Chapter 13

Immunotherapy for Fungal Infections

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Abstract

Opportunistic fungal infections are a major health problem being appointed by some studies as the fourth main cause of hospital-acquired infection in susceptible populations. The constantly growing incidences of these diseases are associated with the growing number of susceptible individuals, such as immunocompromised individuals (leukemia, AIDS, etc) and treatment-induced immunodeficiency (hematopoietic stem cell, solid organ transplant, anticancer therapy). Furthermore, other advances in medical care, patient's long-term hospitalization and antimicrobial therapies have created several vulnerable populations to fungal infections. Currently, antifungal drug therapies are several times inefficient, and the poor outcomes are linked to difficulties in the early diagnosis of fungal infections and drug resistance among fungal pathogens. In this context, novel therapeutic approaches are welcome to stimulate efficiently the host immune response to eliminate the fungal pathogen. This chapter is intended to review advances in immunotherapy strategies for fungal infections.

Keywords: immunotherapy, vaccination, immune enhancement, fungal infections, antifungal

1. Introduction

Fungi are eukaryotic organisms ubiquitous in the environment, are considered the major decomposers in certain ecosystems, are essential to the survival of many organisms which they can be associated and are sources of food, several enzymes and drugs. There is an estimate of the existence of more than 5 million of fungal species, of that around 70,000 were described [1]. However, only a few hundred of them can also cause disease in healthy and immunocompromised humans [2, 3]. It was accepted that fungi and other pathogenic microorganisms can cause disease all by themselves through four basic conditions, also known as

"virulence factors": (i) tolerance to the host body temperature; (ii) ability to colonize and/or invade the host; (iii) production of secreted components such as toxin and proteolytic enzymes associated with the processes of lysis and absorption of host tissue and (iv) evasion and/or resistance to the host immune system [2, 4].

Based on this germ theory, antimicrobial therapies options targeted these virulence factors and have focused on the development of pharmacological products designed to kill the pathogenic microorganisms and immunological interventions that overcame the deleterious effects of their virulence factors [5]. Interestingly, the Damage-Response Framework theory, which was first proposed in 1999, emphasizes that disease state is not unidirectional and that both the pathogenic microorganism and the host contribute to pathogenicity and virulence [6]. Additionally, recent studies on the human mycobiome, that is, the collection of fungi distributed across and within the body, show that despite being as low as ≤0.1% of the total microbiota, these microorganisms can participate and modify several physiological functions of the host, including the maintenance of microbiota community structure, metabolic functions and in the development and function of the immune system [7].

Fungal infections contribute substantially to human morbidity and mortality, and despite the availability of several antifungal drugs, high rates of mortality associated with invasive fungal infections often exceed 50% [8]. In this context, in order to achieve a reduction in the global burden of fungal infections, is urgent the development of new safer and more effective antifungal drugs [9], as well as novel immunotherapeutic strategies that allow the restoration of the host immune system [10] and maintain or improve the favorable interactions between microbiota and host [11]. In this context, immunotherapy represents a therapeutic modality that attempts to augment host immune response and to control the established infection. In this chapter, we review the principles of antifungal immunotherapy.

2. Immunological aspects of fungal infections

The host defense mechanisms against fungi range from the protective mechanisms provided by skin, mucosa and innate immunity to sophisticated adaptive mechanisms (adaptive immunity), which are specifically induced during the fungal infection/disease. The activation of the innate immunity is the first line of host antifungal defenses and is mediated by phagocytic cells (polymorphonuclear and mononuclear leukocytes and dendritic cells (DCs)), cellular receptors and several humoral factors that act by (i) direct destruction of the fungi through phagocytic process or secretion of microbicide compounds and/or (ii) initiation and subsequent direction of adaptive immune responses through the production of pro-inflammatory mediators (chemokines and cytokines), induction of co-stimulatory activity by phagocytic cells and uptake, processing and presentation of antigens [12, 13].

The components of the fungal cell wall are the first structures that interact with the host innate immune system, and the response to a fungal invasion is initiated through recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) present during infection by pattern recognition receptors (PRRs). The PRRs are

present in the immune cells of the myeloid lineage (dendritic cells, macrophages, monocytes and neutrophils) and nonmyeloid (epithelial and endothelial cells) [12]. The binding of PRR with fungal PAMPs, such as polysaccharides (chitin, α and β-glucans, mannans) and fungal DNA, results in the activation of intracellular signaling pathways promoting phagocytosis, cytokine production, respiratory burst and cell maturation [14].

Several families of PRRs are related to the recognition of cell wall components of fungi through the use of distinct ligand recognition domains, including Toll-like receptors (TLR), C-type lectin receptors (CLR) and proteins of galectins family [12, 15]. The PRRs present in different phagocytic cells initiate intracellular events that promote the activation of the immune system and clearance of fungi, with the specific immune response generated depending on the cell type involved (monocytes, macrophages and neutrophils) [16]. Induction of innate immunity by means of PRR activation provides the basis for developing a subsequent adaptive immune response, and dendritic cells form the interface between the innate and adaptive immune system, since these cell lineages are able to acquire antigens in peripheral tissues, mature and migrate to lymphoid organs, where they provide appropriate signals to T lymphocytes [17].

The adaptive immunity is generated by clonal selection of lymphocytes in response to specific microbial antigens, which can result in the development of immunological memory. The production of cytokines that occurs after the interaction of PRRs with fungal PAMPs drives in the differentiation of naïve T lymphocyte in different subtypes, such as T helper (Th) 1, Th2, Th17 and T regulatory (Treg). During the response process and after the immune activation, T cell subtypes express different pro- and anti-inflammatory chemokines and cytokines, which mediate different effector functions [18].

