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REVIEW ARTICLE

Fungal vaccines

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ABSTRACT

Invasive fungal disease continues to be a cause of significant life-threatening morbidity and mortality in humans, particularly in those with a diminished immune system, such as with haematological malignancies. The mainstay of treating such life-threatening fungal infection has been antifungal drugs, including azoles, echinocandins and macrocyclic polyenes. However, like antibiotic resistance, antifungal resistance is beginning to emerge, potentially jeopardizing the effectiveness of these molecules in the treatment of fungal disease. One strategy to avoid this is the development of fungal vaccines. However, the inability to provoke a sufficient immune response in the most vulnerable immunocompromised groups has hindered translation from bench to bedside. This review will assess the latest available data and will investigate potential *Aspergillus* antigens and feasible vaccine techniques, particularly for vaccination of high-risk groups, including immunocompromised and immunosuppressed populations.

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Introduction

Vaccines have historically played an important role in helping to control infectious diseases, especially those of a viral and bacterial aetiology. Whilst these vaccines have played a major societal role in containing and preventing such diseases, both in humans and in animals, there is still no effective licenced fungal vaccine for indications in humans, either locally, nationally or internationally. This is due largely to the molecular complexity of eukaryotic fungal pathogens, as immunological targets alongside their capacity to evade both naturally acquired and vaccine-induced immunity. The optimized development of fungal vaccines could help circumvent the necessity for antifungal drugs and subsequent driving of antifungal resistance, as seen in azole-resistant Invasive Aspergillosis with immunocompromised patients [1,2]. Only one fungal vaccine has been successfully licenced to date to reduce clinical signs, aid recovery and help prevent ringworm in cattle. Its origin began in Russia in 1967, where a vaccine was produced from live immunogenic cells of an attenuated strain of 130 *Trichophyton verrucosum*, leading on to a fully licenced live fungal vaccine in the early 2000s, called *Bovilis® Ringvac* had been made for prophylactic and therapeutic vaccination of cows against the fungus *Trichophyton verrucosum* [3]. This vaccine possesses many practical and logistical aspects that an *Aspergillus* vaccine would need [4]. Currently, there are no *Aspergillus* vaccines in development. The only fungal vaccine trialled on Clinicaltrials.gov was the vulvovaginal *Candida* vaccine in 2012, with no results reported.

Aspergillus is one of the many opportunistic fungi that can critically infect immunocompromised groups such as HIV patients, haematopoietic transplant recipients and chemotherapy patients [5]. While antifungal drugs such as azoles, echinocandins, allylamines and polyenes exist to treat these groups, no such fungal vaccine exists that can stimulate protective immunity [4] Whereas these antifungal drugs treat a variety of fungi, they are slowly driving resistance and being negated through their over usage in farming as fungicides, as well as in cases of poor compliance to antifungal usage, resulting in exposure of the fungal pathogen to subinhibitory concentrations of drug [6]. However, with a fungal vaccine, the threat of complete fungal resistance to each class of antifungal can be mitigated as vaccines can produce protective immune responses in patients. It is estimated that at least three million people are affected by chronic pulmonary aspergillosis worldwide [7] and if treatment options are nullified by resistance, their situation could become fatal. However, fungal pathogens primarily afflict lethality in their invasive and nosocomial forms, and those who are vulnerable to such pathogens are primarily immunocompromised patients [8]. A major obstacle for the production of a fungal vaccine is stimulating a weakened immune system. Such patients may not recognize an inactivated subset or attenuated form of the fungus as foreign and thus produce no immune response; or as exploited by invasive fungi, produce, an inappropriate detrimental

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immune response [9]. The typical course of an invasive aspergillosis infection is illustrated in Figure 1, a vaccine would stimulate immunity and bypass the compromising stages of invasive aspergillosis infection. If immunity can be stimulated in immunocompromised patients, then the fatal effects of the infection can be subdued.

Additionally, *Aspergillus*-infected patients tend to be severely immunocompromised [10]. Evidence exists that immunocompromised patients can mount an immune response due to the plasticity of the immune system, as defined by the ability of the immune system to adapt to both phenotype and function in response to a dynamic environment. However, complete cytokine and immune restoration through plasticity has not occurred in immunocompromised patients. Mortality rates for invasive aspergillosis can reach 80% [11]. A vaccination plus a booster may be more economical. Reduced immunocompromised patients' lifespans, cost of in-hospital readmissions post-surgery and the emergence of azole resistance promote the clinical need for vaccine development.

However, the translation of *Aspergillus* vaccines from bench to bedside has not occurred. Vaccines typically evoke a protective response in healthy patients; however, the heavily fungal-nosocomial-afflicted groups encompass the immunocompromised and immunosuppressed, which show variability in their immune responses [10]. This review assesses published data on experimental *Aspergillus* vaccines, their effect on healthy and immunocompromised groups and what immune responses are desirable for fungal protection.

