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VACCINES AND IMMUNOTHERAPY AGAINST FUNGI: THE NEW FRONTIER

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Joshua D. Nosanchuk and
Carlos P. Taborda

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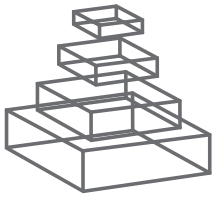
MICROBIOLOGY



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ISSN 1664-8714
ISBN 978-2-88919-116-1
DOI 10.3389/978-2-88919-116-1

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VACCINES AND IMMUNOTHERAPY AGAINST FUNGI: THE NEW FRONTIER

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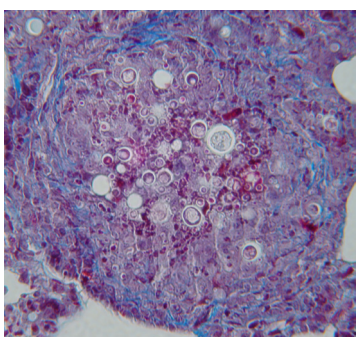

Invasive fungal diseases have increased many fold over the past 50 years. Current treatment regimens typically require prolonged administration of antifungal medications that can have significant toxicity. Moreover, our present potent antifungal armamentarium fails to eradicate fungal pathogens from certain compromised hosts. Additionally, invasive fungal diseases continue to have unacceptably high mortality rates. A growing body of work has focused on the utility of vaccines and/or immunotherapy as a powerful tool in combating mycoses, either for the active treatment, as an adjuvant, or in the prevention of specific fungal pathogens. This Research Topic will detail the exciting progress in developing vaccines and immunotherapy for fungi.

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frontiers in
MICROBIOLOGY

EDITORIAL
published: 25 January 2013
doi: 10.3389/fmicb.2013.00006

Vaccines and immunotherapy against fungi: the new frontier

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Invasive fungal diseases have increased many fold over the past 50 years. Current treatment regimens typically require prolonged administration of antifungal medications that can have significant toxicity. Moreover, our present potent antifungal armamentarium fails to eradicate fungal pathogens from certain compromised hosts. Additionally, invasive fungal diseases continue to have unacceptably high mortality rates. A growing body of work has focused on the utility of vaccines and/or immunotherapy as a powerful tool in combating mycoses, either for the active treatment, as an adjuvant, or in the prevention of specific fungal pathogens. This Research Topic "Vaccines and Immunotherapy against fungi: a new frontier" in *Frontiers in Fungi and their Interactions* details the exciting progress in developing vaccines and immunotherapy for fungi.

The critical requirement for understanding the degrees of engagement of host defense pathways in responding to fungal invasion has led to an increased focus on host-pathogen interactions. In this Research Topic, Carvalho et al. (2012) review our current progress on this endeavor and underscore the need for coordinated cross-disciplinary future efforts. A major focus of the special Topic is the advance of vaccine strategies against major fungal pathogens. To this extent, the issue focuses on developments in vaccine strategies against *Candida albicans* (Vecchiarelli et al., 2012), *Aspergillus fumigatus* (Diaz-Arevalo et al., 2012), *Cryptococcus neoformans* (Hole and Wormley, 2012), and *Paracoccidioides brasiliensis* (Travassos and Taborda, 2012). Progress in optimizing adjuvants for a vaccine against *P. brasiliensis* is also presented (Mayorga et al., 2012). Shifting host responses to facilitate fungal clearance is shown in work utilizing ArtinM, a D-mannose binding lectin from *Artocarpus heterophyllus*, which modulates immunity against *P. brasiliensis* (Ruas et al., 2012). Along this line, information is presented regarding the immunomodulatory effects of fungal immunogens and how they impact disease (Rodrigues and Nimrichter, 2012). The utility of antibody based therapeutic approaches is presented against a specific fungus, *Histoplasma capsulatum* (Nosanchuk et al., 2012), and as a broad-spectrum therapeutic using antibody labeled with fungicidal nuclides (Nosanchuk and Dadachova, 2012). A therapeutic monoclonal antibody in early phase research for sporotrichosis is also detailed (Almeida, 2012). Moreover, the broad potential of antibody-derived "killer peptides" is presented (Magliani et al., 2012). Finally, new information about the antifungal activity of "old" drugs is discussed. Hydroxyurea is shown to impact the sphingolipid pathway underscoring the role of these compounds in fungal biology (Tripathi et al., 2012). The diverse activities of Amphotericin B on both fungi, impacting ergosterol stability and cellular morphology, and host cells, engaging pattern receptors, is critically summarized (Mesa-Arango et al., 2012).

In sum, these articles broadly paint the current spectrum of investigations on host-pathogen interactions and provide a review of the state-of-the-art in vaccinology and immunotherapy against fungi. The information presented also underscores the rich areas for future study, all promising improved therapeutics against fungal invaders.

ACKNOWLEDGMENTS

The authors sincerely acknowledge the efforts of all of the authors who contributed to this Research Topic. Joshua D. Nosanchuk is supported in part by NIH grant AI52733 and an Irma T. Hirschl/Monique Weill-Caulier Trust Research Award. Carlos P. Taborda is supported by grants 07/58750-0, 2011/17267-4 from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Received: 22 November 2012; accepted: 06 January 2013; published online: 25 January 2013.

*Citation: Nosanchuk JD and Taborda CP (2013) Vaccines and immunotherapy against fungi: the new frontier. Front. Microbio. **4**:6. doi: 10.3389/fmicb.2013.00006*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 10 May 2012
doi: 10.3389/fmicb.2012.00176

Host defense pathways against fungi: the basis for vaccines and immunotherapy

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Fungal vaccines have long been a goal in the fields of immunology and microbiology to counter the high mortality and morbidity rates owing to fungal diseases, particularly in immunocompromised patients. However, the design of effective vaccination formulations for durable protection to the different fungi has lagged behind due to the important differences among fungi and their biology and our limited understanding of the complex host–pathogen interactions and immune responses. Overcoming these challenges is expected to contribute to improved vaccination strategies aimed at personalized efficacy across distinct target patient populations. This likely requires the integration of multifaceted approaches encompassing advanced immunology, systems biology, immunogenetics, and bioinformatics in the fields of fungal and host biology and their reciprocal interactions.

Keywords: fungi, immune responses, vaccines, immunotherapy, vaccinomics

INTRODUCTION

Fungal diseases are epidemiological hallmarks of distinct settings of at-risk patients, not only in terms of their underlying condition but in the spectrum of diseases they develop (Segal, 2009). Although fungi are responsible for pulmonary manifestations and cutaneous lesions in apparently immunocompetent individuals, their impact is most relevant in patients with severe immune dysfunction, in which they can cause severe, life-threatening forms of infection. As an increasing number of immunocompromised individuals resulting from intensive chemotherapy regimens, bone marrow or solid organ transplantation, and autoimmune diseases has been witnessed in the last decades, so has the incidence of fungal diseases (Segal, 2009). Therefore, fungal vaccination has been regarded as a particularly promising strategy in these groups of highly susceptible individuals. Indeed, the fact that a number of well-defined risk factors manifest before the onset of infection affords a window of opportunity to vaccinate. However, many challenges confront the development of fungal vaccines for humans. Among them, the insufficient understanding of the critical immune defects that predispose to pathogen-specific vulnerability in primary or secondary immunodeficient patients and the historical assumption that the immune system of these patients would not respond properly to strategies relying on immunological memory. However, it is noteworthy that the immunogenicity and efficacy of vaccines has been confirmed even in patients with profound lymphocyte defects, such as the case of human immunodeficiency virus (HIV)-infected patients (Klugman et al., 2003). However, a further degree of complexity has been recently provided by the acknowledgment that immune responses critically rely on individual genetic profiles (Carvalho et al., 2010). Hence, and despite the obvious advantages of "universal vaccine" strategies to address protection from fungi (Cassone and Rappuoli, 2010), immunogenetic-based approaches have also revealed the significant contribution of the host's genetic background to efficient vaccine responses (Carvalho et al., 2012b), thereby suggesting that a more personalized approach would ultimately be of additional interest. The purpose of this review is therefore to present an update of concepts relevant for the design of ideal antifungal vaccines and the challenges faced in delivering them to specific target populations.

DECODING ANTIFUNGAL IMMUNITY INTO VACCINATION STRATEGIES

Although the global incidence of fungal diseases is currently rivaling those of many of the best known bacterial diseases, humans have coevolved with ubiquitous or commensal fungi in host–fungus relationships that for the most part are positive or neutral (Romani, 2011). This is illustrated by a number of cases, including that of *Candida albicans*, *Pneumocystis jiroveci*, and *Malassezia* spp., that live as benign commensals in one or more body locations in a majority of healthy individuals. As opportunistic pathogens, they are poised to overgrow cavities and penetrate tissue in response to alterations in host physiology that presumably compromises the complex mechanisms of immune adaptation that normally suppress their growth. Most fungi, however, such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, and the thermally

dimorphic fungi (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Coccidioides immitis*, *Penicillium marneffei*, and *Sporothrix schenckii*) are found ubiquitously in nature and can cause a wide spectrum of diseases ranging from acute pulmonary manifestations and cutaneous lesions in immunocompetent individuals to allergic syndromes and severe life-threatening infections in patients with primary or secondary immune dysfunction.

It is now clear that the clinical manifestations of a given fungal disease depend, to a great extent, on the immune ability of the host (Casadevall and Pirofski, 2003; Romani, 2011). Indeed, the paradoxical association of fungal diseases with either deficient or hyper-reactive states of immune activation is closely related with the two types of defense mechanisms a host can evolve to increase its fitness when challenged with a pathogen: resistance and tolerance (Schneider and Ayres, 2008). Mechanisms of resistance delineate the host's ability to limit the fungal growth by directly countering pathogens through recognition and elimination systems. Mechanisms of tolerance, however, regulate the self-harm that can be caused by an overactive immune response and other mechanisms not directly related to immune resistance. Given the different pathological and epidemiological effects these mechanisms may prompt, a further detailed understanding of the wide spectrum of host–pathogen interactions and immune responses will ultimately be paramount for the design of effective vaccination formulations affording comprehensive and durable protection to different fungi (**Figure 1**). The design of fungal vaccines is however not only constrained by the nature of the target populations, which may be genetically and immunologically different – not necessarily immunocompromised – but also by the dynamics of fungal diversity. Indeed, and even considering the premise that a fungal vaccine would be feasible even in patients with severe immune dysfunction (Spellberg, 2011), no examples can be cited up until today. Attempts at fungal vaccination have been restricted to pre-clinical research essentially because of safety concerns, as complex and ill-defined antigenic mixtures do not cope with present day safety restrictions. However, whole genome sequencing and proteomic approaches have made available most – if not all – fungal proteins, thereby allowing the selection of a discrete number of fungal antigens to test for protection. This has directed interest in subunit antigens, which however lack the natural adjuvant properties of whole-cell or live vaccines, and consequently optimal immunogenicity properties. In addition, the human microbiota and their role in programming human metabolism is currently emerging as a key component required for the definition of immune responses to fungi, in particular adaptive immunity (Littman and Pamer, 2011). Thus, the symbiotic relationship of the microbial species with the host requires a tuned response that prevents host damage while tolerating the presence of potentially beneficial microbes, meaning that the host and the fungus exert control over each other in a way that fungal commensalism ultimately benefits the host (Bonifazi et al., 2009). As a corollary, the shaping of intestinal and lung immune responses by microbiota to achieve protection to vaccines will likely become an area of intense research.

The growing understanding that fungal pathogens may thrive in regulatory environments has to be integrated within the protective immune responses developed in a context of vaccination. Although protective immunity may be accomplished by means of preventing regulatory T (T_{reg}) cell induction or function – as T_{reg} cells can indeed control the intensity of secondary responses to fungal infections (Cavassani et al., 2006; Deepe and Gibbons, 2008; Loures et al., 2009) – their presence upon secondary antigen exposure may prevent immunopathology in the context of vaccination and favor long-term memory (Romani and Puccetti, 2006; Bozza et al., 2009). This notion is crucially exemplified in infections spawned by the reactivation of latent commensal organisms, in which a vaccine candidate is expected to elicit protective memory responses in a T_{reg}-rich environment – that is, long-lasting sterilizing immunity through the generation of effector T cells is not needed here. This can be achieved by concurrently focusing on effector mechanisms of resistance as well as on manipulation of tolerance to restrain immunopathology. However straightforward this approach may sound, these mechanisms lie on a precarious balance that may differ with each fungal pathogen and even sites of infection. This demands for a fine prediction and definition of fungal antigens and adjuvants that trigger the most appropriate classes of resistance and tolerance mechanisms as well as the selection of sites for vaccination where their contribution to protective memory could be properly and most significantly achieved.

FUNGAL VACCINES: CHALLENGES AND PROMISES

A successful vaccination relies on the eliciting of pathogen-specific immune responses and consequent immunological memory that mediates long-term protection from infection or disease. A plethora of chemical and antigenic formulations has already been considered for active vaccination against all major fungal pathogens in pre-clinical models of infection (**Table 1**; Cassone, 2008) and it is well accepted nowadays that the immunogenic potential of fungal stimuli critically relies on their innate immune recognition, particularly by pattern recognition receptors (PRRs). The most well-known PRRs for fungi include Toll-like receptors (TLRs), C-type lectin receptors and the nucleotide binding domain leucine-rich repeat containing receptors which detect a vast array of fungal molecules or danger signals (Romani, 2011). In this regard, systems biology analyses of naïve to effector to memory transition has revealed changes in expression of innate immune receptors to be one major early molecular signature upon vaccination (Pulendran et al., 2010).

Given the array of fungal ligands present at the cell surface, as well as those that become available to immune sensing upon processing of the fungus by phagocytic cells, it is now clear that vaccine-induced protection to attenuated fungal strains occurs through distinct PRRs and downstream signaling adapters (Wuthrich et al., 2011; De Luca et al., 2012). For instance, T helper (Th)17-induced acquisition of vaccine immunity to live attenuated strains of *B. dermatitidis*, *H. capsulatum*, and *C. posadasii* was found to require myeloid differentiation primary response gene 88 (MyD88) signaling (Wuthrich et al., 2011), whereas Th1-induced protection to *A. fumigatus* relied on TIR-domain-containing adapter inducing interferon-β (De Luca

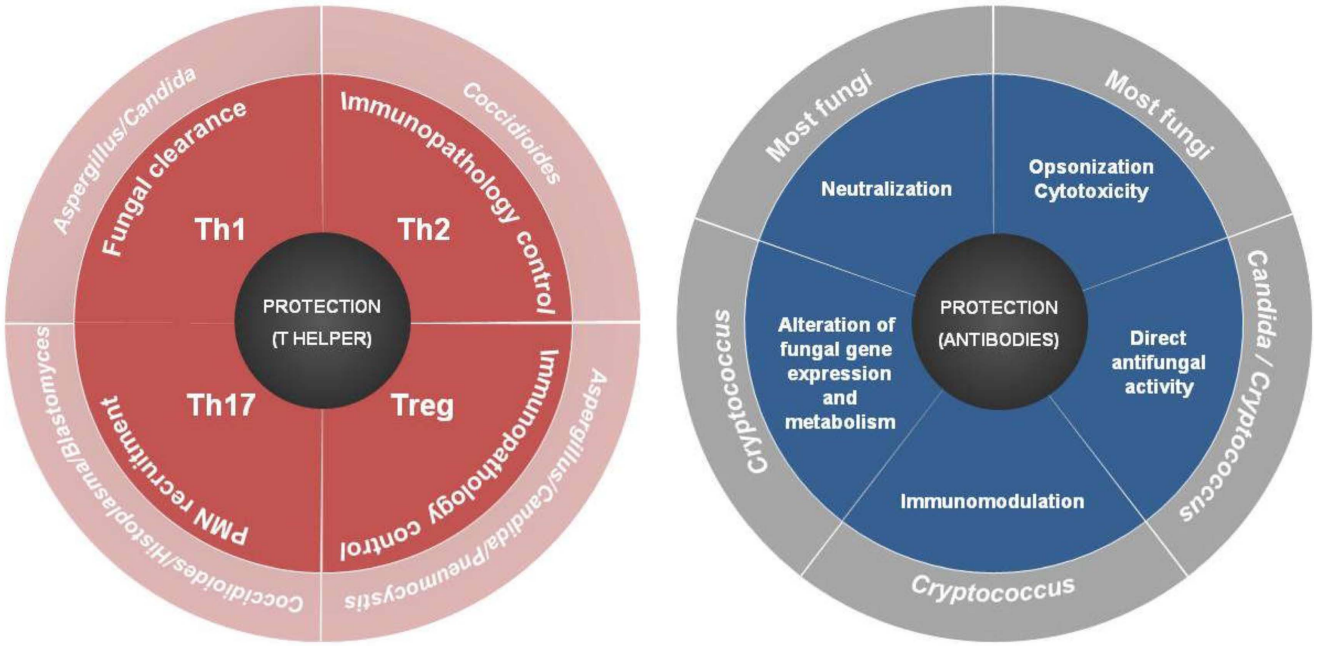

FIGURE 1 | Varieties of T helper (Th) and antibody cell responses to fungi and their correlates of protection.

et al., 2012). Of interest, vaccination with purified *A. fumigatus* antigens was found to be dependent on the MyD88 pathway in the presence of the appropriate adjuvant (Carvalho et al., 2012b; De Luca et al., 2012), a finding pointing to the crucial role of adjuvants in promoting T cell differentiation along specific effector pathways. Thus, fungal innate sensing is one critical step in mounting immune responses eventually defining appropriate effector responses to maximize protection (Levitz and Golenbock, 2012). Moreover, given the intricacies of the complex innate immune signaling networks activated in response to fungal antigens (Romani, 2011), the use of individual PRRs or in combinations will have to be weighed in order to achieve the best vaccine-specific responses appropriate for each fungal pathogen.

The thorough dissection of mechanisms regulating the magnitude, quality, and persistence of vaccine-induced humoral and T cell dependent immunity will add to a more rational design of potentially useful vaccines (Pulendran and Ahmed, 2011). Examples of such approaches include the development of a novel vaccine platform consisting of hollow yeast-derived β-glucan particles that combine adjuvancity and high load antigen delivery to induce strong humoral and Th1- and Th17-biased T cell responses (Huang et al., 2010) and the glycoconjugate vaccines which elicit B-cell responses of increased potency by provision of immunogenic epitopes to $CD4^+$ T cells (Torosantucci et al., 2005; Rachini et al., 2007; Xin et al., 2008; Bromuro et al., 2010). T cells are critical for protective immunity, as they monitor host cells for infection and mobilize appropriate effector functions, either by inducing cytokines and effector cytolytic molecules or by attracting professional phagocytes to the site of microbial deposition, where they activate their antimicrobial capacities. Although $CD4^+$ Th1 cells have been historically considered the cornerstone of cell-mediated defense against intracellular fungi, $CD8^+$ T cells have also been found to perform effector functions against these pathogens (Cutler et al., 2007). Indeed, in a mouse model of vaccination against blastomycosis, both the numbers and function of protective antifungal memory $CD8^+$ T cells were maintained even in the absence of $CD4^+$ T cell help (Nanjappa et al., 2012). In any case, Th1-mediated protection has been reported across nearly all clinically relevant fungal infections. For example, crude antigen preparations from *A. fumigatus* or recombinant fungal antigens alone (Diaz-Arevalo et al., 2011) or in conjunction with CpG oligonucleotides as adjuvants (Cenci et al., 2000; Ito et al., 2006; Bozza et al., 2009; Stuehler et al., 2011), mannosylated cryptococcal antigens (Lam et al., 2005), *B. dermatitidis* adhesin antigen (Wuthrich et al., 2003), heat shock protein 60 from *P. brasiliensis* (de Bastos Ascenco Soares et al., 2008) and *H. capsulatum* (Deepe and Gibbons, 2002) and the multivalent vaccines, comprised of complexes of protein antigens of *Coccidioides* spp., administered in combinations with adjuvants (Shubitz et al., 2006; Tarcha et al., 2006) have been associated with induction of strong Th1 responses.

The persistence of immunological memory and how it pertains to vaccination strategies is also a question of central importance. Memory T cells are derived from normal T cells that have learned how to overcome a pathogen by "remembering" the strategy used to defeat previous infections (Sallusto et al., 2010). In addition to central memory T cells present in secondary lymphoid organs which scrutinize the presence of remote pathogens via dendritic cells (DCs), effector memory T cells reside in peripheral non-lymphoid tissues such as the skin and mucosa. The latter

Table 1 | Types of vaccines for fungal diseases and associated mechanisms of protection.

Type of vaccines	Fungal diseases	Mechanism(s) of protection
Whole cells and cell extracts	Candidiasis Aspergillosis Cryptococcosis Blastomycosis Histoplasmosis Coccidioidomycosis Sporotrichosis	Antibodies; Th1/Th2/Th17 immunity; $CD8^+$ T cells
Subunits and glycoconjugates	Candidiasis Aspergillosis Cryptococcosis Blastomycosis Histoplasmosis Coccidioidomycosis Paracoccidioidomycosis Pneumocystosis	Antibodies; Th1/Th17/T_{reg} immunity
DNA	Candidiasis Coccidioidomycosis Paracoccidioidomycosis Pneumocystosis	Antibodies; Th1/Th2 immunity
Idiotypes and mimotopes	Candidiasis Cryptococcosis	Antibodies
Antigen-pulsed dendritic cells	Candidiasis Aspergillosis Cryptococcosis Paracoccidioidomycosis Pneumocystosis	Antibodies; Th1 immunity

are heterogeneous in terms of homing receptor expression and effector function and comprise the Th1, Th2, Th17, and Th22 cell subsets, as well as T_{reg} cells and cytotoxic T lymphocytes. Although Th1 and Th17 cells mediate vaccine-induced protection from fungal infection through a variety of antifungal effector mechanisms, Th22 cells are instead required for antifungal resistance at mucosal surfaces (De Luca et al., 2010). Memory $CD8^+$ cytotoxic T cells are also induced in fungal infections (Nanjappa et al., 2012) and exhibit a pleiotropic activity by mediating protection via production of IFN-γ and cytolytic activity against fungus-laden cells or the fungus itself (Carvalho et al., 2012b; De Luca et al., 2012). As such, $CD8^+$ T cells, especially if long-lasting, are regarded as ideal candidates for expansion at mucosal surfaces by vaccination strategies. The recent evidence proposing a role for metabolism (Pearce et al., 2009) and bioenergetic stability (van der Windt et al., 2012) in harnessing T cell memory opens up new perspectives on how epigenetic and environmental mechanisms modulate memory differentiation and quality, thus opening new avenues for vaccine development. Finally, additional subsets of T cells may also become important targets for new vaccines, such as the newly described invariant natural killer T cells that activate antifungal responses through the recognition of fungal cell wall β-1,3 glucans (Cohen et al., 2011).

ACTIVE VERSUS PASSIVE IMMUNOTHERAPY

Fungal vaccines are active immunotherapies in the sense they boost the immune system to specifically attack fungi, honing in on one or more specific fungal antigens (Carvalho et al., 2012a). Alternatively, passive immunotherapy strategies are comprised of laboratory-synthesized antibodies or other immune system components that are administered to patients. Thus, passive immunotherapies do not stimulate the immune system to "actively" respond to infection in the way a vaccine does. In this regard, a number of monoclonal human recombinant antibodies and their fragments have already been tested in experimental fungal infection (**Table 1**). Antifungal vaccines are known to exploit the redundancy in the immune system to afford protection through a multiplicity of mechanisms (Cutler et al., 2007; Cassone, 2008). Indeed, antibody responses are induced by most antifungal vaccines and antibody titer threshold may therefore predict vaccine efficacy and may serve as a vaccine surrogate marker even when the mechanism of protection is cell-mediated (Spellberg et al., 2008). Indeed, and even though protection against intracellular pathogens might be prevalently provided by $CD4^+$ and $CD8^+$ T cells, antibodies are now known to participate in all aspects of the immune response, globally contributing to the optimal function of T cell-mediated immunity (Casadevall and Pirofski, 2012). This also suggests that administering antibodies together with a vaccine may be potentially exploited to further enhance or modulate the immune response. Given that passive antibody administration has been deemed effective against fungal infection, it is now accepted that vaccine-mediated protection not only relies on the production and maintenance of specific antibodies, but also on their direct activity (Cutler et al., 2007; Cassone, 2008). This is the case of anti-β-glucan antibodies generated by immunization with laminarin, a β-glucan from algae, conjugated with a genetically detoxified diphtheria toxin (Bromuro et al., 2010) or antibodies generated through idiotypic vaccination (Magliani et al., 2005) that proved to be protective in passive vaccination experiments in different fungal infection settings by acting directly on fungal cells. Because of quantity restrictions, high cost, and the limited effectiveness inherent to a pure antibody approach, it is difficult to envisage antibody therapy against fungal infections in a near future. Indeed, the development of efungumab (Mycograb), a monoclonal recombinant antibody fragment against fungal HSP90 (Matthews et al., 2003) has recently been discontinued. This may have been related with concerns regarding specificity, affinity, and even isotype. For instance, different immunoglobulin G (IgG) subclasses with identical variable regions but different capacities to bind Fc receptors displayed distinct efficacy in terms of protection from cryptococcosis (Beenhouwer et al., 2007). In addition, given that immunocompromised patients may lack efficient effector functions, the use of antibodies that inhibit fungal growth or viability should be favored in these patients.

PATIENT-TAILORED VACCINATION: THE COMING OF AGE OF VACCINOMICS

The deciphering of the complexity of immune responses to vaccines demands for the integration of advanced immunology approaches, systems biology, immunogenetic profiling, and bioinformatics in the areas of pathogen biology, host biology,

and the interaction between the two. Vaccinomics is an emergent term in the field of vaccinology that encompasses the use of immunogenetics to the appreciation of mechanisms of heterogeneity in immune responses to vaccines (Poland and Oberg, 2010). A number of genetic variants in immune genes has already been disclosed as major determinants of the immune response to fungi (Carvalho et al., 2010) and are regarded as promising targets to exploit toward improved diagnosis and therapy of fungal diseases, particularly in immunocompromised patients (Cunha and Carvalho, 2012). A systematic evaluation of the functional impact of genetic variability in the immune system will pave the way to the discovery and interpretation of immunogenetic signatures and immune profiles that may be used to discriminate response efficiencies to antifungal vaccines. The recent finding that genetic deficiency of TLR3 was associated with susceptibility to invasive aspergillosis and concomitant failure to activate memory protective $CD8^+$ T cells in allogeneic stem cell transplanted patients is one first example (Carvalho et al., 2012b). Given the high degree of complexity in human immune responses, overcoming the many challenges currently restraining accurate prediction of vaccination efficiencies has been recently proposed to rely on five state-of-the-art approaches (Kennedy and Poland, 2011). By using whole genome immunogenetics, next generation sequencing, cutting-edge "omics" techniques, advanced bioinformatics, and systems biology applied to immune profiling and vaccine responses, it may be possible to identify the best predictors of vaccine efficacy or adverse responses – predictive vaccinology – in each target population, thereby improving the management of these severe, often fatal diseases.

ACKNOWLEDGMENTS

We thank Cristina Massi Benedetti for editorial assistance. The studies were supported by the Specific Targeted Research Project "ALLFUN" (FP7-HEALTH-2009-260338) and the Fondazione per la Ricerca sulla Fibrosi Cistica (FFC#21/2010, with the contribution of Francesca Guadagnin, Coca Cola Light® Tribute to Fashion and Delegazione FFC di Belluno). Agostinho Carvalho and Cristina Cunha were financially supported by fellowships from Fundação para a Ciência e Tecnologia, Portugal (contracts SFRH/BPD/46292/2008 and SFRH/BD/65962/2009, respectively).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 April 2012; accepted: 21 April 2012; published online: 10 May 2012.

*Citation: Carvalho A, Cunha C, Iannitti RG, Casagrande A, Bistoni F, Aversa F and Romani L (2012) Host defense pathways against fungi: the basis for vaccines and immunotherapy. Front. Microbio. **3**:176. doi: 10.3389/fmicb.2012.00176*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 13 August 2012
doi: 10.3389/fmicb.2012.00294

New approaches in the development of a vaccine for mucosal candidiasis: progress and challenges

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The commensal fungus *Candida albicans* causes mucosal candidiasis in the rapidly expanding number of immunocompromised patients. Mucosal candidiasis includes oropharyngeal, esophageal, gastrointestinal, and vaginal infections. Vulvovaginal candidiasis (VVC) and antimycotic-refractory recurrent VVC is a frequent problem in healthy childbearing women. Both these mucosal infections can affect the quality of life and finding new therapeutical and preventive approaches is a challenge. A vaccine against candidal infections would be a new important tool to prevent and/or cure mucosal candidiasis and would be of benefit to many patients. Several *Candida* antigens have been proposed as vaccine candidates including cell wall components and virulence factors. Here we discuss the recent progress and problems associated with vaccination against mucosal candidiasis.

Keywords: mucosal candidiasis, *C. albicans*, vaccine, fungal infections, candidiasis

INTRODUCTION

Candida albicans is a dimorphic fungus that colonizes different areas of the body from the gastrointestinal tract to oral and vaginal mucosa. It is usually a commensal microorganism but in immunocompromised or otherwise debilitated hosts it can cause disseminated and mucosal candidiasis. Systemic candidiasis is the fourth most common hospital-acquired infection in the USA (Pfaller et al., 1998a,b). The associated mortality depends on host conditions, and ranges between 30 and 50% (Pfaller et al., 1998b; Kibbler et al., 2003). Invasive fungal infections are common and severe in patients with hematologic malignancies, leading to particularly high mortality. Somewhat different from systemic infections, mucosal infections are common not only in immunocompromised patients but also in apparently normal subjects (Kirkpatrick, 2001). Their most common sites are the oral cavity, and the gastrointestinal and vaginal tracts. Oral thrush is one of the most frequent clinical forms of mucocutaneous candidiasis. It occurs at all ages with aggressive symptoms especially in infants and elderly people (Lopez-Martinez, 2010).

Vulvovaginal candidiasis (VVC) is a common distressing infection that affects up to 75% of childbearing women worldwide at least once in their life. Up to 7% of these women suffer from frustrating recurrent infection (RVVC) defined as at least three or four episodes of acute VVC in 1 year (Fidel, 2007; Sobel, 2007). Two forms of RVVC have been described: idiopathic (Id) primary RVVC with unknown predisposing factors, and secondary RVVC which occurs under predisposing conditions such as antibiotic treatment, oral contraceptive use, or diabetes mellitus (Fidel, 2004). These infections affect the quality of life, and require frequent antimycotic treatment enhancing the risk of acquiring drug resistance (Cassone, 2007).

Overall, the growing impact of fungal diseases has resulted in renewed interest in new approaches for improving their control. Among these new approaches, the generation of fungal vaccines is recognized as a priority. A mucosal vaccine would improve the quality of life of a long list of target populations of women suffering from RVVC. The aim of this review is to discuss the recent progress and problems associated with the development of a protective mucosal vaccine against candidiasis.

INNATE IMMUNE RESPONSE AT MUCOSAL SURFACES

The immunopathology associated with mucosal candidiasis is still not completely understood. The presence of *C. albicans* as a commensal on the mucosal surface does not go unnoticed or tolerated by the host – humoral and cellular factors of the innate and acquired immune response play an important role in limiting the growth of the fungus and neutralizing the activity of the virulence factors (Cassone et al., 2007). Indeed the transition from asymptomatic colonization to symptomatic infection occurs when the local defenses are impaired and when the fungus becomes more aggressive causing epithelial damage and inflammation through the production of virulence factors. In addition, alteration of the normal microbiota plays an important role.

The mucosal microbiota is acquired by colonization when passing through the birth canal, and soon after birth. The commensals establish their residence in mucosal niches where they replicate and play a crucial role in the development of the immune system. The immune response to the microbiota at the mucosal surface helps to maintain barriers to potentially harmful microorganisms (Pirofski and Casadevall, 2012). *C. albicans* as a commensal microorganism is part of normal microbiome in specific niches such as the oral cavity, vagina, and gut in at least 50% of healthy individuals. However it can cause pathology following alterations of the local environment and/or impairment of the immune response. To persist within the human host and cause disease at the mucosal surfaces, *C. albicans* has acquired several distinct factors enabling these microorganisms to adhere to and invade host cells.

Epithelial cells (ECs) lining the mucosa play an essential role in the defense against *C. albicans*. Indeed at the mucosal surface ECs sense the presence of *Candida* and elaborate a number of mechanisms to tolerate its presence and limit adherence, penetration, and damage. It is clear that ECs recognize the fungus and are capable of distinguishing its different forms of growth (Moyes et al., 2010), however the relative role of pattern recognition receptors (PRRs) in ECs is still largely unclear, despite the intensive research in PRR-mediated interaction of *C. albicans* with myeloid cells (Netea et al., 2006, 2008). The recognition of *C. albicans* by these cells has largely been elucidated and the major PRRs and their putative ligands derived from *C. albicans* have been extensively studied (Roeder et al., 2004a,b; Netea et al., 2006). It is known that ECs express PRRs: toll-like receptors (TLRs), dectin-1, and galectins (Backhed and Hornef, 2003; Hornef and Bogdan, 2005; Weindl et al., 2007). TLR2 and TLR5 are expressed at particularly high levels and these receptors have been associated with biological functions of ECs such as growth, survival, and repair (Rhee et al., 2005; Shaykhiev et al., 2008).

CANDIDA RECOGNITION AT THE MUCOSAL SURFACE

Compelling evidence shows that *C. albicans* recognition by TLR4 on monocytes/macrophages results in release of proinflammatory cytokines such as IL-1 and TNF-α (Netea et al., 2006) and also the stimulation via TLR2 and FcγRII leads to TNF-α production (Netea et al., 2008). TLR4, which is highly expressed on myeloid cells and plays a critical role in driving immune response against *C. albicans* (Netea et al., 2006), is poorly expressed in ECs at the oral mucosal surface (Backhed and Hornef, 2003), and it seems that TLR4 does not have a role in cytokine induction in response to *Candida* (Li and Dongari-Bagtzoglou, 2009) and to other TLR4 ligands such as LPS (Naglik and Moyes, 2011).

At present, which fungal cell wall structures are recognized in oral ECs is also unknown, however a recent study has established that human oral ECs have a direct antifungal activity demonstrating the important role of these cells in mucosal protection (Weindl et al., 2007). Indeed, the oral epithelium is able to induce various defense effector molecules (Diamond et al., 2008) and to orchestrate an immune response to activate immune cells in the submucosal layers to clear the invading pathogens (Cutler and Jotwani, 2006). It has also been reported that after fungi recognition, infected ECs are activated and they produce proinflammatory cytokines and chemokines including IL-8 which induces neutrophil recruitment (Weindl et al., 2007; Moyes and Naglik, 2011) into the mucosa surface. This could be considered a mechanism of immune surveillance (Moyes and Naglik, 2011). However, despite the beneficial role of these cells in protection against systemic candidiasis, PMNs seem to exert no protective role in vaginal candidiasis. Neutrophils are abundantly recovered during vaginal candidiasis, but their presence was not associated to reduction of fungal burden (Fidel et al., 1999). On the contrary, it has been hypothesized that ECs have high sensitivity to *Candida* and secrete S100 alarmin resulting in a vigorous PMN migration which results in an inflammatory response and symptoms of infection (Yano et al., 2010). In agreement with this, a live challenge model in humans recently revealed that symptomatic vaginitis is caused by an aggressive innate response (neutrophil infiltrates and elevated fungal burden in the vaginal lumen); the protection has been associated to a non-inflammatory innate response (Fidel, 2007). Therefore the capacity of ECs to recruit and activate neutrophils might be a double-edged sword, with both favorable and unfavorable consequences depending on the specific niche.

There is no doubt, therefore, that many aspects of EC interaction with *C. albicans*, including the recognition of specific antigens and regulation of the complicated network of regulatory activities that characterize commensalism and infection in different niches, remain to be studied.

T CELL RESPONSE IN MUCOSAL CANDIDIASIS

The role of T cells in mucosal candidiasis has only been partially elucidated, and the attempt to integrate the defense response to systemic and mucosal candidiasis remains inconclusive (Ashman et al., 2011). There is a wealth of studies reporting that Th1-type immunity is dependent on the presence of IL-12 in the milieu (Trinchieri, 2003) and compelling evidence indicates that the Th1 response is protective against mucosal candidiasis. The beneficial role of the Th1 response in oral candidiasis in particular has been underlined (Schaller et al., 2004). In agreement with these findings is the marked susceptibility of HIV-infected subjects to oral candidiasis when $CD4^+$ T cells are depleted, suggesting a role for both Th1 and Th17 cell functional CD4 subsets. In addition, depletion of IL-12 resulted in acute susceptibility to oral infection in a mouse experimental model (Farah et al., 2002; Fidel, 2006; Zakikhany et al., 2007). In a report by Conti et al. (2009) susceptibility to oropharyngeal candidiasis (OPC) in mice with impaired Th1 and/or Th17 responses was compared. In this study fungal infections of the tongue were less severe in mice lacking IL-12p35 than in mice lacking IL-23p19, suggesting that Th17 responses play a central role in control of infection. However, the fungal burden in both IL-12p35- and IL-23p19-deficient mice was substantially higher than in controls, evidencing that the absence of either Th1 or Th17 cells impaired the ability of the mice to limit fungal growth (Pirofski and Casadevall, 2009). As well as driving innate immunity and neutrophil responses, Th17 cells have also been shown to drive antibody responses at mucosal surfaces, in particular secretory IgA (Moyes and Naglik, 2011).

HUMORAL RESPONSE IN MUCOSAL CANDIDIASIS

For many years antibodies have been considered irrelevant in the host defense against invasive candidiasis, but evidence for this mode of protection has been mounting over the last two decades. Indeed a number of antibodies or their engineered derivatives directed against *C. albicans* cell wall compounds have been shown to confer protection (Cutler et al., 2007, 2011; Cassone and Rappuoli, 2010; Xin and Cutler, 2011). The discovery of numerous antigens on the fungal cell wall that elicit protective antibody responses raises the possibility of vaccines designed with multiple antigens, and/or passive therapies that combine antibodies with different specificities (Casadevall et al., 2004; Casadevall and Pirofski, 2007).

Protective antibodies have been reported for *C. albicans* cell wall polysaccharides, proteins, and peptides (De Bernardis et al., 1997;

Matthews et al., 2003; Cutler, 2005; Yang et al., 2005; Xin et al., 2008). As a prevention strategy, protection against disease may be actively or passively acquired by vaccination and the administration of preformed monoclonal antibodies (Xin and Cutler, 2011).

In early studies protection against vaginal candidiasis was associated with the presence of protective antibodies against *Candida* constituents in the vaginal fluids and increased number of activated lymphocytes in the vaginal mucosa (De Bernardis et al., 1997; de Bernardis et al., 2000). More recently the same group demonstrated that vaginal B cells play an important role in protection against vaginal candidiasis suggesting that the contribution of T cells is predominantly that of helping B cells in protective antibody production (De Bernardis et al., 2010). Recently, treatment with synthetic peptides whose sequences are identical to fragments from the constant region of different classes of antibodies has been shown to provide protection against experimental vaginal candidiasis (Polonelli et al., 2012), and a protective effect in vaginal candidiasis was also noticed after passive administration of anti-β-glucan mAbs (Torosantucci et al., 2009). Unfortunately, the role of Abs has rarely been investigated in other mucosal infections such as oral candidiasis.

ANTI-*CANDIDA* VACCINES AND PROTECTION MECHANISMS

Recent papers support the idea that it may be possible to develop an antifungal vaccine that grants protection against multiple fungal pathogens (Cassone and Rappuoli, 2010; Stuehler et al., 2011; Wuthrich et al., 2011). Some studies have focused on cell-mediated immunity as the mechanism of protection (Spellberg et al., 2008), which is in accordance with the literature (Xin et al., 2008), whereas others (Cutler et al., 2007; Karwa and Wargo, 2009; Torosantucci et al., 2009) have determined that antibodies specific to certain *Candida* antigens are protective. Because of the variegate nature of *Candida* virulence and participation of different host responses in protection, it may well be possible that both humoral and cellular responses play a role in vaccination, providing the necessary collaboration to grant protection.

Several papers suggest that the induction of strong cellular immunity plays a paramount role in protection against candidiasis. One approach for stimulating cellular immunity was priming *ex vivo* dendritic cells (DCs) with fungal cells and re-administering the loaded DCs to the host (Roy and Klein, 2012). Less recent papers showed that DCs transfected with fungal RNA adoptively transferred into otherwise susceptible recipients, did in fact confer protection against *C. albicans* by inducing a protective Th1 response (Bozza et al., 2004; Perruccio et al., 2004). Recently a novel antigenic peptide, derived from a cell wall-associated adhesion and recognized by a major population of all *Candida*-specific Th cells isolated from infected mice, was isolated and sequenced from infected DCs. This peptide contributes to fungal pathogenicity and it is conserved in many clinically important *Candida* species such as *C. dubliniensis* and *C. glabrata*. Of note is the fact that human Th cells also responded to stimulation with the peptide. Furthermore when this peptide is used in combination with an adjuvant inducing IL-17A secretion, it acts as an efficient vaccine by protecting mice from fatal candidiasis (Bar et al., 2012).

The fungal-specific antibody-mediated protection may occur not only through classical immunological responses such as phagocytosis and killing and complement activation, but also through direct antibody actions on fungal cells, and even though antibody protection is considered specific many fungal antigens may be used for vaccination and to obtain therapeutic immunoglobulins (Casadevall and Pirofski, 2012). The novel fully synthetic β-(Man)3-Fba glycopeptide vaccine that represents two epitopes of *C. albicans* cell surface has been shown to be protective in an experimental model of systemic candidiasis (Xin et al., 2008). The protection was afforded with a strong antibody response that was achieved by using a DC/Complete Freund Adjuvant (CFA) combination (Xin et al., 2008). Indeed the antigen-pulsed DC proved to be a powerful means to induce a potent immune response that was not achieved by using small carbohydrate and peptide antigens of *C. albicans*. More recently, by coupling β-(Man)3-Fba glycopeptide to tetanus toxoid to render this vaccine entirely compatible with human use, a high degree of antibody-mediated protection was observed (Xin et al., 2012). These vaccine formulations also proved protective in mucosal models of infection (Xin et al., 2012). Another important effort to induce protection against *C. albicans* was made by Li et al. (2011) who showed that treatment with recombinant enolase, an important glycolytic enzyme located on the cell wall of *C. albicans*, conferred a protective effect against systemic challenge evaluated by fungal burdens in target organs, titers of specific antibodies to enolase, and levels of Th1/2 cytokines in serum.

VACCINE CANDIDATES FOR MUCOSAL CANDIDIASIS

Strategies to elaborate a vaccine for mucosal candidiasis should take into account what type of immune response is protective under natural conditions, but should not necessarily be limited to mimicking the natural history of mucosal infection and protection. Hence, several studies have focused on different strategies to induce protection against vaginal candidiasis. In particular, it has been reported that vaccination with the recombinant N terminus of the candidal adhesin rAls3p-N protects mice against disseminated and oropharyngeal and vaginal candidiasis by a cell-mediated immune response (Th1/Th17). Antibodies have also been generated but their titers did not correlate with protection. The rAls3p-N vaccine is a promising new vaccine candidate for further exploration to prevent systemic and mucosal candidal infections (Spellberg et al., 2006). It has recently completed a Phase 1 clinical trial and proved to be safe and immunogenic. In particular, this research group reported that vaginal and systemic protective responses could be achieved by vaccination with rAls3-N which stimulates Th1/Th17 lymphocytes to produce high levels of IFN-γ and IL-17A, as well as the chemokines KC and MIP-1. These cytokines enhance the capacity of phagocytes to kill the pathogen. This vaccine protects immunocompetent mice from both vaginal candidiasis and lethal disseminated candidiasis and it also significantly reduces oral fungal burden in the corticosteroid-treated mouse model of OPC. Human trials with the rAls3-N vaccine are in final preparation (Liu and Filler, 2011). An additional approach to achieve protection against mucosal infection was immunization with *C. albicans* dsDNA which induces host resistance in newborn mice against gastrointestinal *C. albicans*

infection. The protective properties of dsDNA are related to an increased number of $CD4^+$ T cells secreting IFN-γ (Remichkova et al., 2009).

One attempt to develop a mucosal vaccination was by using an aspartyl proteinase (Sap2), a very well known enzyme belonging to a family of virulence factors of *C. albicans* (Naglik et al., 2003). Previous clinical and experimental work strongly suggested that Sap2 and possibly other Saps were involved in vaginal infection (Cassone et al., 1987; De Bernardis et al., 1990). Mice immunized with Sap2 showed significantly reduced fungal burdens both orally and vaginally, and rats receiving intravaginal administration of anti-Sap2 antibodies were protected by an intravaginal challenge by *C. albicans*. These studies demonstrated that Sap2 is an immunogenic antigen capable of inducing protective responses against *C. albicans* colonization and infection, and tentatively supports its targeting as a potential vaccine candidate (Rahman et al., 2007). The role of Sap2 in inducing protection against mucosal candidiasis has more recently been underscored by Sandini et al. (2011). These authors generated a recombinant truncated Sap2 protein (rSap2t) and reported that intravaginal immunization with this antigen and cholera toxin as an adjuvant protected from the challenge of a highly vaginopathic strain of *C. albicans*. Protection was possibly due to the elicitation of specific antibodies IgM and IgG anti-rSap2t. More recently rSap2t was incorporated into influenza virosomes, an adjuvant/carrier formulation already used in other human vaccines, which avoids the necessity for a toxin adjuvant. This formulation generated a potent serum antibody response in the mouse and rat following intramuscular immunization. In a rat model of candidal vaginitis the intravaginal or intramuscular administration of rSap2t induced production of anti-Sap2 IgG and IgA in the vaginal fluid, which conferred a consistent degree of protection against vaginal *C. albicans* infection (De Bernardis et al., 2012).

