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Review Article

Review Article

Adjuvants and delivery systems for antifungal vaccines: Current state and future developments

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Abstract

Mycoses are gaining increasing attention in modern medicine because of the increase in diseases associated with opportunistic fungal infections. Despite the recognized role of the immune system in the control of fungal infections, no antifungal vaccines are currently licensed for use in humans. However, numerous vaccine candidates are being developed in many laboratories, as proof of the renewed interest in integrating or replacing chemotherapy with vaccines to reduce antibiotic use and consequently limit drug resistance and toxicity. In the effort to use safer and simpler fungal antigens for vaccinations, adjuvants have become relevant as immunostimulators to elicit successful protective immune responses. To address the relevant role of adjuvants as determinants in the balance of vaccine efficacy and safety, an updated and critical review of the adjuvants used in preclinical antifungal vaccines is presented, and prospective trends are addressed. Selected recent papers and other historically relevant and innovative strategies using adjuvants in experimental fungal vaccines are highlighted.

Key words: adjuvant, antifungal vaccine, efficacy, toxicity.

Introduction

The incidence rates of fungal diseases are notably increasing worldwide, with an associated increase in immunocompromised patients due to AIDS, cancer, diabetes, immunosuppressant use for transplantation and autoimmune diseases, senescence, and similar diseases. The elevated use of prescription antifungals is giving rise to resistant fungi, resulting in low effectiveness and high toxicity of conventional treatments [1]. To date, the most important advances in vaccines have been related to the prevention of viral and bacterial diseases. However, antifungal and antiparasitic vaccines have not had similar success. The lack of a good understanding of fungal–host interactions including the innate and specific immune responses, together with other obstacles such as an underappreciation of the magnitude of opportunistic and endemic fungal infections, have largely hindered the development of vaccines against pathogenic fungi in recent decades [2].

Recent elucidation of the immune mechanisms that provide protective immunity against fungal diseases and the increased incidence of chronic diseases associated with opportunistic mycoses and emergent fungal diseases such as sporotricosis have renewed interest in the development of antifungal vaccines [3–6]. There has been extensive research relating to development of veterinary and human vaccines for both opportunistic and endemic fungal infections, especially candidiasis, cryptococcosis, coccidioidomycosis, blastomycosis, histoplasmosis, paracoccidioidomycosis, and infections caused by Pneumocystis, and, more recently, aspergillosis. However, none have yet been approved by regulatory agencies for either active or passive immunization in humans [2–4,7,8]. Edwards has listed several reasons for the lack of approval of human antifungal vaccines [2]. Two factors considered by this author are the high cost of preparing antigens for use in human studies and the high cost of toxicology studies. Consequently, the selection of adequate adjuvants for vaccine formulations is an important aspect of optimizing the use of antigens and improving their immunogenicity, though the influence of adjuvants on toxicity must be considered [9,10]. Advances in the understanding of the biology of dendritic cells and innate immune activation have allowed for more rational approaches to the selection of adjuvants for prophylactic and therapeutic vaccines. In parallel, the interest in developing antifungal vaccines is growing. This fact is reflected in the increase in articles, both original works and reviews, discussing this topic (Fig. 1). Today, two antifungal vaccines are in clinical development; one containing ALs3p-N formulated with an aluminum hydroxide adjuvant (NDV-3 vaccine) directed against *Candida albicans* and *Staphylococcus aureus* and the other containing rSap2p plus virosome adjuvant (PEV-7) directed against vulvovaginitis [2,11]. No still antifungal vaccines are licensed for use.

Here, our objective was to provide an update on the use of adjuvants in antifungal vaccines while analyzing strategies to improve efficacy in the future. Despite adjuvants that cannot be used in humans, such as Freunds adjuvants, and do nothing directly to advance the vaccine antigen into clinical development, they help to advance in mechanistic studies. This review does not include cell-based or cytokine- and antibody-based therapies or other adoptive therapy techniques discussed in another recent review [12].

Adjuvant overview and mechanism of action

Adjuvants are molecules, compounds, or macromolecular complexes that can increase and/or modulate the intrinsic immunogenicity of an antigen and elicit strong and long-lasting immune responses. They have been important components of human and veterinary vaccines for more than 80 years [13]. Adjuvants include alum, a universally accepted adjuvant for human use, although more recently, several adjuvant formulations have been accepted for specific vaccines [9,14]. In fact, any adjuvanted vaccine must be more efficacious than the aqueous vaccine, and this benefit must outweigh its risk. A number of new substances with documented adjuvant activity have been reported in the literature in recent years [15–17]. Recently, the trend in vaccine development has been to move from using whole inactivated organisms without adjuvants to less complex antigens such as purified, recombinant, or synthetic proteins or even peptides that need adjuvants for effective immune induction (Fig. 2).

In addition to their immunostimulatory properties, several post-vaccination toxicity reactions have been described in adjuvanted vaccines [18]. For this reason, very few adjuvants have been approved for use in preventive human vaccines [9,14].

Adjuvants can be grouped as follows: antigen delivery systems that promote antigen uptake by antigen-presenting cells (APCs) such as liposomes and micro- or nanoparticles and immunopotentiators that activate APCs mainly

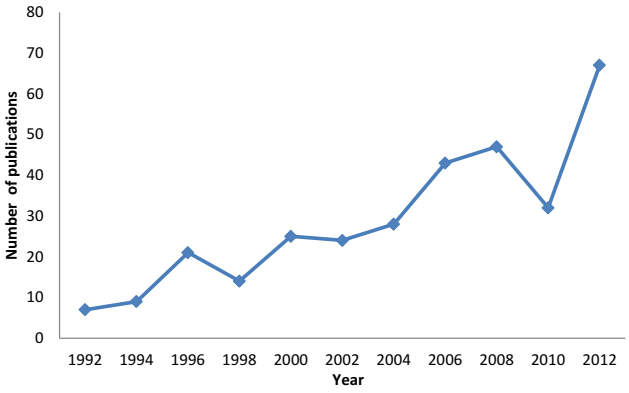

Figure 1. Evolution of the number of publications related to fungal vaccines (1992–2012). Data obtained from PubMed.

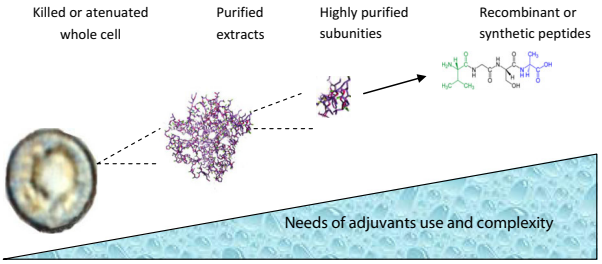

Figure 2. Antigen complexity and need for adjuvants on antifungal vaccines. The earliest vaccines were based on complete microorganisms. The new generation of vaccines comprise simpler but poorly immunogenic antigens, for example, highly purified subunities, recombinant or synthetic peptides, or DNA vaccines. Therefore, adjuvants are proportionally required to become effective vaccine.

Table 1. Side effects described for experimental and licensed immunoadjuvants.

Frequency	Manifestation
Frequent	Local: inflammation, local pain, granulomas Systemic: flu-like symptoms and other acute phase responses
Less frequent or rare	Local: lysis, ulcer, cyst, Arthus reactions, macrophagic myofasciitis, Bell's palsy (intranasal route), tumors Systemic: allergy, vascular leak syndrome, modification of hepatic metabolism, induction or worsening of autoimmune system, embryo fetal immunotoxicity

through innate immune receptors that either directly or indirectly induce the expression of cytokines and chemokines, thereby modulating the local microenvironment to activate and stimulate immune cells [19]. Thus, an adjuvant's efficacy largely relies on its ability to activate innate immune responses and regulate the interplay between the innate and acquired immune systems to cause the desired specific immune response [20].

