Advances in combating fungal diseases: vaccines on the threshold

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Abstract | The dramatic increase in fungal diseases in recent years can be attributed to the increased aggressiveness of medical therapy and other human activities. Immunosuppressed patients are at risk of contracting fungal diseases in healthcare settings and from natural environments. Increased prescribing of antifungals has led to the emergence of resistant fungi, resulting in treatment challenges. These concerns, together with the elucidation of the mechanisms of protective immunity against fungal diseases, have renewed interest in the development of vaccines against the mycoses. Most research has used murine models of human disease and, as we review in this article, the knowledge gained from these studies has advanced to the point where the development of vaccines targeting human fungal pathogens is now a realistic and achievable goal.

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For many years, the concept of developing vaccines against fungal diseases attracted little interest, but this has changed in the past fifteen years (reviewed in REFS 1–6) because of the dramatic increase in the incidence rates of fungal diseases worldwide. Carbohydrate and, especially, protein antigens that exert protective immunity against various fungal diseases have now been identified. Fungal carbohydrates can induce the production of antibodies that enhance host resistance in many ways and, fuelled by advances in cellular and molecular biology, numerous fungal proteins that trigger T-cell-mediated immunity and that are immunogenic in murine models of fungal disease have been identified (TABLE 1). A vaccine based on one or more of these candidate antigens could prevent disease by inducing protective antibodies, T-cell-mediated immunity or a combination of both of these aspects of the host immune response. In this Review, we will assess the state of fungal vaccine development, both prophylactic and therapeutic.

Overview of the immune response to fungi

Successful resolution of the diseases caused by pathogenic fungi is crucially dependent on the coordinated interactions of many constituents of the host immune response. The host response to these organisms varies, as would be expected for such a heterogeneous group of pathogens, which differ morphologically, genetically and biochemically. Therefore, the effector molecules and cells that are important for combating opportunistic fungi such as *Candida* and *Aspergillus* spp. are less important for primary pathogens such as *Coccidioides* spp. and *Histoplasma capsulatum* (BOX 1).

The innate response to fungi. As with all pathogens, the innate immune system is a crucial determinant in the antifungal response (FIG. 1). Host cell-surface receptors are instrumental in the initial contact with fungi, which can bind to or engage several receptors, including Toll-like receptors, dectin-1, the mannose receptor, Fc receptors and integrins[7–13]. Engagement of these receptors by fungi such as *Candida* spp. can lead to the release of inflammatory mediators and the activation of innate immunity or, in the case of *H. capsulatum*, inhibition of innate immunity[14–16]. Binding of *H. capsulatum* to the CD11b cell-surface glycoprotein suppresses the production of interleukin 12 (IL-12) by macrophages and results in a suboptimal or absent T-helper 1 (T_H1) response[14] (BOX 2).

Neutrophils, macrophages and dendritic cells (DCs) constitute the cellular effectors of innate immunity against fungal pathogens. Neutrophils are essential for host defence against *Candida*, *Fusarium* and *Aspergillus* spp., and their absence is a major risk factor for the acquisition of infection and development of disease[17]. Although neutrophils seem to be essential, they can also impair the host response. For example, murine neutrophils expressing the CD80 cell-surface marker inhibit the expression of the T_H1 response in an experimental model of candidiasis[18], and depletion of mouse neutrophils in an experimental model of cryptococcosis enhances rather than diminishes protective immunity[19]. The main contribution of neutrophils resides in their phagocytic and microbicidal functions, but they can produce cytokines and chemokines that can modulate the protective immune response.

Table 1 | **Fungal protective antigens and immunogens that rely on antibody and/or cell-mediated immunity**

Antigen or immunogen	Proposed mechanism of protection	Refs
Candida *spp.*		
β-1,2-linked mannobiose and mannotriose	Antibody; antibody-dependent complement opsonization	33,46
β-1,3-linked glucan	Antibody; possibly directly candidacidal	40,69
Heat shock protein 90 (Hsp90); epitope LKVIRK of Hsp90	Antibody; possibly by neutralizing effects of Hsp90 on host proteins	143,156
Antibody idiotope specific for yeast killer toxin	Anti-idiotypic antibody; directly candidacidal	43
Unknown, possibly protein	Antibody (mAb C7); antibody-dependent complement opsonization and possibly directly candidacidal	44,80
Secreted aspartyl proteinase	Antibody	157
Mannoprotein 65	Antibody	157
Agglutinin-like sequence 1 and 3	Unknown	158
Aspergillus *spp.*		
β-1,3-linked glucan	Antibody; possibly directly candidacidal	40,69
Aspf16	Unknown	89
Cryptococcus neoformans		
Glucuronxylomannan (GXM) and GXM peptide mimotopes	Antibody; enhance effectiveness of CMI by various mechanisms	5
Polysaccharide deacetylase	IFN-γ dependent	90
Coccidioides *spp.*		
Proline-rich antigen (also known as Antigen 2)	IFN-γ dependent	91
ELI antigen (unknown function)	Unknown	110
Aspartyl transferase	Unknown	
Gel-1 (β 1,3 glucosyltransferase)	Unknown	159
Urease	Unknown	93
Peroxisomal matrix protein	Unknown	160
Chimeric proline-rich antigen plus *Coccidioides*-specific antigen	Unknown	103
Chimeric protein-aspartyl proteinase, phospholipase B and α mannosidase	Unknown	104
Live attenuated strain harbouring deletion of two chitinase genes	Unknown	G. Cole, personal communication
Histoplasma capsulatum		
Histone-H2B-like protein	mAb; unknown mechanism	36
Hsp60	Induction of IFN-γ and IL-10; $CD4^+$ T-cell dependent	87
H antigen	Unknown	161
80-kilodalton antigen	Unknown	162
Sec31 homologue	Unknown	163
Blastomyces dermatitidis		
BAD1	Unknown	88
BAD1-deleted live attenuated strain	$CD4^+$ T cells, $CD8^+$ T cells and select T_H1 cytokines, depending on host's immune status	See text
Pneumocystis carinii		
Major surface glycoprotein (also known as gp120)	Antibody and T-cell dependent	164
p55	Antibody and T-cell dependent	165
Kexin	Antibody and CD40 ligand-dependent	166
Paracoccidioides brasiliensis		
gp43	T-cell dependent	115

BAD1, *Blastomyces* adhesin 1; CMI, cell-mediated immunity; ELI, expression library immunization; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; T_H1, T-helper 1.

Box 1 | **Fungal pathogens: primary pathogens and opportunists**

Medically important fungi can be categorized as opportunists or primary pathogens. The opportunists rarely cause disease in an immunocompetent host whereas the primary pathogens do. The opportunists are *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans* and *Pneumocystis jirovecii*. The primary pathogens referred to in this review are *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii* and *Blastomyces dermatitidis*. However, the primary pathogens can become opportunists when host immunity wanes. The distinction between primary pathogen and opportunist is not well-defined, although some have argued that susceptibility of the mould form to toxic oxygen radicals might distinguish a primary pathogen from an opportunist[145]. Generally, exposure of the host organisms to non-lethal challenge with a primary pathogen leads to resolution of infection. However, in the case of *H. capsulatum* or *B. dermatitidis*, the organism can establish a dormant state and reactivate spontaneously or when the host's immune system is impaired. Recovery is dependent on the activation of a T-helper 1 (T_H1) response. The opportunists manifest heightened virulence only when the host's defence mechanisms are impaired.

Macrophages are an important phagocytic population in host defence against fungi. Not only can they ingest organisms that have been opsonized with antibody, complement or collectins, they can also phagocytose unopsonized fungal elements through recognition receptors such as the integrins[7,8]. This phagocytic population has many functions, including fungistatic and fungicidal activities, the production of cytokines and chemokines, and antigen presentation to both $CD8^+$ T cells and $CD4^+$ T cells. For some intracellular fungal pathogens, such as *Cryptococcus neoformans*, *H. capsulatum* and *Blastomyces dermatitidis*, their intracellular location protects them from host defences, and these organisms thrive within macrophages.

Immature DCs can engulf and kill several fungal species[20–22]. One mechanism of killing is the increased mobilization of phagolysosomes, which generates an inhospitable environment for fungal survival[22]. Most often, ingestion of fungi or fungal antigens leads to DC maturation and increased efficiency of fungal antigen presentation. DCs can also distinguish between different fungal morphotypes. These cells ingest both the yeast and hyphal forms of *Candida albicans* and the conidial and hyphal forms of *Aspergillus* spp.[21] DCs use distinct receptors to recognize each form of a particular fungus, thereby activating different signalling pathways with distinct functional consequences.

The non-cellular effectors of innate immunity comprise collectins, complement and natural antibodies. These molecules mediate opsonization and therefore promote the ingestion of fungi by phagocytes. One member of the collectin family, pentraxin 3, is necessary for the response to *Aspergillus* spp.[23], and the pulmonary collectins surfactant proteins A and D not only cause aggregation of fungi but also have fungicidal activity[24]. The fate of opsonized fungi can differ from that of unopsonized organisms, probably because in phagocytes, opsonized fungi traffic through a different pathway to opsonized organisms.

Toll-like receptors
A family of receptors present on mammalian cells that recognize pathogen-associated molecular patterns.

