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Prospects of vaccines for medically important fungi

This is the fourth of a series of reviews that detail the basic science progress in the quest to achieve vaccines for several of the medically important fungi.

Review

Prospects of vaccines for invasive aspergillosis

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Invasive aspergillosis is a disease of immunocompromised hosts and the pathogenesis of this disorder is heavily dependent upon the defect within a given host. Consequently, vaccine development is limited by our understanding of effective host responses and by limitations in our knowledge of fungal molecules that elicit protective immunity. Nonetheless, the past few years have witnessed advances in our understanding both of the immune response to this organism and in the relationship between antigenicity and the ability to confer protection. Manipulations that promote the development of T_H1-associated responses correlate with increased resistance to disease, at least partly because of consequent enhancement of innate cellular effector function. Two areas of investigation most actively being pursued include the search for adjuvants that will allow products of *Aspergillus fumigatus* to become effective vaccine candidates, regardless of the form of immunity they ordinarily induce, and the identification of the specific antigens that will most effectively elicit beneficial responses. Strategies using antigen-exposed dendritic cells as adjuvants appear to be particularly promising. Though we currently are far away from a candidate that is applicable for human trials, recent progress is encouraging.

Keywords invasive aspergillosis, vaccine, *Aspergillus fumigatus*

Introduction

Aspergillus fumigatus is a ubiquitous mold that causes invasive disease in immunocompromised hosts. The incidence of invasive aspergillosis has been rising since the 1960s because of advances in medical technologies, particularly in transplantation, and because of the AIDS epidemic [1–3]. Infection is acquired by inhalation of conidia (spores) and the lung is the most common site of primary infection, followed by the sinuses. In the lung, conidia swell and then germinate, initiating the polarized hyphal growth pattern that defines molds (Fig. 1). Disseminated disease, which occurs most commonly in the brain and kidney, may follow angioinvasion, or could result from migration of conidia-containing pulmonary immune cells, though the latter mechanism has not been proven [4,5]. Limitations of currently available antifungal agents include high toxicity, complex drug interactions and limited efficacy, such that mortality rates for proven

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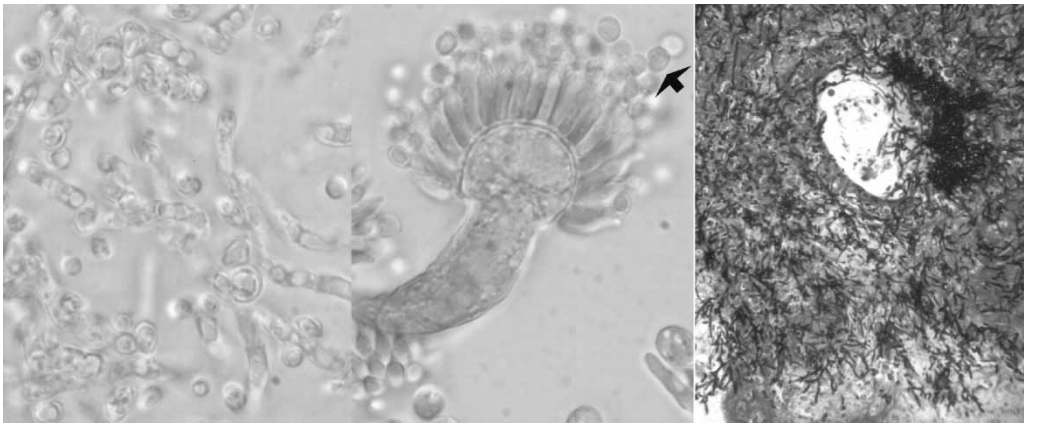

Fig. 1 Life cycle of *Aspergillus fumigatus*. Left. *A. fumigatus* hyphae. The classical description of vegetative growth of the organism is the appearance of clear walled, septate hyphae that branch at 45° angles, shown here by light microscopy after growth in Sabouraud's dextrose agar *in vitro*. Middle. *A. fumigatus* conidiophore. At air/water interfaces and during other "stress" conditions, footpads form from hyphae and result in the formation of conidiophores, which produce conidia. Conidia (arrow) are easily aerosolized and are the infectious particle of the organism. Though conidiophores are not usually seen in most forms of invasive disease in humans, their appearance has been reported more commonly in avian disease. Right. Hyphal growth in tissue. Disease results from germination of the organism, with resumption of hyphal growth, as seen in this Gomori Methenamine silver stained section of the lung of an immunosuppressed mouse two days after infection. Arrow, *A. fumigatus* conidia. *In vitro* images were provided by Shane Stephens-Romero (Albert Einstein College of Medicine).

invasive disease continue to range from 60–90%, depending upon the host and the site(s) of involvement [6]. Therefore, the development of strategies that target the host response are needed to combat this pathogen.

Though *A. fumigatus* is the most common cause of invasive aspergillosis in most cases, several other members of this genus are also increasingly important causes of this disease, including *A. flavus*, *A. terreus*, *A. nidulans*, and *A. niger* [7]. Because most immunologic study and consideration of a vaccine has involved *A. fumigatus*, this review is focused on this species, with the caveat that development of a vaccine that can protect against disease due to all *Aspergillus* spp. is desirable even though the results presented here may not be applicable to all species. Accordingly, this review focuses on our current understanding of the host response in invasive aspergillosis, the challenges that are engendered by the search for a vaccine for an organism whose pathogenesis is heavily dependent upon the host, prior studies applicable to vaccines for invasive aspergillosis, and newer strategies that may hold promise for development of vaccines for invasive aspergillosis.

Overview of the host response to *A. fumigatus*

The response to any infectious process can be divided into innate and adaptive immunity. In both of these phases, soluble and cellular components work in concert to produce host responses that, when maximally effective, rid the host of pathogenic microorganisms while producing minimal damage due to an inflammatory response. Innate immune responses are characterized by the use of germline DNA-encoded receptors, called pattern recognition receptors, for recognition of conserved microbial components or products [8]. In contrast, adaptive immunity relies on the generation of highly diverse and specific antigen receptors on T and B cells by gene rearrangement and somatic mutation. Adaptive responses strengthen and regulate the host response, while allowing for the development of memory, the induction of which is an important goal of vaccination. Stimulation of adaptive immune responses, particularly in T_H1-associated patterns, is dependent upon innate immune system activation at least partly through ligation of toll-like receptors (TLRs) [9].