It is important to emphasize that a fine balance between pro- and anti-inflammatory signals is a prerequisite for successful control of infection. If on one hand, the early inflammation prevents or limits the fungal infection, on the other hand, the uncontrolled inflammatory response may eventually act in opposition to eradicate the disease [19]. Thus, a successful immune response against a fungal infection requires (i) resistance mechanisms, that is, ability to reduce pathogen burden through innate (e.g., dendritic cells) and adaptive (e.g., Th1, Th2, Th9, Th17 and Th22 cells) immunity and (ii) tolerance mechanisms, that is, ability to protect and/or limit the host from immune- or pathogen-induced damage (e.g., Treg cells and enzymes involved in tryptophan metabolism) [18, 20].

The consensus is that Th1 cellular responses are the main defense mechanism of the host against pathogenic fungi, whereas Th2 responses are associated with susceptibility to infections or allergic responses [21]. Th1 cells are predominantly related to protective immunity against fungi and effective antifungal vaccines [12]. Interferon-γ (IFN-γ) is the main cytokine produced by Th1 cells and is important for the stimulation of the antifungal activity of neutrophils [21]. Interleukin (IL)-4, IL-5 and IL-13 are cytokines produced during Th2 immune responses, promote an alternative route of macrophage activation, favor fungal infections and allergic responses associated with the fungus and may be related to disease recurrence [12].

Th17 responses are characterized by the production of IL-17, working in the host defense against certain extracellular bacteria and fungi [22], and are related to protective vaccine responses against experimental fungal infections [23]. However, Zelante et al. [24] described

that IL-23 and the Th17 pathway can also promote inflammation and impair antifungal resistance. Treg cells by IL-10 production have the function of moderating the inflammatory response during infection to limit damage to the host cells and restore homeostasis [25]. However, this response may limit the effectiveness of the protective immune response as a result of the reduction of pro-inflammatory activity generating persistence of the fungus in the tissue and can lead to immunosuppression [21].

3. Cell-based therapies

3.1. Granulocyte transfusion

Polymorphonuclear leukocytes are specialized cells found in the bloodstream that can directly attack microorganisms through phagocytosis, release of soluble antimicrobials and generation of neutrophil extracellular traps, playing an essential role in host defense against pathogenic bacteria and opportunistic fungal pathogens [26, 27]. Granulocyte transfusion is reserved for patients with prolonged neutropenia and life-threatening infections that are resistant to conventional treatment and is intended to improve the side effects of neutropenia and enhance repopulation of granulocytes [28–31].

Early studies of Strumia [32], Brecher et al. [33] and Freireich et al. [34] are among the pioneering research work to propose the infusion of neutrophilic granulocytes from donors as an option to enhance host defenses in patients with neutropenia. Pedersen et al. [35] reported that the combination of granulocyte transfusion with trimethoprim-sulfamethoxazole resulted in the successful treatment of a refractory *Pneumocystis carinii* pneumonia in an 11-year-old girl with chronic granulomatous disease. In a retrospective study, Bhatia et al. [36] evaluated the efficacy of granulocyte transfusion in 87 bone marrow transplant recipients during the first 100 days following the transplantation. No clinical benefit of granulocyte transfusion among 50 of these patients could be shown in the resolution of candidiasis or noncandidal infections. In a meta-analysis of 32 studies, Strauss [37] described that from 63 patients with invasive fungal infections and receiving granulocyte transfusion, only 18 (29%) had successful outcomes.

Based on these and other contradictory results, for a period of time, it has been suggested that there is no strong evidence that granulocyte transfusion consistently brings benefits for the treatment of invasive fungal infections. However, the advances in the cytapheresis technology in the last decades optimize the allogeneic or autologous collection of several types of blood leucocytes to be used in transfusion therapies, which renewed the interest in granulocyte infusions [38–40].

Price et al. [41], in a phase I/II trial of neutrophil transfusions from donors stimulated with granulocyte colony-stimulating factor (G-CSF) and dexamethasone, demonstrated that four of the seven patients with candidemia cleared the infection and, in contrast, none of the patients with aspergillosis (n = 5) or fusariosis (n = 3) were able to clear the infection. Mousset et al. [42] in a prospective, nonrandomized study demonstrated a good clinical efficacy of granulocyte transfusions to prevent recurrence of severe fungal infections during hematopoietic stem cell

transplantation or intensive chemotherapy (23 episodes). However, in a randomized phase III study with 74 neutropenic patients, Seidel et al. [29] concluded that there was no effect on survival of 55 patients with invasive fungal infection up to day 100.

Several case series and case reports [31, 43–46] provide evidence for the safety and feasibility of granulocyte transfusions in patients with severe neutropenia and uncontrolled fungal infections. Thus, although the role of therapeutic granulocyte transfusions remains controversial, future randomized controlled studies will clarify the use of the granulocyte transfusion as a life-saving treatment option [47].

3.2. Dendritic cell therapy

Dendritic cells (DCs) are the bridge between the innate and adaptive immune system by sensing the fungal pathogen via their PRRs, phagocytizing fungal particles, processing and secreting cytokines and chemokines into the environment and presenting antigens to Th cells to induce an adaptive immune response [48]. This remarkable functional plasticity of DCs has been explored for the development of fungal vaccines [49], whereas DCs transfected with yeast cells, yeast RNA or conidia (but not hyphae or hyphal RNA) induce a protective Th1 response [50].

