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VACCINES FOR EMERGING PATHOGENS: FROM RESEARCH TO THE CLINIC. PART 2

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REVIEW ARTICLE

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A novel vaccine platform using glucan particles for induction of protective responses against *Francisella tularensis* and other pathogens

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Summary

Vaccines are considered the bedrock of preventive medicine. However, for many pathogens, it has been challenging to develop vaccines that stimulate protective, long-lasting immunity. We have developed a novel approach using β-1,3-D-glucans (BGs), natural polysaccharides abundantly present in fungal cell walls, as a biomaterial platform for vaccine delivery. BGs simultaneously provide for receptor-targeted antigen delivery to specialized antigen-presenting cells together with adjuvant properties to stimulate antigen-specific and trained non-specific immune responses. This review focuses on various approaches of using BG particles (GPs) to develop bacterial and fungal vaccine candidates. A special case history for the development of an effective GP tularaemia vaccine candidate is highlighted.

Keywords: beta-glucans, fungal, tularemia, vaccine

Introduction

β-1,3-D-glucans (BG) as a biomaterial for vaccines

BGs are abundant polysaccharides naturally found in fungal, bacterial and algae cell walls, without any mammalian counterparts [1]. Structurally, they are composed of linear chains of β-1-3 glucopyranosyl residues with periodic β-1,6-linked branches. Based on the source, growth and isolation conditions, BGs with varying cell wall constituents (mannose, chitin), different branching patterns and sizes can be obtained. Each of these biomaterials act as a pathogen-associated molecular pattern (PAMP) engaging different receptors of antigen-presenting cells (APC), leading to varying immune responses [2–4]. Additionally, they are classified as 'generally regarded as safe' (GRAS) materials and used orally as a nutraceutical. Based on their solubility, BGs can be classified as soluble glucans or insoluble glucan particles (GPs), each of which may act as a biological response modifier (BRM) [5]. This review mainly focuses on the use of yeast-derived GPs as a vaccine development platform.

Mechanisms of immune modulation by BGs

The power of using BGs for vaccine development lies in their ability to stimulate all three arms of immunity: innate, trained and adaptive. Like other fungal components, BGs act as PAMPs that are recognized by macrophage- or dendritic cell (DC)-specific transmembrane pattern recognition receptors (PRRs) such as Dectin-1 or complement receptor 3 (CR3) [6]. Glucan binding to these PRRs leads to a cascade of signalling events resulting in phagocytosis of the glucan shell, release of proinflammatory cytokines, chemokines, anti-microbial proteins (lysozyme, defensins) and enhanced oxidative burst. Formation of the Dectin-1-GP phagocytic synapse is crucial for phagolysosomal maturation and release of cytokines by spleen tyrosine kinase (Syk) [7]-dependent caspase recruitment domain family member 9 (CARD9), nuclear factor kappa B (NF-κB)-inducing kinase (NIK), nuclear factor of activated T cells (NFAT) and independent pathways (Raf-1) [7–12]. Dendritic cell (DC) activation via glucan stimulation of the dectin-1-Syk-CARD9 pathway results in production of proinflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-12p40 in a Toll-like receptor (TLR)-independent manner [13]. Such an activation, together with transforming growth factor [(TGF)-β, secreted by T regulatory cells], polarizes $CD4^+$ T cells towards a T helper type 1 (Th1) and Th17 fate upon fungal infection *in vivo*. In human DCs, Th17 cell expansion is also mediated by glucan stimulation of prostanoid lipid mediator [prostaglandin E_2 (PGE_2)] expression, which in turn results in enhanced IL-23 production [14]. Dectin-1-Syk activation also triggers light chain 3-associated autophagy, which augments epitope presentation by recruitment of major histocompatibility complex class II (MHC-II) to the phagosomes of APCs [15]. BGs also potently activate the alternative pathway of complement, resulting in deposition of fragments of the third component of complement (C3) on the surface of GPs, which are then recognized by complement receptors on phagocytes [16]. Additionally, BG-activated leucocytes together with anti-tumour antibodies (natural or transferred) result in enhanced cytotoxicity against C3-opsonized (iC3b) tumour cells, thereby increasing the tumoricidal potential of antibodies and targeting tumours that are CR3-cytotoxic resistant [17,18].

Apart from Dectin-1- and CR3-mediated signalling, there are PAMPs recognized by host TLRs. The immunomodulatory properties of BG differed when co-administered with TLR agonists. For example, combining lipopolysaccharide (LPS) with GPs magnified the production of proinflammatory cytokines such as TNF-α in a myeloid differentiation primary response 88 (Myd88)-dependent manner [19]. This synergy was unaffected by type-II interferon (IFN)-γ priming in murine and human DCs [19].

During the past few years many studies have reported that innate immune cells, upon encountering a pathogen during infection or vaccination, can be trained to exhibit a heightened non-specific but protective immune response during reinfection or secondary stimulation by the same or a separate pathogen [20,21]. This innate immunological memory, often called trained innate immunity (TII), has been demonstrated by the protective effects of pretreatment of BG prior to pathogen infection. Glucan uptake by monocytes and macrophages results in induction of TII by stable epigenetic reprogramming that alters the cell's metabolic state [a shift towards glycolysis through the protein kinase B/mammalian target of rapamycin/hypoxia-inducible factor-α (Akt/mTOR/HIFα) pathway] and heightened cytokine production [20,22,23]. Unlike TII induction by bacillus Calmette–Guérin (BCG) vaccination, which lasted up to a year [24], BG priming immune responses are short-lived, as they were not observed 20 days post-dosing with BG [25].