Efficacy–toxicity balance of adjuvanted vaccines

The search for new, more potent, and safe adjuvants is a scientific challenge today. Obviously, the overstimulation of the immune response by adjuvants can be associated with local and systemic effects (Table 1) [18,21,22]. Many adjuvants were developed under empirical conditions. However, today, a better understanding of the innate immune response and its connections with the adaptive immune system permits the rational design of new adjuvants with higher efficacy and lower toxicity [10,23–25].

Fungal molecules involved in immune activation

For a long time, adaptive immunity has been regarded as the major player in the protection against most fungal infections. Nevertheless, in recent years, innate immunity has received special attention because, despite its low specificity compared with the adaptive immune system, it effectively distinguishes host cells (self) from pathogens (non-self) and activates adaptive immune mechanisms by providing specific signals [26,27]. The evidence suggests that both innate and adaptive responses integrate to produce effective antifungal protection [28,29].

Pathogenic fungi contain a number of pathogen-associated molecular patterns (PAMPs) such as carbohydrates (β-glucans), glycoproteins (mannans), nucleic acids (DNA and RNA), glycolipids (lipopolysaccharides), peptidoglycans (PGs), and diacyl and triacyl lipopeptides (lipoproteins) that are recognized by receptors that activate innate immune cells [30–32]. These PAMPs are often essential for fungal survival and pathogenesis and are not found on mammalian cells [33,34].

Knowledge about these naturally occurring fungal molecules and how they stimulate the immune system allows the selection of immunological adjuvants that cause these responses in antifungal vaccines (Tables 2 and 3) [35–37].

Adjuvants and delivery systems used in antifungal vaccines

The manipulation of the antifungal immune response is becoming an important strategy for the prevention and treatment of systemic mycoses, and is an alternative to conventional toxic drug-based treatments [4]. Many different immunological adjuvants have been used in vaccine formulations to achieve effective and long-lasting protection by improving the protective immune response. The evaluation of most of these adjuvants has only been done in animals. Following is a summary of representative experiments with various adjuvants in experimental antifungal vaccines, with a brief discussion of the results.

Emulsions

Freund's adjuvant. Freund's adjuvants are water-in-oil emulsions. Freund's complete adjuvant (FCA) is a mixture of mineral oil (Marco 52) and emulsifier (Arlacel A, mannide monooleate) prepared as an emulsion of 85% mineral oil and 15% emulsifier with 500 μg of heat-killed and dried *Mycobacterium tuberculosis* per milliliter of emulsifier mixture. Freund's incomplete adjuvant (FIA) lacks the mycobacterial component, making it safer but less potent [38,39]. Investigators have frequently considered FCA to be the gold standard of adjuvants used in testing new vaccine candidates used in animals [38].

Often FCA is used in the first immunization dose, and FIA is used for boosting [40]. These formulations are potent adjuvants with the ability to elicit both T helper (Th)1 (FCA) and Th2 (incomplete Freund's adjuvant [IFA]) responses. The mycobacterial components, which elicit the Th1 response of FCA, also activate Th17 cells in murine cell culture and *in vivo*, which is accompanied by elevated interleukin (IL)-6, IL-23, and transforming growth factor–β levels [41].

Many reports on Freund's adjuvants in antifungal experimental vaccines exist. FCA has been formulated with P10, a synthetic peptide derived from gp43, a glycoprotein of 416 amino acids from the cell wall of the

Table 2. TLR-independent fungal PAMPs and corresponding known adjuvants´ ligands

PRRs	Selected fungal PAMP(s)/ligands	Pathogenic fungi examples	PRRs agonist as adjuvants	Selected references
C-type lectin receptor family				
Mannose receptor (CD206)	Fucose and terminal mannose structures	*C. albicans* *C. neoformans*, *Pneumocystis* *P. jirovecii*	Mannans Chitin/chitosan Lipoarabinomannan	[165] [29] [117]
Dectin 1	1,3-β-glucan	*C. albicans*	Laminarin (β -1,3- and β -1,6-glucan), Curdlan (1,3-β-glucan), Glucan particles (GPs) puriffied *S. cerevisiae* cell walls	[166] [167] [168] [169] [170]
Dectin 2	α-mannan	*H. capsulatum*, *P. brasiliensis*, *C. neoformans* *C. albicans* *Microsporum audouinii*	Mannans	[171] [172] [31] [173] [174]
MBL	1,3-β-glucan	*Blastomyces dermatitidis*	Mannans β-glucan	[175] [117]
Mincle	α -mannose	*Candida sp.*, *Malassezia sp.* *Fonsecaea pedrosoi*	TDM (cord factor)	[176] [165] [31]
DC-SIGN	mannoproteins Conidia High-mannose structures	*C. neoformans* *A. fumigatus* *C. albicans*	Lipoarabinomannan	[177] [178] [179] [180]
MMR	Mannoproteins *Manosa, fucosa*	*C. neoformans* *S. cereviciae*	Glucans, dextrans, lentinans, glucomanans, galactomanans, Levans y xylans	[181] [10]
Galectin-3	b-1,2-mannosides	*C. albicans*	?	[182]
SCARF1/CD36	b-1,3-glucan	*C. albicans*	?	[183]
SP- A	120-kD surface glycoprotein	*P. carinii*	?	[184]
SP- D	1,3-β-glucan	*B. dermatitidis*	?	[185]
NLR receptors family				
NLRP3*	?	*C. albicans*	Alum	[186] [187] [188] [79]
	Zymosam and mannan	*S. cerevisiae*		[189]
Scavenger receptor cysteine-rich (SRCR) domains family				
CD5 ectodomain	β-glucan	*S. cerevisiae*, *C. neoformans*	?	[190]

CD, leukocyte cluster of differentiation; DC-SIGN, dendritic cell specific ICAM-3 grabbing non-integrin; MBL, mannose-binding lectins; MMR, macrophage mannose receptor; NLR, NOD-like receptor; NLRP3, NLR family, pyrin domain containing 3; PRR, pattern recognition receptors; SCARF1/CD36, scavenger receptors; SP-A and SP-D, collectins (collagen-containing C-type lectins); TDM (Cord factor), trehalose 6,6′-dimycolate; ?, unknown.

Table 3. TLR-dependent fungal PAMPs and corresponding known adjuvants' ligands

PRRs (TLR family)	Selected fungal PAMP(s)/ligands	Pathogenic fungi examples	PRRs agonist as adjuvants	Selected references
TLR 1	?	*A. fumigatus*		[191]
TLR 2	Conidia, phospholipomannan	*C. albicans*	Zymosan (b-1,3-glucan from	[32]
	Conidia and hyphae	A. niger and	*Saccharomyces*)	[192]
	Zymosan	A. fumigatus		[193]
	Gucuronoxylomannanl (GXM)	*S. cerevisiae*∗		[194]
	Cell wall protein Yps3p	*C. neoformans*		[195]
	?	*H. capsulatum*		[196] [197]
		P. brasiliensis		[198]
				[199]
				[200]
				[70]
	Cell wall proteins and lipids	*S. schensckii*	Artin M, muramyl dipeptide (MDP)	[117]
TLR 3	Double-stranded RNA	*A. fumigatus*	poly(I:C)	[201]
				[202]
TLR 2/ TLR1	GXM	*C. neoformans*	Triacylated synthetic	[203]
	?	A. fumigatus	lipoprotein (Pam3CSK4)	[191]
				[202]
TLR 2/ TLR 6	Zymosan	*S. cerevisiae*	MDP	[204]
	GXM	*C. neoformans*	Pam2Cys	[203]
	?	*A. fumigatus*		[191]
TLR 4	Conidia, mannans	*C. albicans*	Lipid A monophosphoryl (MPL),	[30]
	Conidia and hyphae	*A. niger*	RIBI,	[205]
	LPS	*S. schensckii*	MDP	[192]
	GXM	*C. neoformans*		[206]
	Conidia	*A. fumigatus*		[207]
				[204]
				[191]
				[50]
				[70]
TLR 5			*Salmonella enterica* FliC flagellin	[107]
				[68]
TLR 7	Single-stranded RNA	*C. albicans*	Imiquimod	[196]
				[202]
TLR 9	Oligodeoxynucleotides (CpG) DNA	*C. neoformans*	Oligodeoxynucleotides (CpG), CpG 1668	[208]
		A. fumigatus		[96]
				[209]
				[98]