Induction of an adoptive immunity to *Aspergillus* using DCs pulsed with live conidia or transfected with conidial RNA [51] or primed with CpG oligodeoxynucleotides and pulsed with Aspf16 antigens [52] triggers specific and protective Th1 response in murine models of hematopoietic stem cell transplantation (HSCT). DCs transduced with an adenovirus vector encoding the cDNA of IL-12 and pulsed with heat-inactivated *Aspergillus fumigatus* induce a protective response (lower fungal burdens and higher survival rate) against a model of invasive pulmonary aspergillosis [53]. Stimulation of Asp f16-specific T cell responses are more effective by using a protocol of antigen presented on DC followed by Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (BLCL) as antigen-presenting cells [54].

Protection against disseminated candidiasis in mice was observed with DCs pulsed with cell wall proteins expressed during infection, particularly those derived from fructose-bisphosphate aldolase, which induced a robust antibody-dependent protective responses against *Candida albicans* [55]. Bone marrow-derived dendritic cells pulsed with an acapsular *Cryptococcus gattii* strongly induced cytokine-producing CD4 T cells and multinucleated giant cells, and these were associated with a protection against a *Cryptococcus gattii* model of pulmonary cryptococcosis [56].

3.3. Adoptive T-cell transfer

Adoptive T cell transfer is an immunotherapy strategy used to treat cancer and chronic infections by intravenous injection of autologous T cells, which was, after isolated from the donor, stimulated *in vitro* with antigens or modified with a gene encoding a specific antigen receptor and expanded to a large quantity before infusion back into the patient [57]. Th1 lymphocyte responses confer significant protection against fungal infections [18], and the pivotal role of

$CD4^+$ T cell has led to increasing interest and investigation of the use of adoptive transfer of $CD4^+$ cells in the prophylaxis and treatment of invasive fungal infections [58].

Perruccio et al. [59] used heat-inactivated conidia of *A. fumigatus* to generate specific T-cell clones *in vitro*, which was infused soon after hematopoietic transplantation in 35 patients. High-frequency T-cell responses to pathogen within three weeks infusion were associated with control of *Aspergillus* antigenemia and infectious mortality. In contrast, spontaneous pathogen-specific T cells in 46 transplant recipient patients who did not receive adoptive therapy occurred in low frequency as late as 9–12 months after transplantation and displayed a nonprotective, type-2 cytokine profile. However, commonly used immunosuppressants such as cyclosporine A, mycophenolic acid and methylprednisolone decreased the number of anti-*Aspergillus* Th1 cells and the expression of CD154 by anti-*Aspergillus* Th1 cells, which may limit using this type of immunotherapy for organ transplant recipients [60].

Prolonged survival rates were observed in a model of mice invasive pulmonary aspergillosis after the adoptive transfer of splenic $CD4^+$ T cells from mice previously sensitized with a crude culture filtrate antigens of *A. fumigatus* [61]. In BALB/c mice, the cell glucanase Crf1 from *A. fumigatus* induces memory $CD4^+$ Th1 and cross-protection against lethal infection with *C. albicans* [62]. In addition to Crf1, *A. fumigatus* proteins Gel1 and Pmp20 are described as strong inducers of Th1 responses in healthy individuals [63]. Tramsen et al. [64] reported a clinical scale generation of multi-specific antifungal T cells protocol based on the use of cellular fungal extracts of *A. fumigatus, C. albicans* and *Rhizopus oryzae* that allow the generation of numerous activated memory Th1 cells that respond to a broad spectrum of fungal pathogens. The data from these and similar studies [65, 66] support the development of adoptive T-cell transfer protocols for the therapy of multiple microbial pathogens as well as in prophylaxis/vaccination protocols [67].

Despite the main role of $CD4^+$ Th1 cells for host defense against pathogenic fungi, current findings highlight the effector functions of $CD8^+$ T cells against these pathogens [10, 68]. Adoptive transfer of *Aspergillus* f16 peptide-specific $CD8^+$ T cells extended the overall survival time of *A. fumigatus*-infected immunocompromised mice [69], supporting alternative adoptive T-cell treatments for hosts with progressive depletion of $CD4^+$ T lymphocytes and at high risk of invasive fungal infections.

3.4. B cell and natural killer cell treatment

B cells and natural killer (NK) cells are other cell lineages that have been evaluated for its antimicrobial activity through adoptive transfer procedures. Hoyt et al. [70] demonstrated that adoptive transfer of B cells into mice lacking both lymphocytes and type I IFN receptor and with *Pneumocystis murina* lung infection maintained early hematopoietic progenitor activity during immune responses against the infection, thus promoting replenishment of depleted bone marrow cells in an IL-10- and IL-27-dependent manner mechanisms possibly by stimulation of dendritic cells/macrophages.

NK cells are innate lymphocytes that exhibit both adaptive and innate features and that can be activated in the presence of infected cells, allogeneic cells or transformed cells, for acting

on antigen-specific recognition and mounting rapid effector responses such as rapid cytolytic and cytokine activity and antibody secretion [71] as well as in the immunological memory process [72]. Park et al. [73] described that the NK cells mediate their protective effect in the lungs of neutropenic mice with invasive aspergillosis by acting as the most important source of IFN-γ during the early stages of infection. Additionally, the transfer of activated NK cells from a wild-type host to both IFN-γ-deficient and wild-type recipients resulted in a more rapid clearance of *A. fumigatus* from the lungs. Bouzani et al. [74], similarly, concluded that NK cells mediate anti-*Aspergillus* activity through an alternative mechanism involving IFN-γ and tumor necrosis factor (TNF)-α secretion and not through degranulation of their cytotoxic proteins.

