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RESEARCH ARTICLE

A glucan-particle based tularemia subunit vaccine induces T-cell immunity and affords partial protection in an inhalation rat infection model

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Abstract

Tularemia is a zoonotic disease caused by the facultative intracellular gram-negative bacterium *Francisella tularensis*. *F. tularensis* has a very low infection dose by the aerosol route which can result in an acute, and potentially lethal, infection in humans. Consequently, it is classified as a Category A bioterrorism agent by the US Centers for Disease Control (CDC) and is a pathogen of concern for the International Biodefence community. There are currently no licenced tularemia vaccines. In this study we report on the continued assessment of a tularemia subunit vaccine utilising β-glucan particles (GPs) as a vaccine delivery platform for immunogenic *F. tularensis* antigens. Using a Fischer 344 rat infection model, we demonstrate that a GP based vaccine comprising the *F. tularensis* lipopolysaccharide antigen together with the protein antigen FTT0814 provided partial protection of F344 rats against an aerosol challenge with a high virulence strain of *F. tularensis*, SCHU S4. Inclusion of imiquimod as an adjuvant failed to enhance protective efficacy. Moreover, the level of protection afforded was dependant on the challenge dose. Immunological characterisation of this vaccine demonstrated that it induced strong antibody immunoglobulin responses to both polysaccharide and protein antigens. Furthermore, we demonstrate that the FTT0814 component of the GP vaccine primed $CD4^+$ and $CD8^+$ T-cells from immunised F344 rats to express interferon-γ, and $CD4^+$ cells to express interleukin-17, in an antigen specific manner. These data demonstrate the development potential of this tularemia subunit vaccine and builds on a body of work highlighting GPs as a promising vaccine platform for difficult to treat pathogens including those of concern to the bio-defence community.

Introduction

Tularemia is a disease caused by the facultative intracellular gram-negative bacterium *Francisella tularensis*. It is a zoonotic disease most commonly encountered in rodents and

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lagomorphs in nature. Human infections, although rare, can occur through accidental exposure to material from infected animals. The most acute disease presentation in humans is from respiratory exposure to aerosols. By this route, the human infectious dose of *F. tularensis* with high virulence strains has been determined to be < 50 colony forming units (CFU) [1] and in the absence of antibiotic treatment, case fatality rates can be up to 30% [2]. Due to its low infectious dose and the high morbidity and mortality following inhaled infection, *F. tularensis* has been used in historic biological weapon defence programmes, including the reported production of antibiotic resistant strains [2, 3]. *F. tularensis* is classified as a Category A bioterrorism agent by the US Centres for Disease Control (CDC) and remains a pathogen of concern for the International Biodefence community. Development of safe and effective medical countermeasures to treat tularemia remains an enduring requirement.

Currently there is no licensed vaccine for tularemia. A live attenuated vaccine strain (LVS) derived from the low virulence *F. tularensis* subspecies *holarctica* was developed in the 1950s and has demonstrated efficacy in people. In human exposure studies, protection against disease at aerosol challenges up of up to 20,000 CFU has been demonstrated [4]. Yet, whilst LVS has been used historically to vaccinate at-risk individuals such as laboratory workers [5], it remains unlicensed due to safety concerns such as reversion to virulence and mixed colony morphologies [6]. Despite the promise shown by more recent defined live-attenuated strains such as the *F. tularensis clpB* gene-deletion strain [7, 8], the increased human safety concerns associated with live attenuated vaccines are likely to remain a barrier to their licensure at this time.

The non-replicating nature of killed whole-cell and sub-unit vaccines would suggest that the safety-compliance acceptance requirements would be easier to achieve. However, the challenge to date has been in achieving the efficacy needed. Killed whole cell preparations have been found to be reactogenic and to have limited efficacy [9, 10]. The most promising subunit vaccine-antigen identified so far is the lipopolysaccharide (LPS) antigen of *F. tularensis*. In murine infection studies, LPS remains the only single subunit antigen to date that has provided protection of immunised animals in simple adjuvanted delivery formats, albeit primarily to the lower virulence *Francisella* strains [11–14]. However, protection afforded by LPS may be limiting due to it being a T-cell independent antigen and the known importance of cell-mediated immunity in controlling *F. tularensis* infections [15]. Indeed, the potential of protein co-formulation to enhance *F. tularensis* LPS protective efficacy was first demonstrated in a murine model of tularemia infection using bovine serum albumin conjugated LPS [11]. Furthermore, bio-synthetic conjugation of the O-antigen of *F. tularensis* to the carrier protein *Pseudomonas aeruginosa* ExoProtein could protect Fischer 344 (F344) rats against challenge with the high virulence *F. tularensis* strain SCHU S4 [16]. However, it was hypothesised that the lack of an *F. tularensis* conjugate protein antigen in this vaccine may have limited its efficacy potential.

In exploring strategies to combine *F. tularensis* protein antigens with LPS, we previously reported on the potential of β-glucan particles (GPs) as a vaccine delivery platform for immunogenic *F. tularensis* antigens [17]. GPs are purified *Saccharomyces cerevisiae* cell walls, treated so that they are primarily β1,3-D-glucans (BGs) which are free of mannans, proteins and nucleic acids. BGs act as pathogen associated molecular patterns (PAMPs) that are recognised by receptors on macrophages and dendritic cells such as Dectin-1 and complement receptor 3 (CR3) [18]. GPs are a versatile vaccine antigen delivery platform as they can combine high antigen loading capacity of multiple antigens within their hollow porous structure with immunostimulatory properties [19–22]. The utility of GPs as an efficacious vaccine platform has been demonstrated for a variety of fungal [23–25], bacterial [17] and recently viral [26] microbial pathogens. Furthermore, there is growing recognition of yeast BGs for their ability to stimulate trained immunity of the innate immune system. BG uptake by monocytes and

macrophages can elicit an epigenetic effect leading in faster and more robust innate immune responses to secondary challenges (reviewed in [27]).

In our previous work, we developed a GP vaccine that encapsulated LPS together with a down-selected *F. tularensis* protein antigen FTT0814. Using a Fischer rat 344 (F344) infection model, we demonstrated that this GP based sub-unit vaccine could protect rats against a lethal aerosol challenge with the *F. tularensis* strain SCHU S4 [17]. This was the first demonstration of a *F. tularensis* protein antigen augmenting protection in a lethal rodent *F. tularensis* SCHU S4 challenge model. We have now extended the efficacy and immunological characterisation of this GP-based vaccine. In this paper, we report on 1) the efficacy assessment of this vaccine at more stringent aerosol challenge doses, 2) our attempts to enhance efficacy through co-formulation with the toll-like receptor 7 (TLR7) agonist adjuvant imiquimod and 3) provide a more detailed characterisation of vaccine-mediated cellular immunity induced by this vaccine in the F344 rat model of tularemia.

Materials and methods

Strains and culture conditions

F. tularensis LVS vaccine ampoule (NDBR 101, Lot 4) was reconstituted in phosphate buffered saline (PBS, pH 7.2) and cultured overnight at 37˚C on supplemented blood cysteine glucose agar (BCGA). Single use aliquots were prepared and stored at ˉ80˚C prior to use. *F. tularensis* SCHU S4 [28] was cultured at 37˚C on BCGA, or in modified cysteine partial hydrolysate (MCPH) broth. For preparation of cultures for aerosol infection studies, *F. tularensis* SCHU S4 was first grown for 24 h on BCGA and then used to inoculate MCPH broth and incubated for a further 48 h at 37˚C, shaking at 180 rpm. To allow bacterial enumeration, cultures were serially diluted in PBS and inoculated onto BCGA. For enumeration of *F. tularensis* SCHU S4 from animal tissues, BCGA plates were supplemented with lincomycin, colistin sulphate, amphotericin B and trimethoprim (LCAT) selective supplement (Thermo Scientific).

