S. cerevisiae* in 1963 [236]; this was followed by the identification of toxins produced by *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Ustilago, Pichia*, and other fungal genera. For example, KT produced by the fungus *Pichia anomala* is toxic against a wide variety of microorganisms including hyphomycetes, bacteria (*M. tuberculosis*), and important opportunistic pathogens (*C.*
Direct use of KTs as therapeutic agents against fungal infections is of little promise given their potential toxicity to host cells and the fact that they are labile (temperature and pH sensitive) proteins that remain stable inside the host for short periods of time (often few minutes). To put them to good therapeutic use therefore, a number of anti-idiotypic mAbs have been generated against fungal KTs. The rationale being that fungal species sensitive to KT activity circumvent it in the presence of anti-KT (competing Abs) or anti-KT receptors (KTR) on sensitive fungal cells (blocking Abs). Therefore, anti-idiotypic mAbs that neutralize anti-KT or anti-KTR activity release KT and/or its receptor allowing KT to become active against pathogenic fungi. An IgM anti-idiotypic mAb generated in mice immunized with the anti-KT mAb (KT4) exhibited potent killing activity against KT-sensitive C. albicans strains [238]. A single-chain recombinant anti-idiotype (KT-scFv) acting as a functional internal image of P. anomala KT was synthesized and evaluated for its antimicrobial activity using KT-susceptible C. albicans as a model pathogen [239]. A killer decapetide (KP) containing the first three amino acids of the CDR1 region in KT-scFv anti-idiotype light chain has shown strong candidacidal activity in vitro [239]. In rats with vaginal candidiasis caused by fluconazole-susceptible or -resistant strains of C. albicans, post-challenge local administration of KP resulted in rapid clearance of the infection. Administration of KP into BALB/c mice challenged with lethal intravenous doses of C. albicans significantly prolonged survival (>60 days) compared with that in control mice (3-5 days) [239]. An IgG1κ neutralizing mAb raised against the HM-1 KT (hence the name nmAb-KT) produced by yeast Williopsis saturnus var. mrakii IFO 0895 has been described [240]. This Ab binds to HM-1 at the sequence 41GSTDGK46 and reduces its killing and glucan synthase inhibitory effects. Treatment of neutropenic T cell-depleted allogeneic bone marrow transplanted-mice with aspergillosis using nmAb-KT prolonged survival, decreased pathology, and significantly inhibited A. fumigatus growth and hyphal development in lung tissues [240]. In a subsequent study by the same group [241], it was further confirmed that recombinant anti-idiotypic scFv Abs can exert antifungal growth activity by inhibiting β-1,3-glucan synthase activity. In that, a series of scFv anti-idiotypes were produced from splenocytes of mice immunized with nmAb-KT; tested fragments were able to inhibit the activity of fungal β-1,3-glucan synthase at IC₅₀ values inhibitory to Candida species growth.

ANTIBODY-BASED RADIOIMMUNOTHERAPY

Radioimmunotherapy (RIT) uses Abs labeled with radionuclides to deliver cytotoxic radiation hits to target cell. There are two essential criteria upon which the success (efficacy and safety) of RIT depends. The first pertains to the expression, on target cells, of unique antigens that can be specifically recognized and targeted by specific radiolabeled-molecular probes. Abs are favored over other types of molecular probes because of their exquisite antigen-specificity. This feature enables RIT to target intended cell populations expressing the particular antigen with minimal bystander effects. The second criterion concerns the availability of safe radionuclides that can emit tightly-measured lethal radiation hits against intended targets. Several radionuclides are currently available for targeted radiation therapy ranging in half-life from days (Yttrium ⁹⁰Y, 2.7 days; Iodine ¹³¹I, 8 days) to hours (Rhenium ¹⁸⁸Re, 16.9 hrs) to minutes (Bismuth ²¹¹Bi, 60 min; indium ¹¹¹In, 7.7 min). RIT was first
developed to treat certain forms of human cancer; the first RIT clinical trial was conducted in the mid 1980s to test the safety and efficacy of this approach against human hepatomas [242]. Since then, several RIT-based anti-cancer drugs have been developed and approved. For example, ibritumomab tiuxetan (Zevalin®), an anti-CD20 mAb conjugated to tiuxetan which chelates isotopes like 90Y or 111In, has been granted FDA approval in 2002 to treat B cell non-Hodgkin's lymphoma. Tositumomab (Bexxar®), also an anti-CD20 mAb covalently-linked to 131I, was approved by the FDA in 2003 to treat relapsed follicular lymphomas or those refractory to rituximab (rituxan®) immunotherapy.

With regard to fungal infections, numerous fungal antigens, whether shared by different fungi or unique to one species, set fungi apart from host cells thus providing for a high degree of discrimination in radiation delivery protocols. The availability of a pan-Ab that can recognize an antigen common to a whole class of pathogens is possible (e.g. 18B7 and D6 mAbs) and desirable. Desirable because it eliminates the need for the expensive and time-consuming routine of generating, radio-processing, and optimizing a wide array of pathogen-specific Abs [243, 244]. Optimal affinity of antifungal Abs as delivery vehicles of radiation is of paramount importance for RIT to be effective. By comparing the quantity of delivered radiation dose, biodistribution and killing activity of anti-C. neoformans Abs 18B7 (IgG) and 13F1 (IgM), it was evident that 18B7 was far superior to 13F1 in delivering effective radiation killing activity to lung-localized pathogens [245]. Microorganisms including fungi are susceptible to “direct-hit” and “cross-fire” radiation from a number of α and β particle emitters [244, 246]. The α-emitter 213Bi (half-life = 60 min), which delivers radiation in short bursts with short emission track in tissues, can effectively irradiate and kill small and fast-dividing bacterial pathogens (1μm in diameter, <½ hr doubling time) but not large slow-dividing fungi (≥10μm in diameter, 2-3 hr doubling time). Hence, the use of α- plus β-emitters like 188Re (half-life >16 hrs) might be a better choice to effectively and comprehensively irradiate and kill fungal pathogens [244]. Tightly-measured and highly-targeted radiation delivery is very important to minimize radiation damage to radiation-susceptible host cells. While cell-walled bacteria and fungi require several hundred to several thousand gray (Gy) to be lethally irradiated, mammalian cells can be killed by only few Gy [247].

RIT has been tried to treat experimental C. neoformans and H. capsulatum infections with some success and with only transient side effects [37, 243, 244, 248-250]. The approach developed by Dadachova and co-workers involves the use of organism-specific mAbs radiolabeled with an imaging radionuclide (e.g. 99mTc or 111In) to localize the site of infection. This is then followed by the administration of mAbs radiolabeled with particle emitters such as 188Re or 90Y to irradiate detected pathogen-infested sites [243]. While radiation activities of up to 250 μCi were well tolerated by healthy A/JCr mice for 213Bi- or 188Re-labeled 18B7 mAb therapy, radiation activities of up to 150 μCi in C. neoformans-infected counterparts produced only transient toxicity with the lungs of treated mice being free of radiation fibrosis [251]. Antifungal RIT was also shown to reduce mortality in high-burden cryptococcal infections without inducing radiation-resistant cells [252]. The mechanism of action of antifungal RIT varies depending on the radiation dose delivered, which ultimately depends on the type of radionucleotide and its delivery vehicle and how close or distant the complex docks on the surface of target cells. In general terms however, RIT involves direct killing and decreased metabolic activity of target cells. Additionally, RIT has been shown to associate with the induction of apoptosis-like cell death and the occurrence
of marked changes in the concentration of IL-2, IL-4, IL-10, TNF-α and IFN-γ suggesting some sort of cooperation between RIT and cellular immunity [253].