Glucan particles

GPs are highly purified 3–4 μm hollow porous cell wall microspheres composed primarily of BG, typically isolated from *Saccharomyces cerevisiae*, using a series of hot alkaline, acid and organic extractions [26]. Owing to their immunomodulatory properties, GPs have been explored for vaccine delivery and stimulating the immune system. There are three general approaches to using GPs in vaccines (Fig. 1): (i) as a co-administered adjuvant with antigen(s) to enhance T and B cell-mediated immune responses, (ii) chemically cross-linked with antigens to provide for both antigen delivery and adjuvant functions and (iii) as a physical delivery vehicle of antigens trapped inside the hollow GP cavity, to provide targeted antigen delivery to APCs for tailored T and B cell-mediated immune responses. Each of these strategies is further explained in the following sections.

Vaccination with GPs and antigens results in enhanced antigen-specific $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (CTL), with a bias towards Th1 (IFN-γ) and Th17 (IL-17) proinflammatory responses [27]. Apart from these responses, GPs also enhance antibody responses after vaccination. The immune responses are often long-lasting and can persist throughout the lifetime of the vaccinated animal. Thus, GP-based vaccines stimulate well-rounded immune responses via a combination of their adjuvant and antigen delivery properties.

GPs co-administered with vaccines/antigens/adjuvants

Antigen-specific adaptive immune responses can be enhanced by co-administering BG together with antigens [28,29]. In this strategy, both innate as well as adaptive immune responses are activated to exert protective responses against pathogens. Immunizations with a killed *Trypanosoma cruzi* vaccine adjuvanted with GPs resulted in 85% survival of mice challenged with *T. cruzi* [30]. In contrast, controls that received dextrose, glucan or vaccine alone had 100% mortality [30]. Oral or subcutaneous immunizations with zymosan (a crude preparation of *S. cerevisiae* cell walls that contain BG and mannans) and dinitrophenyl-keyhole limpet haemocyanin in chicks led to induction of protective antigen-specific antibodies [31]. GPs enhanced the efficacy of a Venezuelan equine encephalitis vaccine more effectively than other adjuvants, including Freud's complete adjuvant, highlighting the use of GP as an adjuvant in boosting immunity [32]. Synergistic effects of enhanced proinflammatory cytokine release and expression of co-stimulatory markers were also seen in mice treated with zymosan–polyriboinocinic:polyribocytidylic acid [poly(I:C)] and inactivated influenza vaccine [33]. Thus, GPs not only serve as an adjuvant, but also can enhance the activity of other adjuvants.

GPs covalently cross-linked to antigens

The carbohydrate surface of GPs can be covalently modified using sodium periodate ($NaIO_4$) oxidation–borohydride reduction, carbodiimide-cross-linking or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate-mediated

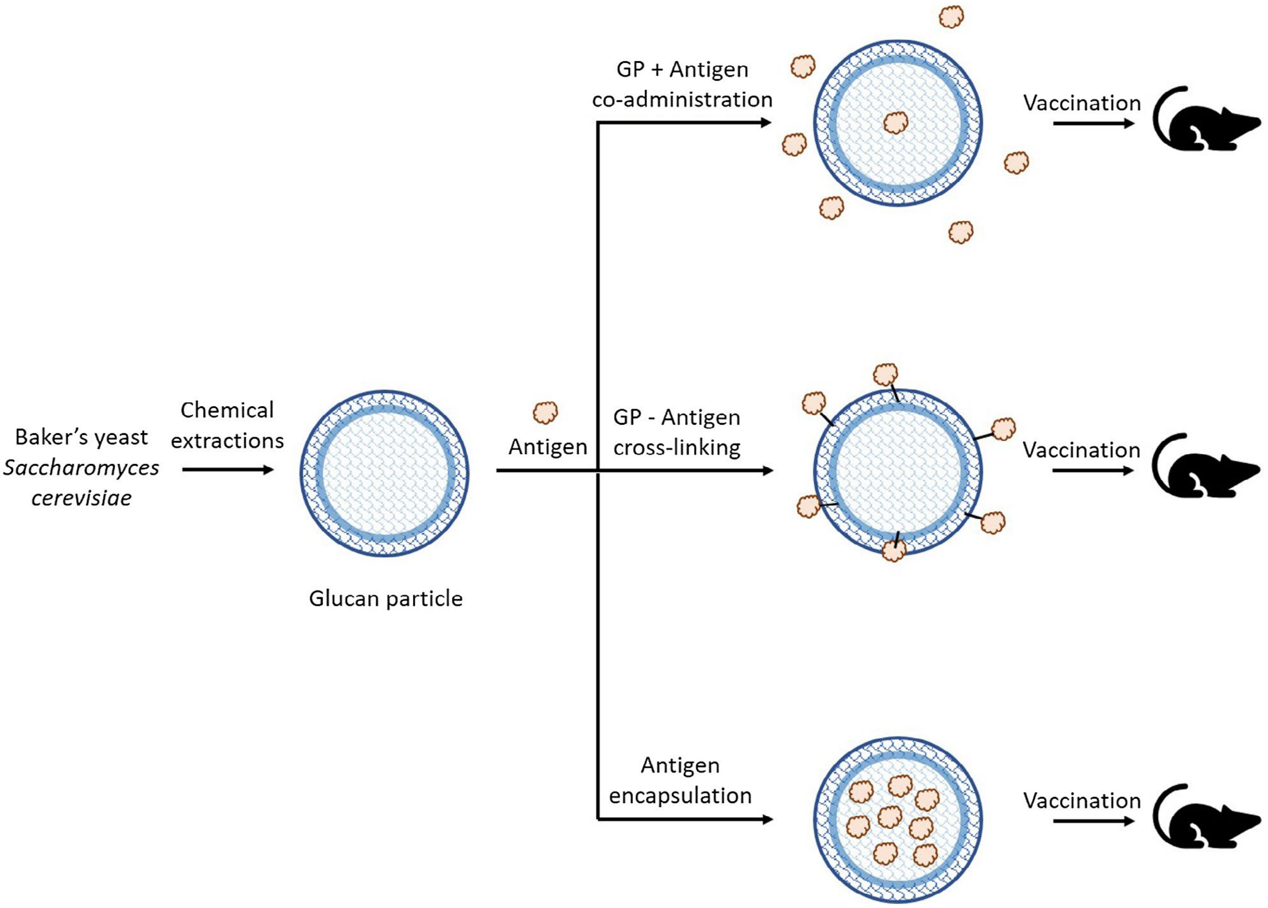

Fig. 1. Diagram showing different methods of vaccinations using β-1,3-D-glucans (BG) particles (GPs). Purified GPs can be employed as a vaccination platform by co-administration, cross-linking and encapsulation of antigens.

