Exploitation of bacterial N-linked glycosylation to develop a novel recombinant glycoconjugate vaccine against *Francisella tularensis*

Jon Cuccui¹, Rebecca M. Thomas², Madeleine G. Moule¹, Riccardo V. D’Elia², Thomas R. Laws², Dominic C. Mills¹, Diane Williamson², Timothy P. Atkins²-³, Joann L. Prior² and Brendan W. Wren¹

¹Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
²Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire SP4 0JQ, UK
³School of Biosciences, University of Exeter, Devon, UK

1. Summary

Glycoconjugate-based vaccines have proved to be effective at producing long-lasting protection against numerous pathogens. Here, we describe the application of bacterial protein glycan coupling technology (PGCT) to generate a novel recombinant glycoconjugate vaccine. We demonstrate the conjugation of the *Francisella tularensis* O-antigen to the *Pseudomonas aeruginosa* carrier protein exotoxin A using the *Campylobacter jejuni* PglB oligosaccharyltransferase. The resultant recombinant *F. tularensis* glycoconjugate vaccine is expressed in *Escherichia coli* where yields of 3 mg l⁻¹ of culture were routinely produced in a single-step purification process. Vaccination of BALB/c mice with the purified glycoconjugate boosted IgG levels and significantly increased the time to death upon subsequent challenge with *F. tularensis* subsp. *holarctica*. PGCT allows different polysaccharide and protein combinations to be produced recombinantly and could be easily applicable for the production of diverse glycoconjugate vaccines.

2. Introduction

Vaccines more than any other medical intervention measure have improved the lives of mankind. When selecting a vaccine, there are a number of choices. Live attenuated vaccines carry the advantage of exposing the host to an array of immunogenic epitopes, often leading to generation of humoral and cellular immunity; however, they also carry the risk of reversion to full virulence and they cannot be administered to immunosuppressed individuals. Killed vaccines are cheaper to store than live attenuated vaccines; however, only humoral immunity is induced resulting in the need to vaccinate individuals multiple times. A defining characteristic of a successful vaccine is the ability to evoke long-lasting protective immunity with minimal side effects. One strategy that has been particularly successful in
Figure 1. Principles of protein glycan coupling technology in E. coli. An E. coli cell is transformed with three plasmids to generate the cloned glycoconjugate protein (GP). The plasmids carry the oligosaccharyltransferase PglB, the biosynthetic polysaccharide locus and the carrier protein. The polysaccharide is synthesized on an undecaprenyl pyrophosphate lipid anchor (blue/black circle) within the cytoplasm; this is transferred to the periplasmic compartment where PglB recognizes the lipid linked reducing end sugar and transfers the polysaccharide en bloc onto an acceptor sequon (D/E-X-N-X-S/T) on the carrier protein to produce the GP. IM, inner membrane; OM, outer membrane.
afforded protection against subsequent *F. tularensis* LVS challenge in the murine tularemia infection model [25]. The prevalent immune response was humoral [26], although recently *F. tularensis* LPS has been found to cause proliferation of a rare subset of B-1a lymphocytes cells in the absence of T-cell help or TLR4 stimulation in mice, indicating that the immune response to this antigen cannot be classified either as innate or adaptive [27]. The polysaccharide moiety alone is insufficient for long-lasting immunological memory either as innate or adaptive [27]. The polysaccharide moiety that the immune response to this antigen cannot be classified in the absence of T-cell help or TLR4 stimulation in mice, indicating that the immune response to this antigen cannot be classified either as innate or adaptive [27].

In this study, we clone and express the *F. tularensis* O-antigen coding region in *E. coli* and transfer it to the acceptor protein ExoA from *P. aeruginosa* using PGCT. We show for the first time that the Francisella tularensis O-antigen can be transferred by PGCT, and have produced a novel candidate vaccine in the murine *F. tularensis* infection model. The purified recombinant glycoconjugate vaccine was easily purified in high yields and was capable of providing significant protection against subsequent challenge with a virulent wild-type strain of *F. tularensis* subsp. *holarctica*, demonstrating a considerable improvement over the LPS unconjugated vaccine. This demonstrates that PGCT can be used to generate efficacious glycoconjugate vaccines, and the technique could be adapted to a variety of other pathogens.

