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Ambily Abraham

University of Massachusetts Medical School

Et al.

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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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A. Abraham,* G. Ostroff,* S. M. Levitz* and P. C. F. Oyston  †

*University of Massachusetts Medical School, Worcester, Massachusetts, USA, and †CBR Division, Defence Science and Technology Laboratory, Porton Down, Salisbury, UK

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Correspondence: P. C. F. Oyston,

Microbiology, B07 Room 201, CBR Division, Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK.

E-mail: pcoyston@dstl.gov.uk

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,

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

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- κ B)-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

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prostanoid lipid mediator [prostaglandin E₂ (PGE₂)] 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/HIF α) 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 μ m 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–polyribonocinic: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 (NaIO₄) oxidation–borohydride reduction, carbodiimide-cross-linking or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate-mediated

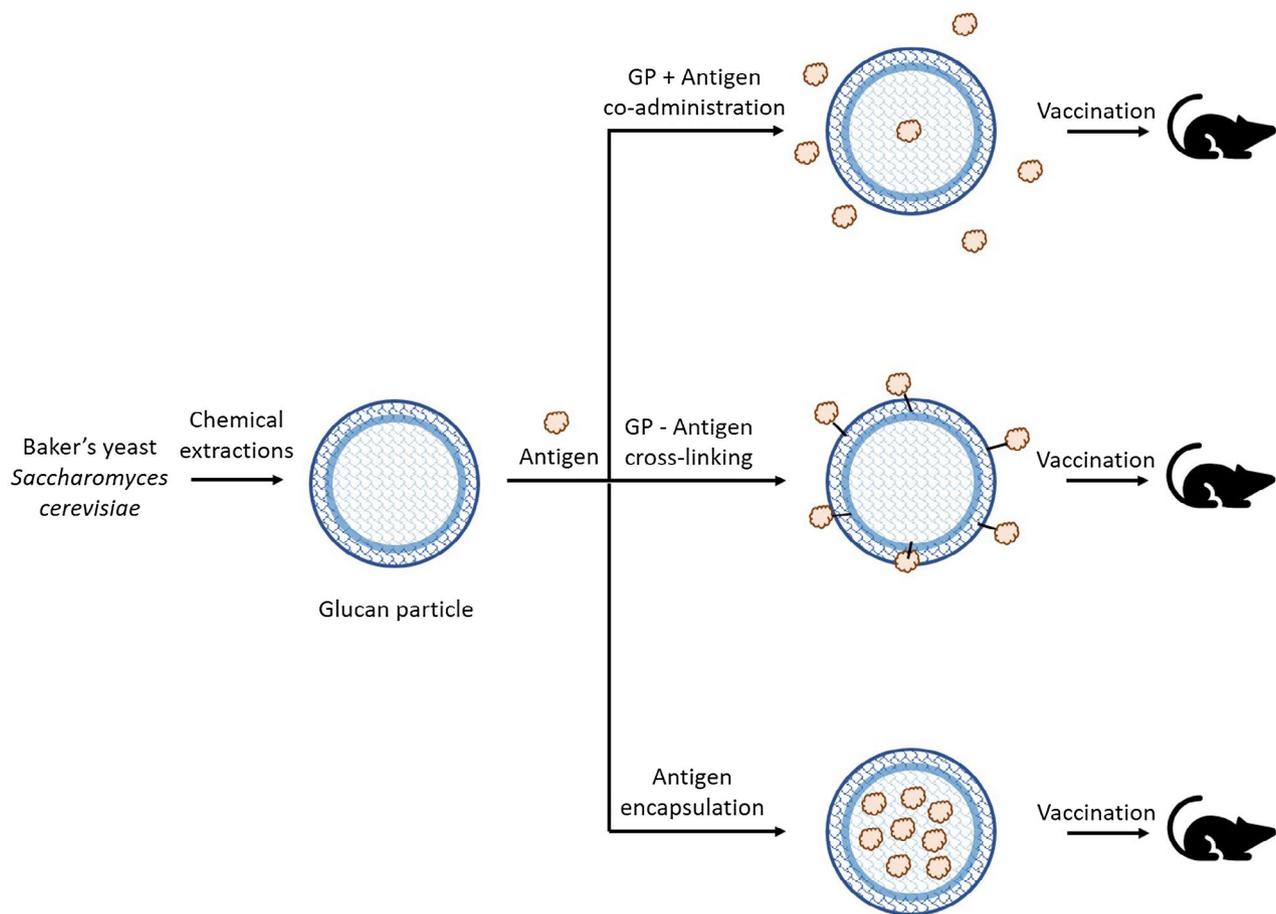


Fig. 1. Diagram showing different methods of vaccinations using β -1,3-D-glucans (BG) particles (GPs). Purified GPs can be employed as a vaccination platform by co-administration, cross-linking and encapsulation of antigens.

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-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
<i>Cryptococcus neoformans</i>	GP	Soluble alkaline extracts from <i>C. neoformans</i> 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]
<i>Histoplasma capsulatum</i>	GP	Soluble alkaline extracts from <i>H. capsulatum</i>	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]
<i>Francisella tularensis</i>	GP	Recombinant FTT0814, <i>Francisella</i> 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]
<i>Coccidioides posadasii</i>	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]
<i>Blastomyces dermatitidis</i>	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 IgIC 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 IgIC 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 1.6×10^3 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

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