Innate host defense

Invasive aspergillosis occurs in the setting of defective innate immunity. Alveolar macrophages form the first line of host defense against *A. fumigatus* and are the immune cellular component most important for host interactions with conidia [10]. Following binding, macrophages ingest conidia, which then break dormancy and swell. Host proteins that enhance macrophage phagocytosis of conidia include surfactant protein D and the long pentraxin PTX 3 [11,12]. Conidial activation of the alternative complement

pathway enhances macrophage phagocytosis, though complement deposition has been associated both with enhancement and inhibition of killing *in vitro* [13,14]. Until the onset of swelling, macrophage conidiocidal mechanisms are ineffective [15]. Though killing by alveolar macrophages had been thought to proceed via non-oxidative pathways, the importance of reactive oxygen intermediates was demonstrated more recently *in vitro* [16–18]. Signaling responses by macrophages in response to conidia involve ligation of TLR2 and signaling through the adapter protein MyD88 results in production of tumor necrosis factor-α [19], a key pro-inflammatory cytokine for host defense against *A. fumigatus* pulmonary infection [20].

Neutrophils are rapidly recruited to the lung in response to experimental pulmonary infection with *A. fumigatus* and effectively kill hyphae resulting from conidia that escape killing by macrophages [10,21]. Resting conidia are relatively resistant to killing by either reactive oxygen intermediates or neutrophil cationic peptides and their ingestion triggers neutrophil degranulation and the respiratory burst only weakly [22–24]. Once conidial swelling has occurred, the response of neutrophils is enhanced, such that phagocytosis, degranulation and reactive oxygen species production are increased and conidia are killed more readily [22]. As with macrophages, neutrophil killing of conidia is accomplished through myeloperoxidase-dependent and -independent pathways, while hyphal damage depends upon both the myeloperoxidase-dependent pathway and granule contents [25].

Natural killer (NK) cells, an additional component of innate responses, also function in host defense against invasive aspergillosis in an experimental murine pulmonary model [26]. NK cell depletion in neutrophil-depleted mice increases mortality in association with increased fungal burden. Early NK cell recruitment is partly dependent upon the interaction of NK cell chemokine receptor CCR2 with the chemokine MCP-1/CCL2. The concentration of this chemokine increases in the lungs of neutropenic, but not immunocompetent, mice early after infection, suggesting that other components of innate responses are sufficient to control infection in normal hosts. Thus, NK cells form a redundant function that may be important in neutropenic hosts [26].

TLR signaling

TLRs are a family of conserved receptors that mediate cellular responses to structurally conserved pathogen-associated molecular patterns. These receptors are present on a wide range of cells, including those of the classical immune system, as well as on airway epithelium [27,28]. As noted above, these receptors participate in the activation of innate immune cells and the resulting cell signaling not only affects the pattern of activation of these cells, but influences the development of adaptive responses. With regard to the latter, TLR signaling in dendritic cells is particularly important because of their trafficking patterns and high antigen presenting capacity. Further, as will be discussed below, understanding the differences in outcome of TLR signaling in response to the organism through the individual family members is important because of the potential utility of TLR ligands as vaccine adjuvants.

Signal transduction through the adapter protein MyD88 is required for the production of inflammatory responses following TLR ligation with many, but not all, ligands [27]. Though, in the absence of immune suppression, TLR- and MyD88-deficient mice are not more susceptible to infection, cyclophosphamide pretreatment increases mortality in TLR-4- and MyD88-deficient mice compared to wild type following intranasal (i.n.) infection. In this model, mortality is unchanged in TLR-2-deficient mice, but their resistance to secondary infection may be reduced [29]. Though both TLR-2 and TLR-4-deficient mice exhibit increased lung fungal burden following infection with *A. fumigatus*, interestingly, unlike TLR-4-deficient mice, who develop inflammatory pathology similar to that seen in wild type mice, the inflammatory response in TLR-2-deficient mice is markedly reduced [25].

With regard to specific effector cell function, while conidia induce neutrophil expression of both TLR-2 and TLR-4, only TLR-4 is induced upon expression to hyphae. As seen in study of neutrophils from TLR-deficient mice, TLR4 is essential for neutrophil killing of both conidia and hyphae, though signaling through TLR-2 also participates in conidial killing. Signaling through both TLR-2 and TLR-4 induces TNF-α secretion, though signaling through TLR-4 induces IL-10 secretion, which may be important for down-regulation of inflammatory responses in response to infection [30].

Dendritic cells

Dendritic cells are key components at the interface of innate and adaptive immunity because of their capacity to present antigen and to determine the outcome of T cell priming [31]. Dendritic cells in the lung ingest conidia within 2 h after experimental pulmonary infection. Dendritic cells respond differently to conidia than they do to hyphae. Though hyphae are efficiently

killed, conidia are not, and trafficking of pulmonary dendritic cells to the regional lymph nodes and spleen follows rapidly [5]. Conidial uptake has been reported to occur via mannose receptor- and DC-SIGN-mediated recognition of cell wall galactomannan, while complement receptor 3 and Fcγ receptors participate in hyphal ingestion, a process that is partially inhibited by cell wall β-glucan [5,32,33]. Following phagocytosis, conidia induce dendritic cell production of IL-12p70 and prime T cells for T_H1-associated responses, which are associated with resistance to invasive aspergillosis [34,35], while hyphal ingestion induces IL-4 and IL-10 production and primes T_H cells toward production of T_H2-associated responses [5]. Of the TLRs, conidia induce only TLR4 expression by murine lung and human myeloid dendritic cells *in vitro* and are not themselves sufficient stimulus for dendritic cell maturation [36].

Adaptive immunity

Early experiments in nude mice demonstrate that T cells are unnecessary for protection against disease in the host with intact innate immunity [37]. Though mice deficient in T_H1-associated cytokines survive experimental pulmonary infection, they demonstrate difficulty in fungal clearance and enhanced inflammatory responses [38]. After i.v. infection, lethal inocula stimulate T_H2-associated responses characterized by high concentrations of IL-4 production and lower concentrations of IFN-γ by splenocytes than that which follows sublethal inoculation [35]. Resistance to invasive pulmonary aspergillosis in the setting of suppressed innate effector cellular function is associated with the development of a T_H1-like environment.

The cytokine milieu, partly determined by T_H cells, affects the effector activity of innate immune cells, as neutrophils from IL-4-deficient mice, which exhibit enhanced resistance to infection that correlates with lower fungal loads, have markedly increased anti-hyphal activity [38]. The abrogation of resistance in these mice following IL-12 neutralization, the inverse correlation between IL-12 or IFN-γ concentration and fungal burden in experimental pulmonary infection, and the absence of a correlation with IL-4, support the view that resistance is dependent more upon the presence of a T_H1-like environment, rather than on the absence of a T_H2-like milieu.