Glucan particle (GP) preparation

GPs were prepared from *S. cerevisiae* (Fleischmann's baker's yeast) as previously described [22, 29]. Briefly, following centrifugation and washing in water, *S. cerevisiae* was subjected to two rounds of hot alkali extraction by heating for 1 h at 90˚C in 1 M NaOH. The particles were suspended in water at pH 4.5, heated at 75˚C for 1 h, and then successively washed with water (x 3), isopropanol (x 4), and acetone (x2) and finally dried. To enumerate GPs, a 10 μg/ml suspension of particles in 0.9% saline was sonicated, counted using a hemocytometer, and stored in aliquots at ˉ20˚C until use. One microgram of GPs contains approximately $5x10^5$ particles.

GP vaccine formulations

The recombinant FTT0814 protein used in GP formulations was expressed and purified from *Escherichia coli* as described previously [17]. In summary, the hypothetical protein identified as FTT_0814c in the *F. tularensis* SCHU S4 Genome, NCBI GenBank Accession number CAG45447.1 (LVS homolog xxxxx). The gene was cloned into the plasmid vector pGEX-4T-3 (GE Healthcare) and expressed as a glutathione-A-transferase (GST) fusion in *Escherichia coli BL21 DE3* pLysS (Invitrogen). Expression was induced by IPTG and expressed protein purified GSTrap HP chromatography column (GE Healthcare). The purity of the purified GST-cleaved recombinant protein was confirmed by SDS-PAGE analysis [17]. For consistency with our previous publication, the recombinant protein has been referred to as FTT0814. The LPS used in GP formulations was isolated from heat inactivated *F. tularensis* SCHU S4 and was a kind

gift from Wayne Conlan, National Research Council-Canada. Vaccines were prepared in 50-dose amounts following methods previously reported [21]. Glucan Lipid Particles (GLPs) were used in place of GPs to allow the loading of LPS. GLPs were prepared by the same method used to prepare GPs [21] except that alcohol and acetone extraction steps were replaced by lyophilization to prepare the dry GLP powder. GLPs retain the non-saponifiable lipid fraction of the yeast cell facilitating hydrophobic compound encapsulation. Briefly, 50 μL of a 10 mg/mL solution of the FTT0814 antigen, or Ovalbumin (Worthington), and then mouse serum albumin were loaded sequentially into 10 mg dry GLPs for 30 minutes, frozen and lyophilized. Then the particles were swollen in 2–50 μL additions of a 25 mg/mL solution of yeast RNA (Sigma) and then resuspended by sonication in 4.9 ml of 5 mg/mL yeast RNA to trap the protein antigens in the GLP shells. After washing the formulation in 10 mL 0.9% saline the supernatants were collected and frozen for antigen analysis. The vaccine pellet was collected, frozen and lyophilized. Next to the vaccines containing LPS, 50 μL of a 10 mg/mL emulsion of LPS was added to the GLPs to swell and absorb the LPS, followed by freezing and lyophilization. Next, 5 μL of a 100 mg/mL solution of MSA was added and then 50 μL chitosan (10 mg/mL in 0.1M acetic acid) was added to trap the encapsulated LPS, and the formulations frozen and lyophilized. Next Imiquimod VacciGrade (Invivogen) (50 μL of a 25 mg/mL solution in water) was added to the indicated vaccines and the GLPs were allowed to swell and absorb the Imiquimod, and then frozen and lyophilized. Finally, another layer of MSA complexed with yRNA was added as described above. The formulated vaccines were then diluted to a final volume of 5 mL with saline, aliquoted and flash frozen. Each vaccine dose contained as indicated: 200 μg GLP, 10 ug Ovalbumin or FTT0814, 50 μg mouse serum albumin, +/- 10 μg LPS and +/- 10 μg Imiquimod. Antigen identity and loading were determined by SDS-PAGE electrophoresis and densitometry. Immunoactivation by LPS and Imiquimod were measured by incubating murine BMDCs with vaccines (5 particles/cell) for 3 hours and secreted TNFα measured by ELISA [29].

Animal procedures

Ethics statement. All animal procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Codes of Practice for the Housing and Care of Animals used in Scientific Procedures 1989. The licence application underwent approval by the local ethical review process with the Dstl Animal Welfare and Ethical Review Body (AWERB) before submission and approval with the UK Home Office and Animal Procedures Committee (an independent committee that offers advice to The UK Secretary of State of the ethics of the proposed work). All animal experiments were also performed under the authority of an application approved by the US Animal Care and Use Review Office (ACURO).

Animals. Inbred female Fischer 344 rats, 6–8 weeks of age and 90 g (+/- 20 g) upon arrival, were sourced from Envigo, UK. No animals were excluded from the study. Groups of 3–5 rats were randomly assigned to cages, and subsequent treatment groups, upon arrival. Rats were acclimatised to each housing facility for a minimum of 10 days before any procedures were performed. The rats were housed under a 12 h light/dark cycle (350 to 400 Lux during the day, 10 Lux during the night with a ramp up and ramp down period at 'dawn' and 'dusk') at 19 to 23˚C and 45 to 65% relative humidity. Free access to food and water was provided. Cages contained 8/10 and 10/14 grade corn cob (International Product Supplies, UK) as a nesting material with additional environmental enrichment. During immunisation and the subsequent rest period, rats were housed in a conventional animal unit in rooms supplied with rough filtered air giving 20 to 25 air changes/h. For challenge with *F. tularensis* SCHU S4, all animals were handled under UK Advisory Committee on Dangerous Pathogens animal

containment level 3 conditions within a half-suit isolator compliant with British Standard BS5726, supplied with an inward flow of HEPA-filtered air giving 35 to 45 air changes/h.

Immunisations. Rats were vaccinated with either LVS or GP vaccine formulations as follows. A single injection of 1 x 10^7 CFU of LVS was administered in a 100 μL volume via the sub-cutaneous (sc) route. For the GP-formulated vaccines, F344 rats received 3 immunisation 2 weeks apart via the sc route. Each GP immunisation contained 10 μg of each component antigen and 200 μg of GP particles in a 100 μL volume. To allow measurement of pre-challenge vaccine immunity, a tail-bleed was performed 2 weeks after the final GP vaccination or 4 weeks after LVS vaccination. Six weeks following the final vaccination, rats were either killed by anaesthetic overdose with sodium pentobarbitone delivered by the intraperitoneal route or they were challenged with *F. tularensis* SCHU S4 by the aerosol route.

Aerosol challenge. Aerosols were generated using a 3-jet Collison nebuliser (CH Technologies) containing a volume of 20 mL of *F. tularensis* SCHU S4 in PBS controlled and conditioned to 70% (± 5%) relative humidity by an AeroMP platform system (Biaera Technologies, Hagerstown, MD, USA) [30]. Animals were exposed to the aerosol for a total of 10 min, with sampling achieved for 1 min at mid-point of the challenge (4.5–5.5 min) using an all-glass impinger (AGI-30; Ace Glass, Vineland, NJ, USA) containing 10 mL PBS. The flow rates through the Collison, impingee and exposure chamber were 7 l/min, 6 L/min and 30 L/min respectively. The system was operated at the ambient temperature of the animal isolator (21˚C $\pm$ 2˚C) and the system pressure was maintained in the range of -150 to -250 Pascals for the duration of each exposure run.

$$concentration\ per\ L\ of\ air = \frac{(conncentration\ per\ ml)x(sampler\ volume)}{(sampler\ flow\ rate)\ x\ (sampling\ duration)}$$

A maximum of 6 rats were exposed in any single aerosol exposure. Rats weighed 190g (+/-30g) at the time of challenge. Animals were physically restrained in holding tubes enabling nose-only exposure to the aerosol. The presented challenge dose was calculated following enumeration of bacteria in serially diluted impinged aerosol samples and using Guyton's formula for the respiratory volumes of laboratory animals (100mL min-1) [31].

$$Presented\ dose\ =\ concentration\ per\ L\ of\ air\ x\ minute\ respiratory\ volume\ x\ challenge\ duration$$

Following aerosol challenge, clinical signs were recorded a minimum of twice daily and weights were recorded daily. Rats were monitored for up to 19 days post-infection and culled if they reached pre-determined humane end-point criteria. Clinical signs recorded included piloerection, hunched posture, reduced mobility, abnormal breathing and closure of eyelids. Animals that were moribund and deemed incapable of recovery were culled according to pre-determined humane end-point criteria. Cull criteria were lack of mobility, paralysis of any limb, problems with both eyes and/or $>$ 15% weight loss.