Dectin-1
A C-type lectin pattern recognition receptor mainly found on phagocytes that is involved in the innate immune response to fungal pathogens by recognizing β-glucans.

Mannose receptor
A lectin-like pattern recognition receptor expressed on the surface of macrophages, endothelial cells and immature dendritic cells that recognizes microbial glycans.

Fc receptor
Surface molecules on various cells that bind to the Fc regions of immunoglobulins, thereby initiating effector functions.

Integrins
A large family of heterodimeric transmembrane glycoproteins that function as adhesins, mediating adhesion to other cells, either microbial or host.

Complement
Proteins found in the serum that can be activated by proteolytic cleavage, resulting in the generation of molecules that can bind to specific surface receptors on mammalian cells and can lead to the formation of a terminal cell-lytic complex in the membrane of a target cell. Complement fragments such as those derived from C3 and C5 have important pro-inflammatory properties, such as vasodilation, chemotaxis and opsonization.

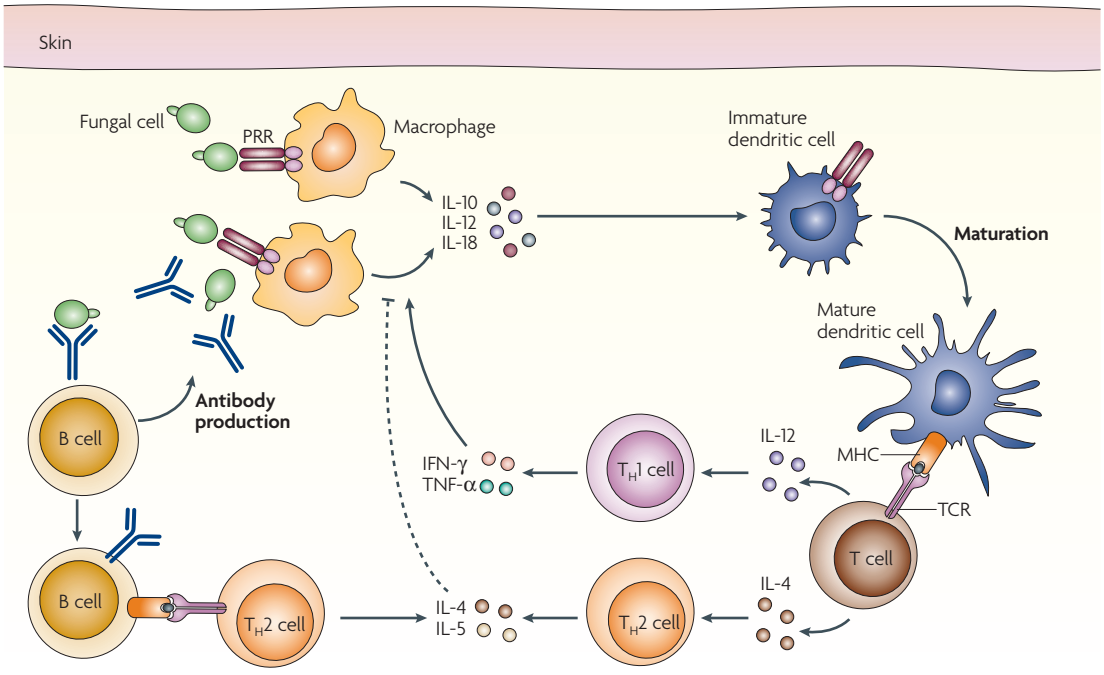

Figure 1 | **The host response to fungi.** The figure shows the complex interaction between fungi or fungal antigens and the host immune response. Dendritic cells (DCs) process and present antigens through class I or class II major histocompatibility complex (MHC) molecules to antigen-specific clones of T cells endowed with the capacity to recognize the peptide epitopes through specific T-cell receptors (TCR). The production of interleukin (IL)-12 by DCs leads to the outgrowth of T-helper 1 (T_H1) cells that produce interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or both. The T_H1 response leads to enhanced fungistatic and fungicidal activities by phagocytes. The induction of IL-4 (and failure to produce IL-12) by DCs leads to a T_H2 response, which blunts the generation of protective immunity. PRR, protein recognition receptor.

Box 2 | The T_H1/T_H2 response in fungal diseases.

The induction of a dominant T-helper 1 (T_H1) response is crucially important in the host response to naturally acquired infection with pathogenic fungi. The T_H1 cytokines IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α are required for the clearance of infection with most, if not all, of these pathogens, especially in primary disease[26]. By contrast, progressive disease in immunodeficient or susceptible mice is associated with a shift in the balance between T_H1 and T_H2, towards the T_H2 response[26]. The latter is characterized by upregulation in IL-4, IL-5 and IL-10, an increase in tissue eosinophils and elevated levels of IgE. Neutralization of IL-4, IL-5 and IL-10 *in vivo* can sometimes restore protective immunity[84, 146,147]. A dominant T_H2 response is evoked when the host fails to synthesize sufficient amounts of T_H1 cytokines such as IFN-γ and TNF-α[84,148,149]. For example, the neutralization of TNF-α or granulocyte–macrophage colony-stimulating factor (GM–CSF) in mice infected with *Histoplasma capsulatum* results in a sharp increase in the levels of IL-4 and IL-10 (REFS 146,147). Both cytokines block the expression of a protective response. Aside from the absence of a cytokine, a T_H2 response can also be elicited if a receptor for a T_H1 cytokine (for example, the IFN-γ receptor) is blocked or absent[150]. However, the elaboration of at least some T_H2 cytokines also helps to balance the immune response.

Collectins
C-type lectins that have a collagen-like domain, including mannose-binding lectin and the two mucosal-associated proteins, surfactant protein A and D.

CD8⁺ T cell
A subpopulation of T cells that express the CD8 receptor. $CD8^+$ T cells recognize antigens that are presented on the surface of host cells by major histocompatibility complex class I molecules, leading to their destruction, and are therefore also known as cytotoxic T cells.

CD4⁺ T cell
A subpopulation of T cells that express the CD4 receptor and respond to antigens presented on the surface of host cells that have major histocompatibility complex class II molecules. Two distinct subsets of activated $CD4^+$ T cells have been described. T-helper 1 (T_H1) cells produce interferon γ, tumour-necrosis factor α and interleukin (IL-)12, and support cell-mediated immunity. T_H2 cells produce IL-4, IL-5 and IL-13, support humoral immunity and downregulate T_H1 responses.

Phagolysosome
An intracellular vesicle that results from the fusion of phagosomes that enclose ingested extracellular material and lysosomes, which contain lytic enzymes.

Opsonization
The deposition of antibody or complement products on the surface of microorganisms that can facilitate recognition and uptake.

The adaptive response to fungi. For all fungi, T-cell activation is a crucial element in the development of optimal protective immunity (FIG. 1). Both $CD4^+$ and $CD8^+$ T cells are necessary for the elimination of fungal pathogens; however, in the primary stages of disease, for many fungi the presence of $CD4^+$ T cells is vital for the survival of the host, whereas $CD8^+$ T cells are necessary to restrict infection[25]. In secondary disease, each T-cell subset is dispensable, therefore, the absence of either $CD4^+$ or $CD8^+$ T cells does not lead to overwhelming disease.

The main effector mechanisms of T cells are cytotoxicity and cytokine secretion. The role of cytotoxicity in host defence against fungi is not well delineated, however the activity of cytokines is better understood. For all of the pathogenic fungi, a T_H1 response is the dominant adaptive response[26–28] (BOX 2). The absence of the T_H1 cytokines interferon (IFN)-γ or tumour necrosis factor (TNF)-α leads to overwhelming disease. The T_H2 response is often associated with a subversion of the host response to fungi. Increases in the T_H2 cytokines IL-4 and IL-10 are commonly observed in progressive disease, and neutralizing their activity restores protective immunity[26]. One possible beneficial effect of T_H2 cytokines is that they dampen the damage associated with an exuberant inflammatory response.

Nearly all fungi elicit an antibody response, yet there is little evidence that these antibodies modulate pathogenesis. The acquisition of antibodies to *C. albicans* and *C. neoformans* occurs early in life, yet there is little evidence that these antibodies either confer protection or exacerbate disease[29]. The generation of numerous antibody specificities in response to infection and disease results in a heterogeneous polyclonal antibody population. Individually, these antibodies might enhance immunity, dampen immunity or exert no effect. The algebraic sum of their activities defines the ultimate function of the antibody response *in vivo*. The fact that polyclonal antibodies do not confer protection does not exclude the possibility that protective antibodies are generated; identifying those protective antibodies often requires examination at the monoclonal level.

Evidence for protective antibodies against fungal disease. Why has a role for antibodies in host defence against fungal disease been questioned? As alluded to above, one reason is that patients typically have antifungal antibodies before and/or during disease. Experimentally, only a smattering of reports have shown evidence of beneficial antibodies being generated following immunization with whole fungal cells[30,31]. Evidence now abounds, however, that protective antibodies must be of the correct specificity, isotype and titre (for examples, see REFS 32–34). The enormous antigenic complexity of fungal cells probably precludes the production of antibodies that fulfil these criteria whether during colonization, tissue invasion or immunization with whole cells.