**ANTIBODY-BASED ANTIFUNGAL DRUGS**

Encouraging developments towards the introduction of therapeutic mAbs to treat fungal infections notwithstanding, data gaps pertinent to the efficacy and safety of the majority of experimental antifungal mAbs are still considerable with the exception of few specific examples. Efficacy of the recombinant human mAb specific for HSP90 Mycograb® (Efungumab) as an adjunctive drug in patients with culture-proven invasive mycosis has been amply demonstrated [233, 234]. A case report describing prospective treatment of fungal sepsis in a critically ill child with Mycograb® in combination with liposomal AMB or caspofungin was reported to be effective and well-tolerated [254]. Currently, the FDA lists Mycograb® as a drug with an orphan designation for the treatment of invasive candidiasis. Omalizumab (Xolair, Genetech, Novartis), a recombinant DNA-derived humanized IgG1k mAb that selectively binds to human IgE, is an approved drug to treat patients with moderate-to-severe allergic asthma that is uncontrollable with corticosteroid therapy. Recently, it was reported that treatment with anti-IgE (omalizumab) of a cystic fibrosis patient with her third exacerbation of ABPA improved pulmonary symptoms and lung function; outcomes not achievable with antibiotics or corticosteroids (prednisone) alone [255]. A phase I clinical trial that has evaluated the safety and efficacy of the anti-C. neoformans capsular polysaccharide mAb (18B7) when used as an adjunctive therapy in HIV patients with prior history of cryptococcal meningitis has yielded encouraging results [256]. In that, serum antigen titers decreased by a median of 2- and 3-fold at weeks 1 and 2 post-infusion respectively. Side effects in subjects receiving a single infusion of 1-2mg/kg body weight of the drug were minor and the serum half-life of the drug was about 53h.

**SIDE EFFECTS ASSOCIATED WITH ANTIBODY-BASED THERAPEUTICS**

The emergence of mAb therapy has been associated with a variable spectrum of adverse effects resulting from cross reactivity and immune responsiveness as well as complications associated with biological consequences of target-specific Abs. Allergy, infusion-related reactions (hypotension, rigors, fever, shortness of breath, broncho-spasm, chills, and rash), compromised immunity, and gastrointestinal- and cardiovascular-related side effects that range in severity from mild to fatal were all reported in cancer patients who have received Ab therapy [257, 258]. The jury is still out however with regard to side effects that may associate with antifungal mAbs therapy. However, few points are worth considering especially with the potential arrival of more antifungal mAbs and in light of recent developments in antifungal RIT.

Immune responsiveness to the Fc region of foreign Abs and to the Fab region of both foreign and humanized Abs has been a major obstacle in mAb immunotherapy. Approaches to deal with this problem are scarce but some have been tested and, in some instances, proven more effective and less harmful than whole Abs [259]. Chief among these is the development
of humanized mAbs or alternative Ab formats lacking the Fc region altogether. In theory, absence of Fc minimizes interspecies related immune reactivity but does little to minimize the risks of appearance of problematic anti-idiotypes within the host. Nonetheless, Ab modalities like Fabs, scFvs, dAbs, mini-bodies and multi-bodies are gaining increasing appeal to replace whole Abs. For instance, the variable sequence of an anti-idiotypic recombinant killer Ab was used to synthesize CDR-related peptides with differential \textit{in vitro} and \textit{in vivo} anti- \textit{C. albicans} activity [259]. Another set of side effects that could potentially associate with mAb therapy arise from complications related to nonimmunological activities of antibodies as they react with cell surface or cytoplasmic proteins within host cells. For instance, cancer patients and patients suffering from autoimmune and inflammatory diseases can be treated with TNF-\(\alpha\) inhibitors. However, as TNF-\(\alpha\) is an essential component of the innate immune response, blocking its activity associates with increased risk of fungal infections. A recent case of primary cutaneous cryptococcosis in a rheumatoid arthritis patient being treated with the TNF-\(\alpha\) inhibitor adalimumab in combination with methotrexate and hydroxychloroquine has been reported [260]. Furthermore, the FDA has announced on September 4th, 2008, that makers of TNF-\(\alpha\) blockers [Cimzia (certolizumab pegol), Enbrel (etanercept), Humira (adalimumab), and Remicade (infliximab)] must strengthen existing warnings on the risk of developing fungal infections as a number of patients with invasive infections due to treatment with these drugs have died.

\section*{ANTIMICROBIAL PEPTIDES}

Most kingdoms of life secret AMPs or host defense peptides as evolutionarily conserved components of the innate immune response. AMPs are potent, broad spectrum biologics with demonstrated ability to kill enveloped viruses, bacteria, mycobacteria, fungi, and transformed and cancerous cells. On average, AMPs are 12-50 amino acids in length with few positively charged residues (arginine, lysine, histidine) interspersed throughout large portions (>50\%) of hydrophobic residues. AMPs occur as \(\alpha\)-helixes, \(\beta\)-pleated sheets, linear or cyclic structures. Moderately hydrophobic AMPs with a net positive charge adopt an amphipathic arrangement with the hydrophobic and positively-charged faces occurring in opposite orientations across the plasma membrane of target cells. While the majority of AMPs target pathogens at the cell membrane or cytoplasmic levels, hence their classification here as such, some AMPs have the potential to modulate host immunity as discussed previously.

\section*{MECHANISM(S) OF ACTION}

The activity of AMPs against pathogenic fungi is dependent upon their ability to cause membrane disruption and permeabilization, disrupt and block various biochemical processes, or adversely interact with cytoplasmic and nuclear target molecules (figure 3). The ability of AMPs to differentially localize across the membrane lipid bilayer and/or translocate into the cytoplasm, are largely responsible for determining their mechanism of action against target cells. For example, using a series of cyclic peptides of variable ring size that were modeled after gramicidin S 10 (GS10), an analogue of GS, it was demonstrated that biological activity
correlates with ring size, degree of beta-structure disruption, net charge, hydrophobicity, amphipathicity, and affinity for lipid membranes [261]. Not only is the chemical composition of AMPs responsible for determining their antimicrobial activity but that of membrane lipids of target cells as well. While increasing the hydrophobicity of analogues of the 26-residue amphipathic α-helical AMP D-V13K results in dramatic decrease in antifungal activity against zygomycetes, increased hydrophobicity dramatically increases their antifungal activity against ascomycetes [262]. Additionally, increased hydrophobicity greatly enhances the hemolytic activity of D-V13K analogues against both zygomycetes (by 1569-fold) and ascomycetes (62-fold). Activity of the L-enantiomer of the winter flounder-derived AMP pleurocidin (Ple) is far more potent (up to 16-fold) against bacterial pathogens and less potent (2-fold) against fungi as compared with that of the D-enantiomer [263]. This is possibly due to the fact that while the L-enantiomer is potent at compromising the integrity of negatively charged liposomes (mimics of bacterial membranes), the D-enantiomer is more potent at causing leakage effects in positively charged liposomes (mimics of fungal membranes). Moreover, the capacity of AMPs to translocate into the cytoplasm (via receptor-mediated or zipper type phagocytosis or other internalization mechanisms) and the presence/absence of target molecules to which translocated AMPs can bind with, endows them with the unique ability to alter select biochemical or metabolic processes inside target cells.