NK cells, directly and indirectly (through IFN-γ), showed killing activity against *A. fumigatus* hyphae, but lack activity against infecting conidia [75]. Based on these observations, adoptive immunotherapy with NK cells represents a potential alternative to be used alone or in combination with other antifungal therapies, but should have limited role in prophylactic strategies against aspergillosis.

4. Cytokine therapy

Cytokines are intercellular regulatory polypeptides or glycoproteins that promote growth, differentiation and activation of normal cells and play an essential role on immunomodulation and inflammatory processes. If on one hand, the determination of a patient cytokine profile may indicate the status of the disease, on the other hand, the therapeutic administration of cytokines can result in a favorable immunomodulation for the treatment of autoimmune, neoplastic and infectious diseases [76].

4.1. Colony-stimulating factors (CSFs)

Granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and GM-CSF are used to accelerate myelopoiesis in neutropenic patients and as immune enhancing agents. G-CSF is widely used during chemotherapy neutropenia in clinical practice to prevent immune dysregulation and accelerated functional immune recovery [77]. G-CSF not only increases neutrophil production but also significantly enhanced polymorphonuclear-mediated killing of *A. fumigatus* and *Rhizopus arrhizus* but not against *C. albicans* [78]. In a mice model of experimental disseminated candidiasis, the treatment with recombinant G-CSF (rG-CSF) leads to significantly reduced mortality. However, it is less effective in subacute or chronic disseminated candidiasis. Additionally, combination of rG-CSF and fluconazole results in an additive effect on the reduction of fungal load in the organs [79]. Combination of G-CSF and caspofungin or caspofungin plus amphotericin B-intralipid reduced the fungal burden in organs, decreased the detection of serum galactomannan and increased survival rate up to 78.9% of infected mice with *A. fumigatus* [80]. Favorable clinical response was also observed in 15 of 18 patients with mucormycosis [81]. While G-CSF clearly reduces the neutropenic period of patients, more data about clinical outcomes in fungal infections are needed [82, 83].

Human macrophage colony-stimulating factor (hM-CSF) slightly prolonged survival of lethal *C. albicans* infection in mice and enhanced the efficacy of amphotericin B by enhancing the growth-inhibitory activities of both macrophages and neutrophils against *Candida* [84]. In a neutropenic rabbit model of pulmonary aspergillosis, M-CSF administered prophylactically significantly increased survival and decreased pulmonary injury, probably through increased phagocytosis of *A. fumigatus* conidia by alveolar macrophages [85]. Recombinant human macrophage colony-stimulating factor (rhM-CSF) associated with standard antifungal therapy, into a phase I/II trial, was administrated to 46 bone marrow transplant patients from day 0 to 28 after determination of progressive fungal disease. Survival of these patients was higher (27% *v* 5%) when compared with 58 similar historical controls [86].

GM-CSF significantly enhanced both the killing by neutrophils and monocytes and the collaboration of these cells with voriconazole for killing *C. albicans* [87]. GM-CSF blocks the *in vivo* immunosuppressive effects of dexamethasone on bronchoalveolar macrophages, killing of *A. fumigatus* conidia and suggests its use in patients at risk of pulmonary aspergillosis in the course of dexamethasone treatment [88]. Similarly, Quezada et al. [89] demonstrated that GM-CSF administered intranasally to immunosuppressed mice infected with pulmonary aspergillosis reduced the lung fungal burden compared to the control.

Wildbaum et al. [90] related a patient with chronic mucocutaneous candidiasis that quickly went to a complete clinical remission with improvement in his/her monocyte and neutrophil functions after intravenous GM-CSF treatment (leucomax, 800 mg twice a week). Safdar et al. [91] retrospectively assessed 66 patients in whom GM-CSF was given during antifungal therapy, for which more than half of partial or complete response was observed. Wan et al. [92], in a prospective multicenter randomized phase IV trial with 206 patients, showed that GM-CSG for prophylaxis of infection after allogeneic transplantation was more effective than G-CSF alone in decreasing 100-day cumulative-, transplantation- and infection-related mortalities. Further studies to assess the use and efficacy of CSFs in the treatment of invasive fungal infections are needed.

4.2. Pro-inflammatory cytokines

Several studies demonstrate that immunomodulation with a variety of cytokines can enhance the antifungal activity of neutrophils and monocytes/macrophages as well as upregulation of protective Th1 immune response [93, 94]. Of these, interferon-γ (IFN-γ) produced by T and NK cells is a key cytokine in both the innate and adaptive immune response against invasive fungal infections, for which there are randomized controlled clinical trials [95, 96].

IFN-γ, *in vitro*, effectively primed neutrophils and mononuclear cells for enhanced fungal damage against *A. fumigatus, Fusarium solani* and *C. albicans*, as well as the stimulation of other cytokines [97]. In experimental animal models of fungal infections, IFN-γ had efficacy in a systemic cryptococcosis infection in mice, especially in combination with amphotericin B [98], and enhances host resistance against acute disseminated *C. albicans* in mice [99] and in murine invasive models of aspergillosis [100, 101]. However, inconsistent results were observed in a murine model of candidiasis when different strains of mice were used [102].