Immunological assays

Cell isolation and culture. Single-cell suspensions of splenocytes were isolated by maceration of each rat spleen through a 40 μm sieve into RPMI1640 medium (Life Technologies) supplemented with 10% Foetal Bovine Serum (Sigma), non-essential amino acids (Life Technologies, UK), 50μM 2-mercaptoethanol (Life Technologies, UK), 100 U/mL penicillin and 100 mg/mL streptomycin sulphate (Life Technologies, UK). Red blood cells were lysed using ACK lysis buffer (Sima-Aldrich) in accordance with manufactures recommendations. Briefly, splenocytes were re-suspended in ACK lysis buffer and then incubated for 5 min at ambient room temperature. Cells were then washed twice in PBS, with centrifugation for 5 min at 400 g

between washes, prior to resuspension in RPMI1640 complete medium. Peripheral blood mononuclear cells (PBMCs) were isolated from blood collected by either tail vein (250–1000 μL) or cardiac puncture (2–3 mL) collection procedures. Blood was diluted into an equal volume of PBS and then overlayed onto Ficoll-Paque 1.084 gradient density centrifugation media (GE Healthcare, UK) using a 1:1 volume overlay ratio. Samples were centrifuged at 800 g for 40 min and recovered PBMCs then washed two further times with PBS. Following isolation, splenocytes or PBMCs were enumerated using a haemocytometer and then incubated at 37˚C/5% CO_2 in the presence of either RPMI1640 medium alone, FTT0814 (5 μg/ml, Dstl), LVS lysate (10 μg/ml, Dstl), Endograde OVA (5 μg/ml, BioVendor) or Concanavalin-A (ConA, 5 μg/ml, Sigma-Aldrich). Incubation times and cell concentrations for the different assays are described below.

Measurement of IFNγ by Enzyme Linked Immunosorbent Assay (ELISA). PBMCs ($2x10^5$ / well) were cultured in duplicate wells in 96-well microtitre plates in the presence of antigen for 72 hours and supernatants harvested and stored at -20˚C prior to use. The expression of IFNγ was quantified in supernatants using a commercial rat IFNγ ELISA kit (Mabtech) with responses determined by measurement of optical density at 450 nm (OD_{450nm}). Rat IFNγ quantification standards were included on each assay plate to allow transformation of OD_{450nm} measurements to normalised concentrations (ng/mL).

Measurement of IFNγ by Enzyme Linked Immunospot (ELISPOT) assay. PBMCs ($2x10^5$/well) or splenocytes ($4x10^5$/well) were cultured in duplicate wells of a commercial 96-well ELISPOT plate pre-coated with rat IFN capture antibody (Mabtech). Rat IFNγ ELISPOT responses were measured after 20 h of antigen-stimulation using the companion kit reagents (Mabtech). The assay was performed in accordance with kit instructions and spot enumeration was performed using an AID automated reader. IFNγ ELISPOT responses were only reported for those animals/samples where functional viability of cells was confirmed by demonstration of strong mitogen (ConA) stimulated responses.

Flow cytometry. Splenocytes ($2x10^6$ cells per assay well) were cultured in 48-well microtitre plates in the presence of antigen for 20 hours. Brefeldin-A (10 ug/mL, Sigma-Aldrich, UK) added to culture medium for the final 4 hours of the culture. Cells were harvested by centrifugation (300 g/5 min) and stained using the following anti-rat surface marker antibodies; CD3-Brilliant Violet 421 (clone IF4, BD Biosciences), CD4-FITC (clone OX35, eBioscience) and CD8-PerCP eFluor710 (clone OX8, eBioscience). Cells were stained for 15 minutes at 4˚C in the presence of a fixable yellow (405nm) cell viability dye (Life Technologies) and then fixed for 16 hours at 4˚C in Cytofix fixation reagent (BD Biosciences). Fixed cells were permeabilised in BD Biosciences Permeabilistation Buffer and then stained intracellularly for 30 minutes at 4˚C using anti-rat antibodies IFNγ-PE (clone DB-1, BD Biosciences) and IL17A-APC (clone eBio17B7, eBioscience). Stained samples were analysed using a FACSCanto II analyser equipped with 405, 488 and 633 nm lasers (BD Biosciences). An example gating strategy for measurement of intracellular expression of IFNγ and IL-17 is given in supplementary information (S3 Fig). All antibodies were titrated prior to use to ensure optimal staining. To ensure quality of data reporting, animals were excluded from the analysis if cell viability of stained splenocytes stimulated with only RPMI1640 media was less than 40%. Median number of live-singlet lymphocyte cell events on which analyses were performed was 226,000 (± 34,009 SD). Graphical data presentation and analysis was performed using FlowJo v10 software (TreeStar, USA).

IgG antibody assays. Plasma was isolated from blood samples by centrifugation at 2000g for 10 minutes and stored at -20˚C prior to use. Maxisorb (Nunc) 96-well microtitre plates were coated overnight with 5 μg/ml of test antigen. Additionally, each assay plate included wells coated with goat anti-rat IgG (R5130, Sigma-Aldrich) to allow preparation of a standard

curve for IgG response quantification. Serial dilutions rat plasma, or respective rat IgG standard (NI04, Sigma-Aldrich), were added to the respective wells of coated plates and incubated for 2 h at ambient room temperature (20–22˚C). Bound antibody was detected using a sequential combination of horse radish peroxidase conjugated goat anti-rat IgG (A9037, Sigma) followed by 3,3′,5,5′-Tetramethylbenzidine (Sigma-Aldrich) development substrate. Responses were detected by measurement at OD_{450nm} on a Multiscan Ascent plate reader and antibody concentrations calculated using Ascent software.

Statistical analysis

All statistical analysis of animal study data was performed in Graphpad Prism software. All data sets were tested for normality and appropriate statistical tests applied. Statistical tests, where performed, are stated in the appropriate results section and/or in the supporting figure legend.

Results

GP vaccine provides partial protection against a high stringency *F. tularensis* aerosol challenge

We have previously demonstrated that a GP based vaccine containing a combination of the *F. tularensis* antigens FTT0814 and LPS could protect F344 rats in a lethal model of inhalational tularemia [17]. The aerosol challenge dose reported in this previous study was an average of 1.6×10^3 CFU per F344 rat which equated to a presented dose of 4.0×10^3 CFU per rat. To progress the development and evaluation of this promising vaccine, we considered it important to assess its efficacy at a more stringent challenge dose. In addition, we wished to determine if a TLR agonist adjuvant could enhance efficacy. The TLR7/8 agonist imiquimod was selected as a candidate adjuvant for use in these studies. Groups of F344 rats (n = 5) were vaccinated with imiquimod formulated GP-vaccines containing FTT0814 alone or FTT0814 with *F. tularensis* LPS. Control treatment groups included rats sham-immunised with PBS (n = 5) or with imiquimod-formulated empty GP vector (n = 4). There were only 4 rats in the imiquimod empty GP group as one rat had to be culled for welfare reasons which were unrelated to the vaccine. Rats received 3 vaccine immunisations, 2 weeks apart. An additional group of rats (n = 5) was immunised with a single inoculation of the live attenuated tularemia strain LVS. All rats were challenged with an aerosol of *F. tularensis* SCHU S4 6-weeks post vaccination. The average presented aerosol challenge dose over 6 inhalation exposure runs was confirmed to be 2.8×10^4 CFU (range 2.1×10^4–3.2×10^4 CFU). All PBS sham-immunized and empty GP/imiquimod vaccinated rats reached the pre-defined humane lethal endpoint between days 5–7 post infection. There was no significant difference between the Kaplan Meier survival curves for these groups (Fig 1A). LVS vaccination provided complete protection against the lethality of the infection. The GP vaccine combination of FTT0814, LPS and imiquimod provided a significant delay in the time-to-death in comparison with the PBS control group ($p = 0.002$. Log-rank test) but there was no protection against the lethality of the challenge. The GP vaccine combination of FTT0814 and imiquimod alone did not provide any significant protection in comparison with the control groups. All rats which reached the humane lethal endpoint were colonised with high bacterial burdens of *F. tularensis* in lung and spleen tissues (Fig 1B). In these groups, only those rats vaccinated with GPs containing the combination of FTT0814, LPS and imiquimod had significantly lower bacterial burdens compared with the PBS control group. The LVS vaccinated rats were culled 14 days post-infection. Whilst no clinical signs were observed in any of these rats at this time point, low level *F. tularensis* colonisation was still present in lung and/or spleen tissues in all LVS vaccinated rats (Fig 1B).