The most compelling data for a role for antibodies in protective immunity against fungal diseases come from studies on the opportunistic mycoses, especially cryptococcosis and candidiasis, but also aspergillosis. The aetiological agents *in vivo* are primarily extracellular, but *C. neoformans*, the cause of cryptococcosis, is considered a facultative intracellular pathogen[35]. Research has focused on fungal cell-surface carbohydrates, including the capsular material of *C. neoformans* and the cell-wall polysaccharides of *Candida* and *Aspergillus* species (discussed in more detail in the next section).

Antibody protection can also occur through recognition of specific proteins, such as heat shock protein 90 (Hsp90) and mannoprotein 65 (MP65) in *C. albicans* and the histone-H2B-like surface protein found on tissue-phase yeast cells of the endemic fungus *H. capsulatum* (TABLE 1). The latter finding, based on the isolation of a protective monoclonal antibody[36], is intriguing because the yeast form of *H. capsulatum* is found in macrophages, implying that an appropriate antibody response could augment host defences against intracellular pathogens[37]. Some researchers have raised the question of whether protective antibodies also function against other endemic dimorphic fungi[38], such as *Coccidioides* spp., *B. dermatitidis*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii* and *Penicillium marneffei*.

A role for antibodies in defence against fungal disease might seem to be in conflict with the underlying T_H1-directed response that is the most widely accepted explanation for host-acquired specific immunity against fungi. On closer examination, however, in at least one case, T_H2-derived antibodies have a protective effect by somehow augmenting cell-mediated immunity[39]. In other cases, antibodies can function as opsonins, promoting fungal ingestion and even killing by phagocytes, and several antibodies are directly fungicidal[40–47] (TABLE 1). These kinds of activities are complementary, rather than exclusive, to cell-mediated mechanisms. The central importance of granulocytes and macrophages in innate defence against opportunistic fungal pathogens, and of activated neutrophils and macrophages against fungi in general, is consistent with an expectation that opsonic antibodies facilitate host defence. Likewise, fungicidal antibodies acting independently of additional host factors would be of obvious benefit to the host.

Box 3 | **Adjuvants and conjugates**

An adjuvant can be defined as a substance that can enhance the antigenicity of an immunogen. A conjugate is a substance that can be fused with an immunogen and promotes its effect. The injection of a protein or carbohydrate into a host often does not lead to an immune response as assessed by T- or B-cell activation. Under these circumstances, adding an adjuvant or conjugating an immunogen to an antigen can substantially elevate the potency of the protein or carbohydrate antigen.

In vaccinology, two adjuvants have been used over the years. In mice, Freund's adjuvant consisting of killed *Mycobacteria* suspended in an oil emulsion was the standard bearer. Although highly useful, this adjuvant is no longer recommended for use in animals. The other adjuvant, alum, has been used in humans for years. However, this adjuvant often elicits a T-helper 2 (T_H2) response, which is not the type of response necessary to combat most fungal diseases.

There has not been a systematic study of adjuvants or conjugates in fungal vaccine development. Their use would be limited to material that is non-replicating. CpG DNA, which engages Toll-like receptor 9 (REF. 151) and commercial preparations consisting simply of oil or an admixture of mycobacterial constituents and lipid A have been used. In addition, delivery of carbohydrates in liposomes has also been shown to provoke an antibody response[58]. Heat shock protein 70 has been reported to possess adjuvant activity but its use in fungal vaccines has not been explored[152]. β-glucan, a constituent of most fungal cell walls, has long been recognized for its potent adjuvant properties[153].

The use of conjugates is another route to enhance antigenicity. As reported in the text, the glycoconjugate vaccine that fuses the poorly immunogenic β-glucan laminarin to the diphtheria toxoid CRM 197 is one such example. Here, the toxoid serves as a carrier protein to enhance the immunogenicity of β-glucan. The glucan–CRM conjugate proved to be immunogenic and protective against *Candida* and *Aspergillus* spp.[40] Multivalent vaccines have been shown to be more potent than single antigens. The linking of two or three antigens or antigenic epitopes confers superior protection in models of coccidioidomycosis[103]. However, this vaccine preparation still requires an adjuvant.

As many antigens from fungi are glycosylated, the influence of the carbohydrate molecule has been unclear. Evidence points to the fact that glycosylation enhances immunogenicity of the model protein ovalbumin, as assessed by T-cell proliferation[102]. Moreover, antigens from *Cryptococcus neoformans* contain mannose residues and use the mannose receptor for entry[9]. The absence of the receptor sharply reduces the recognition of the antigen. Therefore, entry into the mannose-receptor pathway seems to be an important determinant for subsequent antigen processing and presentation.

One challenge facing investigators interested in vaccine development is developing vaccines that induce protective, rather than non-protective, antibody responses, as discussed below.

Carbohydrate antigens and protective antibodies

The accessibility of antigens to the immune response, rather than their chemical composition, is a key consideration in selecting a carbohydrate antigen for vaccine development. The major capsular polysaccharide of *C. neoformans*, glucuronoxylomannan (GXM), conjugated to the tetanus toxoid resulted in anti-GXM protective antibody responses[48,49], and monoclonal antibodies specific for GXM protect against experimental cryptococcosis[50]. However, the pleiotropic effects of GXM on host immunity (reviewed in REFS 51,52), and the variable protective responses to GXM–carrier conjugates[53] (BOX 3), militate against the use of intact GXM in human vaccine development. Coupling a heptasaccharide that is thought to be the major GXM immunodeterminant to a protein carrier induced antibodies against the heptasaccharide[53]. Presumably, the heptasaccharide will not have GXM-like side effects, but this presumption requires testing, as does the question of whether the antibodies are protective (BOX 4). The GXM peptide mimotope P13 conjugated to the tetanus toxoid prolonged the survival of cryptococcal-infected transgenic mice owing to the production of human P13-specific IgG2 (but not IgG1)[54]. The effects of immunoglobulin isotype correlate with the clinical observations, as IgG2 is commonly produced in response to bacterial capsular polysaccharides[45] and by normal adults in response to GXM, whereas IgG1 is associated with paediatric AIDS patients at risk of developing cryptococcosis[55].

In *C. albicans*, short-chain β-1,2-linked oligomannosides, either as part of the phosphomannan complex that is *N*-linked to cell-wall matrix proteins[56] or as cell-wall-associated phospholipomannan complexes[57], are recognized by anti-mannan antibodies that are protective against experimental candidiasis[58–60]. These oligomannosides are produced by *C. albicans* serotype A and B strains, *Candida tropicalis*, most *Candida glabrata* strains[61,62] and *Candida lusitaniae*[63]. Antibodies against β-1,2-linked mannotriose or mannobiose protect mice against hematogenously disseminated candidiasis involving both *C. albicans* serotypes and *C. tropicalis*[58], and protection is expected to be elicited against other species of *Candida* that constitutively produce β-1,2-linked oligomannosides. *In vitro* synthesis of β-1,2-oligomannosides[64] has led to the ability to mass produce this epitope[65] and prototype vaccines consisting of synthetic β-trimannose coupled to protein carriers have been produced[66]. Normal rabbits produce high antibody titres to a trimannose–tetanus toxoid conjugate and, when rendered immunocompromised, they show enhanced resistance to disseminated candidiasis (David Bundle, personal communication), which is consistent with antibody protection in neutropenic mice[67]. These, and further studies showing the effectiveness of beneficial antibodies against experimental *C. albicans* vaginal infection of mice[68], have prompted discussion on the need for a vaccine against candidiasis, including the safety concerns regarding the induction of an immune response that might negatively impact on a fungus that is a member of the human normal microbiota[1].

Recently, another fungal cell-wall polysaccharide — β-glucan — has been identified as a possible target for the induction of protective antibodies[40]. This finding

Mimotopes
An epitope derived from a peptide library that mimics the stimulatory activity of a natural epitope.

Box 4 | **Immunoglobulin classes or isotypes**

There are five main classes or isotypes of human immunoglobulins (Igs) produced by B cells: IgM, IgD, IgG, IgA and IgE. In addition, there are four subclasses of human IgG, namely IgG1, IgG2, IgG3 and IgG4, and two subclasses of IgA. Monomeric IgM and IgD are antigen receptors on B cells and, following an encounter with antigen, the B cells can differentiate into plasma cells that produce and secrete pentameric IgM, which can be detected in the blood. In the presence of appropriate T helper (T_H) cell cytokines, these same B cells become stimulated to rearrange the DNA of the genetic elements that code for the Ig heavy chains and shift from being IgM-producing cells to cells that can produce IgG, IgA or IgE. In the ontogeny of the immune response, IgM is the first antibody to appear in the bloodstream following antigenic stimulation. If the antigen has a high density of repeating epitopes, as typified by polysaccharides, which are specifically recognized by the B-cell receptor, T_H cell interactions are minimal and IgM remains the primary Ig class in response to these so-called T-cell-independent type antigens. If the antigen is a protein or a polysaccharide antigen covalently coupled to a protein carrier, these T-cell-dependent antigens require the involvement of T_H cells that secrete cytokines. Depending on the cytokine produced, the Ig response can shift from the IgM to the IgG subclasses, and to IgA and IgE. In addition to inducing an Ig class shift, the cytokines promote B-cell clonal expansion, resulting in a heightened or memory-cell response on future encounters with the T-cell-dependent antigen.