Studies that have investigated the influence of structural modifications on the activity of AMPs have also shed some light on the mechanism(s) of action of AMPs and the factors influencing these mechanisms. P-113 peptide and the Trp-rich peptide Ac-KWRRWVRWI-NH(2) have strong antifungal activity against yeast pathogens only at low-salt concentration. However, antifungal activity of the modified Pac-525 peptide (D-Nal-Pac-525) was retained in media with low or high salt concentration [264]. End-tagging of the kininogen-derived peptides GKHKNKGKKNGKHNGWK (GKH17) and HKHGHGHGKHKNKGKKK (HKH17) with hydrophobic oligopeptide stretches enhanced their C. albicans killing activity [265]. Increased tag length and the use of large Trp and Phe (but not aliphatic) amino acid stretches resulted in enhanced cytocidal activity, better peptide binding to artificial phospholipid membranes, and increased capacity to rupture anionic and cholesterol-void liposomes. Tagging was also shown to render the peptides less toxic and more resistant to degradation by human leukocyte elastase, staphylococcal aureolysin and V8 proteinase [265]. Furthermore, decreasing hydrophobicity and/or increasing cationicity of Ple by Arg or Ser amino acid substitutions at the hydrophobic face did not affect antifungal activity but decreased hemolytic activity of Ple [266]. In its native form, the peptide trappin-2 (P2 or pre-elafin) is an endogenous serine protease inhibitor with potent antimicrobial activity against several microbial and fungal pathogens. Similar antimicrobial activity is achievable with trappin-2 A62D/M63L, a trappin-2 variants lacking antiprotease properties, indicating that trappin-2 exerts its antimicrobial activity through mechanisms independent of its intrinsic antiprotease potential [267].

**Natural vs. Synthetic AMPs**

Natural AMPs occur in bacteria (iturin, bacillomycin, syringotoxins, cepacidines), fungi (echinocandins), insects (cecropins A and B, drosomycin, dermaseptin) and plants (zeamatin,
the cyclopeptide alkaloids amphibe H, frangufoline, nummularine) [268]. The mammalian innate immune system also secretes numerous classes of potent AMPs including those of human origin (histatins, α- and β-defensins, HNP peptides, gallinacin, cathelicidin cationic peptides [hCAP-18], LL-37, protegrins, dermcidin), mouse origin (CRAMP), rabbit origin (CAP18), pig origin (protegrins and prophenin), and cattle origin (indolicidin) [207, 268, 269]. Of the hundreds of natural AMPs discovered so far, some 150 peptides have antifungal activity; hence the term antifungal peptides (AFPs) [5, 20]. Furthermore, the list of synthetic and semisynthetic peptides that have been modeled according to naturally occurring ones is already extensive. The recombinant defensin Tfgd1 synthesized from a cDNA cloned from *Trigonella foenum-graecum* was reported to have broad spectrum antifungal activity [270]. D4E1, a synthetic peptide, is active against *Aspergillus* with the 50% lethal dose (LD₅₀) being achieved at a concentration of 2.1-16.8μg/ml [268]. Treatment with the Paracoccidioid gp43 antigen-derived peptide P10 in combination with AMB, fluconazole, ketoconazole, or itraconazole at 2-30 days following intratracheal challenge of adult mice with virulent isolates of *P. brasiliences* resulted in significant additive protective effects [271]. Based on these findings, it was suggested that this treatment protocol can improve the outcome of chemotherapy, shorten its duration, and bring down the high rates of relapse of *P. brasiliences* infections [268]. The synthetic peptide PLD-118 (BAY 10-8888) has demonstrated a dose-dependent antifungal activity in treating fluconazole-resistant oropharyngeal and esophageal candidiasis [272]. Although the spectrum of activity of intravenous caspofungin (one of several derivatives of the semisynthetic lipopeptide pneumocandin; collective name is echinocandins) is focused on *Candida* species (*C. albicans* and *C. glabrata* but not *C. parapsilosis*), other fungi like *Saccharomyces* species, *P. carinii* and *Aspergillus* species are also susceptible to caspofungin activity (table 1) [273, 274]. FDA approval of caspofungin, micafungin, and anidulafungin in the last decade is proof of the promise of synthetic AMPs (or AFPs) in fighting fungal infections.

**AMPS TARGETING FUNGAL CELL MEMBRANE**

The majority of AMPs kill fungi by membrane permeabilization and pore formation, which compromise membrane integrity and cause cytoplasmic leakage and cell lysis. Minimum inhibitory concentration (MIC) values of the halocidin synthetic peptide analogue di-K19Hc against clinical isolates of *C. albicans* and *Aspergillus* species are 8μg/ml respectively [275]. The peptide rapidly (<30s) kills *C. albicans* by binding to cell wall β-(1,3)-glucan with the subsequent formation of ion channels. Fungicidal activity of the synthetic lactoferrin-derived peptide (Lfpep) and the kaliocin-1 peptide against fluconazole- and AMB-resistant *C. albicans* strains is dependent upon their strong membrane-permeabilization potential [276]. KKVVFKVKFKK is a membrane-active peptide (MP) that inhibits the growth of fluconazole-resistant strains of *C. albicans* at concentrations in the range of 2-32μg/ml [277]. Synthetic analogues of the bactericidal domain of the cationic antimicrobial polypeptide CAP37 (19NQGRHFCGGALIHARFVMTAASCFQ45) has strong fungicidal activity against fluconazole-sensitive and -resistant isolates of *C. albicans* blastoconidia, *C. guilliermondii*, *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis*, and *C. dubliniensis* [278]. The chicken egg white-derived, affinity-purified peptide cystatin (CEWC)
was able to inhibit the growth of azole-sensitive *C. albicans*, *C. parapsilosis* and *C. tropicalis* (but not *C. glabrata*) isolates at MIC values comparable with those of fluconazole or the human saliva-derived AMP histatin 5 (0.8-3.3 μmol/L) [156]. Cystatin was also fungicidal to azole-resistant *C. albicans* isolates overproducing the multidrug efflux transporters Cdr1p and Cdr2p [156].