Methods

Peer-reviewed fungal vaccines papers (n = 136) were searched for through PubMed's search engine under

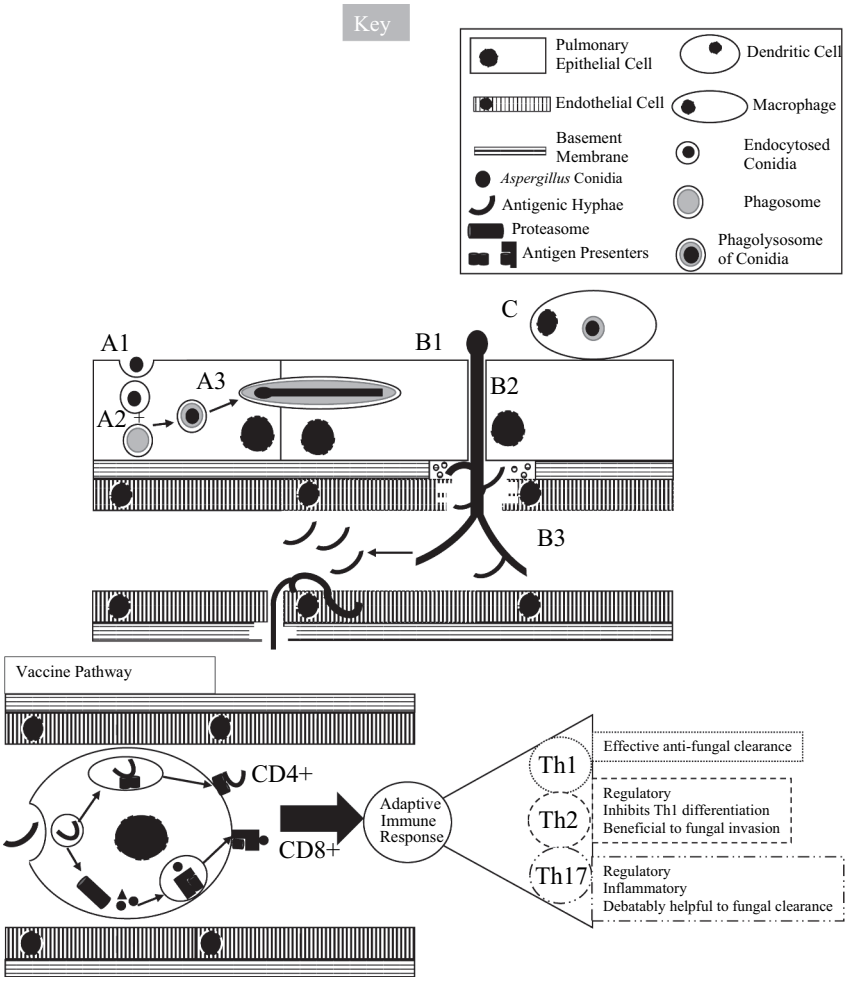

Figure 1. Three routes of Invasive Aspergillosis (IA) infection: route A:A1) Conidial adhesion to pulmonary epithelial cells, A2) Endocytosis of conidia and fusion with phagosome, A3) Phagolysosome fungicidal failure and subsequent germination within phagolysosome. Route B: B1) Conidial adhesion, B2) Hyphal extension and angioinvasion, B3) Aspergillus dissemination. Route C) Phagocytic uptake by macrophage and phagolysosomal fungicidal failure leads to systemic spread. Vaccine pathway: hyphal fragments are endocytosed by dendritic cells which are presented on MHC I and II receptors to stimulate CD4+ or CD8 + T-Helper cells to create either a Th1, Th2 or Th17 adaptive immune response. For most patients, the main portal of entry to the body and site of infection for *Aspergillus fumigatus* is the respiratory tract. Resulting pulmonary diseases can be classified according to (i) the site of the disease within the respiratory tract and (ii) the extent of mycelial colonization or invasion, both of which are influenced by the immunological status of the host. A vaccine would stimulate immunity and bypass the compromising stages of IA infection. If immunity can be stimulated in immunocompromised patients, then the fatal effects of IA may be lessened, with improved clinical outcomes.

the terms of 'fung*, vacc* & *Aspergillus*', as shown (Figure 2). The studies were screened by full-text analysis. Qualitative and quantitative papers were included: qualitative papers that hypothesized fungal immune responses in immunocompetent and immunocompromised patients, as these papers can aid in the screening of potential *Aspergillus* vaccine quantitative papers. Subsequently, 106 papers were rejected due to exclusion criteria, including non-English language, accessibility issues and not relevant to *Aspergillus*, leaving 30. Further screening identified 11 quantitative scientific studies of *Aspergillus*-relevant vaccination from these 30 scientific articles. The data in each paper ranged from model mortality to the number of antibodies produced by the model. This data was standardized into the binary category (Y/N) for immunity into a tick table. Additionally, 9 studies list the post-vaccination infection mortality rate, making these papers comparable. However, 3 papers did not list their mortality but quoted chemokine responses or did not supply information to calculate mortality. This data was screened for with additional context, such as the type of immunity displayed. Nineteen papers were excluded due to lack of quantitative data, leaving 11, as detailed in Table 1 [12–22].