conjugation of antigens to the GP shell. GPs cross-linked to ovalbumin (OVA) using the carbodiimide method activated bone marrow-derived DCs to prime OVA-specific $CD4^+$ and $CD8^+$ T cells *in vitro* [34,35]. OVA can be cross-linked to periodate-oxidized GPs with 20% coupling efficiency (calculated on a weight basis). This is equivalent to $\sim 5 \times 10^5$ OVA/GP. When mice were subcutaneously immunized with GP-OVA and then challenged with OVA-expressing E.G7 lymphoma cells, significant reductions in tumour size were observed compared to groups receiving OVA or GP alone [34]. GP-OVA were found in the DCs ($CD11c^+MHC\text{-}II^+$) in lymph nodes 12 and 36 h post-subcutaneous injection [34]. The tumour protective effects were associated with an increase in total immunoglobulin (Ig)G titre, enhanced MHC-II and co-stimulatory molecule (CD80, CD86) expression and heightened CTL responses [34,35]. In infection models, administration of GPs conjugated to bovine serum albumin (BSA) at a lower dose (0·6 mg) protected mice challenged with the fungal pathogens *Aspergillus fumigatus* and *Coccidioides posadasii* marginally more effectively than GP-alone immunization [36,37]. The exact reason for a slightly better protective response is unclear, although few of the colony-stimulating factors, cytokines and chemokines were marginally enhanced in the whole glucan particles (WGP)-BSA vaccinated mice, suggesting that the protective effects were governed by activation of both innate and adaptive immunity. Interestingly, there were no substantial changes in the anti-β-glucan antibodies in these vaccinated mice, implying that antibodies against glucans do not contribute significantly to the protective immune response. Soluble glucan (laminarin) conjugated to a detoxified mutant diphtheria toxin (CRM197) has been tested as a pan fungal vaccine [38] for generation of efficient anti-glucan antibodies. One of the major limitations of surface conjugation methods is the low coupling efficiency (20%) compared to antigen encapsulation in GPs, limiting the number of vaccine candidates utilizing this strategy. Additionally, immune response to surface- *versus* core-loaded antigens may differ significantly, as exemplified in the case of *Francisella tularensis* vaccine explained later in this review.

GPs with non-covalently encapsulated antigens for vaccine delivery

The elegance of using GPs as a vaccine delivery platform is that it can serve as both a carrier of antigens in its hollow core, target antigens to APCs via receptor-mediated phagocytosis and concurrently acts as an adjuvant, thereby reducing the antigen dosage by ~100-fold [27,39]. Moreover, it can encapsulate one or more antigens/DNA/RNA/adjuvants/drugs/combinations with greater than 90% loading efficiency. The strategy of encapsulation is dictated by the type of payload and the mode of delivery. Antigens can be encapsulated in the hollow cavity of the GPs using polymer nano-complexation methods [26]. Using this strategy, Huang *et al* reported that mice vaccinated with GP–OVA showed strong $CD4^+$ T cell lymphoproliferation, a Th1 and Th17 skewed T cell-mediated immune response together with high IgG1- and IgG2c-specific antibody responses [39]. The non-covalent encapsulation strategy elicited stronger immune responses compared to GPs co-administered with antigen. Moreover, antigen-specific T cell and antibody responses remained robust 20 months following the last immunization [39].

Subunit vaccines with GPs encasing soluble alkaline extracts of *Cryptococcus neoformans* acapsular strain (cap59) protected mice challenged with lethal doses of highly virulent *C. neoformans* (60% survival) by inducing an antigen-specific $CD4^+$ T cell response (positive for IFN-γ, IL-17a) that reduced the fungal colony-forming units (CFU) more than 100-fold from the initial challenge dose [40,41]. A similar strategy of vaccinating mice with GP encapsulating antigens proved efficacious against *Histoplasma capsulatum* [42], *F. tularensis* [43], *Blastomyces dermatitidis* [44] and *C. posadasii* [45]. The versatility of using GPs vaccines against microbial pathogens is summarized in Table 1.

Identification of immunologically relevant epitopes aids the development of recombinant peptides/polypeptides that can be encapsulated within GPs for generating antigen-specific protective immunity. For example, recombinant chimeric poly-epitope antigen (rCpa1), consisting of a combination of three different antigens and five MHC-II binding pathogen-derived peptides, was designed for a *C. posadasii* vaccine [46]. When administered together with a TLR-9 agonist [DNA containing unmethylated cytosine–phosphate–guanine oligonucleotides (CpG-ODN)], a non-protective immune response was induced in a humanized human leucocyte antigen (HLA)-DR4 transgenic mouse model. However, immunization with a GP-rCpa1 formulation resulted in an enhanced Th1- and Th17-based protective immune response. Upon further optimization, yeast particles containing different cell wall constituents were used to synthesize similar encapsulated rCpa1 vaccines. Vaccines prepared with glucan chitin particles (GCPs) showed enhanced protection compared to GP or GP mannoprotein-based vaccines, suggesting that additional PAMPs in these more complex particles enhanced protective immune responses [46]. A single conserved antigen can also confer cross-protection to different fungal pathogens, as demonstrated by protective responses of calnexin encapsulated in GP vaccine against *B. dermatitidis* and *C. posadasii* [44].