In another study, murine mAb (KT4, IgG1) was used, neutralizing *in vitro* the anti-*Candida* activity as an Id vaccine to elicit Abs. An effective protection that correlated with a significant decrease in vaginal *Candida* CFU was obtained in Id-vaccinated animals compared with controls. The protection was associated with rising vaginal titers of anti-idiotypic Abs (IdAb), prevalently of the IgA isotype, that were able to passively transfer the protective state to non-immunized animals (Polonelli et al., 1994). Since the receptor of the killer toxin recognized by the mAbKT4 is a beta-glucan molecule, it is possible that the protection conferred by the Id vaccine somewhat parallels the protection conferred by the β-glucan-conjugate vaccine (see below).

In a further work, immunization was performed with *C. albicans* heat shock protein 90 kDa (hsp90-CA). Intradermal priming with recombinant hsp90-CA protein, followed by an intranasal or intradermal booster with recombinant hsp90-CA protein, induced significant increases of specific IgG and IgA antibodies in both serum and vaginal fluid. The specific IgG isotype increased after vaginal *Candida* infection, suggesting that *Candida* has the ability to induce a local hsp90-specific antibody (IgG) response during VVC (Raska et al., 2008).

A vaccine composed of β-glucan has been considered a candidate against *Candida* and other fungi. A non-fungal source of β-glucan, laminarin, has been used in these studies. Because polysaccharides are poor immunogens, laminarin was conjugated with the diphtheria toxoid CRM197. This novel glyco-conjugate vaccine administered with human-compatible adjuvant resulted immunogenic and protective as a prophylactic against experimental systemic and mucosal infections by *C. albicans* (Torosantucci et al., 2005). The protection has been ascribed to the production of antibodies to β-(1,3)-glucan (Bromuro et al., 2010). The protective capacity of this β-glucan-conjugate vaccine formulated with the human-compatible MF59 adjuvant was assessed in a murine model of vaginal candidiasis exploiting an *in vivo* imaging technique to monitor the infection. The vaccine conferred significant protection, which was associated to anti-β-glucan IgG antibodies in the serum and in the vagina. The efficacy of the antibodies was demonstrated by the passive transfer of the immune vaginal fluid or anti-β-glucan monoclonal antibodies to naïve mice before infection (Pietrella et al., 2010). The main vaccine candidates are reported in **Table 1**.

CONCLUSION

Despite several promising approaches, the achievement of a vaccine against mucosal infections in humans still faces a number of problems and challenges. The major challenge remains the difficulties in translating results from rodents to humans, as rodent models can represent at best only part of the variegate patterns of vaginal *Candida* infections in women (Sobel, 2007). In fact most of the data indicating that protection against vaginal infection has been obtained in the rat model, and is not considered by some to be fully representative of the human infection (Fidel and Cutler, 2011). In addition, very few vaccination studies have addressed oral and other types of mucosal candidiasis. Another issue is that vaccine-induced protection must occur in specific niches where *C. albicans* is tolerated as a commensal organism, and it cannot be excluded that affecting fungus colonization by a vaccine could induce some harm in the host by affecting the mucosal microbiome. Other issues concern the search for new vaccine immunogens and adjuvants by existing advanced technologies. It should be taken into account that the most studied vaccines, particularly those in clinical trials (the virosomal Sap2 and the Als3 peptide) have been generated by classical "empiric" approaches, including clinical impressions. Now, a large variety of covalently attached proteins has been found in fungal walls and these proteins can rapidly change their expression in response to different environmental conditions. The knowledge of the specific proteome of the fungus in the host is fundamental for the identification of new vaccine candidates (Klis et al., 2011). Not only proteomics but also complementary post-genomic tools such as transcriptomics and metabolomics in a systems biology context will be useful tools to study pathogen-host interaction, to identify the protective response during infection and the immune response after vaccination. In a recent study, through an integrated analysis of genome-wide transcriptome, Lindqvist et al. determined which signature pathways, processes, and networks are shared by or are exclusive to two classes of experimental vaginal adjuvants, the TLR-9 agonist CpG-ODN and alpha-galactosylceramide in the mouse vagina. These molecular signatures could be used to develop potent mucosal adjuvants that can be used in the female genital tract of a mammal (Lindqvist et al., 2011).

Table 1 | Vaccine candidates for protection against candidiasis.

Components	Reference	Protective immunity	Protection
1,3-β-glucan	Torosantucci et al. (2009), Cassone et al. (2010), Bromuro et al. (2010)	Abs	Systemic
rHyr1p-N	Luo et al. (2011)	Abs	Systemic
Mannoproteins	De Bernardis et al. (2010)	B cells	Vaginal
	Pietrella et al. (2002)	Th1	Systemic
β-1,2-mannotriose-Fba	Xin et al. (2012)	Abs	Systemic
Heat shock proteins 90	Raska et al. (2008)	Abs	Systemic and vaginal
C. albicans surface protein, Als3p	Spellberg et al. (2008)	Th1–Th17	Systemic
(Agglutinin-Like Sequence 3) rAls3-N	Baquir et al. (2010), Liu and Filler (2011)	Abs	Vaginal, systemic, and oral
Phosphoglycerate kinase	Calcedo et al. (2012)	Abs	Oral
Secreted aspartic proteases 2	De Bernardis et al. (2012), Cassone and Casadevall (2012)	Abs	Mucosal and vaginal
Yeast derived-β glucan particles (GPs)	Huang et al. (2010)	Th1, Th17, Abs	NT
DCs transfected with fungal RNA	Bozza et al. (2004), Perruccio et al. (2004)	T cell response Th1	Systemic
Nanoparticle-mediated target DCs	Roy and Klein (2012)	T cell response	NT
Liposome-mannan (L-mann)	Han et al. (2000)	Abs to β-1,2-mannotriose	Systemic and vaginal
Daucosterol	Lim et al. (2007)	Th1	Systemic
Enolase	Li et al. (2011)	Abs Th1	Systemic
Glycopeptide or a peptide synthetic vaccine, (*Candida albicans* cell wall-derived)	Xin et al. (2012)	Abs	Systemic
Fba, (peptide derived from fructose bisphosphate aldolase which has cytosolic and cell wall distributions in the fungus)	Cutler et al. (2011)	Abs	Systemic
C. albicans dsDNA	Remichkova et al. (2009)	T cells (Th1)	Gastrointestinal

NT, not tested.

Knowledge of the host mechanisms involved in candidiasis protection, and understanding of the fungal virulence factors, will allow the development of a novel topical mucosal vaccine.

ACKNOWLEDGMENTS

This paper was supported by a grant, No. 2010.011.0398, from the Fondazione Cassa di Risparmio di Perugia. We thank Catherine MacPherson for editorial assistance.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 July 2012; accepted: 24 July 2012; published online: 13 August 2012.
*Citation: Vecchiarelli A, Pericolini E, Gabrielli E and Pietrella D (2012) New approaches in the development of a vaccine for mucosal candidiasis: progress and challenges. Front. Microbio. **3**:294. doi: 10.3389/fmicb.2012.00294*
This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 22 August 2012
doi: 10.3389/fmicb.2012.00299

Protective effector cells of the recombinant Asp f3 anti-aspergillosis vaccine

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An *Aspergillus fumigatus* vaccine based on recombinant Asp f3-protein has the potential to prevent aspergillosis in humans, a devastating fungal disease that is the prime obstacle to the success of hematopoietic cell transplantation. This vaccine protects cortisone acetate (CA)-immunosuppressed mice from invasive pulmonary aspergillosis via $CD4^+$ T cell mediators. Aside from these mediators, the nature of downstream fungicidal effectors is not well understood. Neutrophils and macrophages protect immunocompetent individuals from invasive fungal infections, and selective neutrophil depletion rendered mice susceptible to aspergillosis whereas macrophage depletion failed to increase fungal susceptibility. We investigated the effect of neutrophil depletion on rAsp f3-vaccine protection, and explored differences in pathophysiology and susceptibility between CA-immunosuppression and neutrophil depletion. In addition to being protective under CA-immunosuppression, the vaccine also had a protective effect in neutrophil-depleted mice. However, in non-immunized mice, a 10-fold higher conidial dose was required to induce similar susceptibility to infection with neutrophil depletion than with CA-immunosuppression. The lungs of non-immunized neutrophil-depleted mice became invaded by a patchy dense mycelium with highly branched hyphae, and the peribronchial inflammatory infiltrate consisted mainly of $CD3^+$ T cells and largely lacked macrophages. In contrast, lungs of non-immunized CA-immunosuppressed mice were more evenly scattered with short hyphal elements. With rAsp f3-vaccination, the lungs were largely clear of fungal burden under either immunosuppressive condition. We conclude that neutrophils, although important for innate antifungal protection of immunocompetent hosts, are not the relevant effectors for rAsp f3-vaccine derived protection of immunosuppressed hosts. It is therefore more likely that macrophages represent the crucial effectors of the rAsp f3-based vaccine.

Keywords: Asp f3, aspergillosis, *Aspergillus fumigatus*, corticosteroid immunosuppression, macrophages, neutropenia, neutrophils, vaccine

INTRODUCTION

The filamentous fungus is widely present in the environment and is responsible for most fatal cases of invasive aspergillosis (IA) that occur in patients with hematologic malignancies, especially recipients of hematopoietic cell transplantation (HCT), a common treatment for leukemia, lymphoma, and other hematological malignancies. Patients at highest risk of developing IA are those who experience prolonged neutropenia and those who undergo prolonged immunosuppressive treatments, for example to control graft-vs.-host disease after HCT (Marr et al., 2002; Cordonnier et al., 2006; Segal et al., 2007; Baddley et al., 2010; Kontoyiannis et al., 2010).

In an immunocompetent host, macrophages and neutrophils mediate the innate immune response against fungal pathogens. Previously, macrophages were postulated as the first line of defense against conidia, and neutrophils were recognized as the key cell population that provides protection against hyphae and swollen conidia through oxidative and non-oxidative mechanisms (Schaffner et al., 1982; Diamond, 1983; Diamond et al., 1983; Roilides et al., 1993; Feldmesser, 2006; Park and Mehrad, 2009; Hasenberg et al., 2011). However, more recent studies have demonstrated that neutrophils provide anti-conidial defense at early time points following *A. fumigatus* infection. For example, *A. fumigatus* infected mice in which neutrophils are depleted before or within 3 h after fungal infection exhibit a high mortality rate. In contrast, neutrophil depletion at later time points post-infection is associated with survival (Mircescu et al., 2009). Similarly, a comparison of different immunosuppressive regimens showed that neutrophil recruitment, rather than recruitment of alveolar macrophages, is essential for early host defense against aspergillosis (Ibrahim-Granet et al., 2010).

An efficient and safe strategy to protect immunosuppressed patients from fungal infections could involve specific reconstitution of the immune response with an antifungal vaccine. Therefore, in recent years, several approaches have been taken to develop an *A. fumigatus* vaccine. Immunizations with the recombinant Asp f3-protein and truncated portions thereof protected cortisone acetate (CA)-immunosuppressed mice against pulmonary IA (Ito

et al., 2006). Moreover, the *A. fumigatus* cell wall glucanase Crf1 protected mice against both *A. fumigatus* and *Candida albicans* (Stuehler et al., 2011). Furthermore, a protein designated Asp f16, when combined with unmethylated CpG, was able to induce Th1 priming and resistance to the fungus (Bozza et al., 2002). However, it is controversial whether Asp f16 actually exists (Bowyer and Denning, 2007) or instead is a splice form of the crf1 gene, together with the sequence-related vaccine candidates Asp f9 and Crf1 (Schutte et al., 2009). In another approach, it was shown that heat-killed *Saccharomyces* can protect immunosuppressed mice against systemic aspergillosis (Liu et al., 2011), and immunizations with *Laminaria digitata* β-glucan proved to be protective against *C. albicans* and systemic aspergillosis (Torosantucci et al., 2005, 2009). Despite these promising research results, no aspergillosis vaccine has made it into clinical use thus far. The difficulty lies in the question on how to vaccine-protect severely immunocompromised individuals. Because patients who receive HCT have initially low counts of T and B cells, it is usually not advisable to vaccinate HCT-receiving patients immediately after transplantation (Ljungman et al., 2009). For example, the guidelines for preventing infectious complications among HCT recipients advise waiting 6–12 months post-HCT to deliver the first bacterial vaccines, and 2 years post-HCT for attenuated viral vaccines (Tomblyn et al., 2009). Detailed knowledge about the mediators and effectors of an antifungal vaccine's mechanism is required to devise a safe and effective immunization strategy for HCT recipients.

$CD4^+$ T cells are recognized as key mediators of the protective immune response for the control of IA (Beck et al., 2006; Tramsen et al., 2009; Chaudhary et al., 2010; Diaz-Arevalo et al., 2011). Functionally active $CD4^+$ T cells in combination with antigen presenting cells contributed to enhance the neutrophil effector function that caused hyphal damage in an *in vitro* study with *A. fumigatus* (Beck et al., 2006). Likewise, stimulation of polymorphonuclear leukocytes (PMNs) with Th1 cytokines allowed hyphal damage in the presence of hydrocortisone (Roilides et al., 1993) and TNF-alpha augments the ability of PMNs to damage *Aspergillus* hyphae, and increases macrophage phagocytic activity against conidia (Roilides et al., 1998).

In the studies described herein, we studied the role of innate effector cells in a pulmonary IA model with rAsp f3-vaccinated mice. We present novel data on both antibody-induced neutropenia and corticosteroid-induced immunosuppression, as well as their effects on susceptibility to *A. fumigatus* infection, protection conferred by the rAsp f3-vaccine, the role of neutrophils in vaccine derived protection, and the resulting differences in IA pathophysiology under the two conditions.

MATERIALS AND METHODS

ANIMALS, STRAINS, AND REAGENTS

Female CF-1 mice (20 g, 6–8 weeks old) were purchased from Charles River Laboratory and housed in a biosafety level 2 control facility. The mice were cared in accordance with animal care regulation and use protocols approved by the City of Hope Institutional Animal Care and Use Committee. Animal numbers (n, between 8 and 26) per group are indicated in the figure captions.

Aspergillus fumigatus strain AFCOH1 (Ito et al., 2006; Ito et al., 2009; Diaz-Arevalo et al., 2011) was used in all experiments and was cultured on potato dextrose agar (BD/Difco, *Aspergillus fumigatus*Franklin Lakes, NJ, USA) for 7 days at 37°C. Resting conidia were harvested by rinsing from the cultured fungus, and resuspended in Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS, Mediatech, Inc., Manassas, VA, USA) prior to infection. Conidia were quantified with a Countess Automated Cell Counter (Invitrogen, Eugene, OR, USA), and their viability was measured by colony forming unit assay.

VACCINATION, IMMUNOSUPPRESSION, AND *A. FUMIGATUS* CHALLENGE

CF-1 mice were immunized twice, once per day on days 0 and 14, with subcutaneous injections of N-terminal truncated rAsp f3-based vaccine (15 μg) suspended in TiterMax (TiterMax, Inc., Norcross, GA, USA) as previously described (Ito et al., 2006). For neutrophil depletion assays, mice were injected with the vaccine a third time on day 60. As controls, mock vaccinated mice were injected with PBS plus TiterMax. Prior to infection, one of the following immunosuppression treatments was applied.

Cortisone acetate treatment

Cortisone acetate (CA) immunosuppression was performed as described previously (Diaz-Arevalo et al., 2011). Briefly, 5 weeks after the second vaccination the mice received daily injections of CA (2.5 mg/mouse, TCI America, Portland, OR, USA) suspended in methylcellulose (0.5%, Sigma, Aldrich, St. Louis, MO, USA) and Tween 80 (0.1%) for 10 days prior to fungal infection.

In vivo depletion of neutrophils

For neutrophil depletion, vaccinated mice received intraperitoneal injection of anti-GR1-specific monoclonal antibody RB6-8C5 (100 μg, Bio X Cell, West Lebanon, NH, USA) or, as a control, rat IgG (Sigma, Aldrich, St. Louis, MO, USA), 1 day before and after fungal challenge. Neutrophil depletion was monitored by flow cytometric analysis of tail blood (50 μl) from two randomly selected mice from each group. Neutrophils were stained with allophycocyanin (APC)-labeled anti-GR1 (RB6-8C5), or APC-labeled anti-Ly6G (1A8), and PerCP-Cy5.5-conjugated anti-Ly6C antibody (eBioscience, Inc., San Diego, CA, USA). T cells were label with PE anti-CD3 conjugated and FITC labeled anti-CD8. Data were analyzed using FlowJo 7.5 (Tree Star, Inc., Ashland, OR, USA), and paired t test was used for statistical analysis.

In addition, one group of mice was immunosuppressed with CA for 10 days, after which mice received the anti-GR1 antibody two and 1 day before infection as described above. Control mice were immunosuppressed with CA followed by injection of non-specific rat IgG antibody at identical time points.

To prevent bacterial infection, mice were maintained on acidified water containing sulfamethoxazole (0.8 mg/ml) and trimethoprim (0.16 mg/ml; Hi-Tech Pharmacal Co., Inc., Amityville, NY, USA) during the immunosuppression and infection period.

Prior to intranasal (i.n.) inoculation with *A. fumigatus* conidia, mice were anesthetized by subcutaneous injection of ketamine-xylazine. Mice were then intranasally challenged with 3–30 million viable conidia (VC) in DPBS (30 μl). Infected mice were observed every 2 h during the day, and their weight and body temperature were monitored twice per day for 4–12 days after infection,

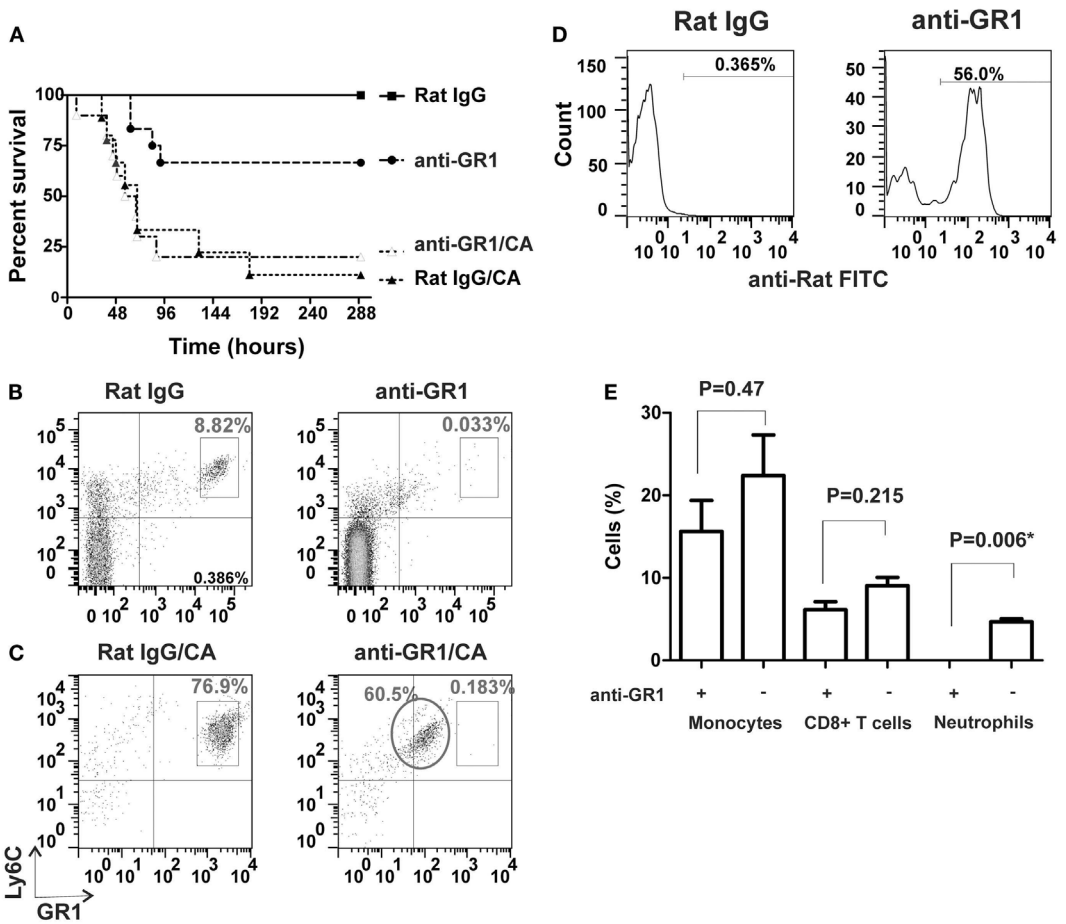

FIGURE 1 | Comparison of CA-immunosuppression and neutrophil depletion and their effects on IA survival. (A) Kaplan–Meier survival plots of CF-1 mice i.n. infected with three million viable *A. fumigatus* conidia. Curves show CA-immunosuppressed mice with non-specific rat IgG as controls (Rat IgG/CA, triangles, $n = 9$), CA-immunosuppressed mice with anti-GR1 antibody (anti-GR1/CA, open triangles, $n = 10$), neutrophil-depleted mice (anti-GR1, circles, $n = 12$), and non-immunosuppressed mice with non-specific rat IgG (Rat IgG, squares, $n = 12$). **(B,C)** FACS analysis of Ly6C$^+$ GR1$^+$ neutrophils in tail blood from control mice (left panels, Rat IgG) and neutrophil-depleted mice (right panels, anti-GR1; **(B)**] without CA-immunosuppression and **(C)** with CA-immunosuppression. The circle marks the population of neutrophils coated with the unlabelled anti-GR1 antibody. **(D)** Binding of anti-rat antibody to anti-GR1 IgG-coated neutrophils in CA-immunosuppressed mice from the circled population in **(C)** (right panel, anti-GR1) compared to the isotype control (left panel, Rat IgG). **(E)** Effect of anti-GR1 antibody on the depletion of monocytes, CD8$^+$ T cells and neutrophils in mouse blood, $n = 3$; *statistically significant, $p < 0.05$.

depending on the type of experiment (Ito et al., 2006; Diaz-Arevalo et al., 2011). Survival data was plotted by the Kaplan–Meier method and statistical analyses performed using Fisher's exact test and Graph Pad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Vaccinated mice that survived were euthanized 5 days after *A. fumigatus* infection and their lungs collected and immediately fixed in 10% formalin and embedded in paraffin. The lungs of non-surviving mice were collected from four mice at the time of death. Lung sections were stained with hematoxylin and eosin (HE), and Gomori methenamine silver staining was used to stain hyphal mycelium and remaining conidia.

Immunohistochemistry was performed using anti-CD3 (Dako Inc., Carpinteria, CA, USA), anti-GR1, and anti-F4/80 (eBioscience, Inc., San Diego, CA, USA) primary antibodies and horseradish peroxidase (HRP) conjugated anti-rat IgG (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) as the secondary antibody. Imaging was performed on an Olympus AX70 model U-MPH microscope (Tokyo, Japan) with a QImaging RETIGA EXi camera. Data were acquired with Image ProPlus v5.1 software.

RESULTS

CORTISONE ACETATE IMMUNOSUPPRESSION RENDERS MICE MORE SUSCEPTIBLE TO PULMONARY ASPERGILLOSIS THAN NEUTROPHIL DEPLETION

We first assessed the susceptibility of mice to invasive pulmonary aspergillosis after induction of either CA-induced immunosuppression or antibody-mediated neutrophil depletion. When challenged with three million VC, only 10–20% of CA-immunosuppressed mice survived (**Figure 1A**, curve Rat

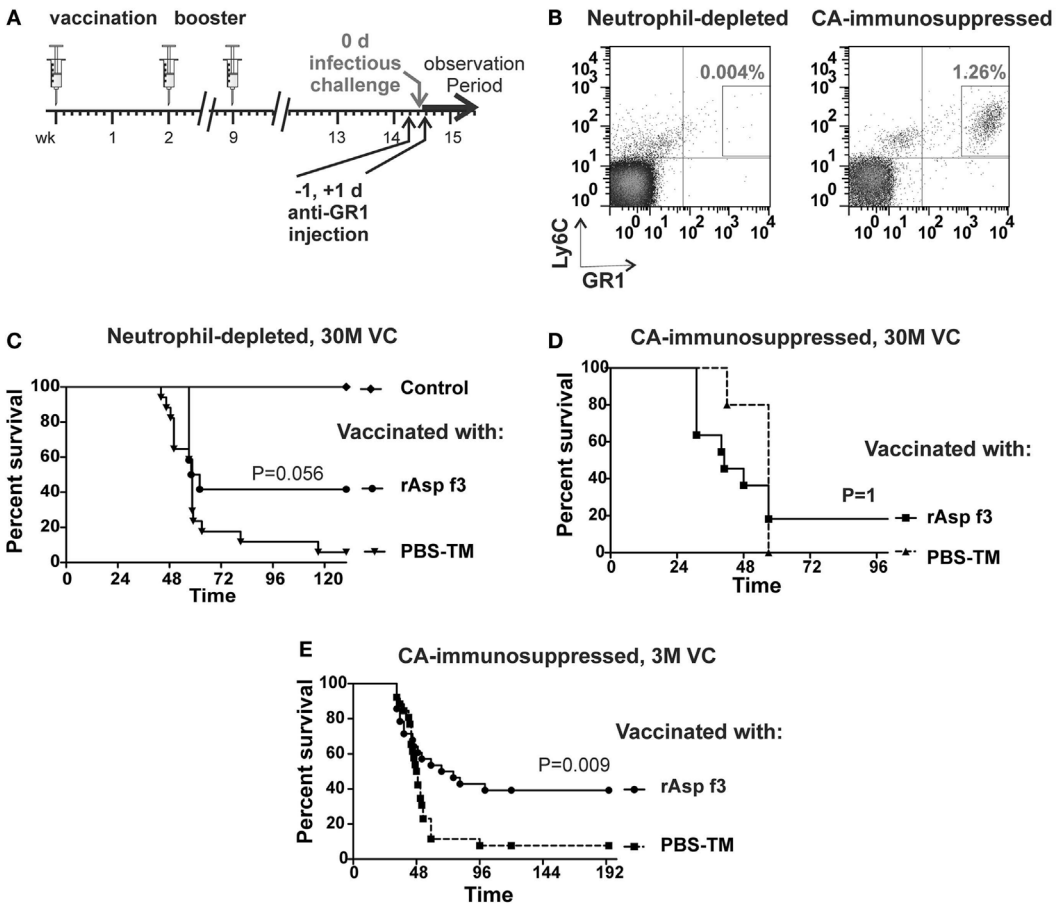

FIGURE 2 | The rAsp f3-based vaccine protects neutrophil-depleted mice. **(A)** Timeline of immunization, neutrophil depletion and *A. fumigatus* challenge. **(B)** FACS analysis of $Ly6C^{+}/GR1^{+}$ neutrophils in tail blood of rAsp f3-vaccinated mice after anti-GR1 antibody-mediated neutrophil depletion (left panel) compared to that of CA-immunosuppressed mice (right panel). **(C)** Kaplan–Meier survival curves of neutrophil-depleted mice challenged with 30 million viable conidia (VC) after mock vaccination with buffer and TiterMax (PBS-TM, triangles, $n = 17$) or rAsp f3-vaccine (rAsp f3, circles, $n = 12$), and non-immunosuppressed, non-vaccinated controls (Control, diamond, $n = 12$). **(D)** Survival of CA-immunosuppressed mice challenged with 30 million VC after rAsp f3-vaccination (rAsp f3, squares, $n = 12$) or mock vaccination (PBS-TM, triangles, $n = 5$). **(E)** CA-immunosuppressed mice challenged with three million VC, rAsp f3-vaccinated (circles, $n = 28$), or mock vaccinated (squares, $n = 26$).

IgG/CA). In contrast, ∼67% of the neutrophil-depleted mice survived the infection (**Figure 1A**, curve anti-GR1). However, at an infectious dose of 30 million VC, the survival of neutrophil-depleted mice was comparable to that of CA-immunosuppressed mice that received only three million spores (6% survival vs. 10–20% survival, respectively; **Figure 2C** vs. **Figure 1A**). Therefore we concluded that, CA treatment made the mice more susceptible to *A. fumigatus* infection than neutrophil depletion. Because we depleted neutrophils with a rat anti-GR1 antibody, we treated CA-immunosuppressed mice with a non-specific rat IgG as a control, which had no significant effect on survival. In addition, the control group that received only rat IgG and no further immunosuppression survived the infection entirely (**Figure 1A**). We also combined CA-immunosuppression with anti-GR1-mediated neutrophil depletion, but this did not further increase the susceptibility of the mice to *A. fumigatus* infection (**Figure 1A**). To confirm that neutrophils were depleted, we monitored neutrophil counts by FACS analysis using Ly6C and GR1 as markers. Twenty-four hours after injection of anti-GR1 antibody, neutrophil counts were reduced to 0.033% of normal levels (**Figure 1B**). Treatment with non-specific rat IgG did not change the neutrophil population of the mice (**Figure 1B**).

When we attempted to deplete neutrophils in CA-immunosuppressed mice, we observed that a population of neutrophils was still present after treatment, but their staining with an APC-labeled anti-GR1 antibody had shifted to lower intensities in the FACS analysis (**Figure 1C**). Furthermore, the circulating neutrophils were still coated with the anti-GR1 antibody that was given to induce their depletion (**Figure 1D**). Others have observed a similar shift in the population of labeled neutrophils when attempting to antibody-deplete neutrophils after reduction of peritoneal macrophage numbers with Clodronate liposomes (Mircescu et al., 2009). No significant reduction of monocytes and $CD8^{+}$ cells was observed after neutrophil depletion (**Figure 1E**).

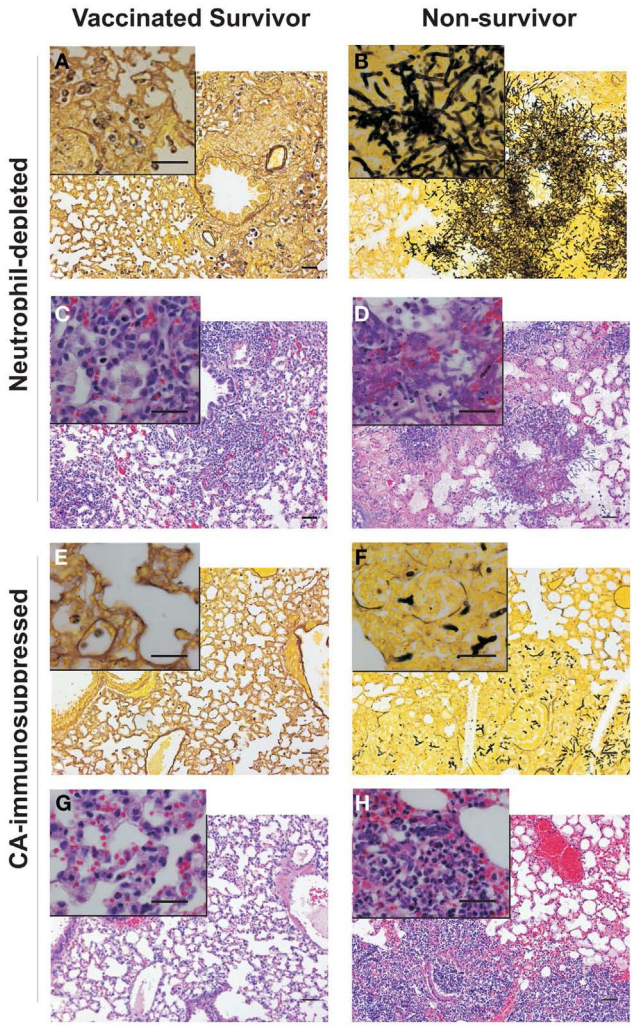

FIGURE 3 | Histopathologies of pulmonary IA. Micrographs of Gomori silver **(A,B,E,F)** and hematoxylin and eosin **(C,D,G,H)** staining of lung tissues from rAsp f3-vaccinated survivors **(A,C,E,G)** and mock vaccinated non-survivors **(B,D,F,H)** from neutrophil-depleted mice **(A–D)**, and CA-immunosuppressed mice **(E–H)**. Scale bars are 50 μm. The insert is a 4× magnification of the underlying micrograph.

Asp f3-VACCINE PROTECTION IS NOT ABOLISHED AFTER NEUTROPHIL DEPLETION

To study the role of neutrophils in rAsp f3-vaccinated mice, we depleted neutrophils with anti-GR1 antibody (**Figure 2A**). Administration of the antibody led to systemic depletion of neutrophils, down to 0.004% of the population, while neutrophils composed 1.26% of the population in mice that were immunosuppressed with CA (**Figure 2B**). Neutrophil depletion partially affected the survival of vaccinated mice in that 40% of the mice remained protected after *A. fumigatus* infection. In contrast, non-immunized, neutrophil-depleted mice were highly susceptible to the infection (**Figure 2C**). Although the observed difference in survival lacks strict statistical significance ($p = 0.056$) a trend of partial vaccine protection of neutrophil-depleted mice is apparent.

As a control, we immunosuppressed rAsp f3-vaccinated mice with CA and then challenged them with 30 million conidia. Only 20% of these mice survived *A. fumigatus* infection, and no statistical difference in survival ($p = 1$) was observed between rAsp f3-immunized mice and non-vaccinated mice (**Figure 2D**). We attribute the high mortality of vaccinated mice to the very high number of conidia (30 M) used for challenge. Consistent with our previous observations (Ito et al., 2006; Diaz-Arevalo et al., 2011), vaccinated, CA-immunosuppressed mice challenged with three million conidia had significantly enhanced survival ($p = 0.009$) over that of non-vaccinated mice (**Figure 2E**).

DIFFERENCES IN PULMONARY PATHOLOGY BETWEEN CA-IMMUNOSUPPRESSED AND NEUTROPHIL-DEPLETED MICE

Histological analysis using Gomori methenamine silver staining of the lungs of non-immunized, neutrophil-depleted mice showed extensive hyphal tissue invasion (**Figure 3A** vs. **Figure 3B**) characterized by hemorrhagic foci, with inflammatory infiltrates, necrotic tissue, and edema (**Figure 3C** vs. **Figure 3D**). The fungi were located in a few patchy areas in the lungs, and the mycelium contained multiply branched, densely grown hyphae (**Figure 3B**). In contrast, CA-immunosuppressed, non-vaccinated mice, had hyphal elements throughout the entire lung, but the mycelium consisted of only short hyphal fragments with very few branches (**Figure 3F**). In contrast, lungs of rAsp f3-vaccinated CA-immunosuppressed (**Figure 3E**) and neutrophil-depleted mice (**Figure 3A**) had no hyphae and only a small number of non-germinated conidia were present in the lung parenchyma.

Lungs of neutrophil-depleted, rAsp f3-vaccinated mice showed only mild infiltration of macrophages and $CD3^+$ T cells in the peribronchial tissue (**Figures 4A–C**). Vaccinated CA-immunosuppressed mice had a similar type of peribronchial infiltrate that included neutrophils (**Figures 4G–I**). The infiltrate of immune cells in neutrophil-depleted, non-immunized mice was characterized by presence of $CD3^+$ T cells and absence of neutrophils and alveolar macrophages (**Figures 4D–F**). The lungs of non-vaccinated, CA-immunosuppressed mice exhibited extensive infiltration of PMNs and $CD3^+$ T cells, but almost completely lacked alveolar macrophages (**Figures 4J–L**), and exhibited edema and focal hemorrhage (**Figure 3H**).

DISCUSSION

THE ROLE OF NEUTROPHILS IN ANTIFUNGAL PROTECTION

In the current study, we demonstrated that rAsp f3-vaccination shows a protective trend in neutrophil-depleted mice (40% survival) challenged with pulmonary *A. fumigatus* infection. Consistent, with previous work by others (Mircescu et al., 2009; Ibrahim-Granet et al., 2010), our data suggest that neutrophils are essential for the innate immune protection of *immunocompetent* animals against *A. fumigatus*. Furthermore, the protective effect of the vaccine can only be observed in immunosuppressed mice, as it has not been possible to induce fatal pulmonary *A. fumigatus* infections in immunocompetent mice. However, neutrophils do not appear to be the key effector cells that provide vaccine protection against *A. fumigatus*, because neutrophil depletion did not abolish the protective vaccine effect entirely.

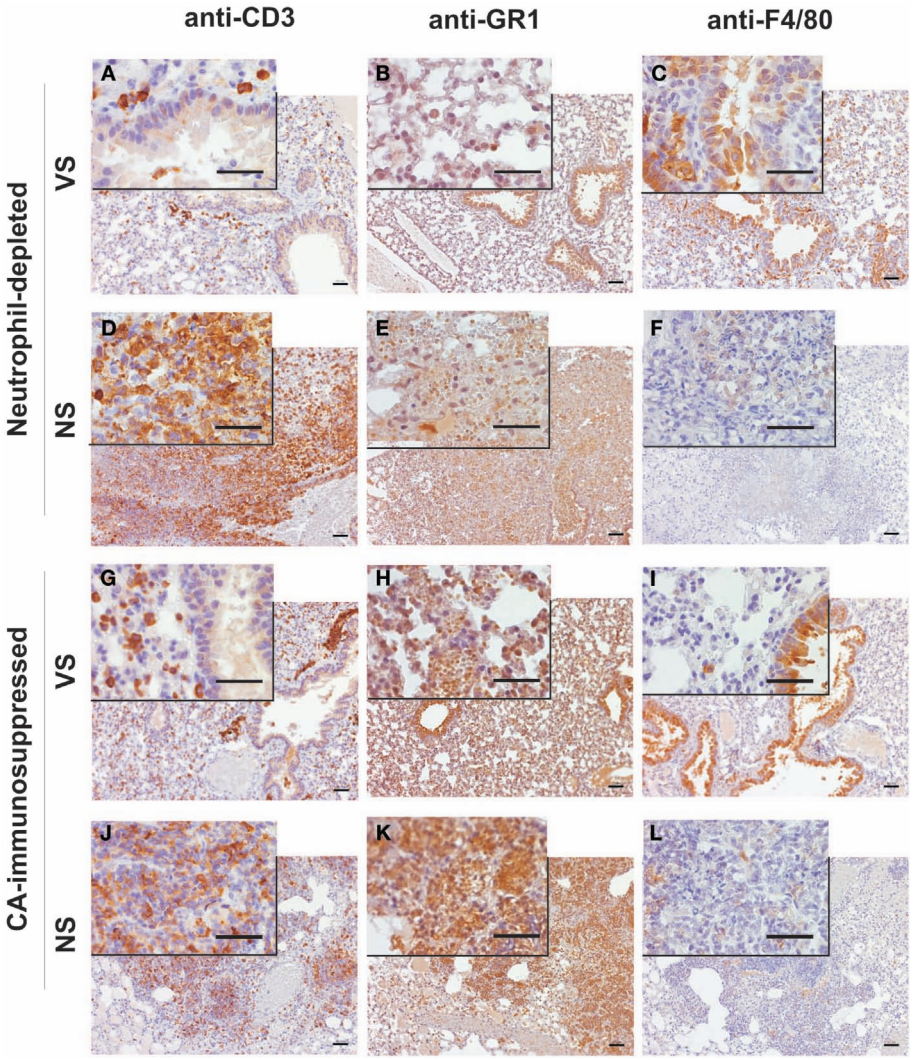

FIGURE 4 | Immunohistochemical characterization of the inflammatory infiltrates in lungs of mice with IA. Micrographs of *A. fumigatus* infected lungs of rAsp f3-vaccinated survivors [VS **(A–C,G–I)**] and mock vaccinated non-survivors [NS **(D–F,J–L)**] from neutrophil-depleted **(A–F)** or CA-immunosuppressed mice **(G–L)**. T cells were stained with anti-CD3 antibody **(A,D,G,J)**, neutrophils with anti-GR1 (RB6-8C5) antibody **(B,E,H,K)** and alveolar macrophages with anti-F4/80 antibody **(C,F,I,L)**, followed by anti-rat IgG-HRP antibody and 3,3′-diaminobenzidine staining (brown). Lungs of VS were collected 5 days after *A. fumigatus* infection. Scale bars are 50 μm. The insert is a 4 × magnification of the underlying micrograph.

A secondary auxiliary role in antifungal protection was previously attributed to neutrophils in an *in vitro* study in which specific T cells in combination with antigen presenting cells and neutrophils enhanced the damage to cultured *A. fumigatus* hyphae (Beck et al., 2006). It must be pointed out that the immune system of immunosuppressed mice can be overwhelmed when the infectious dose is sufficiently large, regardless of vaccination status. However, the markedly higher susceptibility of CA-immunosuppressed mice as compared to that of neutrophil-depleted mice indicates that macrophages contribute substantially to innate antifungal protection, as CA is known reduce the killing potential of macrophages (Schaffner, 1985; Ibrahim-Granet et al., 2003; Philippe et al., 2003), and it elicits a broad effect on the immune system by reducing the numbers of immune cells and cytokine signaling (Almawi and Melemedjian, 2002).

ANTIBODY-MEDIATED NEUTROPHIL DEPLETION

The RB6-8C5 antibody is known to bind to the neutrophil cell surface antigens Ly6G and Ly6C, the latter with lower affinity (Fleming et al., 1993). Because Ly6C is not only present on neutrophils, but also on monocytes (Henderson et al., 2003), plasmacytoid dendritic cells (Nakano et al., 2001), and a subpopulation of $CD8^+$ cells (Matsuzaki et al., 2003), we also analyzed PBMCs for a reduction of monocytes and $CD8^+$ cells, but found it not to be statically significant (**Figure 1E**) and consistent with previous observations (Mircescu et al., 2009). Although the more specific anti-Ly6G antibody 1A8 can alternatively be used to deplete neutrophils (Daley et al., 2008), we chose the RB5-8C5 antibody, which is readily available, has been well characterized, and used extensively to study the role of neutrophils in *A. fumigatus* infection (Mehrad et al., 1999; Mircescu et al., 2009; Ibrahim-Granet et al., 2010).

HISTOPATHOLOGY OF rAsp f3-VACCINATED AND NON-IMMUNIZED ANIMALS

The IA histopathology of CA-immunosuppressed mice was markedly different from that of neutrophil-depleted mice. The absence of a robust macrophage response in the non-immunized neutrophil-depleted mice may explain why *A. fumigatus* was able to invade the lungs and grow into dense patches with highly branched hyphal mycelium. Furthermore, the presence of a strong neutrophil infiltrate in CA-immunosuppressed mice, may explain why the hyphal elements appeared much shorter, because they may be constantly damaged by neutrophils, although this damage is not sufficient for fungal clearance. This observation is consistent with the suggestion that steroid-induced immunosuppression may lead to an inflammatory response that is destructive to lung tissue (Balloy et al., 2005; Stephens-Romero et al., 2005). However, neutrophil-depleted mice were not protected from IA either, and their inflammatory infiltrate at the time of death consisted mainly of $CD3^+$ T cells and largely lacked macrophages. It is possible that, due to the larger infectious inoculum used, the macrophages were simply overwhelmed or their resident population was exhausted. Interestingly, the $CD3^+$ T cell infiltrate was unable to recruit further macrophages, whereas in mice immunized with the rAsp f3-vaccine, T cell recruitment of macrophages to the point of infection appears to be associated with successful clearance of the fungal pathogen (**Figures 3** and **4**). Our observation does not exclude the possibility that other cell populations such as $CD8^+$ T cells and natural killer (NK) cells could contribute to vaccine based antifungal protection; however, the selective depletion of $CD4^+$ cells readily abolishes rAsp f3-based vaccine protection (Diaz-Arevalo et al., 2011). The involvement of NK cells in other type of vaccinations was demonstrated recently; for example the production of IL-2 by antigen specific $CD4^+$ T cells enhanced NK activation after vaccination either with *Plasmodium falciparum* (Horowitz et al., 2010b; McCall et al., 2010), rabies virus (Horowitz et al., 2010a), or simian immunodeficiency virus (Vargas-Inchaustegui et al., 2012). Therefore, although the effector role of macrophages appears to be very likely, further investigation will be required to understand if $CD8^+$ T or NK cells participate in the mechanism of the rAsp f3-based *A. fumigatus* vaccine.

CONCLUSION

Our results suggest CA-immunosuppressed mice are much more susceptible to *A. fumigatus* infection than neutrophil-depleted mice. Neutrophils, although important for innate antifungal protection of immunocompetent hosts, are not the relevant effectors for rAsp f3-vaccine derived protection in immunosuppressed hosts. Considering our immunohistochemical observations, it appears to be far more likely that macrophages (or possibly other cell types) are the crucial effectors of the rAsp f3-based vaccine. Further research will be required to refine this model.