![Figure 3. Targets and consequences of AMPs with antifungal activity. AMPs target both the pathogen, at the plasma membrane and cytoplasm/nuclear levels, and the host. Depending on the target, which is dictated by peptide molecular composition as well as its cationicity and amphipathicity, the consequences of AMP activities can halt pathogen growth and activity, cause cell lysis and death, or modulate the immune response to better deal with fungal pathogens.](image3)

The membrane binding, *Hevea brasiliensis*-derived small (4.7KD) cysteine-rich protein hevein was able to inhibit the growth of *C. albicans*, *C. tropicalis* and *C. krusei* at MIC values of 95, 12 and 190 μg/ml respectively [279]. Growth inhibition potential of hevein against such oral pathogens was also demonstrated by the disk diffusion method. At a concentration of 30μg/ml, hevein was able to cause a Ca²⁺-dependent aggregation of *C. tropicalis* yeast cells [279]. The histidine-rich glycoprotein (HRG), an abundant and multimodular plasma protein, binds *Candida* cells and induces breaks in the cell wall rather than the cell membrane. This activity was demonstrated by the finding that HRG lysed ergosterol- but not cholesterol-containing liposomes [280]. The ultra-short antimicrobial lipopeptide palmitoyl-lys-ala-DAla-lys was shown to exert detergent-like effects and membranolytic activity against *A. fumigatus in vitro* [281]. *In vivo* studies involving intranasal administration of DsRed-labeled *A. fumigatus* conidia into immunosuppressed C57BL/6 wild-type mice followed by lipopeptide treatment have demonstrated the potential of the lipopeptide to degrade and clear hyphal forms and residual conidia in infected lungs. Ple, exhibits strong antifungal activity by disrupting the plasma membrane of target fungal cells [266]. A 13-mer synthetic peptide variant (pEM-2) derived from myotoxin II, a
phospholipase A(2) homologue present in Bothrops asper snake venom, was shown to exert potent fungicidal activity against pathogenic Candida species [282]. Membrane-permeabilization by pEM-2 resulted in 100% killing at a 5µM concentration. The fungicidal effects of piscidin 2 (P2), a striped bass mast cell-derived 22-residue cationic member of the piscidin family of peptides, against human pathogenic fungi was shown to be dependent upon its capacity to cause massive membrane damage and pore formation [283]. Histatins represent a unique family (at least 12 members) of small, cationic, histidine-rich peptides secreted in human saliva by the parotid and submandibular–sublingual glands [284, 285]. Different members of the histatin family can act on fungal plasma membranes or fungal cytoplasmic proteins. Histatins 1 (38 amino acids), 3 (32 amino acids), and 5 (24 amino acids), participate in various biological processes like the formation of enamel pellicle of the teeth, induction of histamine release, inhibition of hemagglutination, inhibition of protease activity and neutralization of lipopolysaccharide 284, 285]. These Histatins exhibit fungicidal activity against several Candida species (C. albicans, C. glabrata and C. krusei), S. cerevisiae, C. neoformans and A. fumigatus. They kill or inhibit Candida species at near physiological concentrations (15-30µM).

**AMPS TARGETING THE CYTOPLASM**

While the majority of AMPs kill fungi by compromising the integrity of the plasma membrane, some can inhibit the growth of fungi by interacting with cytoplasmic or nuclear targets to disrupt various intracellular processes [268]. For example, the weak amphipathic structure of histatin-5 enables the peptide to be internalized into the cytoplasm of C. albicans cells through receptor-mediated endocytosis or through a translocation transport system in a non-lytic manner. Histatin 5 targets the mitochondria and initiates the loss of transmembrane potential; therefore, inhibition of mitochondrial ATP synthesis protects C. albicans against the fungicidal activity of histatin 5 mainly due to reduced accumulation of the peptide [284, 285]. Once internalized, histatin-5 exerts potent candidacidal activities on intracellular targets [286] by, for example, forming free oxygen radicals [287]. In vitro studies of the antifungal activity of a cationic peptide derived from the N-terminal portion of human mucin MUC7 peptide (MUC7) against pathogenic C. albicans strains has been associated with the upregulation of several glycolytic enzymes within target cells [288]. Trappin-2, an endogenous serine protease inhibitor in neutrophils that controls excess proteolysis during inflammation, exhibits potent antifungal activity against A. fumigatus and C. albicans at a 5-20µM concentration range [267].

**SELECTIVE TOXICITY OF AMPs**

The majority of naturally occurring and synthetic AMPs are nonselective; they are toxic to pathogen and host cells alike. However, two aspects of AMP activity are worth considering in this context. First, AMP toxicity is strongly pronounced when used across species; AMPs derived from the same species are much less toxic to host cells. In the case of humans, a large number of human-derived AMPs (histatins, defensins, cathelicidin, etc.) have highly potent
antifungal activity against a whole host of fungal pathogens. Therefore, use of human-derived AMPs is one of the possible means by which AMP toxicity can be avoided or at least minimized. Second, some AMPs, irrespective of the source species, have specific antifungal selective toxicity owing to factors like host vs. pathogen membrane structural variations and the availability (or lack of it thereof) of molecular targets that enable AMPs to mediate their action against specific target cells. For example, IC₅₀ of the MP AMP for NIH 3T3 and Jurkat cells is about 100-fold higher than the MIC for C. albicans ATCC strain 36232. This suggests that MP is selectively active against fungus but not host cells. Additionally, synthetic non-amphipathic cationic AMPs (CAPs) have been shown to possess selective fungicidal activity against fluconazole-resistant strains of C. albicans, C. tropicalis and C. glabrata in the µM range [289]. The fact that histatins selectively bind to C. albicans but not to mammalian cell membranes enables them to express selective candidacidal activity with minimal or no toxic side effects. Substitutions of amino acids that increase the hydrophobicity of cationic AMPs tend to increase antifungal but not anti-host activity [290]. The antifungal activities of the human lactoferrin (hLF)-derived peptide hLF(1-11) and the histatin 5 peptide analogue dhvar5 are comparable to that of AMB against A. fumigatus hyphae and conidia [291]. However, whereas hLF(1-11) does not lyse human erythrocytes, dhvar5 at concentrations ≥16 µM is hemolytic.

**IMMUNOSUPPRESSIVE THERAPY AND FUNGAL INFECTIONS**

In theory, immunosuppression is very undesirable as it exacerbates existing infections and increases the susceptibility to opportunistic infections. That unless the action of immunosuppressive drugs against host cells can be replicated in fungal pathogen cells. Immunosuppression can also be of value to counter exuberant inflammatory responses during the course of invasive fungal infections.