In summary, GP-encapsulated antigen vaccines provide stronger immune responses than co-administered GP or GP-cross-linked antigen vaccination strategies. Importantly, GP vaccines stimulate Th1/Th17-biased immune responses, which are being recognized as necessary for protection against a growing number of pathogens. The adjuvanticity of glucans can sometimes be enhanced by loading GPs with other adjuvants such as alum and TLR agonists, etc. [47]. These attributes demonstrate the potential of the GP encapsulation technology for antigen discovery and vaccine development. As an example, our work on the development a GP-based vaccine against tularaemia is discussed below.

Tularaemia: a challenge for vaccinology

F. tularensis is an intracellular pathogen and the causative agent of the disease tularaemia. Capable of infecting a wide range of hosts, its normal zoonotic hosts are rodents and lagomorphs, but humans can be accidental hosts. The most common form of human tularaemia is ulceroglandular tularaemia, which arises following the bite of an infected insect or arthropod vector. However, it was reported recently that almost half the isolates studied from human cases in Nebraska were cat-associated, with transmission by bites and scratches [48]. In humans, the most acute presentation is respiratory or pneumonic tularaemia, following inhalation of infectious aerosols. The organism has a very low aerosol infectious dose for humans, requiring fewer than 50 CFU to establish respiratory infection [49]. Following inhalation, the most highly virulent strains can have a case fatality rate of up to 30% if untreated, but appropriate antibiotic therapy reduces this to approximately 2% [50]. Diagnosis based on symptoms is difficult, as the presentation can range from mild pneumonia to an acute infection with high fever, malaise, chills, cough, delirium and pulse–temperature dissociation, all of which are extremely non-specific. The high aerosol infectivity, morbidity and mortality led to the organism being developed as a biological weapon by various nations, including the reported production of antibiotic-resistant strains [50,51]. Tularaemia responds well to timely antibiotic therapy. Aminoglycosides, particularly streptomycin, have been used extensively for the treatment of tularaemia, but streptomycin is rarely used now due to adverse side effects, and gentamicin is a suitable alternative which has been used to treat cases

Table 1. β-1,3-D-glucan particles (GP) encapsulated vaccines against microbial pathogens. Different types of GPs can be formulated with a variety of antigens to stimulate antibody, T helper type 1 (Th1)- and Th17-biased immune responses that protect against numerous microbial pathogens in separate animal models

Pathogen	Type of particle	Antigen/ adjuvant	Vaccination strategy	Immunological response	Result	Ref
Cryptococcus neoformans	GP	Soluble alkaline extracts from *C. neoformans* cap59	Three subcutaneous injections followed by fungal challenge 2 weeks later in C57BL/6 mice	Robust Th1 and Th17 T cell recall response	60% mice survival	[40]
	GP	Recombinant Cda2	Three subcutaneous injection followed by fungal challenge 2 weeks later in C57BL/6 mice and DR4 mice	Possible-Th1 and Th17 T cell response	90–100 % mice survival	[41]
Histoplasma capsulatum	GP	Soluble alkaline extracts from *H. capsulatum*	One intranasal installation and two subcutaneous booster injections followed by fungal challenge 2 weeks later in C57BL/6 mice	Th1 and Th17 T cell response in lungs and lymph nodes, enhanced IFN-γ^+ CD8$^+$ T cells	75% mice survival	[42]
Francisella tularensis	GP	Recombinant FTT0814, *Francisella* LPS	Three subcutaneous injections 2 weeks apart followed by aerosol challenge 6 weeks after final dose in Fischer 344 rats	Intracellular LPS might engage NOD-like receptors, Strong IgG response, T cell-mediated IFN-γ response	100% rat survival	[43]
Coccidioides posadasii	GP	Recombinant epitopes, CpG-ODN adjuvant	Three immunizations followed fungal challenge after 4 weeks in HLA-DR4 mice	Lung infiltration of Th1 and Th17 T cells	Lung CFU reduction. Marginal increase in mice survival	[45]

Table 1. *Continued*

Pathogen	Type of particle	Antigen/ adjuvant	Vaccination strategy	Immunological response	Result	Ref
	GCP	rCpa1	Two subcutaneous immunizations followed by intranasal fungal challenge after 4 weeks in C57BL/6 and HLA-DR4 mice	Increased lung infiltration of Th1 and Th17 T cells	100% protection in C57BL/6 mice, 60% protection in HLA-DR4 mice	[46]
Blastomyces dermatitidis	GMP	Calnexin, adjuplex adjuvant	Three subcutaneous vaccinations 2 weeks apart, intratracheal challenge 2 weeks after final dose in C57BL/6 mice	Increased $CD4^+$ T cells in the lungs and draining lymph nodes, Th1 and Th17 response	3000-fold reduction in the lung CFU compared to control mice vaccinated with GMP-adjuplex	[44]

GCP = GP containing chitin, GMP = GP containing mannose; Cda2 = chitin deacetylase 2; CpG-ODN = ssDNA with unmethylated cytosine–phosphate–guanine (CpG) oligonucleotides; HLA-DR4: transgenic mice containing a hybrid major histocompatibility complex class II (MHC-II) with human leucocyte antigen peptide binding domains; rCpa1: recombinant chimeric polypeptide antigen; Th = T helper; CFU = colony-forming units; IFN = interferon; Ig = immunoglobulin; NOD = nucleotide oligomerization domain.

of pneumonic tularaemia on Martha's Vineyard [52]. However, aminoglycosides are reserved for the most serious cases due to the requirement for parenteral dosing and monitoring of serum levels. Ciprofloxacin is currently the preferred choice of drug for the oral treatment of uncomplicated tularaemia [50]. Relapse is common following short courses and some patients may require respiratory support and intensive care should sepsis develop. Suppurating nodes are a common cause of treatment failure, and these may require draining [53]. Failure can also arise as a result of delayed initiation of antibiotics or if therapy is withdrawn prematurely (reviewed by Caspar *et al.* [54]) As such, there is strong interest in developing effective medical countermeasures to prevent and treat tularaemia, particularly vaccines.