Results

All the different types of *Aspergillus* vaccinations induced non-toxic immunity in the murine models. Using semi-qualitative and quantitative data from these results, suitable candidates for further research can be deduced from these vaccines. Different types of immunity were induced in these murine models: A T-helper 1 (Th1) response incurred the most effective antifungal immunity [23], whereas a T-helper 2 response (Th2) is counterproductive to antifungal immunity [24] and a T-helper 17 (Th17) response is debatable [25–27].

These vaccines were screened for their toxicity, the Titermax adjuvant and Complete Freund's Adjuvant used in Studies 1,7,11a and 11b (Table 1) are not toxic in murine models, they are toxic in human models, respectively; therefore, they cannot be considered for immunocompromised or immunocompetent patients. Conveniently, in Study 4, an alternative Asp f 3 vaccine was used with a minimally toxic Incomplete Freund's adjuvant and succeeded in their immunocompromised murine model, as it can be mixed with other adjuvants, potentiating its adjuvanticity. Additionally, Study 8's sonicated and filtrated Crude Culture Filtrate Antigens (CCFA) would not be endorsed for clinical trials. An unknown mixture of different antigens would fall outside of Good Manufacturing Practice (GMP) standards, as only defined preparations that can be reproduced and standardized with exact antigen concentrations can be accepted. Although Study 8 only produced semi-qualitative information, it is conclusive that immunization with antigens from CCFA is capable of producing antifungal immunity, a foundation for further *Aspergillus* antigen research.

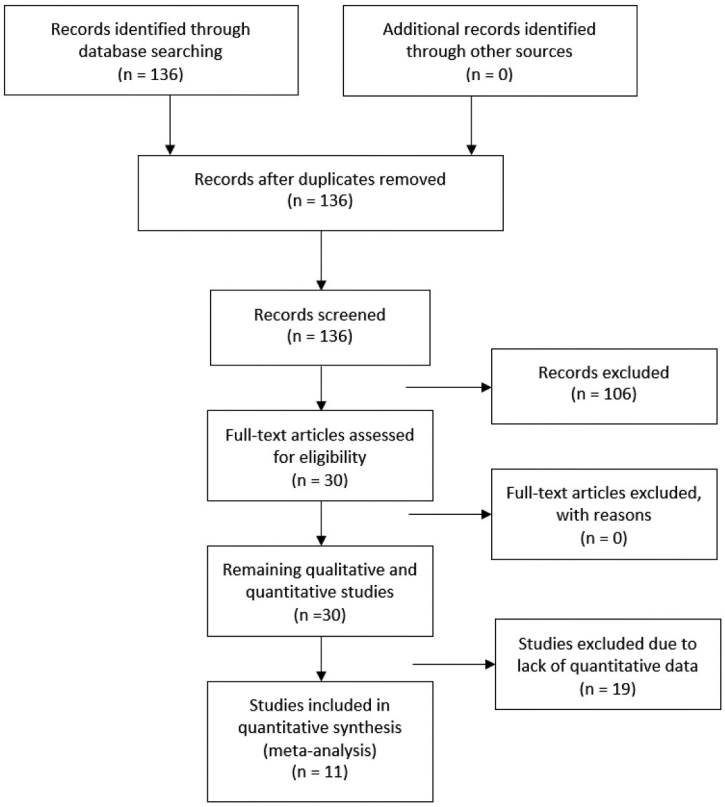

Figure 2. A PRISMA analysis of literature searches relating to this review.

Table 1. Analysis of 11 reports of *Aspergillus*-related vaccines.