Production of IL-10, a T_H2-associated regulatory cytokine, is associated with susceptibility to invasive aspergillosis, as IL-10-deficient mice are more resistant to both pulmonary and systemic disease [39,40]. IL-10 may have both stimulatory and inhibitory effects on innate cell function, as this cytokine enhances human peripheral blood monocyte phagocytosis of conidia and somewhat increases the ability of these cells to inhibit germination [41]. However, IL-10 does not increase their conidiocidal activity, but does reduce their capacity for hyphal killing. Thus, the impact of IL-10 could be either stimulatory or inhibitory, as enhanced macrophage phagocytosis with ineffective killing could increase the intracellular reservoir of live organisms. As discussed later, some findings in vaccine studies suggest that IL-10 may play a beneficial role, which could occur through downregulation of inflammatory responses after infection is controlled. The contribution of regulatory T cell subsets to IL-10 production in invasive aspergillosis has not yet been defined.

The antibody response in invasive aspergillosis remains poorly characterized, though numerous B cell antigens have been identified. The lack of an association between the appearance of serum antibody and clinical improvement has been used to argue against a role for antibody in immunity [42]. Earlier studies indicate that severely immunocompromised patients do not make *A. fumigatus*-specific immunoglobulin until immunologic recovery, but a more recent study using recombinant antigens suggests that antibody responses may be more prevalent in those with invasive disease [43]. Blockade of FcγRII and FcγRIII inhibits hyphal phagocytosis by murine dendritic cells *in vitro* [5], though the implication of this finding has not been fully explored. In μMT mice, which lack mature B cells, the fungal burden in the lung that follows primary infection is reduced compared to that seen in wild type mice, implicating a potentially harmful role for antibody. However, administration of normal serum to these mice further lowers fungal burden in μMT mice, but not in wild type mice, suggesting that antibody may contribute to fungal clearance early after infection. Nonetheless, when μMT mice are further immunosuppressed by administration of cyclophosphamide, prolongation of survival is demonstrable as well [44,45].

In summary, our current understanding of the host response to *A. fumigatus* is that innate immunity is critical for prevention or control of disease, with essential effector roles for alveolar macrophages and neutrophils. Dendritic cells, key determinants of the nature of adaptive responses, respond differently to various forms of the organism. Development of T_H1-associated responses is associated with enhanced resistance, which is believed to result from consequent augmentation of innate effector function. The mechanisms by which interactions of *A. fumigatus* with host

cells induce beneficial responses are active areas of investigation, among which is the role played by engagement of individual cell surface receptors and downstream molecules.

Challenges to the development of vaccines for invasive aspergillosis

Several features of invasive disease due to *Aspergillus* pose special challenges for the development of vaccines, as has recently been reviewed [46]. Unlike the dimorphic fungi, such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*, for which virulence factors have been defined, identification of similar fungal properties for *A. fumigatus* has been difficult. Such knowledge has allowed for the development of conjugate vaccines aimed at eliciting effective immunity to these targets, such as the glucuronoxylomannan-conjugate for *C. neoformans* [47], or for the development of attenuated strains by targeted disruption of these molecules that can function as live attenuated vaccines, such as the *B. dermatitidis BAD-1*-deleted strain [48]. *A. fumigatus* is ubiquitous in the environment and is an important colonizer of compost, where it is thought to play a major role in leaf degradation [6]. A study based upon the generation of mutant strains with targeted disruption has identified a limited number of genes that, when deleted, result in decreased virulence in animal models. These include genes encoding proteins implicated in environmental sensing, such as the ras-related protein rhbA that is involved in nitrogen sensing [49], the fos1 of a two component histidine kinase [50], and components of the cyclic AMP signaling pathway, including GPAB, a G protein α subunit, and PKAC1, a putative catalytic subunit of protein kinase A [51]. CgrA, a nucleolar protein involved in thermotolerance [52] and PKSP, a polyketide synthase involved in melanin biosynthesis that also plays a role in reducing phagolysosomal fusion in macrophages [53–55] also affects virulence. However, the presence of similar pathways in fungi that are considered less or non-pathogenic and the ability to induce experimental invasive mold infection with species that have not been reported to cause human disease has led to the suggestion that the higher prevalence of disease due to *A. fumigatus* compared to other invasive molds reflects the relative predominance of this species in air combined with its growth properties, rather than inherent pathogenicity [6]. Some, but not all, epidemiologic evidence supports this hypothesis outside the setting of an epidemic [56,57]. Nonetheless, the difficulty in detecting fungus-defined virulence factors likely reflects the fact that occurrence of invasive disease in an apparently immunocompetent host is an exceptional event. This feature has important implications for the identification of fungal targets that will elicit protective immunity, as prediction of molecules that are more likely to be effective is more difficult. However, as discussed below, beneficial host responses could be induced by fungal immunogens that are not necessarily directly involved in virulence, making investigation of antigens preferentially expressed during disease an alternative approach.

The second feature is a corollary of the overwhelming importance of the host as a determinant of the outcome of infection with *A. fumigatus*, namely, that the specific defect of a given host plays a key role in defining the pathogenesis of infection. Host defects that result in predisposition to invasive aspergillosis include neutropenia, defective neutrophil function (such as that seen in patients with chronic granulomatous disease), receipt of corticosteroid therapy, or immunosuppressive agents for solid organ or graft vs. host disease and late-stage HIV infection. Further, immune reconstitution is a time of particular vulnerability in invasive disease, as, for example, bone marrow recovery has been associated with pulmonary cavitation and hemoptysis in patients with leukemia that received chemotherapy [58]. In the pre-HIV era, over one third of reported cases of immunorestitution disease were described in patients with invasive aspergillosis [59,60]. The pathogenesis of disease in these diverse hosts likely is quite different and may vary with the specific defect, as has been seen both in autopsy series and animal models [61–63]. Therefore, pathogenesis in the persistently neutropenic host may result more from fungal growth or toxicity of fungal products, while in other cases, the inflammatory response may be responsible for the majority of host damage [64]. Along these lines, our knowledge of the pathology of invasive aspergillosis in some of these patient populations, such as those with graft vs. host disease, is very limited.

That invasive aspergillosis occurs in immunocompromised hosts points to an additional challenge – the need to be able to induce effective immune responses in the setting of defective immunity. However, for some of the patient populations susceptible to invasive aspergillosis, such as those receiving chemotherapy, the period of susceptibility may be easily identifiable and relatively circumscribed. Thus, one could, for example, vaccinate a patient that will be receiving a solid organ transplant prior to the time of transplantation, allowing the immune response to develop before the therapeutic manipulation that will induce susceptibility. Along these lines, the feasibility of therapeutic vaccines

for invasive aspergillosis recently has been proposed as a means of augmenting the host response to established disease, providing additional time for immune reconstitution [65].

