Recent Advancements in Combinational Antifungal Therapy and Immunotherapy

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Abstract

The most predominant opportunistic fungal pathogens Candida sp, Aspergillus and Cryptococcus sp are emerging as a global problem of great concern as it is associated with increased morbidity and mortality. Although there are different classes of approved antifungal agents like polyenes, pyrimidines, azoles, and echinocandins, the morbidity and mortality rate due to systemic infection remains high, due to its toxicity, narrow spectrum of activity and tolerability. The continuous usages of these drugs are associated with resistance development and thus this is another area of emerging global problem. In addition, biofilm formation by fungal pathogens are proven to be an important virulence factor and are resistant to host defense mechanism and antifungal agents. Combination therapy has shown to be a promising choice to overcome the emergence of drug resistance, minimizing the toxic effects by reducing the drug dosage, and to increase the spectrum of killing. In this chapter, we have reviewed the last 5 years' developments made in combinational antifungal therapy with special emphasize on biofilm related infections and have presented a reappraisal of the current practices associated with immunotherapy to treat fungal infections and future challenges.

Keywords

Combinational therapy • Immunotherapy • Fungal infections • Biofilm

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6.1 Introduction

The improvement in medical advances incidentally has led to an increase in the incidence of opportunistic fungal infections. The most predominant opportunistic fungal pathogens are *Candida* sp., *Aspergillus* sp., and *Cryptococcus* sp. (Paramythiotou et al. 2014). During the last decade, there has been an emergence of other pathogens like *Zygomycetes*, *Fusarium*, etc., (Pagano et al. 2011). These pathogens take the chance to cause disease when the immune system of an individual is altered due to a plethora of reasons like organ transplantation, cancer therapies, diabetes mellitus, immature birth, dietary factors, and nutrient deficiency (Riddell and Shuman 2012). The other contributing factor for the increase of fungal infections includes surgical problems with prolonged antibiotic treatment (Miceli et al. 2011).

Although there are different classes of approved antifungal agents like polyenes, pyrimidines, azoles, and echinocandins, the morbidity and mortality rate due to systemic infection remains high, due to its toxicity, narrow spectrum of activity, and tolerability (Bal 2010). The continuous treatment with similar kind of drugs evolves different drug resistance mechanisms in fungi, and the emergence of drug-resistant strains is a growing global problem (Allen 2010). The common drug resistance mechanisms established in fungi are (I) transport alteration (ATP-binding cassette transporters or major facilitator superfamily transporter), (II) target alteration (by mutation or structural change in target protein), (III) gene upregulation (overexpression of target protein) and utilization of compensatory and catabolic pathway, (IV) biofilm formation, (V) efflux of antifungal drug, and (VI) decreased uptake of antifungal drug (Kontoyiannis and Lewis 2002; Sanglard et al. 2009).

Combination therapy has shown to be a promising choice to overcome the emergence of drug resistance, minimizing the toxic effects by reducing the drug dosage, and to increase the spectrum of killing. The drug combinations are always selected based on the two different modes of actions of the antifungals. These are inhibitors of cell wall synthesis and inhibitors of protein/DNA synthesis or inhibitors of the cell membrane. The drug interactions in the combinational therapy are classified as synergistic, antagonistic, and additive (Chen et al. 2014). Some proposed mechanisms for synergistic drug interactions are inhibition of biochemical pathway at different stages, for example, ergosterol biosynthesis and weakening of the fungal cell membrane by combinational drug therapy using terbinafine and azoles (Barchiesi et al. 1998; Johnson et al. 2004); increased penetration of combined drugs by impairing the cell wall or cell membrane activity, for example, combination of fluconazole or amphotericin B with quinolones and rifampin allows these antibacterial agents to penetrate fungal cell to target DNA synthesis (Medoff 1983); transport interaction of antifungal drug with the help of another antifungal drug, for example, the cell membrane degradation by amphotericin B via oxidative decay allows flucytosine into the cell (Beggs and Sarosi 1982); and instantaneous inhibition of multiple fungal cell targets (Bartizal et al. 1997). Antagonism among antifungal agents might occur in one of several ways. Administration of antifungal drug at the same site and time will decrease the efficacy of drug, for example, amphotericin B and azole: amphotericin B binds with ergosterol in the cell membrane and usually blocks the synthesis of ergosterol (Cosgrove et al. 1978). Adsorption of one antifungal agent to the cell surface inhibits binding of another antifungal agent to target site of activity, for example, itraconazole and ketoconazole are lipophilic azole that bind with the cell surface and inhibit binding of amphotericin B (Johnson et al. 2004). Modification of a drug target upon previous exposure to an antifungal agent reduces the susceptibility, for example, exposure of pathogen to an azole compound results in alteration of membrane ergosterol with methylated sterol derivative, which will reduce the activity of amphotericin B (Currie

et al. 1995). The selection of drugs in combination should accomplish these criteria for better treatment option.

Considering these facts, the therapy should also incorporate the appropriate dosage for eradication of fungal biofilms. Biofilm formation is an important contributing factor for virulence and an emerging global problem of great concern. The biofilms are resistant to host defense mechanism and antifungal agents (Martinez and Fries 2010). The three main drug resistance mechanisms proposed in biofilms are (I) incomplete or slow penetration of antimicrobials due to exopolysaccharide polymers; (II) resistant phenotype, known as persister cells characterized by a slower growth rate and greater resistance to antimicrobials; and (III) an altered environment that may affect the antibiotic action such that fungal biofilm persists even when appropriate antifungal treatment is given (Chatzimoschou et al. 2011). For instance, fluconazole and amphotericin combination therapy was unsuccessful in the treatment of *Cryptococcus neoformans*-infected hip joints due to poor penetration of drugs through the biofilm (Johannsson and Callaghan 2009). Catheter-related fungal infections are serious infectious complications that worsen the patient outcomes. The guidelines of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) have suggested removing the catheter, but a recent report, by analyzing 842 patients from two randomized clinical trials, reveals that the removal of the catheter does not improve the patient outcome (Nucci et al. 2010). Hence, development of new strategies for fungal infection management is required. One such approach is identifying an antiquorum sensing molecule which comprises targets of biofilm, virulence factors, and hyphal formation. The most studied quorum sensing molecule in fungi is farnesol, which has a control over growth, morphogenesis, and biofilm formation. The fungus quorum sensing research is still in infancy, and further research could lead to the development of new antifungal agents (Albuquerque and Casadevall 2012). Immunotherapy is based on the premise that most opportunistic fungal infections are a result of compromised immune system. And thus, any means of modulating the immune system using immune or immunogenic components could prove to be useful in achieving immune activation in the patient that will enable pathogen clearance. Such strategy minimizes the toxic effects of conventional antifungal drugs (and in some cases even synergizes with the drug or potentiates the activity of the drug), thereby opening up a new way of combating multidrug-resistant fungal pathogens.

In this chapter, we have reviewed the last 5 years' developments made in combinational antifungal therapy with special emphasis on biofilm-related infections and have presented a reappraisal of the current practices associated with immunotherapy and future challenges.