### 3. Material and methods

#### 3.1. Bacterial strains and plasmids

*Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C, with shaking. Antibiotics were used at the following concentrations: tetracycline 20 μg ml⁻¹, ampicillin 100 μg ml⁻¹, spectinomycin 80 μg ml⁻¹ and chloramphenicol 30 μg ml⁻¹. The host strain for initial cloning experiments was *E. coli* XL-1, subsequent strains used for glycoconjugate production were *E. coli* DH5α and CLM24 (see the electronic supplementary material, table S1). For efficacy studies, mice were challenged with 10^8 CFU of *E. coli* DH5α cells, fixed on glass cover slips by air-drying and washed in PBS.

#### 3.2. Cloning, sequencing and expression of the *Francisella tularensis* O-antigen coding region

DNA was prepared from the *F. tularensis* subsp. *holarctica* strain SchuS4 by phenol extraction as described by Karlsson et al. [31]. The O-antigen coding region was amplified using the primers FTfragment2rev (5'-GGATCATTAATAGCCAAATGTAGTGCT-3') and Oant1fwd (5'-TTTGTGAATTCTACAGGCTGTCAATGGAGAATG-3') using the following cycling conditions: 94°C, 15 s, 55°C, 15 s, 68°C, 20 min; 35 cycles using Accuprime Taq Hifi (Invitrogen, UK). This was cloned into the TA cloning vector pGEM-T Easy to generate the vector pGAB1. The plasmid pGAB1 was digested with EcoRI in order to subclone the insert into the vector pLAFL to generate the construct pGAB2.

#### 3.3. Immunofluorescence imaging of *Escherichia coli* cells carrying *Francisella tularensis* O-antigen-coding region

Approximately 1 × 10^8 CFU of *E. coli* DH5α cells were fixed on separate glass cover slips by air-drying and washed in PBS. Cover slips were then incubated in 5% v/v fetal calf serum (FCS) in PBS for 2 h. After three 2 min washes in PBS, cover slips were incubated with IgG2a mouse monoclonal antibody (mAb) FB11 (1 μl ml⁻¹) in 5% v/v FCS/PBS for 1 h at 37°C and, following three more washes in PBS, bacteria were air dried on a glass coverslip and probed with Alexa Fluor 488 goat anti-mouse IgG (whole molecule, Invitrogen Life Technologies Corp.). Cover slips were mounted on a glass slide using Vectashield mounting medium containing the DNA-specific counter stain 4', 6-diamidino-2-phenylindole (DAPI). The level of fluorescence for each isolate was observed under fluorescent microscopy using an Olympus Fluoview laser scanning microscope (Olympus Imaging and Audio Ltd).

#### 3.4. Immunoblot analysis

To verify the transfer and presence of the *F. tularensis* O-antigen, samples were analysed by western blotting. *Escherichia coli* cells were grown overnight in 10 ml LB broth and subsequently diluted to an OD_{600 nm} of 1.0. Cells were isolated by centrifugation at 12,100×g for 10 min, the supernatant was removed and cells were resuspended in 100 μl Laemmli buffer. Cells were lysed by boiling for 10 min after analysis by western blotting or silver staining. Mouse anti-*F. tularensis* O-antigen monoclonal antibody FB11 (AbCam, UK) was used at a dilution of 1:1,000, and rabbit anti-HIS monoclonal antibody was used to detect ExoA at a dilution of 1:10,000. Secondary antibodies used were goat anti-mouse IRDye800 and IRDye900 conjugates used at 1:5,000 dilutions. Signal detection was undertaken using the Odyssey LI-COR detection system (LI-COR Biosciences GmbH).