A final challenge relates to the nature of the host response to *Aspergillus*. For *A. fumigatus*, many antigens have been defined in the context of allergy and the current list of allergens of the International Union of Immunological Societies defines 22 *A. fumigatus* proteins as allergens (www.allergen.org). By definition, these fungal molecules elicit host responses that are damaging. However, fungal cell wall carbohydrates may induce T_H2-associated responses through adjuvant actions, as they are not, themselves, the target of IgE responses in an experimental model of allergy [66]. Therefore, by manipulating the immune response through, for example, the use of different adjuvants, one potentially can convert harmful responses to those that confer host benefit. A striking example is contained in the work of Bozza *et al.* [34], which is discussed further below.

How would vaccines against invasive aspergillosis work? The traditional view of the mechanism of action of vaccines had been that induced IgG responses are sufficient to confer protection [67]. Until recently, little evidence supported the ability of induced antibody immunity to protect against invasive aspergillosis, though most studies had not evaluated parameters that affect outcome in other systems, including antibody specificity, concentration, and isotype [47]. However, the demonstration that an antiidiotypic monoclonal antibody of *Pichia anomala* killer toxin, which binds to cell wall β-glucan, delays germination of swollen conidia, kills hyphae and prevents experimental murine infection with *A. fumigatus* provides support for the ability to induce protective antibody responses [68]. This monoclonal antibody primarily affects hyphal growth and has broad activity against bacteria, fungi and parasites [69–71]. A second strategy that has resulted in protective antibody responses has been reported recently. Immunization of mice with a crude cell wall preparation from *A. fumigatus* hyphae induces an antibody response to a >95 kDa glycoprotein that also may stimulate antibody responses in patients with aspergillosis [72]. A monoclonal antibody that binds to this protein made using a vaccinated mouse prolongs survival in systemically infected mice and may have direct antifungal activity. These two examples provide strong evidence that protective antibody responses can be induced, despite limited support that those generated during disease are important for host recovery.

Nonetheless, the past decade has seen marked evolution in this view of vaccinology, as induction of cell-mediated immunity is seen as critical for efficacy in disease prevention for some vaccines, such as the live attenuated vaccine used to prevent herpes zoster [73], and either $CD4^+$ or $CD8^+$ T cells can mediate protection to whole cell vaccination with *B. dermatitidis* and *H. capsulatum* [74]. As might be anticipated based upon the importance of T_H1-associated responses for control of invasive aspergillosis, induction of this response by vaccines is emerging as important for protection against this disease [34,75,76]. Nonetheless, our understanding of the inter-relatedness between humoral and cellular immunity also has grown, and antibody responses could affect vaccine-induced immunity through enhancement, modulation, or reduction of inflammation [77].

Prospects of vaccines for avian aspergillosis

For the birds?

Aspergillus spp. have been recognized as an important cause of pulmonary disease in birds since the early 1800s and, as in humans, *A. fumigatus* is the most frequent cause of disease [78]. In contrast to mammals, birds are highly susceptible to invasive aspergillosis. Though all birds are considered susceptible, disease is particularly common among waterfowl, birds of prey, captive penguins, some parrot species and poultry [79–83]. Losses in turkey flocks are consequential because aspergillosis in adult turkeys most commonly occurs late in the growing cycle, resulting in mortality or the need for carcass condemnation at slaughter inspection [82]. The current actual prevalence of aspergillosis in turkeys is difficult to assess, as production by the US turkey industry is controlled by commercial companies. The concentration of turkeys on fewer farms has increased the risk of turkey health problems, which are now primarily handled within the companies [84]. However, prior estimates from the University of Georgia in 1986 and collected yearly by Iowa State University from 1983 through 1994 suggest a mean prevalence of over 8% among flocks, with peaks of over 17% [84,85]. In Iowa, estimates of dollars lost showed a mean of $5,260 per affected flock, which were considered conservative but were second only to those due to fowl cholera [84]. Though successful treatment of birds with antifungal agents has been reported, such treatment is not considered cost-effective for poultry and prevention remains the preferred strategy [78]. Thus, the availability of a vaccine that can prevent disease in poultry or in susceptible avian species in zoological parks may have the potential for significant economic impact.

The reason for the increased susceptibility of some avian species is unclear at this time. However, age is important, as penguin and chicken chicks, as well as turkey poults are more susceptible than are adult birds and their disease typically is more acute [81,86]. A study of avian aspergillosis in chicks found that the appearance of serum antibody reactive with hyphal fragments correlates with the development of resistance to infection and the appearance of precipitins coincides with clearance of hyphae from air sac and lungs, suggesting a role for antibody in acquired resistance [86]. The increased incidence accompanying captivity, some farming practices or mating has been noted repeatedly, leading to the hypothesis that susceptibility is stress-induced [81,87]. In a recent review, a number of distinguishing features have been proposed to play a role, such as anatomic differences from mammals and differences in innate immune cells, including a lack of surface macrophages in the lung and dependence upon heterophils, which, unlike mammalian neutrophils, do not have myeloperoxidase and oxidative mechanisms for killing hyphae [88].

Prior vaccine studies in birds

Vaccine studies of avian species have shown variable results. In penguin chicks, intramuscular vaccination with a killed suspension of *A. fumigatus* did not alter the prevalence of disease or the attributable mortality rate [81]. In a study of turkeys challenged by transcutaneous injection into the posterior thoracic air sac, delivery of a second challenge five weeks postinoculation resulted in more severe pulmonary lesions than occurred after primary challenge, indicating that prior infection may enhance susceptibility [82]. However, subcutaneous vaccination of ducks with conidia can confer substantial protection against i.v. infection, as a reduction in mortality from 60 to 17% has been observed. Though vaccinated ducks initially become ill, most recover clinically and ultimately clear their infection, suggesting that, though disease is not prevented in this model, the subsequent host response can control the infection [89].