CpG, cytosine guanine dinucleotide; GXM, gucuronoxylomannan; LPS, lipopolysaccharide; MDP, muramyl dipeptide; PRR, pattern recognition receptors; RIBI, RIBI adjuvant system (ARS); TLR, toll-like receptor; Yps3p, *H. capsulatum* yeast phase-specific protein; ?, unknown.
∗*S. cereviciae is non-pathogenic but use in anti-fungal vaccines.*

thermodimorphic fungus *Paracoccidioides brasiliensis*, the causative agent of paracoccidioidomycosis (PCM) [42–44]. Additionally, Spellberg et al. report that immunization with a recombinant protein (rAls3p-N) from *C. albicans* mixed with Freund's adjuvant can protect mice from lethal *Candida* infection [45]. Another experimental vaccine against *Cryptococcus neoformans* was prepared using glucuronoxylomannan–tetanus toxoid formulated with FCA. The survival of infected mice after immunization was significantly improved compared with controls during the 301 days after infection [46].

Similarly, subcutaneously administered soluble antigenic fractions from *Histoplasma capsulatum* emulsified in FCA protected mice from a lethal challenge in msurine models. This protection was linked to the *in vitro* production of interferon-gamma (IFN-γ) [47].

The use of FCA has been limited to experimental immunizations in animal studies due to the severity of adverse reactions [21,40], whereas IFA was used successfully in numerous human trials in the 1950s. However, the use of IFA in vaccines was discontinued because of safety concerns based on animal trials [41,48]. In addition, it is currently unacceptable to compare experimental antifungal vaccines to those formulated with FCA for ethical reasons because this emulsion is very toxic and is not used in human vaccines.

Ribi adjuvant system. This adjuvant is a formulation of squalene, Tween 80, and monophosphoryl lipid A and is used in various experimental antifungal vaccines. Ribi and two variants using monophosphoryl lipid-squalene (MPL-SE) or MPL-AF (an aqueous micellar suspension of MPL dispersed in dipalmitoyl phosphatidylcholine) formulated with the rAg2 fusion protein from *Coccidioides* showed variable but effective results regarding the reduction of the fungal burden in the liver, lungs, and spleen and enhanced survival after fungal challenge [49,50]. These results are discussed later.

In another report, both antigens separated by concanavalin A affinity chromatography into adherent (mannoprotein [MP]) and nonadherent (flowthrough [FT]) fractions from *C. neoformans* strain B3501 culture filtrates formulated with Ribi adjuvant, were administered intraperitoneally to C57BL/6 and CBA/J mice. Mice that received two inoculations of MP and FT exhibited prolonged survival and reduced brain and kidney fungal loads following intravenous challenge with the pathogenic fungi in comparison with adjuvant alone. On the other hand, CBA/J mice also benefited from immunization with FT and MP; however the benefits were less evident than those observed in C57BL/6 mice. Furthermore, MP and FT immunization also protected B-cell–deficient, but not T-cell–deficient, mice, suggesting that protection was mediated by T-cell–dependent mechanisms [51].

Montanide oil adjuvants. Mineral oils in water–oil emulsions, such as IFA, stay at the injection site and produce local inflammation [21]. Because IFA has not been accepted as a commercially viable adjuvant due to safety concerns based on animal studies [48], a proprietary, highly refined emulsifier from the mannide monooleate family in a natural metabolizable oil solution called Montanide ISA 51 was developed by SEPPIC (Paris, France). This adjuvant contains mannide oleate in a mineral oil solution (DRAKEOL 6VR). These formulations are generally well tolerated systemically, though several instances of mild to severe local reactions have been reported [52,53]. Other low-viscosity formulations containing highly purified oils and injectable emulsifying agents that have high immunopotentiation capacity and milder side effects are available. For example, ISA 720 is an emulsion containing a highly refined emulsifier from the mannide monooleate family in a natural metabolizable oil solution [52,54]. SEPPIC has developed another family of adjuvants called IMS that are composed of nanoparticles suspended in aqueous solutions combined with an immunostimulatory compound [55]. One of the more closely studied is the IMS 1313 N VG PR (IMS 1313) adjuvant, which consists of water-soluble liquid nanoparticles combined with an immunostimulatory compound. Because this adjuvant has an aqueous phase, it is suitable as a mucosal delivery vehicle [56,57].

The ISS/Montanide adjuvant was evaluated for safety, immunogenicity, and efficacy using the recombinant Ag2/PRA106 + CSA chimeric fusion protein (CFP) expressed in *Saccharomyces cerevisiae*. The vaccine was administered via the intramuscular route in adult female cynomolgus macaques challenged with *Coccidioides posadasii*. Animals received three immunizations using two doses of CFP plus adjuvant or adjuvant alone on days 0, 28, and 112 and were intratracheally challenged 28 days following the final immunization with arthroconidia (*C. posadasii* strain Silveira). At the time of the challenge, all animals showed evidence of disease. Animals vaccinated with the highest doses of CFP were protected and showed evidence of enhanced sensitization compared with adjuvant controls and animals vaccinated with lower doses of CFP. This observation was based on higher serum anti-CFP titers, enhanced secretion of IFN-γ from stimulated bronchoalveolar lavage mononuclear cells, reduced pulmonary radiologic findings following intratracheal challenge, reduced terminal complement fixation titers, and reduced necropsy findings. Overall the vaccine was well tolerated [58].

TiterMax. TiterMax (TM) is a water-in-oil emulsion consisting of squalene, an emulsifier (sorbitan monooleate 80), block copolymers of polyoxypropylene and polyoxyethylene, and microparticulate silica. This formulation allows for the incorporation of a wide variety of antigens for antibody production and vaccines. TM presents antigen to the immune system in a highly concentrated form that often produces antibody titers comparable to or higher than FCA. This adjuvant stimulates complement activity and increases class II major histocompatibility complex expression on macrophages. Toxicity is lower than other water-in-oil adjuvants such as FCA [40].

TM was used by Ito et al. in an experimental vaccine against *Aspergillus fumigatus* when the following two antigens were evaluated: recombinant Asp f 3 (rAsp f 3) and Asp f 1. The rAsp f 3 vaccine induced a protective immune response only in the presence of the TM adjuvant. Subcutaneous injections of Asp f 3 with or without TM and the mock immunizations with either