#### 3.5. Determination of glycosylation sequon occupancy within exotoxin A

To establish if the asparagine residues at amino acid positions 262 and 404 within ExoA were both capable of being glycosylated with the *F. tularensis* O-antigen, these residues were altered to glutamine (262DNNSN264 altered to 262DNQNS264 and 402DNQRT406 altered to 402DQQRT406) using an Invitrogen QuickChange XL kit according to manufacturer’s instructions (Invitrogen Life Technologies Corp.). The primers a826c_t828g 5'- gacctggcatcaaggataactaca-3' and a826c_t828g_an 5'- atgaccgtgggagtagaattctgatt-3' were used to mutate the 262DNNSN264 to yield pGVNX150260 DNQNS264 and the primers a1252c_t1254g 5'- tggccgcgtgcctcaagaatacaagaaaggggtgt-3' and a1252c_t1254g_a 5'- cattcctcttagttctgtgatcttggaaggggca-3' to mutate...
then loaded into 10 ml polypropylene columns (Thermo Scientific). His-tagged ExoA was purified by the addition of an elution buffer according to manufacturer’s instructions (QIA expressionist, QIAGEN) containing 250 mM imidazole with the addition of 20 per cent glycerol and 5 per cent glucose. Protein yields were estimated using a bichinchonic acid assay kit according to manufacturer’s instructions (Pierce Biotechnology BCA Protein Assay Kit, USA).

For large-scale protein purification, material was isolated using GE Healthcare HIS trap columns and an AKTA purifier with an imidazole gradient of 30 to 500 mM. The collected fraction containing ExoA glycosylated with *F. tularensis* O-antigen was further purified using a resource Q anionic exchange column (GE Healthcare) with a NaCl gradient from 0 to 500 mM in 20 mM Tris HCl pH 8.0. This generated a typical yield of 2–3 mg ml⁻¹ of glycoconjugate per 1 l of E. coli culture.

The same techniques were used for the generation of the ‘sham’ *C. jejuni* heptasaccharide ExoA glycoconjugate. The plasmid coding for this heptasaccharide was pACYCpgl carrying the entire Cpgl cluster from *C. jejuni* 81116 [5].

### 3.7. BALB/c mouse challenge studies

Female Balb/c mice were obtained from Charles River Laboratories (Kent, UK) at six to eight weeks of age. The pilot study was done in groups of 10 mice immunized with either 0.5 µg *F. tularensis* LPS, 0.5 µg *F. tularensis* glycoconjugate, 0.5 µg *F. tularensis* glycoconjugate + Sigma Adjuvant System (SAS), 0.5 µg ‘sham’ glycoconjugate + SAS, 0.5 µg ‘sham’ glycoconjugate or SAS only. In addition, one group of mice were left untreated as challenge efficacy controls. The amount of SAS used was a 1:1 ratio (for example, 50 µl bioconjugate in PBS + 50 µl SAS).

The SAS solution was made by re-suspending 1 vial containing 0.5 mg monophosphoryl lipid A (detoxified endotoxin) from *Salmonella minnesota* and 0.5 mg synthetic trehalose dicorynomycolate in 2 per cent oil (squalene)–Tween 80–water in 1 ml PBS.

Immunizations occurred on days 0, 14 and 28 via the IP route. Mice were challenged 35 days after the last immunization with 100 CPU of *F. tularensis* strain HN63 by the IP route, delivered in 0.1 ml. Subsequent experiments used the same vaccination schedules except for increasing the number of mice per group to 15 and increasing doses to 10 µg of material per animal per immunization. Four weeks following final vaccination, five mice from each group were tail bled to obtain sera for antibody analysis. On day 3 post-infection, the same five mice from each group were culled, and spleens were harvested and analysed for bacterial load and cytokine response. For organ load determination, spleen samples were homogenized in 2 ml of PBS through 40 µm cell sieves (BD Biosciences). Cell suspension of 100 µl aliquots were plated onto BCGA plates for the enumeration of bacteria. All work was performed under the regulations of the UK Home Office Scientific Procedures Act (1986).

### 3.8. Immunology

*Francisella tularensis* LPS-specific IgM and total IgG levels were determined in serum samples by ELISA as previously described [32]. Spleen supernatants were assessed using mouse inflammatory cytometric bead array kit for interleukin (IL)-10, IL-12p70, interferon-γ, IL-6, tumour necrosis factor-α,
4. Results

4.1. Expression of the Francisella tularensis SchuS4 O-antigen in Escherichia coli DH5α cells

The bacterial strains and vectors used in this study are summarized in the electronic supplementary material, table S1. The 20 kb F. tularensis SchuS4 O-antigen coding region was PCR amplified and cloned into pGEM-T Easy to generate the plasmid pGAB1. To confirm O-antigen expression and transport to the outer cell surface of E. coli, pGAB1 was transformed into DH5α cells and probed by immunofluorescence using mAb FB11, specific to the F. tularensis O-antigen. Figure 2c demonstrates the expression of the O-antigen on the surface of E. coli DH5α cells, which is absent in the vector-alone control (figure 2d). The presence of E. coli DH5α cells was confirmed by staining the respective samples with DAPI to visualize nucleic acid (figure 2a,b).