Many different approaches have been explored in the quest for a safe effective vaccine to protect against tularaemia. Crude culture extracts [55–58], subunit vaccines and attenuated strains have all been evaluated, but none meet the criteria of efficacy and safety required for a modern vaccine. The most promising candidates were purified LPS and the 'Live Vaccine Strain' (LVS). Purified Francisella LPS induced a humoral response that was able to protect mice against low virulence strains of *F. tularensis* [59,60], but only extended the time to death following challenge with more virulent strains. Despite extensive screening, no protein antigens were identified to supplement the protection induced by LPS. In contrast, LVS, having been used in many thousands of humans under Investigational New Drug status, seems to be effective for the prevention of respiratory tularaemia in humans [61], but has safety concerns associated with its use, and thus the LVS strain has yet to be approved by regulatory bodies. We hypothesized that subunit vaccines are most attractive due to their defined nature and thus good safety profiles, but that we needed to deliver promising candidate antigens in a manner that induced both humoral and cellular immune responses to achieve protection, as only a balanced humoral and cellular immune memory response, supported by innate immune mechanisms, protects against tularaemia (reviewed by Roberts *et al.* [62] and Krokova *et al.* [63]). We therefore decided to employ the GP vaccine delivery platform described above to address this challenge [43]. This work is summarized below.

A panel of 28 *Francisella* proteins was selected for evaluation in the GP platform, the majority of which had been identified by the approach described in [64]. Many of these had been evaluated previously as potential vaccine antigens using other adjuvants without success. These proteins were expressed recombinantly in *Escherichia coli* and purified. Initial expression analysis showed some of the proteins to be relatively insoluble, and thus our standard GP loading conditions were modified to include 6 M urea, which was subsequently removed by washing [65]. In addition, LPS was loaded either onto the surface of the GPs or into the core. However, the GP core-loaded *F. tularensis* LPS formulations were more immunostimulatory than surface-linked *F. tularensis* LPS GP formulations or free biotinylated *F. tularensis* LPS. Therefore, core-loaded *Francisella* LPS was selected for further evaluation as a component of the GP-delivered vaccine.

The mouse is a good model for immunological analysis of tularaemia vaccines, but its acute susceptibility means that it is difficult to induce protection. The LVS strain can induce protection in mice, and correlates of protection are being determined [66,67]. However, no correlates in mice have been identified for subunit vaccines, other than the need for antibody titres against LPS [59,60], which are not sufficient on their own to protect against infection with fully virulent strains of *F. tularensis* [59]. The selection of promising candidates to progress to the next round of screening was primarily influenced by a combination of the development of antigen-specific IFN-γ enzyme-linked immunospot (ELISPOT) responses and/or the detection of an antibody response, particularly where an IgG2a bias was observed. As there are currently no robust correlates of protection known for tularaemia vaccines, we were interested in selecting candidates that represented a variety of immune response profiles, albeit with a bias towards cell-mediated immunity. This allowed us to identify seven proteins of interest: IgIC, FTT0071, FTT0289, FTT0438, FTT0814, FTT0890 and FTT1043. As IglC has been previously reported to induce partial protection in animals (reviewed by Roberts *et al.* [62] and Krokova *et al.* [63]) this was also included, even though the IglC GP vaccine induced poor immune responses. However, consistent with our selection rationale, it did not perform well later, and was subsequently dropped. T cell memory recall responses induced in splenocyte cultures from immunized C57Bl/6 mice showed that FTT0071, FTT0814 and FTT0890 were the most potent inducers of IFN-γ responses. FTT0814 stimulated the strongest and most consistent IL-10 response. However, there was no protection induced in immunized mice against challenge with a similar dose of *F. tularensis*.

The Fischer 344 rat has been proposed as a more appropriate model for *F. tularensis* vaccine efficacy testing, as it is more resistant to tularaemia than the highly susceptible mouse model, and overall the pathogenesis of respiratory tularaemia in the rat model appears to replicate tularaemia in humans [68]. Strain SchuS4 is a highly virulent strain, and injection of mice with a dose of 1 CFU results in 100% mortality. As rats are more resistant, a higher bacterial dose of $1{\cdot}6 \times 10^3$ CFU of *F. tularensis* SchuS4 delivered via the respiratory route was determined to achieve 100% lethality in PBS-treated controls. An immunological bridging study was first undertaken to determine the hierarchy of immunological responsiveness of the seven down-selected *F. tularensis* antigens in rats. Responses to the carrier protein, OVA, included in each vaccine were lower than seen in the mouse, but it has previously been reported that Fischer rats are 'low immunological responders' to OVA even when compared with other rat strains, such as Wistar and Sprague–Dawley rats [69]. While the hierarchy of immune responsiveness in mouse and rat models was largely overlapping, FTT0071 was a notable exception, and while this antigen was immunodominant with regard to IgG and IFN-γ responses in mice, it induced poor responses in rats. Further evaluation of responses in rats showed that immunization with the FTT0814-based GP vaccine induced the strongest and most consistent antigen-specific IgG response and the strongest T cell-mediated IFN-γ responses. In challenge studies, all GP-encapsulated *F. tularensis* antigen combinations containing LPS were able to protect rats against an otherwise lethal aerosol challenge of *F. tularensis* SchuS4. Only the GP-FTT0814-LPS vaccine was able to prevent the development of any clinical scores in rats to the same extent as LVS (Fig. 2). This is suggestive that FTT0814 may supplement the protection induced by LPS when delivered by GPs. This is an impressive step forward towards developing a subunit vaccine to prevent tularaemia and demonstrates the broad immunological responses that can be induced by GP technology. It also highlights the importance of using an appropriate animal model for efficacy studies *versus* immunogenicity screens.