Aspergillus	Type of Vaccine	Adjuvant	Toxicity in Model (Y/N)	Model	Type of Immunity	Route of Administration	Formulation	Post-Vaccination Infection	Dosage	Study	Reference
Asp f3	Recombinant Subunit	Titermax	N	Corticoid-treated BALB/c mice	Humoral Th1 cells	Subcutaneous	Injection	0	2.5 mg x 6 days	1	[12]
Asp f16	Recombinant subunit Pulsed Dendritic Cells	CpGs ODNs	N	Cyclophosphamide immune Suppressed BALB/c mice	Th1 Cells	Intranasal	Injection	5.5	2 × 10exp7 conidia/20 µl saline	2	[13]
Beta glucan	Heat Killed Yeast Subunit	None	N	BALB/c mice	Humoral, Th1, Th2, Th17 cells	Subcutaneous	Injection	-	6 × 10exp7 HKY cells split dose of 0.75 µl once a week for 3 weeks.	3	[14]
Asp f3	Recombinant Subunit	Incomplete Freund's Adjuvant	N	Corticoid-treated BALB/c mice	Humoral, Th1 cells	Subcutaneous	Injection	39	2 doses of 15 µg twice 2 Weeks apart	4	[15]
Crf1	Recombinant Subunit	CpG ODNs	N	BALB/c mice	Th1 cells Anti-glucan /mannans	Intranasal	Injection	-	Once 2 × 10exp7 *Aspergillus* resting Conidia/20 µL saline,	5	[16]
Lam-CRM	Conjugate	Complete Freund's Adjuvant		CD2F1 mice	IgGs	Subcutaneous	Injection	68–71	7 µg with adjuvant 1 week later 7 µg without Adjuvant	6	[17]
Aspergillus Crude culture Filtrate Ags	Sonicate and filtrate	None	N	BALB/c mice	Th1, Th2 cells	Intranasal	Injection	-	2 × 10exp7 conidia in 20 µl of Sterile saline	7	[18]
Conidial RNA Pulsed DCs	Recombinant Subunit Pulsed Dendritic Cells	CpG ODNs	N	Murine: BALB/c, C57BL6 C3H/HeJ	Th1 cells	Subcutaneous	Injection	0	DCs (5 × 10exp5/each injection) Injected subcutaneously in 20 µL	8	[19]
A.fumigatus Conidia	Sonicate* Ags Filtrate^ Ags	None	N	CF-1 mice	Th1 cell cytokine bolstering	Subcutaneous Intranasal	Injection Injection	*0, ^50 *29, ^75	2 injections 2 weeks apart, Either intranasally with 30 µL or subcutaneously with 100 µL	9	[20]
Whole Glucan Glucan Particles (WGP)	Conjugate	Bovine Serum Albumin (BSA)	N	CD-1 mice	Adaptive T cell, Innate Enhancement	Subcutaneous	Injection	20	3 weekly 0.6/12 mg of	10	[21]
N and C Double f 3 (15–142)	Recombinant Subunit	Titermax/Free	N	Corticoid-treated CF-1 mice	IgG antibodies, Cellular Activation	Subcutaneous	Injection	43	40 µL twice 2 weeks apart	11a	[22]
N-Truncated rAsp f 3	Recombinant Subunit	Titermax	N	Corticoid-treated CF-1 mice	IgG antibodies, Cellular Response, macrophage	Subcutaneous	Injection	13	40 µL twice 2 weeks apart	11b	[22]

Additional vaccine screening criteria may be the route of administration. In Study 9, which performed two subcutaneous and intranasal injections of sonicated and filtrated versions two weeks apart, intranasally with 30 µL or subcutaneously with 100 µL, the subcutaneous route had lower mortality compared to the intranasal route in sonicate and filtrate versions (0%, 50% and 29%, 75%, respectively). Targeting the highly dense blood vessels and mucosal layers in the nose for immediate vaccination and concentration of immunity may be worth researching, as encounters with *Aspergillus* spores often occur at in the mucosal tract [28]. However, currently, Study 9 showed that the subcutaneous route is a superior to an intranasal route. These studies utilized five different fungal vaccine forms (Table 2). Some have been utilized previously in bacteria, viruses and parasites [29]. However, Ag Pulsed Dendritic Cells (DCs) are a novel and promising concept for immune restoration in immunocompromised patients. The forms of subcutaneous injections lend themselves for a mobile potential mass vaccine rollout. Additionally, the subcutaneous layer has few blood vessels, the vaccine components diffuse slowly at a prolonged rate of absorption; this continuous delivery of vaccine components may explain the superiority of the subcutaneous injection route [30].

Discussion

Immunocompetent and immunocompromised models: A particular barrier to the development of a vaccine is that patients with invasive aspergillosis tend to be highly immunocompromised, which could make vaccination more problematic. Data may have more weight if the models used in experimental fungal vaccinations are corticoid or cyclophosphamide-treated models, in parallel to the immunocompromised state that leads to Invasive Aspergillosis in humans [31]. An issue with murine models is their inbred laboratory-controlled strains: BALB/c, CD2F1, CF-1, CD-1, C57/BL6, C3H. Inbred laboratory mice live in highly controlled environments and have no natural exposure to *Aspergillus*. In humans, constant exposure may induce a degree of tolerance and the immune system may not recognize commonly encountered *Aspergillus* antigens as foreign [4]. A successful vaccination in these inbred murine models may not translate to success in human models because of *Aspergillus* allergen normalization [32]. In a contemporary review, a diversification of animal models, humanized mice models and *in vitro* human testing were posed as solutions to the inbred murine model problem. However, results from murine models are still valuable, as they exist as precursors to human models, to test for safety and drug efficacy [4]. These murine models may have to be used as the safety of using immunocompromised human models for experimental trials is of concern. Ultimately, the use of immunocompromised human models can fully test the pharmacokinetics of a vaccine and provide greater clinical significance for fungal vaccines [33]. Safety validation through Phase I trials in humans is the first tentative step towards fungal vaccine clinical trials in the immunocompromised host [33].