A systematic study of whole cell vaccination in turkey poults was performed by Richard and colleagues, who examined the ability of vaccines made from culture filtrate, killed conidia, germlings and mycelia to protect against aerosol challenge [90]. They found that, though administration of a mycelia vaccine somewhat delays the time to death after administration of an inoculum that kills 100% of control animals, only vaccination with a germling vaccine results in a significantly higher percentage of turkeys surviving until the end of the observation period. Lung and brain lesions in surviving animals do not differ histologically among the vaccine groups, again suggesting that, rather than preventing disease, vaccination decreases severity. The nutritional medium used for growth of *A. fumigatus* affects germling vaccine efficacy [91,92], suggesting that protective antigen expression is medium-dependent. Route of vaccination and/or adjuvant administration further alters the relative efficacy of the different germling vaccines, administration of which results in between 29 and 57% reductions in mortality [91]. When examining the impact of different adjuvants on germling vaccine efficacy, these investigators found that poults receiving LPS as adjuvant have less severe lung lesions following sublethal infection than do those receiving intralipid, N-acetylmuranyl-L-alanyl-D-isoglutamine or aviridine [92]. This finding is particularly intriguing in view of our more recent understanding of the importance of TLR-4 signaling, as discussed earlier, because LPS is an important TLR-4 ligand [93]. Thus, these studies provide support that poultry vaccination may be feasible.

Murine models for invasive aspergillosis

Several animal species have been used to emulate human disease due to *A. fumigatus*, including mice, rats, guinea pigs, rabbits and, more recently, drosophila [94,95]. Mice are the predominant species that have been used to evaluate vaccine efficacy. Because pathogenesis may vary with the route of infection or the specific mechanism of immune suppression, these models are briefly reviewed here.

Intravenous infection

In systemic infection models, conidia are administered intravenously and lethal infection occurs without the need for administration of immunosuppressive agents [96]. These models are useful for the study of disease outside of the lung, as, particularly in mice, dissemination is not reliably observed following pulmonary infection. The disadvantage of this model is that conidia are introduced into the peripheral circulation, a compartment that they are unlikely to contact during the course of naturally occurring infection. Thus, the early events after infection in this model do not mimic human disease, as serum components could affect germination and the organism gains access to the lung from the capillary beds, rather than the airspace.

Pulmonary infection

Consequently, a variety of pulmonary infection models have been developed, in which mice are infected intranasally (i.n.), intratracheally (i.t.) or via a variety of aerosol systems [97,98]. In these models, the maximum numbers of conidia that can be introduced do not result in reliable mortality. Therefore, different methods for immunosuppression are used that are selected, in part, to parallel the range of immune disorders that result in enhanced susceptibility to disease in humans.

Neutropenia

Most vaccine studies have used either cyclophosphamide or the granulocyte-depleting MAb RB6-8C5 to induce neutropenia. Cyclophosphamide is a common component of human chemotherapeutic regimens. This drug induces bone marrow aplasia and dissolution of lymphoid tissue by alkylating the 7 nitrogen of guanine residues, leading to G-T base pairing, DNA chain scission and interchain cross linking [99]. Thus, this agent is very broadly immunosuppressive, severely affecting both innate and adaptive cellular components. The use of MAb RB6-8C5, which binds to Ly6-G on granulocytes, resulting in subsequent depletion, has been used to induce more selective neutropenia [100]. However, at commonly used doses, lymphocytes are depleted as well [61]. In mice in whom neutropenia is induced either with cyclophosphamide or with MAb RB6-8C5, the inflammatory response that occurs at the time of recovery may be sufficient to be responsible for mortality, rather than extensive fungal growth and invasion [61].

Glucocorticoids

Corticosteroids also are broadly immunosuppressive, but act primarily by altering function of immune cells, rather than by depleting them. For monocyte/macrophages and dendritic cells, antigen presenting and effector functions are impaired via effects that include reduction in phagocytic ability, co-stimulatory molecule expression, superoxide anion production and prostaglandin synthesis [101–103]. Pro-inflammatory cytokine and chemokine production are diminished through mechanisms that operate mainly, but not exclusively, at the level of transcriptional control [104,105]. Glucocorticoids may alter cell surface expression or function of Fc and complement receptors [106,107]. However, glucocorticoids also may increase IL-10 production, resulting in selective suppression of T_H1-associated responses and a shift toward a T_H2-associated pattern [108,109]. Regarding direct effects on lymphocytes, corticosteroid administration results in sequestration of $CD4^+$ T cells in the reticulendothelial system, inhibition of IL-2 secretion and down-regulation of T and NK cell granzyme production, an effect associated with reduced cytotoxicity [110–112].

Regarding our current understanding of which of these mechanisms are important for enhanced susceptibility to invasive aspergillosis, dexamethasone reduces the ability of both alveolar macrophages and peripheral blood mononuclear cells to inhibit germination *in vitro*, without impairment of phagocytosis, while the effect of cortisol is less potent [113]. Similarly, hydrocortisone impairs monocyte- and neutrophil-mediated hyphal damage [114,115]. The demonstration of these effects, combined with lack of enhanced mortality in corticosteroid-treated athymic mice following i.v. or pulmonary infection [10], has resulted in the view that corticosteroids increase susceptibility to invasive aspergillosis primarily via impairment of innate cellular function. The actions of the various glucocorticoid compounds may vary quantitatively or qualitatively [116] and extensive systematic comparison of their effects on host interactions with *A. fumigatus* has not been done. Regarding the course of infection in corticosteroid-treated mice, administration of repeated doses of cortisone acetate allows for 100% mortality with 10^7 to 10^8 conidia reaching the lung following i.n. infection [117]. Early studies showed that increased susceptibility correlates with abundant hyphal growth in corticosteroid-treated mice early after infection [10,118]. However, a more recent study examining terminally ill mice suggests that mice die from tissue damage due to enhanced neutrophil recruitment [63]. Interestingly, in this study, neither TNF-α nor IL-10 was detected in bronchoalveolar lavage fluid from cortisone-treated mice, suggesting that T_H2-associated responses are not increased in this system.

Bone marrow transplantation

More recently, an elegant model for bone marrow transplantation has been applied to the study of vaccine candidates. In this model, lethally irradiated mice are transplanted with T cell-depleted bone marrow hematopoietic cells. Transplanted mice show stable donor-type hematopoietic chimerism [68]. The timing of infection after transplantation is important as, once neutrophil recovery occurs, mice are no longer susceptible to invasive aspergillosis. Reproducible lethality follows three daily infections with large conidial inocula. Susceptibility to infection correlates with extensive fungal growth in the lungs and bronchial

wall damage one day after the last inoculation and the mean survival time after infection is approximately five days [36,68].