ACKNOWLEDGMENTS

This study was supported by the Hermann Foundation and the Tim Nesvig Lymphoma Fellowship and Research Fund.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 May 2012; accepted: 28 July 2012; published online: 22 August 2012.
*Citation: Diaz-Arevalo D, Ito JI and Kalkum M (2012) Protective effector cells of the recombinant Asp f3 anti-aspergillosis vaccine. Front. Microbio. **3**:299. doi: 10.3389/fmicb.2012.00299*
This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 28 August 2012
doi: 10.3389/fmicb.2012.00291

Vaccine and immunotherapeutic approaches for the prevention of cryptococcosis: lessons learned from animal models

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Cryptococcus neoformans and *C. gattii*, the predominant etiological agents of cryptococcosis, can cause life-threatening infections of the central nervous system in immunocompromised and immunocompetent individuals. Cryptococcal meningoencephalitis is the most common disseminated fungal infection in AIDS patients, and *C. neoformans* remains the third most common invasive fungal infection among organ transplant recipients. Current anti-fungal drug therapies are oftentimes rendered ineffective due to drug toxicity, the emergence of drug resistant organisms, and/or the inability of the host's immune defenses to assist in eradication of the yeast. Therefore, there remains an urgent need for the development of immune-based therapies and/or vaccines to combat cryptococcosis. Studies in animal models have demonstrated the efficacy of various vaccination strategies and immune therapies to induce protection against cryptococcosis. This review will summarize the lessons learned from animal models supporting the feasibility of developing immunotherapeutics and vaccines to prevent cryptococcosis.

Keywords: *Cryptococcus neoformans*, cryptococcosis, *Cryptococcus*, fungal pathogenesis, host-fungal interactions, fungal vaccines

INTRODUCTION

Cryptococcus neoformans and C. gattii, the etiological agents of cryptococcosis, are encapsulated fungal pathogens that cause fungal pneumonia and life-threatening infections of the central nervous system (CNS) (Mitchell and Perfect, 1995). Cryptococcal meningoencephalitis remains the most common disseminated fungal infection in AIDS patients (Powderly, 1993). Global estimates suggest that one million cases of cryptococcal meningitis due predominantly to *C. neoformans* occur each year resulting in approximately 620,000 deaths (Park et al., 2009). *C. gattii* primarily causes life-threatening fungal meningitis and infections of the lung and skin in otherwise healthy individuals (Jarvis and Harrison, 2008). The geographical distribution of *C. gattii* was believed to be limited to tropical and subtropical climates such as Australia, New Zealand, and Southeast Asia (Kwon-Chung and Bennett, 1984). However, *C. gattii* infections began to occur within animal and human populations on Vancouver Island, British Columbia, Canada, a temperate climate. Subsequently, *C. gattii* disseminated onto the mainland of British Columbia and proceeded throughout the Pacific Northwest of the United States (Datta et al., 2009a,b; Centers for Disease Control and Prevention, 2010; Galanis and Macdougall, 2010).

The administration of highly active antiretroviral therapy (HAART) has resulted in a decrease in the number of cases of AIDS-related cryptococcosis in developed countries, but in developing countries where HAART is not readily available, *Cryptococcus* is still a major problem (Dromer et al., 2004). Those who are successfully treated for AIDS-associated cryptococcal meningitis oftentimes require life-long anti-fungal therapy due to a high relapse rate (Bozzette et al., 1991; Vibhagool et al., 2003). *C. neoformans* also remains the third most common invasive fungal infection among organ transplant recipients (Husain et al., 2001; Singh et al., 2005; Pappas et al., 2010). Studies have shown that 2.8% of organ transplant recipients can develop cryptococcal infections resulting in an overall estimated death rate of 42% (Husain et al., 2001). Also, cryptococcosis accounted for 8% of the invasive fungal diseases in solid organ transplant recipients in the Transplant Associated Infection Surveillance Network database from March 2001 to March 2006 (Pappas et al., 2010). The acute mortality rate is between 10 and 25% in medically advanced countries (Powderly, 1993), and at least one third of patients with cryptococcal meningitis who receive appropriate therapy will undergo mycologic and/or clinical failure (Van Der Horst et al., 1997; Saag et al., 2000). Thus, there remains an urgent need for therapies and/or vaccines to combat cryptococcosis.

The current clinical picture of cryptococcosis suggests that immunosuppressed patients or individuals predicted to be at an exceptionally high risk for developing cryptococcosis are likely targets for vaccination to prevent the development of cryptococcal disease. Additional target populations for vaccination as a prophylactic measure include immune competent persons in high exposure areas as highlighted by the recent *C. gattii* outbreak in Vancouver Island, British Columbia, Canada, or those at high risk for exposure to HIV. Animal models of cryptococcosis have been important tools for elucidating the mechanisms of protection and for testing the efficacy of various antibody

or antigen-based vaccine candidates. This review will focus on the lessons learned from animal models of cryptococcal disease that support efforts toward developing immunological therapies and/or vaccines against cryptococcosis.

HOST RESPONSE TO *CRYPTOCOCCUS*

The consensus is that cell-mediated immunity (CMI) primarily by Th1-type $CD4^+$ T cells directs the protective host immune response against *C. neoformans* (Huffnagle et al., 1991; Casadevall, 1995; Zhang et al., 2009), whereas a Th2-type immune response is considered detrimental to the host (Arora et al., 2005; Jain et al., 2009). A Th1-type response is characterized by the production of interleukin (IL)-2, IL-12, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) (Cherwinski et al., 1987). These cytokines induce granuloma formation in the lung to wall off the yeast and are responsible for initiating delayed-type hypersensitivity (DTH) reactions that lead to enhanced uptake, killing by neutrophils and dendritic cells, and the induction of classically activated macrophages (Cher and Mosmann, 1987; Zhang et al., 2009; Arora et al., 2011). A Th2-type response is characterized by the production of IL-4, IL-5, IL-9, IL-10, and IL-13 (Cherwinski et al., 1987), and is associated with eosinophilic inflammation and dissemination of cryptococci to the CNS (Huffnagle et al., 1994; Olszewski et al., 2001; Muller et al., 2007). A Th17-type response consisting of IL-17, IL-21, and IL-22 (reviewed in Onishi and Gaffen, 2010) has been suggested to be important in protection against *C. neoformans*, however, it seems to play a secondary role to the Th1-type response (Kleinschek et al., 2010). When a Th17-type response is blocked, there is no effect on survival of mice with experimental pulmonary *C. neoformans* infection (Wozniak et al., 2011a).

The predominant studies evaluating host immune responses to cryptococcal infections have been performed using *C. neoformans* as the model organism. However, several studies now suggest that the mechanism for mediating protection against *C. neoformans* infections differs from that which induces protection against *C. gattii*. Experimental studies suggest that *C. gattii* may thrive in immune competent hosts by suppressing host immune responses (Dong and Murphy, 1995; Wright et al., 2002; Cheng et al., 2009; Kronstad et al., 2011). *C. gattii* strain R265, the predominant isolate of the Vancouver Island outbreak, has been shown to be more suppressive in mice compared to those infected with *C. neoformans*. *C. gattii* infected mice had decreased neutrophil recruitment and decreased pro-inflammatory cytokine production (Dong and Murphy, 1995; Wright et al., 2002; Cheng et al., 2009).

While the patient population that historically develops cryptococcal infections includes individuals with defects in CMI, patients with defects in antibody-mediated immunity (AMI) such as hypogammaglobulinemia and hyper-IgM syndrome also have an increased probability of developing cryptococcosis (Gupta et al., 1987; Iseki et al., 1994; Tabone et al., 1994; Casadevall, 1995; Antachopoulos et al., 2007). The role of AMI in cryptococcal infections is complicated in that antibodies can be protective, non-protective, or can exacerbate the infection (Casadevall, 1995; Mukherjee et al., 1995a,b; Yuan et al., 1998). Antibody protection against cryptococcosis is dependent on the antibody isotype, concentration, and specificity (reviewed in Casadevall, 1995). Comparisons of variable-region-identical mAbs to the glucuronoxylomannan (GXM) component of the *C. neoformans* polysaccharide capsule of the murine IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 isotypes have consistently shown that all IgG isotypes, except IgG_3 are protective against *C. neoformans* infection in mice (Yuan et al., 1995, 1998; Nussbaum et al., 1996). Resistance to disease may depend upon the proportion of protective anti-cryptococcal antibodies produced during infection. However, the efficacy of anti-cryptococcal antibodies of a protective isotype can be lost if induced or passively administered in excess creating a prozone-like effect (Taborda and Casadevall, 2001; Taborda et al., 2003). Other major determinants of the effectiveness of antibody isotype against *C. neoformans* infection include mouse genetics (Rivera and Casadevall, 2005; Zaragoza et al., 2007), T cell function (Yuan et al., 1997), and the presence of Th1- and Th2-related cytokines (Beenhouwer et al., 2001). Despite what we know from animal studies concerning protective antibody isotypes, the efficacy of various immunoglobulin isotypes against *C. neoformans* may vary between humans and mice (Beenhouwer et al., 2007) necessitating some caution in trying to translate findings in animal models to the clinic. Nevertheless, anti-cryptococcal antibodies can act as opsonins, participate in antibody-dependent cellular cytotoxicity, augment Th1-type polarization, and help to eliminate immunosuppressive polysaccharide antigen from serum and tissues. They also inhibit cryptococcal biofilm formation, alter lipid metabolism resulting in increased sensitivity to anti-fungal drugs, have direct anti-fungal activity, and modulate the immune response to prevent host damage (Martinez and Casadevall, 2005; Casadevall and Pirofski, 2007; Mcclelland et al., 2010; Brena et al., 2011). The role of antibodies in the host defense against fungal infection remains controversial; however, some recent studies continue to show a protective role of antibodies against cryptococcosis. For example, naturally occurring IgM (nIgM) is essential for the prevention of cryptococcosis in mice (Subramaniam et al., 2010). Cryptococcal disease is more severe in mice lacking serum IgM and in sIgM knockout mice. Experimental pulmonary *C. neoformans* infection of sIgM knockout mice results in increased mortality and higher blood and brain fungal burden compared to that observed in wild-type infected mice. While it is generally agreed that the protective host immune response to *C. neoformans* is dependent on inducing protective CMI, AMI certainly contributes in the protection against cryptococcosis.

CRYPTOCOCCAL PROTEIN TARGETS

Significant efforts have been made towards identification of cryptococcal antigens that induce *Cryptococcus*-specific Th1-type immune responses. This effort is based on the predominant role for CMI responses to mediate protection against *C. neoformans* infections. Protein antigens can induce T-dependent immune responses and thus be highly immunogenic. Additionally, proteins are biochemically defined, thus allowing for the production of large quantities of recombinant proteins. There is also no fear of protein "reversion" that could possibly occur using an attenuated strain to induce immunity. Immunization of mice with *C. neoformans* culture filtrate antigen (CneF), which contains secreted proteins as well as cell wall-associated proteins, resulted in the stimulation of DTH responses (Murphy et al., 1988). Fractionation

of CneF revealed its mannoprotein (MP) fraction as the primary antigenic component responsible for DTH and T-dependent immune responses in mice (Murphy et al., 1988; Levitz and Specht, 2006). Mice given two immunizations of purified cryptococcal MP (MP98) experienced prolonged survival and had reduced CFUs in the organs when compared to the control mice receiving adjuvant alone (Mansour et al., 2002). Cryptococcal MP induced Th1-type responses in immunized mice upon infection with *C. neoformans* (Mansour et al., 2002). Four protective MP, one being a chitin deacetylase (MP98) (Levitz et al., 2001), one of unknown function (MP88) (Huang et al., 2002), and an additional two, MP84 and MP115 (Biondo et al., 2005) have been found.

In addition to the MP found in the cell wall, other potential targets include the heat shock protein (hsp) family (Kakeya et al., 1997; Nooney et al., 2005), fungal glucosylceramide (Rodrigues et al., 2007), and melanin (Rosas et al., 2001). MAbs to melanin have been shown to prolong the survival of and reduce fungal burden in lethally infected mice and reduce the growth rate of the fungus *in vitro* (Rosas et al., 2001). In the hsp family, hsp70 has been found to be highly immunogenic (Kakeya et al., 1997). Mycograb, a recombinant antibody targeting an epitope within the hsp90 of *Candida albicans* that is conserved in *Cryptococcus*, leads to enhanced fungicidal activity when combined with amphotericin B treatment of infected mice (Nooney et al., 2005). Targeting antigens common to multiple fungal species such as hsps or melanin may serve to extend protection to multiple disparate fungal pathogens.

CYTOKINE IMMUNOTHERAPIES

Adaptive immunity against cryptococcosis is orchestrated via cytokine/chemokine production by Th1-type $CD4^+$cells. Consequently, the historic objective of most immune-based therapies has been to skew the non-protective immune responses toward a protective Th1-type immune response. For example, IL-2 given along with a CD40 agonist antibody significantly prolonged the survival of *C. neoformans*-systemically infected mice (Zhou et al., 2006, 2007). In addition, mice depleted of $CD4^+$ T cells were protected when stimulated with CD40 and IL-2 compared to depleted mice without these stimulations. This model also showed significant reduction of fungal burden in the organs and increased levels of IFN-γ and TNF-α (Zhou et al., 2006, 2007). Furthermore, mice treated with recombinant TNF-α experienced significantly prolonged survival times compared to non-treated control mice (Kawakami et al., 1996a). Additionally, treatment of mice with a TNF-α-expressing adenoviral vector after experimental challenge of *C. neoformans* resulted in a significant decrease in fungal burden, an increase in IFN-γ levels, and an increase in infiltration of macrophages and neutrophils (Milam et al., 2007). Treatment with IL-12 also led to significant increases in survival of mice with experimental cryptococcosis, but the timing of the treatment is important. Treating mice with IL-12 for 7 days post infection resulted in 90% survival, but delaying IL-12 treatment until day 7 post infection negated the effect of the IL-12, and the mice had similar survival rates compared to the untreated mice (Kawakami et al., 1996c). Administering IL-23, a cytokine necessary for the proliferation and stabilization of the Th17-type cells (reviewed in Onishi and Gaffen, 2010), significantly extended the survival of intraperitoneally infected mice (Kleinschek et al., 2010). However, when compared to their positive control group that received IL-12 and showed 100% survival at day 100, it was concluded that IL-23 may play only a limited role in the protection against *C. neoformans* (Kleinschek et al., 2010). Treating mice with IL-2 and anti-CD40, TNF-α, or IL-12 led to an increase in IFN-γ levels (Kawakami et al., 1996c; Zhou et al., 2006; Milam et al., 2007). Giving mice daily injections of IFN-γ prolonged their survival and reduced the fungal burden in the lungs and the brain (Kawakami et al., 1996b).

Studies in humans indicated that the cytokine profile at the site of infection determines the rate of clearance. Patients with higher IFN-γ at the site of infection had a faster rate of clearance compared to those with lower levels (Siddiqui et al., 2005), suggesting that the microenvironment at the site infection is more important than the macroenvironment. Considering this study, a highly virulent strain of *C. neoformans*, H99, was modified to produce murine IFN-γ. Mice given an experimental pulmonary infection with the IFN-γ producing *C. neoformans* strain, designated H99γ,were capable of completely resolving the infection and showed 100% protection upon re-challenge with wild-type cryptococci (Wormley et al., 2007). This protection was shown to be T cell dependent and B cell independent (Wozniak et al., 2009). T cell knock out (KO) mice succumbed to an experimental pulmonary infection with H99γ whereas B cell KO mice survived and showed 100% protection upon challenge with the wild-type *C. neoformans* strain H99 (Wozniak et al., 2009). Mice immunized with the H99γ strain and then depleted of either $CD4^+$ or $CD8^+$ T cells prior to and during subsequent challenge with wild-type *C. neoformans*, remained 100% protected (Wozniak et al., 2009). Remarkably, when the same experiment was performed with depletion of both $CD4^+$ and $CD8^+$ T cells during the challenge with wild-type cryptococci, there was still 80% survival (Wozniak et al., 2011b). These results suggest that if immune competent individuals are immunized against *C. neoformans* and then become T cell deficient they may still be protected. This system also produced high amounts of IL-17A, but when IL-17A deficient or IL-17A receptor KO mice were immunized with the H99γ strain there was no difference in protection compared to the wild-type mice (Hardison et al., 2010; Wozniak et al., 2011a). Thus, IL-17A may contribute to, but ultimately be dispensable for, protection against *C. neoformans* infection.

Cytokine treatment has also shown promising results when administered along with conventional anti-fungal drugs. In a systemic infection model, treating mice with IL-12 along with fluconazole significantly lowered the fungal burden in the brain compared to mice treated with fluconazole alone (Clemons et al., 1994). Administering IFN-γ along with amphotericin B during a lethal systemic infection model significantly lowered the fungal burden in the brain compared to amphotericin alone (Lutz et al., 2000). In a sub-lethal systemic infection model amphotericin B with IFN-γ led to complete clearance of the infection (Lutz et al., 2000). Fluconazole and IFN-γ significantly lowered the fungal burden in both the lethal and sub-lethal systemic infection models but failed to completely resolve the infection in any of the models (Lutz et al., 2000). Cytokine treatment may be necessary considering the immune suppressed status of the typical cryptococcosis patient. However, the possible induction of *C. neoformans*-related

immune reconstitution inflammatory syndrome (IRIS) which is the induction of over-exuberant pro-inflammatory responses following the resolution of immune suppression necessitates caution when proceeding with such approaches.

ANTIBODY-MEDIATED IMMUNOTHERAPIES AND ANTIBODY VACCINES

One of the main virulence factors of *Cryptococcus* is the polysaccharide capsule that is comprised primarily of the polysaccharides GXM and galactoxylomannan (GalXM) and to a much lesser extent, <1%, MP (Zaragoza et al., 2009). Cryptococcal polysaccharides such as GXM have profound suppressive effects on immune responses and elicit little to no antibody responses making it an unlikely choice as an antigen (reviewed in Zaragoza et al., 2009). GXM can bleb off the fungus and direct immune responses away from infection, while GXM and GalXM both can non-specifically diminish lymphocyte proliferation and induce apoptosis (Yauch et al., 2006; Cutler et al., 2007; Chow and Casadevall, 2011; Vecchiarelli et al., 2011). However, an entirely plausible strategy to induce protection against cryptococcosis may be to develop antibodies against specific capsular components. Mouse models have been utilized to investigate the efficacy of cryptococcal conjugate vaccines in which GXM or GalXM has been conjugated to protein carriers (Casadevall et al., 1992, 1998; Devi, 1996; Chow and Casadevall, 2011). GXM conjugated to the tetanus toxoid resulted in anti-GXM protective antibody responses and antibodies specific for GXM which help protect against cryptococcosis (Casadevall et al., 1992, 1998; Devi, 1996). Two GalXM conjugates where tested and found to be useful in generating high titers of anti-GalXM antibodies in the serum, but the responses were not protective (Chow and Casadevall, 2011). Casadevall developed an antibody to the capsule, 18B7, which was found to bind to all serotypes, enhance phagocytosis, and help with the clearance of the blebs of polysaccharide released by the fungus (Casadevall, 1995). Recently, MAb 18B7 has been conjugated to the therapeutic radioisotopes ^{188}Rhenium or ^{213}Bismuth (Dadachova et al., 2003; Bryan et al., 2010) and studied as a potential radioimmunotherapy (RIT) against *C. neoformans*. Administration of radiolabeled MAb 18B7 to lethally infected mice resulted in prolonged survival, reduced organ fungal burden, and was a more effective therapy compared to mice treated with amphotericin B alone.

A potential strategy to circumvent the immunosuppressant effects of GXM and GalXM may be to focus on specific epitopes within the polysaccharides that elicit protective antibody responses. An approach using small peptides that mimic defined GXM or GalXM epitopes may elicit protective antibody responses where using total polysaccharides has immunosuppressive effects. Peptides which are able to induce antibodies that are capable of binding to the native antigen when administered as immunogens are termed mimotopes. A peptide mimetic (P13) of GXM was developed that is recognized by human anti-GXM antibodies (Zhang et al., 1997). Vaccination with P13-protein conjugates in mice elicited an antibody response to GXM (Zhang et al., 1997). Additionally, mice immunized with the conjugates experienced prolonged survival after an otherwise lethal *C. neoformans* challenge compared to controls (Fleuridor et al., 2001) or following establishment of a chronic infection (Datta et al., 2008). Immunization with P13-protein conjugates also prolonged survival in mice transgenic for human immunoglobulin loci (Fleuridor et al., 2001) and passive immunization with serum from P13-conjugate vaccinated mice to naïve mice conferred partial protection to a lethal cryptococcal challenge (Maitta et al., 2004; Datta et al., 2008). Nonetheless, the therapeutic efficacy of conjugate vaccines containing a peptide mimotope of GXM appears to depend on the genetic background of the host and the carrier protein employed (Datta et al., 2008). Conjugation of P13 to keyhole limpet marine mollusk (KLH) elicited an anti-GXM response to non-protective epitopes possibly due to the generation of non-protective antibodies against an epitope within the carrier protein that is cross-reactive to GXM (May et al., 2003).

Development of antibodies targeting essential components within the fungal cell wall may be an additional method of antibody-mediated protection against cryptococcosis. Treatment with an anti-β-glucan antibody, MAb 2G8, which targets β(1,3) glucans of the brown algae, *Laminaria digitata*, was shown to inhibit capsule formation and growth of *C. neoformans in vitro* and mediate a significant reduction in fungal burden in mice given an experimental systemic cryptococcal infection (Rachini et al., 2007). Also, passive immunization with MAbs against melanin or glucosylceramide prolonged the survival of mice given a lethal infection with *C. neoformans* (Rosas et al., 2001; Rodrigues et al., 2007). Again, targeting fungal antigens that are common among multiple fungal pathogens is an appealing strategy toward generating cross-protection using one immune-therapy and/or vaccine.

CONCLUSION

The need for novel treatments to combat cryptococcosis is expected to rise. This is due to an increasing population of immune compromised patients, a high relapse rate, and increased percentage of patients predicted to experience mycologic and/or clinical failure. Animal models of cryptococcosis have been important tools for elucidating the mechanisms of protection against cryptococcosis and for testing the efficacy of various antibody or antigen-based vaccine candidates against cryptococcal infection. Overall, animal models have provided a means for studying strategies for modulating T helper responses and evaluating antibodies and radioimmunotherapies. They have also proved useful in developing approaches for inducing cell responses using peptide mimotopes and specific cryptococcal antigens. Perhaps a shift in approach from defining targets for anti-fungal drug development toward devising immune therapies and/or vaccines that enhance host anti-fungal immune responses is a direction requiring more focus. Such studies will concentrate more on the host and less on the pathogen. Still, *Cryptococcus* is a disease in which the host and pathogen are intimately intertwined. Consequently, animal models will surely be required to understand the relationship and devise plausible treatments and/or vaccines to prevent cryptococcal infections.

ACKNOWLEDGMENTS

Dr. Wormley and Mr. Hole are supported by the Army Research Office of the Department of Defense under Contract No.

W911NF-11-1-0136 and grants RO1 AI071752-05 and R21 AI083718-02 from the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the Department of Defense or NIAID of the NIH.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 May 2012; accepted: 24 July 2012; published online: 28 August 2012.
Citation: Hole CR and Wormley FL Jr. (2012) Vaccine and immunotherapeutic approaches for the prevention of cryptococcosis: lessons learned from animal models. Front. Microbio. ***3****:291. doi: 10.3389/fmicb.2012.00291*
This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 12 June 2012
doi: 10.3389/fmicb.2012.00212

New advances in the development of a vaccine against paracoccidioidomycosis

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Paracoccidioidomycosis (PCM) is an endemic Latin American mycosis caused by *Paracoccidioides brasiliensis* and also by the recently described *P. lutzii*. The systemic mycosis is the 10th leading cause of death due to infectious diseases in Brazil. As published, 1,853 patients died of PCM in the 1996–2006 decade in this country. The main diagnostic antigen of *P. brasiliensis* is the 43 kDa glycoprotein gp43, and its 15-mer peptide QTLIAIHTLAIRYAN, known as P10, contains the T-CD4$^+$ epitope that elicits an IFN-γ-mediated Th1 immune response, which effectively treats mice intratracheally infected with PCM. The association of peptide P10 with antifungal drugs rendered an additive protective effect, even in immunosuppressed animals, being the basis of a recommended treatment protocol. Other immunotherapeutic tools include a peptide carrying a B cell epitope as well as protective anti-gp43 monoclonal antibodies. New delivery systems and gene therapy have been studied in prophylactic and therapeutic protocols to improve the efficacy of the recognized antigens aiming at a future vaccine as co-adjuvant therapy in patients with PCM.

Keywords: *P. brasiliensis, P. lutzii*, paracoccidioidomycosis, vaccine, immunotherapy

INTRODUCTION

Paracoccidioidomycosis (PCM) appears to be caused by a complex group of fungi within the *Paracoccidioides* genus comprising four distinct phylogenetic lineages known as PS2, PS3, S1, and Pb01-like (Carvalho et al., 2005; Matute et al., 2006). Based on clinical and genetic studies, the Pb01 isolate differs from the other strains and has been included in a new species known as *Paracoccidioides lutzii* (Teixeira et al., 2009).

The disease is endemic in a broad region from Mexico to Argentina. About 80% of diagnosed patients are from Brazil. Most patients are rural workers but cases in urban centers located on the route of migration movements are also found (Restrepo, 1985; McEwen et al., 1995). The infection starts by inhalation of conidia that subsequently transform into infective yeast forms in the lung. Although acquisition of the fungus typically results in asymptomatic infection, it can progress in susceptible individuals and give rise to acute, subacute, and chronic clinical forms of the disease (Franco et al., 1993). Systemic dissemination of the fungus can be fatal. A mortality evaluation of *P. brasiliensis* showed that it is the 10th most common cause of death owing to chronic/recurrent infections and parasitic diseases in Brazil. When analyzed as the underlying cause, 51.2% of deaths were due to PCM, which is then one of the most lethal among systemic mycoses. In the 1996–2006 decade, the most severe cases of PCM occurred in the 30–59 years-of-age range, predominantly (87%) in men (Prado et al., 2009). Besides the mortality data, it is important to consider the morbidity associated to the disease, which invariably leads to withdrawal of the patients from labor activities or school. In the severe cases, hospitalization of patients is necessary for long periods of time with high costs.

Antifungal chemotherapy is required for PCM treatment, although there is no assurance, even after treatment, of complete destruction of the fungus. Initial treatment depends on the severity of the disease and may last from 2 to 6 months; it includes sulfonamides, amphotericin B, or azoles. In severe cases endovenous amphotericin B or sulfonamides are required and when there is clinical improvement, it can be switched to oral sulfonamides or azoles. Extended periods of treatment are often necessary, up to 2 or more years, with a significant frequency of relapsing disease. According to Brazilian guidelines, oral itraconazole is the drug of choice (Shikanai-Yasuda, 2005; Shikanai-Yasuda et al., 2006; Travassos and Taborda, 2011).

Although chemotherapy stands as the basic treatment of PCM, therapeutic vaccination with fungal antigens or passive transfer of specific monoclonal antibodies may boost the cell immune response and add to the protective effect of chemotherapy, eventually counteracting a relapsing disease and reducing fibrotic sequels. Both the innate immune response and the adaptive immunity are important for the antifungal protective effect. The immune system recognizes fungal antigens with subsequent eliciting of antibodies and T cell protective responses. Cytokines and chemokines are produced. IFN-γ-activated macrophages have increased fungistatic and fungicidal activities. Antigens of *P. brasiliensis*, gp43 or P10, depend on IFN-γ for their protective activity (reviewed in Travassos et al., 2008).

The aim of this review is to update the new concepts and methodologies used in the attempt to develop a therapeutic vaccine against PCM.

THE USE OF FUNGAL WHOLE CELLS AS VACCINE

The use of low-virulence yeast cells as the immunization tool has been investigated. Subcutaneous infection with *P. brasiliensis* Pb265 induced cellular immunity with high T cell reactivity in susceptible mice which resulted in immunoprotection or disease exacerbation depending on the route of a secondary infection (Arruda et al., 2007a). Immunoprotection with aseptical cure was shown in the pre-immunization procedure and required a combination of $CD4^+$ T cells and $CD8^+$ T cells and the production of endogenous IFN-γ and IL-12 as well as increased levels of anti-*P. brasiliensis*-specific IgG1 and IgG2a antibodies (Arruda et al., 2007b).

Radio-attenuated yeast cells lost their virulence after the exposure to 6.5 kGy. The irradiated cells were examined by scanning, and also transmission electron microscopy. When examined 2 h after irradiation the cells showed deep folds or collapsed. The plasma membrane and cell wall were intact, but an extensive DNA fragmentation was found (Demicheli et al., 2007). The use of attenuated yeast cells by gamma-irradiation induced a long lasting protection in BALB/c mice (Demicheli et al., 2006; do Nascimento Martins et al., 2007). For this purpose, BALB/c mice were immunized twice in the ocular plexus with 10^5 viable cells. Animals were then challenged, by the same route, with yeast cells after 30, 45, and 60 days after the last immunization. Thirty days after the challenge, a significant reduction in the fungal burden was observed in the lung, spleen, and liver. A 99.5% decrease in CFU was obtained 90 days post challenge. The animals showed high levels of IFN-γ and IgG2a with very low production of IL-10 and IL-5, suggestive of a Th1 immune cell response (do Nascimento Martins et al., 2007). The number of immunizations with radio-attenuated yeast cells also interferes with the immune response. Authors found that mice immunized once developed a mixed Th1/Th2 response, which was less efficient in the control of the infection, whereas a Th1 pattern was obtained with two immunizations resulting in the elimination of *P. brasiliensis* yeast cells (do Nascimento Martins et al., 2009).

UNDEFINED SOLUBLE ANTIGENS

Soluble antigens of *P. brasiliensis* and fractions obtained by ion exchange chromatography of *P. brasiliensis* extract (F0, FII, and FIII), showed variations in the induction of a protective immune response. Mice immunized with F0 and FII developed benign chronic PCM restricted to the lung, associated with low mortality rates and the presence of compact granulomas with few fungal cells. Significant enhancement of IFN-γ and high levels of IgG2a and IgG3 were found in animals immunized with F0. Immunizations with FII induced significant production of IFN-γ and IL-10 associated with high levels of IgG1 and IgG2a. In contrast, mice immunized with FIII developed a progressive disease with dissemination to spleen and liver. These animals did not control the spread of the fungus showing granulomas with a high number of viable fungal cells (Diniz et al., 2004). A fraction of approximately 380 kDa designated high-molecular-mass (hMM) fraction was able to induce lymphocyte proliferation and production of IFN-γ but not IL-4 when incubated with spleen cells from BALB/c mice infected intravenous with *P. brasiliensis* (isolate Pb18). Animals previously immunized s.c. with hMM and infected with virulent yeast cells showed a significant reduction of the fungal burden in the lungs and spleen (Pavanelli et al., 2007).

PURIFIED ANTIGEN AND PEPTIDE

The major diagnostic antigen gp43 was isolated from *P. brasiliensis* culture supernatant fluids in 1986 (Puccia et al., 1986). It reacted with antibodies from virtually 100% of patients with PMC, except some patients exposed to *P. lutzii*, who showed irregular reactivity to gp43 (Batista et al., 2010). Epitopes in gp43 that elicit a strong antibody response are peptidic in nature (Puccia and Travassos, 1991) and different isoforms of gp43 vary in their reactivity with patients sera. The gp43 gene was cloned and sequenced (Cisalpino et al., 1996); gene expression and polymorphism have been shown (Travassos et al., 2004a,b).

The first evidence that gp43 carried an immunodominant epitope able to elicit DTH reactions was shown in guinea pigs (Rodrigues and Travassos, 1994) and later in patients (Saraiva et al., 1996) using the purified antigen. The T cell epitope responsible for DTH reactions, and $CD4^+$ T cell proliferation, was mapped to a peptide called P10 with the sequence QTLIAIHTLAIRYAN (Taborda et al., 1998). The hexapeptide HTLAIR has been shown to be essential for priming the cellular immune response. In *P. lutzii*, there is, however, an important mutation in this hexapeptide (Teixeira et al., 2009). Peptide P10 showed to be promiscuous in its presentation by MHC class II molecules from three different mouse haplotypes (Taborda et al., 1998). This was extended to most Caucasian HLA-DR molecules (Iwai et al., 2003). By using the TEPITOPE algorithm neighboring peptides to P10 were also recognized with high affinity HLA-DR binding (Iwai et al., 2007). The protective effect of P10 using complete Freund's adjuvant (CFA) against intratracheally infected mice is shown in **Figure 1**.

The association of peptide P10 immunization and chemotherapy was tested in i.t. infected BALB/c mice using two protocols. Mice infected with yeast cells of the highly virulent *P. brasiliensis* (Pb18) underwent P10 and/or drug treatment starting after 48 h or 30 days of infection. The treatment continued for 30 days, during which groups of mice received intraperitoneal doses of itraconazole, fluconazole, ketoconazole, sulfamethoxazole, or trimethoprim/sulfamethoxazole every 24 h. Amphotericin B was administered every 48 h. Immunization with P10 was carried out weekly for 4 weeks, once in CFA and three times in incomplete Freund's adjuvant (Marques et al., 2006). Animals were sacrificed at different times of infection and significant reduction in the fungal load was observed in both groups, with an additive protective effect obtained with the combination of P10 and antifungal drugs (Marques et al., 2006). Unexpectedly, animals treated with sulfamethoxazole, showed early protection followed by relapse, but the association of sulfamethoxazole and P10 vaccination successfully controlled the infection. The detection of cytokines in lung homogenates from mice vaccinated with P10, showed a typical Th1 response, rich in IFN-γ and IL-12 but without suppression of Th2 cytokines (Marques et al., 2006).

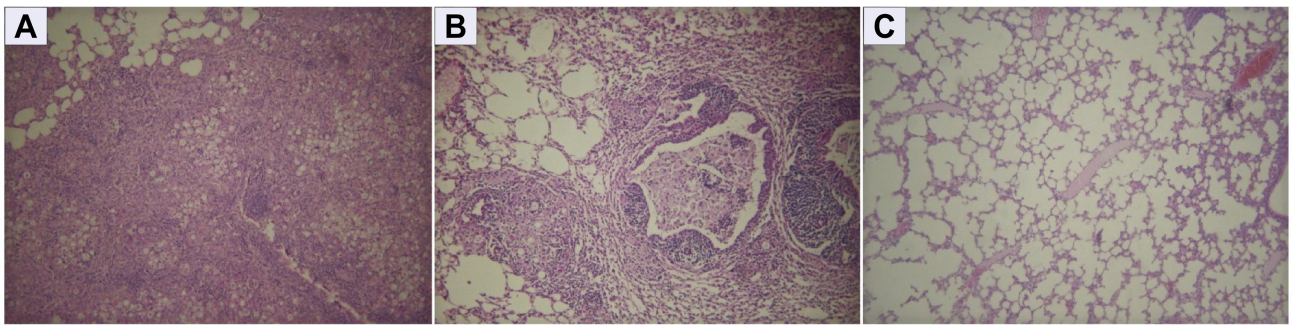

FIGURE 1 | Representative histopathology of lung lesions caused by *P. brasiliensis* Pb18 strain in mice immunized with P10 in presence of Freund's complete adjuvant (CFA). Tissue samples were collected two months after i.t. challenge with the Pb18 strain. **(A)** Lung section from infected mouse, with a granuloma containing multiple viable fungal cells; **(B)** Lung section from mouse treated with CFA, showing the extensive granulomatous lesion with intense cellular infiltration and large number of multiplying fungal cells; **(C)** Lung section from mouse immunized with P10 admixed with CFA showing preserved alveolar structure, absence of granulomatous lesions as well as of fungal cells. All sections were amplified 10-fold and stained with hematoxylin–eosin.

In an attempt to reproduce a general anergic state, BALB/c mice were treated with dexamethasone-21 phosphate added to the drinking water. After 30 days animals showed negative DTH to fungal antigens. Mice were then infected with virulent *P. brasiliensis* (Pb18) and after 15 days were subjected to chemotherapy and/or P10 immunization. The association of drugs and P10 immunization conferred additive protection. A significant increase in IL-12 and IFN-γ and decrease of IL-4 and IL-10 were observed in mice immunized with P10 alone or in association with antifungal drugs, indicating that also in this case of immune suppression, P10 immunization can be helpful (Marques et al., 2008).

Different ways of peptide delivery using formulations that did not include CFA have also been investigated. The first attempt involved a multiple antigen peptide (MAP) construction. A tetramer of truncated P10 was designated M10 and had four equal LIAIHTLAIRYAN (N-terminal QT-less P10) chains synthesized on a branched lysine core containing glycine at the C-terminal position. Mice immunized with a single dose of M10 without adjuvant and challenged intratracheally with *P. brasiliensis* showed significantly fewer lung, spleen, and liver CFUs and few or no yeasts in lung sections histopathology (Taborda et al., 2004).

The therapeutic or prophylactic protective effect of P10 was also tested with the peptide admixed with different adjuvants, bacterial flagellin, aluminum hydroxide, cationic lipid in comparison with CFA. A vaccine formulation based on intranasal administrations of gp43 or P10 with the *Salmonella enterica* FliC flagellin was evaluated in BALB/c mice. Animals were immunized with recombinant purified flagellins genetically fused with P10, either or not flanked by two lysine residues, or with the synthetic P10 admixed with purified FliC. After the last immunization, mice were i.t. infected with *P. brasiliensis* (Pb18). BALB/c mice immunized with the chimeric flagellins and particularly those immunized with P10 admixed with FliC had reduced fungal burden in the lungs and elicited a predominantly Th1-type immune response (Braga et al., 2009). Other adjuvants were also compared in terms of protective immune response to P10 immunization. Reduction of the pulmonary fungal burden was obtained with aluminum hydroxide, CFA, flagellin, and cationic lipid in intratracheal infected BALB/c mice. The cationic lipid proved to be very efficient in the clearance of fungal load and reduction of fibrotic areas in the lung (Mayorga et al., 2012).

The combination of P10 with sulfamethoxazole/trimethoprim entrapped within poly- (lactic acid–glycolic acid) nanoparticles (PLGA) was tested in the experimental therapeutic protocol of PCM. The incorporation of P10 into PLGA reduced the amount of this peptide necessary to decrease the fungal load in the infected animals and avoid disease relapse when compared with P10 emulsified in Freund's adjuvant (Amaral et al., 2010).

The potential use of the recombinant protein of 27 kDa (rPb27), present in the soluble fraction F0 (Reis et al., 2008), has been investigated. Immunization of rPb27 in the presence of *Propionibacterium acnes* and aluminum hydroxide prior to intravenous infection by the orbital plexus with virulent *P. brasiliensis* (Pb18), was able to efficiently protect BALB/c mice. Recently, another recombinant protein, rPb40, was used associated with fluconazole and shown to reduce the fungal burden in the lungs of BALB/c mice (Fernandes et al., 2011, 2012).

DNA VACCINE AND IMMUNOTHERAPY WITH DENDRITIC CELLS

Immunization of BALB/c mice with a mammalian expression vector (VR-gp43) carrying the full gene of gp43 with CMV promoter induced B and T cell-mediated immune responses protective against the intratracheal challenge by virulent *P. brasiliensis* yeast forms. The cellular immune response in mice immunized with VR-gp43 induced IFN-γ and the response was maintained for at least 6 months although reduced to half of the stimulation index obtained 15 days after immunization (Pinto et al., 2000). In order to develop a more specific DNA vaccine based predominantly on a T cell-mediated immune response, a plasmid encoding the P10 minigene in pcDNA3 expression vector was tested in intratracheally infected BALB/c and B10.A mice. The vaccination with plasmid encoding P10 induced a significant reduction in the fungal burden in the lung. Co-vaccination with a plasmid encoding mouse IL-12 proved to be even more effective in the elimination of the fungus with virtual sterilization in the long-term infection and treatment

assay, using the more susceptible B10.A mice. The immunization elicited significant production of IL-12 and IFN-γ (Rittner et al., 2012). Such immunization with plasmid encoding P10 induced memory cells as well as T regulatory cells (Amorim, 2010), that might help reduce the tissue cell damage of the protective immune response.

The use of heterologous DNA plasmid encoding HSP65 from *Mycobacterium leprae* was used in a prophylactic protocol. Intramuscular immunization with DNAhsp65 induced an increase of Th1 cytokine levels and reduction of the fungal burden with marked reduction of collagen and lung remodeling (Ribeiro et al., 2009). Similar results were obtained with a therapeutic model (Ribeiro et al., 2010).

A competent $CD4^+$ T cell response producing IFN-γ is usually the chief protective mechanism in fungal infections, particularly in PCM, and dendritic cells are able to initiate the response in naïve T cells.

The use of transfected DCs with a plasmid (pMAC/PS-scFv) encoding a single chain variable fragment (scFv) of an anti-Id antibody that is capable of mimicking gp43 from *P. brasiliensis* was used to subcutaneously immunize BALB/c mice. After 7 days, the scFv peptide was presented to the regional lymph node cells and was capable to activate proliferation resulting in a decrease of fungal burden (Ferreira et al., 2011).

Using an experimental model in BALB/c mice, P10-primed DCs were administrated prior to (subcutaneous vaccination) or weeks after (subcutaneous or intravenous injection) *P. brasiliensis* infection, and showed to significantly reduce the fungal burden. The protective response mediated by the injection of primed-DCs was mainly characterized by increased production of IFN-γ and IL-12 and reduction in IL-10 and IL-4 compared to infected mice that received saline or unprimed-DCs (Magalhães et al., 2012).

PASSIVE TRANSFERENCE OF ANTIBODIES

The exacerbated humoral immune response in PCM has been associated to a poor prognosis since patients with acute and subacute forms of the disease show high antibody titers. It has been reported, however, that monoclonal antibodies against gp70 were protective against experimental PCM (Mattos Grosso et al., 2003). The same group of researchers also showed that a monoclonal antibody against surface 75 kDa protein was able to inhibit fungal growth (Xander et al., 2007). The protective effect of anti-gp43 mAbs injected intraperitoneally on the i.t. infection by *P. brasiliensis* was examined in BALB/c mice (Buissa-Filho et al., 2008). Using a panel of monoclonal antibodies, protective and non-protective monoclonal antibodies with similar reactivity with gp43 on ELISA, were found. The reactivity of mAb 3E, the most efficient mAb in the reduction of fungal burden, that was able to enhance phagocytosis, was mapped to the sequence NHVRIPIGYWAV (Buissa-Filho et al., 2008). This peptide could thus represent, together with P10, another candidate for a peptide vaccine against PCM. In a P10-pre-immunization protocol, mAbs were tested as protective agents. The association of P10-pre-immunization and mAb 3E administered 24 h before i.t. challenge with virulent *P. brasiliensis* and *P. lutzii* yeasts, resulted in additive protection using short-term protocols in comparison with a non-protective mAb (data not published).

FINAL REMARKS

While chemotherapy has the chief role of reducing the fungal burden in mycotic infections, the long-term control and eventual sterilization involve an effective immune response. The combination of chemotherapy and an effective vaccine against PCM should ideally treat the more serious cases of the systemic mycosis aiming at a shorter period of treatment, prevention of relapses and of fibrotic sequels. A highly immunogenic antigen of *P. brasiliensis* and the peptide containing a T cell epitope have repeatedly proved to be protective in prophylactic and therapeutic models with massively infected mice. A new peptide also from the gp43 contains a B cell epitope that reacts with a protective monoclonal antibody. Recently, a DNA vaccine expressing the P10 peptide showed a remarkable protective effect in a long-term infection protocol using mice highly susceptible to PCM. Immunized mice had memory T cells as well as T regulatory cells that prevented tissue cell damage due to the initial pro-inflammatory protective response involving T effector cells. These encouraging results, along with other protective immunizations using new adjuvants, delivery systems, and dendritic cells, point to a next stage of experimentation aiming at the clinical use of the peptide vaccines.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 May 2012; paper pending published: 11 May 2012; accepted: 24 May 2012; published online: 12 June 2012.

Citation: Travassos LR and Taborda CP (2012) New advances in the development of a vaccine against paracoccidioidomycosis. Front. Microbio. ***3****:212. doi: 10.3389/fmicb.2012.00212*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 04 May 2012
doi: 10.3389/fmicb.2012.00154

The role of adjuvants in therapeutic protection against paracoccidioidomycosis after immunization with the P10 peptide

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Paracoccidioidomycosis (PCM), a common chronic mycosis in Latin America, is a granulomatous systemic disease caused by the thermo-dimorphic fungus *Paracoccidioides brasiliensis*. The glycoprotein gp43 is the main antigen target of *P. brasiliensis* and a 15-mer internal peptide (QTLIAIHTLAIRYAN), known as P10, defines a major $CD4^+$-specific T cell epitope. Previous results have indicated that, besides having a preventive role in conventional immunizations prior to challenge with the fungus, protective anti-fungal effects can be induced in *P. brasiliensis*-infected mice treated with P10 administered with complete Freund's adjuvant (CFA). The peptide elicits an IFN-γ-dependent Th1 immune response and is the main candidate for effective immunotherapy of patients with PCM, as an adjunctive approach to conventional chemotherapy. In the present study we tested the therapeutic effects of P10 combined with different adjuvants [aluminum hydroxide, CFA, flagellin, and the cationic lipid dioctadecyl-dimethylammonium bromide (DODAB)] in BALB/c mice previously infected with the *P. brasiliensis* Pb18 strain. Significant reductions in the number of colony forming units of the fungus were detected in lungs of mice immunized with P10 associated with the different adjuvants 52 days after infection. Mice treated with DODAB and P10, followed by mice treated with P10 and flagellin, showed the most prominent effects as demonstrated by the lowest numbers of viable yeast cells as well as reductions in granuloma formation and fibrosis. Concomitantly, secretion of IFN-γ and TNF-α, in contrast to interleukin (IL)-4 and IL-10, was enhanced in the lungs of mice immunized with P10 in combination with the tested adjuvants, with the best results observed in mice treated with P10 and DODAB. In conclusion, the present results demonstrate that the co-administration of the synthetic P10 peptide with several adjuvants, particularly DODAB, have significant therapeutic effects in experimental PCM.