Maintenance therapy in transplant patients consists of various regimens of calcineurin inhibitors (CIs) that block T cell activation and inhibit cytokine production (FK506 and CsA), inhibitors of nucleotide synthesis (azathioprine, and mycophenolate mofetil), and inhibitors of intracellular signal transduction (the anti-IL-2 signal blocking and mTOR-targeting agents sirolimus and everolimus). The capacity of certain immunosuppressive agents to interfere with the cellular signaling apparatus of eukaryotic cells has already been explored and utilized as potential antifungal therapy. Calcineurin is a Ca²⁺/calmodulin-dependent serine/threonine-specific phosphatase that plays a central role in T-cell activation and IL-12 production [14-16]. The macrolide tacrolimus (FK506) and cyclosporine A (CsA) are the two main CIs, hence their wide use to treat solid organ transplant patients. FK506 and CsA can bind with cyclophilin A and the cytosolic protein FK-binding protein 12 (FKBP12), thereby producing drug-protein complexes that inhibit calcineurin activity [17, 18]. Consequently, T cell proliferation, IL-12 production, and ultimately T cell-mediated immunity are inhibited. CIs have been reported to inhibit calcineurin function in the three main human fungal pathogens (C. albicans, C. neoformans, and A. fumigatus) [9-12]. Inhibition of calcineurin activity in C. albicans cells results in enhanced susceptibility to azoles, cationic (Ca²⁺, Na⁺, and Li⁺) stress, decreased survival in serum and avirulence in systemic murine candidiasis [10, 12]. Further antifungal potential of FK506 and CsA derives from their capacity to
interrupt calcineurin-dependent biosynthesis of fungal cell wall 1,3-β-D-glucan contents especially in *A. fumigatus* [292]. This aspect of CIs antifungal activity can enhance the antifungal activity of echinocandins (caspofungin), which block the activity of 1,3-β-D glucan synthase and inhibit 1,3-β-D-glucan synthesis. Therefore, CIs have been used in the past as adjunctive therapies in combination with conventional antifungals [13, 293, 294]. Synergism between CIs and fluconazole is attributed to the ability of CIs to enhance the fungicidal activity of fungistatic azoles. Furthermore, CIs are known to enhance fluconazole-mediated inhibition of ergosterol biosynthesis causing massive fungal cell membrane perturbations, hence increasing their own intracellular influx and concentration. FK506 inhibitory effects on *C. neoformans* is temperature-dependent; in that *C. neoformans* is resistant to FK506 antifungal activity at 24 °C but not at 37 °C [295]. Although *C. albicans* biofilms are resistant to FK506, CsA or fluconazole in separation, the pathogen is very sensitive to combinations of fluconazole and FK506 or CsA [13]. While *C. albicans* strains lacking FKBP12 or those expressing a dominant FK506-resistant calcineurin mutant subunit (Cnb1-1) are resistant to FK506-fluconazole combinations, they are susceptible to CsA-fluconazole combinations. Lastly, azole-CI combinations have found use as salvage therapies in cases of difficult-to-treat or unresponsive invasive fungal infections [293, 296].

HSP90, the chaperone of molecular chaperones, can influence the path of species evolution by unleashing previously silent genetic variations in response to environmental changes. The remarkable benefits conferred by HSP90 on fungal cells are well manifested in its role of potentiating the evolution of drug resistance through coupling new mutations with immediate phenotypic consequences. The exquisite antifungal activity of HSP90 inhibitors plays on the theme of abrogating the evolution of resistant fungi [155] in response to chemotherapy. The naturally occurring HSP90 inhibitor radicicol (monorden), an inhibitor of Ca²⁺-signal-dependent cell-cycle regulation in yeast, was reported to minimize the deleterious effects of external CaCl₂ influx in *S. cerevisiae* by inducing the expression of constitutive active calcineurin [297]. Whether treatment with radicicol could restore calcineurin activity in patients on FK506 or CsA immunosuppressive therapy is yet to be full addressed.

Despite their desirable immunosuppressive and antifungal activities, treatment with CIs and other biological inhibitors has its limitations. Two such limitations are of interest in the context of this discussion. The fist involves the potential risk of adverse drug-drug interactions between biological inhibitors and azoles (fluconazole in particular) due to potent inhibitory effects of azoles on the mitochondrial P450 enzyme system [298, 299]. The second involves immunosuppression-related increased susceptibility to opportunistic infections [300-303]. Resistant strains of *C. neoformans* have been isolated from patients with solid organ transplants [300]. FK506 can penetrate the CNS and exacerbates cryptococcal meningitis in rabbits [295]. The nonimmunosuppressive FK506 analogue L-685,818 is also toxic to *C. neoformans in vitro* at 37 °C and is active against FK506-sensitive, but not FK506-resistant, strains of *C. neoformans* [295]. Further work is still needed to establish the full range of antifungal activity of this analogue against invasive and systemic fungal infections. Unlike tacrolimus, the immunosuppressant sirolimus (rapamycin or rapamune), which was first developed as an antifungal drug, inhibits the response to IL-2 instead of calcineurin, thereby blocking activation of T and B cells. Sirolimus binds with FKBP12 and the sirolimus/FKBP12 complex inhibits the mTOR pathway. Since its introduction, sirolimus and related derivatives (everolimus) has proven less likely to render patients susceptible to opportunistic infections [304, 305].
MISCELLANEOUS AGENTS WITH ANTIFUNGAL IMMUNOMODULATORY ACTIVITIES

Wide spectrum antifungal potential has been associated with a diverse set of miscellaneous drugs, biological inhibitors, probiotics, biologics, antineoplastics and the likes. Although such promising agents have a long way to go before delivering clinically valuable drugs, they are becoming part of an ever increasing number of agents amenable to rigorous screening and testing as antifungals. The diverse means by which different agents kill fungal pathogens or inhibit their growth is telling of the potential vulnerabilities of pathogenic fungi. Although many such agents directly target fungal pathogens, some have immunomodulatory potential, making them the more interesting. It is hard to classify these agents into any set of categories given their diverse origins, structures, mechanisms of action and so on. Therefore, the brief discussion to follow is meant to, instead of exhaustively surveying the long list of miscellaneous antifungal agents, make the point that despite our deep understanding of fungal biology and immunity, our understanding of the vulnerabilities or points of attack against pathogenic fungi still falls short of complete.

DRUGS AND BIOLOGICAL INHIBITORS

Deferasirox, an FDA-approved oral iron chelator, is used to reduce chronic iron overload in patients on long-term blood transfusion therapy for anemia. Administration of deferasirox into diabetic ketoacidotic or neutropenic mice with mucormycosis results in improved survival and decreased tissue fungal burden at levels comparable to that of LAMB [306]. Treatment with deferasirox in combination with LAMB significantly enhanced treatment outcome as compared with treatment with either agent alone [306]. Intraperitoneal administration of daucosterol (a beta-sitosterol glycoside) before intravenous challenge with viable C. albicans cells induced Th2 protective immunity, prolonged survival and partially cleared kidney fungal load in mice with disseminated candidiasis [307]. Pentraxin 3 (PTX3), a member of the long pentraxins subgroup of the pentraxin family, is an essential component of innate immunity against pulmonary aspergillosis [308]. PTX3 was reported to modulate cytokine production and enhance complement responses to pulmonary aspergillosis. When administered intranasally or parenterally before, during or after intranasal inoculation with A. fumigatus conidia, PTX3 can render mice transplanted with allogeneic bone marrow resistant to aspergillosis at levels equal or superior to that induced by AMB or LAMB [308]. Co-administration of PTX3 with suboptimal doses of either antifungal potentiates therapeutic efficacy associated with accelerated recovery of lung phagocytes and Th cells. Indinavir (IDV), conventionally used as an antiretroviral protease inhibitor (PI), was shown to induce the expansion of splenic CD8α+ DCs in hosts infected with C. neoformans [306]. Upregulated expression of costimulatory molecules (CD40 and CD80) and proinflammatory cytokines (IL-12) was also noted on splenic CD11c+ DCs in mice treated with IDV. The capacity of IDV to modulate the immune response and enhance T cell activity through its effects on splenic DCs is interesting [309].
As mentioned previously, immunosuppressants and signaling or metabolic inhibitors also exhibit wide spectrum antimycotic activities. The topoisomerase I inhibitor camptothecin plus some of its derivatives [310] and the irreversible suicide inhibitor of ornithine decarboxylase eflornithine [311] have strong anti-\textit{C. neoformans} activity. The sodium channel blocker amiodarone, conventionally used to treat arrhythmia, has potent anti-\textit{S. cerevisiae} activity [312, 313]. Its effects are particularly pronounced in yeast mutants lacking key calcium transporter proteins as unreplenished internal Ca^{2+} stores are depleted in a gradual but sustained manner [314]. Combination therapy with miconazole or fluconazole plus low doses of amiodarone greatly (>90%) reduces survival rates of \textit{S. cerevisiae} and induces significant (>90%) in vitro killing of \textit{C. albicans} and \textit{C. neoformans} respectively [315].