6.2 *Cryptococcus* Meningitis

Cryptococcus meningitis (CM) is an opportunistic neurological infection in immunocompromised individuals. It has emerged as the leading cause of morbidity and mortality in HIV-positive and HIV-negative patients (Muzoora et al. 2012). Recently, the Center for Disease Control estimated approximately one million new cases of cryptococcal meningitis each year, resulting in the death of 625,000 people worldwide. The standard therapy for CM consists of three phases: induction, consolidation, and maintenance (Vaidhya et al. 2015). The current standard initial therapy consists of amphotericin B and flucytosine (5-FC), but the availability of 5-FC is an issue in the countries of Asia and Africa, where CM is common (Perfect et al. 2010). Hence the probability of effective antifungal combination therapies is being investigated by the researchers.

The primary objectives of the therapies are to reduce the high infectious dose in the lungs and brain tissues which in turn increase the intracranial pressure and tend to have a worse prognosis. The induction therapy with amphotericin B deoxycholate (0.7–1.0 mg/-kg) combined with flucytosine (100 mg/kg/day for 2 weeks), followed by further consolidation therapy with

fluconazole, is considered a standard management regime in the developed world (Denning and Hope 2010). An alternate but promising treatment strategy includes administering amphotericin B and fluconazole (both at 800 mg/14 days), followed by administration of fluconazole alone (800 mg/56 days). However, validation of this strategy is required through randomized phase III clinical trial. Fluconazole resistance is not common if it is used as a part of amphotericin B induction therapy. But continuous use of fluconazole has been shown to result in other resistant yeast infections, for example, fluconazole resistant (Govender et al. 2013). Toward the end of these antibiotic therapies, protein abnormalities may persist for years, which would cause adverse effects including nephrotoxicity (Fine and Atta 2007). Hence the combination therapy of fluconazole and flucytosine was recommended and has greater antifungal activity compared with fluconazole monotherapy, even though fluconazole has high penetration in CSF (Yao et al. 2014). However, the effect of this combination in comparison with amphotericin B is unknown (Denning and Hope 2010). Although the lipid-based formulations of amphotericin B have excellent efficiency by reducing its nephrotoxicity, the major drawback is its cost. To execute significant measures to overcome drug resistance, few studies have attempted to search for an alternative antifungal drug (Nikapitiya 2012). In search of new antifungal agents, in vivo efficacy of miltefosine, alkylphosphocholine, was evaluated for its usage as monotherapy and in combination with fluconazole and amphotericin. The monotherapy does not significantly improve survival of mice. Combination with miltefosine revealed neither synergy nor antagonism in both models, suggesting caution with the utility of miltefosine for anticryptococcal therapy (Wiederhold et al. 2013).

Apart from cost, the lipid formulation of amphotericin B is unable to pass through the blood-brain barrier. In CNS fungal infections, the permeability of lipid amphotericin B is enhanced by the inflammatory process. However, the inflammation is associated with the risk of intracranial pressure. Hence drugs directly administered into the CNS could be a suitable therapy for CN infections, especially in those cases where conventional therapy fails or a relapse of the infection occurs. Gazzoni et al. (2012) compared the in vivo efficacy of intrathecal administration of L-AmB alone and in combination with voriconazole in a murine model of cryptococcal meningitis. The animals were treated with liposomal amphotericin B administered intrathecally at 0.006 mg/kg weekly or intravenously at 10 mg/kg daily or with voriconazole administered orally at 30 mg/kg per dose twice daily or with combinations of both drugs, i.e., L-AmB i.t.c. + voriconazole or L-AmB i.v. + voriconazole at the same doses as used in the monotherapies. All treatments significantly increased the survival and reduced fungal burden in comparison with the control group, with voriconazole being less effective in comparison with all other treatments. The combination of L-AmB i.t.c. + voriconazole showed a synergistic effect in the reduction of fungal load that was significantly superior to any tested therapy (Gazzoni et al. 2012).

The increase of fungal burden in the CSF is a major issue to be focused to increase the survival. Studies were conducted to determine the therapeutic value of amphotericin B and voriconazole (alone and in combination) that would support reduction of fungal burden. Preclinical studies were conducted using BALB/c-SCID mice infected with fluconazole-resistant strains of *Cryptococcus neoformans* var. *grubii*. The survival curves and yeast quantifications indicate that amphotericin B was effective in increasing the survival of the animal without reducing the fungal burden in the lung and brain tissue (Silva et al. 2011). The rate of clearance of *C. neoformans* from cerebrospinal fluid is facilitated by amphotericin B and flucytosine as first-line therapy. But flucytosine is not available in many resource-limited countries (Bisson et al. 2013). Thus, combinations with different modes of action and different doses were tried with AmB + fluconazole (800 mg/day), AmB + fluconazole (600 mg twice per day), and AmB + voriconazole (300 mg twice per day) that

provides a means to compare the antifungal activity of alternate regimens in small phase II studies. The combination therapy comprising rifampin and amphotericin B for treating fungal diseases is of past interest to eradicate the toxic effect of conventional AMB. In vivo studies were not conducted due to the lack of laboratory testing standards, of *Cryptococcus* sp. (Loyse et al. 2012).

Gamaletsou et al. (2012) experimented salvage therapy for two patients with HIV-related, refractory *Cryptococcus* meningitis as there are no potential comparative studies for salvage therapy. The salvage regimen provides a successful treatment, consisting of the combination of liposomal amphotericin B (3 mg/kg), intravenous voriconazole, and subcutaneous recombinant interferon γ-1b (200 μg thrice weekly). And to overcome interactions with co-administered ritonavir, voriconazole was given at an increased dose (5 mg/kg, twice daily). Oral fluconazole was given as maintenance therapy. The salvage therapy eliminates the fungal burden in the CSF after 10 weeks of therapy, also no adverse side effects, and no recurrence of infections has been observed even after 4 years of follow-up. Further validation is needed to use this strategy as initial therapy for HIV-related CM.

Recently, Yao et al. (2014) conducted a meta-analysis for prospective cohort studies to compare the survival benefits between fluconazole and 5-flucytosine given in combination with AmB as the initial therapy for CM. The survival rate was similar in case of AmB in combination with high-dose fluconazole, and therefore this combination is alternative for 5-flucytosine for the management of HIV-associated CM therapy, which is not available in resource-limited countries (Yao et al. 2014). Clinical data disclose that high-dose fluconazole shows no adverse liver function abnormalities (Vaidhya et al. 2015). However, high-dose fluconazole might lead to the emergence of drug-resistant strains. For patients without CNS involvement with limited pulmonary cryptococcosis and non-pulmonary cryptococcal infections in immunocompetent patients, initial therapy with fluconazole (400 mg per day) could be considered. The standard therapy of amphotericin B and flucytosine is not recommended for disseminated cryptococcosis (Probst et al. 2010).