Keywords: *Paracoccidioides brasiliensis*, paracoccidioidomycosis, P10, adjuvants, dioctadecyl-dimethylammonium bromide, FliC flagellin, aluminum hydroxide, complete Freund's adjuvant

INTRODUCTION

Paracoccidioidomycosis (PCM) is a systemic mycosis that typically starts as a granulomatous pulmonary disease subsequent to the inhalation of conidia of the dimorphic fungus *Paracoccidioides brasiliensis*. When it is not diagnosed and treated properly, *P. brasiliensis* yeast cells can spread rapidly to lymph nodes, tegument, spleen, liver, and lymphoid organs of the digestive tract (Shikanai-Yasuda et al., 2006). PCM is endemic in Latin America, mostly affecting rural workers in Brazil, Colombia, and Venezuela (Wanke and Londero, 1994), and the majorities are involved in agricultural activities (Blotta et al., 1999; Restrepo et al., 2008). In Brazil, approximately 1,853 (∼51.2%) of 3,583 confirmed deaths due to systemic mycoses from 1996 to 2006 were caused by PCM (Prado et al., 2009).

gp43 is a glycoprotein of 416 amino acids (Puccia et al., 1986; Cisalpino et al., 1996). A specific T-$CD4^+$ cell epitope was mapped to a 15-amino acid sequence designated P10, which is recognized by T cells from mice infected with *P. brasiliensis*. Immunization of previously intratracheally infected BALB/c mice with P10 reduces the fungal load in the lungs more than 200-fold as compared to non-immunized animals (Taborda et al., 1998). P10 immunized animals produced greater amounts of IFN-γ and interleukin (IL)-12. These mice also had significantly reduced damage to lung tissue. In fact, the immune response elicited by P10 prevents the rapid spread of *P. brasiliensis*, and we have hypothesized that P10 administered as with newer adjuvants might enhance the immunoprotection by the peptide. Hence, we have begun to investigate the efficacy of different adjuvants co-administered with

P10. Along this line, we have found FliC flagellin, derived from *Salmonella enterica*, can significantly modify the Th-1 immune response associated with P10 (Braga et al., 2009).

Aluminum hydroxide (Alum) is another adjuvant widely used in human and veterinary vaccines, which is highly effective in eliciting primary immune responses to target antigens. Concerning secondary responses, the adjuvant stimulates a Th-2 immune response that mainly stimulates the production of antibodies. In this sense it is perhaps less suitable for vaccines against intracellular microorganisms (HogenEsch, 2002). Alum has been associated with severe local reactions such as erythema, subcutaneous nodules, and contact hypersensitivity (Baylor et al., 2002).

Promising results have been observed with complete Freund's adjuvant (CFA) and P10 (Marques et al., 2008). However, this adjuvant causes a variety of side effects such as localized injection-site granulomas, hepatic and renal granuloma formation, and necrotizing dermatitis. Therefore, the use of CFA has been limited to experimental immunizations in animal studies. Due to the severity of adverse reactions, this adjuvant has been banned for use in humans as well as for non-experimental veterinary administration (Stills Jr., 2005).

The idea of using cationic lipids (dioctadecyl-dimethylammonium bromide, DODAB) as adjuvants arose from the effective uptake of microparticles by both dendritic cells and macrophages (Lincopan et al., 2009). Cationic polymer particles carry antigen to these phagocytes and can efficiently stimulate antibody production and activate cytotoxic T cells at low antigen dose (Singh et al., 2000; Lincopan et al., 2009). DODAB also induces maturation of dendritic cells (Thiele et al., 2001; Little et al., 2004) with high levels of IL-12 and IFN-γ production, which may be an important benefit in the design of an anti-*Paracoccidioides* vaccine.

In the present work, a comparative appraisal of the various adjuvants is presented aiming to identify which compound produces the most effective immune response to P10 using murine models of PCM.

MATERIALS AND METHODS

ANIMALS

Six male BALB/c mice per group (6- to 8-week old) were housed in polypropylene cages under specific pathogen free conditions. Animals used in this study were bred at University of São Paulo animal facility. All experiments involving animals were conducted and approved by the Ethics Committee of University of São Paulo and conducted in accordance with international recommendations.

FUNGAL STRAIN

Virulent *P. brasiliensis* Pb18 yeast cells were used to infect the animals. The strain was maintained by weekly passage on solid Sabouraud medium at 37°C and yeast cells were used after 7–10 days of growth. Before the experimental infection, the fungus was grown in modified McVeigh–Morton medium at 37°C for 5–7 days (Restrepo and Arango, 1980). Fungal cells were washed in phosphate-buffered saline (PBS, pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions was determined by staining with trypan blue (Sigma, St. Louis, MO, USA) and was always higher than 90%. The virulence of the Pb18 strain was checked in each experiment by infecting BALB/c mice i.t. and recovering the yeast cells from the infected organs.

INTRATRACHEAL INFECTION OF BALB/c MICE

BALB/c mice were inoculated i.t. with 3×10^5 virulent Pb18 yeast cells/animal, grown on Sabouraud agar and suspended in sterile saline (0.85% NaCl). A maximum volume of 50 μl was inoculated per mouse. Briefly, mice were anesthetized i.p. with 200 μl of a solution containing 80 mg/kg ketamine and 10 mg/kg of xylazine (both from União Química Farmacêutica, Brazil). After approximately 5 min, their necks were hyperextended, and the tracheas were exposed at the level of the thyroid and injected with 3×10^5 yeast cells.

PEPTIDE SYNTHESIS AND PURIFICATION

Peptide synthesis and purification was carried out at the Department of Biophysics, UNIFESP as described previously (Taborda et al., 1998). HPLC analysis showed that the synthetic P10 in the amidated form was >90% pure.

IMMUNIZATION OF MICE

Immunization of BALB/c mice (6- to 8-week old males) was initiated 30 days after infection and repeated on days 37 and 44, by the subcutaneous route, with 20 μg of P10 in presence of the respective adjuvant. The adjuvants used were CFA with subsequent immunizations with incomplete Freund's adjuvant (IFA); Alum 100 μg/ml; FliC flagellin 5 μg/animal, and cationic lipid at 0.1 mM/animal. All adjuvants were vortexed with the peptide before immunization. The animals were sacrificed 7 days after the last immunization, at day 52 of infection.

COLONY FORMING UNITS

For each mouse, the lungs, spleen, and liver were excised and weighed immediately after sacrifice. Tissues were individually homogenized in PBS and 100 μl of this suspension was plated on brain heart infusion medium (BHI; Difco Laboratories, Detroit, MI, USA), supplemented with 4% fetal bovine serum (Gibco, NY, USA) and 5% of the spent culture medium of *P. brasiliensis* 192 isolate (Castañeda et al., 1988), streptomycin/penicillin 10 IU/ml (Cultilab, Brazil), and cycloheximide 500 μg/ml (Sigma, St Louis, MO, USA). The plates were incubated at 37°C for a period of 10 days. The numbers of colonies were counted and results expressed in colony forming unit (CFU) per gram of tissue.

CYTOKINE ANALYSIS

Lungs of each mouse were macerated with protease inhibitor (Sigma, St Louis, MO, USA) and centrifuged; supernatants of these samples were used for cytokine detection. IL-4, IL-10, TNF-α, and IFN-γ were measured using ELISA kits (BD Biosciences, San Diego, CA, USA). The detection limits of the assays were as follows: 7.8 pg/ml for IL-4, 31.25 pg/ml for IL-10 and IFN-γ, and 15.6 pg/ml for TNF-α, as previously determined by the manufacturer. Cytokine levels present in the supernatant preparations were analyzed using GraphPad Prism 5.

HISTOPATHOLOGY

The lungs were excised, fixed in 10% buffered formalin (Merck, Germany) and submitted to histopathological analysis [(hematoxylin and eosin (H&E) and Masson's trichrome].

STATISTICAL ANALYSIS

Statistics was done using GraphPad Prism 5 software (San Diego, CA, USA). The results were expressed as mean values and standard deviations (SDs) of the indicated values. The non-parametric Tukey's honestly significant difference test was employed. *p*-Values of ≤ 0.05 indicated statistical significance.

RESULTS

COLONY FORMING UNITS IN INFECTED BALB/c MICE IMMUNIZED WITH PEPTIDE 10 (P10)

After 30 days of infection, immunizations were initiated with three weekly doses of P10 with or without the different adjuvants. After 52 days of infection, the fungal load was evaluated by enumeration of CFUs from recovered organs (lung, spleen, and liver). Lungs of mice immunized with P10 along with each of the different adjuvants had a significantly reduced number of CFU compared to controls (**Figure 1**). The order of efficacy for CFU reduction was cationic lipid and P10 >> CFA/IFA > Alum > FliC. In this model, we did not recover any fungal cells from the spleens or livers of mice that received any of the immunizations with P10 (data not shown), demonstrating the effectiveness of the peptide in the control of experimental PCM. In contrast, the fungal burdens were 625 ± 60 CFU/g of tissue in the spleens and 296 ± 59 CFU/g of tissue in the livers of unimmunized mice.

CYTOKINE PATTERN INDUCED BY IMMUNIZATION WITH P10 ASSOCIATED WITH ADJUVANTS

In comparison with control infected animals, IFN-γ was significantly increased in mice that received P10 with either cationic lipid or FliC (**Figure 2A**). TNF-α levels were significantly increased only in mice treated with P10 and cationic lipid (**Figure 2B**). P10 immunization with either cationic lipid or FliC significantly reduced levels of IL-4 (**Figure 2C**) and IL-10 (**Figure 2D**). Immunization with P10 and Alum also reduced IL-10 levels, but did not significantly alter the levels of the other cytokines analyzed.

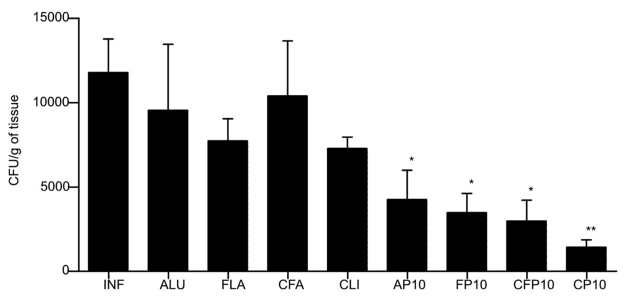

FIGURE 1 | Colony forming units (CFU) from lungs of BALB/c mice infected intratracheally with 3 × 10^5 yeast cells of Pb18 and immunized at 30, 37, and 44 days after infection with the different adjuvants with or without P10. Animals were sacrificed after 60 days of infection. Control animals were infected by not immunized (IFN). The adjuvants used were: aluminum hydroxide alone (ALU) or with P10 (AP10), FliC flagellin alone (FLA) or with P10 (FP10), complete Freund's adjuvant alone (CFA) or with P10 (CF10), and cationic lipid alone (CLI) or with P10 (CP10). Significant difference $*p < 0.05$, $**p < 0.01$.

LUNG HISTOPATHOLOGY IN BALB/c MICE INFECTED (i.t.) WITH Pb18 AND IMMUNIZED WITH P10

Lung tissues from mice immunized with cationic lipid with or without P10 were stained with H&E and compared with unimmunized infected tissues as well as uninfected lungs (**Figure 3**). As expected, the non-infected group (**Figure 3A**) showed normal lung tissue, and the unimmunized infected lungs (**Figure 3B**) showed dense cell infiltrates with high numbers of fungal cells disseminated throughout the lung parenchyma. In the case of mice immunized only with the cationic lipid, we observed the formation of loose granulomas with many fungal cells (**Figure 3C**). In contrast, lungs of mice immunized with cationic lipid and P10 showed significantly preserved lung parenchyma without fungal cells (**Figure 3D**). The histological appearances of lungs from mice immunized with either FliC (**Figure 3E**) or Alum (**Figure 3G**) alone were similar to that with cationic lipid alone (**Figure 3C**). Immunization of mice with P10 and FliC resulted in improved granuloma formation in the lungs, which prevents the spread of the fungus, and increased preservation of normal lung parenchyma (**Figure 3F**). Although there was a slight increase in normal lung parenchyma in mice immunized with P10 and Alum, there was poor granuloma formation and large numbers of yeast cells within areas of inflammation (**Figure 3H**).

The amount of pulmonary collagen type I in mice immunized with P10 and FliC, Alum, or cationic lipid were compared with unimmunized infected controls using Masson's trichrome staining. Tissues from control mice revealed abundant collagen I fibers within cellular infiltrates containing large numbers of fungal cells (**Figure 4A**). Although no fungal cells were visualized, lungs of mice immunized with FliC flagellin and P10 nevertheless diffusely displayed increased amounts of collagen (**Figure 4B**). Mice immunized with Alum and P10 showed large granulomas containing fungal cells and the formation of collagen fibers on the granuloma's periphery (**Figure 4C**). In contrast, mice immunized with cationic lipid and P10 displayed preserved lung tissue without increased collagen (**Figure 4D**).

DISCUSSION

The P10 peptide (QTLIAIHTLAIRYAN) has important immunoprotective properties that make it a leading candidate for the development of a therapeutic vaccine (Taborda et al., 1998). We have also shown that P10 immunization can be utilized concomitantly with standard antifungal drugs in the treatment of PCM and that co-administration may also prevent disease recurrence (Marques et al., 2008).

The protective effect of P10 is related to the induction of an INF-γ dependent Th-1 immune response (Taborda et al., 1998; Travassos et al., 2007), so it may be stimulated by adjuvants or nanoparticle encapsulation that increase the efficiency of P10 uptake by dendritic cells resulting in the enhanced presentation of the peptide for cellular immune responses (Amaral et al., 2010; Magalhães et al., 2012). In the present work, we show that the association of P10 with cationic lipids led to a significant reduction of

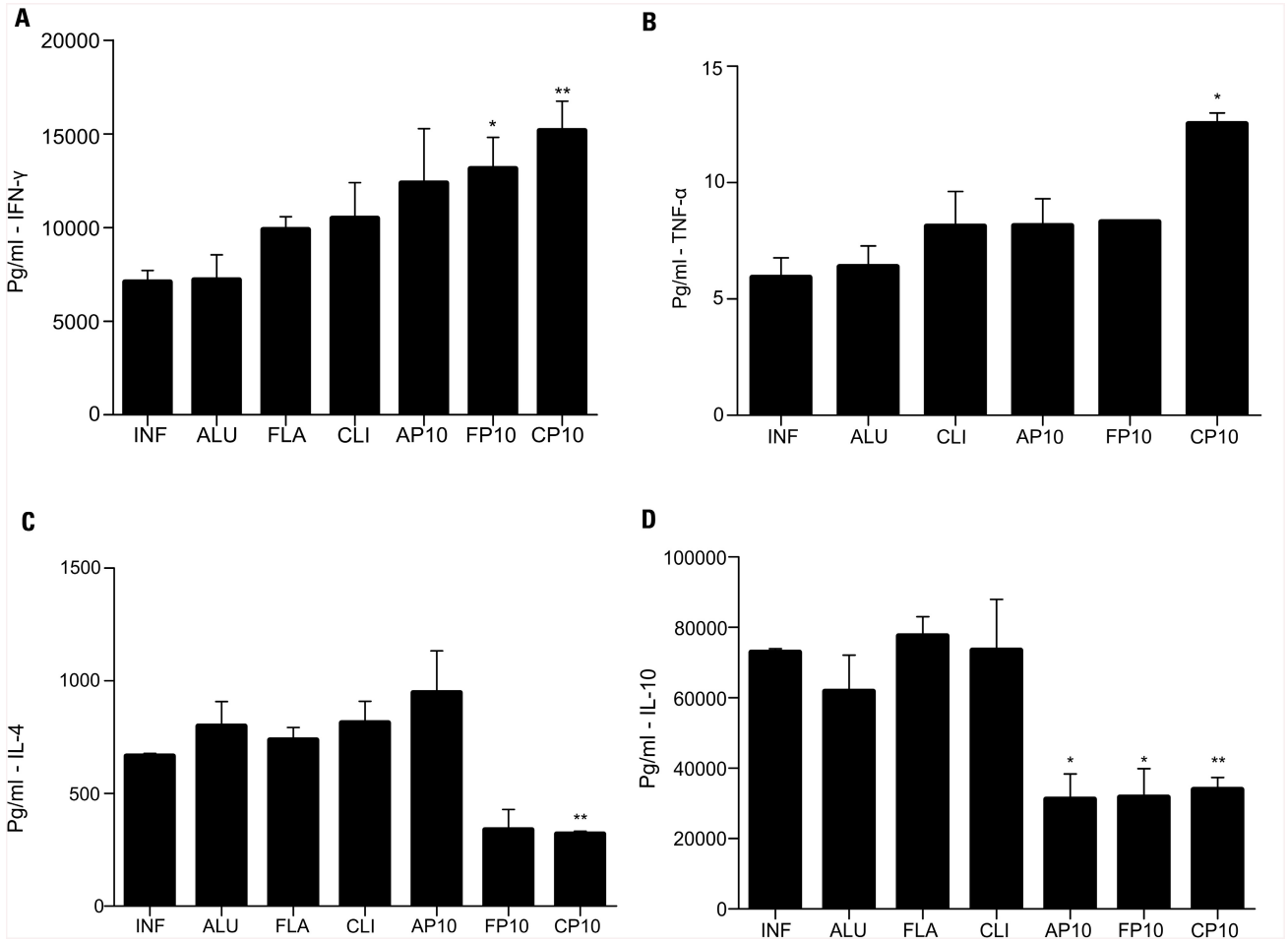

FIGURE 2 | Cytokine detection was assayed in the lung tissue from BALB/c mice 52 days after infection. Analyzed cytokines were: **(A)** IFN-γ, **(B)** TNF-α, **(C)** IL-4, and **(D)** IL-10. Each group was infected i.t. with 3×10^5 yeast cells and immunized at 30, 37, and 44 days after infection with different adjutants with or without P10. The groups of mice included unimmunized, infected control mice (INF); animals infected and immunized with aluminum hydroxide alone (ALU) or with P10 (AP10), FliC flagellin alone (FLA) or with P10 (FP10), and cationic lipid alone (CLI) or with P10 (CP10). *$p < 0.05$: significance $p < 0.05$ compared to control mice (only infected). **$p < 0.01$: compared to control mice (only infected).

CFU in the lungs of 52-day infected animals (**Figure 1**). In contrast, animals immunized only with P10 (without adjuvants), had minimal reductions in fungal burden (not shown data). Hence, combining adjuvants, such as cationic lipids, with P10 can generate augmented immune responses mediated by Th-1 cells, directly related to the secretion of IFN-γ (**Figure 2A**), which leads to peritoneal and lung macrophage activation as well as enhancing their fungicidal effect on yeasts and conidia of *P. brasiliensis* (Buissa-Filho et al., 2008). Immunization performed with P10 associated with antifungal drugs in animals infected with Pb18, has been shown to induce a significant reduction in IL-4 (Marques et al., 2008), which we now show also occurs in the setting of immunizations with cationic lipid and P10 (**Figure 2C**). Moreover, P10 administration with cationic lipid also significantly reduces levels of IL-10 (**Figure 2D**). It is worth noting, however, that depending on the degree of inflammation generated by the Th-1 response, Th-2 cytokines are essential for balancing the immune response and reducing the risk of self-damage (Travassos et al., 2008).

Studies with cationic lipids associated with recombinant HSP of *Mycobacterium leprae* have demonstrated the ability of the adjuvant to promote antigen presentation in lymph node cells with production of high levels of IL-12 and INF-γ, suggesting that cationic lipid can be useful in the formulation of vaccines against intracellular bacteria, as well as against protozoa (Lincopan et al., 2009). IFN-γ is required for the synthesis of TNF-α by macrophages and is essential for the accumulation of these cells and their subsequent differentiation into epithelioid cells. INF-γ is also responsible for granuloma's formation and maintenance, and, therefore, it plays a vital role in the control of dissemination by fungi such as *P. brasiliensis* (Souto et al., 2000).

Although the cationic lipid/P10 association has demonstrated the best protective response in the setting of short-term infection in our murine model, it is relevant also to consider the protective effect of the association of P10 and FliC flagellin. A significant reduction in the number of CFU was observed and the cytokine profile was similar to that achieved with cationic lipid and P10

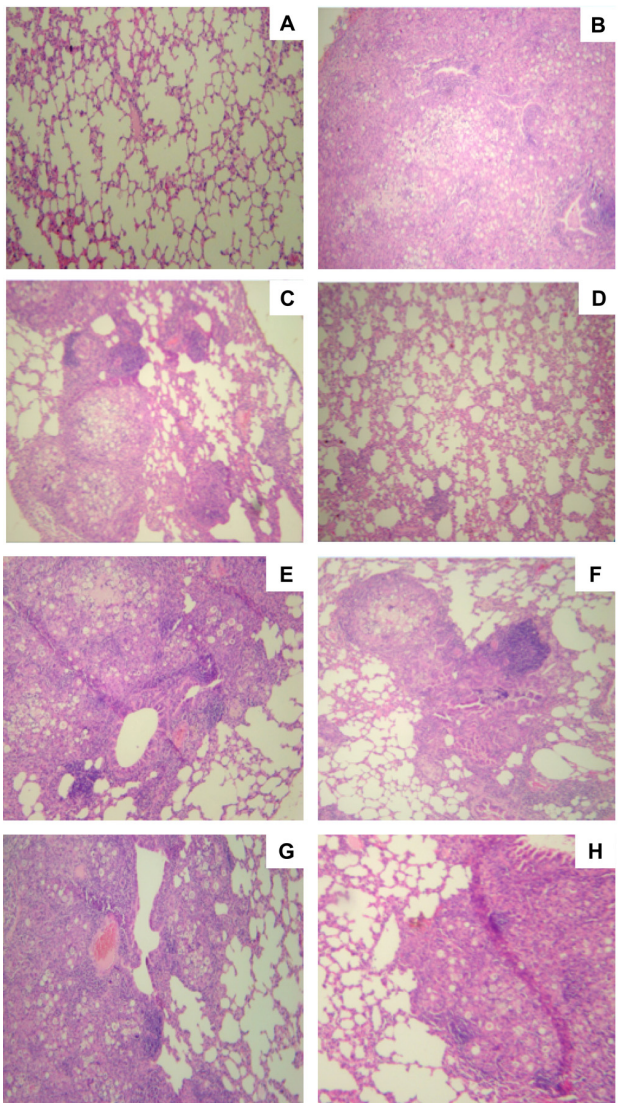

FIGURE 3 | Histological sections of murine lungs from i.t. infected BALB/c mice submitted to immunization with P10 associated to cationic lipid. Groups of mice included **(A)** uninfected controls, **(B)** unimmunized infected controls, **(C)** infected and immunized only with the cationic lipid, **(D)** infected and immunized with cationic lipid plus P10, **(E)** infected and immunized only with FliC flagellin, **(F)** infected and immunized with FliC flagellin plus P10, **(G)** infected and immunized only with aluminum hydroxide, **(H)** infected and immunized with aluminum hydroxide plus P10 after 52 days of infection. H&E staining, ×10 magnification.

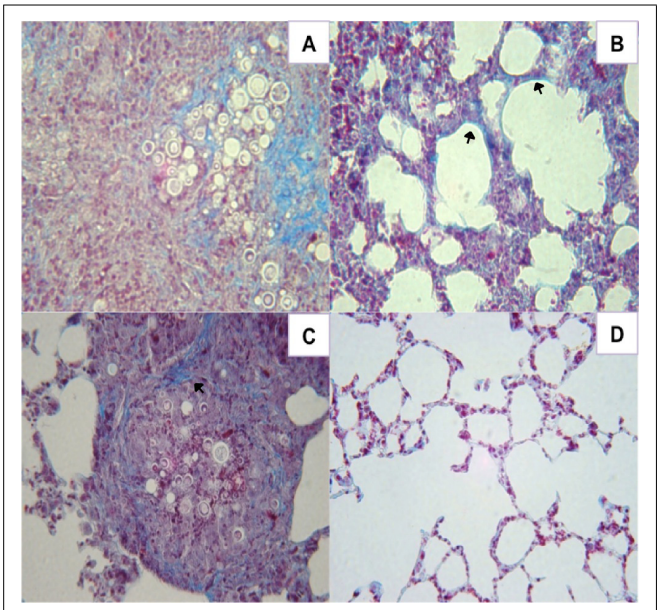

FIGURE 4 | Histological sections of murine lungs from i.t. infected BALB/c mice immunized after 30 days of infection with P10 associated with FliC flagellin, aluminum hydroxide, or cationic lipid. (A) infected, unimmunized control mice, **(B)** infected and immunized with FliC flagellin plus P10, **(C)** infected and immunized with aluminum hydroxide plus P10, and **(D)** infected and immunized with cationic lipid plus P10 after 52 days of infection. Masson's trichrome staining, ×40 magnification. Blue staining and arrows indicate type I collagen fibers.

(**Figures 1 and 2**). We have previously shown that prophylactic experiments performed with FliC flagellin have demonstrated the effectiveness of the association of this adjuvant with P10 allowing for the control of fungal infection in vaccinated mice (Braga et al., 2009). Intranasal immunization carried out with this formulation induced high levels of IFN-γ and IL-12 production by lung cells and suppressed the production of Th-2 cytokines. The formation of compact granulomas (**Figure 3F**) as well as cytokine levels obtained with FliC Flagellin and P10, can be associated to the administration route, since present immunizations were performed subcutaneously, according to Braga et al. (2009), better results are obtained with intranasal immunizations.

In the lung parenchyma, *P. brasiliensis* induces chronic damage leading to the development of pulmonary fibrosis, which is presumably due to persistent antigenic stimulation and an ongoing active immune response. Granulomatous inflammation can increase the formation of connective tissue rich in collagen type I and III, leading to functional changes and subsequent fibrosis in the lung (Naranjo et al., 2010). Since fibrosis is a well-known sequela of PCM, it is therefore important that the adjuvants used do not induce an exacerbated inflammatory response. In fact, the association of cationic lipid and P10 resulted in a significant reduction of pulmonary fibrosis in mice infected with Pb18 (**Figure 4D**).

In summary, we have shown that the examined regimens combining P10 with different adjuvants results in significantly different protective responses. Administration of P10 with cationic lipid provided the most effective response profile, with the greatest reduction in fungal burden and a Th-1 biased cytokine response that maintained pulmonary architecture without inducing fibrotic injury.

ACKNOWLEDGMENTS

This work was supported by grants from FAPESP 11/17267-4, 09/15823-7, 09/53354-9, 07/58750-4 and CNPq 146809/2010-6. Carlos P. Taborda, Luiz R. Travassos, Luis C. S. Ferreira, and Nilton Lincopan are research fellows of the CNPq. We acknowledge the valuable scientific inputs from Dr. Joshua D. Nosanchuk.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 April 2012; accepted: 03 April 2012; published online: 04 May 2012.

Citation: Mayorga O, Muñoz JE, Lincopan N, Teixeira AF, Ferreira LCS, Travassos LR and Taborda CP (2012) The role of adjuvants in therapeutic protection against paracoccidioidomycosis after immunization with the P10 peptide. Front. Microbio. ***3****:154. doi: 10.3389/fmicb.2012.00154*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 15 June 2012
doi: 10.3389/fmicb.2012.00218

ArtinM offers new perspectives in the development of antifungal therapy

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The thermally dimorphic fungus *Paracoccidioides brasiliensis* is the causative agent of paracoccidioidomycosis (PCM), the most frequent systemic mycosis that affects the rural populations in Latin America. Despite significant developments in antifungal chemotherapy, its efficacy remains limited since drug therapy is prolonged and associated with toxic side effects and relapses. In response to these challenges, it is now recognized that several aspects of antifungal immunity can be modulated to better deal with fungal infections. A common idea for halting fungal infections has been the need to activate a cell-based, pro-inflammatory Th1 immune response to improve the fungal elimination. ArtinM, a D-mannose binding lectin from *Artocarpus heterophyllus*, has the property of modulating immunity against several intracellular pathogens. Here, we review the immunomodulatory activity of ArtinM during experimental PCM in mice. Both prophylactic and therapeutic protocols of ArtinM administration promotes a Th1 immune response balanced by IL-10, which outstandingly reduces the fungal load in organs of the treated mice while maintaining a controlled inflammation at the site of infection. A carbohydrate recognition-based interaction of ArtinM with Toll-like receptor 2 (TLR2) accounts for initiating the immunomodulatory effect of the lectin. The precise identification of the TLR2 *N*-glycan(s) targeted by ArtinM may support novel basis for the development of antifungal therapy.

Keywords: *Paracoccidioides brasiliensis*, ArtinM, immunomodulation

BALANCING RESISTANCE AND TOLERANCE TO FUNGI

The two main components of the host immune response to fungi, namely, resistance (the ability to limit fungal burden) and tolerance (the ability to limit host damage caused by immune response or other mechanisms) highlight the bipolar nature of the inflammatory process in fungal infections. Both components must be considered when developing any therapeutic or prophylactic antifungal procedure.

Dendritic cells (DCs) are primarily responsible for antigen recognition, decoding this information, and then stimulating various T cell pathways using specific cytokine signals. The T cell subsets in turn secrete cytokines that mediate protective or detrimental/pathogenic effects on phagocytes and the inflammatory process. The primary protective response against fungal disease is the cell-mediated Th1 response (Calich and Kashino, 1998; Netea et al., 2004; Zhang et al., 2009; Ito, 2011). Th1 lymphocytes produce IFN-γ, which stimulates the antifungal activity of PMN and macrophages.

Otherwise, some cytokines such as IL-4 and IL-13 provide signals that favor a Th2-mediated immune response by lymphocytes. By diminishing the Th1 cell response and promoting antibody production and T regulatory cells, it favors fungal infections, fungus-associated allergic responses, and disease relapse (Benard et al., 1997; Netea et al., 2004; Müller et al., 2007). Therefore, Th2 immunity is associated with severe and disseminated forms of fungal infections. This pattern is well established and reported in cryptococcosis (Müller et al., 2007), paracoccidioidomycosis (PCM; Calich and Kashino, 1998; Ruas et al., 2009), and candidiasis (Netea et al., 2004; Haraguchi et al., 2010).

In this review, we discuss the role of a plant lectin named ArtinM in a murine model of *Paracoccidioides brasiliensis* infection, highlighting its immunomodulatory properties and the importance of the modulation of a cell-mediated immune response in the resistance to the fungus. We discuss the aspects that make this lectin an excellent candidate for further studies as a potential therapeutic for severe cases of PCM in human patients or for development as a prophylactic for individuals at risk for severe disease.

IMMUNITY TO *PARACOCCIDIOIDES BRASILIENSIS* INFECTION

The most common human systemic mycosis in Latin America is PCM, which is caused by the dimorphic fungus *P. brasiliensis*. Infection occurs by inhalation of fungal spores or particles, which transform into the pathogenic yeast form after reaching the pulmonary alveolar epithelium (Restrepo-Moreno, 1993). Yeast can either be eliminated by immune-competent cells or disseminate to other tissues through lymphatic and hematogenous routes, resulting in a wide spectrum of clinical manifestations, which vary from asymptomatic, benign and localized to severe and disseminated forms (Borges-Walmsley et al., 2002). Clinical and experimental evidences indicate that, similar to other systemic mycosis, Th1 immunity exerts a singular role in the asymptomatic form of PCM, while a Th2 pattern is associated with progression to

the severe disease form (Cano et al., 1998; Karhawi et al., 2000; Benard et al., 2001; Oliveira et al., 2002; Peraçoli et al., 2003; Ruas et al., 2009).

These immune patterns of resistance or susceptibility to fungal infections have been studied in murine models of infection that simulate human mycosis. Animal models facilitate the study of immune response mechanisms involved in PCM. Resistant mice produce early and sustained levels of IFN-γ and IL-2, whereas susceptible mice produce low levels of IFN-γ, but significant levels of IL-5 and IL-10 (Calich and Kashino, 1998; Kashino et al., 2000). Murine models have also showed that IFN-γ and TNF-α activate macrophages to exert effects against *P. brasiliensis* (Brummer et al., 1989; Gonzales et al., 2003). The essential role of these cytokines has been further demonstrated using mice that are genetically deficient in either the IFN-γ or the TNF-α receptor (Cano et al., 1998; Souto et al., 2000). Indeed, the presence of cytokines accounting for the activation of macrophages, which is necessary for fungal killing, has been consistently documented (Gonzales et al., 2000; Moreira et al., 2008, 2010).

The importance of innate immunity in the recognition of fungi has been extensively reviewed elsewhere (Roeder et al., 2004; Romani, 2004), and it has been recently characterized for *P. brasiliensis* infection (Loures et al., 2009, 2010, 2011). The lack of the receptors Toll-like receptor 2 (TLR2) or TLR4 did not alter the survival rates of mice infected with *P. brasiliensis*. TLR2 knockout (KO) mice infected with *P. brasiliensis* presented with increased Th17 immunity, associated with an impaired regulatory T cell expansion, which resulted in an uncontrolled inflammatory reaction. Therefore, the authors concluded that the presence of TLR2 in *P. brasiliensis* infection is important to downregulate Th17 immunity and lung pathological condition (Loures et al., 2009). TLR4-deficient mice presented lower fungal loads than the TLR4-normal mice, but these mice were unable to clear the infection completely owing to enhanced regulatory T cells and low inflammation (Loures et al., 2010).

Current treatment for PCM relies on antifungal chemotherapy to control the disease. Clinically, the antifungal drugs most commonly used for PCM treatment include amphotericin B, sulfa derivatives, and azoles, but their toxicity can be a limiting factor in the treatment (Mendes et al., 1994). Treatment regimens with these agents often require extended periods of maintenance therapy, which may range from months to years, and are usually associated with relapses (Shikanai-Yasuda et al., 2006). Moreover, even after prolonged administration of these drugs, there is no guarantee that the fungus will be completely eradicated.

Based on these data, there is a strong need for alternative clinical treatments to chemotherapy. Researchers have focused their efforts in investigating fungal components able to promote cellular immune responses and host protection. Immunization with heat-shock proteins (HSPs) from *P. brasiliensis* has also been shown to provide some degree of protection against experimental disease (Soares et al., 2008; Ribeiro et al., 2009, 2010). Recently, it was shown that plasmid immunization with a peptide derived from the 43-kDa glycoprotein antigen from the fungus, called P10, was shown to be protective against PCM, inducing a reduction in fungal load in the lungs of experimentally infected mice (Rittner et al., 2012). Although these studies focused on the use of fungal components to immunize mice against *P. brasiliensis* infection, it was shown that immunotherapy with a Th1-inducing adjuvant that was independent of Pb antigens has a beneficial effect against PCM (Oliveira et al., 2008). A single-dose administration of the adjuvant in infected mice was sufficient to restore their ability to mount an effective immune response to the fungus. These data support that stimulation of the host Th1 immune response is a promising approach toward expanding available treatment options for systemic fungal diseases, including PCM. Moreover, Th1 stimulation may be achieved irrespective of whether *P. brasiliensis* antigens are used, providing new possibilities for the use of alternative drugs against the disease

IMMUNOMODULATION BY ArtinM

ArtinM (also known as KM^+ or Artocarpin) (Pereira Da Silva et al., 2008) is a lectin from *Artocarpus heterophyllus* seeds that specifically recognizes the trisaccharide Manα1–3 [Manα1–6] Man core of *N*-glycans. ArtinM is a homotetramer formed by 13-kDa subunits, each one corresponding to a β-barrel, with a β-prism folding, which includes a carbohydrate-recognition domain (CRD). ArtinM cDNA has been cloned and heterologously expressed in *Saccharomyces cerevisiae* and *Escherichia coli* (Silva et al., 2005). Native (ArtinM) and recombinant (rArtinM) proteins share the same sugar recognition specificity and are equivalents in terms of the kinetics of binding affinity to a glycoligand (Pesquero et al., 2010). The ArtinM CRD is preserved in rArtinM, and the recombinant protein retains the same biological properties as the native form, with the advantage that it does not form oligomers. ArtinM possesses many relevant biological properties in cells of the immune system, which is reflected in the modulation of immunity during infection with intracellular pathogens. The lectin acts on mast cells and induces degranulation (Moreno et al., 2003). It also acts on neutrophils and induces haptotactic migration, as well as phenotypic and functional changes, which include intracellular tyrosine phosphorylation, shedding of L-selectin, release of inflammatory mediators, phagocytic and cell-killing activities, and increased expression of TLR2 (Ganiko et al., 2005; Toledo et al., 2009).

The pioneering observation on the ArtinM immunomodulatory activity was its ability to induce IL-12 production in murine macrophages. This cytokine production then promoted a switch in the BALB/c mouse immune response from Th2- to Th1-mediated immunity against *Leishmania major* antigens. Cytokine production was dependent on the CRD of the lectin, since IL-12 production was selectively inhibited by D-mannose, which is an ArtinM-specific ligand (Panunto-Castelo et al., 2001).

Additional studies have shown that the benefits provided by the immunomodulation induced by ArtinM can be extended to several infections in which a Th1-biased immunity is necessary for resistance, including the murine model of *Candida albicans* infection. Infected mice that were treated with ArtinM developed Th1- and Th17-mediated immune responses; their macrophages and neutrophils exhibited increased phagocytical and candidacidal activities (Custodio et al., 2011). The augmented phagocytosis of yeast cells by macrophages from ArtinM-treated mice occurred via mannose and dectin-1. This effect explains

the faster clearance of *C. albicans* in the initial phase of infection in mice, which favors ArtinM-induced protection against disseminated candidiasis (Loyola et al., 2012).

Knowledge about the immunomodulatory effects of ArtinM on PCM is derived from studies that used an experimental model developed in BALB/c mice that had been intravenously infected with *P. brasiliensis*. Trials involving several protocols for the therapeutic and prophylactic administration of ArtinM showed that the most effective therapeutic protocol consisted of a single subcutaneous injection of ArtinM 10 days after infection, whereas the best prophylaxis was attained by the administration of two subcutaneous injections of ArtinM on day 10 and day 3 before infection. The beneficial effects of therapeutic and prophylactic regimens (Coltri et al., 2008, 2010) of ArtinM on the severity of *P. brasiliensis* infection, which manifested on day 30 post-infection, included marked decrease in fungal burden and absence of granulomas in the lungs, which exhibited a well-preserved bronchoalveolar architecture. This pattern was in contrast to what was observed in the untreated mice, which had disseminated infection and multiple sites of focal and confluent epithelioid granulomas with lymphomonocytic halos circumscribing a high number of viable and non-viable yeast cells (**Figure 1**). The lesions were larger and still disseminated on day 60 after infection, while ArtinM-treated mice had no granulomas or yeast cells in the liver, spleen or lung tissue.

The advantages of ArtinM administration correlate with an adequate milieu of pulmonary mediators. Lung homogenates from mice that were infected with *P. brasiliensis* and then subjected to prophylactic or therapeutic ArtinM regimen showed higher levels of the pro-inflammatory cytokines IL-12 and TNF-α, and NO. ArtinM administration drove cytokine production from a Th2 immune response pattern to a Th1 immune response pattern. High concentrations of IL-4 and low concentrations of IFN-γ were detected in untreated control mice, whereas in ArtinM-treated mice, lower IL-4 and higher IFN-γ concentrations were stably produced during the course of the disease, as illustrated in **Figure 2**. It was clear that a drive toward Th1-mediated immunity is stimulated *in vivo* by ArtinM. Interestingly,

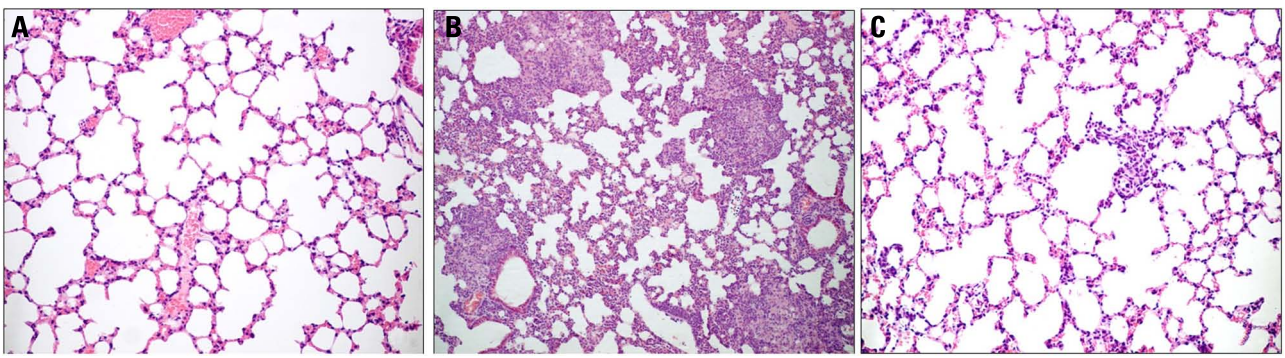

FIGURE 1 | ArtinM administration prevents pulmonary lesions in *P. brasiliensis*-infected mice. Lung histopathology of uninfected mice **(A)**, *P. brasiliensis*-infected mice **(B)**, and *P. brasiliensis*-infected mice treated with ArtinM **(C)**. *P. brasiliensis*-infected mice display extensive and confluent lesions in the lungs, with epithelioid granulomas surrounding a large number of yeast cells. Infected mice treated with ArtinM present no granulomas, and lung architecture is similar to that of uninfected mice. The lung sections were stained with H&E (Modified from Coltri et al., 2010).

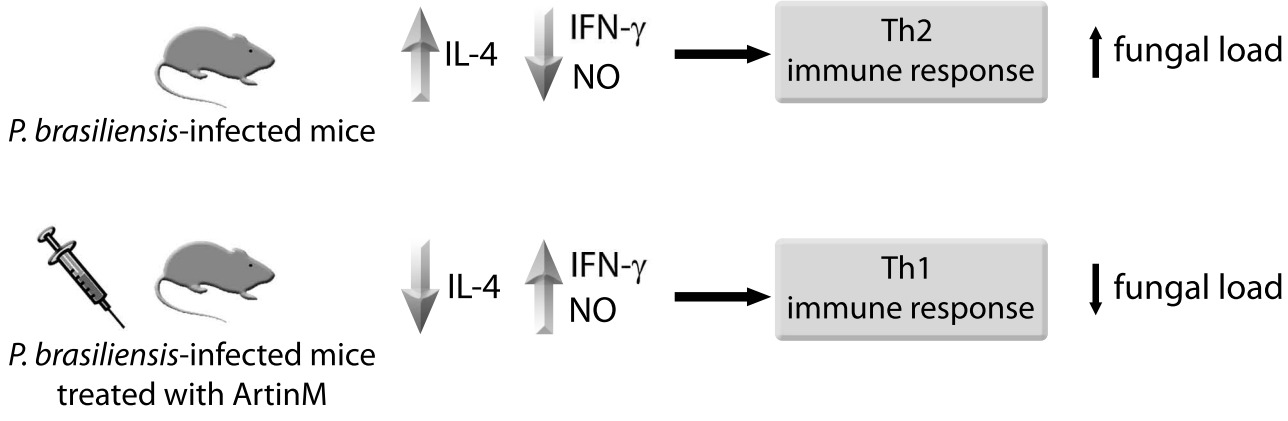

FIGURE 2 | Treatment with ArtinM induces Th1 immunity. Mice were infected with *P. brasiliensis* yeast cells, and then treated or not with ArtinM. On day 30 after infection, the mouse lung tissue was analyzed for IL-4, IFN-γ and NO concentrations. ArtinM treatment was associated with lower IL-4 and higher IFN-γ and NO pulmonary levels, which reflected in lower fungal load.

stable IL-10 production was also verified in the ArtinM-treated mice, indicating that the induced Th1 response is balanced by the effects of this anti-inflammatory cytokine. Studies involving IL-12 KO mice have demonstrated the importance of IL-12 for ArtinM-mediated beneficial effects on experimental PCM. When these mice were infected with *P. brasiliensis*, treatment with ArtinM exerted no protective effects against the infection. The parallel utilization of an ArtinM recombinant form to treat the *P. brasiliensis*-infected mice has provided evidence that the administration of ArtinM or its recombinant form (rArtinM) exerts an equally protective effect against *P. brasiliensis* infection (Coltri et al., 2008, 2010).