Future of GP vaccines

Heat-killed yeast expressing antigens (intracellularly or surface displayed) have been used as a vaccine vector to generate antigen-specific adaptive immune responses [70]. Clinical trials confirm that these vaccinations result in minimal toxicity to humans [71]. However, such a mode of vaccination can result in delivery of yeast-derived peptides into the host cell and the presence of other components (such as mannans, chitins) might have additional role in immune responses. As glucans are the major immunomodulatory component of the yeast cell walls, the use of purified GPs as a vaccine vector have been investigated. A stepwise process for development of GP-based vaccine is depicted in Fig. 3. Briefly, the first step involves discovery of protective antigens and *in-vivo* testing of recombinantly expressed antigens that are encapsulated within GPs. After identification of lead antigens, the GP-based formulations will be further optimized

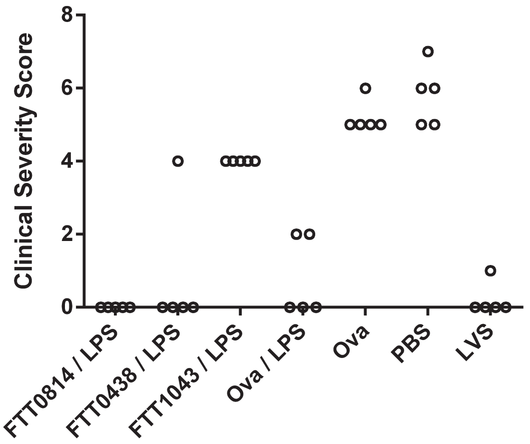

Fig. 2. Clinical score at acute stage of infection in rats immunized with β-1,3-D-glucan particle (GP) vaccines. Clinical scores for each individual rat, within each respective treatment group, are presented at day 4 post-infection. Significance was determined using non-parametric analysis of variance (ANOVA) with Holm–Sidak's multiple comparisons test. Signs exhibited by animals vaccinated with FTT0814/LPS, FTT0438/LPS and ovalbumin/lipopolysaccharide (OVA/LPS) were not significantly different from clinical signs in the live vaccine strain (LVS) group, which is indicative of protection.

with respect to type of GP, antigen(s)/adjuvant(s) and mode of vaccination. The lead vaccine candidate/candidates will be manufactured under cGMP. An Investigational New Drug (IND) application will be submitted to the Food and Drug Administration (FDA), upon approval of which the vaccines can be tested in humans. Following successful clinical trials, a Biological License Application (BLA) will be submitted that enables commercialization of the vaccine.

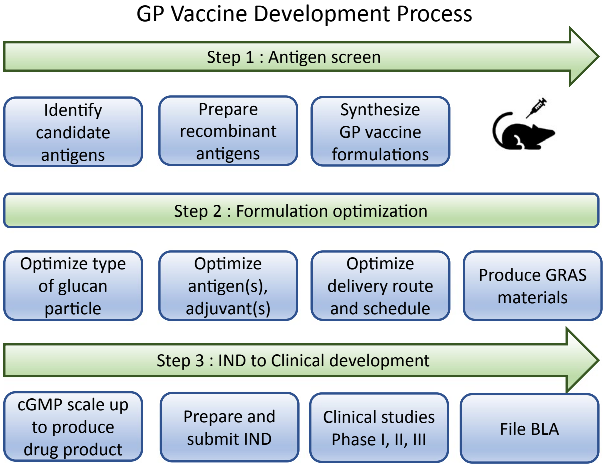

Fig. 3. Overview of β-1,3-D-glucan particle (GP) vaccine development process. GP-based vaccine development employs three major phases of antigen screen, formulation optimization and clinical translation of the vaccine. GRAS = generally regarded as safe; IND = investigational new drug; BLA = Biological License Application.

GPs offer significant advantages over other adjuvants and vaccine delivery systems. GP vaccines have been shown to be protective against bacterial, fungal and viral infections, as well as in animal models of cancer. Immune responses to GP vaccines have been elicited following multiple routes of delivery, including subcutaneous, intramuscular, pulmonary and oral [72]. While most studies have been preclinical, there have been promising Phase I/II clinical trials demonstrating that BGs [73] and GPs [74] are well tolerated in humans. Understanding the basis of protective host immunity for specific infections, and how BGs interact with immune cells to enhance immunity, will hopefully pave the way for development of optimal BG-based vaccines.

References

1 Thompson IJ, Oyston PC, Williamson DE. Potential of the beta-glucans to enhance innate resistance to biological agents. Expert Rev Anti Infect Ther 2010; **8**:339–52.

2 Du B, Lin C, Bian Z, Xu B. An insight into anti-inflammatory effects of fungal beta-glucans. Trends Food Sci Technol 2015; **41**:49–59.

3 Wang Q, Sheng X, Shi A *et al*. Relationships between modification, conformation and functional activities. Molecules 2017; **22**.

4 Sletmoen M, Stokke BT. Higher order structure of (1,3)-beta-D-glucans and its influence on their biological activities and complexation abilities. Biopolymers 2008; **89**:310–21.

5 Bohn JA, BeMiller JN. (1→3)-β-d-Glucans as biological response modifiers: a review of structure–functional activity relationships. Carbohyd Polym 1995; **28**:3–14.

6 Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. Immunol Rev 2009; **230**:38–50.

7 Mansour MK, Tam JM, Khan NS *et al*. Dectin-1 activation controls maturation of beta-1,3-glucan-containing phagosomes. J Biol Chem 2013; **288**:16043–54.

8 Goodridge HS, Reyes CN, Becker CA *et al*. Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature 2011; **472**:471.

9 Hara H, Ishihara C, Takeuchi A *et al*. The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. Nat Immunol 2007; **8**:619.

10 Gross O, Gewies A, Finger K *et al*. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature 2006; **442**:651–6.

11 Gringhuis SI, den Dunnen J, Litjens M *et al*. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-kappaB activation through Raf-1 and Syk. Nat Immunol 2009; **10**:203–13.