In a phase II, double-blind, placebo-controlled study, Pappas et al. [96] evaluated the safety and antifungal activity of adjuvant therapy with recombinant IFN-γ in patients with AIDS and acute cryptococcal meningitis. The results suggested that recombinant IFN-γ induces a more rapid sterilization of the cerebrospinal fluid and better clinical outcome. Similar results were observed in a randomized controlled trial conducted by Jarvis et al. [95]. The addition of short course IFN-γ (100 or 200 μg) to conventional antifungal therapy significantly increased the rate of negative cerebrospinal fluid culture. Guidelines for management of cryptococcal disease suggest that the adjunctive immunological therapy with recombinant IFN-γ can be considered for refractory cases [103].

The adjunctive immunotherapy with recombinant IFN-γ partially restored cell-mediated immunity and enhanced antifungal immunity in a prospective case series describing eight patients with invasive *Candida* or *Aspergillus* infections [104]. Other case reports have described the successful treatment of invasive aspergillosis with the combination of IFN-γ and antifungal therapy [105–108]. Adjunctive therapy with interferon-γ, GM-CSF or G-CSF showed good functional outcome in a case of *Scedosporium apiospermum* otomastoiditis and in a case of *Mucor* sinusitis and orbital cellulitis refractories to treatment with conventional antifungals [109]. However, for invasive mold infections, more data and randomized controlled clinical trials are needed.

IFN-γ, in combination with antimicrobials drugs, is currently recommended in prophylaxis of patients with chronic granulomatous disease (CGD), a population for which invasive filamentous fungal infections are a persistent problem [108, 110].

IL-12, IL-15 and TNF-α are other pro-inflammatory cytokines that have been assessed in preclinical trials as candidate adjuvant since they also upregulate the antifungal Th1 response [94]. IL-12 plays an obligatory role for the development of a Th1 response to *Candida* [111] and *Cryptococcus neoformans* [112], enhances the antifungal capacity of monocytes against *A. fumigatus in vitro* and has also been explored in immunotherapeutic proposals for various animal models of cryptococcal infections [113]. IL-12 gene therapy enhanced the host response against experimental coccidioidomycosis [114] and accelerated the clearance of infection in a murine *Pneumocystis* pneumonia model [115]. However, the use of IL-12 as immune enhancer remains controversial because this cytokine may paradoxically increase the susceptibility of the host to fungal pathogens [116, 117].

IL-15 is involved in the innate immunity against fungal infections and, similarly like IL-12, enhances the antifungal activity of granulocyte or monocyte cells against *C. albicans, A. fumigatus, Fusarium* spp. and *Scedosporium* spp. [118–121]. Although IL-15 has been potential as a new therapeutic option against invasive fungal infections, more information from future preclinical and clinical trials is needed.

TNF-α is necessary for the development of effective immunity to fungal infections [94], enhancing the activity of granulocyte cells against *A. fumigatus, C. albicans* and *Cryptococcus neoformans* [93, 122–124]. In a murine invasive pulmonary aspergillosis model, antibody-mediated neutralization of TNF-α increases mortality, whereas the intratracheal administration of a TNF-α agonist peptide improved survival [100]. Additionally, stimulatory/protective effect of TNF-α is also described against cryptococcal infections [113].

4.3. Other cytokines

Several other cytokines have been shown to be involved in the immune process during fungal infections and may be targets for future immunotherapy proposals. Gresnigt et al. [125] described the biological relevance of IL-36 in a pathway involved in the induction of Th responses by *A. fumigatus*. Nlrp3, Asc and caspase-1 mediated *Paracoccidioides brasiliensis*-induced IL-18, and the activation of the inflammasome is associated with a strong Th1-mediated immune response and, consequently, host antifungal defense against *Paracoccidioides brasiliensis* [126]. IL-7 is potent immunotherapeutic that acts at multiple levels to improve host immunity. In mice infected intravenously with *C. albicans*, the treatment with IL-7 weakened the infection and improved the host survival rates [127]. IL-18 contributes to host defense against *Cryptococcus neoformans* and *Paracoccidioides brasiliensis* [126, 128].

5. Antibody-based therapy

Antibodies or immunoglobulins are heterodimeric proteins composed of two heavy and two light chains, which are associated with the specific humoral immunity and primary defense against several infectious diseases [129–132]. The protective potential of antibodies produced during fungal infections can be accomplished by indirect and direct mechanisms and can vary depending on certain factors, such as the isotype, subisotype and title of antibodies and major histocompatibility complex background of the host [132, 133].

The interest in the potential benefit of antibody-based therapy for invasive fungal infections starts with Dromer et al. [134] describing that the intraperitoneal administration of a monoclonal IgG1 anti-*Cryptococcus neoformans* antibody could be used as a passive serotherapy, participating in the prevention or treatment of experimental cryptococcosis and with Gigliotti and Hughes [135] describing that the use of the monoclonal antibody (mAb) M5E312 was capable of hindering the development of an experimental murine *Pneumocystis carinii* infection.

Efungumab (Mycograb®) and the 18B7 (mAb) are two examples of antifungal mAbs evaluated in clinical trials. 18B7 mAb is a murine IgG1 that demonstrated acceptable safety in a phase I dose-escalation study in subjects with treated cryptococcal meningitis [136], but efficacy data for this therapy have not been generated. However, as reviewed by Larsen et al. [136], the administration of mAb against *C. neoformans* capsular polysaccharide to infected mice prolonged survival, reduced tissue burden, enhanced granuloma formation and enhanced antifungal activity of amphotericin B, fluconazole and flucytosine. These data support the continued investigation of this mAb.