Table 4 Adjuvant formulations used in human-licensed vaccines

Adjuvant	Basic composition	Manufacturer	Indication	Tested in experimental anti-fungal vaccine
Adjuvant used as part of licensed human vaccines				
Alum	Al(OH)3 or $Al_4(OHPO4)_3$, or $Al(OHPO_4)SO$	Several	Several	Yes
Calcium phosphate	$Ca_3(PO_4)_2$	Sanofi	DTP	No
MF59	squalene polysorbate 80, sorbitan triolate, sodium citrate	Novartis	Seasonal Flu	Yes
MPL	O-desacyl-4-monophosphoryl lipid A, derived from LPS of *S. minnesota* R595	Allergy Therapeutics	Allergy	Yes
AS03	Squalene, DL-α-tocopherol (vitamin E) polysorbate 80	GSK	Pandemic flu	No
AS04	MPL/Alum	GSK	HBV, HPV	No
RC529	Synthetic MPL/Alum	Berna Biotech	HBV	No
Virosome (VLP, IRIV)	Phosphatidylcholine bilayer liposomes	Berna Biotech	HAV	Yes
AF03		Sanofi	Influenza	
AFPL1^{TM}	Outer membrane vesicles (*Neisseria meningitidis B*)	Finlay Institute	Meningococcal disease	No
Tested in clinical trials but not yet licensed				
CpG	Unmethylated motif of DNA bacterial		HBV, Influenza Cancer	Yes
IC31			TB	No
Imiquimod	TLR7/8 agonist		Cancer	No
Flagellin	TLR 5 agonist		Influenza	Yes
AS01	MPL/ liposomas/QS21	GSK	Malaria	No
AS02	MPL/w/o emulsion/QS21	GSK	Malaria, TB, Cancer	No
AS15		GSK	Cancer	No
Iscomatrix	Phospholipid/colesterol/saponin		HCV, influenza, HPV, cancer	No
Montanide ISA51, ISA720	o/w emulsion	Seppic	Malaria, HIV, cancer	Yes
LT LTK63	Heat-labile enterotoxin from *E. coli*		Influenza, ETEC	No
Virosome (VLP, IRIV)	rSap2, a truncated, recombinant aspartyl proteinase-2	Pevion Biotech AG	Influenza, TB,HIV	Yes
Alum	Candidal Als3p adhesin	NovaDigm Therapeutics	PEV7, Candida NDV-3, Candida and *S. aureus*	Yes

Alum, aluminum adjuvants; AS, adjuvant system; CpG, CpG oligodeoxynucleotides (or CpG ODN); DTP, diphtheria, tetanus, pertussis; GSK, GlaxoSmithKline; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; IC, IRIV, immunostimulating reconstituted influenza virosomes; ISA, montanide incomplete Seppic Adjuvants; LPS, lipopolysaccharide; LT, labile toxin; LTK, adjuvant nontoxic derivatives of heat-labile enterotoxin of *E. coli*; MF59, immunologic adjuvant; MPL, monophosphoryl lipid A; NDV, NovaDigm Therapeutics; PEV, pharmaceutical company Pevion Biotech; QS21; saponin adjuvant derived tree *Quijalla saponaria* Molina; rSAP, recombinant aspartyl proteinases-2 of *C. albicans*; TLR, Toll-like receptor; VLP, virus-like particle.

phosphate buffered saline (PBS) or TM alone were not protective. The lungs of Asp f 3–vaccinated survivors were free of hyphae and showed only a patchy low-density infiltrate of mononuclear cells. In contrast, the nonimmunized animals died and showed invasive hyphal elements and a compact peribronchial infiltrate of predominantly polymorphonuclear leukocytes [59]. Recent work from this group has focused on the protective mechanisms of this vaccine, in particular, the roles of antibodies and especially CD4+ T cells [60]. TM has shown good immunostimulatory properties under experimental conditions but is too toxic for use in humans.

MF59. MF59 is an oil-in-water (o/w) system from Novartis that consists of 4.3% squalene, a metabolizable oil from shark liver; 0.5% polysorbate 80; 0.5% sorbitan triolate; and 10 mM sodium citrate, with a size of 160 nm [24,61]. Although the mode of action is still unclear, the mechanism of adjuvanticity by MF59 is proposed to be attributed, in part, to its depot effect and cellular infiltration, including the recruitment of APCs to the injection site, enhancement of antigen uptake into APCs, and activation of innate immunity without activating Toll-like receptor (TLR) pathways [14,16,62]. MF59 has been used in a licensed influenza vaccine (Fluad) with good safety in numerous countries for more than 15 years. This vaccine exhibits an adequate protection and safety balance, with acceptable, mild, and transient local injection site reactions [63,64].

MF59 has been tested in combination with laminarin from *Laminaria digitata* (Lam), a β-glucan from a nonfungal source that has a molecular structure resembling that of fungal β-glucans, to generate various experimental glycoconjugate vaccines using the nontoxic diphtheria toxin mutant CRM197. These laminarin–diphtheria toxoid conjugates were either natural (Curd-CRM197) or synthetic linear (15mer-CRM197) or β-(1,6)-branched (17mer-CRM197) β-(1,3)-oligosaccharides. All of these conjugates combined with the MF59 adjuvant resulted in immunogenicity and elicited antibodies against both Lam and the native β-glucan from *Candida*. However, Curd-CRM197 and 15mer-CRM197 conjugates induced high anti-β-(1,3)-glucan immunoglobulin G (IgG) titers, but no antibodies against β -(1,6)-glucan, that conferred protection to mice challenged with a lethal dose of *C. albicans*. In contrast, the 17mer-CRM197 conjugate, which induced anti-β-(1,6)-glucan antibodies in addition to the anti-β-(1,3)-glucan IgG, was nonprotective [65].

MF59 is regarded as a potent and safe adjuvant that has been used in licensed human vaccines with excellent results, making it an interesting adjuvant for future antifungal vaccines.

Cationic lipids. Cationic lipid (dioctadecyldimethylammonium bromide [DODAB]) adjuvants are able to carry antigens, efficiently stimulate antibody production, and activate cytotoxic T cells at a low antigen dose [66,67]. DODAB also induces dendritic cell maturation and the production of high levels of IL-12 and IFN-γ [68,69]. DODAB is another adjuvant that was evaluated in the previously mentioned experiment. The best results were observed with the combination P10 + DODAB in the immunized mice 30 days after infection with *P. brasiliensis* [70].

Sesame oil. Sesame oil, also known as gingelly oil, is an edible vegetable oil derived from sesame seeds. Its adjuvant properties were demonstrated by Kimura as part of a water-in-oil-in-water emulsion [71]. Sesame oil (Sigma–Aldrich Inc., St. Louis, MO, USA) was used by the Stevens group in an experimental vaccine against coccidioidomycosis using heat-killed *S. cerevisiae* (HKY) as the antigen. As happened with other adjuvants used, this oil did not improve the protection against systemic coccidioidomycosis in vaccinated animals [72].

In comparison, o/w emulsions seem to be safer than water-in-oil emulsions [16].

Aluminum compounds

In 1926, Glenny and colleagues discovered that a suspension of alum-precipitated diphtheria toxoid had a much higher immunogenicity than the fluid toxoid [13]. Historically, the word "alum" has been used in the adjuvant literature to describe both aluminum phosphate ($AlPO_4$) and aluminum hydroxide $Al(OH)_3$ gels, but this use of terminology is incorrect. Potassium alum, $KAl(SO_4)^{2-}12H_2O$, which has not been used as an adjuvant, conforms to the chemical definition of an alum, whereas neither $Al(OH)_3$ nor $Al(PO)_4$ is chemically considered an alum [73].

Despite their long period of use, a flurry of activity aimed at understanding the adjuvant action of these compounds has occurred only within the past two decades. Initially, the proposed mechanism of action was the depot effect in the local site of inoculation and slow antigen release [13,74]. Currently, the depot effect has been shown to not be a relevant mechanism of alum adjuvanticity [75]. In contrast, the cytotoxicity of aluminum salt was suggested to cause the release of uric acid [76] and DNA [77] from dying host cells *in vivo*, which acts as a damage-associated molecular pattern required for their adjuvant activity through caspase-1 activation. This process is mediated by the nucleotide oligomerization domain-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) and apoptosis-associated speck-like protein containing a CARD (N-terminal caspase recruitment) domain collectively known as the NLRP3 inflammasome [78,79].

Alum has been tested in several experimental vaccines against fungi. A vaccine against coccidioidomycosis was tested using HKY as antigen. Alum administration proved not to be beneficial and, in fact, appeared detrimental to the protective capacity of the HKY [72]. However, other anti-*Candida* vaccines based on synthetic, short β-(1→2)-linked mannose oligosaccharide haptens conjugated to tetanus toxoid admixed with alum were able to induce higher opsonizing antibody titers and a significant reduction in the fungal burden in vital organs after two injections in a standard model of invasive candidiasis [80]. Concurrently, this group also reported that a disaccharide from *C. albicans* conjugated with chicken serum albumin induces

a secondary immune response, as demonstrated by the detection of high levels of IgG antibodies specific for the cell wall β-mannan and reduced fungal burden in rabbits challenged with live *C. albicans* [81].