ArtinM TARGETS TLR2 *N*-GLYCANS TO INDUCE IL-12 PRODUCTION

The role of the 70-kDa heterodimeric cytokine IL-12 in the activation of type 1 immune response is largely recognized. Its bioactive IL-12p70 form is composed of two disulfide-linked subunits: a 40-kDa heavy chain of (p40) and a 35-kDa light chain (p35). Macrophages and DCs are the major cell types producing this cytokine, which is released as the biologically inactive peptide IL-12p40 as well as the biologically active IL-12p70. IL-12 acts on T lymphocytes and natural killer (NK) cells, and induces IFN-γ production. This hallmark Th1 cytokine is responsible for T cell proliferation and enhancement of macrophage cytotoxic activity (Kobayashi et al., 1989; Wolf et al., 1991).

IL-12 production by phagocytes is generally initiated by the interaction of cell-surface TLRs with pathogen-associated molecular patterns (PAMPs). TLRs constitute a protein family of cellular receptors that mediate recognition of microbial pathogens and subsequent inflammatory response in vertebrates. These receptors confer PAMP recognition and their signaling triggers synthesis followed by release of pro-inflammatory cytokines, and induces expression of co-stimulatory molecules for promoting activation of adaptive immunity during antigen presentation (Janeway and Medzhitov, 2002). Upon recognition of respective PAMPs, TLRs recruit a specific set of adaptor molecules that harbor TIR domains, such as MyD88 and TRIF, and initiate downstream signaling events that lead to the activation of the transcription factor and its translocation into the nucleus to induce the expression of pro-inflammatory genes, including the IL-12-coding gene. Inflammatory cytokines are released from the cell into the extracellular matrix, and they promote the recruitment of neutrophils to the site of infection, activation of macrophages, and induction of IFN-γ-stimulated genes, resulting in direct killing of invading pathogens. Moreover, activation of TLR signaling leads to the maturation of DCs, which contributes to the induction of adaptive immunity (West et al., 2006).

The involvement of MyD88-mediated signaling in the enhanced secretion of ArtinM-induced IL-12 was proved by the fact that macrophages from MyD88KO mice did not respond to *in vitro* stimulation with the lectin. To investigate whether TLR2 was involved in ArtinM-induced IL-12 production, an *in vitro* assay was performed to quantify the IL-12 concentrations released by ArtinM-stimulated macrophages from the TLR2KO or TLR4-deficient mice. Macrophages from TLR2KO mice, distinctly from those from TLR4-deficient or WT mice, were unable to produce IL-12 in response to ArtinM stimulus. Moreover, IL-12 production by ArtinM-stimulated macrophages was inhibited by D-mannose, which indicates that its production is dependent on the lectin CRD. These results demonstrate that TLR2 plays a critical role in ArtinM-mediated production of IL-12 (Coltri et al., 2008).

Potential *N*-linked glycosylation sites have been revealed by amino acid sequencing analysis of all known TLRs. Several lines of evidence indicate that oligosaccharides attached to TLRs play important roles in the recognition of PAMPs, and in the formation of a functional receptor complex on the cell surface (Ohnishi et al., 2001, 2003; da Silva Correia and Ulevitch, 2002; Weber et al., 2004). Concerning human TLR2, its ectodomain contains *N*-glycans linked to the residues Asn114, Asn199, Asn414, and Asn442; among them, the glycan linked to Asn442 was reported to contribute to efficient secretion of the TLR2 ectodomain (Weber et al., 2004) and cellular recognition of PAMPS (Kataoka et al., 2006). Direct interaction of ArtinM with TLR2 was further demonstrated by a gene reporter assay involving TLR2-transfected cells (unpublished data). Currently, TLR2 mutants for the ectodomain glycosylation sites (generated in Dr. Nicholas Gay's laboratory, University of Cambridge, UK) are being used to identify the glycan(s) targeted by ArtinM.

CONCLUDING REMARKS AND PERSPECTIVES

ArtinM administration interferes with the outcome of *P. brasiliensis* infection by modulating host immunity according to the following events:

(a) recognition of TLR2 glycans by the lectin,
(b) induction of IL-12 production,
(c) generation of Th1-balanced immunity, and
(d) protection against *P. brasiliensis*, mainly manifested by the occurrence of milder lung lesions and low fungal burden.

IL-12-dependent mechanism of protection is a process triggered by the MyD88/TLR2 signaling pathway. Detection of IL-10 production in ArtinM-treated animals reveals that the induced Th1-prone immune response is regulated in a way that prevents systemic immune pathology, as indicated by the absence of exacerbated inflammatory lesions in ArtinM-treated animals (Coltri et al., 2008, 2010). As part of a study on the pleiotropic activities of ArtinM, we are trying to identify the IL-10-producing cells.

Observations concerning the immunomodulatory effects of ArtinM support the use of this protein, in its native or recombinant form, as an immunomodulatory agent that can stimulate balanced Th1 immunity, which is required to protect the host against fungal infection. Otherwise, complete characterization of the *N*-glycan(s) recognized by ArtinM in TLR2 molecules may provide an adequate target for the development of novel antifungal therapies.

ACKNOWLEDGMENTS

We thank Dr. Maria Aparecida de Souza for helpful discussions. Research was supported by grants of FAPESP (00/09333-2 and 06/60642-2) and CNPq (350418/00-4).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 March 2012; paper pending published: 05 April 2012; accepted: 29 May 2012; published online: 15 June 2012.

Citation: Ruas LP, Carvalho FC and Roque-Barreira M-C (2012) ArtinM offers new perspectives in the development of antifungal therapy. Front. Microbio. ***3****:218. doi: 10.3389/fmicb.2012.00218*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 09 July 2012
doi: 10.3389/fmicb.2012.00249

In good company: association between fungal glycans generates molecular complexes with unique functions

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The biological properties of fungal immunogens have historically utilized testing of isolated molecules. Recent findings, however, indicate that fungal glycans differing in structure and function can interact to form hybrid complexes with unique properties. In the pathogenic yeast *Cryptococcus neoformans*, chitin-like molecules associate with capsular glucuronoxylomannan (GXM) to form functionally distinct glycan complexes. Such interactions between glycans that result in the formation of structures with different functions strongly suggest that additional molecular complexes with unknown properties may exist in fungal pathogens. Moreover, the identification of these novel complexes has stimulated the search of new immunogens with potential to protect human and animal hosts against systemic mycoses.

Keywords: chitin, *Cryptococcus neoformans*, glucuronoxylomannan, polysaccharides, glycan association

The surface of fungal cells is rich in polysaccharides and protein- or lipid-bound oligosaccharides (Nimrichter et al., 2005) that are called glycans (Bertozzi and Rabuka, 2009). In the fungal cell wall, polysaccharides, glycoproteins and glycolipids form complex carbohydrate networks that play key physiological functions (Nimrichter et al., 2005), such as providing structural support and regulating extracellular secretion (Rodrigues et al., 2008b; Casadevall et al., 2009). Notably, structural aspects of fungal glycans differ considerably from those found in mammalian cells (Fukazawa et al., 1995; Nimrichter et al., 2005). Therefore, the uniqueness of wall glycans makes these molecules promising targets for antimicrobial drugs, as extensively reviewed in the literature (Fukazawa et al., 1995; Nimrichter et al., 2005; Doering, 2009; Goldman and Vicencio, 2012).

Fungal glycans have diverse effects in the interplay between the fungus and the host (Nimrichter et al., 2005). Carbohydrate-rich molecules can effectively stimulate protective immune defenses (Pirofski, 2001; Casadevall and Pirofski, 2006), but they can also down-regulate host effector responses (Zaragoza et al., 2009). To date, there has been an extraordinarily rich spectrum of fungal glycans identified with activities ranging from activation of innate responses and induction of humoral and cell-mediated functions to inhibiting host effector cell recruitment and dysregulating cytokine responses (Casadevall and Pirofski, 2005, 2006; Lee et al., 2008; Li et al., 2009; Sorgi et al., 2009; Mora-Montes et al., 2011; Vecchiarelli et al., 2011). Examples of fungal glycans showing contrasting biological activities are available in a number of comprehensive reviews and the impact of glycans from the human pathogenic *Cryptococcus neoformans* have especially been investigated (Fukazawa et al., 1995; San-Blas et al., 2000; Pirofski, 2001; Zaragoza et al., 2009; Vecchiarelli et al., 2011).

Experimental models describing structural and functional aspects of fungal glycans have historically used purified molecules, mutants lacking genes coding for glycan-synthesizing enzymes, and specific glycan-binding probes, including antibodies, lectins, and peptides. These classic approaches have traditionally focused on isolated molecules for structural and/or functional testing. Microscopic techniques, however, have clearly revealed a number of molecular associations at the cell surface of fungi (Maxson et al., 2007a,b; Rodrigues et al., 2008a; De Jesus et al., 2009; Fonseca et al., 2009b; Zaragoza et al., 2009; Jesus et al., 2010), which suggests that the study of isolated molecules is insufficient to fully elucidate the functional impact of these complex structures. Inter and intramolecular non-covalent associations keep cell wall structures compacted and prevent extracellular release. These molecular complexes differ in structure and composition from isolated molecules, implying that functional differences may occur. To illustrate this hypothesis, we will focus on *C. neoformans*, in which glycan complexes with unique functions have been recently described.

The surface of *C. neoformans* is mainly composed of glycans that include complex polysaccharides, protein-bound oligomannosides, *N*-acetyl-glucosamine-rich oligosaccharides and glucosylceramides (Rodrigues et al., 2000; Reese and Doering, 2003; Nimrichter et al., 2005; Reese et al., 2007; Zaragoza et al., 2009; Nimrichter and Rodrigues, 2011). The most striking feature of *C. neoformans* is an external glycan capsule, which plays a number of significant functions during infection and is crucial for disease progress (Zaragoza et al., 2009). Classically, the capsule has been defined as a complex surface network composed of mannoproteins and the heteropolysaccharides glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal). GXM, the main component of the capsule, is a potent immune modulator that has been suggested as a vaccine candidate (Pirofski, 2001). Interestingly, a monoclonal antibody targeting GXM (Casadevall et al., 1998) has undergone phase I clinical testing

for use in the treatment of cryptococcosis (Larsen et al., 2005). GXMGal, a minor capsular component, can induce apoptosis in immune cells (Villena et al., 2008). Cryptococcal mannoproteins are efficient stimulators of T cell-mediated immune responses (Levitz and Specht, 2006). These molecules are stably connected to the cell wall and require γ-radiation or DMSO treatment to be detached from the fungal surface (Maxson et al., 2007a,b).

During the last five years, a number of studies have demonstrated that the complexity of the *C. neoformans* capsule is greater than previously thought (Rodrigues et al., 2009). For instance, GXM can self-aggregate (Nimrichter et al., 2007), producing polysaccharide samples that differ in both biophysical and serological properties from fractions obtained through classical biochemical methods (Frases et al., 2008). In addition, microscopic analyses in combination with gene deletion and biochemical approaches strongly suggest that, within the capsular microenvironment, GXM interacts with other glycans, including α1,3 glucan (Reese and Doering, 2003; Reese et al., 2007), GXMGal (De Jesus et al., 2009), mannoproteins (Jesus et al., 2010), and chitin-like structures (Rodrigues et al., 2008a). These studies have led investigators to question the prior models of the structure of the *C. neoformans* capsule, and have led us and others to ask whether the association of GXM with other glycans produces functionally different molecules. This question has been initially addressed in an experimental model testing the association of GXM with chitin-like structures (Ramos et al., 2012), as detailed below.

Chitin is composed of β1,4 linked units of *N*-acetylglucosamine. This water-insoluble polysaccharide is a scaffold component of the fungal cell wall (Nimrichter et al., 2005), that is not normally accessible to the immune system. During cell division, chitin is hydrolyzed through the activity of chitinases, resulting in the formation of chitooligomers (Kuranda and Robbins, 1991; Adams, 2004). In *Saccharomyces cerevisiae*, these molecules accumulate in bud scars (Powell et al., 2003). However, the distribution of cell wall chitooligomers in *C. neoformans* seems to be unique, as these molecules are intercalated within the capsular network (**Figure 1**) (Rodrigues et al., 2008a). The wide distribution of GXM in the capsule, in fact, supports the hypothesis that this polysaccharide has the potential to interact with peripheral components, including chitin oligosaccharides.

The supposition that GXM and chitin-derived structures interact has been confirmed by a number of approaches (Rodrigues et al., 2008a; Fonseca et al., 2009a,b; Ramos et al., 2012). Using chromatographic and serologic methods in combination with dynamic light scattering, GXM has been shown to interact with chitin and chitooligomers based on the facts that: (1) complexes containing both structures have been isolated from *C. neoformans* cultures, (2) chitooligomers promoted enlargement of GXM fibrils, and (3) exposure of *C. neoformans* cells to an inhibitor of *N*-acetylglucosamine synthesis caused a decrease in capsular dimensions (Fonseca et al., 2009b). Although these studies were in agreement with the ability of *C. neoformans* to form glycan complexes composed of chitin-derived structures and GXM, their production during infection, impact on the host's immune system, and structural determinants regulating this glycan-glycan interaction were unknown until very recently.

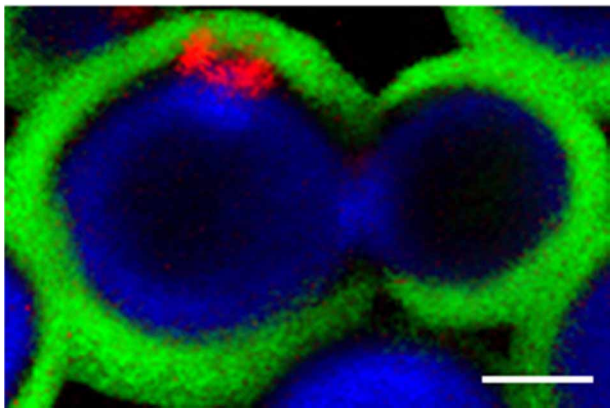

FIGURE 1 | Confocal section of budding *C. neoformans* cells. Cell wall chitin (blue fluorescence), capsular GXM (green fluorescence) and chitin oligosaccharides at the cell wall-capsule interface (red fluorescence) were stained as described in Rodrigues et al. (2008a). The image demonstrates that chitin oligosaccharides interact with GXM. Scale bar, 1 μm.

A recent study (Ramos et al., 2012) has demonstrated that chitin-GXM association involves non-covalent bonds, large GXM fibers, and depends on the *N*-acetyl amino group of chitin, but not on carboxyl and *O*-acetyl groups of GXM. Importantly, this study shows that glycan complexes formed by GXM and chitin-derived molecules also arise during macrophage infection. Injection of either isolated molecules or the glycan complexes into mice induced distinctly different cytokine responses. In fact, the glycan complexes were efficient in inducing the production of lung IL-10, IL-17, and TNFα, while the cytokine profiles of mice challenged with either GXM or chitin oligomers alone were similar to cytokine levels in control animals. The fact that glycan complex structures produce enhanced immunosuppressive and pro-inflammatory cytokine responses while chitin oligomers and GXM alone did not suggested that cell-associated *C. neoformans* glycans form hybrid structures with unique functions.

The discovery of the formation of functionally distinct glycan complexes raises a number of puzzling questions. For instance, the surface of fungal pathogens is decorated with many different glycans that coexist in several microenvironments (Nimrichter et al., 2005). In fact, many of these molecules are also released into the extracellular space (Rodrigues and Djordjevic, 2012). Therefore, isolated and complexed molecules may interact simultaneously but discordantly with the immune system. Considering the differential response of lung cells to isolated and hybrid molecules in the *C. neoformans* model (Ramos et al., 2012), it is reasonable to postulate that different receptors may be involved in the immune response to each molecular species. The physiologic events regulating the formation of the components of the glycan complex have largely been elusive. For instance, hybrid glycan complexes composed of chitin oligosaccharides and GXM are found in the capsule (Fonseca et al., 2009b) and in the extracellular space (Ramos et al., 2012), implying the requirement

of secretory mechanisms for transporting these macromolecules across the fungal cell wall. In fact, GXM is secreted to the cell surface and to the extracellular space by vesicular mechanisms (Yoneda and Doering, 2006; Rodrigues et al., 2007), but secretory processes resulting in the export of chitin oligosaccharides are not known. In this context, it has been established by a number of studies that polymerization and hydrolysis of fungal polysaccharides are surface-associated events (Adams, 2004), with GXM being the only well-known exception (Yoneda and Doering, 2006; Rodrigues et al., 2011). Consequently, it would be reasonable to suppose that the generation of soluble oligosaccharides participating in glycan interactions depends on hydrolytic enzymatic activity.

Chitooligomers are the products of enzymatic hydrolysis of chitin. Chitinase expression is induced during pulmonary cryptococcosis in rodents (Vicencio et al., 2008) and in the bronchoalveolar lavage fluid of asthmatic children (Goldman et al., 2012). The surface distribution of chitooligomers in *C. neoformans* is in fact increased in the lungs of infected rats (Fonseca et al., 2009b). It is also likely that chitooligomers produced through the activity of chitinase are released to the extracellular space, considering their high hydrophilicity and consequent solubility in water. GXM, on the other hand, is constitutively secreted extracellularly (Zaragoza et al., 2009). The concentration of hybrid glycans is severely reduced in cultures with methylxanthine, an inhibitor of fungal chitinases (Ramos et al., 2012). The reduced formation of hybrid glycan complexes as a consequence of chitinase inhibition is in accord with *in vivo* observations demonstrating that chitooligomer detection and capsule enlargement are more evident in host tissues manifesting higher activity of this enzyme (Fonseca et al., 2009b). Therefore, a putative synergistic or additive activity of host and fungal chitinases cannot be discarded.

GXM has the potential to associate with a number of hydrophilic components, mainly because of its high efficiency in the formation of hydrogen bonds. Thus, GXM-chitin interactions probably have other counterparts in *C. neoformans*. Microscopic examinations of *C. neoformans* yeast cells, in fact, support this possibility. Co-staining of cryptococci with antibodies raised to GXM and to α1,3 glucan reveal that α1,3 glucan is widely distributed in the capsule (Cordero et al., 2011). Nevertheless, α1,3 glucan is well known as a cell wall polysaccharide responsible for anchoring *C. neoformans* GXM (Reese and Doering, 2003; Reese et al., 2007), and has not previously been considered as a capsular component. Such unexpected cellular distribution may be linked to enzyme-dependent generation of α1,3 glucan fragments. Glucans are dynamically polymerized and hydrolyzed during cell wall remodeling and yeast replication (Adams, 2004), resulting in the production of soluble glucan oligosaccharides as a natural consequence of cell division. In *C. neoformans*, the presence of the capsule is well-known to slow down the molecular traffic across the cell surface (Nosanchuk et al., 1998; Rodrigues et al., 2000), supporting the possibility that glucan-derived oligosaccharides could be retained within the capsular network after enzymatic hydrolysis. Such mechanism would result in the formation of hybrid microenvironments composed of GXM and glucan-derived oligosaccharides that are compatible with the fluorescence profile observed by Cordero and colleagues (2011) and illustrated in **Figure 2**. Structural determinations regulating GXM-glucan interactions are still unknown, although hydrogen bonds are likely involved in polysaccharide-polysaccharide interactions (Fonseca et al., 2009b; Ramos et al., 2012). Importantly, these molecules have the potential to form unique glycan complexes, as observed for GXM-chitin oligosaccharides. Such rationale could be also be applicable to other cell wall and capsular components, including β-glucans, GXMGal and mannoproteins.

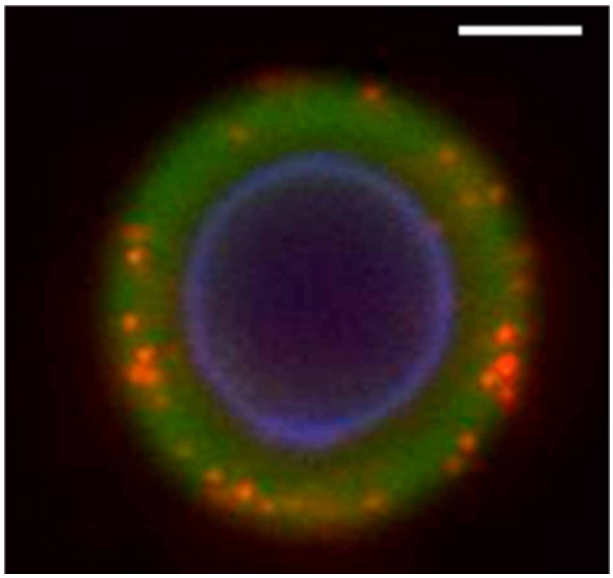

FIGURE 2 | Fluorescence microscopy of *C. neoformans* after staining for cell wall chitin (blue fluorescence), capsular GXM (green fluorescence) and α1,3 glucan (red fluorescence). The capsular distribution of α1,3 glucan suggests that the glycan is interacting with GXM and supports the hypothesis that the fungus forms hybrid polysaccharides. For experimental details, see Cordero et al. (2011). Image provided by Dr. Radames, J. B. Cordero. Scale bar, 1 μm.

Surface molecules do not exist in their isolated form in cellular systems and approaches investigating interacting molecules can provide a deeper understanding of complex biological processes than the study of individual purified molecules. The discovery of hybrid glycans with previously unknown functions suggests new venues of investigation on the roles of polysaccharides and glycoconjugates in fungal infections. In addition, the connections between glycan association and functional variation strongly indicate that molecular complexes with still unknown properties may exist in fungal pathogens. This conclusion encourages new perspectives on models aiming at the discovery of protective immunogens.

ACKNOWLEDGMENTS

Marcio L. Rodrigues and Leonardo Nimrichter are supported by grants from the Brazilian agencies CNPq, CAPES, and FAPERJ. We are thankful to A. Casadevall and L. R. Travassos for many suggestions on the roles of hybrid glycans in the *C. neoformans* model. We are also grateful to Radames J. B. Cordero for the image used in **Figure 2** and also for constant support and suggestions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 May 2012; paper pending published: 14 June 2012; accepted: 20 June 2012; published online: 09 July 2012.

Citation: Rodrigues ML and Nimrichter L (2012) In good company: association between fungal glycans generates molecular complexes with unique functions. Front. Microbio. ***3****:249. doi: 10.3389/fmicb.2012.00249*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 02 February 2012
doi: 10.3389/fmicb.2012.00021

Antibody therapy for histoplasmosis

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The endemic human pathogenic fungus *Histoplasma capsulatum* is a major fungal pathogen with a broad variety of clinical presentations, ranging from mild, focal pulmonary disease to life-threatening systemic infections. Although azoles, such as itraconazole and voriconazole, and amphotericin B have significant activity against *H. capsulatum*, about 1 in 10 patients hospitalized due to histoplasmosis die. Hence, new approaches for managing disease are being sought. Over the past 10 years, studies have demonstrated that monoclonal antibodies (mAbs) can modify the pathogenesis of histoplasmosis. Disease has been shown to be impacted by mAbs targeting either fungal cell surface proteins or host co-stimulatory molecules. This review will detail our current knowledge regarding the impact of antibody therapy on histoplasmosis.

Keywords: *Histoplasma capsulatum*, histoplasmosis, antibody, histone 2B, heat shock protein 60, M antigen, co-stimulation

INTRODUCTION

The most commonly encountered endemic mycoses in the Americas are due to *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Coccidioides immitis/posadasii* (Lockhart et al., 2009; Prado et al., 2009). As is the case in other endemic fungi, *H. capsulatum* infection is typically acquired by inhalation of fungal propagules after disturbances of contaminated soil or excreta (Guimaraes et al., 2006). The clinical manifestation of the disease range from asymptomatic infection or a mild influenza-like illness to a disseminated sepsis form that may involve virtually any tissue (Meloan, 1952; Goodwin and Des Prez, 1978; Fojtasek et al., 1994; Bradsher, 1996). These manifestations depend mainly on the magnitude of exposure (i.e., the number of fungal particles inhaled), the immunological status of the host (i.e., patients with AIDS or individuals receiving steroids or chemotherapy), and the virulence of the infective strain, indicating that environmental and genetic factors influence the manifestation of disease (Goodwin et al., 1981; Kauffman, 2007). The vast majority of infected persons have either no symptoms or a very mild illness that is never recognized as being histoplasmosis (Wheat et al., 2007). In fact, 95–99% of the primary infections are not recognized or detected in immunologically normal hosts in endemic areas (Saliba and Beatty, 1960; Isbister et al., 1976; Goodwin et al., 1981). Although the majority of symptomatic infections follow primary exposures to *H. capsulatum*, reactivation of latent infection can result in significant disease, particularly in the setting of immunosuppression (Kauffman, 2007). Furthermore, reactivation disease can be developed in liver transplant recipients with disease originating from latent infections in the transplanted organs (Limaye et al., 2000). Additionally, reactivation histoplasmosis has increasingly occurred in patients receiving anti-cytokine therapies, especially inhibitors of INF-γ and TNF-α (Deepe, 2005; Deepe et al., 2005; Scheckelhoff and Deepe, 2005).

As infection with *H. capsulatum* is not a mandatory reportable event, the actual incidence of clinically significant histoplasmosis is not known. Epidemiological studies have estimated that 500,000 individuals acquire *H. capsulatum* annually in the USA and over 80% of young adults in endemic areas have been infected with the fungus (Edwards et al., 1969). A national survey of hospital discharge diagnoses from 2002 identified 3,370 patients hospitalized for histoplasmosis in the USA with a crude mortality rate of 8% (Chu et al., 2006). Notably only 14% of the patients were immunocompromised and this percentage was similar in those who died. Given the nature of the survey, it only represented a "fraction of the burden of all morbidity and mortality" (Chu et al., 2006) related to *H. capsulatum*. This study also documents that hospital charges for the identified patients were well over $100 million. Hence, histoplasmosis is a significant and costly cause of morbidity and mortality in otherwise healthy individuals and in immunodeficient patients. Despite the potency of current antifungal drugs, they nevertheless fail to prevent mortality in nearly 1 in 10 patients hospitalized with histoplasmosis.

Although *H. capsulatum* has previously been considered to consist of three varieties, *capsulatum*, *duboisii*, and *farciminosum* (Darling, 1906; Dodd and Thompkins, 1934; Medoff et al., 1987), recent molecular work has shown that these distinctions are phylogenetically meaningless, but instead, there are genetically distinct geographical populations or phylogenetic species (Kasuga et al., 2003). *H. capsulatum* is a dimorphic fungal pathogen with two distinct morphological forms, filamentous and yeast, depending on the nutritional factors and temperature (Maresca and Kobayashi, 1989). *H. capsulatum* is found in nature primarily as a saprophytic mold, and exists in soils enriched with organic nitrogen sources

such as animal excrements, or when grown in the laboratory at less than 35°C (Emmons, 1950, 1956a,b; Zeidberg et al., 1952; Alteras, 1966; Emmons et al., 1966; Disalvo et al., 1970; Smith, 1971a,b). The mold form is composed of hyaline septate hyphae that produce two different asexual reproduction structures, macroconidia and microconidia. The microconidia are the purported infectious propagule, as their size, 2–6 μm, is well suited for deposition into distal alveoli. Upon entry to a susceptible host, the microconidia rapidly convert to the pathogenic single, budding yeast-like form, which can also be cultivated in laboratory medium at 37°C.

As a facultative intracellular parasite, the interaction of *H. capsulatum* with macrophage cells is a critical component of the host response to infection (Newman, 2005) and is a complex and obscure phenomenon. Heat shock protein 60 (Hsp60) is the major *H. capsulatum* surface ligand that engages CD11b/CD18 (CR3) integrin on the surface of phagocytes resulting in phagocytosis (Long et al., 2003; Habich et al., 2006). *H. capsulatum* yeasts have critical interactions with inflammatory neutrophils, and with dendritic cells (DCs) in the lung and other organs. Indeed, recent new evidence suggests that DCs may be the key antigen-presenting cells that initiate cell-mediated immunity (Deepe et al., 2008). *H. capsulatum* yeast cells must survive and/or subvert the hostile antimicrobial environmental within phagocytes (Allendoerfer et al., 1997), including fungicidal mechanisms such as reactive oxygen species and products of the nitric oxide synthase (NOS) pathway (Eissenberg and Goldman, 1987). The yeast form actively inhibits phagolysosomal fusion, thereby preventing exposure to the acidic hydrolytic enzymes of the lysosomes. *H. capsulatum* also prohibits accumulation of vacuolar ATPase, which is important for proton accumulation in phagosomes, and the fungus can actively alkalinize phagosomal pH to 6.5 (Strasser et al., 1999). Within the phagocytic cells, viable yeast may travel to hilar and mediastinal lymph nodes where they gain access to the blood circulation for dissemination to various organs, such as the liver and spleen (Wheat and Kauffman, 2003).

The therapeutic approach to patients with histoplasmosis is well documented in a 2007 "practice guideline" by the Infectious Diseases Society of America (Wheat et al., 2007). Azole drugs, such as itraconazole and voriconazole, and amphotericin B are the drugs of choice for clinically significant disease. However, as detailed above, these potent therapeutics fail to prevent mortality in a significant proportion (∼10%) of hospitalized patients. Additionally, the antifungal agents are given for protracted periods or even life-long in settings of ongoing immunocompromise. Hence, new therapeutic approaches have been investigated. One of the different avenues of study has been the application of antibodies to modify the pathogenesis of histoplasmosis.

ANTIBODIES IN HISTOPLASMOSIS

Passive immunization with polyclonal antibodies is controversial for mycoses (Louria and Kaminski, 1965). However, there is a growing consensus that antibodies collaborate with phagocytic cells and T cells for the enhancement of the immune response during systemic mycosis (Yuan et al., 1997; Vecchiarelli and Casadevall, 1998; Huffnagle and Deepe, 2003). Moreover, experiments with polyclonal sera have produced conflicting results that have raised questions regarding antibody efficacy in fungal disease (Louria and Kaminski, 1965; Mukherjee et al., 1992; Casadevall, 1998). In the 1970s, adoptive transfer experiments of serum from mice immunized with Hc ribosomes or live yeast cells failed to protect mice infected with the fungus, whereas transfer of filtered spleen or peritoneal cells from the immunized animals were protective (Tewari et al., 1977).

Recently, studies with monoclonal antibodies (mAbs) strongly have suggested that divergent results obtained with polyclonal antibodies preparations may be a result of the relative proportions of protective, non-protective, and inhibitory antibodies in immune sera (Dromer et al., 1987; Mukherjee et al., 1992) since animal experiments with mAbs to the capsular polysaccharide of *Cryptococcus neoformans* (Cn) have revealed the existence of protective, non-protective, and disease-enhancing mAbs. A protective mAb may even have a reduced efficacy if administered in high amounts, due to a prozone-like effect (Taborda and Casadevall, 2001; Taborda et al., 2003). In addition, protective efficacy of mAbs is determined by several variables including pathogen inoculum (Taborda et al., 2003), genetic background of both microbe (Mukherjee et al., 1995b) and host (Lendvai and Casadevall, 1999; Zaragoza et al., 2007), host immunological status (Yuan et al., 1997), both epitope specificity and isotype of mAb (Mukherjee et al., 1992, 1995a), timing of antibody administration (Casadevall, 1998; Casadevall et al., 1998), and route of infection (Briles et al., 1992a,b).

The antibody response to infection with *H. capsulatum* has been characterized. In humans, infection induces an increase in IgM by 2 weeks, followed by rising titers of IgA and IgG (Chandler et al., 1969). The IgG fraction contains complement-fixing and precipitating antibodies (Chandler et al., 1969). Murine experiments show that *Histoplasma*-specific serum immunoglobulin levels peak by day 21 (Fojtasek et al., 1993). Studies on the inflammatory reactions in the lungs of mice infected with *H. capsulatum* demonstrate that the number of B cells increase in the first week of infection, albeit to a lesser degree than other myeloid cells lines (Fojtasek et al., 1993; Cain and Deepe, 1998). Subsequently, the number of B cells continues to increase as other myeloid lines decrease (Cain and Deepe, 1998). The number of B cells in the spleen does not significantly change until the end of the second week of infection when all cell subsets nearly double (Fojtasek et al., 1993). In histoplasmosis, the current paradigm for host control of infection relies most heavily on activation of cellular immunity, since, in the absence of effector cells, progressive disease with dissemination occurs (Allendorfer et al., 1999). However, B-lymphocytes can impact histoplasmosis. Depletion of CD4+ and CD8+ T cells in B-lymphocyte knockout mice induced a markedly higher *H. capsulatum* burden in organs when compared with T cell depletion in wild-type animals in a secondary histoplasmosis model (Allen and Deepe, 2006), corroborating previous studies showing that antibodies can modify the pathogenesis of mycoses (Pirofski and Casadevall, 1996; Casadevall, 1998; Casadevall et al., 1998).

HISTOPLASMA CAPSULATUM FUNGAL CELL SURFACE: TARGETS FOR ANTIBODY

The fungal cell wall is rich in targets for the immune system (Nimrichter et al., 2005). As with most other pathogenic fungi, *H.*

capsulatum possesses a rigid, polysaccharide composed cell wall structure, with four different glycans: soluble galactomannan, α-1,3-glucan, β-1,3-glucan, and a fibrillar chitin skeleton (Domer, 1971; San-Blas et al., 1978). Displayed on the surface are a variety of proteins that have been associated with virulence or utilized in diagnosis of the fungus (**Figure 1**). MAbs have been generated that bind *H. capsulatum* cell wall antigens, including melanin (Nosanchuk et al., 2002), histone 2B (Nosanchuk et al., 2003), Hsp60 (Guimaraes et al., 2009), M antigen (Guimaraes et al., 2008), and a 70-kDa protein (Lopes et al., 2010).

HISTONE 2B

Immunization with heat-killed *H. capsulatum* yeast cells resulted in the generation of a panel of IgM isotype mAbs that specifically bound cell surface expressed histone 2B (Nosanchuk et al., 2003). Although the identification of histone 2B as a cell surface antigen was surprising, histones are increasingly described on the cell surface of diverse host cells as well as pathogens; such as the cell surface of *Mycobacterium leprae* (Pessolani et al., 1993; Marques et al., 2000, 2001) and *Mycobacterium smegmatis* (Pethe et al., 2001) that are associated with binding to host cells and, specifically in the case of *M. leprae*, cell invasion (Shimoji et al., 1999). The administration of the IgM mAbs to *H. capsulatum* histone 2B was hindered by the large molecular weight of the mAbs and the difficulty with their entry into the alveolar space. However, co-incubation of mAb with *H. capsulatum* yeast cells prior to infection of mice revealed that the mAbs reduced the severity of disease. The protective effect was augmented by the administration of sub-inhibitory concentrations of amphotericin B, which stimulates host immune responses by engaging TLR2 and CD14 (Sau et al., 2003). Overall, the IgM mAbs to histone 2B reduced fungal burdens, decreased pulmonary inflammatory damage, and prolonged murine survival.

The protective effect of these mAbs was associated with an alteration in the intracellular fate of *H. capsulatum* (Nosanchuk et al., 2003; Shi et al., 2008). The mAbs reduced the capacity of the fungus to replicate intracellularly. Although *H. capsulatum* typically tightly regulates the phagosomal pH of macrophages, yeast opsonized with mAb to histone 2B were unable to maintain a neutral pH in their host macrophage cells. The inability to regulate the pH resulted in fungal cell killing, increased processing of fungal antigens and increased T cell activation (Shi et al., 2008).

HEAT SHOCK PROTEIN 60

As described above, Hsp60 is the surface protein that is the major ligand that mediates attachment of *H. capsulatum* to macrophage CR3 (CD11b/CD18; Long et al., 2003). Hsp60 is also an immunodominant antigen expressed on the surface of *H. capsulatum* yeasts and is able to induce protective immunity upon vaccination (Deepe et al., 1996; Deepe and Gibbons, 2002; Scheckelhoff and Deepe, 2002). Using recombinant Hsp60, we generated IgG isotype mAbs (Guimaraes et al., 2009). Interestingly, IgG2a and IgG1 mAbs to Hsp60 were protective whereas a IgG2b was disease-enhancing. Protection was characterized by a reduction of fungal burden, decreased tissue damage, and prolongation of survival. Cytokine analyses revealed that the protective mAbs induced a strong Th1-type host response. Notably, the IgG2b recognized the same region on the protein to which a protective IgG1 also bound in a competitive manner, suggesting that protection was regulated by isotype. This finding was supported by data from mice treated with methamphetamine that developed significant increases in their IgG2b levels and also had accelerated and exacerbated histoplasmosis (Martinez et al., 2009).

As with the mAbs to histone 2B, the protective mAbs to Hsp60 modified the intracellular fate of the yeast cells. Phagosomal

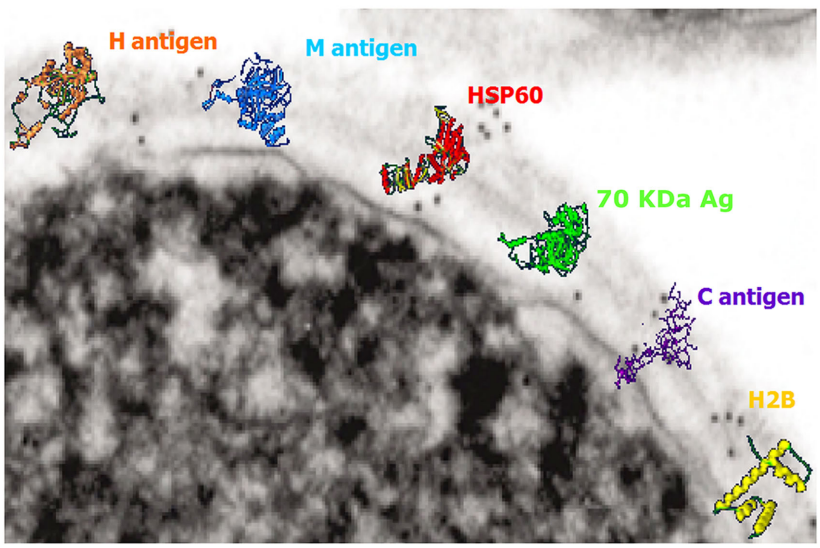

FIGURE 1 | Transmission electron micrograph of the surface of a *H. capsulatum* yeast cell with overlaying cartoons depicting the hypothetical structures of the H antigen, M antigen, heat shock protein 60, 70 kDa antigen, C antigen, and histone 2B. The antigen structures are based on molecular modeling as described in Guimaraes et al. (2008).

maturation was significantly increased in the presence of the protective mAbs and this correlated with a reduction in intracellular yeast cell survival. In contrast, yeast cultured with the disease-enhancing IgG2b mAb replicated at an enhanced rate compared to controls.

Interestingly, the protective mAbs were noted to induce aggregation of *H. capsulatum* yeast cells (Guimaraes et al., 2011a). Agglutination of yeast cells caused by antibodies had been reported previously for *C. neoformans* (Kozel and McGaw, 1979). However, agglutination was evaluated with IgG-opsonized strain 602 of *C. neoformans*, by using a monospecific antiserum to IgG heavy chains, in order to confirm association of IgG antibodies to the yeast and specifically determine that these molecules were binding directly at the yeast surface. With *H. capsulatum* we observed that agglutination occurred only when cells are brought together due to a result of a Brownian movement during which cellular collision permits interaction. However, the surface of *H. capsulatum* yeast is typically negatively charged due to the high amounts of α-1,3-glucans on the cell wall. The negative charge increases the electrostatic potential surrounding the cells leading to repulsion between cells. *H. capsulatum* yeast aggregation was an effect of concentration, but the magnitude of aggregation efficiency was dependent on the dissociation constant of each mAb characterized. Additionally, we used an optical tweezer to measure real-time interactions between single cells in the presence of opsonins and found a correlation of time for aggregation (characteristic time) and binding constant, with the protective mAb being more effective than the non-protective mAb. Interestingly, blockade of CR3 receptors resulted in an additional drop of phagocytosis rate of larger aggregates, suggesting a cooperative function of Fc and CR3 receptor for the phagocytosis of large particles. Overall, it is unclear what the impact of agglutination potential of the antibodies is during infection. However, it is possible that the antibodies may keep replicating cells agglutinated, which can reduce the dissemination of the fungus, and these clusters of cells may be more effectively targeted by host responses.

We have also recently demonstrated that Hsp60 is involved in the presentation of a diverse range of proteins on the fungal cell surface, including proteins associated with oxidative stress responses (Guimaraes et al., 2011c). Hence, the binding of Hsp60 by antibody may dysregulate the chaperone functions of the protein. Disruption of this interactome, especially during stress response conditions, could impact the capacity of the fungus to cause disease.

M ANTIGEN

The M antigen is a glycoprotein that is well known as a diagnostic antigen for acute histoplasmosis as it induces the first precipitins during disease (Pizzini et al., 1999). We have documented that live fungal cells secrete only small amounts of the protein and interestingly, we demonstrated that the M antigen was also expressed at the *H. capsulatum* cell surface and that it functions as a catalase (Guimaraes et al., 2008). In the course of this study, we produced three mAbs to the M antigen, one IgM and two IgG2a isotype mAbs. Opsonization of *H. capsulatum* with the IgM or IgG2a mAbs to the M antigen enhances yeast cell phagocytosis by macrophages and two of the three mAbs also promote host cell cidal activity (**Figures 2A,B**). All three mAbs also altered the pathogenesis of experimental murine histoplasmosis as mice challenged with opsonized yeast cells uniformly survived a lethal challenge with *H. capsulatum* (**Figure 2C**).

70 kDa ANTIGEN

In addition to the fact that the IgG2b mAb to Hsp60 was not protective, we have demonstrated that an IgG1 mAb specific for a 70-kDa cell surface antigen was non-protective (Lopes et al., 2010). The mAb was previously shown to be highly specific for *H. capsulatum* and is a candidate for use in the serological diagnosis and management of histoplasmosis (Gomez et al., 1997, 1999). The finding that the IgG1 to the 70-kDa antigen was non-protective indicates that isotype is not the only determinant for a protective response, but that the antigen target can also influence the outcome of disease.

DISCUSSION

The incidence of clinically relevant mycoses is rising, mainly due to recent advances in modern medicine, including the use of intravascular devices, broad spectrum antibiotics, organ transplantation, the use of chemotherapeutic and anti-inflammatory drugs, and the increasing number of individuals with HIV infection (Pfaller and Diekema, 2010). Given the difficulties in combating severe fungal diseases with our current antifungal medications, novel approaches, including administration of mAbs, are being pursued. Studies with antibody and *H. capsulatum* reveal that protective and non-protective antibodies exist. This finding is also extremely relevant to vaccine development, as an adverse outcome of vaccination might be the generation of disease-enhancing antibodies, even if the latter are a subset of those induced.

The protective mAbs to *H. capsulatum* identified to date mediate protection by modifying the intracellular fate of the yeast cells. *H. capsulatum* is remarkable for its capacity to tightly regulate the intracellular milieu of macrophage phagosomes. The protective mAbs to histone 2B and Hsp60 enhance macrophage fungistatic/fungicidal responses, subverted *H. capsulatum*'s ability to modify the phagosomal environment, resulting in augmented antigen processing and T cell activation, and control of the fungal disease. The opsonization by the antibody on *H. capsulatum* can also impact the fungus' ability to traffic surface proteins. Future research is needed to better characterize the fundamental influences on protection of antibody in histoplasmosis, including defining the importance of isotype on the protective efficacy, determining the role of the quantity of surface antigen on protection, and the direct effect of antibody on *H. capsulatum* transcriptional regulation.

In addition to exploring fungal antigens as targets for antibody therapy, we have examined the impact of antibody on host cell co-stimulation as a new alternative for controlling the pathogenesis of histoplasmosis. We determined that *H. capsulatum*

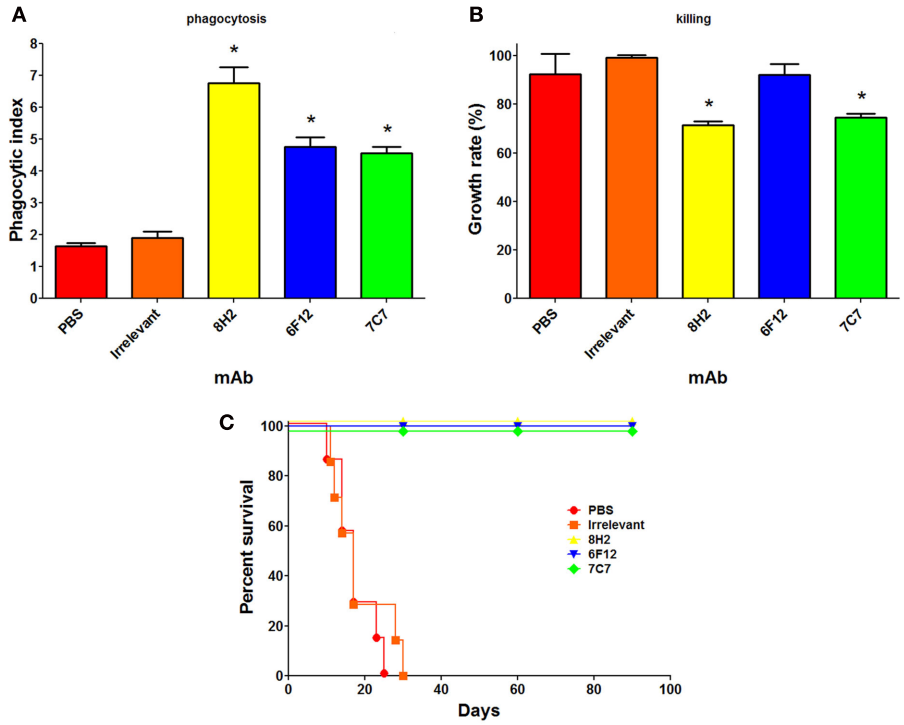

FIGURE 2 | Monoclonal antibodies (mAbs) to the M antigen can modify the pathogenesis of experimental histoplasmosis. The generation of the mAbs is described in Guimaraes et al. (2008). The mAbs produced are mAb 7C7 (IgM isotype), mAb 6F12 (IgG2a), and mAb 8H2 (IgG2a). Using methods described in Guimaraes et al. (2011b), the phagocytosis rates **(A)**, and killing capacity **(B)** of J774.16 macrophage-like cells reveal that the mAbs significantly enhanced the uptake of yeast cells by the phagocytes ($p < 0.05$ vs. controls) and that two of the three mAbs enabled the killing of yeast cells by the J774.16 cells ($p < 0.05$ for mAbs 8H2 and 7C7 vs. controls). **(C)** C57Bl/6 mice infected with 1.25×10^7 opsonized yeast cells had a 100% survival whereas control infected mice died within 1 month after infection ($p < 0.001$ for mAbs 8H2, 6F12, and 7C7 vs. controls).

altered the PD-L expression on macrophages, which resulted in a dysregulation of T cell activation (Lazar-Molnar et al., 2008). The PD-1/PD-L pathway is involved in maintenance of self-tolerance and T cell regulation. Mice deficient in PD-1 were protected against lethal challenges of *H. capsulatum*. Administration of mAb that blocks PD-1 similarly protected wild-type mice against lethal infection. Hence, antibody targeting host responses can modify histoplasmosis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 November 2011; accepted: 12 January 2012; published online: 02 February 2012.