Table 2. Fungal vaccine forms that exploit fungal antigens and the immune system.

1. Recombinant subunit vaccines: An immunogenic defined protein, polysaccharide or carbohydrate part of a microorganism.
2. Antigen pulsed dendtritic cell vaccine: Immature dendritic cells primed with microorganism antigens and can internalised them through protein pattern receptors such as C-type lectins, Toll-like receptors, NOD-like receptors and RIG-I-like receptors.
3. Conjugate vaccines: Typically, consists of a poorly immunogenic antigen and a strongly coupled protein. This highlights the weakly immunogenic antigen against which specific antibodies can be produced.
4. Sonicated and filtrated antigens: This process of ultrasound and filtration is used to obtain specific antigens from a crude mixture.
5. Live/attentuated forms: The application of a weak/dead version of the pathogen to active long-term and strong immune responses.

Th1 immunity: The type of immunity evoked from each vaccine is vital in optimizing a protective response in immunocompromised patients, as the most effective antifungal immunity must be evoked, which is Th1 immunity [34]. All these studies, with the exception of Study 6, induced Th1-mediated immunity. Furthermore, this work has confirmed that recombinant protein antigens from *Aspergillus* can induce type 1 cell-mediated immune responses that protect mice from invasive aspergillosis. The induction of a dominant Th1 response is significantly important in the host response to naturally acquired infection with pathogenic fungi [34]. The Th1-produced cytokines IL-12, IFN-γ and TNF-α are required for the clearance of infection with the most pathogenic fungi, specifically in primary infection [35]. CD4 + T cells mediate the control and clearance of fungi, with Th1 responses having an essential role in antifungal vaccine immunity [36]. There is a positive consensus on the significant importance of a Th1 immune response against intracellular fungi, as well as the main partner-type CD8 + T cells. These cells can perform conidiocidal activity against fungus-laden cells [35]. Thus, Th1 cells contribute to vaccine-induced resistance in murine aspergillosis [37]. However, within immunocompromised patients, Th1 responses are weakened by Th2 responses which dominate and promote inflammation for fungal infiltration of tissues and the bloodstream [38].

Th2 immunity: Antifungal vaccines must be careful not to stimulate Th2-mediated immunity, as progressive disease in immunodeficient or susceptible mice is associated with a shift in the balance between Th1 and

Th2, towards the Th2 response [38]. Th2 immunity is counterproductive towards initial Th1 antifungal immunity, as the Th2 response may play a role in post-infection regulation of a Th1 response. Fungal hyphae may preferentially stimulate IL-4 and IL-10 pathways, as it is not effective against *Aspergillus* [39].The production of IL-4 by Dendritic Cells and the production of IL-10 by Th2 cells blunts the generation of protective immunity [40]. The progression of *Aspergillus* infection is related to a decrease in Th1-type response and an increase in the response mediated by Th2 cells, which produces a lymphoproliferative positive feedback cytokine, such as IL-4, IL-5 and IL-10 [9].

Although the Th2-type response is associated with aggravation of fungal infections with eotaxins, cytokines such as IL-10 may have a regulatory role for exaggerated inflammatory responses [34]. In Study 7, this response was induced alongside a Th1 response in CCFA, and since there is no defined concentration of antigens, it is unknown what caused this Th2 response and what antigens should be avoided for fungal vaccination. *Aspergillus* intentionally induces a predominant Th2 response to blunt an effective Th1 host response. Consequently, it is imperative for an effective *Aspergillus* vaccine to balance the Th1 and Th2 arms of the immune system. Figure 3 shows the imbalance and subsequent balancing of the Th1 and Th2 arms of the immune system, Th2 antagonists and IFN-γ injections [41,42]. In light of CCFA and the different immune responses, fungal vaccine studies have been performed with definitive, and defined concentrations of *Aspergillus* antigens and produced favourable Th1 immune responses in immunocompetent and immunocompromised patients.