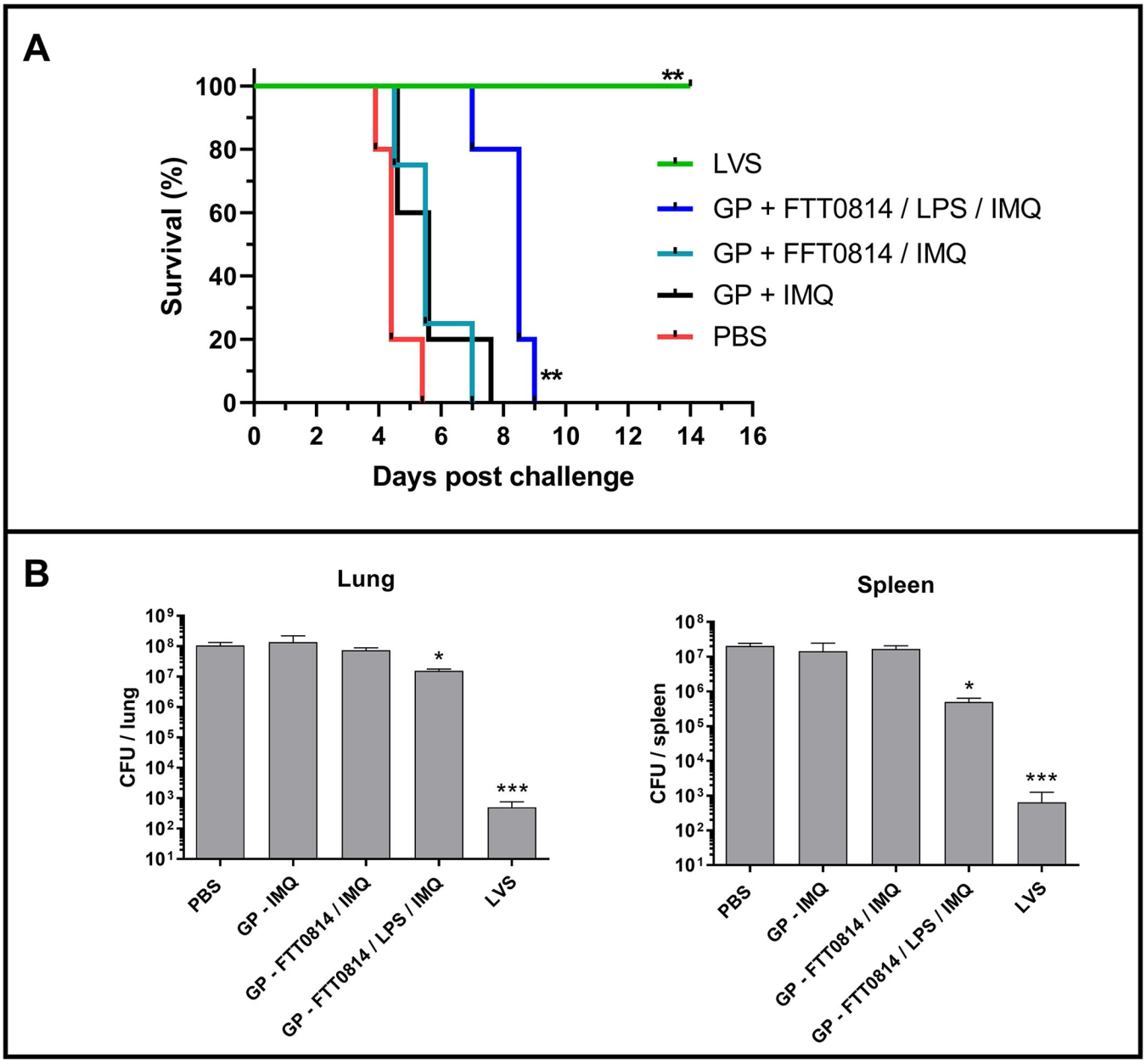

Fig 1. GP-FTT0814/LPS vaccine provides delayed time-to-death in a high-challenge F344 rat model of inhaled tularemia. F344 rats were vaccinated with GP vaccine combinations that included FTT0814, LPS and imiquimod (labelled IMQ on graphs) adjuvant. Groups sizes = 5 rats except the GP/IMQ vector/adjuvant control group where n = 4. Groups of rats vaccinated with the live attenuated vaccine *F. tularensis* LVS and sham-inoculated with PBS were included as controls. Rats were challenged with an aerosol of *F. tularensis* SCHU S4 (2.8×10^4 CFU) 6-weeks post vaccination. **Panel A**: F344 rats were monitored for 14 days post-infection and culled if they reach predefined humane-endpoint criteria. Data is presented on the Kaplan-Meier survival curve and any significant difference between the PBS and treatment-group survival curves is indicated (Log rank test with Bonferroni correction, significance threshold $p < 0.0125$, ** $p = 0.002$). **Panel B**: Enumeration of *F. tularensis* in lungs and spleens of F344 rats was determined either on the day that rats reached their humane endpoint, or for the LVS vaccinated rats, at 14 days post infection. For each treatment group, the bars represent the mean CFU in respective tissues (± SEM). Significant differences in bacterial colonisation between PBS and vaccine groups were assessed using non-parametric Kruskal-Wallis test with Dunn's multiple comparison test (* $p<0.05$, *** $P<0.001$).

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Imiquimod fails to enhance protective efficacy of GP-based tularemia vaccine

We next wished to determine any efficacy contribution of imiquimod to the GP formulated FTT0814/LPS vaccine in light of the results from the challenge above. F344 rats (n = 6) were vaccinated with GP vaccines formulated with these *F. tularensis* antigens with and without imiquimod. Control treatment groups (n = 6) included rats sham-immunised with PBS, or with empty GP particles formulated with, or without, imiquimod. Rats received 3 vaccine immunisation, 2 weeks apart. A tail-bleed was performed 2 weeks after the third vaccination to allow measurement of vaccine induced immunological responses in these rats prior to *F. tularensis* challenge. Measurement of immune responses in these blood samples demonstrated that vaccination of rats with the GP vaccines containing FTT0814 and LPS resulted in generation of antigen-specific FTT0814 and LPS IgG antibodies (Fig 2A and 2B). Expression of interferon-γ (IFNγ) from FTT0814 stimulated peripheral blood mononuclear cells (PBMCs, Fig 2C) also demonstrated antigen-specific induction of T-cell immunity. These rats were then challenged with an aerosol of *F. tularensis* SCHU S4 6-weeks after the final vaccination. In comparison with the previous efficacy study, we chose to use a lower challenge dose to increase our ability to determine differences between treatment groups, particularly with respect to protection against disease lethality. An LVS vaccine group was not included in this study since full protection against lethality had already been demonstrated at a higher challenge dose. The average presented *F. tularensis* infection dose over 5 inhalation exposure runs was 7.8 x 10^3 CFU (range 5.8 x 10^3–1.0 x 10^4 CFU). Comparison of Kaplan-Meier survival curves demonstrated that only those groups of rats vaccinated with GP-FTT0814/LPS, formulated either with or without imiquimod, were significantly protected (Fig 3A, Log-rank test). However, protection against lethality was only partial for these treatment groups and the GP vaccine formulated without imiquimod provided improved survival advantage. One rat vaccinated with the empty GP-Ova vector failed to demonstrate any weight loss following exposure indicating no infection was established (S1 Fig). This indicates a failed challenge in this animal. All other animals