Citation: Nosanchuk JD, Zancopé-Oliveira RM, Hamilton AJ and Guimarães AJ (2012) Antibody therapy for histoplasmosis. Front. Microbio. ***3****:21. doi: 10.3389/fmicb.2012.00021*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 12 January 2012
doi: 10.3389/fmicb.2011.00283

Radioimmunotherapy of fungal diseases: the therapeutic potential of cytocidal radiation delivered by antibody targeting fungal cell surface antigens

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Radioimmunotherapy is the targeted delivery of cytocidal radiation to cells via specific antibody. Although mature for the treatment of cancer, RIT of infectious diseases is in preclinical development. However, as there is an obvious and urgent need for novel approaches to treat infectious diseases, RIT can provide us with a powerful approach to combat serious diseases, including invasive fungal infections. For example, RIT has proven more effective than standard amphotericin B for the treatment of experimental cryptococcosis. This review will discuss the concepts of RIT, its applications for infectious diseases, and the strides made to date to bring RIT of infectious diseases to fruition. Finally, we will discuss the potential of PAN-FUNGAL RIT, the targeting of conserved fungal cell surface antigens by RIT, as a treatment modality for fungi prior to the formal microbiological identification of the specific pathogen. In sum, RIT provides a mechanism for the targeted killing of drug susceptible or resistant fungi irrespective of the host immune status and may dramatically reduce the length of therapy currently required for many invasive fungal diseases.

Keywords: ***Histoplasma capsulatum, Cryptococcus neoformans, Candida albicans*, antibody, heat shock protein 60, beta-glucan, melanin**

INTRODUCTION

Fungal diseases are increasingly common major causes of human disease, especially in immunocompromised individuals and hospitalized patients with underlying medical conditions (Patterson, 2005; Caston-Osorio et al., 2008; Richardson and Lass-Florl, 2008; Pfaller and Diekema, 2010). In fact, since 1979 there has been a >200% increase in the annual number of cases of invasive fungal infections (IFI) in the United States. IFIs rates have risen in large part due enhanced interventions in intensive care units (ICUs), increased survival rates of individuals with immunodeficiencies, and the increased administration of potent therapeutics, chemotherapeutics, and biologicals, that compromise the immune responses of our patients. This increase is best exemplified by the example of *Candida* spp.: *Candida* were once infrequent causes of invasive disease, whereas they are currently the fourth leading cause of nosocomial bloodstream infection in the United States, responsible for 8–15% of all such hospital acquired infections. However, despite the increased prevalence of many mycotic diseases, there remains an enormous gap in knowledge and our current therapeutic armamentarium all too often fails to eradicate these insidious pathogens.

Although they have powerful activities, the number of available medications for mycoses is significantly less than for bacterial diseases. At present, there are three main medication categories for IFI: azoles (fluconazole, itraconazole, voriconazole, and posaconazole), polyenes (primarily formulations of amphotericin B), and echinocandins (caspofungin, micafungin, and anidulafungin). Notably, both the azoles and polyenes target cell membrane sterols, with azoles inhibiting sterol synthesis and the polyenes purportedly disrupting the membrane structure. The echinocandins inhibit cell wall production by interfering with beta-1,3-glucan synthesis. In addition to these drugs, flucytosine, an antimetabolite, is utilized primarily in combination with amphotericin B for the treatment of cryptococcosis. Notably, the echinocandins are the last new class of antifungal drug, with caspofungin gaining FDA approval in by the FDA in 2001. Unfortunately, there is no antifungal medication poised to enter clinical medicine for the foreseeable future. Hence, there is a consensus that new approaches are needed to combat IFI.

Radioimmunotherapy (RIT) uses antigen–antibody interactions to deliver cytocidal amounts of ionizing radiation to specific cell targets. Currently, RIT is clinically utilized in the treatment of primary, refractory, and recurrent non-Hodgkin lymphoma using the radiolabeled mAbs Zevalin® and Bexxar®. It is important to note that RIT offers several significant advantages over standard antifungal therapy. Firstly, RIT delivers lethal radiation, such that it does not merely interfere with a single cellular pathway but completely destroys targeted cells. As such, RIT is less subject to drug resistance mechanisms. Moreover, RIT is cidal in immunologically compromised individuals as the nuclides are equally able to destroy cell targets in immunologically intact individuals or those with HIV or other immunodeficiencies, either primary or drug induced. RIT does not suffer the drug–drug

interactions that clinically trouble clinicians caring for complex patients, such as azole or echinocandin interactions with commonly prescribed immunosuppressive drugs, like cyclosporine or tacrolimus. Finally, in contrast to weeks, months, or years required for the treatment of certain mycoses with standard antifungals, RIT may permit single dose or a limited number of doses to combat fungal diseases.

What are the barriers for translating RIT into treatment approaches for infectious diseases? Cell surface antigens are well defined for diverse pathogens, including viruses, bacteria, parasites, and fungi. Moreover, monoclonal antibodies exist that target microbial cell surface antigens. Additionally, the technology for linking radionuclides to mAbs is well established, so the approaches can be readily translated from oncology into infectious diseases. Additionally, the US hospitals that are now regularly using RIT to treating cancer patients are fully equipped for initiating Infectious Diseases RIT. Included in this ability, imaging of patients receiving RIT to ascertain the targeting of radiolabeled mAbs in Infectious Diseases RIT can be readily achieved using portable imaging equipment that is standard in these hospitals. Hence, the time is "now" for developing RIT to combat IFI.

RIT OF INFECTIOUS DISEASES

Our laboratories were the first to demonstrate that microorganism-specific mAb-RIT is highly effective for the treatment of experimental fungal, bacterial, and viral infections, as well as virally induced cancers (**Table 1**). Although the initial RIT work utilized *Cryptococcus neoformans* for proof-of-principle studies in 2003 (Dadachova et al., 2003), RIT of bacterial and viral pathogens also has rapidly progressed. In 2004, we established the feasibility of RIT for invasive bacterial infection using a mouse pneumococcal disease model (Dadachova et al., 2004a). An IgM isotype mAb to serotype 8 *Streptococcus pneumoniae* capsular polysaccharide was conjugated to the alpha-particle emitter Bismuth-213 (^{213}Bi) and we showed that an 80-μCi dose was sufficient to protect 60% of animals from an otherwise lethal challenge. More recently, in 2009, mAbs to the protective or lethal antigens of *Bacillus anthracis* labeled with either ^{213}Bi or the beta-particle emitter rhenium-188 (^{188}Re) were shown to prolong the survival of mice infected with *B. anthracis* Stern bacterial cells, but not spores (Rivera et al., 2009). Treatment with ^{213}Bi was more effective than when less powerful beta-particles were used.

In 2006, HIV infected cells were found to be effectively targeted by RIT (Dadachova et al., 2006a). Radiolabeling of antibody to the HIV-1 envelope glycoproteins gp120 and gp41 with ^{213}Bi or ^{188}Re selectively killed HIV infected cells *in vitro* and eliminated the majority of HIV infected cells injected into SCID mice. Notably, the mAb to gp41 is fully human. It is possible that, used in combination with anti-retroviral drugs, that RIT may provide a means to eradicate HIV infection (Casadevall et al., 2007). RIT has also proven to be highly effective in the treatment of virally induced cancers (Dadachova et al., 2007). Using a human papillomavirus (HPV) type 16-associated cervical cancer model in mice, RIT with a human mAb targeting the HPV16 E6 antigen significantly impeded tumor growth compared to cancers in control animals (Wang et al., 2007). Interestingly, RIT targeting cancer cells expressing low levels of HPV16 E6 was also successful (Phaeton et al., 2010a). Furthermore, pre-treatment of the cervical tumors with a proteosome inhibitor, MG-132, and unlabeled antibody to E6 resulted in an increase in the levels of E6 for targeting by ^{188}Re-labeled mAb and enhanced the efficacy of RIT (Phaeton et al., 2010b). These studies also suggest that RIT could potentially eradicate infected cells prior to their malignant transformation. In addition to cervical cancer, RIT proof-of-principle experiments with HPV-associated head and neck squamous cell carcinoma suggest that this modality may be effective for this difficult to combat disease (Harris et al., 2011).

RIT OF CRYPTOCOCCOSIS

Cryptococcus neoformans is a yeast-like encapsulated fungus with a global distribution that is responsible for ∼600,000 deaths annually (Park et al., 2009), particularly in individuals with HIV infection. As noted above, the first RIT experiments published in 2003 utilized *C. neoformans* as a model organism (Dadachova et al., 2003). The initial studies with *C. neoformans* demonstrated that a mAb specific for glucuronoxylomannan (GXM), the major component of the pathogen's polysaccharide capsule, radiolabeled with either ^{213}Bi or ^{188}Re efficiently killed cryptococcal cells *in vitro* and significantly reduced fungal burdens in and prolonged the survival of lethally infected mice (Dadachova et al., 2003). Most impressively, 60% of infected mice treated with 100 μCi ^{213}Bi were alive at

Table 1 | List of monoclonal antibodies that have been used in experimental studies of radioimmunotherapy for infectious diseases.

Antibody	Isotype	Antigen recognized	Reference or source
D11	IgM, human	Serotype 8 pneumococcal polysaccharide	Dadachova et al. (2004a), Zhong et al. (1999)
7.5G	IgG2b, mouse	*B. anthracis* protective antigen	Rivera et al. (2006, 2009)
10F4	IgG1, mouse	*B. anthracis* protective antigen	Rivera et al. (2006, 2009)
14FA	IgG2b, mouse	*B. anthracis* lethal antigen	Rivera et al. (2009)
18B7	IgG1, mouse	GXM, cryptococcal polysaccharide	Dadachova et al. (2003, 2004b, 2006b), Bryan et al. (2009, 2010)
246-D (cluster I)	IgG1, human	HIV gp41	Dadachova et al. (2006a)
C1-P5	IgG1, mouse	HPV E6	Wang et al. (2007), Phaeton et al. (2010a,b), Harris et al. (2011)
2G8	IgG2b, mouse	Beta (1,3) glucan	Torosantucci et al. (2009), Rachini et al. (2007)
4E12	IgG2a, mouse	Hsp60	Guimaraes et al. (2009)
6D2	IgM, mouse	Melanin	Rosas et al. (2000)
B11	IgM	Fungal glucosyl ceramide	Rhome et al. (2011)

day 75 after RIT. Importantly, systemically infected mice tolerated activities of up to 150 μCi of mAb labeled with either nuclide with platelet counts normalizing by 14 days post-treatment (Dadachova et al., 2004b). The reduction in platelets in the infected mice was similar to that seen in the setting of cancer therapy (Sharkey et al., 1997; Behr et al., 1999). Additionally, these mice had no evidence of pulmonary fibrosis 5 months after receiving RIT. The lack of severe toxicities due to radiation in the mice with cryptococcosis treated with RIT is presumably due to the high specificity of the mAb.

Chemotherapy for severe cryptococcosis is based on the administration of amphotericin B. However, RIT was recently shown to be more effective than amphotericin B for the treatment of disseminated cryptococcosis (Bryan et al., 2010). In the murine model examined, amphotericin B was able to prolong survival, but fungal burdens were not significantly reduced. In contrast, RIT significantly reduced the fungal burdens in the tissues examined, regardless of the melanization state of the yeast cell. This is an important observation as *C. neoformans* can produce melanin along the yeast cell wall and this pigment can protect the fungus from injury from external radiation (Nosanchuk and Casadevall, 2006; Dadachova et al., 2008). Interestingly, ∼10 times less mAb 18B7 conjugated to ^{213}Bi was required for killing melanized *C. neoformans* yeast cells than ^{188}Re conjugated antibody (Bryan et al., 2010), demonstrating the superior penetrating power of the alpha-particle versus the beta-particle emitter. Combining RIT with amphotericin B was more effective than amphotericin B alone (Bryan et al., 2010). However, combination therapy was less potent than RIT alone if melanized fungal cells were used for the infectious challenge. It is not clear whether this result is due to the potential toxicities of the amphotericin or the inflammatory responses that are induced upon infection with melanized yeast cells (Mednick et al., 2005).

Although drug resistance is a concern in the treatment of fungal infections, there is no literature on pathogen resistance to RIT. Nevertheless, experiments have been performed to assess whether resistance can develop (Bryan et al., 2009). *C. neoformans* yeast cells were harvested from infected mice that received RIT and subjected to treatment with ^{213}Bi or ^{188}Re conjugated mAbs. Notably, the RIT and radiation naive *C. neoformans* yeast cells were similarly radiosensitive to the radiolabeled mAbs. Additionally, no differences in survival occurred comparing mice infected with RIT *C. neoformans* to radiation naive yeast cells.

MECHANISM

Although the precise mechanism of action is not known, there are major radiobiological probabilities for the effectiveness of RIT. The first and most targeted effect occurs by a "direct hit," when the radiolabeled antibody binds to a cell and the emitted particles kill the cell (**Figure 1A**). However, "cross-fire" effects, killing of a cell by particles emitted from a radiolabeled antibody bound to a distant cell, can be equally efficient (**Figure 1B**). Both of these mechanisms lead to cell apoptosis and cell cycle redistribution (Macklis, 2004). It is also highly probable, that a radiolabeled antibody could kill by both of these processes. Notably, the "cross-fire" mechanism would be expected to play a large role in combating fungal biofilm diseases or situations where some fungi are extracellular, whilst others are intracellular.

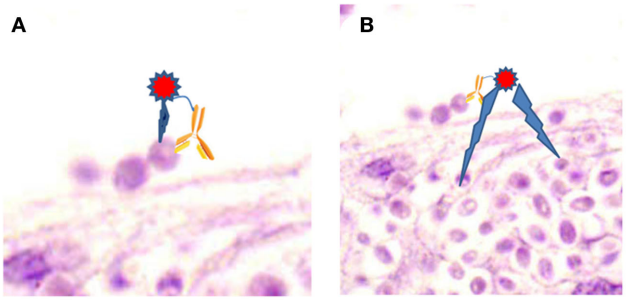

FIGURE 1 | Proposed mechanism of RIT. (A) "Direct hit," where emitted particles kills the cell bound by radiolabeled antibody. **(B)** "Cross-fire," where emitted particles kill cells distant from the cell bound by radiolabeled antibody. Image is epithelial tissue infected with *Candida parapsilosis*.

Certain mechanisms of radiation effects on fungi have been studied using *C. neoformans*. *C. neoformans* yeast cells can be directly killed by gamma, beta, and alpha radiation in a dose and time dependent manner (Bryan et al., 2008). Notably, although membrane permeability did occur, it does not appear to be the primary cause of death. Gamma radiation induced significantly more membrane disruption than either beta or alpha radiation. All forms of radiation induced cellular apoptosis, although external gamma radiation and ^{188}Re-labeled antibody induced more apoptosis than ^{213}Bi-labeled antibody. However, ^{213}Bi-labeled antibody significantly reduced the metabolic activity of the yeast cells, whereas the other forms of radiation did not. Hence, the cellular effects of radiation on yeast are dependent on dose and time as well as the form of radiation administered. In a more recent study, gamma radiation, as well as UV and visible light, were shown to affect ATP levels in *C. neoformans* yeast cells, and this effect was more pronounced in melanized compared to non-melanized cells (Bryan et al., 2011a). Another study assessed whether "direct hit" or "cross-fire" predominated in the killing of *C. neoformans* yeast cells (Dadachova et al., 2006b). As expected, given the power of alpha radiation, ^{213}Bi-labeled antibody resulted in significant "direct hit" killing. However, ^{213}Bi-labeled antibody bound to heat-killed yeast cells also led to "cross-fire" fungicidal effects. In contrast, the killing with the lower energy beta-particles produced by the ^{188}Re-labeled antibody was primarily due to "cross-fire."

RIT TOXICITIES

As briefly described above, the primary toxicity identified in our RIT experiments is a modest thrombocytopenia that resolves within 14 days post therapy, which is similar to that seen in RIT in cancer patients (Macklis and Pohlman, 2006). Importantly, thrombocytopenia prediction models are developed for RIT and dose-fractionated RIT strategies can be implemented to minimize the nadir and duration of the thrombocytopenia (Shen et al., 2002). Importantly, since microbial cells in otherwise sterile sites are foreign to hosts, the microbes display antigens that are distinct from those expressed by host cells. These distinct microbial antigens are the primary targets for infectious diseases RIT. In contrast, RIT of cancers targets tumor-associated antigens that are also expressed on normal cells, which clearly increases toxicity

risks. Therefore, in addition to leading to a theoretically higher therapeutic index in infectious diseases RIT relative to that for cancer, RIT of infectious diseases should have a lower toxicity risk than RIT for cancer.

There is an important issue of "free antigen" as pathogens release/secrete many substances, including proteins, carbohydrates, and lipids. Hence, antibody directed to released or potentially secreted antigen has the potential to produce off-target effects. Previously, we have studied this issue in murine cryptococcosis, since *C. neoformans* releases large amounts of capsular polysaccharide and we were concerned that the radiolabeled antibody to the capsule would interact with non-organism associated polysaccharide. However, we determined that radiolabeled antibody had higher affinity for mice-associated polysaccharide than for its soluble form (Dadachova et al., 2003). Nevertheless, unlabeled antibody can be administered prior to RIT in order to potentially clear circulating antigens.

BIOFILMS

Biofilms are complex communities of pathogens that adhere to one another and are typically surrounded by a matrix of extracellular materials, particularly exopolysaccharides. Fungal biofilms can be especially tenacious and can exacerbate disease by leading to increased drug resistance or enhanced adherence to foreign bodies or tissues. RIT using mAb to *C. neoformans* GXM conjugated to ^{213}Bi can effectively damage cryptococcal biofilms (50% reduction in metabolic activity; Martinez et al., 2006). Notably, beta and gamma forms of radiation did not have a significant effect on the fungal biofilms. It was not determined whether RIT of biofilms synergized with antifungal drugs, as the alterations in biofilm could enhance drug penetration facilitating the activities of the drugs on fungal cells. Hence, RIT may be a means for combating fungal biofilms with or without concomitant antifungal drugs.

CONCEPT OF PAN-FUNGAL RIT

To dramatically drive the treatment of infectious diseases forward with RIT, we need to make a "quantum leap" by identifying antigens shared by major IFI-causing pathogens (PAN-FUNGAL antigens) in order to deliver RIT without the need for specific mycological diagnosis or fear of drug resistance. To this end, we have been exploring the possibility of targeting common cell wall associated antigens, which also happen to constitute major virulence factors for these fungi. We have begun exploring the utility of four cell surface antigens. The majority of fungal cells, yeast, and hyphal, display beta-glucans on their cell surface and Cassone and colleagues have generated a mAb to beta-glucan that provides significant protection against *Candida albicans, C. neoformans*, and *Aspergillus fumigatus* in animal models if given prior to infection (Torosantucci et al., 2005, 2009; Rachini et al., 2007). Heat shock protein 60 (Hsp60) is a key regulator of virulence in *Histoplasma capsulatum* and mAbs directed to this protein are protective in murine histoplasmosis (Guimaraes et al., 2009). Recently, we demonstrated that mAbs to *H. capsulatum* Hsp60 also bound other pathogenic fungal species (Bryan et al., 2011b), but does not react with human Hsp60 (Guimaraes et al., 2009). Melanin is present in the cell wall of diverse human fungal pathogens and a monoclonal to fungal melanin has been shown to bind *C. neoformans, H. capsulatum, Aspergillus* spp., *C. albicans, Scytalidium dimidiatum, Sporothrix schenckii, Paracoccidioides brasiliensis, Coccidioides posadasii,* and *Blastomyces dermatitidis* (reviewed in Nosanchuk and Casadevall, 2006; Taborda et al., 2008). Glucosylceramide is another common exposed cell surface antigen on fungi and a mAb to glucosylceramide has been shown to bind glucosylceramide in *C. neoformans* and *C. albicans,* but not mammalian glucosylceramide (Rhome et al., 2011).

Selected mAbs to these four surface antigens were used for radiolabeling and tested for their capacity to kill *C. neoformans* and *C. albicans* (Bryan et al., 2011b). ^{213}Bi-labeled >90% of mAbs 2G8, IgG2b to beta-galactan, and 4E12, IgG2a to Hsp60, but the labeling efficiency of mAb 6D2, IgM to melanin, was only 30%. ^{213}Bi did not effectively label mAb B11, IgM to glucosylceramide, but the mAb was labeled by ^{188}Re. Radiolabeled mAb to beta-glucan killed 100% of *C. neoformans* yeast cells, whereas *C. albicans* pseudohyphal cells were significantly more resistant as the treatment produced a 30% reduction in CFUs. Targeting Hsp60 produced > 90% reductions in CFUs of both *C. neoformans* and *C. albicans*. The poor ^{213}Bi radiolabeling of the mAb to melanin resulted in the delivery of three to four times less radiation than the ^{213}Bi-labeled mAbs to beta-glucan and Hsp60, and ~4 μCi/5 million yeast cells was the maximal radiation delivered resulting in only 15 and 22% reduction for *C. albicans* and *C. neoformans*, respectively, but the decrease was only significant for *C. neoformans*. The ^{188}Re-labeled mAb to glucosylceramide decreased the CFU of *C. neoformans* by 40%. Hence, we have demonstrated that we can radiolabel diverse antibodies to distinct conserved surface antigens and deliver cytocidal radiation to important fungal pathogens by RIT. It is noteworthy that the efficacy of killing was achieved with significantly lower (~1,000 times) concentrations than were required for biological efficacy with unlabeled antibody, such as with mAb 2G8 (Torosantucci et al., 2009). Moreover, the data demonstrated that the powerful alpha particles from ^{213}Bi efficiently killed fungal cells even when there were relatively few binding sites on their cell surface (~5% mAb binding). Additional experimentation is required to develop more efficient labeling of IgM isotype mAbs and perhaps improve the labeling of IgG isotype mAbs. Nevertheless, the PAN-FUNGAL concept is validated by these proof-of-principle experiments and further study is clearly warranted.

DISCUSSION

The drive to develop RIT of infectious diseases is fueled by the facts that (1) our current antifungal armamentarium often fails to eradicate IFI, (2) the last new category of antifungals was FDA approved in 2001, (3) no new antifungal drugs are close to clinical availability, and (4) RIT is a means to specifically target microbes in a cytocidal manner, irrespective of the host immune status, current drug regimen, or potential resistance mechanisms of the microbe. With RIT of fungi, especially with PAN-FUNGAL RIT, we can potentially reduce treatment durations, prevent the toxicities of the prolonged administration of (possibly ineffective) antifungals, minimize concerns of drug–drug interactions, permit the preservation of current immunomodulating medications (i.e., for transplant patients), and eliminate the need for a specific mycological

diagnosis. Given the well developed applications of RIT in Oncology, the barriers to initiating RIT for infectious diseases, especially IFIs that continue to have unacceptably high mortality rates, are indeed surmountable. The work to date demonstrates that we have in hand a diverse set of specific mAbs and mAbs with reactivity to several leading fungal pathogens that can be harnessed for RIT. We are hopeful that the upcoming years will see a broader acceptance of this approach and a rapid growth in pre-clinical work that will allow the translation of RIT from the basic science laboratory to our patients.

ACKNOWLEDGMENTS

E. Dadachova is a Sylvia and Robert S. Olnick Faculty Scholar in Cancer Research and is supported by the NIH grant R56 AI060507-06. Joshua D. Nosanchuk is in part supported by NIH grant AI52733.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 October 2011; accepted: 28 December 2011; published online: 12 January 2012.

Citation: Nosanchuk JD and Dadachova E (2012) Radioimmunotherapy of fungal diseases: the therapeutic potential of cytocidal radiation delivered by antibody targeting fungal cell surface antigens. Front. Microbio. ***2****:283. doi: 10.3389/fmicb.2011.00283*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 28 November 2012
doi: 10.3389/fmicb.2012.00409

Therapeutic monoclonal antibody for sporotrichosis

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Sporotrichosis is a chronic subcutaneous mycosis that affects both humans and animals worldwide. This subcutaneous mycosis had been attributed to a single etiological agent, *Sporothrix schenckii*. *S. schenckii* exhibits considerable genetic variability, and recently, it was suggested that this taxon consists of a complex of species. Sporotrichosis is caused by traumatic inoculation of the fungus, which is a ubiquitous environmental saprophyte that can be isolated from soil and plant debris. The infection is limited to cutaneous forms, but recently, more severe clinical forms of this mycosis have been described, especially among immunocompromised individuals. The immunological mechanisms involved in the prevention and control of sporotrichosis are not well understood. Some studies suggest that cell-mediated immunity plays an important role in protecting the host against *S. schenckii*. In contrast, the role of the humoral immune response in protection against this fungus has not been studied in detail. In a previous study, we showed that antigens secreted by *S. schenckii* induced a specific humoral response in infected animals, primarily against a 70-kDa molecule, indicating a possible role of specific antibodies against this molecule in infection control. In another study by our group, we produced a mAb against a 70-kDa glycoprotein of *S. schenckii* to better understand the effect of the passive immunization of mice infected with *S. schenckii*. The results showed a significant reduction in the number of CFUs in various mice organs when the mAb was injected before or during *S. schenckii* infection. Similar results were observed when T-cell-deficient mice were used. The drugs of choice in the treatment of sporotrichosis require long periods, and relapses are frequently observed, primarily in immunocompromised patients. The strong protection induced by the mAb against a 70-kDa glycoprotein makes it a strong candidate as a therapeutic vaccine against sporotrichosis.

Keywords: fungal infection, immunology, medical mycology, monoclonal antibody, sporothrix, sporotrichosis, vaccine, yeast

INTRODUCTION

Sporotrichosis is a chronic fungal infection that is endemic to Brazil, and it is the most common subcutaneous mycosis in South America (Schubach et al., 2008). The disease is mainly caused by the dimorphic fungus *Sporothrix schenckii*. In its saprophytic stage or when cultured at 25°C, it assumes a filamentous form, and at 37°C, it assumes a yeast form (Bustamante and Campos, 2001; Barros et al., 2011). *Sporothrix* is widely distributed in nature and exists in a saprophytic mycelial form in plant debris and soil. The traumatic inoculation of the conidia and hyphae of this fungus results in the development of subcutaneous mycoses; within the infected tissue, the fungus differentiates into its yeast form and may spread to other tissues (Ramos-e-Silva et al., 2007; Barros et al., 2011). Since the 1980s, domestic cats have been a source of mycosis transmission to humans (Nusbaum et al., 1983; Dunstan et al., 1986a,b; Larsson et al., 1989; Fleury et al., 2001). The largest epidemic of sporotrichosis due to zoonotic transmission was described in Rio de Janeiro between 1998 and 2004, in which 759 humans were diagnosed with sporotrichosis (Barros et al., 2004; Freitas et al., 2010). Recently, Marimon et al. (2007) suggested that *S. schenckii* should not be considered the only species that causes sporotrichosis on the basis of a combination of phenotypic and genetic features. The group described four new species: *S. globosa*, *S. brasiliensis*, *S. mexicana*, and *S. luriei* (Marimon et al., 2008). These new species have been defined as having a worldwide distribution, whereas *S. brasiliensis* is apparently restricted to Brazil, and *S. mexicana* is restricted to Mexico. Due to difficulties in classifying strains belonging to the *Sporothrix* complex, the same group (Marimon et al., 2008) proposed an identification key that includes the analysis of conidial morphology, auxanogram analysis using raffinose and sucrose and genotyping via polymerase chain reaction (PCR) amplification of the calmodulin gene.

Sporotrichosis has diverse clinical manifestations. The most frequent clinical form (approximately 80% of cases) is the lymphocutaneous form (Bonifaz and Vazquez-Gonzalez, 2010). It starts with a nodular or ulcerated lesion at the site of fungal inoculation and follows a regional lymphatic trajectory characterized by nodular lesions that ulcerate, fistulate, and heal, representing true gummae. Another common clinical manifestation is the fixed cutaneous form. In general, the fixed cutaneous form is characterized by infiltrated nodular, ulcerated, or erythematosquamous lesions located on exposed areas on which the fungal inoculation occurred (Schechtman, 2010). The

systemic form of sporotrichosis may evolve from an initial cutaneous lesion or be associated with the inhalation of conidia (Gutierrez-Galhardo et al., 2010). More severe clinical forms of this disease have been associated with immunocompromised patients, such as human immunodeficiency virus (HIV)-infected patients, suggesting that *S. schenckii* is an emerging opportunistic pathogen (Galhardo et al., 2010). In contrast, disseminated cutaneous sporotrichosis has been reported in an immunocompetent individual (Yap, 2011), which demonstrates that although it is common in immunosuppressed patients, disseminated cutaneous sporotrichosis can also present in immunocompetent patients.

Different drug protocols are used for the treatment of sporotrichosis, including potassium iodide, itraconazole, terbinafine, fluconazole, and amphotericin B (Song et al., 2011). The treatment choice is based on the individual's clinical condition, the extent of the cutaneous lesions, the assessment of drug interactions and adverse events, and systemic involvement. Some adverse events, such as nausea, vomiting and diarrhea, headache, abdominal pain, hypersensitivity reactions, and liver dysfunction, may be observed (Lopez-Romero et al., 2011).

Both the pathogenic characteristics of the individual *S. schenckii* strains and the immunologic status of the host can determine the clinical manifestations of sporotrichosis. However, the factors involved in the pathogenesis of *S. schenckii* and mechanisms to determine the strain's virulence remain unclear.

IMMUNE RESPONSE

The host defense against pathogenic microorganisms includes innate and acquired immunity. The innate immune system is the first line of defense against invading pathogens; it is activated within minutes after the invasion of the host and is responsible for defense during the initial hours and days of the infection. In contrast to the rapid activation of the innate immune system, the activation of specific acquired immunity, which is primarily mediated by T and B lymphocytes, requires at least 7–10 days before a proper cellular or humoral response occurs. The ability to discriminate "non-self" from "self" is an essential component of innate immunity and is achieved through germ line-encoded receptors that recognize highly conserved microbial structures: pathogen-associated molecular patterns (PAMPs).

Several classes of recognition receptors mediate the recognition of fungal pathogens. Because of the structure of the fungal cell wall, which is mainly composed of carbohydrate chains, such as mannans and glucans, the first group of fungal recognition receptors discovered was the lectin receptors (Brown, 2008; Netea et al., 2008). PAMPs are recognized by pattern recognition receptors (PRRs), which are expressed on various antigen-presenting cells (APCs), such as dendritic cells (DC) and macrophages. PRRs play an important role in recognizing microbial pathogens, activating the innate immune system, and releasing pro-inflammatory cytokines.

Recent studies have demonstrated that lipid extracts from the yeast form of *S. schenckii* bind to TLR4, and this interaction leads to the induction of an oxidative burst against the fungus (Sassa et al., 2012).

Uenotsuchi et al. (2006) showed that *S. schenckii* of cutaneous origin but not visceral origin binds to TLR2 and/or TLR4 and induces JNK, ERK, and p38 MAPK activation and IL-6 and TNF-α release. Although both TLR2 and TLR4 ligation can induce these pro-inflammatory cytokines, TLR2 signals are also known to mediate an anti-inflammatory effect by directing the release of IL-10. Thus, the authors speculate that TLR4 but not TLR2 might be the main receptor for recognizing *S. schenckii* of cutaneous origin and inducing a strong Th1 immune response.

T cell-mediated immunity has been described as being fundamental in the defense against *S. schenckii*. In experimental infections, nude mice are more susceptible to sporotrichosis, and acquired immunity against *S. schenckii* is primarily mediated by macrophages that have been activated by T cells (Hachisuka and Sasai, 1981). Both $CD4^+$ T cells and macrophages are required in the granulomatous skin lesions of sporotrichosis; the presence of IFN-γ-producing $CD4^+$ T cells is an essential component of host defense against this pathogen (Tachibana et al., 1999). These findings indicate that the Th1 response mediates granuloma formation in sporotrichosis.

Cell-mediated and innate immunity are considered the most important mechanisms of host defense against fungal infections. In contrast, the role of the humoral immune response in protection against this fungus has not been studied in detail. Recent studies have demonstrated that antibodies with defined specificity show different degrees of protection against mycosis (Casadevall and Pirofski, 2005). The administration of mAbs-mediated protection against *Paracoccidioides brasiliensis* (Buissa-Filho et al., 2008), *Candida albicans* (Polonelli et al., 2003), *Histoplasma capsulatum* (Nosanchuk et al., 2003) and *Cryptococcus neoformans* (Rivera et al., 2005) infection in mice.

THERAPEUTIC MONOCLONAL ANTIBODIES AND FUNGAL INFECTION

Monoclonal antibodies are attractive biologic drugs because of their specificity and well-understood mechanisms of action, which result in a higher predictability and lower attrition rate compared with other drugs. The mechanisms of antibody action against infectious disease include complement-mediated lysis, the enhancement or inhibition of phagocytosis, Fc-mediated cytokine release, and direct antimicrobial effects (Casadevall and Pirofski, 2005). In terms of fungal infection, it is clear that antibody-mediated immunity can be decisive for host defense against *C. neoformans* (Casadevall and Pirofski, 2012). However, several factors could affect the function of these antibodies, such as antibody isotype and quantity.

For many years, the protective role of antibodies in fungal infection was contested. A consensus has now emerged that the inability of immune sera to mediate protection against fungi reflects inadequate amounts of protective antibody and/or the simultaneous presence of protective and non-protective antibodies rather than a fundamental inability of antibodies to protect against fungal pathogens. Recently, several studies have established that some antibodies are protective against fungi (Xander et al., 2007; Buissa-Filho et al., 2008; Toledo et al., 2010; Zhang et al., 2011). Moreover, some MAbs designed to protect against cryptococcosis (Larsen et al., 2005) and candidiasis

(Pachl et al., 2006) have been approved for clinical evaluation. An HIV+ patient with cryptococcal meningitis was treated with the murine MAb 18B7, which is directed against the capsular polysaccharide of *C. neoformans*, with excellent results. The MAb infusion had a half-life in the serum of approximately 53 h and reduced the fungal circulating antigen (Larsen et al., 2005). The phase I trial of Mab 18B7 has been completed. Additional phase II and III studies will be required to demonstrate whether adjunctive therapy with anti-*Cryptococcus* antibody confers additional benefit over conventional antifungal therapy. In another study, a human recombinant MAb to heat shock protein 90 was used in the treatment of patients with invasive candidiasis (Pachl et al., 2006). Recently, Krenova et al. (2010) reported the successful treatment of life-threatening *Candida peritonitis* in a child with abdominal non-Hodgkin lymphoma using a MAb against heat shock protein 90 in association with amphotericin B.

In a previous study, we demonstrated that mice infected with *S. schenckii* are able to produce specific IgG1 and IgG3 antibodies against a 70-kDa fungal protein during experimental infection, indicating that specific antibodies against this molecule may participate in controlling infection (Nascimento and Almeida, 2005). To better understand the role of the antibody response in sporotrichosis, our group produced an IgG1 mAb, P6E7, against a 70-kDa glycoprotein (gp70) of *S. schenckii*. Immunolocalization using this anti-gp70 antibody showed that the antigen was preferentially localized on the cell surface and that it could be a putative adhesin for fibronectin and laminin. To analyze the protective effect of the mAb *in vivo*, *S. schenckii*-infected mice were passively immunized. Our results showed a significant reduction in the number of CFUs in the spleen and liver of mice when the mAb was injected before and during *S. schenckii* infection (Nascimento et al., 2008). Furthermore, in a second experiment, the mAb was injected after infection was established, and again, we observed a significant reduction in CFUs. IFN-γ was detected at high levels in the organs of mice that received P6E7. These results indicate that treatment with the P6E7 mAb may induce a protective cell-mediated immune response via the production of IFN-γ (Nascimento et al., 2008). In a recent study, our group showed that yeast cells opsonized with mAbs against gp70 had an increased phagocytic index and TNF-α production (Franco et al., 2012).

A possible limitation to the use of vaccines in immunosuppressed patients is that these patients may not mount protective responses, but passive immunization with protective antibodies may well be a rapid and effective preventive or even therapeutic measure. The efficacy of this immunoprophylaxis can be augmented when it is used in combination with conventional antifungal therapy. Because the disseminated cutaneous forms of sporotrichosis have mainly been observed among immunosuppressed patients, especially HIV+ individuals, we analyzed the protective effect of the anti-gp70 mAb in deficient nude animals. We demonstrated that in this model, the mAb was efficient at controlling the dissemination of fungal infection (Nascimento et al., 2008).

The results of these studies may facilitate the development of an efficient therapy for sporotrichosis. Currently, our laboratory is in the process of developing a humanized P6E7 mAb. The humanized antibodies could be an alternative therapy for the treatment of patients with sporotrichosis.

CONCLUSION

Therapeutic monoclonal antibodies, which are primarily used as treatment for cancer and less frequently for infection, are among the most active area of research and development in the pharmaceutical industry. mAbs can be used as conjugates with target drugs and can be fragmented or humanized. A better understanding of the mechanism of action of successful mAbs will be critical for the development of more active and less toxic mAbs.

The development of a therapeutic vaccine that has the ability to induce a strong protective response to *S. schenckii* could therefore be more advantageous than existing treatments. The development of a new mAb-based therapy is an exciting challenge that may lead to novel approaches in the treatment and immunoprophylaxis of sporotrichosis.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 May 2012; paper pending published: 21 June 2012; accepted: 13 November 2012; published online: 28 November 2012.

*Citation: Almeida SR (2012) Therapeutic monoclonal antibody for sporotrichosis. Front. Microbio. **3**:409. doi: 10.3389/fmicb.2012.00409*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 01 June 2012
doi: 10.3389/fmicb.2012.00190

Antibody peptide based antifungal immunotherapy

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Fungal infections still represent relevant human illnesses worldwide and some are accompanied by unacceptably high mortality rates. The limited current availability of effective and safe antifungal agents makes the development of new drugs and approaches of antifungal vaccination/immunotherapy every day more needed. Among them, small antibody(Ab)-derived peptides are arousing great expectations as new potential antifungal agents. In this topic, the search path from the study of the yeast killer phenomenon to the production of Ab-derived peptides characterized by *in vitro* and *in vivo* fungicidal activity will be focused. In particular, Abs that mimic the antimicrobial activity of a killer toxin ("antibiobodies") and antifungal peptides derived from antibiobodies (killer peptide) and other unrelated Abs [complementarity determining regions (CDR)-based and constant region (Fc)-based synthetic peptides] are described. Mycological implications in terms of reevaluation of the yeast killer phenomenon, roles of antibiobodies in antifungal immunity, of β-glucans as antifungal targets and vaccines, and of Abs as sources of an unlimited number of sequences potentially active as new immunotherapeutic tools against fungal agents and related mycoses, are discussed.

Keywords: antifungal antibodies, killer toxin, antibiobodies, synthetic CDRs, killer peptides, antifungal peptides

INTRODUCTION

Fungal infections still represent relevant human illnesses worldwide and often are sentinel markers of immunological primary disorders or induced suppression (Vinh, 2011). Some mucocutaneous infections can be persistent or refractory and involve much of the population, such as vulvovaginal candidiasis that may affect up to 75% of women at least once in their childbearing age (Sobel, 1997). More problematic are invasive fungal infections (IFIs) that are dramatically increasing and are often severe, difficult to treat, and accompanied by unacceptably high mortality rates. Aspergillosis, cryptococcosis, and invasive candidiasis are among the most widespread, less treatable, and life-threatening IFIs. They are emerging with increasing frequency, typically in the setting of immunocompromised patients, even those treated with new antifungal drugs (Wisplinghoff et al., 2004; Kauffman, 2006; Binder and Lass-Flörl, 2011). This poses a serious threat to public health, taking into account that the currently available antifungal agents are limited in number, and often their prolonged administration can have significant toxicity. Even newer antifungal agents have important limitations related to their spectrum of activity, pharmacokinetics, and drug–drug interactions. The increasing resistance to old and new antifungals makes the situation even more complicated and far from satisfactory. On the one hand, therefore, the development of new antifungal drugs is becoming every day more demanding (Zhai and Lin, 2011), the other, much attention has been paid in recent years to new approaches of antifungal vaccination and/or immunotherapy. In this Research Topic, the search path from the study of the yeast killer phenomenon to the production of antibodies (Abs) that mimic the antimicrobial activity of a killer toxin (KT) to Ab-derived peptides characterized by fungicidal activity will be focused.

FROM KILLER TOXIN TO "ANTIBIOBODIES"

The demonstration that the killer effect, which was previously considered to be restricted to conspecific yeasts, was extensible to taxonomically unrelated fungi opened unannounced perspectives in antifungal therapy (Polonelli and Morace, 1986). KTs are exotoxins, generally proteins or glycoproteins, that exert their antifungal activity with different mechanisms of action by means of a preliminary, basic interaction with specific cell-wall receptors (KTRs; Magliani et al., 1997b). The therapeutic effect of a wide-spectrum KT, produced by *Wickerhamomyces anomalus* (formerly *Pichia anomala* and *Hansenula anomala*; *Pa*KT), in the topical treatment of experimentally induced pityriasis versicolor-like lesions suggested the possible use of KTs as potential new antifungals (Polonelli et al., 1986). This was later ruled out because of the characteristics of *Pa*KT in terms of toxicity, as well as antigenicity and instability in the physiological milieu (Pettoello-Mantovani et al., 1995). The production and characterization of a monoclonal Ab (mAb KT4), capable of neutralizing the fungicidal activity of *Pa*KT (Polonelli and Morace, 1987), allowed its use as an immunogen in rabbit for the production of anti-idiotypic (anti-Id) Abs that competed with *Pa*KT for the binding site of mAb KT4 and, most importantly, were able to kill *in vitro* cells of *Candida albicans*, adopted as fungus model, thereby mimicking the effect of *Pa*KT (Polonelli and Morace, 1988). *Pa*KT-like anti-Id Abs allowed to visualize, by immunofluorescence, *Pa*KTRs on *C. albicans* cell wall, but not on mammalian cells (Polonelli et al., 1990). The preferential location of *Pa*KTRs in budding scars and germ tubes, where inner cell-wall components are synthesized and exposed on the surface before being buried beneath the dense mannoprotein outermost coat, confirmed other observations on the greater susceptibility to *Pa*KT of cells in their active phase of growth and then

suggested a role of inner components, such as β-glucans (BGs), as *Pa*KTR constituents (Guyard et al., 2002). Affinity chromatography purified anti-Id Abs were likewise able to visualize *Pa*KTRs also in *Pa*KT-producing cells and to kill them, which are normally resistant to the activity of their own KT. The killer activity of anti-Id Abs could be neutralized by pre-incubation with mAb KT4, thus supporting the specificity of their action. *Pa*KT-like Abs able to exert a direct fungicidal activity, without the intervention of other factors or cells of the immune system, were defined "antibiobodies" (antibiotic-like Abs; Polonelli et al., 1991).

Antibiobodies showed to compete with *Pa*KT for both the combinatorial site of the neutralizing mAb KT4 and *Pa*KTRs of susceptible microorganisms suggesting, therefore, their three-dimensional structural and functional homology. As antibiobodies could be considered to mimic in some way the fungicidal activity of *Pa*KT, the combinatorial site of mAb KT4 could be considered as a mimic of *Pa*KTR. Based on these considerations, studies on "idiotypic vaccination," using mAb KT4 as parenteral or mucosal immunogen to stimulate the production of antibiobodies in different formats and experimental conditions, were carried out. Polyclonal antibiobodies elicited in mice or rats immunized with mAb KT4 induced protection against experimental systemic or vaginal infections, respectively, caused by *Pa*KT-susceptible *C. albicans* cells. The protection was associated with rising titers of circulating or mucosal antibiobodies. MAb KT4 affinity chromatography purified antibiobodies were capable of killing *C. albicans* cells *in vitro* and were able to passively transfer the protective state to non-immunized animals (Polonelli et al., 1993, 1994). *Pa*KT-like antibiobodies were also produced in unlimited quantities of indefinitely available formats. Thus, monoclonal (mAb K10) and recombinant single-chain (scFv H6) antibiobodies were produced by immunization of rats and mice, respectively, with mAb KT4 using established hybridoma and recombinant DNA technologies. Both antibiobodies formats proved to be candidacidal *in vitro* and to compete with *Pa*KT for the specific *Pa*KTR on *C. albicans* cells. The fungicidal activity of mAb K10 and scFv H6 was neutralized by mAb KT4 and, when administered at the time of challenge or postchallenge in an experimental model of vaginal candidiasis, they proved to exert a significant therapeutic activity (Magliani et al., 1997a; Polonelli et al., 1997).