4.2. Oligosacharyltransferase can transfer Francisella tularensis O-antigen to the acceptor protein exotoxin A

In order to generate a strong IgG response and lasting immunity, a highly immunogenic protein is required as a carrier for the F. tularensis O-antigen. The selected carrier protein was an inactivated form of the ExoA variant (L552V, ΔE553) [15]. The protein was modified to carry two N-glycosylation sequons (N262 and N404), a C-terminal His tag and a signal peptide sequence from the E. coli protein DsbA fused to the N-terminal to localize ExoA to the periplasm of the E. coli host cell [15]. The F. tularensis O-antigen coding region was subcloned from pGAB1 into the unique EcoRI site of the low copy vector pLAFR1 [33] to yield the vector pGAB2. The plasmids pGAB2, pGVXN114 and pGVXN150 containing the O-antigen, CjPglB and ExoA, respectively, were transformed into E. coli strain CLM24 that has a deletion in the waaL gene rendering the E. coli host strain ligase negative and unable to transfer UndPP-linked glycan to lipid A, therefore generating a lipid-linked substrate specific for CjPglB. As negative glycosylation controls, CLM24 cells were transformed with pGVXN150 alone and also with the combination of pGAB2, pGVXN150 and pGVXN115, the latter coding for a version of CjPglB with point mutations within a domain involved in acceptor sequon recognition from 457WWDGY462 to 457WAAYGY462 [5]. Following overnight induction of CjPglB and exoA expression with 1 mM IPTG and 0.2% L-arabinose (w/v), respectively, cells were lysed and His-tagged ExoA purified using a nickel column. Four elution fractions from each sample were separated by SDS-PAGE and tested by immunoblotting using the mAb FB011 specific for LPS. A band matching the expected size of ExoA and an O-antigen-like pattern could only be purified when a functional CjPglB was present (figure 3, lanes 2 and 2b). In the absence of a functional CjPglB there was no cross-reaction with mAb FB11 (figure 3, lanes 1 and 3). Typical yields were 3 mg ml⁻¹ of protein after the one step purification procedure. The His-tagged ExoA F. tularensis O-antigen conjugate (now termed glycoconjugate) was purified using Ni-NTA agarose (QIAGEN) and digested with Proteinase K. The disappearance of the O-antigen ladder was confirmed after Proteinase K treatment but not in the untreated sample confirmed that the O-antigen was anchored to ExoA (see the electronic supplementary material, figure S1).

In order to test if F. tularensis O antigen was capable of glycosylating ExoA at both glycosylation sequons we generated site-directed mutants of the asparagine residues found at amino acid positions 262 and 404 and modified them to glutamines. A version of ExoA with both asparagines altered to glutamines...
was also generated as a negative control. The results from a 4 h induction of protein expression and small-scale protein purification demonstrated that both the 260DNNNS264 and 402DQNRT406 sequons can be occupied by the polysaccharide (see the electronic supplementary material, figure S2).

4.3. Vaccination with the glycoconjugate provides significant protection against Francisella tularensis subsp. holarctica infection in mice

In a pilot study we compared LPS alone against the glycoconjugate vaccine and monitored antibody levels and murine survival. In order to demonstrate the specificity of the glycoconjugate, we used controls that included mice with SAS adjuvant alone, unvaccinated naive mice and mice that were vaccinated with a ‘sham’ glycoconjugate control (C. jejuni heptasaccharide conjugated to ExoA). In this pilot study, the only vaccinated groups of mice that demonstrated increased survival compared with the appropriate controls were the 0.5 µg test glycoconjugate + SAS (p < 0.05) and 0.5 µg LPS (p < 0.001), determined by log rank test (see the electronic supplementary material, figure S3). These candidates were selected for further assessment at higher doses and a further group consisting of LPS + SAS was also added to determine if this combination matched the glycoconjugate + SAS group. Protection was compared between mice immunized with either 10 µg glycoconjugate + SAS, 10 µg LPS or 10 µg SAS. All three vaccines were protective when compared with the unvaccinated mice (p < 0.001), and the SAS adjuvant alone did not elicit any protection (p > 0.05; figure 4). This experiment also indicated that LPS + SAS did not elicit the same level of protection as the glycoconjugate + SAS combination (p < 0.05) and thereafter LPS + SAS was deemed unnecessary for testing. The study was repeated in order to provide further bacterial organ load and immunological response data.