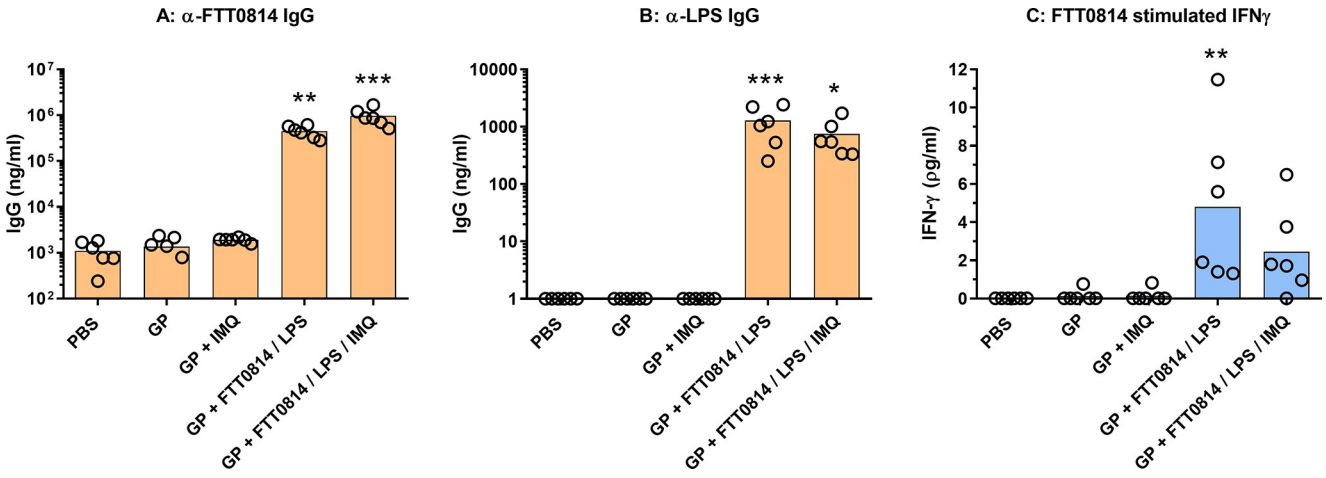

Fig 2. GP-FTT0814/LPS vaccine induces antigen specific IgG and cell-mediated immunity. Blood was collected from F344 rats (n = 5) 2 weeks after a third vaccination with GP vaccines containing combinations of FTT0814, LPS and imiquimod (IMQ) adjuvant. Control groups included rats sham inoculated with PBS and with the empty GPs. Plasma IgG responses to FTT0814 (graph A) and to LPS (graph B) are reported for individual rats (closed circles) with the mean response for each treatment group shown by the respective bars. IFNγ from FTT0814 stimulated PBMCs (graph C) are reported for individual rats (open circles) with the mean response for each treatment group shown by the respective bars. Each respective vaccine treatment group is indicated on the x-axis. Significant differences in responses between PBS and vaccine groups was assessed using one-way ANOVA with Dunnett's multiple comparison post-analysis test (* $p<0.05$, ** $p<0.01$, *** $P<0.001$).

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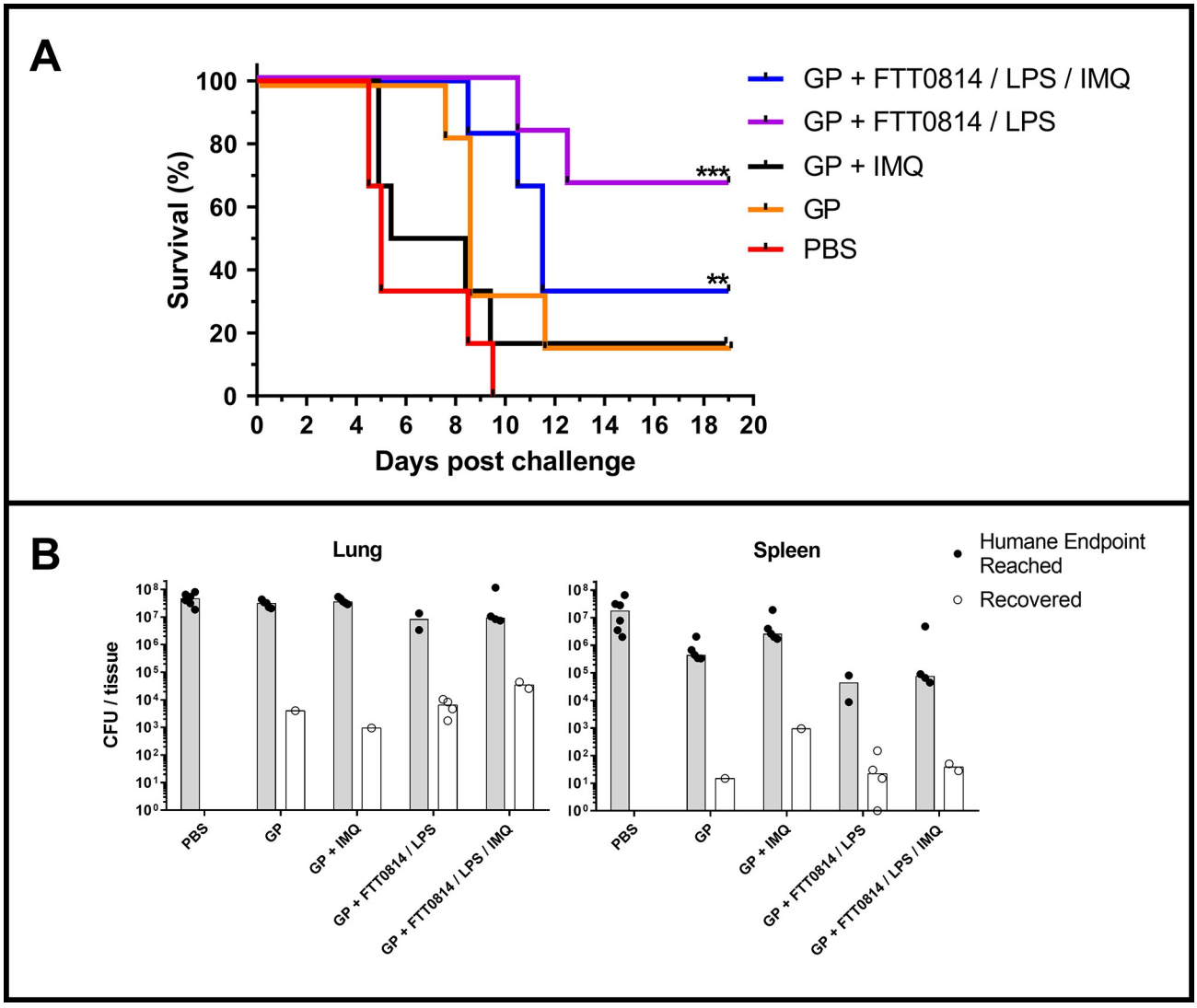

Fig 3. Imiquimod fails to enhance protective efficacy of the tularemia GP-FTT0814/LPS vaccine. F344 rats were vaccinated with GP vaccine combinations that included FTT0814, LPS and imiquimod (IMQ) adjuvant. Groups of rats vaccinated with the live attenuated vaccine *F. tularensis* LVS, sham-inoculated with PBS and empty GPs were included as controls. Rats (n = 6) were challenged with an aerosol of *F. tularensis* SCHU S4 (7.8×10^3 CFU) 6-weeks post vaccination. **Panel A**: F344 rats were monitored for 14 days post-infection and culled if they reach predefined humane-endpoint criteria. Data is presented on the Kaplan-Meier survival curve and any significant difference between the PBS and treatment-group survival curves is indicated (Log rank test with Bonferroni correction, significance threshold $p < 0.01$, ** $p = 0.0023$, ***$p = 0.0005$). **Panel B**: Enumeration of *F. tularensis* in lungs and spleens of F344 rats was determined either on the day that rats reached their humane endpoint (grey bars, closed circles) or at 19 days post infection for rats that recovered and survived to the study end (white bars, open circles). The respective vaccine treatment groups are shown on the x-axis. The circles represent the CFU for each sample and the bar is the median for the group. Comparison of lung and spleen bacterial burdens using 2-way ANOVA analysis did not demonstrate any significant differences between bacterial burdens in the any of the vaccinated groups in comparison with the PBS control group (bacterial burdens in rats that reached the humane endpoint, and those that recovered, were analysed separately).