As an obvious corollary, natural antireceptor antibiobodies were detected in the serum or secretions of animals and humans undergoing experimental or natural infections caused by *Pa*KT-susceptible *C. albicans* cells. Rising titers of fungicidal Abs could be detected, after intravaginal or intragastric inoculations of *Pa*KTR-bearing *C. albicans* cells, in vaginal fluids of rats previously vaccinated or never immunized with mAb KT4. Antireceptor antibiobodies were also consistently found in the vaginal fluid of women afflicted with recurrent vaginal candidiasis, as well as in the serum, saliva, and/or bronchial washing of HIV positive patients with oral or lung infections caused by *Pa*KTR-bearing fungi. Similar to what previously observed, affinity chromatography purified human natural antibiobodies were capable of killing *C. albicans* cells *in vitro* and their activity was neutralized by mAb KT4. These antibiobodies were also able to passively transfer the protective state to non-immunized animals (Polonelli et al., 1996).

The natural existence of candidacidal Abs as part of the Ab response against *C. albicans* added significance to the growing evidence on the importance of Ab-mediated acquired immunity for host defense against candidiasis and other relevant fungal infections (Casadevall et al., 1998). The availability, moreover, of reproducible antibiobodies in different formats and unlimited amounts, potentially free of undesired toxic effects, suggested the feasibility of new therapeutic approaches for the immunotherapy of candidiasis (Magliani et al., 2002). Based on the wide antifungal spectrum of *Pa*KT and the potential diffusion of *Pa*KTRs (Magliani et al., 1997b), antibiobodies should display a fungicidal activity against various fungal agents. While *Pa*KT's activity was severely limited by the environmental conditions, being manifested at acidic pH (4.6) and temperatures around 28°C, antibiobodies proved to be active in physiological conditions (pH 7 and 37°C). Conversely, the idiotype of mAb KT4 or purified *Pa*KTR could be suggested as potential antifungal vaccines.

Human natural antibiobodies proved to exert *in vitro* a strong and specific inhibitory activity against rat-derived *P. carinii* organisms, in terms of attachment to cultured cells and infectivity to nude rats. This activity could be abolished by their previous incubation with mAb KT4. Immunofluorescence studies of competition with *Pa*KT showed that antibiobodies efficiently bound to specific *Pa*KTRs on the surface of *P. carinii* cells (Séguy et al., 1997). Pneumocystosis (PCP) extension was significantly reduced by aerosol administration of mAb K10 in a PCP experimental nude rat model (Séguy et al., 1998). In a murine model of allogeneic T-cell-depleted bone marrow transplantation, treatment with mAb K10 protected mice with profound neutropenia from experimental invasive pulmonary aspergillosis in terms of long-term survival and decreased pathology associated with inhibition of fungal growth and chitin content in the lungs. This finding was supported by the *in vitro* effect of mAb K10 against *Aspergillus fumigatus* swollen conidia (inhibition of the hyphal development and metabolic activity; Cenci et al., 2002). A Gram-positive generally recognized as safe bacterium, *Streptococcus gordonii*, was engineered to produce scFv H6 as molecules secreted or displayed on the bacterial surface. Recombinant bacteria were able to stably colonize vaginal mucosa, and proved to be as efficacious as fluconazole in rapidly abating the fungal burden and in curing the infection in a rat model of experimental candidiasis (Beninati et al., 2000).

FROM ANTIBIOBODIES TO KILLER PEPTIDE

Synthetic peptides derived from the sequence of scFv H6 could still display *in vitro* candidacidal activity. In particular, a decapeptide containing the first three amino acids of the light chain (L) complementarity determining region (CDR)1, with an alanine replacement of its first residue (AKVTMTCSAS), proved to exert a strong candidacidal activity *in vitro*, and was therefore designated killer peptide (KP). Significantly, KP competed with mAb K10 for binding to germinating cells of *C. albicans*. Furthermore, KP demonstrated a significant therapeutic activity against infections caused by fluconazole-susceptible or -resistant *C. albicans* strains in a rat model of vaginal candidiasis as well as against systemic candidal infections in immunocompetent or severely immunocompromised mice (Polonelli et al., 2003). Thus, KP proved to act

as functional mimotope of *Pa*KT. KP demonstrated a broad antifungal spectrum without any detectable toxicity (Magliani et al., 2004a). Rapid candidacidal activity of KP was confirmed in time-killing studies and proved to be inhibited, in a dose-dependent fashion, by laminarin, a soluble 1,3-BG (Magliani et al., 2004b). KP was able to kill, *in vitro*, both capsular and acapsular *Cryptococcus neoformans* cells and impaired the production of specific virulence factors, such as the capsule, rendering the fungus more susceptible to natural effector cells. More importantly, KP reduced significantly the fungal burden in immunosuppressed mice with cryptococcosis and protected most of them from an otherwise lethal experimental infection (Cenci et al., 2004). KP demonstrated a significant activity against *Paracoccidioides brasiliensis* and experimental paracoccidioidomycosis being fungicidal *in vitro*, even in its D-isomeric form, and therapeutic *in vivo* by markedly reducing the fungal load in target organs (liver, lung, spleen) of infected animals (Travassos et al., 2004).

Killer peptide exerted a strong dose-dependent candidacidal activity against a large number of candidal strains isolated from saliva of adult diabetic and non-diabetic subjects, regardless of their species and pattern of resistance to conventional antifungal drugs (Manfredi et al., 2005). KP showed killing activity on *C. albicans* cells even adhered to sanded acrylic resin disks, a major condition in which candidal biofilms are formed (Manfredi et al., 2007).

The spectrum of KP activity was subsequently extended to phytopathogenic fungal agents, such as *Botrytis cinerea* and *Fusarium oxysporum*. KP was expressed in an active form in plants (*Nicotiana benthamiana*) by using a Potato virus X-derived vector. KP-expressing plants showed enhanced resistance to an experimental bacterial challenge with *Pseudomonas. syringae* pv. *tabaci* (Donini et al., 2005).

Killer peptide, moreover, was able to bind selectively to murine dendritic cells (DCs) and, to a lesser extent, to macrophages, possibly through major histocompatibility complex (MHC) class II, CD16/32, and cellular molecules recognized by anti-specific intercellular adhesion molecule-grabbing non-integrin R1 Abs. The peptide proved to modulate the multiple functions of DCs, improving their capacity to induce better immune antimicrobial response (Cenci et al., 2006).

The fungicidal activity of KP was apparently based on a new mechanism of action as no resistant mutant was found by testing a wide *Saccharomyces cerevisiae* non-essential gene deletion strain library that included isolates resistant to conventional antifungal drugs such as caspofungin and fluconazole (Conti et al., 2008).

Even though the precise molecular mechanism of action has still to be clarified, KP caused in *C. albicans* the appearance of significant internal alterations, such as cell-wall swelling, plasma membrane collapse, and condensation and fragmentation of nuclear material, similar to those observed by treatment of the yeast cells with classical apoptotic agents (Magliani et al., 2008b). KP proved to be very stable in its lyophilized form and, when solubilized in non-reducing conditions, due to the presence of a cysteine residue, it could easily dimerize by formation of disulfide bridges. KP dimer turned out to be the functional unit as confirmed by the instant and total candidacidal effect showed by the dimeric molecule synthesized *ad hoc*. After dimerization, KP revealed its ability to spontaneously and reversibly self-assemble in an organized network of fibril-like structures that resembled physical hydrogels. This process was catalyzed by the addition of 1,3-BG, as soluble laminarin or *C. albicans* cells exposing BGs on their surface, that caused an immediate conformational conversion of the peptide from random coil to antiparallel β-sheet. This self-assembled state was concentration- and temperature-dependent and could provide protection against proteases and assure a release of the active form over time. KP was proposed as paradigmatic of a new class of autodelivering therapeutic peptides (Pertinhez et al., 2009).

FROM KILLER PEPTIDE TO Ab-DERIVED ANTIFUNGAL PEPTIDES

All the peptides reproducing the six CDRs of scFv H6 showed candidacidal activity *in vitro*, even if to a lesser extent compared to KP (Polonelli et al., 2003). Other Abs have been reported meanwhile as characterized by direct antifungal activity: a human anti-heat shock protein 90 recombinant Ab (Mycograb; Matthews et al., 2003); a mAb (C7), directed to a protein epitope of a *C. albicans* cell-wall stress mannoprotein, that, besides its candidacidal activity, proved to exert inhibition of both adhesion and filamentation as well as blockage of the reductive iron uptake pathway of the yeast (Moragues et al., 2003; Brena et al., 2011); a scFv and a scFv-derived peptide able to mimic the fungicidal activity of the *H. anomala* HM-1 KT (Selvakumar et al., 2006; Kabir et al., 2011). The existence of a family of antifungal Abs, from which new innovative wide-spectrum fungicidal tools could be properly derived, was suggested (Magliani et al., 2005).

As seen in available databases, the sequence of P6, the peptide from which KP was derived, was present within the V regions of many unrelated Abs. On this basis, it was speculated that CDR-related peptides may display antifungal activity regardless of their specificities. Synthetic peptides with sequences identical to the CDRs of mAb C7 were proved for candidacidal activity in comparison to the CDRs of two unrelated Abs, whose variable region sequences were deposited and available. A murine IgM (mAb pc42), directed to a synthetic peptide containing the surface antigen of hepatitis B virus and the T-helper-cell epitope from the circumsporozoite protein of *Plasmodium falciparum*, was selected because it shared CDR H1 and H2 with mAb C7. A human IgM (mAb HuA), specific for difucosyl human blood group A substance, was selected because not sharing any sequence homology with either mAb C7 or mAb pc42 CDRs and because representing an Ab widely diffused in normal population. When tested in *in vitro* and *in vivo* experimental models against *C. albicans*, some CDR peptides showed differential fungicidal and therapeutic activities. Alanine substituted derivatives of candidacidal CDR peptides showed further differential increased, unaltered, or decreased candidacidal activity. Thus, short synthetic CDR-related peptides may display fungicidal activity irrespective of Ab specificity for a given antigen, conceivably involving different mechanisms of action. Alanine substitution can be used to increase variability of CDR peptides' fungicidal activity (Polonelli et al., 2008). A synthetic peptide representative of CDR H3 of a murine mAb (MoA) conspecific with HuA and representing

the different ways by which the same epitope can be recognized by different immune systems though presenting unrelated primary sequences, showed no candidacidal activity *in vitro*. MoA H3, however, was able to induce a significant increased production of proinflammatory cytokines, IL-6, and TNF-α, in murine splenocytes and peritoneal macrophages (PMs), but not in peritoneal neutrophils. Further characterization of MoA H3 allowed to visualize its binding and uptaking by PMs. This activated the Akt pathway in correlation to an increased production of TNF-α, and significantly up-regulated TLR-4 gene and protein expression. The state of PM activation could explain the therapeutic effect observed by treatment with MoA H3 in the mouse experimental model of systemic candidiasis in terms of survival and impressive decrease of candidal recovery from kidneys (Gabrielli et al., 2009).

MYCOLOGICAL IMPLICATIONS

These studies contributed to the advancement of knowledge on various aspects of treatment and control of fungal diseases. In particular, they suggested unusual considerations and perspectives on potential therapeutic and prophylactic approaches based on the yeast killer phenomenon, idiotypic vaccination, antibiobodies, and Ab-derived peptides.

REEVALUATION OF THE YEAST KILLER PHENOMENON

Given the impossibility of directly using KTs as antifungal therapeutic agents, their fungicidal properties have been harnessed by generating Ab derivatives. The production of antibiobodies and Ab-derived antimicrobial peptides (Magliani et al., 1997b, 2008b; Selvakumar et al., 2006; Kabir et al., 2011) suggests that very similar approaches can be applied with other KTs. Different antifungal molecules could be obtained, thereby taking advantage of the mimic of a widely spread natural phenomenon. Unraveling their mechanisms of action could result in the discovery of new potential targets for antifungal agents and/or immunoprevention, such as 1,3-BG, the suggested target of antibiobodies and Ab-derived antimicrobial peptides.

ANTIBIOBODIES AND HUMORAL ANTIFUNGAL IMMUNITY

The relative importance of cell-mediated (CMI) and humoral immunity against fungal infections has been longly debated. While CMI continues to be rightly considered the primary mechanism for antifungal defense, Ab response has been increasingly taken into consideration (Polonelli et al., 2000). In particular, antibiobodies were shown to occur in the Ab repertoire mounted during fungal infections caused by *Pa*KT-sensitive fungi. Their clinical relevance, however, still needs to be determined. They may represent only a minor part of the plethora of Abs produced during experimental or natural infections by *Pa*KTR-bearing fungal organisms and they could be very scarcely produced *in vivo* being unable to reach protective titers. The occurrence of interfering Abs of different specificities and isotype, moreover, could explain the negative results often achieved in active and passive immunoprotection experiments based on humoral immunity. The interplay between protective and interfering Abs could dictate the outcome of fungal infections and may also help to explain why subjects with elevated anti-*Candida* Ab titers could remain nonetheless susceptible to candidiasis (Bromuro et al., 2002). The observations made in the past decade, showing that Abs can function as direct effector molecules against fungi, suggest the need for new conceptual approaches in the understanding of humoral immunity to fungal infections (Casadevall and Pirofski, 2011).

β-GLUCANS AS CRITICAL VIABILITY MOLECULES, ANTIFUNGAL TARGETS, AND VACCINES

BGs, 1,3-BG in particular, have emerged as viability-critical inner components of many fungal cell walls and were reasonably suggested as *Pa*KTRs constituents. While 1,3-BGs are biosynthesized by a wide range of fungal species, they are not produced by mammalian cells (Magliani et al., 2008a). In the fungal cell wall, 1,3-BGs are usually masked beneath the dense mannoprotein outermost layer and this may protect them by recognition of Abs. When exposed on the surface, mainly during the active phase of growth, such as in budding cells and germ tubes in *C. albicans* (Iorio et al., 2008), BGs can represent a relevant fungal virulence factor being recognized as major pathogen associated molecular pattern able to act as potent proinflammatory molecules. Their critical structural role was underscored by the discovery of a new class of antifungals, the echinocandins, that are fungicidal by inhibiting the 1,3-BG synthesis (Denning, 2003) and by reports on antibiobodies and Ab-derived peptides. An innovative antifungal vaccine composed of laminarin, a soluble poorly immunogenic linear polymer of 1,3-BG purified from the brown alga *Laminaria digitata*, conjugated with diphtheria toxoid CRM197, was developed. In animal models, the elicited Abs proved to protect against ascomycetous and basidiomycetous fungal agents, such as *A. fumigatus*, *C. albicans*, and *C. neoformans* (Torosantucci et al., 2005; Rachini et al., 2007; Bromuro et al., 2010). As outlined by Casadevall and Pirofski, these observations introduced a fungal heresy into the immunological dogma that effective immune responses should be pathogen specific and that Abs to "common," "universal," or "cross-reactive" antigens may not be protective. In the case of 1,3-BG, over all derived from a non-fungal source, a single vaccine induced protection against three major fungal pathogens. Furthermore, this provides a vulnerable Achilles heel for Ab-mediated antifungal protection, suggesting the possibility to develop Abs for passive therapy of the diseases caused by each of these fungal pathogens (Casadevall and Pirofski, 2007). Recently, radiolabeled Abs to BG and other common fungal antigens as well as plant-derived recombinant Abs to BG have been described and proposed as universal tools for fungal disease (Capodicasa et al., 2011; Bryan et al., 2012). 1,3-BG-conjugated vaccines can be seen as "universal" vaccines that could be administered to patients who share risk factors (e.g., neutropenia) to immunize them, before they become debilitated and immunocompromised, against all of the main opportunistic fungal agents (Cassone and Rappuoli, 2010). Further studies will hopefully clarify all the different aspects in this field, including the role that anti-BG Abs, that are ubiquitous at low levels in human sera, may play in determining susceptibility or resistance to fungal infections (Chiani et al., 2009).

Ab-DERIVED PEPTIDES AS NEW IMMUNOTHERAPEUTIC TOOLS

The concept that short synthetic peptides corresponding to segments of variable region of immunoglobulins (Igs), CDRs

particularly, may display antifungal activities regardless of the specificity of the belonging Ab was claimed. This opened new perspectives in the field of antifungal therapy and encouraged to continue research on Abs as source of fungicidal peptides. Peptides encompassing sequences of the constant region of mammalian Abs (Fc-peptides) belonging to different isotypes (IgG, IgM, IgA), putatively released *in vivo* by proteolysis of Igs, were synthesized. Selected Fc-peptides proved to exert a fungicidal activity *in vitro* against pathogenic yeasts, such as *C. albicans*, *C. glabrata*, *C. neoformans*, and *Malassezia furfur*, including caspofungin and triazole resistant strains, without any hemolytic, cytotoxic, and genotoxic effect. An Fc-peptide (N10K), included in all human IgGs and selected as a proof-of-concept, displayed a therapeutic activity when administered in consolidated mouse models of systemic and vaginal candidiasis. N10K proved to spontaneously aggregate in a rich β-sheet structure and this possibly contributed to its *in vivo* therapeutic activity. The decapeptide bound to the surface of *Candida* cells, without causing major lysis. However, gross alterations in the morphology of yeast cells, with disruption of internal organelles, were seen (Polonelli et al., 2012). N10K, moreover, was able to induce in human monocytes, *in vitro*, IL-6 secretion, pIkB-α activation and up-regulation of Dectin-1 expression, leading to an increased activation of BG-induced pSyk, CARD9, and pIkB-α, and an increase in the production of proinflammatory cytokines, such as IL-6, IL-12, IL-1β, and TNF-α (manuscript submitted for publication). These findings may be of great interest from an immunological point of view. While significant amounts of specific fragments from the Ab variable regions, such as CDRs, are unlikely to be released *in vivo*, Fc-peptides could potentially occur *in vivo* and influence the antifungal immune response in a way reminiscent of molecules of innate immunity. Ongoing studies using mass spectrometry-based approaches are aimed to search for the presence of Fc-peptides in human sera from individuals in various clinical conditions. Positive results would shed new light on the role that Ab fragments could exert in the antifungal homeostasis. Furthermore, the reported high frequency of Ab-derived fungicidal peptides suggests that Abs, irrespective of their isotype and specificity for a given antigen, may be the source of potentially active and therapeutically exploitable molecules for devising new immunotherapeutic tools against pathogenic fungi (Magliani et al., 2009).

CONCLUSION

From the study of the interesting, but apparently therapeutically impracticable, yeast killer phenomenon, fungicidal antibiobodies, antibiobody-derived peptides, and Ab-derived CDR – as well as Fc-peptides were produced. Like many other proteins, such as bactericidal proteins (D'Alessio, 2011), hemoglobin (Catiau et al., 2011), *Helicobacter pylori* ribosomal protein L1 (Park and Hahm, 2012), human lactoferrin (Brouwer et al., 2011), human milk lysozyme (Ibrahim et al., 2011), human salivary protein (Gorr et al., 2011), and thrombin (Kasetty et al., 2011), longicin (Galay et al., 2012), thymic stromal lymphopoietin and kininogen (Sonesson et al., 2011a,b), ubiquitin (Pasikowski et al., 2011), among the most recently reported, Abs should be considered as containing many hidden peptides, known as "cryptides" (Ng and Ilag, 2006; Pimenta and Lebrun, 2007; Ueki et al., 2007; Samir and Link, 2011) in both their variable and constant regions. They can exert biological effects that cannot be predicted based on the activity of the precursor protein (Polonelli et al., 2012). These observations call into question the traditional distinction between acquired and innate immunity, suggesting a further close link between them. Ab-derived antifungal peptides, on the other hand, may be promising molecules for future therapeutic developments. Their easy production, engineering, and chemical optimization, through aminoacidic substitutions, peptidomimetics, etc., can greatly expand the possibilities of obtaining effective antifungal immunotherapeutic tools. These approaches may reasonably fall within the "fragment-based drug discovery," i.e., the design of good-quality lead compounds from fragment hits that can be developed into clinical candidates (Foloppe, 2011).

Future studies on Ab-derived peptides will be addressed to better clarify their molecular mechanisms of fungicidal action, presumably leading to the discovery of cellular targets for new therapeutic antifungal approaches in the never ending war against fungal diseases.

ACKNOWLEDGMENTS

Part of the work described in the manuscript has been supported by funding from the Italian Ministry of Health in the frame of the National AIDS Research Program (last grant number 40H39). The funder had no role in decision to publish or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 April 2012; paper pending published: 08 May 2012; accepted: 10 May 2012; published online: 01 June 2012.

Citation: Magliani W, Conti S, Giovati L, Zanello PP, Sperindè M, Ciociola T and Polonelli L (2012) Antibody peptide based antifungal immunotherapy. Front. Microbio. ***3****:190. doi: 10.3389/fmicb.2012.00190*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 24 May 2012
doi: 10.3389/fmicb.2012.00187

Hydroxyurea treatment inhibits proliferation of *Cryptococcus neoformans* in mice

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The fungal pathogen *Cryptococcus neoformans* (*Cn*) is a serious threat to immunocompromised individuals, especially for HIV patients who develop meningoencephalitis. For effective cryptococcal treatment, novel antifungal drugs or innovative combination therapies are needed. Recently, sphingolipids have emerged as important bioactive molecules in the regulation of microbial pathogenesis. Previously we reported that the sphingolipid pathway gene, *ISC1*, which is responsible for ceramide production, is a major virulence factor in *Cn* infection. Here we report our studies of the role of *ISC1* during genotoxic stress induced by the antineoplastic hydroxyurea (HU) and methyl methanesulfonate (MMS), which affect DNA replication and genome integrity. We observed that *Cn* cells lacking *ISC1* are highly sensitive to HU and MMS in a rich culture medium. HU affected cell division of *Cn* cells lacking the *ISC1* gene, resulting in cell clusters. *Cn ISC1*, when expressed in a *Saccharomyces cerevisiae* (*Sc*) strain lacking its own *ISC1* gene, restored HU resistance. In macrophage-like cells, although HU affected the proliferation of wild type (WT) *Cn* cells by 50% at the concentration tested, HU completely inhibited *Cn isc1*Δ cell proliferation. Interestingly, our preliminary data show that mice infected with WT or *Cn isc1*Δ cells and subsequently treated with HU had longer lifespans than untreated, infected control mice. Our work suggests that the sphingolipid pathway gene, *ISC1*, is a likely target for combination therapy with traditional drugs such as HU.

Keywords: *Cryptococcus*, *ISC1*, hydroxyurea, morphology

INTRODUCTION

Cryptococcus spp. are environmental fungal pathogens afflicting immunocompromised patients as well as immunocompetent individuals, causing life-threatening meningoencephalitis (Idnurm et al., 2005; Jarvis et al., 2008; Dadachova and Casadevall, 2011; Del Poeta and Casadevall, 2011; Kronstad et al., 2011; Kozubowski and Heitman, 2012). *Cryptococcus* causes approximately one million annual cases of meningoencephalitis globally among AIDS patients, leading to nearly 625,000 deaths (Park et al., 2009). Despite major developments in HIV treatment *Cryptococcus* infection still remains a major threat to AIDS patients, especially in sub-Saharan Africa (Warkentien and Crum-Cianflone, 2010).

Cryptococcus neoformans (*Cn*) is a ubiquitous fungus, found in tree hollows and pigeon droppings. It is present in the environment and in human hosts predominantly in the yeast form; however, *Cn* can assume hyphal and other shapes depending upon its life cycle state or environmental influences (Zaragoza et al., 2010; Kronstad et al., 2011; Kozubowski and Heitman, 2012). Pathogenic *Cn* infection initiates upon the inhalation of infectious *Cn* particles, which initially disseminate to the lungs and subsequently to the central nervous system via the circulation if the host's immune response does not control fungal proliferation within the lung (Kronstad et al., 2011). An intracellular facultative pathogen, *Cn* can grow and replicate within the phagolysosome of phagocytic cells, such as alveolar macrophages (AMs) and it can also grow in extracellular spaces, such as within the alveoli or in the bloodstream (Feldmesser et al., 2000; Goldman et al., 2000; Levitz, 2001; Steenbergen et al., 2001; Shea et al., 2006). Because the pathogen rapidly develops drug resistance (Morschhauser, 2010), and because the number of immunocompromised patients is increasing, there is a constant need for innovative and effective antifungal therapies.

Hydroxyurea (HU), an antineoplastic drug used for treatment of HIV, cancer, and myeloproliferative diseases (Kovacic, 2011) slows the progression of DNA replication machinery by reducing the cell's deoxyribonucleotide (dNTP) pool (Katou et al., 2003). HU treatment of the budding yeast *Saccharomyces cerevisiae* (*Sc*) results in DNA replication fork slowing, and the formation of a fork-protection complex to guard the cell's replication machinery, activating the replication checkpoint (Alcasabas et al., 2001; Katou et al., 2003; Zegerman and Diffley, 2003; Bando et al., 2009). In the absence of replication proteins, yeast cells become HU sensitive. Interestingly, in addition to DNA replication genes, ∼300 genes from various other pathways have been shown to play role in resistance to HU toxicity and the absence of these genes gives

rise to HU sensitivity (Chang et al., 2002; Hartman and Tippery, 2004; Parsons et al., 2004; Woolstencroft et al., 2006).

Recently, lipid signaling, especially sphingolipid metabolism, has gained recognition for its role in fungal pathogenesis (Shea and Del Poeta, 2006; Rhome and Del Poeta, 2010; Singh and Del Poeta, 2011). All yeast cells, including *Cn*, produce inositol-containing sphingolipids instead of choline-containing sphingolipids (e.g., sphingomyelin), and the deletion of the inositol sphingophospholipid phospholipase C 1 (*ISC1*) gene in *Sc* (*Sc isc1*Δ) causes accumulation of inositol-containing sphingolipids (Sawai et al., 2000; Shea et al., 2006). *Cn* is a pathogenic yeast, and deletion of *ISC1* renders it incapable of causing meningoencephalitis (Shea et al., 2006). Isc1 has been characterized in *Sc* (Sawai et al., 2000) and *Cn* (Henry et al., 2011) and in *Leishmania* (Zhang et al., 2009), indicating that this sphingolipid metabolizing enzyme has unique biochemical characteristics. The absence of the *ISC1* gene in *Sc* increases fungal sensitivity to HU and methyl methanesulfonate (MMS) accompanied by cell division arrest and morphological aberrations (Chang et al., 2002; Matmati et al., 2009; Tripathi et al., 2011). Here, we report our studies into the role of *Cn ISC1* in the fungal resistance to HU and MMS and their specific effects on the virulence of the pathogenic fungus *Cn*. We show that *Cn* cells lacking the *ISC1* gene are highly sensitive to HU and MMS and form cell clusters upon HU exposure. The absence of *ISC1* in conjunction with HU treatment synergistically reduced *Cn* infection of macrophage-like cells and immunocompetent mice.

MATERIALS AND METHODS

STRAINS AND PLASMIDS

Wild type (WT) *Cn* (var. *grubii* serotype A strain H99) and its *isc1*Δ derivative were used in the current study and have been described previously (Shea et al., 2006; Henry et al., 2011). The *Sc* strain *Jk9-3d a* (*MATa trp1 leu2-3 his4 ura3 ade2 rme1*) and its *isc1*Δ derivative were used and have been described previously (Matmati et al., 2009; Tripathi et al., 2011).

EXPOSURE TO HU AND MMS

YPD plates (1% yeast extract, 2% peptone, and 2% glucose plus 2% agar) containing appropriate concentrations of HU (Sigma; 0, 25, 50, 100, and 200 mM) or MMS (Sigma; 0.033%) were prepared and used within 48 h. Overnight cultures were inoculated in fresh medium at A_{600} of 0.2 and grown at 30°C. Log-phase cultures were adjusted to A_{600} of 0.4 before making 10-fold serial dilutions and plate spotting (2.5 μl). Plasmids pYES-*Sc ISC1* and pYES-*Cn ISC1* that express *Sc ISC1* and *Cn ISC1* genes respectively have been described previously (Henry et al., 2011). The two plasmids and a control vector were transformed into appropriate strains (WT and *isc1*Δ of *Sc*) and plated on SD/Ura$^-$ plates. Then, 10-fold serial dilutions of log-phase liquid cultures in SD/Ura$^-$ liquid medium were spotted on SD/Ura$^-$ and SD/Ura$^-$/HU plates, and the plates were incubated at 30°C, and analyzed at appropriate times before recording the data.

CELLULAR MORPHOLOGY

Cells were grown to log-phase as described above, HU (25–200 mM) or MMS (0.033% v/v) was added, and cells were incubated for 5 or 22 h before they were fixed with 3.7% formaldehyde. Cells were washed with phosphate buffered saline (PBS, 50 mM, pH 7) and further suspended in PBS before analyzing them under a Nikon Eclipse (TE2000-5) microscope with a 40×/100× objective lens.

EFFECT OF HU ON INTRACELLULAR GROWTH OF *Cn*

The murine reticulum sarcoma macrophage-like cell line J774A.1 cells were used up to passage #8. Cells were then plated in 96-well cell culture plates in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. WT *Cn* (H99) and its *isc1*Δ derivative were grown overnight in YPD at 30°C. Cells were washed three times in PBS and counted. Approximately 10^5 cells in DMEM + FBS medium were added with 10 μg/ml of anti-GXM monoclonal antibody 18B7 (kindly provided by Dr. Arturo Casadevall) with 1 mM HU or without HU. Meanwhile the macrophage-like cells were washed off the non-adhered cells and activated with 50 units/ml of recombinant murine gamma interferon (IFNγ) and 0.3 μg/ml of lipopolysaccharide (LPS). The antibody-opsonized *Cn* cells were added to the macrophage cells at an effector-to-target ratio of 1:1. After incubation for 2 h, extracellular *Cn* cells were washed with three changes of warm DMEM medium and fresh medium without or with 1 mM of HU. For one set of the experiments 200 μl sterile water was added to each well and the macrophage-like cells were lysed by pipetting several times. The samples were diluted and an aliquot was spread on YPD agar plate for determining colony forming units (CFUs); this set served as the time-point "zero." The other time points were 6, 12, and 24 h, at which points the supernatant was aspirated and cells were rinsed once with DMEM. Macrophage cells were lysed by adding 200 μl of sterile water and pipetting several times. The samples were diluted and spread on YPD agar plate for determining the CFUs.

For the phagocytic indices (PI) and for photographs, the conditions were same as above except that the macrophage-like cells were grown on glass cover slips. After 2 h of the *Cn* challenge, the cells were washed three times with PBS, fixed with ice-cold methanol, and stained with Giemsa. For the 24-h experiment, cells were washed three times and fresh medium without HU or with 1 mM HU was added and incubated at 37°C in 5% CO_2. After 24 h the cells were washed three times with PBS, fixed with ice-cold methanol, and stained with Giemsa. Photographs were taken using a Zeiss microscope equipped with charged-coupled device camera. Results for 0 and 24 h time points are shown in the text.

SURVIVAL STUDIES IN MOUSE MODELS

Mice were anesthetized with a xylazine–ketamine mixture (60 μl, i.p., 5 mg/kg xylazine, 95 mg/kg ketamine). All *Cn* strains were grown in YPD medium for 16–18 h at 30°C. Cells were washed and re-suspended in PBS. Mice were challenged intranasally with 20 μl of the inoculum containing 5×10^5 *Cn* cells. After *Cn* infection, mice were administered HU (0.8 mg/kg every 48 h). Mice were fed *ad libitum* and monitored twice daily for signs of morbidity or pain or clinical signs suggesting meningoencephalitis. Mice exhibiting any of these signs were immediately sacrificed using CO_2 inhalation followed by cervical dislocation.

STATISTICAL ANALYSIS

All data were analyzed by standard Student's *t*-test with *P* values shown in appropriate figures.

RESULTS

ABSENCE OF *ISC1* CAUSES SENSITIVITY TO HU AND MMS IN *Cn* CELLS

Cryptococcus neoformans WT and *isc1*Δ cells were analyzed for their response to long-term exposure to HU and MMS. As shown in **Figures 1A,B**, whereas WT *Cn* cells recovered from HU and MMS stress, *isc1*Δ cells were highly sensitive to both HU and MMS. The HU sensitivity of *Cn isc1*Δ cells was almost comparable to that of *Sc isc1*Δ cells (**Figure 1A**). To ensure that the sensitivity to HU and MMS was due to loss of the *ISC1* gene, we examined a *Cn isc1*Δ strain containing reconstituted *ISC1* for HU and MMS tolerance. As shown in **Figures 1A,B**, the reconstituted strain was resistant to HU and MMS similar to the WT strain, strongly suggesting a role for *Cn ISC1* in HU/MMS tolerance. In addition to testing the role of the reconstituted strain for HU and MMS sensitivity, we tested the role of *Cn ISC1* in HU tolerance independently: we expressed the *Cn ISC1* gene in an *Sc isc1*Δ strain and examined whether the former complemented HU sensitivity. As shown in **Figure 1C**, whereas the *Sc* WT strain containing a vector showed HU resistance, its *isc1*Δ derivative containing the vector was HU sensitive. In contrast, the *Sc isc1*Δ strain expressing either *Sc ISC1* or *Cn ISC1* (in pYES vector; Henry et al., 2011) showed HU resistance, albeit with minor differences (**Figure 1C**). All these results show that *Cn* Isc1 plays a key role in HU/MMS tolerance.

ABSENCE OF *ISC1* AFFECTS CELL MORPHOLOGY AND CELL DIVISION IN *Cn* CELLS UPON EXPOSURE TO HU AND MMS

We examined whether *Cn ISC1* controls cell division and cellular morphology under HU stress. *Cn* WT and *isc1*Δ cells were grown in the presence of various concentrations of HU in liquid media and cell morphology was analyzed microscopically. As shown in **Figures 2A,B**, HU did not affect cell division of WT cells at 25–50 mM concentrations; however, the bud size was relatively large compared to the untreated WT cells. At higher concentrations of HU (100–200 mM) some WT cells had defects in cell division resulting in cell chains. In contrast to the WT cells, cell division in *isc1*Δ cells was severely inhibited at low HU concentrations (25–50 mM) resulting in cell chains and lawns (**Figures 2A,B**); a few misshapen cells were also seen (data not shown). These data suggest a synergism between HU and the absence of *ISC1* in inhibiting cell division.

SYNERGISTIC EFFECTS OF HU AND *ISC1* DELETION ON MACROPHAGE INFECTION

One mechanism by which Isc1 protects *Cn* cells against the host immune response is by increasing the resistance to antifungal activity of macrophages by favoring fungal intracellular growth (Shea et al., 2006). HU treatment of macrophages infected by *Toxoplasma gondii*, *Leishmania amazonensis*, *Trypanosoma cruzi*, and *L. mexicana* has been shown to drastically reduce the number of infected cells (Melo and Beiral, 2003; Martinez-Rojano et al., 2008). Therefore, we tested whether HU would compromise the intracellular growth of *Cn* cells. We first allowed macrophages to internalize *Cn* cells and then treated the macrophages with HU. HU treatment diminished the intracellular growth of WT *Cn* by ~3.5-fold (**Figure 3A**). Interestingly, HU treatment completely abolished the growth of *Cn isc1*Δ cells within the macrophages suggesting a strong synergism between HU and *ISC1* deletion. Representative macrophages with *Cn* infection are shown in **Figure 3B**. Importantly, inhibition of intracellular growth was not due to HU's effect on phagocytosis because the drug did not inhibit macrophage ingestion of *Cn* (data not shown). We have already demonstrated that a *Cn isc1*Δ strain reconstituted with *Cn ISC1* behaves like the WT *Cn* strain in macrophages (Shea et al., 2006). Because the *in vitro* experiments were carried out at 30°C and *in vivo* experiments were performed at 37°C, we needed to ensure that the loss of *isc1*Δ cell viability in macrophages was not temperature dependent. Thus, we grew WT and *isc1*Δ cells at 37°C and compared these data with those obtained at 30°C. We observed that the growth pattern of WT and *isc1*Δ at 37°C was similar to those patterns observed at 30°C (data not shown).

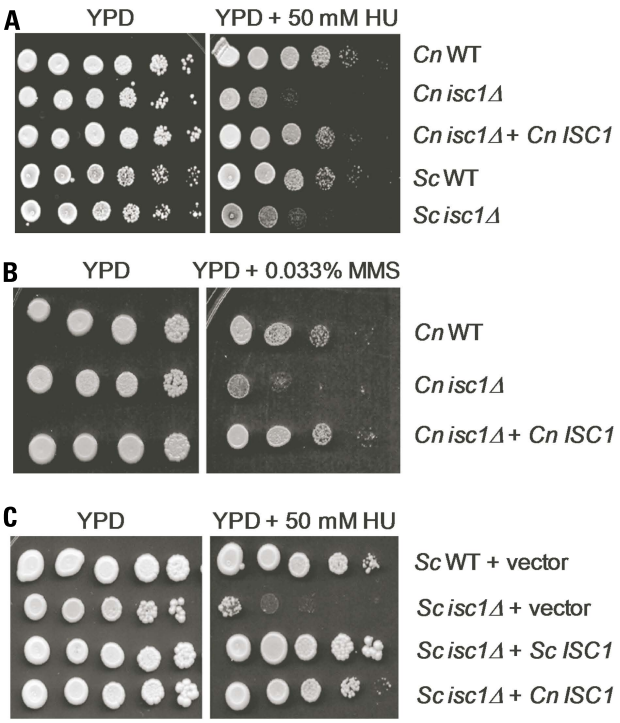

FIGURE 1 | *Cryptococcus neoformans* cells lacking the *ISC1* gene are sensitive to HU and MMS. (A) Ten-fold serial dilutions of log-phase cultures of *C. neoformans* (WT, *isc1*Δ, and *isc1*Δ + *Cn ISC1* reconstituted) and *S. cerevisiae* (WT and *isc1*Δ strains) were spotted on plates containing YPD and YPD + HU (50 mM). **(B)** Ten-fold serial dilutions of log-phase cultures of *C. neoformans* (WT, *isc1*Δ, and *isc1*Δ + *Cn ISC1* reconstituted) were spotted on YPD and YPD + 0.033% MMS plates. **(C)** *Cn ISC1* complements HU sensitivity of the *S. cerevisiae isc1*Δ strain. Ten-fold serial dilutions of following log-phase cultures were spotted on SD/Ura$^-$ and SD/Ura$^-$/HU plates: *Sc* WT cells containing pYES vector, *Sc isc1*Δ cells containing the pYES vector, *Sc isc1*Δ cells containing pYES-*Sc ISC1* and *Sc isc1*Δ cells containing pYES-*Cn ISC1*.

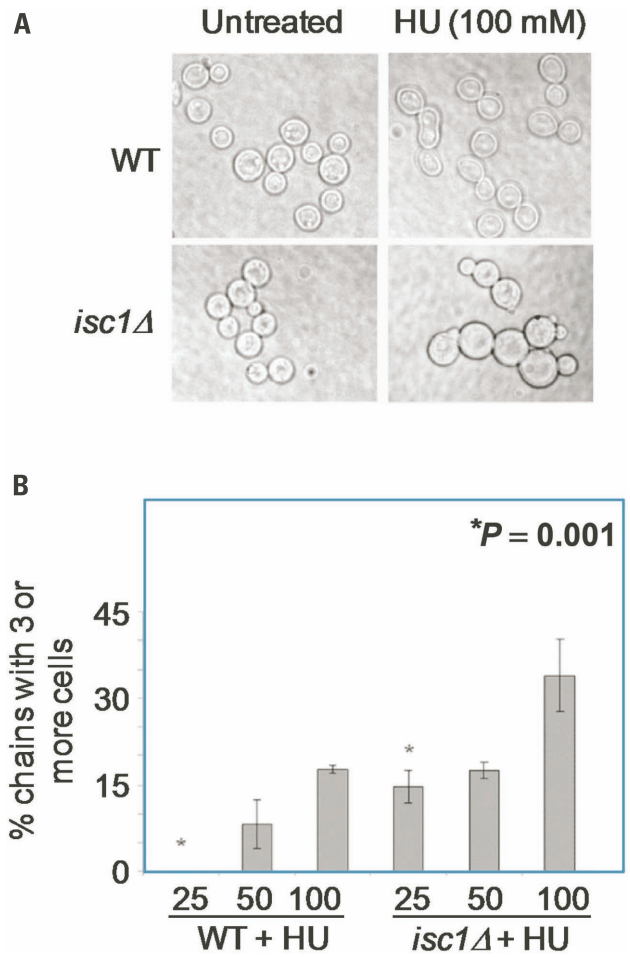

FIGURE 2 | *Cryptococcus neoformans* cells show HU-induced defects in morphology and cell division. (A) *Cn* WT and *isc1*Δ cells were treated with HU overnight and observed under phase contrast microscope (×1000). *Cn isc1*Δ cells formed cell chains and clumps upon HU exposure. **(B)** Bar diagrams showing morphological aberrations in untreated and HU-treated *Cn* WT and *Cn isc1*Δ cells.

HU TREATMENT INHIBITS GROWTH OF *Cn* WT AND *isc1*Δ CELLS IN MICE

We tested the effects of HU on survival and virulence of *Cn* WT and *isc1*Δ strains in mice. Mice were infected with fungal cells and treated with HU as described in Section "Materials and Methods." Interestingly, HU inhibited the proliferation of WT *Cn* cells in mice and significantly prolonged their survival. In addition, we observed that HU acted synergistically when the *ISC1* gene was absent to inhibit *Cn* cell growth. We performed survival and tissue burden studies in mice by infecting them intranasally with *Cn* WT or the *isc1*Δ strain and then treated the mice with HU (0.8 mg/kg every other day). As expected, untreated mice died within 30 days whereas HU-treated mice survived up to 60 days (**Figure 4A**). Interestingly, mice infected with *Cn isc1*Δ cells also survived for 60 days regardless of HU treatment (**Figure 4B**). HU significantly reduced fungal burden, especially in the lung tissue. Specifically, HU-treatment reduced the number of CFUs in the lung infected with *Cn* WT by ∼10-fold from the initial inoculum (**Figure 4C**). Remarkably the number of *Cn isc1*Δ CFU decreased by ∼1,700 fold in HU-treated mice compared to untreated mice. These data suggest a synergistic effect of HU treatment with *ISC1* deletion in increasing host survival by decreasing organ fungal load. The survival of mice infected with WT *Cn* and treated with HU suggests that exposure to HU (at the HU concentration tested) slows down DNA replication and growth of WT cells, allowing the host's immunity to take over the pathogen. The role of *Cn ISC1* in mice experiments was revealed by the CFU of HU-treated WT *Cn*, untreated *isc1*Δ and HU-treated *isc1*Δ cells recovered from lung tissues.

DISCUSSION

Our results show that HU slows down growth of WT *Cn* cells, which helps both mice and macrophages to inhibit further pathogenic growth. This effect is enhanced by deletion of *ISC1*, suggesting that Isc1 and the sphingolipid metabolic pathway in general should be exploited as novel targets for antifungal drug development, either alone or in combination with existing drugs (e.g., HU) to better control cryptococcosis. Of note, all experiments conducted with mice ended on the 60th day of infection when all surviving mice were sacrificed. Thus, we observed no differences in survival between WT and *isc1*Δ cells upon HU treatment; the *isc1*Δ has a significant defect in virulence. However, treatment with HU profoundly diminished *Cn* proliferation in the lung environment compared to untreated cells.

At present, the molecular mechanism of HU inhibition of *Cn* growth is unknown. We hypothesize that *isc1*Δ may be more susceptible than the WT strain because it controls phytoceramide generation (Garcia et al., 2008) and its decrease in the deletion mutant could affect membrane permeability and thus HU transport. However, this hypothesis was not supported by studies in budding yeast in which the inhibition of DNA synthesis by HU was not enhanced by deletion of *ISC1* (Matmati et al., 2009).

Of note, HU has been shown to have an anti-proliferative activity on T cells (Benito et al., 2007) and to cause neutropenia in humans and mice (Hermans et al., 1999). Because neutropenia is associated with prolonged survival of *Cn*-infected mice (Mednick et al., 2003) HU could increase mice survival through neutropenia. However, the neutropenic effect of HU is remarkably linked to the administered HU dose. Almost all patients will develop neutropenia when the administered dose is 20–30 mg/kg/day or greater. However, several studies have shown that such toxicity can be dramatically reduced if the HU dose is decreased to 4–5 mg/kg/day. In mice, HU at 50 mg/kg/day in a sickle cell model does cause a moderate neutropenia, whereas a lower dose of 25 mg/kg/day does not cause neutropenia (Lebensburger et al., 2012). The dose used in our mouse experiment was 0.8 mg/kg/every other day, a dose that is 10-fold less that the HU dose that does not produce neutropenia in people and 25- to 50-fold less than the dose that does not produce neutropenia in mice. Thus, due to the very low dose of HU used in our experimentations, we hypothesize that the increased mouse survival is not due to an effect of HU on neutrophils.