4.4. Mice vaccinated with test glycoconjugate and challenged with Francisella tularensis subsp. holarctica have lower bacterial loads and pro-inflammatory cytokines 3 days post-challenge

Three days post-challenge, five mice per group were sacrificed and bacterial loads in the spleens and the inflammatory

Figure 3. F. tularensis O-antigen is conjugated to ExoA by CjPglB in E. coli CLM24 cells. Two-colour immunoblots performed on His-tag purified ExoA using mouse mAb FB11 (green) and rabbit anti-6× His-tag antibody (red). Lane 1, E. coli CLM24 carrying pGAB2, pGVXN150, pGVXN115 (non-functional CjPglB control); lane 2, pGAB2, pGVXN150, pGVXN114 (functional CjPglB); lane 3, pGVXN150 only; panel 2b, close up view of His-tagged purified ExoA attached to various chain lengths of F. tularensis O-antigen. M = marker IRDye 680/800 protein marker. pGVXN150 contains ExoA, pGAB2 contains F. tularensis O-antigen, pGVXN114 and pGVXN115 carry a functional and non-functional CjPglB respectively.

Figure 4. Vaccination with test glycoconjugate increases host survival compared with LPS and controls. Balb/C mice were vaccinated with three doses, two weeks apart, with glycoconjugate vaccine or relevant controls (n = 10 per group). Mice were challenged five weeks following final vaccination with 100 CFU of F. tularensis strain HN63 via the IP route. Mice were vaccinated with 10 µg of test glycoconjugate + SAS, LPS + SAS or 10 µg LPS and the data were analysed by stratified log rank test. Both LPS, LPS + SAS and test glycoconjugate provided improved protection when compared with the relevant unvaccinated controls (p < 0.001) and the SAS alone provided no survival benefit (p > 0.05). The test glycoconjugate provided significantly better protection than the LPS alone or LP + SAS vaccine (p < 0.001 and p = 0.025, respectively).

Figure 5. Mice vaccinated with test glycoconjugate show a reduced bacterial load in spleens compared with LPS and controls. Unvaccinated, SAS vaccinated, 10 µg LPS, or 10 µg test glycoconjugate + SAS vaccinated mice were challenged with 100 CFU of F. tularensis strain HN63 via the IP route. Spleens were removed 3 days post-infection from each group (n = 5) and assessed for bacterial CFUs. Logarithm data were analysed using a general linear model and Bonferroni’s post-tests. There was no difference in bacterial load between SAS vaccinated and unvaccinated mice (p > 0.05) but the 10 µg LPS or 10 µg test glycoconjugate vaccinations had significantly decreased bacterial load when compared with relevant controls (p < 0.001). Mice vaccinated with the test glycoconjugate + SAS had significantly reduced bacterial numbers in the spleen compared with LPS (p < 0.05).
responses were evaluated (figure 5). Figure 5 shows the bacterial loads from the spleens of mice vaccinated with 10 μg of each candidate. In both experiments, mice that were immunized with the glycoconjugate + SAS or LPS had significantly decreased bacterial loads in spleens ($p < 0.01$) when compared with the SAS and unvaccinated controls. In addition, when mice vaccinated with glycoconjugate + SAS were compared with those vaccinated with LPS alone, significantly less bacteria were enumerated in spleens ($p < 0.05$). Inflammatory cytokine profiles between the different vaccine groups were also analysed (see the electronic supplementary material, figure S4). Mice vaccinated with glycoconjugate + SAS and LPS alone had reduced levels of inflammatory cytokines when compared with the SAS and unvaccinated controls ($p < 0.05$), corresponding with the decreased bacterial loads. There was no significant difference between cytokine profiles for both experiments ($p > 0.05$).