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demonstrated weight loss commencing day 2–3 post infection. The rats that did not meet the humane lethal endpoint cull criteria began to regain weight around day 12 post-infection (S1 Fig). All rats that reached the humane lethal endpoint were colonised with high bacterial burdens of *F. tularensis* in lung and spleen tissues (Fig 3B). All remaining rats that demonstrated disease recovery, and which were culled at the study endpoint 19 days post-infection, were still colonised with *F. tularensis* in lung and spleens at this time point, although at lower bacterial concentrations than observed in animals at their humane lethal endpoint (Fig 3B).

Comparison of lung and spleen bacterial burdens (2-way ANOVA) did not demonstrate significant differences between bacterial burdens in the any of the vaccinated groups in comparison with the PBS control group.

FTT0814/LPS GP-vaccine induces antigen specific IgG, Th1 and Th17 memory immunity

To further characterise the immune responses to the FTT0814/LPS GP vaccines, both with and without imiquimod, groups of rats (n = 6) were immunised with GP vaccine preparations using the same protocol as that used for efficacy evaluation studies. Reference control groups included LVS immunised rats (n = 6), rats immunised with empty GPs with and without imiquimod (n = 4) and sham-PBS immunised rats (n = 4). IgG responses were measured at weeks 2 and 6 after the third vaccination using tail bleed and terminal cardiac puncture samples respectively. IgG antibodies that recognised FTT0814 and LPS were only detected in those rats vaccinated with the GP vaccines containing these antigens (Fig 4A). Formulation of the FTT0814 and LPS containing GP vaccine with imiquimod did not significantly alter the magnitude of respective IgG responses at either 2 or 6 weeks post vaccination (one-way ANOVA with Holm-Sidak's multiple comparison test).

Splenocytes were isolated 6 weeks after the final vaccination to allow characterisation of T-cell mediated immunity using antigen re-stimulation assays. The isolated splenocytes were first screened for functional viability by measurement of IFNγ following exposure to a mitogen (ConA) and non-responsive samples were excluded from the analysis. Thus, responses are only reported for 3/4 rats in the PBS, GP-Ova and GP-Ova/IMQ control groups and in 5/6 rats in the GP based FTT0814/LPS vaccine and LVS groups. Few or no FTT0814-stimulated IFNγ Enzyme Linked Immuno-Spots (ELISPOTs) were detected in the PBS or GP-vector control rats confirming the antigen-specific nature of the responses generated by FTT0814 formulated GP vaccines (Fig 4B). Splenocytes from the LVS vaccinated rats generated the strongest IFNγ ELISPOT recall response to LVS-lysate re-stimulation. The LVS vaccinated rats also demonstrated a recall response to FTT0814. Antigen-stimulated IFNγ responses from PBMCs isolated from blood samples 6 weeks after the final vaccination were also measured by ELISA. The PBMC IFNγ responses (S2 Fig) were broadly comparable with the reported splenocyte IFNγ ELISPOT responses (Fig 4B).

A multifunctional intracellular cytokine staining (ICS) protocol was used to phenotype the cells responsible for vaccine induced expression of IFNγ. Furthermore, the staining panel developed allowed quantification of co-expression of interleukin-17 (IL17). Antigen stimulated IFNγ expression from both $CD4^+$ and $CD8^+$ T-cells was observed for FTT0814 and also for the Ova component of the GP vaccines (Fig 5). IL17 expression was predominantly detected from the $CD4^+$ T-cell populations. Cells expressing IFNγ and IL17 were largely mutually exclusive. Formulation of the FTT0814 and LPS containing GP vaccine with imiquimod did not enhance the magnitude or alter the proportions for IFNγ and IL17 expression from $CD4^+$ and $CD8^+$ T-cells.

Discussion

There is currently no licensed vaccine available to prevent tularemia. To address this need, we report on our continued developmental assessment of a novel GP-based subunit tularemia vaccine. We have used a F344 rat model of tularemia to assess vaccine efficacy as it considered to be the most relevant rodent model for extrapolating vaccine efficacy data to humans [32, 33]. We previously reported that a GP vaccine that delivered the *F. tularensis* protein antigen FTT0814 together with *F. tularensis* LPS provided complete protection of F344 rats in a lethal