Cryptococcus neoformans biofilms are becoming common due to the increased use of brain valves and other medical devices such as ventriculoatrial shunt catheters, peritoneal dialysis fistula, cardiac valves, and prosthetic joints. The knowledge on the mechanism of biofilm formation by *Cryptococcus* is still in infancy; hence, there are very limited reports on the evaluation of antifungals on *C. neoformans* biofilms. An in vitro study reveals that amphotericin B and caspofungin are effective against the mature biofilms of *C. neoformans*; however, the efficacy profile is not similar for melanized *Cryptococcus* biofilms. Recently, antibiofilm activity of antifolate drugs (sulfamethoxazole-trimethoprim and sulfadiazine-pyrimethamine) alone and in combinations with amphotericin B was studied (Liao et al. 2015). However clinical study and combination therapy for cryptococcal shunt infections are lacking.

6.3 Candidiasis

Candida albicans is the most prevalent infectious species among the 20 different species of *Candida* (Centre for Disease Control 2014). It is the normal commensal of the alimentary tract, vagina, and oral cavity. The emergence of *Candida* sp. as the important nosocomial pathogen is owing to the increased use of medical devices such as central venous catheters and various implanted devices (Kim et al. 2011). Although *C. albicans* is the most prevalent, non-albican species, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, and *C. kefyr* are also increasing recently, which is evident in a recent 5-year prospective study and population-based surveys of candidemia (Alexander et al. 2013). For candidemia management, the most frequently administered antifungal agent is fluconazole and echinocandins. The effectiveness of the antifungal agent varies with the species of *Candida*

isolated from different clinical samples (Pfaller et al. 2012; Sardi et al. 2013).

Andes et al. (2012) conducted an individual patient-level quantitative review of randomized trials consisting of 1,915 patients for treatment of invasive candidiasis. They identified that the use of an echinocandin was associated with reduced mortality when compared to the use of a drug from either the triazole or polyene classes. And their study also supports the greater toxic effects associated with amphotericin B than triazole or echinocandin therapy. These findings lend support to recent treatment guidelines that recommend echinocandin as a first-line choice for invasive candidiasis particularly for patients who are critically ill and those with previous exposure to triazole (Andes et al. 2012).

The treatment for *Candida* infections varies with patient's underlying conditions. For instance, the delayed treatment of candidemia in septic shock is shown to be an important factor of patient outcome. Septic shock due to invasive candidiasis is a near-fatal condition. Retrospective cohort studies of septic shock patients with *Candida* infections reveal the risk of death is exceptionally high among patients with septic shock associated with *Candida* infection. The antifungal therapy regime is needed to improve the clinical outcomes (Kollef et al. 2012). Invasive candidiasis is also a leading cause of mortality and morbidity in immune-compromised neonates. A comparative outcome of antifungal treatment of candidiasis in infants was provided by a large multicenter cohort study. Infants treated with L-AmB had a higher mortality when compared to infants treated with either amphotericin B deoxycholate or fluconazole. This finding may be related to inadequate penetration of L-AmB into the kidneys. There are no recommendations for combination therapy to treat life-threatening infection and deep tissue infection as the comparative outcome data are limited (Tragiannidis et al. 2015). In case of candidiasis in hematologic malignancies, echinocandins are recommended as initial therapy, in non-neutropenic patients with candidial infections, initial therapy with the echinocandin followed by fluconazole, is recommended. There are no reports on combination therapy for invasive candidiasis in leukemic patients (Walsh and Gamaletsou 2013). The summary of recent treatment recommendations for different types of candidiasis is given in Table 6.1.

The important factor for drug resistance in *Candida* sp. is their ability to grow and adhere as biofilms on various surfaces including the medical devices. *Candida* biofilms are the leading cause for intravascular catheter-related infections. The presence of biofilms on intravascular devices makes the treatment of CR-BSI difficult and resists the action of clinically important drugs including amphotericin B and azole (Chatzimoschou et al. 2011). Also a recent randomized clinical trial reveals the higher toxicity effects of amphotericin B with fluconazole (Sanati et al. 1997). Thus, many researchers have turned their focus to evaluate the antifungal therapy comprising different combinations including antimicrobial peptides, natural products, and non-antifungal agents. And many studies are being carried out to demonstrate the antimicrobial lock therapy to treat catheter-related blood infections. Antimicrobial lock therapy involves the instillation of a small volume of the highly concentrated antibiotic solution, which is allowed to stay in the lumen of the catheter for 12–72 h, to eradicate biofilm formed on the inner surface of the device. Ku et al. (2010) determined the efficacy of antibiofilm and persistence of the activity for the lock solutions of caspofungin, micafungin, and posaconazole in in vitro model of *Candida* biofilm on silicone catheter. Micafungin and caspofungin had significant inhibitory activity against mature *C. albicans* and *C. glabrata* biofilms, but caspofungin displayed less persistence in *C. glabrata* biofilms. Even though posaconazole was less effective against *C. albicans* biofilms, its activity was maintained. They had suggested that echinocandin lock therapy may be useful to control candidiasis in catheterized patients (Cateau et al. 2011). Until recently, the guidelines of the Infectious Diseases Society of America (IDSA) had suggested the use of antibiotic lock therapy only under special circumstances as a salvage treatment. For the management of catheter-

Table 6.1 Summary of recent treatment recommendations for different types of candidiasis

Type	Organism	Symptoms	Estimated statistics	Treatment	References
Oral candidiasis	*Candida* sp., *Candida albicans* (69.23 %)	Soreness and redness in infected area, difficulty in swallowing, dryness at the corners of mouth	5–7 %, babies less than 1 month; 9–31 %, AIDS patients; ~20 %, cancer patients	Clotrimazole with fluconazole or itraconazole	Pieralisi et al. (2014), Jensen and Peterson (2014), Center for Disease Control and Prevention
Vulvovaginal candidiasis	*Candida albicans* (87.5 %), C. *glabrata* (6.8 %), other *Candida* species less than 3 %	Woman (itching, burning, and vaginal discharge)	75 % adult woman	Oral medication (clotrimazole with fluconazole), suppositories, and creams	Mendling (2015), Center for Disease Control and Prevention
Invasive candidiasis	*Candida albicans* (70 %), C. *glabrata* (11 %)	Fever, chills, and failure of antibiotics	8–10 cases per 100,000 person, 10–20 % in new born child (less than 1 month)	Neonates (amphotericin B), child and adults (fluconazole, micafungin, caspofungin, anidulafungin, and echinocandin)	Cornely et al. (2011), Kobayashi et al. (2015), Laua et al. (2015), Center for Disease Control and Prevention
Genital candidiasis	*Candida albicans* (87.5 %), *C. glabrata* (6.8 %), other *Candida* species less than 3 %	Man (itchy rash), woman (itching, burning, and vaginal discharge)	75 % adult woman, men (rare occasion)	Oral medication, suppositories, and creams	Mendling (2015), Center for Disease Control and Prevention

related bloodstream infections, tigecycline, an antibacterial agent, was evaluated alone and in combination with fluconazole, amphotericin B, or caspofungin against *Candida* biofilms. It has been found that high-dose tigecycline is highly active against in vitro *Candida* biofilms; however, the combination with antifungals is not significant. Further investigation is required to utilize the antimicrobial lock therapy containing tigecycline for the management of CR-BSI (Ku et al. 2010). The antibiofilm activity of amphotericin B was found to be significantly enhanced when used in combination with polyamine biosynthesis inhibitors, 1,4-diamino-2-butanone (DAB) and difluoromethylornithine (DFMO). Further studies have shown that DAB and DFMO also enhanced the antibiofilm activities of several other antifungal agents by enhancing the formation of reactive oxygen species in biofilm cells.