Th17 immunity: Study 3 utilized HKY as an *Aspergillus* vaccine, whilst a Th1 response was produced, Th17 cells were inadvertently produced. Th17 cells have a debatable role within *Aspergillus* infections, such as the release of IL-17, matrix metalloproteinase 9 (MMP 9) and myeloperoxidase [25]. These cytokines cause chronic lung inflammation advantageous to invasive aspergillosis IL-17 impairs the antifungal activity of polymorphonucleocytes in the presence of the Th1-stimulatory IFN-γ chemokine [25]. This Th17 response counters and outweighs the productive antifungal Th1 immune response and worsens the patient's condition through the induced inflammatory pathology. Th2 and Th17 responses appear to exacerbate inflammation to the benefit of invasive fungi, as their involvement complicates and potentially attenuates a productive Th1 effector cell response in immunocompetent and immunocompromised patients. However, Th17 cells are necessary for antifungal vaccine immunity [43,44]. IL-17 has been shown to be particularly important in resistance to *Aspergillus* infection in an IL-6-deficient animal model. IL-17 is upregulated as a neutrophilic chemokine for neutrophil recruitment into the lungs in *Aspergillus* infection, this may also explain the ease of invasion by invasive aspergillosis as inflammation

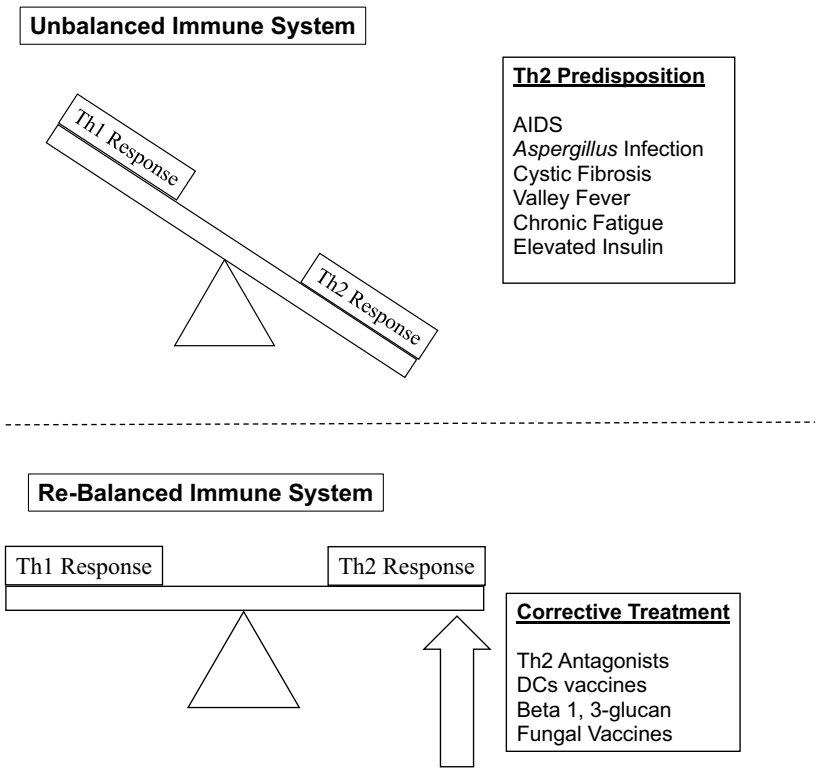

Figure 3. Factors influencing a Th2 dominant, counterproductive response can be corrected by antigen-pulsed dendritic cell vaccination and drugs attenuating Th2 proliferation.

would be exacerbated [45]. Study 3's vaccine protected against systemic aspergillosis, coccidioidomycosis, candidiasis and cryptococcosis. This split opinion on the role of Th17 cells in antifungal vaccines and immunity can only be resolved with further research. A question to be answered is 'Does Th17-induced inflammation aid or worsen invasive aspergillosis?' The answer can only benefit *Aspergillus* vaccines and immunity.

Dendritic cell (DC)vaccines and ODN-CpG adjuvants: The initial detection and defence against pathogenic fungi and fungal surfaces are performed by pattern recognition receptors, such as Dectin-1-Receptors and Toll-like Receptors (TLRs), on myeloid cells, in particular DCs. These antigen-presenting cells internalize, process and present pathogenic antigens to CD4+ cells, evoking a pathogen-specific immune response. DCs bridge innate and adaptive immune responses to co-ordinate the most effective immune responses. DCs immune co-ordination and antigen presentation have become targets for the development of innovative vaccines [46]. This has led to *Aspergillus* vaccines such as Antigen-Pulsed DCs, pulsed with specific antigens *in vitro* and are injected into immunocompromised patients to restore immunocompetency. Adoptive transfer of DCs primed T-cells for Th1 terminal differentiation and proliferation readjusts the immune system by DCs re-educating CD4+ cells and evoking Th1 differentiation and proliferation [47]. Within Study 8, the course and outcome of the *Aspergillus* infection was changed by Antigen-Pulsed DCs in mice; compared to the control, the infection survival rate was 100%. Whilst Study 8 did not use immunocompromised murine models, the natural course of invasive aspergillosis imbalances the immune system to produce a Th2 immune response. This vaccine tilted the balance of the immune system from a counterproductive Th2 response to the effective Th1 response. However, transplanting foreign human DCs does risk inducing Graft Vs Host Disease and may disrupt an already compromised immune system [48].