Prior study of vaccines for invasive aspergillosis in mammals

Whole cell vaccines and systemic infection

From the late 1930s through the 1990s, investigators reported the effect of either whole cell vaccination with *A. fumigatus* or with poorly defined antigen preparations made from disrupted organisms or culture filtrate. Whole cell vaccine efficacy has been examined in both systemic and pulmonary infection models in rabbits and mice, utilizing a variety of adjuvants and routes of vaccination, with varying results. Of the models examining systemic disease induced by i.v. infection, Tilden *et al.* found that neither prior sublethal infection nor immunization with heat-killed conidia protects rabbits against intravenous infection with large numbers of conidia [119]. In contrast, in murine models of systemic disease, protection by whole cell vaccination against i.v. infection with conidia has been observed by several investigators. Two studies found that viability of conidia, whether administered i.v. or i.n., is required to confer resistance [76,120]. The ability to obtain protection against disease by prior i.v. immunization with conidia despite cortisone treatment of mice led to the hypothesis that antibody dependent cellular cytotoxicity may be involved in the observed prevention of disease, as immune serum did not directly affect conidial germination in this study [121]. Vaccination experiments in nude mice demonstrate that prior subcutaneous vaccination with a sublethal inoculum of live conidia confers protection against subsequent i.v. infection in heterozygotes but not in homozygotes, implicating an important role for T cells in the protective memory response [37]. De Repentigny *et al.* found that increased survival induced by i.v. vaccination with a sublethal conidial inoculum correlates with statistically significant, though small, reductions in fungal burden in the lung, kidney and liver [122]. In this model, adoptive transfer of splenocytes, but, again, not serum, confers protection despite the presence of *A. fumigatus*-specific antibody. Rather than T cells, protection is abrogated by depletion of an adherent cell population that consists mainly of macrophages. Peritoneal and splenic macrophages from pre-infected mice have enhanced capacity for phagocytosis, but not killing, and hydrogen peroxide production by splenic macrophages in response to PMA is reduced. Thus, though the authors speculated that enhanced effector function is involved, the mechanism by which macrophages confer protection in this system is unclear, but one possibility is the terminal effector function of a T_H1-type response.

Whole cell vaccines in pulmonary infection

Conclusions regarding the efficacy and mechanism for protection against experimental invasive pulmonary aspergillosis have also varied. Kisch *et al.* found that subcutaneous (s.c.) immunization with live conidia with complete Freund's adjuvant induces both antibody and cellular responses and protects cortisone-treated mice against subsequent aerosol infection [123]. Interestingly, passive transfer of rabbit antiserum also prolongs survival in their model. However, their investigations have been published only in abstract form. A more recent study found that prior pulmonary infection with conidia prevents mortality in mice that then are treated with cortisone [124]. Nonetheless, a vaccine made from sonicated mycelia, when administered s.c., but not i.n., is more effective in preventing mortality. The ability of repeated i.n. conidial inoculations to protect mice subsequently treated with cyclophosphamide against pulmonary infection also has been seen and, as in i.v. infection, conidial viability is required to induce protection [76].

A live, attenuated vaccine

The above studies used isolates for immunization that were virulent in the varying models tested. One attempt at using a live attenuated conidial vaccine has been reported [96]. Vaccination of mice with an avirulent *p*-aminobenzoic acid (PABA) auxotrophic mutant, produced by chemical mutagenesis, protects against systemic infection with the virulent parental strain. Though i.v. vaccination reduces mortality from 90 to 50%, with organ cultures from all dead mice growing *A. fumigatus*, an intramuscular (i.m.) vaccination protocol in mice is substantially more effective, resulting in 15% mortality and no positive organ cultures. However, a caveat with attributing the difference to route of administration is that mice that were vaccinated i.m. received a larger number of vaccinations with a higher inoculum.

Thus, studies of vaccines that used whole cells, either in the form of conidia, or that included disrupted whole germlings or mycelia support the view that protective immunity against pulmonary or systemic infection can be induced. The requirement for viability of conidia, but not other forms, suggests that the antigens that elicit protection may not be present or accessible in the other morphologic form of the organism. Experiments

in animals that were subsequently immunosuppressed provide evidence that induction of immunity prior to immunosuppression may be effective in immunocompromised hosts. As has been seen generally in vaccine studies, route of administration may be an important factor in determining outcome.

Vaccines using uncharacterized antigen preparations

Henrici reported the use of a thermolabile "endotoxin", consisting of partially purified extracts from ground mycelia, that, by itself, is highly toxic to rabbits, guinea pigs and mice [125]. Immunization with this material in incrementally increasing doses resulted in protection of rabbits against death due to i.v. inoculation with 10 times the minimal lethal dose of conidia. However, Tilden *et al.* were unable to reproduce this result, despite substantial characterization of the toxin [119,126,127].

Varying results have been obtained with the use of culture filtrate preparations, which are comprised of molecules released by the organism during growth in liquid culture. Though prior investigators had not found efficacy in turkeys or mice [90,124], Cenci *et al.* obtained better protection by administration of crude filtrate preparations than by vaccination with live conidia in immunocompetent mice infected i.v. or in cyclophosphamide-treated mice infected i.n. [76]. Protection in the pulmonary model is associated with: (i) reduced lung fungal burden; (ii) altered inflammatory cellular recruitment, with a shift in predominance from neutrophils toward macrophages and lymphocytes; (iii) reduced pulmonary pathology; (iv) activation of $CD4^+$ T cell populations capable of antigen-specific lymphoproliferation; (v) shift of lung cytokine profiles and antibody responses toward a T_H1-associated pattern, such that bronchoalveolar lavage fluids contain higher concentrations of IL-12p70 with lower concentrations of IL-10 and IgE compared to unvaccinated mice or those that received non-protective antigens; and (vi) more IFN-γ and IL-2 in the supernatant of lung cells stimulated with anti-CD3 *ex vivo*. Further support for the relationship between T_H1-associated patterns and protective efficacy comes from the findings that culture filtrate antigens do not induce protective responses in IL-12p40$^{-/-}$ or IFN-$\gamma^{-/-}$ mice, but do in IL-4$^{-/-}$ mice. Protection could be induced by passive transfer of $CD4^+$ T cells stimulated with antigen and dendritic cells from immunized mice. The component(s) of culture filtrate antigens that are responsible for the development of protective responses have not been defined. However, these findings provide an important rationale for the goal of subsequent investigation, namely, the identification of strategies that enhance T_H1-associated responses.

More recent studies have proceeded in three important directions: toward focus on manipulation of the immune response with different adjuvants, identification of specific antigens capable of inducing protective responses and the development of a vaccine with efficacy against a broad range of pathogens, including *A. fumigatus*.