In vitro, the combination of caspofungin or anidulafungin with antimicrobial peptides is very effective in killing *Candida* sp.; however, the efficacy was not improved in a murine model of systemic candidiasis, wherein the combination of caspofungin and antimicrobial peptide, ranalexin, was used. The enhanced efficacy was observed in the same animal model when the combination therapy of amphotericin B and lactoferrin was used. There is no literature support for the synergism between antimicrobial peptides and echinocandins. Rossignol et al. (2011) investigated the tryptophan-rich, ApoEdpL-W peptide, derived from human

ApoE apolipoprotein shown to have less antifungal activity against mature biofilms and partially inhibited biofilms on medical devices.

Synergism of natural products with antibiotics is the currently developing area of phytopharmaceuticals. Many natural products including essential oils, peptides, and polyphenols are active against different shapes of *Candida* biofilms. Recently the effectiveness of the antifungal activity of the essential oils of *Ocimum sanctum* was evaluated for the fluconazole-resistant *Candida* sp. Besides its selective cytotoxicity, the synergism with the fluconazole and ketoconazole showed a better antifungal activity in in vitro fluconazole-resistant *Candida* strains (Amber et al. 2010). Similar synergistic interactions were observed in farnesol with micafungin, fluconazole, and amphotericin B against in vitro *C. albicans* biofilms (Katragkou et al. 2015). The in vitro effect of essential oil components of eugenol and methyleugenol alone and in combination was checked against fluconazole-sensitive and fluconazole-resistant strains of clinical *Candida* sp. The synergistic interactions of eugenol and methyleugenol with fluconazole are very effective killing fluconazole-resistant strains (Ahmad et al. 2010a). Mandal et al. (2011) demonstrated the activity of Tn-AFP1 from *Trapa natans* against biofilm inhibition of *C. tropicalis* in vitro. Similarly, Delattina et al. (2014) identified OSIP108, a nontoxic decapeptide from the model plant *Arabidopsis thaliana*, that inhibits *C. albicans* biofilm. These recent reports support the synergism of natural products with antifungal agents in controlling the *Candida* biofilms. However, the major drawback of natural products' utility is the unestablished toxicity profiles. One such well-established compound, fulvic acid, was proven to be nontoxic in an epithelial cell line and rat model alone (Nett 2014). The information from several studies indicating synergistic interactions of non-antifungal drugs in combination with fluconazole are summarized in Table 6.2

Due to the high cost and side effects of combination therapy, the focus of research recently turned to examine the combinations with non-antifungal agents, including tetracyclines (Loreto et al. 2011), quinolones (Wang et al. 2012), calcineurin inhibitors (Chen et al. 2011), heat shock protein 90 inhibitors, calcium homeostasis regulators, and traditional medicines (Muñoz et al. 2012).

One such combination is azole with calcineurin inhibitor. The calcineurin signaling pathway is a key mediator of stress responses in *C. albicans* as a potential drug target for systemic candidiasis and candidal keratitis. Calcineurin inhibitors when given as a monotherapy were not suitable for other candidial infections of oropharyngeal, pulmonary, and vaginal tract. The virulence pathway inhibitor in combination with azole would increase the efficiency of the antifungal drug. The establishment of virulence pathway varies with the species. Hence, identifying suitable drug targets and finding its synergism with the antibiotics would be potential therapeutic options (Reedy et al. 2010). However, drugs targeting the inhibition of virulence expression would be suitable targets to enhance the current antifungal therapy and to reduce the evolution of drug-resistant strains. The synergism shown by calcineurin inhibitors with the antifungal agents amphotericin B and caspofungin is potentially good as the combination lock therapy in the rat catheter infection model; however, its utility is limited as the drugs have immunosuppressive effects that prevents the systemic administration.

Diclofenac, aspirin in combination with caspofungin and amphotericin B, was evaluated to treat infections of *Candida albicans* and *C. parapsilosis*. Bink et al. (2012) screened 1,600 compounds of a drug-repositioning library that enhance antibiofilm activity against *C. albicans*. They have found out that hexachlorophene, pyrvinium pamoate, and artesunate can act synergistically with miconazole in eradicating C. *albicans* biofilms. They suggested miconazole and artesunate as the potential combination therapy to treat oral and vulvovaginal candidiasis. In vivo toxicity studies are required to execute the potentiality of the miconazole and artesunate (De Cremera et al. 2015). Finasteride, a

Table 6.2 Synergism of fluconazole with non-antifungal agents

Combined drug	Clinical *Candida* spp.	Methods and results	Antifungal mechanisms/targets
Antimicrobial agents			
Minocycline	Azole-susceptible *C. albicans* strains	FICI, E method, indifference	Minocycline significantly enhances the amount of FLC penetrating *C. albicans* biofilms as well as interrupting the cellular calcium balance
	Azole-resistant *C. albicans* strains	FICI, E method, synergy	
Doxycycline	*C. albicans* (SC5314), FLC-resistant strain	FICI, synergy	Interference with iron homeostasis
	C. albicans biofilm	FICI, synergy	Inhibition of biofilm formation
	C. albicans biofilm	FICI, E method, synergy	Inhibition of biofilm formation is associated with calcium
Ciprofloxacin, trovafloxacin	*C. albicans*, *C. tropicalis*, *C. glabrata*	FICI, in vivo, prolonged the survival of mice	Possibly inhibits fungal topoisomerase
	Calcineurin inhibitors		
FK506 (tacrolimus)	Azole-susceptible *C. albicans* isolates	FICI, E method, time-kill curves, synergy, or indifference	CsA and FK506 bind to cyclophilin A and FKBP12, respectively, to form protein–drug complexes that inhibit the calcineurin-mediated stress response, cause damage to the cell membrane, and increase fungal cell membrane permeability, thus increasing the concentration of azoles in fungal cells to achieve a fungicidal effect
	Azole-resistant *C. albicans* isolates	FICI, E method, time-kill curves, synergy	
	C. albicans wild type	FICI, synergy	
	C. albicans mutants	FICI, synergy	
	C. albicans wild-type biofilm	FICI, SEM, synergy	
	C. albicans biofilms (mutants: cnb1/cnb1, crz1/crz1)	FICI, indifference, or antagonism	
Cyclosporine A(CsA)	*C. albicans* (standard strain, ATCC90028) planktonic cells	FICI, synergy	
	C. albicans (standard strain, ATCC90028) biofilm cells	FICI, synergy	
	C. albicans wild type	FICI, synergy, or indifference	
	C. albicans mutants	FICI, synergy, or indifference	
	C. albicans biofilm (wild-type SC5314)	FICI, synergy	
	C. albicans (mutants: cnb1/cnb1,crz1/crz1)	FICI, synergy	
	C. albicans biofilms (mutants: cnb1/cnb1, crz1/crz1)	FICI, indifference, or antagonism	
Heat shock protein 90 (Hsp90) inhibitors			
Geldanamycin	*C. albicans*	FICI, synergy	Inhibit the stress responses induced by Hsp90 and reverse fungal azole resistance