12 Plato A, Willment JA, Brown GD. C-type lectin-like receptors of the dectin-1 cluster: ligands and signaling pathways. Int Rev Immunol 2013; **32**:134–56.

13 LeibundGut-Landmann S, Groß O, Robinson MJ *et al*. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol 2007; **8**:630.

14 Gagliardi MC, Teloni R, Mariotti S *et al*. Endogenous PGE2 promotes the induction of human Th17 responses by fungal ss-glucan. J Leukoc Biol 2010; **88**:947–54.

15 Ma J, Becker C, Lowell CA, Underhill DM. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class II presentation of fungal-derived antigens. J Biol Chem 2012; **287**:34149–56.

16 Agarwal S, Specht CA, Huang H *et al*. Specificity and role of properdin in activation of the alternative complement pathway by fungal glycans. mBio 2011; **2**:e00178–e211.

17 Hong F, Hansen RD, Yan J *et al*. Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. Cancer Res 2003; **63**:9023–31.

18 Cheung NK, Modak S, Vickers A, Knuckles B. Orally administered beta-glucans enhance anti-tumor effects of monoclonal antibodies. Cancer Immunol Immunother 2002; **51**:557–64.

19 Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM. Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists. Infect Immun 2009; **77**:1774–81.

20 Netea MG, Joosten LAB, Latz E *et al*. Trained immunity: a program of innate immune memory in health and disease. Science 2016;**352**:aaf1098.

21 Netea MG, van der Meer JWM. Trained immunity: an ancient way of remembering. Cell Host Microbe 2017; **21**:297–300.

22 Saeed S, Quintin J, Kerstens HH *et al*. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. Science 2014; **345**:1251086.

23 Cheng SC, Quintin J, Cramer RA *et al*. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. Science 2014; **345**:1250684.

24 Kleinnijenhuis J, Quintin J, Preijers F *et al*. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. J Innate Immun 2014; **6**:152–8.

25 Garcia-Valtanen P, Guzman-Genuino RM, Williams DL, Hayball JD, Diener KR. Evaluation of trained immunity by β-1, 3 (d)-glucan on murine monocytes *in vitro* and duration of response *in vivo*. Immunol Cell Biol 2017; **95**:601–10.

26 Mirza Z, Soto ER, Dikengil F, Levitz SM, Ostroff GR. Beta-Glucan particles as vaccine adjuvant carriers. Meth Mol Biol 2017; **1625**:143–57.

27 Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust Stimulation of humoral and cellular immune responses following vaccination with antigen-loaded β-glucan particles. mBio 2010; **1**.

28 Williams DL. Overview of 1,3-D-glucan immunobiology. Mediat Inflamm 1997; **6**:247–50.

29 Jin Y, Li P, Wang F. beta-glucans as potential immunoadjuvants: a review on the adjuvanticity, structure–activity relationship and receptor recognition properties. Vaccine 2018; **36**:5235–44.

30 Williams DL, Yaeger RG, Pretus HA, Browder IW, McNamee RB, Jones EL. Immunization against *Trypanosoma cruzi*: adjuvant effect of glucan. Int J Immunopharmacol 1989; **11**:403–10.

31 Abou Elazab MF, Inoue Y, Kamei H, Horiuchi H, Furusawa S. Zymosan A enhances humoral immune responses to soluble protein in chickens. J Vet Med Sci 2017; **79**:1335–41.

32 Reynolds JA, Kastello MD, Harrington DG *et al*. Glucan-induced enhancement of host resistance to selected infectious diseases. Infect Immun 1980; **30**:51–7.

33 Ainai A, Ichinohe T, Tamura S *et al*. Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. J Med Virol 2010; **82**:476–84.

34 Pan Y, Li X, Kang T *et al*. Efficient delivery of antigen to DCs using yeast-derived microparticles. Sci Rep 2015; **5**:10687.

35 Berner VK, duPre SA, Redelman D, Hunter KW. Microparticulate β-glucan vaccine conjugates phagocytized by dendritic cells activate both naïve CD4 and CD8 T cells *in vitro*. Cell Immunol 2015; **298**:104–14.

36 Clemons KV, Danielson ME, Michel KS *et al*. Whole glucan particles as a vaccine against murine aspergillosis. J Med Microbiol 2014; **63**(Pt 12):1750–9.

37 Clemons KV, Antonysamy MA, Danielson ME *et al*. Whole glucan particles as a vaccine against systemic coccidioidomycosis. J Med Microbiol 2015; **64**:1237–43.

38 Torosantucci A, Bromuro C, Chiani P *et al*. A novel glyco-conjugate vaccine against fungal pathogens. J Exp Med 2005; **202**:597–606.

39 Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Characterization and optimization of the glucan particle-based vaccine platform. Clin Vaccine Immunol 2013; **20**: 1585–91.

40 Specht CA, Lee CK, Huang H *et al*. Protection against experimental cryptococcosis following vaccination with glucan particles containing cryptococcus alkaline extracts. MBio 2015; **6**:e01905–e1915.

41 Specht CA, Lee CK, Huang H *et al*. Vaccination with recombinant cryptococcus proteins in glucan particles protects mice against cryptococcosis in a manner dependent upon mouse strain and cryptococcal species. mBio 2017; **8**:e01872–e1917.

42 Deepe GS Jr, Buesing WR, Ostroff GR *et al*. Vaccination with an alkaline extract of *Histoplasma capsulatum* packaged in glucan particles confers protective immunity in mice. Vaccine 2018; **36**:3359–67.

43 Whelan AO, Flick-Smith HC, Homan J *et al*. Protection induced by a *Francisella tularensis* subunit vaccine delivered by glucan particles. PLOS ONE 2018; **13**:e0200213.