Mice also produce the five basic isotypes or classes of Igs, but the subclasses of IgG are IgG1, IgG2a or IgG2c, IgG2b and IgG3. The effector functions of mouse IgG subclasses are not directly comparable to human IgG subclasses, but there are some important similarities, as pointed out below.

The Ig shift that can occur in response to T-cell-dependent antigens is essential from several standpoints[154]. The epitope specificity of the original IgM and subsequent Ig shifts that occur within a given B cell remain the same, but the affinity for the epitope can be increased by a process known as affinity maturation. Also, the Fc region of the heavy chains, that is, the part of the Ig not directly involved in combining with the epitope, has important effector functions. Whereas the Fc region of IgM, IgG1 and IgG3 in humans, and of IgM and IgG3 in mice, engenders these Igs with the ability to activate the classical or antibody-dependent complement activation pathway, IgG2 and IgG4 of humans and IgG1 of mice interact poorly with complement[154,155]. IgA is known as secretory antibody as it is found as a protease-resistant dimer primarily on mucosal surfaces, but it is found as a monomer in the bloodstream and breast milk, and fixes complement only weakly[154]. The Fc region of the IgE heavy chains is recognized by high-affinity Fcε receptors on mast cells and basophils[154]. The combination of antigen with specific IgE adsorbed onto mast cells can result in degranulation of these cells and the release of pharmacologically active mediators of type I (immediate) hypersensitivity reactions. There are other important effector functions of Igs, such as transplacental passage primarily of IgG subclasses; in humans IgG1 and IgG3 subclasses pass especially well[154]. Therefore, an ideal antibody-inducing vaccine will cause the production of antibodies that must not only have the correct specificity, but are also of the correct class or subclass to ensure protective effector function.

is striking for at least two reasons. First, the discovery was made by immunizing mice with the algal glucan laminarin, indicating that the β-glucan structure is phylogenetically conserved. Second, glucan is common to many fungi and the antibodies protected mice against experimental candidiasis and aspergillosis. This observation prompted the suggestion that β-1,3-glucan could be part of a universal antifungal vaccine[40,69].

Mechanisms of antibody protection

Antibodies protect the host from infectious processes by various complementary mechanisms (FIG. 2). The simplest example is direct interaction between the antibody and the foreign material, causing its neutralization. In other cases, additional factors that interact with antibodies include constituents of the innate immune system such as complement and cellular components, most notably neutrophils, monocytes and macrophages. The most complex mechanism, which is the least understood, involves the linking of antibody function to T-cell-dependent immunity.

The oldest recognized protective function of antibodies is toxin neutralization. The formation of an antibody–toxin complex subverts the interaction of the toxin with host-cell receptors. This function is shown by antibody neutralization of clostridial toxins and is dependent on the specific interaction of the antibody-combining site with the toxin[70]. Although this interaction prevents toxin entry into susceptible host cells, recent work on the differential effects of protective antibodies against the A and B chains of the toxin ricin, which is an A/B type toxin (as are the diphtheria, cholera and pertussis toxins), indicates that the mechanisms involved in antibody neutralization of toxins could be more complex than simply preventing the binding of the toxin to its receptor[71]. A related mechanism is antibody-mediated viral neutralization; high-affinity binding of antibodies to some virions interferes with their attachment to host receptors, preventing entry of the virions into susceptible host cells[72].

Antibody-dependent opsonization is another protective mechanism. A capsule characteristically prevents host phagocytic cells from ingesting and killing encapsulated microorganisms, such as *Streptococcus pneumoniae*[73]. Through a process called opsonization, which has been known for more than a century, capsule-specific IgM and IgG antibodies (BOX 4) promote ingestion of encapsulated microorganisms[73]. Protection by antibodies is markedly enhanced in the presence of complement, as ingestion involves both phagocyte Fc receptors, which recognize the heavy chain of IgG, and complement receptors, which recognize the complement molecule C3b, which is degraded to iC3b following deposition or fixation of C3b onto the capsular surface[74].

Fc region
The region of an antibody that is responsible for binding to antibody receptors on cells and the C1q component of complement.

Type I (immediate) hypersensitivity reaction
An immediate or type I hypersensitivity reaction is a pattern of allergy that can be largely attributed to IgE and a sub-population of immune cells, mast cells and basophils. These cells de if sufficient antigen reacts with IgE antibodies, which function as receptors on the cell's surface, resulting in a range of allergic symptoms.

Some infectious organisms, most notably certain Gram-negative bacteria, but not fungi, are lysed following activation and deposition of late-acting complement components (C5b–C9) onto their cell surface. This might occur as a result of direct activation of complement factor C3 by the bacterial surface through the alternative pathway of complement activation, or by activation of complement factor C1, the classical or antibody-dependent pathway of complement activation that occurs after the antibody reacts with the bacterial cell surface[75].

Antibodies can have direct microbicidal or microbistatic action on attachment to microbial surfaces. As demonstrated by recent work on fungi (see below), antibodies can directly inhibit the growth of fungal cells following an interaction with the fungal surface. This is an exciting finding because of the potential for the application of pre-formed or vaccine-induced antibodies in immunodeficient patients.

As discussed below, there could be additional ways by which antibodies interact with fungal cells, such as *C. neoformans*, that somehow promote T-cell-mediated immunity against the fungus. Furthermore, studies on this fungus and parallels found with other intracellular parasites indicate that antibodies might have a role in regulation, both up and down, of inflammatory responses[76].

Most of our insights into the mechanisms of antibody protection against fungal diseases have been gleaned through controlled studies using monoclonal antibodies, rather than attempting to sort out the polyclonal responses generated *in vivo*. The mechanisms of antibody protection are complex, incompletely understood and vary depending on the antibody isotype and the mycosis (TABLE 1). A few examples have been chosen that show the complexity of this topic.

The role of opsonizing antibodies in protection against encapsulated bacteria, such as *S. pneumoniae*[73], leads one to expect that the anti-phagocytic capsular polysaccharide GXM of *C. neoformans*[77] would be opsonically neutralized by protective antibodies. In an elegant experiment, a set of three mouse monoclonal antibody isotypes was generated, all derived from the same B-cell parent and with identical GXM specificity but different opsonic activity[78] (BOX 4). Surprisingly, the mouse isotypes with known high opsonic activity, IgM and IgG3, had low protective activity against murine cryptococcosis and the isotype with low opsonic activity, IgG1, exhibited the highest level of protection[78]. This is consistent with an earlier report[79], and the data indicate that IgG1-mediated protection occurs through enhanced $CD4^+$ T-cell-dependent immunity and increased organization of the cell-mediated inflammatory response[39]. Certain IgM antibodies specific for GXM can also be protective, possibly by altering the structure of the capsule and preventing sloughing of GXM, while at the same time having non-complement-dependent opsonic activity[10].

The anti-mannan antibody-mediated response against experimental candidiasis, on the other hand, depends on the activation of complement and the rapid deposition of complement factor C3 onto the fungal cell surface[46,47]. In these experiments, β-1,2-mannotriose-specific IgM and IgG3 showed the highest protection, whereas IgG1 with the same epitope specificity had little or no protective value. Furthermore, protection by the IgM and IgG3 isotypes was dependent on the presence of C3 in the test mice[46].

The mechanism of protection conferred by anti-glucan antibodies is unknown, but the reported *in vitro* direct candidacidal activity[40] is potentially exciting. That is, if antibodies generated by a glucan vaccine are candidacidal in humans, then these antibodies would be expected to benefit all patients, regardless of their immunocompromised condition.

The finding that antibodies directly inhibit the growth of *C. albicans* is not unique. Antibodies can suppress fungal respiration[41] and inhibit the yeast-to-hyphal transition[41,42]. An anti-idiotypic antibody specific for a yeast killer toxin is candidacidal[43], presumably because it has killer toxin activity. The monoclonal antibody C7 (REF. 44) protects mice against candidiasis[80] apparently by mechanisms similar to antibodies specific for anti-mannotriose. In addition, however, C7 directly inhibits the growth of *C. albicans*, but by unknown mechanisms[44]. Of particular interest is the fact that C7 inhibits isolates of *C. lusitaniae*, *C. neoformans*, *Aspergillus fumigatus* and *Scedosporium prolificans*[44], but whether the antibody is active against these agents *in vivo* is not known. The specificity of C7 has not been defined, but the epitope is resistant to periodate oxidation[44], suggesting that it is specific for a protein epitope.