(continued)

Table 6.2 (continued)

Combined drug	Clinical *Candida* spp.	Methods and results	Antifungal mechanisms/targets
Calcium homeostasis regulators			
Amiodarone (AMD)	FLC-susceptible *C. albicans* strains	FICI, indifference	AMD elicits an immediate, dose-dependent hyperpolarization of the membrane, causing Ca2+ influx and release from internal stores, inducing a disruption of calcium homeostasis
	FLC-resistant *C. albicans* strains	FICI, synergy	
	FLC-resistant *C. albicans*	FICI, E method, synergy	
	FLC-susceptible *C. albicans* strains	FICI, E method, indifference, or antagonism	
	C. albicans SC5314	FICI, synergy	
Traditional Chinese medicine			
Pseudolaric acid B	*C. albicans*	FICI, synergy	–
	C. krusei, *C. tropicalis*, *C. dubliniensis*	FICI, indifference	–
	C. glabrata, *C. guilliermondii*		–
	FLC-resistant *C. albicans*	FICI, E method, time-kill curves, synergy	–
	FLC-sensitive *C. albicans*	FICI, E method, time-kill curve, synergy, or indifference	–
Berberine chloride	FLC-resistant *C. albicans*	FICI, time-kill curves, synergy	–
Baicalein	FLC-resistant clinical *C. albicans*	FICI, time-kill curves, synergy	Inhibition of efflux pumps thus may increase the susceptibility of cells to antifungals. Exerts a synergistic effect with FLC by affecting some other factors on resistance, which requires further research
Eugenol, methyleugenol	FLC-sensitive *Candida* isolates	FICI, synergy, or indifference	Target the sterol biosynthetic pathway, altering fluidity and permeability
	FLC-resistant *Candida* isolates	FICI, synergy, or indifference	
	FLC-sensitive *Candida* isolates	FICI, synergy, or indifference	
	FLC-resistant *Candida* isolates	FICI, synergy, or indifference	

Source: Liu et al. (2014)

5-α-reductase inhibitor, commonly used for the treatment of benign prostatic hyperplasia was tested alone and in combination with amphotericin B and fluconazole against *C. albicans* urinary biofilms. It exhibits synergistic activity in combination with fluconazole. However, further investigation is required to explore the clinical utility of finasteride in the prevention of candidiasis (Chavez-Dozal et al. 2014). Synergistic interactions were observed during in vitro combination studies of allicin and flucytosine against *C. albicans*, *C. glabrata*, and *C. tropicalis* (Alexander et al. 2013). L-AmB in combination with heat shock protein, HSP 90 inhibitor (Mycograb), also provides promising therapy; however, the antibody-based inhibitor is not available for clinical trials (Pachl et al. 2006). The synergistic interactions of fluconazole with non-antifungal agents are summarized in Table 6.2.

The present review recommends the necessity for standard guidelines for the management of fungal biofilm infection.

6.4 Aspergillosis

Aspergillus exposure to human is quite common due to their ubiquitous nature. In immune-compromised host, it causes aspergillosis. Invasive aspergillosis (IA) has become increasingly common in recent days resulting in a mortality ranging from 40 % to 80 % (Ascher et al. 2012; Aguado et al. 2015). The predominant causative agent is *A. fumigatus* which also finds concurrence in a recent literature study reported from 1985 to 2013. The study comprised of 116 liver transplant recipients with invasive aspergillosis. The most common infecting species are *Aspergillus fumigatus* (73 %), *A. flavus* (14 %), and *A. terreus* (8 %). Voriconazole improves the survival among the commonly used AMB and itraconazole (Barchiesi et al. 2015; Ogawa et al. 2015). However, an increase in drug resistance restricts the usage of azole for treatment and necessitates the combinational therapy approach.

A randomized, double-blind, placebo-controlled, multicenter trial comprising 454 patients with hematologic malignancies and hematopoietic stem cell transplantation suspected with invasive aspergillosis were treated with voriconazole and anidulafungin. The study showed the improvement of survival when compared with monotherapy (Marr et al. 2015). In another retrospective study that was conducted between 1993 and 2008, the combination of L-AmB and echinochandins for the management of IA in hematological malignancies was analyzed, and the results suggested that the combination therapy offered no improvement in terms of response and mortality (Mihu et al. 2010).

Though sinus aspergillosis was believed to be a rare disease for many years, recently there has been an increase in the number of cases. Chronic invasive aspergillosis of the sinus occurs in mildly immunodeficient patients. Two of the most important treatment regimens for invasive aspergillosis of the sinus are surgical debridement and antifungal therapy. The pharmacodynamic interactions of amphotericin B and voriconazole were assessed using conventional in vitro tests that mimic human serum concentrations. The combination was more effective than monotherapy regimens (Siopi et al. 2015).

Various formulations of L-AmB differ in terms of toxicity and efficacy which concurs with a recent finding by Olson et al. (2015) while comparing, AmBisome and Lambin, in *Aspergillus fumigatus* mice model. Lambin was more toxic than the other, emphasizing the adequacy requirement to compare liposomal formulations. The in vitro combination studies of nystatin-intralipid with caspofungin demonstrated effective synergistic interaction against *A. terreus* when compared with voriconazole and 5-fluorocytosine (Semis et al. 2015; Shadomy et al. 1975). In a murine aspergillosis model, the combinations of L-AmB, micafungin, and deferasirox improved the fungal clearance and patient survival. The in vitro efficacy of PTX3, a glycoprotein produced by monocytes in combination with voriconazole, improved patient survival and clearance of fungal burden (Giudice et al. 2012). Erythropoietin in combination with AMB improved the outcome of in vivo disseminated aspergillosis.

The synergistic interactions vary with the species and resistance mechanism. In a similar kind of study, the synergistic interactions of posaconazole and caspofungin varied with 20 different wild and resistant types of *A. fumigatus*, with ten different resistant mechanisms. The interactions are insignificant when compared with wild isolates (Mavridou et al. 2015). Similarly, while investigating the efficacy of the combination of voriconazole and anidulafungin in an in vitro model of invasive pulmonary mice infected with triazole-resistant *Aspergillus*, the addition of anidulafungin does not significantly improve the voriconazole activity, whereas another study suggests the combination of triazole and echinocandin as the treatment strategy for triazole-resistant isolates (Lepak

et al. 2013). The efficacy varied with the type of strain and the drug resistance pattern evolved. A study by Jeans et al. (2012) has proven the efficacy of voriconazole and caspofungin in an in vivo model of pulmonary aspergillosis infected with different species of *Aspergillus*. The synergism between *A. flavus* and *A. niger* was found to be significant while not in the case of *A. fumigatus* (Zhang et al. 2014). In a neutropenic *A. fumigatus* murine model, the synergistic interactions of voriconazole and anidulafungin were observed in voriconazole-susceptible strain, but additive in resistant type, which creates concern for the management of infections by voriconazole-resistant strains.