**PROBIOTIC THERAPY**

Probiotic treatment involving the administration of living microorganisms in adequate quantities has been suspected of conferring some health benefits on the host since the days of \textit{Eli Metchnikoff}. Lactic acid bacteria (LAB), bifidobacteria and some yeast species are currently used as probiotics against a wide array of infections and inflammatory diseases. Specific examples include \textit{Lactobacilli} (\textit{L. acidophilus, L. brevis, L. bulgaricus, L. casei, L. gasseri, L. paracasei, L. plantarum, L. rhamnosus, L. salivarius}, and \textit{L. lactis}), \textit{Streptococci} (\textit{S. thermophilus}), \textit{Bifidobacteria} (\textit{B. bifidum, B. breve, B. infantis, B. lactis, and B. longum}), and some yeasts (\textit{S. cerevisiae}). Probiotics are thought to benefit the host by improving microbial balance that inhibits pathogens and toxin-producing bacteria, alleviate chronic intestinal inflammatory diseases, prevent and treat pathogen-induced diarrhea, urogenital infections, and atopic diseases [316]. A study that has reviewed probiotic therapy literature published between 1966-1995 [317] has concluded that placebo-controlled studies have established that biotherapy can successfully prevent and/or treat antibiotic-associated diarrhea (\textit{L. casei GG, B. longum with L. acidophilus, and S. boulardii}), acute infantile diarrhea (\textit{B. bifidum with S. thermophilus}), recurrent \textit{Clostridium difficile} disease (\textit{S. boulardii}), and various other diarrheal illnesses (\textit{Enterococcus faecium} SF68, \textit{L casei GG}, and \textit{S boulardii}). The study also concluded that there exists ample evidence to support the contention that \textit{L. acidophilus} probiotic therapy is effective at preventing vaginal candidiasis. Criticisms of probiotic therapy have focused on sample size and degree of information masking in clinical trials, significant patient to patient benefit variations, cost effectiveness, and most importantly the generality of efficacy-gauging parameters employed [317]. For example, several studies and reviews have concluded that probiotic therapy can modulate anti-infection immunity [318-321]. In one study which used specific immune parameters however [322], out of 19 different biomarkers tested in subjects under academic examination stress, intake of milk fermented with yogurt cultures plus \textit{L. casei} DN-114001 was only able to modulate the number of lymphocytes and that of CD56^+ (mostly NK) cells. Therefore, the ability of probiotic therapy to modulate immunity under stress conditions (infections or otherwise) is in doubt to put it mildly. Additionally, safety concerns especially in the immunocompromised and/or critically ill are yet to be resolved despite the claims that probiotic therapy is safe when given in oral [323] or topical forms [317]. For example, cases of \textit{lactobacillus} septicemia and allergic reactions in immunocompromised hosts have been reported [321, 324].
With regard to fungal infections, the majority of basic and clinical studies have focused on the potential of oral and topical use of different combinations of probiotics to prevent or reverse the dominance of pathogenic Candida species in the gastrointestinal and urogenital tracts [317, 325-329]. Probiotic therapy using a combination of L. rhamnosus GR-1 and L. reuteri RC-14 has proven effective at restoring and maintaining a normal vaginal microbiota through modulating host immunity, reducing pathogen ascension from the rectum, and interfering with colonization and survival of pathogens [318, 319]. Topical treatment with Lactobacillus–rich yogurt was shown to significantly reduce vaginal fungal burden in mice with estrogen-dependent vaginal candidiasis (EDVC) [327]. Furthermore, metronidazole-treated naïve mice developed persistent C. albicans vaginal colonization at levels significantly lower than that in untreated or metronidazole-treated EDVC mice suggesting that antibiotic therapy minimizes the benefits of probiotic therapy. A pilot, randomized, double-blind, placebo-controlled trial in human preterm neonates has demonstrated that L. casei GG administered in the first month of life significantly reduces enteric Candida colonization [326, 330]. During a 16-week, randomized, double-blind, placebo-controlled study with 136 elderly people consuming daily doses of 50g probiotic dairy products and 140 counterparts consuming daily doses of 50g cheese, the prevalence of Candida counts (≥10 CFU/ml) decreased in the probiotic group from 30% to 21% and increased in the control group from 28% to 34% [326, 330]. Probiotic intervention reduced the risk of high yeast counts by 75% and the risk of hyposalivation by 56%. The incidence of systemic candidiasis was significantly reduced and rates of survival were significantly improved in adult and neonatal athymic bg/bg-nu/nu mice by the presence of probiotic bacteria (L. acidophilus, L. reuteri, L. casei GG, or B. animalis) in the gastrointestinal tract [320]. Although no complete prevention of mucosal candidiasis was achieved, B. animalis was able to reduce incidence and severity of the disease. Furthermore, these probiotics were shown to modulate Ab- and cell-mediated immune responses to C. albicans [320].

**SYNTHESIS**

While the list of pathogen- and host-targeting immune-based therapies is extensive, stories of success are few and far in between. Lack of progress despite massive efforts begs the question why. Several technical and nontechnical problems continue to hinder progress in this area. On the technical side, several issues come together to complicate the utility of immune-based antifungal therapy. To name some: (i) serious fungal infections are primarily secondary to underlying diseases. (ii) Fungal infections tend to elicit cellular rather than humoral immune responses. (iii) The risk of blunting immunity by vaccination or other forms of immunotherapy in the immunocompromised is high. (iv) The risk of vaccine-induced pathogen strain replacement is real. (v) Universalism versus species-specific vaccine activity is a “muddling” issue that requires further exploration. (vi) Fungal pathogens, owing to their eukaryotism, sophisticated genetic makeup, and ability to switch form endows them with, a yet to be fully appreciated, potential to evade immunity and resist the effects of immunotherapy. (vii) Toxic side effects that may associate with, some if not all, immune-based therapies have yet to be addressed in any meaningful way.
All in all, the search for fungal vaccines and immunotherapies seems to be going through a repetitive cycle. Published literature on the subject has focused, for the most part, on the same organisms, same protocols, same variations (type of adjuvant, type of animal model, route of administration, chemotherapeutic combination to use, etc.), and same means of efficacy evaluation. Focusing on the same set of questions for so long has left other important questions unaddressed and unanswered. This, it seems, is beginning to deplete the field of new concepts and new ideas. As for the nontechnical issues, they pertain to some of the practices of concerned regulatory agencies, funding, and most importantly capital investment.