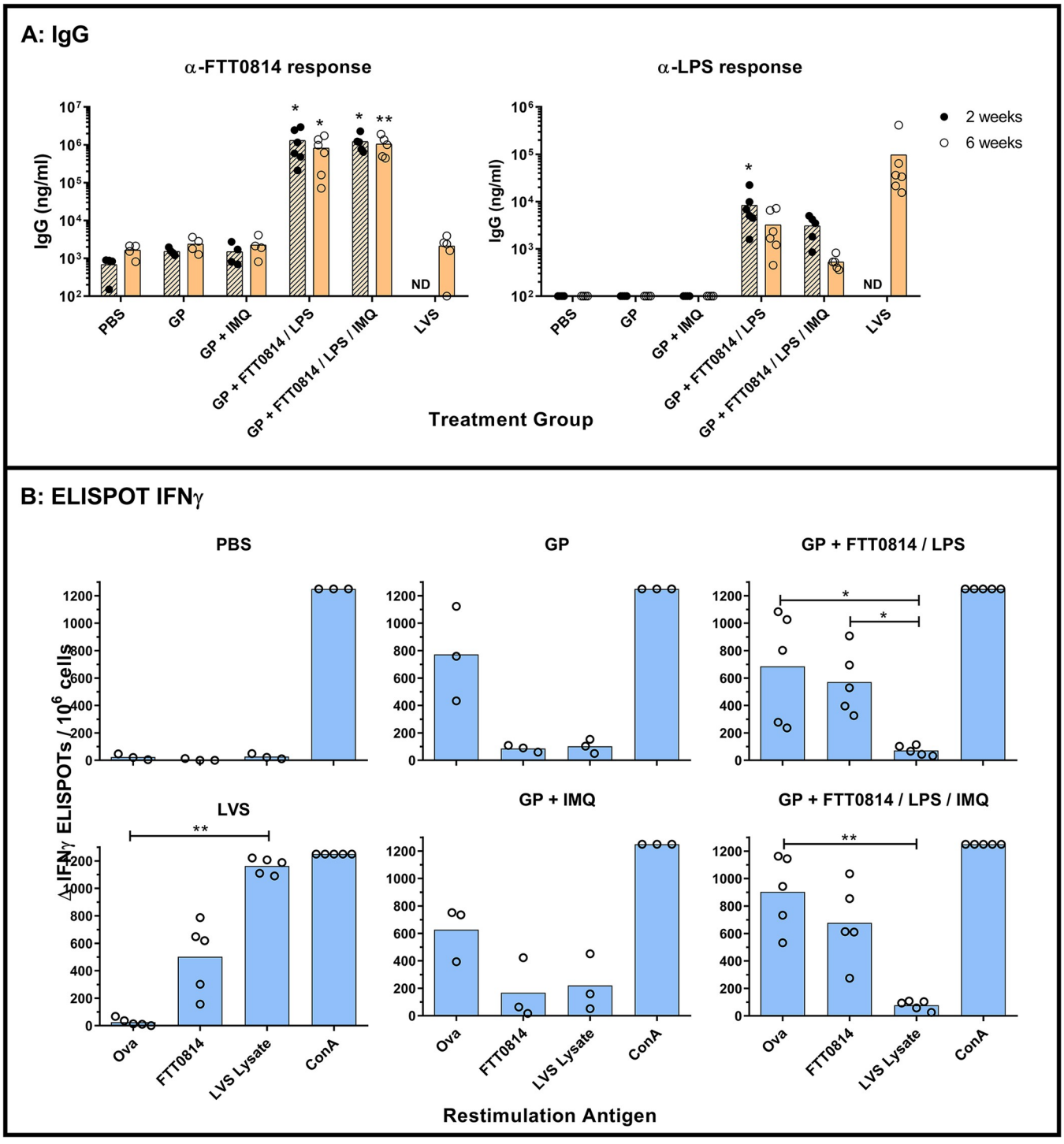

Fig 4. IgG and IFNγ ELISPOT responses in vaccinated F344 rats. F344 rats were vaccinated with GP vaccine combinations that included FTT0814, LPS and imiquimod (IMQ) adjuvant. Additional group included of rats vaccinated with the live attenuated vaccine *F. tularensis* LVS, sham-inoculated with PBS and empty GPs. **Panel A:** Plasma IgG recognizing FTT0814 or LPS was measured by ELISA in blood collected by tail-bleed 2 weeks after the third vaccination (grey bars/black circles) or by cardiac puncture (CP) 6 weeks after the third vaccination (white bars/white circles). The vaccine treatment groups are shown on the x-axis. The bars represent the mean IgG response for the group with responses for individual rats plotted as circles. The LVS vaccinated rats did not undergo the tail bleed procedure (ND). Where a significance differences in the IgG response between each of the respective vaccine groups and the PBS control group was identified, this is indicated on the figure (one-way ANOVA with Holm-Sidak's multiple comparison test, * $p < 0.05$, ** $p < 0.01$). IgG data for blood samples collected 2 and 6 weeks after the final vaccination were analysed separately. **Panel B:** Splenocytes were isolated from rats 6-weeks after the final vaccination. Splenocytes were stimulated with the antigens shown on the x-axis and expression of IFNγ was measured by ELISPOT. Each graph reports the ELISPOT

response for individual animals for each vaccine treatment group. Delta responses are reported after subtraction of any response detected in media-only stimulated cultures for each respective rat. Responses are only reported for those animals/samples where post-processing functional viability was confirmed by measurement of mitogen (ConA) responsiveness. The grey bars represent the mean response for the group with responses for individual rats plotted as circles. Where significant differences in the magnitude of ELISPOT responses were observed when comparing delta responses for either the Ova, FTT0814 or LVS Lysate stimulated cultures, this is indicated on the corresponding graph (non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$).

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model of inhaled tularemia [17]. In that proof-of-concept efficacy study, rats were challenged with a presented *F. tularensis* SCHU S4 aerosol challenge dose of 4 x 10^3 CFU (calculated retained dose = 1.6 x 10^3 CFU). In the current study we have evaluated this GP encapsulated antigen combination at more stringent aerosol challenges doses to determine the limits of protective efficacy afforded in this model. At the presented challenge dose of 2.8 x 10^4 CFU the GP-FTT0814/LPS vaccine did not provide protection against lethality, but it did provide a significant survival advantage with respects to increasing the time to death of rats compared with control groups. Notably, the GP vaccine that contained FTT0814 in the absence of LPS failed to show any efficacy advantage confirming the protective benefit provided by inclusion of the LPS antigen. At this challenge dose, the whole-cell LVS provided complete protection against the lethality of the challenge demonstrating inferiority of our GP subunit vaccine under these stringent challenge conditions. The observed efficacy of LVS in F344 rats challenged with *F. tularensis* SCHU S4 is consistent with data reported by others by others [33–35]. When F344 rats were challenged with an aerosol of 7.8 x 10^3 CFU, the GP-FTT0814/LPS vaccine provided partial protection against the lethality of this challenge. Therefore, these data have helped us define the limitations of the protection provided by this vaccine in this aerosol challenge model.

In an attempt to enhance the efficacy of our GP vaccine, we evaluated the inclusion of the TLR7/8 agonist imiquimod in our formulation. Signals from TLR agonists can promote the efficient generation of a $CD4^+$ T-cell response by dendritic cells (DCs) [36]. In a murine model of *Francisella* infection TLR-mediate macrophage stimulation has been shown to be important to induce optimal protection [37]. Imiquimod was selected as our candidate adjuvant as it can synergise with GPs to enhance TNFα expression from mouse and human DCs [29]. Furthermore, imiquimod can activate antigen presenting cells (APCs), induce humoral and cellular immunity and to enhance T helper 1 (Th1) T-cell responses [38]. Despite our hypothesis that imiquimod might enhance vaccine induced immunity, we failed to observe any immunological or efficacy benefit following its inclusion in our GP formulations. Indeed, rats vaccinated with GP-FTT0814/LPS in the absence of imiquimod had a marginally improved survival advantage when challenged with an aerosol of 7.8 x 10^3 CFU *F. tularensis* SCHU S4. However, animal group sizes used in this study were not sufficiently powered to determine the significance of subtle differences on efficacy between these formulations. A possible explanation for the absence imiquimod benefit may be due to potential dysregulation of IL-12p70 signalling. Whilst imiquimod can synergise with GPs to enhance TNFα expression from DCs, it has also been observed that the presence of GPs can also inhibit expression of IL-12p70 [29]. In view of the role of IL-12p70 in initiating Th1 $CD4^+$ T-cell responses [39], its potential dysregulation may have hindered rather then helped the generation of appropriate protective immunity. IL-12p70 expression was not measured in in the current study so we are unable to confirm if this was an inhibitory mechanism. Interestingly, whilst GP synergised inhibition of IL-12p70 expression by DCs was also previously observed for other TLR agonists including Pam_3CSK_4, poly:IC and CpG it was not demonstrated for LPS from *Escherichia coli* [29]. Therefore, there could be further scope to investigate alternative adjuvants to enhance GP antigen immunity in the future.