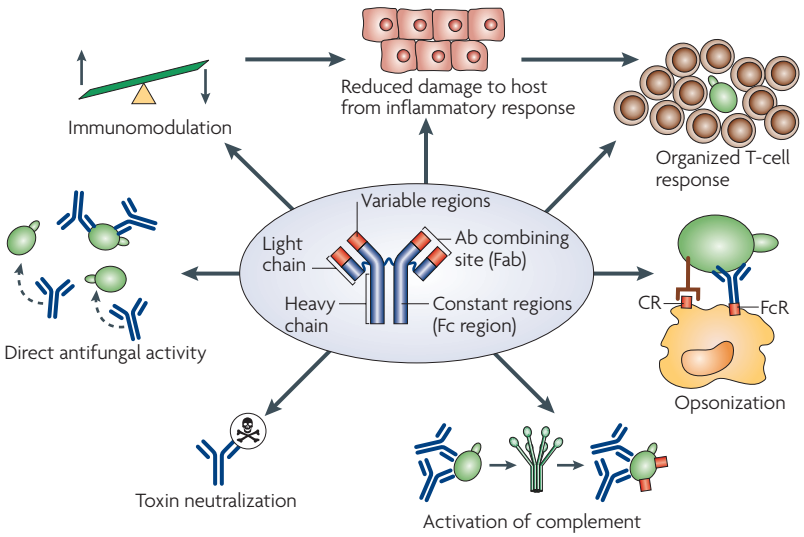

Figure 2 | **Antibody-mediated protection against fungal disease.** The mechanisms described for antibody-mediated defence against bacterial agents are presumed or proven to also be operative against fungi, including direct antibody (Ab) neutralization of fungal toxins and extracellular enzymes, and direct inhibition of fungal growth. Antibodies can indirectly inhibit fungi by functioning as an opsonin, either alone or in conjunction with complement factor C3, which is activated and deposited as C3b and which degrades to iC3b on the fungal surface. Antibody and complement-coated fungal cells interact with Fc receptors (FcR) and complement receptors (CR) on host phagocytic cell membranes, resulting in prompt ingestion of the fungal cell and which can lead to the death of the ingested fungal cell. In defence against intracellular fungal pathogens, such as *Cryptococcus neoformans*, protective antibodies seem to have a role in modulating host inflammatory responses and enhancing the organization of T-cell responses.

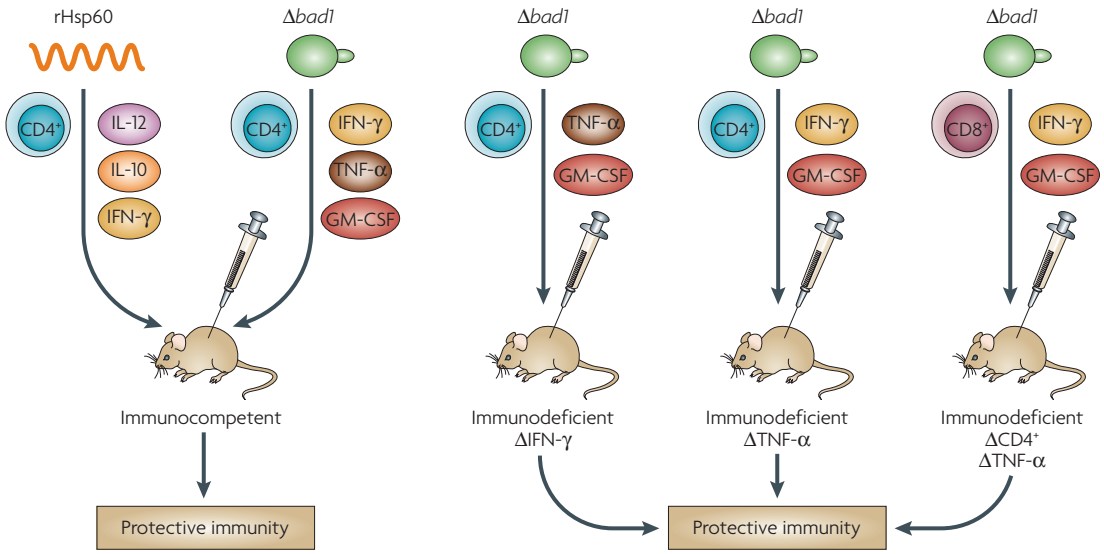

Figure 3 | **The crucial role of the T-helper 1 (T_H1) response in vaccine-induced immunity to fungi.** In experimental models, recombinant heat shock protein 60 (rHsp60) induces resistance in immunocompetent animals to lethal challenge with *Histoplasma capsulatum* (left). $CD4^+$ T cells and the T_H1 cytokines interleukin (IL)-12, IL-10 and interferon γ (IFN-γ) are required for this resistance. In immunocompetent animals, the resistance that is induced against experimental blastomycosis by a live attenuated strain of *Blastomyces dermatitidis* (with a mutation in the *Blastomyces* adhesin 1 (*bad1*) gene) has similar requirements for $CD4^+$ T cells and T_H1 cytokines (right). However, the immune system shows some plasticity and can compensate for the absence of particular cells or cytokines if they are absent during the induction or afferent phase of the vaccine-induced immune response (rather than being eliminated during the expression or efferent phase of the response). For example, immunodeficient animals that lack either $CD4^+$ T cells or selected T_H1 cytokines at the time of vaccination could control the live attenuated vaccine following subcutaneous administration, and use other T cells or T_H1 cytokines to engender vaccine resistance to lethal experimental disease. The absence of IFN-γ is compensated by tumour necrosis factor α (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM–CSF), and the loss of $CD4^+$ T cells is compensated by $CD8^+$ T cells that produce type 1 cytokines. Δ, gene knockout or missing owing to antibody depletion.

Protein antigens and cell-mediated immunity

The principal underlying tenet of vaccine development is that cell-mediated immunity, and in particular T_H1-mediated immunity, is pivotal in the action of candidate vaccines. Substantial evidence indicates that, for each of the fungi listed in TABLE 1, the T_H1 response has a role in resolving disease that is acquired naturally, even for the opportunistic pathogen *A. fumigatus*, which most often afflicts those in whom neutrophils and macrophages are either absent or functionally impaired. Conversely, the T_H2 response is largely associated with unrestrained growth of the fungus leading to progressive disease, although the underlying mechanisms have not been fully characterized[81–86].

Vaccination should therefore direct the immune response to a T_H1 response, with the induction of IL-12 and IFN-γ, the two principal cytokines of the T_H1 response, of paramount importance (FIG. 3). In this regard, vaccination with several protein antigens that confer protective immunity stimulates the production of IL-12 or IFN-γ within days of vaccination[85,87–92]. For example, recombinant forms of Hsp60 from *H. capsulatum* (but not *Coccidioides immitis* Hsp60)[93], the proline-rich antigen Ag2/PRA and urease from *Coccidioides* spp., and the *C. neoformans* polysaccharide deacetylase induce the production of these cytokines when analysed *ex vivo*[87,90,91,93]. However, despite the finding that these cytokines are associated with protective immunity, there is less evidence that they are necessary for vaccine-induced immunity.

Two lines of evidence support the importance of IL-12 and IFN-γ in protective immunity against fungal diseases. First, in studies that compared the immune response to a protective fungal protein, *H. capsulatum* Hsp60, and a non-protective fungal protein, *H. capsulatum* Hsp70, Hsp70 either induced a lower level of T_H1 cytokine protection, or did not induce T_H1 cytokines at all during either immunization or infection[87]. These results indicate that the ability to stimulate the production of IL-12, IFN-γ or both could be a reliable surrogate marker for identifying candidate protein antigens for further study. A second approach has been to investigate the necessity of IL-12 and IFN-γ for the efficacy of an immunogen *in vivo*. The neutralization of IFN-γ or IL-12 *in vivo* during vaccination with *H. capsulatum* Hsp60 abolished the protective efficacy of the vaccine[87]. Endogenous IFN-γ, but not IL-12, was essential for protection elicited by the polysaccharide deacetylase from *C. neoformans*[90]. Along these lines, protection conferred by immunization with Ag2/PRA requires IL-12, IFN-γ and class II, but not class I, major histocompatibility complex (MHC)-restricted T cells[94].