Efungumab (Mycograb®) is a human genetically recombinant antibody that binds to the *Candida* heat-shock protein 90 (HSP90), preventing a conformational change needed for fungal viability [137]. Mycograb® showed protective activity against several *Candida* species and synergized with antifungal drugs when evaluated *in vitro* and in preclinical studies [138–140]. In a clinical trial, the treatment with lipid-associated amphotericin B in combination with Mycograb® produced significant clinical improvement in outcome for patients with invasive

candidiasis [141] when compared with the lipid-associated amphotericin B monotherapy. However, due to production difficulties, safety and quality issues, the drug was refused to grant marketing authorization.

Another immunoglobulin-based strategy was the production of anti-idiotypic monoclonal antibodies [142]. These mAbs that specifically reacting with killer toxins (KT) from *Pichia anomala* and *Williopsis mrakii* have broad antimicrobial spectrum and demonstrated *in vitro* similar activity to polymorphonuclear neutrophils against the hyphae and germinated conidia of *A. fumigatus* and *in vivo* protected immunocompromised mice with invasive aspergillosis from infection [143]. Similar results were observed against *C. albicans in vitro* and in vaginal and systemic murine models of candidiasis [144, 145].

Radioimmunotherapy uses the interactions between a fungal antigen and antibodies labeled with radionuclides to deliver cytocidal amounts of ionizing radiation to the specific target [146]. The advantages attributed to this immunotherapeutic method over standard antifungal therapy include: (a) it completely destroys the target cell by lethal radiation, delivers without the need of interaction with specific metabolism of the pathogen; (b) it is less subject to drug resistance mechanisms and does not suffer the drug-drug interactions that can be observed with some antifungals and others drugs; (c) it may permit single or a limited number of doses for the treatment of fungal diseases in contrast to weeks, months or years required to combat certain mycoses with antifungal drugs; (d) mAbs can be radiolabeled to bind antigens shared by many pathogenic fungi, such as heat-shock protein 60, β-(1,3)-glucan, ceramide and melanin [146–148].

Protection against experimental *Cryptococcus neoformans* and *Histoplasma capsulatum* infections was described using the radioimmunotherapy, whose mechanisms include killing of microorganism cells by "direct hit" and "cross-fire" effects, promotion of apoptosis-like death, cooperation with macrophages and modulation of the inflammatory response [149, 150]. The effects of this therapy on bystander mammalian cells demonstrated minimal effects on host cells [151]. Reviews conducted to discuss the efficacy, toxicity, radiation resistance, radiobiological mechanisms and comparison with standard antifungal treatments [152] and the possibility of developing "panantibodies" for a universal treatment of the fungal diseases [148] suggest that this immunotherapeutic modality is a promising alternative for the treatment of invasive fungal infections.

Antibodies like anti-β-glucans demonstrated protection against *A. fumigatus*, *C. albicans* and *Cryptococcus neoformans* [153–155]. Similarly, anti-melanin antibodies inhibit the growth and protection against *C. neoformans* and *Fonsecaea pedrosoi* and potential cross-resistance against various other fungi [156, 157].

Although most studies evaluate the antibodies that neutralize or kill the fungal pathogens [132], is growing the interest in the development of antibodies that can modulate the immune system and bring benefits to the host. For instance, through different immunomodulatory processes, 3B4 antibody protected mice from *C. albicans*-induced death in passive immunization [158], an agonist antibody CD40 prolonged the survival time of mice infected with *Cryptococcus neoformans* [159], anti-CD25 treatment decreased disease severity in the progressive and regressive forms of paracoccidioidomycosis [160], anti-PD-1 and anti-PD-L1

mAbs improved survival in fungal sepsis by *C. albicans* [161] and anti-CD3 antibody rapidly reversed the pathologic immune response caused by *Pneumocystis carinii* in a murine model of pneumonia [162].

6. Antifungal vaccines

The evaluation of antifungal vaccines has a great interest, but their development is challenging [10]. Antifungal vaccines are becoming a need in clinical settings, principally of immunocompromised and debilitated patients who are more prone to develop aggressive fungal infections. However, its use is limited by the weak immune response of these patients to respond vigorously to vaccination [163]. In this way, researchers made considerable progress in the last years, leading to more specific and well-characterized vaccines. Indeed, many antifungal vaccine candidates had been reported, and some have undergone preclinical evaluation and at least two are on the phase I clinical trials [164].

Studies on vaccination against fungal infections are conducted with whole-cell inactivated, live or attenuated fungi, cell wall subunits and the transfer of passive or adoptive immunity [165]. Usually, whole-cell inactivated vaccines are poorly immunogenic and have the disadvantage of having a complex chemical composition making it difficult to standardize [163]. In contrast, live virulence-attenuated vaccines are the best immunogens to achieve protection, but are unsafe in immunocompromised or otherwise debilitated patients, and even attenuated microorganisms can sometime cause disease in this subset [165]. In this way, subunit vaccines could be the best choice with regard to standardization and safety, but it lacks the natural adjuvant properties of whole-cell or live vaccines and usually needed an adjuvant to increase immunogenicity [10]. In animal models, the use of adjuvants is not a problem, since various adjuvants, such as Freund and aluminum hydroxide among others, have been very useful, but in humans, there is a scarcity of good adjuvants suitable for use in clinical practice [165]. Several adjuvants have been tested in recent years like the use of liposomes, virosomes, fungal immunogenic moieties and other bioengineered preparations and have proven useful [165, 166].