4.5. Vaccination with the Francisella tularensis glycoconjugate induces a greater IgG immune response

The levels of LPS-specific IgG were assessed in mice 7 days prior to challenge for both experiments. Increased LPS-specific IgG was observed in the glycoconjugate + SAS vaccinated group when compared with animals vaccinated with LPS only ($p < 0.001$). Although experiment 2 had higher levels of antibody ($p < 0.01$), we observed no evidence for the pattern between vaccination groups differing between experiments ($p > 0.05$; figure 6). No significant differences were observed between LPS-specific IgM levels from the glycoconjugate and LPS vaccine groups (see the electronic supplementary material, figure S5).

5. Discussion

Traditional glycoconjugate vaccine production using chemical conjugation requires that the glycan from the pathogenic organism is isolated and detoxified by stripping components such as lipid A, and that sufficient material is present to be chemically linked to a protein. The procedures involve harsh chemical treatments, can be extremely time consuming, often produce low yields, and are relatively expensive. In addition, the material generated at each step needs to be verified for purity and variation between vaccine batches is common. This process is also particularly difficult and hazardous for Francisella tularensis subsp. tularensis and holarctica owing to risks associated with aerosol generation and the low infectious dose. In this study, we used recombinant technology for the continuous generation of Francisella tularensis O-antigen within a safe laboratory strain of E. coli (figure 1).

The Francisella tularensis subsp. tularensis and subsp. holarctica repeating O-antigen subunit consists of a tetrasaccharide with the structure 4-α-β-GalNAcAN-(1-4)-α-β-GalNAcAN-(1-3)-β-α-QuiNac-(1-2)-β-α-Qui4NFMf(1-). The GalNAcAN is 2-acetamido-2-deoxy-O-β-galacturonamide, Qui4NFMf is 4,6-dideoxy-4-formamido-6-o-glucose and the reducing end group QuiNac is 2-acetamido-2,6-dideoxy-O-β-glucose [30]. Exploiting the relaxed sugar substrate specificity of CjPglB and the ability to target protein glycosylation, we generated a glycoconjugate vaccine by glycosylating ExoA from P. aeruginosa with the F. tularensis O-antigen repeat unit. This glycoconjugate vaccine was purified to a typical yield of 3 mg ml$^{-1}$ of protein from a 21 starting culture of E. coli. Thus, we demonstrate that the relaxed substrate specificity seen to be required by CjPglB extends to the ability to transfer glycans containing QuiNac at their reducing ends.

PGCT has previously been used to produce a purified S. dysenteriae O-antigen/ExoA combination but the vaccine potential of this conjugate has not been reported [15]. More recently Iwashkiw et al. [14] used PGCT to link a Y. enterocolitica 09 polymer composed of N-formyl perosamine to a multidrug efflux pump component protein, AcrA, from C. jejuni. The construct was shown to be a useful diagnostic tool for Brucella abortus in bovine sera. The Francisella tularensis O-antigen/ExoA glycoconjugate produced in this study is the first report of a recombinantly generated glycoconjugate to be protective in an infectious disease model.

Vaccination with the test glycoconjugate was shown to provide protection against challenge with Francisella tularensis subsp. holarctica strain HN63, providing both an increased time to death compared with vaccination with LPS alone and significantly reduced bacterial loads (figures 4 and 5). The mice also had reduced inflammatory cytokine levels (see the electronic supplementary material, figure S4), correlating with the reduced bacterial load. This is indicative of an appropriate early host immune response to counteract the pathogen. Importantly, mice vaccinated with the glycoconjugate showed an altered immunological response compared with mice vaccinated with LPS alone. While the immune response against LPS is primarily a humoral immune response characterized by a dominance of IgM rather than IgG antibodies, the glycoconjugate + SAS vaccinated mice showed a large increase in the production of IgG (figure 6). Although increased IgG antibody level cannot be taken to mean direct evidence of a shift to a T-cell-dependent response, our results tentatively represent a proof of principle for the use of glycoconjugate vaccines against Francisella tularensis by demonstrating the expected shift in immune response from humoral to cell-mediated when the LPS O-antigen is conjugated to a protein and administered with an appropriate adjuvant.