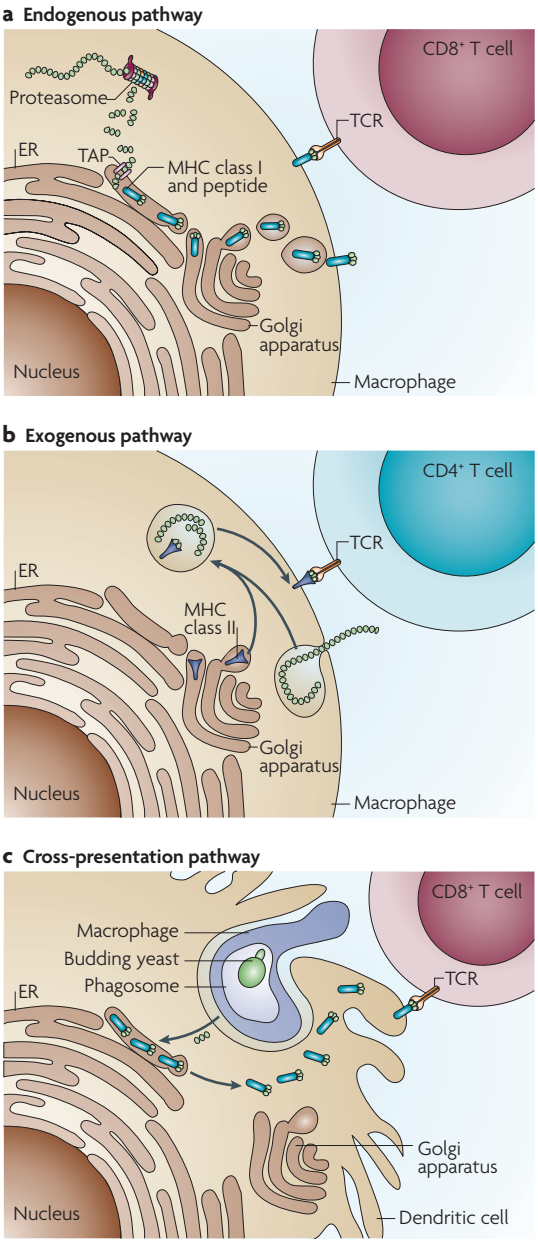

Figure 4 | **Antigen processing and presentation to T cells by major histocompatibility complex (MHC) class I and class II molecules. a** | In the endogenous processing pathway, proteins produced in the cytosol of phagocytic or non-phagocytic cells are cleaved in the proteasome into short peptide fragments of 8–10 amino acids and transported into the endoplasmic reticulum (ER) through TAP1 and TAP2, where they bind to MHC class I molecules. The peptide–MHC class I complex is transported through the Golgi apparatus to the cell surface where it is recognized by $CD8^+$ T cells. **b** | In the exogenous processing pathway, antigen-presenting cells such as macrophages or dendritic cells take up extracellular proteins and other microbial products by endocytosis or phagocytosis. In acidified endosomes, MHC class II molecules that have been transported from the Golgi bind fragmented antigenic peptides that are 10–25 amino acids in length. Peptide–MHC class II complexes are displayed on the cell surface for recognition by $CD4^+$ T cells. **c** | During cross-presentation, dendritic cells take up particles or even phagocytes that have internalized microorganisms. Through an ill-defined process, proteolytically cleaved fragments of antigenic peptide from the phagosome 'cross over' and enter into the MHC class I pathway, probably binding molecules from the ER. These peptide–MHC class I molecules move to the cell surface where they can be recognized by $CD8^+$ T cells. TCR, T-cell receptor.

The robust induction of the T_H2 cytokine IL-10 was an unexpected response to the *H. capsulatum* Hsp60 protective immunogen, as this cytokine principally functions in the inhibition of inflammation and cellular immunity[87]. Neutralization of IL-10 during immunization abolished the efficacy of the Hsp60 vaccine. The importance of IL-10 has not been thoroughly explored for other fungal vaccines. Recombinant urease from *Coccidioides* spp. does not induce the transcription of the *IL10* gene by T cells although it can promote IL-10 production by macrophages, which are the main generators of this cytokine in response to Hsp60 (REFS 87,93). An increase in *IL10* transcription is seen in the lungs of urease-vaccinated mice infected with *C. immitis*[93], but the influence on the course of disease is unknown. The importance of IL-10 for the efficacy of vaccine-related immunity should be contrasted with its role in disease exacerbation[27,28]. *A priori*, the induction of T_H2 cytokines should limit the use of a vaccine that must provoke a T_H1 response to be effective. However, a vaccine that strictly induces a T_H1 response alone might induce an overly exuberant inflammatory response that can damage the host when the infectious agent is encountered. A vaccine that incorporates both T_H1-stimulating and T_H2-stimulating epitopes could provide a balance between immunity and inflammation.

Protein-bearing antigens ingested by antigen-presenting cells (APCs) most often traffic through the exogenous antigen-processing pathway (FIG. 4), in which the protein is digested and the peptides are displayed on the APC surface in the context of class II MHC molecules for subsequent interaction with $CD4^+$ T cells[95]. Therefore, it is not surprising that the use of a protein-containing vaccine for fungi is thought to be strictly dependent on the presence of $CD4^+$ T cells (FIG. 3). This postulate is supported by the finding that the protective efficacy of the *H. capsulatum* Hsp60 vaccine was abolished when $CD4^+$ T cells, but not $CD8^+$ T cells, were depleted before immunization[87]. The reliance on this cell population for protective immunity can be disadvantageous, especially if one seeks to vaccinate individuals with altered $CD4^+$ T-cell function, such as those receiving immunosuppressive agents or those with reduced $CD4^+$ T-cell numbers such as AIDS patients.

The T-cell-dependent response to *H. capsulatum* Hsp60 or the fragment of the protein that contains the protective domain (F3) is highly biased at the T-cell receptor (TCR) level[96,97]. Most T cells contain two heterodimeric chains, α and β, that confer the specificity for antigen recognition. The β chain consists of variable (V), diversity (D), joining (J) and constant (C) regions, whereas the α chain contains only V, J and C regions. The gene segments encoding these regions undergo somatic rearrangement to produce a functional TCR. In mice, there are 20 Vβ chains and 50–100 Vα chains. This provides T cells with sufficient diversity to recognize the numerous antigens that the host encounters[98].

Immunization with Hsp60 induces a dominant population of T cells that express a particular TCR, the Vβ 8.1/8.2 TCR. This population of T cells is required for protective immunity as its depletion eliminates the

protective efficacy of Hsp60. Among this population, protection is confined to a small subpopulation that both releases IFN-γ and reacts to the F3 fragment of Hsp60. Interestingly, vaccination with the F3 fragment induces Vβ6$^+$ and not Vβ 8.1/8.2$^+$ cells, with protection confined to Vβ$^+$ T cells that secrete T_H1 cytokines[97]. The reason or reasons for the expansion of two disparate Vβ T-cell populations will most likely be found at the level of antigen processing and presentation, as the immunogenic peptides generated by digestion of the F3 fragment and Hsp60 must be different. These findings raise concerns about vaccination in large populations. If the efficacy of a protein antigen is entirely dependent on a specific subset of T cells, it is possible that a certain proportion of the population that lacks the reactive T cells would fail to respond to vaccination.

Engaging CD4$^+$ T cells is crucial for generating T-cell-dependent antibody formation, but the necessity for humoral immunity in vaccine efficacy remains unknown. In fact, all approved human vaccines rely on the induction of antibodies to mediate immunity. Therefore, the ability to induce humoral immunity could add to the effectiveness of a protein-containing vaccine. Studies in several pathogenic fungi have shown that antibodies against cell-surface molecules, whether complex carbohydrates or proteins, can be used in immunotherapy[36,58,99]. Many of these studies have shown that only specific B-cell clones can produce the proper antibody that is capable of transferring protective immunity. The challenge is inducing only antibody isotypes that are protective and not those isotypes that inhibit host defences or exert no positive effect on immunity.

An alternative approach to vaccination is to deliver T cells or APCs such as DCs. T cells from mice that have been immunized with protein antigens can vaccinate *Aspergillus*-infected mice[83,100]. The transfer of DCs loaded with the Ag2/PRA antigen induces protective immunity in an IFN-γ-dependent manner[100]. Likewise, antigen-loaded DCs confer protection in an animal model of human aspergillosis[101].

Many proteins that are generated by pathogenic fungi are glycosylated, but most of the studies with recombinant proteins in the medical mycology field have used proteins generated in *Escherichia coli*. Although recombinant proteins produced by this bacterium lack glycosylation, they do mediate protective immunity[87,88,93]. This finding, however, does not imply that glycosylation is unimportant for immune protection by protein antigens. There are virtually no studies that compare the protective efficacy of glycosylated and non-glycosylated recombinant proteins. Mannosylation of the model antigen ovalbumin dramatically enhances its antigenicity, as assessed by T-cell proliferation[102]. If these findings can also be extended to the production of cytokines, it is quite likely that the addition of carbohydrate moieties to immunogenic protein antigens could enhance their potency or reduce the amount required to achieve protection[102].

Another approach to potentially augment the potency of fungal vaccines is to create vaccines that contain more than one antigen. The use of such an approach would be to enhance the potency of a single antigen and engage a broader repertoire of T-cell families. The constituents of a multivalent vaccine should be at least additive, if not synergistic. Vaccination with a recombinant protein consisting of a fusion between the Ag2/PRA antigen from *C. immitis* with the *Coccidioides*-specific antigen from *C. posadasii* provided superior efficacy to vaccination with either recombinant protein alone, although surviving mice had a higher burden of infection (10^4 colony-forming units) in the lungs[103]. Likewise, in experimental coccidioidomycosis, a trivalent vaccine consisting of recombinant phospholipase B, aspartyl proteinase and α-mannosidase exhibited superior efficacy to each protein alone. The number of colony-forming units was markedly decreased in surviving mice[104]. Therefore, the data suggest that this approach is feasible and provides another approach to improve the efficacy of fungal vaccines.

Stimulating multiple arms of immunity

The foregoing discussion underscored the importance of stimulating CD4$^+$ T cells and their soluble T_H1 cytokines for the efficacy of vaccines containing purified protein antigens that mediate protective immunity to fungi (FIG. 3). CD4$^+$ T cells have a central role in both primary and secondary immunity to disease caused by *H. capsulatum*[105]. This T-cell subset is also responsible for vaccine-induced resistance to *B. dermatitidis* disease in immunocompetent hosts[106]. These and other studies of fungal vaccines suggest that CD4$^+$ T cells must be recruited for vaccine efficacy, even where antibodies participate in resistance. As indicated above, this recruitment should be feasible in healthy hosts who are given a purified protein vaccine, but perhaps might not be feasible in hosts in which CD4$^+$ T cells are depleted in number or their function is altered.