The role of adjuvant

Advances in the understanding of the biology of dendritic cells and TLR have allowed for somewhat more rational approaches to the selection of adjuvants. Thus, Bozza *et al.* have examined the ability of unmethylated CpG oligodeoxynucleotides (ODNs) to induce T_H1 polarization of vaccine-induced responses [34]. CpG ODN motifs are pathogen-associated molecular patterns that interact with TLR 9 on plasmacytoid dendritic cells and B cells, with subsequent NK and T cell activation [128]. These motifs skew host responses toward a T_H1-associated phenotype in response to bacterial, viral and fungal antigens in a sequence-specific manner and are active in the development both of systemic and mucosal immunity [129]. These investigators found that co-administration of CpG ODN i.n. with Asp f16, a 43 kDa protein of unknown function that is bound by IgE and IgG4 of patients with allergic aspergillosis [130], markedly enhances survival of cyclophosphamide-treated mice infected i.n. with *A. fumigatus* [34]. No effect is seen for immunization with either CpG or Asp f16 alone. Prolongation of survival by co-administration of CpG and Asp f16 again correlated with reduced fungal burden, less inflammation and the development of a more polarized T_H1-associated response in the lungs. The exposure to Asp f16 in combination with CpG ODN further increases lymph node dendritic cell maturation beyond that seen after administration of CpG alone. Exposure to CpG ODNs enhances both the phagocytic and killing capacity of alveolar macrophages and peritoneal neutrophils for resting conidia *in vitro*, indicating that this vaccine may enhance innate effector functions. The demonstration that a potent immunogen that ordinarily elicits T_H2-associated responses can be induced to evoke a protective T_H1-associated response suggests that a broader range of defined candidate antigens might be of use than previously was considered possible, which is an important advance. The question of whether any antigen can be converted to a protective one by manipulation of the adjuvant and/or immunization protocol is an

intriguing one that is unanswered at this time. That similar benefit is not seen with all of the allergens tested may simply reflect the particular vaccination scheme, the adjuvant used or a requirement for unknown properties of fungal molecules in order for them to elicit protective immunity. The specific use of CpG as an adjuvant for human vaccines may, however, be limited by the recently demonstrated toxicity associated with repeated administration [131].

Subsequent investigation to dissect the mechanism for protection by this antigen examined the Asp f16 epitopes responsible for induction of protective immunity. Using a protein-spanning pool of overlapping pentadecapeptides, Ramadan *et al.* determined that appropriately primed dendritic cells pulsed with several defined regions of the protein induce MHC class II - restricted T_H1 and cytotoxic $CD4^+$ cell, as well as class I-restricted cytotoxic $CD8^+$ cell, responses [132,133]. Cytotoxic $CD4^+$ and $CD8^+$ cells exhibit some ability to directly kill conidia and hyphae. Thus, several regions of this molecule are capable of inducing host responses associated with beneficial cellular immunity. These studies are also the first demonstration of direct fungicidal activity by T cells against *A. fumigatus*.

Another adjuvant that has attracted attention is thymosin α1, a naturally occurring thymic peptide that, in synthetic form, has been used in clinical trials for treatment of viral infections and malignancy and that had been shown in the early 1980s to protect mice against *Candida albicans* infection [134–137]. Repeated thymosin α1 administration beginning prior to infection prolongs survival both in a murine model of allogeneic bone marrow transplantation and in cyclophosphamide-treated neutropenic mice infected i.n. [36]. Markedly less, though significant, prolongation of survival is seen in cyclophosphamide-treated NOD/SCID and IFN-γ-deficient mice, while maximal protection occurs in IL-4-deficient mice, suggesting a role for T_H1 cells in the mechanism of action [36]. Administration of the granulocyte-depleting mAb RB6-8C5 to NOD/SCID mice obliterates protection, a finding in accordance with the view that T_H1-associated responses act by stimulating neutrophil function. Less fungal growth is observed in bone marrow-transplanted mice treated with thymosin α1, an effect that is dependent upon signaling through MyD88 [36]. *In vitro*, exposure of murine lung dendritic cells to synthetic thymosin α1 enhances ingestion of conidia, while stimulating maturation and IL-12 production in a MyD88-dependent manner [36]. In human dendritic cell subsets, differential cytokine production is stimulated, such that IL-12p70 and IL-10 production are induced by myeloid and plasmacytoid dendritic cell subsets, respectively. Pre-exposure to thymosin α1 also increases both phagocytosis and conidiocidal activity of murine bronchoalveolar macrophages and peripheral blood neutrophils *ex vivo* [36]. Thus, thymosin α1 appears to stimulate effective immunity via both innate and adaptive responses, the latter involving dendritic cell priming toward a polarized T_H1 response, but also possibly through regulatory effects on the inflammatory response, and this agent has been proposed for use as an adjuvant for vaccines against invasive aspergillosis.

Dendritic cells as adjuvant

In view of the importance of dendritic cells for determining the pattern of host response to infection with *A. fumigatus*, Bozza *et al.* have examined the ability of a dendritic cell vaccine to confer protection in the murine bone marrow transplant model [75]. After hematopoeitic cell transplantation, s.c. administration of dendritic cells that had been pulsed with conidia or transfected with conidial RNA significantly prolonged survival in mice subsequently infected i.v. or with a very high conidial inoculum i.t. Though, under these conditions, mice that receive either no or unstimulated dendritic cells have 100% mortality, 95–100% of the mice that receive dendritic cell vaccines survive. No protection was observed in granulocyte-depleted mice infected i.t., suggesting that neutrophils are critical for the efficacy of this vaccine. Protection is associated with reduced fungal burden in mice infected by either route and in skewing of cytokine patterns in cell populations from vaccinated mice toward T_H1-associated patterns. In contrast, no protection is seen when mice are vaccinated with dendritic cells that have been exposed to hyphae or transfected with hyphal RNA and cell populations from these mice demonstrate T_H2-associated cytokine patterns, a finding that is in agreement with a prior study [5,75]. However, more IL-10-producing $CD4^+$ T cells are isolated from the lungs of mice that receive dendritic cells that have been stimulated with conidia or conidial RNA, suggesting possible participation of an inflammation regulatory mechanism in protection. In this model, though infusion of antigen-specific T cells prolongs survival, all mice ultimately die, indicating superior efficacy of dendritic cell vaccination. These results are in concert with prior work from these investigators showing that T_H1-polarization is associated with resistance to invasive aspergillosis and demonstrate the potential promise of dendritic cell vaccination as an approach to immune-based prophylaxis. The rationale for developing this vaccine will be further strengthened if, as suggested, T_H priming and phagocytosis are severely impaired in

immature DC subsets from hematopoeitic cell transplant recipients.