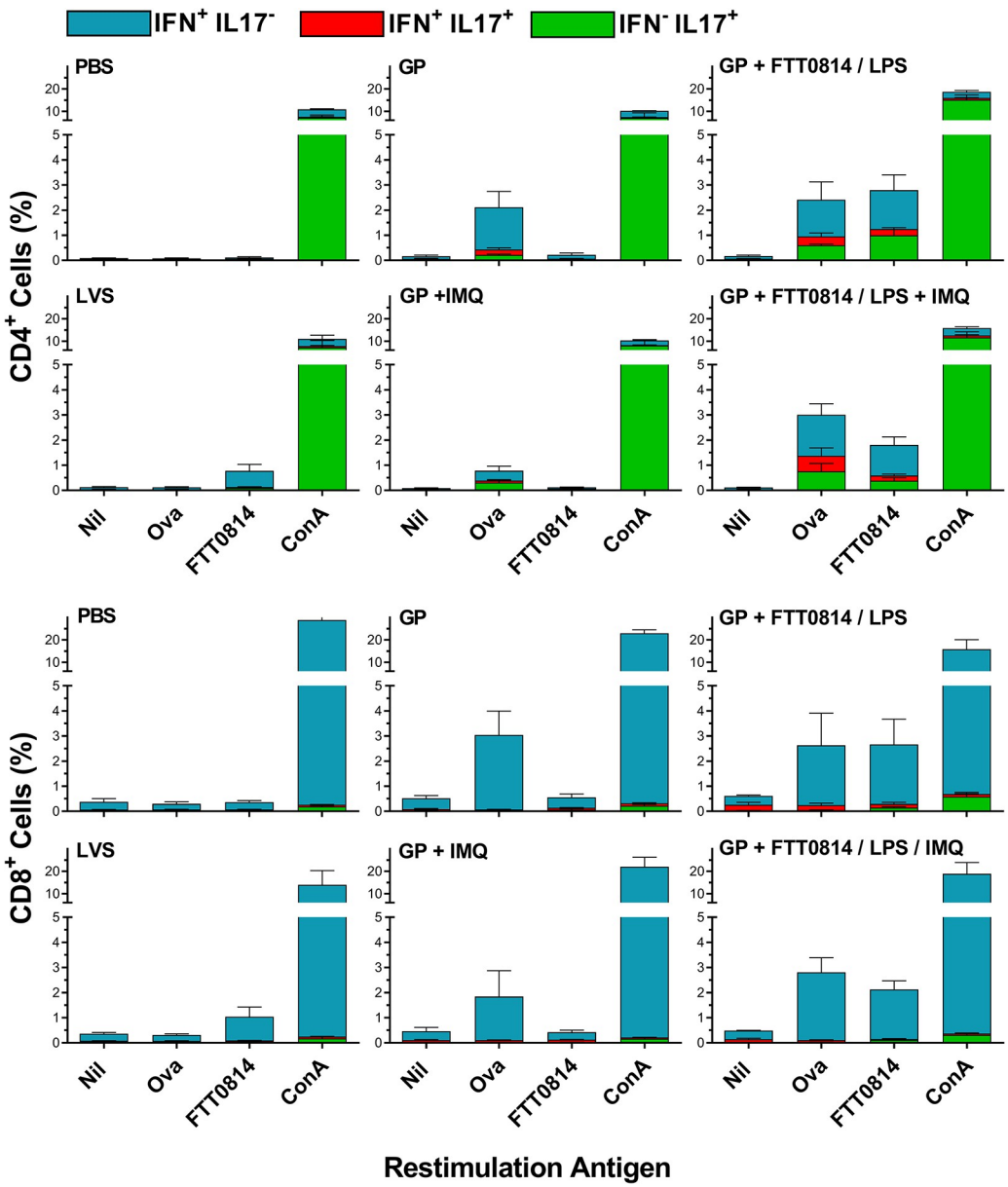

Fig 5. ICS phenotyping of IFNγ/IL17 expression of T-cells from vaccinated F344 rats. F344 rats were vaccinated with GP vaccine combinations that included FTT0814, LPS and imiquimod (IMQ) adjuvant. Additional group included of rats vaccinated with the live attenuated vaccine *F. tularensis* LVS, sham-inoculated with PBS and empty GPs. Splenocytes were isolated 6 weeks after the final vaccination and stimulated for 20 hours with each of the antigen shown on the x-axis of the bar graphs. Brefeldin A was included in the last 4 hours of culture. A minimum of $1x10^5$ singlet, live, lymphocyte cell events were acquired for each sample. The stacked bar graphs show the mean percentage of either $CD4^+$ or $CD8^+$ cells expressing either only IFNγ (blue bars), only IL17 (green bars) or both IFNγ and IL17 (red bars) with error bar representing the SEM for the group. Responses are only reported for those animals/samples where post-processing functional viability was confirmed by measurement of mitogen (ConA) responsiveness.

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Understanding the nature of the immunity required to protect against infection is an essential aspect of any vaccine development process. Passive transfer approaches have demonstrated that antibodies can provide a protective response in lethal murine LVS and *F. tularensis* SCHU S4 challenge models [37, 40–42]. In F344 rats, an O-antigen glycoconjugate vaccine that did not include a *F. tularensis* protein antigen component protected rats from a lethal low-dose

inhaled infection with *F. tularensis* SCHU S4 [16]. These data provide examples where humoral immunity can provide a protective response in tularemia infection models. Yet, T-cell depletion studies have demonstrated an essential role for cell mediated immunity in *Francisella* vaccine efficacy studies [43–47]. Furthermore, recent work has demonstrated that both tissue resident and circulating T-cells are required for protective immunity against *F. tularensis* [48]. Therefore, humoral and T-cell mediated responses are likely to be required for optimal vaccine induced immunity against *Francisella* infections.

Recent work to identify tularemia vaccine-induced correlates of protection have compared the immune response induced by live attenuated vaccines displaying different efficacies in *F. tularensis* infection models. Vaccine-induced IFNγ has been consistently correlated with protective immunity in these models [35, 49, 50]. It is therefore encouraging that in addition to LPS and FTT0814 specific antibodies, our GP vaccine primed T-cells from F344 rats to express FTT0814-stimulated IFNγ *ex vivo*. Whilst we demonstrated this previously [17], in the currently study we extended our immunological characterisation to identify both $CD4^+$ and $CD8^+$ T-cells as sources of IFNγ expression. Furthermore, we identified FTT0814 stimulated $CD4^+$ T-cells as a source of IL17 expression. GP vaccines for coccidioidomycosis have also been shown to promote antigen specific Th1 (IFNγ) and Th17 (IL17) responses [51, 52]. The role of IL17 as a modulator of protective immunity to *Francisella* infection is unclear. IL17 has been reported to be important for promoting Th1 cell mediated protective immunity in murine LVS infection models [53]. Also, elevated IL17 responses correlated with improved tularemia vaccine efficacy in an *ex vivo* F344 rat co-culture system [50]. However, whilst IL17 was important for protection against primary infection of mice with LVS, unlike IFNγ, it was not associated with protective immunity against secondary LVS challenge [54]. Furthermore, although IL17 could had a protective role in LVS infected mice, it was not sufficient in the absence of IFNγ to protect against high virulence strains of *F. tularensis* [55]. From these data, whilst vaccine induced generation of Th17 immunity may not be an essential protective component, it is likely to have a beneficial contribution in controlling *Francisella* infections.

Our data demonstrates that our GP-based vaccine can induce both IgG and T-cell mediate memory immune responses and we hypothesise that both components of this adaptive immunity are contributing to the protection observed. However, it remains to be determined what relative efficacy contribution that each of these immunological responses provide as this was not addressed in the current study. Furthermore, assessing the duration of humoral and cellular immunity beyond 6 weeks post vaccination will be an important consideration for future developmental assessment of this vaccine.

Concluding remarks

We have demonstrated that a GP-based subunit vaccine delivering the *Francisella* antigens LPS and FTT0814 can protect F344 rats from an inhaled challenge with *F. tularensis* SCHU S4. Our immunological characterisation of this vaccine candidate demonstrates that it can prime for antigen specific IgG, Th1 and Th17 responses, all of which are likely to contribute to the protective immunity observed. We have expanded on previous work to determine the limits of protection provided by this vaccine in the inhaled tularemia F344 challenge model. Whilst the protection provided by our GP-based vaccine proved to be inferior to that provided by LVS, the demonstration that we could protect F344 rats at a lethal aerosol challenge dose of *F. tularensis* SCHU4, albeit a low one, is still a notable achievement for a subunit tularemia vaccine. As such, it provides further evidence of the potential of GPs as a vaccine platform for difficult to treat pathogens including those of concern to the bio-defence community.

Supporting information

S1 Fig. Weight change of vaccinated F344 rats following aerosol infection with *F. tularensis* SCHU S4. The weight change is presented as % weight loss relative to the weight of each rat on the day prior to infection. Weight change for rats culled due to reaching either clinical severity or 15% weight loss are showed by red and black lines respectively. Weight change for rats which recovered from exposure and survived to the end of the study are shown by the blue lines.
(TIF)

S2 Fig. IFNγ ELISA PBMC responses 6-weeks post vaccination. PBMCs were isolated from F344 rats 6-weeks after the final vaccination. PBMCs were stimulated with the antigens shown on the x-axis and expression of IFNγ was measured by ELISA. Each graph shows the IFNγ response for individual animals for each vaccine treatment group. The grey bars represent the mean response for the group with responses for individual rats plotted as circles.
(TIF)

S3 Fig. ICS gating strategy. The hierarchical gating strategy used to quantify the expression of IFNγ and/or IL17 from lymphocytes expressing the T-cell surface markers CD4 or CD8 is shown using ConA stimulated splenocytes from a PBS control rat as an example.
(TIF)

S1 Data.
(XLSX)

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