The inappropriate use of antibiotics is another major factor for the emergence of drug-resistant strains. A recent cross-sectional cohort study states that systemic antifungal therapy was administered irrespective of the presence of invasive fungus infection (Ascher et al. 2012; Olson et al. 2015). Based on this, a recent survey study conducted revealed the weakness in the knowledge on current guidelines of invasive candidiasis and invasive aspergillosis among the European physicians (Valerio et al. 2015).

A. fumigatus biofilms are difficult to manage with conventional antifungal agents; hence, even after antimycotic therapy, these infections are not eradicated and are emerging as life-threatening infections (Mowat et al. 2007). Melanin produced by *A. fumigatus* during infection is required for lung tissue invasion, conferring less susceptibility to drugs (Bowyer et al. 2011). Biofilms of *A. fumigatus* are resistant to itraconazole and, some extent, to caspofungin due to its extracellular polymeric substances (EPS) (Singh et al. 2011). An in vitro finding reveals *A. fumigatus* are not responding to amphotericin B, when they have a prior exposure to voriconazole, suggesting the improper usage of azole will be a hindrance for treatment with amphotericin B. Synergistic interactions were observed in combination with caspofungin and amphotericin B or voriconazole against *Aspergillus* biofilms (Liu et al. 2012). Another in vitro study with combinations of amphotericin B or Lamb with alginate lyase, the degrading enzyme of EPS (Bugli et al. 2013; Rajendran et al. 2015), suggesting the EPS as a suitable target for antibiofilm activity. There are very limited clinical studies that are focused on the management of biofilm-associated *A. fumigatus* infections.

In a recent literature review, data addresses the significance of combination antifungal therapy in the treatment of invasive aspergillosis. The information suggests that evidence is not convincing enough to support the use of combination antifungal therapy (Garbati et al. 2012). Another systematic review comprising animal and clinical studies addressed the efficacy of combination therapy of triazole and echinocandin in treatment of invasive aspergillosis, suggesting a development toward improved overall survival (Day et al. 2013; Marr et al. 2015).

In summary, combination therapies appear to be conflicting in some studies, and the limited clinical data are not convincing to support combination antifungal therapy over monotherapy. In addition, well-sketched clinical trials focusing on management of biofilm-related fungal infections are immediately needed to overcome the emergence of drug resistance in these pathogens.

6.5 Immunotherapy Against Fungal Infections

Opportunistic fungal infections become a major challenge to health care since a majority of them have affected immune-compromised individuals such as HIV, hypersensitive, autoimmune, or transplant patients. Most of these infections in the case of normal persons are easily combated by the immune system, which is not the case with patients whose immune system is suppressed. Added to this are the problems associated with hospital multiple drug-resistant pathogens. Opportunistic fungal infections such as candidiasis, aspergillosis, and cryptococcosis are three of the leading causes of significant mortality in both hospital-derived infections and in those of immune-compromised patients (Truan et al. 1994). Studies also clearly suggest that due to modified lifestyles and exposure, these fungal infections are set to increase and dominate

the concerns of medical industry in the future (Kauffman 2001). This creates the need for either combinatorial therapies or rapid discovery of novel antibiotics. However, most such strategies are replete with adverse side effects and may not be favorable, though the benefits far outweigh the risks. One way forward to combat such opportunistic fungal infections, with nil or minimal side effects, is immunotherapy (Ahmad et al. 2010b).

Majority of the immunotherapy strategies rely on immunomodulation of the patient's immune system to prime it against the fungal pathogen. This indirect method has many advantages, including lack of side effects and resistance development (Ahmad et al. 2010b; Owais et al. 2010), and more importantly such treatment to some extent can be catered to suit an individual patient's needs. However, it's important to recognize that host-fungi interaction (Ahmad et al. 2010b), immune responses of the host to fungi, and, importantly, immune escape mechanisms of fungi are quite complicated and the underlying mechanisms of fungal infections are beginning to be unraveled. With better understanding of these components, there is a better hope of developing much more effective immune modulators. In the next few sections, we will primarily focus on the use of immunotherapy as the latest and futuristic treatment for three of the major opportunistic pathogenic fungi: *Candida*, *Aspergillus*, and *Cryptococcus*.

6.5.1 Antimicrobial Proteins

These form one of the first lines of innate immune defense against a variety of pathogens and are widely distributed in animal kingdom. These are usually cationic and amphipathic peptides (Owais et al. 2010) and expressed in cells of the innate immunity such as macrophages, mast cells, and neutrophils, as well as in nonimmune cells such as keratinocytes and epithelial cells. The antimicrobial proteins can either directly defend the pathogens (Braff et al. 2005) or stimulate cells such as neutrophils (Sorensen et al. 1997) to degranulate resulting in inflammatory responses (Hancock and Diamond 2000). These antimicrobial peptides comprise cathelicidins (Sorensen et al. 1997), β-defensins, substance P, RANTES (Braff et al. 2005), LL-37 (Larrick et al. 1995; Nagaoka et al. 2001), etc. Apart from immune cell activation, these peptides can also activate epithelial cells upon microbial invasion leading to proinflammatory cytokine signaling (Van Watering et al. 1997) and toll-like receptor (TLR) activation (Akira et al. 2006). This could end up activating and recruiting immune cells to the site of infection and thus boosting the host immune defense. Thus, components of the innate immunity can be tweaked that could prove to be effective not only in controlling the spread of infection but also end up activating the adaptive immune components. However, the effectiveness of the use of these antimicrobial peptides as a therapy against opportunistic fungal pathogens needs more attention.

6.5.2 Cytokines

Both pro- and anti-inflammatory cytokines are regulators of innate and adaptive immune responses (Iwasaki and Medzhitov 2015), including cell recruitment, immune cell activation, and immune cell differentiation. Nowhere is this more pronounced than in the interplay of cytokines during development of T helper subsets (Romani 2000). Studies have shown the importance of cytokines in not only modulating immune responses against fungal pathogens (Mencacci et al. 1998) but have also demonstrated mechanisms involved in hijacking of the host cytokine network for immune escape by the fungal pathogens (Netea et al. 2006). Cytokines such as proinflammatory TNF-α and IFN-γ are unregulated (Rodriguez-Adrian et al. 1998) upon infection by *Candida* and *Aspergillus*, resulting in the activation of phagocytic cells. Cytokines can modulate immune cells very effectively if properly applied and are one of the potential immunotherapeutic strategies against opportunistic fungal infections. TNF is a proinflammatory cytokine and has been found to be very effective against *A. fumigatus* (Steinbach

and Stevens 2003). TNF has pleiotrophic effect on phagocytic cells, including cell activation and phagolysosomal killing of fungal pathogens (Cisalpino et al. 1996). The study by Steinbach and Stevens (2003) has shown the importance of TNF-α in suppressing immunodeficiency induced by dexamethasone, and the mechanism consists of upregulation of TLR and IFN-γ and preventing leukocyte apoptosis (van der Meer et al. 2005). Although both Th1 and Th2 are essential for anticryptococcal immunity, Th1 are very effective (Hoag et al. 1997). Thus, higher levels of Th1 cytokines such as TNF-α and IFN-γ are associated with increased fungal clearance (Milam et al. 2007; Wormley et al. 2007). More importantly, TNF-α is necessary for IFN-γ production (Bekker et al. 2001) which results in macrophage activation. So, it is clear that TNF therapy could potentially stimulate IFN activity leading to better antifungal responses. Apart from this, pathogen-associated molecular patterns (PAMPs) are potent antigens recognized by innate pattern recognition receptors (PRRs) such as TLRs. Both TNF-α and IFN-γ synergize to increase the cell surface expression of TLRs (Bosisio et al. 2002) resulting in better recognition and clearance of *Candida*, *Aspergillus* (Tsiodras et al. 2008), and *Cryptococcus* (Pappas et al. 2004). Thus, TNF-α immunotherapy could be a potential therapeutic strategy against fungal pathogens.