In another study, Lin et al. reported that vaccination with the candidal Als3p adhesin (rAls3p-N) with aluminum hydroxide ($Al(OH)_3$) adjuvant reduced the tissue infectious burden of mice subsequently infected intravenously with *C. albicans* or methicillin-resistant *S. aureus*. They also showed that the mechanism of protection was a Th1/Th17 response, resulting in recruitment and activation of phagocytes at sites of infection and more effective clearance of *S. aureus* and *C. albicans* from tissues [82].

A first-in-human phase 1 clinical trial involving 40 healthy adult subjects showed that this vaccine was generally well tolerated, causing only local site reactions characteristic of an intramuscular vaccine formulated with an aluminum adjuvant. This reaction was characterized by transient injection site pain and swelling that increased with higher doses. Systemic effects were generally mild and resolved quickly without sequelae [83].

Other vaccines prepared with different adjuvant combinations using alum are discussed later.

Bacterial-derived adjuvants

Monophosphoryl lipid A. Monophosphoryl lipid A (MPL) is composed of a series of 4′-monophosphoryl lipid A species that vary in the extent and position of their fatty acid substitutions. It is isolated from the lipopolysaccharide of *Salmonella minnesota* R595 and retains much of the immunostimulatory properties of the parent lipopolysaccharide without the inherent toxicity [84–86]. MPL is a TLR4 and TLR2 agonist on dendritic cells and macrophages that preferentially signals via TRIF (TIR-domain-containing adapter-inducing interferon-β) over MyD88 [87]. Moreover, MPL stimulates the production of the Th1 cytokines IL-2 and IFN-γ and IgG2a antibodies and, to a lesser extent, the Th2 cytokines IL-4 and IL-5 and IgE and IgG1 antibodies in mice. Furthermore, it is capable of upregulating human leukocyte antigen–DR, CD80, CD86, and CD40 and the activation marker CD83 expressed on dendritic cells *in vitro* [41,88,89]. In clinical trials with more than 10c000 healthy subjects, MPL was shown to have an acceptable tolerability and was effective in inducing IL-2, IFN-γ, and cytotoxic T cells [90].

Two inbred strains of mice (BALB/c and C57BL/6) were vaccinated with recombinant spherule-derived proline-rich antigen protein (rPRA) from *Coccidioides immitis* in MPL. Four weeks after vaccination, mice were infected intraperitoneally with arthroconidia. By 2 weeks, immunized mice had significantly lower pulmonary fungal burdens, ranging from 3.0 to 4.5 log10 fewer colony-forming units. *In vitro* immunologic markers of lymphocyte proliferation and IFN-γ release after splenocytes were stimulated with rPRA correlated with protection. Additionally, the plasma concentrations of rPRA-specific IgG1 and IgG2a showed increases in vaccinated mice [49].

Two formulations of MPL that have been used are MPL-SE and MPL-AF. MPL-AF augments humoral and cellular immune responses to vaccines administered by the intranasal route. These two variants have been explored in experimental vaccines against coccidioidomycosis using recombinant Ag2 fusion protein (rAg2/PRA) as the antigen [91]. The efficacy of rAg2/PRA in MPL-SE was compared with rAg2/PRA in FCA. Mice immunized with the former were significantly more protected than mice immunized with the latter after intranasal challenge with 30 arthroconidia. Moreover, the administration of rAg2/PRA in MPL-AF adjuvant significantly protected BALB/c and C57BL/6 mice against pulmonary challenge with *Coccidioides* [49,50].

In other more recent studies, mice immunized with the 14-mer peptide Fba, which is derived from the N-terminal portion of the *C. albicans* cytosolic/cell surface protein fructose-bisphosphate aldolase, mixed with MPL as the adjuvant conferred significant protection, which was associated with the production of anti-Fba peptide antibodies in the sera of immunized mice [92,93]. MPL is also used in several adjuvant combinations, which are discussed below.

Cytosine guanine dinucleotide oligodeoxynucleotide (CpG-ODN). Bacterial DNA contains cytosine guanine dinucleotide (CpG) motifs and, unlike vertebrate DNA, activates innate immune cells because the recognition of CpG motifs in vertebrate DNA is suppressed by methylation. These motifs have the general structure of two 5′ purines, an unmethylated CpG motif, and two 3′ pyrimidines and occur much more commonly in bacterial DNA than in mammalian DNA [94,95].

CpG ODNs are recognized by TLR9, which is expressed exclusively on human B cells and plasmocytoid dendritic cells (pDCs). This receptor expression pattern induces Th1-dominated immune responses, making CpG ODNs useful as vaccine adjuvants for peptide antigens as well as numerous microbial proteins. This adjuvant has been evaluated in neonates in a variety of species [96–98].

Romani et al. have shown that the combined intranasal delivery of CpG ODN and the Asp f 16, but not the Asp f 3 Aspergillus allergen, resulted in the functional maturation and activation of airway DCs capable of inducing Th1 priming and that this immunization was protective against invasive pulmonary aspergillosis in neutropenic BALB/c mice. CpG ODN predominantly induces Th1 activity and is a potent adjuvant for vaccine-induced protection against fungi [99].

Another study compared the protection obtained in mice against a respiratory coccidioidal infection by immunizing mice with vaccines containing rAg2/PRA truncations and a chimeric fusion protein composed of Ag2/PRA1–106 linearly coexpressed with CSA formulated with CpG ODN and MPL-SE. Mice vaccinated with either combination survived longer than mice given single antigens, exhibited high titers of specific IgG, and yielded interferon-gamma-producing splenocytes in response to either antigen [100].

Additionally, IFA mixed with either CpG1668 or curdlan has been used. T-cell responses in the draining lymph nodes were analyzed on day 7 after immunization. Alternatively, mice were challenged with *C. albicans* 3 weeks after immunization. In this study, the choice of vaccine adjuvant appeared to be critical because mice that were immunized using CpG were not protected from candidiasis despite the priming of *Candida*-speciffic CD4+ T cells. In this group, T cells primed in the presence of CpG differentiated into IFN-γ–secreting Th1 cells that lacked the ability to produce IL-17, whereas mice immunized with curdlan favored a Th17 response and were protected from fatal candidiasis [101]. Capilla et al. [72] reported similar results. They separately evaluated different adjuvants, including CpG in combination with HKY (heat-killed *S. cerevisiae*), to examine protection against systemic murine coccidioidomycosis. CD-1 mice received HKY subcutaneously or by oral gavage with or without adjuvants once weekly 3 or 4 weeks prior to infection; oral live *Saccharomyces* was also studied. Subcutaneously administered CpG appeared detrimental rather than beneficial to the protective capacity of the HKY, while HKY alone appeared to be the most effective [72].

In another recent study, female C57BL/6 mice and homozygous *Tlr3*$^{-/-}$ mice (genetically deficient in TLR3) were treated with *A. fumigatus* conidia or the protective recombinant fungal Ag Crf1p using CpG as an adjuvant. Resistance to subsequent infection was determined by assessing survival, fungal growth, and patterns of cytokine gene expression. In contrast to control mice, *Tlr3*$^{-/-}$ mice failed to develop vaccine-induced resistance in response to conidia, as revealed by their inability to survive infection and restrict fungal growth, their susceptibility to pulmonary aspergillosis, and their failure to produce protective IFN- γ and IL-10. *Tlr3*$^{-/-}$ mice also failed to develop MHC class I–restricted CD8^{+} T-cell responses after vaccination. However, surprisingly these mice developed full resistance after Crf1p vaccination [102].

These conflicting results obtained using CpG in different formulations are valuable examples that one simple and universal adjuvant effective for all types of antigens and conditions is elusive.