Alongside DCs that have a 1,000-fold more efficient T-cell activation than typical adjuvants [9], these studies included the novel immunoadjuvant unmethylated CpG oligodeoxynucleotides (CpG ODNs). It appears that CpG-ODNs of different classes, through the activation of innate TLRs, act as adjuvants for Th1-tailored vaccination. In Studies 2, 5 and 8, this type of adjuvant was used. All studies with this adjuvant produced Th1-dominant immune responses and Study 8 reported the lowest infection mortality rate at 0%. This survival success may be due to CpG-ODN exposure mimicking immunological aspects of live bacterial infection, provoking a Th1-dominant response [49]. Furthermore, Study 2 utilized cyclophosphamide to immunosuppress their murine models, and the subsequent infection mortality rate was higher than Study 8 at 5.5%. However, Study 2 may prove more valuable than Study 8 as immunosuppressed models are predominantly invasive aspergillosis afflicted. This demonstrates that Antigen Pulsed DCs can work in immunosuppressed murine models, which contributes to the ultimate aim of effective and safe *Aspergillus* vaccines for immunosuppressed human patients.

In Studies 2, 5, and 8, in response to their respective *Aspergillus* antigens, the cytokines IL-12 and IFN-γ were produced by functionally mature DCs and may have contributed to the induction of a Th1 immune response. Also, the TLR9 specifically recognizes CpG-ODNs. This internalization of CpG-ODN adjuvant promotes internalization of the whole vaccine molecule and may have provoked a more effective antigen presentation for Th1 differentiation [50]. CpG-ODNs are a non-toxic adjuvant that primed DCs which may restore immunocompetence in immunocompromised patients. Adoption of this adjuvant for fungal vaccines would be beneficial to immunocompromised patients.

Recombinant subunit vaccines: Studies 2, 5 and 8 utilized specific antigenic segments of the *Aspergillus* cell wall. These unique cell wall fragments allow the vaccines to provoke a specific Th1 mediated response to *Aspergillus*. Cell wall fragments (Asp f 3, Asp f 16 and Crf1) in the vaccines allow the patients to produce responses specific to *Aspergillus*. However, Asp f antigens are allergenic fragments [51]. Vaccination with these fragments in hyper-sensitivity Type IV patients may cause anaphylactic shock and further patient complication. However, Studies 11a and 11b utilized recombinant subunit vaccines to bypass IgE site recognition of allergens by truncating the N and C terminals. Vaccination of the murine models with the single N- and double N- and C-truncated rAsp f 3 produced specific effective antibodies and immunity. However, infection survival rates were 13% and 43% for single N- and double N- and C- terminal truncated Asp f 3 vaccines. Truncation may remove resemblance to the human allergenic IgE-binding epitope and produce a non-allergenic immunoprotective vaccine. However, the editing of the primary structure of the antigen may have resulted in different immunogenic properties and conformational properties. With trial and error, the optimum truncation may produce the optimum immunogenic non-allergenic *Aspergillus* antigen for immunocompromised patients.

Additionally, the Titermax-free, particulate version of the rAsp f 3 vaccine was as immunoprotective as the rAsp f 3/TM preparation and the production of specific anti-Asp f 3 antibodies were similar. This adjuvant free rAsp f 3 displayed non-toxicity and may be a more eligible *Aspergillus* vaccine candidate for human clinical trials. *Aspergillus* vaccines that utilized Asp f 3, Asp f 16, rAsp f 3 and Crf1 were achieved using recombinant engineering. These antigens/allergens are malleable

and, in rAsp f 3, truncation of N- and C- terminals can improve immunogenicity and decrease allergenicity of these antigens/allergens. Recombinant vaccines have exploited the utility of antigens by maximising their immunogenicity and lessening the pathogenicity, virulence and toxicity. Therefore, the non-toxic and designer selling point of these vaccines becomes even more vital for immunocompromised patients. However, there are issues with reproducibility with immunocompromised patients, high costs to human clinical trials and fungi *in vivo* antigen glycosylate their Asp f allergens; the degree of glycosylation in the fungal antigen may cause immunization discrepancies [52–54].