**Technical Issues to Consider**

Based on the classical definition, the goal of vaccination is to elicit pathogen-specific immune responses with lasting immunologic memory capable of protecting the host against subsequent infections by the same pathogen. Potential application of cross-kingdom vaccines [129, 133, 134] may expanded the goal of vaccination to target scores rather than single pathogenic species. In fact, experimentation with vaccines protective against antigenically- or immunogenically-related fungal pathogens is becoming increasingly fashionable [88, 95, 131-135]. Furthermore, the goal of vaccination by modern-era vaccines, especially those against certain forms of cancer and fungal infections, is being expanded to encompass treatment in addition to or in place of prevention. This does not necessarily come always by design; it is often the case that the efficacy of experimental vaccines is carried out in already infected animals and is evaluated by measuring its capacity to clear up existing infections [120-124]. Collectively, attempts to develop fungal vaccines with far reaching goals in terms of target and/or scope, vis-à-vis prevention and treatment, apply to a significant portion of published work on the subject. As to whether expanding the goal and/or scope of vaccination has helped or hindered fungal vaccine development is debatable. For one thing though, unlike viruses and bacteria, fungi are unique in their capacity to elicit diverse pathogen-specific immune response profiles. Granted that immune response profiles are similar, subtle differences may produce significantly different outcomes. Subtleties in the modes, mechanisms, and factors of virulence utilized by different pathogenic fungi bear heavily on the overall kinetics of pathogen-specific immune responses [19].

According to the classical definition of vaccines, immunocompetence of the host prior to, during, and after vaccination is a prerequisite for the exercise to be of benefit to the host. As noted previously, common opportunistic fungal infections are secondary to immune incompetence precipitated by underlying disease states. In other words, those at risk of contracting opportunistic infections hardly meet the immunocompetence requirement. The question hence becomes, if a yet-to-be-developed fungal vaccine is to succeed in eliciting protective log-lasting immunological memory in the healthy, will those at risk benefit from it? The collective failure to develop fungal vaccines that work in the healthy may partially explain why this question is rarely raised [5]. There exist strategies to design vaccines that could stimulate effective immune responses in immunocompromised hosts [95, 129, 142] but fears of blunting the immune response in high-risk patients persist. The next question then is how to overcome the risk of overwhelming the immune response in the immunocompromised? The capacity of algal beta-glucan vaccine (laminarin) to induce the
production of protective Abs that kill or inhibit the growth of fungal cells is interesting as it invites further work into the potential of passively immunizing immunocompromised hosts against fungal infections.

Current trends in fungal epidemiology and fungal vaccine research suggest that the number of clinical vaccine studies will dramatically increase. The role of Ab immunity in fighting and clearing fungal infections notwithstanding, most fungal infections and antigens tend to elicit cellular immune responses. Therefore, classical vaccination approaches that conventionally target humoral immunity might be of limited efficacy. Work on vaccination strategies that could effectively target CMI is still lacking and exact parameters of what constitutes vaccines that target CMI are yet to be fully established? Manipulating the molecular structure of putative vaccines [99, 100, 142], the use of immunomodulating DCs as fungal antigen delivery systems that could enlist adaptive immunity [102, 104, 107, 108], and the inclusion of immunostimulatory adjuvants [85, 91-96, 125] hold some promise in this regard.

In evolutionary terms, development of pathogenic resistance due to chemotherapy can be viewed as a response to selective pressures exerted by drugs. In this case, some cells of the target pathogen undergo genetic changes that enable them and their progeny to survive at much higher doses of the drug (less susceptible) or to become totally resistant to the action of the drug. Cells exhibiting this ability quickly replace the more drug-susceptible ones, which leads to the appearance of resistant strains. In real life, development of resistance to bacterial and fungal pathogens due to chemotherapy is common knowledge. It is now recognized that host immunity (and possibly immunomodulation) imposes selection pressures on pathogens similar (though not necessarily identical) to those imposed by drugs [151]. By definition, vaccination modifies the pattern and pace of selection by protecting the host against specific strains of an organism at the risk of opening the door for other strains to emerge as dominant pathogenic strains, a phenomenon known as vaccine-induced pathogen strain replacement [151]. Vaccination-related induction of viral and bacterial, but not fungal, resistance has been documented [152-154]. In comparative terms, while chemotherapy induces resistance by directly acting on and manipulating the genetic makeup of the pathogen, immunity induces pathogen strain replacement (roughly equivalent to resistance by effect) by acting on a population of cells of a heterogeneous genetic mix and selecting for cells endowed with the ability to endure or evade the immune response (figure 4). Like in chemotherapy, the pace and scope of immunity-driven dominant pathogen strain replacement should, in theory, be proportional to the genetic complexity of the pathogen. This is well manifested in the immense capacity of fungi to develop resistance to chemotherapy [118, 155-157]. It is therefore possible that fungi are more likely than not to develop robust resistance to immunomodulation given their sophisticated genetic makeup and their proven potency at devising immunoevasive and resistance to therapy strategies [reviewed in 158]. Currently, whatever role host immunity plays in initiating or accelerating the induction of resistance or pathogen strain replacement is yet to be fully addressed. Furthermore, the role of vaccines and other immunemodulatory agents in inducing fungal resistance or its equivalents by effect is beyond any meaningful evaluation at this point.

The majority of studies (preliminary and subsequent) conducted to test the therapeutic efficacy of experimental antifungal immunotherapies have focused on the antifungal activity of a specific modality (pathogen- or host-targeting) at a time. By doing so and without taking into account the overwhelming complexity of fungal infections and the intricacies
precipitated by fungal pathogenesis on host immunocompetence, this approach may have made poorer the whole antifungal immunotherapy enterprise.

Figure 4. Drug-induced microbial resistance vs. vaccine-induced pathogen strain replacement. The model proposed here is an attempt to differentiate the general mechanism involved in pathogen-targeting therapy-induced resistance from the one involved in host-targeting immunomodulation-induced pathogen strain replacement. Although both phenomena result in resistance, mechanistic differences may entail variable rates of resistance, temporal differences in its appearance, and possibly variations in pathogen vulnerability to these two distinct processes.