Muramyl dipeptide. Muramyl dipeptide (MDP) is an adjuvant that was first identified in a mycobacterial peptidoglycan fraction known to have potent adjuvant activity. It is composed of N-acetylmuramic acid linked by its lactic acid moiety to the N-terminus of an L-alanine D-isoglutamine dipeptide [103]. MDP is recognized by nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a member of the NLR family, a member of the NLR family. The characterization of Nod2 as the first pathogen-recognition molecule that detects MDP has helped to unravel the well-known biological activities of this immunomodulatory compound [104]. MDP also acts via the TLR pathway, providing synergistic coactivation that leads to the potent activation of nuclear factor-κB (NF-κB), the master immune regulator, and the induction of inflammatory cytokine production [105,106]. MDP was another one of the adjuvants evaluated by Capilla et al. in combination with HKY using the same conditions to CpG, and it was also ineffective against systemic infection with *Coccidioides* [72].

Flagellin. Flagellin is a highly conserved structural component of the flagellar filament in bacteria. Flagellin obtained from *Salmonella enterica* has been successfully used as a vaccine adjuvant to generate antigen-specific antibodies and T cells either when administered to mice as native purified protein or as a hybrid protein genetically fused to the target antigen, inducing both humoral and cellular responses. Its strong adjuvant activity is mediated by TLR5 on CD11+ dendritic cells, linking innate and adaptive immunity. Flagellin exposure leads to a strong cellular response accompanied by high IFN-γ secretion, indicating a Th1-biased response. Furthermore, flagellin has been recently shown to promote Th17 differentiation in a certain subset of dendritic cells (CD11chigh/CD11bhigh) [107–109].

FliC, a flagellin protein derived from *S. enterica* serovar Dublin, significantly alters the Th1 immune response associated with P10 of *P. brasiliensis*. BALB/c mice were intranasally immunized with gp43, a secreted fungal cell wall protein of *P. brasiliensis*, or a 15-amino acid peptide called P10, which contains a CD4^{+} T-cell–specific epitope, in combination with FliC flagellin. Immunization with purified recombinant flagellin genetically fused with P10 or the synthetic P10 peptide admixed with purified FliC elicited a predominantly Th1-type immune response based on lung cell–secreted type 1 cytokines. These immunized mice exhibited reduced *P. brasiliensis* growth and lung damage after intratracheal challenge with *P. brasiliensis* yeast cells in comparison with those immunized with gp43 and FliC, which suffered increased fungal proliferation and lung tissue damage [110]. In another experiment from this group, the therapeutic effect of several adjuvants, including FliC formulated with P10, a 15-mer internal peptide of the

glycoprotein gp43, the main antigen target of *P. brasiliensis*, were evaluated. Mice subcutaneously immunized with P10 and FliC 30 days after intratracheal infection and boosted on days 37 and 44 showed low numbers of viable yeast cells as well as reductions in granuloma formation and fibrosis. Concomitantly, in contrast to IL-4 and IL-10, the secretion of IFN-γ and tumor necrosis factor-alpha (TNF-α) was enhanced in the lungs of immunized mice, and significant therapeutic effects were observed for experimental PCM [70]. These results show that *S. enterica* FliC flagellin represents a promising alternative for the generation of preventive or therapeutic anti-PCM vaccines.

Bacterial enterotoxins. The heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* have been extensively studied for their contributions to virulence in microbial infections and for their immunomodulatory properties. Thus, ADP-ribosylating enterotoxins, including cholera toxin (CT), heat-labile enterotoxin (LT) from *E. coli* and their mutants or subunits, are the best-studied mucosal adjuvants. These enterotoxins promote the induction of antigen-specific IgA antibodies and long-lasting memory to coadministered antigens when administered mucosally or transcutaneously [111].

An MP extract and secreted aspartyl proteinase (Sap) of *C. albicans*, with or without CT as a mucosal adjuvant were formulated to immunize oophorectomized estrogentreated rats by the intravaginal or intranasal route. Both routes of immunization were equally effective in inducing anti-MP and anti-Sap vaginal antibodies and conferred a high degree of protection against vaginal infection by the fungus [112].

This same group developed a recombinant *C. albicans* vaccine consisting of a truncated 6xhis-tagged, enzymatically inactive Sap2 that lacks the N-terminus 76 amino acids (rSap2t) plus CT. This vaccine was used to intravaginally immunize oophorectomized estradiol-treated rats. These animals received this formulation three times at weekly intervals. At the end of the experiment, the immunized rats produced local anti-rSap2t IgG and IgA antibodies and were protected from the challenge of a highly vaginopathic strain of the fungus. In independent experiments, the passive transfer of immune vaginal fluid and the protective effects of passive vaccination with anti-rSap2t IgM and IgG monoclonal antibodies showed that protection was possibly due to the specific antibodies [113].

Cárdenas-Freytag et al. reported the effectiveness of a mucosal vaccine composed of heat-killed *C. albicans* (HK-CA) or *C. albicans* culture filtrate (CaCF) in conjunction with the mucosal adjuvant LT (R192G) against vulvovaginal candidiasis in an estrogen-dependent murine model. Mice vaccinated intranasally with HK-CA + LT (R192G) exhibited a significant but short-lived protection accompanied by high titers of circulating (but not in vaginal secretions) *C. albicans*–specific antibodies and a vigorous delayed-type hypersensitivity response. Vaginal priming with *C. albicans* before vaccination did not alter the protective outcome, while CaCF + LT (R192G) administered intrarectally but not intranasally induced a modest level of protection [114]. However, in a previous report from this group, use of killed *C. albicans* in conjunction with LT (R192G) administered intranasally to male CBA/J mice resulted in significant levels of protection after intravenous challenge with viable *C. albicans* and relevant stimulation of specific antibodies and delayed-type hypersensitivity tested in the footpad with *C. albicans* mannan [115].

Torosantucci et al. [116] developed a vaccine formulation to protect against a variety of human pathogenic fungi based on laminarin (Lam), a well-characterized but poorly immunogenic beta-glucan from the brown alga *Laminaria digitata* conjugated with the diphtheria toxoid CRM197 as the carrier protein and FCA as an adjuvant for systemic immunization via the intravenous route and CT for mucosal (intravaginal) immunization. This Lam–CRM conjugate was immunogenic and protective against both systemic and vaginal infections by *C. albicans* in mice. Moreover, passive transfer of whole immune serum, the immune vaginal fluid, and the affinity-purified anti-glucan IgG fractions provided protection to naive mice. This passive protection was prevented by adsorption of antibodies on *Candida* cells or glucan particles before transfer. Additionally, *in vitro* inhibition of *Candida* by pretreatment with anti-glucan antibodies generated in immunized mice was observed. Interestingly, Lam–CRM-vaccinated mice were also protected from a lethal challenge with *A. fumigatus* conidia, and their serum also bound to and markedly inhibited the growth of *A. fumigatus* hyphae. These experiments showed the protective role of the anti–beta-glucan antibodies [116].

In spite of the efficacy demonstrated for CT and LT and their derivatives as mucosal adjuvants, safety issues have prevented the full realization of the potential of this class of potent mucosal adjuvants [111,117,118].

Carbohydrate-based adjuvants

Several natural complex carbohydrates are able to stimulate immune cells. The main source of these polysaccharides are plants and fungi. Polysaccharides that have adjuvant activity include inulin, glucans, dextrans, lentinans, glucomannans, galactomannans, chitin/chitosan, levans, and xylans [119,120].

Macrophages have glucan and mannan receptors, and the activation of these receptors stimulates phagocytosis and cytokine secretion in addition to the release of leukotrienes and prostaglandins. *In vitro*, mannan activates

monocytes and macrophages to secrete IFN, TNF, GM-CSF (Granulocyte-macrophage colony-stimulating factor) IL-1 and IL-6. Acemannan, a natural polysaccharide extracted as a mucilaginous gel of *Aloe barbadensis*, stimulates the generation of specific antibodies, cytotoxic T lymphocytes, and the cytotoxic activity of natural killer cells in laboratory animals [119,121–124]. A carbohydrate derived from plant roots of the Compositae family, γ-inulin, is effective at boosting cellular immune responses without toxicity. The complement pathway is activated by γ-inulin, increasing activated C3 production and thereby activating macrophages. This adjuvant can be combined with a variety of other adjuvants, for example, aluminum hydroxide (Algammulin). This combination induces a higher ratio of Th2 to Th1 activity than γ-inulin alone [119].