44 Wuthrich M, Brandhorst TT, Sullivan TD *et al*. Calnexin induces expansion of antigen-specific CD4(+) T cells that confer immunity to fungal ascomycetes via conserved epitopes. Cell Host Microbe 2015; **17**:452–65.

45 Hurtgen BJ, Hung CY, Ostroff GR, Levitz SM, Cole GT. Construction and evaluation of a novel recombinant T cell epitope-based vaccine against Coccidioidomycosis. Infect Immun 2012; **80**:3960–74.

46 Hung C-Y, Zhang H, Castro-Lopez N *et al*. Glucan-chitin particles enhance Th17 response and improve protective efficacy of a multivalent antigen (rcpa1) against pulmonary *Coccidioides posadasi*i infection. Infect Immun 2018; **86**:e00070–e118.

47 Liu H, Jia Z, Yang C *et al*. Aluminum hydroxide colloid vaccine encapsulated in yeast shells with enhanced humoral and cellular immune responses. Biomaterials 2018; **167**:32–43.

48 Larson MA, Fey PD, Hinrichs SH, Iwen PC. *Francisella tularensis* bacteria associated with feline tularemia in the United States. Emerg Infect Dis 2014; **20**:2068–71.

49 Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. Tularemia vaccine study. II. Respiratory challenge. Arch Intern Med 1961; **107**:702–14.

50 Dennis DT, Inglesby TV, Henderson DA *et al.* Tularemia as a biological weapon. Medical and public health management. JAMA 2001; **285**:2763–73.

51 Oyston PCF, Sjostedt A, Titball RW. Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol 2004; **2**:967–78.

52 Matyas BI, Nieder HS, Telford SR. Pneumonic tularemia on Martha's Vineyard – clinical, epidemiologic, and ecological characteristics. Ann NY Acad Sci 2007; **1105**:351–77.

53 Karlı A, Şensoy G, Paksu Ş, Korkmaz MF, Ertuğrul Ö, Karlı R. Treatment-failure tularemia in children. Korean J Pediatr 2018; **61**:49–52.

54 Caspar Y, Maurin M. *Francisella tularensis* susceptibility to antibiotics: a comprehensive review of the data obtained *in vitro* and in animal models. Front Cell Infect Microbiol 2017; **7**:122.

55 Foshay L. A comparative study of the treatment of tularemia with immune serum, hyperimmune serum and strepomycin. Am J Med 1946:180–8.

56 Foshay L. Tularemia. Ann Rev Microbiol 1950; **4**:313–30.

57 Foshay L, Hesselbrock WH, Wittenberg HJ, Rodenberg AH. Vaccine prophylaxis against tularemia in man. Am J Public Health 1942; **32**:1131–45.

58 Eigelsbach HT, Downs CM. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J Immunol 1961; **87**:415–25.

59 Fulop M, Mastroeni P, Green M, Titball RW. Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of *Francisella tularensis*. Vaccine 2001; **19**:4465–72.

60 Conlan JW, Shen H, Webb A, Perry MB. Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine 2002; **20**:3465–71.

61 McCrumb FR. Aerosol infection of man with *Pasteurella tularensis*. Bacteriol Rev 1961; **25**:262–7.

62 Roberts LM, Powell DA, Frelinger JA. Adaptive immunity to *Francisella tularensis* and considerations for vaccine development. Front Cell Infect Microbiol 2018; **8**:115.

63 Krocova Z, Macela A, Kubelkova K. Innate immune recognition: implications for the interaction of *Francisella tularensis* with the host immune system. Front Cell Infect Microbiol 2017; **7**:446.

64 Mayers C, Duffield M, Rowe S *et al*. Analysis of known bacterial protein vaccine antigens reveals biased physical properties and amino acid composition. Comp Funct Genomics 2003; **4**:468–78.

65 Cole GT, Hung C-Y, Sanderson SD *et al*. Novel strategies to enhance vaccine immunity against Coccidioidomycosis. PLOS Pathog 2013; **9**:e1003768.

66 De Pascalis R, Mittereder L, Kennett NJ, Elkins KL. Activities of murine peripheral blood lymphocytes provide immune correlates that predict *Francisella tularensis* vaccine efficacy. Infect Immun 2016; **84**:1054–61.

67 Roberts LM, Wehrly TD, Crane DD, Bosio CM. Expansion and retention of pulmonary CD4(+) T cells after prime boost vaccination correlates with improved longevity and strength of immunity against tularemia. Vaccine 2017; **35**:2575–81.

68 Hutt JA, Lovchik JA, Dekonenko A, Hahn AC, Wu TH. The natural history of pneumonic tularemia in female Fischer 344 rats after inhalational exposure to aerosolized *Francisella tularensis* subspecies *tularensis* strain SCHU S4. Am J Pathol 2017; **187**:252–67.

69 Tada N, Itakura K, Aizawa M. Genetic-control of antibody-response in inbred rats. J Immunogenet 1974; **1**:265–75.

70 Ardiani A, Higgins JP, Hodge JW. Vaccines based on whole recombinant *Saccharomyces cerevisiae* cells. FEMS Yeast Res 2010; **10**:1060–9.

71 Habersetzer F, Baumert TF, Stoll-Keller F. GI-5005, a yeast vector vaccine expressing an NS3-core fusion protein for chronic HCV infection. Curr Opin Mol Ther 2009; **11**:456–62.

72 De Smet R, Allais L, Cuvelier CA. Recent advances in oral vaccine development. Hum Vaccines Immunother 2014; **10**:1309–18.

73 Kushner BH, Cheung IY, Modak S, Kramer K, Ragupathi G, Cheung NK. Phase I trial of a bivalent gangliosides vaccine in combination with beta-glucan for high-risk neuroblastoma in second or later remission. Clin Cancer Res 2014; **20**:1375–82.

74 Feldman S, Schwartz HI, Kalman DS *et al*. Randomized phase II clinical trials of wellmune WGP[R] for immune support during cold and flu season. J Appl Res 2009; **9**:30+.