Are there other ways to induce immunity that recruits additional T-cell subsets, for example CD8$^+$ T cells, and a broader profile of immunity than is feasible with purified protein vaccines? Perhaps other recombinant approaches, such as vaccination with nucleic acids or live attenuated fungi, might help fill this gap (FIG. 5). Genetic immunization involves the delivery of nucleic acid encoding a gene(s) of interest into a host by one of several methods; naked DNA injection into muscle and particle bombardment into the skin by a gene gun are the most common[107]. The early 1990s witnessed initial reports of naked DNA administration into muscle for gene expression *in vivo*[108]. These exciting studies are now moving towards the development of vaccines against infectious diseases and cancer. One advantage of genetic immunization is the ability to control the orientation and magnitude of the immune response to a gene product. This advantage stems partly from the fact that the gene products are expressed inside host cells, and therefore engage the endogenous pathway of antigen processing and presentation (FIG. 5). This pathway leads to the recruitment of CD8$^+$ T cells[107,109], which also mediate resistance to fungi (see below). Released or secreted antigens from transfected or dying cells can

also facilitate antigen processing and presentation by the exogenous or class II MHC pathway, which preferentially stimulates $CD4^+$ T cells. A DNA-encoded antigen can be targeted to additional locations, such as the cell membrane or the extracellular milieu, and can also be introduced with additional sequences encoding cytokines or adjuvants, such as immunostimulatory sequences. These features might be useful for vaccination against fungi, as broader immune responses including robust $CD8^+$ T cell responses can be recruited by these methods.

Nucleic acid vaccination has been achieved in experimental models of invasive fungal disease due to *C. immitis*, *P. brasiliensis*, *A. fumigatus* and *P. marneffei*[91,93,109–117]. Most studies so far have examined *C. immitis* antigens, especially Ag2/PRA, the expression library immunization-antigen 1 (ELI-Ag1) and urease[3,118]. DNA vaccines have generally been more effective than the respective recombinant-protein vaccines. Studies of DNA vaccination against fungi have pointed out the role of several variables in the efficacy of DNA vaccines, including the amount of DNA administered[114], the route of DNA administration[116], the value of IL-12 sequences as an adjuvant[111] and even the inclusion of DNA encoding a signal sequence in the case of the Ag2/PRA DNA vaccine[114]. Studies in other disease models have also shown that larger quantities of DNA can be more immunogenic and that the location of DNA delivery — lung, epidermis or muscle — can influence the orientation of the T_H cell response[114]. Early studies have been encouraging, but most *C. immitis* DNA vaccines have used a model of intra-peritoneal infection for initial screening. Whether this vaccine approach is useful against the natural pulmonary route of infection with *C. immitis* has yet to be tested. Presumably, these fungal DNA vaccines function by stimulating cellular immunity, particularly the response of $CD4^+$ T cells and T_H1 cytokines, but they also induce strong antibody responses[91,112,114]. Their mechanism of action and the contribution of antibody and $CD8^+$ T cells has not been explored, and are significant areas of ongoing investigation. By contrast, recombinant Ag2/PRA given in conjunction with immunostimulatory oligodeoxynucleotides protects mice in a manner that requires IL-12, IFN-γ and MHC class II restricted T cells, whereas $CD8^+$ T cells are not required[94]. Because human clinical trials of DNA vaccines have reported low immunogenicity, regimens to increase vaccine potency, such as a DNA prime and protein boost regime, are under investigation[119].

DCs are among the most potent APCs for stimulating immune responses, prompting investigators to harness these cells for the delivery and presentation of fungal antigens, including nucleic acid. DCs transfected with nucleic acid from *C. albicans* or *A. fumigatus* were able to induce resistance against experimental disease with the respective pathogens[117]. In another study, a DC line was transfected with *C. immitis* PRA and introduction of these cells into the lungs of mice produced a T_H1 cytokine response and resistance against experimental disease with this fungus[100].

Live attenuated fungi as vaccines

In the field of vaccinology, there are several examples of highly efficacious infectious-disease vaccines derived from live attenuated infectious agents, principally viruses, including polio, measles, mumps, rubella, varicella, influenza and, most recently, rotavirus[120]. The advantages of using a live agent are that the pathogen replicates at the site of infection and induces strong, broad responses involving multiple arms of the immune response (FIG. 5), which recapitulates natural immunity to disease. By contrast, protein or killed agents might not access the endogenous antigen-processing pathway and induce $CD8^+$ T-cell responses. Such immune responses contribute to protective immunity against many pathogenic fungi, including *H. capsulatum*, *B. dermatitidis*, *P. brasiliensis*, *C. neoformans*, *Pneumocystis carinii* and perhaps others as well[85,121–125].

Work on *B. dermatitidis* has demonstrated the efficacy of live attenuated fungi for vaccination, and the benefit of using live organisms to recruit multiple arms of the host immune response. It had been postulated that stimulating an immune response to a major virulence factor, such as *Blastomyces* adhesin 1 (BAD1) from *B. dermatitidis*, could provide the host with a selective advantage and protect against disease, either through prompt pathogen recognition or possibly by neutralization of virulence factor function. However, when recombinant BAD1 was used to vaccinate mice[88,126] the vaccine efficacy was not striking, even when combined with IL-12 as an adjuvant[126].

The inability of BAD1 to rescue mice from a lethal pulmonary challenge with *B. dermatitidis* prompted other approaches to vaccination against blastomycosis. A genetically engineered *BAD1* null mutant is sharply attenuated in a mouse model[127]. This finding suggested that it might induce an immune response in mice and so vaccine protection. When live yeast of the *BAD1* null mutant were injected subcutaneously in the absence of adjuvant, most of the vaccinated mice survived a lethal challenge and evidence of sterilizing immunity was obtained[128]. This report established that a genetically engineered fungus strain could be used as a vaccine. Experimental vaccine studies had previously been carried out with natural fungal variants that exhibited loss of pathogenicity; that is, a strain of *C. albicans* that had lost its ability to filament and a temperature-sensitive variant of *C. immitis* that grew poorly at 37°C (REFS 129,130). A concern with natural variants, however, is the potential for reversion to virulence.

Studies of the protective mechanisms of the live attenuated *B. dermatitidis* vaccine uncovered plasticity in the residual immune elements in compromised hosts at both the molecular and cellular level[106] (FIG. 3). In immunocompetent mice, $CD4^+$ T cells mediated resistance mainly by the production of TNF-α and IFN-γ[106]. Although both of these cytokines are crucial mediators of the expression or efferent phase of vaccine immunity, the efficacy of vaccination with the mutant strain did not require the pre-existing presence of IFN-γ or TNF-α. The efficacy of vaccination in IFN-$\gamma^{-/-}$ or TNF-$\alpha^{-/-}$ mice required either the reciprocal cytokine or granulocyte–macrophage colony-stimulating factor (GM–CSF).