The prophylactic effects of both the d-mannose-binding lectin ArtinM, which is extracted from the seeds of *Artocarpus integrifolia* (jackfruit), and its recombinant counterpart during the course of experimental paracoccidioidomycosis induced in mice were evaluated. The best effect was obtained after administration of two native or recombinant ArtinM doses on days 10 and 3 before challenge with *P. brasiliensis*. This treatment strategy reduced the fungal burden and lung granuloma incidence and augmented the levels of IL-12, IFN-γ, TNF-α, and nitric oxide (NO), favoring Th1 immunity in comparison with the untreated infected mice [125].

Liu et al. [126] studied an acid-stable cell-wall mannan (α-1, 6-linked backbone highly branched with α-1, 2; α-1, 3; and β-1, 2-linked manno-oligomers) derived from *C. albicans*, conjugated or not to bovine serum albumin (BSA), as a vaccine against systemic aspergillosis. They demonstrated that mice vaccinated subcutaneously with three doses of mannan or mannan-BSA conjugate weekly 2 weeks prior to infection with *A. fumigatus* conidia exhibited protection and reductions in the fungal burdens in the brains and kidneys in a dose-dependent manner, and conjugation with BSA improved the protection approximately 40-fold [126]. This result corroborates a report by Bystricky et al., who demonstrated that mannans alone do not induce sufficient levels of protective antibodies compared with a mannan–human serum albumin conjugate in immunized rabbits, which elicits a booster response with a significant increase in the serum IgG level and strong inhibition of *in vitro Candida* growth in the second case [127].

It is interesting that many carbohydrates derived from fungi can be used as antigens as well as adjuvants because they contain PAMPs that stimulate innate cells. This characteristic is a good opportunity to explore molecules with common structures in various pathogenic fungus to design universal antifungal vaccines [128].

Micro and nanoparticles

Liposomes. Liposomes are composed of natural, biodegradable, nontoxic, and nonimmunogenic phospholipids in which the antigen is either enclosed within the aqueous core or intercalated into the lipid layer. Due to their flexibility with regard to size, composition, charge, and bilayer fluidity, as well as their ability to incorporate large amounts of antigens and a variety of hydrophilic or hydrophobic compounds, liposomes are widely used as antigen delivery systems. In vaccine applications, their main functions are to protect antigens from clearance in the body and to deliver the antigens to professional antigen-presenting cells. Thus, they are an attractive alternative for the mucosal delivery of antigens [129,130]. Liposomes can facilitate the *in vivo* migration of antigens and deliver encapsulated antigen into the cytosol of antigen-presenting cells for both cell-mediated and humoral immune responses. The uptake of liposomes is generally believed to occur through a phagocytic or endocytic process, not by fusing with cellular membranes. Charged liposomes can readily bind to antigens and enhance their uptake and the efficiency of their presentation. Liposomes also upregulate several chemokine genes including CCL2, CCL3, and CCL4 in dendritic cells [16,131].

A mannan extract from *C. albicans* that functions as an adhesin was encapsulated in multilamellar liposomes and used to vaccinate mice over a 5- to 6-week period with an initial vaccine dose and weekly booster immunizations. Circulating agglutinins specific for this fraction correlated with increased resistance to disseminated candidiasis [132]. Another liposomal vaccine was formulated with *C. albicans* ribosomes and the lipids dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol (9:1 molar ratio). Some of the vaccines contained lipid-A as an additional adjuvant. The efficacy of these vaccines in mice was evaluated using the survival rate against a challenge with *C. albicans*; the induction of delayed type hypersensitivity (DTH) and the anti-*Candida* antibody titer. Unimmunized mice and mice vaccinated with ribosomes supplemented with IFA were used as controls. The results indicate that the liposomal vaccines were at least as effective as the IFA-based vaccine [133]. Similar results were observed by Lambros et al. [134] for a cryptococcal vaccine when they evaluated anticryptococcal DTH reactivity and the clearance of cryptococci from groups of mice immunized with liposome-encapsulated CneF (CneF-liposome) compared with those of mice immunized with CneF-CFA. The CneF-liposome formulation induced a positive anticryptococcal DTH response and increased the clearance of *C. neoformans* from tissues compared with the saline-liposome formulation [134]. More recently, groups of mice were immunized with

C. albicans cytosolic proteins (Cp) that were unencapusulated, liposome-encapsulated or liposome-encapsulated, and further entrapped in fibrin cross-linked plasma beads. Among various Cp-based vaccines investigated, the preparation containing liposomized Cp entrapped in plasma beads was more immunogenic and imparted superior protection in immunized mice, as evaluated by the survival rate and fungal burden in systemic circulation and vital organs, compared with other antigen delivery systems [135]. These results suggest that liposomes may function as valuable immunoadjuvants and delivery systems for generating protective immunity against fungal infection, with the possibility of being used in different antifungal vaccines.

Virosomes. Virosomes developed by Crucell, which are used as adjuvants and delivery systems, are reconstituted viral envelopes derived from the influenza virus that are devoid of viral RNA but retain the viral components necessary for target cell binding and entry, including hemagglutinin-mediated fusion activity. The antigen of interest can be displayed on the surface of virosomes in a highly immunogenic context owing to the virosomes combined action as a carrier and adjuvant, strongly promoting antigen presentation [136]. Virosomes are used in vaccines against inffiuenza (Inffiexal V) and hepatitis A (Epaxal) [137,138].

A novel vaccine candidate (PEV7; Pevion Biotech), which consists of rSap2, a truncated, recombinant aspartyl proteinase-2 of *C. albicans*, was developed as a virosome formulation [139]. PEV7 generated a potent serum antibody response in mice and rats following intramuscular immunization. Rats were immunized using intravaginal or intramuscular plus intravaginal administration of PEV7. Anti-Sap2 IgG and IgA were detected in the vaginal fluid after vaccination. In a rat model of candidal vaginitis, PEV7 induced significant, long-lasting, likely antibody-mediated protection following intravaginal immunization. PEV7 was also found to be safe in a repeated-dose toxicological study in rats [139].

Poly(lactic acid-glycolic acid). The biodegradable and biocompatible polyesters known as poly(lactic acid-glycolic acid); PLGA) have been used in humans for many years as suture materials and as controlled-release delivery systems for peptide drugs and are the primary candidates for the development of microparticles as vaccines [140]. The adjuvant properties of PLGA are due to small microspheres (<10 gm) that may be phagocytosed to enhance antigen presentation. In addition, studies have shown that microparticles also exert an adjuvant effect for cell-mediated immunity, including the induction of cytotoxic T-cell responses following both systemic and mucosal administration [141,142]. However, the primary use of PLGA for vaccine delivery is based on their ability to control the release of antigen after administration because their degradation rate and antigen release can be predicted, thereby eliminating or reducing the need for boost immunizations [143–145]. Unfortunately, the potential of microparticles as vaccine adjuvants has been limited by several studies that describe the degradation and denaturation of proteins during microencapsulation [146,147].