Conjugate vaccines: Studies 3 and 10 attached a poorly immunogenic substrate to a highly immunogenic protein. The concept of conjugation allows a poorly antigenic substrate to be highlighted and recognized as foreign by the immune system. Without the Diphtheria toxoid conjugation in Study 3, specific anti-glucan IgGs would not be produced and effective immunity could not be reached. Additionally, the Bovine Serum Albumin (BSA) conjugation in Study 10 produced a 20% infection mortality rate which was lower than the positive control of Heat Killed Cerevisiae. Study 3 attempted to create a pan-fungal vaccine, which will be discussed below, and Study 10 attempted to create an *Aspergillus* specific vaccine. Whilst both studies use β-glucan, Study 3 produced anti-β-glucan antibodies and Study 10 produced, primarily, a cell-mediated immune response and innate immune elevation, without inducing anti-β-glucan antibodies. Study 10 conjugated BSA to Whole Glucan Particles and produced a 20% infection survival rate. The conclusion of innate and cell-mediated immunity was inferred through the elevation of IL-10 and IL-17, Th2 and Th17 immune responses. Nevertheless, as previously discussed, Th2 may have a counter-productive role and Th17 has a debatable role within antifungal immunity [24,25]. However, the elevation of innate cytokines such as granulocyte-CSF, granulocyte macrophage-CSF, TNF-α, MCP-1, MIP-1α, IL-6 and IFN-γ can be definitively denoted as constructive for this immunity. The immunity may be attributed to the activity elevation of polymorphonucleocytes and macrophages. Ethically, the use of BSA is controversial and its usage within cell culture has been criticized, there have been attempts to produce alternatives for cell culture and scrutiny may fall onto mass production of vaccines with BSA.

Pan-fungal vaccines and humoral immunity: A safer and more profitable antigen to explore is the β-Glucans for a Pan-Fungal Vaccine. These wall fragments are present in all fungi and Study 6 attempted to make this ideal vaccine. Poorly immunogenic β-glucans isolated from Laminarin were conjugated with highly immunogenic Diphtheria toxoid CRM197 to produce a Pan-Fungal Vaccine. Their murine model produced anti-β-glucan IgGs and bound efficiently *Aspergillus* and *Candida* hyphae; these hyphae are structurally invasive fungal extensions that spread the fungi's area of infection. Antibodies that opsonise and block binding sites on hyphae could inhibit fungal adherence and tissue invasion. Additionally, mice were given passive protection with anti-β-glucan antibodies. Moreover, these β-glucan antibodies may shift the cytokine profile towards the protective Th1 pattern, as demonstrated in experimental cryptococcosis [55]; stimulating both cellular and humoral arms of the immune system is integral to fungal clearance. However, for this vaccine to possess universal application for the immunocompromised groups, a non-toxic alternative to Complete Freund's Adjuvant and further testing in immunocompromised murine/human models are required.

Alongside this pan-fungal vaccine, Study 3 experimented with an attenuated fungal vaccine, Heat Killed Yeast (HKY). Subcutaneous injection of HKY into mice produced significant upregulation of key antifungal cytokines: IFN-γ, IL-6 and IL-17A by spleen cells and lymph node cells. IFN-γ causes hyphal damage and enhances monocyte function against *Aspergillus* [56]. Models and patients immunodeficient in IL-6 are more susceptible to invasive pulmonary aspergillosis [57], upregulation of this deficient cytokine is beneficial for immunocompetent restoration. A vaccination that restores aspects of immunocompetency and provides all-encompassing fungal protection may make this a desirable candidate for immunocompromised patients, as aspergillosis is one of the many opportunistic fungal infections.

Conclusions

Whilst there may be no current clinical trials for *Aspergillus* vaccines, in both immunocompetent and immunocompromised patients, there exists a common groundwork and a market for their development. Molecular engineering has produced recombinant techniques to tailor antigens for vaccines: high immunogenicity, Th1-provocative immune response and no toxicity. Additionally, the adoptive transfer of pulsed DCs can restore immunocompetence in immunocompromised patients and boost innate immunity. Experimental fungal vaccine trials can be supported by using immunocompromised models and a diverse range of animal models, Phase I trials in humans, all supported by adequate and sufficient funding. The development of *Aspergillus* vaccines requires greater funding, which would enable immunocompromised murine and human subjects to accurately detail pharmacokinetic and pharmacodynamic data, as well as the Therapeutic Index of *Aspergillus* vaccines. The use of non-toxic adjuvants may accentuate immunogenicity for immunocompromised patients, which is preferable to non-adjuvant vaccines.

Recent advances in fungal vaccine discovery have focussed on employing fungal cell wall polysaccharides for the development of glycoconjugate vaccines based on synthetic oligosaccharides [58]. This approach to vaccine development focuses on the structure-based rational design of target fungal epitopes, combined with optimized conjugation and formulation technology [58]. Another novel vaccine approach has been employed for nanoparticles, including polymeric nanoparticles, phospholipid-based vesicles, nanostructured lipid carriers, dendrimers, nanoemulsions and metallic and magnetic nanoparticles. Nanoparticles can act as a delivery tool capable of improving the stability of antigens, such as peptides and the immunogenicity of the antigen, as well as possible immunostimulant adjuvants and magnetic nanoparticles [59,60].

Medicine has invented antimicrobials to compensate for the human body's inability to overcome disease. However, the over-reliance and abuse of azole antifungals and other classes of antifungal drugs is beginning to drive antifungal resistance in *Aspergillus*.

Disclosure statement

No potential conflict of interest was reported by the authors.

Ethics

Not required. This study did not involve any human or animal subjects.

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