We predict that HU (at the concentration tested here) slows *Cn* DNA replication and cell division while host immunity overtakes

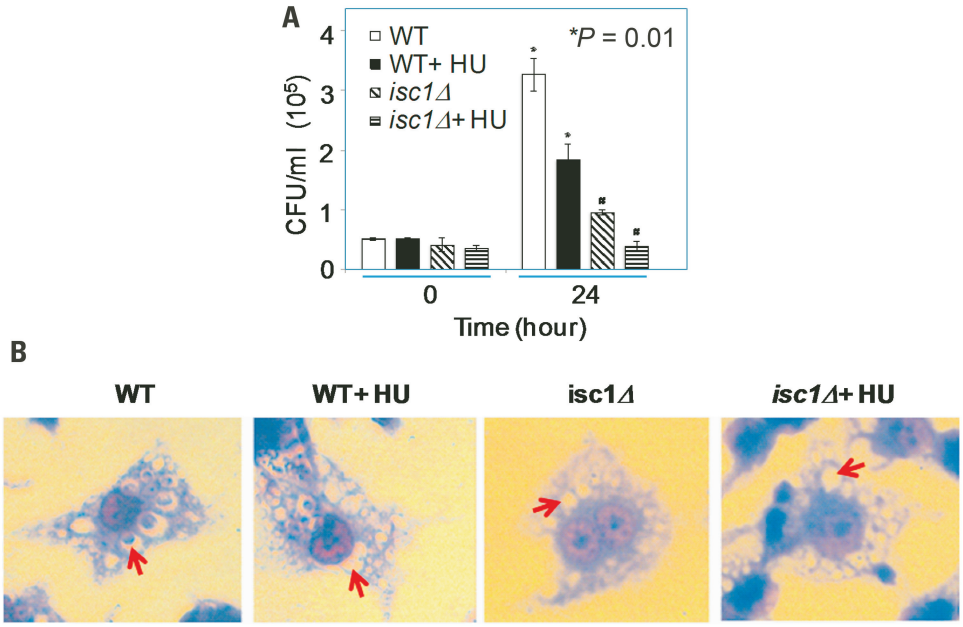

FIGURE 3 | Synergistic role of HU and *ISC1* deletion on *Cryptococcus* infection of the macrophage-like cell line J774A.1. (A) Phagocytosis assay with macrophage-like cell line J774A.1 cells infected with *Cn* WT or *isc1*Δ cells in the presence/absence of HU (see Materials and Methods). **(B)** *Cn* cells phagocytosed by J774A.1 were grown in the presence/absence of HU and stained with Giemsa after 24 h. Photographs were taken with a Zeiss microscope equipped with a CCD camera. All images were captured under oil immersion (×100). Red arrows indicate the presence/absence of *Cryptococcus* in the phagolysosomes of macrophages. Most phagolysosomes in untreated WT cells contained *Cn* cells, whereas some HU-treated WT cells still contained *Cn* cells. In contrast, most macrophages did not contain *Cn isc1*Δ cells regardless of HU treatment.

the pathogen. This effect appears to be enhanced when *Cn* cells are intracellular. This hypothesis is supported by our experiment with macrophage-like cell line showing that HU significantly suppresses fungal cell division within macrophages. Very interestingly, HU has been tested against various intracellular parasites such as *Toxoplasma gondii*, *L. amazonensis*, *Trypanosoma cruzi*, and *L. mexicana* (Melo and Beiral, 2003; Martinez-Rojano et al., 2008). Not only did HU induce morphological changes in these parasites, but also it inhibited intracellular multiplication of these microbes, similar to the phenotype observed with *Cn* in macrophages illustrated in this paper. In addition, in *L. mexicana* HU induced cell cycle arrest suggesting that the mechanism by which HU inhibits the synthesis of the DNA replication and cell division has been maintained in different microbial species.

A recent study points to an important aspect concerning the use of HU to control cryptococcosis. The authors found that HU enhances post-fusion hyphal extension in *Cn* cells, but not in haploid cells (Zulkifli et al., 2012). HU is known to induce morphological changes such as hyphal generation in *Candida albicans* (Shi et al., 2007; Sun et al., 2011) but in *Sc* it generates limited morphological aberrations (in 1–3% cells) and extensive morphological aberrations are seen in *Sc* mutants on checkpoint, budding and, notably, in the *isc1*Δ deletion mutant (Jiang and Kang, 2003; Enserink et al., 2006; Tripathi et al., 2011). It seems that *Cn* behaves similar to *Sc*; morphological changes by HU (at low concentrations) only occur in *isc1*Δ cells and not in WT cells. In another study it has been shown that certain *Cn* mutants such as *ras1*Δ were sensitive to HU and MMS (Maeng et al., 2010).

Intriguingly, the morphological changes ascribed to HU are strictly linked to defects in yeast cell division. Possibly, in addition to the inhibition of DNA synthesis, HU also affects actin polymerization/depolymerization during *Cn* cell division and cell wall synthesis (Enserink et al., 2006; Tripathi et al., 2011). This hypothesis is supported by our previous studies in *Sc* in which we showed that actin depolymerization is inhibited by HU especially in conditions in which *ISC1* is deleted. This will ultimately block cell division or cell proliferation (Tripathi et al., 2011), possibly through the regulation of morphogenesis and DNA integrity checkpoint proteins. The latter hypothesis is also supported by the results presented in this paper in which we show that HU delays the separation of *Cn isc1*Δ daughter cells from the mother cell (**Figures 2A,B**). This delay in cell division of *Cn* cells may expose the *isc1*Δ cells for longer time to intracellular inhibitors (e.g., hydrogen peroxide, nitric oxide) rendering *isc1*Δ even more susceptible to the intracellular compared to the extracellular environment. Considering that the *Cn isc1*Δ is already hypersusceptible to hydrogen peroxide and nitric oxide (Shea et al., 2006), the treatment with HU may render the *isc1*Δ cells even more sensitive than WT cells. Thus, it is possible that HU does increase the killing capacity of macrophages indirectly by increasing the exposure of undivided *Cn* cells to intracellular toxins. Finally, in *Sc* HU affects chitin deposition on the cell wall (Tripathi et al., 2011) particularly when *ISC1* is deleted. This observation further supports a role for Isc1 under HU stress in cell division as chitin is an important regulator of cell morphology and cell division in yeasts

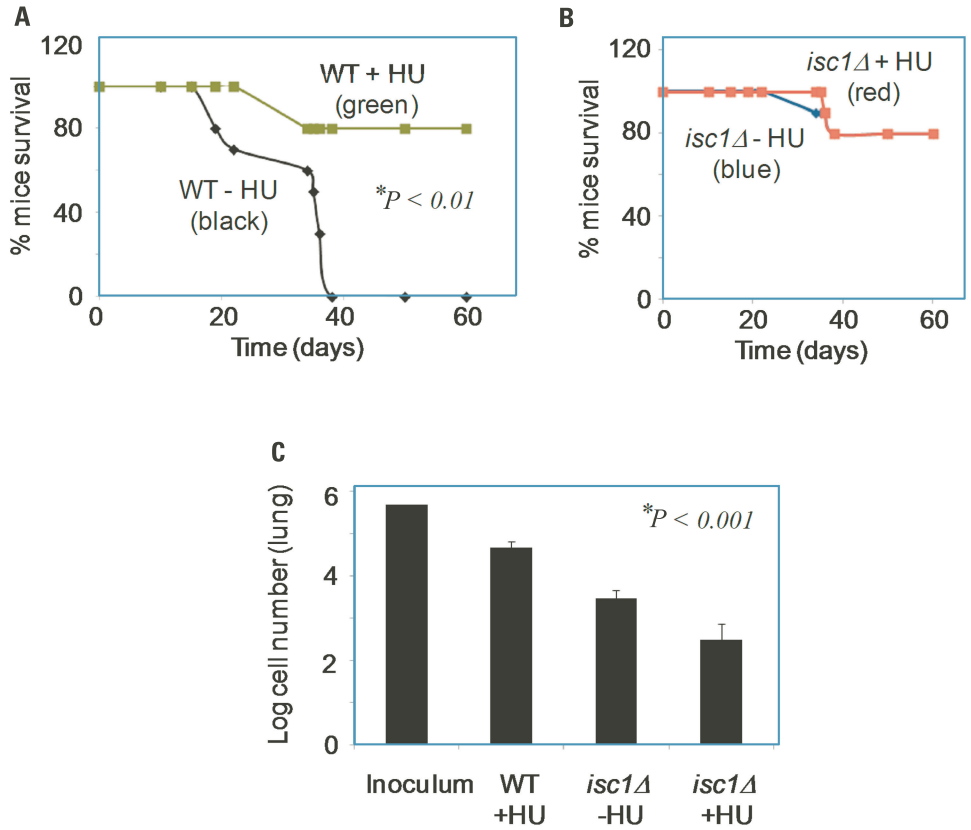

FIGURE 4 | Role of HU and *ISC1* deletion on the survival of mice and on lung tissue burden. (A) Survival curve of CBA/J mice upon infection with WT *Cn* in the presence (green) or absence (black) of HU; **(B)** Survival curve of CBA/J mice upon infection with *Cn isc1*Δ cells in the presence (red) or absence (blue) of HU. **(C)** Survival of *Cn* cells in lung tissue of CBA/J mice after 60 days of infection. Lung tissues were collected from mice and *Cn* CFU was counted by plating on YPD plates.

(Roh et al., 2002). These are exciting possibilities that we will explore in the future.

ACKNOWLEDGMENTS

We thank Aimee Guillotte in Del Poeta's laboratory for helping with the animal experiments. We thank Dr. Jennifer Schnellmann and Dr. Ashley Cowart for editing the manuscript. This work was performed in part by the National Institute of Health (NIH) awards AI056168, AI071142, AI078493, and AI087541 to Maurizio Del Poeta and, in part, by pilot research funding from an American Cancer Society Institutional Research Grant (ACS-IRG# 97-219-08) awarded to the Hollings Cancer Center, Medical University of South Carolina, and in part by a Center of Biological Research Excellence (COBRE P20 RR17677) pilot grant to Bidyut K. Mohanty. Kaushlendra Tripathi is thankful to the American Society for Biochemistry and Molecular Biology (ASBMB) for a travel award. This work was conducted in a facility constructed with support from the NIH C06 RR015455 from the Extramural Research Facilities Program of the National Center for Research Resources. Maurizio Del Poeta is a Burroughs Welcome New Investigator in the Pathogenesis of Infectious Diseases.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 April 2012; accepted: 08 May 2012; published online: 24 May 2012.
Citation: Tripathi K, Mor V, Bairwa NK, Del Poeta M and Mohanty BK (2012) Hydroxyurea treatment inhibits proliferation of Cryptococcus neoformans in mice. Front. Microbio. ***3****:187. doi: 10.3389/fmicb.2012.00187*
This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 08 August 2012
doi: 10.3389/fmicb.2012.00286

It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug

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"*Amphotericin B acts through pore formation at the cell membrane after binding to ergosterol*" is an accepted dogma about the action mechanism of this antifungal, and this sentence is widely found in the literature. But after 60 years of investigation, the action mechanism of Amphotericin B is not fully elucidated. Amphotericin B is a polyene substance that is one of the most effective drugs for the treatment of fungal and parasite infections. As stated above, the first mechanism of action described was pore formation after binding to the ergosterol present in the membrane. But it has also been demonstrated that AmB induces oxidative damage in the cells. Moreover, amphotericin B modulates the immune system, and this activity has been related to the protective effect of the molecule, but also to its toxicity in the host. This review tries to provide a general overview of the main aspects of this molecule, and highlight the multiple effects that this molecule has on both the fungal and host cells.

Keywords: amphotericin B, pore, oxidative damage, immunomodulation, fungal infection

INTRODUCTION

The control of invasive fungal infections is based on the use of antifungal drugs, being polyenes, azoles, and echinocandins the main families used in clinical practice. Among these, polyenes are the drugs that have been in use for a longer time, since they were first described in the middle of the twentieth century (Oura et al., 1955). The main polyene used as antifungal drug is Amphotericin B (AmB), which is an amphipatic macrolide. This molecule was discovered in 1950s after a broad screening of *Streptomycete* cultures that contained antifungal activity. The AmB-producing organism was isolated from a soil sample taken from the Orinoco River region (Venezuela) and was identified as *Streptomyces nodosus* (Trejo and Bennett, 1963). An intravenous presentation was introduced in the market in 1958 as a sodium deoxycholate solution (D-AmB) (Fungizone-Squibb), which forms a micellar suspension when reconstituted in glucose solution.

AmB has been used for the treatment of fungal infections and, despite the toxicity and the development of other antifungals, such as azoles and echinocandins, this drug remains as the first line treatment for severe and life threatening systemic infections such as cryptococcal meningitis and invasive zygomycosis (Saag et al., 2000; Waness et al., 2009). AmB is also effective for other mycoses such as aspergillosis, candidiasis, histoplasmosis, blastomycosis, coccidioidomycosis, sporotrichosis, fusariosis, and phaeohyphomycosis in the cases of lack of response to azoles or echinocandins. (Ellis, 2002; Davis and Porter, 2005; Metcalf and Dockrell, 2007; Chandrasekar, 2008; Gomez-Lopez et al., 2008; Muhammed et al., 2011). Additionally, AmB has activity against parasites as *Trypanosoma cruzi*, *Schistosoma mansoni*, *Echinococcus multilocularis*, and *Leishmania* spp, being the second drug of choice for the treatment for visceral leishmaniasis when antimonials fail or cannot be used (Yardley and Croft, 1999; Reuter et al., 2003; Mone et al., 2010; Paila et al., 2010). Also an amphotericin-derived drug, MS8209, has effect against HIV-1 infection avoiding virus entry to the cell (Pleskoff et al., 1995).

The D-AmB formulation has been considered the gold standard for many years and it has broad-spectrum activity. Unfortunately, this formulation is highly nephrotoxic and shows side effects as fevers, malaise, weight loss, headache, hypotension, abdominal pain, nausea, vomiting, diarrhea, normochromic normocytic anemia, and myalgia (Sabra and Branch, 1990; Meunier et al., 1991; Ringden et al., 1991; Gulati et al., 1998; Laniado-Laborin and Cabrales-Vargas, 2009). For this reason, new formulations have been introduced in the last years (Lopez-Berestein et al., 1985; Bohme and Hoelzer, 1996; Gulati et al., 1998; Rust and Jameson, 1998; Walsh et al., 1998; Dupont, 2002). The new presentations have reduced toxicity because they are lipid-carried presentations. These last formulations include a colloidal dispersion with cholesterol sulphate (CD-AmB, Amphotec), a lipidic complex with two phospholipids (LC-AmB, Abelcet) and liposomal AmB (L-AmB, Ambisome), which is integrated into true unilamellar liposomes (Veerareddy and Vobalaboina, 2004; Torrado et al., 2008). These different formulations differ in their price and in the associated toxicity. Lipid-based formulations, and in particular, L-AmB, have reduced nephrotoxicity and have superior efficacy than conventional AmB (Gulati et al., 1998; Saliba and Dupont, 2008).

MECHANISM OF ACTION OF AMPHOTERICIN B

The mechanism of action of AmB still is not completely elucidated. AmB has effects on the fungal cell at two different levels: Binding to the ergosterol at the membrane, inducing pore formation and ergosterol sequestration, and induction of oxidative damage. In the following sections we will summarize how AmB exerts these two effects on the fungal cells, which are also summarized in **Figure 1**.

EFFECTS ON THE FUNGAL MEMBRANE: PORE FORMATION AND ERGOSTEROL SEQUESTRATION

Early studies suggested that AmB inserts into the fungal lipid bilayer through the hydrophobic domains that bind to ergosterol. As a consequence, multimeric pores are formed, with the lipophilic polyene chains of the antifungal in contact with membrane lipids (Finkelstein and Holz, 1973; Brajtburg et al., 1990). AmB pores increase the permeability of the fungal membrane to small cations as K^+, Ca^{2+}, and Mg^{2+} promoting the rapid depletion of intracellular ions and fungal cell death (Kinsky, 1970). AmB can also bind to other sterols, such as cholesterol, but with a lower affinity (Hsuchen and Feingold, 1973).

Recently, it was proposed that AmB can exert its action through two complementary mechanisms depending on the interaction of AmB and sterols: membrane permeabilization and sterol sequestration (Palacios et al., 2011). In this sense, it has been proposed that cholesterol sequestration in the host membrane avoids macrophage–parasite interaction in *Leishmania* infection as a novel mechanism for AmB in visceral leishmaniasis (Chattopadhyay and Jafurulla, 2011).

Analytical studies have demonstrated that AmB forms two different types of pores, which differ in their substrate specificities and that are formed at different moments. Moreover, they participate differentially in the killing effect of the molecule [see seminal review in Cohen (2010) and Hartsel et al. (1994); Romero et al. (2009)]. After addition of AmB to the cells, the first type of pores that are formed are non-aqueous, which are permeable to monovalent cations and have lower permeability to monovalent anions (Ramos et al., 1996; Romero et al., 2009). Afterwards,

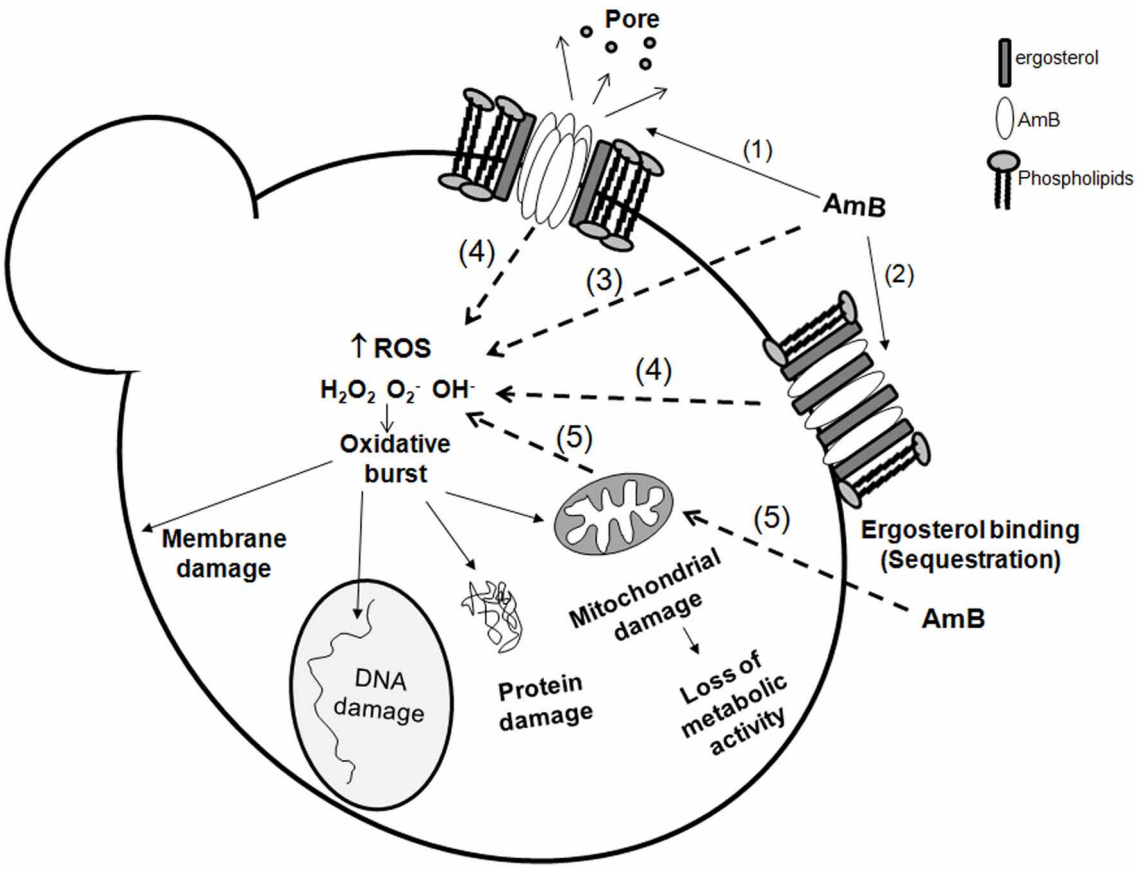

FIGURE 1 | Amphotericin B action mechanisms on fungal cells. Amphotericin B exerts its action at different levels on the cell: membrane effects and intracellular effects. At the membrane, it can bind to ergosterol (1) and form pores, or merely induce ergosterol sequestration (2) resulting in membrane stability disruption. In the cell, AmB also induces an oxidative burst. The mechanism of this induction remains unknown, but there are several possibilities: AmB can act directly as a prooxidant (3) and induce accumulation of reactive oxygen species (ROS). However, it is also possible that this intracellular effect requires previous binding to ergosterol (4). Since ROS are natural products of the respiratory chain, it cannot be discarded that AmB influences the mitochondrial activity (5), and contribute in this way to the oxidative burst. The accumulation of free radicals has multiple deleterious effects on the essential components of the cell (membrane, proteins, DNA and mitochondria) resulting in cell death.

aqueous-pores are formed, which are permeable to monovalent cations and anions and large electrolytes, such as glucose (Cohen and Gamargo, 1987; Ramos et al., 1989; Cohen et al., 1990; Cohen, 1992). The formation of pores is a very rapid process, and occurs in milliseconds. Furthermore, although AmB has affinities for both ergosterol and cholesterol (Hsuchen and Feingold, 1973), pore formation is delayed in liposomes formed with cholesterol (Mouri et al., 2008). The ergosterol and cholesterol content also determines the concentration at which AmB forms aqueous or non-aqueous pores, indicating that membrane composition has a profound effect on the AmB action (Mouri et al., 2008).

Ergosterol is required for multiple processes, such as endocytosis, vacuole fusion, and stabilization of proteins at the cell membrane (Heese-Peck et al., 2002; Zhang et al., 2010). So binding of AmB to these molecules could account for the toxic effect of the antifungal by a mechanism that involves ergosterol sequestration. This idea is supported by a recent work (Gray et al., 2012) that demonstrated that channel formation by AmB is a secondary mechanism that enhances the activity of the drug, but is not required to induce killing in the fungal cells. Using different forms of AmB that had been chemically modified, it was found that modifications that affect pore formation do not affect its antifungal activity. In this sense, it has been shown that other polyenes, such as natamycin, have antifungal effects that are not related to pore formation (Te Welscher et al., 2010).

INDUCTION OF OXIDATIVE DAMAGE

Although it is well established that AmB binds to sterols and forms pores, there are numerous articles that indicate that increased permeability might not be the only mechanism responsible for the killing effect of the molecule. Early studies found that there was not correlation between the lethal effect of different polyenes on *C. albicans* and the degree of potassium release by the cells, suggesting that pore formation does not correlate with killing of the cells (Chen et al., 1978; Sokol-Anderson et al., 1986). This finding indicates that the formation of non-aqueous (cation-selective) pores is not enough to induce killing of the cells, and suggests that AmB elicits other killing mechanism. In this sense, it has been observed that the biological effect of AmB is very complex and depends on a variety of factors, such as the growth phase of fungi (Gale, 1974; Gale et al., 1975; Mowat et al., 2008) and the presence of oxygen (Gale et al., 1977; Sokol-Anderson et al., 1986). These data suggest that AmB action depends on metabolic factors, and indicate that the action mechanism is more complex that binding to ergosterol and pore formation. In fact, some studies argue against the idea that pore formation is the main killing mechanism. Chemical modifications of the AmB molecule that interfere with its ability to form pores do not affect its fungicidal activity (Palacios et al., 2007), which provides strong evidence that pore formation is not essential for the function of the molecule.

In agreement with the idea that AmB has other toxic mechanism than pore formation at the membrane, it has been shown that this antifungal induces oxidative stress in the cells (Sokol-Anderson et al., 1986; Haido and Barreto-Bergter, 1989; Sangalli-Leite et al., 2011). An early study demonstrated that addition of free radicals scavengers, such as catalase and/or superoxide dismutase, protects *C. albicans* protoplasts from the lytic effect of AmB (Sokol-Anderson et al., 1986). Genome-wide expression analysis confirmed that AmB, not only has an effect on the expression of genes involved in ergosterol synthesis pathway, but also induces the expression of stress genes (Liu et al., 2005), providing another evidence that AmB has pleiotropic effects in the fungal cells.

The induction of oxidative damage in the cells has been frequently reported in the literature using independent approaches. The direct production of free radicals by AmB has been measured using probes that emit fluorescence after being attacked by the free radicals, such as dihydrofluorescein diacetate or dihydrorhodamine 123 (Phillips et al., 2003; Sangalli-Leite et al., 2011). Lipid peroxidation, protein carbonylation, and apoptotic-like phenotypes (such as DNA fragmentation and anexin V staining) have been also used as indicators of oxidative stress generated by AmB in fungal cells (Phillips et al., 2003; Mousavi and Robson, 2004; Blum et al., 2008; Al-Dhaheri and Douglas, 2010; Sharma et al., 2010; Sangalli-Leite et al., 2011).

The role of oxidative damage in the antifungal effect of AmB is still unknown, but different studies suggest that this mechanism participates in this effect. A recent study demonstrates that killing of *C. neoformans* cells, measured by propidium iodide uptake, occurs after the induction of an oxidative burst. The mechanism by which AmB induces oxidative burst in the cells remains unknown. Several studies demonstrate that AmB can autooxidize, which suggests a mechanism by which AmB induces oxidative stress in the cells (Lamy-Freund et al., 1985; Sokol-Anderson et al., 1986). On the other hand, it has been demonstrated that AmB can also act as an antioxidant similar to carotenoid and retinoids (Osaka et al., 1997).

AmB induces oxidative damage in organisms others than fungi (Haido and Barreto-Bergter, 1989). Moreover, this feature of the antifungal has been related to the reduction in virulence observed in some parasite infection models. For example, AmB does not have a direct effect on development of the miracidia (larval stages) and sporocyst of the parasite *Schistosoma mansoni*, but it decreases its infectivity through a process linked to the oxidative damage induced by the antifungal that impaired the response of the parasite during infection (Mone et al., 2010).

MECHANISMS OF RESISTANCE TO AmB

Acquired resistance to AmB is very low despite its widespread use. Secondary resistance has been described in *C. tropicalis*, *C. parapsilosis*, *C. lusitanie*, and *C. haemulonii* (Powderly et al., 1988; Ellis, 2002). In contrast, in the last years, there has been an increase in the incidence of infections caused by fungi intrinsically resistant to AmB, such as *A. terreus*, *Fusarium* spp, and *Scedosporium prolificans* (Cuenca-Estrella et al., 1999; Sutton et al., 1999; Khan et al., 2007; Rogasi et al., 2007).

Resistance to this antifungal is achieved in different ways. Decrease in ergosterol content results in resistance to this compound (Kim and Kwon-Chung, 1974; Kim et al., 1974; Woods et al., 1974; Safe et al., 1977; Drutz and Lehrer, 1978; Merz and Sandford, 1979; Kreiner et al., 1993; Kelly et al., 1994; Currie et al., 1995; Ghannoum and Rice, 1999; Walsh et al., 2003; Vandeputte et al., 2007). Most of these studies showed alterations in the ergosterol synthesis pathway and accumulation of sterol intermediates.

Moreover, in biofilms (which are microbial populations that grow attached to a surface and have reduced susceptibility to antimicrobials), resistance to AmB has been associated not only to a decrease in the ergosterol content, but also to changes in the cell wall (Khot et al., 2006). Since azoles inhibit ergosterol synthesis, cross resistance between azole and AmB has been described in the literature (Sud and Feingold, 1983; Kelly et al., 1996, 1997; Nolte et al., 1997; Sanglard et al., 2003).

However, other studies did not find a correlation between ergosterol content and susceptibility to AmB (Joseph-Horne et al., 1996a,b; Dannaoui et al., 2000). In agreement, it has been shown that pre-exposure of *C. albicans* cells to fluconazole can protect the yeasts from AmB treatment, and this effect is still present when ergosterol is added to the medium, suggesting that this resistance phenotype does not depend on ergosterol (Vazquez et al., 1998). Interestingly, it has been recently shown that subinhibitory concentrations of fluconazole induce a response in yeast that confer resistance to oxidative and nitrosative stress (Arana et al., 2010), which supports the idea that adaptation to oxidative stress can result in AmB tolerance. Resistance to AmB has been also studied using genome-wide expression analysis in *C. albicans* (Barker et al., 2004). This work demonstrated that resistance to AmB and fluconazole was associated, not only with an increase in the expression or *ERG* genes, but also with the induction of stress genes such as catalase, and reduction of mitochondrial enzymes, such as cytochrome c oxidase and acetyl CoA synthetase, suggesting that resistance to AmB could be associated to a decrease in mitochondrial activity and reactive oxygen intermediates (ROIs) production.

A strong support for the role of oxidative damage in the antifungal activity of the drug is provided by the relationship between resistance to AmB and to oxidant stress. This was first described in *C. albicans*, where it was observed that resistant strains to AmB had reduced susceptibility to H_20_2 (Sokol-Anderson et al., 1988). In this work, resistance to AmB and H_2O_2 correlated with increased catalase activity. Another evidence of the importance of the oxidative damage was provided in the filamentous fungi *Aspergillus terreus,* which is considered intrinsically resistant to AmB. This fungus has similar ergosterol levels than a susceptible species, such as *A. fumigatus* (Dannaoui et al., 2000; Blum et al., 2008). However, AmB did not induce lipid peroxidation in *A. terreus*, suggesting that this fungus has an induction in antioxidant mechanisms. In agreement, catalase activity in *A. terreus* was significantly higher in this fungus than in *A. fumigatus* (Blum et al., 2008).

The respiratory chain in the mitochondria plays a key role in the production of free radicals in the cells because these molecules are subproducts of the respiration. So it is tempting to correlate the effect of AmB with the mitochondrial activity. Little is known about this correlation, but it has been demonstrated that disruption of respiratory function results in increased resistance to AmB in *C. albicans* (Geraghty and Kavanagh, 2003). This finding is very relevant, especially because the mitochondria is not only required for the accumulation of free radicals, but also because it is necessary for ergosterol biosynthesis, so changes in mitochondrial activity can influence the antifungal activity of AmB at multiple levels.

AmB AS A MOLECULE WITH IMMUNOMODULATORY PROPERTIES

Antifungal drugs are derived from natural compounds with complex structure, and many of them have other effects than growth inhibition or killing of fungi. In this sense, antifungals have inmmunomodulatory properties [see review in Ben-Ami et al. (2008)]. AmB is a good example about this type of drugs. Besides the direct action on the fungal cell, several studies have shown that AmB has a potent immunomodulatory effect on the host cells. This has been demonstrated *in vitro* in different cellular lines from human and murine models, such as polymorphonuclear neutrophils (PMNs), macrophages, NK cells, T, B, and tumoral cells, but also *in vivo* in animal models. The immunomodulatory properties offer an alternative action mechanism for this antifungal by enhancing the immune response of the host. But at the same time, this effect has been related to toxicity associated to this drug. In the following sections, we will briefly review the main immunomodulatory properties of AmB.

IMMUNOMODULATION *IN VITRO* AND *IN VIVO*

Multiple studies performed *in vitro* and *in vivo* have demonstrated that AmB has an effect on the host, not only in the presence of the pathogen, but also when uninfected cell lines or animals are treated with the antifungal. AmB stimulates transcription and production of multiple mediators of the immune system (such as cytokines, chemokines, and prostaglandins) and ICAM-1 in murine and human cells (Borden and Leonhardt, 1976; Sculier and Body, 1991; Cleary et al., 1992; Louie et al., 1994; Saxena et al., 1999; Rogers et al., 2000; Sau et al., 2003; Camacho et al., 2004; Simitsopoulou and Roilides, 2005; Simitsopoulou et al., 2005). Moreover, this antifungal upregulates the expression of genes involved in angiogenesis (Lin et al., 2009). AmB also induces the accumulation of nitric oxide (NO) (Mozaffarian et al., 1997) and ROIs (Wilson et al., 1991). Most of these effects are summarize in **Figure 2**. In endothelial activated cells, AmB increases iNOS expression mediated by endogenous IL-1 and, in consequence, AmB augments the production of NO, which plays important role in vasodilation and protection against pathogens (Suschek et al., 2002).

The immunomodulatory properties and the proinflammatory effect induced by AmB have been associated with protective effects during infection. AmB enhances the antifungal activity of PMN and pulmonary alveolar macrophages against conidia and/or hyphal phase of *A. fumigatus* (Roilides et al., 2002). Similar results were published with murine peritoneal macrophages pretreated with IFN-γ and different doses of AmB. In this case AmB induced the production of NO, TNF-α, and IL-1, that enhanced the anticryptococcal activity of these cells (Tohyama et al., 1996).

Macrophage oxidative burst, leading to O_2^- release, is activated *in vivo* after intraperitoneal injections of recombinant IFN-γ and TNF-α or AmB (Wolf and Massof, 1990). Moreover, when AmB was combined with IFN-γ, a synergic effect was observed, suggesting that IFN-γ may serve as a useful adjuvant during the treatment of intracellular fungal infections.

AmB also produces oxidative burst in macrophages following stimulation with phorbol myristate acetate. This effect was

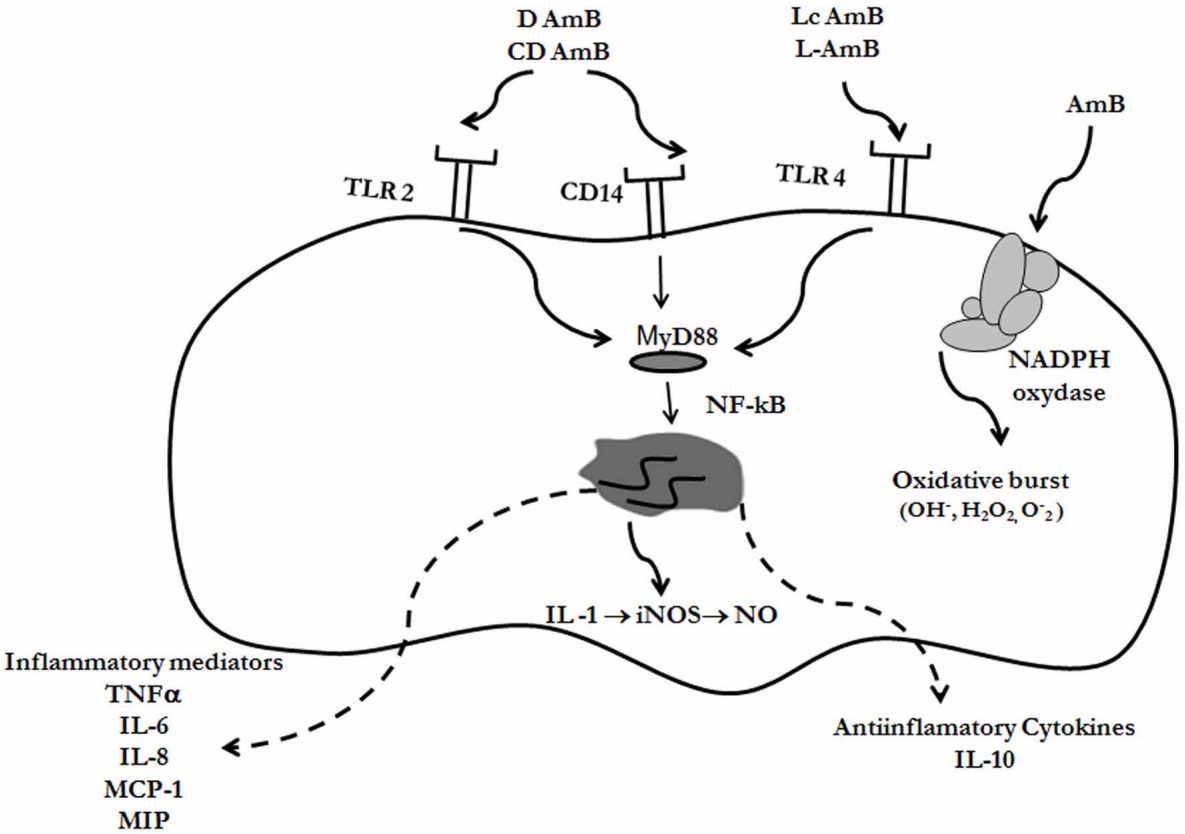

FIGURE 2 | Immunomodulatory effects of AmB. Different formulations of AmB can bind to Toll-like receptors (TLR-2 or TLR-4) or CD14, resulting in immunomodulation of the cell. The signal is transduced through the adaptor protein MyD88, and as a final effect, NF-kB is activated and translocated to the nucleus. In this way, cytokines are expressed, which can be pro- or anti-inflammatory, depending on the AmB formulation, and receptors involved (see text for further details). AmB also induce the accumulation of free radicals (reactive oxygen intermediates, ROIs, and nitric oxide, NO) through induction of NO synthase and NADPH oxidase

related to the binding to the antifungal to the membrane that could in turn induce conformational changes that activate membrane enzymes involved in the induction of oxidative burst, such as NADPH oxidase (Chapman and Hibbs, 1978; Wilson et al., 1991). AmB also has a cooperative effect with IFN-γ in enhancing the candidastatic activity of the macrophages through a process that involves the accumulation of ROIs (Coste et al., 2002). However, the same authors also noticed that AmB had a cooperative effect with IL-13, but this effect was independent of ROIs, indicating that AmB can activate macrophages in different ways.

The outcome of systemic and mucosal fungal infections depends on the Th response of the host. Th1 response (which depends on proinflamamatory cytokines TNF-α, IFN-γ, IL-1, IL-6) leads to resistance because it primes the immune system with macrophage inflammatory activation and superoxide and NO production. In contrast, a Th2 response (IL-10, IL-4, IL-2, IL-13, and IL-5) is associated with susceptibility to infection and disease enhancement (Puccetti et al., 1995; Romani and Howard, 1995). To evaluate the effect of AmB on the Th cell response, mice with disseminated or gastrointestinal candidiasis were treated with antifungal alone or in combination with an IL-4 antagonist, and the production of IFN-γ (Th1) and IL-4 (Th2) was evaluated. AmB induced a protective Th1 response with concomitant IL-4 depletion (Cenci et al., 1997). Similar results were observed in Balb/c mice infected with *A. fumigatus* spores and treated with AmB (Saxena et al., 1999). In agreement, it was described that AmB induces up-regulation of IL-1β and TNF-α in mouse kidney (Falk et al., 2005). The idea that AmB exerts part of its effect through immunomodulation was supported by the fact the antifungal shows a defect in the protection when mice receive neutralizing TNF-α antibodies (Louie et al., 1995).

Since changes in the immune response could have profound consequences in the host, the immunomodulatory properties of AmB can explain some of the secondary effects of the molecule. For example, the increase in proinflammatory cytokines has been correlated with the toxicity of AmB (Chia and McManus, 1990; Cleary et al., 1992; Arning et al., 1995; Shadkchan et al., 2004). In addition, direct renal toxicity has been described by the induction of apoptosis and alterations in the expression of the constitutive NO synthase (Suschek et al., 2000; Falk et al., 2005; Yano et al., 2009).

AmB has been occasionally described to have immunosupressor effects. In the human THP-1 monocytic cell line, pretreatment

with AmB and challenge with *A. fumigatus* conidia results in reduced expression of TNF-α (Choi et al., 2010). Also Becker et al. observed decrease of IL-6, macrophage inflammatory protein (MIP-2), and monocyte chemoattractant protein (MCP-1) in neutropenic rats with invasive pulmonary aspergillosis treated with AmB (Becker et al., 2003).

MECHANISMS BY WHICH AmB INDUCES IMMUNOMODULATION

The mechanism by which AmB produces immunomodulation and induction of ROIs and NO is not fully elucidated. As stated above, AmB binds to the mammalian membrane because it presents affinity to cholesterol, and in this way, it could induce conformational changes that activate the NADPH oxidase enzyme (Chapman and Hibbs, 1978; Wilson et al., 1991) (**Figure 2**). But the mechanism that better explains the immunomodulatory effects of AmB is mediated through the Toll-like receptor (TLRs) signaling pathway (**Figure 2**). TLRs are members of a conserved family of mammalian receptors that recognize microbial products, being TLR2 and TLR4 the best characterized. TLR2 presents affinity for Gram-positive bacteria, peptidoglycan, lipoteichoic acid, and zymosan, whereas TLR4 ligands include LPS from Gram-negative bacteria, Taxol, and *Cryptococcus neoformans* capsular polysaccharide (Shoham et al., 2001; Janeway et al., 2005). AmB can bind to TLR, resulting in cytokine and chemokine release. Binding to TLR2 has been associated to release of proinflammatory cytokines, while binding to TLR4 produced release of anti-inflammatory (Bellocchio et al., 2005). Binding of AmB to the TLRs triggers polymerization of receptors which results in recruitment of the adaptor protein, MyD88. This signaling produces the nuclear translocation of NF-kB, which induces the expression of genes involved in macrophage activation. In addition, AmB also exerts its immunomodulatory effect through CD14 (Trajkovic et al., 2001; Sau et al., 2003), which is a receptor that activates the TLR signaling pathway after binding to LPS.

EFFECT OF THE AmB FORMULATIONS ON THE IMMUNOMODULATORY PROPERTIES

The immunomodulatory properties of AmB depend on the clinical presentation used in the treatment. In a study using plasma of patients treated with different presentations, it was found that D-AmB and L-AmB increased TNF-α, IL-6, and IL-1-RA, but this effect was not observed when patients were treated with CD-AmB (Arning et al., 1995). In human monocytes D-AmB and CD-AmB induced up-regulation of inflammatory cytokines such as IL-1, TNF-α, monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 1 (MIP-1), while LC-AmB lipid complex and L-AmB down-regulated or had no effect on the gene expression of these proinflammatory cytokines (Simitsopoulou et al., 2005). Moreover, D-AmB is more effective than LC-AmB in enhancing PMN oxidative activity and D-AmB induced higher expression of CD11b/CD18 integrin (Mac-1) (Sullivan et al., 1992). On the other hand, using antibody arrays, both D-AmB and CD-AmB induced proinflammatory cytokines in the THP-1 monocytic cell line (IL-8, TNF-γ, MCP-1, and RANTES) while LC-AmB and L-AmB had no effect (Turtinen et al., 2004). This difference between the AmB formulations can be explained by the type of TLR to which the different AmB presentations bind. D-AmB binds to TLR2, which induces a proinflammatory response, in contrast to L-AmB which induces anti-inflammatory effect after binding to TLR4 in PMNs (Bellocchio et al., 2005).

CONCLUSIONS AND FUTURE PERSPECTIVES

AmB is still an enigmatic molecule, and although it has been vastly used for the treatment of fungal infections during decades, there are still aspects about its action mechanism that remain unknown. Although the first studies demonstrated that this drug binds to sterols and in particular, ergosterol, and forms pores at the membrane, it has been also shown that AmB induces oxidative damage in the cells. There are contradictory data about the importance of these mechanisms. The fact that resistance to this antifungal correlates with different mechanisms, such as a reduction in ergosterol content or induction of antioxidant enzymes indicates that most probably both are required for the killing effect of the molecule. At the moment, it is not possible to know if these mechanisms are related or are independent, although a tempting hypothesis is that binding to ergosterol is necessary not only for pore formation, but also for the induction of oxidative damage. Further studies are required to clarify the importance of each mechanism. In addition, the induction of different killing mechanisms is in agreement with the fact that AmB is the antifungal drug with a stronger fungicidal activity. To make the situation more complex, AmB has also strong immunomodulatory properties, and in particular, it induces proinflammatory responses. This effect has been associated with protective effects, but also with the toxicity. The immunomodulatory properties of the antifungal open many questions about how AmB acts during infection, not only on the pathogen, but also on the host. This issue is of particular interest because patients affected by fungal infections are immunocompromised. So it is important to consider that AmB may have different effects on patients with different immunological states and therefore, the antifungal treatment could have unpredicted consequences in the outcome of the disease. Although AmB is one of the most effective treatments for fungal infections and secondary clinical resistance remains low, there is an increase in the incidence of pathogens that have intrinsic resistance to this antifungal, such as *Trichosporon* spp, *A. terreus* and *Scedosporium prolificans*, so more studies are required to understand the basis of intrinsic resistance and to provide an efficient strategy for the management of these infections.

ACKNOWLEDGMENTS

Ana C. Mesa-Arango is funded by a grant from Fundación Carolina and University of Antioquia, Medellin, Colombia, and Liliana Scorzoni is supported by fellowship from the Agencia Española de Cooperación Internacional para el Desarrollo. Oscar Zaragoza is funded by grant SAF2011-25140 from the Spanish Ministry for Economics and Competitivity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 May 2012; paper pending published: 28 May 2012; accepted: 21 July 2012; published online: 08 August 2012.

Citation: Mesa-Arango AC, Scorzoni L and Zaragoza O (2012) It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. Front. Microbio. ***3****:286. doi: 10.3389/fmicb.2012.00286*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.