Recently, PLGA was used as a sustained delivery system for the immunomodulatory peptide P10 for reducing the *in vivo* degradation of the peptide and to elicit a protective immune response against paracoccidioidomycosis [148]. BALB/c mice were infected with *P. brasiliensis* yeast to mimic the chronic form of paracoccidioidomycosis. The animals were treated daily with sulfamethoxazole/trimethoprim alone or combined with peptide P10, either emulsified in Freund's adjuvant or entrapped in PLGA nanoparticles at different concentrations. After 30 days of treatment, animals given combined chemotherapy and P10 nanotherapy presented a marked reduction in fungal load in the lungs accompanied by high levels of IFN-γ in the lungs compared with untreated animals. In addition, this combined therapy was more effective than "free" P10 emulsified in Freund's adjuvant. As an additional advantage, P10 incorporation into PLGA nanoparticles dramatically reduced the amount of peptide necessary to elicit a protective effect, in turn, reducing costs of production.

In another experimental vaccine, cytosolic proteins (Cp) from the pathogen *C. neoformans* were used as an antigen in combination with PLGA microspheres further coencapsulated into the biocompatible fibrin cross-linked plasma beads (Fib-PLGA-Cp) for immunization of mice. This formulation mediated the cytosolic delivery of antigen to the target cells by both endocytosis as well as membrane fusion, thus helping in the activation of both CD4(+) and CD8(+) T cells. A protective response associated with Th1/Th2 polarization in favor of type-1 cytokines such as IFN-γ and IL-2 and high stimulation of specific IgG l and IgG 2a isotype responses was observed; the animals successfully cleared the fungal burden in vital organs, and the survival rate of immunized animals was increased [149].

The adjuvant effect of PLGAs can be further enhanced by their coadministration with additional adjuvants [150].

Conclusions and perspectives

Here, we discussed numerous reports that have experimentally demonstrated that modulating the host antifungal immune response for prophylactic and treatment of different forms of mycoses is possible. The immune response required to control fungal growth is delicately balanced to minimize tissue damage associated with the immune response, and this balance varies with each fungal pathogen and site of infection [6]. To achieve a protective and nontoxic vaccine,

the correct selection of the antigen–adjuvant combination in conjunction with the immunopathology of the specific fungi is necessary.

For a long time, the search for and development of adjuvants had been empirical. However, in the last 15 years, improved knowledge of the mechanisms of activation of innate immunity and their connection with adaptive immunity [36,79,151,152] have fostered improved results, and new adjuvants have been incorporated into human vaccines (Table 3) [9,17,153]. The increased knowledge of TLR biology has favored the development of new TLR-dependent adjuvant candidates, although more studies must be performed on polymorphisms in TLR receptors to account for variations in vaccine efficacy [36,154]. Additionally, the discovery of TLR-independent activation pathways, especially those associated with the NLRP3 inflammasome complex, aid in the understanding of mechanisms of other many adjuvants such as alum, which has been used empirically since 1926 [20,78,155]. Other important recent discoveries regarding memory of innate immunity (also called trained immunity) [156,157] can be relevant for the future rational design of adjuvanted vaccines [158,159].

Regarding antifungal vaccines, several aspects are being considered for the rational design of future vaccines. A major challenge is the elucidation of what constitutes protective immunity against the different pathogenic fungi and how to efficiently elicit the adaptive immune response with minimal reactogenicity. Undoubtedly, one of the more important factors for success in the endeavor to develop future vaccines is the educated selection of adjuvants that can drive the immune response toward the desired protective response for each fungus [10].

Regarding prophylactic vaccines, antiviral and antibacterial vaccines are administered widely, whereas fungal vaccine prophylaxis is performed according to risk criteria. For example, fungal vaccines might be used in people occupationally exposed to pathogenic fungus, close family of patients with systemic mycoses, before long-lasting and high doses of immunosuppression therapy, and before organs transplants. [2]. Prophylactic vaccines might also be used to immunize animals with the capacity to transmit some fungal diseases to humans in certain geographic areas, for example, sporotricoses in areas with high prevalence.

To develop therapeutic antifungal vaccines, it is necessary to consider that patients with systemic mycoses are often severely immunocompromised, and a number of factors could influence the host's response to antifungal drug therapy adversely, including relapsed/refractory hematological malignancy, granulocytopenia, myeloablative antineoplastic chemotherapy, high-risk allogeneic hematopoietic transplantation, the use of high-dose immunosuppressive agents and systemic corticosteroids, which can result in a poor response to antifungal therapy [160]. Although therapeutic antifungal vaccines have still not been used clinically, assessing these risk factors before applying these vaccines in patients with all of these associated factors is logical. On the other hand, various adoptive therapies using hyperimmune serum, monoclonal antibodies, cytokines, and cell-based immunotherapy seem to be better options for critical patients suffering from invasive fungal infections [12,132,161,162].

As was reviewed here, different adjuvants have been tested under different experimental conditions. These studies have helped us to understand the mechanisms of immune antifungal defense and to address proposals for future vaccines candidates. However, if the aim is to quickly develop a vaccine for clinical use, the early, correct selection of the adjuvants for the formulation is a very important decision. Adjuvants are considered an integral part of the finished vaccine product by regulatory agencies. Consequently, they are not licensed *per se*, including those that were used with co-administered antigens. Fortunately, in recent years, the survey of adjuvants licensed in human clinical trials for specific vaccines has been expanded (Table 3). This offers new opportunities to select better products or, alternatively, to design novel formulations with compositions comparable to those of recognized adjuvants. These previous experiences are important because they increase the likelihood of acceptance for clinical use from the regulatory viewpoint.

If the objective is a human preventative vaccine, we must begin evaluating adjuvants that have already been used in other human vaccines [9]. Despite several recent concerns about possible risks associated with its clinical use [163–166], alum is still the gold standard for the evaluation of new adjuvants for human use, while considering the recommendations to reduce its toxicity [167]. Alum has been evaluated in various antifungal vaccines, with variable but generally acceptable results. Other adjuvants acceptable for human use that have been evaluated with antifungal vaccines include MPL, virosomes, and MF59, while others such as calcium phosphate, AS03, AS04, and RC529 have not been explored to date. For veterinary vaccines, the selection criteria are more flexible, and a wide spectrum of available adjuvants can be used [168]. In this regard, a real problem currently is the limited commercial use of many adjuvants due to patent rights. Most vaccine companies develop their own adjuvant formulations and keep the proprietary of the patented product only with few vaccine products of their interest. Consequently, this limits the development of the adjuvant for other vaccine applications [9,169].

Because almost all systemic mycoses enter the host via mucosal surfaces (eg, upper respiratory, gastrointestinal, vaginal, or urinary tracts), the induction of mucosal

immune responses (ie, secretory IgA) is an exciting line of development in the search for adjuvants for mucosal immunization (oral, intranasal, and others routes), allowing the appropriate delivery of the antigen to the mucosal associated lymphoid tissue. This approach is safer, cheaper, and more feasible and would greatly simplify rapid massive vaccination. However, a major obstacle in the development of an effective mucosal vaccine is the requirement of breaching the mucosal epithelial barrier, presenting the antigen efficiently to the mucosal immune system, and overcoming natural tolerance at mucosal surfaces. In fact, the adequate selection of adjuvants and delivery systems is critical to obtain optimal protective mucosal immune response [170–172].

Another interesting field of research is the development of a broadly protective "universal vaccine" to provide protection against the most widespread fungal infections using common antigens, e.g., HSP60 and β-glucan, raising the possibility of achieving cross-protective immunization against several fungi with a single antigenic formulation [4,128]. However, several potential limitations to universal vaccines exist, such as balancing the dominance of antigenic determinants without excessive inflammation and carefully dissecting beneficial host immunity from harmful responses. In addition, these broadly specific immune responses could cause the excessive elimination of commensal microorganisms, affecting the local control of other pathogens [173]. The use of potent, well-selected adjuvants and delivery systems would permit the modulation of the immune response toward the desired function [10].

Here, we reviewed the more relevant results obtained with different adjuvants in experimental antifungal vaccines. This review can serve as a valuable reference for researchers who are working in this promising field. The selection and rational development of new adjuvants and delivery systems for antifungal vaccines demand special attention and is undoubtedly an important factor of success in the balancing the efficacy and toxicity of a final product today.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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