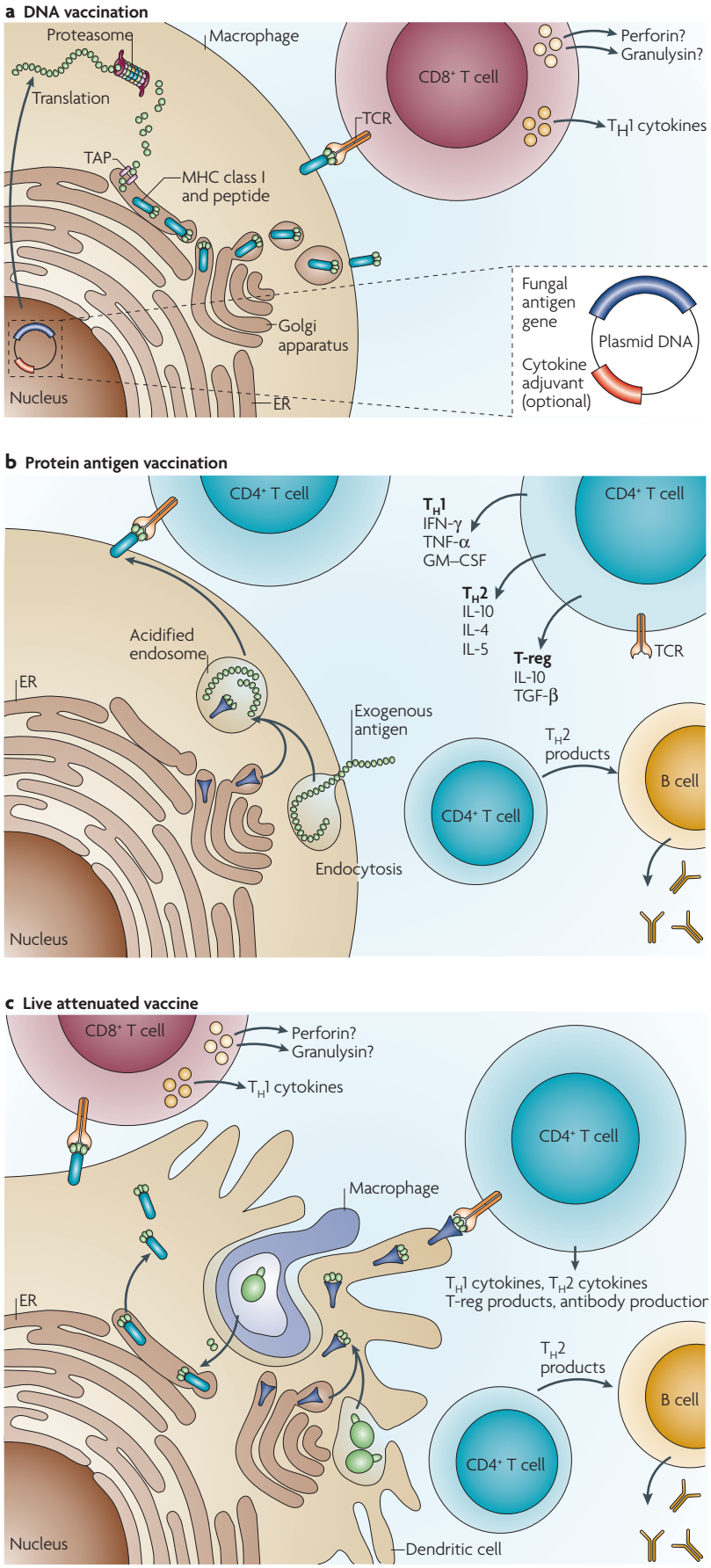

Figure 5 | **The profiles of the anti-fungal immune responses induced by various immunogens. a** | In DNA vaccination, the plasmid DNA contains a fungal gene that is often under the control of an active promoter. Other genes can be co-expressed, for example cytokines such as interleukin (IL)-12 that can exert adjuvant effects. The fungal proteins are translated and from the cytosol enter the endogenous route of antigen processing, as shown in Fig. 4a. Therefore, this vaccine strategy primes $CD8^+$ T cells, which function by producing T-helper 1 (T_H1) cytokines, and perhaps lytic products such as perforin or granulysin. A secretion signal (not shown) can be engineered onto the antigen sequence so that the expressed gene product is exported out of the cell, and thereby enters the exogenous antigen-processing pathway, as shown in Fig. 4b, priming $CD4^+$ T cells and their soluble products. **b** | In vaccination with a protein antigen, the soluble protein and an adjuvant enter the exogenous antigen-processing pathway, which chiefly primes $CD4^+$ T cells. These cells perform helper and regulatory functions by releasing various soluble products, which arm phagocytes, balance the unrestrained vigor of the immune response, and help B cells produce antibody. **c** | A live attenuated vaccine can enter antigen-presenting cells, especially dendritic cells, by multiple routes. The vaccine primes $CD4^+$ T cells through the exogenous antigen-processing pathway pathway and $CD8^+$ T cells through cross presentation. This engages multiple arms of the immune response, including multiple T-cell subsets and their accompanying products, and B cells and antibody production. GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; MHC, major histocompatibility complex; TCR, T-cell receptor; TGF, transforming growth factor; TNF, tumour necrosis factor; T-reg, regulatory T cell.

The vaccine required the presence of TCR $\alpha\beta^+$ cells but functioned unexpectedly well in the absence of either $CD4^+$ or $CD8^+$ T cells. This study demonstrated that vaccination of immunodeficient hosts can be accomplished. This finding has implications for developing vaccines for use in hosts that are vulnerable to fungal disease owing to the loss of immune function.

These studies were extended to *H. capsulatum* and to additional immunodeficient hosts, and it was found that the efficacy of the live attenuated vaccine in $CD4^{-/-}$ mice relies on the presence of competent $CD8^+$ T cells[85]. The $CD8^+$ T cells interact with MHC class I antigens. Therefore, antigens from the live attenuated yeast were either accessing the endogenous pathway of antigen processing, or were being cross-presented and cross-priming $CD8^+$ T cells (FIG. 5). $CD8^+$ T cells can function appropriately in the absence of either TNF-α or IFN-γ. In the absence of TNF-α, GM–CSF compensates and in the absence of IFN-γ, TNF-α and GM–CSF regulate the expression of immunity. Vaccine-mediated immunity to *B. dermatitidis* and *H. capsulatum* thereby overcame a requirement for $CD4^+$ T-cell help, and this immunity was durable in mice for at least 8 weeks post-vaccination. This is in contrast to the findings of recent studies on $CD4^+$ T-cell-independent $CD8^+$ memory

T-cell responses against viral and bacterial diseases[131,132], which waned over a similar interval of time, indicating that $CD8^+$ T-cell memory to fungi might be longer lasting and biologically distinct from $CD8^+$ T-cell memory against viruses and bacteria.

These studies show the efficacy of using live attenuated fungi for vaccination, the ability of these immunogens to recruit multiple arms of the immune response, including both $CD4^+$ and $CD8^+$ T cells, and the unexpected finding that immunodeficient hosts can control a live attenuated vaccine and compensate for the loss of elements of the immune response by substituting other elements to confer protective immunity.

There are other examples of live attenuated vaccines against fungi. A targeted mutant of *C. immitis* in which two chitinase genes were deleted is unable to convert from an infectious arthroconidial form into an endosporulating, pathogenic spherule form, and is avirulent after intranasal injection in mice (G. Cole, personal communication). This strain elicits immunity and resistance in subcutaneously vaccinated mice against a lethal pulmonary disease with wild-type arthroconidia of *C. immitis*. These findings support the observations above with *B. dermatitidis* and *H. capsulatum*, and extend the results to a novel type of genetically engineered mutant, which is impaired in morphogenesis to a virulent form. The fungus is physically unable to mature into a virulent state, which might increase its safety. Another live attenuated fungal vaccine has been developed for prevention of ringworm caused by *Trichophyton verrucosum* in cattle[133]. The attenuated strain has been tested in controlled challenge–exposure experiments and in field trials. In studies of >400,000 vaccinated cattle over a 5-year period, it was concluded that trichophytosis in cattle can be controlled by immunoprophylaxis with negligible side effects.

We emphasize that there are theoretical and real concerns in using live pathogens for vaccination. Even if 'unnatural immunity' can be recruited to confer vaccine efficacy in immunocompromised hosts[134], a formidable issue is whether vulnerable patients can contain even an attenuated live pathogen. Live non-pathogenic fungi have been explored as vaccine vehicles for the delivery of immunogens, including heterologous antigens. This concept was tested in *Saccharomyces cerevisiae*[135]. This yeast, when genetically engineered to express the model antigen ovalbumin, could access DCs and prime $CD4^+$ T cells in addition to cross priming $CD8^+$ T cells. As well as priming multiple arms of immunity, this strategy adds the potent adjuvant properties of the yeast β-glucan and, because of the genetic tractability of this yeast, it also offers the ability to deliver multiple vaccine antigens in a single immunogenic vehicle.

Perspectives

The level of our understanding of fungal–host interactions has progressed to the point where vaccines against both primary fungal pathogens and the prevalent opportunistic fungi are becoming a reality. Prototypic antigens, both subcellular and whole cells, have been identified, against which protective immunity can be induced. The mechanisms of immunity vary from antibody-mediated immune responses to cell-mediated responses, and even a combination of both of these main arms of the acquired immune system. As we move closer to the reality of human clinical vaccine trials, a range of theoretical questions regarding the safety and efficacy of fungal vaccines have been raised. These questions include whether such vaccines are necessary[1,3–5], whether they can be made efficacious in immunodeficient hosts[1,2,85], whether, as in the case of vaccines against *C. albicans*, they can prevent disseminated disease without affecting *C. albicans* as a member of the normal microbiota[1,136], and whether fungal vaccines against agents commonly encountered by humans will result in, or possibly prevent, allergic manifestations[1,136–139]. Answers to these questions will not be known until human vaccine trials are initiated. So far, a killed spherule vaccine against coccidioidomycosis is the only human fungal vaccine field trial ever conducted[140]. The negative protection results obtained were more likely caused by the inability to use immunogenic doses of the vaccine because of toxic manifestations of the appropriate dose, than to the vaccine being unable to induce protection[140,141]. In spite of the theoretical questions posed above, the results of preliminary vaccine studies on the prevention of coccidioidomycosis should encourage, rather than stifle, enthusiasm to pursue vaccine development against the ever-growing problem posed by fungal diseases. Although mouse models of human mycoses have served us well in defining candidate antigens and vaccine formulations, it is time to move towards clinical trials in humans.

Is a universal vaccine against mycotic disease possible? The experiments involving protective antibody responses against β-1,3-glucan that have been carried out so far are limited in scope, but suggest that a universal fungal vaccine could be developed. The fact that β-glucans are found in many fungal pathogens (see above), along with the immunomodulation activities of this polysaccharide[142], implies that β-glucan would be an important vaccine component. The inclusion of other oligosaccharides, such as the GXM heptasaccharide epitope and *C. albicans* β-linked mannotriose, could improve the vaccine effectiveness. Additional epitopes that should be considered are GXM peptide mimotopes and the LKVIRK epitope of the *C. albicans* Hsp90 antigen[143,144]. Prudent choice of the protein carrier for the carbohydrate epitopes could further broaden the spectrum of coverage by using fungal proteins that fulfil the carrier function and induce protective T_H1-dependent responses or additional protective antibody responses. For example, the two or three carbohydrate moieties could be coupled to a mixture of Hsp60 from *H. capsulatum*, BAD1 from *B. dermatitidis*, Ag2/PRA protein from *C. immitis* and, perhaps, the protein from *C. albicans* that led to the isolation of the C7 monoclonal antibody[44,80]. Of course a myriad of questions would need to be addressed before such a vaccine was developed, but the ever increasing problems with fungal diseases and the level of our understanding of fungal–host interactions now make this a prospect worthy of consideration.

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Competing interests statement
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