Protective immunity triggered by vaccination depends on both T-cell responses, particularly Th1 and/or Th17, and antibody responses which in turn are dependent on the kind of immunogens delivered to immune system [165]. A study conducted with a hyphal sonicate of *A. fumigatus* administered subcutaneously in corticosteroid immunosuppressed mice demonstrated that it was capable of conferring protection against invasive pulmonary aspergillosis [167]. Several studies have been conducted using the culture hypha filtrate, sonicated or vortexed hypha and demonstrated some degree of protection [168–171]. PitiumVac®, a licensed immunotherapy to treat equine pythiosis in Brazil, is an example of the use of disrupted hyphae, and its use reaches a cure rate of 70–80% [172, 173]. Despite the good curative properties of PitiumVac®, it does not present protective activity [174].

Various investigations have reported that heat-killed yeasts (HKY) of *Saccharomyces cerevisiae* given subcutaneously in mice are protective against fungi from five genera: *Aspergillus,*

Coccidioides, Cryptococcus, Candida and *Rhizopus* [175–179]. Analysis of the underlying immune responses associated with HKY-induced protection of the host suggested that HKY vaccination induces significant and specific Th1 response and antibodies to glucan and mannan [180]. The components of HKY responsible for the cross-protective response against these infections are not known, but several homologous proteins and key cell wall glycan components shared among fungi have been described [177, 181, 182]. These results in combination with studies that demonstrated the safety of yeast-based vaccines in humans are suggestive that a pan-fungal yeast-based vaccine is possible [178, 183].

A recombinant live attenuated strain of *Blastomyces dermatitidis* null for the adhesion BAD1 (identified as a virulence factor) given subcutaneously as a vaccine protects mice from a lethal blastomycosis infection, and a Th1 response was linked with vaccine-induced resistance [184]. A study about safety, toxicity and immunogenicity of this vaccine in dogs proved to be save, well tolerated and the cytokine profile observed belonging to Th1 response (INF-γ, TNF-α and GM-CSF) [185]. Subsequent study, with this same vaccine, demonstrated that a Th1 response is dispensable and that Th17 cells are sufficient for vaccine-induced protection against a lethal pulmonary blastomycosis in mice [23]. Indeed, other two different live attenuated vaccines prepared with strains of *Histoplasma capsulatum* and *Coccidiodes posodassi* were tested and were found to protect mice from these endemic mycoses by a mechanism dependent upon Th17 cells [23, 186]. Another experimental live attenuated vaccine has been tested against hematogenously disseminated candidiasis, and it is based on a genetically engineered *C. albicans* tet-NRG1. Under certain conditions, this strain remains in the yeast phase and is nonpathogenic when administered to mice as a vaccine and resulted in substantial protection from virulent strain [187].

7. Fungal antigens

Components from fungal cell are able to modulate the Th response, particularly the molecules from their cell wall components, notably carbohydrates and glycoproteins, which are related to the induction of a Th1 and/or Th17 responses [12, 188]. These carbohydrates include β-glucans (β-1,3-linked polymers of glucose with β-1,6 branches), chitin (homopolymer of N-acetylglucosamine) and mannans (mannose chains of varying lengths and configurations added to fungal proteins through N- or O-linkages) [16]. In addition, some fungal proteins appear to activate monocytes and can be used as adjuvants in specific immunotherapy.

Fungal β-glucans activity has been researched for over 50 years with the focus principally on the glucans from yeasts (*S. cerevisiae*) and mushrooms (*Lentinan edodes, Schizophyllun commune* and *Ganoderma lucidum*) [189, 190]. Most of the fungal β-glucans markedly stimulate the immune system, and they are considered as biological response modifiers with pronounced immunomodulating activity against infectious disease and cancer [191]. Different immunological pathways have been related to the biological activities of β-glucans, as well as improvement in phagocytosis and proliferative activities of professional phagocytes (i.e., granulocytes, monocytes, macrophages and dendritic cells), T and NK cells stimulation, and

activation of the alternative pathway of complement [192]. In mammals, β-glucans are recognized by dectin-1 and complement receptor 3 (CR3), and its preparations derived from fungi have a record of safety in both preclinical and human trials [193, 194].

Torosantucci et al. [154] were the first to link the use of a β-glucan and the induction of protective antibodies against different fungal infections. In their study was used the algal glucan laminarin conjugated with the diphtheria toxoid CRM197 for mice immunization that showed the ability of this glycoconjugate vaccine to confer protection against both lethal infections of *C. albicans* and *A. fumigatus*. This ability of a nonfungal β-glucan to induce specific anti β-glucan antibodies against two different pathogenic fungi highlights the possibility of the development of a single vaccine protecting against different fungal infections [195, 196]. This β-glucan CRM197 conjugated vaccine is in preclinical phase of development for aspergillosis, candidiasis and cryptococcosis [197]. Recent works using highly purified particulate β-glucan from *S. cerevisiae* alone or conjugated with bovine serum albumin (BSA) demonstrated the pluripotent activity of this vaccine in protection against experimental aspergillosis and coccidioidomycosis [198, 199]. Once β-glucans are highly conserved molecules, common to many fungi and that are able to induce protective antibodies, this implies that β-glucan would be an important vaccine component. Therefore, all these findings provide the basis for the future development of a pan-fungal conjugate vaccine [197].