Moreover, studies that have considered the efficacy of combinatorial regimens have addressed specific immunotherapeutic modalities in combination with conventional chemotherapy. This was based on the rationale that adjunctive chemo/immunotherapy could capitalize on the advantages of both approaches, vis-à-vis reducing the scope of chemotherapy (time and dose wise) by complementing it with immunotherapy. However, such an approach, while more promising than the “solo chemo- or immunotherapy” approach, is bound to add an additional layer of complexity by bringing to bear the disadvantages and shortcomings of chemotherapy. For instance, given that immunotherapy and chemotherapy have the potential to cause side effects and induce resistance it becomes extremely difficult to sort out the contribution(s) of each therapy to these problems when both therapies are used in combination. Worse yet, while positive synergism has been reported with regard to chemo/immunotherapy combinations [13, 31, 84, 120, 170, 171, 190, 191, 196, 231, 233, 234, 254], little is known about negative synergism. In other words, issues pertinent to the cumulative effects of chemotherapy and immunotherapy on the induction of resistance and precipitation of side effects are have not been addressed.
On the other hand, the eukaryotic nature of fungi, the significant biochemical and metabolic similarities they share with host cells and their sophisticated genetic makeup make them hard-to-deal-with pathogens. Fungi are endowed with significant potential to evade immunity, resort to resistance, and cause severe inflammatory reactions. Therefore, it is highly unlikely for single-modality therapies (chemical or otherwise) to do the trick. Evolution seems to have come to this conclusion a long time ago; else why the multilayered, multifaceted, and complex antifungal immune response. This realization may have been responsible, in part, for the appearance of drug-drug or drug-immunotherapy combination treatment approaches. Surprisingly, little effort has been directed at evaluating the therapeutic potential of combinations consisting of various pathogen- and host-targeting modalities (figure 5). In fact, insofar as one can tell by reviewing the literature, this approach has yet to be adopted. This although parallels between fungal immunity and immunotherapeutic approaches that combine pathogen- and host-targeting modalities are discernable. For without stating the obvious, fungal immunity involves such a diverse set of innate and adaptive components that modulate immunity (regulatory and helper responses) and target the pathogen (effector responses) (figure 1). Therefore, adopting pathogen- plus host-targeting combination treatment approaches can mimic the immune response if only as a distant approximation.

Figure 5. Benefits of pathogen- plus host-targeting immunotherapy combinations. While pathogen targeting therapies directly act on the pathogen, host-targeting therapies modulate the immune response of the host to better deal with the pathogen. Although pathogen-targeting therapy applies to both chemotherapy and immunotherapies, it is combinations involving pathogen and host targeting immune-based therapies that are yet to be tested.

Focusing on pathogen- and host-targeting components as complementary to one another rather than to chemotherapy [20] could serve as a new source for the development of new antifungal immunotherapies. It must be emphasized that, while this approach may in theory minimize problems associated with chemotherapy or those precipitated by classical combination therapies, it is by no means a guaranteed solution. Only diligent experimental work will be the true judge.

**Nonotechnical Issues**
Lack of capital investment in developing, testing, and translating bench findings into bedside immunotherapeutics is a major hurdle. Following requests submitted by some drug manufacturers, the FDA has designated a number of antifungal drugs as orphan drugs (OD) (table 3). Drug developers and manufacturers often brush away the so called orphan diseases, those affecting fewer than 5 individuals per million, as commercially non-feasible areas for capital investment and drug development. Therefore, having a considerable percentage of fungal diseases receiving the orphan disease designation may partially explain the lack of capital investment in developing and commercializing antifungal drugs. This is no truer than in the case of designer antifungals to which the vast majority of antifungal immunotherapeutics belong. It is worth noting that out of 13 antifungal ODs, only one immunotherapeutic agent was included, namely the recombinant human anti-HSP90 mAb Mycograb®. Current designation of most fungal diseases as ODs notwithstanding, rising trends in fungal infections and the ever growing number of immunocompromised hosts susceptible to opportunistic fungal infections warrant periodic reconsideration.

### Table 3. List of antifungal products with an FDA Orphan Drug designation

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Designated Indication</th>
<th>Date of Designation</th>
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<tr>
<td>AMB lipid complex (Abelcet)</td>
<td>Treatment of invasive fungal infections.</td>
<td>1991</td>
</tr>
<tr>
<td>AMB inhalation powder</td>
<td>Prevention of pulmonary fungal infections in patients at risk for aspergillosis due to immunosuppressive therapy</td>
<td>2005</td>
</tr>
<tr>
<td>Itraconazole suspension</td>
<td>Topical treatment of fungal otitis externa (otomycosis)</td>
<td>2008</td>
</tr>
<tr>
<td>Liposomal nystatin (Nyotran)</td>
<td>Treatment of invasive fungal infections.</td>
<td>2000</td>
</tr>
<tr>
<td>AMB lipid complex (Abelcet)</td>
<td>Treatment of invasive coccidioidomycosis, zygomycosis, and candidiasis.</td>
<td>1996</td>
</tr>
<tr>
<td>Itraconazole suspension</td>
<td>Topical treatment of fungal otitis externa (otomycosis)</td>
<td>2008</td>
</tr>
<tr>
<td>Meclorethamine or nitrogen mustard</td>
<td>Treatment of mycosis fungoides</td>
<td>2004</td>
</tr>
<tr>
<td>Methotrexate with laurocapram Methotrexate(azone)</td>
<td>Topical treatment of mycosis fungoides.</td>
<td>1990</td>
</tr>
<tr>
<td>Nikkomycin Z</td>
<td>Treatment of coccidioidomycosis</td>
<td>2006</td>
</tr>
<tr>
<td>Posaconazole (Posoril)</td>
<td>Treatment of zygomycosis</td>
<td>2004</td>
</tr>
<tr>
<td>Recomb. Hum. mAb to HSP90 or Efungumab (Mycograb®)</td>
<td>Treatment of invasive candidiasis (Currently in phase III clinical trial)</td>
<td>2002</td>
</tr>
<tr>
<td>Liposomal AMBAmbisome</td>
<td>Treatment of cryptococcal meningitis</td>
<td>1996</td>
</tr>
<tr>
<td>Clindamycin (Cleocin)</td>
<td>Prevention of Pneumocystis carinii pneumonia in AIDS patients.</td>
<td>1988</td>
</tr>
</tbody>
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Source of preliminary data: http://www.fda.gov/downloads/ForIndustry/DevelopingProductsforRareDiseasesConditions/HowtoapplyforOrphanProductDesignation/UCM162066.xls

The original product list included more than 2200 drugs.

Orphan Drug: A product that treats rare disease affecting fewer than 1 in 200,000 people

Notwithstanding these difficulties and setbacks, research and review articles on the subject often conclude with one of several overused clichés like “remarkable antifungal activity” “effective fungal vaccines” “alternative to chemotherapy” and so on (citation of the long list of references falling into this category is withheld). If nothing else, such hype is a turnoff to manufacturers willing to consider developing novel antifungal therapies. For misreading or misrepresenting the real state of affairs of antifungal immunotherapy can, by no means, convince savvy entrepreneurs to commit significant capital to murky investments. Therefore, overoptimism in reaching the long-awaited goal of developing antifungal vaccines and immunotherapeutics in the near future is premature to say the least.

CONCLUSION

Variations in pathogenesis and host immune responsiveness to fungal infections make it unlikely for a single approach whether chemical or immunological to be fully effective. Limitations imposed by antifungal chemotherapy necessitate the engagement of different forms of immunotherapy to complement and hopefully replace conventional therapies. Work on developing antifungal immunotherapeutics is still, for the most part, in the basic research phase. However, recent developments in fungal vaccines and antifungal monoclonal antibodies as respective representatives of host- and pathogen-targeting immunotherapies are promising. As host- and pathogen-targeting immunotherapies represent divergent counter measures against fungal diseases and as each have their own advantages and disadvantages, exhaustive and systematic testing of the efficacy and safety of various combinations from these two general categories is needed.

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DECLARATION OF COMMERCIAL INTERESTS

The authors declare that they are not associated with and have no commercial interests in any of the companies that manufacture or market any of the drugs or agents described in this review.
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Antifungal Immunotherapy: A Reality Check


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