The Sigma Adjuvant System was selected for use in this study because monophosphoryl lipid A (MPL)-based adjuvants have been demonstrated to be safe and efficacious immunostimulators in a number of vaccines including Cervarix (GlaxoSmithKline Ltd) [34]. The human papillomavirus vaccine Cervarix is licensed with an adjuvant combination of MPL and aluminium hydroxide. Furthermore, previous studies...
have shown that SAS is a suitable adjuvant for polysaccharide vaccine candidates for other intracellular pathogens [35].

Currently, the LVS-attenuated strain is the gold standard vaccine for *F. tularensis*. However, there are significant safety concerns before this vaccine can be licensed for use in humans. Sebastain et al. [36] attempted to incorporate the immunogenic components of the *F. tularensis* LVS vaccine whilst removing the adverse effects of the vaccine by combining an O-antigen–tetanus toxoid chemical conjugate with a highly attenuated *F. tularensis* LVS mutant that is unable to generate O-antigen. Unfortunately, while this vaccine improved the safety of the live vaccine, it conferred only partial protection in mice against intranasal challenge with wild-type strain SchuS4 [36]. Recently, Kim et al. [37] demonstrated that the *F. tularensis* LVS strain can be further attenuated by removing O-antigen polymerase function (Wzy) leading to a bacterium that is coated with a single repeat unit. This strain was capable of inducing high-level protection against type B *F. tularensis* in Balb/C mice following intranasal infection, but it did not match the LVS strain in terms of protection against type A *F. tularensis* challenge. However, the study demonstrates the importance of the O-antigen as an inducer of immunological response and the necessity to include this component in the vaccine, implying the necessity for a vaccine that induces both cellular and humoral immunity [37], such as a glycoconjugate.

The results from the present study represent a significant advance in the application of bacterial PGCT to produce successful glycoconjugate vaccines. The versatility and recombinant nature of this system will facilitate vaccine design. For example, additional glycosylation sites can be engineered into ExoA to improve the ratio of glycan to protein in the glycoconjugate. In these studies 10 μg of purified LPS were compared with 10 μg of total glycoconjugate measured by bicinchonic acid assay, an assay that detects only the concentration of protein and not of glycan. The mice vaccinated with glycoconjugate will have received substantially lower doses of O-antigen than the mice vaccinated with LPS. Increasing the amount of conjugated O-antigen given to mice could substantially improve efficacy. In addition our results demonstrate that both glycosylation sequons grafted within ExoA are capable of being occupied (see the electronic supplementary material, figure S2). The abundance of glycan signal seen near the size where unglycosylated ExoA normally migrates on an SDS PAGE gel shows that a significant amount of glycoprotein carries single repeat units. It is likely therefore that in this system, CjPglB competes with the *F. tularensis* O-antigen polymerase (Wzy). We are currently working on modulating the level of CjPglB to ensure that higher polymer length glycans are attached. An advantage of this technology over traditional chemical conjugation methods is that the protein to be glycosylated can easily be ‘swapped out’ and replaced with another protein of choice. This step requires only two protein modifications, consisting of a signal sequence to ensure it is delivered to the periplasm and the addition of a ‘glycosylation tag’ sequon at the N- or C- terminus. This means that several glycoconjugate combinations can be rapidly produced and purified and combined to provide multiple immunogenic epitopes within the vaccine. This is particularly significant as the intracellular pathogen *F. tularensis* has been notoriously difficult in deriving candidate antigens that protect against infection.

We are currently working towards modifying a number of immunogenic *F. tularensis* proteins to generate a dual-specific immune response against both the glycan and the protein. Future work will focus on using glycotags to optimize the efficacy of recombinant *F. tularensis* glycoconjugate vaccines. This study demonstrates the efficacy of a recombinant glycoconjugate vaccine produced by PGCT and provides a foundation for a new era in the generation of glycoconjugate vaccines.

6. Acknowledgements

We thank Dr Michael Wacker and Dr Michael Kowarik (Glyco-Vaxyn) for providing PGVXN114, pGVXN115, pGXVN150, pLAFR1 and invaluable suggestions. This work was supported by the Defence Science and Technology Laboratories and the Biotechnology and Biological Research Council, United Kingdom.

References