Mannans, another carbohydrate from the fungal cell wall, have been implicated a long time ago with the host protective response against candidiasis [200]. Pioneering studies demonstrated that a vaccine containing a liposome-encapsulated mannan from *C. albicans* was protective in disseminated candidiasis in mice and that the specific antibodies produced were at least partly responsible for the protection with a β-1,2-linked mannotriose being the active epitope [201, 202]. Liposomal encapsulation was required because the extract alone was poorly immunogenic, and in an attempt to improve this formulation, new studies were made by conjugating mannan extract to protein (BSA) [203, 204]. This same *C. albicans* mannan conjugated to BSA presented cross-protective activity against systemic murine aspergillosis [205]. A study with *S. cerevisiae* mannan demonstrated the change of a T-cell-independent response when the polysaccharide was administered alone for a T-cell-dependent response when it was conjugated to human serum albumin (HSA). This study was conducted in mice and showed the increase in specific IgG isotype antibodies, mainly Th1 associated (IgG_{2a} and IgG_{2b}), making it a vaccine candidate for preventive immunomodulation treatment [206]. A synthetic β-mannan trisaccharide conjugated to different peptides found in *C. albicans* cell wall proteins was demonstrated to induce protective immunity in mice against candidiasis [55]. A tricomponent conjugate vaccine that associated the β-mannan trisaccharide, tetanus toxoid and laminarin was capable to promote multiple immune pathways leading to DC activation, induction of Th17 response and a potent immunization [207].

Glucuronoxylomannan (GXM), the major capsular polysaccharide of *Cryptococcus neoformans*, exerts many immunoregulatory effects, and in humans, the development of anticapsular antibodies is correlated with improved prognosis [208]. Conjugated vaccines of GXM-tetanus toxoid elicit protective antibodies in mice [209, 210], although deleterious antibodies can also be induced [211, 212]. This contradictory effect (protective *v* deleterious antibodies) can be

avoided using GXM epitopes that elicit only protective antibodies, and it was observed coupling a derived GXM heptasaccharide to a protein carrier [212] and this vaccine is in phase I clinical trial [197]. The mimotope-based immunization can elicit an antibody response to a protective epitope on the native antigen [213]. The GXM peptide mimotope P13 conjugated to a protein carrier has demonstrated its effectiveness in prolonging survival of mice after a lethal challenge with *C. neoformans*, and the protection was associated with a reduction of serum levels of GXM and the production of antibodies to GXM [214–216].

Once carbohydrates are known to produce T-cell-independent immune responses with a poor-quality antibody response, the conjugation with immunogenic protein carriers is a strategy to overcome this poor immunogenicity [217, 218]. Immunization with glycoconjugated vaccines elicit T cell help for B cells that produce IgG antibodies and can induce memory B-cell development and T-cell memory [219]. The main carrier proteins used in licensed conjugate vaccines are the enzymatically inactive and nontoxic variant of diphtheria toxin (CRM197), diphtheria toxoid (DT) and tetanus toxoid (TT) [220, 221].

On the other hand, protein antigens are highly immunogenic, elicit a strong T-cell response and are biochemically defined, which facilitate the large-scale production of recombinant proteins [222]. Indeed, two vaccines based on recombinant proteins of *C. albicans* are under phase I clinical trials [165]. The first vaccine is based on a recombinant N-terminus of the candida adhesion, Als3p (rAls3p-N) with alum as adjuvant, and elicits Th1 and Th17 cells [223]. The target of this vaccine included systemic and mucosal candidiasis, and the study in human subjects has been performed by NovaDigm Therapeutics [224]. Data from safety and immunogenicity have been posted on the web (www.novadigm.net). The second vaccine is based on the recombinant secretory aspartyl proteinase2, Sap2 [225] and appears to confer protection by Sap-neutralizing antibodies. This active vaccine, named PEV7, is targeted to prevent recurrent vulvovaginitis and is being developed by Pevion Biotech. Initial report about safety and immunogenicity profile of PEV7 in women is strongly encouraging (www.pevion.com).

Protein and glycoprotein from different pathogenic fungi have been studied, and particularly, heat-shock proteins (HSPs) represent an attractive candidate due to their association with both innate and adaptive immunity [226, 227]. Immunization with HSPs from *Paracoccidioides brasiliensis* has been shown to provide some degree of protection against experimental disease [228, 229]. Protective immune response has been also observed with native or recombinant (r) HSP60 from *Histoplasma capsulatum*, and rHSP60 reduced fungal burden and improved survival in experimental pulmonary histoplasmosis [230, 231]. Immunization with whole glycoprotein gp43 from *P. brasiliensis* demonstrated a dual response, inducing Th1 and Th2 cells, whereas the P10 (15-mer peptide derived from gp43) elicited Th1 response that protected mice from experimental paracoccidioidomycosis [232].

Summarizing all these studies with several pathogenic fungi has shown that antibodies against cell surface molecules could be implicated in the protective response to infection. The goal is to identify which molecules or epitopes are able to produce only protective antibodies and not those isotypes that inhibit host defenses or exert a negative effect on immunity. Despite a growing medical need and all efforts made by researchers, there is still no approved vaccine against any fungal disease.

8. Conclusion

Breakthroughs in our understanding of how homeostasis is established, maintained or disrupted during fungal exposure and/or colonization should help to guide the development of new therapeutics that target specific inflammatory or metabolic end points. For example, limiting inflammation—through PRR agonism or antagonism—to stimulate a protective immune response to fungi should pave the way for the rational design of novel immunomodulatory therapies.

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