IFN-γ synergizes with TNF-α in a variety of immune functions including Th1 differentiation (Milam et al. 2007), TLR expression (Tsiodras et al. 2008), and phagocyte activation (Bekker et al. 2001). In rodents, the use of IFN-γ has shown good results against systemic cryptococcosis (Lutz et al. 2000), and the same cytokine was also successfully tested in one human patient (Netea et al. 2004). Studies have shown the effectiveness of IFN-γ against a variety of fungal pathogens including *Candida*, *Aspergillus*, and *Cryptococcus* (Rodriguez-Adrian et al. 1998; Shahid et al. 2010). IFN-γ has been shown to stimulate phagocytic activity and intracellular killing of *A. fumigatus* (Steinbach and Stevens 2003; Shahid et al. 2010), through stimulating generation of free radical production (Steinbach and Stevens 2003). In murine models, immunotherapy using TNF-α and IFN-γ was shown to produce significant reduction in mortality due to *Aspergillus* infection (Shahid et al. 2010). However, one issue with IFN-γ usage is that it reduces anticryptococcal activity of human macrophage and thus its use in humans is debatable (Reardon et al. 1996). Nevertheless, in 2010, the Infectious Diseases Society of America has approved recombinant IFN-γ against persistent cryptococcosis (Antachopoulos and Walsh 2012). Gallin et al. (1995) have shown that the use of IFN-γ under clinical settings is very effective in the case of patients suffering from chronic granulomatous disease (CGD) and who are especially prone to *Aspergillus* (Antachopoulos and Roilides 2005). Unlike anticryptococcal action, macrophage anti-*Candida* activity is enhanced by IFN-γ treatment (Redmond et al. 1993; Baltch et al. 2005). But other studies in rodents have shown lack of an effect of IFN-γ (Brummer and Stevens 1987; Marcil et al. 2002) in case of pulmonary macrophages, but peritoneal or peripheral blood PMNs (from both human and murine) showed good anti-*Candida* activity after IFN-γ treatment (Djeu et al. 1986; Kullberg et al. 1993). In spite of the lack of strong evidence for IFN-γ immunotherapy, Dignani et al. (2005) showed in the case of three patients with candidiasis that IFN-γ therapy was actually beneficial. Similarly, IFN-γ immunotherapy of an HIV-infected patient proved to be beneficial (Riddell et al. 2001; Bodasing et al. 2002). Nonetheless, large-scale clinical trials are the need of the hour to better understand the efficacy of this cytokine for immunotherapy.

IL-12, like TNF-α and IFN-γ, is a Th1 cytokine (Milam et al. 2007; Wormley et al. 2007), and Th1 activation and responses are vital for strong antifungal responses against *Candida*, *Aspergillus*, and *Cryptococcus*. IL-12 has been shown to inhibit Th2 responses, thereby increasing anti-candidiasis response of the host (Morrison et al. 1986; Deepe 1997). Other studies have shown the potential of IL-12 in enhancing the antifungal efficacy of fluconazole against *Candida* (Afonso et al. 1994), murine cryptococcosis (Muchmore et al. 1968), and

aspergillosis (Allendoerfer et al. 1996). From these studies, it is recognized that one way by which IL-12 could exert its antifungal effect is through enhancement in IFN-γ levels (Antachopoulos and Roilides 2005; Kawakami et al. 1996; Jacobson et al. 2000), synergization with IL-18 in the induction of anticryptococcal activity (Qureshi et al. 1999), and suppression of IL-4 activity (Qureshi et al. 1999). However, IL-12 usage has been shown to exacerbate mucosal disease in mouse (Morein et al. 1984) and produce adverse reactions on the immune system (Murphy et al. 1993). Thus, more studies are warranted to check the efficacy of this cytokine as an immunotherapy agent.

Colony-stimulating factors (CSFs) are essential for phagocyte development (especially in the case of neutropenia) and function and have been approved for humans (Rodriguez-Adrian et al. 1998). Being able to facilitate antifungal activity of phagocytes, CSFs are one of the promising immunotherapy agents. Approved and accepted since 1991 for patient use (Welte et al. 1996), granulocyte colony-stimulating factor (G-CSF) is effective in prolonging phagocyte and respiratory burst activity against *Aspergillus* infection (Polak-Wyss 1991; Liles et al. 1997) and *Candida hyphae* (Gaviria et al. 1999; Kullberg et al. 2004). However, when compared to other CSFs, G-CSF also produces anti-inflammatory cytokines and could be useful against endotoxins in immune-compromised patients (Gomez et al. 1995). G-CSF also favors a Th2 response and so can be beneficial against *Candida* (Condon et al. 1996) and *Aspergillus* (Cisalpino et al. 1996). However, anticryptococcal immunity requires a robust Th1 activation, and thus G-CSF may not prove to be successful here. However, neutrophils from G-CSF-treated HIV patients have been shown to be effective against *Cryptococcus* and *Candida* (Han and Cutler 1995), and this was shown to be due to enhanced superoxide anion generation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is very important for the development and activation of neutrophils (Rodriguez-Adrian et al. 1998) and macrophage (Steinbach and Stevens 2003). In murine models, Brummer and Stevens (1987) demonstrated the usefulness of GM-CSF in rescuing dexamethasone-induced suppression of macrophage and synergistically working with TNF-α (in potentiating anti-*Aspergillus* function of macrophage (Bodey 1994)). Rowe et al. (1995) have shown that GM-CSF was effective against aspergillosis in clinical trials, but issues remain (Kong and Levine 1967; Bodey et al. 1993; Groll et al. 1996). However, when compared to other CSFs, studies have shown that GM-CSF is more effective (Kirkland and Fierer 1985; Kirkland et al. 1991) and thus is more promising. In combination with antifungal therapy, GM-CSF administration was shown to improve clinical outcome of *Candida* infection (Vazquez et al. 2000), but controlled trials are the need of the hour as far as GM-CSF is concerned.