An additional approach to the use of dendritic cell vaccines for invasive aspergillosis has been reported recently. Shao *et al.* studied the efficacy of administering dendritic cells transduced with an adenovirus vector encoding IL-12 that then were pulsed with heat-inactivated *A. fumigatus* conidia [138]. Transfection with this vector results in constitutive overproduction of IL-12. Cyclophosphamide-treated mice that are vaccinated with either IL-12-transfected dendritic cells or those that had been pulsed with heat-inactivated conidia show markedly reduced mortality seven days after i.n. infection, in comparison to those that received either naïve dendritic cells or those transfected with empty vector, all of which die by six days after infection. Mice that received dendritic cells pulsed with heat-inactivated conidia had intermediate survival rates. Reduced mortality correlates with lower fungal burden three days after infection, which is lowest in mice that receive dendritic cells that both are transfected with the IL-12 vector and exposed to heat-inactivated conidia. Pulmonary pathology is reduced in mice that receive dendritic cells that either have been transfected with the IL-12 vector and/or exposed to heat-inactivated conidia. *In vitro* and *ex vivo* data support the association of vaccine efficacy with enhanced maturation of T_H1-associated responses. However, some of the reported data suggest that vaccination with IL-12-transfected, conidia-exposed dendritic cells results in very high constitutive expression of IFN-γ in the spleen, raising concern about the safety of the combined treatments. Further, mice that received dendritic cells transfected with the IL-12 vector develop splenomegaly, which reportedly occurs after IL-12 DNA vaccination, as well [138,139]. Thus, this approach to a vaccine for invasive aspergillosis is logical, but unlikely to be clinically useful.

Identification of specific antigens

As noted above, the other important direction for pursuit is the identification of specific antigens that induce protective responses. Toward this end, Denikus *et al.* have screened an *A. fumigatus* cDNA expression library derived from young hyphal mRNA in order to identify antigens that elicit antibody responses during systemic infection [140]. These investigators found that, compared to pre-immune sera, stronger Ab responses are induced in cortisone-treated rabbits to fractions enriched for cell wall components, compared to those enriched for intracellular components. Thirty six antigens have been identified by this technique, most of which do not contain signal sequences for protein secretion. These investigators hypothesize that there are either additional cellular locations for these antigens or that non-classical secretion mechanisms may be operative, justifying their exploration as putative vaccine candidates. However, they also appropriately postulate that immune responses to these antigens may be beneficial by modulating inflammatory responses or by promoting fungal clearance.

A multi-fungal vaccine?

An additional approach toward vaccine development has stemmed from the finding of broad-spectrum protective efficacy of antiidiotypic monoclonal antibody of killer toxin, discussed above [68]. The investigators reasoned that, because the target of this protective antibody is involved in β-glucan synthesis, the development of host responses to β-glucan could be beneficial. Beta glucans are important constituents of the cell wall of many fungi, including *Aspergillus* spp., which, like many carbohydrates, are themselves poorly immunogenic. Therefore, a novel vaccine was synthesized using laminarin, a linear β-1,3-linked glucan backbone with occasional β-1,6-linked branches from the brown alga *Laminaria digitata*, conjugated to the diphtheria toxoid CRM197, a protein carrier that has been used in other human vaccines [141]. Though investigation has focused primarily on protection against systemic and vaginal candidiasis, preliminary study suggests that this vaccine also significantly prolongs survival in mice infected systemically with *A. fumigatus*. Sera from vaccinated mice significantly reduce hyphal elongation *in vitro*, suggesting a mechanism for protection. However, though these investigators clearly demonstrate that antibody is responsible for the efficacy of this vaccine against *Candida*, similar dissection has not yet been reported for *A. fumigatus*. Interestingly, the antibody response to this vaccine consists overwhelmingly of IgG1, though low titers of the other IgG subclasses also are induced and their enrichment is suggested to augment the efficacy of the vaccine for invasive candidiasis. Though antibody isotype alone cannot be used to determine T_H subset patterns, this pattern suggests the induction of a mixed T_H1, T_H2 response with possible T_H2 predominance. Thus, the mechanism of protection may differ significantly from that induced by the other vaccines discussed earlier. The potential of this vaccine as one that could protect against a variety of organisms make it especially attractive, particularly as other fungal pathogens emerge as important causes of disease, either because of medical interventions targeting more com-

mon pathogens or changes in the host, such that immunosuppression is even more profound [142].

Vaccine efficacy in immunocompromised hosts

To summarize our knowledge of vaccine efficacy in the setting of immune compromised conditions with vaccines that have been studied to date, neither corticosteroid (whole cell vaccine) nor cyclophosphamide (whole cell, crude culture filtrate and Asp f16 vaccines) administration after vaccination eradicates subsequent protection, even though both agents are broadly immunosuppressive. In contrast, though the effect of MAb RB6-8C5 is somewhat more limited to granulocytes, its administration prevents the efficacy of dendritic cell vaccination in bone marrow transplanted mice, which could be due either to induction of more profound neutropenia or to rebound at the time of immune recovery. T cells are required for development of protective immunity by whole cell vaccine, and IFN-γ and IL-12 are required for that induced by extracts. Whether T cells and cytokines are required for development of memory or for the effector arm of the secondary response is unknown at this time, as depletion in mice that are vaccinated while these components are present has not yet been reported. Thus, we are early in our understanding of the host immune components that are necessary for protective efficacy and the specific requirements may differ with the vaccine strategy. Testing in multiple models may be necessary to be able to predict the susceptible hosts that may benefit.

Conclusion

In recent years, significant advances have been made in our understanding of host defense against invasive aspergillosis and in methods for inducing protective responses. Though progress is being made in identifying the fungal molecules important for disease production, these may not represent unique targets and the development of clinically relevant vaccines likely will need to take into consideration the varied host populations that are affected. The repeated demonstration in animal models that protection following vaccination can be maintained during subsequent immunocompromised conditions holds promise for the efficacy of vaccines for patients in whom immunosuppression can be predicted. The development of vaccines to be administered to those already at risk will require further dissection of innate immunity and memory responses to *A. fumigatus*. Nonetheless, the demonstration that dendritic cell vaccination can confer protection in at least some immunocompromised hosts is exciting. Continued progress in the rational design of vaccines to prevent or treat invasive aspergillosis will likely follow advances in our understanding of both the specific antigens of *A. fumigatus* and mechanisms for effective manipulation of responses to these antigens with cellular or cell-free adjuvants.

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