Macrophage colony-stimulating factor (M-CSF), important for mononuclear phagocyte functions (Shahid et al. 2010), has been demonstrated to be very effective against *Candida* infections (Mencacci et al. 1996). A study employing rabbits (Gonzalez et al. 2001) has shown that M-CSF is very effective against pulmonary aspergillosis and resulted in higher numbers of activated alveolar macrophages. But its usefulness, when compared to G-CSF or GM-CSF, is less clear (Steinbach and Stevens 2003). Currently, human recombinant G-CSF and GM-CSF are available for clinical use but not so in the case of M-CSF (Hubel et al. 2002). Thus, extensive studies are needed to confirm the effectiveness of these three CSFs as potent immunotherapy agents.

6.5.3 Antibodies

Antibody represents one of the best effector arms of the humoral immune response and is important for establishing a robust secondary immune response. Being highly specific in their action, antibodies are receiving a lot of interest as adjuncts for antifungal therapy. Antibody-mediated immunity (AMI) thus is a potential immunotherapeutic strategy and can be activated by direct mechanism or by indirect mechanism

(Casadevall and Pirofski 2012). In the case of direct action, antibodies can interfere with the fungal pathogen and inhibit *Cryptococcus* virulence production (Martinez et al. 2004) and resistance development (Martinez and Casadevall 2005), *Candida* replication (Brena et al. 2007), gene expression in *Cryptococcus* (McClelland et al. 2010), and metabolism in *Candida* (Brena et al. 2011). By their indirect action, antibodies have been shown to enhance antifungal responses against *Cryptococcus* by inducing phagocytic activity (Schlagetter and Kozel 1990), complement activation (Shapiro et al. 2002), NK cell-mediated killing (Nabavi and Murphy 1986), and proinflammatory responses (Feldmesser et al. 2002). Thus, for both *Candida* and *Cryptococcus*, a variety of monoclonal antibodies have been developed (Ullmer et al. 1993; Beno et al. 1995) but only very few against *Aspergillus* (Torosantucci et al. 2009). In most of these studies, it is clear that administration of mAbs produces a variety of antifungal effects including opsonization (Rachini et al. 2007), inhibition of cell growth (Rodrigues et al. 2000), reduction of capsule/cell wall thickness (Rachini et al. 2007), regulating cytokine production and interplay (Beenhouwer et al. 2001), and in general triggering inflammation (Rivera et al. 2005). But, patient response to mAb administration remains inconsistent since their function is affected by dose and immune parameters of the patient (Casadevall and Pirofski 2006). In addition, the actual antigen load can also influence antibody activity (Casadevall and Pirofski 2003). Under these circumstances, studies have started to show the importance of idiotypic antibodies, especially against *Candida* (Van De Veerdonk et al. 2010), primarily because these produce highly specific immunotherapy and affect the so-called "natural antibody" network.

6.5.4 Transfusion of Leukocytes

Opportunistic infection by fungal pathogens such as *Candida*, *Aspergillus*, and *Cryptococcus* is high in the case of immune-compromised patients, including those with neutropenia. The use of blood transfusion, especially using leukocytes, represents an effective strategy for immunotherapy. In spite of early failures, leukocyte transfusion is gaining importance now due to advances in our understanding of leukocyte development and function. Thus, for example, use of CSFs along with leukocyte transfusion increases the effectiveness of immunotherapy (Casadevall 1995; Deepe 1997). Indeed, clinical trials have shown that the use of G-CSF-pretreated leukocytes for transfusion is very effective against *Candida* (Condon et al. 1996) and *Aspergillus* (Cisalpino et al. 1996) infections. In fact phase 1/2 trial using G-CSF primer leukocytes has been reported to be effective (Cox et al. 1988). Bozza et al. (2004) have reported that transfer of antigen-primed dendritic cells results in enhanced adaptive immune response against *Candida*. In vitro priming of leukocytes, by appropriate cytokine or fungal antigen, and subsequent transfer of the patient represent a simple strategy for antifungal immunotherapy (Bacci et al. 2002). It is recognized that neutrophils are effective against disseminated candidiasis, whereas cell-mediated immunity is triggered against mucosal candidiasis (Deepe 1997). In the case of *Aspergillus* infection of mononuclear cells (Deepe 1994) and cryptococcosis, macrophages (Rohatgi and Pirofski 2015) appear to be important. Tramsen et al. (2007) have shown the effectiveness of anti-*Candida* T cells after transfusion, especially against the hyphal forms and synergize with neutrophils to restrict *Candida* infection (Tramsen et al. 2007). In the case of cryptococcosis, Th1 responses are vital (Hoag et al. 1997) and higher levels of Th1 cytokines such as TNF and IFN are associated with increased fungal clearance (Milam et al. 2007; Wormley et al. 2007). In addition to this, a recent work by Muller et al. (2007) has demonstrated the importance of IL-17 and the associated Th-17 response in immune modulation of *C. neoformans*-infected mice. Moreover, such adoptive transfer of leukocyte also has the added advantage of increasing the activity of specific components of either innate or adaptive

immune system, resulting in a robust response. Thus, it is clear that transfusion of a specific type of leukocyte after priming, based on the nature of fungal infection, could be an important immunotherapy strategy in the case of immune-compromised patients.

6.5.5 Vaccines

Antigens from fungus have been successfully isolated and have been used as vaccine candidates. An excellent review by Casadevall and Pirofski (2012) shows that some of these antigens include β-glucan (Torosantucci et al. 2009) and cell wall glycoprotein (Chaturvedi et al. 2005) from *A. fumigatus*, Hsp 90 (Matthews et al. 1991), aspartyl proteinase (Sandini et al. 2011), phosphoglycerate kinase (Calcedo et al. 2011) from *C. albicans* and melanin (Rosas et al. 2001), glucosylceramide (Rodrigues et al. 2000), β-glucan (Torosantucci et al. 2009), and glucuronoxylomannan (Beenhouwer et al. 2007) from *C. neoformans*. These studies have shown that most of these antigens elicit excellent antibody-mediated immunity in the host and thus are quite successful (for more information, please refer to specific reviews on antifungal vaccines). However, a successful vaccination requires intact cellular components and thus might be an issue in the case of immune-compromised individuals. However, vaccination strategy has worked well in murine models of both systemic and mucosal candidiasis (Torosantucci et al. 2005), using diphtheria toxoid CRM197 and laminarin conjugate. Similarly, vaccine conjugates containing mannan too have been successful against disseminated candidiasis and vaginal infection by *Candida* in mice (Han et al. 1999). The use of inert antigens from the fungal pathogens appears to be preferred since it is safer when compared to attenuated or live fungus. But, such a strategy may not be very effective in terms of the antigenicity or purity of the antigen preparation. Under these circumstances recombinant technology provides an excellent opportunity to precisely select and express the desired epitope to be used as an effective vaccine. Nevertheless, the use of adjuvants, such as cytokines, has opened up huge possibilities in antifungal vaccine development (Deepe 1997), which allows us to modulate the cellular response postvaccination. Another way forward is DNA vaccines, as has been shown for virulent bacterial infections such as *Mycobacterium* (Tascon et al. 1996). But antifungal DNA vaccines are yet to be reported for major fungal pathogens. In addition, extensive studies are needed to analyze antifungal vaccines in humans to better understand the mechanisms involved and the therapeutic effectiveness reached. This will enable us to develop an effective antifungal vaccination for use in the general population.

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